Materials and Methods

Animals. Male C57BL/6 wild type mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Male Sprague Dawley rats were obtained from Taconic (Hudson, NY). Animals were housed for one week prior to use and had access to water and standard rodent chow *ad libitum*. 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. Euthanasia was achieved by isoflurane inhalational anesthesia (1.5%) and concurrent cervical dislocation (mice) and or CO_2 inhalation from a pressurized tank followed by cervical dislocation (rats).

Reagents and cells. Human renal tubular epithelial cells (rTEC) and rat aortic VSMC were purchased from Lonza (Switzerland) and maintained in recommended medium. Cells were used between passages 3-7. TSP1 was purchased from Athens Research & Technology (Athens, GA). SIRP-α and CD47 morpholino oligonucleotides complementary to a 5′-UTR sequence rat mRNAs and corresponding 5 base mismatched control morpholino were purchased from GeneTools, Inc. (Philmonth, Oregon). The TSP1-derived peptide 753 was synthesized by Dr. Henry C. Krutzsch and kindly provided by Dr. David D. Roberts (NCI, NIH, Bethesda, MD). The SIRP-α monoclonal Ab (clones C20 for treatment applications and A1 for Western immunoblot) and monoclonal CD47 Ab (clones OX101) were purchased from Santa Cruz Biotechnology and the β integrin blocking Ab (clone Ha2/5) was purchased from BD Biosciences.

Murine rTEC cultures. Primary murine wild type and CD47 null cells were harvested as previously published with modifications[5]. Briefly, mice were euthanized, flushed with PBS, and kidneys removed. The renal cortex was manually resected into pieces of approximately 1 mm^3

and digested at 37°C for 25 minutes in DMEM/F12 medium (Invitrogen, Carlsbad, CA) with 1mg/ml type II collagenase (Worthington Chemicals), BSA (Sigma Aldrich, St Louis, MO) and DNase (Sigma Aldrich). The kidney digest was washed through a 40 µm sieve and spun down at 300 *g* for 5 minutes. The cell pellet was resuspended in defined K1 medium: DMEM/F12 medium supplemented with 25 ng/ml epidermal growth factor (Sigma Aldrich), 1 ng/ml PGE_1 (Cayman Chemicals, Ann Arbor, MI), 5 × 10–11 M triiodothyronine (Sigma Aldrich), 5 × 10–8 M hydrocortisone (Sigma-Aldrich), insulin–transferrin–sodium selenite media supplement (Sigma Aldrich), 1% penicillin/ streptomycin (Cellgro, Manassas, VA), 25 mM HEPES (Invitrogen), and 5% FCS (Invitrogen). The cell suspension was then cultured on collagen-coated Petri dishes (BD Biosciences) in K1 medium until epithelial colonies were established. Experiments were commenced after the cells had reached 90% confluence, which was usually between 3-4 days after the isolation procedure.

Protein binding assays. The recombinant human extracellular domain of human SIRP-α fused to a modified human Fc domain was a gift of Dr. William Frazier (Washington University, St. Louis, MO). Recombinant SIRP-α was labeled using ^{Na125}I by the iodogen method as we previously published[24]. Immulon 2HB Removawells (Thermo, Franklin, MA) were coated with 50 µl of TSP1 at several concentrations for 16-20 h at 4ºC. Nonspecific binding was blocked by incubating the wells with DPBS with Ca^{2+} and Mg^{2+} and 1% BSA for 2 h at room temperature. Radio-labeled SIRP-α was then added and incubation conducted for 2 h at room temperature. Following extensive washing, bound radioactivity was quantified.

Co-immunoprecipitation. Immunoprecipitation was performed as we have published with minor modification.[2] Arterial vascular smooth muscle cells (passages 2-8) were grown in full media and then collected in NP-40 lysis buffer with protease inhibitor cocktail (Sigma Aldrich).

Protein levels were quantitated using a DC^{TM} assay (Bio-Rad, Hercules, CA). Two hundred micrograms of protein was precipitated using either a TSP1 antibody (clone 6.1, Abcam, Cambridge, UK) or mouse IgG_1 control antibody (Santa Cruz Biotechnology, Dallas, TX), or a SIRP-α (clone C20) or goat IgG control antibody (both Santa Cruz Biotechnology). For the phosphotyrosine immunoprecipitation, vascular smooth muscle cells or human rTEC (passages 2-6) were serum starved over 24 h, and then treated with TSP1 (2.2 nmol/L) for 1 h, lysate prepared and measured, and then precipitated with a SIRP-α antibody (clone C20, Santa Cruz Biotechnology). Immune complexes were recovered with Protein G Plus-Agarose (Thermo Scientific Pierce, Rockford, IL), washed four times and centrifuged at 600*g* at 4°C for 5 min. Pellets were suspended in sample buffer and boiled at 95°C for 5 min. Protein was then separated via gel electrophoresis and transferred to membranes and blotted against TSP1, SIRP-α (clones C20, Santa Cruz Biotechnology), or phosphotyrosine (Millipore, Billerica, MA).

Western protein analysis. Following 24 h of serum starvation, the indicated treatments of cells were carried out at 37°C in fetal calf serum- and growth factor-free medium with 0.1% BSA. Following treatment, the cells were rinsed with ice-cold PBS and lysed at 4°C in lysis buffer that contained: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1X protease inhibitors cocktail (Sigma) and 1X phosphatase inhibitors cocktail (Roche Applied Science, Hercules, CA). Cell lysates were centrifuged at 17,000*g* for 20 min. A DC^{TM} assay (BioRad, Life Sciences Research, Hercules, CA) was used to quantify total protein. Cell lysates mixed with SDS sample buffer were boiled at 95°C for 5 min, electrophoretically separated on 7.5% PAGE gels for approximately 1 h at 150 V, and transferred to nitrocellulose membrane (BioRad) for 2 h at 400 mA. Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE)

and incubated overnight at 4°C with primary Ab. The following Ab were employed – anti-SIRP-α, anti-SHP1, anti-SHP2, anti-CD47 and anti-Nox1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine and phosphoserine Abs were obtained from Millipore (Billerica, MA). Anti-phospho-S591 SHP1, phopho-Y536 SHP1, phosphor-Y542 SHP2, phosphor-Y580 SHP2 and anti-Nox2 Ab were obtained from Abcam (Cambridge, MA). The β-actin Ab was obtained from Cell Signaling Technology (Danvers, MA). The intensity of the bands was quantified using the Odyssey software or 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 (1 µg) 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 10 µl volumes in triplicate with gene-specific primers and probed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), according to the 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. The following primer sequences were employed: TSP1 (Mm01335418_m1), CD47 (Mm00495005_m1), SIRP-α (Mm00455928_m1), SHP1 (Mm00442278_m1), SHP2 (00448434_m1), Nox1 (Mm00549170_m1), and Nox2 (Mm01287743_m1), TNF-α (Mm00441889_m1), IL-6 (Mm00446190_m1), CCL2 (Mm00441242_m1), CXCL2 (Mm00436450_m1), IL-1β (Mm00434228_m1) and HPRT1 (Mm00446968_m1) (Taqman,

Applied Biosystems).

Assessment of p47^{phox} phosphorylation. Phosphorylation of p47^{phox} is consistent with increased Nox2 or 1 activity in cells. The phosphorylation of p47^{phox} (critical cytosolic subunit of Nox) in p47^{phox} immunoprecipitates was determined using an anti-phosphoserine Ab as we published.[65] Briefly, cells were lysed and protein extracted. Equal amounts of protein (300 μg) from the cytosolic fraction as determined by the Bio-Rad protein assay (Life Science Research, Hercules, CA) were incubated overnight at 4°C with anti-p47^{phox} Ab (6 μg, Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were recovered with Protein G Plus-Agarose (Thermo Scientific Pierce), washed four times and centrifuged at 600*g* at 4°C for 5 min. Pellets were suspended in sample buffer and boiled at 95°C for 5 min. Immunoprecipitates were then subjected to Western blotting with a phospho-serine Ab (1:1000, clone 4A4, Millipore).

Measurement of $O_2^{\bullet-}$ via cytochrome *c* reduction. Cells were suspended in 200 µl of ice-cold disruption buffer (8 mM potassium, sodium phosphate buffer, pH 7.0, 131 mM NaCl, 340 mM sucrose, 2 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail). The suspension was lysed by five freeze/thaw cycles and passed through a 30-gauge needle five times[14]. Cell disruption was confirmed by phase-contrast microscopy. The cell lysate was centrifuged at 1000*g* for 10 min at 4°C to remove unbroken cells, nuclei, and debris. The supernatant was transferred to another eppendorf tube and was centrifuged at 28,000*g* for 15 min at 4°C to collect the membrane fraction pellet, which was resuspended in 50 µl disruption buffer. Throughout all these procedures, extreme care was taken to maintain the lysate at a temperature of 4°C. Membrane fractions (2 μg/well) were added to cytochrome *c*-containing oxidase assay buffer (65 mM sodium phosphate buffer, pH 7.0, 1 mM EGTA, 10 μM FAD, 1 mM $MgCl_2$, 2 mM NaN_3, and 0.2 mM cytochrome *c*). After a 5 min baseline

measurement, NADPH (180 μM) was added to initiate the reaction. $O_2^{\bullet -}$ was measured as the initial linear rate of SOD-inhibitable cytochrome *c* reduction quantified at 550 nm using the extinction coefficient 21.1 mM^{-1} cm^{-1} [13].

siRNA transfection. Cells were plated the day before transfection to achieve 30–50% confluence in DMEM medium containing 10% fetal calf serum without antibiotics. The small interfering RNA (siRNA) transfections were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum free Opti-MEM according to the manufacturer's instructions and were carried out in 10-cm^2 plates with a final siRNA concentration of 46 nM. DMEM containing 5% FBS was added to culture plates 10 h post-transfection. At 48 h post transfection, the cells were serum and growth factor starved with 0.1% BSA medium for 24 h followed by treatments as indicated in the figure legends. Confirmation of target suppression was provided by Western blot analysis.

Morpholino oligonucleotide protein suppression of CD47. Cells were seeded onto 60-mm^2 plates in DMEM medium containing 10% fetal calf serum. CD47 morpholino was transfected with a final concentration of 10 μM using the delivery agent Endoporter (6 μl/ml, Genetools, Philomath, OR) according to protocol instructions. At 48 h post transfection, the cells were serum and growth factor starved with 0.1% BSA medium for 24 h followed by treatments as indicated in the figure legends. Gene silencing was monitored at the protein level by Western blotting of cell lysates collected 72 h following transfection.

Tissue histology and microscopy. Kidneys embedded in paraffin were sectioned at 3μm and stained with haematoxylin and eosin 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 in "blinded" fashion on 6 randomly selected corticomedullary fields (magnification x200). Light microscopy images were acquired under identical settings using a Zeiss Axiovert 40CFL microscope and Axiovision v4.8 software (Zeiss, Oberkochen, Germany). Displayed tissue section images are at 200x magnification, and insets, are included, at 400x magnification.

Immunofluorescence of arterial rings, human rTEC and murine kidneys. Cryostat sections (5 μm) of vessels were washed three times with phosphate buffered saline (PBS), followed by 3 washes of 0.5% BSA in PBS. Sections were blocked with 2% BSA solution for 30 min. The slides were incubated for 1 h at room temperature (RT) with primary anti-TSP1 Ab (AMSBio, Abingdon, UK; 1:200 in 0.5% BSA solution). Slides were washed three times with BSA solution and incubated for 1 h at RT with a CY3 goat anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories; 1:500 in 0.5% BSA solution) in combination with 1:250 dilution of F-actin dye Rhodamine Phalloidin (Invitrogen). For rTEC, cytospins were generated and fixed with ethanol. Slides were blocked with 5% goat serum and then incubated overnight at 4°C with SIRP-α Ab (clone A-1), 1:50. Slides were washed with PBS and incubated with anti-mouse PE (Immunotec Research Inc, Swanton, VT) 1:500 for 1 h at room temperature. Nuclei were stained with Hoechst dye (bisbenzamide 1mg/100ml water) for 30 sec. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a). For whole organs cryostat sections (5 μm) of mouse kidneys were fixed for 20 minutes with 2% paraformaldehyde. Sections were then washed three times with phosphate buffered saline (PBS), followed by 3x washes with solution of 0.5% BSA in PBS. Sections were blocked with 2% donkey serum in BSA solution for 30 minutes. The slides were incubated for 2 hours at room temperature (RT)

with combined primary antibodies for TSP1 (PA1-29196, Thermo Scientific) at 1:100 and SIRP-α (clone C-20, Santa Cruz) at 1:200 in 0.5% BSA solution. Slides were washed three times with BSA solution and incubated for 1 hour at RT with CY3 donkey anti goat secondary antibody (705165147, Jackson Immuno) diluted 1:500, combined with donkey anti rabbit Alexa 488 (A-21206, Invitrogen) diluted 1:1000 in BSA solution. Nuclei were stained with Hoeschts dye (bisbenzamide 1mg/100ml water) for 30 seconds. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 2.01).

Arterial myography. Myography of arterial segments was performed as previously published with minor modifications[1, 6]. Animals were anesthetized with pentobarbital 50 mg/kg i.p. To exsanguinate the abdominal vena cava was transected and the vasculature was gently flushed with chilled 4ºC PBS buffer via puncture of the left ventricle. Thoracic aortas of mice and rats were cleared of adherent adipose tissue and excised. The endothelial layer was removed by gently rubbing the luminal side of the vessel along the rough surface of a blunt needle. Arterial segments approximately 2 mm in length were mounted on myograph pins (Danish Myo Technology, Atlanta, GA) in 5ml incubation buffer maintained at 37°C, pH 7.4, gassed with 95% O_2 and 5% CO_2, and brought to an optimal resting tension by increasing tone every 10 sec. Rings were allowed to stabilize for 1 h, replacing the PSS solution every 20 min. Viability of the vessels was ascertained by a contractile response to potassium chloride (100 mM KCl in PSS solution) for 30 min. Rings were then washed 3 times with PSS and allowed to stabilize to baseline. Phenylephrine (PE; Sigma-Aldrich) concentration–response curves (10^{-9} to 10^{-5} M) were generated by measuring contraction plateaus at each concentration. After vessel segments reached a stable plateau phase induced by a PE concentration producing 80% maximum

contraction (EC_{80}), a single concentration of acetylcholine (ACh; Sigma-Aldrich, 10^{-6} M) was added to assess endothelium function. Aortic rings were then washed and incubated in the presence or absence of a SIRP-α monoclonal blocking Ab (1 μg/ml, 15 min) ± TSP1 (2.2 nmol/L) ± Tempol (30 μM). Vessels contracted with an EC_{80} of PE were allowed to reach a stable plateau and endothelium-independent vasodilation to sodium nitroprusside (SNP, Sigma-Aldrich, 10^{-10} to 10^{-5} M) was then tested.

Detection of ROS in renal tissue sections. Assessment of ROS in renal tissue sections was done using the several techniques. The cell permeable agent dihydroethidium (DHE, 10μM, Molecular Probes) was applied to unfixed frozen sections, incubated in a light-protected humidified chamber at 37°C for 30 minutes, washed with PBS and mounted with Gelvital mounting medium. In other experiments cryostat sections (5 micron) of kidneys were washed three times with PBS, followed by 3x washes with solution of 0.5% BSA in PBS. Sections were blocked with 2% BSA solution for 30 minutes. The slides were incubated for 1 h at room RT with primary antibody for 4-HNE (Alpha Diagnostics), 1:100 in 0.5% BSA solution. Slides were washed three times with BSA solution and incubated for 1 h at RT with CY3 goat anti-rabbit secondary antibody (Jackson Immuno), diluted 1:500 in BSA solution, in combination with 1:250 dilution of F-actin dye Rhodamine Phalloidin (Invitrogen). Nuclei were stained with Hoeschts dye (bisbenzamide 1mg/100ml water) for 30 sec. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a).

Cell apoptosis and viability analysis. Human rTEC were grown to 80% confluence, serum starved in 0.1% FBS overnight, and treated with TSP1 (2.2nM) and/or SIRP-α antibody (clone C-20) for 24 h. Cells in 60mm plates were washed with cold PBS, and collected in RIPA buffer

for protein quantification. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose membrane and probed for caspase-3 (Cell Signaling Technology). Additionally, cell viability was assessed in a 96-well plate using the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes). Calcein (0.5µM) and ethidium homodimer-1 (2µM) were added to the cells for 30 minutes and fluorescence read using a microplate reader as per manufacturer instructions.

Ischemia reperfusion injury. Mice were anaesthetized using isoflurane and oxygen titrated to effect, and body temperature maintained at 36°C with the aid of a rectal temperature probe, warming pad and lamp. A microaneurysm clip was placed to occlude the renal pedicles for 20 min after which the clip was removed. The abdomen was closed with 5/0 monofilament suture. Mice were used for Laser Doppler experiments 30 min or 24 h after reperfusion, and euthanized. Blood was collected and kidney tissue snap frozen, placed in RNA*later*, embedded in OCT compound, or fixed in 10% neutral buffered formalin.

Laser Doppler blood flow analysis. Real time kidney perfusion and blood flow was measured using laser Doppler imaging (MoorLDI-2%, Moor Instruments, Devon, UK) as we have published [3, 4]. Briefly, animals were anesthetized and core temperature maintained at 36°C. Organ 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.

***In vivo* SIRP-α Ab treatment.** Wild type C57BL/6 mice were randomized to receive either a SIRP-α monoclonal Ab (clone C20, Santa Cruz Biotechnology, 0.4µg/gram body weight i.p. in 100 µl sterile PBS) or an IgG isotype-matched control Ab (Santa Cruz Biotechnology) 90 min before surgery.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software. Data were analyzed by 1-way ANOVA followed by Tukey test for multiple comparisons. For grouped analysis, data were analyzed by 2-way ANOVA followed by Bonferroni post hoc test. A p value of < 0.05 was assumed to be significant.

References

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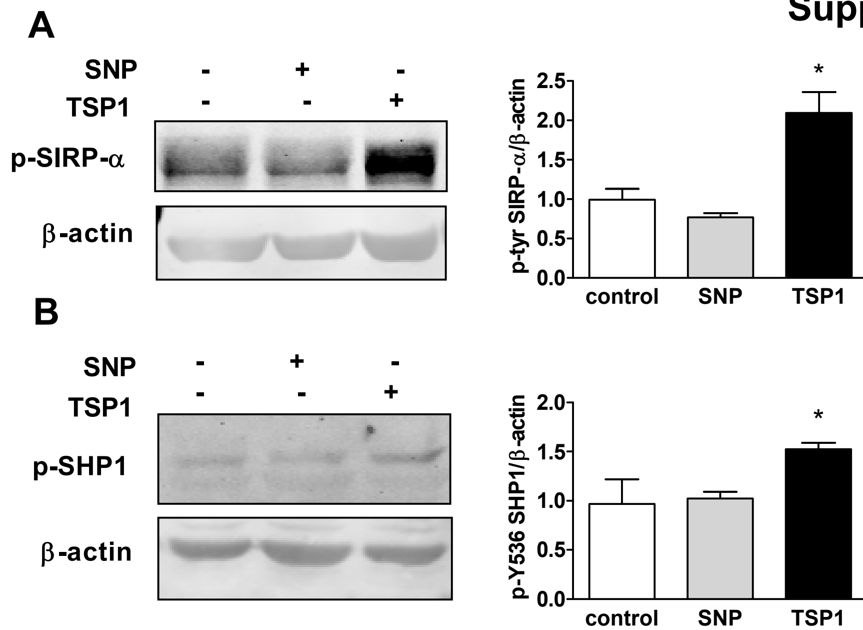
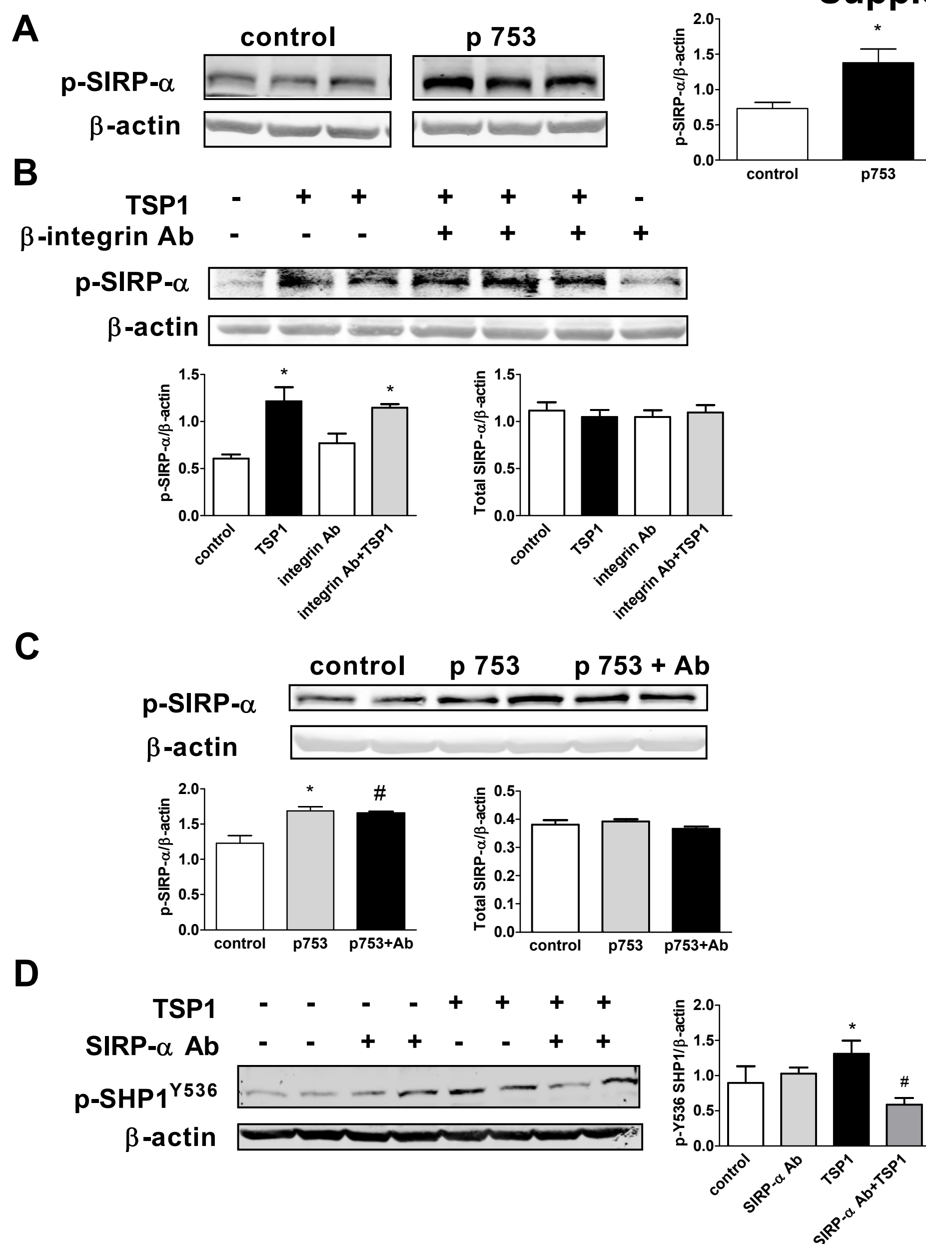
Supplemental Figure 1



Supplemental Figure 1. Treatment of VSMC with the NO pro-drug sodium nitroprusside does not stimulate phosphorylation of SIRP-α or SHP1. VSMC in minimal medium were treated with TSP1 (2.2 nmol/L) or the NO pro-drug sodium nitroprusside (SNP, 10 uM) for 10 min, cells lysed and proteins separated and blots probed for p-SIRP-α (**A**, **C**) and p-SHP1 (**B**) or β-actin. Representative data from 4 independent experiments are presented. Densitometry is presented as mean ratio of p-tyr-SIRP-α to β-actin (± S.E.M.) and as the mean ratio p-SHP1 to β-actin (± S.E.M.). * = statistically significant difference ($p < 0.05$) TSP1 compared to SNP or control (untreated).

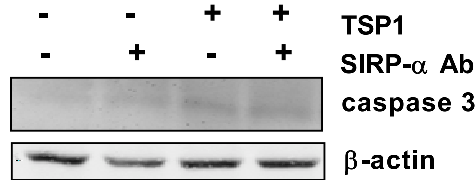
Supplemental Figure 2



Supplemental Figure 2. TSP1 activation of SIRP-α does not require β integrins. (**A**) VSMC in basal medium were treated with peptide 753 (10 μM), or a β integrin blocking Ab (1 μg/ml) ± TSP1 (2.2 nmol/L) for 10 min (**B**), cell lysate prepared and Western blot analysis performed. Densitometry is presented as the mean ratio of p-SIRP-α to β-actin (± S.E.M.)(A, B) or total SIRP-α to β-actin (± S.E.M.) (B). (**C**) VSMC in basal medium were treated with peptide 753 (10 μM) ± a β integrin blocking Ab (1 μg/ml) for 10 min, cell lysate prepared and Western blot analysis performed. Densitometry is presented as the mean ratio of p-SIRP-α to β-actin (± S.E.M.) or total SIRP-α to β-actin (± S.E.M.). (**D**) VSMC in basal medium were treated with TSP1 (2.2 nmol/L) ± a SIRP-α blocking Ab (1 μg/ml) for 10 min, cell lysate prepared and Western blot analysis performed. Results are representative of 4 separate experiments. Densitometry is presented as the mean ratio of p-SHP1 to β-actin (± S.E.M.). All demonstrated results are representative of 4 separate experiments; * = statistically significant difference ($p < 0.05$) compared to untreated and Ab treated and # = statistically significant difference ($p < 0.05$) compared to TSP1 treated.

Supplemental Figure 3

A



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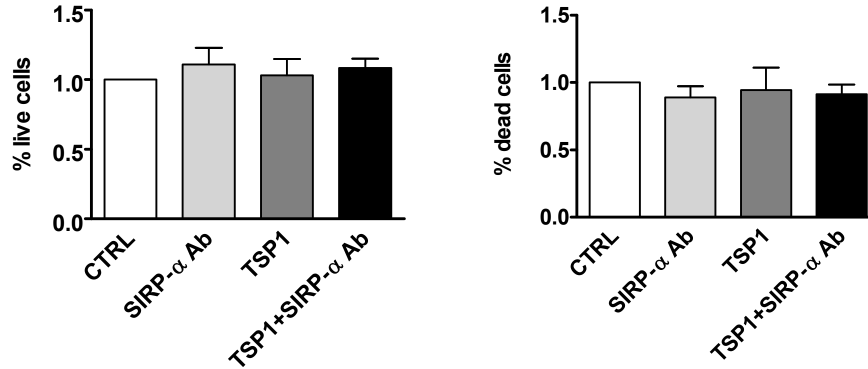
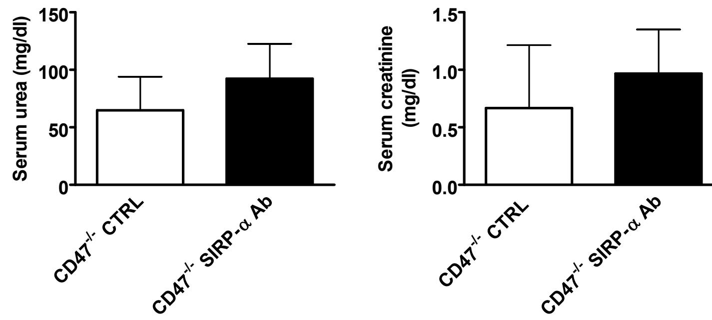


Supplemental Figure 3. TSP1 treatment dose not stimulate cell apoptosis or cell death in human rTEC. (**A**) Human rTEC were treated in minimal medium with 0.1% BSA with TSP1 (2.2 nmol/L) ± a SIRP-α Ab (1μg/ml) for 24 h and protein expression of caspace 3 determined via Western immunoblot. A representative blot from 3 separate experiments is shown. Data are presented as the means ratio of target protein to β-actin (± S.E.M.). (**B**) Human rTEC were cultured in minimal medium with 0.1% BSA and treated with TSP1 (2.2 nmol/L) for 24 h and cell viablility determined using the LiveDead Cell Viability kit (Molecular Probes) as per the manufacture's instructions. Data are presented as the means (± S.E.M.) of the % live and % dead cells.

Supplemental Figure 4



Supplemental Figure 4. SIRP-α Ab treatment does not improve serum urea or creatinine in IRI-challenged CD47 -/- mice. CD47 null age matched male mice were treated with a SIRP-α blocking Ab or isotype IgG control (CTRL) Ab (0.4 μg/g body weight i.p.) and challenged with 20 min of renal ischemia and 24 h of reperfusion, n=8 per group. Serum urea and creatinine were measured 24 h after reperfussion. Results are presented as the means (± S.E.M.).