Microbiome modulation after severe acute kidney injury accelerates functional recovery and decreases kidney fibrosis

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Targeting gut microbiota has shown promise to prevent experimental acute kidney injury (AKI). However, this has not been studied in relation to accelerating recovery and preventing fibrosis. Here, we found that modifying gut microbiota with an antibiotic administered after severe ischemic kidney injury in mice, particularly with amoxicillin, accelerated recovery. These indices of recovery included increased glomerular filtration rate, diminution of kidney fibrosis, and reduction of kidney profibrotic gene expression. Amoxicillin was found to increase stool *Alistipes*, *Odoribacter* and *Stomatobaculum* species while significantly depleting *Holdemanella* and *Anaeroplasma*. Specifically, amoxicillin treatment reduced kidney $CD4^+$T cells, interleukin (IL)-17 $^+CD4^+$T cells, and tumor necrosis factor-α double negative T cells while it increased $CD8^+$T cells and $PD1^+CD8^+$T cells. Amoxicillin also increased gut lamina propria $CD4^+$T cells while decreasing $CD8^+$T and $IL\text{-}17^+CD4^+$T cells. Amoxicillin did not accelerate repair in germ-free or CD8-deficient mice, demonstrating microbiome and $CD8^+$T lymphocytes dependence for amoxicillin protective effects. However, amoxicillin remained effective in CD4-deficient mice. Fecal microbiota transplantation from amoxicillin-treated to germ-free mice reduced kidney fibrosis and increased $Foxp3^+CD8^+$T cells. Amoxicillin pre-treatment protected mice against kidney bilateral ischemia reperfusion injury but not cisplatin-induced AKI. Thus, modification of gut bacteria with amoxicillin after severe ischemic AKI is a promising novel therapeutic approach to accelerate recovery of kidney function and mitigate the progression of AKI to chronic kidney disease.

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Translational Statement

Acute kidney injury (AKI) leads to increased morbidity and mortality, with no specific therapy. Previous studies demonstrated that changing the microbiome preinjury modified outcomes from moderate experimental AKI. However, most patients are diagnosed after acute injury. We demonstrate that modifying gut microbiota with amoxicillin after severe AKI accelerated recovery of kidney function and reduced fibrosis through gut bacteria and $CD8^+$ T cells. Modification of the microbiome with amoxicillin holds promise to accelerate recovery of kidney function after AKI.

Acute kidney injury (AKI) refers to an abrupt decrease in kidney function and leads to significant mortality, as high as 23% to 35% in critically ill patients.[1–3] The pathogenic mechanisms of AKI include inflammation, organ crosstalk, and many other processes.[4,5] AKI can lead to chronic kidney disease (CKD).[6] There is no specific therapy for AKI except for supportive care and dialysis. The bidirectional relationship between kidney immune cells, gut microbiota, and short-chain fatty acids (SCFAs) has recently emerged as an important mechanism in AKI.[7,8] Immune cells, particularly T cells, mediate kidney injury and repair from AKI, and have been invoked in microbiome effects.[9–12]

The gut microbiota has an important role in preserving homeostasis in the human gastrointestinal tract, while contributing to mucosal immunity and nutrient metabolism in the intestine.[13,14] Gut and kidney communicate via microbiota-derived metabolites and immune systems.[15,16] Despite many reports on the relationships between kidney disease and the gut microbiota,[17–20] the precise mechanisms remain unknown. The direct link between gut microbiome and kidney in the context of AKI was initially demonstrated when germ-free (GF) mice with ischemic AKI exhibited worsened

kidney injury with kidney T-cell changes compared with normal mice.[21] Fecal transplantation from normal mice to GF mice reversed the AKI phenotypes, directly implicating a role for the gut microbiome. Gut microbiota depletion with broad-spectrum antibiotics (ABX) before ischemia-reperfusion (IR) injury in mice significantly attenuated renal damage.[22]

Although previous studies assessed the effect of antibiotic treatment in prevention models, the effect of antibiotic treatment on kidney repair when given *after* severe AKI has not been investigated. This is important because most patients are diagnosed after the injury causing AKI, and severe AKI leads to pronounced and short- and long-term effects. We therefore used a murine model of severe AKI and administered ABX after the insult. We found amoxicillin accelerated recovery of glomerular filtration rate (GFR) and decreased kidney fibrosis when given after severe AKI. Other ABX had variable effects. We explored both kidney and gut T cells as well as potential cellular and molecular mediators, including SCFAs. These findings can lead to clinical trials for ABX-based therapy for treating AKI, enhance post-AKI kidney repair, and reduce AKI to CKD progression. These data are also relevant for other acute organ injuries, such as myocardial infarction and stroke.

METHODS

Experimental animals

Male C57BL/6J (wild-type), B6.129S2-Cd4tm1Mak/J ($CD4^{-/-}$), and B6.129S2-Cd8atm1Mak/J ($CD8^{-/-}$) mice were housed under specific pathogen-free conditions. C57BL/6J GF mice were maintained at the Johns Hopkins GF mouse core facility.

Murine AKI models

In the severe unilateral IR injury (UIRI)–induced AKI model, the left renal pedicle was bluntly dissected and clamped for 50 minutes before release. In the moderate bilateral IR injury (BIRI) model, both renal pedicles were clamped for 30 minutes before release. In the cisplatin AKI model, a single 25-mg/kg dose of cisplatin was injected in mice i.p.

End points

GFR was measured by transcutaneous fluorescein isothiocyanate–labeled sinistrin or estimated by serum creatinine. Kidney histology was evaluated by hematoxylin and eosin and Masson trichrome staining. Kidney and colon immune cells were evaluated by tissue digestion and flow cytometry. Profibrotic genes were measured using reverse transcriptase polymerase chain reaction. SCFAs were measured by gas chromatography/mass spectroscopy. Gut microbiota were quantified with 16S rRNA gene sequencing. Detailed information about study design, animal experiments, methods, and statistics is provided online in the Supplementary Methods.

RESULTS

ABX administered after severe ischemic injury improved kidney function and reduced fibrosis

Different ABXs were administered orally after severe UIRI (Figure 1a). There were significant increases in GFR in mice treated with amoxicillin at 1 (900 ± 25.9 vs. 748.7 ± 31.24 μl/min per 100 g; $P < 0.01$), 2 (922.7 ± 28.8 vs. 707 ± 24.6 μl/min per 100 g; $P < 0.01$), 3 (921.6 ± 21.3 vs. 810.5 ± 25.8 μl/min per 100 g; $P < 0.01$), and 4 (931.3 ± 23.4 vs. 757.6 ± 24.3 μl/min per 100 g; $P < 0.01$) weeks after UIRI compared with control (Figure 1b and c). Mice treated with metronidazole had a significant increase in GFR at 1 (968.5 ± 25.8 vs. 748.7 ± 31.24 μl/min per 100 g; $P < 0.01$) and 2 (855.9 ± 41.7 vs. 707 ± 24.6 μl/min per 100 g; $P < 0.01$) weeks after UIRI. However, no significant differences were observed at 3 and 4 weeks compared with the control (Figure 1b and c). There were significant increases in GFR in mice treated with combination of ABX at 1 (982.6 ± 34.6 vs. 748.7 ± 31.24 μl/min per 100 g; $P < 0.001$), 2 (916.1 ± 48.5 vs. 707 ± 24.6 μl/min per 100 g; $P < 0.01$), 3 (972 ± 34.8 vs. 810.5 ± 25.8 μl/min per 100 g; $P < 0.01$), and 4 (938.4 ± 24.1 vs. 757.6 ± 24.3 μl/min per 100 g; $P < 0.05$) weeks after UIRI compared with control (Figure 1b and c).

Mice treated with amoxicillin had decreased fibrosis in kidney cortex compared with metronidazole (5.5 ± 1.2 vs. 22.3 ± 3; $P < 0.01$) and combination of ABX (5.5 ± 1.2 vs. 25.1 ± 7.6; $P < 0.01$) as well as outer medullary regions compared with the control (6.5 ± 0.6 vs. 13.8 ± 1.2; $P < 0.01$), metronidazole (6.5 ± 0.6 vs. 13.6 ± 1.6; $P < 0.01$), and combination of ABX (6.5 ± 0.6 vs. 15.5 ± 1.7; $P < 0.01$; Figure 1d and e). The expression of profibrotic genes *TGFβ*, collagen, type I α 1 (*Col1α1*), and α-smooth muscle actin (*αSMA*) was used to assess the ABX effect on kidney fibrosis–associated genes (Table 1). *Col1α1* mRNA expression was significantly increased in the kidneys of mice treated with combination of ABX (4.1 ± 1.2 relative fold change) compared with metronidazole (1.7 ± 0.2 relative fold change; $P < 0.05$), amoxicillin (0.8 ± 0.1 relative fold change; $P < 0.001$), and control (1.1 ± 0.1 relative fold change; $P < 0.01$) groups. Although no differences were observed in mRNA expression of *TGFβ* profibrotic gene between the groups, mice treated with metronidazole exhibited increased *aSMA* mRNA expression compared with amoxicillin-treated mice (1.6 ± 0.2 vs. 0.8 ± 0.1 relative fold change; $P < 0.01$; Supplementary Figure S1).

Antibiotic effects on stool microbiome after AKI

We compared the effects of different antibiotic combinations on the gut microbiome after AKI with 16S rRNA gene-sequencing analysis (Table 2 and Figure 2a). α Diversity analysis using Chao1, Shannon, and InvSimpson revealed species richness in mice treated with amoxicillin after 1 week from UIRI was higher than controls, which was at the same level as baseline (Figure 2b). Principal coordinate analysis of the samples using the Bray-Curtis distance metric A exhibited clearly different patterns (Figure 2c). Genus-level taxonomic assignment revealed increases in abundance of *Alistipes*, *Odoribacter*, and *Stomatobaculum* in mice treated with amoxicillin at 1 week after UIRI compared with baseline, control, and those treated with metronidazole and combination of ABX. Metronidazole reduced most bacterial species, except *Barnesiella* and *Parasutterella*. Combination of ABX reduced the abundance of *Barnesiella* (Figure 2d).

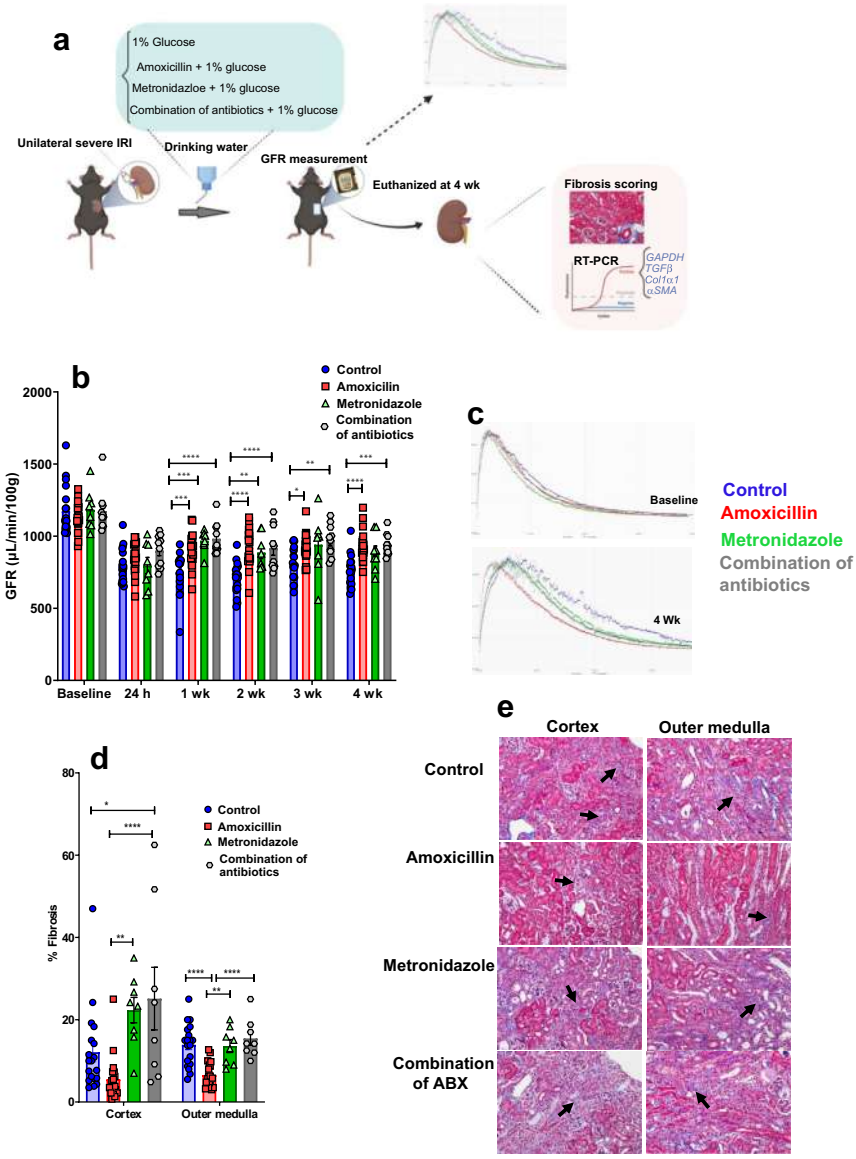


Figure 1 | Amoxicillin provided the most consistent protection of acute kidney injury to chronic kidney disease compared with metronidazole or a combination of antibiotics (ABX). (**a**) Experimental design. (**b**) Kidney function at 24 hours and 1, 2, 3, and 4 weeks after unilateral ischemia-reperfusion injury (UIRI) in control mice (n = 9) versus mice treated with amoxicillin (n = 10), metronidazole (n = 8), and a combination of ampicillin, metronidazole, neomycin, and vancomycin ABX (n = 10). (**c**) Representative glomerular filtration rate (GFR) at baseline and after 4 weeks after UIRI in control (free ABX), and mice treated with amoxicillin, metronidazole, and combination of ABX. (**d**) Kidney fibrosis scores of kidney cortex and outer medulla in mice served as control (n = 9) versus mice treated with amoxicillin, metronidazole, and combination of antibiotics at 4 weeks after UIRI. (**e**) Representative kidney fibrosis (arrows) in mice kidneys 4 weeks after UIRI. Data in (**b**) and (**d**) displayed as mean ± SEM; multiple comparisons by 2-way analysis of variance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. RT-PCR, reverse transcriptase polymerase chain reaction. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Differentially abundant amplicon sequence variants were identified between baseline and 1-week samples (all antibiotic groups; Figure 2e) as well as baseline and control, amoxicillin, metronidazole, and combination of ABX (Supplementary Figure S2) using DESeq2 on normalized counts (adjusted $P < 0.05$) and visualized by volcano plot.

Amoxicillin accelerated kidney tissue repair in mice

Histologic evaluation on the kidney sections stained with Masson trichrome after 1, 2, and 4 weeks of reperfusion was performed to assess amoxicillin effects on kidney structure. There was significantly higher percentage of fibrosis in both cortex and outer medullary regions of control mice compared with those treated with amoxicillin at 2 (9 ± 0.6 vs. 5.1 ± 1.1 [$P < 0.05$] and 13.6 ± 1.4 vs. 6.8 ± 0.7 [$P < 0.01$], respectively) and 4 weeks (12.1 ± 2.3 vs. 5.5 ± 1.2 [$P < 0.05$] and 13.9 ± 1.2 vs. 6.6 ± 0.7 [$P < 0.01$], respectively) after UIRI (Figure 3).

We measured SCFAs in plasma by gas chromatography/mass spectroscopy. There were no significant differences in

Table 1 | Forward and reverse primers for *TGFβ*, *Col1α1*, and *αSMA* profibrotic genes

Gene	Forward primer	Reverse primer
GAPDH	CCTTCCGTGTTCCTACC	CCACCTGGTCCTCAGTGTA
TGFβ	GCAACAATTCCTGGCGTTACC	CGAAAGCCCTGTATTCCGTCT
Col1α1	TGACTGGAAGAGCGGAGAGT	GTTCGGGCTGATGTACCAGT
αSMA	AGGGCTGGAGAATTGGATCT	CCAGCAAAGGTCAGAGAAGG

propionic acid, succinic acid, lactic acid, isovaleric acid, and butyric acid levels at 1 week after UIRI in control versus mice treated with amoxicillin. However, a significant decrease (183.1 ± 8.7 vs. 238.2 ± 7.7 μM; $P < 0.001$) was observed in acetic acid in mice treated with amoxicillin compared with the control (Supplementary Figure S3).

Amoxicillin effects on kidney and colon T-lymphocyte populations

Amoxicillin effects on T-lymphocyte populations in kidney and gut were measured with tissue digestion followed by flow cytometry. We observed significant increases in the proportion of $CD8^+$ T cells (61.17 ± 3.4 vs. 46.37 ± 2; $P < 0.01$) and programmed cell death protein 1 $(PD1)^+CD8^+$ (37.56 ± 5.2 vs. 15.6 ± 4; $P < 0.01$) in kidneys of mice treated with amoxicillin compared with control. The proportion and absolute number of $CD4^+$ T cells (31.35 ± 2.8 vs. 45.33 ± 1.9 [$P < 0.01$] and 6938 ± 929 vs. 17,173 ± 3114 [$P < 0.01$], respectively), interleukin (IL)-17^+ $CD4^+$ (2.22 ± 0.19 vs. 5.22 ± 0.86 [$P < 0.01$] and 153.7 ± 26.8 vs. 912.13 ± 290 [$P < 0.05$]), as well as tumor necrosis factor $(TNF)^+$ double-negative T cells (24.18 ± 1.61 vs. 31 ± 1.1 and 368.2 ± 54 vs. 902.7 ± 135 [$P < 0.01$], respectively) were significantly decreased in kidneys of mice treated with amoxicillin compared with control (Figure 4a). The proportion and absolute number of macrophages were significantly decreased (3 ± 0.5 vs. 4.9 ± 0.7; $P < 0.05$) in kidneys of mice treated with amoxicillin compared with control (Supplementary Figure S4). No significant differences were observed in the proportion and absolute number of B cells, dendritic cells, and neutrophils in kidney of mice treated with amoxicillin at 4 weeks after UIRI (Supplementary Figure S4).

The proportion of $CD4^+$ T cells was significantly increased (33 ± 1.9 vs. 23 ± 2.4; $P < 0.01$) in colon of mice treated with amoxicillin compared with the control, whereas the proportion of $CD8^+$ T (41.3 ± 0.6 vs. 52.3 ± 2.1; $P < 0.001$) cells as well as IL-17^+CD4^+ T cells (3.23 ± 0.4 vs. 5.21 ± 0.6; $P < 0.05$) was significantly decreased (Figure 4b). However, the absolute numbers of $CD4^+$, $CD8^+$, and IL-17^+CD4^+ T cells were comparable (Figure 4b). No differences were observed in proportions of kidney and colon T-cell receptor beta–positive cells, $PD1^+CD4^+$, and double-negative T cells between groups (data not shown). We observed a significant decrease in IL-17 in whole kidney tissue of mice treated with amoxicillin compared with control (3.4 ± 0.2 vs. 5 ± 0.4; $P < 0.01$) 2 weeks after UIRI (Figure 5a). No significant differences were observed in TNF-α in kidney tissue of mice treated with amoxicillin compared with the control at 2 and 4 weeks after UIRI (Figure 5a). There was a trend for decrease in IL-17 levels in serum of mice treated with amoxicillin compared with the control at 4 weeks after UIRI; however, it was not statistically significant (Figure 5b). Serum IL-17 and TNF-α levels were comparable between the groups at 4 weeks after UIRI (Figure 5b).

Table 2 | Forward and reverse costume primers for 16s analysis

Variable	Primer
16S V3 forward PCR primer	AATGATACGGCGACCACCGAGATCTACACTATGG TAATTGTTCCTACGGGAGGCAGCAGT
16S reverse indexed PCR primer	CAAGCAGAAGACGGCATACGAGATGNNNNNNNN NNNNTCAGTCAGCCGGACTACHVGGGTWT CTAAT
Read 1 primer	TATGGTAATTGTGTGCCAGCMGCCGCGGTAA
Read 2 primer	AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
Index sequencing primer	ATTAGAWACCCBDGTAGTCCGGCTGACTGACT

PCR, polymerase chain reaction.

$CD8^+$ T cells are a mediator of amoxicillin effects on kidney repair

To directly test the mechanistic role for $CD8^+$ and $CD4^+$ T cells in amoxicillin-mediated enhancement of kidney repair, we performed UIRI on $CD8^{-/-}$ and $CD4^{-/-}$ mice and treated them with amoxicillin followed by monitoring GFR at 24 hours and 1, 2, 3, and 4 weeks after UIRI (Figure 6a and Figure 7a). Amoxicillin did not improve GFR of kidneys of $CD8^{-/-}$ mice compared with the control at different time points (Figure 6b and c). In addition, percentage of fibrosis in cortex and outer medullary regions as well as the expression of *Col1α1*, *TGFβ*, and *aSMA* profibrotic genes were comparable between the groups (Figure 6, d–h). However, there was still a significant improvement in kidney function of $CD4^{-/-}$ mice treated with amoxicillin compared with the control at 3 (958.4 ± 34.7 vs. 735.4 ± 66 μl/min per 100 g; $P < 0.05$) and 4 (1028.1 ± 46.0 vs. 802.3 ± 42.5 μl/min per 100 g; $P < 0.05$) weeks after UIRI (Figure 7b and c). No significant differences were observed in expression of *Col1α1*, *TGFβ*, and *aSMA* profibrotic genes between the groups (Figure 7d–f).

Amoxicillin accelerated kidney repair through gut microbiota

To directly test if protective mechanisms of amoxicillin were through the gut microbiota, we tested the effect of amoxicillin on GF mice (Figure 8a). The UIRI surgery in GF mice was performed in a GF facility with longitudinal monitoring of GF status following the surgery. Microbial testing using both fecal cultures and molecular analysis confirmed mice remained GF at 24 hours, 1 week, and before sacrificing at 2 weeks (Figure 8b and c). We observed no significant differences in GFR in mice treated with amoxicillin compared with control at 2 weeks (Figure 8d and e). No differences were observed in expression of profibrotic genes *TGFβ*, *Col1α1*, and *aSMA* in GF mice with or without amoxicillin (data not shown). The percentages of T-cell receptor, $CD4^+$, $CD8^+$, DN, and PD1 on $CD4^+$, $CD8^+$, and DNT cells were comparable between the groups (Figure 9).

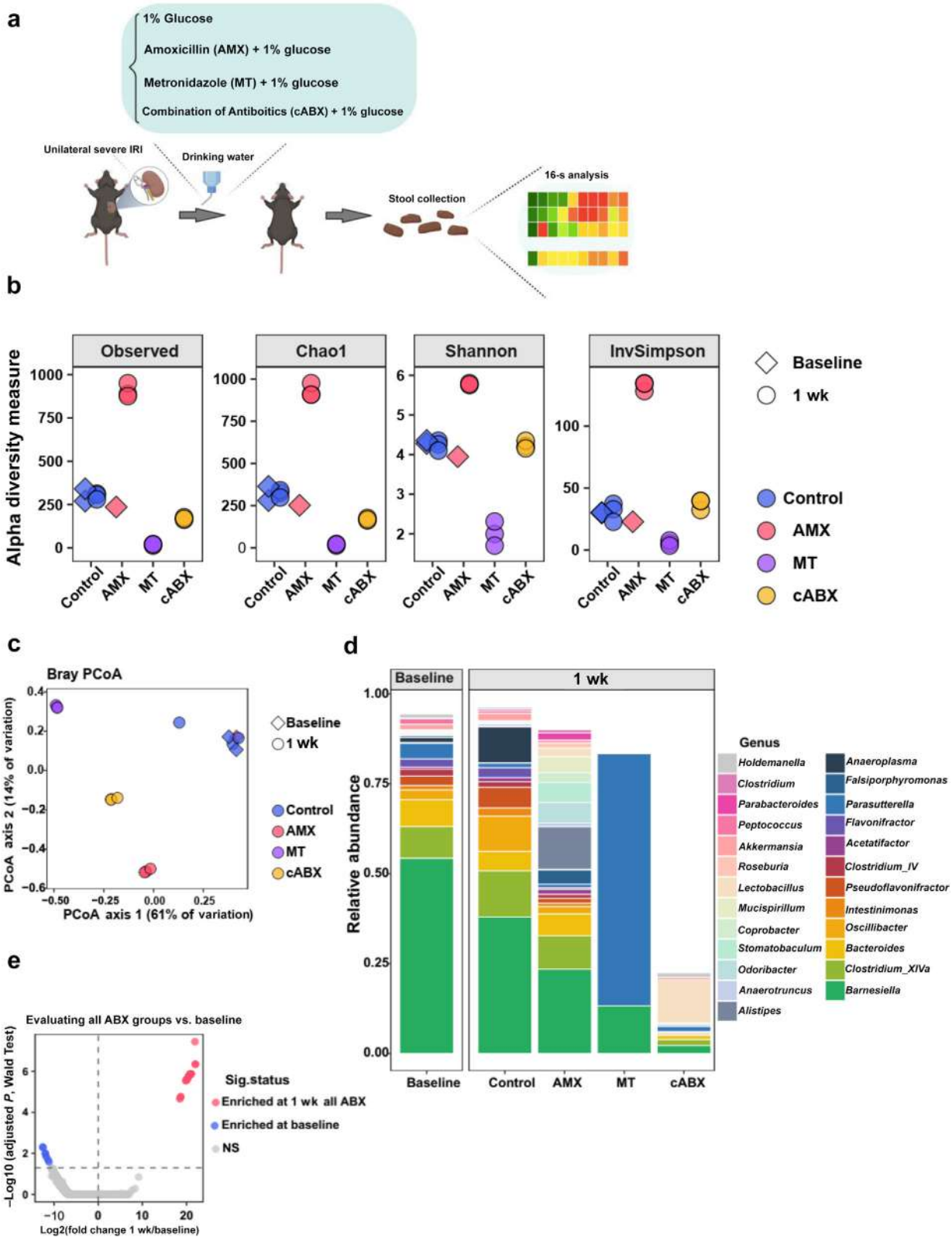


Figure 2| Gut microbiota analysis demonstrated major changes by amoxicillin (AMX) in severe acute kidney injury. (**a**) Experimental design. (**b**) α Diversity estimators by time point and treatment (AMX, metronidazole [MT], or combination of antibiotics [cABX]), including total amplicon sequence variants (ASVs; observed), the Chao1 richness estimator, the Shannon diversity index, and the inverse Simpson index. (**c**) Principal coordinates analysis (PCoA) of the samples using the Bray-Curtis distance metric. Axis labels provide the percentage of variation explained by each principal coordinate axis. (**d**) Genus-level stacked bar plot for the most abundant genera across the sample set. (**e**) Volcano plot displaying differentially abundant ASVs between baseline samples and the all-antibiotics groups (amoxicillin, metronidazole, and combination of antibiotics [ampicillin, metronidazole, vancomycin, and neomycin]) at 1 week. Horizontal dashed line indicates a false discovery rate–adjusted (adj) *P* value of 0.05. IRI, ischemia-reperfusion injury; NS, not significant.

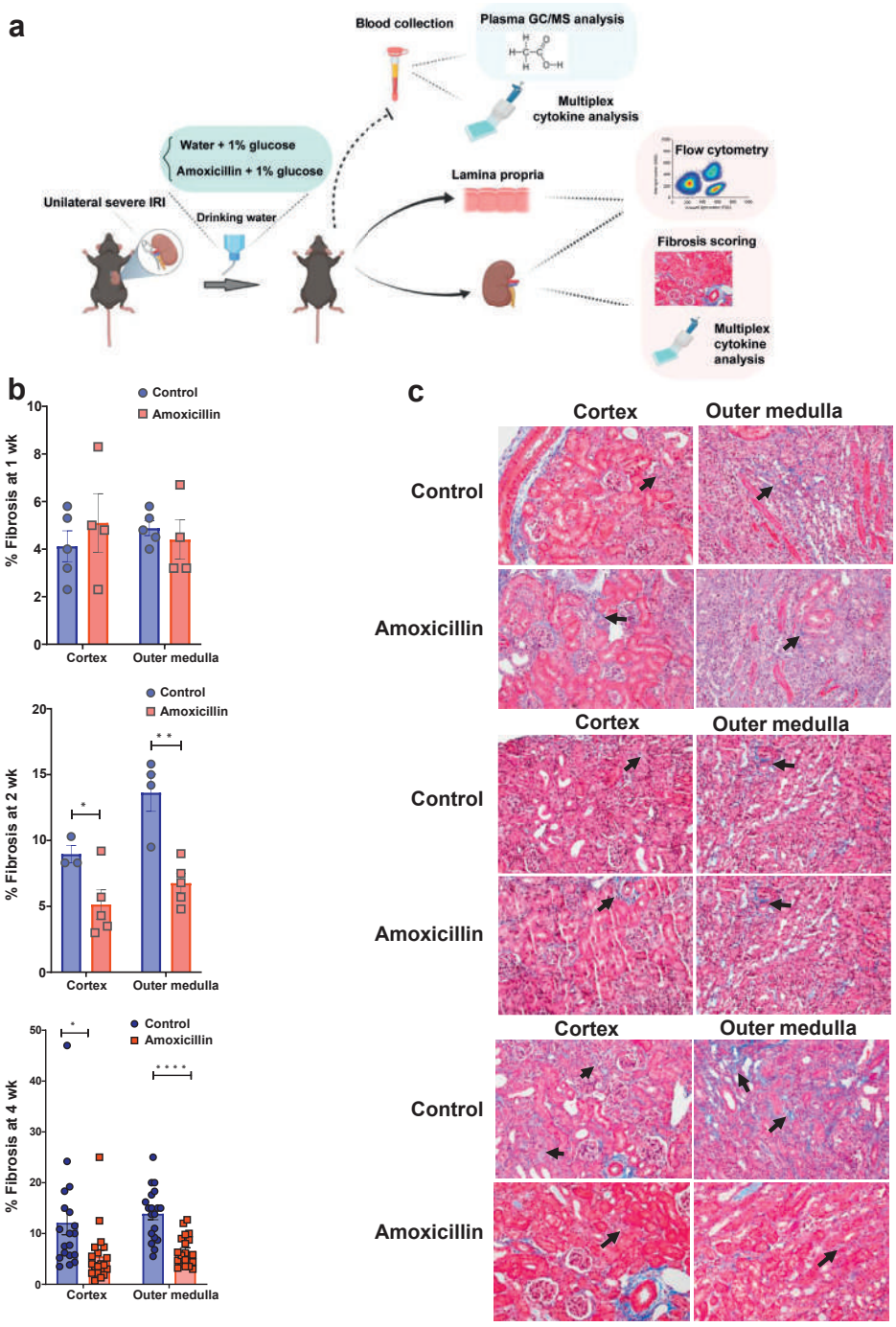


Figure 3 | Effects of amoxicillin on mice kidney during severe ischemia-reperfusion injury (IRI) at the different time points. (**a**) Experimental design. (**b**) Kidney fibrosis scores of kidney cortex and outer medulla in mice serving as control versus mice treated with amoxicillin at 1, 2, and 4 weeks after unilateral IRI (UIRI). (**c**) Representative kidney fibrosis (arrows) in mice kidneys at 1, 2, and 4 weeks after UIRI. Data in (**b**) displayed as mean ± SEM; multiple unpaired *t* test. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$. GC/MS, gas chromatography/mass spectroscopy. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Thus, absence of gut microbiome in GF mice removed the protective effect of amoxicillin.

Fecal microbiota transplantation (FMT) has been studied as an effective and safe intervention for intestinal microbiota reprogramming.[23] FMT from amoxicillin-treated mice (donor) to GF mice via oral gavage after severe UIRI (Figure 10 a) led to a significant decrease in percentage of fibrosis in their kidney outer medulla (24 ± 1.9 vs. 46.6 ± 5.3; $P < 0.05$) compared with control (Figure 10d and e). No significant changes were observed in the percentage of fibrosis in outer medulla of kidney of mice treated with FMT from normal mice compared with other groups. No significant changes were observed in the percentage of fibrosis in cortex between the groups (Figure 10d and e). A trend for GFR

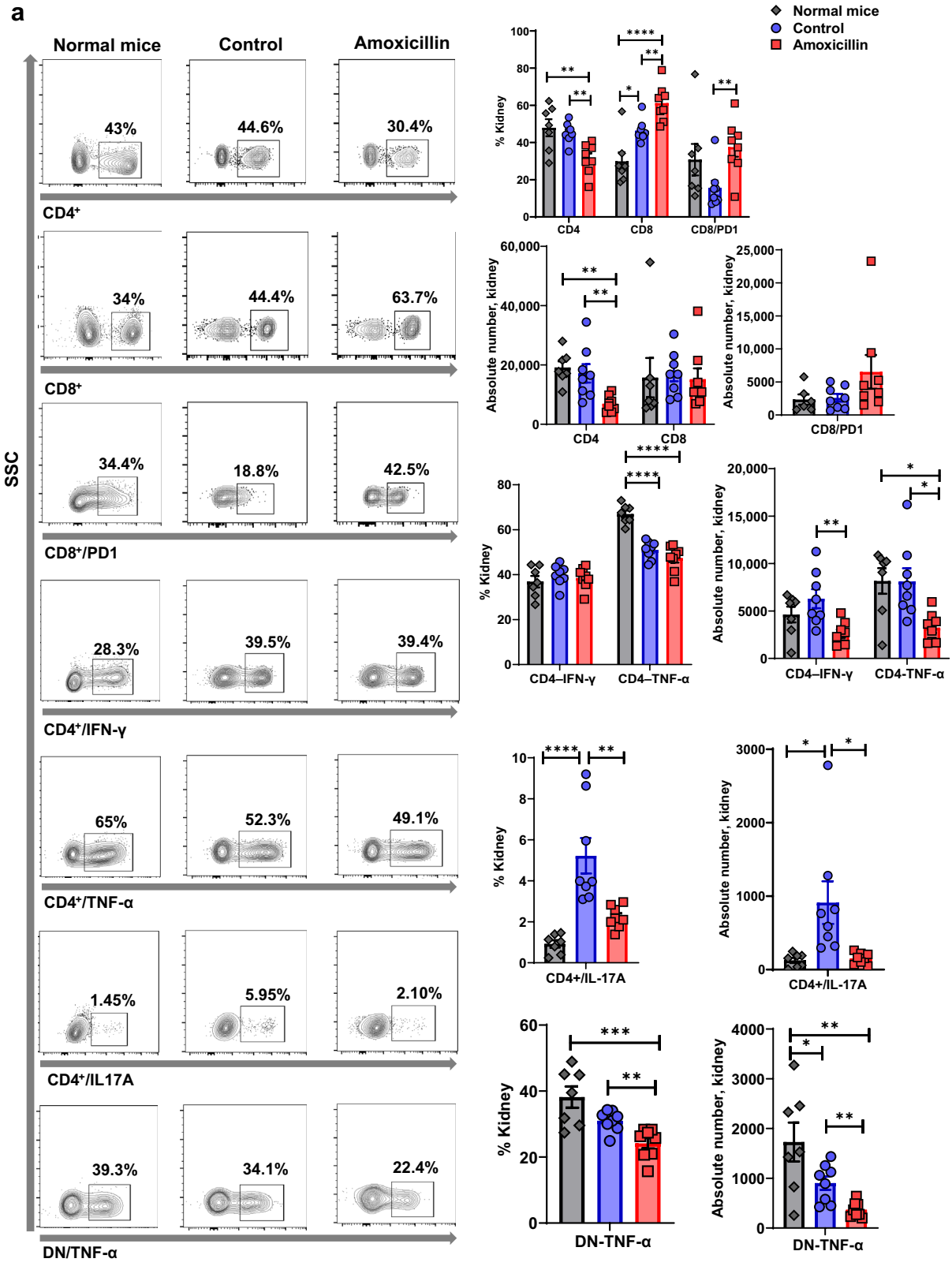


Figure 4| Effects of amoxicillin on mice kidney and colon T lymphocytes during severe ischemia-reperfusion injury (IRI). Proportion and absolute number of T lymphocytes in (**a**) kidney and (continued)

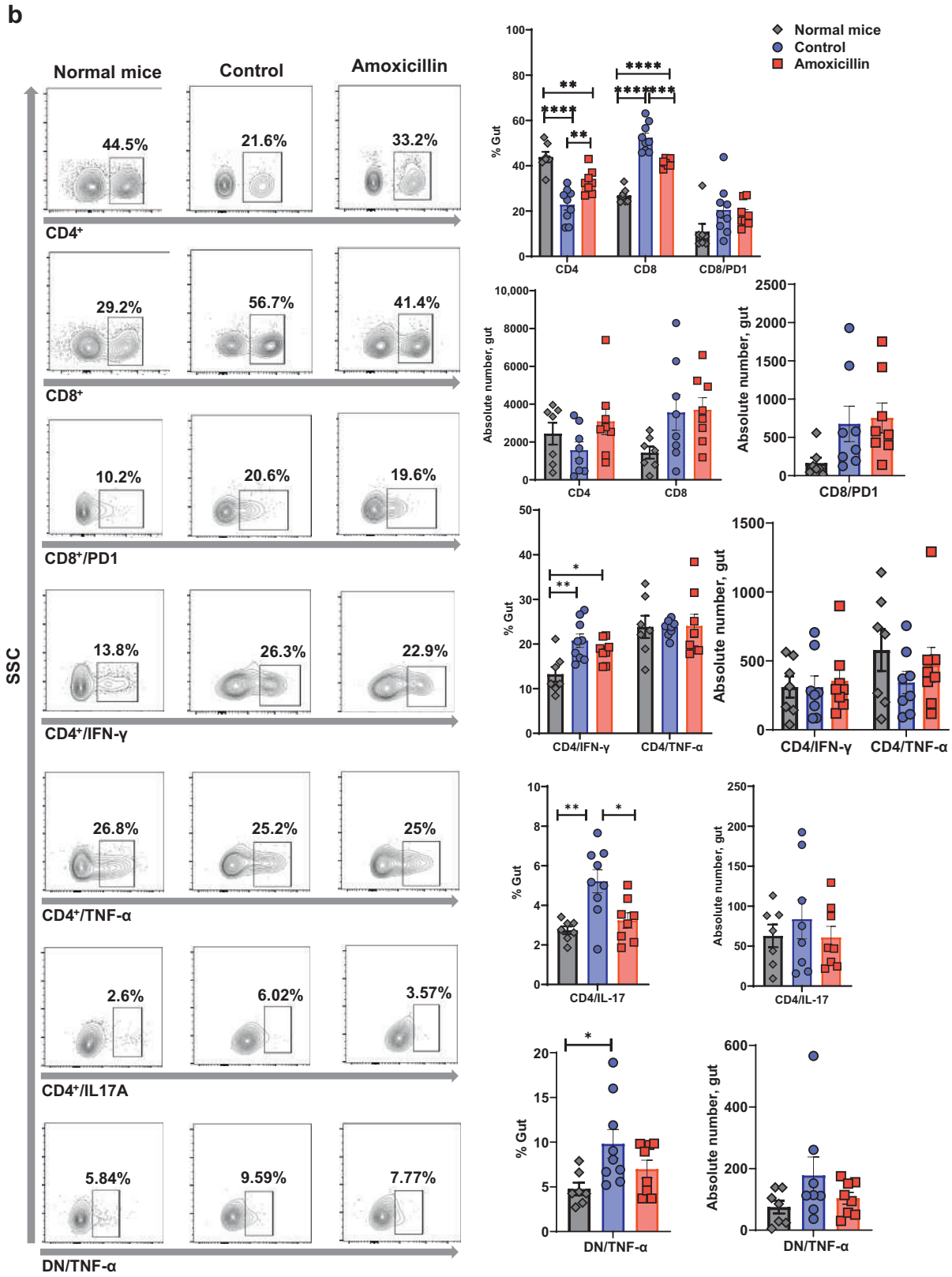


Figure 4| (continued) (**b**) gut of normal mice (n = 7) as well as control (free of antibiotics; n = 7), and mice treated with amoxicillin (n = 10) at 4 weeks after unilateral IRI. Data displayed as mean ± SEM; multiple comparisons by 1-way analysis of variance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. DN, double negative; IFN-γ, interferon-γ; PD1, programmed cell death protein 1; SSC, standard saline citrate; TNF-α, tumor necrosis factor-α.

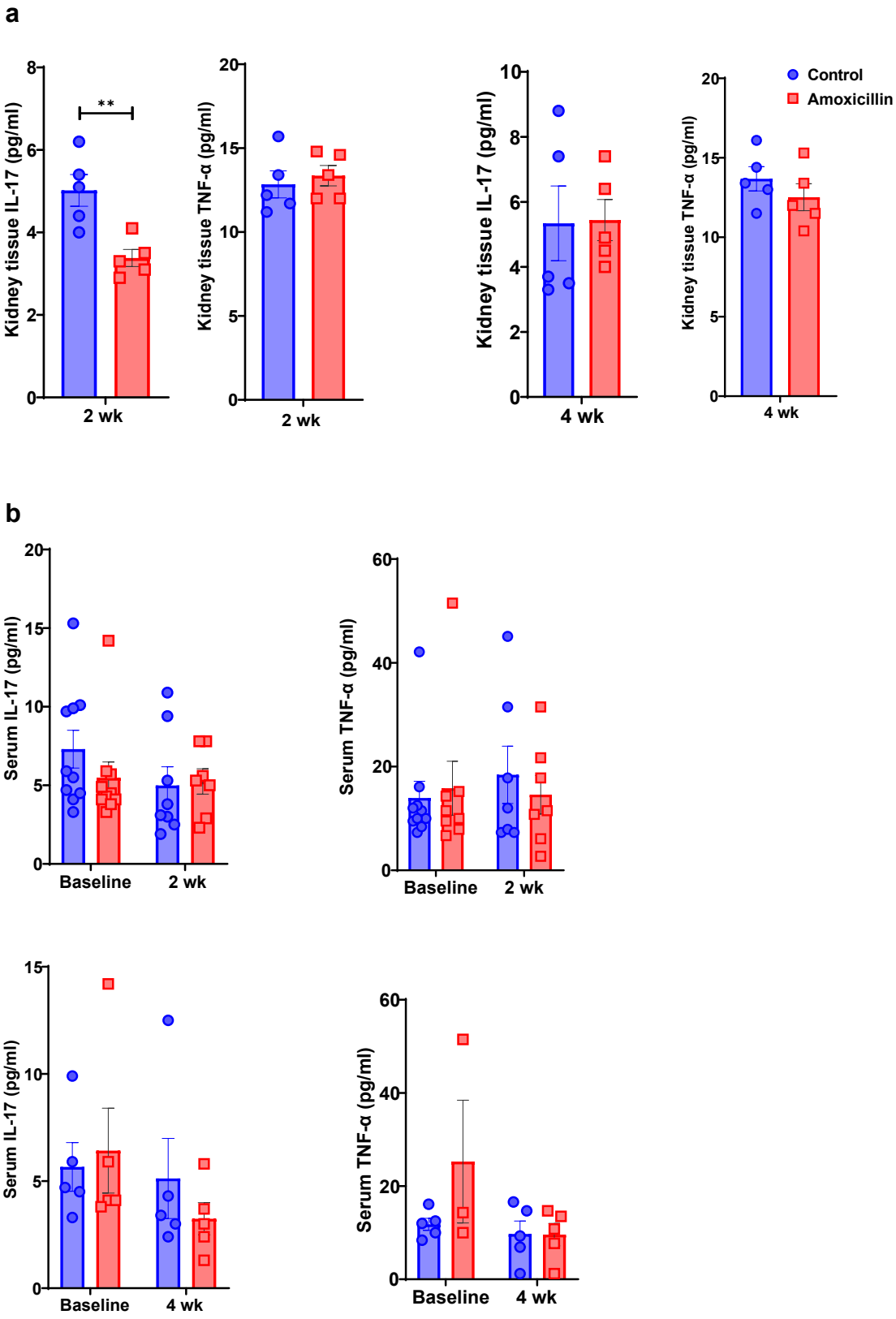


Figure 5 | Effects of amoxicillin on IL-17 and tumor necrosis factor (TNF)-α in protein level in mice serum and kidney during severe ischemia-reperfusion injury (IRI). Levels of IL-17 and TNF-α at 2 and 4 weeks after unilateral IRI in (**a**) kidney and (**b**) serum of mice treated with amoxicillin compared with control. Data in (**a**) displayed as mean ± SEM; multiple unpaired *t* test. $^{**}P < 0.01$.

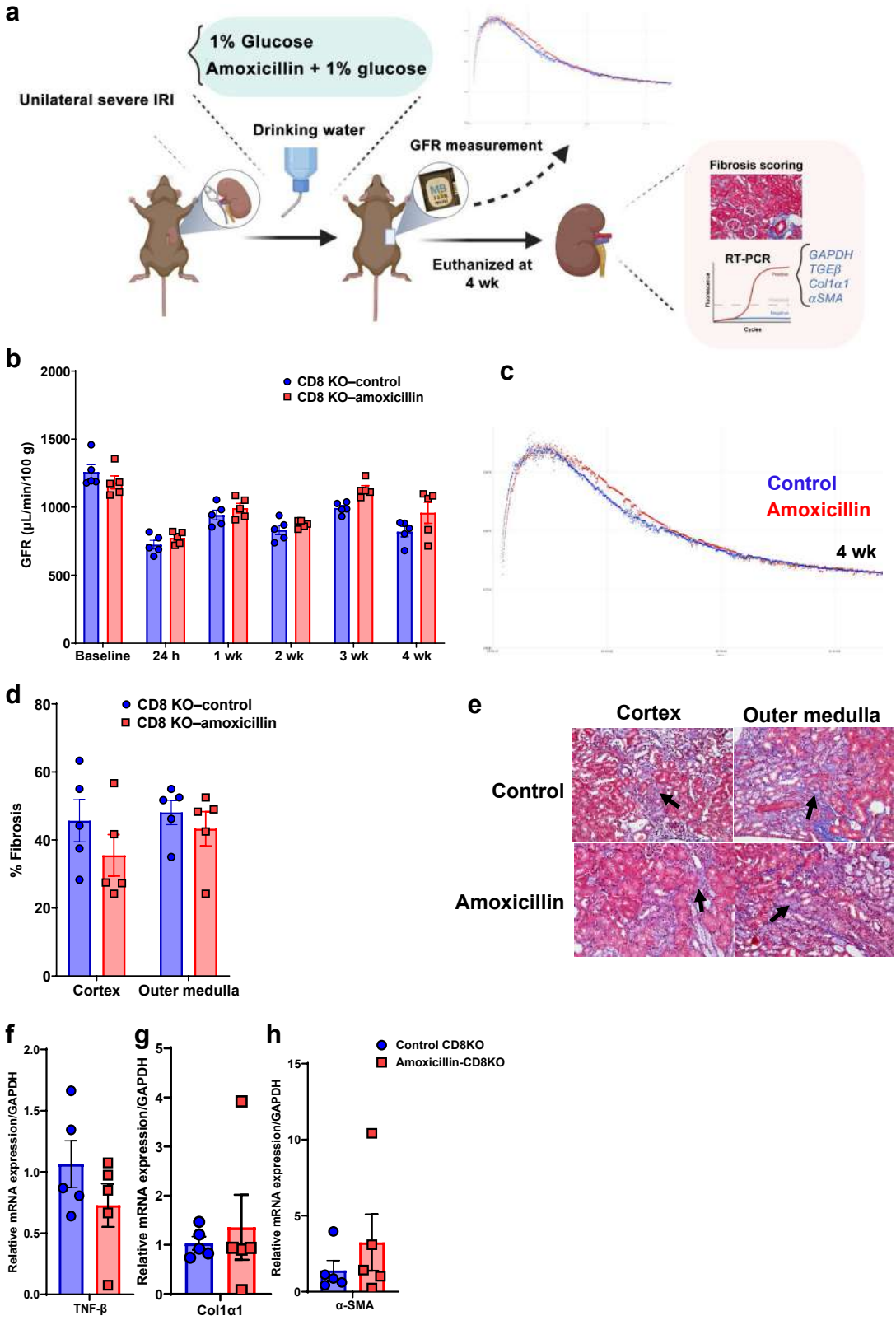


Figure 6 | Effects of amoxicillin on CD8 knockout (KO) mice kidney during severe ischemia-reperfusion injury (IRI). (**a**) Experimental design. (**b**) Kidney function at 24 hours and 1, 2, 3, and 4 weeks after unilateral IRI (UIRI) in CD8 KO control mice (n = 5) versus CD8 KO mice treated with amoxicillin (n = 5). (**c**) Representative glomerular filtration rate (GFR) at baseline and after 4 weeks after UIRI in CD8 KO control (free of antibiotics), and CD8 KO mice treated with amoxicillin. (**d**) Kidney fibrosis scores of kidney cortex and outer medulla in CD8 KO mice serving as control (n = 5) versus CD8 KO mice treated with amoxicillin (n = 5) at 4 weeks after UIRI. (**e**) Representative kidney (continued)

improvement by FMT was not statistically significant (Figure 10b and c).

The proportion of kidney $CD8^+$ T cells was significantly increased (62 ± 3.9 vs. 41.5 ± 3.6; $P < 0.01$) in kidneys of mice treated with FMT from normal mice. Proportion and absolute number of forkhead box p3 $(Foxp3)^+CD8^+$ T cells were significantly increased (2.79 ± 0.6 vs. 0.8 ± 0.3; $P < 0.05$) in kidneys of mice treated with FMT from amoxicillin-treated mice compared with control. No significant changes were observed in proportion and absolute number of $CD4^+$, $CD4^+Foxp3^+$, dendritic cells, and B cells (Figure 10f).

Amoxicillin pretreatment protects mice against kidney bilateral IR injury

To study whether amoxicillin treatment can affect baseline kidney function in the absence of kidney injury, we treated mice with amoxicillin and monitored the kidney function for 4 weeks. We observed no significant differences in baseline GFR in kidneys of mice treated with amoxicillin compared with control (Supplementary Figure S5A and B). Proportion and absolute numbers of T-cell receptor$^+$, $CD4^+$, $CD8^+$, DN, $PD1^+CD4^+$, $CD8^+$, and DNT cells were comparable between the groups (Supplementary Figure S5C). Given the beneficial effects of amoxicillin administered after severe ischemic injury on kidney function and structure, we pursued studies on the preventive effect of amoxicillin in moderate BIRI and cisplatin-induced AKI models (Figures 11a and 13a). Serum creatinine level was significantly decreased (1.5 ± 0.3 vs. 2.3 ± 0.1; $P < 0.05$) in mice pretreated with amoxicillin for 2 weeks before BIRI compared with control (Figure 11b). Kidney damage scored with the percentage of necrotic tubules among total tubules at 24 hours after BIRI revealed a significant decrease (63.9 ± 2 vs. 75.9 ± 2.1; $P < 0.01$) in percentage necrosis in outer medullary regions of kidneys of mice treated with amoxicillin compared with control (Figure 11c and d). Proportion and absolute number of $PD1^+CD8^+$ T cells were significantly increased (35.2 ± 6.4 vs. 10.3 ± 1.5 [$P < 0.01$] and 511.1 ± 175.2 vs. 85.4 ± 25 [$P < 0.05$], respectively) in kidneys of mice treated with amoxicillin compared with control (Figure 11e). No significant difference was observed in serum creatinine level of mice pretreated with amoxicillin for 1 week before BIRI-induced AKI and the control group (Figure 12a). Serum creatinine and proportion of T lymphocytes, except CD8 T cells, were comparable between mice pretreated with amoxicillin for 2 weeks before cisplatin injection to the control (Figure 13b and c).

DISCUSSION

Experimental studies focusing on the early injury phase and prevention strategies in mild to moderate AKI are essential. However, most patients present after the initiation of AKI, and severe AKI leads to multiple consequences as well as long-term risk of CKD. We therefore used a model of severe AKI in mice for kidney function assessment coupled with tissue molecular and histologic analysis and focused on the challenge of intervening after severe AKI to accelerate recovery. We tested the hypothesis that modifying the gut microbiome would accelerate repair from AKI using ABX (separately or in combination) started *after* severe ischemic injury. We found that post-AKI amoxicillin treatment, compared with other ABX, accelerated kidney functional recovery and mitigated fibrosis. Amoxicillin did not accelerate kidney repair in GF mice, demonstrating that it was working through bacteria. It was not effective in $CD8^{-/-}$ mice, but it was effective in $CD4^{-/-}$ mice, demonstrating amoxicillin mediated its effect, in part, through $CD8^+$ T lymphocytes. Stool microbiome 16s sequencing identified specific bacterial changes for the amoxicillin effects. FMT from amoxicillin-treated mice to GF mice reduced fibrosis. Amoxicillin pretreatment for 2 weeks but not 1 week protected mice against moderate bilateral IR injury–induced AKI.

Comparison of different postsevere AKI antibiotic regimens, including metronidazole, combination of ABX, or amoxicillin, demonstrated that amoxicillin-treated mice had the highest GFR at 4 weeks. Amoxicillin reproducibly reduced the expression of kidney fibrosis and profibrotic genes. Similar attenuation in profibrotic gene expression was reported in mice treated with amoxicillin in a model of chronic rhinosinusitis.[24] Although there are reports of beneficial pretreatment effects of modifying gut microbiota and organ injuries in kidney,[17–20] brain,[25] liver,[26] and heart,[27] we are not aware of studies demonstrating the benefit of postsevere injury microbiota modification to accelerate repair. Depletion of gut microbiota using pretreatment ABX was protective from kidney IR injury in murine models, associated with a reduced maturation of $F4/80^+$ kidney macrophage, monocytes, and an attenuated T-helper 17 and T-helper 1 response.[22,28] Depletion of gut bacteria using combination of ABX for 4 weeks ameliorated kidney fibrosis in unilateral ureteral obstruction mice, and FMT counteracted this effect.[29] Tubular damage, fibrosis, macrophage infiltration in the kidney, expression levels of *Tgfb1*, *Fn1*, and *Col1a1* fibrosis-related genes, as well as *Emr1*, *Il1a*, and *Tnf* inflammation-related genes were less severe in mice treated with adenine diet and combination of ABX than those in the adenine group with no ABX.[30] In another study, amoxicillin pretreatment for 10 days prevented acute ischemic liver injury in mice.[31]

In addition to serum creatinine and GFR, kidney injury marker-1, neutrophil gelatinase-associated lipocalin, and soluble urokinase plasminogen activator receptor are

Figure 6 | (continued) fibrosis (arrows) in mice kidneys 4 weeks after UIRI. (**f**–**h**) mRNA expression of *TGFβ*, *Col1α1*, and *αSMA* profibrotic genes in CD8 KO control and CD8 KO mice treated with amoxicillin 4 weeks after UIRI (n = 5 mice per group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

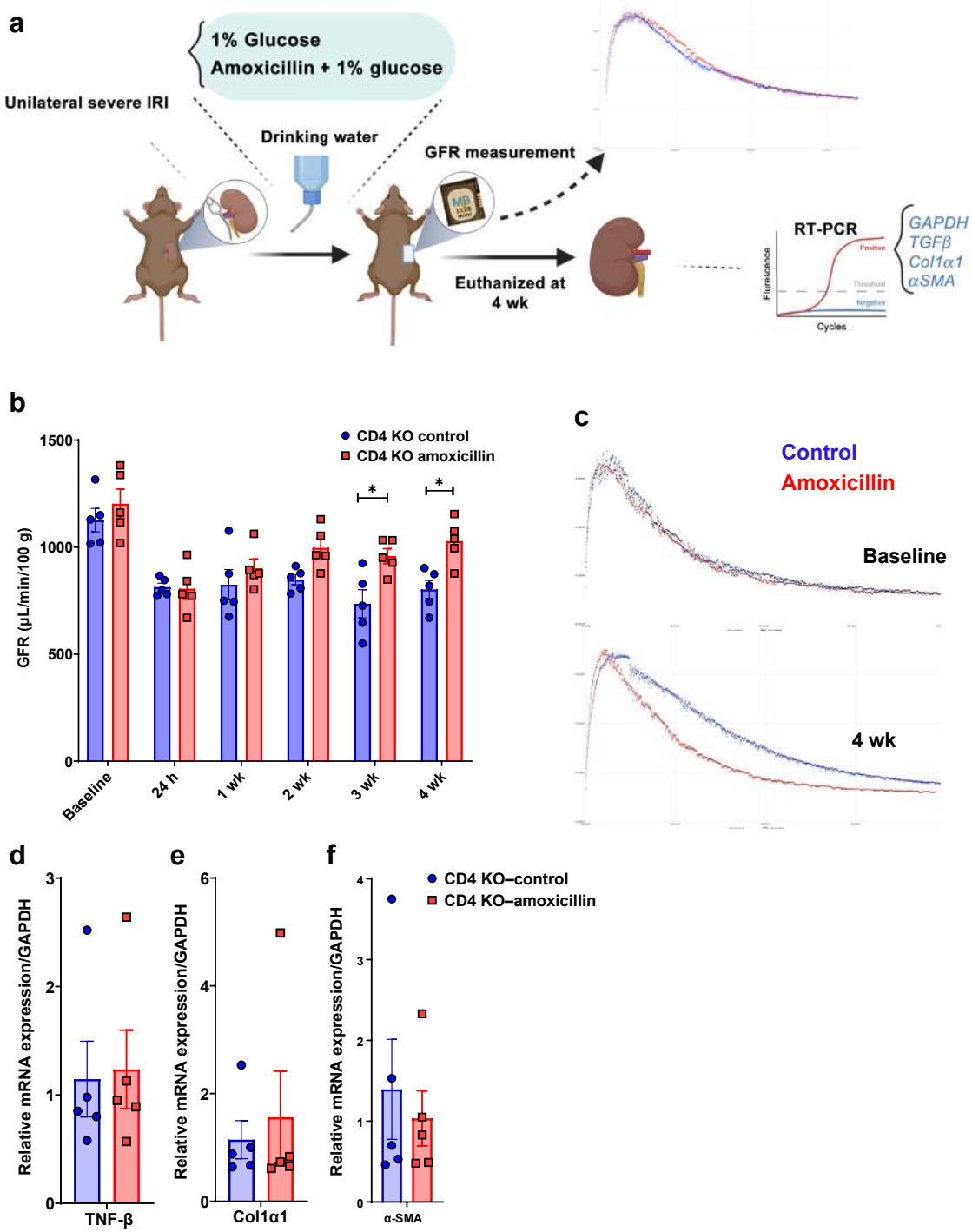


Figure 7 | Effects of amoxicillin on CD4 knockout (KO) mice kidney during severe ischemia-reperfusion injury (IRI). (**a**) Experimental design. (**b**) Amoxicillin remained protective of kidney function after unilateral IRI (UIRI) in CD4 KO (n = 5 mice per each group). (**c**) Representative of glomerular filtration rate (GFR) at baseline and after 4 weeks after UIRI in CD4 KO control and CD4 KO mice treated with amoxicillin. (**d**–**f**) mRNA expression of *TGFβ*, *Col1α1*, and *αSMA* fibrosis genes in CD4-deficient control-treated and CD4-deficient mice treated with amoxicillin at 4 weeks after UIRI (n = 5 mice per group). Data in (**b**) displayed as mean ± SEM; multiple comparisons by 2-way analysis of variance. *$P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction.

biomarkers and pathophysiological mediators of AKI. Depletion of gut bacteria using amoxicillin could be reducing oxidative stress and inflammation by decreasing neutrophil gelatinase-associated lipocalin, kidney injury marker-1, and soluble urokinase plasminogen activator receptor levels. A previous study depleting gut microbiota using combination of ABX led to prevention from kidney I/R injury, as assessed by measuring the kidney expression of neutrophil gelatinase-

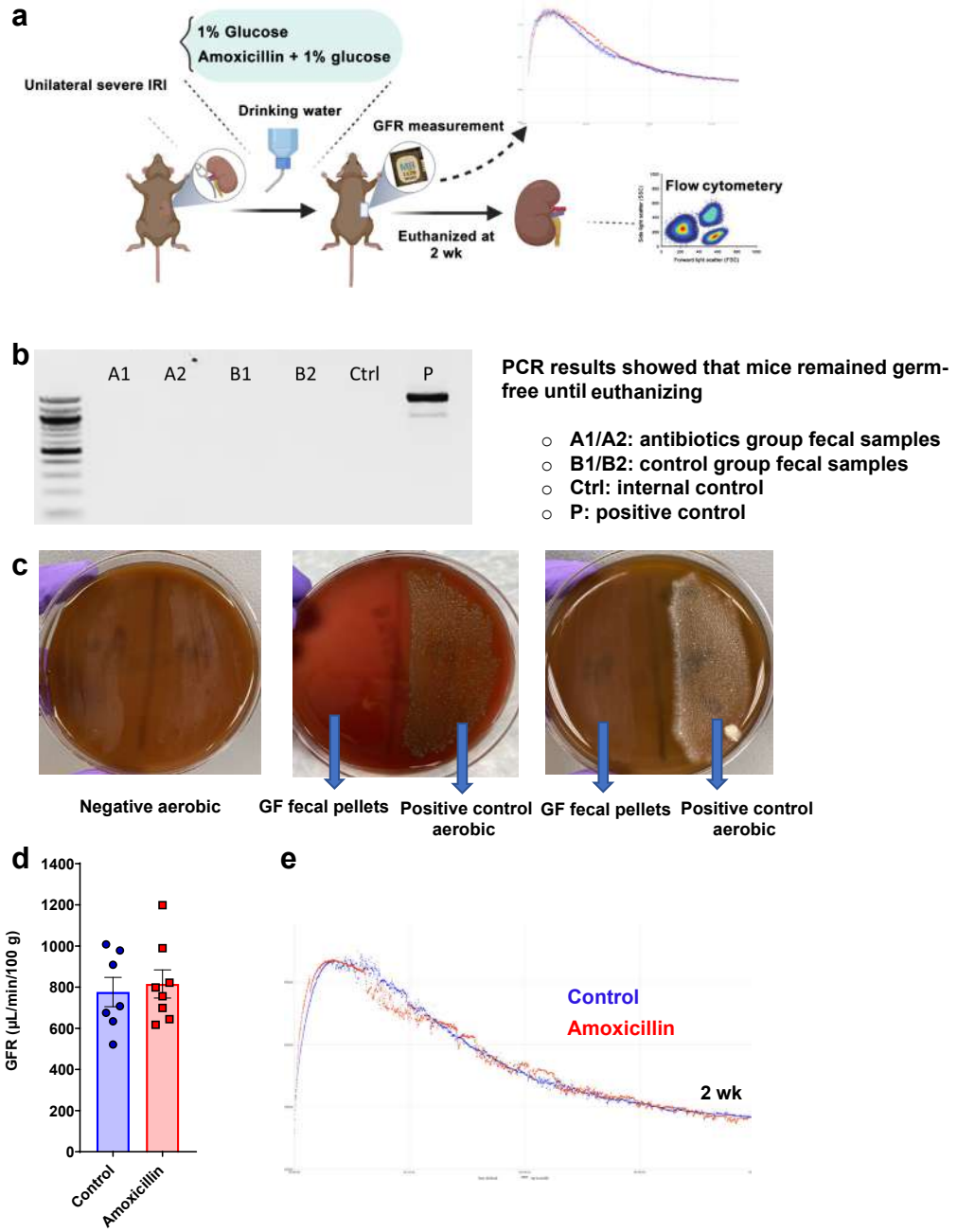


Figure 8 | Germ-free (GF) mice were not protected by amoxicillin during severe acute kidney injury. Representative of microbial investigation of mice stool. (**a**) Experimental design. (**b**) Polymerase chain reaction (PCR). (**c**) Representative of bacterial culturing methods at 24 hours and 1 week and before euthanizing at 2 weeks. (**d**) Kidney function at 2 weeks after unilateral ischemia-reperfusion injury (UIRI) in GF control mice (n = 7) versus GF mice treated with amoxicillin (n = 8). (**e**) Representative of glomerular filtration rate (GFR) at baseline and after 2 weeks after UIRI in GF control (free antibiotic) and GF mice treated with amoxicillin.

associated lipocalin at 24 hours after I/R injury.[22] Kidney injury marker-1 and soluble urokinase plasminogen activator receptor have not been well studied in kidney microbiome studies and need further investigation.

We evaluated the gut microbiome changes after amoxicillin post treatment of severe AKI with 16s sequencing. We identified an increased abundance of *Alistipes*, *Odoribacter*, and *Stomatobaculum* compared with the baseline and other groups. In addition, metronidazole significantly reduced nearly all bacterial species, except *Barnesiella* and *Parasutterella*. *Alistipes* are anaerobic bacteria that have been found in the healthy human gut.[32] Increased *Alistipes* abundance in mice treated with curcumin[33,34] led to reduced bowel inflammation via enhanced IL-10 production by $CD4^{+}$latent-associated peptide $(LAP)^{+}Foxp3^{-}$ cells.[35] *Odoribacter splanchnicus* was identified as a single bacterial strain that induced T-helper 17 cells and protected mice against colitis and colorectal cancer.[36]

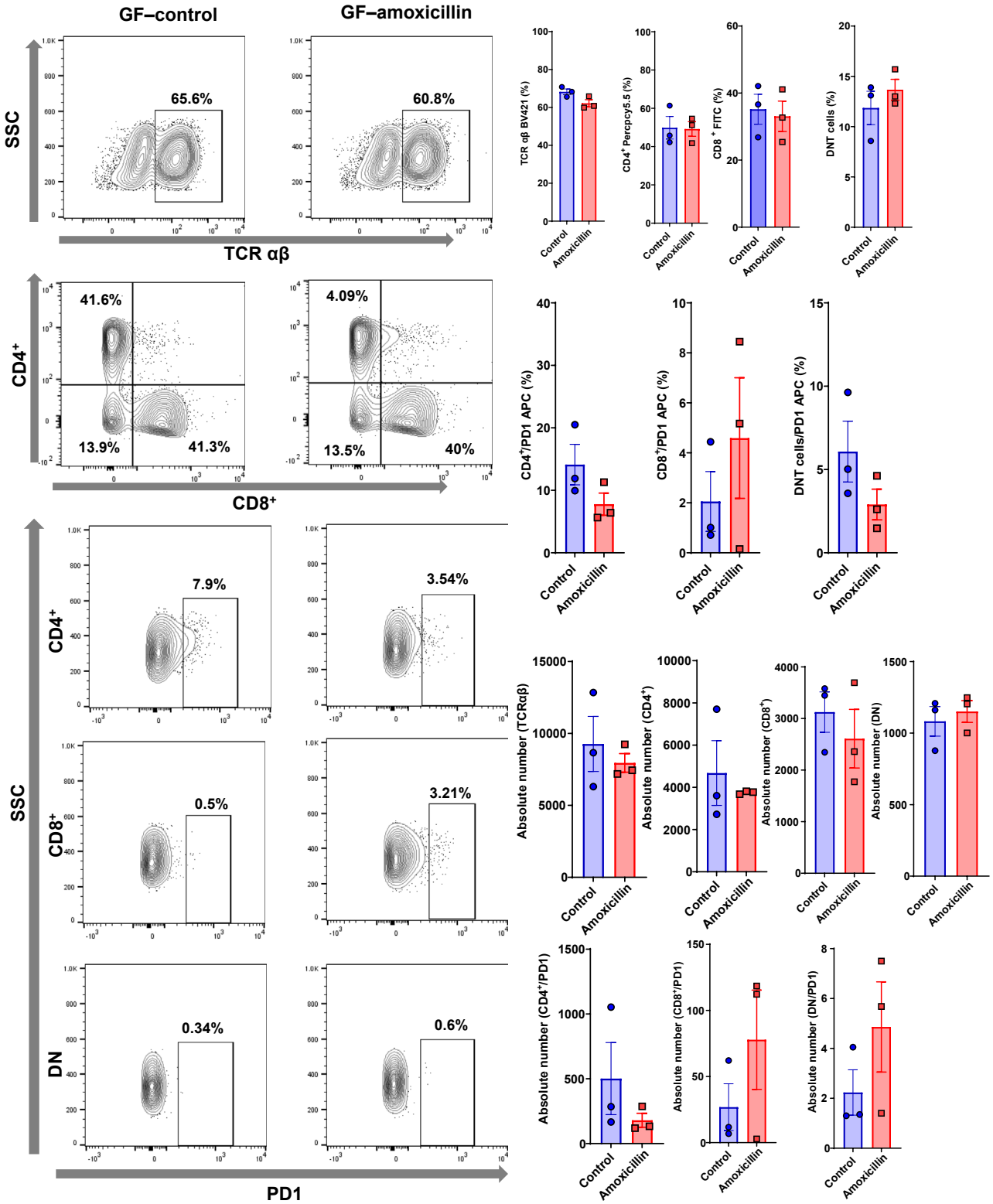


Figure 9| Proportion of T lymphocytes at 2 weeks after unilateral ischemia-reperfusion injury in germ-free (GF) control (n = 3) mice and GF mice treated with amoxicillin (n = 3). APC, antigen-presenting cell; DN, double negative; DNT, double-negative T cell; FITC, fluorescein isothiocyanate; PD1, programmed cell death protein 1; SSC, standard saline citrate; TCR, T-cell receptor.

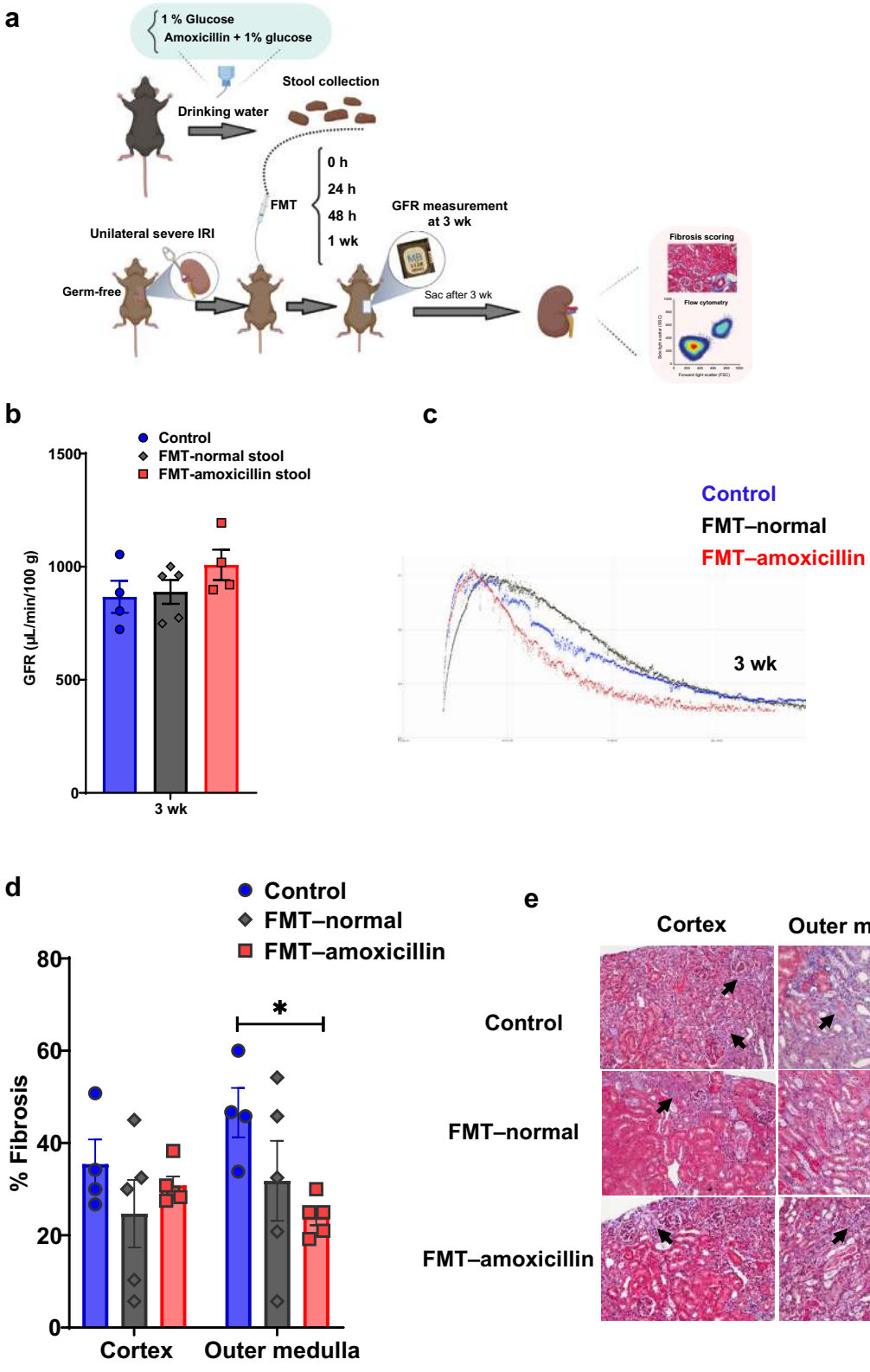


Figure 10 | Kidney function improved after fecal microbiota transplantation (FMT) from amoxicillin-treated mice (donor) to germ-free (GF) mice after unilateral ischemia-reperfusion injury (UIRI). (**a**) Experimental design. (**b**) Kidney function at 3 weeks after UIRI in GF control mice (n = 4) versus GF mice treated with FMT from normal mice (n = 5), and GF mice treated with FMT from amoxicillin-treated mice (n = 4). (**c**) Representative of glomerular filtration rate (GFR) at 3 weeks after UIRI. (**d**) Kidney fibrosis scores of kidney cortex and outer medulla in GF mice serving as control versus GF mice treated with FMT from normal mice and GF mice treated with FMT from amoxicillin-treated mice. (**e**) Representative kidney fibrosis (arrows) in GF mice kidneys 3 weeks after UIRI. (continued)

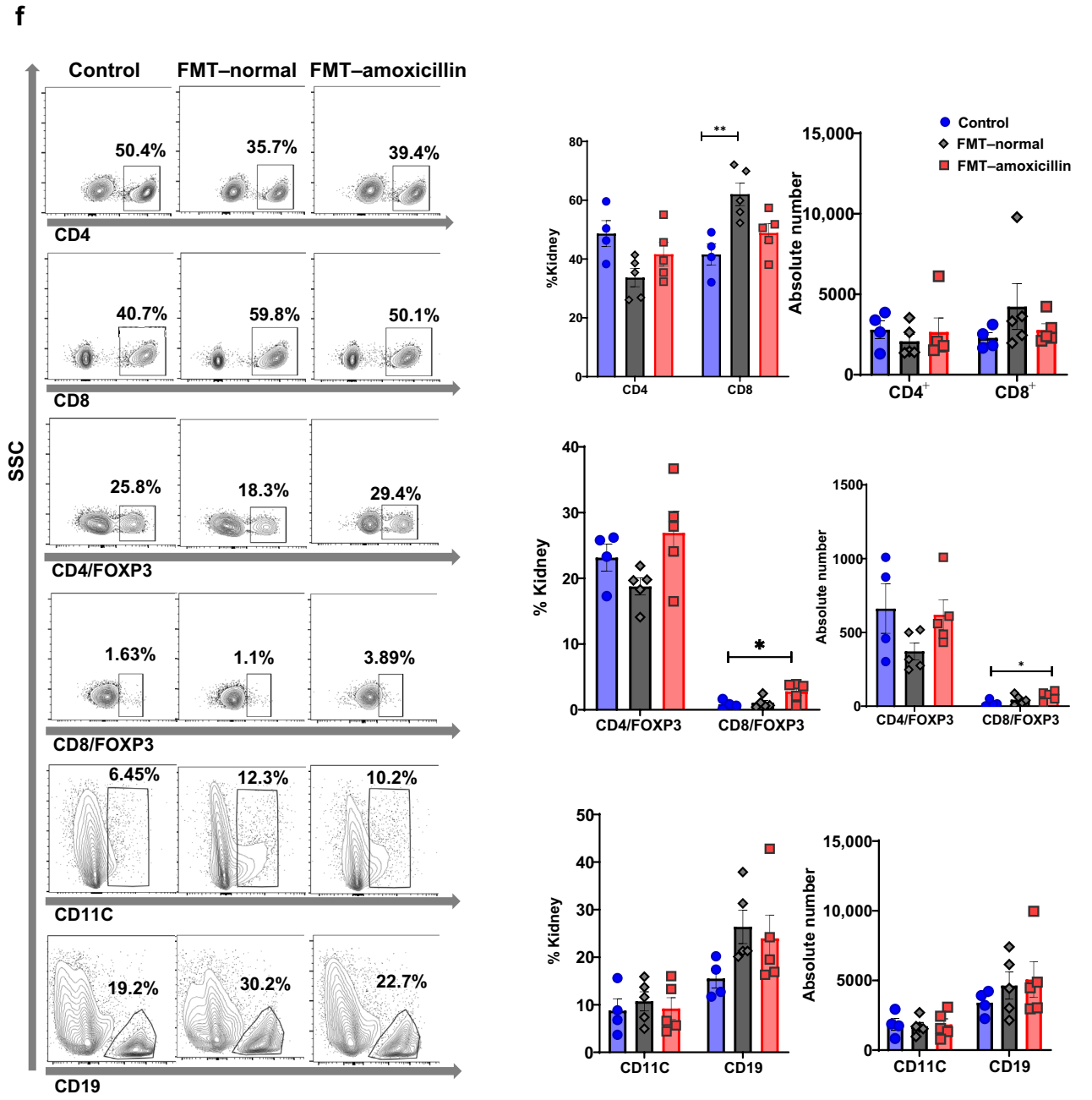


Figure 10 | (continued) (**f**) Proportion and absolute number of T lymphocytes, dendritic cells, and B cells in kidney of GF mice. Data in (**d**) and (**f**) displayed as mean ± SEM; multiple comparisons by 1-way analysis of variance. *$P < 0.05$ and **$P < 0.01$. FOXP3, forkhead box p3; SSC, standard saline citrate. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Our study revealed that metronidazole increased the abundance of *Parasutterella* in mice. High abundance of *Parasutterella* was identified in patients with inflammatory bowel disease.[37] We found that the combination of ABX reduced the abundance of *Barnesiella*, which is associated with protection against vancomycin-resistant enterococci in patients receiving allogeneic hematopoietic stem cell transplants.[38]

The intestinal epithelial barrier plays a key role in maintaining homeostasis of intestinal microbiota.[16] Increasing urea concentration during CKD leads to alterations in the intestinal flora that can enhance production of gut-derived toxins and alter the intestinal epithelial barrier. Several bacterial species, including *Lactobacillus*, positively impact the epithelial barrier and mucus layer.[39] A recent study revealed supplementation of *Lactobacillus reuteri* and *Clostridium butyricum* had a protective effect on cisplatin-induced nephrotoxicity. This intervention improved the cisplatin-induced intestinal epithelial barrier impairment.[40] Amoxicillin may have a positive impact on epithelial barrier and mucus layer as a mechanism of action.

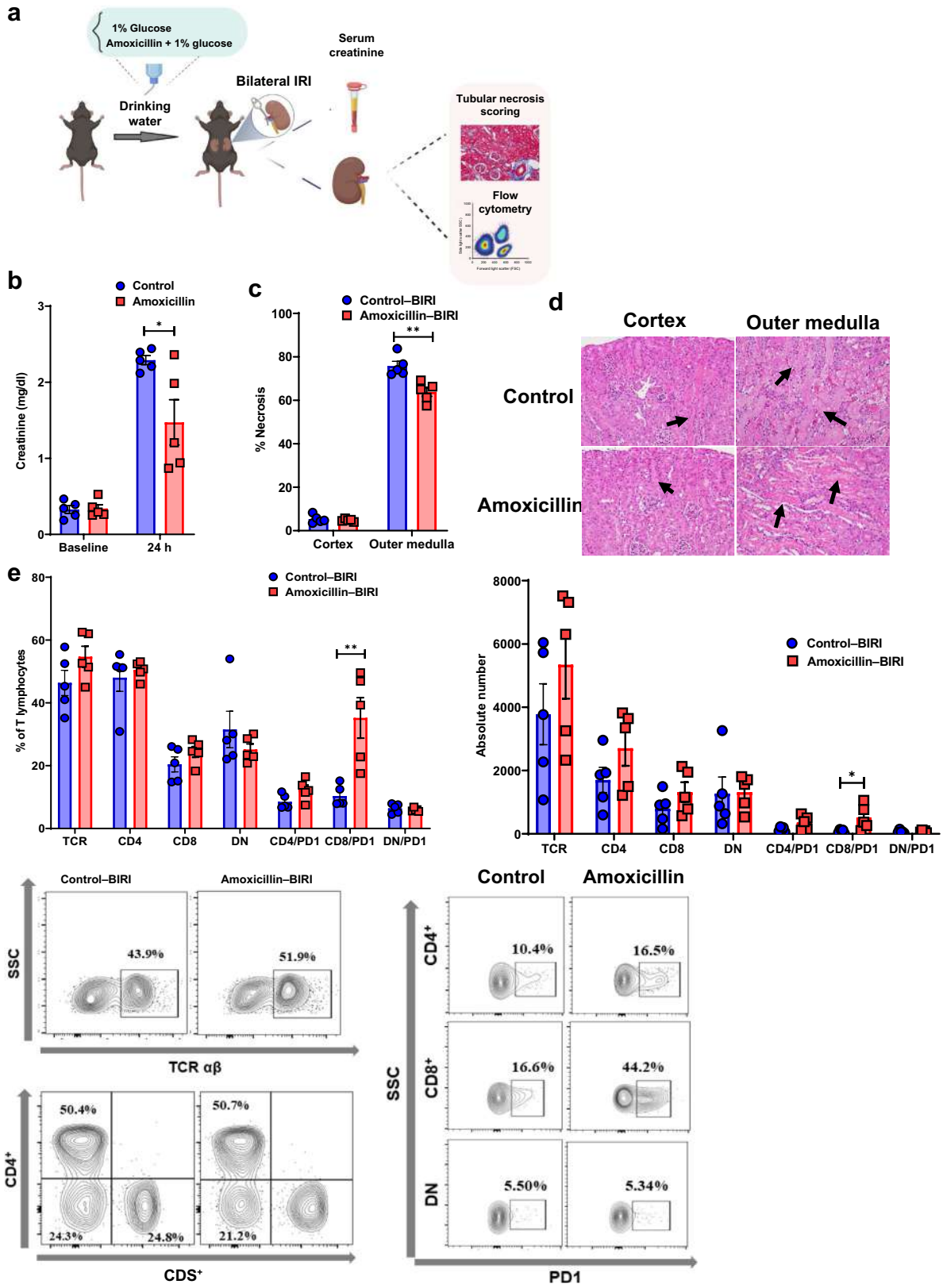


Figure 11 | Two weeks of amoxicillin pretreatment protected mice against kidney bilateral ischemia-reperfusion injury (BIRI)–induced acute kidney injury. (**a**) Experimental design. (**b**) Serum creatinine level at 24 hours after BIRI in control mice (n = 5) versus mice pretreated treated with amoxicillin (n = 5). (**c,d**) Evaluation of kidney damage scored with the percentage of necrotic tubules among total tubules at 24 hours after BIRI in control mice (n = 5) versus mice pretreated treated with amoxicillin (n = 5). (**e**) Proportion of T lymphocytes at 24 hours after BIRI in control mice (n = 5) versus mice pretreated treated with amoxicillin (n = 5). Data in (**b**), (**c**), and (**e**) displayed as mean ± SEM; multiple unpaired *t* test. **P* < 0.05 and ***P* < 0.01. DN, double negative; PD1, programmed cell death protein 1; TCR, T-cell receptor. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

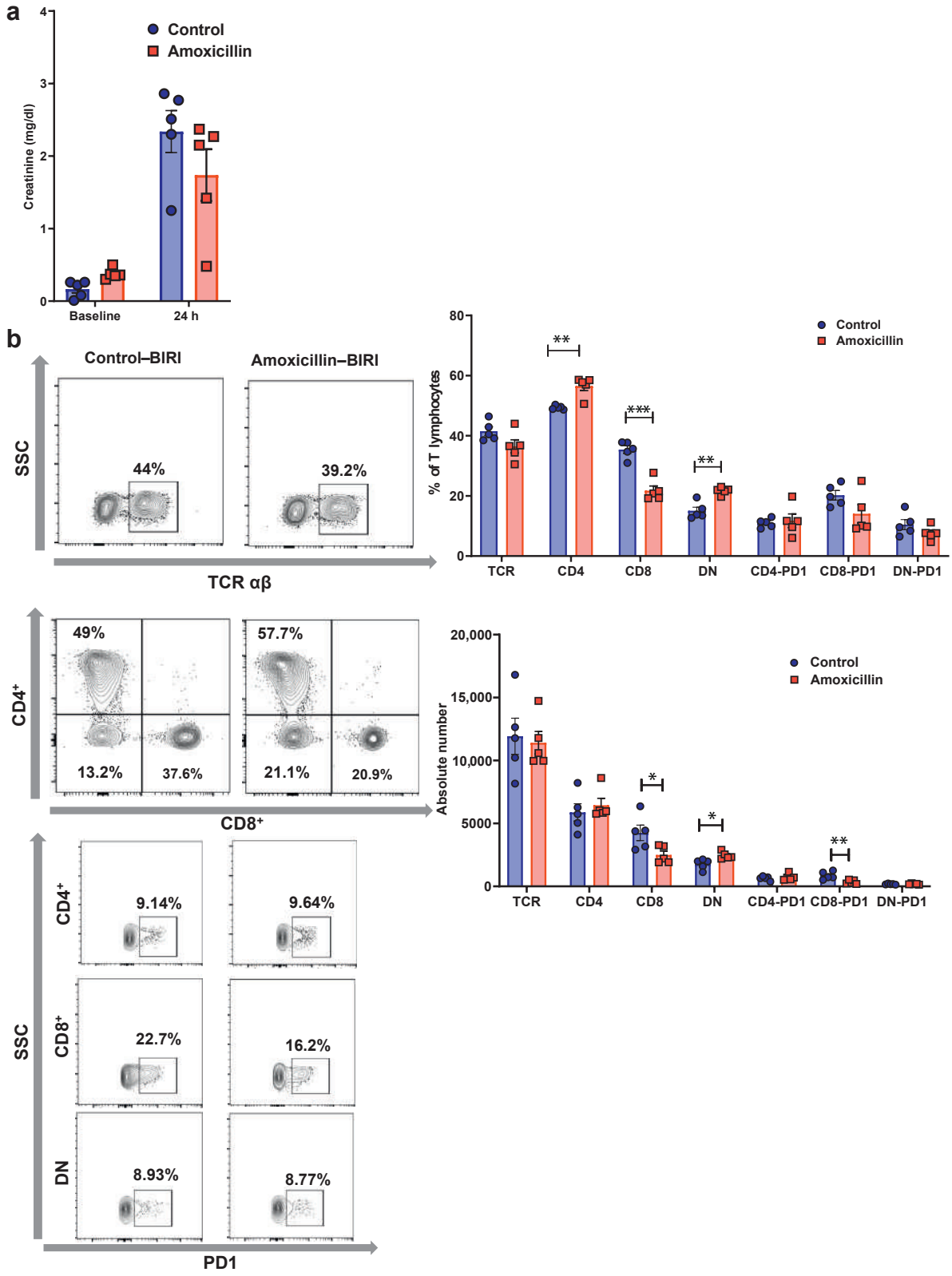


Figure 12| One week of amoxicillin pretreatment did not protect mice against bilateral ischemia-reperfusion injury (BIRI)–induced acute kidney injury. (**a**) Serum creatinine level at 24 hours after BIRI in control mice (n = 5) versus mice 1 week pretreated with amoxicillin (n = 5). Data in (**b**) displayed as mean ± SEM; multiple unpaired *t* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. DN, double negative; PD1, programmed cell death protein 1; SSC, standard saline citrate; TCR, T-cell receptor.

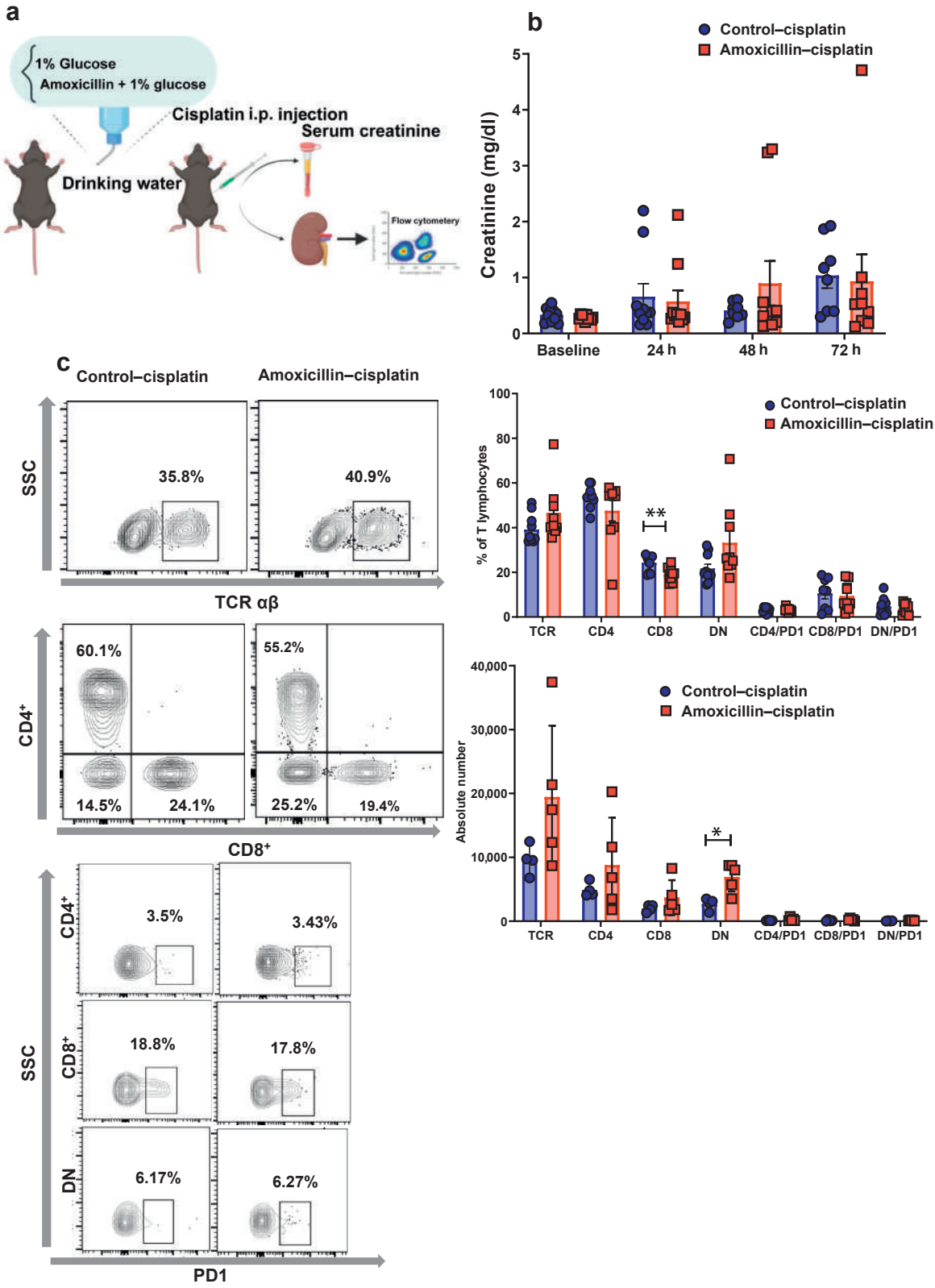


Figure 13 | Effects of amoxicillin pretreatment in cisplatin-induced acute kidney injury. (**a**) Experimental design. (**b**) Serum creatinine level at 24, 48, and 72 hours after cisplatin injection in control mice (n = 5) versus mice pretreated treated with amoxicillin (n = 5). (**c**) Proportion of T lymphocytes at 72 hours after cisplatin injection in control mice (n = 5) versus mice pretreated treated with amoxicillin (n = 5). Data in (**c**) displayed as mean ± SEM; multiple unpaired *t* test. **P* < 0.05 and ***P* < 0.01. DN, double negative; PD1, programmed cell death protein 1; SSC, standard saline citrate; TCR, T-cell receptor.

Both preclinical and clinical studies have demonstrated gut microbiota modulation could be a promising strategy for enhancing the effectiveness of immunotherapies.[41] There is an important modulatory role of T cells during AKI.[10] Gut microbiota affect T-cell homeostasis and functions, and gut dysbiosis resulted in imbalance of T-cell subpopulations.[7] *Bifidobacterium*-treated mice displayed significantly improved effects on B16.SIY melanoma in comparison with their non–*Bifidobacterium*-treated counterparts, which was accompanied by robust induction of tumor-specific T cells in the periphery and increased accumulation of antigen-specific $CD8^+$ T cells within the tumor.[42]

Intestinal microbiota and colon mucosal immunity could be important modifiers of AKI outcome as they have been linked to kidney inflammation and injury.[28] We therefore examined the effects of amoxicillin treatment after severe AKI on kidney and colonic T cells, and tested if $CD8^+$ or $CD4^+$ T cells were direct mediators of the amoxicillin effects. We found increases in kidney $CD8^+$ T cells and increased $PD1^+CD8^+$ T cells in mice treated with amoxicillin. PD1 is an inhibitory receptor expressed by T cells during activation. It also regulates T-cell effector functions during physiological responses, including acute and chronic infection.[43] It is also well-known that PD1 expression on antigen-specific T cells reflects the antitumor reactivity of these T cells and has anti-inflammatory properties.[44] Studies on mice with unilateral ureteric obstruction demonstrated that increased infiltration of $CD8^+$ T cells attenuates kidney fibrosis in mice and CD8 deficiency worsened it, whereas adoptive transfer of $CD8^+$ T cells into CD8 knockout mice reduced kidney fibrosis in mice.[45,46] We found that amoxicillin led to a reduction in kidney $CD4^+$ T cells as well as IL-17 expression by $CD4^+$ and TNF-α expression by DNT cells. IL-17 produced by T-helper 17 cells promotes inflammation by directly causing tissue injury and enhancing secretion of proinflammatory cytokines and chemokines.[47] TNF-α is also a potent mediator of inflammation, which has been invoked in the pathophysiology of CKD.[48] Thus, amoxicillin could potentially accelerate kidney repair by reducing IL-17 and TNF-α. We also found an increase in $CD4^+$ T cells in the colons of mice treated with amoxicillin compared with control, whereas $CD4^+$IL-17 as well as $CD8^+$ T cells were decreased in colon. No significant changes were detected in kidney IL-17 at 4 weeks as well as kidney and serum TNF-α at 2 and 4 weeks after UIRI between the groups. IL-17 and TNF-α may not have been released in sufficient quantities to be detected in the plasma or in whole tissue samples. Given changes in both $CD8^+$ and $CD4^+$ T cells after amoxicillin, we tested the direct role for these cells in amoxicillin effects. $CD8^{-/-}$ mice treated with amoxicillin lost the protective effect of amoxicillin. However, $CD4^{-/-}$ mice retained protection by amoxicillin. Thus, $CD8^+$ cells were likely participating in the protective amoxicillin effects.

To test if amoxicillin was working through the gut bacteria or with direct effects on the kidney, experiments were conducted in GF mice. We found that GF mice were not protected by amoxicillin, demonstrating that amoxicillin accelerated kidney repair through bacteria. We found a significant decrease in fibrosis in outer medullary region and increase in proportion and absolute number of $Foxp3^+CD8$ T cells when GF mice had fecal transplants from amoxicillin-treated mice. We previously demonstrated that $Foxp3^+CD4^+$ regulatory cells directly participated in kidney repair from AKI.[11]

We also studied the effects of amoxicillin prevention in AKI and found 2 weeks but not 1 week of amoxicillin pretreatment protected mice against kidney BIRI-induced AKI. However, 2 weeks of amoxicillin pretreatment was insufficient to protect from cisplatin-induced AKI. We previously studied the effect of ischemic versus cisplatin AKI on gut microbiota and found key differences between models.[7] Differences in how the models are performed, pathophysiological differences in the kidney, as well as key differences in microbiome responses between these models could be contributing to differences in amoxicillin response.

Our study had many limitations, including the following: (i) The severe murine AKI model, unlike in humans, has a remaining kidney intact, which was needed for mice to survive long-term without dialysis. (ii) We used a validated technique for measuring SCFAs but may have missed important time points of where changes occurred in the 4-week studies. (iii) Despite identifying a key role for CD8 T cells and microbiome in the protective effects of amoxicillin, we still do not know the complete molecular mechanisms by which gut bacteria communicate with kidney during AKI. (iv) We described a large number of amoxicillin-induced changes of gut bacteria at genus level but did not identify exact species involved in repair from severe AKI. These and other limitations will be addressed in future studies.

In summary, our results demonstrate that amoxicillin treatment significantly improved kidney function and structure even when given after severe AKI. These findings are rapidly translatable to test in humans and could be useful for patients with AKI as well as other acute organ injuries like myocardial infarction and stroke.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

SG, SN, and HR designed the study. SG, KL, and SN performed the experiments. LJA scored histology slides and took representative pictures. SG, KL, AMN-R, JX, SKP, MG, JP, CP, DSR, SN, and HR analyzed and interpreted the data, prepared figures, and drafted the manuscript. All authors helped with manuscript preparation and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods. Experimental animals and kidney unilateral-ischemia reperfusion model, assessment of kidney function, tissue histologic analysis, measurement of gene expression, analysis of gut microbial burden, measurement of short-chain fatty acids, isolation of kidney and colon lamina propria mononuclear cells, flow cytometry, measurement of serum and kidney cytokines, evaluation of kidney function and structure in germ-free mice with acute kidney injury (AKI), fecal microbiota transplantation, and statistical analyses.
Supplementary Methods References.
Supplementary Figure S1. mRNA expression of *TGFβ*, *Col1α1*, and *αSMA* profibrotic genes in control mice (n = 9) versus mice treated with amoxicillin, metronidazole, and combination of antibiotics at 4 weeks after unilateral ischemia-reperfusion injury (UIRI) (n = 9, 10, 8, and 8 mice per group, respectively). Data displayed as mean ± SEM; multiple comparisons by 2-way analysis of variance (ANOVA). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
Supplementary Figure S2. Volcano plot displaying differentially abundant amplicon sequence variants (ASVs) between baseline samples and at 1 week. Horizontal dashed line indicates a false discovery rate (FDR)–adjusted *P* value of 0.05.
Supplementary Figure S3. Plasma short-chain fatty acids after amoxicillin treatment. (**A**–**F**) Acetic acid, propionic acid, succinic acid, lactic acid, isovaleric acid, and butyric acid levels at 1 week after unilateral ischemia-reperfusion injury (UIRI) in control mice (n = 8) versus mice treated with amoxicillin (n = 8). Data in (**A**) displayed as mean ± SEM; multiple comparisons by 2-way analysis of variance (ANOVA). $^{*}P < 0.05$.
Supplementary Figure S4. Proportion and absolute number of B cells, dendritic cells, neutrophils, and macrophages in kidney of mice served as control (free of antibiotics [ABX]) and mice treated with amoxicillin at 4 weeks after unilateral ischemia-reperfusion injury (UIRI). Data displayed as mean ± SEM; multiple unpaired *t* test. $^{*}P < 0.05$.
Supplementary Figure S5. Amoxicillin had minimal effects on normal kidney function and immune cells. (**A**) Kidney function at 24 hours and 1, 2, 3, and 4 weeks in normal control mice (n = 5) versus normal mice treated with amoxicillin (n = 5). (**B**) Representative glomerular filtration rate (GFR) at baseline and after 4 weeks after amoxicillin treatment in normal control mice and those treated with amoxicillin. (**C**) Proportion of T lymphocytes in normal control mice and normal mice treated with amoxicillin at 4 weeks (n = 5).

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