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Supporting Information for
Kidney-resident innate-like memory γδ T cells control chronic *Staphylococcus aureus* infection

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Supporting Information: Material and methods

Mice

Tcrd$^{-/-}$ mice (1), *TCRdH2BeGFP* mice (2), *TCRdGDL* mice (3) and *Foxp3*^{RFP}×*Il17a*^{eGFP}×*Ifng*^{Kat} mice (4, 6, 6) were on a C57BL/6 background. Transgenic mice and wild-type C57BL/6 mice were housed under specific pathogen-free conditions at the University Medical Center Hamburg-Eppendorf. Animal experiments were approved by the local committee for animal experiments of the City of Hamburg Experiments (registration numbers: N078/2019 and N082/2019). Age and sex matched mice were used. Mice were monitored on daily basis and mice with severe disease were eliminated. The housing was done under standard conditions with food and water ad libitum in individually ventilated cages. For parabiosis experiments C57BL/6 (CD45.2) mice and CD45.1 congenic mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/*BoyJ*) were housed under specific pathogen-free conditions at the Centro Ciencia & Vida. Animal experiments were approved by the local committee for bioethical guidelines of the Centro Ciencia & Vida (registration number 19322-FCS-UCH). Mice were monitored daily and mice with signs of distress were eliminated.

S. aureus infection

Mice were infected with *Staphylococcus aureus* strain (SH1000) (7, 8). Mice received 1-5×10^{7} colony-forming units (CFU) in 100 µl sterile PBS via the tail vein. The inoculum was controlled by serial dilutions on LB-Agar plates. Bacterial clearance was achieved by adding ampicillin (1g/l) to the drinking water for 2 weeks. (Ampicillin sensitivity of the strain was confirmed by the Microbiology Department of the University Medical Center Hamburg-Eppendorf.) Bacterial numbers in kidney, lung, liver, and spleen were quantified by serial dilutions of homogenized organs in sterile PBS. Suspensions were plated on LB-agar and incubated at 37°C to analyze CFUs after 16 hours.

Depletion of γδ T cells

γδ T cells were depleted in *TcrdGDL* mice by the i.p. injection of diphtheria toxin (DT, Merck KGaA, Darmstadt). 1 µg DT diluted in 100 µl PBS was administered twice in an interval of 48h. Control mice only received PBS. The depletion was controlled by flow cytometry (3).

Parabiosis

Parabiosis was performed as described before (7). Briefly, CD45.1 mice were infected with *S. aureus.* After 2 weeks, infected and non-infected CD45.2 C57BL/6 control mice were treated for one week with ampicillin in the drinking water. On day 30 post infection, two mice (CD45.1 and CD45.2) were surgically joined according to standard protocols (9). After 28 days, mice were sacrificed and analyzed separately.

Histology

Formalin fixed, paraffin embedded murine kidney samples were cut to a thickness of 2 or 3 µm using a microtome and then placed on SuperFrost Plus slides (Fisher Scientific, Schwerte, Germany), after which tissue recovery using xylene, followed by an ethanol series was performed. Antigen retrieval was performed using 10 mM citrate buffer pH 6.1 or DAKO Target Retrieval Solution pH 9 (Agilent, Santa Clara, CA) in combination with a Braun FS20 steam boiler (Braun, Kronberg, Germany), followed by an incubation in Agilent DAKO Wash Buffer Solution (Agilent). Unspecific binding was blocked in 5% horse serum and 0.05% TritonX-100 for 30 min. Primary antibodies (anti-GFP Ab (ab6658, OriGene, Herford, Germany), anti-CD44 mAb (2211D, BD Biosciences, San Jose, CA), or anti-NFκB p65 mAb (4764S, Cell Signaling, Frankfurt, Germany)) were applied according to the vendors' guidelines for 30 min at room temperature or overnight at 4°C. Secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 586 or Alexa Fluor 647 were incubated for 1h at room temperature. Nuclei were visualized using Hoechst, overall kidney structure using biotinylated wheat germ agglutinin (WGA) followed by AF647-streptavidin. Imaging was performed with the LSM800 with airyscan for high-resolution confocal microscopy using ZENblue software (all Zeiss, Oberkochen, Germany).

Isolation of cells from the spleen, kidney lung and liver and antibody staining

To distinguish between resident and circulating cells, intravascular leukocytes (termed $CD45iv^{+}$) were marked by i.v. injection of fluorochrome-conjugated anti-CD45 mAb (clone 30-F11, 2,5 µg/mouse) 3 min before sacrificing the mice (10).

Unless stated otherwise, cells were isolated, antibody stained and analyzed as bulk cell populations without prior enrichment of T cells. Cells from spleens were isolated by pressing the organ successive through 70 and 40 µm cell strainers. Cells from the kidney, lung and liver were digested for 40 min at 37°C with 10 U/ml DNaseI (Sigma-Aldrich, St.

Louis, MO) and 400 µg/ml Collagenase D (Roche, Mannheim, Germany). Afterwards, leukocytes were processed by density gradient centrifugation (37,5% Easycoll, Merck Millipore, Darmstadt, Germany) and an additional filtration with a 30 µm strainer. Erythrocytes in all organs were depleted using lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 µM EDTA, pH 7.2).

Unspecific antibody staining was prevented by incubation for 5 min with 10 µg/ml anti-CD16/32 mAb (clone 2.4G2; BioXcell, West Lebanon, NH) and 1:100 rat serum in PBS. Surface proteins were stained using mAbs (SI Appendix, Table S1) and fixable live dead staining (pacific orange succinimidyl ester; Gibco, Darmstadt, Germany or live/dead™ fixable near-IR dead cell stain kit; Life Technologies, Carlsbad, CA). For staining of intranuclear proteins, cells were fixed and permeabilized using the Foxp3 transcription factor staining kit (EBioscience, San Diego, CA) according to the manufacturer's protocol. For intracellular cytokine staining, cells were washed with PBS and fixed for 20 min with PBS, 2% paraformaldehyde at room temperature. Cells were washed with PBS, 0.2% BSA, permeabilized with PBS, 0.1% BSA, 0.3% saponin (Sigma, Aldrich), and incubated in this buffer with 1:100 rat serum. After 5 min, fluorochrome-conjugated antibodies were added. After further 20 min on ice, cells were washed with PBS.

The monoclonal anti-Vγ5/Vδ1 IgM antibody (clone: 17D1) also detects $Vγ6^+$ T cells that are preincubated with an anti-γδTCR antibody (clone: GL3) (11). Cells were extracellularly stained as described earlier with an antibody mix including an anti-γδTCR antibody (clone GL3). After 30 minutes, the 17D1 antibody was added. Finally, the cells were secondarily stained with an anti-IgM antibody (clone: HIS40). Since $Vγ5^+$ T cells are exclusively found in the epidermis, staining of kidney cells with 17D1 can be used to identify $Vγ6^+$ T cells in spleen and kidney.

For flow cytometric analysis of cytokine production by murine T cells, cells were stimulated for 4 hours *in vitro* in IMDM medium supplemented with 5% FCS, L-glutamine, pyruvate, gentamicin and 2-mercaptoethanol with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma Aldrich) and ionomycin (1 µM, Sigma Aldrich). To prevent cytokine secretion 10 µg/ml Brefeldin A (Sigma-Aldrich) was added. *Foxp3*^{RFP}×*Il17a*^{eGFP}×*Ifng*^{Kat} mice were analyzed without further stimulation directly after the isolation of leukocytes and surface mAb staining.

Flow Cytometry and cell sorting

FACSCanto II, FACSCelesta, FACS LSR II or LSRFortessa (all by BD Bioscience, San Jose, CA) were used for flow cytometric analysis. Data were analyzed by FlowJo V10.8.1 (Becton Dickinson, Franklin Lakes, NJ) or FACSDiva software (BD Bioscience, San Jose, CA). Cell sorting was performed on FACS AriaFusion or AriaIIIu (BD Biosciences, San Jose, CA). T cells from *TCRdH2BeGFP* mice were pre-enriched using EasySeps Mouse T Cell Isolation Kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer´s manual and γδ T cells were subsequently sorted based on their GFP expression.

In vitro Stimulation of γδ T cells

Prior to isolation of T cells from mice, anti-Art2b nanobodies (clone S+16, 50 µg in 100 µl PBS, kindly provided by Dr. Koch-Nolte) were administered 30 min before sacrificing the mice by i.p. injection to prevent NAD-induced cell death (12). γδ T cells were isolated from kidneys of previously infected and subsequently ampicillin-treated *TCRdH2BeGFP* mice. γδ T cells were enriched by magnetic negative selection and then purified by FACS sorting. 14000 γδ T cells were cultured in 40 µl of IMDM medium containing 10% FCS, streptomycin, penicillin, 2-mercaptoethanol and mIL-2 (10 ng/ml) and mIL-7 (10 ng/ml). Cytokines were purchased from Biolegend, San Diego, CA. Cells were stimulated with either Dynabeads (Thermo Fisher Scientific, Waltham, MA), a cocktail of mIL-1β (20 ng/ml), mIL-6 (20 ng/ml) and mIL-23 (40 ng/ml), Pam_3Cys-Ser-$(Lys)_4$, or heat-killed *S. aureus*. After 3 days, cytokines in the supernatant were determined using the Legendplex assay (mouse Th17 panel, Biolegend) according to the manufacturer's protocol.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 9 (GraphPad, Software Inc, San Diego, CA). Results were analyzed by the statistically test indicated in the figure legends. * p <0.05, ** p <0.01, *** p <0.001, ****p <0.0001

SI References

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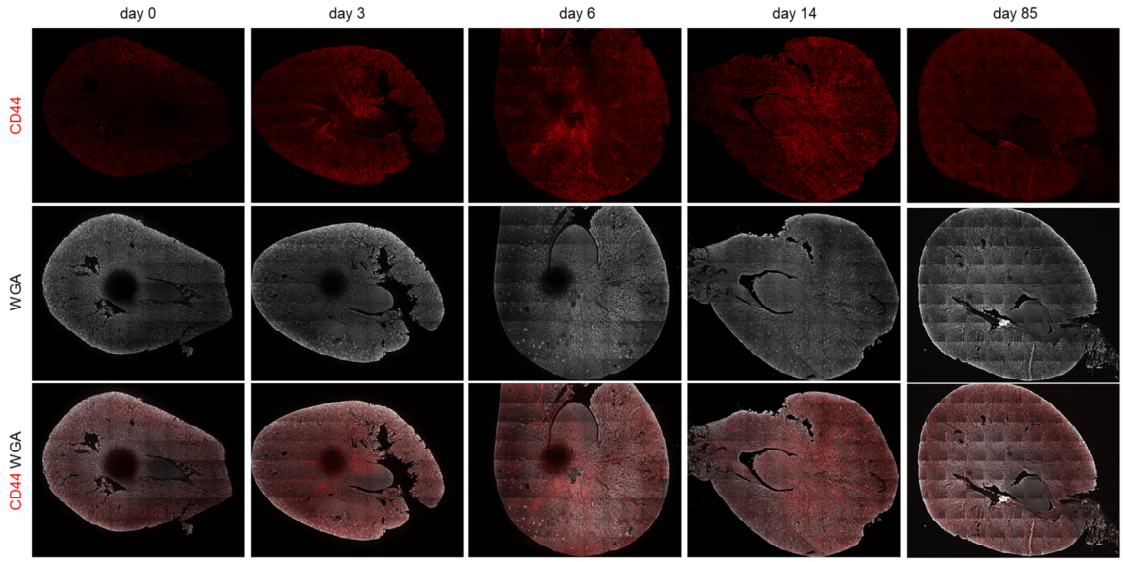

Fig. S1. *S. aureus* causes substantial renal inflammation.

Mice were infected as described in Fig. 1A. At the indicated time points p.i., renal cross sections were stained with wheat germ agglutinin (WGA, white) to provide basal kidney structure information and with anti-CD44 mAb (red) to identify inflamed areas. Representative staining for sections from 5-7 mice per time points.

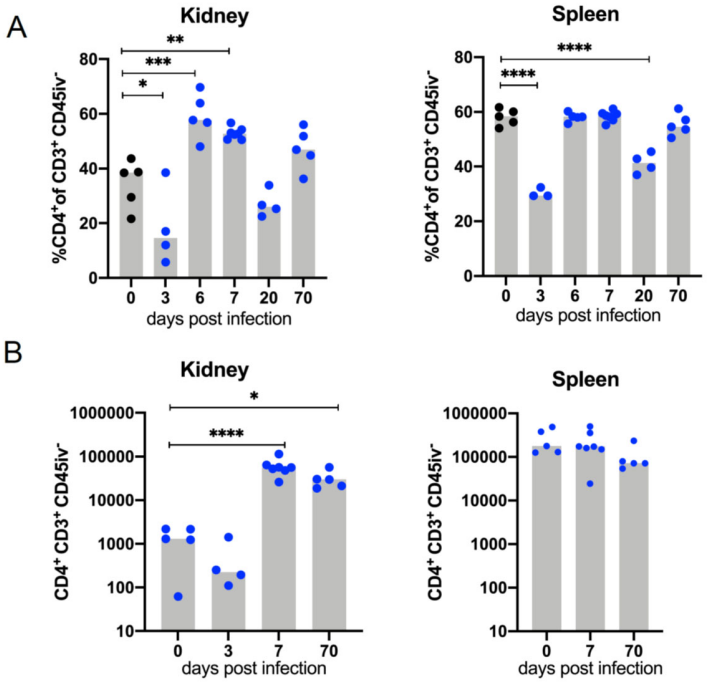

Fig. S2. *S. aureus* and induces a strong renal CD4⁺ T cell response.

Foxp3^{RFP}×*Il17a*^{eGFP}×*Ifng*^{Kat} mice were infected and T cells from different tissues were analyzed as described in Fig. 1B. (**A**) Percentages of CD4⁺ T cells and (**B**) CD4⁺ T-cell counts in kidney and spleen. Results from one of two independent experiments with 3-7 animals per time point. Symbols represent individual mice and bars show median values. Statistical analysis was performed by one-way ANOVA test and Dunnett's multiple comparison post-test.

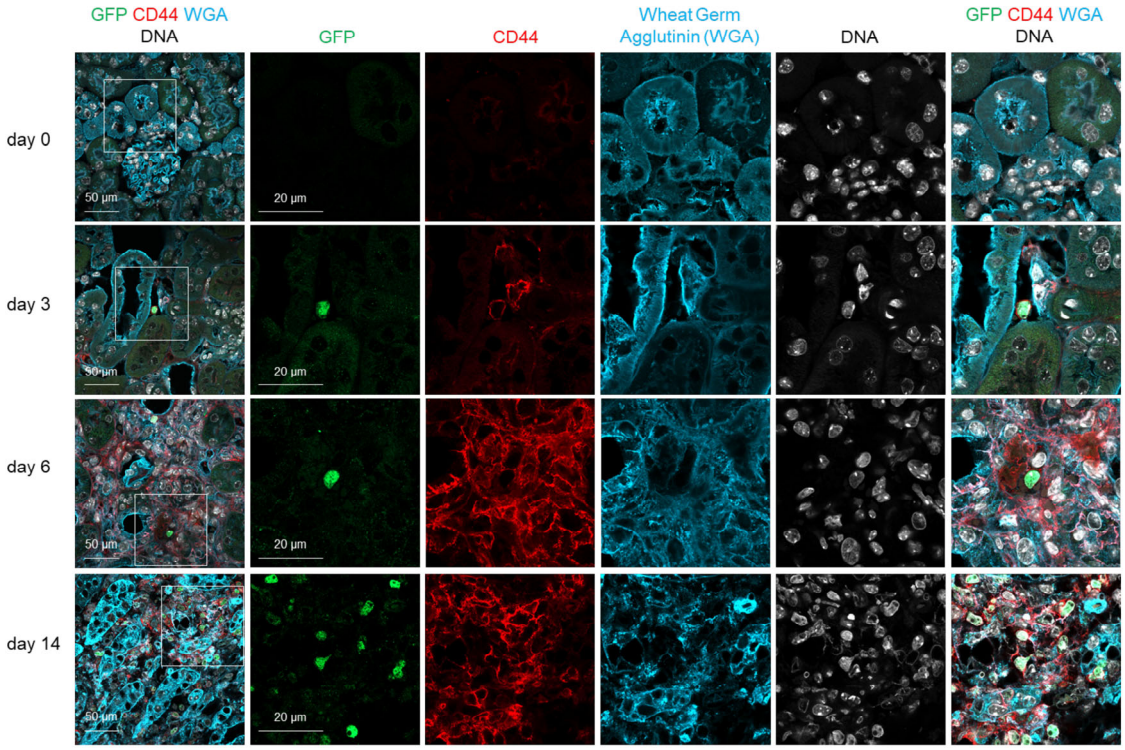

Fig. S3. Renal accumulation and distribution of γδ T cells following *S. aureus* infection.

TCRdH2BeGFP mice were infected as described in Fig. 1. At the indicated time-points p.i., renal sections were stained with anti-GFP Ab to identify GFP⁺ γδ T cells (green), anti-CD44 mAb to mark inflamed areas (red) as well as wheat germ agglutinin (WGA) for tubular structures (blue) and Höchst for nuclear DNA (white), respectively. Representative staining for sections from 5-7 mice per time points.

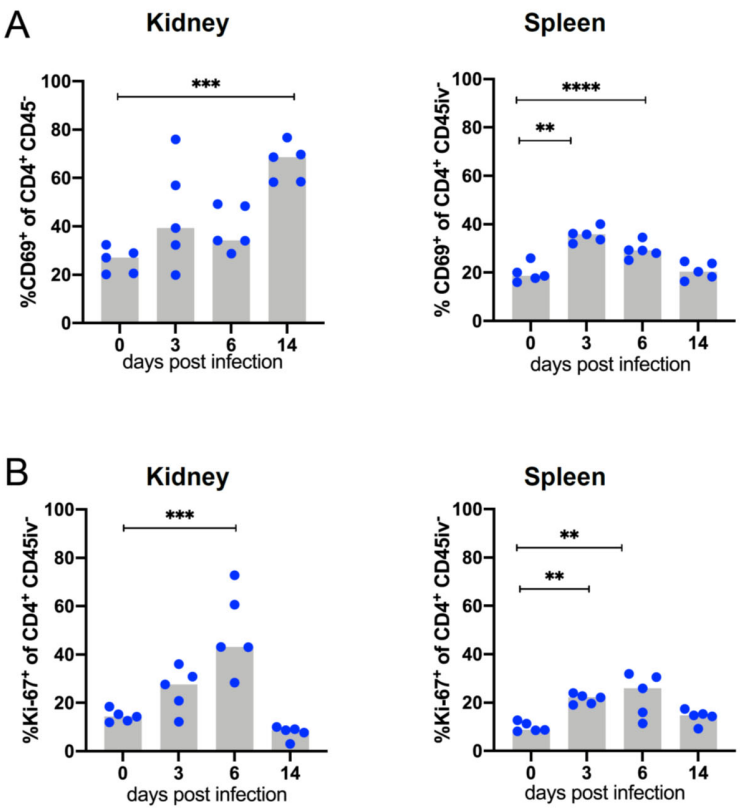

Fig. S4. After *S. aureus* infection, renal CD4⁺ T cells proliferate and acquire a tissue-resident phenotype.

TCRdH2BeGFP mice were infected and analyzed as described in Fig. 3. (**A**) Percentages of CD69⁺ and (**B**) of Ki-67⁺ CD4⁺ T cells in spleen and kidney. Results are from one of two independent experiments with 4-5 animals per time point. Symbols represent individual mice and bars show the median. Statistical analysis was performed by one-way ANOVA test and Dunnett's multiple comparisons post-test.

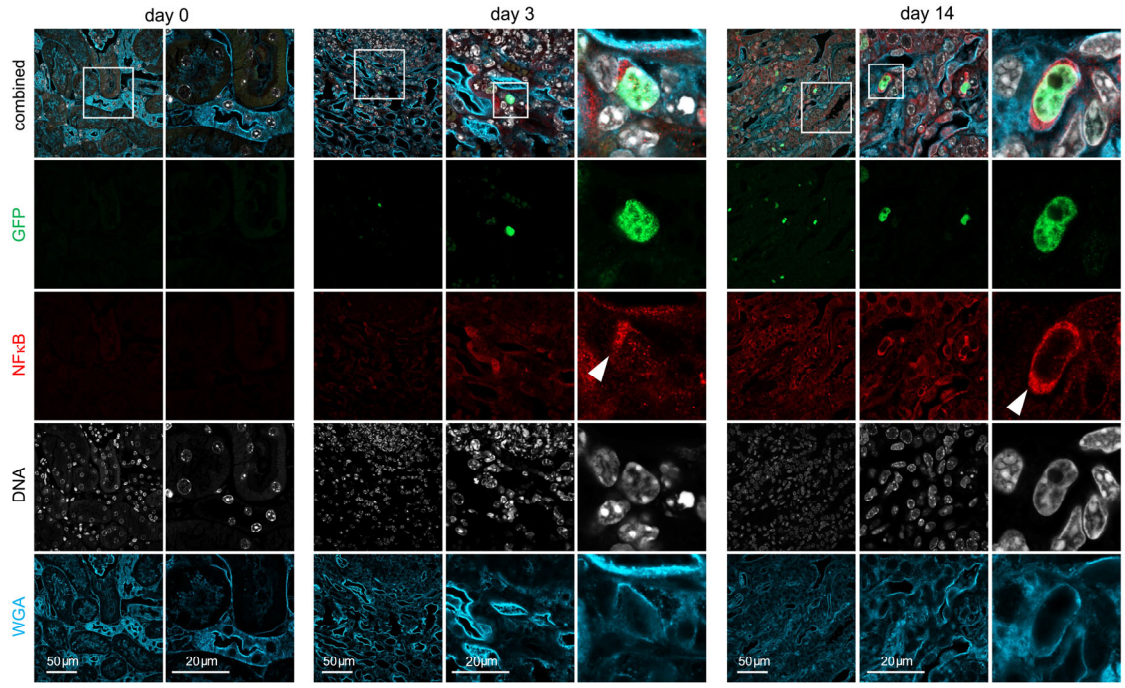

Fig. S5. NFκB response in renal γδ T cells following *S. aureus* infection.

TCRdH2BeGFP mice were infected and analyzed as described in Fig. 4. Renal sections were stained with anti-GFP Ab to identify GFP⁺ γδ T cells (green), anti-NFkB Ab (red), DNA (Hoechst, white) and wheat germ agglutinin (WGA, blue). Large magnifications show nuclear GFP⁺ γδ T cells with cytoplasmic NFκB staining (arrow head). Representative staining for sections from day 0, 3 and 14 are shown. Sections are representative for 5-7 mice per time-point.

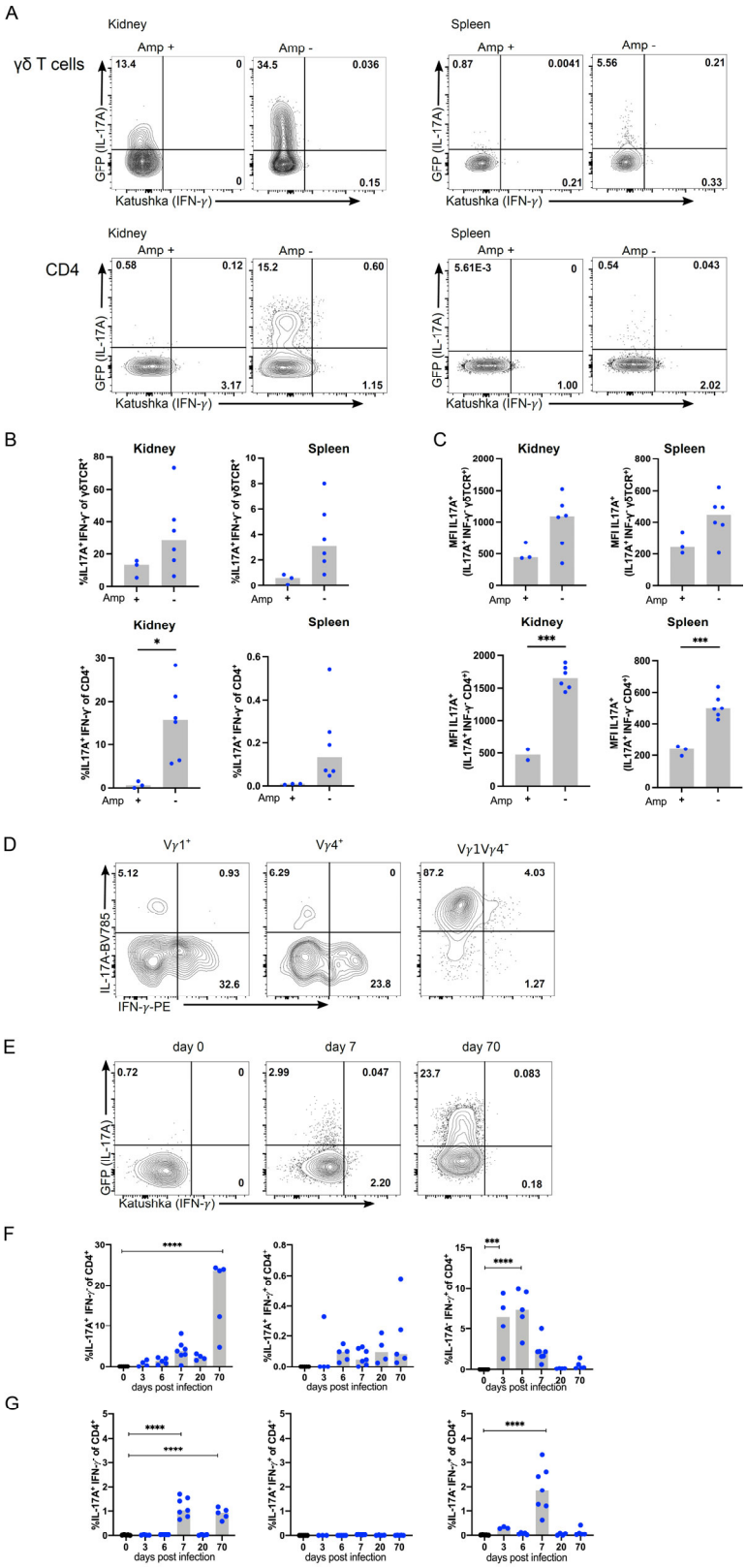
A
Kidney
Spleen
Amp +
Amp -
γδ T cells
CD4
GFP (IL-17A)
Katushka (IFN-γ)
13.4
0
0
34.5
0.036
0.15
0.87
0.0041
0.21
5.56
0.21
0.33
0.58
0.12
3.17
15.2
0.60
1.15
5.61E-3
0
1.00
0.54
0.043
2.02
B
C
%IL17A+IFN-γ+ of γδTCR+
MFI IL17A+ (IL17A+ INF-γ+ γδTCR+)
%IL17A+IFN-γ+ of CD4+
MFI IL17A+ (IL17A+ INF-γ+ CD4+)
Kidney
Spleen
Amp
*
***
D
Vγ1+
Vγ4+
Vγ1Vγ4−
IL-17A-BV785
IFN-γ-PE
5.12
0.93
32.6
6.29
0
23.8
87.2
4.03
1.27
E
day 0
day 7
day 70
0.72
0
0
2.99
0.047
2.20
23.7
0.083
0.18
F
G
%IL-17A+ IFN-γ+ of CD4+
days post infection
****
***

Fig. S6. Cytokine production by renal T cells during chronic *S. aureus* infection.
(**A-C**) *Foxp3*^{RFP}×*Il17a*^{eGFP}×*Ifng*^{Kat} mice were i.v. infected with *S. aureus*. After two weeks, one group of mice was treated for 2 weeks with ampicillin in the drinking water to clear the infection. On day 85, γδ T cells and CD4$^+$ T cells in spleen and kidney were analyzed for IL-17A and IFN-γ production without further *in vitro* stimulation. (**A**) Representative results for cytokine production of γδ T cells and CD4$^+$ T cells. (**B**) IL-17A expression in γδ T cells and CD4$^+$ T cells. (**C**) MFI of GFP/IL-17A in GFP$^+$ γδ T cells. (**D**) C57BL/6 mice were infected with 10^7 CFU of *S. aureus.* On day 14 p.i., renal γδ T cells were stimulated for 4h with PMA and ionomycin and analyzed by flow cytometry. Cells gated for CD3 and γδ TCR were analyzed for Vγ1 and Vγ4 as well as for IFN-γ and IL-17A expression. Representative dot plots for cytokine expression are shown. (**E-G**) *Foxp3*^{RFP}×*Il17a*^{eGFP}×*Ifng*^{Kat} mice were infected and analyzed as described in Fig. 5. (**E**) Representative dot plots for Katushka (IFN-γ) and GFP (IL-17A) expression in renal CD4$^+$ T cells. (**F, G**) Percentages of IL-17A$^+$IFN-γ$^-$, IL 17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ CD4$^+$ T cells in kidney (**F**) and spleen (**G**). Representative result of one (**A-D**) or two (**E-G**) independent experiments with 3 to 7 mice per group. Symbols represent individual mice and bars show median values. Statistical analysis was performed by one-way ANOVA test and Dunnett's multiple comparisons post-test or student t test.

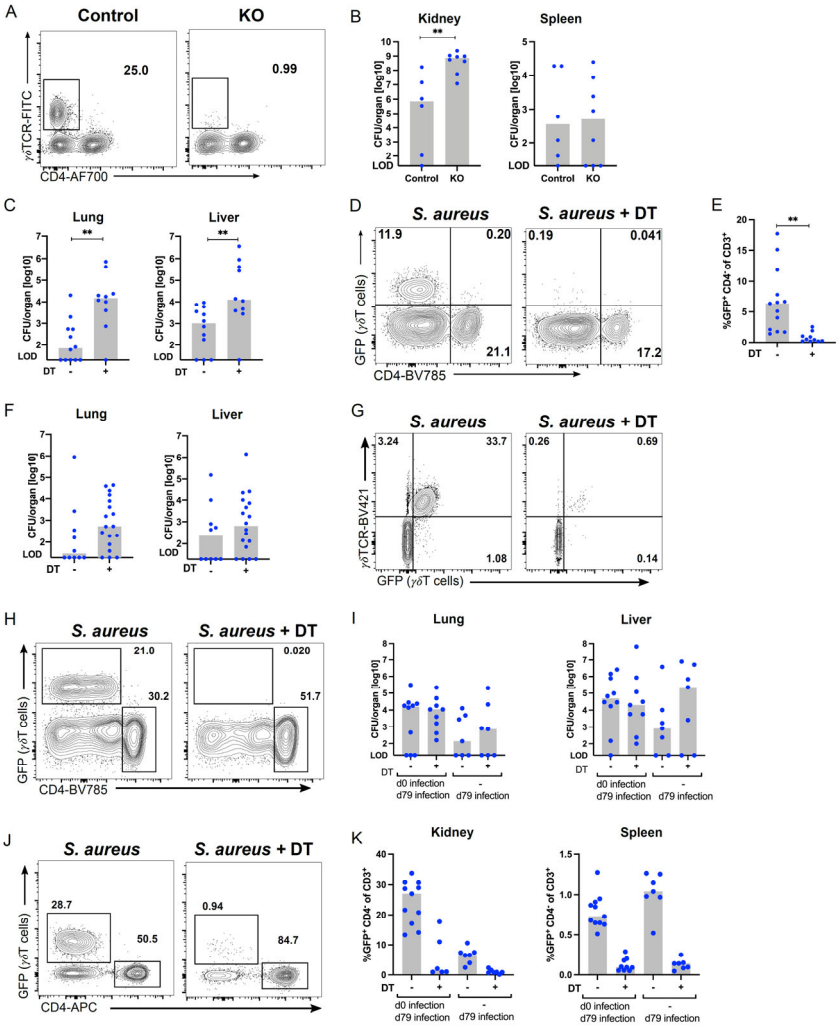

Fig. S7. Depletion of γδ T cells during *S. aureus* infection.

(**A, B**) *Tcrd*$^{+/-}$ and *Tcrd*$^{-/-}$ mice were infected with 10^7 CFU of *S. aureus*. On day 7 p.i., bacterial numbers in kidney and spleen were determined by plating serial dilution of tissue homogenates. Absence of γδ T cells in tissues was controlled by FACS. (**A**) Representative dot plot for renal CD4$^+$ and γδTCR$^+$ T cells (gated for CD3$^+$CD45iv$^-$ cells). (**B**) CFU of *S. aureus* in kidneys and spleen. Data are representative of two independent experiments (n = 6 and 8 animals per group). (**C-E**) *TcrdGLD* mice were treated and analyzed as described in Fig. 7A-B. (**C**) Bacterial counts in lung and liver. (**D**) Representative dot plot for renal GFP$^+$ γδ T cells (gated for CD45$^+$CD3$^+$CD19$^-$ cells) and (**E**) percentages of renal γδ T cells of infected mice with and without DT treatment. (**F-H**) *TcrdGLD* mice were treated and analyzed at day 27 as described in Fig. 7C. (**F**) Bacterial counts in lung and liver. (**G**) Representative dot plots of renal CD45$^+$CD3$^+$CD19$^-$ cells for GFP expression and anti-γδ TCR mAb staining and (**H**) for GFP expression and anti-CD4 mAb staining. (**I-K**) *TcrdGLD* mice were treated and analyzed as described in Fig. 7E. (**I**) Bacterial count in lung and liver. (**J**) Representative FACS plots of renal CD3$^+$ cells for GFP expression and anti-CD4 mAb staining and (**K**) percentages of renal γδ T cells of infected mice with and without DT treatment. (**B**, **C**, **E**, **F, I, K**) Each dot represents one mouse. Bars show median values. Limit of detection (LOD) = 20 colony forming units (CFU). Statistics were performed with Mann-Whitney-U test (**B**, **C, F**), student t test (**E**),

Kruskal-Wallis test and Dunn's multiple comparisons post-test (**I**), or ANOVA test and Dunnett's multiple comparisons post-test (**K**).

Table S1. Antibodies

Antigen	Clone	Fluorochrome	Provider
CD3ε	500A2	BV421, AF700	eBioscience Biolegend
CD4	RM4-5	BV605, BV785, AF700, BV785, APC	Biolegend
CD19	6D5	BV510	Biolegend
CD45	30-F11	PerCP, PeCy7, APCCy7, BV510	Biolegend
CD69	HI1.2F	BV605	Biolegend
γδ TCR	GL3	BV605, FITC, BV421	Biolegend, eBioscience
Vγ1	2.11	PerCPCy5.5	Biolegend
Vγ4	UC3-10A6	FITC	Biolegend
Vγ6	17D1	-	Purified
IgM	HIS40	FITC, PE	eBioscience
RORγt	Q31-378	APC, PE	BD
IFN-γ	XMG1.2	APCCy7, PE	Biolegend
IL-17A	TC1118H10.	BV785, PE	Biolegend
KI-67	SolA15	BV421	eBioscience
TCRβ	H57-597	APC-Cy7	Biolegend
GFP	ab6658	-	OriGene
CD44	2211D	-	BD
NFκB p65	4764S	-	Cell Signaling