Supplemental Material:

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*$^{-/-}$, *Apoe*$^{-/-}$ mice (both on C57Bl/6 background) (Jackson Labs, Bar Harbor, ME), CD11c^{YFP},[1] kindly provided by Dr. M. Nussenzweig, Rockfeller University, NY and crossed with *Apoe*$^{-/-}$ to obtain double-deficient mice, and mice lacking IL-17A (*Il17a*$^{-/-}$),[2] 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.[3]

Bone marrow transplantation, nephrectomy and measurement of renal function

Lethal irradiations were performed in a ^{137}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*$^{-/-}$ mice was conducted after two weeks when peripheral blood neutrophils have started to recover,[4] high fat diet was started on week later. Injection with DiI-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.[5] Angiotensin II was determined by ELISA (RayBio, Norcross, GA, detection limit: 20pg/ml) according to the manufacturer's description in serum from *Apoe*$^{-/-}$ mice after 12 weeks high fat diet.

Quantification of atherosclerosis and histologic analysis

For en face staining, mice were sacrificed by CO_2 suffocation and aortas were excised, fixed and stained with SudanIV.[6] 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 20x 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.[7, 8] 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_{H0}), in the presence of 16 ng/nl IL-12 (T_{H1}) 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_{H17} polarization as described.[9] 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.[9]

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 erythematodes, 3 IgA nephropathy, 3 FSGS, 2 membranous GN, 2 minimal change GN, 6 other and unknown.

RNA isolation and Real Time PCR

RNA was isolated using NucleoSpin® RNAII Kit (Macherey-Nagel, Duren, Germany) and reversely transcribed with M-MLV-RT (Promega, Mannheim, Germany) according to the manufacturer's instructions. Realtime PCR was performed on a LightCycler480 using Sybr-Green (Roche, Grenzach-Wyhlen, Germany).
Primers were selected using PrimerBank as follows: ABCG1: fw: GCTCCATCGTCTGTACCATCC, rev: ACGCATTGTCCTTGACTTAG, CD36: fw: AGAAGGCGGTAGACCAGAC, rev: GTAGGGGGATTTCTCCTTGGA, ABCA: fw: AAAACCGCAGACATCCTTCAG, rev: CATACCGAAACTCGTTCACCC, SRA: fw: TTCACTGGATGCAATCTCCAAG, rev: CTGGACTTCTGCTGATACTTTGT, T-box 21: fw: CAACAACCCCTTTGCCAAAG, rev: TCCCCCAAGCAGTTGACAGT, GATA3: fw: CTCGGCCATTCGTACATGGAA, rev: GGATACCTCTGCACCGTAGC, RORgt: fw: CCGCTGAGAGGGCTTCAC, rev: TGCAGGAGTAGGCCACATTAC, Foxp3: fw: ACTGGGGTCTTCTCCCTCAA, rev: CGTGGGAAGGTGCAGAGTAG, HPRT: fw: CAGTCCCAGCGTCGTGATTA, rev: AGCAAGTCTTTCAGTCCTGTC. Products were confirmed by melting curve and gel electrophoresis. Transcript levels were normalized to HPRT using the ΔCt method.

Flow cytometry

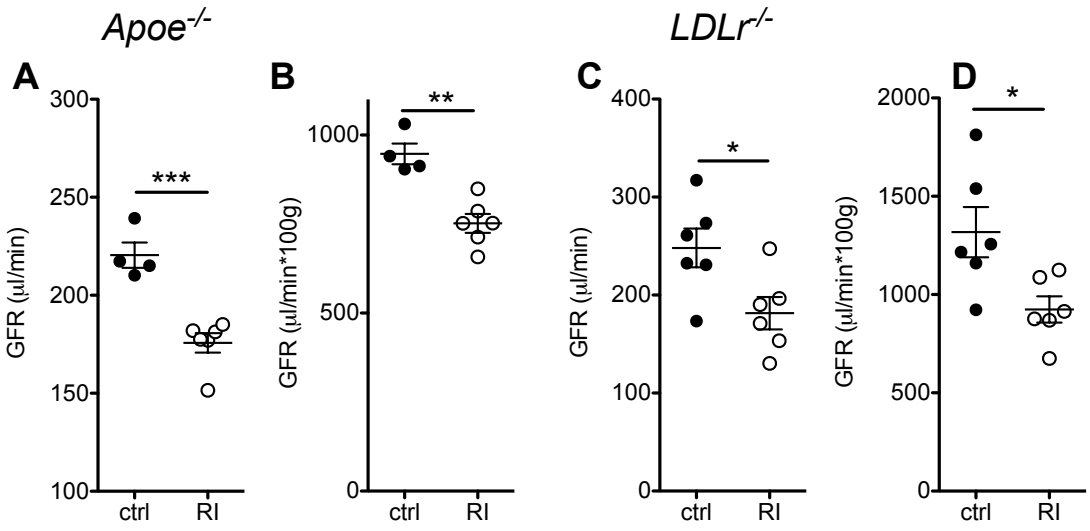
The following antibodies were used: Anti- mouse: anti-CD45 (30-F11) (Becton-Dickinson), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD19 (1D3), anti-IL-17RA (5G4) (eBioscience, San Diego, CA), anti-Foxp3 (150D), anti-IL17A (TC11-18H10.1), anti-IFNγ (XMG1.2), anti-TCRβ (H57-597)(Biolegend, San Diego, 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 Pharmingen, 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.[10] Splenic $CD4^+$ T cells were purified by Robosep negative selection (StemCell Technology, Vancouver, CA), labeled with SNARF (Molecular Probes, 2.5mM) and resuspended at $1.5x10^6$/ml. T cells were incubated with the explanted aortic arch and thoracic aorta of $CD11c^{YFP}$*Apoe*$^{-/-}$ 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_2/5% CO_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.[11] Velocities were observed over the whole time of acquisition to avoid potential bias from manual classification of interacting versus non-interacting cells.

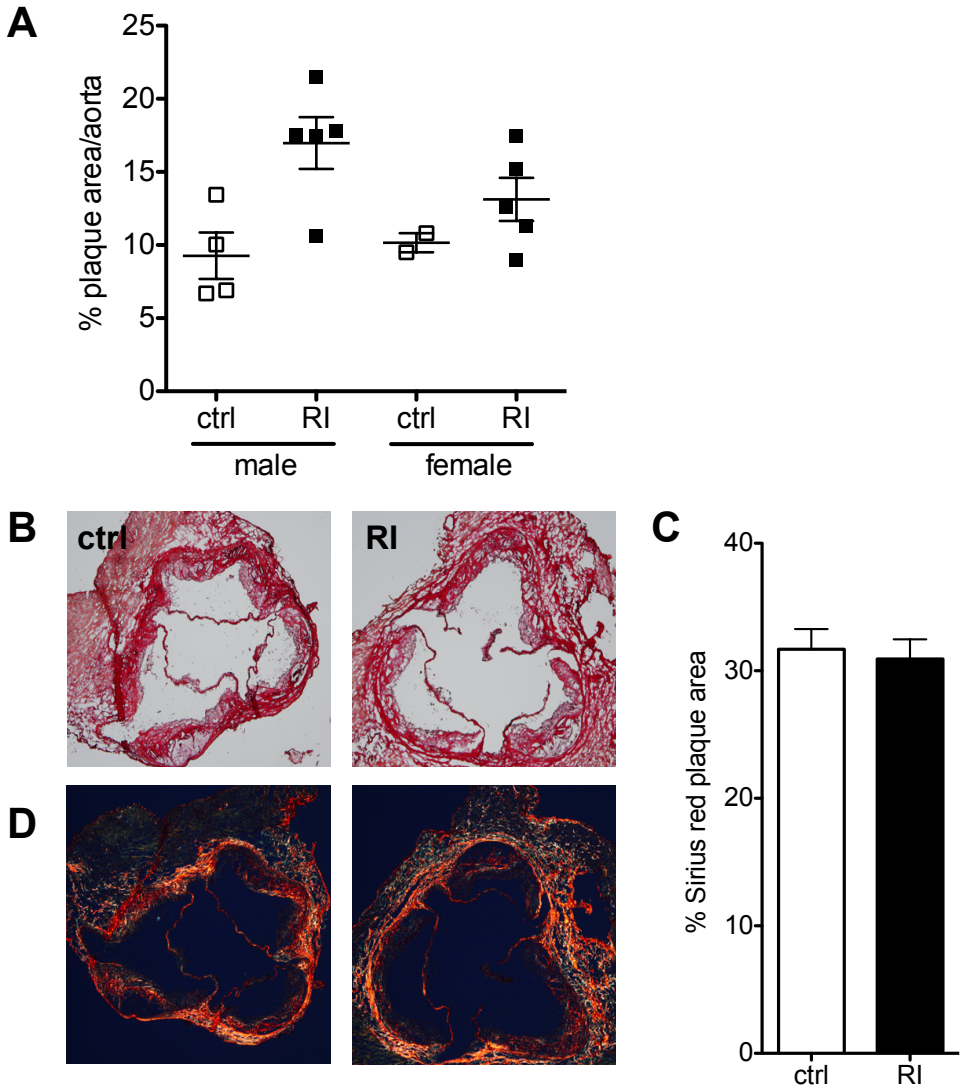
Suppl. figure I



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*$^{-/-}$ and *LDLr*$^{-/-}$ (C,D) mice. Results are given as total GFR (A,C) and per 100g of body weight (B,D) (n= 4-6 *Apoe*$^{-/-}$, n=6 *LDLr*$^{-/-}$ mice, unpaired t-tests).

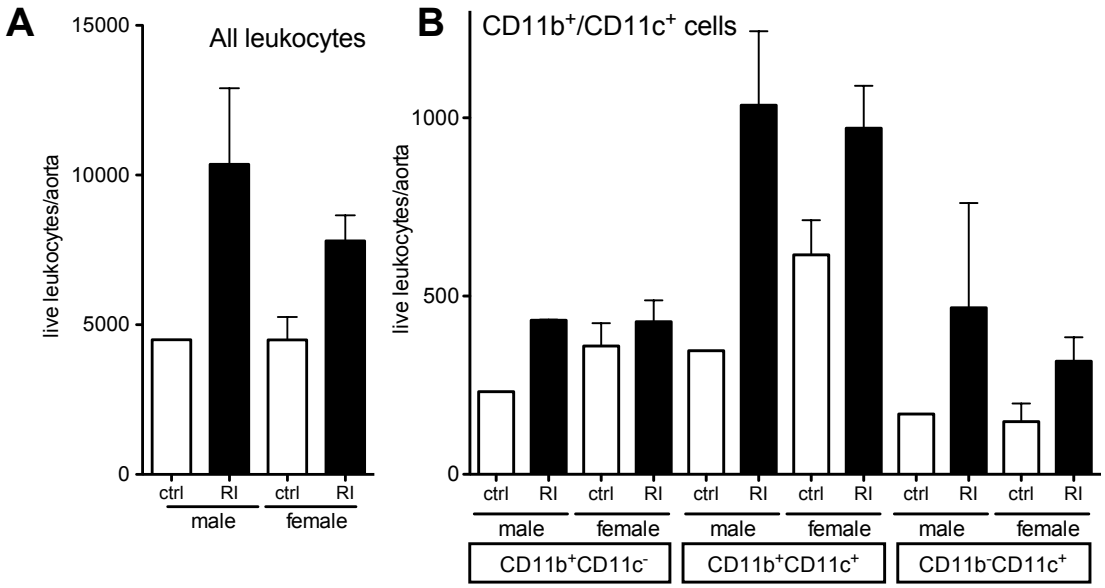
Suppl. figure II



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).

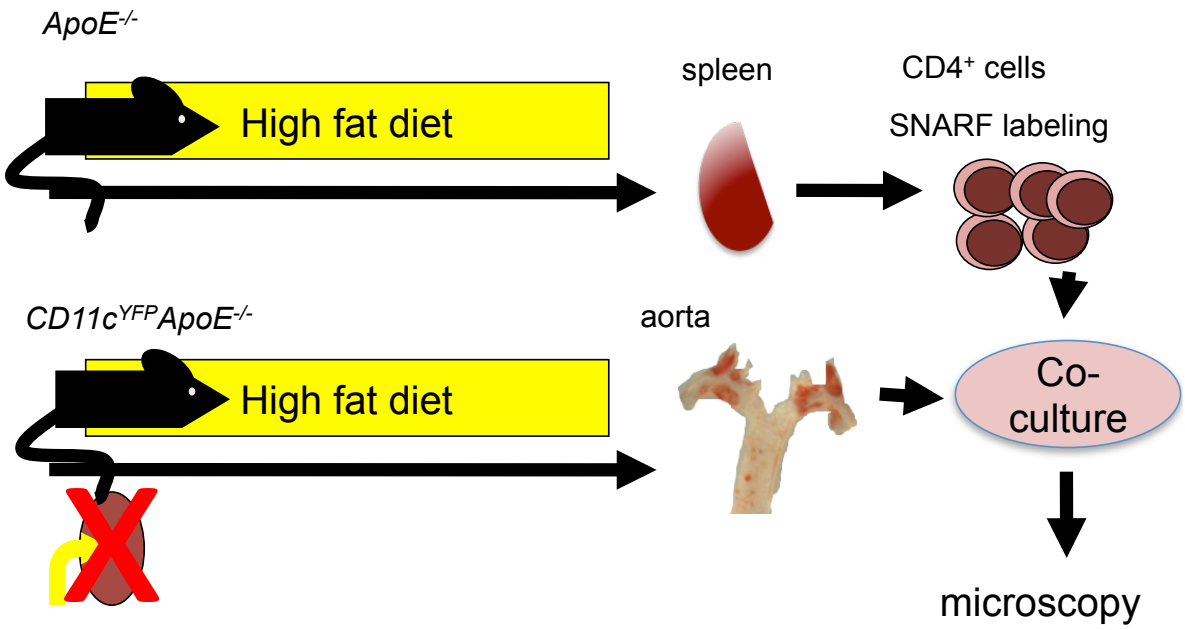
Suppl. figure III



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.

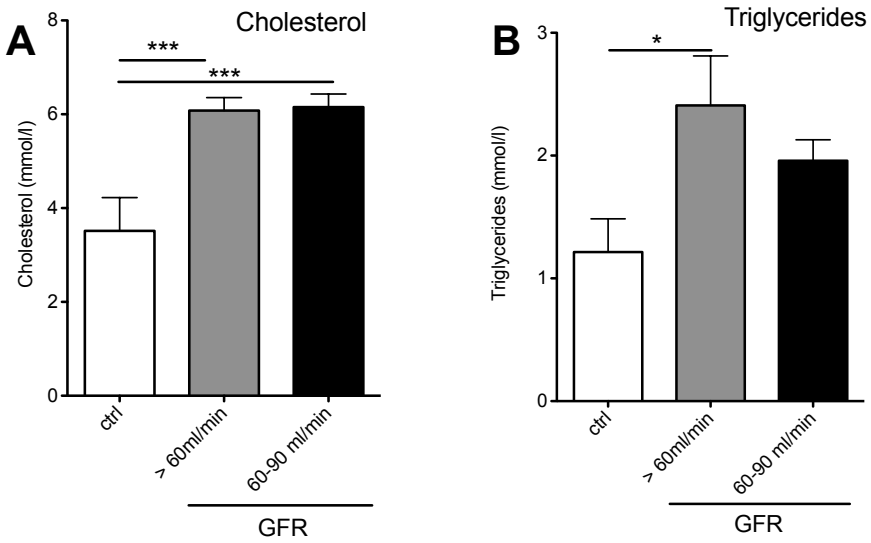
Suppl. figure IV



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 $CD11c^{YFP}$*Apoe*$^{-/-}$ 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% CO_2.

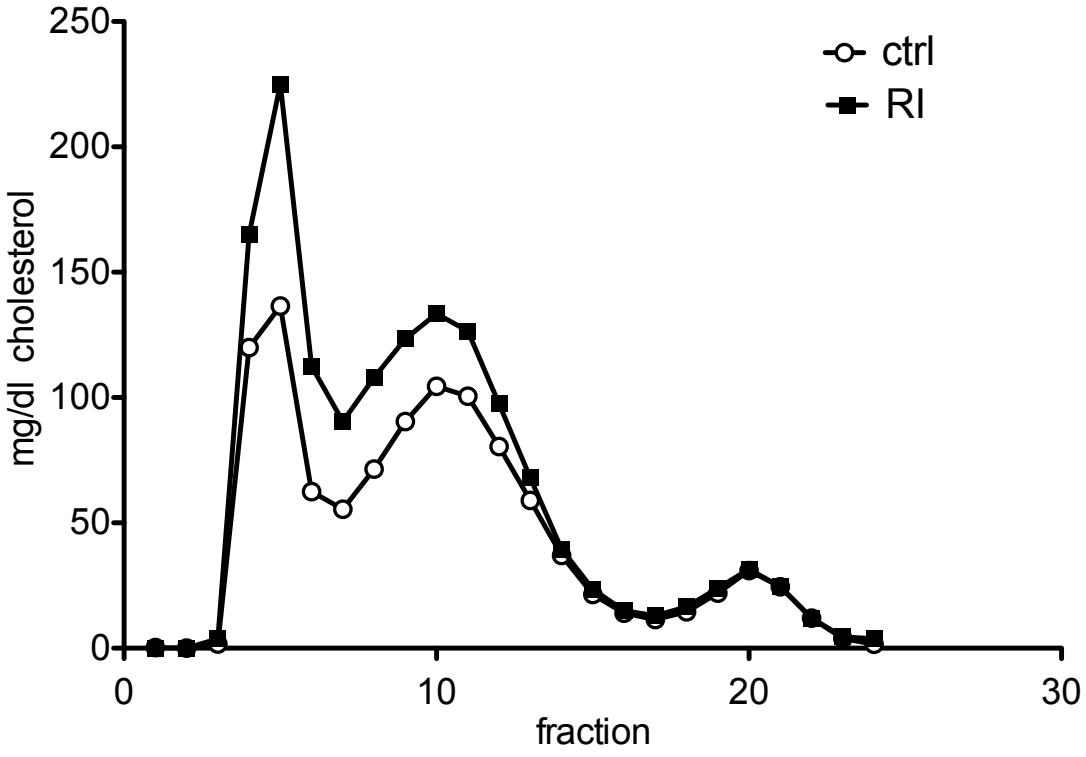
Suppl. figure V



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).

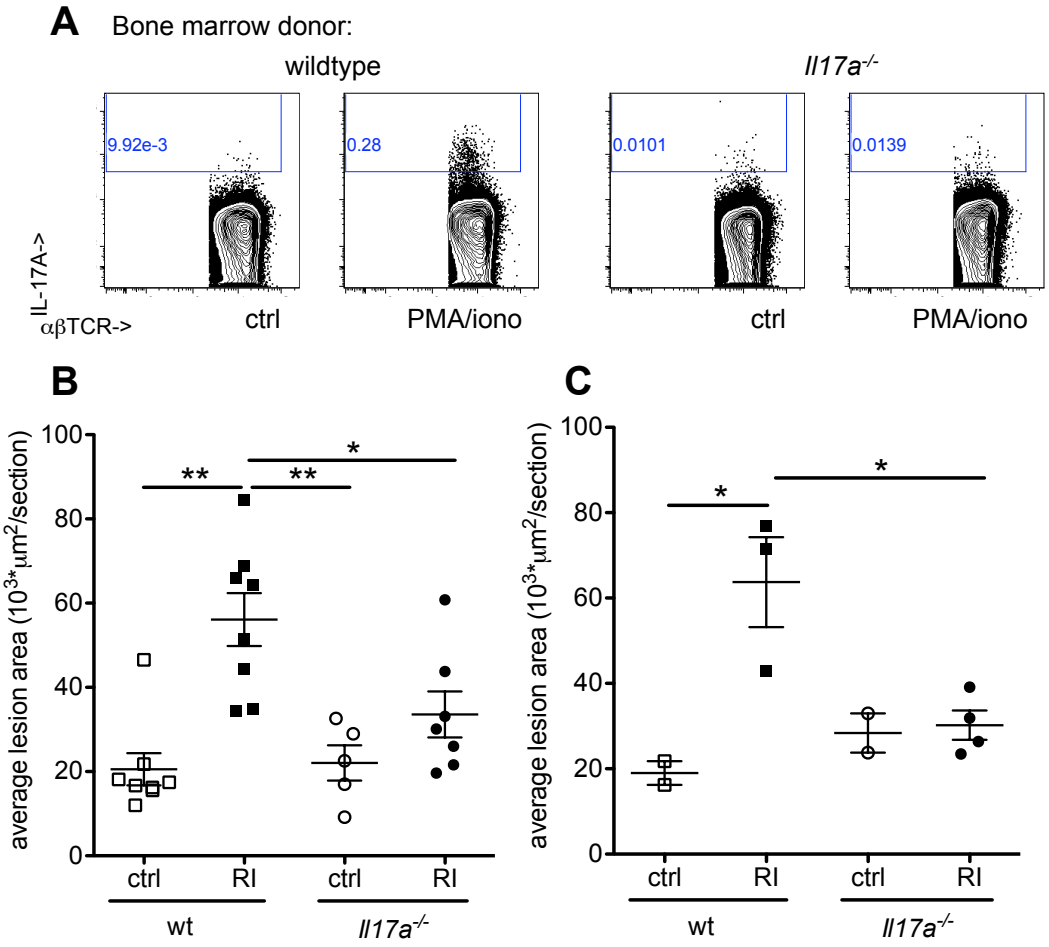
Suppl. figure VI



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.

Suppl. figure VII



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 αβTCR^{+} 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*$^{-/-}$ mice after 12 weeks high fat diet

	control	Renal impairment	p-value
Body weight (g)	29.4±1.7 (13)	26.2±1.1 (20)	0.11
Spleen weight (g)	0.21±0.01 (10)	0.20±0.02 (12)	0.7
Kidney weight (g)	0.19±0.005 (10)	0.22±0.009 (12)	0.007**
Serum creatinine (mg/dl)	0.13±0.01 (6)	0.18±0.01 (8)	0.01**
Serum calcium (mg/dl)	9.2±0.7 (7)	9.8±0.4 (9)	0.48
Serum phosphorus (mg/dl)	8.2±0.5 (7)	8.8±0.5 (9)	0.46
Blood leukocytes (10^3/µl)	8.5±0.48 (15)	9.7±0.28 (21)	0.2
Blood monocytes (/µl)	503±56 (15)	550±33 (21)	0.5
Blood thrombocytes (10^6/µl)	931±71 (15)	1111±126 (21)	0.06
Blood erythrocytes (10^6/µl)	9.1±0.25 (15)	9.3±0.24 (21)	0.27
Total cholesterol (mg/dl)	1251±91 (7)	1642±107 (7)	0.02*
Triglycerides (mg/dl)	131±7 (7)	200±21 (7)	0.01**

Values given as ± SEM (n)

Suppl. table II: Characteristics of *LDLr*$^{-/-}$ mice after 6 weeks high fat diet

	control		Renal impairment		p-value (ctrl vs RI)		p-value (wt vs *Il17a*$^{-/-}$)	
BM genotype	wt	*Il17a*$^{-/-}$	wt	*Il17a*$^{-/-}$	wt	*Il17a*$^{-/-}$	ctrl	RI
Body (g)	18±1(8)	19±1(7)	17±1(10)	20±1 (11)	0.5	0.6	0.4	0.05
Spleen (g)	0.09±0(8)	0.07±0(4)	0.09±0(10)	0.08±0(7)	0.5	0.16	0.3	0.8
Kidney (g)	0.12±0(8)	0.12±0(4)	0.13±0(10)	0.16±0(7)	0.4	0.0	0.9	0.6
Creatinine	0.28±0(7)	0.33±0(4)	0.32±0 (5)	0.34±0(7)	0.5	0.7	0.5	0.5
Leukocytes (10^3/µl)	16±2.2(8)	12±1.6(5)	17±2.1(10)	15±1.7(8)	0.8	0.2	0.2	0.5
Monocytes (10^3/µl)	1.4±0.2(8)	0.9±0.2(5)	1.5±0.2(10)	1.2±0.1 (8)	0.5	0.2	0.13	0.16
Thrombocytes (10^6/µl)	293±64 (8)	364±83 (5)	283±67 (10)	409±71 (8)	0.4	0.6	0.6	0.8
Erythrocytes (10^6/µl)	7.8±1 (8)	8.0±1 (5)	8.2±0 (10)	8.8±1 (8)	0.6	0.5	0.9	0.4
Cholesterol	1436±143 (8)	1477±69 (4)	1549±181 (6)	1622±170 (7)	0.6	0.6	0.9	0.8
Triglycerides	500±65(8)	551±62(4)	570±54(6)	647±81 (7)	0.4	0.5	0.6	0.4

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:

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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