Detailed Methods

In vivo model of renal IRI

All studies were approved by the Genzyme Institutional Animal Care and Use Committee. C57BL/6 male mice (8-10 weeks) purchased from Taconic (Germantown, NY) were housed in a virus- and parasite-free barrier facility with a standard 12:12hr light-dark cycle and had ad libitum access to water and standard chow. For surgery, the procedure of Clements et al was followed.[11] Briefly, animals were anesthetized with sodium pentobarbital (50-70 mg/kg, IP), aseptically prepped, and positioned on homeothermic operating tables to rigorously maintain body temperature at 37^{o}C through rectal probe. The kidneys were exposed by bilateral flank incisions and the renal pedicles were cleaned of adherent connective tissue. The renal artery and vein of each kidney were clamped with non-traumatic microaneurysm clamps (Roboz Surgical, Holliston MA) for 28 minutes. Clamps were released and reflow of each kidney was visually confirmed. After suturing, 1 ml of sterile saline was injected IP and the animals were recovered on surgical heating pads at 37ºC for 24-48 hrs. Buprenorphine (0.05 mg/kg, SC) was administered 2.5 hrs prior to surgery and again at 24, 36 and 48 hrs after the initial dose. Sham surgeries were identical without the bilateral clamping.

At various times post-reperfusion, blood was collected from the retro-orbital sinus followed immediately by euthanasia and whole body perfusion with Hank's Balanced Salt Solution (HBSS). Kidneys were harvested and the kidney capsules were removed. One kidney was snap frozen in liquid nitrogen for molecular analysis while the second kidney was divided in half, one half for flow cytometric analysis and one half fixed in 10 mM sodium periodate-75 mM L-lysine-2% paraformaldehyde. Plasma creatinine and blood urea nitrogen (BUN) values were measured on a Roche Integra 400 Bioanalyzer (Roche Diagnostics, Indianapolis IN). Whole blood was analyzed for circulating monocytes (Sysmex XT-V Automated Hematology Analyzer, Sysmex, Japan).

To determine monocyte/macrophage function in the different phases following renal IRI, a small molecule inhibitor of c-fms kinase activity was administered at 30 mg/kg, PO, following the dosing regimens described in Figures 3 and 4 to reduce monocyte/macrophage proliferation, differentiation and survival, based on published reports.[30, 57]

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To extract total RNA, snap frozen tissues were lysed for 2 min at RT in RLT Lysis Buffer containing β-mercaptoethenol (Qiagen, Valencia, CA) using zirconium beads placed in a bead beater (Biospec, Bartlesville, OK) at 1:5 ratio. Lysate was then purified using RNAeasy column and dissolved in RNAse free water. RNA quality and concentration was determined. Total RNA (2 ug) was reverse-transcribed to cDNA by Clontech Sprint RT Reagent (Clontech, Mountain View, CA) using random hexamer primers with the following cycling conditions: 42°C for 60 min, 70°C for 15 min, 4°C end. qRT-PCR was done using 4 ug of newly synthesized cDNA, TaqMan RT-PCR Mastermix (Applied Biosystems, Foster City, CA), and FAM/TAMRA labeled primers/probes (Applied BioSystems) following standard RT-PCR reaction. Probes and primers specific for mouse were as follows: IL-6 (Mm00446190_m1), CXCL1 (Mm04207460_m1), fibronectin (Mm01256744_m1), TGFB1 (Mm01178820_m1), KIM-1 (Mm00506686_m1), NGAL (Mm01324470_m1), CXCL3 (Mm01701838_m1),

IGF1 (Mm00439560_m1), C3AR1 (Mm02620006_s1), PARP1 (Mm01321084_m1), CD74 (Mm00658576_m1), MMP8 (Mm00439509_m1), MMP9 (Mm00442991_m1) and 18S (Hs99999901_s1). Data were normalized to 18S and expressed as fold change compared to normal.

Morphology

Fixed kidneys were used for all histological and immunohistochemical analysis following paraffin embedding, sectioning (5μm) and deparaffinization, as previously described.[11, 58, 59] To detect fibrosis, sections were stained with picrosirius red.[11]

To identify proliferating cells, sections were washed with 0.05% Tween-20 in PBS, quenched in 10 mM citric acid buffer containing 0.05% Tween-20, pH 6 by boiling for 30 min, washed again and blocked in 10% normal donkey serum. Samples were then incubated with 1 μg/mL rabbit anti-PCNA (proliferating cell nuclear antigen) antibody (Abcam, Cambridge, MA) followed by 25 μg/mL Alexafluor 488 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hr each at RT. Samples were mounted with hard set mounting medium containing DAPI after washing (Vector, Burlingame, CA) and coverslipped.

To detect cell death, TUNEL positive nuclei were detected by an ApopTag Red Apoptosis Determination kit (Millipore, Billerica, MA) according to the manufacturer's recommendations.

To quantify cell proliferation and cell death, four to six random fields within the cortex and outer medulla per animal were photographed with a Nikon Eclipse 80i microscope (Nikon, Melville, NY) at 20X magnification. Metamorph analysis (Molecular Devices, Sunnyvale, CA) was used to quantify PCNA, TUNEL and DAPI positive nuclei in kidney tubules. Data are expressed as mean ± standard error (SEM) of percent PCNA or TUNEL positive cells per total cell number.

Flow cytometric analysis

Single cell preparations of the kidney were made by digesting the tissue in dissociation media (HBSS containing 1 mg/mL collagenase B, 1.2 U/mL dispase, 5 U/mL DNase II) for 45 min at 37ºC with agitation. The digested homogenate was passed through an 18 gauge needle and filtered through a 100 μm cell strainer. After washing with ice cold PBS, cells were centrifuged at 2000 rpm for 5 min. The cell pellet was lysed in BD Pharm Lyse buffer (BD Bioscience, Franklin Lakes, NJ) for 3 min at RT to remove red blood cells. Lysed cells were washed twice with PBS containing 2% fetal bovine serum (FBS), resuspended in PBS and filtered through a 40 μm cell strainer. The number of cells in each kidney homogenate was counted (Vicell, Beckman Coulter, Brea CA) followed by incubation of single cells (1×10^6) with RPMI containing 10% normal mouse serum/0.05% NaAzide, for 10 min at RT to block Fc receptors. Cells were then incubated for 20 min at RT in the dark with an antibody cocktail which included, Alexa Fluor 488 rat anti-mouse CD11b (BD Bioscience) APC rat anti-mouse F4/80 (eBioscience, San Diego, CA) and APC Cy7 rat anti-mouse Ly6C (BD Bioscience), each at a 1:20 dilution. Cells were washed with PBS, 2% FBS followed by incubation with UV-fixable live-dead dye (Invitrogen, Carlsbad, CA) for 30 min at 4ºC in the dark to allow gating only of viable cell populations. After washing, cells were fixed in 1%

methanol-free formaldehyde and analyzed on a BD LSRII flow cytometer (BD Bioscience) using FlowJo 7.6 software (TreeStar, Ashland, OR).

For gating, only live cells were included in the analysis followed by dot plots to identify cell populations of Ly6C vs CD11b and F4.80 vs CD11b. Once the $CD11b^{+}Ly6C^{high,\ int\ and\ low}$ populations were gated, each population was then plotted for CD11b vs F4/80 to determine the expression levels of F4/80 of each individual population. To accurately measure cell number, counting beads (Invitrogen) were used following the manufacturer's recommendation. Data are expressed as total cell number +/- standard deviation.

Microarray analysis

Timepoints for microarray analysis were selected based on when the Ly6C population of interest dominated to optimize mRNA isolation and amplification. Cell populations were sorted on a BD FACSARIA II cell sorter (BD Bioscience) based on $CD11b^{+}$/Ly6C high, intermediate or low into RLT Lysis Buffer containing β-mercaptoethanol for RNA isolation (Qiagen). Total RNA was extracted with RNeasy Micro kit (Qiagen) with on-column DNase I digestion. RNA quality control was tested with Bioanalyzer running RNA Pico 6000. Samples were linearly amplified with NuGen Ovation Pico WTA v2 (NuGen Technologies, San Carlos, CA) to obtain a sufficient quantity of cDNA for downstream microarray application. The amplified cDNA library was further fragmented and labeled with NuGen Encore Biotin Module (NuGen Technologies) then hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 array. Subsequent washing and scanning were performed according to a validated Affymetrix protocol in a CLIA certified service lab.

Raw CEL files were RMA (robust multi-array average) preprocessed in Affymetrix Expression Console and imported into Array Studio v6.1 (OmicSoft, Cary, NC) for further downstream statistical analysis. In short, the expression dataset was filtered to reduce background noise. Probe intensities <5 in log2 scale in at least 67% of any individual group were removed. 30512 (67.7%) probes were kept from 45101. The data was median normalized across filtered probes. Primary separation for Principal Component Analysis was between ischemia and sham kidneys. Inference reports (t-tests) were generated for I/R and sham group separated by population and time course as well as overall I/R vs. sham. Similarly, hierarchical clustering was performed to show differentially expressed genes (DEG) between I/R vs. sham. Statistical significance was set at $p < 0.01$ with no multiple testing correction. DEG lists (FC +/- 1.5, $p<0.01$) from comparative analysis were correlated to public gene expression repository with commercial software NextBio (NextBio, Cupertino, CA). Furthermore, links between genes and cell processes/phenotype were identified with Pathway Studio (Ariadne Genomics).

Statistical analysis

One way ANOVA followed by the Newman-Keuls multiple comparison test and two way ANOVA followed by Sidak's multiple comparison test or t-tests were used when appropriate to determine statistical differences using GraphPad Prism v6.0 software (GraphPad Software, Inc, Lo Jolla, CA). Data are expressed as mean ± standard deviation (SD), unless otherwise indicated.