Hypoxia-Inducible Factor-2α Limits NKT Cell Cytotoxicity in Renal Ischemia/Reperfusion Injury

Supplementary Results

There Was a Constitutive HIF-2α Stabilization in the Thymus, Which Can Be Abrogated by Lck-Cre-mediated HIF-2α Knockout

Genomic PCR analysis indicated efficient recombination of the HIF-2α conditional allele in NKT cells (Supplementary Figure 1). Western blot analysis showed that without any treatment wild-type (WT) thymus had a relatively high expression of HIF-2α, which couldn't be detected in the mutants (Supplementary Figure 2). Measurements of renal function parameters and erythropoiesis revealed no significant difference between HIF-2α$^{-/-}$ mutants and WT littermates without treatment (Supplementary Table 1).

Complete methods

Mice

The Cre/loxp recombination system was used to generate HIF-2α knockout mice, as described previously.[1] Lck-Cre (stock number: 003802), Mx1-Cre (003556) transgene mice, as well as HIF-2α floxed mice (008407) were all from the Jackson Lab (Bar Harbor, Maine USA). After a mating of HIF-2α$^{loxP/loxP}$ with these Cre transgene strains and a second mating of their progeny, mice that were homozygous for the HIF-2α floxed allele and also carried the Cre transgene were generated. Lck-Cre^{+}HIF-2α$^{loxp/loxp}$ mice were referred to as HIF-2α$^{-/-}$ mutants with mutated HIF-2α in T-lineage cells, including NKT cells. Mx1-Cre^{+}HIF-2α$^{loxP/loxP}$ mice that had received 3 injections of an interferon inducer poly deoxyinosinic/deoxycytidylic acid (pIpC, 400μg) were referred to as Mx1-HIF-2α$^{-/-}$ mice. Cre-negative littermates were used as WT controls. Rag1KO mice (002216) were also from the Jackson Lab (Bar Harbor, Maine USA)

Genotyping and the confirmation of target gene excision were as described previously.[2] [3] Briefly, the WT, 2-loxp, and 1-loxp allele (KO allele) were distinguished by a multiplex PCR (P1: 5'-CAGGCAGTATGCCTGGCTAATTCCAGTT-3'; P2: 5'-CTTCTTCCATCATCTGGGATCTGGGACT-3'; P3: 5'-GCTAACACTGTACTGTCTGAAAGAGTAGC-3'). The WT allele produced a 410-bp fragment (P1 and P2), the 2-loxp allele produced a 444-bp fragment (P1 and P2), and the 1-loxp allele produced a 340-bp fragment (P1 and P3). To confirm the successful HIF-2α knockout in different tissues by Cre recombinase, thymus, liver, spleen, lumph node samples, as well as purified NKT cells from Mx1-HIF-2α$^{-/-}$, Lck-HIF-2α$^{-/-}$ and Cre^{-}HIF-2α$^{loxP/loxP}$ mice were subjected to PCR analysis, and a typical result was shown in Supplementary Figure 1.

Male mice, 8-14 weeks of age and weighing 20-28g, were used in the present study. All animal experiments have been conducted according to NIH guide for the care and use of laboratory animals and the institutional guidelines of Shanghai Jiaotong University

School of Medicine. All the procedures described were approved by the Animal Use and Care Committee of Shanghai Jiaotong University School of Medicine (approval number: SYKX-2012-0013). All surgery was performed under sodium pentobarbital anesthesia. Analgesia used was bupivacaine (0.5%), a long acting local analgesic, immediately after surgery and only once. Several drops of bupivacaine were dripped on the suture line after the muscle layer was closed, and before the closure of skin wound. All these efforts were made to minimize suffering.

Renal Ischemia-Reperfusion (IR) and Drug Treatment

The selective adora2a agonist, CGS21680 (0.7mg/kg, from Sigma-aldrich), was administered i.p. at 24 h before renal IR. In some experiments, CGS21680 was given together with lipopolysaccharide (LPS, 3mg/kg, i.p., from E. coli serotype 055:B5, Sigma-aldrich).

A warm renal IR model was used as described previously,[1, 2] with minor modifications. The surgical procedures were carried out by an experienced investigator with no prior information regarding the previous treatments and genetic background of the animals. Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight i.p.) and placed on a temperature-controlled heating table with a rectal thermometer probe attached to a thermal feedback controller (ALC-HTP Homeothermic System, Shanghai Alcott Biotech Co. Ltd, China) to maintain rectal temperature at 36ºC. Following a midline abdominal incision, right nephrectomy was performed. After intraperitoneal injection of heparin (50 U/kg), left renal pedicle was localized and clamped for 20 or 25 min using an atraumatic micro-vascular clamp. After inspection for signs of ischemia, animals were covered with surgical dressing to keep stable intraperitoneal temperature. After removal of the clamp, restoration of blood flow was inspected visually. Mice underwent same surgical procedures but without vascular occlusion, hereafter were referred to as sham controls. Animals were killed 3 h, 6 h, 9 h, 12 h or 24 h after reperfusion by exsanguination, to obtain blood and renal samples for further analyses.

Separate groups of mice were used in the survival experiments and survival was recorded daily. If an animal was considered possibly morbid during the observation period, the condition of the animal was monitored every two hours. The presence of morbid symptoms was determined by an experienced observer with no prior information regarding the treatments and genetic background of the animals. Animals were considered morbid if they were severely immobile, hunched in posture, experiencing severe hypothermia, and/or unresponsive to noise. After signs of morbidity were detected, death was considered unavoidable and the animal was euthanized via exsanguinations under anesthesia. Renal failure was confirmed by macroscopic and microscopic examination. The animals that survived to 7 days after reperfusion were euthanized via exsanguinations under anesthesia, and the successful recovery of renal function was confirmed by serum and histological analyses.

Preparation of Single-Cell Suspensions from Lymphoid Organs, Oxidative Stress Treatment and Flow Cytometry Analysis

After mice were euthanized under anesthesia, spleens, thymuses and livers were removed. Splenocytes and thymocytes were prepared by running specific gentleMACS programs on a gentleMACS dissociator (Miltenyi Biotec Inc, Bergisch Gladbach, Germany), according to the manufacturer's optimized protocols. Liver MNCs were prepared by using the gentleMACS dissociator according to the manufacturer's protocol and a previous report,[4] with modifications. Briefly, the liver was perfused in situ through the portal vein with room temperature PBS. Then the liver was cut into small pieces in prewarmed dissociation mix solution (Krebs-Ringer-Buffer solution supplemented with $CaCl_2$, $MgCl_2$, Collagenase IV and DNase I). The liver was transferred into the C Tube, which was attached upside-down onto the sleeve of the gentleMACS dissociator and run the gentleMACS program m_liver_01.02. The sample was incubated for 30 min at 37°C under slow continuous rotation using the MACSmix Tube Rotator. Then run the gentleMACS Program m_liver_02.02. The liver sample was pressed gently through a 70μm cell strainer (BD Falcon, #352350), and suspended in 40 ml of cold PBS/FBS/Az solution. The pellet was then resuspended in a 37.5% isotonic Percoll solution and centrifuged at 680–700g for 12 min at room temperature. The cells of interest would form a pellet at the bottom of the solution. The supernatant was discarded and the pellet containing RBC and lymphocytes was washed in cold PBS/FBS/Az solution. After the RBCs were lysed, the cells were resuspended in 5 ml PBS/FBS/Az and filtered through 70um cell strainer again to remove the debris. Finally, the mononuclear cells (MNCs) were resuspended in PBS/FBS/Az solution, tissue culture medium, or staining buffer, according to the downstream experiment. When purified from an untreated mouse, an average of $4–5\times10^6$ MNCs (60–70% of them being lymphocytes) can be recovered with this method.

The single-cell suspensions were incubated with anti-mouse CD16/CD32 blocking antibody prior to staining. Then samples were labeled using combinations of the following antibodies: anti-NK1.1 APC (17-5941, eBioscience), anti-TCR-β FITC (11-5961, eBioscience), anti-FasL PE(12-5911, eBioscience) or anti-adora2a PE (sc-32261, Santa Cruz). Because the adora2a antibody was against the adora2a protein epitope mapping to the intracellular loop, it was added to the samples after the fixation and permeabilization step by using the intracellular fixation and permeabilization buffer set (88-8824, eBioscience) according to the manufacturer's protocol. Immunofluorescence staining was analyzed using a FACSCalibur instrument (BD Biosciences). The lymphocytes were gated using forward and side scatter to exclude debris and dead cells, then 30,000 events were acquired in each assay for analysis.

In some experiments, CGS21680 (10μmol/L) or DMSO (as vehicle) were added to the culture medium of suspended thymocytes at 1h before H_2O_2 (25μmol/L) was added. 4 hours later, the cells were labeled with the antibodies (NK1.1, TCR-β and FasL) and subjected to FACS analysis.

Isolation of the Infiltrating Inflammatory Cells from the Ischemic Kidneys

Inflammatory Cells from the ischemic mouse kidneys were isolated by using the gentleMACS Dissociator (Miltenyi Biotec), according to the protocol described by the manufacturer. In brief, HIF-$2\alpha^{-/-}$ mice and their WT littermates were subjected to

bilateral renal ischemia (20 min), followed by 3 h of reperfusion. Then both kidneys were harvested and capsule was removed. After the kidneys were transferred into the digest solution in the gentleMACS C Tube, which was attached to the gentleMACS Dissociator, we ran certain programs (m_lung_01 and m_spleen_04) according to the instructions, to dissociate the kidneys. Then a cell strainer (70 μm mesh size) was applied to remove the tissue debris. The obtained cells were counted and magnetically labeled with CD45 microbeads (Miltenyi Biotec, 130-052-301). The unwanted cells were subsequently depleted by separation over a MACS Column, which was placed in the magnetic field of the MACS Separator. After removal of the column from the magnetic field, the magnetically retained $CD45^+$ cells were eluted and subjected to FACS analysis as described above.

NKT Cell Purification from Splenocytes and Adoptive Transfer

NKT Cell isolation was performed in a two-step procedure from mouse spleens using a commercially available $NK1.1^+$ iNKT Cell Isolation Kit (130-096-513, Miltenyi Biotec Inc, Bergisch Gladbach, Germany), according to the manufacturer's protocols. Briefly, HIF-2$\alpha^{-/-}$ mice and their WT littermates were euthanized under anesthesia and spleens were harvested. Splenocytes were passed through a 40-mm nylon cell strainer (BD Biosciences) and collected in phosphate buffered saline. Red blood cells were lysed, and the non-$NK1.1^+$ iNKT cells are labeled with a cocktail of biotin-conjugated antibodies, anti-Biotin MicroBeads. The labeled unwanted cells, including NK cells, B cells, macrophages, CD8+, and $TCR\gamma\delta^+$ T cells were subsequently depleted by separation over a MACS Column, which was placed in the magnetic field of the MACS Separator. Then the pre-enriched $NK1.1^+$ iNKT cells were labeled with anti-NK1.1-APC and anti-APC microbeads and then positively selected by magnetic separation to produce cell populations of ≥90% $NK1.1^+$ iNKT Cells (Supplementary Figure 3). About 3×10^5 NKT cells (from 4-6 mice) were adoptively transferred into a Rag1KO mouse via tail vein injection at 4 d before renal IRI. Successful reconstitution was confirmed by FACS analysis of splenocytes collected after 24 h of reperfusion. Control animals received vehicle injections which didn't contain cells.

Measurement of Intracellular Cyclic AMP (cAMP) Accumulation

Splenocytes, liver MNCs or thymocytes were treated with 50μM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, USA) for 30 min at 4°C, followed by culture in 37°C incubator. Then CGS21680 (10μmol/L) or the equivalent amount of vehicle (DMSO) was added to the culture medium. At 60 min after the treatment, the culture medium was removed and 1N HCl was added to stop endogenous phosphodiesterase activity and achieve adequate cell lysis. Then the mixture were boiled for 5 minutes and centrifuged at 10000 rpm for 5 minutes at 4°C. The supernatant was collected and the cAMP level was determined by using a Monoclonal Anti-cAMP Antibody-Based Direct cAMP ELISA Kit (NewEast Biosciences Inc., Malvern, USA).

Histology and Histomorphological Scoring of Acute Tubular Injury

Kidney tissues were fixed in 10% neutral buffered formalin overnight, dehydrated, embedded in paraffin and sectioned at 3 μm. For histological analysis, sections were stained with Periodic Acid-Schiff (PAS). Samples were analyzed for tubular cell necrosis, tubular dilation, intratubular cell detachment, and cast formation (original magnification ×200) and were all evaluated in a blinded manner by a nephropathologist. Abnormalities were graded by a semiquantitative histomorphological scoring system from 0 to 4, as described previously.[1, 2] At least 3 fields per section were evaluated.

Polymorphonuclear Leukocyte Infiltration (MPO Activity)

Renal sections were processed for immunohistochemical localization of myeloperoxidase (MPO, polyclonal rabbit antibody; Novus Biologicals, NBP1-42591), and were then visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. Polymorphonuclear leukocyte (PMN) infiltration was scored semiquantitatively on a scale of 1 (none) to 4 (severe), as described previously.[1, 2]

Terminal Deoxynucleotidyl Transferase-Mediated 2'-Deoxyuridine 5'-Triphosphate Nick-End Labeling Assay (TUNEL)

Apoptotic cells in formalin-fixed, paraffin-embedded kidney tissue sections were identified with ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Chemicon International), according to the manufacturer's protocol. Cells with nuclear positive staining by fluorescent antibodies for DNA fragmentation were visualized directly by a fluorescence microscopy and counted (original magnification ×200). At least 3 fields per section were examined.

Western Blot Analyses

Western blot analysis of HIF-1α/HIF-2α was performed as described previously.[1, 2] Nuclear extracts were isolated from harvested thymuses and spleens using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Inc., USA.), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Indiana, USA). Protein samples were separated on 10% SDS-PAGE gels and then transferred to a nitrocellulose membrane (Whatman) by standard procedures. The membranes were then incubated with rabbit polyclonal primary antibodies against HIF-1α (1:500, NB100-134, Novus Biologicals), HIF-2α (1:500, ab199, Abcam) or a loading control TATA binding protein (TBP, 1:2000, ab818, Abcam), and then with secondary antibodies (1:10000; LI-COR Biosciences). Signals were visualized and detected using an Odyssey infrared imaging system (LI-COR Biosciences). Samples were corrected for background and quantified using Odyssey software. All values were normalized to the loading control and expressed as fold increase relative to control.

For adora2a and FasL measurements, harvested kidneys, spleens and thymuses were homogenized and lysed with cell lysis buffer, which contained 1 protease inhibitor cocktail tablet per 10 mL of Lysis Reagents (Complete; Roche, Indianapolis, IN). Solutions were then clarified by centrifugation (25 min at 16,000g). Solubilized proteins were then resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Whatman). After blocked with LI-COR blocking buffer, blots were

incubated with adora2a (1:500, sc-13937, Santa Cruz), FasL (1:200, ab15285, Abcam) and anti-β-actin (1:2000, Santa cruz) antibodies. After incubation with secondary antibodies, blots were developed as described above.

Electrophoretic Mobility Shift Assay (EMSA)

Protein-DNA interaction was detected by using an Odyssey Infrared EMSA Kit (LI-COR, Lincoln, NE), according to the manufacturer's protocols. Briefly, nuclear extracts were isolated from harvested thymus using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Product Number 78833, Pierce Biotechnology, Inc., USA.), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Indiana, USA). For EMSA, 10μg of nuclear protein were assembled with 5×Gel Shift Binding buffer (20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl), 0.25 mg/ml poly(dI)-poly(dC), and 5' DyLight 680-labeled oligonucleotide from mouse adora2a HRE (Takara, Dalian, China), which was located in the promoter sequence of mouse adora2a (about 34 bp upstream of exon 1).[5] The sense sequence was GGACGCGTGGACCTGAAGCGCCCACGTTGGGG. Both sense and antisense DNA oligonucleotides were labeled with DyLight 680 at 5' end and annealed to form a double-stranded DNA fragment by placing the oligonucleotide set in a 100°C heat block for 5 minutes. After incubation at room temperature for 30 minutes, the samples were loaded on a pre-run 8% polyacrylamide gel and electrophoresis was continued at 30 mA for 90 min. The signal was then detected and quantified with Odyssey infrared imaging system (LI-COR). Supershift assays using HIF-1α or HIF-2α antibodies were also conducted to confirm the specificity of HIF/DNA-binding activity. For loading control, 10μg of nuclear proteins from each sample were subjected to western blot analysis, which showed equal loading.

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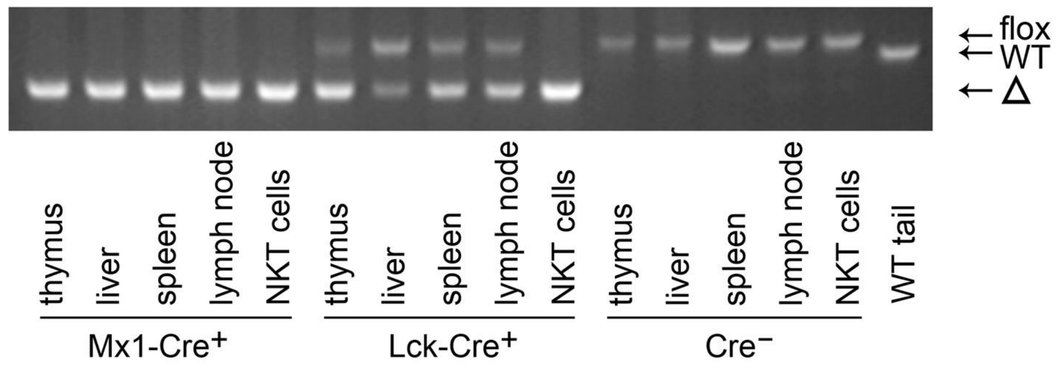
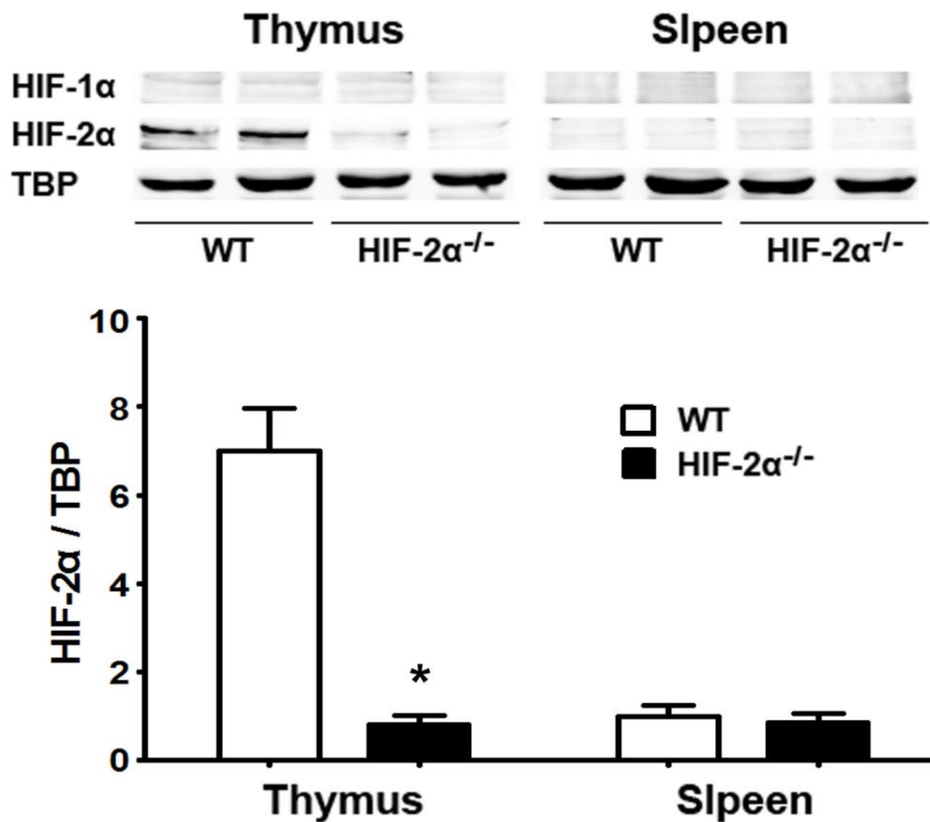
Supplementary Table 1. Analysis of physiologic parameters in 10-week-old male HIF-2α$^{-/-}$ mice and the Cre^{-} (WT) littermates.

Parameter	Cre^{-}	Lck-HIF-2α$^{-/-}$	Mx1-HIF-2α$^{-/-}$	P value
Body weight (g)	24.7±1.6	25.1±1.7	24.1±2.1	>0.05
Kidney weight (mg)	192±14.3	197±13.6	195±10.6	>0.05
Hgb (g/L)	136±9.8	133±8.8	138±10.8	>0.05
WBC (10^9/L)	4.04±0.8	4.12±0.6	4.32±1.1	>0.05
PLT (10^9/L)	535±86	516±92	576±104	>0.05
BUN (mmol/L)	11.6±0.9	10.8±1.0	12.1±1.2	>0.05
Cr (μmol/L)	32±5.6	31±6.7	35±6.7	>0.05

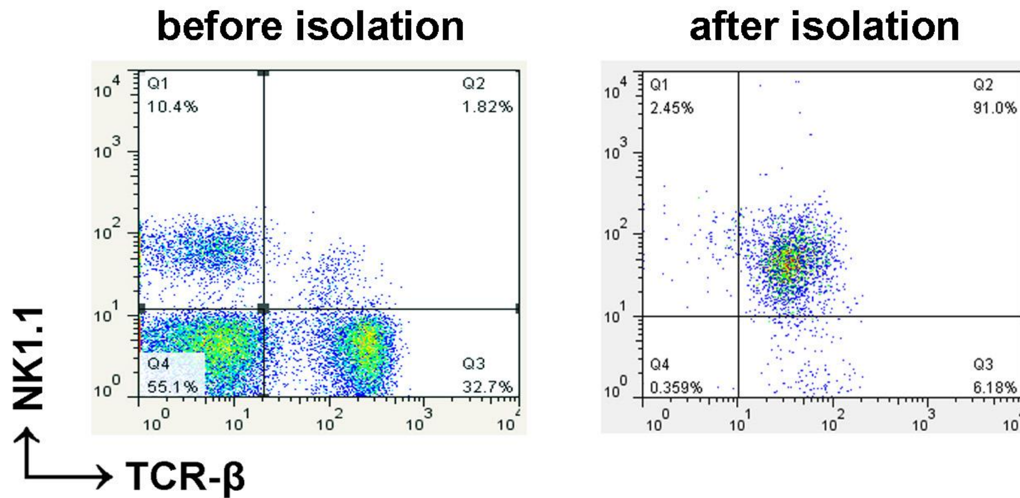
Values represent means and SD of 4 mice of each group.



Supplementary Figure 1. Confirmation of HIF-2α knockout by polymerase chain reaction (PCR). Thymus, liver, spleen, lumph node samples, as well as purified NKT cells from Mx1-Cre^{+}HIF-2α$^{loxP/loxP}$, Lck-Cre^{+}HIF-2α$^{loxp/loxp}$ and Cre^{-}HIF-2α$^{loxP/loxP}$ mice were subjected to a multiplex PCR, which produced a 410-bp fragment in WT allele, a 444-bp fragment in the 2-loxP allele and a 340-bp fragment in the 1-loxP allele (Δ). DNA from the tail of a wild-type mouse was used to show where wild-type HIF-2α was (WT, 410bp).



Supplementary Figure 2. (A) HIF-1α/HIF-2α expression in the thymuses and spleens of HIF-2α$^{-/-}$ mice and WT littermates. The mice received no previous treatments before the thymuses and spleens were harvested. Nuclear HIF-1α/HIF-2α expressions were evaluated by western blot analysis and co-detection of TBP was performed to assess equal loading (n = 4 for each group). No stabilization of HIF-1α was observed in the thymuses or spleens. HIF-2α protein bands were quantified and normalized to TBP. Data were expressed as means ± SD. *, $P < 0.05$ versus WT thymuses.



Supplementary Figure 3. Purification of NKT cells from splenocytes. After the single-cell suspensions from spleens were prepared and before the purification procedures, the cells were subjected to FACS analysis and a typical result was shown on the left. Then NKT cell isolation was performed in a two-step procedure from splenocytes using a commercially available NK1.1^{+} iNKT Cell Isolation Kit. A typical post-isolation result was shown on the right. Typically this method produced cell populations of >90% NKT cells from splenocytes.