Myeloid CCR2 Promotes Atherosclerosis after AKI

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ABSTRACT

Background. The risk of cardiovascular events rises after AKI. Leukocytes promote atherosclerotic plaque growth and instability. We established a model of enhanced remote atherosclerosis after renal ischemia-reperfusion (IR) injury and investigated the underlying inflammatory mechanisms.

Methods. Atherosclerotic lesions and inflammation were investigated in native and bone marrow–transplanted LDL receptor–deficient (LDLr−/−) mice after unilateral renal IR injury using histology, flow cytometry, and gene expression analysis.

Results. Aortic root atherosclerotic lesions were significantly larger after renal IR injury than in controls. A gene expression screen revealed enrichment for chemokines and their cognate receptors in aortas of IR-injured mice in early atherosclerosis, and of T cell–associated genes in advanced disease. Confocal microscopy revealed increased aortic macrophage proximity to T cells. Differential aortic inflammatory gene regulation in IR-injured mice largely paralleled the pattern in the injured kidney. Single-cell analysis identified renal cell types that produced soluble mediators upregulated in the atherosclerotic aorta. The analysis revealed a marked early increase in Ccl2, which CCR2+ myeloid cells mainly expressed. CCR2 mediated myeloid cell homing to the post-ischemic kidney in a cell-individual manner. Reconstitution with Ccr2−/− bone marrow dampened renal post-ischemic inflammation, reduced aortic Ccl2 and inflammatory macrophage marker CD11c, and abrogated excess aortic atherosclerotic plaque formation after renal IR.

Conclusions. Our data introduce an experimental model of remote proatherogenic effects of renal IR and delineate myeloid CCR2 signaling as a mechanistic requirement. Monocytes should be considered as mobile mediators when addressing systemic vascular sequelae of kidney injury.

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AKI frequently affects patients who are critically ill, especially in the intensive care setting. For the majority of patients, renal function recovers and patients require RRT only briefly, if at all. However, AKI significantly increases all-cause mortality. Remote adverse effects influence a range of organs.1,2

An elevated cardiovascular risk after survived AKI was observed in meta-analyses including >50,000 patients.3–5 This is supported further by a more recently reported large cohort.6 The effect
of CKD on cardiovascular disease is clearly established.\textsuperscript{7–9} Studies consistently demonstrate an association of CKD with cardiovascular disease after correction for common risk factors at an eGFR of ≤60 ml/min.\textsuperscript{10} In the setting of AKI, stratification for severity and common risk factors appears to be more challenging than in CKD, given the smaller patient numbers and frequently incomplete follow-up data. In addition, the definition of AKI includes patients with a functional decrease in urine output and those with structural renal injury. To distinguish these entities can be clinically challenging, especially at mild disease stages. Animal models were developed to investigate remote AKI effects and underlying mechanisms in lung, brain, gut, and granulopoiesis.\textsuperscript{2,11–14} Such a model is also required for systematic evaluation of atherosclerosis after AKI.

Inflammatory leukocytes are central in atherosclerosis initiation and progression.\textsuperscript{15} Arterial wall leukocyte numbers increase in patients with CKD stages 3–4.\textsuperscript{16–18} Also, in murine atherosclerosis models, moderate reduction of kidney function by unilateral nephrectomy enhanced atherosclerotic inflammation.\textsuperscript{19–22} Recent clinical trials demonstrate that patients with CKD receiving anti–IL-1 and anti–IL-6 anti-cytokine therapy experience fewer cardiovascular events.\textsuperscript{23,24} AKI not only reduces renal function, but also leaves an injured kidney in place. Renal inflammation can be detected for at least a year after a single episode of ischemia-reperfusion (IR) injury.\textsuperscript{25} Whether an injured kidney modulates atherosclerotic inflammation and specific soluble or cellular mediators has not been addressed.

Monocytes are precursors of atherosclerotic plaque macrophages and myeloid antigen-presenting cells.\textsuperscript{15} The C-C motif ligand 2 (CCL2) receptor (CCR2) is highly expressed by classic, so-called inflammatory monocytes in mice and humans. It mediates their liberation from the bone marrow.\textsuperscript{26} CCL2 levels were elevated in a cohort of 3200 patients with CKD, who were investigated as part of the Dallas Heart Study, and correlated with cardiovascular death, also after correction of multiple risk factors.\textsuperscript{27} CCR2 expression on circulating monocytes correlated with human aortic wall glucose uptake, an in vivo marker of inflammation.\textsuperscript{28} Experimental pharmacologic CCL2 inhibition was only moderately beneficial in some models of severe atherosclerosis without kidney injury.\textsuperscript{29–31} The mechanistic role of CCL2 in enhanced atherosclerosis in CKD or after AKI has not been delineated.

Renal CCL2 expression rises in a multitude of kidney diseases.\textsuperscript{32} CCR2-deficient mice were protected from renal damage and inflammation after ischemia and reperfusion.\textsuperscript{33,34} Similar results were obtained using pharmacologic receptor blockers. Indeed, tubular CCL2 correlated with monocyte infiltration after renal ischemia reperfusion.\textsuperscript{35} However, the concept of detrimental renal inflammation via CCL2 was challenged by excess mortality in CCL2-deficient mice after bilateral renal IR.\textsuperscript{36} As a potential underlying mechanism, increased tubular apoptosis and more inflammatory, instead of reparative, macrophages were reported. In our recent work, we delineated differential effects of tubular and monocyte CCL2.\textsuperscript{37} Tubular CCL2 was required for renal healing after injury. How the monocyte CCR2 signal affects long-term outcome, both locally in the kidney and remotely in the atherosclerotic aorta, has not been determined.

Here, we introduce a murine model of increased atherosclerotic plaque load after a single episode of unilateral AKI. Our results show a mechanistic role for bone marrow–derived CCR2\textsuperscript{2+} cells in enhanced atherosclerotic plaque size and aortic inflammation after kidney injury, without discernible effects on renal outcome. These data introduce a concept of remote inflammation for aggravation of atherosclerosis after kidney injury.

**METHODS**

**Animals**

Male wild-type (CD45.1 or CD45.2), LDL receptor–deficient (LDLr\textsuperscript{−/−}), and Ccr2\textsuperscript{−/−} mice (both CD45.2, all on C57Bl/6 background; Jackson Laboratories, Bar Harbor, ME) were genotyped by PCR, used in age-matched groups, and kept in specific-pathogen-free conditions. Animal experiments were approved by Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Lower Saxony, Germany) in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mice were kept on high-fat diet (Harlan Teklad 88137) for indicated periods of time. Euthanasia was by isoflurane overdose (5%) and ensured by cervical dislocation.

**Bone Marrow Transplantation and Kidney Surgery**

Mice were reconstituted with unfractionated bone marrow after lethal irradiation in a caesium-137 irradiator. For kidney surgery, mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg), and atropine (0.025 mg/kg). The vessels of the left kidney were clamped for 27 minutes, taking care to avoid damage of the adrenal gland and surrounding organs. Control mice

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**Significance Statement**

AKI impairs excretory function, but also leaves a damaged kidney. We demonstrate that a single episode of unilateral ischemia-reperfusion injury significantly promotes atherosclerotic plaque formation in mice. Renal inflammation preceded expression of myeloid and T cell genes in the atherosclerotic aorta. The chemokine receptor CCR2 was instrumental in inflammatory monocyte recruitment to the kidney, renal and aortic inflammatory macrophage marker CD11c expression, and enhanced aortic plaque formation after AKI. Delineating underlying mechanisms in atherosclerosis support the concept of a “toxic kidney” that promotes remote inflammation after ischemic reperfusion injury.
Figure 1. Renal IR injury increases atherosclerotic lesion size and inflammation. (A–G) Male LDLr−/− mice were subjected to unilateral renal IR injury. (A) One week later, mice started being fed a high-fat diet for induction of atherosclerotic lesions. (B) Aortic...
received an identical abdominal incision and brief handling of the kidney. Postoperative analgesia was with subcutaneous metamizole, as needed. Kidney surgeries were performed 2 weeks after bone marrow transplantation.

Serum urea, creatinine, electrolytes, and lipids were measured in an Olympus AU400 Chemistry Immuno Analyzer (Olympus, Hamburg, Germany), and full blood counts were assessed in an automated analyzer (VetABC; ScilVet, Viernheim, Germany).

Histology and Flow Cytometry
Atherosclerotic lesion size was assessed in frozen 5-μm sections, obtained in 50-μm intervals, stained with Oil Red O staining with hematoxylin and light green counterstain, as described. Extent of renal damage was determined as proportion of intact tubuli in cortex and outer stripe of outer medulla in hematoxylin and eosin–stained whole kidney images. Image analysis was conducted in GIMP (version 8). Antibodies, flow cytometry, and microscopy equipment are detailed in the Supplemental Appendix.

RNA Isolation, Quantitative PCR, and Microarray-Based mRNA Expression Analysis
RNA isolation, primer sequences, microarray specifications (ID026652; Agilent Technologies), and tissue preparation for microarray and single-cell analysis methods are detailed in the Supplemental Appendix.

For analysis of aortic gene expression, aortas of three male mice per condition were pooled. Immune genes (ILs and IL receptors, chemokines, integrins, and CD molecules) with a baseline above detection limit were analyzed. For string analysis (https://string-db.org), all genes upregulated at least two-fold in aortas from IR mice after 3 or 10 weeks of a high-fat diet, compared with 3- or 10-week control aortas, are shown. Interactions and similarities are indicated as follows: cyan for curated databases, pink for experiments, green for gene neighborhood, light green for text mining, and black for coexpression. Long-term renal post-ischemic gene expression was extracted from Lui et al. (n=3–4 per time point).

Renal single-cell sequencing data from the kidneys of three male C57Bl/6 mice 7 days after unilateral renal IR and control mice without surgery were analyzed for target gene expression in cell types identified using Uniform Manifold Approximation and Projection. We analyzed renal RNA sequencing data after IR and in controls from a public database and IR kidneys from male LDLr⁻/⁻ mice reconstituted with wild-type and Ccr2⁻/⁻ bone marrow after 3 weeks on a high-fat diet. In mice reconstituted with wild-type versus Ccr2⁻/⁻ bone marrow, regulated gene groups among ≥1.5-fold upregulated and ≥0.75-fold downregulated genes were identified using the PANTHER database (annotation dataset: complete Gene Ontology biological processes).

Statistical Analysis
For continuous biologic variables, a normal distribution was assumed because most follow a Gaussian distribution. Statistical analysis was conducted using GraphPad Prism (version 8). Two-tailed t test with a Welch correction in case of unequal variance was used to compare two conditions. If more than two conditions were compared, the Sidak or Dunnett test of selected conditions was applied after ANOVA. Data are expressed as mean±SEM. P values <0.05 were considered significant and are indicated as *P<0.05, **P<0.01, and ***P<0.001.

RESULTS
Renal IR Injury Promotes Atherosclerotic Plaque Development
To investigate the effect of a damaged kidney on atherosclerosis development, we subjected LDLr⁻/⁻ mice to unilateral renal IR injury (Figure 1A). After 10 weeks on a high-fat diet for induction of atherosclerotic lesions, animals were euthanized. Body and spleen weights did not differ significantly between the groups (Supplemental Table 1). After IR, the contralateral kidney expectedly hypertrophied. Serum levels of the major proatherogenic factors, triglycerides, cholesterol, and phosphate did not differ. Serum urea, but not creatinine, significantly increased, consistent with a mildly impaired excretory renal function. Differential blood counts were unaffected by the procedure.

Atherosclerotic lesion size was assessed in the aortic root (Figure 1B). Lesion size was significantly larger in mice
Figure 2. Parallels in aortic and renal post-ischemic inflammatory gene expression. (A–C) Differentially expressed immune genes in atherosclerotic aortas from mice after renal IR and controls (c or ctrl.) were grouped in genes upregulated two-fold or more only
after AKI by unilateral IR than in control-operated mice. Confocal microscopy after immunostaining revealed marked accumulation of CD11b⁺ myeloid cells expressing the inflammatory macrophage and antigen-presenting cell marker CD11c in the plaques after IR (Figure 1C, specificity controls in Supplemental Figure 1A). These data demonstrate enhanced atherosclerosis in animals with preceding renal injury.

**Upregulation of Aortic Chemokine and T Cell Genes after Renal IR Injury**

To delineate differentially regulated inflammatory pathways after AKI, we analyzed atherosclerotic aorta by gene array. Aortas were harvested early and late during atherosclerosis development (i.e., after 3 and 10 weeks on a high-fat diet). Similar to the later time point (Supplemental Table 1), mice at the early stage showed contralateral renal hypertrophy, elevated urea, nonsignificant changes in creatinine, and no significant alterations in lipid levels or complete blood counts (Supplemental Table 2).

Inflammatory gene expression in the atherosclerotic aortas after AKI was compared with otherwise identically treated animals after control surgery. String analysis revealed upregulation of chemokines and their cognate receptors in early atherosclerosis (Figure 1D). In established plaques, in addition to chemokine pathways, T cell genes were upregulated (Figure 2A). Indeed, confocal imaging and three-dimensional reconstruction of the plaque revealed close spatial proximity of CD3⁺ T cells and F4/80⁺ macrophages in the established aortic root plaques in mice after an AKI episode (Figure 1, F and G). This was not observed in control animals.

This analysis shows upregulation of chemokine, followed by T cell pathways in response to AKI in the atherosclerotic aorta.

**Parallel Regulation of Inflammatory Genes in the Atherosclerotic Aorta and the Post-Ischemic Kidney**

To identify candidate pathophysiologic links between renal post-ischemic and aortic atherosclerotic inflammation, we compared differentially regulated aortic inflammatory genes with a dataset of longitudinal measurements in the post-ischemic kidney.25

Genes were grouped according to time of their regulation during atherosclerosis development. Two-fold or more relative upregulation in aortas from AKI versus control-operated LDLr⁻/⁻ mice only in early (Figure 2A) or only in established atherosclerosis (Figure 2B) is shown. These patterns were reflected in the gene regulatory pattern in the post-ischemic kidney (Figure 2, D and E) by upregulation either days (Figure 2D) or weeks (Figure 2E) after AKI. In many cases, elevated expression was sustained even a year after injury. A relatively large number of aortic inflammatory genes were upregulated both by progression of atherosclerosis and after AKI at each time point (Figure 2C). In most cases, they belong to the group affected late in the postischemic kidney and sustained their elevated expression long term (Figure 2F). We also searched for aortic genes regulated after AKI without overall change during atherosclerosis progression. Up- and downregulated genes were identified. These patterns were, however, not reflected by their renal expression (Supplemental Figure 2).

Parallel regulation of inflammatory genes in the atherosclerotic aorta of mice after AKI and in the kidney itself proposes an effect of renal inflammation on the vessel.

**Renal Myeloid Cells Upregulate Inflammatory Mediators That Increase in the Atherosclerotic Aorta**

To determine how renal inflammation remotely affects the atherosclerotic aorta, we investigated which cells in the postischemic kidney expressed the soluble mediators markedly upregulated specifically in early atherosclerosis of mice after AKI (Figure 2A). Single-cell sequencing was used to define individual cell types. Expectedly, the number of myeloid inflammatory cells in the post-ischemic kidney increased 7 days after AKI (Figure 3A). Expression of the aortic regulated genes Cxcl3, Il24, Il1a, Cxcl1, Ccl2, and Cxcl17 was analyzed in different cell types (Figure 3B). Il19 was below the detection limit. Expression of these soluble inflammatory mediators was highest in leukocytes. Ccl2 increased most markedly (Figure 3B). Most CCL2-expressing cells also expressed its receptor CCR2 (Figure 3C).

These data show a significant role of CCR2⁺ myeloid cells in production of their ligand, CCL2, in the post-ischemic kidney.
gating in Supplemental Figure 3). This was reflected by myeloid cell infiltration in confocal imaging (Figure 4D, specificity controls in Supplemental Figure 1B).

To investigate whether CCR2 was required for leukocyte accumulation after renal IR injury, we generated mixed chimeras in LDLr\(^{-/-}\) mice using wild-type and Ccr2\(^{-/-}\) bone marrow (Figure 4E, Supplemental Figure 4). Genotypes were distinguished by the syngenic markers CD45.1 and CD45.2. This approach allowed us to study the homing of individual cells with and without CCR2 in an identical milieu, including the presence of possible downstream mediators. Our results after 3 weeks of a high-fat diet show that CCR2 conferred an advantage in myeloid cell accumulation in the post-ischemic kidney, compared with blood, the contralateral kidney and spleen, and also the aorta (Figure 4F). This was not observed in mice that underwent control surgery and an otherwise identical treatment and demonstrates CCR2 promotes myeloid cell accumulation in a cell-individual manner after renal IR injury.

Myeloid CCR2 Promotes Leukocyte Infiltration and Inflammatory Gene Expression in the Injured Kidney

To study downstream effects of CCR2-mediated renal leukocyte accumulation, we generated complete bone marrow chimeras in LDLr\(^{-/-}\) mice with 100% wild-type or Ccr2\(^{-/-}\) bone marrow (Figure 5A). The latter ablated renal Ccr2 expression, demonstrating highly effective replacement of recipient cells (Supplemental Figure 5). Expectedly, less monocytes were observed in blood in the absence of CCR2 (Supplemental Tables 3 and 4). Differential histologic assessment after 3 and 10 weeks of a high-fat diet revealed no difference in the proportion of intact tubuli (Supplemental Figures 6 and 7), consistent with very similar functional parameters (Supplemental Tables 3 and 4).

Renal leukocyte infiltration was characterized by flow cytometry and compared with otherwise identically treated mice reconstituted with wild-type bone marrow. IR significantly enhanced leukocyte infiltration in both genotypes early and late after injury (Figure 5, B and C). At the early
Figure 4. CCR2 is persistently upregulated in the post-ischemic kidney and promotes macrophage accumulation in a cell-specific manner. (A–C) In male LDLr<sup>−/−</sup> mice after IR and controls (ctrl.), followed by 3 and 10 weeks of a high-fat diet as indicated,
time point, less monocyte infiltration and a trend toward less CD11b+ mononuclear myeloid infiltration was detected in mice reconstituted with Ccr2−/− bone marrow, consistent with the situation in mixed chimeras (Figure 4(F)).

To address secondary renal mediators with a potential effect on systemic atherosclerosis development, gene expression analysis was performed after 3 weeks of atherosclerosis induction. Genes relatively increased in the post-ischemic kidneys of LDLr−/− mice with wild-type bone marrow were enriched for immune system and inflammatory pathways (Figure 5D). We next investigated regulation of aortic inflammatory gene groups with differential regulatory patterns depicted in Figure 2 in the kidney. In the presence of wild-type bone marrow, a number of genes were overexpressed that had been found to be upregulated in the atherosclerotic aortas of IR mice at the early and the late time point, and to also rise continuously with atherosclerosis development (Figures 2, C and F, and 5E), but not in the other patterns. Significant upregulation was found for Itgax, which codes for CD11c, Cxcr4, Ccl6, Ccl17, and Cd101 within this gene group.

In summary, myeloid CCR2 upregulates an inflammatory transcriptome in the post-ischemic kidney in proatherogenic conditions.

**Myeloid CCR2 Mediates Excess Atherosclerosis after AKI**

To test for a mechanistic role of CCR2 in excess aortic plaque growth, lesions were analyzed in LDLr−/− mice lethally irradiated and reconstituted with either wild-type or Ccr2−/− bone marrow before AKI after 10 weeks of a high-fat diet (Figure 6A).

Aortic root lesion size significantly increased after IR in mice reconstituted with wild-type bone marrow (Figure 6B). This is in line with our results in native LDLr−/− mice (Figure 1). The absence of myeloid CCR2 completely abrogated the enhancement of atherosclerosis after AKI. AKI significantly promoted aortic expression of Ccr2 and its ligand, Ccl2, in LDLr−/− mice reconstituted with wild-type bone marrow (Figure 6, C and D). After reconstitution with CCR2-deficient bone marrow, aortic CCR2 expression remained below the detection limit, consistent with the results in the kidney (Supplemental Figure 5), indicating highly effective reconstitution. The increase of aortic Ccl2 after AKI was abrogated in the absence of bone marrow CCR2 (Figure 6D).

Also, aortic CD11c mRNA was depressed in the absence of CCR2 (Figure 6E). We used confocal microscopy to further investigate immune cells in the atherosclerotic plaques. Less CD11b+CD11c+ macrophages and less spatial proximity of CD3+ T cells and F4/80+ macrophages were observed in the absence of bone marrow CCR2 (Figure 6, F and G).

These experiments determine a mechanistic role for myeloid CCR2 in enhanced atherosclerosis and aortic CD11c expression after renal ischemic injury.

**DISCUSSION**

Here, we describe a novel in vivo model of remote atherosclerosis enhancement after AKI. Our data identify myeloid CCR2 signaling as a mechanistic requirement.

Clinical data indicate an elevated cardiovascular risk during follow-up after AKI.3–6 In our experiments, unilateral renal IR injury significantly enhanced atherosclerotic lesion size in LDLr−/− mice. This occurred despite an only moderate increase in serum urea and no significant change in serum creatinine (i.e., without a major loss of excretory kidney function). Our data demonstrate ischemic AKI causes a parallel rise of inflammatory mediators in the post-ischemic kidney and the atherosclerotic aorta. The clinical definition of AKI as decreased urine output and rise in serum creatinine includes a physiologic response to prerenal volume depletion without structural renal damage and acute tubular injury. There is a dichotomy in renal gene regulation in these conditions: although IR injury promoted renal inflammatory gene expression, renal response to volume depletion suppressed it.27 In clinical practice, these entities are not always clearly separated, especially in less severe forms. Underlying pathology needs to be considered when evaluating the effect of AKI on patients’ cardiovascular outcomes.3–5

Ablation of the CCL2/MCP-1 receptor CCR2 on myeloid cells prevented aggravation of atherosclerosis after AKI.
Figure 5. CCR2 promotes leukocyte infiltration and inflammation after renal IR injury. (A–E) Male LDLr−/− mice underwent renal IR or control (ctrl.) surgery after reconstitution with either wild-type (wt) or Ccr2−/− bone marrow (BM), as depicted in (A). (B and C) Renal leukocytes were assessed by flow cytometry after 3 (B) and 10 (C) weeks of atherosclerosis induction by a high-fat diet. Proportions of live leukocytes, non-neutrophil CD11b+ myeloid cells, and monocytes among all cells are shown (gating strategy in Supplemental Figure 3). n=4–6 mice per group from five independent experiments in (B), n=5–11 mice per group from eight independent experiments in (C), Dunnett test after ANOVA. (D and E) Differentially regulated renal genes after reconstitution with wild-type or Ccr2−/− bone marrow and renal IR in in LDLr−/− mice were studied by microarray after 3 weeks on a high-fat diet (n=4 mice per group). (D) Functional gene groups that were significantly upregulated in kidneys in the presence of wild-type bone marrow (Fisher exact test, false discovery rate of <0.05). (E) Volcano plot of gene regulation in IR kidneys of wild-type versus Ccr2−/− bone marrow recipients. Cutoffs of ≥1.5 times up- or ≥0.75 times downregulation expressed as −log2(fold change [wild type/Ccr2−/−]) relative to −log2(fold change [Ccr2(wild type)/Ccr2−/−]) is shown. t test. Inflammatory genes continuously upregulated in the atherosclerotic aorta (Figure 2C) are marked in red; significantly regulated genes are annotated. betw., between; biol., biologic; BmTx, bone marrow transplantation; con., contralateral kidney; GO, Gene Ontology; inv., involved; org., organisms; proc., process. *P<0.05, **P<0.01, and ***P<0.001.

in our experiments. Despite encouraging early results of reduced atherosclerotic lesion size in CCR2-deficient mice, only some pharmacologic interventions decreased atherosclerotic plaque size in the absence of kidney injury. Consistently in our experimental setup, reduction of plaque size by the deletion of bone marrow CCR2 remained non-significant in the absence of kidney injury. This may suggest that this receptor’s proatherosclerotic function is
Figure 6. Myeloid CCR2 is required for enhanced atherosclerotic plaque formation after AKI. (A–G) Male LDLr<sup>−/−</sup> mice were lethally irradiated and reconstituted with either wild-type (wt) or Ccr2<sup>−/−</sup> bone marrow (BM) before renal IR or control (ctrl.) surgery.
exacerbated in the presence of local inflammation, as in our data for AKI and possibly beyond. Our results add a mechanism to the correlation of systemically elevated CCL2 in CKD with cardiovascular death, which remained stable after correction of multiple risk factors, and the fact that CCR2 expression associated with vascular inflammation in humans with excess risk, including CKD. Our results suggest CCR2 inhibition should be tested as an anti-inflammatory intervention targeted to this patient group. Timing and duration will need to be carefully evaluated, given the long-term kinetics of atherosclerotic lesion formation.

Myeloid CCR2 significantly promoted renal monocyte recruitment in response to IR and was required for enhanced atherosclerotic lesion size. Renal, but not aortic, cell recruitment was mediated on an individual monocyte level by CCR2. Our results rather propose that separate secondary mediators are instrumental for aortic myeloid cell accumulation. Indeed, differential recruitment of inflammatory cells by CCR2 and CCR2 macrophages has recently been observed after myocardial injury. Underlying downstream mechanisms are elusive as yet. We started to address these mechanisms and found upregulation of a range of genes that also increase in the atherosclerotic aorta, including antigen-presenting cell marker CD11c, in agreement with kidneys of completely CCR2-deficient mice. Our data are consistent with a model in which CCR2 promotes monocyte passage into a pathologically altered kidney, which, in turn, modulates secondary cellular or soluble mediators in a proatherogenic way that results in excess atherosclerotic lesion size. Whether this or similar mechanisms also apply to other types of kidney injury, such as drug toxicity or glomerular diseases, remains to be investigated.

Regarding the kidney itself, CCL2 is a major proinflammatory factor and a biomarker of detrimental organ development. As a human example, tubulointerstitial CCL2 expression was elevated and closely correlated with CCR2 in diabetic nephropathy and a number of glomerulonephritides. Very recently, CCR2-myeloid cells were also reported as mediators of renal aging. However, beyond reported detrimental effects, renal CCL2 may also affect healing after injury. We recently investigated this issue further and found that renal tubular, rather than myeloid, CCL2 was required for early macrophage influx and renal recovery. In this context, it is encouraging to note that, in our atherosclerotic mice, ablation of myeloid CCR2 did not significantly alter total renal CCL2 expression or renal histologic or functional outcome after AKI at either investigated time point. Alternate CCL2 receptors, such as CCR4, may need to be considered when studying the role of CCL2 in renal repair.

In summary, our results introduce inflammatory cell activation by the damaged kidney as a mechanism of remote organ damage.

DISCLOSURES

S.V. Fleig reports having other interests in, or relationships with, the European Vascular Biology Organization, German Society for Microcirculation and Vascular Biology (GmVB), German Society for Ultrasound in Medicine (DEGUM), and German Society of Nephrology (DGfN). H. Haller reports serving on speakers bureaus for Alexion, Amgen, AstraZeneca, Bayer Pharma, Boehringer, MedWiss, Novartis, Phenos, and Vifor-Fresenius; receiving honoraria from AstraZeneca, Bayer Pharma, Boehringer, MedWiss, Novartis, Phenos, and Vifor-Fresenius; having consultancy agreements with AstraZeneca, Bayer Pharma, Boehringer, MedWiss, Phenos, and Vifor-Fresenius; and serving in an advisory or leadership role for Alexion, Bayer Pharma, Der Internist, and Der Nephrologe. A.M. Hüsing reports previously being employed by Bayer AG (internship from November 2018 to April 2019, not current). R. Schmitt reports receiving honoraria from Fresenius Medical Care and Otaka Pharmaceutical. S. von Vietinghoff reports having consultancy agreements with AstraZeneca, Bayer Vital GmbH, Boehringer Ingelheim, Shionogi GmbH, and Vifor Pharma. All remaining authors have nothing to disclose.

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AUTHOR CONTRIBUTIONS

D. DeLuca, S.V. Fleig, S. Gaedcke, H. Haller, A.M. Hüsing, S. Rong, R. Schmitt, and V.C. Wulfmeyer reviewed and edited the manuscript; D.
of a high fat diet.

DeLaca, S. Gaedcke, and S. von Vietinghoff were responsible for data curation; S.V. Fleig, A.M. Häusing, S. Rong, and V.C. Wulfmeyer were responsible for investigation; H. Haller, A.M. Häusing, and S. von Vietinghoff conceptualized the study; A.M. Häusing and S. von Vietinghoff wrote the original draft; R. Schmitt was responsible for methodology; R. Schmitt and S. von Vietinghoff provided supervision; and S. von Vietinghoff was responsible for formal analysis, funding acquisition, project administration, validation, and visualization.

DATA SHARING STATEMENT

All gene expression studies have been deposited to the Gene Expression Omnibus (accessions GSE193275, GSE193469, and GSE193649).

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2022010048/-/DC Supplemental.

Supplemental Appendix. Supplemental Methods.

Supplemental Table 1. Characteristics of LDLR−/− mice after 10 weeks of a high fat diet.

Supplemental Table 2. Characteristics of LDLR−/− mice after 3 weeks of a high fat diet.

Supplemental Table 3. LDLR−/− mice after reconstitution with wild-type of Ccr2−/− bone marrow followed by renal ischemia reperfusion or control surgery and 10 weeks of a high fat diet.

Supplemental Table 4. LDLR−/− mice after reconstitution with wild-type or Ccc2−/− bone marrow followed by renal ischemia reperfusion or control surgery and 3 weeks of a high fat diet.

Supplemental Figure 1. Immunostaining specificity controls.

Supplemental Figure 2. Parallels in aortic and renal post-ischemic inflammatory gene expression.

Supplemental Figure 3. Flow cytometric gating strategies.

Supplemental Figure 4. Flow cytometric gating strategies for assessment of mixed bone marrow chimeras.

Supplemental Figure 5. Renal Ccr2 expression after ischemia reperfusion injury.

Supplemental Figure 6. Histologic outcome in LDLR−/− mice in the absence and presence of myeloid CCR2.

Supplemental Figure 7. Similar histologic outcome after IR in the absence and presence of myeloid CCR2.

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Suppl. information on:
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Myeloid CCR2 promotes atherosclerosis after acute kidney injury

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Supplemental Methods

Histology

For immunostaining, rat anti-mouse and human CD11b (M1/70), Armenian hamster anti-mouse CD11c (N418) (both biolegend, San Diego, CA), rabbit anti-human CD3 (polyclonal, Dako, Denmark), rat anti-mouse F4/80 (BM8) (Origene, Rockville, MD) were used with corresponding secondary antibodies: AF488 donkey anti-rat IgG, AF488 donkey anti-rabbit IgG, AF555 goat anti-rat IgG, AF555 donkey anti-rabbit IgG (all Invitrogen, Carlsbad, CA), Cy3 goat anti-Armenian hamster IgG (Jackson Immunoresearch, West Grove, PA) followed by DAPI nuclear counterstain (Dianova, Hamburg, Germany). Images were obtained using a HS All-in-One Fluorescence Microscop BZ-9000 (Keyence Itasca, IL) or AxioObserver Z1 (Carl Zeiss Microscopy GmbH, Jena, Germany). Confocal imaging was performed on a Leica TCS SP8 with a 20x multi-immersion objective using LASX (Leica, Wetzlar, Germany).
**RNA isolation and real time PCR**

RNA was isolated using NucleoSpin RNA Plus Kit for cells and NucleoSpin RNA Kit for organs (both Macherey-Nagel, Duren, Germany). Yield and purity were determined in a Colibri Microvolume Spectrometer (Titertek-Berthold, Pforzheim, Germany). After reverse transcription (M-MLV-RT, Promega, Madison, Wisconsin, USA), real-time PCR was performed on a LightCycler 96 using SYBR-Green (FastStart Taq DNA Polymerase dNTPack, Roche, Grenzach-Wyhlen, Germany). Products were confirmed by melting curve analysis and gel electrophoresis. Data were analyzed with HPRT as a reference gene using LinRegPCR software.

Primer sequences were as follows (5′-3′):

- **Ccl2**
  - FP: TTTAAAAACCTGGATCGGAACCAA
  - RP: GCATTAGCTTCAGATTACGGGT

- **Ccr2**
  - FP: ATCCACGGCATCATACTATCAACATC
  - RP: TCGTAGTCATACGGTGTGGTG

- **Hprt**
  - FP: CAGTCCCAGCGTCGTGATTA
  - RP: AGCAAGTCTTTTCAGTCTGTC

- **Itgax**
  - FP: CTGGATAGCCTTTCTTCTGCTG
  - RP: GCACACTGTGTCCGAACCTCA

**Microarray specification**

This study employed a refined version of the Whole Mouse Genome Oligo Microarray 4x44K v2 (Design ID 026655, Agilent Technologies, ‘026655QM_RCUG_MusMusculus’, Design ID 084956) developed by the Research Core Unit Genomics, Hannover Medical School. For aortic mRNA assessment, 150ng total RNA was used. Synthesis of Cy3-labeled cRNA was performed in ¾ reaction volumes with the ‘Low Input Quick Amp Labeling Kit
One-Color’ (#5190-2305, Agilent Technologies) according to the manufacturer’s recommendations. cRNA fragmentation, hybridization and washing steps were carried-out as recommended in the ‘One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling Protocol V6.7’, 2000-2500ng of labeled cRNA were used for hybridization. For renal mRNA assessment, 200ng of total RNA were used to prepare aminoallyl-UTP-modified (aaUTP) cRNA (Amino Allyl MessageAmp™ II Kit; #AM1753; Thermo Fisher Scientific) applying one-round of amplification as directed by the company, with a twofold downscaling of all reaction volumes. 50% of cDNA was used for aaUTP-cRNA synthesis. The labeling of aaUTP-cRNA was performed by use of Alexa Fluor 555 Reactive Dye (#A32756; Thermo Fisher Scientific) as recommended in the manual of the Amino Allyl MessageAmp™ II Kit (twofold downscaled reaction volumes).

cRNA fragmentation, hybridisation and washing steps were carried-out as recommended in the ‘One-Color Microarray-Based Gene Expression Analysis Protocol V5.7’, 750ng of each fluorescently labelled cRNA population were used for hybridization. Slides were scanned using the Agilent Micro Array Scanner G2565CA (pixel resolution 3 µm, bit depth 20). Data extraction was performed with the ‘Feature Extraction Software V10.7.3.1’ by use of the extraction protocol file ‘GE1_107_Sep09.xml’. Measurements of on-chip replicates were averaged using the geometric mean of processed intensity values of the green channel, ‘gProcessedSignal’ (gPS) to retrieve one resulting value per unique non-control probe. Single Features were excluded from averaging, if they i) were manually flagged, ii) were identified as Outliers by the Feature Extraction Software, iii) lay outside the interval of ‘1.42 x interquartile range’ regarding the normalized gPS distribution of the respective on-chip replicate population, or, iv) showed a coefficient of variation of pixel intensities per Feature that exceeded 0.5. Averaged gPS values were normalized by global linear scaling. A lower intensity threshold (surrogate value) was defined based on intensity distribution of negative control features and
fixed at 15 (aorta) or 10 (kidney) normalized gPS units.

**Tissue preparation for single cell RNA sequencing**

Briefly, kidneys from three male C57Bl/6 control mice (no surgery) and three male C57Bl/6 mice seven days after 27min unilateral renal IR were digested as described. Tissues were pooled for each condition. Following red blood cell lysis and dead cell removal using a Miltenyi Dead Cell Removal Kit according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) 10,000 cells per sample were subjected to single cell mRNA-Seq analysis (Chromium Single Cell 3 Reagent Kits v3 User Guide, Document Number CG000183, Rev A; 10x Genomics). Equimolar amounts of libraries were pooled, denatured with NaOH, and finally diluted to 1.8 pM according to the Denature and Dilute Libraries Guide (Document # 15048776 v02; Illumina). 1.3ml of the denatured pool was sequenced on an Illumina NextSeq 550 sequencer using one third of a High Output Flowcell for 75 cycles per sample (#20024906; Illumina). The proprietary 10x Genomics CellRanger pipeline (v3.0.2) was employed with default parameters.

**Bioinformatic analysis of single cell sequencing data**

CellRanger was used to build a “pre-mRNA” reference package from reference genome provided by 10x Genomics (Mouse reference dataset 3.0.0; November 19, 2018; mm 10) as described in https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/advanced/references. Read data were then aligned to the “premRNA” reference package with CellRanger using the aligner STAR to count aligned reads per gene and calculate clustering and summary statistics. Seurat was used to generate and visualize clusters using the standard Seurat workflow: (1) reads were filtered for based on genes and reads per cell as well as mtRNA abundance. (2) Dimensionality reduction with PCA, followed
by network-based clustering. (3) Visualization of clusters was done using T-SNE. (4) Cluster specific marker genes were generated using the fisher statistic.

Clusters were labeled with cell types by comparing the cluster specific marker genes with previously defined cell type marker genes. Cell types were matched to clusters using the Mann Whitney test between the ranking of the cluster marker genes which were part of a given cell type annotation list and those that were not. In the preprocessing cells with less than 200 or more than 8000 expressed genes were removed. Further cells with more than 15% mt-genes were removed. The data was normalized, excluding highly expressed genes and log transformed. The doublets were removed using Scrublet. The data was clustered using Louvain (neighbors=5, PCAs=10) and visualized with UMAP.

**Flow cytometry**

The following antibodies were used: anti-CD115 (AFS98), anti-MHCII (M5/114.15.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-TCRαβ (H57-597), anti-Gr1 (R B6-8C5, anti-CD19 (6D5), anti-CD45 (30-F11), anti-CD45.1(A20), anti-CD45.2(104) (all biolegend, San Diego, CA). Yellow LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen, Eugene, OR) was used according to the manufacturer’s instructions. Flow cytometry analysis was performed on a Becton-Dickinson FACSCanto and LSRII (Franklin Lakes, NJ) and on an ID7000 Spectral Cell Analyzer (Sony Biotechnologies, San Jose, CA). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).
**Suppl. tables**

**Suppl. table 1: Characteristics of LDLr<sup>−/−</sup> mice after 10 weeks of a high fat diet**

<table>
<thead>
<tr>
<th></th>
<th>ctrl.</th>
<th>IR</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>33.3±2.0 (11)</td>
<td>33.3±1.7 (12)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>133.9±10.3 (11)</td>
<td>120.8±7.4 (12)</td>
<td>0.32</td>
</tr>
<tr>
<td>Rel. spleen weight (%)</td>
<td>0.4±0.04 (11)</td>
<td>0.4±0.03 (12)</td>
<td>0.46</td>
</tr>
<tr>
<td>Contralateral kidney (mg)</td>
<td>205.7±8.9 (11)</td>
<td>260.2±10.3 (12)</td>
<td>*** &lt;0.001</td>
</tr>
<tr>
<td>Rel. cont. kidney weight (%)</td>
<td>0.6±0.04 (11)</td>
<td>0.8±0.05 (12)</td>
<td>* 0.02</td>
</tr>
<tr>
<td>IR (sham) kidney (mg)</td>
<td>198.1±3.9 (11)</td>
<td>76.1±7.5 (12)</td>
<td>*** &lt;0.001</td>
</tr>
<tr>
<td>Rel. IR (sham) kidney weight (%)</td>
<td>0.6±0.03 (11)</td>
<td>0.2±0.03 (12)</td>
<td>*** &lt;0.001</td>
</tr>
<tr>
<td>Leukocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>6.0±0.5 (11)</td>
<td>7.2±1.2 (12)</td>
<td>0.35</td>
</tr>
<tr>
<td>Monocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>0.5±0.04 (11)</td>
<td>0.6±0.08 (12)</td>
<td>0.38</td>
</tr>
<tr>
<td>Lymphocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>2.3±0.2 (11)</td>
<td>2.7±0.4 (12)</td>
<td>0.37</td>
</tr>
<tr>
<td>Granulocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>3.2±0.4 (11)</td>
<td>3.9±0.8 (12)</td>
<td>0.42</td>
</tr>
<tr>
<td>Thrombocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>834.8±57.4 (11)</td>
<td>777.3±68.5 (12)</td>
<td>0.53</td>
</tr>
<tr>
<td>Erythrocytes (10&lt;sup&gt;6&lt;/sup&gt;/µl)</td>
<td>10.6±0.5 (11)</td>
<td>11.1±0.5 (12)</td>
<td>0.49</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>9.2±0.9 (7)</td>
<td>13.3±1.2 (9)</td>
<td>* 0.02</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>24.0±3.8 (7)</td>
<td>29.8±4.1 (10)</td>
<td>0.31</td>
</tr>
<tr>
<td>Phosphorous (mmol/l)</td>
<td>2.5±0.3 (9)</td>
<td>2.6±0.1 (12)</td>
<td>0.69</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.7±0.07 (8)</td>
<td>2.8±0.03 (10)</td>
<td>0.24</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>51.5±4.5 (11)</td>
<td>52.7±3.5 (12)</td>
<td>0.84</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>5.7±0.9 (11)</td>
<td>4.5±0.4 (12)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n). P values are given for unpaired t test after Welch’s correction. Ctrl: mice after control surgery; IR: mice after unilateral ischemia reperfusion injury.
### Suppl. table 2: Characteristics of LDLr<sup>-/-</sup> mice after 3 weeks of a high fat diet

<table>
<thead>
<tr>
<th></th>
<th>Ctrl.</th>
<th>IR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>25.9±0.4 (7)</td>
<td>23.6±0.6 (9)</td>
<td>**0.008</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>96.7±7.7 (7)</td>
<td>105.2±6.1 (9)</td>
<td>0.40</td>
</tr>
<tr>
<td>Rel. spleen weight (%)</td>
<td>0.37±0.03 (7)</td>
<td>0.45±0.02 (9)</td>
<td>0.05</td>
</tr>
<tr>
<td>Contralateral kidney (mg)</td>
<td>176.9±9.6 (7)</td>
<td>211.3±6.1 (9)</td>
<td>*0.01</td>
</tr>
<tr>
<td>Rel. cont. kidney weight (%)</td>
<td>0.7±0.03 (7)</td>
<td>0.9±0.05 (9)</td>
<td>** 0.005</td>
</tr>
<tr>
<td>IR (sham) kidney (mg)</td>
<td>171.3±6.9 (7)</td>
<td>72.4±4.4 (9)</td>
<td>*** &lt;0.001</td>
</tr>
<tr>
<td>Rel. IR (sham) kidney weight (%)</td>
<td>0.7±0.02 (7)</td>
<td>0.3±0.02 (9)</td>
<td>*** &lt;0.001</td>
</tr>
<tr>
<td>Leukocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>6.4±0.9 (7)</td>
<td>6.6±0.7 (9)</td>
<td>0.85</td>
</tr>
<tr>
<td>Monocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>0.5±0.07 (7)</td>
<td>0.6±0.06 (9)</td>
<td>0.62</td>
</tr>
<tr>
<td>Lymphocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>2.7±0.4 (7)</td>
<td>3.1±0.4 (9)</td>
<td>0.47</td>
</tr>
<tr>
<td>Granulocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>3.2±0.6 (7)</td>
<td>2.9±0.3 (9)</td>
<td>0.76</td>
</tr>
<tr>
<td>Thrombocytes (10&lt;sup&gt;3&lt;/sup&gt;/µl)</td>
<td>814±58 (7)</td>
<td>697±76 (9)</td>
<td>0.24</td>
</tr>
<tr>
<td>Erythrocytes (10&lt;sup&gt;6&lt;/sup&gt;/µl)</td>
<td>10.6±0.2 (7)</td>
<td>10.6±0.3 (9)</td>
<td>0.84</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>7.9±0.6 (6)</td>
<td>11.1±0.8 (9)</td>
<td>**0.01</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>22.2±2.3 (6)</td>
<td>28.5±5.8 (8)</td>
<td>0.34</td>
</tr>
<tr>
<td>Phosphorous (mmol/l)</td>
<td>2.9±0.2 (7)</td>
<td>3.0±0.2 (9)</td>
<td>0.73</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.4±0.44 (4)</td>
<td>2.8±0.08 (8)</td>
<td>0.40</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>32.3±3.3 (6)</td>
<td>38.2±2.3 (9)</td>
<td>0.18</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>5.3±0.3 (7)</td>
<td>4.5±0.5 (9)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n). P values are given for unpaired t test with Welch’s correction. Ctrl: mice after control surgery; IR: mice after unilateral ischemic reperfusion injury.
**Suppl. table 3: LDLr⁻/⁻ mice after reconstitution with wildtype or Ccr2⁻/⁻ bone marrow followed by renal ischemia reperfusion or control surgery and 10 weeks of a high fat diet**

<table>
<thead>
<tr>
<th></th>
<th>wildtype</th>
<th>Ccr2⁻/⁻</th>
<th>ctrl. vs. IR</th>
<th>wt vs. Ccr2⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ctrl.</td>
<td>IR</td>
<td>ctrl.</td>
<td>IR</td>
</tr>
<tr>
<td><strong>Body (g)</strong></td>
<td>25.4±0.4 (11)</td>
<td>24.3±0.5 (13)</td>
<td>24.6±0.3 (8)</td>
<td>24.1±0.4 (9)</td>
</tr>
<tr>
<td><strong>Spleen (mg)</strong></td>
<td>118.9±9 (11)</td>
<td>111.4±8 (13)</td>
<td>113.3±10 (8)</td>
<td>108.8±10 (9)</td>
</tr>
<tr>
<td>Rel. spleen weight (%)</td>
<td>0.4±0.03 (11)</td>
<td>0.5±0.04 (13)</td>
<td>0.5±0.04 (8)</td>
<td>0.5±0.04 (9)</td>
</tr>
<tr>
<td><strong>Contralateral kidney (mg)</strong></td>
<td>163.1±8 (11)</td>
<td>207.7±11 (13)</td>
<td>155.9±5 (8)</td>
<td>198.6±6 (9)</td>
</tr>
<tr>
<td>Rel. cont. kidney weight (%)</td>
<td>0.6±0.03 (11)</td>
<td>0.9±0.04 (13)</td>
<td>0.6±0.02 (8)</td>
<td>0.8±0.02 (9)</td>
</tr>
<tr>
<td><strong>IR (sham) kidney (mg)</strong></td>
<td>166.9±7 (11)</td>
<td>34.3±3 (13)</td>
<td>147.0±4 (8)</td>
<td>27.5±2 (9)</td>
</tr>
<tr>
<td>Rel. IR (sham) kidney weight (%)</td>
<td>0.7±0.03 (11)</td>
<td>0.1±0.01 (13)</td>
<td>0.6±0.01 (8)</td>
<td>0.1±0.01 (9)</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leukocytes (10⁶/µl)</strong></td>
<td>8.8±1.2 (11)</td>
<td>10.2±1.0 (13)</td>
<td>8.7±0.8 (8)</td>
<td>11.3±1.3 (9)</td>
</tr>
<tr>
<td><strong>Monocytes (cells/µl)</strong></td>
<td>853±139 (11)</td>
<td>801±95 (11)</td>
<td>270±43 (7)</td>
<td>396±59 (6)</td>
</tr>
<tr>
<td><strong>GR1 [HIGH]</strong></td>
<td>317±55 (11)</td>
<td>344±56 (11)</td>
<td>44±18 (7)</td>
<td>55±18 (6)</td>
</tr>
<tr>
<td><strong>Monocytes (cells/µl)</strong></td>
<td>536±106 (11)</td>
<td>457±64 (11)</td>
<td>227±35 (7)</td>
<td>340±55 (6)</td>
</tr>
<tr>
<td><strong>B lymphocytes (cells/µl)</strong></td>
<td>3641±887 (11)</td>
<td>399±1466 (11)</td>
<td>2818±523 (7)</td>
<td>4669±978 (6)</td>
</tr>
<tr>
<td><strong>T lymphocytes (cells/µl)</strong></td>
<td>1665±215 (11)</td>
<td>2055±236 (11)</td>
<td>1248±121 (7)</td>
<td>1635±189 (6)</td>
</tr>
<tr>
<td><strong>Neutrophils (cells/µl)</strong></td>
<td>2285±248 (11)</td>
<td>2597±477 (11)</td>
<td>3156±822 (7)</td>
<td>4028±1367 (6)</td>
</tr>
<tr>
<td><strong>Thrombocytes (10³/µl)</strong></td>
<td>641±46 (11)</td>
<td>716±47 (11)</td>
<td>882±80 (8)</td>
<td>886±132 (9)</td>
</tr>
<tr>
<td><strong>Erythrocytes (10⁶/µl)</strong></td>
<td>10.3±0.6 (11)</td>
<td>9.7±0.3 (13)</td>
<td>9.7±0.6 (8)</td>
<td>8.9±0.4 (9)</td>
</tr>
<tr>
<td><strong>Urea (mmol/l)</strong></td>
<td>7.4±0.5 (7)</td>
<td>11.9±1.0 (7)</td>
<td>7.4±0.6 (6)</td>
<td>11.7±0.6 (8)</td>
</tr>
<tr>
<td><strong>Creatinine (µmol/l)</strong></td>
<td>20.7±2.2 (11)</td>
<td>25.7±1.7 (13)</td>
<td>23.7±2.9 (8)</td>
<td>29.7±2.6 (8)</td>
</tr>
<tr>
<td><strong>Phosphorous (mmol/l)</strong></td>
<td>2.8±0.1 (11)</td>
<td>2.9±0.1 (11)</td>
<td>2.7±0.2 (8)</td>
<td>2.5±0.1 (8)</td>
</tr>
<tr>
<td><strong>Calcium (mmol/l)</strong></td>
<td>2.7±0.03 (11)</td>
<td>2.7±0.05 (11)</td>
<td>2.5±0.18 (7)</td>
<td>2.7±0.04 (8)</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/l)</strong></td>
<td>37.7±4.9 (11)</td>
<td>37.3±2.0 (13)</td>
<td>35.4±5.0 (8)</td>
<td>33.6±2.7 (9)</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/l)</strong></td>
<td>7.9±1.3 (11)</td>
<td>8.2±0.7 (13)</td>
<td>6.3±1.1 (8)</td>
<td>6.8±1.2 (9)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n). P values are given for Dunnett’s test if ANOVA was significant. Ctrl: mice after control surgery; IR: mice after unilateral ischemia reperfusion injury.
Suppl. table 4: LDLr−/− mice after reconstitution with wildtype or Ccr2−/− bone marrow followed by renal ischemia reperfusion or control surgery and 3 weeks of a high fat diet

<table>
<thead>
<tr>
<th></th>
<th>wildtype</th>
<th>Ccr2−/−</th>
<th>ctrl. vs. IR</th>
<th>wt vs. Ccr2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ctrl.</td>
<td>IR</td>
<td>ctrl.</td>
<td>IR</td>
</tr>
<tr>
<td><strong>Body (g)</strong></td>
<td>23.6±0.7 (5)</td>
<td>22.4±0.7 (5)</td>
<td>23.6±0.5 (4)</td>
<td>22.4±0.5 (7)</td>
</tr>
<tr>
<td><strong>Spleen (mg)</strong></td>
<td>112.0±17 (5)</td>
<td>95.9±7 (5)</td>
<td>119.3±10 (4)</td>
<td>117.4±16 (7)</td>
</tr>
<tr>
<td><strong>Rel. spleen</strong></td>
<td>0.5±0.1 (5)</td>
<td>0.4±0.03 (5)</td>
<td>0.5±0.04 (4)</td>
<td>0.5±0.1 (7)</td>
</tr>
<tr>
<td><strong>Contralateral kidney (mg)</strong></td>
<td>154±12 (5)</td>
<td>172±10 (5)</td>
<td>160±13 (4)</td>
<td>184±9 (7)</td>
</tr>
<tr>
<td><strong>Rel. cont. kidney weight (%)</strong></td>
<td>0.7±0.05 (5)</td>
<td>0.8±0.03 (5)</td>
<td>0.7±0.04 (4)</td>
<td>0.8±0.03 (7)</td>
</tr>
<tr>
<td><strong>IR (sham) kid. (mg)</strong></td>
<td>152±11 (5)</td>
<td>48±2 (5)</td>
<td>154±14 (4)</td>
<td>51±2 (4)</td>
</tr>
<tr>
<td><strong>Rel. IR (sham) kid. weight (%)</strong></td>
<td>0.6±0.04 (5)</td>
<td>0.2±0.01 (5)</td>
<td>0.6±0.05 (4)</td>
<td>0.2±0.01 (7)</td>
</tr>
</tbody>
</table>

**Blood**

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes (10³/µl)</th>
<th>Monocytes (cells/µl)</th>
<th>GR1HIGH Monocytes (cells/µl)</th>
<th>GR1LOW Monocytes (cells/µl)</th>
<th>B lymphocytes (cells/µl)</th>
<th>T lymphocytes (cells/µl)</th>
<th>Neutrophils (cells/µl)</th>
<th>Thrombocytes (10³/µl)</th>
<th>Erythrocytes (10⁶/µl)</th>
<th>Urea (mmol/l)</th>
<th>Creatinine (µmol/l)</th>
<th>Phosphorous (mmol/l)</th>
<th>Calcium (mmol/l)</th>
<th>Cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.6±1.5 (8)</td>
<td>1119±238 (8)</td>
<td>549±122 (8)</td>
<td>571±155 (8)</td>
<td>370±629 (8)</td>
<td>1491±230 (8)</td>
<td>2953±479 (8)</td>
<td>648±59 (8)</td>
<td>11.1±0.2 (8)</td>
<td>8.3±0.6 (5)</td>
<td>22.7±1.7 (5)</td>
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<td>28.8±4.2 (5)</td>
<td>4.5±0.9 (4)</td>
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<td>6.4±0.5 (7)</td>
<td>493±36 (7)</td>
<td>228±17 (7)</td>
<td>277±267 (7)</td>
<td>2537±304 (8)</td>
<td>1134±70 (7)</td>
<td>1936±482 (7)</td>
<td>656±34 (7)</td>
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<td>1167±188 (8)</td>
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<td>204±24 (14)</td>
<td>4248±2665 (14)</td>
<td>976±81 (14)</td>
<td>2260±292 (14)</td>
<td>786±46 (14)</td>
<td>10.4±0.3 (14)</td>
<td>12.3±0.8 (6)</td>
<td>30.1±1.0 (7)</td>
<td>2.7±0.2 (7)</td>
<td>2.8±0.04 (5)</td>
<td>25.3±0.9 (7)</td>
<td>4.0±0.4 (7)</td>
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</table>

Values are mean ± SEM (n). P values are given for Dunnett’s test if ANOVA was significant.
Ctrl: mice after control surgery; IR: mice after unilateral ischemia reperfusion injury.
Supplemental figures and figure legends:

**Suppl. figure 1: Immunostaining specificity controls**

(A,B) Confocal images of aortic root (A) and kidney (B) sections imaged as in figures 1, 4 and 6 but stained with omission of the primary antibodies. Secondary antibodies were used as indicated in methods as follows: AF488 donkey anti-rat and AF555 goat anti-rat for F4/80, AF488 donkey anti-rabbit and AF555 donkey anti-rabbit for CD3, AF488 donkey anti-rat for CD11b, Cy3-goat anti-Armenian hamster for CD11c (blue: DAPI, size bars=100µm).
Suppl. figure 2: Parallels in aortic and renal post-ischemic inflammatory gene expression
(A,B) Differentially expressed immune genes in atherosclerotic aortas from mice after renal IR and controls were grouped in genes upregulated upwards (A) or downwards (B) 1.5fold or more in both early and late atherosclerosis as detailed in methods. (C,D) Regulation of these gene sets in the post-ischemic murine kidney (n=3-4/timepoint, expression relative to baseline, x=not detected in the dataset).
Suppl. figure 3: Flow cytometric gating strategies

(A,B) Gating strategies of renal (A) and blood (B) leukocytes are shown.
Suppl. figure 4: Flow cytometric gating strategies for assessment of mixed bone marrow chimeras

(A-D) Gating strategies of blood (A), spleen (B), kidney (C) and aortic (D) leukocyte for CD11b+ myeloid cells and CD45.1 and CD45.2 syngenic markers are shown.
Suppl. figure 5: Renal Ccr2 expression after ischemia reperfusion injury

(A, B) Renal Ccr2 expression was assessed by qPCR in LDLr⁻/⁻ mice after IR or control surgery and after reconstitution with wildtype or Ccr2⁻/⁻ bone marrow as depicted in figure 5A after three (A) and ten weeks (B) of atherosclerosis induction (A: n=4-7 mice per group from 6 indep. exp., B: n=7-13 mice per group from 10 indep. exp., Dunnett’s after ANOVA).
Suppl. figure 6: Histologic outcome in LDLr⁻/⁻ mice in the absence and presence of myeloid CCR2

(A,B) Renal histology after IR was assessed in LDLr⁻/⁻ mice reconstituted with wildtype or Ccr2⁻/⁻ bone marrow after three weeks of atherosclerosis induction (A, Representative HE-stained kidney sections (size bars= 1mm and 100µm, B: proportion of intact tubuli in cortex and outer stripe of outer medulla, n=5 mice per group, n= 5 indep. exp., t-test).
Suppl. figure 7: Similar histologic outcome after IR in the absence and presence of myeloid CCR2

(A–C) Renal histology was assessed in LDLr<sup>−/−</sup> mice after IR or control surgery and after reconstitution with wildtype or Ccr2<sup>−/−</sup> bone marrow as depicted in figure 5A after ten weeks of atherosclerosis induction (A, Representative HE-stained kidney sections (size bars= 1mm and 100µm, B: proportion of intact tubuli in cortex and outer stripe of outer medulla, n=3–4 mice per group, n= 2 indep. exp., Dunnett’s after ANOVA). (C) Fibrosis was assessed by Masson Trichrome-staining (representative. sections, size bars= 100µm).