Supporting Information

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SI Text

Antibodies and Cytokines. For flow cytometry and magnetic cell sorting, the following antibodies and regents were used: Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45 (30-F11, rat IgG2b), FITC-conjugated rat anti-mouse CD11b (M1/70, rat IgG2b), FITC-conjugated rat anti-mouse CD5 (53–7.3, rat IgG2a), R-phycoerythrin (PE)-conjugated rat anti-mouse CD11b (M1/70, rat IgG2b), PE-conjugated rat anti-mouse CD11c (HL3, Armenian hamster IgG1 λ2), PE-conjugated rat anti-mouse CD19 (1D3, rat IgG2a), PE-conjugated rat anti-mouse CD34 (clone RAM43, rat IgG2a), PE-conjugated rat anti-mouse CXCR4 (clone 2B11, rat IgG2b), PE-conjugated rat anti-mouse HLA-IA/IE (M5/114.15.2, rat IgG2b), PE-conjugated rat anti-mouse CD16/32 (2.4G2, rat IgG2b), PE-conjugated rat anti-mouse CD8 (53–6.7, rat IgG2a), allophycocyanin (APC)-conjugated rat anti-mouse Gr1 (RB6–8C5, rat IgG2b), APC-conjugated rat anti-mouse CD4 (RM4–5, rat IgG2a), streptavidin-APC (all from BD Biosciences), PE-conjugated rat anti-mouse CD115 (AFS98, rat IgG2a) (eBioscience), FITC- and PE-conjugated isotype control antibodies, biotin-conjugated rabbit anti-collagen type I, biotin-conjugated rabbit IgG whole molecule (Rockland). For polyclonal T-cell activation, rat anti-mouse CD3 antibody (145–2C11, Armenian Hamster IgG1κ; BD Bioscience) was used. Blocking antibodies against murine cytokines IL-2 (JES6–1A12), IL-4 (30340.11), IL-21 (AF594), TNF (AF-410-NA), IFN-γ (H22), and TGF-β (1D11) were from R&D Systems . The depleting rat anti-mouse CD4 (GK1.5, rat IgG2b) and the isotype control rat IgG2b (A95–1) were from BD Biosciences. Recombinant murine cytokines IL-2, IL-4, IL-13, TNF, and IFN-γ were obtained from PeproTech.

Isolation of Leukocyte Subsets from Spleen and Kidneys. Single-cell suspensions of kidneys were obtained by digestion of defined amounts of renal tissue with 1 mg/mL collagenase type I (Sigma–Aldrich) in Hank's BSS for 30 min at 37 °C and subsequent washing of cells in medium. $CD4^+$, $CD11b^+$, $CD16/32^+$, $CD11c^+$, $CD115^+$, or $Gr1^+$ cells were depleted from total splenocytes with magnetic mircrobeads (Miltenyi Biotec). For that purpose, cell samples were stained with αCD11b-PE, αCD16/32-PE, αCD11c-PE, αCD115-PE, or αGr1-APC followed by a depletion with anti-PE or anti-APC microbeads (Miltenyi Biotec) and LS columns (Miltenyi Biotec) in accordance with the manufacturer's recommendations. For depletion or isolation of $CD4^+$ T cells and $CD19^+$ B cells, microbeads against CD4 or CD19 (Miltenyi Biotec) were used. Mononuclear cells were prepared from splenocytes by Ficoll-Paque centrifugation. Subsequently, cells were stained with αCD11b-PE or αCD16/32-PE, and monocytes were enriched with anti-PE microbeads by positive selection. $CD45^+$ cells were purified from single-cell suspensions of rat renal tissue or spleens by magnetic cell sorting and FACS. Rats were used to obtain sufficient numbers of highly purified $CD45^+$ cells from ligated and nonligated kidneys. Cell were first enriched with microbeads against rat CD45 (Miltenyi Biotec), then stained with PE-conjugated mouse anti-rat CD45 (OX-1, mouse IgG1), and sorted on a FACSAria (BD Biosciences).

Activation of $CD4^+$ T Cells. Supernatant of activated $CD4^+$ T cells (SN) was generated by coculture of 5×10^5 $CD4^+$ T cells, 5×10^5 $CD19^+$ B cells, and 0.5 μg/mL αCD3 antibody for 72 h in flat-bottom 96-well plates in RPMI medium 1640, 10% (vol/vol) heat-inactivated (56 °C, 30 min) FCS (PAA), and 1% penicillin/streptomycin. Where indicated, $CD4^+$ T cells were activated in the presence of cyclosporine A (2 μg/mL; Novartis), tacrolimus (250 ng/mL; Alexis Biochemicals), or rapamycin (40 ng/mL; Wyatt), and supernatant was recovered after 3 days (SN CyA, SN-TAC, SN-Rapa, respectively).

ELISA. To quantify the amount of intracellular collagen I, cells were sonicated in 0.5 M Na-acetate. The cell lysate was applied to a sandwich ELISA with anti-collagen I (Abcam) for coating and biotinylated anti-collagen I (Rockland) followed by streptavidin-HRP (DakoCytomation) and ABTS (Roche) for detection. To measure collagen I secreted onto the culture plate, cultured cells were removed with PBS/EDTA. Culture plates were blocked with 4% BSA (wt/vol), incubated with biotinylated anti-collagen I (Rockland), followed by streptavidin-HRP and ABTS for detection. Absorption values (A_{450}) are depicted.

Immunofluorescence. Cryosections (5-μm) were fixed with ice-cold acetone and blocked with 3% H_2O_2 in methanol, avidin-biotin blocking kit (Vector Laboratories), and SuperBlock buffer (Thermo Scientific). Sections were stained with rabbit anti-collagen I antibody (dilution 1:1,000; Cat No. ab21286-100 Abcam) followed by Alexa fluor 594 goat anti-rabbit IgG (dilution 1:500; Invitrogen-Molecular Probes) and incubation with Hoechst 33342 (dilution 1:50,000; Invitrogen-Molecular Probes) for nuclear staining. Sections were analyzed on a Leica microscope (200× magnification), and the area of red-stained collagen I of 5 hpf/slide was quantified with MetaVue software (Version 6.3r3).

RNA and Protein Isolation. For mRNA and total protein isolation from kidneys and spleens, PeqGold Trifast (Peqlab) was used. For mRNA isolation from cultured fibrocytes, the Qiagen RNeasy Micro kit was used.

Reverse Transcription and Real-Time PCR. Total RNA was reverse-transcribed with oligo(dT) and M-MLV reverse transcriptase (Invitrogen). Real-time RT-PCR was performed on a ABI PRISM 7900HT detection system (Applied Biosystems) or a Light Cycler system 2.0 (Roche) using QuantiTect SYBR Green PCR kit (Qiagen). GAPDH was used as a reference gene. Data were analyzed with SDS 2.2.3 software (Applied Biosystems) or Light Cycler Software version 3.5 (Roche). Sequences of primers were: Collagen I (mouse): 5′-tgt tca gct ttg tgg acc tc-3′ (forward) and 5′-tca agc ata cct cgg gtt tc-3′ (reverse); collagen I (rat): 5′-gtg gac ctc cgg ctc ctg ctc ctc-3′ (forward) and 5′-ctt ctg ggc aga aag gac agc act cgc-3′ (reverse); GAPDH: 5′-gtc gtg gat ctg acg tgc c-3′ (forward) and 5′-gat gcc tgc ttc acc acc tt-3′ (reverse).

Western Blot Analysis. Renal tissue samples with identical concentration of protein were boiled with Laemmli buffer (Bio-Rad Laboratories), separated on a 10% SDS-PAGE, and transferred to nitrocellulose-membranes (Schleicher & Schuell). Membranes were blocked with 5% nonfat dry milk and incubated with mouse anti-rat collagen I (1:1,000; Cat. No. sc-59772Santa Cruz Biotechnologies) followed by a HRP-conjugated goat anti-mouse antibody (1:1,000; Cell Signaling). The enhanced chemiluminescence (ECL) system (Amersham-Bioscience) was used for detection, membranes were exposed to Hyperfilm (Amersham-Bioscience), and the signal intensity was quantified using

the MetaMorph 4.6.9r device from Universal Imaging (Visitron Systems).

For subsequent detection of GAPDH, membranes were stripped for 30 min with β-mercaptoethanol-containing SDS buffer, blocked, and incubated with rabbit anti-GAPDH antibody (1:1,000; Cell Signaling) and HRP-labeled goat anti-rabbit antibody (1:1,000; Cell Signaling).

Tunel Staining. Cryosections (5-μm) of renal tissue were fixed in 4% formaldehyde, and DNA strand breaks were detected using terminal deoxynucleotidyl transferase incorporation of fluorescein-labeled deoxynucleotides (FragEL DNA Fragmentation Detection kit; Calbiochem) in accordance with the manufacturer's recommendations. Nuclear counterstaining was performed by exposition to Hoechst 33342 (dilution 1:50,000; Invitrogen–Molecular Probes). In each UUO kidney, the number of apoptotic cells was quantified in five nonoverlapping fields observed at 200× magnification.

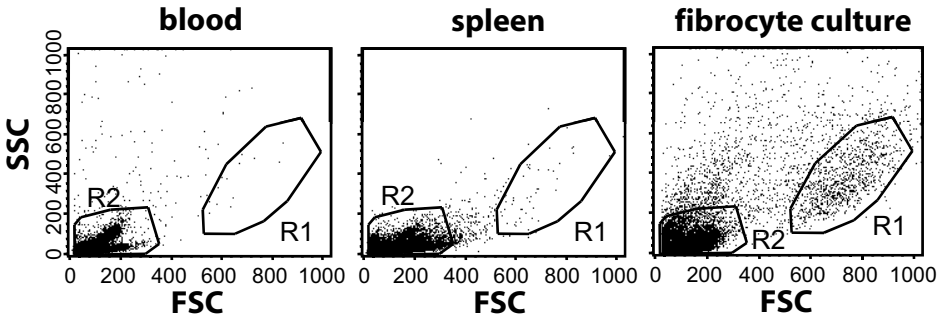

Fig. S1. Light scatter properties of freshly obtained peripheral blood cells (blood), splenocytes (spleen), and fibrocytes that appeared after culture of total splenocytes for 14 days (fibrocyte culture). A distinct population of large cells consisting of fibrocytes (located within gate R1) and numerous smaller cells (located within gate R2) are detectable in the fibrocyte culture. Freshly obtained peripheral blood cells and splenocytes show no distinct population of large cells, but only small cells consisting of lymphocytes, monocytes, and neutrophils (located within gate R2).

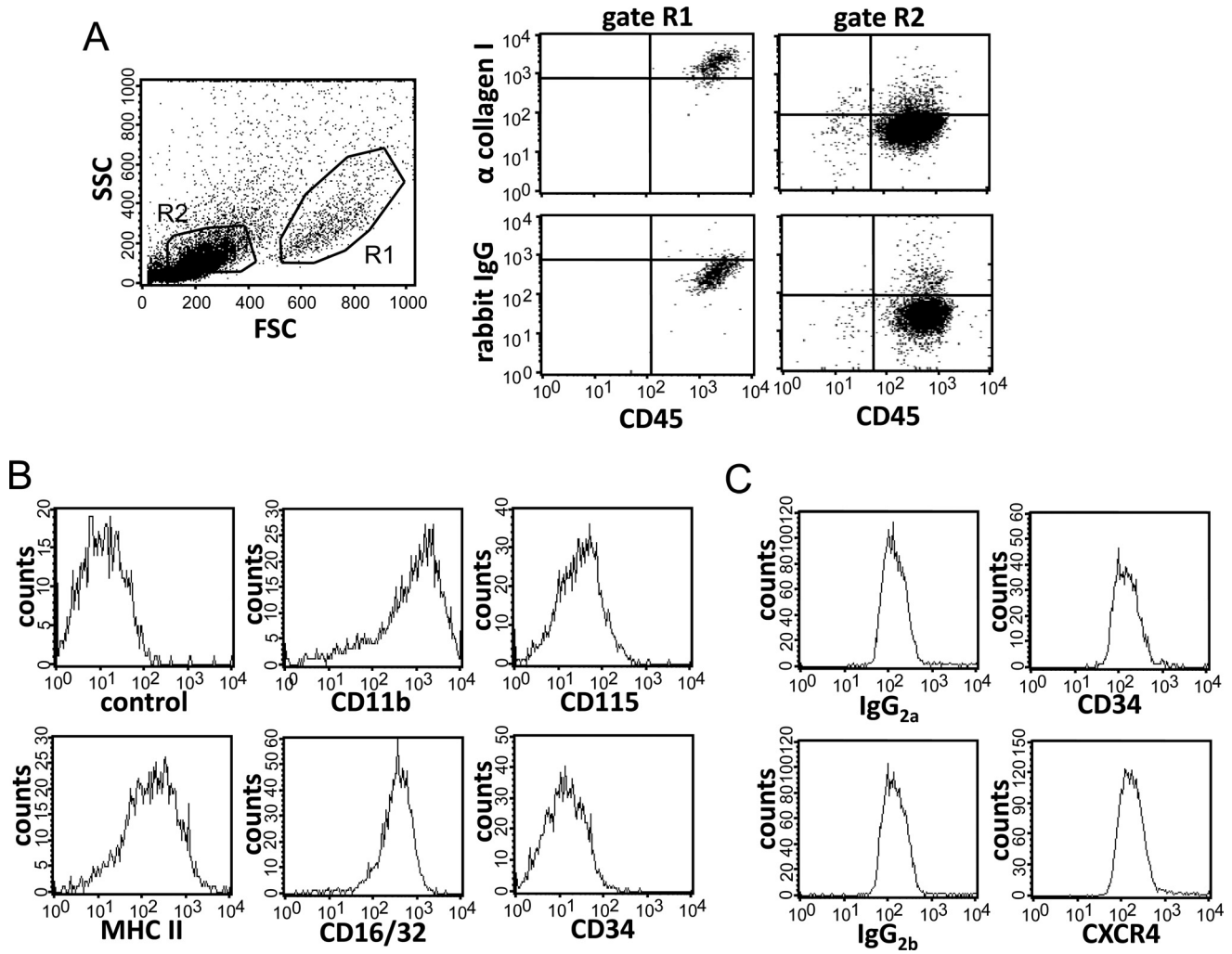

Fig. S2. Flowcytometric analysis of cultured splenocytes. After culture of total splenocytes for 14 days, cells were removed from the culture dish and analyzed by flow cytometry. (*A*) Light scatter analysis (*Left*) shows a distinct population of large cells (located within gate R1) that homogeneously stains positive for intracellular expression of collagen I (*Right*). The population of small cells (located within gate R2 (*Left*) shows no specific intracellular expression of collagen I (*Right*). The small cells most likely consist of remaining leukocytes, dead cells, and cell fragments. (*B* and *C*) Expression of additional surface markers on $CD45^+$ collagen I^+ fibrocytes.

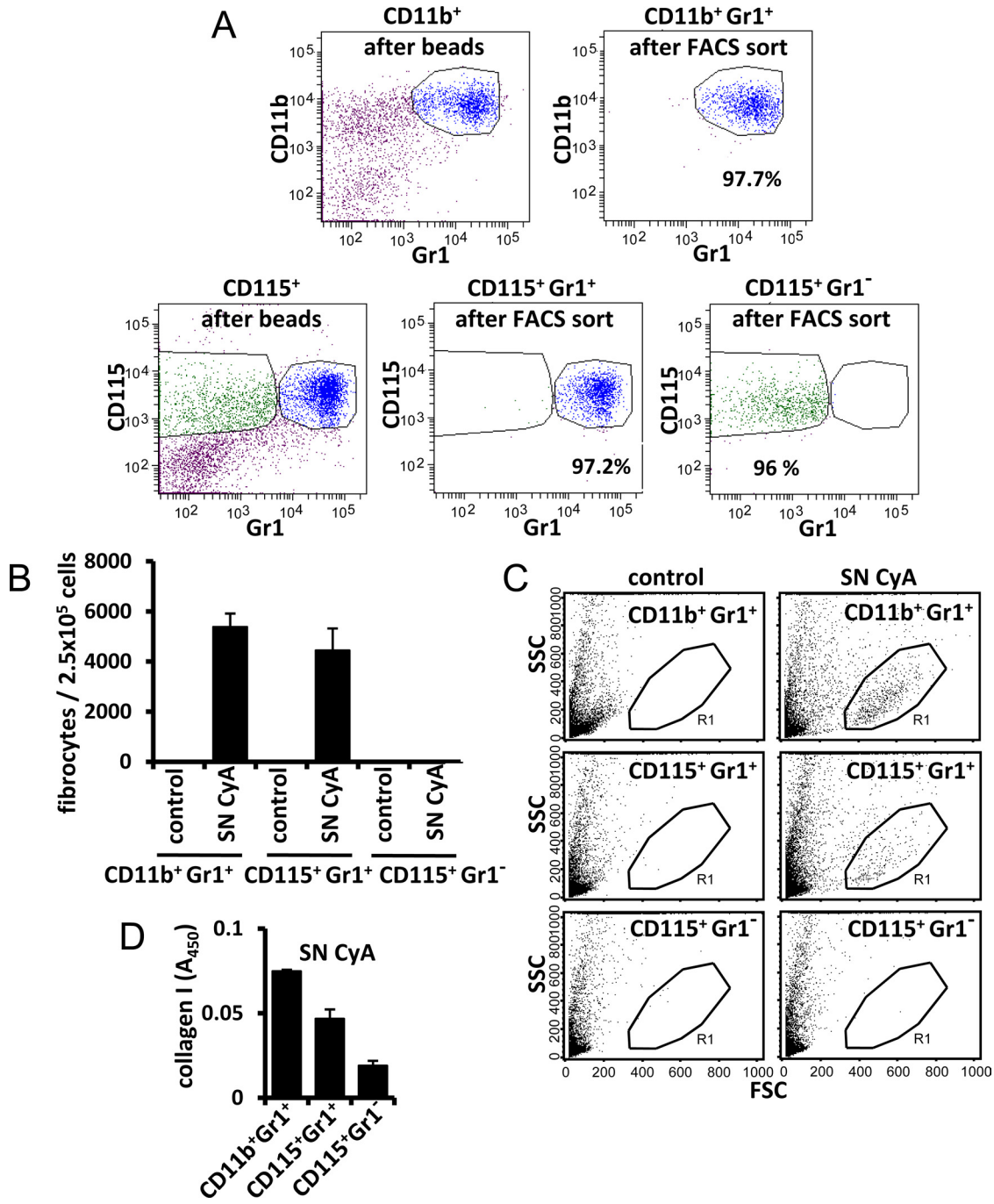

Fig. S3. Differentiation of Gr1$^+$ monocytes into fibrocytes. (*A*) Purification of CD11b$^+$Gr1$^+$, CD115$^+$Gr1$^+$, and CD115$^+$Gr1$^-$ monocytes. Splenocytes were first stained with PE-labeled antibodies against CD11b or CD115 and enriched with magnetic beads against PE (Miltenyi). Subsequently, cells were stained with the APC-labeled antibody Gr1 and further sorted by FACS. Neutrophils were excluded by light scatter properties. The purity of the sorted populations is indicated in the panels. (*B*) CD11b$^+$Gr1$^+$, CD115$^+$Gr1$^+$, and CD115$^+$Gr1$^-$ monocytes were cultured for 14 days with plain medium (control) or SN CyA. The number of spindle-shaped cells per 2.5 $\times$ 10^5 input cells was counted under the microscope, and (*C*) the light scatter properties of cultured cells were analyzed by flow cytometry. (*D*) Secretion of collagen to the culture plate in the presence of SN CyA was measured by ELISA.

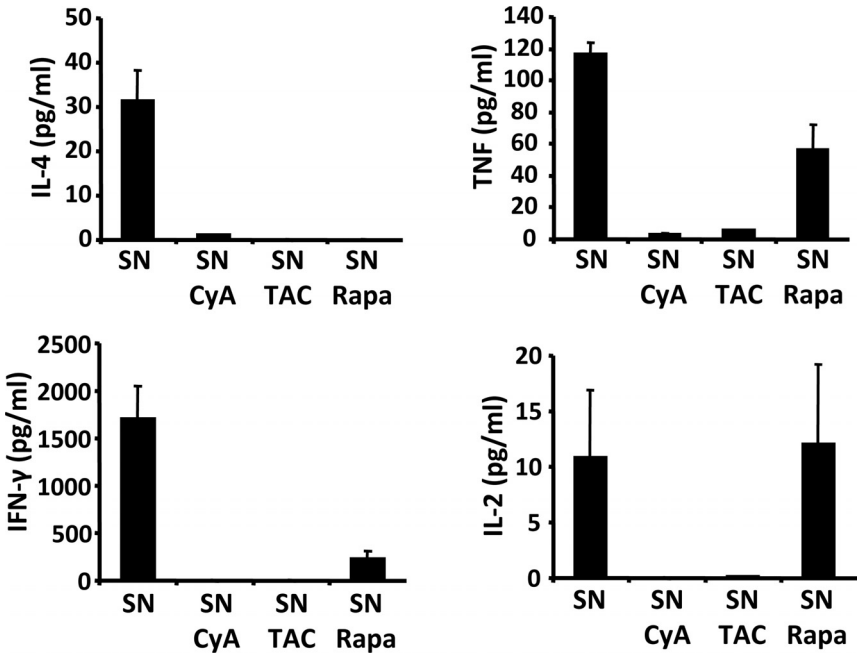

Fig. S4. Influence of calcineurin- and mTOR-inhibitors on the release of cytokines by activated $CD4^+$ T cells. $CD4^+$ T cells were polyclonally activated for 3 days with anti-CD3 in the presence or absence of cyclosporine A, tacrolimus, or rapamycin. Concentrations of IL-4, IL-2, TNF, and IFN-γ were measured in the supernatant by ELISA. Cyclosporine A and tacrolimus almost completely suppressed the release of the cytokines whereas rapamycin had an intermediate effect.

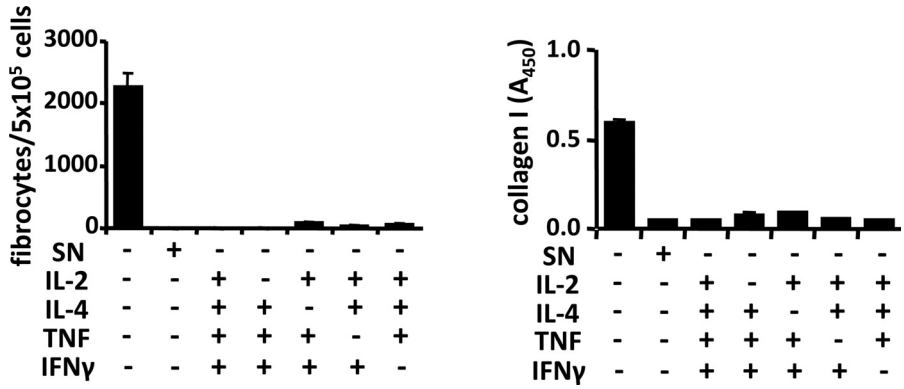

Fig. S5. Influence of recombinant cytokines on the generation of fibrocytes. $CD11b^+$ monocytes were cultured for 14 days with the indicated cytokines. The number of spindle-shaped cells was counted, and secretion of collagen I to the culture plate was quantified by ELISA. Results are expressed as the mean ± SEM of the number of fibrocytes from three pictures of three wells/condition.

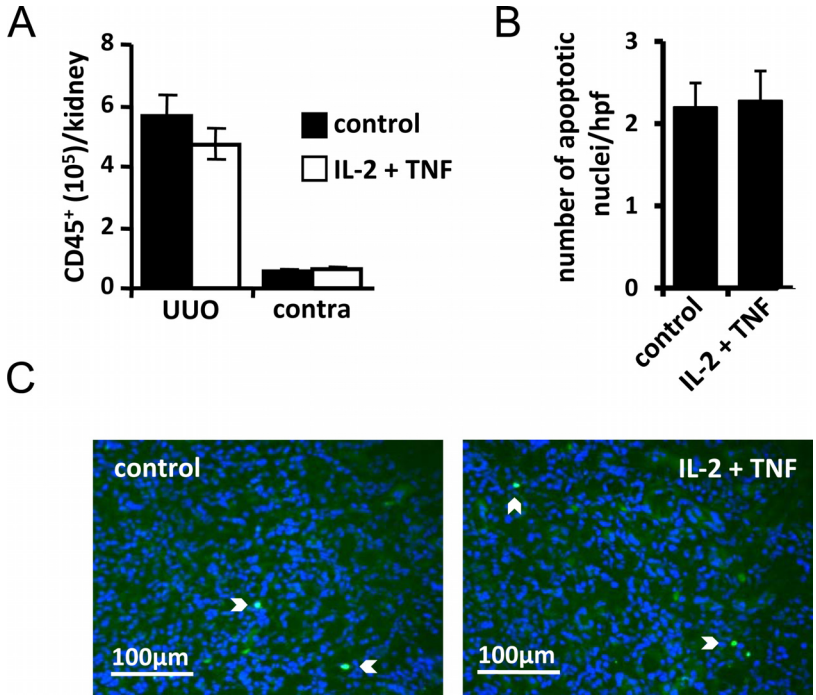

Fig. S6. Analysis of mice treated from day 0 to 6 after induction of UUO with IL-2 and TNF or PBS as control ($n = 5$ per group). (*A*) On day 7 after UUO, the number of $CD45^+$ leukocytes infiltrating the obstructed (UUO) or contralateral kidneys (contra) was quantified by flow cytometry using counting beads. The number of infiltrating cells was markedly higher in the obstructed kidneys, but there was no significant difference between the control group and mice treated with IL-2 and TNF. (*B* and *C*) The number of apoptotic cells per hpf was measured by Tunel-staining and was identical in UUO kidney sections of both groups of mice.

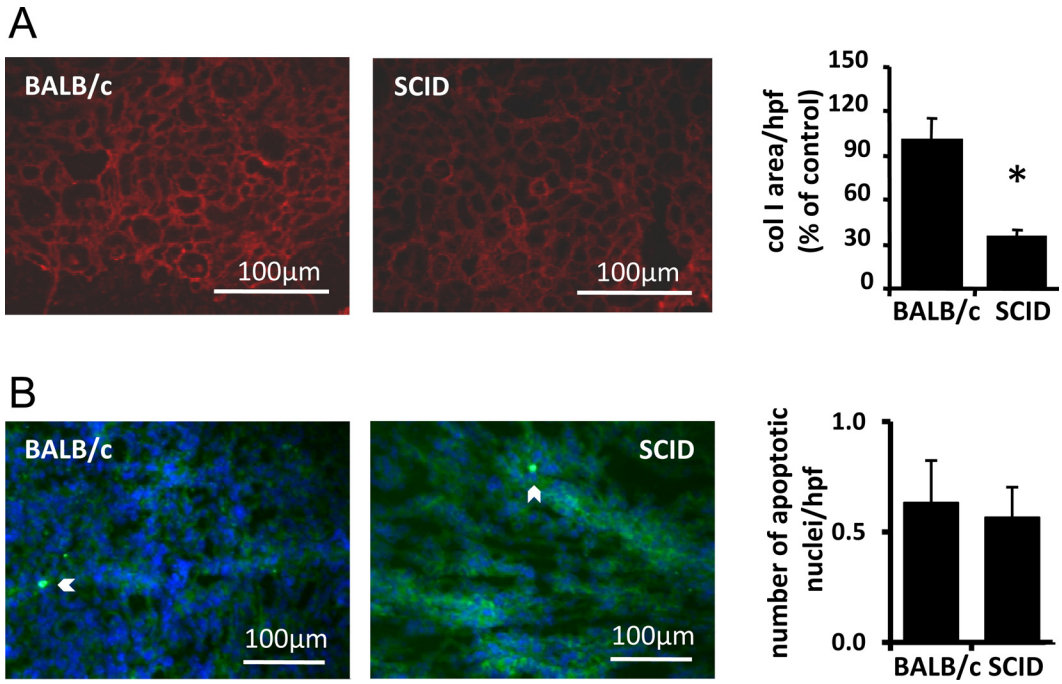

Fig. S7. Analysis of SCID and BALB/c mice on day 7 after induction of UUO. (*A*) To quantify deposition of collagen I, collagen I was stained by immunofluorescence (red). The collagen I positive area in sections of UUO kidneys was significantly reduced in SCID compared with BALB/c mice. (*B*) The number of apoptotic cells per hpf was measured by Tunel-staining and was identical in UUO kidney sections of BALB/c and SCID mice.

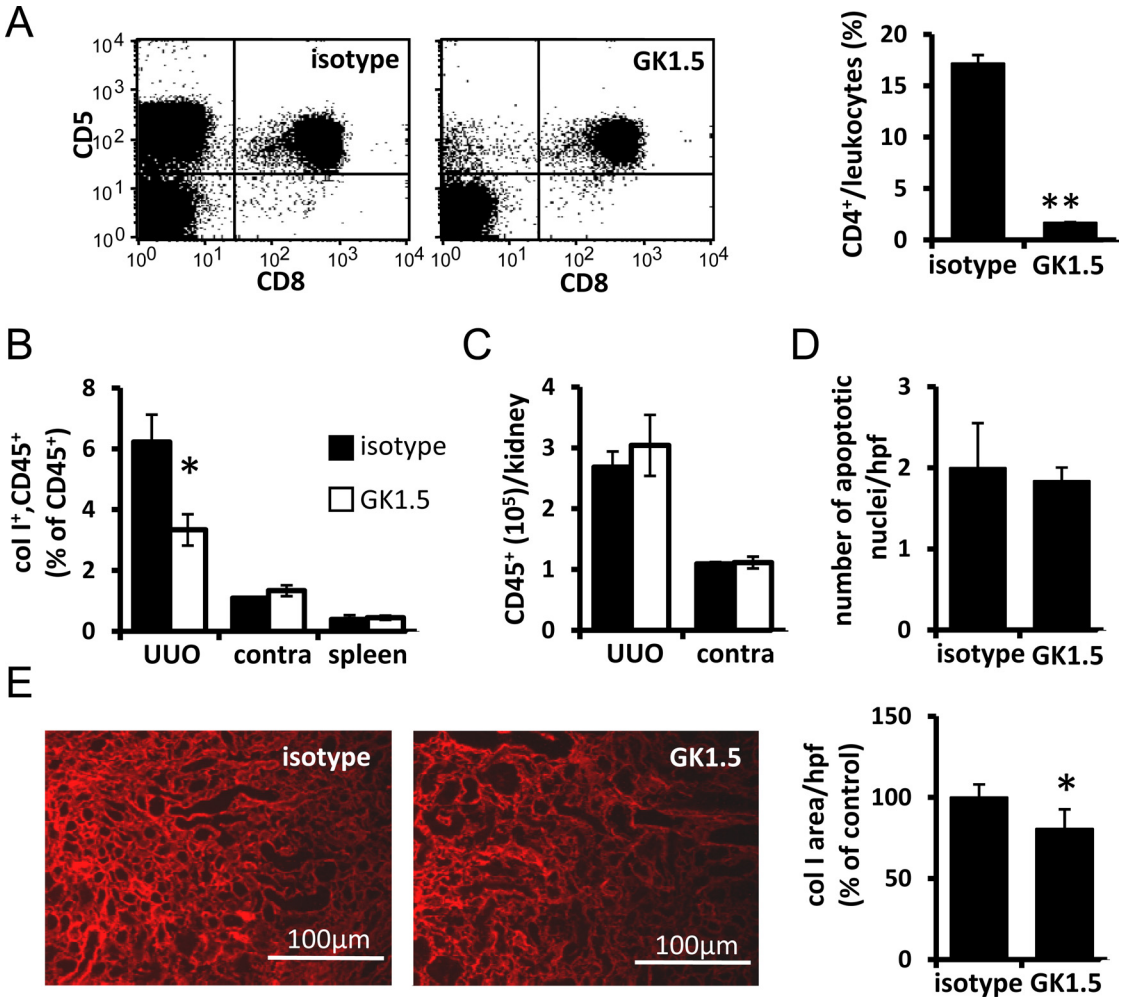

Fig. S8. Depletion of CD4$^+$ T cells in the UUO model. (*A*) C57BL/6 mice were treated on day −3, −2, and −1 with an isotype control antibody (rat IgG2b) or with GK1.5 to deplete CD4$^+$ T cells ($n = 5$ per group). The frequency of CD4$^+$ T cells was determined on day 7 in the peripheral blood by staining cells with antibodies against CD5 and CD8. CD4$^+$ T cells were identified as CD5$^+$CD8$^-$ cells in flow cytometry (*Left*) and were almost completely depleted in the GK1.5 group (*Right*). (*B*) Depletion of CD4$^+$ T cells significantly reduced the number of CD45$^+$ collagen I$^+$ fibrocytes in UUO kidneys but not in the contralateral kidneys (contra) or the spleen on day 7 after UUO. (*C*) The total number of infiltrating CD45$^+$ leukocytes in the kidneys was not significantly altered by treatment with GK1.5. (*D*) The number of apoptotic cells per hpf was measured by Tunel-staining and was identical in UUO kidney sections of both groups of mice. (*E*) Immunofluorescence for collagen I (red) showed a significant reduction of the collagen I positive area in sections of UUO kidneys of mice treated with GK1.5 compared with the isotype control antibody (control).

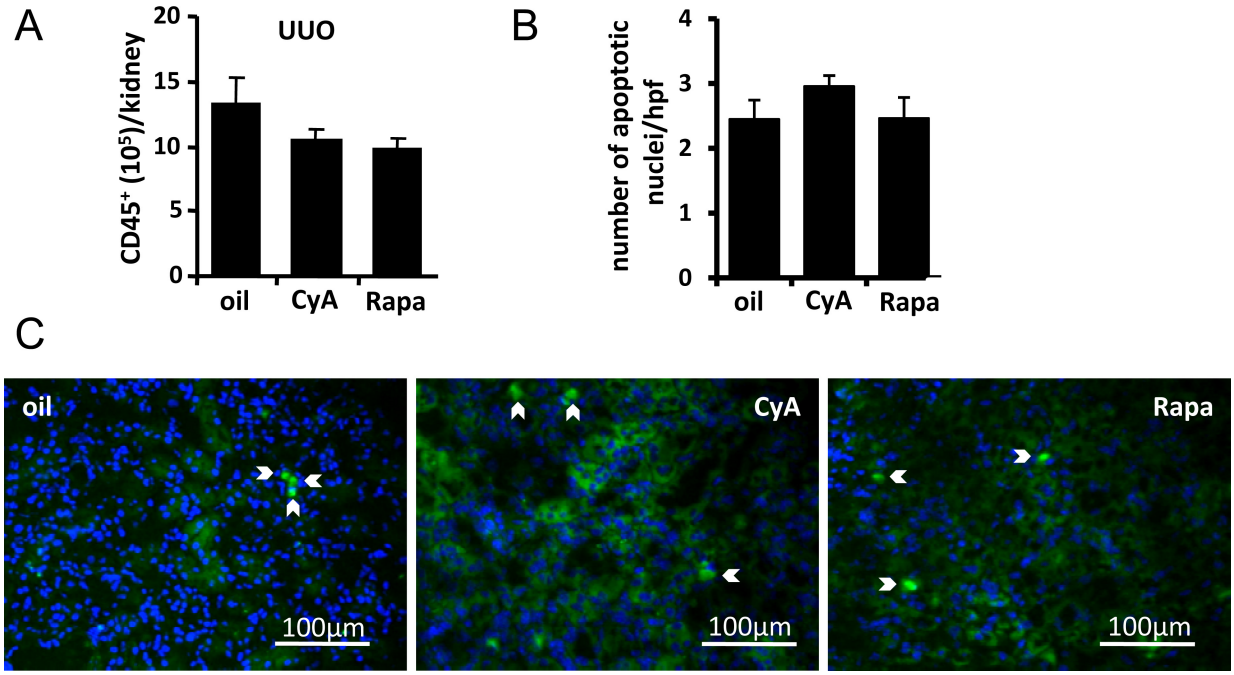

Fig. S9. Analysis of mice treated from day 0 to 6 after induction of UUO with αCD3 together with cyclosporine A (CyA), rapamycin (Rapa), or olive oil (oil) (n = 5 per group). (*A*) The number of infiltrating $CD45^+$ leukocytes was quantified in the obstructed kidneys and showed no significant difference between the three groups. (*B*) The number of apoptotic cells per hpf was measured by Tunel-staining and was identical in UUO kidney sections of the three groups of mice.