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

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Abstract

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 two murine atherosclerosis models, Apolipoprotein E ($Apoe^{-/-}$) and LDL receptor ($LDLr^{-/-}$) deficient mice. The number of aortic myeloid cells increased significantly. They took up less oxidized LDL, while CD11c expression, interaction with T cells and aortic T cell proliferation were significantly enhanced in renal impairment. In human PBMC cultures, chronic kidney disease serum decreased lipid uptake and increased HLAII expression. Supplementation with Interleukin (IL)-17A similarly increased HLAII and CD11c expression and impaired oxLDL uptake. IL-17A expression was increased in atherosclerotic mice with renal impairment. Ablation of IL-17A in $LDLr^{-/-}$ mice by lethal irradiation and reconstitution with $Il17a^{-/-}$ 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 IL-17A in aggravation of vascular inflammation and atherosclerosis in renal impairment.

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DISCLOSURES None.

Keywords

Atherosclerosis; vascular inflammation; leukocyte; renal insufficiency; Interleukin 17

INTRODUCTION

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, patients with kidney disease presenting with less specific symptoms than the general population.[6] At most stages of chronic kidney disease, 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 or less) a significant association with cardiovascular mortality was consistently observed after correction for other risk factors.[1, 3] This degree of chronic kidney disease (CKD stages III and IV) affects about 8% of the adult US population.[7]

Unilateral nephrectomy significantly increased atherosclerotic lesion size in Apolipoprotein E ($Apoe^{-/-}$) mice,[8-11] where, different from wild-type (wt) mice, serum creatinine was significantly elevated after unilateral nephrectomy.[9, 11] Renal glomerular number was decreased in $Apoe^{-/-}$ compared to wt mice, but there was no evidence for accelerated progression of kidney disease.[11] Blood pressure was unaffected by unilateral,[9, 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 anti-atherogenic despite similar blood pressure levels and despite higher cholesterol.

Vascular calcification is prominent in end stage renal disease.[14, 15] In contrast, histology of the atherosclerotic lesions at CKDIII-IV or $Apoe^{-/-}$ mice after unilateral nephrectomy very much resembles lesions in normal kidney function.[8-10] Specific histopathologic features such as medial calcification that are observed in dialysis-dependent patients' arteries[14, 15] and severely uremic murine atherosclerosis models[8, 9, 13, 16] are not found. The mechanism of increased atherosclerosis in patients with mild to moderate renal impairment is incompletely understood.

Vascular wall infiltration by both innate and adaptive immune cells contributes to atherosclerotic lesion progression.[17] Alterations of the immune system by e.g. 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.[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.[17, 18] Their number within the arterial wall increases markedly during atherosclerosis development due to both immigration and local proliferation. Oxidized LDL up-regulates the expression of the β2 integrin subunit CD11c, a marker of antigen presenting cells in mice, on monocytes.[19] CD11c-deficiency decreases atherosclerotic lesion size.[19] Indeed, in atherosclerosis, $CD11b^+CD11c^+$ cells are capable of both lipid phagocytosis and antigen presentation to $CD4^+$ T cells.[20-24] This occurs in lymphatic organs,[21, 22] however, T cells are also located in close proximity to antigen presenting cells in the atherosclerotic arteries of mice and humans[25, 26] and interact with each other in the vascular wall.[27] Close interaction with $CD11b^+CD11c^+$ cells in the vascular wall was limited to $Apoe^{-/-}CD4^+$ T cells and did not occur with $wtCD4^+$ T cells suggesting an antigen specific process.[27] The number of T lymphocytes increases during the atherosclerosis progression in the vessel.[17, 18] T cells are differentially regulated by myeloid and plasmacytoid dendritic cells[24] and can

modulate atherosclerotic inflammation by cytotoxicity and cytokine secretion. While some T cell cytokines such as interferon gamma (IFNγ) have a proven proatherogenic role in diverse settings,[17, 18] the effect of others such as interleukin (IL)-17A is controversial.[28, 29] Enhancement of atherosclerotic lesion formation by IL-17A has been reported in some[30-34] but not all models.[35-38] While some studies were limited by available reagents, the role of IL-17A may also differ depending on the environment, e.g. lesion localization[34, 35] or regulation of other immune mediators.[39] We found an induction of atherosclerosis by IL-17A in pharmacologic immunosuppression[39] and also in IL-17A overproduction in the absence of the IL-27 receptor.[40]

In patients with renal impairment, some serum inflammatory markers are increased and correlate with mortality, among them IL-6, that can enhance IL-17 production and be induced by IL-17 itself.[41] Increased IL-17A in serum in a cohort of patients receiving hemodialysis for renal replacement was recently reported.[42] Cellular immune alterations in renal failure include, among others, peripheral blood lymphopenia and neutrophilia.[43] Circulating dendritic cells are depleted.[44] However, data from the atherosclerotic vascular wall are required for a better understanding of the inflammatory disease process.[45]

Here, we examined the aortic leukocyte infiltrate in atherosclerotic $Apoe^{-/-}$ and LDL receptor ($LDLr^{-/-}$) deficient 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 renal impairment.

METHODS

Detailed methods are available online.

Animals

Wild-type (wt) C57Bl/6, $LDLr^{-/-}$, $Apoe^{-/-}$ mice (both on C57Bl/6 background)(Jackson Labs, Bar Harbor, ME), CD11c^{YFP}, kindly provided by Dr. M. Nussenzweig, Rockfeller University, NY and crossed with $Apoe^{-/-}$, and mice lacking IL-17A ($Il17a^{-/-}$), 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. High fat diet contained 40% of kcal from fat, 1.5% Cholesterol.

Bone marrow transplantation and unilateral nephrectomy

Experiments were commenced at 6 weeks of age. Bone marrow transplantations were performed as described.[46] Unilateral nephrectomy or de-capsulation of the kidney were performed one week before high fat diet was started.

Quantification of atherosclerosis, histologic and aortic leukocyte analysis

Aortic en face staining and histologic assessment of aortic roots, plasma lipid FPLC and measurement of GFR by FITC-Inulin are described in the supplement. Preparation and flow cytometry of aortic leukocytes[39] and two-photon-imaging of aortic CD11c$^+$/T-cell interaction were performed as described.[27]

Cell culture experiments

Cell culture procedures are described online, serum for stimulation experiments was obtained after written informed consent from patients with non-diabetic chronic kidney disease described in the online supplement.

Statistical analysis

Data are expressed as mean ±SEM. Two-tailed student t-test and Mann Whitney test were used to compare two conditions. One-way-ANOVA and post-hoc tests as indicated were used if more than 2 conditions were compared. P-values <0.05 were considered significant. P values are indicated as follows: *p<0.05, **p<0.01, ***p<0.001.

RESULTS

Impaired renal function increases atherosclerotic lesion size and aortic leukocyte infiltration

Unilateral nephrectomy significantly increased serum creatinine (suppl. table I) and decreased glomerular filtration rate (GFR) measured by inulin clearance in *Apoe*^{−/−} mice (suppl. figure I). This degree of renal impairment significantly increased aortic atherosclerotic lesion size in *Apoe*^{−/−} mice (figure 1A,B) with very similar affection of both genders (suppl. figure IIA). Aortic root lesion size also increased (figure 1C,D). Relative collagen contents and composition was essentially un-altered (suppl. figure IIB,C). No significant differences in body or spleen weight occurred, serum calcium, phosphorus, total leukocytes as a marker of systemic inflammation, thrombocyte and erythrocyte counts were un-altered. Total serum cholesterol was increased, due to LDL, but not HDL levels (table 1). No alteration was observed in renal leukocytes (data not shown).

In *Apoe*^{−/−} mice with renal impairment, significantly more leukocytes were recovered from the aorta by flow cytometry analysis as described[39] (figure 1E). All investigated leukocyte types, myeloid cells (CD11b^{+}), B cells (CD19^{+}) and αβTCR^{+} T cells were significantly more abundant (figure 1F,G).

These data show that the increase in atherosclerotic lesion size in *Apoe*^{−/−} mice with renal impairment is accompanied by an extended inflammatory vascular infiltrate.

Enhanced accumulation of aortic myeloid cells and CD11c expression in renal impairment

We further investigated aortic accumulation of myeloid leukocytes in renal impairment. Numbers of CD11b^{+} myeloid cells in the aorta were significantly elevated already early in atherosclerosis development in *Apoe*^{−/−} mice after three weeks of high fat diet (figure 2A). Aortic cell proliferation is highly elevated at this stage in mice with normal renal function.[20, 39] We labeled proliferating cells with BrdU and measured incorporation into CD11b^{+} leukocytes. Renal impairment significantly increased proliferation of aortic myeloid leukocytes (figure 2B). Expression of the antigen presenting cell marker CD11c on aortic CD11b^{+} myeloid cells[19,20, 39] was enhanced in renal impairment (figure 2A).

Both, myeloid cell numbers and the proportion that expressed CD11c further increased in developed lesions after 12 weeks of high fat diet (figure 2C). Very similar results were observed in male and female mice (suppl. figure III). Cell localization in the vascular wall by confocal imaging showed large CD11c^{+} and CD11b^{+} neo-intimal areas (figure 2D) in both lipid rich and cellular plaque areas in the animals with renal impairment.

Renal impairment alters myeloid cell function in the atherosclerotic aortic wall

We next investigated lipid uptake and antigen presenting function of myeloid aortic cells.

Uptake of labeled exogenous oxidized (ox)LDL in aortic leukocytes was measured by flow cytometry (figure 3A). Uptake into total CD11b$^+$ and the CD11b$^+$CD11c$^+$ subgroup of aortic leukocytes was significantly lower in *Apoe$^{-/-}$* mice with impaired renal function (figure 3B).

We studied CD11c$^+$ cell/CD4$^+$ 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[27] (suppl. figure IV) to investigate this in the atherosclerotic aorta of mice with normal and impaired renal function. CD4$^+$ T cells interacted with CD11c$^+$ cells in aortas of mice with renal impairment for an increased proportion of the observation time (11±1% in ctrl, 19±1% in RI, n=2, p=0.02). Second, productive T cell interactions with antigen presenting cells decrease the speed of both cell types.[47] The average speed of both CD11c$^+$ cells and CD4$^+$ T cells was significantly lower in aortas from animals with renal impairment than controls (CD11c$^+$ in figure 3C, suppl. movies 1,2 and CD4$^+$ in figure 3D, suppl. movies 3,4). Third, T cell stimulation by antigen presenting cells induces proliferation. Indeed, aortic CD11b$^+$CD11c$^+$ enhanced CD4$^+$ T cell proliferation during mixed lymphocyte reaction in vitro similar to spleen cells (figure 3E). Renal impairment significantly increased proliferation of aortic αβTCR$^+$ lymphocytes, but not CD19$^+$ B lymphocytes (figure 3F).

Renal impairment and IL-17A alter myeloid cell differentiation in vitro

To test whether the altered aortic myeloid cell phenotype in mice with renal impairment could be replicated in human cells, human adherent PBMC cultures were supplemented with 10% serum from patients with mildly (CKDI,II, GFR>60 ml/min) and moderately (CKDIII, GFR=30-60 ml/min) impaired renal function or healthy controls. While lipid levels were elevated in both patient groups (suppl. figure VA,B), uptake of oxLDL was decreased in cells cultured in CKDIII, but not milder stages of chronic kidney disease (figure 4A). In contrast, HLAII expression as marker of antigen presenting cells was increased compared to healthy controls (figure 4B).

We previously found that IL-17A enhanced accumulation of CD11b$^+$CD11c$^+$ cells in peritonitis,[39] and therefore tested whether IL-17A also up-regulates HLAII as an antigen presenting cell marker of human cells. HLAII expression was significantly higher if cells were exposed to IL-17A during macrophage culture (figure 4C) and, more markedly, dendritic cell polarization by GM-CSF and IL-4 (figure 4D). Similarly, IL-17A supplementation during mouse myeloid derived macrophage differentiation significantly increased CD11c expression (figure 4E). The same result was found when IL-17A effects were tested in *Il17a$^{-/-}$* and *Il17ra$^{-/-}$* cells in parallel to correct for potential unspecific effects of recombinant IL-17A (figure 4F). mRNA expression of genes implicated in cholesterol transport was assessed in *Il17a$^{-/-}$* bone marrow derived macrophages in the presence of IL-17A during differentiation (figure 4G). Both CD36 and ABCA1 expression were significantly decreased. oxLDL uptake by wt macrophages was dose dependently decreased by IL-17A if cell were exposed during differentiation (figure 4H).

IL-17A expression in renal impairment

We next assessed T cell polarization *in vivo*. Among T cell lineage markers, the T$_{H17}$-transcription factor RORγt was significantly higher expressed in aortas of atherosclerotic *Apoe$^{-/-}$* mice with renal impairment (figure 5A) and the proportion of splenic IL-17A producers was significantly increased (figure 5B,D). No significant change was seen in T-

bet and IFNγ, markers of T_{H1} cells and very well described pro-atherogenic mediators (figure 5A,B,C) or Foxp3$^+$ regulatory T cells with known anti-atherogenic function (figure 5A,E). The increase in IL-17 production was not limited to CD4$^+$ T cells but also observed in the CD4$^-$CD3$^+$ compartment that includes multiple lineages (data not shown).[48] In serum from patients with impaired renal function (CKDIII), many IL-17A measurements were below detection limit, similar to cohorts of patients with hypertension and the acute coronary syndrome.[49, 50] However, in 16/32 patients but only 3/9 of healthy volunteers, serum IL-17A was detectable (>6pg/ml) and IL-17A effects on macrophage differentiation started at low concentrations (figure 4C,D). Similarly, most other cytokine concentrations were below detection limit, however, there was a trend towards elevated TNF T_{H17}-related IL-6 levels (data not shown).

Recent data have shown that Angiotensin-induced increase in blood pressure[49, 51] and tissue damage[52, 53] was mediated by IL-17A. Blockade of Angiotensin signaling is among the most successful pharmacologic interventions in renal impairment.[54, 55] Angiotensin II serum concentration was increased in $Apoe^{-/-}$ mice with renal impairment (135±47 pg/ml (RI), 48±14 (ctrl), 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 IFNγ production was observed, but Angiotensin II significantly increased the number of IL-17A producing T cells under T_{H17} polarizing conditions (figure 5F,G). No effect on the proportion of IL-17A was observed if Angiotensin II was applied either without T_{H17} favoring conditions (T_{H0}, figure 5F,G) or during re-stimulation of T cells (data not shown). The effect on T_{H17} 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 renal impairment, we reconstituted $LDLr^{-/-}$ mice with either wt or $Il17a^{-/-}$ bone marrow. Unilateral nephrectomy significantly decreased renal function as measured GFR also in this mouse model of atherosclerosis (suppl. figure I). Suppl. table II shows that no significant differences occurred in body weight, circulating leukocytes and serum triglyceride and total cholesterol levels. In renal impairment, there was a tendency towards an increase in LDL and vLDL levels as determined by FPLC (suppl. figure VI). Aortic lesion size was significantly increased in impaired renal function similar to the $Apoe^{-/-}$ atherosclerosis model (figure 7A-C).

Reconstitution with $Il17a^{-/-}$ bone marrow abolished IL-17A producing cells in the spleen (suppl. figure VIIA). Comparing control $LDLr^{-/-}$ mice transplanted with wt and $Il17a^{-/-}$ bone marrow, aortic root lesion size was very similar. However, in the absence of IL-17A, renal impairment no longer increased atherosclerotic lesion size (figure 6A,B, suppl. figure VIIB,C). Lesional collagen content was very similar in all four experimental groups (figure 6C,D), similar to what was observed in $Apoe^{-/-}$ mice (suppl. figure III). However, lesional macrophage accumulation was significantly enhanced in renal impairment in wt mice (figure 6E). This was abolished in the absence of IL-17A.

Renal impairment 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 more CD11b$^+$CD11c$^+$ leukocytes were detected in the aortas of wt $LDLr^{-/-}$ mice with renal impairment compared to controls with normal renal function (figure 7B). This increase was completely abolished in $Il17a^{-/-}$-reconstituted $LDLr^{-/-}$ mice. Similar to the $Apoe^{-/-}$ model (figure 2), renal impairment significantly increased aortic cell proliferation in control

$LDLr^{-/-}$ mice (figure 7C,D). 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 renal impairment.

DISCUSSION

Kidney disease is a 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.[41, 56] However, inflammatory leukocytes within the vascular wall have not been systematically explored in patients on renal replacement therapy or with lesser degrees of renal impairment. Our study shows a significant increase of total aortic leukocytes and $CD11b^+CD11c^+$ myeloid cells in two 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 renal impairment.

The role of IL-17A in atherosclerosis in general is controversial.[28, 29] A pro-atherogenic role is suggested by anti-atherogenic effects of IL-17-receptor-blockade and IL-17A-blockade in some, albeit not all models[28, 29] and pro-atherogenic IL-17A effects in immunosuppressed $Apoe^{-/-}$ mice where IL-17A participated in myeloid cell accumulation in the arterial wall.[39] The expression 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 functions[30, 31] and macrophage cytokine secretion[57] 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 renal impairment 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 organs[19, 22] could amplify systemic immune response.

Not only the recruitment but also the lipid scavenging function of aortic $CD11b^+CD11c^+$ cells was significantly affected by renal impairment. An earlier report has described impaired efflux of cholesterol from peritoneal macrophages from mice with impaired renal function due 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 renal impairment. 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 chronic kidney disease in vitro. While multiple cytokines and other plasma components lipids may be differentially present in patients with renal impairment, 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 renal impairment as observed here and by others.[8, 9, 13, 16, 58] The changes in circulating lipid levels were significant in $Apoe^{-/-}$, but not $LDLr^{-/-}$ 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. On the other hand, 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 anti-atherogenic than a vasodilator despite higher cholesterol levels.[10] IL-17A influenced molecules responsible for both, cholesterol influx and efflux and therefore may have local pro-atherogenic actions by increasing lipid deposition in the vascular wall that 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 on hemodialysis.[42] Most importantly, renal impairment 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 renal impairment. Several factors may confer atherosclerosis-promoting function to IL-17A in renal impairment not observed under baseline conditions in *LDLr$^{-/-}$* mice here and by others.[37] First, our results show enhancement of IL-17A production by Angiotensin II under T_{H17} polarizing conditions. Angiotensin II blockade is a major disease-modifying factor in patients with kidney disease[54, 55] and more effective than blood pressure treatment with other agents in 5/6[51] and unilaterally nephrectomized mice.[10] Indeed, Angiotensin II levels were elevated in mice with renal impairment. The T_{H17}-regulating cytokine TGFβ 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. On the other hand, an amplification or alteration of the IL-17A signal in renal impairment may also occur by other yet to be determined factors, e.g. cytokines on IL-17A responsive cells such as leukocytes.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviation and Acronyms

Apoe$^{-/-}$	Apolipoprotein E deficient mice
LDLr$^{-/-}$	LDL receptor deficient mice
RI	renal impairment
IL-17A	Interleukin 17A

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Novelty and Significance

What Is Known?

- Chronic kidney disease increases the risk of atherosclerosis and its complications.

- During atherogenesis, leukocytes accumulate in the arterial wall and contribute to plaque growth.

- *Apoe*$^{-/-}$ 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 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.

Large population-based studies have shown that chronic kidney disease increases morbidity and mortality in patients with cardiovascular disease, however; the effect of kidney disease on atherosclerosis has not been systematically investigated. We examined inflammatory cells inflammation in atherosclerotic lesions in moderate renal impairment using a combination of histology, qPCR, flow cytometry and multi-photon imaging. Our data show in two mouse models that arterial inflammation is markedly increased. We characterized myeloid cell phenotype and demonstrate increased antigen-presenting-cell function. Our data show activation of the IL-17 pathway and indicate to its mechanistic importance in leukocyte accumulation and plaque growth in moderate renal impairment, indicating that -IL-17 therapy may be a therapeutic anti-inflammatory target in impaired renal function.
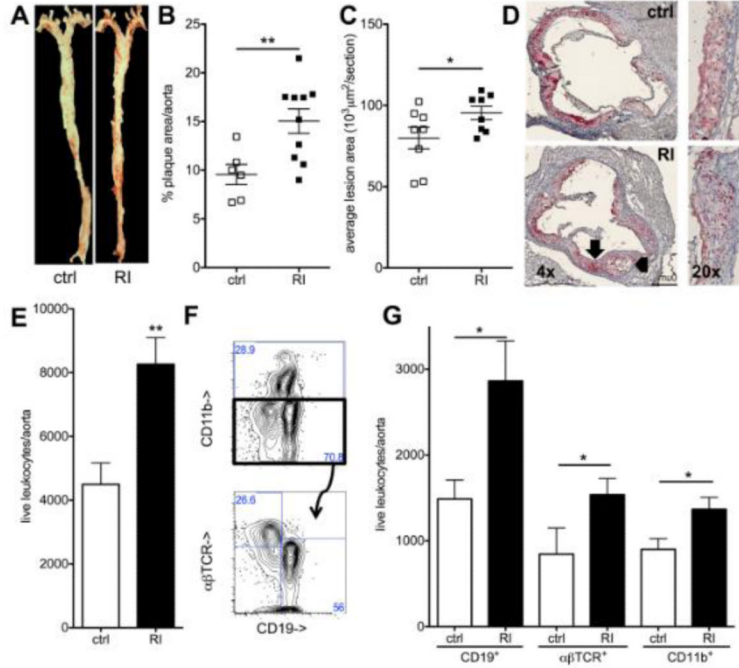
Figure 1. Renal impairment increases atherosclerotic lesion size and leukocyte infiltration
(A,B) En face aortic atherosclerotic lesion size was assessed in *Apoe*$^{-/-}$ mice (12 weeks high fat diet) (A, ctrl: sham operated, RI, renal impairment) and quantified as percent of aortic surface area (B)(n=6-10, 3 independent experiments). **(C,D)** Aortic root lesion size in the 300 μm following the aortic valve (C, n=8, 4 independent experiments, D examples, 4x and 20x 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$^+$ cells; E, n=9-11, 4 independent experiments). Staining for CD11b (myeloid cells), CD19 (B cells) and αβTCR (T cells) (example in F), was used for quantification of these subgroups (G, n=5-11 per group, 4 independent experiments).
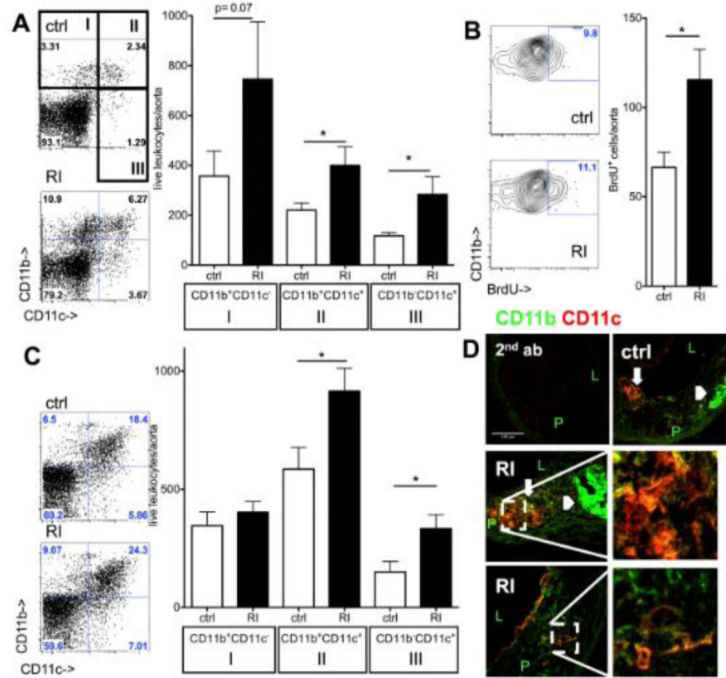
Figure 2. Myeloid cell accumulation in the aorta during atherosclerosis development in renal impairment

(**A**) CD11b and CD11c expression on aortic leukocytes in $Apoe^{-/-}$ mice after three weeks high fat diet by flow cytometric analysis (ctrl=sham-operated, RI=renal impairment, examples and n=11-12, 4 independent experiments). (**B**) Proliferation of aortic myeloid cells by BrdU incorporation at the same timepoint (cell number/aorta, n=8-10 from 3 independent experiments). (**C**) Aortic flow cytometry after twelve weeks high fat diet showed increased CD11b$^+$CD11c$^+$ and CD11c$^+$ cell numbers in renal impairment (n=9-11, 4 independent experiments). (**D**) Localization in established atherosclerotic lesions (12 weeks high fat diet) by confocal imaging revealed CD11c$^+$ (red) cells in the neo-intima. Most of these cells were also CD11b$^+$ (green) in both control and renal impairment (RI) $Apoe^{-/-}$ mice. CD11b$^+$CD11c$^+$ cells were present in foam cell (thick arrows; compare to lipid (figure 1D)) and highly cellular regions. CD11b was also found in a-cellular intimal areas (arrowheads compare to fig. 1D) (secondary antibodies only as negative control, P=plaque, L=lumen, 40x original magnification).
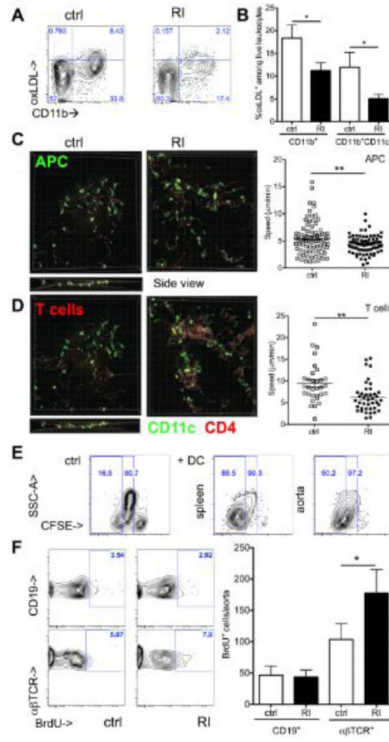
Figure 3. Altered lipid uptake and interaction with T cells of aortic myeloid leukocytes in renal impairment
(A,B) Uptake of DiI-oxLDL by aortic leukocytes was assessed 24h after injection into *Apoe*^{−/−} mice with and without renal impairment (RI) by flow cytometry (A shown in all live leukocytes, B quantified as proportion of CD11b^{+} and CD11b^{+}CD11c^{+} cells, n=4-7, 2 independent experiments). **(C,D)** Aortic antigen presentation was assessed by live cell 2-photon microscopy after co-incubation of explanted aortas from *CD11c*[YFP]*Apoe*^{−/−} mice (ctrl and RI) with SNARF labeled *Apoe*^{−/−} CD4^{+} T cells (red). CD11c^{+} antigen presenting cell (APC, C) and T cell (D) velocities were assessed in control and RI aortas in three dimensions (z axis shown in ctrl, stills from suppl. movies 1-4, available online, 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^{+}CD11c^{+} cells from atherosclerotic *Apoe*^{−/−} mice was assessed by CFSE dilution (1 of 2 independent experiments with similar results, 4-5 aorta donors per group). **(F)** Proliferation of *Apoe*^{−/−} aortic lymphocytes after three weeks high fat diet (BrdU^{+} cells/aorta, n=8-10 from 3 independent experiments).

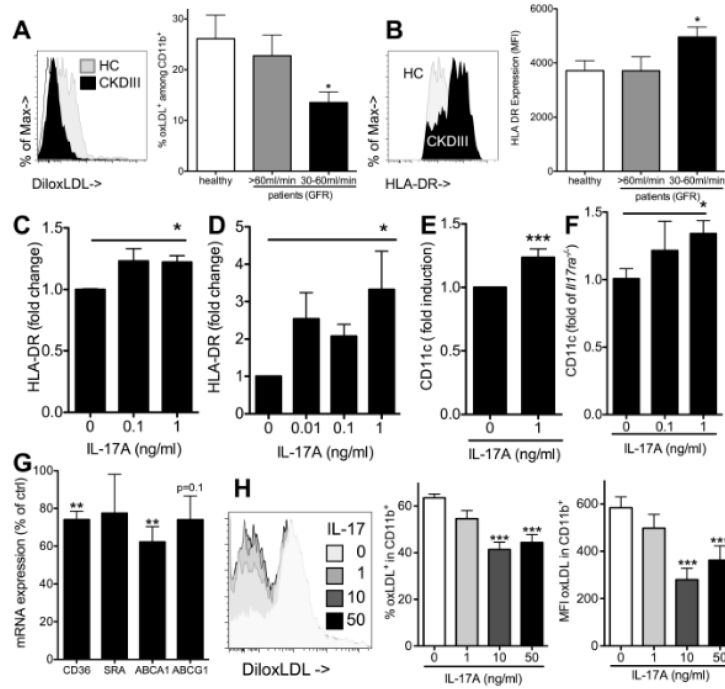**Figure 4. Renal impairment and IL-17A enhance antigen presenting cell marker expression and decrease oxLDL uptake in myeloid cell differentiation**

(A) oxLDL uptake by human PBMC derived macrophages differentiated in the presence of serum from patients with stable chronic kidney disease (GFR above 60 ml/min=CKDI,II, n=5, GFR 30-60ml/min=CKDIII, n=11 and healthy controls (HC), n=9, 3 independent experiments, Bonferroni after One-way-ANOVA) **(B)** HLAII surface expression on human PBMC derived macrophages assessed by flow cytometry (CKDI,II, n=11, CKDIII, n=19, healthy controls, n=8, 6 independent experiments, Bonferroni after One-way-ANOVA).
(C,D) HLAII expression on PBMC derived macrophages cultured with and without recombinant IL-17A (C,n=5, linear trend after One-way-ANOVA) and dendritic cells (D, promoted by culture with GM-CSF and IL-4, n=4, linear trend after One-way-ANOVA).
(E,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 wt (E, 4 independent experiments) and $Il17a^{-/-}$ compared to $Il17ra^{-/-}$ mice (F, t-test after One-way-ANOVA, 3 independent experiments). **(G)** $Il17a^{-/-}$ macrophage mRNA expression was assessed on day three of culture in the presence of recombinant IL-17A (1ng/ml) and compared to control cells (t-tests, 3 independent experiments). **(H)** Wt macrophage oxLDL uptake on day 7 after culture with different concentrations of recombinant IL-17A (Dunnetts after One-way-ANOVA, n=12, 3 independent experiments).
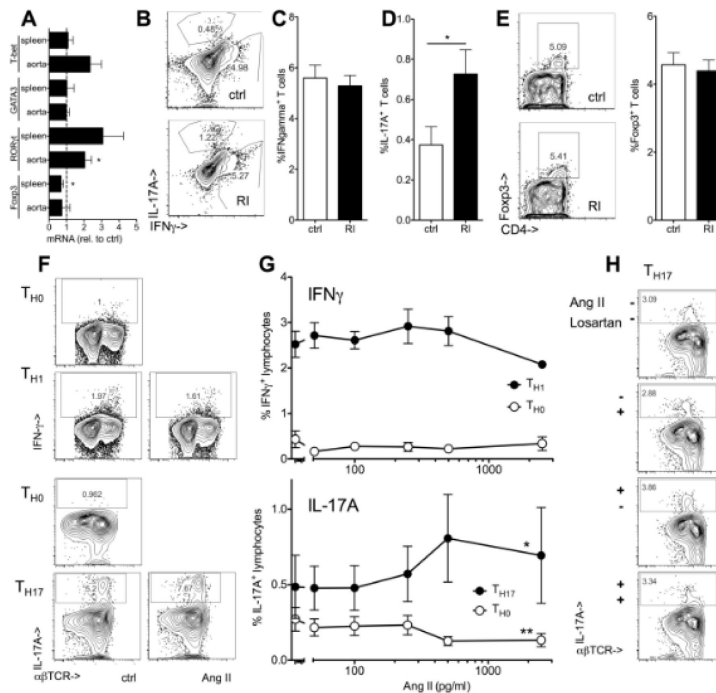
Figure 5. Renal impairment and Angiotensin II enhance TH17 polarization

(A) mRNA expression of markers of T cell lineages (T-bet for the T_{H1}, GATA-3 for T_{H2}, RORγt for T_{H17} and Foxp3 for T_{reg}) was assessed in spleens and aortas of *Apoe*$^{-/-}$ mice after 12 weeks high fat diet (renal impairment relative to controls, n=5, t-tests). **(D-E)** Interferon gamma (IFNγ)(B,C, n=6-8) and IL-17A producing (B,D, n=8-13) T cells and Foxp3^{+} regulatory T cells (E, n=4-5) in spleens from *Apoe*$^{-/-}$ mice with normal and impaired renal function (RI) (5h PMA/ionomycin, % of CD3^{+} cells, *Il17a*$^{-/-}$ spleens used to define IL-17A positivity (<0.1%)). **(F,G)** Mouse splenocytes were cultured under T_{H0}, T_{H1} and T_{H17} polarizing conditions with and without exogenous Angiotensin II (AngII, 250 pg/ml unless indicated) and intracellular cytokine expression was assessed after re-stimulation as described in methods (F, typical examples and G, n=3 independent experiments, One-way ANOVA, *indicates significant slope). **(H)** T_{H17} polarization was carried out in the presence or absence of Angiotensin II and Losartan (1nM) or solvent control (one of 2 independent experiments).
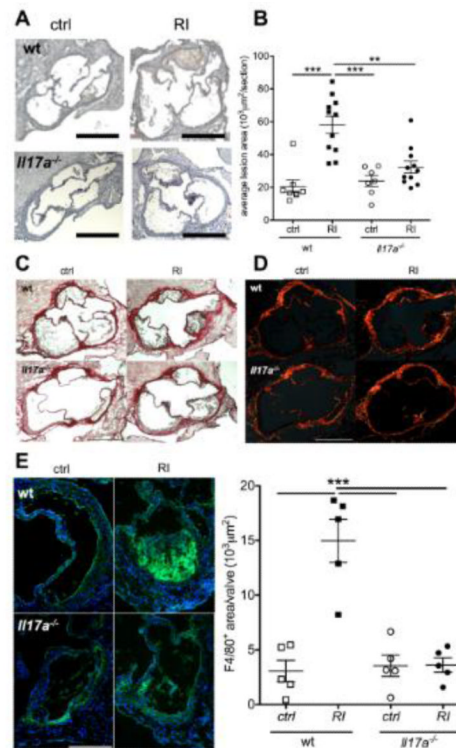
Figure 6. IL-17A ablation abolishes atherosclerosis enhancement in renal impairment
Atherosclerosis development was studied in *LDLr*$^{-/-}$ mice reconstituted with either wt or
Il17a$^{-/-}$ bone marrow and normal and impaired renal function. **(A,B)** Aortic root lesion size
(A, examples, 5x orig. magn., bars=500μm, B means±SEM, n=7-11/group, 4 independent
experiments, Bonferroni after One-way-ANOVA. **(C,D)** Picrosirius-red stain of collagen
contents as translucent image (D) and with the use of polarized light (E, examples of at least
4 mice/group). **(F)** F4/80-macrophage staining (examples and statistical analysis of n=5
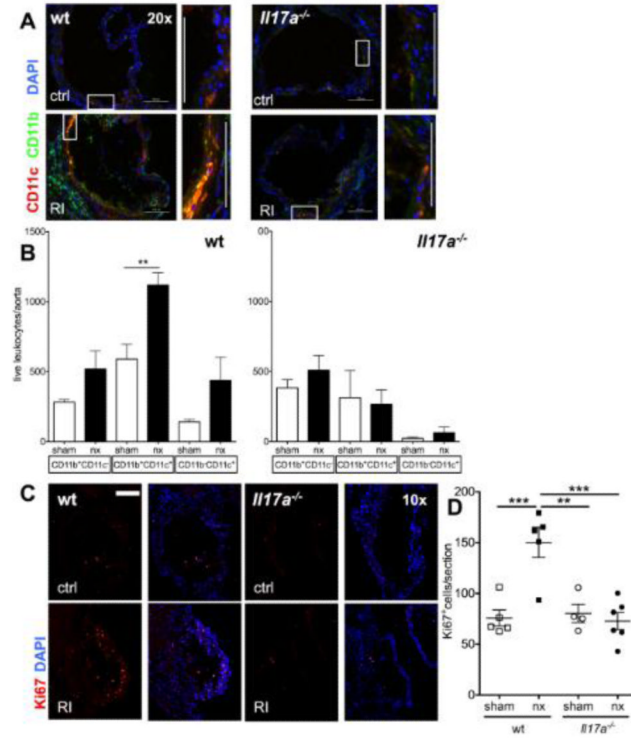mice per group, F4/80 positive area/valvular lesion, Bonferroni after One-way-ANOVA).

Figure 7. IL-17A ablation abolishes enhanced aortic macrophage accumulation in renal impairment

The aortic leukocyte infiltrate was analyzed in $LDLr^{-/-}$ mice reconstituted with either wt or $Il17a^{-/-}$ bone marrow and normal and impaired renal function. **(A)** Immunofluorescence to assess CD11b (green) and CD11c (red) in the aortic root (typical examples of aortic valves, rectangles mark the cell rich intimal regions shown in zoom. 20x orig. magn., bars=100μm). **(B)** Number of total aortic CD11b^{+} and CD11c^{+} cells determined by flow cytometry (n=5-8, 4 independent experiments). **(C,D)** Aortic cell proliferation was assessed by Ki67 staining (E, examples of aortic valve area plaques, 10x orig. magn., bar=200μm, F: mean Ki67^{+} cell numbers/aortic section from n=4 sections/mouse, n=4-6 mice/group, One-way-ANOVA and Bonferroni post-hoc test).