Supplemental information

Expanded Methods

Administration of Sulfatide

To identify the protective effect of sulfatide-reactive NKT cells, sulfatide (10 μg or 20 μg, Sigma-Aldrich, St. Louis, MO, USA) or PBS (300 μl/mouse) was introduced intraperitoneally (i.p.) three hours before IR surgery.

Isolation and adoptive transfer of hepatic NKT cells

For reconstitution of NKT cells, hepatic mononuclear cells (HMCs) were prepared from B6 and B6.Jα18$^{-/-}$ mice as followings. Mice were injected i.p. with/without 20 μg sulfatide (Sigma-Aldrich). After three hours, mice were perfused with chilled PBS. The liver of the mice was smashed, dispersed in loading buffer (1×PBS plus 10% FBS and 1mM EDTA), passed through a stainless steel mesh, overlaid onto lympholyte M (Cedarlane, Hornby, Ontario, Canada), and centrifuged at 950 g at 25^{o}C for 20 minutes. More than 20% of

isolated HMCs was identified as NKT cells defined by double positivity against NK1.1 and TCR-β. Sorted NKT cells were determined using rat anti-mouse PE-conjugated anti-NK1.1 and PE-Cy5-conjugated anti-TCR-β (BD PharMingen, San Diego, CA, USA). Flow cytometric sorting was performed with FACS Calibur instrument using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA) and the purity of sorted cells was > 98%. Sorted NKT cells were transferred into B6, B6.Jα18$^{-/-}$ and B6.CD1d$^{-/-}$ mice intravenously, 1 x 10^6 in 300 μl of PBS/mouse at 3 hours before renal IRI.

Human kidney evaluation

We collected unstained slides from 10 patients who were diagnosed as biopsy-proven ATN to evaluate whether NKT cells have a beneficial effect for ischemia reperfusion injury in human.

The number of CD3$^+$Vα24$^+$cells was measured by con-focal microscopic examination. The number of NKT cell was counted in five randomly selected fields per biopsy tissue. Sections were evaluated in a blinded fashion.

Histological analysis

For histological analysis, 4 μm thick paraffin sections were stained with periodic acid-Schiff reagent. Tubular injury was with the percentage of necrotic tubules and cast among total tubules by a renal pathologist in a blind fashion.

Con-focal microscopic examination

Confocal microscopy was performed with LSM510 META laser confocal microscope (Carl Zeiss, Jena, Germany). Paraffin sections of the kidney were obtained 24 hours after administration of sulfatide (20 μg/mouse, i.p.). For the immunofluorescence study, paraffin-embedded kidney was cut into 4 μm slices. Xylene and ethanol were used for depraffinization and hydration. Apoptosis was estimated by detecting fragmented chromosomal DNA by TUNEL (Roche Mannheim, Mannheim, Germany) assay according to the manufacturer's instructions. TUNEL-positive nuclei were expressed as a percentage of total nuclei per six fields. Nuclei were visualized using 4'-6-Diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR). In another set of experiments, the sections were stained with rabbit anti-mouse CD3 (BD Biosciences), mouse anti-mouse NK1.1 (Biolegend, San Diego, USA), rabbit anti-mouse HIF-1α, rat anti-mouse F4/80 (Serotec, Raleigh, NC, USA), and mouse anti-human Vα24

(Beckman coulter, Marseille, France) in a blocking reagent overnight at 4^{o}C. A second layer of Alexa Fluor® 488-conjugated goat anti-rabbit antibody, Alexa Fluor® 555-conjugated goat anti-rabbit antibody, Alexa Fluor® 555-conjugated anti-mouse antibody, and Alexa Fluor® 555-conjugated anti-rat antibody (Molecular Probes) were used as secondary antibodies, respectively. All sections were washed and incubated for an additional 5 minutes with DAPI for counterstaining. For negative control, primary antibodies were omitted from sections.

Intra-renal localization of NKT cells

The sorted NKT cells were labeled with a fluorescence Cell Tracker^{TM}, CM-DiI (Molecular Probes), by incubation in Hank's balanced salt solution (HBSS) that contained 3 μg/ml CM-DiI and DAPI for 5 minutes at 37^{o}C, and then for an additional 15 minutes at 4^{o}C. Labeled NKT cells were transferred into B6.Jα18$^{-/-}$ mice or B6.CD1d$^{-/-}$ mice intravenously, 1×10^{6} in 300 μl of PBS/mouse 3 hours before IRI. The kidneys were snap-frozen in OCT embedding medium (Miles Inc., Elkhart, IN), cooled to −80^{o}C and 5 μm thick sections were made with cryostat (Leica, Heidelberger, Germany). Frozen sections were fixed for 10

minutes in cold aceton. Intra-renal localization of NKT cells was measured by con-focal microscopic examination.

Flow cytometry analysis

For quantitative flow cytometry analysis, intra-renal mononuclear cells were isolated from B6 mouse kidney homogenates using Stomacher 80 Biomaster (Seward, Worthing, West Sussex, UK). Single-cell suspensions were created by passing tissue through a 40 μm cell strainer. Kidneys were resuspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and overlaid onto 72% Percoll. After 30 minutes of centrifuge at 1000 g at 25^{o}C, renal mononuclear cells were isolated from the interface. Isolated renal mononuclear cells were incubated with PE-conjugated anti-CD44T (BD PharMingen).

Blood was collected from 5 healthy volunteers, and human peripheral blood mononuclear cells (PBMCs) were isolated directly by using Ficolpaque Plus density gradient centrifugation (Amersham Biosciences, Roosendaal, Netherlands). PBMCs were washed in RPMI1640 medium with 1% fetal bovine serum and 1 mM EDTA. Human NKT cells were identified by surface markers [ie., anti-CD3 (for T lymphocytes, BD PharMingen) and anti-CD56 (for NK cells,

BD PharMingen)]. Stained cells were sorted and analyzed using FACS Calibur instrument (BD Biosciences).

Quantitative real-time PCR

Renal tissues were harvested from mice 24 hours after the induction of IRI. Total RNA was extracted from renal tissues and the cytokine mRNA level was assayed by real-time PCR. Briefly, total RNA was isolated from the kidney using the RNeasy kit (Qiagen GmBH, Hilden, Germany) and 1 μg of total RNA was reverse-transcribed using oligo-d(T) primers and AMV-RT Taq polymerase (Promega, Madison, WI, USA). Real-time PCR was performed using Assay-on-Demend TaqMan probes and primers for TGF-β1, IFN-γ, MCP-1, IL-6, IL-4, IL-10, IL-13, HIF-1α, GLUT-1, VEGF, EPO, and GAPDH (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 7500 Sequence Detection System. Vα14Jα18 TCR primers were synthesized by Applied Biosystems Vα14Jα18 TCR: forward, GTGTGGTGGGCGATAGAGGT, reverse, ACAACCAGCTGAGTCCCAGC, and probe, FAM-CAGCCTTAGGGAGGCTGCATTTTGG-TAMRA. The level of mRNA expression of each cytokine was normalized with respect to the level of GAPDH mRNA

expression.

Western blot analysis

Western immunoblot analysis was performed using primary antibodies against to HIF-1α (Novus Biologicals, Littleton, CO, USA) and β-actin (Sigma-Aldrich, Saint Louis, MO, USA). Briefly, Equal amounts (80 μg) of extracted protein were separated by 10% SDS-polyacrylamide gels and transferred onto Immobilon-FL 0.4 μM polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) were used as secondary antibodies. The blots were developed and enhanced using the Super Signal West Pico Chemiluminescent Substrate (Pierce, Woburn, MA, USA). The intensity of the bands was analyzed by Gel documentation system (Bio-Rad Gel Doc 1000 and Multi-Analyst® version 1.1).

Isolation of renal tubular epithelial cells (TECs)

B6 kidneys were flushed with 1×PBS in vivo to remove red blood cells. Kidneys were cut into pieces and then digested with HBSS containing 3 mg/ml of collagenase (Sigma). The kidney digest was washed through a series of sieves

(120, 75, 40 μm) with 1×PBS. Cortical tubular cells were isolated by centrifugation at 500 g over 5 minutes. The cells were incubated in DMEM/F12 medium. After 4 hours, the non-adherent tubules were collected and cultured on collagen-coated petridishes (BD Biosciences) in medium until epithelial colonies were established.[1] Passages 3 to 4 were used for the study. Primary human TECs (hTECs) were isolated from unaffected specimens from surgically resected kidneys in patients diagnosed as renal cell carcinoma, and kidney cortex was dissected mechanically. The same protocol for isolation of mouse cells was applied.

Hypoxia and re-oxygenation

hTECs were grown to confluence in 24-well chamber slides. Cells were placed in serum-free DMEM/F12 medium for 24 hours, and then washed twice with PBS. Transwells with 0.02 μm pore membranes in 24-well plates (Nunc, Roskilde, Denmark) were used. hTECs (1×10^5/well) were incubated in the lower chamber and sorted human NKT cells [2×10^5/well, added sulfatide (20 μg/ml)] were incubated in the upper chamber at a hypoxic condition (1% O_2, 6 hours). After the hypoxic period, cells were placed under a normoxic condition

(20% O_2) for 18 hours. After total 24 hours, cells were harvested. In another set of experiments, Vα14 CD1d-specific NKT hybridoma DN32.D3 (ATCC) cells (1×10^5/well) were cultured with sulfatide (20 μg/ml), with/without an anti-IL-10 Ab (40 μg/ml, R&D), and recombinant IL-10 (500 ng/ml, R&D) in a hypoxic condition. Goat IgG (40 μg/ml, R&D) was used as a control. Cells were also placed back to a normoxic condition. Thereafter, cells were maintained in medium for 24 hours.

Cell proliferation and measurement of cytokine production assay

TECs (3×10^4/well) were co-cultured with B6 or B6.Jα18$^{-/-}$ hepatic NKT cells (6×10^4/well) by Transwell in 96-well plates. Cells were incubated in hypoxia/reoxygenation condition. After a total of 24 hours in culture, proliferation of TECs was determined using colorimetric MTS assay kits (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. NKT cells were activated by sulfatide (10 or 20 μg/ml) and rIL-10 (500 ng/ml). Supernatants were collected after 24 hours of culture. For blocking assay, TECs were incubated with an anti-IL-10 neutralizing antibody (40 μg/ml, R&D) for 1 hour at 37°C before adding sulfatide. Supernatants were then assayed for

cytokines using a multiplex cytokine bead array system (Bio-Plex; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The same protocol was applied for hTECs.

Quantification of systemic sulfatide concentration

To measure the systemic concentration of sulfatide, we obtained plasma samples 1.5 and 3 hours after the induction of IRI from mice treated with sulfatide (20 μg i.p.). Sulfatide concentrations were quantified using a validated high-performance liquid chromatography/mass spectrometry/mass spectrometry/mass (HPLC/MS/MS) method, as previously described.[2] Briefly, plasma sulfatides was measured with 3-Sulfo-β-D-C18-galactosylceramide and N-C18:0-D3-sulfatide, purchased from Matreya LLC (Pleasant Gap, PA, USA) using a method described elsewhere with minor modification. For extraction of serum lipids, 50 μL of plasma or calibrators were extracted with 100 μL of methanol, 50 μL of KH_2PO_4 and 300 μL of chloroform containing 48 nmol of N-C18:0-D3-sulfatide as an internal standard. After vortexing 5 min, lower chloroform layer containing sulfatides was dried under a gently stream of nitrogen. The lipid extract was then reconstituted in 100 μL of methanol. Ten

microliters of each sample was analyzed in a Waters ACQUITY UPLC® (Waters, MA, USA) using a C18 guard cartridge eluted with 5 mmol/L of ammonium acetate in chloroform-methanol-acetic acid (97:2:0.5 by volume) at a constant flow rate of 70 μL/min. Quantification of sulfatides was performed by multiple-reaction monitoring (MRM) in negative ion mode using the transitions of *m/z* 778>97, *m/z* 794>97, *m/z* 806>97, *m/z* 806>97 (internal standard), *m/z* 822>97, *m/z* 834>97, *m/z* 850>97, *m/z* 862>97, *m/z* 876>97, *m/z* 878>97, *m/z* 888>97, *m/z* 890>97, *m/z* 892>97, *m/z* 904>97 and *m/z* 906>97. A Xevo™ TQ mass spectrometry (Waters) was operated with the following settings; capillary voltage, 3.0 kV; cone voltage, 100 V; collision energy 70 V. Quantification was achieved by relating the peak areas of plasma sulfates to the peak area of the internal standard. The sulfatide concentration was calculated from the summed values of each of molecular species. A six-point external calibration was performed in all experiment with the use of 3-sulfo-β-D-C18-galactosylceramide.

References

1. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, Alexander SI, Sharland AF, Chadban SJ: TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest,* 117**:** 2847-59, 2007.

2. Hsu FF, Turk J: Studies on sulfatides by quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes that include an unusual internal galactose residue loss and the classical charge-remote fragmentation. *J Am Soc Mass Spectrom,* 15**:** 536-46, 2004.

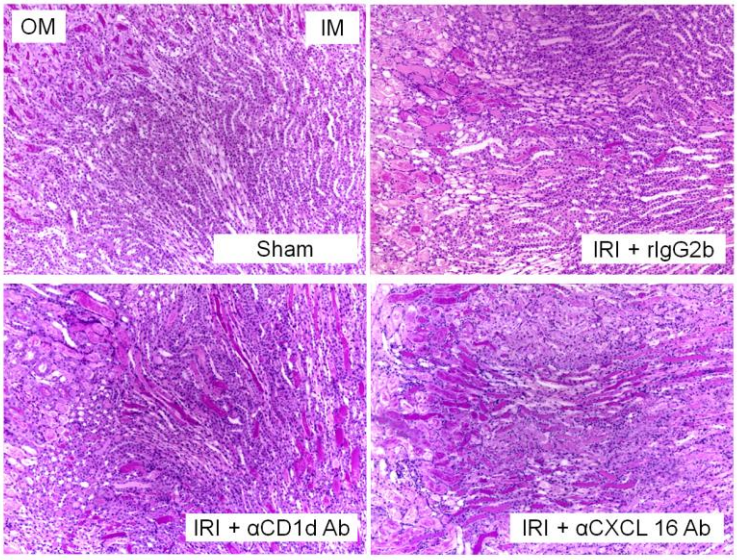
Supplemental figure 1. Blocking of NKT cell recruitment induced severe tubular necrosis B6 mice were injected with anti-CD1d Ab (300 μg/mouse for −1 and 0 days, i.p) or anti-CXCL16 Ab (500 μg/mouse for −1 and 0 days, i.p). NKT cells were activated by sulfatide (20 μg/mouse, i.p) 3 hours before renal IRI. (magnification × 200). Blocking of NKT cell recruitment induced severe tubular necrosis (original magnification × 200). The histological changes were quantified in blinded way by a renal pathologist. IM; Inner Medulla, OM; Outer Medulla

Supplemental figure 2. The changes of various cytokines were traced using culture supernatant in co-culture of human tubular epithelial cells (6×10^4/well) and NKT cells (3×10^4/well). Cells were stimulated with sulfatide (20 μg/ml) for 24 hours. The measurement of IL-8, IL-6, IL-4 and IL-10 productions level by Bio-Plex assay. Samples were triplicated. The changes of various cytokines were traced using culture supernatant in co-culture of human tubular epithelial cells and NKT cells.

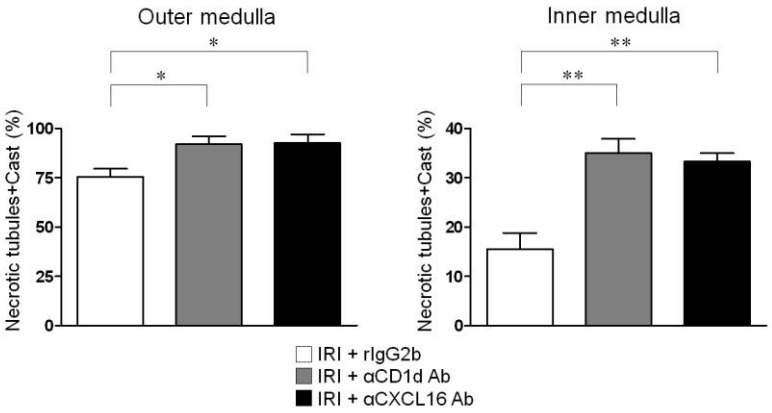
Supplemental figure 3.

Plasma concentration of sulfatide was measured by HPLC/MS/MS. Induction of IRI elevated the plasma concentration of endogenous sulfatide at the early period of IRI, and it was decreased at 3 hours after IRI induction. When we injected exogenous sulfatide, the serum level was maintained as elevated up to 3 hours after IRI. (* $P<0.05$, ** $P<0.01$)

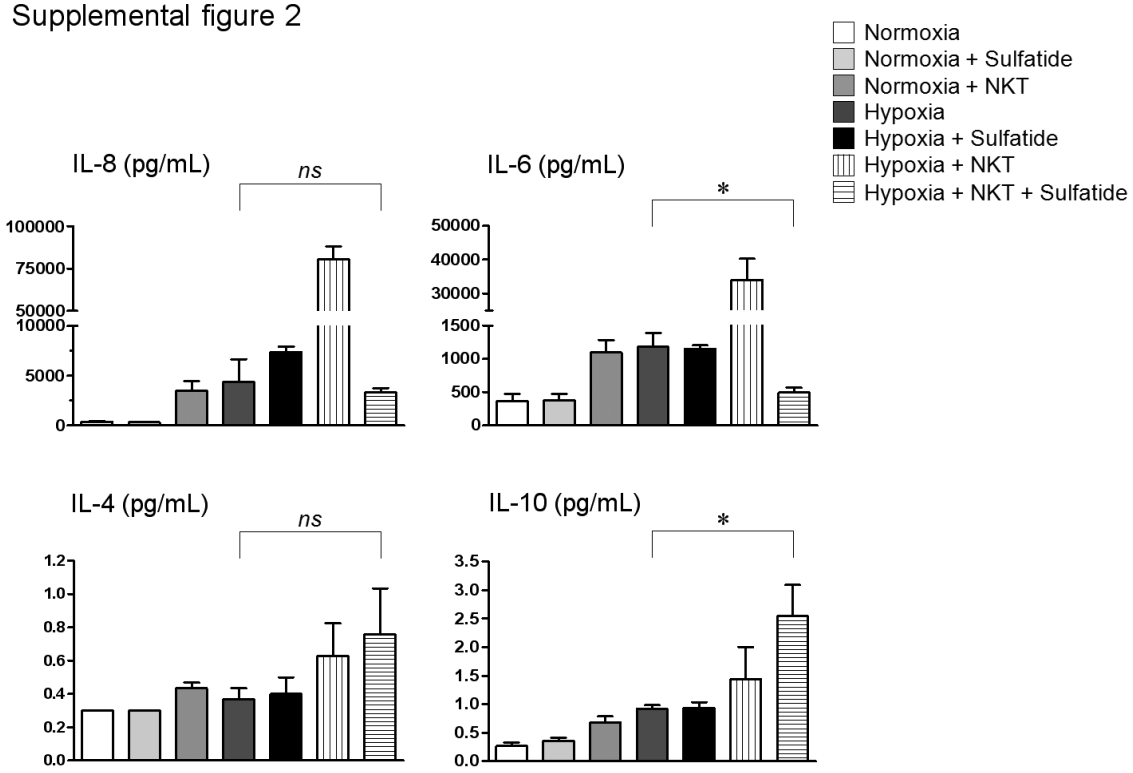
Supplemental figure 1



Blocking of NKT cell recruitment induced severe tubular necrosis
(original magnification x 100) IM; Inner Medulla, OM; Outer Medulla



Supplemental figure 2



The changes of various cytokines were traced using culture supernatant in co-culture of human tubular epithelial cells and NKT cells. Samples were triplicated.

Supplemental figure 3