Double-negative T cells have a reparative role after experimental severe ischemic acute kidney injury

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RUNNING HEAD: Role of double-negative T cells in AKI-repair

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ABSTRACT

T cells mediate organ injury and repair. A proportion of unconventional kidney T cells called double-negative (DN) T cells (TCR^+ $CD4^-$ $CD8^-$), with anti-inflammatory properties, were previously demonstrated to protect from early injury in moderate experimental AKI. However, their role in repair after AKI has not been studied. We hypothesized that DN T cells mediate repair after severe AKI. C57B6 mice underwent severe (40min) unilateral ischemia-reperfusion injury (IRI). Kidney DN T cells were studied by flow cytometry and compared to gold-standard anti-inflammatory $CD4^+$ Tregs. In vitro effects of DN T cells and Tregs on renal tubular epithelial cell (RTEC) repair after injury were quantified with live-cell analysis. DN T cells, Tregs, CD4 or vehicle were adoptively transferred after severe AKI. Glomerular filtration rate (GFR) was measured using FITC-sinistrin. Fibrosis was assessed with Masson's trichrome staining. Profibrotic genes were measured with qRT-PCR. Percentages and the numbers of DN T cells substantially decreased during repair phase after severe AKI, as well as their activation and proliferation. Both DN T cells and Tregs accelerated RTEC cell repair in vitro. Post-AKI transfer of DN T cells reduced kidney fibrosis and improved GFR, as did Treg transfer. DN T cell transfer lowered TGFβ1 and αSMA expression. DN T cells reduced effector-memory $CD4^+$ T cells and IL-17 expression. DN T cells undergo quantitative and phenotypical changes after severe AKI, accelerate RTEC repair in vitro as well as improve GFR and renal fibrosis in vivo. DN T cells have potential as immunotherapy to accelerate repair after AKI.

NEW & NOTEWORTHY

Double-negative (DN) T cells ($CD4^-$ $CD8^-$) are unconventional kidney T cells with regulatory abilities. Their role in repair from AKI is unknown. Kidney DN T cell population decreased during repair after ischemic AKI, in contrast to Tregs which increased. DN T cell administration accelerated tubular repair in vitro, while after severe in vivo ischemic injury reduced kidney fibrosis and increased GFR. DN T cell infusion is a potential therapeutic agent to improve outcome from severe AKI.

Keywords: Acute kidney injury; ischemia-reperfusion injury; lymphocytes; repair; T cells

INTRODUCTION

Acute kidney injury (AKI) is a common and serious clinical problem resulting in high morbidity and mortality world-wide (1). Impaired recovery from AKI can lead to kidney fibrosis and transition to chronic kidney disease (CKD) (2). Although prior studies mostly focused on the prevention of early injury (3), understanding molecular and cellular mechanisms of repair and recovery after AKI is clinically important given that most patients are diagnosed after AKI has occurred (4).

Among the many cellular and molecular pathways involved in the AKI repair process (3, 5-9), immune responses mediated by T cells have been proposed as one of the important pathways (10, 11). Long-term increase in numbers, immuno-phenotypical changes and transcriptomic reprogramming of T cells have been demonstrated in previous studies (12-14), highlighting their potential role in AKI repair or CKD transition. Furthermore, a minor proportion of kidney $CD4^+$ T cells, regulatory T cells (Tregs) that have anti-inflammatory properties not only have a protective role in early injury (15), but also have a reparative role in AKI to CKD transition (16).

An unconventional T cell subset, double-negative (DN) T cells that do not express either CD4 nor CD8 exist in kidneys (17-20). While they are rarely present in lymphoid tissue and peripheral blood, there are significant proportions of DN T cells among total αβ T cells in steady-state as well as post-AKI kidneys (19). DN T cells exhibited a protective role with an anti-inflammatory property in prevention from moderate early injury (19), however little is known about their role in repair after AKI, particularly after clinically significant severe injury. We therefore hypothesized that kidney DN T cells change after severe AKI and can directly participate in repair. We studied effects of DN T cells after severe AKI, effects on renal epithelial cells in vitro

as well as in vivo AKI, comparing them to the "gold standard" anti-inflammatory $CD4^+$ Tregs (18, 20).

MATERIALS AND METHODS

Mice

Seven-week-old male C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions at the Johns Hopkins University animal facility. 8–9-week-old mice were used for experiments using WT mice. 12-week-old *Fasl*^{gld}/J male mice were used as donors for DN T cell isolation as previously described (19, 21). All experiments were performed using experimental protocols approved by the Animal Care and Use Committee of Johns Hopkins University and reported in compliance with the ARRIVE guideline (22).

Severe ischemic AKI model

WT mice were anesthetized with pentobarbital (75 mg/kg; Akorn, Lake Forest, IL) injection intraperitoneally. Mice were placed onto a thermostatically controlled heating table after shaving of abdominal hair. Abdominal midline incision was performed, and left renal pedicles were dissected and clamped for 40 min using a microvascular clamp (Roboz Surgical Instrument, Gaithersburg, MD) to induce severe ischemia. The clamps were released from renal pedicles

after 40 min, and the left kidneys were visually inspected to confirm reperfusion. Mice were kept well hydrated with 1 mL of warm sterile 0.9% saline and at a constant body temperature (37 °C) during the surgery. After being sutured, mice were allowed to recover with free access to chow and water.

Assessment of kidney function

Since serum creatinine is a less sensitive measure of GFR in the unilateral IRI model due to the remaining functional contralateral kidney (23), we directly measured glomerular filtrate rate (GFR) to measure kidney function. GFR was measured by transcutaneous fluorescein isothiocyanate (FITC)-sinistrin (inulin analog) with a fluorometer device (MediBeacon, St. Louis, MO) at baseline, 24hrs, one week, two weeks, and three weeks after reperfusion (24). Briefly, mice were anesthetized with isoflurane (Piramal, Maharashtra, India) and oxygen under an isoflurane vaporizer system (VetFlo, Kent Scientific, Torrington, CT). The background fluorescence signal of skin was recorded for five minutes, and subsequently 0.07 mg/g body weight of FITC-sinistrin (MediBeacon) was injected retro-orbitally. Mice were immediately transferred to separate cages to record FITC-sinistrin clearance in dark. After 1.5 hrs, the devices were gently detached from conscious mice, and raw data from the devices were collected using MB Lab Software (MediBeacon). GFR was calculated using a previously established three-compartment model (25) by Studio2 Software (MediBeacon).

Tissue histological analysis

At 3 weeks after the surgery, mice were anesthetized with intraperitoneal injection of ketamine (130 mg/kg; VetOne, Boise, ID) and xylazine (7 mg/kg; Akorn) mixture. Mice were exsanguinated, and kidneys were collected. Left kidney tissues were fixed with 10% buffered formalin followed by paraffin embedding. Kidney sections were subsequently stained with Masson's trichrome staining. A renal pathologist, blinded to the study groups, scored the degree of fibrosis from the kidney sections.

Isolation of kidney mononuclear cells

For kidney mononuclear cell isolation, post-ischemic kidneys and contralateral kidneys were collected at 1 week and 3 weeks after the IRI surgery. Uninjured intact kidneys from naïve mice were also collected for steady state controls. Kidney mononuclear cells (KMNCs) were isolated using Percoll density gradient protocol described previously (26). Briefly, decapsulated kidneys were incubated in 2 mg/mL collagenase D (Roche, Basel, Switzerland) solution for 30 min at 37 °C. Samples were strained through 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ), washed, and resuspended in 40% Percoll (GE Healthcare, Chicago, IL) followed by gentle overlaying onto 80% Percoll. After centrifugation at 1,800 g for 30 min in brake-off mode at room temperature, KMNCs were collected from the interface between 40% and 80% Percoll. Collected cells were washed and resuspended with Roswell Park Memorial Institute (RPMI) 1640 media (Thermo Fisher Scientific, Waltham, MA) containing 5% fetal bovine serum (FBS,

Thermo Fisher Scientific). Cells were counted on a hemocytometer using trypan blue (Thermo Fisher Scientific) under a microscope (IMT-2, Olympus, Tokyo, Japan).

Spectral flow cytometry

Cells were washed once with phosphate buffered saline (PBS) and stained with viability dye Zombie NIR Fixable Viability (BioLegend, San Diego, CA) for 15 min at room temperature. After washing with Cell Staining Buffer (BioLegend), cells were preincubated with anti-CD16/CD32 Fc receptor blocking antibody (S17011E, BioLegend) for 15 min to prevent nonspecific antibody binding. Subsequently, surface staining was performed with surface staining antibody cocktail in 50 uL of BD horizon^{TM} Brilliant Stain buffer for 30 min at 4 °C: Pacific blue anti-CD44 (IM7, BioLegend), BV510 anti-CD8 (53-6.7, BioLegend), BV570 anti-CD45 (30-F11, BioLegend), BV605 anti-CD69 (H1.2F3, BioLegend), BV650 anti-NK1.1 (PK136, BioLegend), BV711 anti-PD1 (29F.1A12, BioLegend), BV785 anti-TCRβ (H57-597, BioLegend), Alexa Fluor 532 anti-CD3 (17A2, Thermo Fisher Scientific), PE/Dazzel 594 anti-T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (1G9, BioLgend), PE-Cy5 anti-CD122, PE-Cy5.5 anti-CD25 (PC61.5, Thermo Fisher Scientific), PE-Cy7 anti-Ly49 (14B11, BioLgend), Alexa Fluor 647 anti-TCRγδ (GL3, BioLegend), APC-R700 anti-CD62L (MEL-14, BD Biosciences), and APC-Fire810 anti-CD4 (GK1.5, BioLegend). Cells were fixed and permeabilized with Foxp3/Transcription Factor Staining kit (Thermo Fisher Scientific) for 30 min at room temperature and washed with permeabilization/wash buffer (Thermo Fisher Scientific). Intracellular staining was conducted in 50μL of permeabilization/wash buffer with

intracellular staining antibody cocktail for 30 min at room temperature: BV421 anti-Ki67 (16A8, BioLegend), PerCP-efluor 710 anti-FoxP3 (FJK-16S, Thermo Fisher Scientific).

T cell activation and intracellular cytokine analysis

To measure intracellular cytokines, KMNCs were stimulated with pre-mixed leukocyte activation cocktail (BioLegend) containing phorbol 12-myristate-13-acetate, ionomycin, and brefeldin A. After surface staining followed by permeabilization and fixation as described above, cells were stained with the following intracellular antibodies, BV421 anti-Ki67 (16A8, BioLegend), Alexa Fluor 488 anti-TNFα (MP6-XT22, Biolegend), Alexa Fluor 532 anti-IL-2 (JES6-5H4, BD Biosciences), PerCP-efluor 710 anti-FoxP3 (FJK-16S, Thermo Fisher Scientific), PE anti-IL-10 (JES5-16E3, Biolegend), PE-Cy5 anti-INFγ (XMG1.2, Abcam), PE-Cy7 anti-IL17A (TC11-18H10, Biolegend), Alexa Fluor 647 anti-TGF-β (860206, R&D systems, Minneapolis, MN).

After staining, cells were washed with permeabilization/wash buffer then resuspended in Cell Staining Buffer. Samples were analyzed by 4-laser Aurora spectral flow cytometer (Cytek, Fremont, CA). The acquired raw data from the spectral flow cytometer were unmixed by SpectroFlo software (Cytek). Unmixed data was curated and analyzed with FlowJo 10.8 software (BD Biosciences).

Double negative T cell sorting

DN T cells were isolated from *gld* mice lymph nodes. Briefly, single-cell suspension of lymphocytes from lymph nodes was preincubated with anti-CD16/CD32 Fc block (S17011E, BioLegend) and stained in Cell Staining Buffer (BioLegend) with fluorochrome-labeled antibodies: APC-Cy7 anti-CD45 (30-F11), BV421 anti-TCRβ (H57-957), CD4 Alexa Fluor 488 (GK1.5), and CD8 PE (53-6.7) from BioLegend. After staining, cells were washed and resuspended with BD FACS Pre-Sort Buffer (BD Biosciences). Cells were stained with propidium iodide (PI) (Thermo Fisher Scientific) right before sorting for viability assay. PI^- $TCR\beta^+$ $CD4^-$ $CD8^-$ cells were sorted using the MoFlo Legacy or the XDP cell sorter (Beckman Coulter, Brea, CA).

Treg and $CD4^+$ T cell isolation

Tregs and $CD4^+$ T cells were isolated from WT mouse spleens using $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The final eluted fraction containing $CD4^+$ $CD25^+$ cells was used as Treg transfer for both in vitro and in vivo studies. We confirmed that these cells were $FoxP3^+$ $CD4^+$ Tregs by flow cytometry analysis before performing adoptive transfer (Supplemental Figure S1). The flow-through fraction containing $CD4^+$ $CD25^-$ T cells was used for $CD4^+$ T cell transfer (negative control) for the in vitro scratch experiment.

In vitro coculture of renal tubular epithelial cells and T cells to quantify repair after injury

The Boston University mouse proximal tubular (BUMPT-306) epithelial cells (PTECs) were cultured with DMEM with 5% FBS and 100 U/mL penicillin and streptomycin in a 96-well plate (Sartorius, Niedersachsen, Germany). Cultured cells were exposed to hypoxic condition (1% O_2) according to the following protocol. For hypoxia induction, culture plates were placed in a modular incubator chamber, and the chamber was flushed with gas mixture containing 1% O_2, 5% CO_2, and 94% N_2 for 3 min. Subsequently, the chamber was completely sealed and placed into a cell culture incubator for 12h.

To mimic PTEC repair in vitro, we used a scratch wound assay, a technique used for studying the effects of cell-cell interactions on cell migration (27). Homogenous scratch wounds were created to the PTEC monolayer using a 96-pin WoundMaker Tool (Sartorius). Immediately after scratch, activated DN T cells, $CD4^+$ Tregs, and $CD4^+$ T cells were transferred at a 5:1 ratio. Cells were incubated under normoxia and the phase-contrast images were acquired every 2 hr using a real-time cell analysis system (Incuyte Live-Cell Analysis, Sartorius). The wound closure was quantified from time-lapse phase images, and values were expressed as the relative wound density (Incucyte Scratch Wound Analysis Software Module, Sartorius).

T cell adoptive transfer

Vehicle, DN T cells or Tregs were injected twice at 6 h and 48 h after reperfusion via retro-orbital intravenous injection. PBS was used as the vehicle, and isolated cells were resuspended in PBS right before the injection. 5×10^6 DN T cells and 1×10^6 Tregs were injected into each mouse per injection.

Quantification of mRNA by real-time quantitative reverse transcription PCR

After collecting whole kidneys, the upper 1/3 part of each kidney, which includes cortex and medulla, was immediately immersed into RNAlater (Thermo Fisher Scientific). These tissues were subsequently used for RNA isolation followed by RT-PCR. The remaining kidney tissues were used for lymphocyte isolation for flow cytometry or histologic evaluation. Total RNA was extracted from kidney tissue with RNeasy Mini kit (Qiagen, Valencia, CA) and reversed transcribed using high-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Real-time PCR was performed in QuanStudio 12 Flex (Applied Biosystems) using the PowerUp SYBR Green Master mix (Applied Biosystems) for detection of mRNA expression of *Tgfb1*, *Acta2* (encoding α-smooth muscle actin, α-SMA), *Col1a1*, and *Col4a1*. *Gapdh* gene expression was used as the internal control. Relative fold expression values were calculated with a ΔΔ cycle threshold method. The primer sequences for each gene are provided in Supplemental Table 1.

Statistics

Data were expressed as mean ± standard error of mean (SEM). Two group means were compared with two-tailed t test. Three or more group means were compared using one-way ANOVA followed by Tukey's post-hoc analyses. Kruskal-Wallis test followed by Dunn's test was used for non-normally distributed variables. All statistical analyses were performed using GraphPad

Prism version 10 (GraphPad Software, La Jolla, CA). *P* values <0.05 were considered statistically significant.

RESULTS

Kidney DN T cells decreased after severe ischemic AKI

Severe unilateral ischemic AKI was induced in mice then DN T cells and other conventional T cell subsets were evaluated at one week and three weeks after IRI. The gating strategies for kidney T cells are provided in Supplemental Figure S2. DN T cells significantly decreased during recovery phase compared to steady-state level by both percentages (Steady state 18.0±0.5% of αβ T cells; one week 10.1±0.7%, $P<0.001$; three weeks 6.1±0.4%, $P<0.001$) and numbers ($3.9\pm0.2 \times10^5$; $2.8\pm0.2 \times10^5$, $P=0.006$; $1.2\pm0.2 \times10^5$, $P<0.001$), whereas $CD4^+$ Tregs substantially increased (1.5±0.2%; 7.0±0.3%, $P<0.001$; 14.6±1.1%, $P<0.001$) ($1.8\pm0.2 \times10^4$; $10.1\pm1.0 \times10^4$, $P<0.001$; $15.2\pm2.5 \times10^4$, $P<0.001$) (Figure 1). Percentages of $CD8^+$ T cells increased at one week and three weeks, whereas $CD4^+$ T cells remained unchanged (Supplemental Figure S3A). $CD8^+$ Tregs were also studied given the emerging interest as a potential regulatory cell (28-30). CD8 Tregs ($CD8^+$ $Ly49^+$ $CD122^+$) increased at one week and then decreased at three weeks (Supplemental Figure S3B).

Activation and proliferation of DN T cells during repair after severe AKI

To study activation and proliferation of DN T cells during AKI recovery, we measured CD44, CD62L, CD69, and Ki67 expression in DN T cells. Effector memory (EM) phenotype ($CD44^{hi}$ $CD62L^{lo}$) DN T cells decreased (Steady state 92.2±0.5%; one week 70.2±1.1%, $P<0.001$; three weeks 81.3±2.8%, $P<0.001$), whereas central memory (CM) phenotypes ($CD44^{hi}$ $CD62L^{hi}$) increased (6.5±0.4%; 28.8±1.1%, $P<0.001$; 13.1±2.4%, $P=0.038$) in DN T cells during recovery phase. Activation marker CD69 was downregulated (99.2±0.1%; 95.9±0.6%, $P=0.016$; 85.5±1.0%, $P<0.001$). DN T cell proliferation decreased during recovery phase (Ki67, 97.8 ± 0.5%; 95.1±0.9%, $P=0.133$; 90.5±1.2%, $P<0.001$) (Figure 2).

Changes in DN T cell immune checkpoint molecules PD1, TIGIT as well as NK1.1 during repair after severe AKI

$NK1.1^{+}$ and $PD1^{+}$ DN T cells are known to be two major subsets of kidney DN T cells (31). NK1.1 expression (33.4±2.9%; 53.3±1.6%, $P<0.001$, 50.4±2.0, $P<0.001$) and PD1 expression (1.4±0.1%; 1.8±0.2%, $P=0.820$; 3.3±0.7, $P=0.020$) were upregulated at 3 weeks by percentages. However, the absolute numbers of the DN T cells with positive expression of NK1.1 or PD1 did not increase due to decreased total DN T cell numbers. A newly recognized immune checkpoint molecule with a role in early AKI, TIGIT, was also measured (32), and we found TIGIT expression in DN T cells was decreased (1.9±0.2%; 2.3±0.3%, $P=0.461$; 0.9±0.2, $P=0.010$) at 3 weeks after AKI (Figure 3).

DN T cell phenotypes were distinct from $CD4^+$ and $CD8^+$ T cells after severe AKI

Immunologic phenotypes of DN T cells were compared with conventional $CD4^+$ and $CD8^+$ T cells 3 weeks after severe AKI. DN T cells had more central-memory ($CD44^{hi}$ $CD62L^{hi}$) phenotype compared to $CD4^+$ and $CD8^+$ T cells (DN, 13.1±2.4%; CD4, 2.4±0.2% $P<0.001$; CD8, 1.8±0.1%, $P<0.001$) (Figure 4A). When CD44 and CD62L expression was compared with the contralateral normal kidneys, DN T cells from post-AKI kidneys showed lower effector-memory ($CD44^{hi}$ $CD62L^{lo}$) and higher central-memory ($CD44^{hi}$ $CD62L^{hi}$) subtypes compared to those from the non-ischemic kidneys, whereas $CD4^+$ and $CD8^+$ T cells exhibited opposite trends (Figure 4B). In non-ischemic kidneys, there were substantial percentages of naïve phenotypes in $CD4^+$ and $CD8^+$ T cells, but not in DN T cells. There were few naïve phenotypes in post-ischemic kidneys in DN, $CD4^+$, and $CD8^+$ T cells (Figure 4B).

DN T cell Ki67 expression was lower compared to $CD4^+$ and $CD8^+$ T cells during post-AKI repair (90.8±1.1%; 94.4±0.3%, $P=0.001$; 95.0±0.6%, $P=0.001$) (Figure 4C). CD69 expression in DN T cells from post-ischemic kidneys was lower compared to those of non-ischemic kidneys while $CD8^+$ T cells showed the opposite trend. Ki67 expression was lower in $CD4^+$ and $CD8^+$ T cells from non-ischemic kidneys compared to those from ischemic kidneys, whereas it was consistently high in both non-ischemic and post-ischemic kidneys in DN T cells (Figure 4D).

DN T cell NK1.1 expression was substantially higher than $CD4^+$ and $CD8^+$ T cells, and PD1 and TIGIT expression was lower than $CD4^+$ T cells (Supplemental Figure S4).

Cytokine production by DN T cells after severe AKI

Intracellular cytokine analysis of DN T cells was performed after severe AKI. After 3 weeks from severe AKI, DN T cells from postischemic kidneys had significantly lower expression of inflammatory cytokines including IL-17A, IFN-γ, and TNF- α, compared to $CD4^+$ and $CD8^+$ T cells. Profibrotic cytokine TGF-β1 expression was also lower in DN T cells than in $CD4^+$ and $CD8^+$ T cells (Figure 5). When cytokine expression was compared with the contralateral non-ischemic kidneys, there were no significant changes in proinflammatory cytokines in the DN T cells, whereas there was upregulation of IL-17A, IFN-γ, and TNF-α in the $CD4^+$ or $CD8^+$ T cells. TGF-β1 was upregulated in DN T cells from postischemic kidneys than those from nonischemic kidneys. IL-10 remained unchanged after severe AKI (Figure 5). When IL-10 and TGF-β1 expression was compared between DN T cells and Tregs from postischemic kidneys, IL-10 was lower in DN T cells whereas TGF-β1 was higher (Supplemental Figure S5).

DN T cells accelerated proximal tubular epithelial cell repair in vitro

To assess the role of DN T cells in post-AKI repair in vitro, we tested the effect of DN T cell coculture with tubular epithelial cells. PTECs were cultured and then exposed to hypoxia followed by normoxia to model ischemia-reperfusion injury in vitro. After creating consistently sized scratch wounds to tubular epithelial cell monolayer (27), DN T cells were transferred. DN T cells isolated from *gld* donors were used as commonly performed since large numbers of cells are required (19, 21, 33). *gld* DN T cells are known to be similar to kidney DN T cells, and they are capable to suppress T cell proliferation in vitro (33). Tregs were used as a positive control,

313 and $CD25^{-}$ $CD4^{+}$ T cells were used as an additional negative control to exclude nonspecific effect
314 of coculturing with lymphocytes. Kinetic quantification was performed with live-cell analysis
315 system. Cellular density at the wound significantly increased in tubular cells treated with DN T
316 cells or Tregs, whereas $CD4^{+}$ T cell-treated cells did not, compared to the vehicle-treated group
317 (Figure 6).

318

319 **Adoptive transfer of DN T cells after severe AKI increased GFR and decreased kidney**
320 **fibrosis**

321 Given the significant decline of DN T cell numbers in post-severe AKI kidneys and in vitro
322 ability of DN T cells to enhance tubular epithelial cell repair, we performed in vivo adoptive
323 transfer experiments to evaluate whether the DN T cell replenishment could reduce kidney
324 fibrosis and enhance renal recovery. DN T cells isolated from *gld* donors were adoptively
325 transferred at 6h and 48h after reperfusion. We previously demonstrated that adoptively
326 transferred *gld* DN T cells migrate into kidneys (19). CD4 Tregs were also transferred as a
327 positive control. Mice underwent serial GFR measurements during 3-week follow-up. Fibrosis
328 was measured from kidney histology sections, and profibrotic gene expression was quantified at
329 3 weeks after AKI. Kidney T cells were also studied using flow cytometry (Figure 7A). The
330 gating strategy for DN T cell sorting is provided in Supplemental Figure S6.

331 We found significant increases in GFR after 3 weeks from ischemic AKI in DN T cell
332 transferred groups and Treg transferred group (Vehicle 976.7±29.0 μL/min/100 g; DN T cells
333 1092.0±31.4 μL/min/100 g, P=0.012; Tregs 1104.9±42.5 μL/min/100 g, P=0.021) (Figure 7B).

Mice treated with DN T cells exhibited reduced medullary fibrosis (vehicle 76.3±3.2%. DN T cells, 58.1±3.3%, P=0.008; Tregs 48.7±5.7%, P<0.001) compared to the vehicle control group. There were no significant differences in cortex (vehicle 57.4±4.5%; DN T cells 45.8±2.6%, P=0.176; Tregs 41.4±6.0%, P=0.051) (Figure 7C).

We found that kidney expression of profibrotic genes *Tgfβ* (Vehicle 1.46±0.14; DN T cells 1.02±0.07, P=0.011; Tregs 0.92±0.10, P=0.003) and *Acta2* (encoding α-SMA) (Vehicle 1.66±0.15; DN T cells 1.22±0.09, P=0.033; Tregs 1.13±0.12, P=0.014) were reduced in the DN T cell-treated group and Treg-treated group. *Col1α1* was decreased in the Treg transfer group (Vehicle 1.56±0.15; DN T cells 1.24±0.15, P=0.190; Tregs 1.07±0.12, P=0.043). *Col4a1* was comparable between groups (Vehicle 1.53±0.14; DN T cells 1.36±0.10, P=0.527; Tregs 1.30±0.11, P=0.381) (Figure 7D).

DN T cells decrease kidney effector T cells and their IL-17A expression

To begin to elucidate mechanisms by which DN T cells improved GFR and decreased kidney fibrosis after severe AKI, we studied kidney T cells from post-ischemic kidneys at 3 weeks. Although the proportions and numbers of $CD4^+$, $CD8^+$, and DN T cells were comparable between the groups, we found percentages and numbers of effector-memory $CD4^+$ T cells were lower in the DN T cell and Treg-treated groups compared to the vehicle-treated group (Vehicle 96.9±0.3%; DN T cells 94.7±0.2%, P<0.001; Tregs 93.7±0.3%, P=0.021) (Figure 8A). We also measured cytokine expression on kidney $CD4^+$ T cells and found that IL-17A expression was reduced in DN T cell and Treg-treated groups (Vehicle 7.0±1.3%; 3.0±0.4%, P=0.008; 2.8±0.5,

355 *P*=0.020) (Figure 8B). The other cytokines remain unchanged. Thus, a potential mechanism by
356 which DN T cell reduces kidney fibrosis through decreased effector T cells and IL-17A.

357

358 **DISCUSSION**

359 Based on previous data that Tregs mediate organ repair (34) and emerging data on the important
360 role of kidney DN T cells in prevention of AKI (19, 21, 31), we hypothesized that DN T cells
361 undergo functional changes during recovery and could have a reparative role after severe
362 ischemic AKI. Analysis of kidney T cells after severe ischemic AKI using tissue digestion and
363 spectral flow cytometry demonstrated that DN T cells decrease substantially during the repair
364 phase and undergo long-term distinct immunologic changes compared to kidney $CD4^+$, $CD8^+$,
365 and $CD4^+$ Tregs. DN T cell coculture enhanced tubular epithelial cell repair following injury.
366 Addition of DN T cells to injured renal tubular epithelial cells *in vitro* accelerated wound repair
367 comparable to the "gold" standard Tregs. Adoptive transfer of DN T cells administered after
368 severe AKI increased GFR and reduced kidney fibrosis, as well as reduced effector-memory
369 $CD4^+$ T cells and IL-17A expression.

370 DN T cells are rare in lymphoid organs and peripheral blood, but they constitute significant
371 proportions of kidney $TCR\alpha\beta^+$ T cells both in mice and human kidneys (17-20). It was thought
372 that DN T cells originated from $CD4^+$ or $CD8^+$ T cells by loss of receptors, however kidney
373 $TCR\alpha\beta^+$ DN T cells were identified in mice lacking $CD4^+$ T cells (MHC II-deficient mice) and
374 those lacking $CD8^+$ T cells (β2m-deficient mice), which suggest DN T cells can be derived from
375 distinct progenitors (31). Kidney DN T cells expand early after moderate AKI kidneys within the

first 24 h, and then rapidly decrease at 72h below the steady-state level (19). In the present study, we found that kidney DN T cells significantly decrease both in numbers and proportion at later time points until 3 weeks after severe AKI. Interestingly, DN T cell kinetics during the AKI repair were opposite to $CD4^+$ Tregs that showed marked expansion (15, 16, 19, 31).

Though we studied Tregs as a "positive control" anti-inflammatory cell, we found a significant expansion of $CD8^+$ Tregs ($CD8^+$ $CD122^+$ $Ly49^+$), a relatively understudied T cell subset, during repair after severe AKI. The expansion of $CD8^+$ Tregs has also been demonstrated by a recent study using a cerebral IRI model (35). Emerging evidence suggests that $CD8^+$ $CD122^+$ Tregs are involved in immune regulation as their counterpart, $CD4^+$ Tregs (28, 29). Given the recent promising data showing the protective role against other organ IRI (35), $CD8^+$ Tregs' role in AKI is a promising topic of future study.

Previous studies have shown that DN T cells had distinct immunophenotypical features during early injury compared to kidney $CD4^+$ and $CD8^+$ T cells (19, 31). In the present study, we observed late immunologic changes toward more central-memory phenotypes with less proliferation rather than effector-memory phenotypes, an opposing trend with $CD4^+$ and $CD8^+$ T cells during post-AKI recovery. These long-term changes and distinct kinetics/phenotype of DN T cells, compared to Tregs, $CD4^+$, and $CD8^+$ cells, compose the complex immune response to repair and fibrosis after severe AKI.

Consistent with our earlier findings showing that $PD1^+$ subset is a potent early responder in ischemic AKI (31), we observed increased expression of the $PD1^+$ during the recovery phase after severe AKI as well. However, the absolute number of $PD1^+$ DN T remained comparable to normal kidneys due to the substantial decline in the total number of DN T cells despite PD1

upregulation. Besides PD1 expression, we also studied a novel immune checkpoint molecule, TIGIT, given the recent data on its importance on AKI (32). In contrast to upregulation of TIGIT during the early injury phase (32), $TIGIT^+$ DN T cells decreased during the recovery phase. Considering the important role of immune checkpoint molecules in AKI (32, 36, 37) and continued clinical experience of checkpoint inhibitor-associated AKI in cancer patients (38), we believe that findings from our current study have potential clinical relevance.

Proximal tubular epithelial cells are a primary target of ischemic injury and previous studies have demonstrated direct interactions between these cells and T cells in vitro (11). We therefore investigated the reparative capacity of DN T cells by co-culturing them with wounded PTECs. DN T cells significantly enhanced wound healing capacity of PTECs following hypoxic injury. Thus, there could be DN T cell-driven reparative mediators, affecting tubular epithelial cells, which needs to be further explored.

Although the immunologic mechanisms of long-term injury after AKI are less understood than early injury, chronic T cell expansion is frequently seen during AKI repair and fibrosis (12, 13). More specifically, $CD4^+$ T cells skewed toward effector-memory phenotype with upregulated activation markers (12, 13). Taken together with the findings from other organ fibrosis models (39, 40), chronic proinflammatory T cell activation is likely to have an important role in kidney fibrosis and CKD transition. Inhibition of late $CD4^+$ T cell activation could be a potential therapeutic target to reduce kidney fibrosis and CKD transition. In the present study, we observed that post-AKI DN T cell repletion reduced effector-memory $CD4^+$ T cells, consistent with previously found $CD4^+$ T cell suppressive function (19). Another important finding was that DN T cells reduced IL-17 expression in $CD4^+$ T cells. IL-17 and IL-17-producing T cells were

suggested as important contributors to AKI and CKD transition (41-43). There was an increase of IL-17 expressing T cells during post-AKI repair, and genes related to IL-17 pathway were upregulated in kidney fibrosis (14). Elevated IL-17 expression was observed in kidney biopsy specimens from patients with kidney fibrosis (44). Blocking IL-17$^+$ cell activation or deletion of *Il17* gene mitigated renal injury (41-43). Taken together, the reparative effect of DN T cells could be attributed to inhibiting effector CD4$^+$ T cells and IL-17 downregulation. Since the IL-17 downregulation was observed in CD4$^+$ Treg-treated group as well, the IL-17-dependent mechanism could also be involved in the well-known protective effect of Treg-based therapy (45).

The immunoregulatory role of DN T cells has also been shown in other chronic disease models such as allograft rejection, graft-versus-host disease, and type 1 diabetes (46). However, DN T cells' proinflammatory pathogenic roles have also been reported particularly in autoimmune diseases such as lupus, psoriasis, and Sjogren syndrome (47). These discordant findings are likely due to their heterogeneity or plasticity (48). For example, a recent study using single-cell RNA sequencing analysis proposed 5 different subsets of DN T cells (49). Discovering a specific marker for reparative DN T cell subset is needed for a deeper understanding of this population.

The current study has several limitations. First, although we focused on T cell-mediated mechanisms in AKI repair, other types of kidney immune cells such as macrophages, dendritic cells, neutrophils, B cells, and innate lymphoid cells could also play important roles in AKI repair (50-52). Thus, we cannot rule out a collateral effect of DN T cells on other types of kidney immune cells. Second, there are currently no available DN T cell specific markers, thus we were unable to deplete kidney DN T cells or use models lacking DN T cells. The discovery of

reparative kidney DN T cell-specific markers is warranted for future studies to use these techniques to further understand the role of DN T cells. Another limitation was that we used *gld* DN T cells, which are not identical to WT DN T cells. However, since DN T cells are rarely present in lymphoid organs, it is challenging to get enough DN T cells for DN T cell adoptive transfer. We previously demonstrated that adoptively transferred *gld* DN T cells migrate into post-AKI kidneys (19).

Despite these limitations, our study is novel and has important pathophysiologic and therapeutic implications. The decreasing kinetics of DN T cells could be a potential target involved in kidney fibrosis in other diseases. Cell immunotherapy is an increasing reality, particularly for cancer patients. The regulatory cell-based therapy field has flourished with recent ongoing clinical trials in kidney diseases (53). The present study identifies a novel therapeutic approach to administer expanded DN T cells to accelerate recovery and decrease fibrosis in patients after severe AKI.

DATA VAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Figure S1. Flow cytometry analysis of sorted Tregs for adoptive transfer study.

Supplemental Figure S2. Gating strategies for kidney T cells.

Supplemental Figure S3. Changes in $CD4^+$, $CD8^+$ T cells and $CD8^+$ Tregs during repair phase after severe ischemic AKI.

Supplemental Figure S4. Differences in NK1.1 and immune checkpoint molecule expression between DN, $CD4^+$, and $CD8^+$ T cells after severe AKI.

Supplemental Figure S5. IL-10 and TGF-β1 expression in DN T cells and Tregs from postischemic kidneys.

Supplemental Figure S6. Gating strategy for DN T cell sorting from *gld* donors.

Supplemental Table 1. Primer sequences for profibrotic genes.

Supplemental Material link: https://doi.org/10.6084/m9.figshare.24547087

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DISCLOSURES

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

H.R. designed the study. K.L. and H.R. drafted the manuscript. K.L., S.G., J.T.K., A.N.R, and S.N. performed the experiments. K.L., S.N., and H.R. analyzed and interpreted the data. L.J.A. analyzed histology data. S.G., J.T.K, A.N.R., L.J.A, and S.N. revised the manuscript. All authors approved the final version of the manuscript.

REFERENCES

1. **Kellum JA, Romagnani P, Ashuntantang G, Ronco C, Zarbock A, Anders HJ**. Acute kidney injury. *Nat Rev Dis Primers* 7: 52, 2021. doi:10.1038/s41572-021-00284-z

2. **Chawla LS, Eggers PW, Star RA, Kimmel PL**. Acute kidney injury and chronic kidney disease as interconnected syndromes. *N Engl J Med* 371: 58-66, 2014. doi:10.1056/NEJMra1214243

3. **Jang HR, Rabb H**. Immune cells in experimental acute kidney injury. *Nat Rev Nephrol* 11: 88-101, 2015. doi:10.1038/nrneph.2014.180

4. **Thomas ME, Blaine C, Dawnay A, Devonald MA, Ftouh S, Laing C, Latchem S, Lewington A, Milford DV, Ostermann M**. The definition of acute kidney injury and its use in practice. *Kidney Int* 87: 62-73, 2015. doi:10.1038/ki.2014.328

5. **Aufhauser DD, Jr., Wang Z, Murken DR, Bhatti TR, Wang Y, Ge G, Redfield RR, 3rd, Abt PL, Wang L, Svoronos N, Thomasson A, Reese PP, Hancock WW, Levine MH**. Improved renal ischemia tolerance in females influences kidney transplantation outcomes. *J Clin Invest* 126: 1968-1977, 2016. doi:10.1172/JCI84712

6. **Bonventre JV, Yang L**. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest* 121: 4210-4221, 2011. doi:10.1172/JCI45161

7. **Wei Q, Sun H, Song S, Liu Y, Liu P, Livingston MJ, Wang J, Liang M, Mi QS, Huo Y, Nahman NS, Mei C, Dong Z**. MicroRNA-668 represses MTP18 to preserve mitochondrial dynamics in ischemic acute kidney injury. *J Clin Invest* 128: 5448-5464, 2018. doi:10.1172/JCI121859

8. **Yang L, Besschetnova TY, Brooks CR, Shah JV, Bonventre JV**. Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. *Nat Med* 16: 535-543, 531p following 143, 2010. doi:10.1038/nm.2144

9. **Ying WZ, Li X, Rangarajan S, Feng W, Curtis LM, Sanders PW**. Immunoglobulin light chains generate proinflammatory and profibrotic kidney injury. *J Clin Invest* 129: 2792-2806, 2019. doi:10.1172/JCI125517

10. **Burne MJ, Daniels F, El Ghandour A, Mauiyyedi S, Colvin RB, O'Donnell MP, Rabb H**. Identification of the CD4+ T cell as a major pathogenic factor in ischemic acute renal failure. *J Clin Invest* 108: 1283-1290, 2001. doi:10.1172/JCI12080

11. **Rabb H, Daniels F, O'Donnell M, Haq M, Saba SR, Keane W, Tang WW**. Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am J Physiol Renal Physiol* 279: F525-531, 2000. doi:10.1152/ajprenal.2000.279.3.F525

12. **Burne-Taney MJ, Yokota N, Rabb H**. Persistent renal and extrarenal immune changes after severe ischemic injury. *Kidney Int* 67: 1002-1009, 2005. doi:10.1111/j.1523-1755.2005.00163.x

13. **Ascon M, Ascon DB, Liu M, Cheadle C, Sarkar C, Racusen L, Hassoun HT, Rabb H**. Renal ischemia-reperfusion leads to long term infiltration of activated and effector-memory T lymphocytes. *Kidney Int* 75: 526-535, 2009. doi:10.1038/ki.2008.602

14. **do Valle Duraes F, Lafont A, Beibel M, Martin K, Darribat K, Cuttat R, Waldt A, Naumann U, Wieczorek G, Gaulis S, Pfister S, Mertz KD, Li J, Roma G, Warncke M**. Immune cell landscaping reveals a protective role for regulatory T cells during kidney injury and fibrosis. *JCI Insight* 5: 2020. doi:10.1172/jci.insight.130651

15. **Kinsey GR, Huang L, Vergis AL, Li L, Okusa MD**. Regulatory T cells contribute to the protective effect of ischemic preconditioning in the kidney. *Kidney Int* 77: 771-780, 2010. doi:10.1038/ki.2010.12

16. **Gandolfo MT, Jang HR, Bagnasco SM, Ko GJ, Agreda P, Satpute SR, Crow MT, King LS, Rabb H**. Foxp3+ regulatory T cells participate in repair of ischemic acute kidney injury. *Kidney Int* 76: 717-729, 2009. doi:10.1038/ki.2009.259

17. **Ascon DB, Ascon M, Satpute S, Lopez-Briones S, Racusen L, Colvin RB, Soloski MJ, Rabb H**. Normal mouse kidneys contain activated and CD3+CD4- CD8- double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol* 84: 1400-1409, 2008. doi:10.1189/jlb.0907651

18. **Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC**. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* 181: 8761-8766, 2008. doi:10.4049/jimmunol.181.12.8761

19. **Martina MN, Noel S, Saxena A, Bandapalle S, Majithia R, Jie C, Arend LJ, Allaf ME, Rabb H, Hamad AR**. Double-Negative alphabeta T Cells Are Early Responders to AKI and Are Found in Human Kidney. *J Am Soc Nephrol* 27: 1113-1123, 2016. doi:10.1681/ASN.2014121214

20. **Li H, Adamopoulos IE, Moulton VR, Stillman IE, Herbert Z, Moon JJ, Sharabi A, Krishfield S, Tsokos MG, Tsokos GC**. Systemic lupus erythematosus favors the generation of IL-17 producing double negative T cells. *Nat Commun* 11: 2859, 2020. doi:10.1038/s41467-020-16636-4

21. **Gong J, Noel S, Hsu J, Bush EL, Arend LJ, Sadasivam M, Lee SA, Kurzhagen JT, Hamad ARA, Rabb H**. TCR(+)CD4(-)CD8(-) (double negative) T cells protect from cisplatin-induced renal epithelial cell apoptosis and acute kidney injury. *Am J Physiol Renal Physiol* 318: F1500-F1512, 2020. doi:10.1152/ajprenal.00033.2020

22. **Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG**. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412, 2010. doi:10.1371/journal.pbio.1000412

23. **Fu Y, Tang C, Cai J, Chen G, Zhang D, Dong Z**. Rodent models of AKI-CKD transition. *Am J Physiol Renal Physiol* 315: F1098-F1106, 2018. doi:10.1152/ajprenal.00199.2018

24. **Scarfe L, Schock-Kusch D, Ressel L, Friedemann J, Shulhevich Y, Murray P, Wilm B, de Caestecker M**. Transdermal Measurement of Glomerular Filtration Rate in Mice. *J Vis Exp* 2018. doi:10.3791/58520

25. **Friedemann J, Heinrich R, Shulhevich Y, Raedle M, William-Olsson L, Pill J, Schock-Kusch D**. Improved kinetic model for the transcutaneous measurement of glomerular filtration rate in experimental animals. *Kidney Int* 90: 1377-1385, 2016. doi:10.1016/j.kint.2016.07.024

26. **Ascon DB, Lopez-Briones S, Liu M, Ascon M, Savransky V, Colvin RB, Soloski MJ, Rabb H**. Phenotypic and functional characterization of kidney-infiltrating lymphocytes in renal ischemia reperfusion injury. *J Immunol* 177: 3380-3387, 2006. doi:10.4049/jimmunol.177.5.3380

27. **Liang CC, Park AY, Guan JL**. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2: 329-333, 2007. doi:10.1038/nprot.2007.30

28. **Kim HJ, Wang X, Radfar S, Sproule TJ, Roopenian DC, Cantor H**. CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice. *Proc Natl Acad Sci U S A* 108: 2010-2015, 2011. doi:10.1073/pnas.1018974108

29. **Dai Z, Zhang S, Xie Q, Wu S, Su J, Li S, Xu Y, Li XC**. Natural CD8+CD122+ T cells are more potent in suppression of allograft rejection than CD4+CD25+ regulatory T cells. *Am J Transplant* 14: 39-48, 2014. doi:10.1111/ajt.12515

30. **Cantor H, Kim HJ**. A new chapter in the CD8 T reg story. *J Exp Med* 218: 2021. doi:10.1084/jem.20201746

31. **Sadasivam M, Noel S, Lee SA, Gong J, Allaf ME, Pierorazio P, Rabb H, Hamad ARA**. Activation and Proliferation of PD-1(+) Kidney Double-Negative T Cells Is Dependent on Nonclassical MHC Proteins and IL-2. *J Am Soc Nephrol* 30: 277-292, 2019. doi:10.1681/ASN.2018080815

32. **Noel S, Lee K, Gharaie S, Kurzhagen JT, Pierorazio PM, Arend LJ, Kuchroo VK, Cahan P, Rabb H**. Immune Checkpoint Molecule TIGIT Regulates Kidney T Cell Functions and Contributes to AKI. *J Am Soc Nephrol* 34: 755-771, 2023. doi:10.1681/asn.0000000000000063

33. **Hamad AR, Mohamood AS, Trujillo CJ, Huang CT, Yuan E, Schneck JP**. B220+ double-negative T cells suppress polyclonal T cell activation by a Fas-independent mechanism that involves inhibition of IL-2 production. *J Immunol* 171: 2421-2426, 2003. doi:10.4049/jimmunol.171.5.2421

34. **D'Alessio FR, Kurzhagen JT, Rabb H**. Reparative T lymphocytes in organ injury. *J Clin Invest* 129: 2608-2618, 2019. doi:10.1172/JCI124614

35. **Cai W, Shi L, Zhao J, Xu F, Dufort C, Ye Q, Yang T, Dai X, Lyu J, Jin C, Pu H, Yu F, Hassan S, Sun Z, Zhang W, Hitchens TK, Shi Y, Thomson AW, Leak RK, Hu X, Chen J**. Neuroprotection against ischemic stroke requires a specific class of early responder T cells in mice. *J Clin Invest* 132: 2022. doi:10.1172/JCI157678

36. **Schoop R, Wahl P, Le Hir M, Heemann U, Wang M, Wuthrich RP**. Suppressed T-cell activation by IFN-gamma-induced expression of PD-L1 on renal tubular epithelial cells. *Nephrol Dial Transplant* 19: 2713-2720, 2004. doi:10.1093/ndt/gfh423

37. **Jaworska K, Ratajczak J, Huang L, Whalen K, Yang M, Stevens BK, Kinsey GR**. Both PD-1 ligands protect the kidney from ischemia reperfusion injury. *J Immunol* 194: 325-333, 2015. doi:10.4049/jimmunol.1400497

38. **Cortazar FB, Marrone KA, Troxell ML, Ralto KM, Hoenig MP, Brahmer JR, Le DT, Lipson EJ, Glezerman IG, Wolchok J, Cornell LD, Feldman P, Stokes MB, Zapata SA,**

Hodi FS, Ott PA, Yamashita M, Leaf DE. Clinicopathological features of acute kidney injury associated with immune checkpoint inhibitors. *Kidney Int* 90: 638-647, 2016. doi:10.1016/j.kint.2016.04.008

39. **Bresser P, Jansen HM, Weller FR, Lutter R, Out TA**. T-cell activation in the lungs of patients with systemic sclerosis and its relation with pulmonary fibrosis. *Chest* 120: 66s-68s, 2001. doi:10.1378/chest.120.1_suppl.s66

40. **Luzina IG, Atamas SP, Wise R, Wigley FM, Choi J, Xiao HQ, White B**. Occurrence of an activated, profibrotic pattern of gene expression in lung CD8+ T cells from scleroderma patients. *Arthritis Rheum* 48: 2262-2274, 2003. doi:10.1002/art.11080

41. **Chan AJ, Alikhan MA, Odobasic D, Gan PY, Khouri MB, Steinmetz OM, Mansell AS, Kitching AR, Holdsworth SR, Summers SA**. Innate IL-17A-producing leukocytes promote acute kidney injury via inflammasome and Toll-like receptor activation. *Am J Pathol* 184: 1411-1418, 2014. doi:10.1016/j.ajpath.2014.01.023

42. **Mehrotra P, Collett JA, McKinney SD, Stevens J, Ivancic CM, Basile DP**. IL-17 mediates neutrophil infiltration and renal fibrosis following recovery from ischemia reperfusion: compensatory role of natural killer cells in athymic rats. *Am J Physiol Renal Physiol* 312: F385-F397, 2017. doi:10.1152/ajprenal.00462.2016

43. **Mehrotra P, Sturek M, Neyra JA, Basile DP**. Calcium channel Orai1 promotes lymphocyte IL-17 expression and progressive kidney injury. *J Clin Invest* 129: 4951-4961, 2019. doi:10.1172/JCI126108

44. **Coppock GM, Aronson LR, Park J, Qiu C, Park J, DeLong JH, Radaelli E, Susztak K, Hunter CA**. Loss of IL-27Ralpha Results in Enhanced Tubulointerstitial Fibrosis Associated with Elevated Th17 Responses. *J Immunol* 205: 377-386, 2020. doi:10.4049/jimmunol.1901463

45. **Sawitzki B, Harden PN, Reinke P, Moreau A, Hutchinson JA, Game DS, Tang Q, Guinan EC, Battaglia M, Burlingham WJ, Roberts ISD, Streitz M, Josien R, Böger CA, Scottà C, Markmann JF, Hester JL, Juerchott K, Braudeau C, James B, Contreras-Ruiz L, van der Net JB, Bergler T, Caldara R, Petchey W, Edinger M, Dupas N, Kapinsky M, Mutzbauer I, Otto NM, Öllinger R, Hernandez-Fuentes MP, Issa F, Ahrens N, Meyenberg C, Karitzky S, Kunzendorf U, Knechtle SJ, Grinyó J, Morris PJ, Brent L, Bushell A, Turka LA, Bluestone JA, Lechler RI, Schlitt HJ, Cuturi MC, Schlickeiser S, Friend PJ, Miloud T, Scheffold A, Secchi A, Crisalli K, Kang SM, Hilton R, Banas B, Blancho G, Volk HD, Lombardi G, Wood KJ, Geissler EK**. Regulatory cell therapy in kidney transplantation (The ONE Study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. *Lancet* 395: 1627-1639, 2020. doi:10.1016/s0140-6736(20)30167-7

46. **Juvet SC, Zhang L**. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *J Mol Cell Biol* 4: 48-58, 2012. doi:10.1093/jmcb/mjr043

47. **Li H, Tsokos GC**. Double-negative T cells in autoimmune diseases. *Curr Opin Rheumatol* 33: 163-172, 2021. doi:10.1097/bor.0000000000000778

48. **Newman-Rivera AM, Kurzhagen JT, Rabb H**. TCRalphabeta+ CD4-/CD8- "double negative" T cells in health and disease-implications for the kidney. *Kidney Int* 102: 25-37, 2022. doi:10.1016/j.kint.2022.02.035

49. **Yang L, Zhu Y, Tian D, Wang S, Guo J, Sun G, Jin H, Zhang C, Shi W, Gershwin ME, Zhang Z, Zhao Y, Zhang D**. Transcriptome landscape of double negative T cells by single-cell RNA sequencing. *J Autoimmun* 121: 102653, 2021. doi:10.1016/j.jaut.2021.102653

50. **Lee SA, Noel S, Sadasivam M, Hamad ARA, Rabb H**. Role of Immune Cells in Acute Kidney Injury and Repair. *Nephron* 137: 282-286, 2017. doi:10.1159/000477181

51. **Kung CW, Chou YH**. Acute kidney disease: an overview of the epidemiology, pathophysiology, and management. *Kidney Res Clin Pract* 42: 686-699, 2023. doi:10.23876/j.krcp.23.001

52. **Wang C, Li SW, Zhong X, Liu BC, Lv LL**. An update on renal fibrosis: from mechanisms to therapeutic strategies with a focus on extracellular vesicles. *Kidney Res Clin Pract* 42: 174-187, 2023. doi:10.23876/j.krcp.22.159

53. **Rabb H, Lee K, Parikh CR**. Beyond kidney dialysis and transplantation: what's on the horizon? *J Clin Invest* 132: 2022. doi:10.1172/jci159308

FIGURE LEGENDS

Figure 1. Changes in anti-inflammatory T cells during repair phase after severe AKI.

DN T cells significantly decreased after severe ischemic AKI by percentages and numbers, whereas $CD4^+$ Tregs markedly increased. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis ($n = 8$ for steady state kidneys, $n = 10$ for 1 week, $n = 11$ for 3 weeks). Data are from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The steady state group refers to uninjured normal kidneys from naïve control mice.

AKI, acute kidney injury; DN, double-negative; IRI, ischemia-reperfusion injury; Treg, regulatory T cells.

Figure 2. Double-negative T cell activation and proliferation during repair phase after severe AKI.

Effector-memory ($CD44^{hi}$ $CD62L^{lo}$) DN T cells decreased significantly after severe AKI during repair, whereas percentages of central-memory ($CD44^{hi}$ $CD62L^{hi}$) DN T cells increased. Expression of markers for activation (CD69) and proliferation (Ki67) decreased significantly in DN T cells during repair phase. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis ($n = 8$ for steady state kidneys, $n = 10$ for 1 week, $n = 11$ for 3 weeks). Data are from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The steady state group refers to uninjured normal kidneys from naïve control mice.

AKI, acute kidney injury; CM, central-memory; DN, double-negative; EM, effector-memory; IRI, ischemia-reperfusion injury.

Figure 3. Changes in NK1.1 and immune checkpoint molecules in double-negative T cells in the repair phase after severe AKI.

NK1.1 and PD1 expression was upregulated at 3 weeks by percentages, whereas TIGIT expression was decreased at 3 weeks after AKI. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis ($n = 8$ for steady state kidneys, $n = 10$ for 1 week, $n = 11$ for 3 weeks). Data are from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The steady state group refers to uninjured normal kidneys from naïve control mice.

AKI, acute kidney injury; IRI, ischemia-reperfusion injury; TIGIT, T-cell immunoreceptor with Ig and ITIM domains.

Figure 4. Phenotypical differences between double-negative T cells, $CD4^+$ and $CD8^+$ T cells 3 weeks after severe AKI.

(A) DN T cell CD44 and CD62L expression was compared with $CD4^+$ and $CD8^+$ T cells at 3 weeks after severe ischemic AKI. DN T cells had a lower effector-memory phenotype ($CD44^{hi}$ $CD62L^{lo}$) compared to $CD4^+$ T cells during post-AKI repair. There were few central-memory phenotypes ($CD44^{hi}$ $CD62L^{hi}$) in $CD4^+$ and $CD8^+$ T cells. **(B)** When CD44 and CD62L expression was compared to the contralateral normal kidneys, DN T cells from post-AKI kidneys had lower effector-memory and higher central-memory phenotypes compared to those from the contralateral normal kidneys. $CD4^+$ and $CD8^+$ T cells showed opposite trends. There were few naïve phenotype T cells in post-AKI kidneys. Only $CD4^+$ and $CD8^+$ T cells had substantial naïve phenotypes in normal kidneys, and they decreased in the post-AKI kidneys. **(C)** Ki67 expression

among three different T cell subsets was compared in post-AKI kidneys at 3 weeks. $CD8^+$ T cells had the highest expression of CD69. Ki67 expression was lower in the DN T cells compared to $CD4^+$ and $CD8^+$ T cells. **(D)** When CD69 and Ki67 expression was compared to the corresponding subsets from the contralateral normal kidneys, CD69 was lower in the DN T cells from post-AKI kidneys than those from contralateral normal kidneys. Ki69 was higher in $CD4^+$ and CD8 T^+ cells from the post-AKI kidneys, whereas DN T cell Ki67 expression remained unchanged. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis ($n = 11$). Data are from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The empty bars and grey bars represent T cells from ischemic and non-ischemic (uninjured contralateral) kidneys, respectively.

AKI, acute kidney injury; CM, central-memory; DN, double-negative; EM, effector-memory.

Figure 5. Cytokine production by DN T cells during repair phase after severe AKI.

Expression of inflammatory cytokines, IL-17A, IFN-γ, and TNF-α, was significantly lower in DN T cells than $CD4^+$ and $CD8^+$ T cells 3 weeks after severe AKI. TGF-β1 was lower in DN T cells compared to $CD4^+$ and $CD8^+$ T cells. Compared to the corresponding subsets from the contralateral non-ischemic kidneys, IL-17A increased in $CD4^+$ T cells, and IFN-γ and TNF-α increased in $CD8^+$ T cells, whereas IL-17, IFN-γ, and TNF-α remained unchanged in DN T cells. TGF-β1 expression was higher in all T cell subsets from postischemic kidneys compared to those from non-ischemic kidneys. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis ($n = 16$). Data are from 3 independent experiments. *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$. The empty bars and grey bars represent T cells from ischemic and non-ischemic kidneys, respectively.

AKI, acute kidney injury; DN, double-negative; IL, interleukin; IFN, interferon; TGF, transforming growth factor; TNF, tumor necrosis factor.

Figure 6. Double-negative T cell coculture accelerated kidney tubular epithelial cell repair in vitro.

Scratch wounds were created to proximal tubular epithelial cell monolayer after hypoxia exposure. Activated DN T cells, Tregs (positive control), $CD4^+$ T cells (negative control), or vehicle (negative control) were transferred after scratch. Phase-contrast images were acquired serially using a live-cell analysis system. **(A)** Representative wound images at 24h with segmentation lines shown for the initial scratch (blue) and wound (yellow). **(B)** Kinetic quantification showing percent wound density for DN, Tregs, $CD4^+$ T cells, vehicle, and normoxia control groups. The normoxia control group showed higher wound density compared to the other groups exposed to hypoxia (P <0.05 for all time points). DN T cell coculture exhibited better wound repair compared to the Vehicle and $CD4^+$ T cell coculture groups (at 24h, vehicle 56.3±2.1%; CD4 56.1±1.2%, vs vehicle P>0.999; DN 67.5 ± 2.3%, vs vehicle P=0.007, vs CD4 P=0.006; Tregs 62.1±3.0%, vs vehicle, P=0.275, vs CD4, P=0.250). n = 8 replicates /group. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis. *P < 0.05 compared to the vehicle control group

DN, double-negative; Tregs, regulatory T cells.

Figure 7. Double-negative T cell treatment after severe AKI increased GFR and reduced kidney fibrosis.

(A) Schematic of experimental design. DN T cells, Vehicle (negative control), or Tregs (positive control) were adoptively transferred after severe ischemic AKI. GFR was measured with FITC-sinistrin-based method. Kidney sections were stained with Masson's trichrome to assess kidney fibrosis. Fibrosis genes were quantified with quantitative RT-PCR. Kidney T cells were isolated and studied by spectral flow cytometry. **(B)** GFRs at 3 weeks were higher in the DN T cell (n = 17) and Treg (n = 14) transfer groups than the vehicle control group (n = 17). **(C)** Masson's trichrome staining at 3 weeks after severe AKI. Kidney fibrosis in outer medullar was significantly lower in the DN T cell-treated group than the vehicle control group (n = 9 /group). **(D)** Expression of genes encoding TGF-β and αSMA was lower in the DN T cell-treated group (n =14–17 /group). Data are from 3 independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis except for GFR data. The Kruskal-Wallis test followed by Dunn's test was used for GFR due to nonnormal distribution. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

AKI, acute kidney injury; DN, double-negative; GFR, glomerular filtration rate; Treg, regulatory T cells.

Figure 8. Double-negative T cell adoptive transfer after severe AKI decreased effector $CD4^+$ T cells and IL-17 production.

T cells from post-AKI kidneys were analyzed at 3 weeks after severe AKI followed by adoptive transfers. **(A)** Effector-memory phenotype $CD4^+$ T cells were lower in the DN T cell and Treg transfer group. n =10–11 /group. Data are from 2 independent experiments. **(B)** IL-17A expression in $CD4^+$ T cells was lower in the DN T cell (n = 15) and Treg-treated mice (n = 9) than in the vehicle control group (n = 15). Data are from 3 independent experiments. Statistical

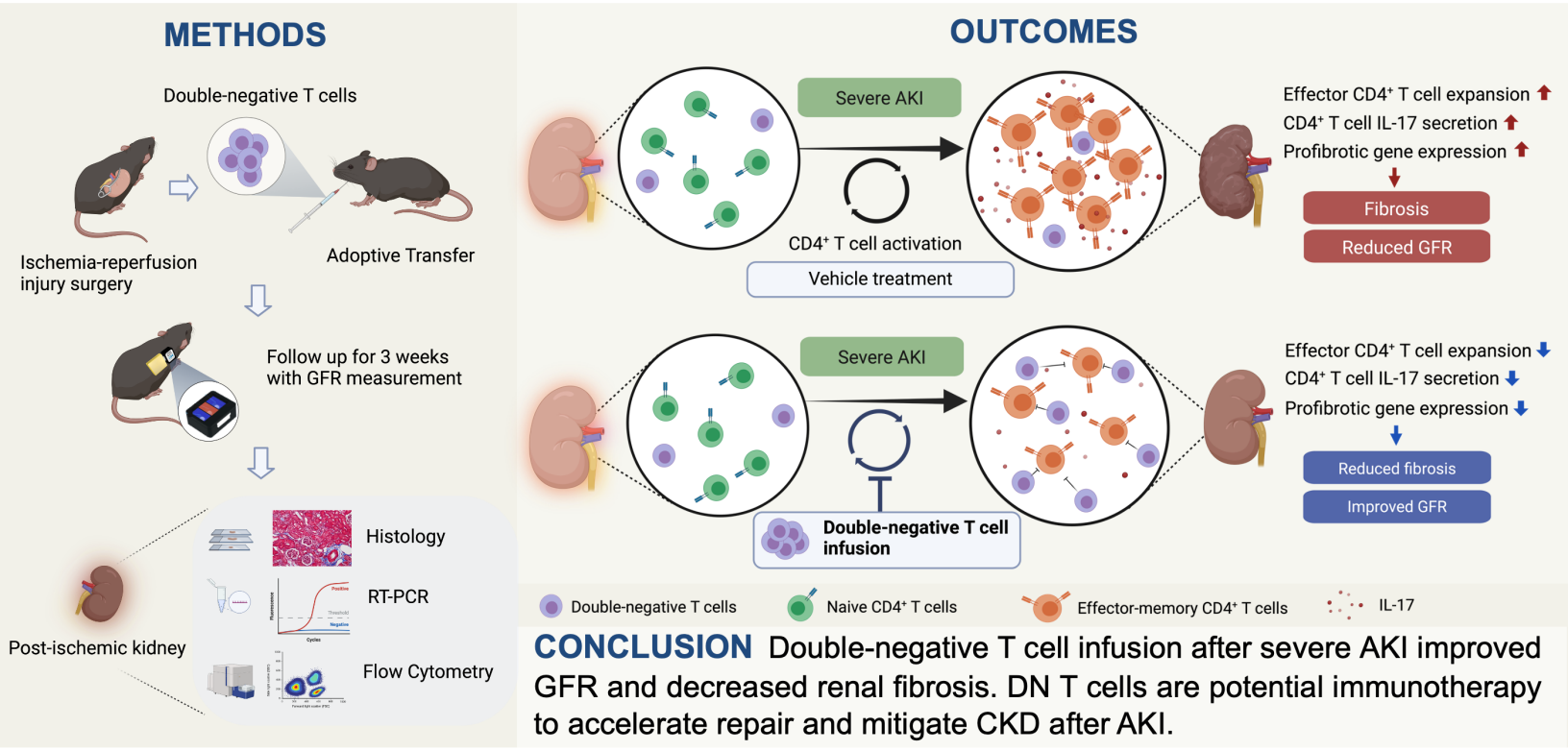
798 analyses were performed using one-way ANOVA followed by Tukey's post hoc analysis. *$P <$

799 0.05; **$P < 0.01$.

800 AKI, acute kidney injury; CM, central-memory; DN, double-negative; EM, effector-memory; IL,

801 interleukin; Treg, regulatory T cells.

Double-negative T cells have a reparative role after severe ischemic acute kidney injury



CONCLUSION Double-negative T cell infusion after severe AKI improved GFR and decreased renal fibrosis. DN T cells are potential immunotherapy to accelerate repair and mitigate CKD after AKI.

Figure 1

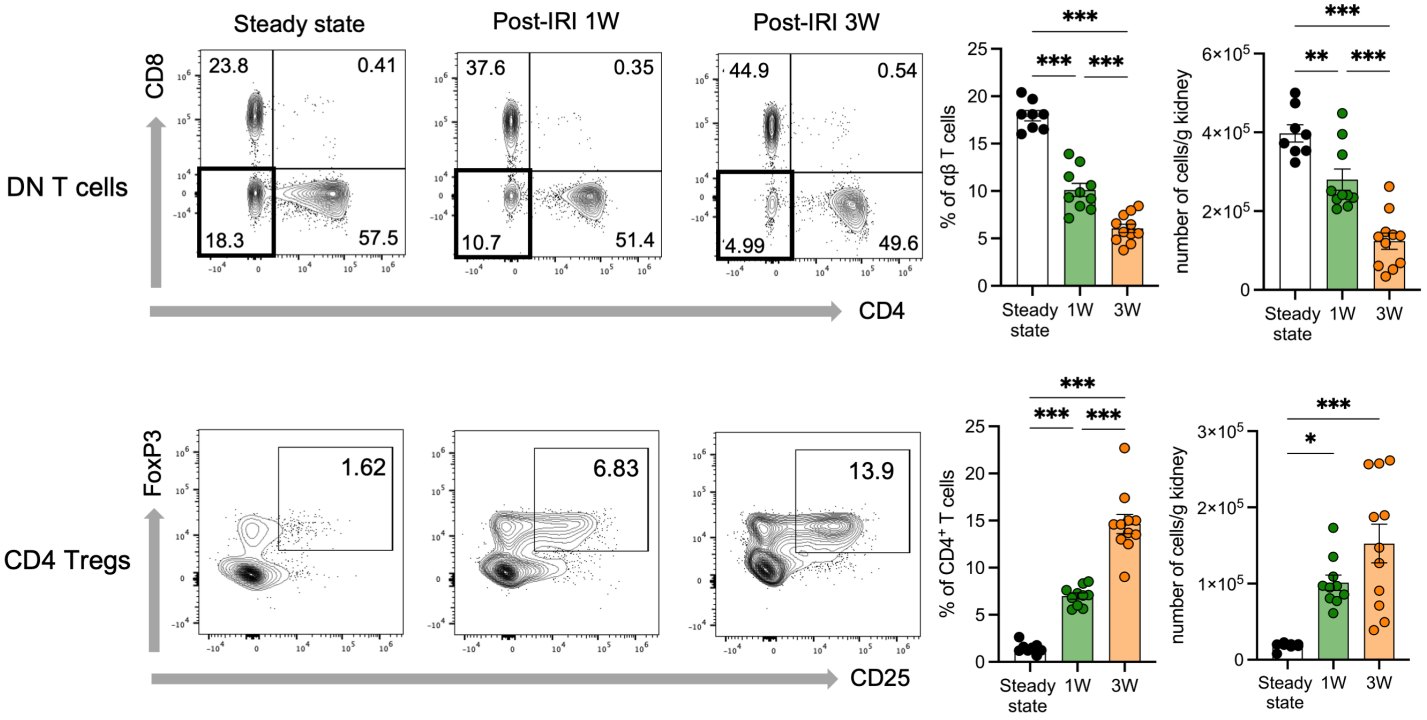
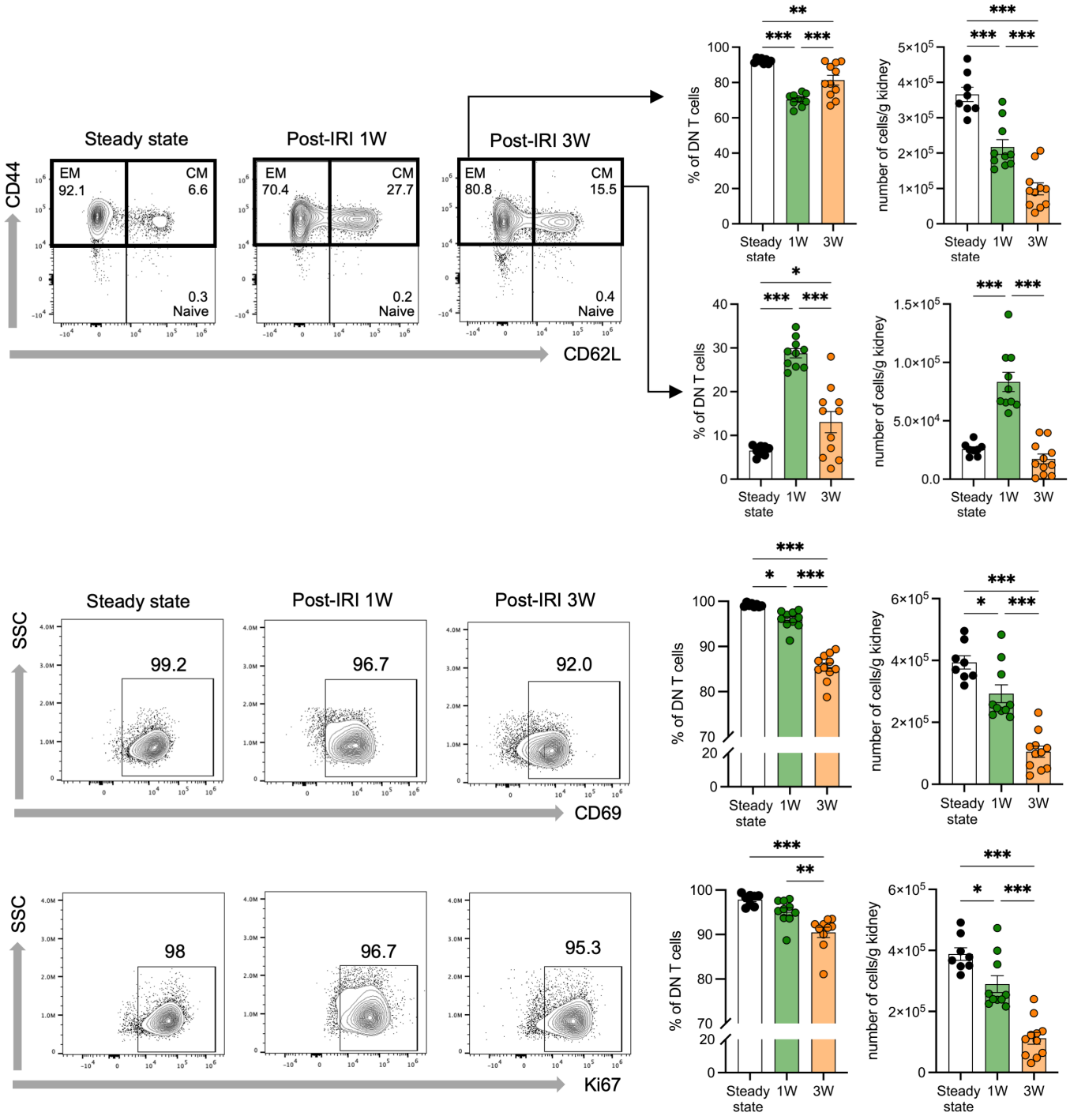


Figure 2



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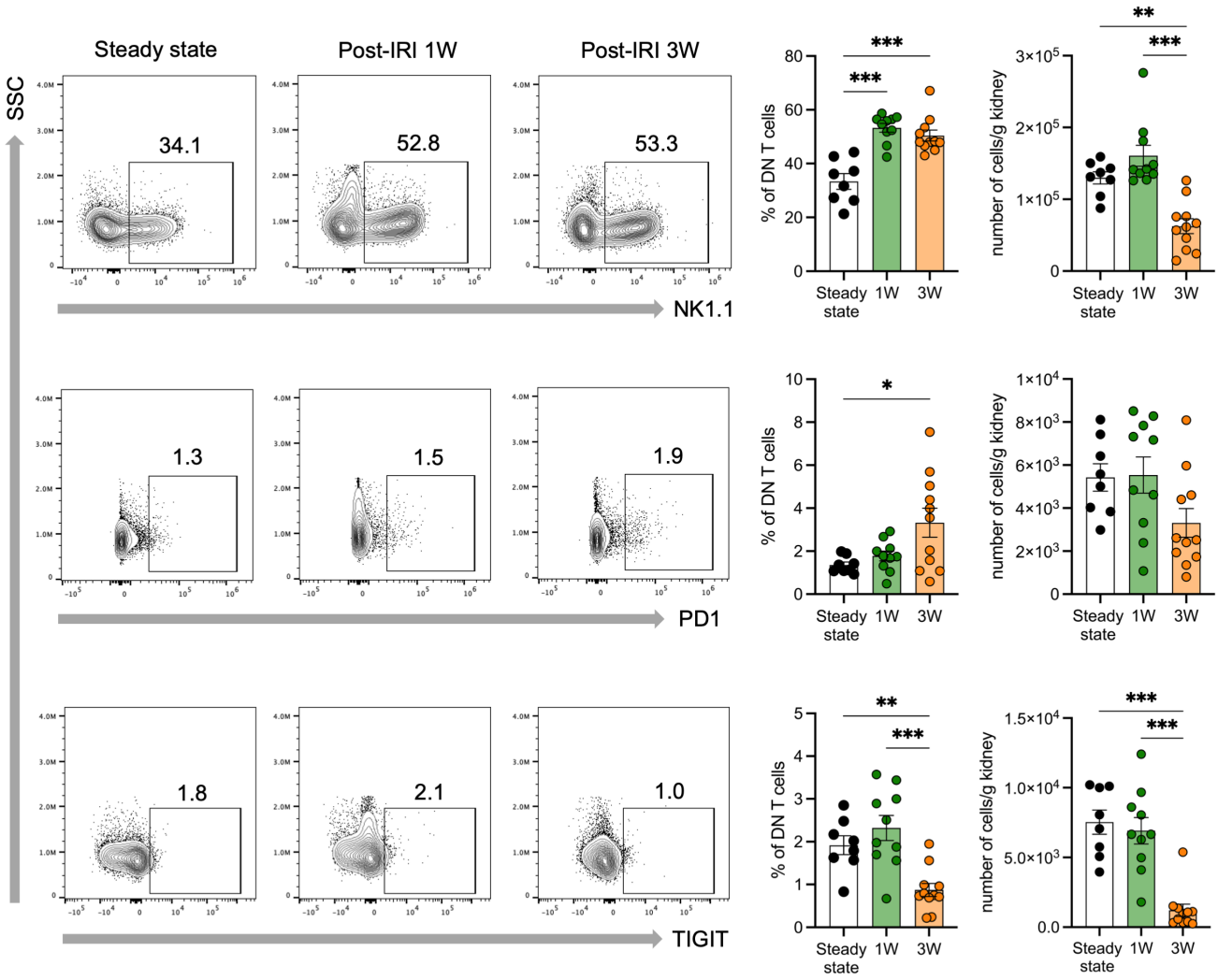

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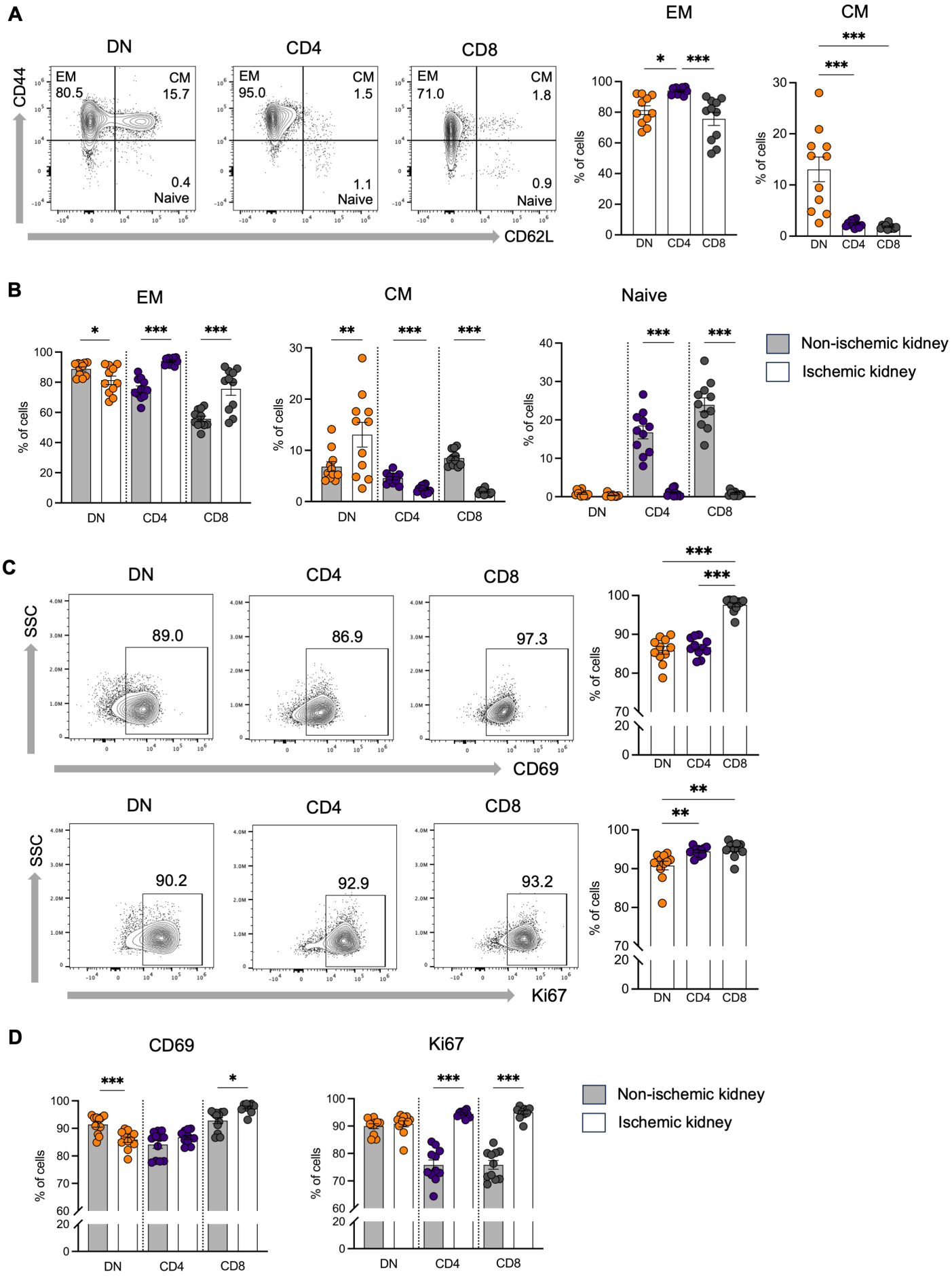

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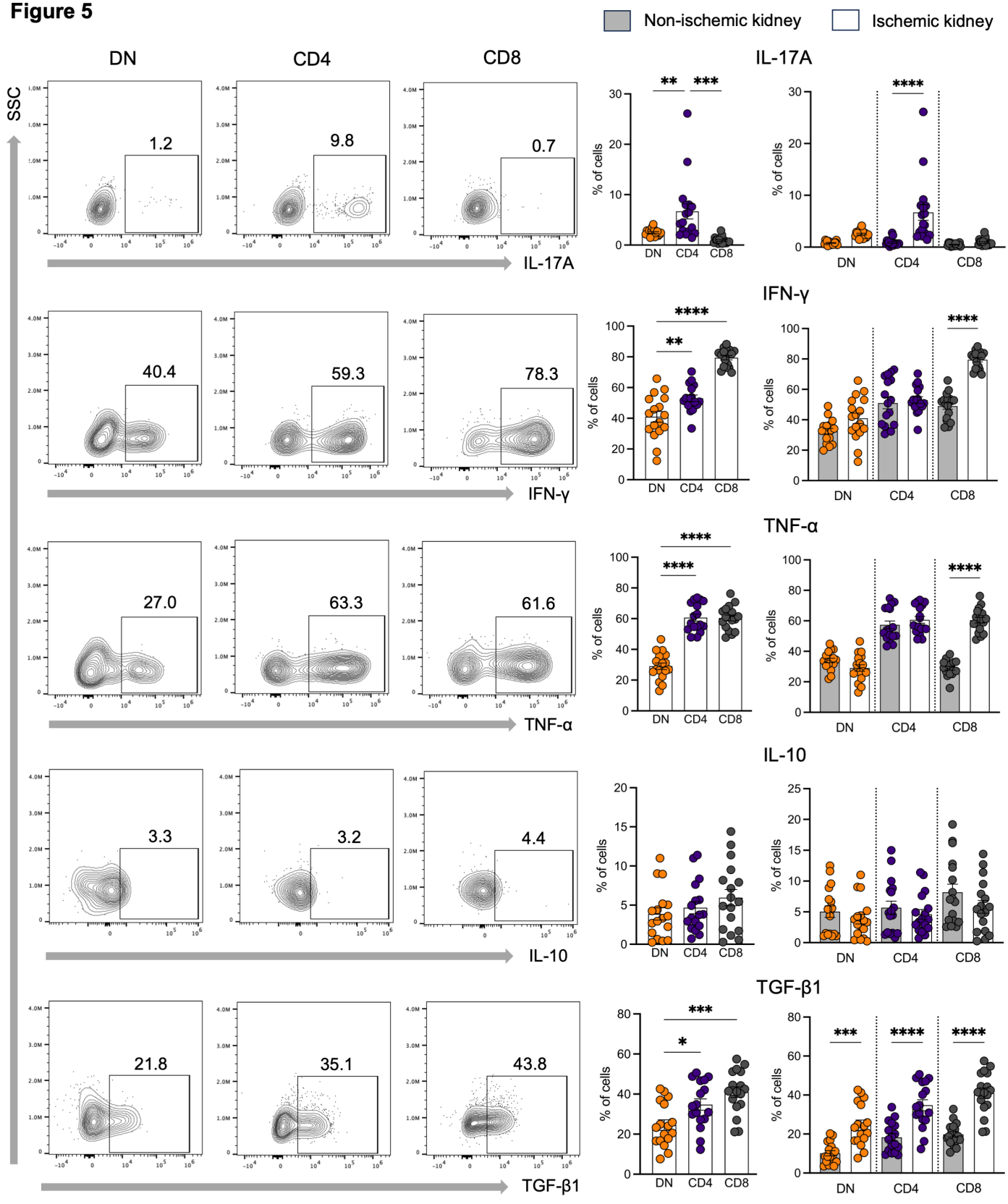
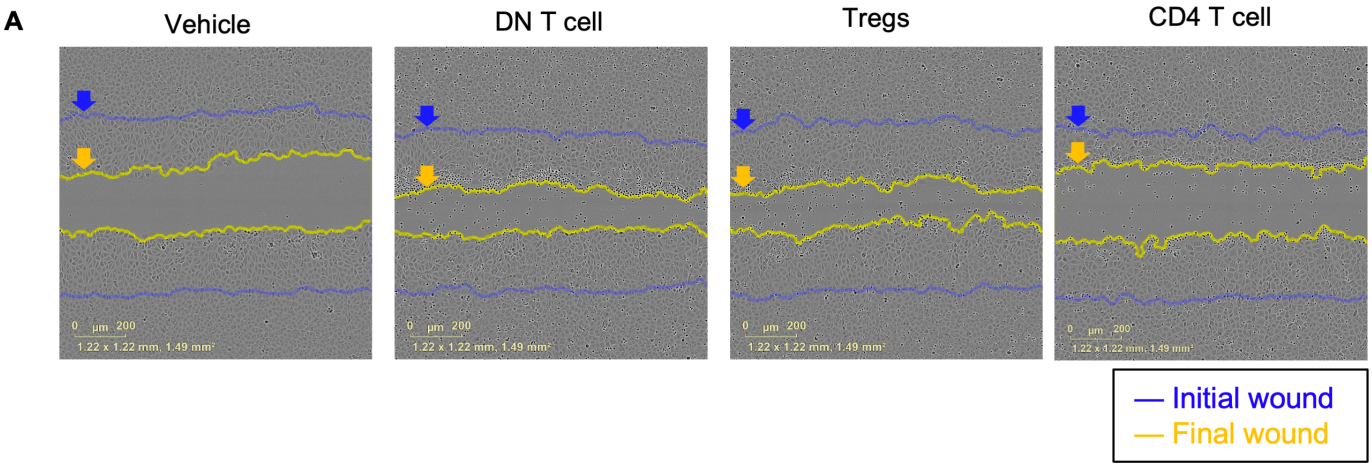
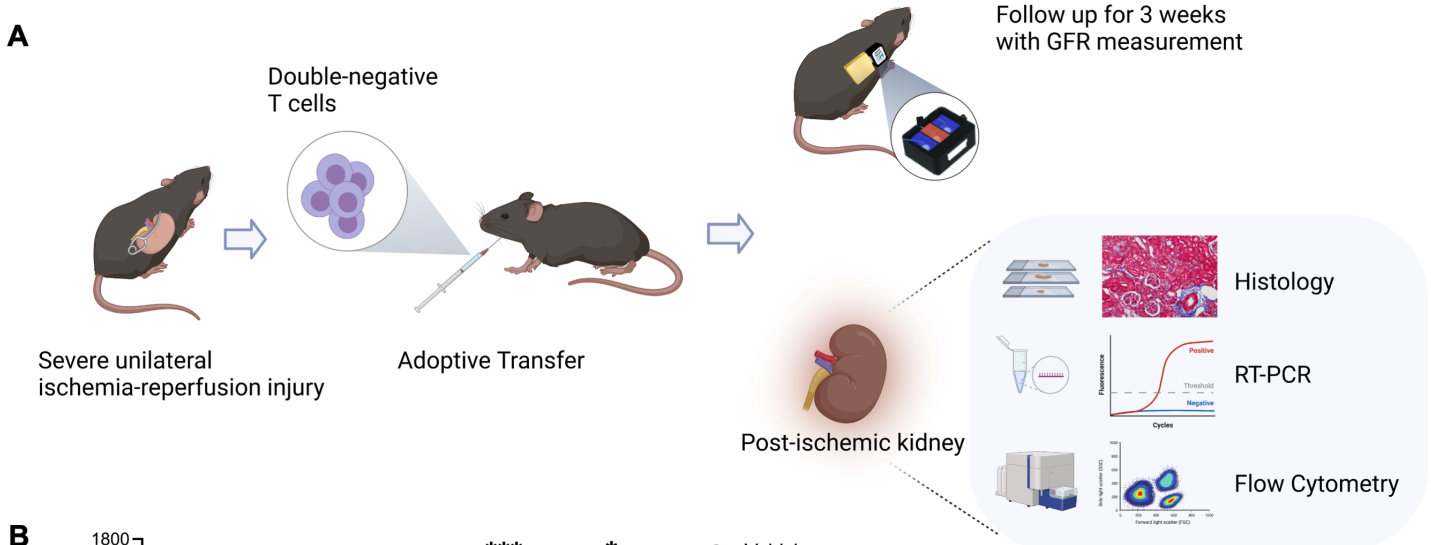
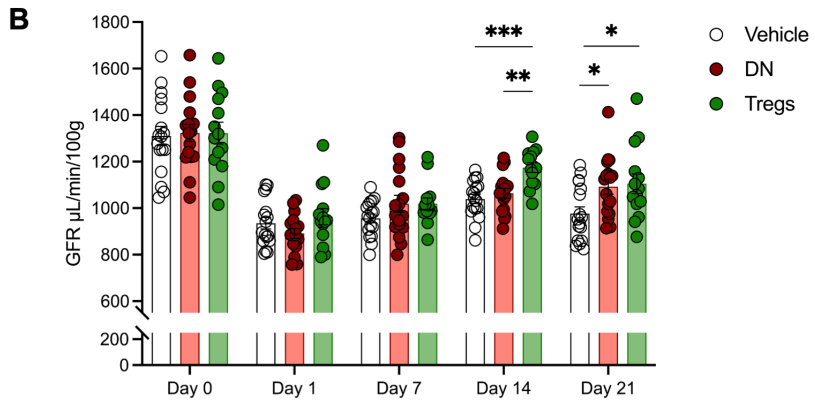
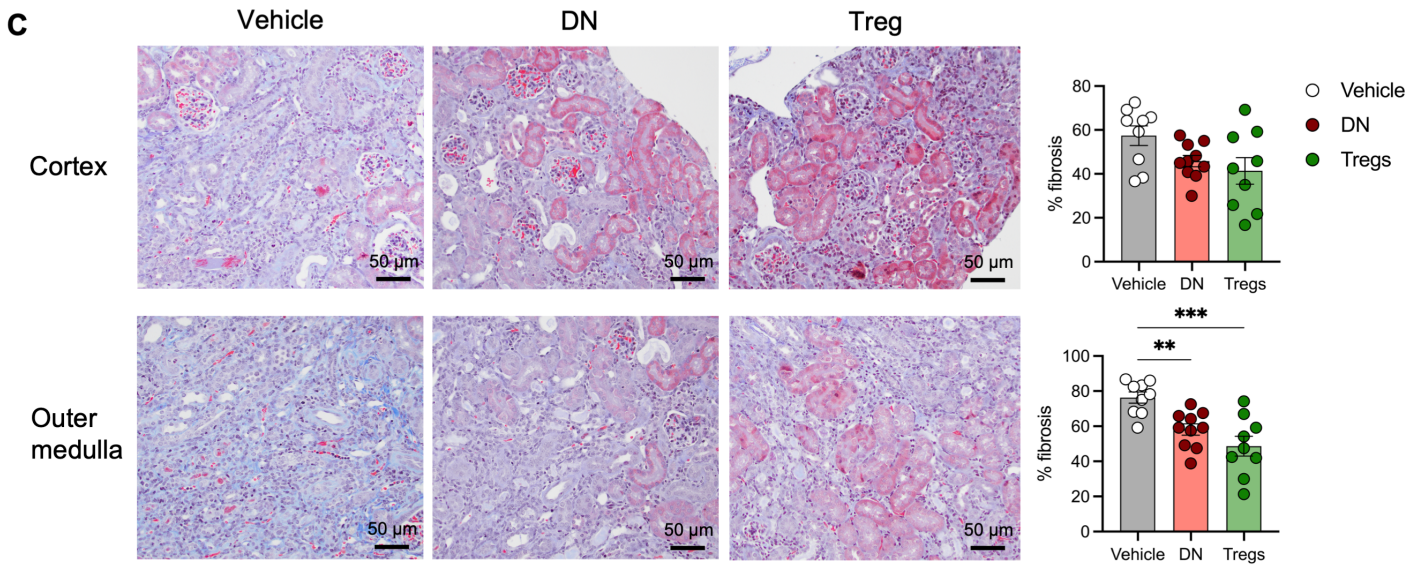


Figure 6









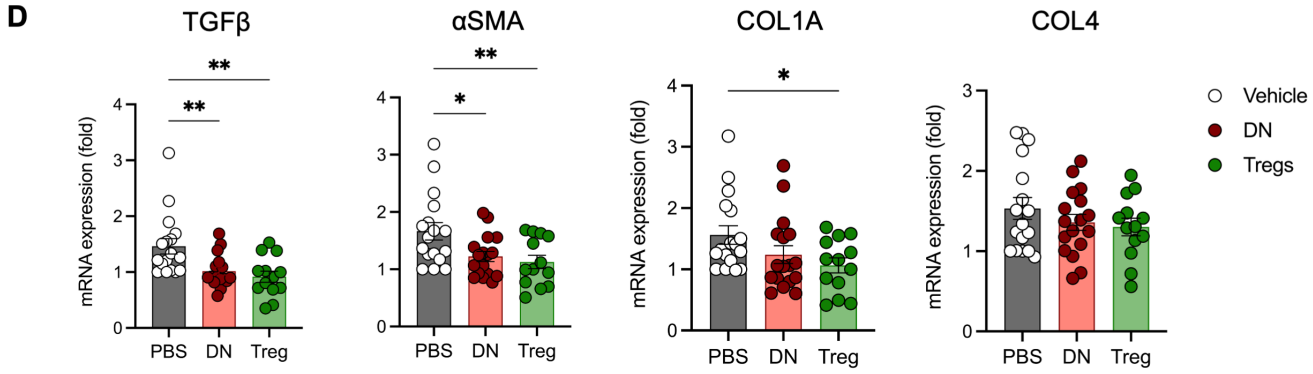


Figure 8