Methods

Animals. Ten week old male C57BL/6 wild type (WT) and CD47$^{-/-}$ mice (stock numbers 000664 and 003173 respectively) were obtained from The Jackson Laboratory (Bar Harbor, ME). All studies were performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh and in accordance with NIH guidelines.

Cell-based experiments. Human renal tubular epithelial cells (Lonza, Basel, SWI) were grown in appropriate media. Cells were serum starved at 80% confluence, subjected to 30 min hypoxia (FiO_2 1%) and then 24 h re-oxygenation and collected for RNA or protein (as described below). For immunofluorescence, cells were subjected to cytospin ($5x10^5$ cells/ml, 400rpm, 5 min), fixed in ethanol, stained with αCD47 antibody (clone B6H12, 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or IgG1 isotype control (Santa Cruz), then secondary antibody AlexaFluor 555 (Molecular Probes, Carlsbad, CA) and 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes).

Ischemia-reperfusion injury. Mice (n=10-12 per group) were anaesthetized using isoflurane and oxygen titrated to effect, and body temperature maintained at 37°C. A midline laparotomy incision was made and microaneurysm clips were placed to occlude both renal pedicles for 22 min. After removal of the clips, the kidneys were inspected for restoration of blood flow. The abdomen was closed with 5/0 monofilament suture. Animals received 600µl 0.9% saline and 0.4mg/kg buprenorphine subcutaneously at the end of the procedure. Mice were euthanized 24 h after reperfusion; blood was collected and kidney tissue snap frozen, placed in RNA*later*, embedded in OCT compound (Sakura FineTek, Torrance, CA), or fixed in 10% neutral buffered formalin. Sham-operated mice

(n=6) were subjected to anaesthesia and midline laparotomy only.

Laser Doppler blood flow analysis. Renal perfusion was measured using laser Doppler imaging (MoorLDI-2%, Moor Instruments, Devon, UK). Briefly, animals were anesthetized and core temperature maintained at 37°C. Renal blood flow was assessed at baseline, in response to ischemia and reperfusion at 30 min and 24 h. Results are expressed as the percent change from baseline control of the region of interest.

CD47 antibody treatment. WT C57BL/6 mice were randomized to receive either rat anti-mouse CD47 monoclonal antibody (clone 301, Santa Cruz Biotechnology, 0.4μg/g IP in 100μl sterile PBS) or an IgG_2a isotype-matched control antibody (Santa Cruz Biotechnology) injected 90 min before surgery. Measurement of creatinine and histology are as described below. WT mice receiving either antibody not subjected to IRI were euthanized and kidneys embedded in OCT compund. Sections were fixed in ethanol and subsequently stained with AF-555 and DAPI.

Assessment of renal function after IRI. Renal function was assessed by measurement of serum creatinine at 24 h after IRI using a Jaffe creatinine picric acid reaction (OSR 6178, Beckman Coulter, Brea, CA) analyzed on an Olympus AU640 analyzer respectively (Beckman Coulter).

Kidney histology. Kidneys embedded in paraffin were sectioned at 3μm and stained with Periodic Acid Schiff (PAS) stain by standard methods. Markers of tubular damage (tubular dilatation, cell necrosis, infarction and cast formation) were scored by calculation of the percentage of tubules in the corticomedullary junction that displayed such features: 0, none; 1, 1-10%; 2, 11-25%; 3, 26-45%; 4, 46-75%; 5>75%. Histological examination was performed blinded on 6 randomly selected corticomedullary fields

(magnification x200).

Immunostaining. Formalin-fixed, paraffin embedded sections of 4μm thickness were deparaffinized and boiled for 30 min in 10mM sodium citrate buffer (pH 6.0). Immunohistochemistry was performed using the following primary antibodies: rat anti-mouse neutrophil (clone Ly6B.2) and macrophage (clone F4/80, both AbDSerotec, Oxford, UK). Sections were exposed to 3% H_2O_2 in methanol to quench endogenous peroxidases and blocked with 10% normal goat serum (Sigma Aldrich, St. Louis, MO) and Avidin/Biotin block (Vector Laboratories, Burlingame, CA). The primary antibody was incubated for 2 h at room temperature, sections were washed with PBS, and then biotinylated goat anti-rat secondary antibody was added in combination with a Vectastain ABC kit (Vector Laboratories) and a metal enhanced 3,3' Diaminobenzidine (DAB) substrate kit (Thermo Scientific, Waltham, MA). Sections were counterstained for hematoxylin (Vector Laboratories), dehydrated and covered. Quantification of the cellular infiltrate was performed in a blinded manner by assessing 20 consecutive hpfs (magnification, x400).

Renal parenchymal cellular apoptosis. TUNEL staining was used to detect apoptosis with a commercially available *in situ* cell death detection kit (Roche, Basel, SWI), performed according to manufacturer's instructions. Tissue sections were subsequently counterstained with DAPI, fixed with 4% paraformaldehyde, mounted with mounting medium (DAKO, Glostrup, Denmark) and imaged.

Renal oxidative stress. Dihydroethidium (DHE, Molecular Probes) was used to evaluate *in situ* production of superoxide. DHE (10μM) was applied to unfixed frozen sections, incubated in a light-protected humidified chamber at 37°C for 30 min, washed with PBS

and mounted with fluorescent mounting medium (DAKO). Images were quantified using Image J (rsbweb.nih.gov/ij/).

RNA extraction and quantification by real-time PCR. Total RNA was extracted using Qiagen RNeasy® Mini Kits (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA was quantified using the Take3 Gen5 spectrophotometer (BioTek, Winooski, VT). One microgram (1μg) of RNA was treated with DNase I (amplification grade, Invitrogen) and then reverse-transcribed using the Superscript III First Strand Synthesis Supermix (Invitrogen). cDNA was amplified using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen) in 20μl volumes in quadruplicate with gene specific primers and probe on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Thermal cycling conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analyzed using the ΔΔCt method with expression normalized to the housekeeping gene and WT-sham-operated animals used as the referent control.

Western blotting. Kidney tissue was homogenized in ice-cold lysis buffer containing NP-40, protease inhibitor cocktail (Sigma Aldrich), 1mM sodium fluoride, 1mM sodium orthovanadate and PhosStop phosphatase inhibitor (Roche), and centrifuged at 12000rpm for 20 min at 4°C. Supernatants were collected and lysates quantified using a Bradford assay (BioRad, Hercules, CA). Thirty micrograms of total protein was resolved by SDS-page and transferred onto nitrocellulose (BioRad). In blots for CD47, non-reducing Laemmli buffer was used with 8% SDS-PAGE. Blots were probed with primary antibody to the respective proteins and visualized on an Odyssey Imaging System (Licor, Lincoln,

NE). The following antibodies were employed – mouse anti-TSP1 (Abcam, Cambridge, UK, 1:500 dilution); goat anti-CD47 C-18 (Santa Cruz, 1:500 dilution), rabbit anti-caspase-3 (Cell Signaling Technology, Danvers, MA, 1:1000 dilution), mouse anti-3-nitrotyrosine (Abcam, 1:1400 dilution) and rabbit anti-βactin (Cell Signaling, dilution 1:5000). The intensity of the bands was quantified using Image J.

Generation of bone marrow (BM) chimeric mice. BM was collected from WT or $CD47^{-/-}$ mice by flushing femurs and tibiae with Hank's balanced salt solution. Recipient mice were lethally irradiated with 2 doses of 5Gy from a $cesium^{137}$ source using a Mark1 irradiator (JL Shepherd & Associates, San Fernando, CA). Six hours after irradiation, recipient mice received 1x10 BM cells retro-orbitally. The animals were allowed to recover for 8 weeks to ensure stable engraftment before being subjected to renal IRI. Full chimerism of each mouse was confirmed by flow cytometry of whole blood stained for CD47 (clone 301 FITC, BD Pharmingen, Franklin Lakes, NJ) run on a FACSCalibur machine and analyzed using CellQuest software (BD Pharmingen).

Statistical analysis. The data are presented as mean $\pm$ SD unless otherwise stated. Data were analysed with Student's t-test (parametric variables) or Mann-Whitney *U*-test (non-parametric variables) for means between two groups or analysis of variance (ANOVA) between multiple (>2) groups using STATA v11.0 (STATA Corporation, College Station, TX). In all cases, $p < 0.05$ was deemed significant.