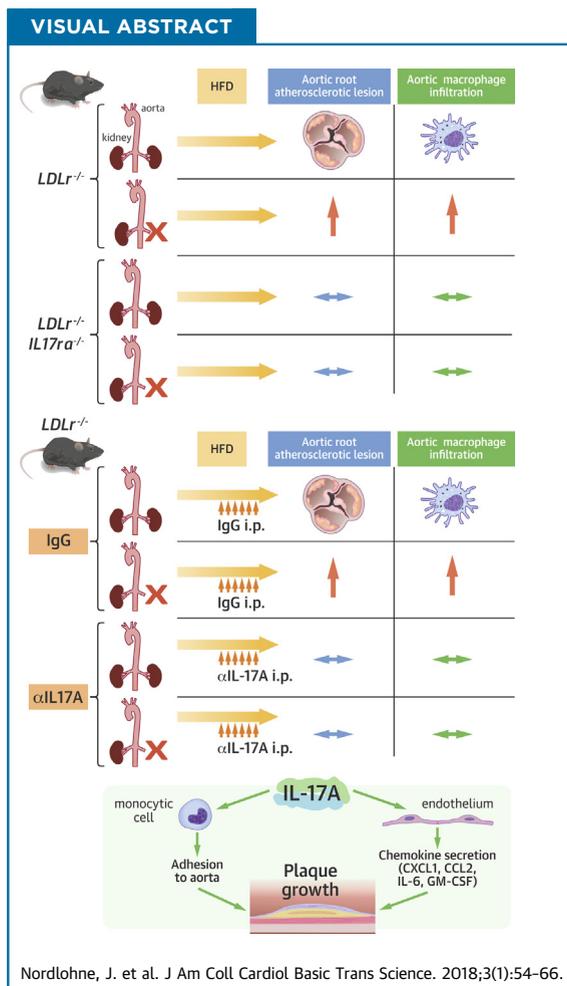**PRECLINICAL RESEARCH**

Aggravated Atherosclerosis and Vascular Inflammation With Reduced Kidney Function Depend on Interleukin-17 Receptor A and Are Normalized by Inhibition of Interleukin-17A

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VISUAL ABSTRACT

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HIGHLIGHTS

- Moderate renal impairment significantly increases atherosclerotic lesion size and leukocyte numbers, most markedly macrophages and T cells, in *LDLr*$^{-/-}$ mice.

- IL-17 receptor A–deficient *LDLr*$^{-/-}$ mice are protected from the growth in lesion size and leukocyte infiltrate in renal impairment.

- Monocytes, especially Ly6C/GR1^{HIGH} cells, express high levels of IL-17 receptor A.

- IL-17A increases monocyte adhesion to the aortic wall and enhances endothelial cell pro-inflammatory cytokine production.

- Ablation of IL-17A or IL-17A blockade normalizes the inflammatory aortic wall infiltrate even in established atherosclerosis.

SUMMARY

Effective therapy of atherosclerotic complications in patients with chronic kidney disease (CKD) is an unmet clinical need. Cardiovascular events are the most common cause of death. At a glomerular filtration rate ≤60 ml/min, these events are increased also after correction for common risk factors. Previous studies have reported enhanced vascular inflammation in mice and recently also in humans. Our current data show, in a mouse model of atherosclerosis in moderate renal impairment, that interleukin-17 receptor A is instrumental in this condition, and blockade of this pathway can normalize arterial inflammation even in advanced atherosclerosis. (J Am Coll Cardiol Basic Trans Science 2018;3:54–66) © 2018 The Authors.
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**ABBREVIATIONS
AND ACRONYMS**

CKD = chronic kidney disease

IL = interleukin

Il17ra = interleukin-17 receptor A (gene name)

LDLr = low-density lipoprotein receptor

PCR = polymerase chain reaction

Renal impairment is a major risk factor for cardiovascular events and death. At a glomerular filtration rate ≤60 ml/min (chronic kidney disease [CKD] stages III through V), a consistent association is found after correction for common risks such as hypertension and diabetes (1,2). The impact of atherosclerotic complications for patients with CKD rises with improved management; stabilization of renal function can now be achieved even in severe CKD, but cardiovascular disease remains poorly controlled (3). Histologically, atherosclerotic lesions are similar to normal kidney function at CKD stages III and IV (4). This scenario is unlike that in patients receiving hemodialysis maintenance therapy. Traditional risk factors such as cholesterol profile are poor prognostic markers in this population (5,6). Thus, it is likely that additional mediators modulate atherosclerosis development in CKD.

Inflammatory leukocytes are central in plaque development (7-9). The most prominent vascular leukocytes in atherosclerosis are myeloid mononuclear phagocytes, mostly termed macrophages. Both lipid scavenging and antigen presentation have been demonstrated for this cell type. In moderate renal impairment, arterial wall inflammation increases in mouse models of atherosclerosis, with a prominent increase in macrophages, their CD11c expression and antigen-presenting function (10,11). An autopsy study found more leukocytes in the arterial wall in patients with nondialysis-dependent CKD than in patients with normal kidney function (12). Two recent studies found increased aortic wall glucose metabolism in human CKD stage III, indicating enhanced vascular wall inflammation also in patients without clinical atherosclerotic endpoints (13,14).

Impaired kidney function profoundly alters immune regulation (15,16). Blood levels of interleukin (IL)-17A-producing T cells were elevated in small cohorts of patients with advanced CKD (17,18). In animal models with defined, more moderate reduction of renal function, we, along with others, have found upregulation of the T-cell cytokine IL-17A in inflammation (11,19). IL-17A and its main receptor subunit, IL-17 receptor A, regulate myeloid response in infection and sterile inflammation (20). IL-17A can directly modulate myeloid cell functions (21-23) but also act on resident vascular cells (24-26). The role of IL-17A in experimental atherosclerosis is diverse. Decisive factors may include the level of IL-17A expression and its balance with other cytokines (8,27,28) resulting in proatherogenic function in nascent plaques in renal impairment (10,11).

In addition to plaque formation, the level of inflammation critically influences further plaque development, determining lesion stability and the risk of plaque rupture (7-9). Animal models have shown distinct macrophage provenance in nascent lesions and established atheroma. Recruitment of monocytic cells from the circulation is largely replaced by local proliferation in mature plaques (29,30). Thus, possible

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

anti-inflammatory interventions will need to be tailored to established lesions.

The aim of this project was to investigate the role of IL-17 receptor A in nascent and established atherosclerosis in renal impairment and its amenability to therapeutic interventions.

METHODS

ANIMALS. Wild-type (wt) C57Bl/6, $LDLr^{-/-}$, $Il17a^{-/-}$ mice (31) (kindly provided by Dr. Y. Iwakura, University of Tokyo), and $Il17ra^{-/-}$ mice (32) (all CD45.2 on C57Bl/6 background), congenic B6.SJL-$Ptprc^aPepc^b$/BoyJ (CD45.1) mice (Jackson Labs, Bar Harbor, Maine), and $Il17ra^{-/-}LDLr^{-/-}$ mice (derived by a standard 4-generation breeding scheme) were genotyped by using polymerase chain reaction (PCR) and used in age- and sex-matched groups. Mice were kept in specific pathogen-free conditions. Animal experiments were approved by Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Lower Saxony, Germany). Mice were maintained on a high-fat diet (Harlan Teklad 88137, Altromin, Lage, Germany) or normal "chow" diet for the indicated time periods. Serum urea, electrolytes, and lipids were measured by using an Olympus AU400 ChemistryImmunoAnalyzer (Olympus, Hamburg, Germany) and blood counts in a VetABC animal blood counter (ScilVet, Viernheim, Germany).

BONE MARROW TRANSPLANTATION AND KIDNEY SURGERY. For unilateral 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 removed after ligation of vessels and ureter, taking care to avoid damage to the adrenal gland and surrounding organs as described elsewhere (11). Control surgery was de-capsulation of the kidney. Post-operative analgesia was performed with metamizole subcutaneously as needed. Lethal irradiation was performed in a ^{137}Cs irradiator (10 Gy), and mice were reconstituted with unfractionated bone marrow. Blood pressure and heart rate were assessed by using a tail-cuff system (Blood Pressure 209000 series, TSE Systems, Bad Homburg vor der Höhe, Germany) after 3 days of training 1 week before the mice were killed. Values represent the mean of at least 3 measurements. Fluorescein isothiocyanate-sinistrin clearance was determined by using the NIC kidney device according to the manufacturer's instructions (Mannheim Pharma & Diagnostics GmbH, Mannheim, Germany). For IL-17A blockade according to a tested protocol (33), 100 μg monoclonal rat-anti-mouse IL-17 (TC11-18H10.1) or immunoglobulin G (IgG) isotype (BioLegend, San Diego, California) in a total volume of 200 μl phosphate-buffered saline was administered intraperitoneally once a week.

QUANTIFICATION OF ATHEROSCLEROSIS AND AORTIC AND HISTOLOGICAL ANALYSES. En face and histological assessment of atherosclerotic lesion size has been described (11). For histological aortic root analysis, frozen 5-μm sections from the aortic valve plane in 50-μm intervals were stained with Oil Red O staining with hematoxylin and light green counterstain. Picrosirius red stain and Masson's trichrome stain with hematoxylin and eosin counterstain were used for assessment of fibrosis. For immunofluorescence, polyclonal anti-mouse CD3 (Dako A0452), anti-mouse B220 (RA3-6B2), anti-mouse CD11b (M1/70), anti-mouse CD11c (N418), polyclonal anti-mouse α-smooth muscle actin (ab15734) (Abcam, Cambridge, United Kingdom, and BioLegend), and the following secondary antibodies were used: donkey-anti-rat-IgG-AF488 (Invitrogen, Carlsbad, California), goat-anti-hamster-Cy3 (Jackson ImmunoResearch, Newmarket, United Kingdom), and donkey-anti-rabbit-IgG-AF555 (Life Technologies A31572, Life Technologies, Carlsbad, California). Images were obtained with a Leica DMI600B or DMI3000B microscope with 5×, 10×, and 20× original magnification using Leica Application Suite version 3.5.0 (Leica, Wetzlar, Germany). Analysis was conducted with NIH ImageJ and GIMP (version 2.8).

CELL CULTURE AND ADHESION ASSAY. Murine cardiac endothelial cells (CELLutions, Eching, Germany) were incubated with 50 ng/ml of IL-17A or IL-17F (R&D Systems, PeproTech, Rocky Hill, New Jersey) for 2 h. Heat-degraded IL-17A or IL-17F (60 min at 80°C) and hydrochloric acid diluent (80 μM final concentration) were used as controls and did not stimulate cytokine expression (n = 2 each; data not shown).

For ex vivo IL-17A stimulation, aortic arches from $Il17a^{-/-}$ and $Il17ra^{-/-}$ mice were co-incubated with $Il17a^{-/-}$ or $Il17ra^{-/-}$ bone marrow myeloid cells after 5 h of adhesion enrichment for 1 h at 37°C with 50 ng/ml of IL-17A (R&D Systems) in a 50/50 mixture of full Roswell Park Memorial Institute (RPMI) and Dulbecco's Modified Eagle Medium with gentle rotation (120 rpm). After washing twice with phosphate-buffered saline, the aortas were digested as described elsewhere (11). Live CD11b$^+$ among all cells defined according to scatter properties was analyzed. Preliminary experiments to assess possible contamination by aorta donor CD11b$^+$ cells were performed by using CD45.1 bone marrow on CD45.2 aortas and vice versa; 94 ± 0.5% of CD11b$^+$ cells were of bone marrow donor origin (n = 8), excluding significant contamination.

RNA ISOLATION AND REAL-TIME PCR. RNA was isolated by using a NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany) and reversely transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR was performed on a LightCycler 96 using SYBR Green (Roche, Grenzach-Wyhlen, Germany).

Primers were as follows (5′-3′): HPRT: forward primer (FP): CAGTCCCAGCGTCGTGATTA, reverse primer (RP): AGCAAGTCTTTCAGTCCTGTC, GM-CSF: FP: TCGTCTCTAACGAGTTCTCCTT, RP: CGTAGACCC TGCTCGAATATCT, IL-6: FP: CTGCAAGAGACTT CCATCCAG, RP: AGTGGTATAGACAGGTCTGTTGG, CXCL1: FP: CTGGGATTCACCTCAAGAACATC, RP: CAGGGTCAAGGCAAGCCTC, CCL2: FP: TTAAAAA CCTGGATCGGAACCAA, RP: GCATTAGCTTCAGATTT ACGGGT, IL-1β: FP: TGTGAATGCCACCTTTTGACA, RP: GGTCAAAGGTTTGGAAGCAG, IL-17F: FP: TGCTACTGTTGATGTTGGGAC, RP: AATGCCCTGG TTTTGGTTGAA, TGF-β: FP: CTCCCGTGGCTTCT AGTGC, RP: GCCTTAGTTTGGACAGGATCTG, TNF-α: FP: CAGCCTCTTCTCATTCCTGC, RP: GGTCTGGGCC ATAGAACTGA, RORγt: FP: CCGCTGAGAGGGCTTCAC, RP: TGCAGGAGTAGGCCACATTAC. IL-17 receptor sub-unit primers were as described (22). Products were confirmed by using melting curve analysis and gel electrophoresis. Data were analyzed with HPRT as a reference gene using LinRegPCR software (Heart Failure Research Centre, Amsterdam, the Netherlands).

ENZYMATIC DIGESTION OF TISSUES AND FLOW CYTOMETRY. Preparation and enzymatic digestion of tissues were as described elsewhere (11). For assessment of IL-17A production, cells were stimulated for 5 h with 10 ng/ml of phorbol 12-myristate 13-acetate and 500 ng/ml of ionomycin (both Sigma-Aldrich, St. Louis, Missouri). The following antibodies were used: anti-mouse anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (6D5), anti-CD31 (390), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD115 (AFS98), anti-F4/80 (BM8), anti-Gr1 (RB6-8C5), anti-Il17ra (PAJ-17R), anti-MHCII (M5/114.15.2), anti-T-cell receptor β (H57-597), and anti-IL-17A (TC11-18H10.1) (BioLegend and eBioscience [San Diego, California]). Aqua and near-infrared LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was used according to the manufacturer's instructions. Flow cytometry analysis was performed on a BD FACSCanto or LSR II (Becton-Dickinson, Franklin Lakes, New Jersey). Data were analyzed by using FlowJo software (Tree Star Inc., Ashland, Oregon). Gating was performed for live, CD45$^+$ events.

STATISTICAL ANALYSIS. In the assessment of continuous biological variables, an assumption of normality was made because most follow a Gaussian distribution. Two-tailed Student *t* tests were used to compare 2 conditions. Paired *t* tests were used if data from individual cell culture experiments were compared. If >2 conditions were compared, Bonferroni's test of selected conditions was applied after analysis of variance (ANOVA). For analysis of aortic root lesion size over the length of the aortic root, 2-way ANOVA with interaction for longitudinal distance and treatment groups was conducted. P values <0.05 were considered significant. Data are expressed as mean ± SEM.

RESULTS

IL-17 RECEPTOR A IS REQUIRED FOR INCREASED ATHEROSCLEROTIC LESION SIZE IN RENAL IMPAIRMENT. *Il17ra*$^{-/-}$ mice were bred to an atherosclerosis-prone *LDLr*$^{-/-}$ background. Unilateral nephrectomy, which induces moderate renal impairment that reduces glomerular filtration rate by ∼30% to 40% in *LDLr*$^{-/-}$ mice, was performed (10,11). Atherosclerosis was induced by a high-fat diet for 20 weeks (**Figure 1A**). There were no significant differences in body weight, blood counts, and plasma lipids between control-operated and unilaterally nephrectomized mice of each genotype (Supplemental Table 1). Total and aortic arch en face lesion size increased in *LDLr*$^{-/-}$ mice but not in *Il17ra*$^{-/-}$*LDLr*$^{-/-}$ mice with renal impairment (**Figures 1B to 1D**). Aortic root atherosclerotic lesions determined by serial section analysis were significantly larger in *LDLr*$^{-/-}$ mice with renal impairment than in all other groups (**Figures 1E to 1G**). Also, at an earlier time point (after 10 weeks of a high-fat diet), atherosclerotic root lesions were significantly larger in *LDLr*$^{-/-}$ mice but not in *Il17ra*$^{-/-}$*LDLr*$^{-/-}$ mice with renal impairment compared with controls (**Figures 2A and 2B,** Supplemental Table 2). Plaque collagen contents and fibrous cap thickness were determined by using Masson's trichrome and Picrosirius red staining (Supplemental Figure 1). There was a tendency for less collagen in the significantly smaller plaques of *Il17ra*$^{-/-}$*LDLr*$^{-/-}$ mice at week 20. No significant differences appeared at week 10 or at either time point in renal impairment. Staining for α-smooth muscle actin in the lesions was very similar (Supplemental Figure 2).

These data demonstrate a profound effect of IL-17 receptor A on atherosclerotic lesion size in renal impairment.

FIGURE 1   IL-17 Receptor A Increases Advanced Atherosclerotic Lesion Size in Renal Impairment
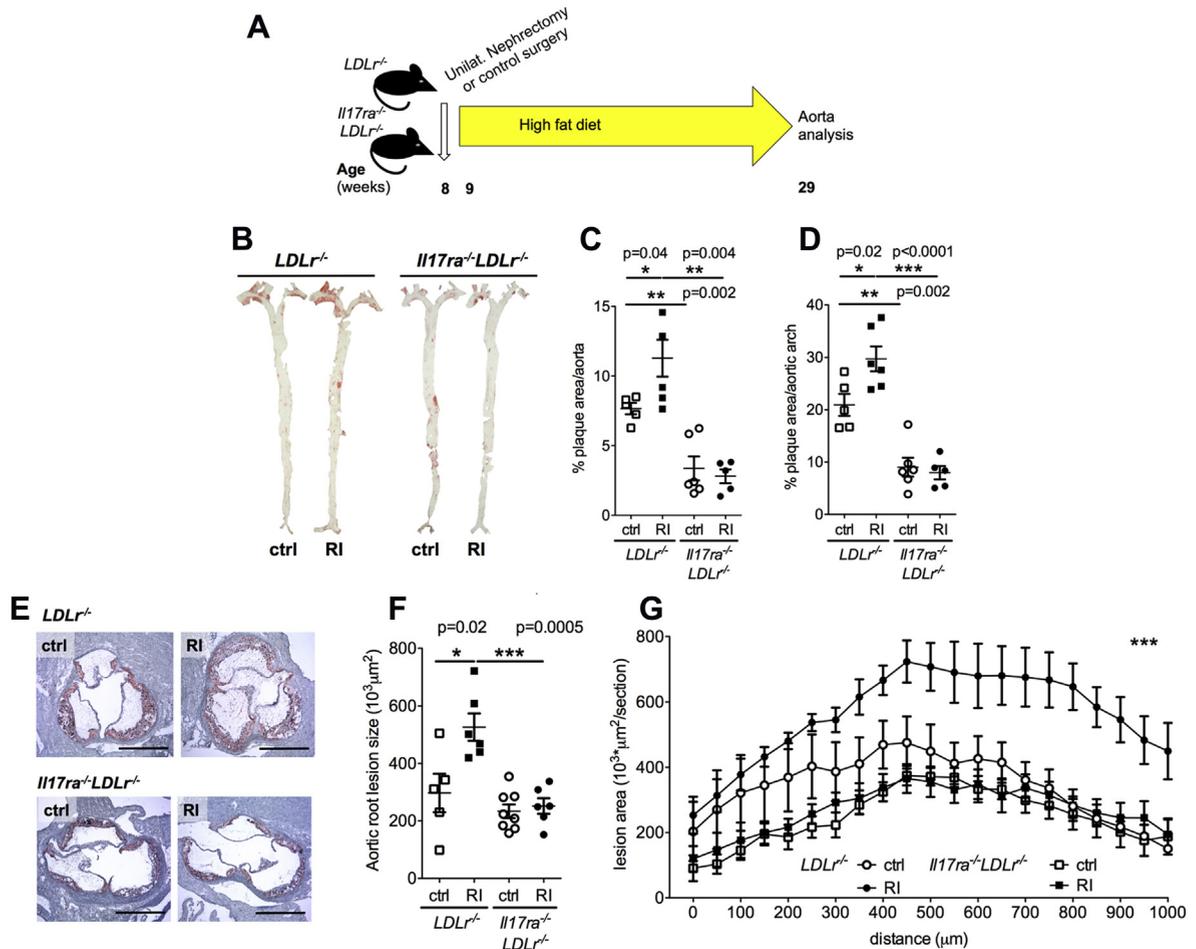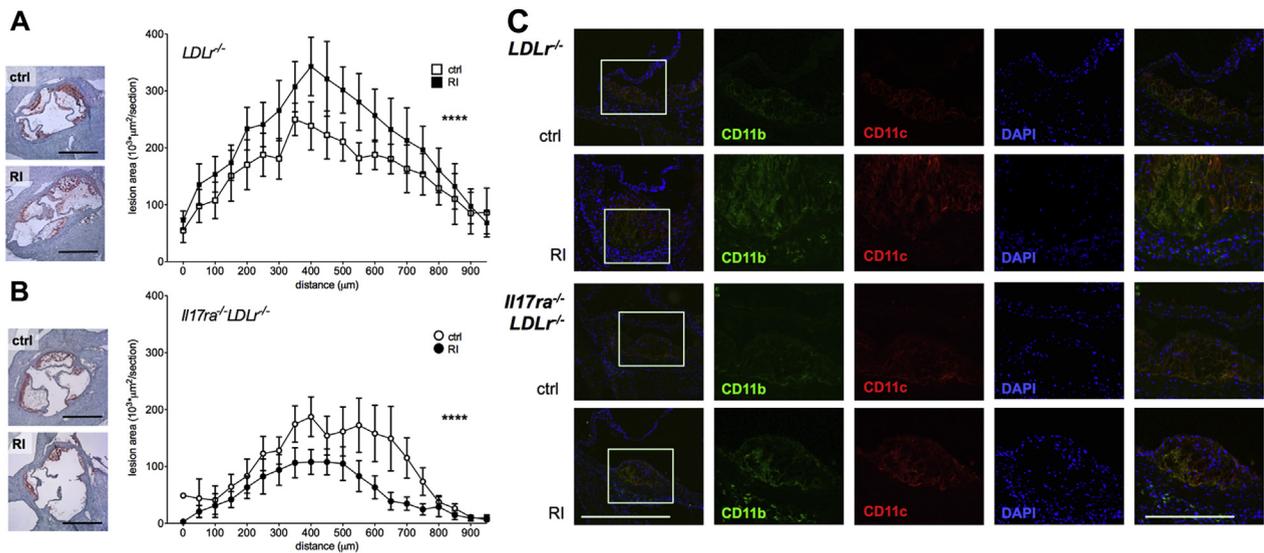

(A to G) $LDLr^{-/-}$ and $Il17ra^{-/-}LDLr^{-/-}$ male mice were kept on a high-fat diet for 20 weeks after unilateral nephrectomy (RI) or control (ctrl) surgery. (B to D) En face atherosclerotic lesion size was analyzed in the total aorta (C) and the aortic arch (D) (n = 5 to 6 per group from 2 independent experiments, Bonferroni after analysis of variance [ANOVA]). (E to G) Aortic root lesions were assessed by histology (E, typical examples, **bar** indicates 500 μm; F, statistical analysis of mean lesion size by Bonferroni after ANOVA). (G) Lesion according to longitudinal distance (2-way ANOVA, significant effects of treatment group [p < 0.0001] and distance [p < 0.0001], no significant interaction) (n = 5 to 8 per group, 2 to 3 independent experiments).

IL-17 RECEPTOR A ENHANCES AORTIC LEUKOCYTE ACCUMULATION. CD11b⁺ mononuclear myeloid cells, mostly termed macrophages, are the most prominent leukocyte to accumulate in intimal atherosclerotic lesions. Altered systemic immunity may be a reason for differential outcome of vascular inflammation. Spleen size was similar (Supplemental Tables 1 and 2). Among splenocytes, the proportion of T cells was increased in $Il17ra^{-/-}LDLr^{-/-}$ mice with renal impairment. A trend toward a higher RORγt and IL-17F messenger ribonucleic acid (mRNA) in the spleen (Supplemental Figure 3) were observed that may indicate increased IL-17A promotion in the absence of IL-17 receptor A (34). Blood concentrations

of B cells, monocytes, and granulocytes tended to be decreased in the absence in $Il17ra^{-/-}LDLr^{-/-}$ mice (Supplemental Figure 4).

In the aorta, immunofluorescence microscopy demonstrated enhanced CD11b⁺ macrophage accumulation in the intimal lesions of wild-type $LDLr^{-/-}$ mice with renal impairment (**Figure 2C**). A large proportion of these cells also expressed the antigen-presenting cell marker CD11c. B and T cells were also localized in the neointima (Supplemental Figure 5). Flow cytometry was performed to quantify leukocytes in aortas carefully freed from peri-aortic and adventitial tissues (10,11). Total aortic leukocytes, including T cells and CD11b⁺ myeloid cells (**Figures 3A and 3B**), and

FIGURE 2 Aortic Root Lesion Formation in Renal Impairment Is Enhanced by IL-17 Receptor A
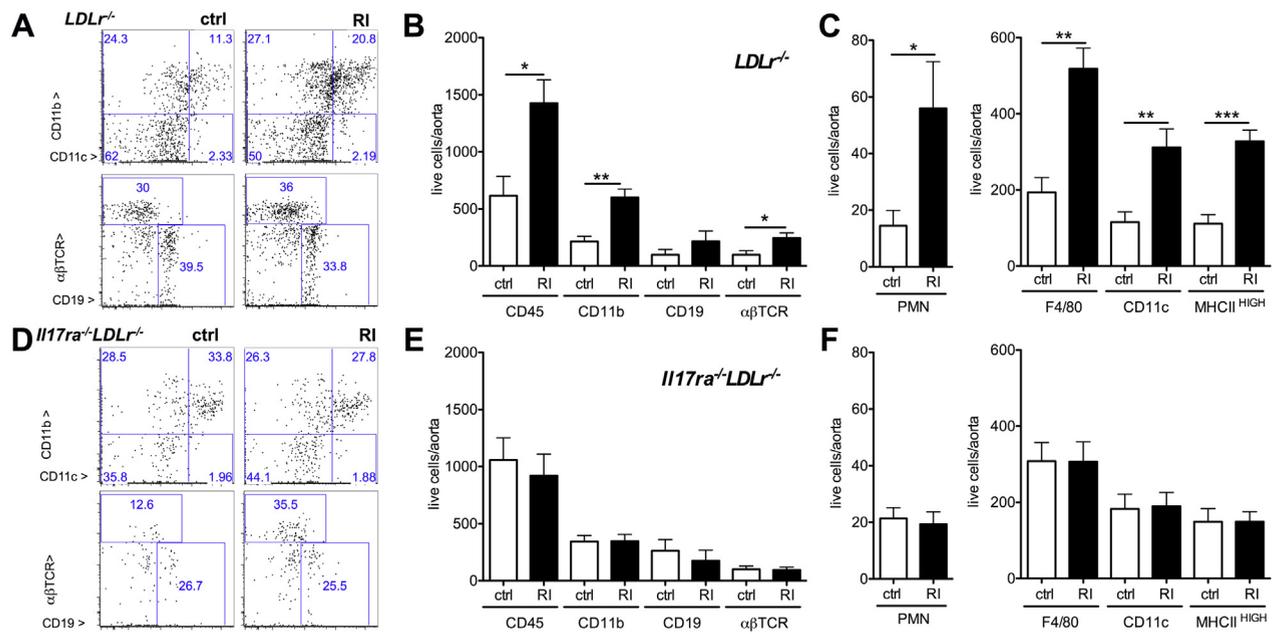
$LDLr^{-/-}$ and $Il17ra^{-/-}LDLr^{-/-}$ male mice were kept on a high-fat diet for 10 weeks after RI or ctrl surgery. **(A and B)** Aortic root lesions were assessed by histology (typical examples, bar indicates 500 μm, and statistical analysis of n = 5 to 6 per group $LDLr^{-/-}$ mice and n = 4 to 5 $Il17ra^{-/-}LDLr^{-/-}$ mice from 3 independent experiments, 2-way ANOVA, significant effects of treatment group [p < 0.0001] and distance [p < 0.0001], no significant interaction for **A or B**). **(C)** Aortic roots were stained for CD11b **(green)** and CD11c **(red)** with nuclear counterstain (4′,6-diamidino-2-phenylindole [DAPI], **blue**) (typical examples, 10× and 20× original magnification, **bars** indicate 500 and 250 μm, respectively). Abbreviations as in **Figure 1**.

among the latter both granulocytes and CD11b+ mononuclear cells (**Figure 3C**) were increased in number in renal impairment. Most CD11b+ mononuclear cells expressed both macrophage marker F4/80 and dendritic cell marker CD11c, as well as high levels of major histocompatibility complex II, suggestive of antigen-presenting function (11,35). The increase in cell numbers and major histocompatibility complex II expression was completely abrogated in $Il17ra^{-/-}LDLr^{-/-}$ mice (**Figures 3D to 3F**), indicating a role for IL-17 receptor A in inflammatory leukocyte accumulation with renal impairment.

IL-17 RECEPTOR A IS EXPRESSED ON CIRCULATING AND AORTIC MYELOID CELLS AND PROMOTES THEIR RECRUITMENT. To investigate possible IL-17A-responsive cells, IL-17 receptor A expression was investigated with flow cytometry by using identically treated $Il17ra^{-/-}$ cells as controls. Among leukocytes, IL-17 receptor A was significantly expressed on monocytes, most prominently Gr1^{HIGH} "inflammatory" monocytes that are progenitors of plaque macrophages during lesion formation (**Figure 4A**) (30). It was detected on myeloid cells also in the aorta (**Figure 4B**).

To determine the functional role of IL-17 receptor A on myeloid versus resident aortic cells, myeloid cell adhesion to the aorta with and without exogenous IL-17A was explored ex vivo (**Figures 4C and 4D**). Alone and in combination with $Il17ra^{-/-}$, $Il17a^{-/-}$ aortas and myeloid cells were used to minimize contamination with endogenous cytokine. IL-17A significantly enhanced adhesion of myeloid cells to aortic arches if IL-17 receptor A was present on the myeloid cells only. There was no detectable role for resident aortic cell IL-17 receptor A in this setting. To investigate $Il17ra^{-/-}$ and wild-type cells in identical pro-atherosclerotic environments in vivo, we generated mixed bone marrow chimeric $LDLr^{-/-}$ mice (50%wt/50%$Il17ra^{-/-}$ bone marrow) (**Figure 4E**). In renal impairment, there was a significant disadvantage for aortic accumulation of $Il17ra^{-/-}$CD11b+CD11c+ myeloid cells compared with CD45.1 wild-type cells (**Figure 4F**). Because any residual bone marrow recipient cells would count into the $Il17ra^{-/-}$ group (CD45.2), this design rather underestimates the effect of myeloid IL-17 receptor A. However, in complete bone marrow chimeric $LDLr^{-/-}$ mice with $Il17ra^{-/-}$ bone marrow (Supplemental Figure 6A, Supplemental Table 3), atherosclerotic lesion size and the number of aortic myeloid CD11b+CD11c+ cells after 10 weeks of a high-fat diet significantly increased in renal impairment (Supplemental Figures 6B to 6D).

FIGURE 3   IL-17 Receptor A Increases Atherosclerotic Inflammation in Renal Impairment

$LDLr^{-/-}$ and $Il17ra^{-/-}LDLr^{-/-}$ mice were kept on a high-fat diet for 10 weeks after RI or ctrl surgery. **(A to F)** Aortic leukocytes were analyzed by using flow cytometry for all leukocytes (CD45), myeloid cells (CD11b), B cells (CD19), and T cells (αβ-T-cell receptor [TCR]). **(A and D)** Typical examples with CD11b+ myeloid cells among all leukocytes are shown in the upper rows and T cells and B cells among all nonmyeloid cells in the lower rows. **(B, C, E, F)** All leukocytes **(B and E)** and among CD11b+ myeloid cells granulocytes (PMN, Gr1^{HIGH}F4/80^{LOW}CD11c^{LOW}) and expression of macrophage marker F4/80 and antigen presenting cell markers CD11c and major histocompatibility complex (MHC) II were investigated **(C and F)** (n = 5 to 6 $LDLr^{-/-}$ and 9 to 13 $Il17ra^{-/-}LDLr^{-/-}$ mice from 3 to 4 independent experiments, Student *t* tests). Abbreviations as in **Figure 1**.
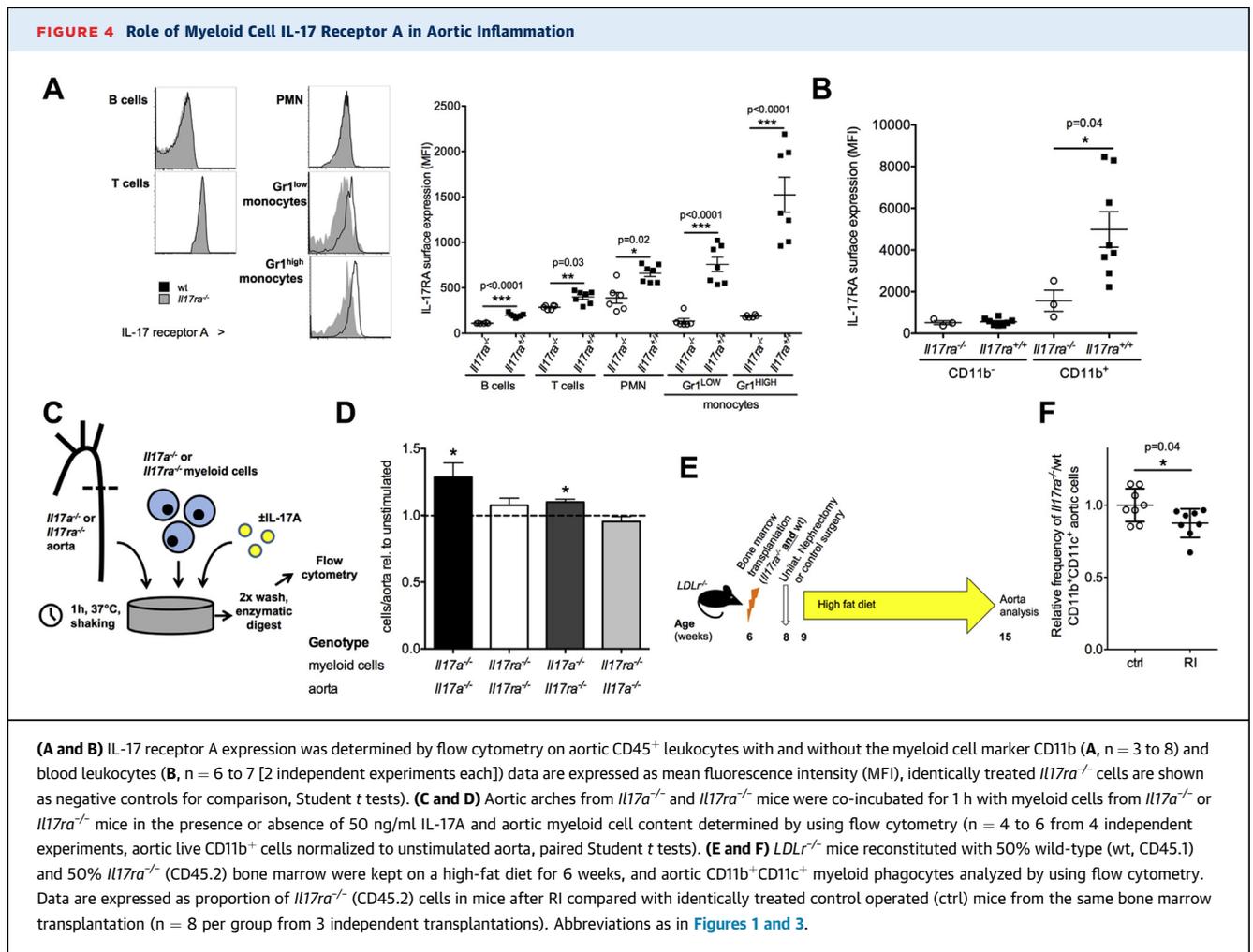
Thus, although the lack of IL-17A receptor signaling also on resident cells was required to prevent enhanced atherosclerotic inflammation in renal impairment, the in vitro results and the competitive disadvantage in vivo demonstrate a role for IL-17 receptor A in individual bone marrow cell homing to the aorta.

ENDOTHELIAL CELL IL-17 RECEPTOR A PROMOTES PRO-INFLAMMATORY CHEMOKINE EXPRESSION.

The endothelium has been implicated in vascular IL-17A effects (25,36,37). It expressed IL-17 receptor A, the main receptor subunit (**Figure 5A**). IL-17 receptor C, the subunit required for IL-17A and IL-17F signaling and IL-17 receptor E mRNA, required for IL-17C signaling, were also detected. IL-17 receptor B (required by IL-17E = IL-25) was below detection limit. The effect of recombinant IL-17A and IL-17F on endothelial cell cytokine and chemokine expression was studied. Indeed, CXCL1, CCL2, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA were significantly upregulated by IL-17A (**Figures 5B to 5E**). An equal dose of IL-17F induced a

significant, but weaker, CXCL1 response (**Figures 5F to 5I**). These data suggest the endothelium as an additional IL-17A signal responder in atherosclerotic inflammation.

Indeed, CXCL1, CCL2, and IL-6 mRNA levels also tended to be higher in the atherosclerotic aortas of $LDLr^{-/-}$ mice with renal impairment than in identically treated $Il17ra^{-/-}LDLr^{-/-}$ animals (Supplemental Figure 7). In addition, IL-17F, which was above mRNA detection limits in atherosclerotic aortas, was lower in $Il17ra^{-/-}LDLr^{-/-}$ mice than in $LDLr^{-/-}$ mice. Decrease of these cytokines that can promote aortic myeloid cell accumulation in atherosclerosis (7-9) is suggestive of interruption of a pro-inflammatory circuit in the absence of IL-17 receptor A.

IL-17A ABLATION IN ESTABLISHED ATHEROSCLEROSIS IN RENAL IMPAIRMENT.

Given the long latency of atherosclerosis, antiatherogenic therapies usually need to target established lesions. Inflammatory cell regulation differs in established and nascent plaques (30). We ablated IL-17A in $LDLr^{-/-}$ mice by lethal irradiation and reconstitution with $Il17a^{-/-}$ or control bone marrow after 6 weeks of atherosclerosis

FIGURE 4   Role of Myeloid Cell IL-17 Receptor A in Aortic Inflammation

(A and B) IL-17 receptor A expression was determined by flow cytometry on aortic CD45$^+$ leukocytes with and without the myeloid cell marker CD11b (A, n = 3 to 8) and blood leukocytes (B, n = 6 to 7 [2 independent experiments each]) data are expressed as mean fluorescence intensity (MFI), identically treated *Il17ra$^{-/-}$* cells are shown as negative controls for comparison, Student *t* tests). (C and D) Aortic arches from *Il17a$^{-/-}$* and *Il17ra$^{-/-}$* mice were co-incubated for 1 h with myeloid cells from *Il17a$^{-/-}$* or *Il17ra$^{-/-}$* mice in the presence or absence of 50 ng/ml IL-17A and aortic myeloid cell content determined by using flow cytometry (n = 4 to 6 from 4 independent experiments, aortic live CD11b$^+$ cells normalized to unstimulated aorta, paired Student *t* tests). (E and F) *LDLr$^{-/-}$* mice reconstituted with 50% wild-type (wt, CD45.1) and 50% *Il17ra$^{-/-}$* (CD45.2) bone marrow were kept on a high-fat diet for 6 weeks, and aortic CD11b$^+$CD11c$^+$ myeloid phagocytes analyzed by using flow cytometry. Data are expressed as proportion of *Il17ra$^{-/-}$* (CD45.2) cells in mice after RI compared with identically treated control operated (ctrl) mice from the same bone marrow transplantation (n = 8 per group from 3 independent transplantations). Abbreviations as in **Figures 1 and 3**.
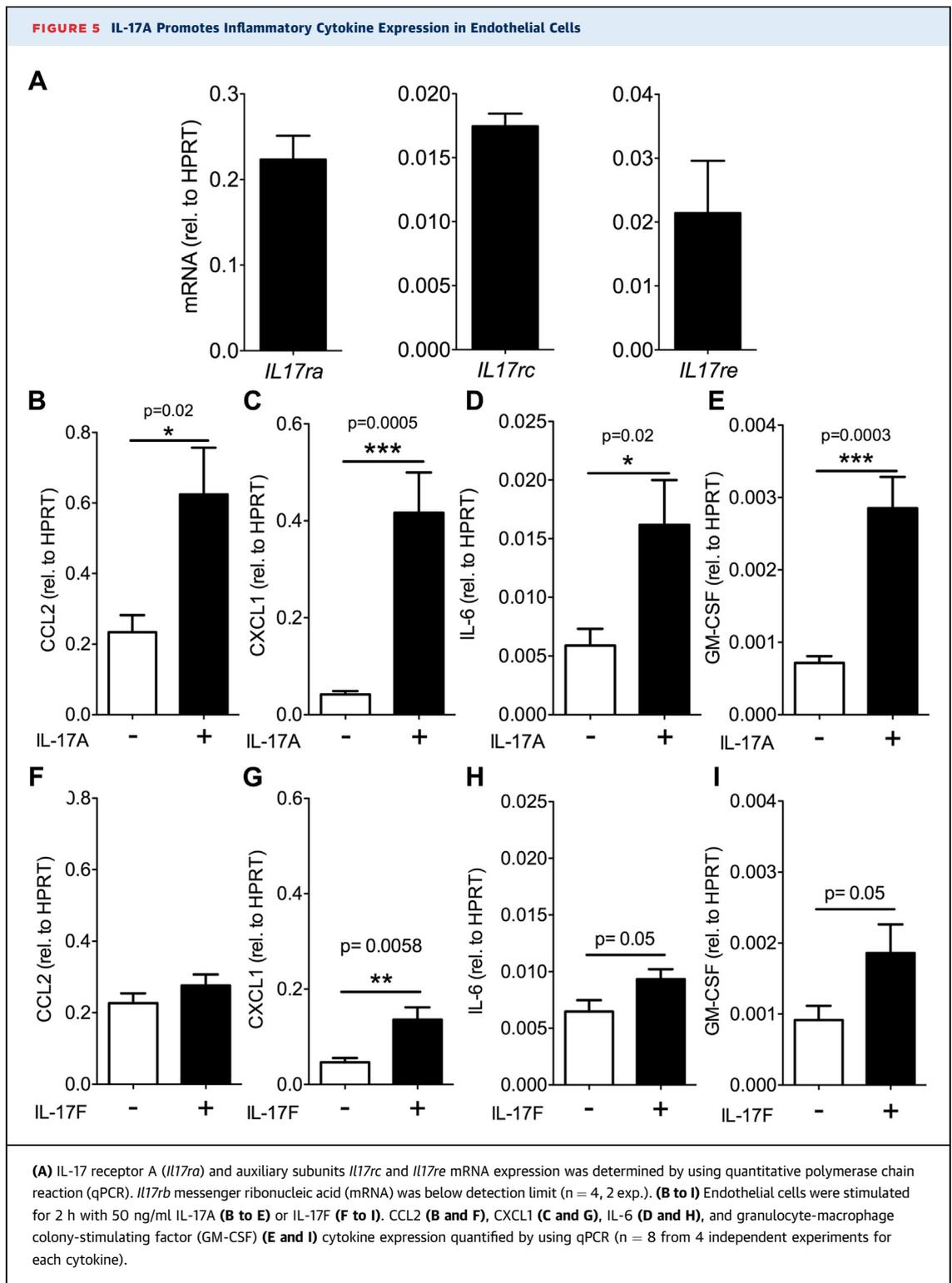
induction by a high-fat diet (Supplemental Figure 8A), a time point when an increase in lesion size and inflammatory cell number in renal impairment was reproducibly observed (10,11). Despite the fact that 4 weeks later, there was still a perceptible increase in lesion size in animals with renal impairment (Supplemental Figure 8B), myeloid cells showed a significant trend toward a decrease after IL-17A ablation (Supplemental Figures 8C and 8D).

IL-17A BLOCKADE NORMALIZES ENHANCED LESION SIZE AND AORTIC INFLAMMATION DESPITE RENAL IMPAIRMENT. As an independent method and possible therapeutic approach of IL-17A antagonism, anti–IL-17A antibody treatment compared with isotype control was used. It was started at the same time point, after 6 weeks of a high-fat diet, and continued for 6 weeks in *LDLr$^{-/-}$* mice with normal and impaired renal function (**Figure 6A**). Supplemental Table 4 illustrates that there was no significant difference between the 4 experimental groups in the following:
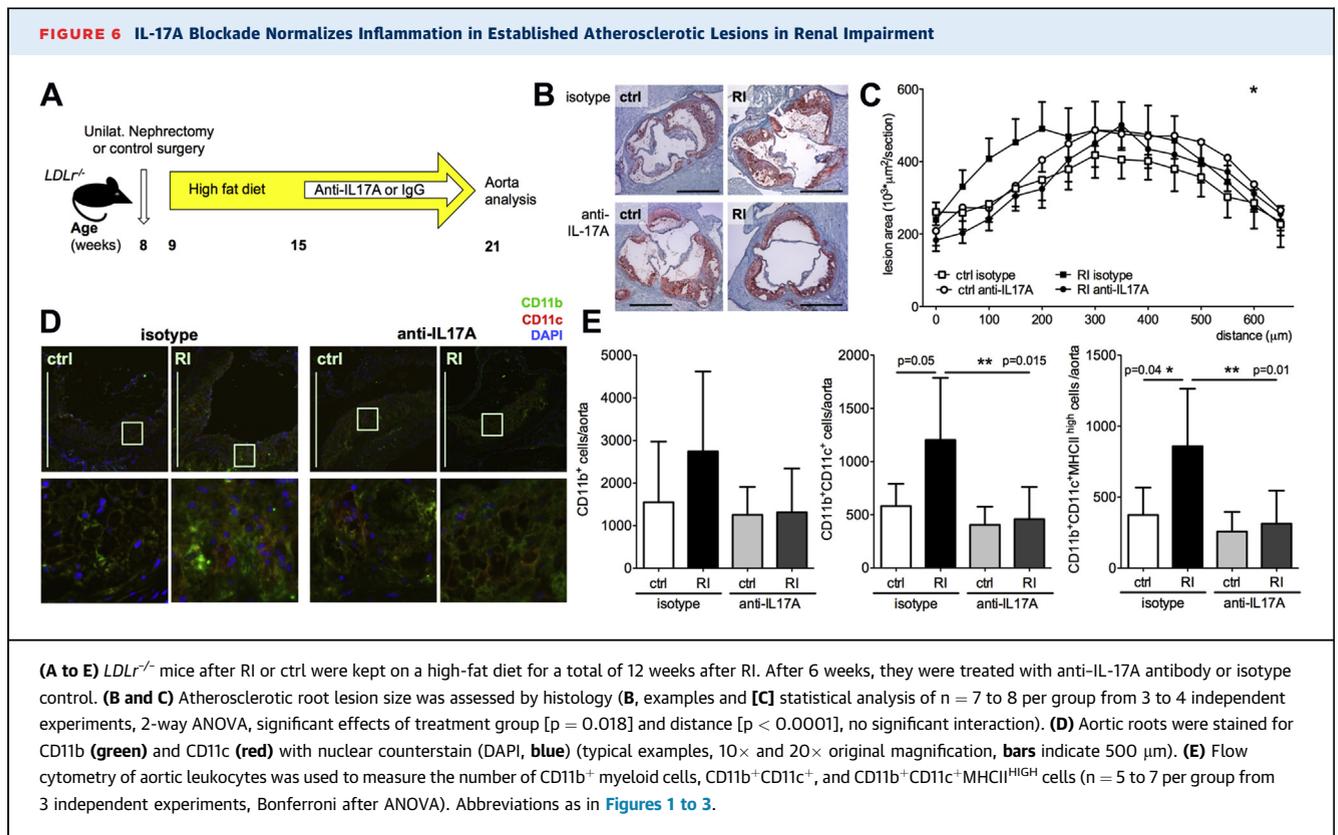
body, heart, or spleen weight; plasma electrolytes; lipids; blood counts; or blood pressure. Remnant kidney weight increased similarly in anti–IL-17A and control IgG–treated groups. Renal function was determined by fluorescein isothiocyanate–sinistrin clearance; it was significantly decreased in the unilateral nephrectomy groups, irrespective of antibody type (Supplemental Figure 9). Peripheral blood and spleen leukocyte composition was not significantly altered (Supplemental Figure 10). With IL-17A blockade, the proportion of splenic IL-17A producers and splenic RORγt mRNA tended to increase (Supplemental Figure 11), whereas GM-CSF as a possible downstream effector was decreased (Supplemental Figure 12).

Atherosclerotic root lesions were far advanced at the end of the experiment (**Figure 6B**). They were significantly larger in mice with renal impairment receiving control isotype IgG compared with all other groups; that is, compared with mice with

FIGURE 5   IL-17A Promotes Inflammatory Cytokine Expression in Endothelial Cells

(A) IL-17 receptor A (*Il17ra*) and auxiliary subunits *Il17rc* and *Il17re* mRNA expression was determined by using quantitative polymerase chain reaction (qPCR). *Il17rb* messenger ribonucleic acid (mRNA) was below detection limit (n = 4, 2 exp.). **(B to I)** Endothelial cells were stimulated for 2 h with 50 ng/ml IL-17A **(B to E)** or IL-17F **(F to I)**. CCL2 **(B and F)**, CXCL1 **(C and G)**, IL-6 **(D and H)**, and granulocyte-macrophage colony-stimulating factor (GM-CSF) **(E and I)** cytokine expression quantified by using qPCR (n = 8 from 4 independent experiments for each cytokine).

normal renal function and mice with renal impairment treated with IL-17A–blocking antibody (**Figure 6C**). Differential histological plaque analysis revealed similar relative distribution of cellular and lipid-rich and fibrotic areas determined by using Masson's trichrome and Picrosirius red stain (Supplemental Figure 13). Staining for α-smooth muscle actin revealed similar contents in the caps of

FIGURE 6 IL-17A Blockade Normalizes Inflammation in Established Atherosclerotic Lesions in Renal Impairment

(A to E) *LDLr⁻/⁻* mice after RI or ctrl were kept on a high-fat diet for a total of 12 weeks after RI. After 6 weeks, they were treated with anti–IL-17A antibody or isotype control. **(B and C)** Atherosclerotic root lesion size was assessed by histology (**B**, examples and **[C]** statistical analysis of n = 7 to 8 per group from 3 to 4 independent experiments, 2-way ANOVA, significant effects of treatment group [p = 0.018] and distance [p < 0.0001], no significant interaction). **(D)** Aortic roots were stained for CD11b **(green)** and CD11c **(red)** with nuclear counterstain (DAPI, **blue**) (typical examples, 10× and 20× original magnification, **bars** indicate 500 µm). **(E)** Flow cytometry of aortic leukocytes was used to measure the number of CD11b⁺ myeloid cells, CD11b⁺CD11c⁺, and CD11b⁺CD11c⁺MHCII^HIGH cells (n = 5 to 7 per group from 3 independent experiments, Bonferroni after ANOVA). Abbreviations as in **Figures 1 to 3**.

the lesions (Supplemental Figure 14). Microscopy after immunofluorescent CD11b and CD11c staining detected myeloid cell accumulation in the lesional intima (**Figure 6D**). The total aortic myeloid cell infiltrate was quantified by using flow cytometry. Both CD11b⁺CD11c⁺ and CD11b⁺CD11c⁺MHCII^HIGH cells significantly increased with renal impairment in the isotype-treated mice. This finding was completely abrogated in mice that received anti-IL-17A during the last 6 weeks of a high-fat diet (**Figure 6E**).

These data show that therapeutic IL-17A blockade is effective in normalization of lesion size and, most notably, inflammatory cell content in established atherosclerotic lesions in renal impairment.

DISCUSSION

Our data show that the IL-17 pathway is required for increased vascular inflammation in both nascent and advanced atherosclerosis renal impairment.

THE IL-17 PATHWAY IN ATHEROSCLEROTIC INFLAMMATION IN NORMAL AND IMPAIRED KIDNEY FUNCTION. IL-17A blockade in established lesions normalized macrophage content in mice with renal impairment. Of note, it did not significantly alter root

lesion size or vascular inflammatory infiltrate in mice with normal kidney function. This finding is consistent with our earlier observation regarding IL-17A ablation before atherosclerosis induction (11). Reported results of IL-17A and IL-17 receptor A blockade or deletion in regard to atherosclerosis development and other types of arterial vascular inflammation (e.g., in aortic aneurysms or hypertension) vary widely (24,27,28,38,39). The *LDLr⁻/⁻* mice investigated here are a less inflammatory atherosclerosis model than *Apoe⁻/⁻* mice and, indeed, effects of IL-17 receptor A deficiency were largely confined to the vessel. We observed that IL-17 receptor A promoted root lesions and en face lesion size in control-operated *LDLr⁻/⁻* mice. This finding is reminiscent of results in *Apoe⁻/⁻* mice (25). It is conceivable that complete receptor deficiency also prevents the signal of other IL-17 isoforms such as IL-17F (38) and thereby has more prominent effects than the absence of 1 ligand. A possible reason for the variable role of IL-17A itself is differential regulation with an increased level in renal impairment (11,19), which is at least partially mediated by angiotensin (11,19). Several recently determined additional regulators of TH17 polarization such as the lipid profile (40-42), salt concentration (43-45), glucose metabolism (46),

and aryl hydroxycarbon receptor agonists (47) are altered in renal impairment in a direction favoring IL-17A. Their individual contributions to the atherosclerosis phenotype remain to be defined.

IL-17 RECEPTOR A FOR PLAQUE COLLAGEN CONTENTS. IL-17A blockade or the absence of IL-17 receptor A did not significantly influence collagen deposition in renal impairment. A decrease in collagen has been described in some (48,49) but not other (33) experimental setups of IL-17A blockade. We observed a decrease only in late atherosclerosis in $Il17ra^{-/-}LDLr^{-/-}$ mice with normal kidney function. However, plaque size was significantly smaller than in wild-type mice at this time point, making direct comparisons difficult. The data in renal impairment, both for $Il17ra^{-/-}LDLr^{-/-}$ and anti–IL17A-treated $LDLr^{-/-}$ mice, offer no indication that plaque stability was not maintained.

VASCULAR EFFECTOR MECHANISMS OF IL-17A. Regarding the type of vascular cell that responds to IL-17A, we found a significant impact of IL-17 receptor A on myeloid cells in both a short-term in vitro model and in identical conditions in mixed bone marrow chimeric mice in vivo. This outcome is consistent with monocyte IL-17 receptor A expression (22,23,39), which was maintained in aortic macrophages in our measurements. However, our data in complete bone marrow chimeric mice support the notion that IL-17 receptor A on both radioresistant and radiosensitive target cells contributes to the severity of atherosclerosis in renal impairment. This result differs from an early publication using a more pro-inflammatory high-fat diet in bone marrow IL-17 receptor A–deficient mice with intact kidneys (39). As a possible mechanism, we found that IL-17A induced inflammatory cytokine expression in the endothelium more than IL-17F in the same concentration. In addition, others have described endothelial cell (25,36,37) or pericyte (50) activation by IL-17A. In the absence of IL-17 receptor A on T cells, increased IL-17A production is found in inflammation, suggesting a short feedback loop (34). Although we did not measure local aortic IL-17A production in the current set of experiments, in mice without IL-17 receptor A on bone marrow-derived cells only, excess IL-17A would have acted on resident cells and thereby may have obscured the role of direct signals on myeloid cells for atherosclerotic inflammation.

CLINICAL APPLICATION OF IL-17A BLOCKADE. Human IL-17 receptor A is central for IL-17A signaling similar to mouse (51). Blocking antibodies for both are now approved for clinical use (52,53). Specific risks and costs continue to be evaluated (54). Clinical trial data in patients with psoriasis (55) and Crohn's disease (56) to date did not detect a signal regarding cardiovascular complications in cohorts with a low event rate. However, long-term follow-up and investigation of their (anti)-atherosclerotic side effects in patients with CKD have not be performed. This approach could be a first step toward a clinical application of our findings.

CONCLUSIONS

We describe a profound anti-inflammatory effect of various anti-IL-17A strategies in enhanced atherosclerosis in renal impairment. The fact that this effect also applies to established lesions may suggest benefit for patients with CKD.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: A moderate decrease in glomerular filtration rate after unilateral nephrectomy significantly increases atherosclerotic lesion size and atherosclerotic vascular inflammation in $LDLr^{-/-}$ mice. IL-17A and its receptor are instrumental in this process and blockade can normalize vascular inflammation even in established disease.

TRANSLATIONAL OUTLOOK: Any anti-inflammatory or immunosuppressive regimen carries an enhanced risk of infection and, possibly, malignancy. Therefore, risks and benefits need to be cautiously considered, especially in a chronic condition such as atherosclerosis. However, many patients with kidney disease require immunosuppression for their underlying kidney disease, a renal (or other) transplant, or other conditions. Thus, optimal tailoring of this therapy may be a first step to addressing enhanced vascular atherosclerotic inflammation in CKD. More specifically regarding IL-17A, subgroup analysis regarding CKD of patients receiving anti-IL17A treatments for other conditions may provide more information regarding whether the murine mechanism also applies to human atherosclerosis.

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KEY WORDS atherosclerosis, chronic kidney disease, interleukin 17, macrophage

APPENDIX For supplemental tables and figures, please see the online version of this paper.