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Depletion of Gut Microbiota Protects against Renal Ischemia-Reperfusion Injury

Diba Emal,* Elena Rampanelli,* Ingrid Stroo,* Loes M. Butter,* Gwendoline J. Teske,* Nike Claessen,* Geurt Stokman,* Sandrine Florquin,*† Jaklien C. Leemans,* and Mark C. Dessing*

*Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and †Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

ABSTRACT

An accumulating body of evidence shows that gut microbiota fulfill an important role in health and disease by modulating local and systemic immunity. The importance of the microbiome in the development of kidney disease, however, is largely unknown. To study this concept, we depleted gut microbiota with broad-spectrum antibiotics and performed renal ischemia-reperfusion (I/R) injury in mice. Depletion of the microbiota significantly attenuated renal damage, dysfunction, and remote organ injury and maintained tubular integrity after renal I/R injury. Gut flora–depleted mice expressed lower levels of F4/80 and chemokine receptors CX3CR1 and CCR2 in the F4/80$^+$ renal resident macrophage population and bone marrow (BM) monocytes than did control mice. Additionally, compared with control BM monocytes, BM monocytes from gut flora–depleted mice had decreased migratory capacity toward CX3CL1 and CCL2 ligands. To study whether these effects were driven by depletion of the microbiota, we performed fecal transplants in antibiotic-treated mice and found that transplant of fecal material from an untreated mouse abolished the protective effect of microbiota depletion upon renal I/R injury. In conclusion, we show that depletion of gut microbiota profoundly protects against renal I/R injury by reducing maturation status of F4/80$^+$ renal resident macrophages and BM monocytes. Therefore, dampening the inflammatory response by targeting microbiota-derived mediators might be a promising therapy against I/R injury.

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AKI is a major clinical problem that affects 5% of hospitalized patients and has a mortality rate of 50%–80% in these patients.[1] Ischemia-reperfusion (I/R) injury is a major cause of AKI which can occur during shock, sepsis, and renal transplantation.[2] Ischemia-induced damage leads to apoptosis and necrosis of renal tubular cells, followed by release of damage-associated molecular patterns which are sensed by pathogen recognition receptors present on tubular epithelial cells (TECs) and renal resident leukocytes.[3–6] Activated TECs and resident leukocytes release cytokines/chemokines to attract inflammatory cells from circulation to the injured kidney to clear cell debris and promote repair.[3,7] However, when the powerful inflammatory response is exaggerated it can also amplify renal damage.[3,8] Thus, inflammation can have profound but disparate roles in the pathogenesis of AKI.

In the past few years, intestinal microbiota–related research has been rapidly expanding, providing important insights into the involvement of the microbiome in modulating systemic immunity and thereby affecting the outcome of several inflammatory diseases.[9–18] For instance, antibiotic-treated mice have impaired innate and adaptive immune responses after exposure to lung infection caused by

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D.E., E.R., J.C.L., and M.C.D. contributed equally to this work.

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Correspondence: Diba Emal, Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands. Email: d.emal@amc.uva.nl

either influenza virus or *Streptococcus pneumoniae*.[9,14,16] Mice lacking intestinal microbiota develop less severe symptoms in autoimmune models of arthritis and experimental autoimmune encephalomyelitis.[17,18]

In regard to renal diseases, a few studies demonstrated an improved uremic state after gut microbiota–directed interventions in CKD and ESRD.[19–21] However, the effect of the microbiome on the disease process of AKI remains unknown, as does a possible mechanism by which microbiota can influence the kidney. We therefore investigated the role of microbiota in I/R injury–induced AKI by depleting gut microbiota with broad-spectrum antibiotic treatment in wild-type mice. We found that microbiota are essential for priming F4/80+ renal resident macrophages and bone marrow (BM) monocytes. Consequently, renal resident macrophages from commensal-depleted mice are less responsive to ischemic injury, resulting in protection against renal I/R injury.

RESULTS

Depletion of Intestinal Microbiota Protects against Renal I/R Injury

After depletion of microbiota, we found a striking and profound protection against renal I/R injury as assessed by scoring the percentage of necrotic tubules and renal expression of neutrophil gelatinase-associated lipocalin (NGAL) 24 hours after I/R injury (Figure 1, A and B). Renal function was significantly preserved in antibiotic-treated mice as indicated by lower plasma creatinine and urea levels compared with control mice (Figure 1, C and D). Lactate dehydrogenase (LDH), Aspartate transminase (ASAT), and Alanine transminase (ALAT) plasma levels are known to increase upon renal I/R injury indicating general tissue injury.[22] Conversely, in antibiotic-treated mice LDH, ASAT, and ALAT levels remain steady after I/R injury (Figure 1, E–G). One day after I/R injury the inflammatory milieu is predominantly characterized by a vast influx of granulocytes,[4,6,7,23] which can play a detrimental role in the postischemic kidney.[24] We found significantly lower influx of granulocytes in antibiotic-treated mice upon I/R injury (Figure 1H). In antibiotic-treated mice renal structural integrity was better maintained as shown by significantly less TEC apoptosis and more TEC proliferation (Figure 1, I and J). Taken together, these data clearly show that depletion of the microbiota protects mice against renal I/R injury.

Decreased Levels of Renal Inflammatory Mediators Early after Ischemic Damage in Commensal-Depleted Mice

To study the mechanism underlying the absence of massive granulocyte influx into the ischemic kidneys after microbiota depletion, we next analyzed the migration capacity of granulocytes and found that it was not affected by microbiota depletion (Supplemental Figure 1A). In addition, the granulocyte phagocytosis and oxidative burst capacities were not altered by microbiota depletion (Supplemental Figure 1, B and C). Because granulocyte motility and function were independent of microbiota, we next investigated the local inflammatory milieu 2 hours after renal I/R injury. At this time point, granulocyte influx did not yet occur (Figure 2A) and renal damage was similar between the groups (Figure 2, B and C). Interestingly, renal inflammatory response was significantly lower as reflected by decreased mRNA levels of TNF-α, IL-6, MCP-1, and MIP-2α in antibiotic-treated mice (Figure 2, D–G). Only levels of KC were similar between control and microbiota-depleted mice (Figure 2H). Upon injury, the sources of cytokine/chemokine release are TECs and renal resident leukocytes, among which macrophages constitute the major population.[8,25,26] After 2 hours of reperfusion the level of macrophages was quantified by expression of classic renal macrophage marker F4/80 on mRNA and at protein level (Figure 2, I and J). Strikingly, F4/80 expression was profoundly lower in commensal-depleted mice.

F4/80+ Renal Resident Macrophages from Commensal-Depleted Mice Are Less Mature and Produce Less MIP-2α in the Early Hours of I/R Injury

FACS analysis of renal resident macrophages at steady state and after 2 hours of reperfusion revealed that F4/80 expression was selectively reduced in the F4/80+ population in commensal-depleted mice (Figure 3, A–C), whereas no changes occurred for CD11B and CD11C, myeloid and dendritic cell markers, respectively (Supplemental Figure 2, A and B). Next, we sorted F4/80+ macrophages at 2 hours of reperfusion and determined their chemokine expression. Strikingly, we found that chemokines MCP-1 and MIP-2α were both decreased in F4/80+ macrophages isolated from antibiotic-treated mice (Figure 3, D–F). KC and MIP-2α are the main chemokines that are produced within the early hours of reperfusion and are known to attract granulocytes from circulation.[7] However, we could not detect any KC in these macrophages, which indicates that KC is not produced by these cells at 2 hours of reperfusion. In conclusion, the reduced granulocyte influx after 24 hours of reperfusion in the antibiotic-treated mice can be explained by the less mature F4/80+ macrophages that produce reduced levels of MIP-2α at 2 hours of reperfusion.

Expression Levels of F4/80 and CX3CR1 in Renal Resident Macrophages Are Reduced by Commensal Depletion and Restored after Reintroduction of Microbiota

We analyzed renal resident macrophages under steady state and found that next to the F4/80 expression (Figure 4A), the chemokine receptors CX3CR1 and CCR2 (Figure 4, C–F), which are important in the trafficking of macrophages, were both reduced after depletion of gut microbiota.[27,28] Despite the reduced expression of the chemokine receptors, the percentage of F4/80+ cells in the total population of CD45+ cells was unchanged in antibiotic-treated mice (Figure 4B). In order to investigate whether the microbiota was responsible for

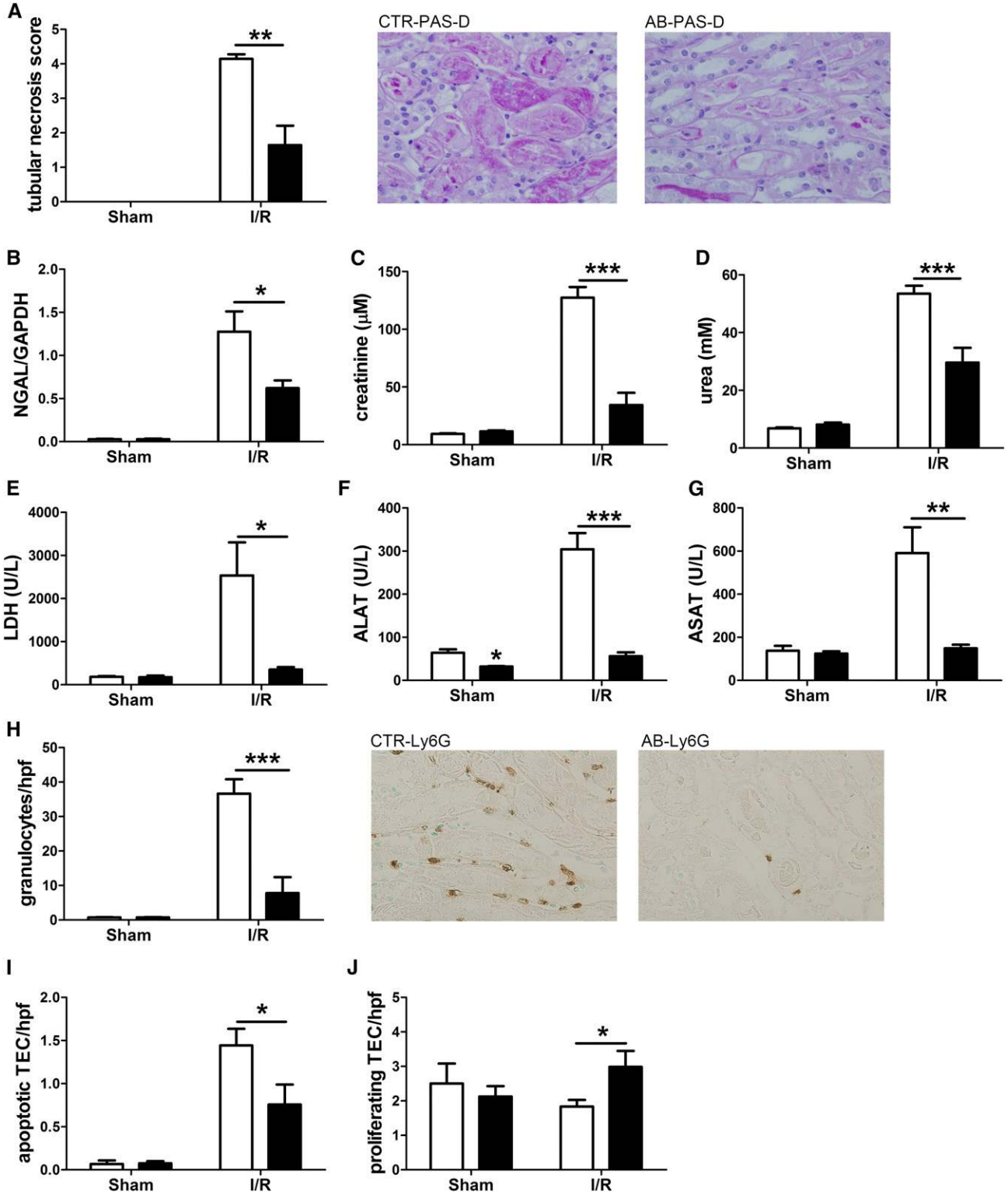

Figure 1. Depletion of gut microbiota protects against renal I/R injury. (A) Renal damage was evaluated by scoring the percentage of necrotic tubules in Periodic acid–Schiff-diastase–stained sections (original magnification, ×400) and by measuring NGAL mRNA levels in total kidney homogenates from control (white bars) and antibiotic-treated mice (black bars) (B). Renal function was quantified by plasma creatinine (C) and urea (D) levels. Plasma levels of general organ damage and liver markers LDH (E), ALAT (F), and ASAT (G) were determined. Influx of granulocytes was assessed by scoring Ly6G$^+$ cells in the corticomedullary region of the kidney (H) (original magnification, ×400). The amount of apoptotic TEC was determined by scoring

affecting expression levels of these markers, we reintroduced microbiota by transplanting healthy fecal samples in mice initially depleted from commensals (Ftx group). Fecal transplantation restored to a certain extent the expression rate of F4/80 and CX3CR1 but not of CCR2 (Figure 4, A and C–F). The reduced levels of F4/80, CX3CR1, and CCR2 were only found on the F4/80+ cells and not on CD11B+ cells (Supplemental Figure 3, A, D, and F), because they did not occur in the CD11B+ F4/80− population (Supplemental Figure 3, E and G). In the total population of CD11B, the majority of cells are F4/80 negative (approximately 60%) whereas the F4/80+ proportion is around 40% (Supplemental Figure 3, B and C). Therefore, changes in the F4/80+ population are no longer visible in the total CD11B+ population. Together these findings show that in the absence of microbiota, only the F4/80+ population of renal resident macrophages is less mature.

Migration Capacity and Expression Levels of F4/80, CX3CR1, and CCR2 Are Reduced in BM Monocytes from Commensal-Depleted Mice

Maintenance of renal resident macrophages under steady conditions depends on a constant supply of mature monocytes from the BM[29,30] and relies on the expression of the chemokine receptor CX3CR1; whereas, the chemokine receptor CCR2 is important for recruitment of monocytes during inflammatory conditions.[31] Therefore, we questioned whether BM monocytes from antibiotic-treated mice, similarly to F4/80+ renal resident macrophages, would display lower levels of these receptors and, hence, poorer migratory capacity. As illustrated in Figure 5, A–D, F4/80, CX3CR1, and CCR2 mean fluorescence intensity levels were markedly reduced in BM monocytes from commensal-depleted mice compared with control mice. In line with these findings, depletion of the microbiota impaired the migration ability of BM monocytes toward CCL2 and CX3CL1 ligands (Figure 5, E and F). Fecal transplantation reversed the phenotype, as shown by higher expression levels of F4/80, CX3CR1, and CCR2 on BM monocytes in the Ftx group compared with antibiotic-treated mice (Figure 5, A–D). Overall, these findings show that F4/80+ BM monocytes, similar to F4/80+ renal resident macrophages, require signals from the gut to terminally differentiate.

Reintroduction of Microbiota into Antibiotic-Treated Mice Abolishes the Protective Effect of Microbiota Depletion upon Renal I/R Injury

Because the low maturation status of F4/80+ renal macrophages that was observed in antibiotic-treated mice was reversed by fecal transplantation (Figure 4A), we wanted to investigate if reintroduction of commensals would also reverse response to renal I/R injury. Indeed, we found no differences in tubular necrosis and NGAL levels between the Ftx and control group, whereas there was a great difference between the control and antibiotic group (Figure 6, A and B). Similarly, fecal transplantation reversed granulocyte influx to a certain extent (Figure 6C). To conclude, depletion of microbiota was indeed responsible for the protective effect against renal I/R injury observed in antibiotic-treated mice (Figure 1).

Liver and Splenic Resident Macrophages Are Less Mature in Commensal-Depleted Mice under Steady Conditions

As we found a marked decrease of F4/80 expression on renal resident macrophages from commensal-depleted mice under steady conditions (Figure 4A), we questioned if resident macrophages of other organs are also affected by depletion of gut microbiota. We found reduced levels of F4/80 expression in liver and spleen tissue as well (Figure 7, A and B). These immature resident macrophages in the liver provide an explanation for the preserved remote liver injury in commensal-depleted mice upon renal I/R injury (Figure 1, F and G). Apparently, gut microbiota do not only induce maturation of renal resident macrophages but also prime resident macrophages in the liver and spleen.

DISCUSSION

In the past decade it has become clear that microbiota play a fundamental role in shaping the immune system outside the gut. An increasing amount of studies show that alterations in the gut microbiome affect the immune response to different inflammatory disorders.[9,10,16] However, little is known as to whether similar processes occur during AKI. Here, we show mechanistic insights into the gut-kidney axis during AKI. Microbiota depletion was protective against renal I/R injury as reflected by reduced renal dysfunction, damage, and inflammation. Mechanistically, microbiota depletion affects macrophage/monocyte maturation status, release of chemokines (MCP-1 and MIP-2α), and primary functions like migration capacity. The reduced macrophage activity can explain the weaker early inflammatory response after I/R injury. The protective effect of microbiota depletion against I/R injury and macrophage alterations was reversed after reintroduction of microbiota after fecal transplantation. This study clearly shows that the microbiome primes macrophage functions that contribute to the initiation of renal inflammation and subsequent nephropathy after renal I/R injury.

Clarke *et al.* were the first to show that microbiota affect systemic innate immunity because granulocytes were less

caspase-3$^+$ TEC (I) and the amount of proliferating TEC (J) was determined by scoring Ki67$^+$ TEC. Data are expressed as mean±SEM. In the I/R group, the two-tailed unpaired *t* test was used in all graphs (n=7/8 per group). *P<0.05; **P<0.01; ***P<0.001. CTR–PAS-D, Control group-Periodic acid–Schiff-diastase staining; AB–PAS-D, Antibiotic group–Periodic acid–Schiff-diastase staining; hpf, high-power field; CTR-Ly6G, Control group-Ly6G staining; AB-Ly6G, Antibiotic group-Ly6G staining.

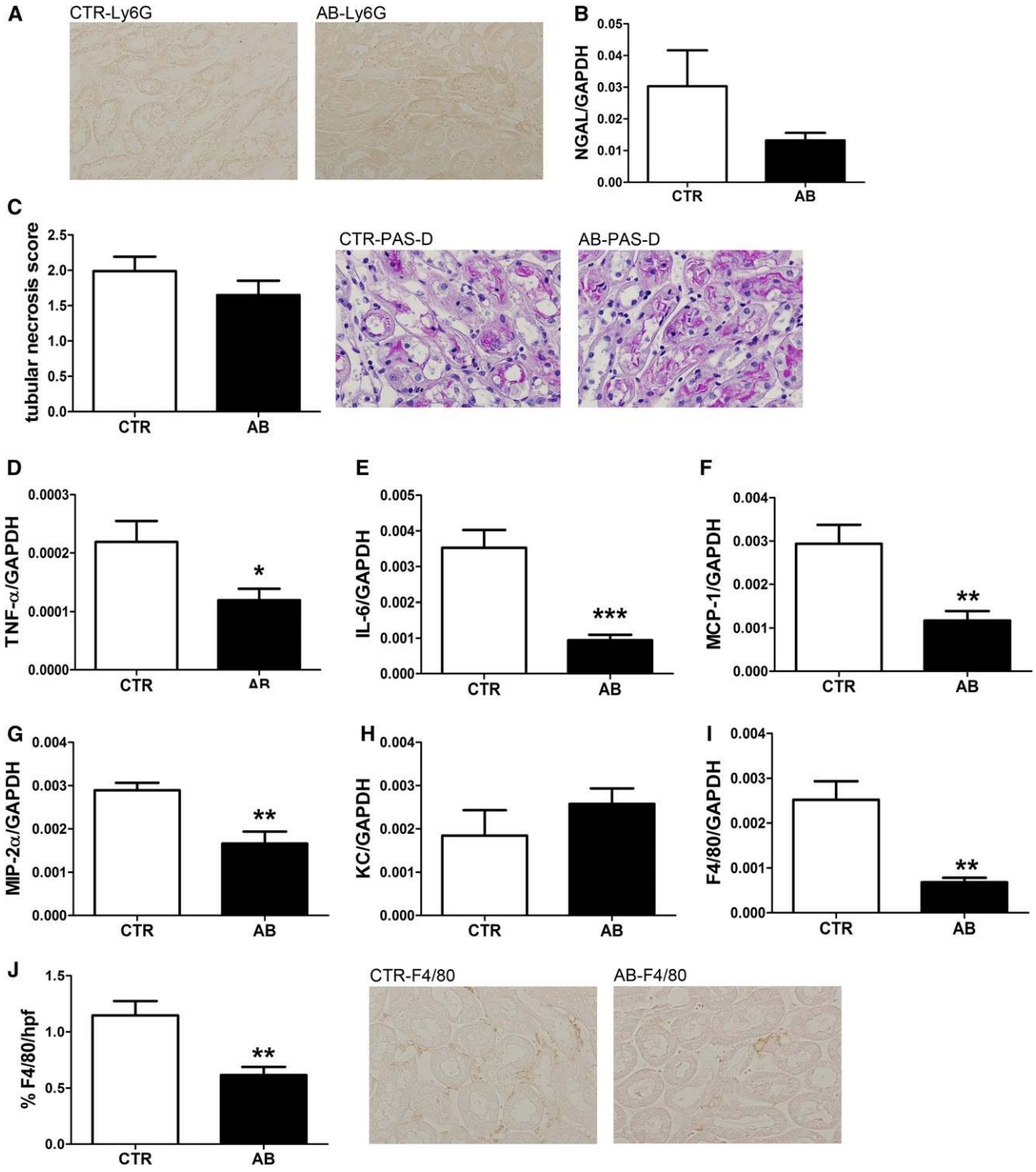

Figure 2. Decreased levels of renal inflammatory mediators early after ischemic damage in commensal-depleted mice. The number of granulocytes was determined by Ly6G$^+$ staining (original magnification, ×400) of kidney tissue (A). Renal damage was assessed by quantification of NGAL mRNA levels in total kidney homogenates (B) and by semiquantitative scoring of the percentage of necrotic tubules in Periodic acid–Schiff-diastase-stained sections (original magnification, ×400) (C). Inflammatory mediators TNF-α (D), IL-6 (E), MCP-1 (F), MIP-2α (G), and KC (H) were measured on mRNA level in kidney homogenates. F4/80 expression was determined by mRNA level in kidney homogenates (I) and by F4/80 staining (original magnification, ×200) of kidney tissue which was digitally analyzed and presented as percentage of staining intensity per high-power field (J). Data are presented as mean±SEM. The two-tailed unpaired *t* test was used in all graphs (n=8/9 per group). *P<0.05; **P<0.01; ***P<0.01. CTR-Ly6G, Control group-Ly6G staining; AB-Ly6G, Antibiotic group-Ly6G staining; CTR, Control group; AB, Antibiotic group; CTR–PAS-D, Control group–Periodic acid–Schiff-diastase staining; AB–PAS-D, Antibiotic group–Periodic acid–Schiff-diastase staining; CTR-F4/80, Control group-F4/80 staining; AB-F4/80, Antibiotic group-F4/80 staining.

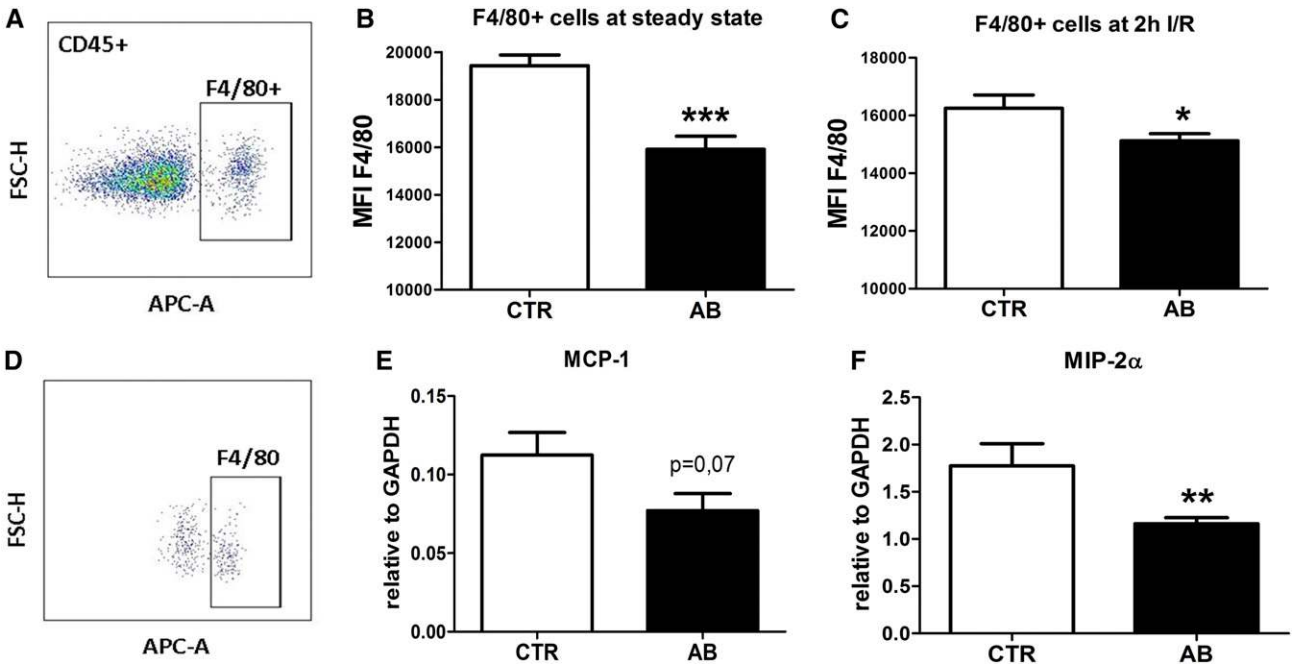

Figure 3. F4/80+ renal resident macrophages from commensal-depleted mice are less mature and produce less MIP-2α in the early hours of I/R injury. A representative FACS plot showing the F4/80+ population on the CD45+ cells (A). Mean fluorescence intensity of F4/80 on F4/80+ cells at steady state (B) and after 2 hours of reperfusion (C). FACS plot illustrating the F4/80+ macrophages after 2 hours of I/R injury that is sorted (D). MCP-1 (E) and MIP-2α (F) mRNA levels in F4/80+ resident macrophages. Data are presented as mean±SEM. The two-tailed unpaired *t* test was used in all graphs (n=5 per group). *P<0.05; **P<0.01; ***P<0.01. FSC-H, Forward scatter-height; APC-A, APC-area; CTR, Control group; AB, Antibiotic group.

efficient in killing *Staphylococcus aureus* and *S. pneumoniae ex vivo* after antibiotic treatment.[12] In renal I/R injury, several immune cells are known to contribute to the severity of kidney injury, including granulocytes, through the release of mediators and enzymes that potentially damage the surrounding tissue.[24] In our hands, granulocyte function (migration, phagocytosis capacity, and oxidative burst) was not affected by antibiotic treatment. This observation is in line with a previous study in which no differences were found in phagocytic ability, reactive oxygen species production, and cell surface adhesion and transmigration markers in granulocytes between control and antibiotic-treated mice.[13] These data imply that the reduced granulocyte content in our antibiotic-treated mice was not due to impaired granulocyte functions but due to alterations preceding granulocyte influx. Indeed, 2 hours after I/R injury (when granulocyte influx is not yet observed), renal inflammation was reduced in microbiota-depleted mice.

Dendritic cells and macrophages are the major resident leukocytes in the kidney, which are present within the renal interstitium and are essential for homeostatic regulation of the kidney environment.[32,33] These resident leukocytes are known to contribute to initiation of inflammation shortly after renal I/R,[8] and depleting macrophages in the kidney before I/R protects mice and rats against renal injury.[34,35] Distinguishing dendritic cells and macrophages from each other is complicated because they have overlapping ontogeny, function, and phenotype.[28] Therefore, we analyzed renal expression of CD11B, CD11C, Ly6C (data not shown), and F4/80 in control and antibiotic-treated mice. We found a reduced expression of F4/80, CX3CR1, and CCR2 only in the F4/80+ resident macrophages, whereas no changes occurred in the expression of CD11B, CD11C, and Ly6C markers. F4/80 is considered as the classic cell surface marker for mature resident macrophages,[25,36,37] whereas CX3CR1 is involved in adhesion, migration, and activation of leukocytes.[27,31,38] Although tissue macrophages originate from the yolk sac,[37] it is believed that the renewal of resident macrophages under steady conditions is dependent on a constant supply of CX3CR1+ monocytes from the BM.[29,30] Indeed, CX3CR1-deficient mice display 50% less renal resident macrophages.[39] BM monocytes from commensal-depleted mice were also less mature as assessed by low expression of F4/80, CX3CR1, and CCR2 markers in the F4/80+ population, and the migration capacity was also reduced. However, we did not find decreased numbers of F4/80+ resident macrophages in the kidneys. As we may speculate, there are several possibilities that can explain this observation; the life span of resident macrophages ranges from weeks to months, it might be that these resident macrophages are not renewed during the 2-week course of antibiotic treatment, so there is no need for migration of the precursors. This hypothesis would mean that the gut flora prime renal resident macrophages and BM monocytes independently from each other, which is possible because circulation reaches both sites

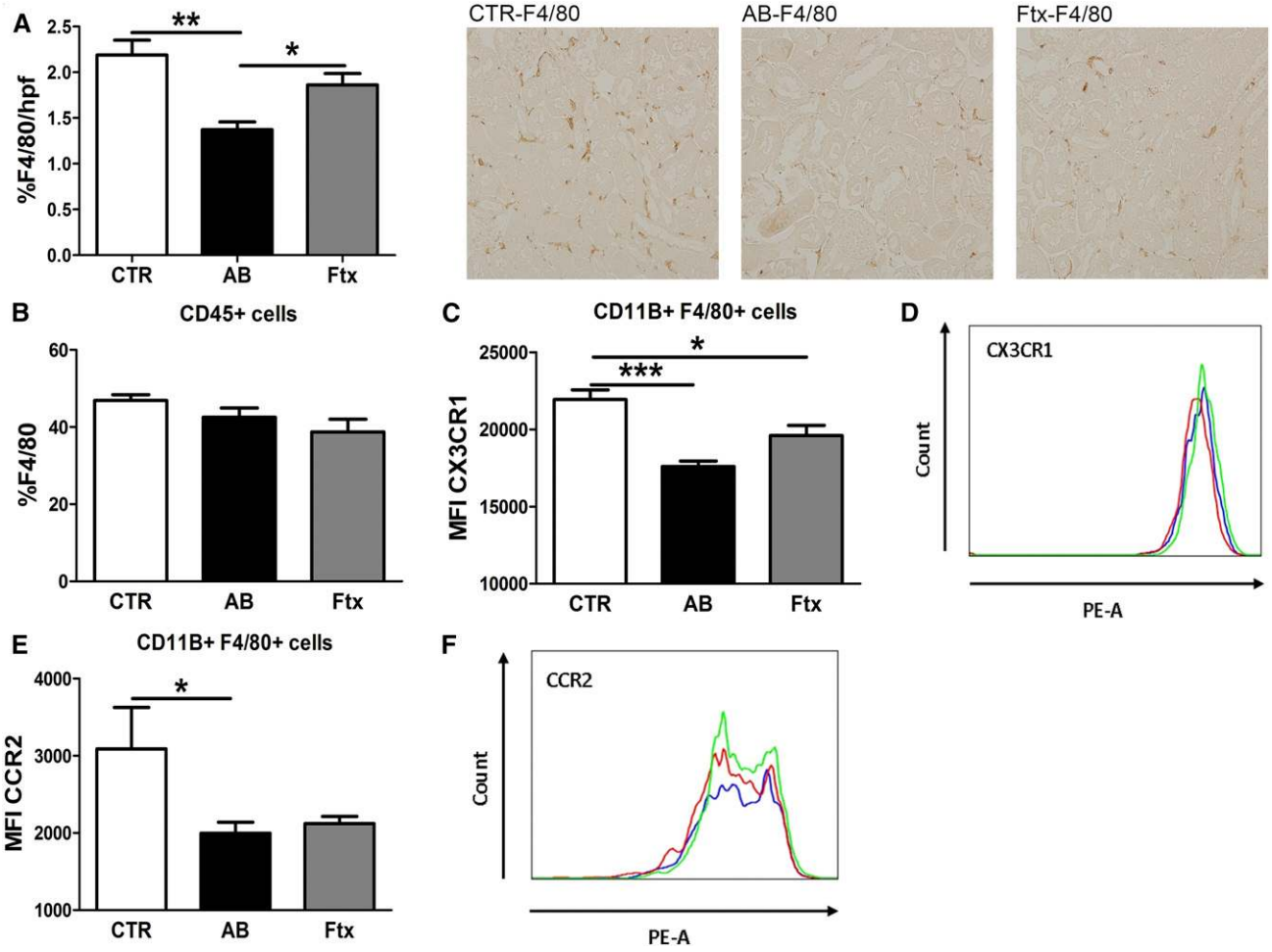

Figure 4. Expression levels of F4/80 and CX3CR1 in renal resident macrophages are reduced by gut flora depletion and restored after reintroduction of microbiota. F4/80 positivity was determined by F4/80 staining (original magnification, ×200) of kidney tissue which was digitally analyzed and presented as percentage of staining intensity per high-power field of kidney tissue (A). The percentage of F4/80 positive cells in the total gate of CD45 positive cells was measured by flow cytometry (B). Mean fluorescence intensity of CX3CR1 positive cells (C) and its FACS plot (D) in CD11B+ F4/80+ gate from control (green), antibiotic (red), and Ftx group (blue). Mean fluorescence intensity of CCR2 (E) and its FACS plot (F) on CD11B+ F4/80+ macrophages from control (green), antibiotic (red), and Ftx group (blue). Data are presented as mean±SEM. One-way ANOVA followed by Bonferroni multiple comparison test was used in all graphs (n=5/6 per group). *P<0.05; **P<0.01; ***P<0.01. CTR, Control group; AB, Antibiotic group; CTR-F4/80, Control group-F4/80 staining; AB-F4/80, Antibiotic group-F4/80 staining; Ftx-F4/80, Fecal transplant group-F4/80 staining; PE-A, PE-area.

(kidneys and the BM). On the other hand, trafficking receptors and the ability to migrate is indeed decreased in BM monocytes from antibiotic-treated mice, however it is not completely diminished. This means that these precursors are still able to migrate (slowly) during renewal of resident macrophages. Longer treatment with antibiotics might result in decreased numbers of macrophages in the kidneys. The last possibility is the sum of both scenarios. The gut microbiota may affect renal resident macrophages directly and indirectly *via* the BM. In this way, 2 weeks of antibiotic treatment is still too short to see changes in macrophage numbers in the kidneys at steady state. The reduced maturation and activation state of macrophages due to microbiota depletion is supported by our finding that F4/80+ resident macrophages produce less MIP-2α after 2 hours of reperfusion. It is MIP-2α, together with KC, that is responsible for the optimal attraction of granulocytes into tissue. Thus, the reduced maturation status of resident macrophages observed in kidneys of antibiotic-treated mice explains the reduced MIP-2α production early after I/R, leading to decreased granulocyte influx and eventually resulting in preserved renal function and attenuated renal damage.

It is of great interest to identify commensal-derived products which are responsible for priming systemic immunity in order to target it. Only one study has revealed that peptidoglycan can translocate from the gut to circulation. This statement was on the basis of an experiment in which guts of germ-free mice were colonized with *Escherichia coli* for

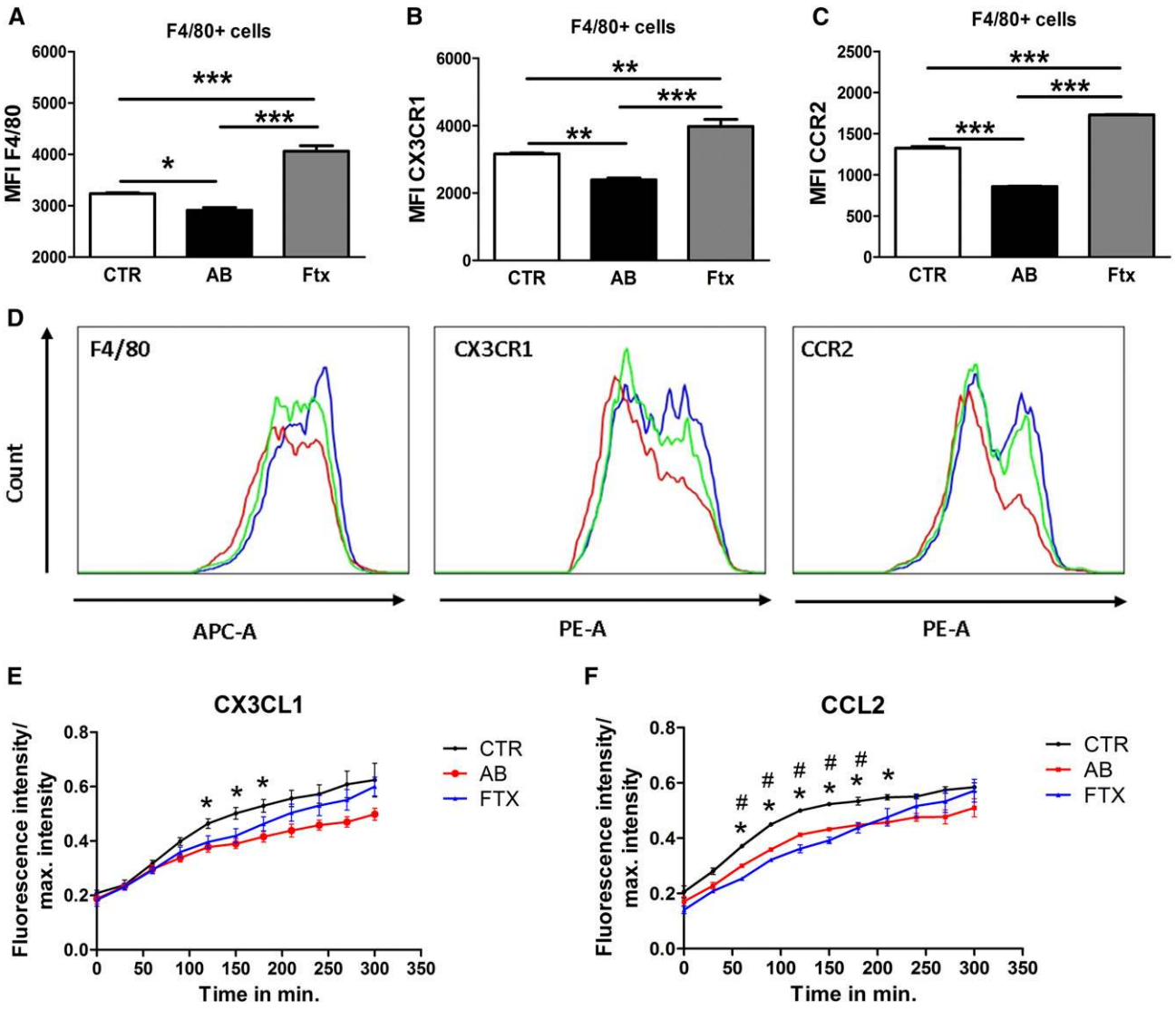

Figure 5. Migration capacity and expression levels of F4/80, CX3CR1, and CCR2 are reduced in BM monocytes from commensal-depleted mice. Mean fluorescence intensity of F4/80 (A), CX3CR1 (B), and CCR2 (C) was determined on isolated F4/80+ BM monocytes and its FACS plots (D) from control (green), antibiotic (red), and Ftx group (blue). Migration capacity of BM monocytes with CX3CL1 (E) and CCL2 (F) as a chemoattractant was quantified in a transwell migration system (D). Data are presented as mean±SEM. One-way ANOVA followed by Bonferroni multiple comparison test was used in all graphs (n=4 per group). Significant difference between control and antibiotic group is indicated with a "*" and differences between control and the Ftx group are given with a "#". *P<0.05; **P<0.01; ***P<0.01. CTR, Control group; AB, antibiotic group; APC-A, APC-area; PE-A, PE-area.

3 days.[12] Therefore, it is difficult to assume that this is also the case in normal conventional mice with complete functional microbiota. We also could not measure detectable levels of LPS and peptidoglycan in the plasma of control mice (data not shown), suggesting that the levels are too low to be detected but are sufficient for a tonic activation of monocytes/macrophages or that other gut-derived products are involved. Short chain fatty acids (SCFAs) are well known candidates that are end products of fermentation by intestinal bacteria. They are known to have anti-inflammatory properties.[40] Recently, it has been stated that SCFAs prevent renal I/R injury.[41] The authors showed that only acetate treatment diminished cellular stress and inflammation in kidney I/R injury. However, in this study SCFAs were used as a treatment in the presence of an intact microbiota, meaning that physiologic concentrations of SCFAs in the circulation under steady conditions do not have substantial anti-inflammatory properties. Identifying microbiota-derived products that modulate systemic immunity remains imperative in future translational research. A first step toward identifying microbiota-derived products that are essential in priming monocytes/macrophages would be reconstitution with specific groups of bacteria instead of total fecal transplantation.

Several studies have demonstrated a beneficial role for microbiota in priming immunity against eradicating infectious

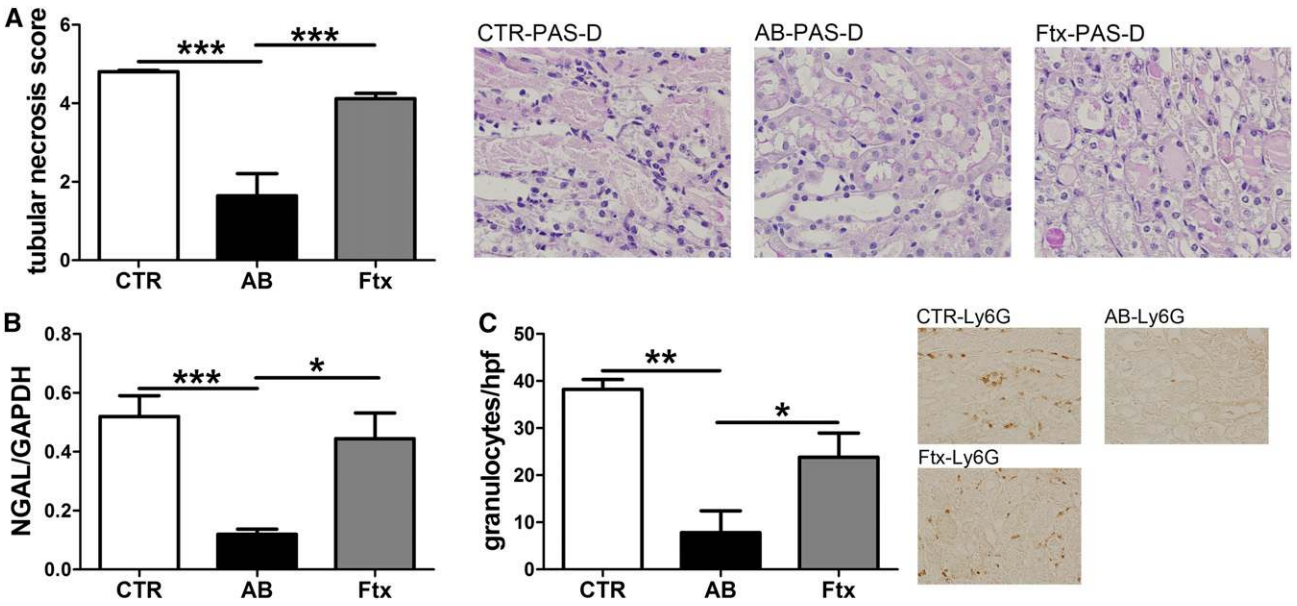

Figure 6. Reintroduction of microbiota into antibiotic-treated mice abolishes the protective effect of microbiota depletion upon renal I/R injury. (A) Renal damage was assessed by semiquantitative scoring of the percentage of necrotic tubules in PAS-D–stained sections (original magnification, ×400) and by determining NGAL mRNA levels in total kidney homogenates (B). Influx of granulocytes was determined by scoring Ly6G$^+$ cells (original magnification, ×400) in the corticomedullary region of the kidney (C). Data are expressed as mean±SEM. One-way ANOVA followed by Bonferroni multiple comparison test was used in all graphs (n=6/7/10 per group). *P<0.05; **P<0.01; ***P<0.01. CTR–PAS-D, Control group–Periodic acid–Schiff-diastase staining; AB–PAS-D, Antibiotic group–Periodic acid–Schiff-diastase staining; Ftx–PAS-D, Fecal transplant group–Periodic acid–Schiff-diastase staining; CTR, Control group; AB, Antibiotic group; Ftx, Fecal transplant group; hpf, High-power field; CTR-Ly6G, Control group-Ly6G staining; AB-Ly6G, Antibiotic group-Ly6G staining; Ftx-Ly6G, Fecal transplant group-Ly6G staining.

pathogens.[9,10,14,16] In contrast, priming innate immunity is detrimental during pathologic conditions in which inflammation is unwanted, including lung[15] and kidney (our study) I/R injury. We show that the microbiome primes macrophage functions to become fully mature and these mature macrophages contribute to the initiation of inflammation and subsequent nephropathy after renal I/R injury. Of note, we are not advocating the use of antibiotics in the treatment of AKI but our findings do present first steps toward understanding how to tailor microbiota-related therapies.

CONCISE METHODS

Mice

C57Bl/6 wild-type mice were purchased from Janvier (Le Genest, France). Mice were housed under specific pathogen-free conditions receiving food and water *ad libitum*. Eight- to 12-week-old male mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all animal experiments.

Microbiota Depletion

Depletion of gut microbiota before renal I/R was achieved by administering mice broad-spectrum antibiotics *via* autoclaved (tap) drinking water (1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin [Sigma Aldrich] and 0.5 g/L vancomycin [Xellia Pharmaceuticals ApS]) supplemented with 1% (wt/vol) glucose. Control drinking water was prepared in the same way only without antibiotics. Drinking water was replaced twice a week. Two weeks of treatment with broad-spectrum antibiotic strategy did not affect gut integrity (data not shown) and led to a significant drop in microbial diversity as described before.[16] Antibiotic treatment did not lead to changes in body weight, temperature, baseline hematologic indices, and food intake (Supplemental Figure 4, B–G). Fluid intake was slightly less in antibiotic group, probably due to the unpleasant taste of antibiotic-containing drinking water but did not lead to dehydration and weight loss (Supplemental Figure 4E). Baseline biochemical indices (ALAT, ASAT, creatinine, urea, LDH, albumin, total protein, and triglycerides) were also comparable between control and antibiotic-treated mice (data not shown).

Fecal Transplantation

A fresh fecal sample from an untreated mouse was collected and resuspended in 1 ml of NaCl. One hundred microliters of this suspension was administrated *via* oral gavage per mouse (Ftx group). Fecal transplantation started 1 day after cessation of antibiotics and lasted for 3 days. Fecal transplantation protocol succeeded, as we did not detect any bacterial colonies in the antibiotic-treated group whereas bacteria were abundantly present in control and Ftx fecal samples (Supplemental Figure 4A).

I/R Injury

I/R injury was induced by unilateral or bilateral clamping (micro aneurysm clamps) of renal pedicles for 25 minutes under general

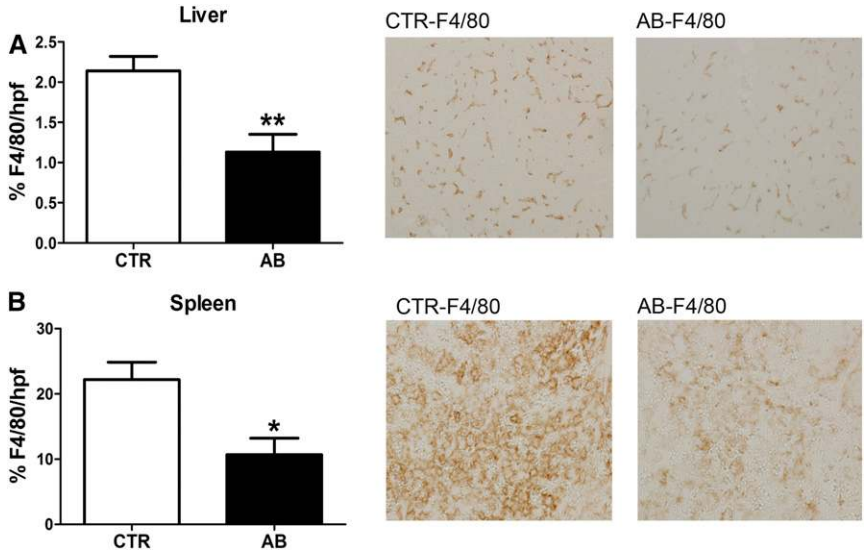

Figure 7. Liver and splenic resident macrophages are less mature in commensal-depleted mice under steady conditions. F4/80 expression was determined by F4/80 staining (original magnification, ×200) of liver (A) and spleen (B) tissue and presented as percentage of staining intensity per high-power field. Data are expressed as mean± SEM. Two-tailed unpaired *t* test was used in all graphs (n=4/6 per group). *P<0.05; **P<0.01. hpf, High-power field; CTR, Control group; AB, Antibiotic group; CTR-F4/80, Control group-F4/80 staining; AB-F4/80, Antibiotic group-F4/80 staining.

inhalation anesthesia (3% isoflurane and oxygen). After removal of clamps, kidneys were inspected for restoration of blood flow. For analgesic purposes, mice received a subcutaneous injection of 0.1 mg/kg buprenorphine (Temgesic; Schering-Plough) 30 minutes before surgery. Sham-operated animals underwent the same procedure excepting clamping of the renal pedicles. At the time of euthanasia, blood was collected by heart puncture in heparin-containing tubes followed by cervical dislocation. Kidneys were snap-frozen in liquid nitrogen and stored at −80°C or fixed in 10% formalin overnight before further processing.

Plasma Biochemical Analysis

Using standard autoanalyzer methods plasma levels of creatinine, urea, LDH, ASAT, and ALAT were determined at our hospital diagnostic facility.

Histology and Immunohistochemistry

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-micrometer-thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid–Schiff reagents after diastase digestion. Injury to tubules was scored by a pathologist in a blinded fashion, semiquantitatively, by determining the percentage of affected tubules per 10 high-power fields according to the percentage of necrotic tubules in the corticomedullary region on a scale from 0 to 5 (0=0%, 1=<10%, 2=10%–25%, 3=25%–50%, 4=50%–75%, and 5=>75%). For immunohistochemical staining, sections were boiled for 10 minutes in 10 mM sodium citrate buffer (pH 6.0) for F4/80, apoptosis, and proliferation detection, or digested with a solution of 0.25% pepsine (Sigma Aldrich) in 0.01 M HCl for granulocyte detection. Subsequently, sections were incubated overnight with FITC-labeled anti-mouse Ly6G (BD Biosciences), rat anti-mouse F4/80 (Biorad), rabbit anti-mouse active caspase-3 (Cell Signaling Technology), or rabbit anti-human Ki67 (Neomarkers) to detect granulocytes, macrophages, apoptosis, or proliferation, respectively. For CD11B and CD11C stainings, frozen renal sections were stained with rat anti-mouse CD11B (BD Pharmingen) and hamster anti-mouse CD11C (eBioscience) overnight. Next, all sections were incubated for 30 minutes with relevant peroxidase-conjugated secondary antibodies and stained using 3,3″-diaminobenzidine dihydrochloride. The number of Ly6G positive cells and the number of capase-3 and Ki67 positive TECs were counted in 10 nonoverlapping high-power fields. F4/80, CD11B, and CD11C expression was digitally quantified with ImageJ software.

RT-PCR

Total RNA was isolated from frozen renal tissue sections with Tri-reagent (Sigma Aldrich) according to the manufacturer's protocol. RNA from sorted renal macrophages was isolated with a Qiagen RNA isolation kit according to manufacturer's instruction (Qiagen). cDNA was synthesized using oligo-deoxy-thymidine as primer. mRNA expression of NGAL, TNF-α, IL-6, MCP-1, MIP-2α, KC, and F4/80 was measured by real-time quantitative RT-PCR performed on a Light Cycler 480 (Roche) with SYBR green PCR master mix (Bioline). Intensity of SYBR green dye was determined by linear regression analysis (LinRegPCR; developed by the Heart Failure Research Center, Amsterdam, The Netherlands). Expression of specific genes was normalized to expression of the house keeping gene GAPDH. Primer sequences are provided in Supplemental Table 1.

Isolation of BM Granulocytes and Monocytes

Femoral and tibial BM was flushed with ice-cold sterile PBS with a 21-gauge needle above a 40 μm pore filter. Monocytes and granulocytes were isolated by means of magnetic labeling using Monocyte Isolation Kit and by anti-Ly6G Microbead Kit, respectively, according to the manufacturer's instructions (Miltenyi Biotec).

Oxidative Burst, Migration, and Phagocytosis Assay

The Amplex Red Hydrogen Peroxide kit (Invitrogen) was used to detect hydrogen peroxide release by BM granulocytes and monocytes stimulated with PMA (100 ng/ml) (Sigma Aldrich) according to manufacturer's instructions. Briefly, 20 μl of cell suspension (0.75×10^6 cells/ml) in Krebs Ringer phosphate buffer was added in a black 96-well plate with a clear bottom (Greiner Bio-One) together with 100 μl reaction buffer containing 0.1 U/ml horseradish peroxidase and 50 μM Amplex Red reagent. The reaction was measured by 530 nm excitation and 585 nm emission with microplate reader CLARIOstar (BMG Labtech). For quantification of the migration

capacity of BM granulocytes and monocytes, 5×10^6 cells/ml were resuspended in HEPES buffer with 4 μg/ml Calceine-AM (Life Technologies) and incubated for 30 minutes at 37°C. Cells were washed once in PBS and resuspended (1×10^6 cells/ml) in HEPES buffer. Migration was measured by the transwell system using a 24-well plate (BD Falcon) and Fluoroblok cell culture insert (BD Falcon) with pore sizes 3 μm (granulocytes) and 8 μm (monocytes). CX3CL1 and CCL2 in HEPES buffer were used as chemoattractants. Migration of cells was measured in time at 37°C with excitation 485 nm and emission 530 nm with microplate reader CLARIOstar (BMG Labtech). Phagocytosis was measured with PHAGOTEST (Glycotope Biotechnology) according to the manufacturer's instructions. Briefly, 100 μl of heparinized whole blood was stained with opsonized FITC-labeled *E. coli* and incubated for 10 minutes in a 37°C water bath, whereas the negative control remained on ice. After phagocytosis was stopped, quenching solution was used to discriminate between surface-bound and internalized *E. coli*. After lysing of the red blood cells and DNA staining, phagocytic capacity was measured by flow cytometry on a FACS Canto (BD Bioscience) and analyzed with FlowJo version 10.

FACS Analysis and Sorting of Renal Macrophages

After renal tissue mechanic and enzymatic digestion, leukocytes were isolated by density gradient centrifugation ($330 \times g$, 30 minutes, room temperature) using a two-density Percoll (GE Healthcare) medium of 40% (upper phase) and 80% (lower phase). Cells at the interface between 40% and 80% Percoll were harvested, washed, blocked with anti-Fc Receptor (Anti-Mouse CD16/CD32; eBioscience), and stained with antibodies anti-CD45.2-Percp Cy5.5, CD11b-FITC, F4/80−APC, CCR2-PE, CX3CR1-PE, and Ly6C-PE (Biolegend). Stainings were visualized using a BD LSRFortessa cell analyzer (BD Bioscience). 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was added to the cell suspension shortly before measurement to discriminate dead cells from live cells. Gating strategies are illustrated in Supplemental Figure 5. Sorting of F4/80+ renal macrophages was performed on a SH800 Cell Sorter (Sony). In brief, kidneys (two kidneys per sample) were mechanically and enzymatically digested, and subsequently leukocytes were isolated by Percoll gradient centrifugation and after Fc-Receptor blocking were stained with anti-CD45–labeled magnetic beads in order to isolate renal CD45+ cells, accordingly to manufacturer's instructions (Miltenyi Biotec). After isolation, CD45+ leukocytes were stained with anti-F4/80−APC, and cells were gated for APC-positivity for sorting. Per kidney pair, between 10,000 and 20,000 macrophages were sorted on average. All flow cytometry data were analyzed using FlowJo v10 (Ashland, OR).

Whole Blood Composition

Absolute numbers of white blood cells and percentages of lymphocytes, monocytes, and granulocytes in whole blood were determined with Scil Vet abc Plus+ (HORIBA Medical) in 30 μl of heparinized blood sample.

Statistical Analyses

All statistical analyses were performed using Graphpad Prism version 5 software. Comparisons between two groups were analyzed using the two-tailed unpaired *t* test. One-way ANOVA followed by Bonferroni multiple comparison test was used for comparison between more than two groups. Results are expressed as mean±SEM. Values of $P<0.05$ were considered to represent a statistically significant difference.

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DISCLOSURES

None.

REFERENCES

1. Gill N, Nally JV Jr., Fatica RA: Renal failure secondary to acute tubular necrosis: Epidemiology, diagnosis, and management. *Chest* 128: 2847–2863, 2005
2. Le Dorze M, Legrand M, Payen D, Ince C: The role of the microcirculation in acute kidney injury. *Curr Opin Crit Care* 15: 503–508, 2009
3. Leemans JC, Kors L, Anders HJ, Florquin S: Pattern recognition receptors and the inflammasome in kidney disease. *Nat Rev Nephrol* 10: 398–414, 2014
4. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest* 115: 2894–2903, 2005
5. Bakker PJ, Butter LM, Claessen N, Teske GJ, Sutterwala FS, Florquin S, Leemans JC: A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion. *Am J Pathol* 184: 2013–2022, 2014
6. Pulskens WP, Teske GJ, Butter LM, Roelofs JJ, van der Poll T, Florquin S, Leemans JC: Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS One* 3: e3596, 2008
7. Stroo I, Stokman G, Teske GJ, Raven A, Butter LM, Florquin S, Leemans JC: Chemokine expression in renal ischemia/reperfusion injury is most profound during the reparative phase. *Int Immunol* 22: 433–442, 2010
8. Akcay A, Nguyen Q, Edelstein CL: Mediators of inflammation in acute kidney injury. *Mediators Inflamm* 2009: 137072, 2009
9. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J, Wherry EJ, Artis D: Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37: 158–170, 2012
10. Benson A, Pifer R, Behrendt CL, Hooper LV, Yarovinsky F: Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell Host Microbe* 6: 187–196, 2009
11. Clarke TB: Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. *Infect Immun* 82: 4596–4606, 2014
12. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN: Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med* 16: 228–231, 2010
13. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'Leary CE, Oliver PM, Kolls JK, Weiser JN, Worthen GS: The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat Med* 20: 524–530, 2014

14. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, Iwasaki A: Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci USA* 108: 5354–5359, 2011
15. Prakash A, Sundar SV, Zhu YG, Tran A, Lee JW, Lowell C, Hellman J: Lung ischemia-reperfusion is a sterile inflammatory process influenced by commensal microbiota in mice. *Shock* 44: 272–279, 2015
16. Schuijt TJ, Lankelma JM, Scicluna BP, de Sousa EMF, Roelofs JJ, de Boer JD, Hoogendijk AJ, de Beer R, de Vos A, Belzer C, de Vos WM, van der Poll T, Wiersinga WJ: The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut* 65: 575–583 2016
17. Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, Kasper DL, Kasper LH: Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. *J Immunol* 185: 4101–4108, 2010
18. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, Littman DR, Benoist C, Mathis D: Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 32: 815–827, 2010
19. Niwa T: Role of indoxyl sulfate in the progression of chronic kidney disease and cardiovascular disease: Experimental and clinical effects of oral sorbent AST-120. *Ther Apher Dial* 15: 120–124, 2011
20. Ranganathan N, Patel BG, Ranganathan P, Marczely J, Dheer R, Pechenyak B, Dunn SR, Verstraete W, Decroos K, Mehta R, Friedman EA: In vitro and in vivo assessment of intraintestinal bacteriotherapy in chronic kidney disease. *ASAIO J* 52: 70–79, 2006
21. Ueda H, Shibahara N, Takagi S, Inoue T, Katsuoka Y: AST-120 treatment in pre-dialysis period affects the prognosis in patients on hemodialysis. *Ren Fail* 30: 856–860, 2008
22. Stroo I, Claessen N, Teske GJ, Butter LM, Florquin S, Leemans JC: Deficiency for the chemokine monocyte chemoattractant protein-1 aggravates tubular damage after renal ischemia/reperfusion injury. *PLoS One* 10: e0123203, 2015
23. Dessing MC, Pulskens WP, Teske GJ, Butter LM, van der Poll T, Yang H, Tracey KJ, Nawroth PP, Bierhaus A, Florquin S, Leemans JC: RAGE does not contribute to renal injury and damage upon ischemia/reperfusion-induced injury. *J Innate Immun* 4: 80–85, 2012
24. Bolisetty S, Agarwal A: Neutrophils in acute kidney injury: Not neutral any more. *Kidney Int* 75: 674–676, 2009
25. Austyn JM, Gordon S: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 11: 805–815, 1981
26. Dessing MC, Tammaro A, Pulskens WP, Teske GJ, Butter LM, Claessen N, van Eijk M, van der Poll T, Vogl T, Roth J, Florquin S, Leemans JC: The calcium-binding protein complex S100A8/A9 has a crucial role in controlling macrophage-mediated renal repair following ischemia/reperfusion. *Kidney Int* 87: 85–94, 2015
27. Umehara H, Goda S, Imai T, Nagano Y, Minami Y, Tanaka Y, Okazaki T, Bloom ET, Domae N: Fractalkine, a CX3C-chemokine, functions predominantly as an adhesion molecule in monocytic cell line THP-1. *Immunol Cell Biol* 79: 298–302, 2001
28. Kawakami T, Lichtnekert J, Thompson LJ, Karna P, Bouabe H, Hohl TM, Heinecke JW, Ziegler SF, Nelson PJ, Duffield JS: Resident renal mononuclear phagocytes comprise five discrete populations with distinct phenotypes and functions. *J Immunol* 191: 3358–3372, 2013
29. Gordon S, Plüddemann A, Martinez Estrada F: Macrophage heterogeneity in tissues: Phenotypic diversity and functions. *Immunol Rev* 262: 36–55, 2014
30. Gordon S, Taylor PR: Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5: 953–964, 2005
31. Geissmann F, Jung S, Littman DR: Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82, 2003
32. Bonventre JV, Yang L: Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest* 121: 4210–4221, 2011
33. Nelson PJ, Rees AJ, Griffin MD, Hughes J, Kurts C, Duffield J: The renal mononuclear phagocytic system. *J Am Soc Nephrol* 23: 194–203, 2012
34. Day YJ, Huang L, Ye H, Linden J, Okusa MD: Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: Role of macrophages. *Am J Physiol Renal Physiol* 288: F722–F731, 2005
35. Jo SK, Sung SA, Cho WY, Go KJ, Kim HK: Macrophages contribute to the initiation of ischaemic acute renal failure in rats. *Nephrol Dial Transplant* 21: 1231–1239, 2006
36. Schaller E, Macfarlane AJ, Rupec RA, Gordon S, McKnight AJ, Pfeffer K: Inactivation of the F4/80 glycoprotein in the mouse germ line. *Mol Cell Biol* 22: 8035–8043, 2002
37. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SE, Pollard JW, Frampton J, Liu KJ, Geissmann F: A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336: 86–90, 2012
38. Landsman L, Bar-On L, Zernecke A, Kim KW, Krauthgamer R, Shagdarsuren E, Lira SA, Weissman IL, Weber C, Jung S: CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood* 113: 963–972, 2009
39. Lionakis MS, Swamydas M, Fischer BG, Plantinga TS, Johnson MD, Jaeger M, Green NM, Masedunskas A, Weigert R, Mikelis C, Wan W, Lee CC, Lim JK, Rivollier A, Yang JC, Laird GM, Wheeler RT, Alexander BD, Perfect JR, Gao JL, Kullberg BJ, Netea MG, Murphy PM: CX3CR1-dependent renal macrophage survival promotes Candida control and host survival. *J Clin Invest* 123: 5035–5051, 2013
40. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR: Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 461: 1282–1286, 2009
41. Andrade-Oliveira V, Amano MT, Correa-Costa M, Castoldi A, Felizardo RJ, de Almeida DC, Bassi EJ, Moraes-Vieira PM, Hiyane MI, Rodas AC, Peron JP, Aguiar CF, Reis MA, Ribeiro WR, Valduga CJ, Curi R, Vinolo MA, Ferreira CM, Câmara NO: Gut bacteria products prevent AKI induced by ischemia-reperfusion. *J Am Soc Nephrol* 26: 1877–1888, 2015

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