Supplemental Experimental procedures

Cre-inducible human CD59 mediates rapid cell ablation after intermedilysin administration

Animals

Animal studies were approved by the Animal Care and Use Committee from the National Institute on Alcohol Abuse and Alcoholism and from Temple University School of Medicine institutional

Generation of transgenic *ihCD59* construct and *ihCD59*$^+$ mice

LoxP-Stop-loxP (LSL)-hCD59 knock-in (*ihCD59*) mice were generated by TARGATT^{TM} service provided by Applied StemCell Inc. Briefly, a chicken beta-actin promoter with a CMV enhancer (CAG promoter) and loxP-Stop-loxP cassette were inserted upstream of the open reading frame of *hCD59* into the pBT378-CAG-LSL plasmid (LSL: loxP-Stop-loxP). NIH 3T3 cells were co-transfected with pBT378-CAG-LSL-*hCD59* vector and Cre expression vector (Cre+) or pBT378-CAG-LSL-*hCD59* vector alone (Cre-), *hCD59* expression was examined by live staining. A mixture of the confirmed construct and in vitro transcribed mRNA was microinjected into the pronucleus of each of 80 zygote in FVB genetic background using TARGATT^{TM} Technology and the injected zygotes were implanted into four CD1 foster mice. Upon recombination between the attB sites on the TARGATT^{TM} vector and attP sites on the chromosome, the transgene, CAG-LSL-*hCD59*, was integrated at the H11 locus (1). Twenty-three mice were born from the microinjection. Successful integration from the found mice was identified by PCR analyses of genomic DNA using a panel of primers. After screening, one female and one male positive founders (*ihCD59* mice) were identified.

Generation of *Cre*$^+$*ihCD59*$^+$ mice

The *ihCD59*$^+$ mice in FVB/NJ background were crossed with *Lck-Cre*$^+$ [Jacksons labs (JAX), stock number: 003802)], *CD4-Cre*$^+$ (JAX stock number: 017336), *CD11c-Cre*$^+$ (JAX stock number: 008068), *Alb-Cre*$^+$ (JAX stock number: 003574), *Lyz-Cre*$^+$ (JAX

stock number: 004781), *Sox9-Cre^{ERT+}* (JAX stock number: 018829) or *mGFAP-Cre^{+}* (JAX stock number: 024098) mice to generate the *Cre^{+}ihCD59^{+}* double transgenic mice. Resulting *ihCD59* offspring mice: *Cre^{+}ihCD59^{+}* and *Cre^{-}ihCD5^{+}* mice were used as experimental and control mice, respectively.

ILY purification. His-tagged recombinant ILY was purified as described previously (2). The concentration and purity of ILY was determined by SDS-PAGE.

Administration of Ad-*AlbCre* virus. *ihCD59^{+}* mice (8-10 weeks) were infected with Alb-Cre or GFP adenoviruses (1-3x10^{11} viral particles per mouse) via tail vein injection. Alb-Cre adenoviruses were kindly provided by Dr. Liangyou Rui (University of Michigan, Ann Arbor, MI) and described previously (3).

Tamoxifen treatment of *Sox9Cre^{ERT+}ihCD59^{+}* mice. *Sox9Cre^{ERT+}ihCD59^{+}* mice were treated with tamoxifen to induce hCD59 expression in BECs. Tamoxinfen was dissolved in corn oil. The mice with treated with tamoxinfen (50mg/kg) every two days for 3 times.

Con A induced liver injury and ILY treatment: Mice received ILY or PBS by i.v injection, 3 h later, Con A was administrated at a dose of 12 mg/kg by i.v. injection. Serum was obtained 24 h after ConA injection for ALT and AST measurement (4). Liver tissue was fixed in formalin for histological analysis.

EAE induction, clinical score evaluation and ILY treatment. The mice were induced by subcutaneous injection of 200 µl of emulsion containing 200 µg of 35-55 myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant with 200 µg of H37Ra Mycobacterium tuberculosis. Bordetella pertussis toxin (50 ng) was injected intraperitoneally (i.p.) on the same day after MOG peptide injection and 48 hrs thereafter. Following immunization, animals were evaluated for clinical EAE scores with the following criteria: 0, no detectable sign of EAE; 1, weakness of the tail; 2, definite tail paralysis and hind limb weakness; 3, partial paralysis of the hind limbs; 4, complete paralysis of the hind limbs; 5, complete paralysis of the hind limbs with incontinence and partial or complete paralysis of forelimbs. Three days after immunization, all the mice in each group received systemic delivery of ILY (100 ng/g/day) via daily subcutaneous injections for 14 consecutive days (until 17 days after immunization).

Biochemical analysis. Serum ALT, AST and total bilirubin levels were determined using an IDEXX chemistry analyzer system (IDEXX Laboratories, Westbrook, ME). White blood cell count, the biochemical test in the serum including blood urea nitrogen (BUN), creatinine (CREA), alkaline phosphatase (AP), ALT, AST, Amylase, creatine kinase (CK) and lactate dehydrogenase (LD) levels in serum were determined by biochemistry analyzer (Department of Laboratory Medicine, NIH).

Brain injury model and stereotaxic injection of ILY

After mice were anesthetized and mounted on a stereotaxic frame, their skulls were exposed by a midline longitudinal skin incision. Based on bregma, two bilaterally symmetrical holes (a 1-1.5 mm diameter, A/P 0.5, M/L ±1.5, D/V -3.5 from the skull) were made by using an electric drill. Cortical injury was made with a 26G needle attached to the controlled cortical impact device by 3 time insertions of the needle through the hole to depth of 3.5 mm. Then, total 4 μl of ILY (10 ng/g) or PBS (vehicle control) were locally injected into the injury sites, separately. One, two, and one μl of ILY or PBS were respectively administered at the depths of 3.0 mm, 2.4 mm, and 1.8 mm while gradually pulling the needle out as shown in the Figure 7A. At the day 5, ILY or PBS were repetitively administered to the mouse with the same injection procedure as in day 1. At day 7, the mice was sacrificed for harvesting the tissue.

Spinal Cord Injury (SCI) model and local injection of ILY.

The spinal cord injury was performed in a dorsal over-hemisection at 0.8 mm at T7 (~1.5 mm in dorsoventral diameter) with a 30-gauge needle and microscissors to completely sever dorsal part of the spinal cord as reported previously (5) (6). The lesion depth of 1 mm was ensured by passing a marked 30-gauge of needle 4 times across the dorsal spinal cord. Immediately after injury and at day 4 after the injury, the mice received local ILY by applying a gelfoam pledget soaked in a solution of ILY (20 ng/ml) to the transection site. Mice were perfused 7 days after SCI and the spinal cord around the lesion was processed GFAP staining. Particularly, the spinal cord extending from 0-4 mm rostral to and caudal to the lesion (8 mm long, containing the injury site) was cut

parasagittally (30 mm). All the parasagittal sections were immunostained with a mouse GFAP antibody (1:400) and an Alexa488-conjugated secondary antibody.

Tissue fixation, brain sectioning & immunohistochemistry

Mice were perfused with PBS followed by 4% paraformaldehyde fixation overnight. Then the brain was transferred and immersed in 30% sucrose for two days. Formalin fixed liver samples were processed and paraffin section of 5 μm thickness were stained with hematoxylin and eosin (H&E) for histological analysis. The coronal sections (40 μm) were used to co-stain with antibodies specific against the human CD59 (PE-conjugated mouse anti-human CD59, eBionscience, Clone OV9A2) and GFAP (rabbit anti-GFAP (Dako) followed by staining with Alexa 488-conjugated donkey anti-rabbit immunoglobulin (Invitrogen Life Technologies) as a secondary antibody for GFAP detection. The tile scans of the staining of the serial sections were taken under the LEICA de-convolution microscope (Leica Microsystems Inc, Buffalo Grove, IL).

BrdU incorporation assay. Mice received 50 mg/kg BrdU (Sigma) in PBS by i.p. injection. 2 hours later, mice were sacrificed and tissues were fixed in formalin. BrdU staining was determined by immunostaining with an anti-BrdU antibody.

ELISA: The serum levels of IFN-γ and IL-4 were measured by using a Quantikine immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Immunohistochemical staining in the liver. For detection of hCD59, paraffin-embedded sections were stained with hCD59 antibody (Sigma) after heat-induced epitope retrieval and visualized by DAB. For detection of α-SMA, paraffin-embedded sections were stained with α-SMA antibody (DAKO) and visualized by DAB. For detection of CK19, paraffin-embedded sections were stained with CK19 antibody (abcam) after 20 min 20μg/ml proteinase K treatment and visualized by DAB.

Immunofluorescence staining: Tissues were fixed in 4% PFA, embedded in O.C.T compound and sectioned (10 μm). The sections were staining with anti-F4/80 (AbD Serotec), anti-BrdU (BD bioseciences), PE-anti-hCD59 (eBiosciences), CK19 (abcam), Sox9 (Sigma) and anti-α-SMA (DAKO) antibodies. Fluorescent labeled secondary

antibodies (Cell Signaling Technology) were used to visualize the primary antibodies. The images were obtained by using LSM 710 confocal microscope (Zeiss, Thornwood, NY).

Reference:

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2. Giddings KS, Zhao J, Sims PJ, and Tweten RK. Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. *Nat Struct Mol Biol.* 2004;11(12):1173-8.
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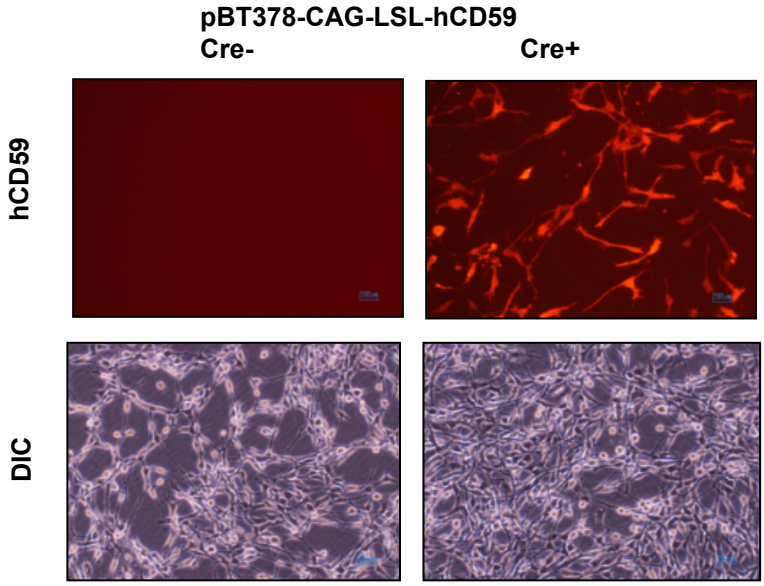
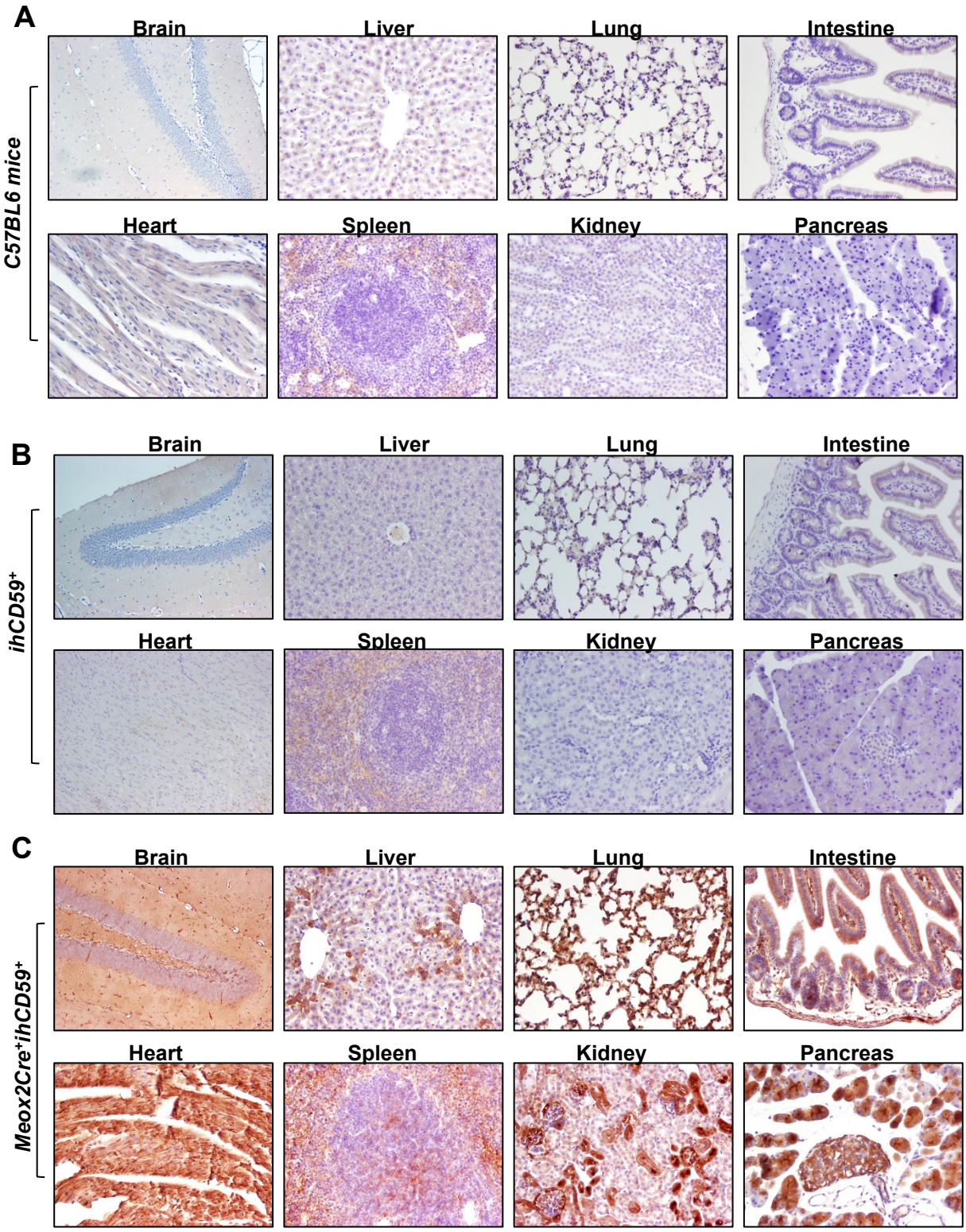
4. Hong F, Jaruga B, Kim WH, Radaeva S, El-Assal ON, Tian Z, Nguyen VA, and Gao B. Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J Clin Invest.* 2002;110(10):1503-13.
5. Fisher D, Xing B, Dill J, Li H, Hoang HH, Zhao Z, Yang XL, Bachoo R, Cannon S, Longo FM, et al. Leukocyte Common Antigen-Related Phosphatase Is a Functional Receptor for Chondroitin Sulfate Proteoglycan Axon Growth Inhibitors. *J Neurosci.* 2011;31(40):14051-66.
6. Ohtake Y, Park D, Abdul-Muneer PM, Li H, Xu B, Sharma K, Smith GM, Selzer ME, and Li S. The effect of systemic PTEN antagonist peptides on axon growth and functional recovery after spinal cord injury. *Biomaterials.* 2014;35(16):4610-26.

Feng et al. Supplemental Figure 1



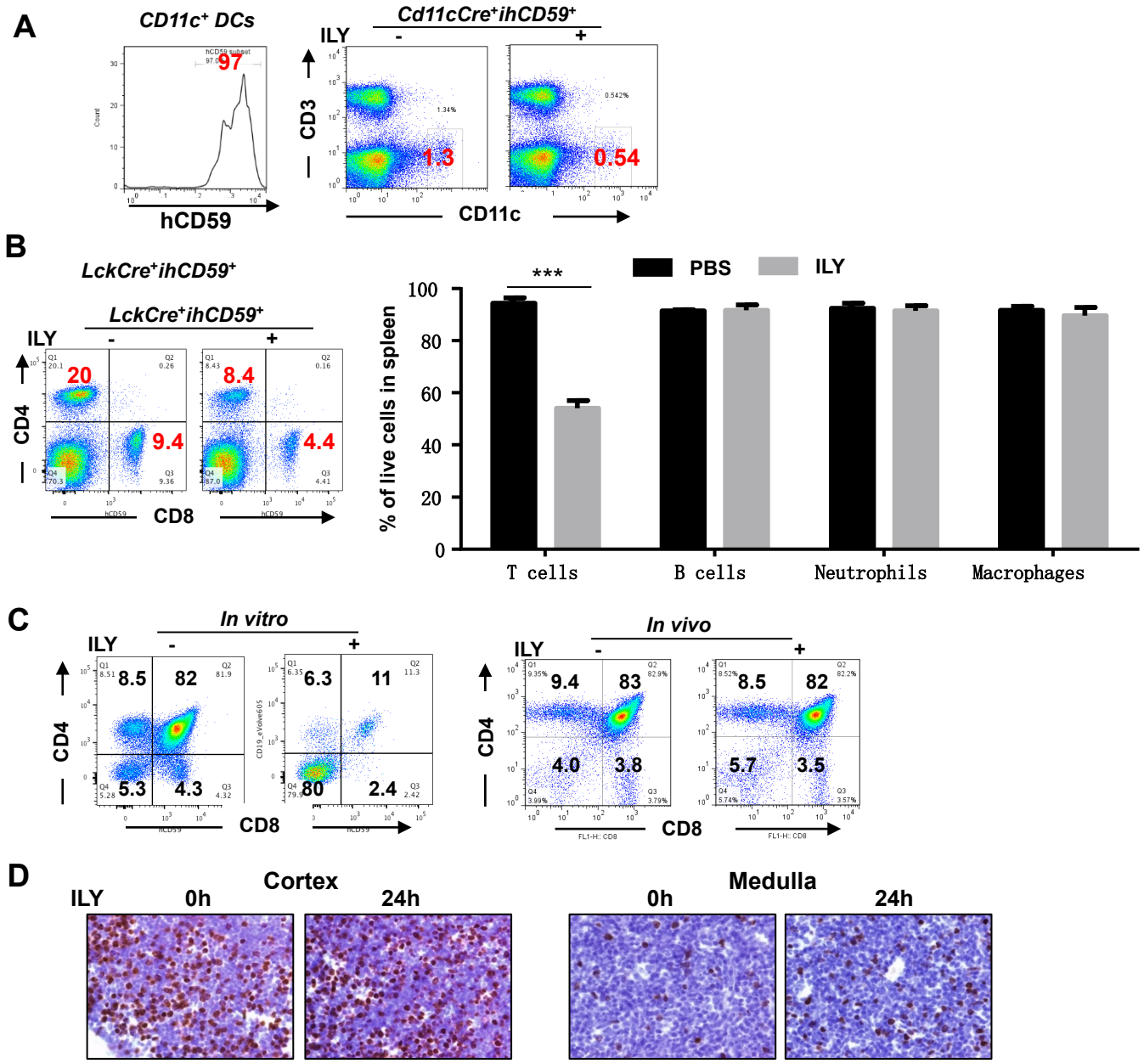
Supplemental Figure 1. Validation of Cre induced hCD59 expression *in vitro*. NIH 3T3 cells were co-transfected with pBT378-CAG-LSL-CD59 vector and Cre expression vector (Cre+) or pBT378-CAG-LSL-CD59 vector alone (Cre-), hCD59 expression was examined by immunofluorescence staining (Representative images from 3 independent experiments). Magnification: X200. DIC: Differential interference contrast.

Feng et al. Supplemental Figure 2



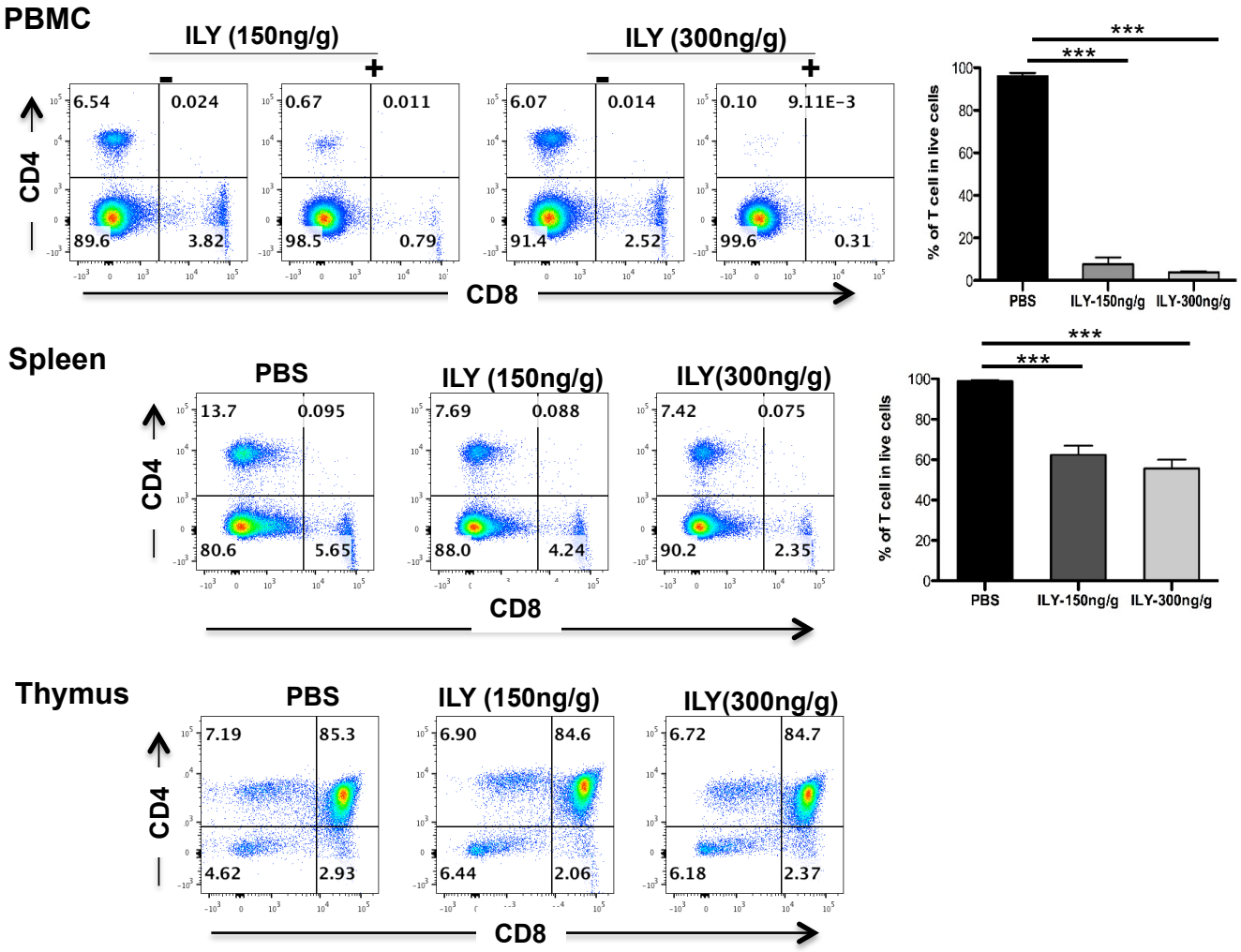
Supplemental Figure 2. hCD59 expression on C57BL6, *ihCD59*$^+$ and *Meox2Cre*$^+$*ihCD59*$^+$ mice. Various organs from negative control C57BL/6 mice (A), Cre negative *ihCD59*$^+$ mice (B) and *Meox2Cre*$^+$*ihCD59*$^+$ mice (C) were obtained for hCD59 immunohistochemical staining. (Representative images from 3 animals in each group). Magnification: Brain: X100, all other organs: X200.

Feng et al. Supplemental Figure 3



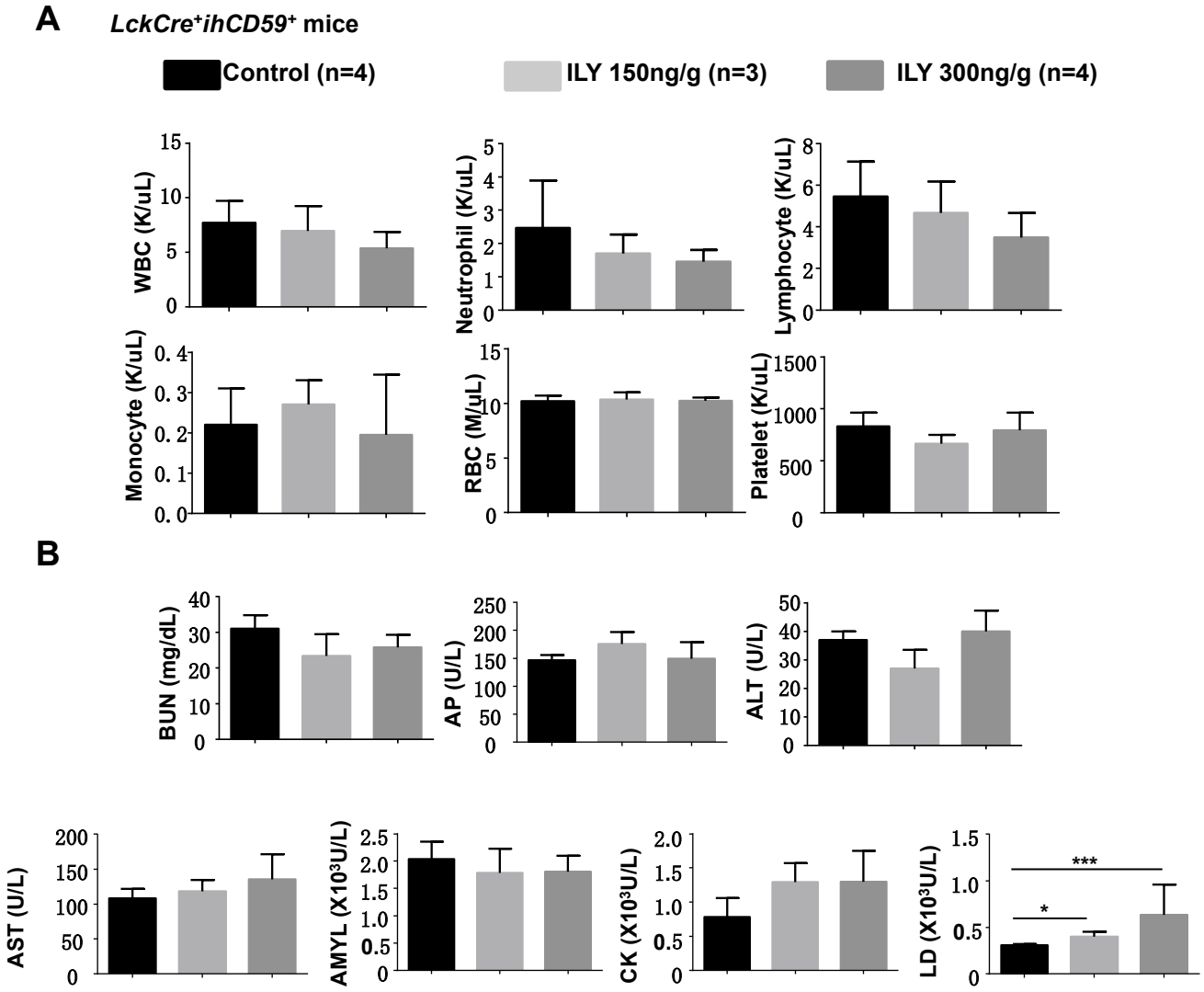
Supplemental Figure 3. Injection of ILY deletes T cells and DCs in *Cre⁺ihCD59⁺* mice. (A) Left panel shows 97% splenic CD11c⁺DCs express hCD59 protein from *Cd11cCre⁺ihCD59⁺* mice. Right panel shows that injection of ILY deletes CD11c⁺ DCs in these mice (Representative graphs from 4 animals). (B) Injection of ILY specifically deletes T cells without affecting other cells in the spleens of *LckCre⁺ihCD59⁺* mice (The values represent the means ± SD, n=6, ***P ≤ 0.001, as determined by 2-tailed Student's *t* test). (C) Treatment of ILY kills T cells in thymus of *LckCre⁺ihCD59⁺ in vitro* but does not affect them *in vivo* (Representative graphs from 4 aniamls). (D) Injection of ILY promotes cell proliferation in the medulla of thymus in *LckCre⁺ihCD59⁺* mice. The mice received a single injection of ILY (200ng/g) for 24h. BrdU (50 mg/kg) was given 2h before euthanization. Immunohistochemical staining with anti-BrdU antibody in thymus. BrdU⁺ cells appear as brown nuclei (Representative images from 4 animals). Magnification: X200.

Feng et al. Supplemental Figure 4



Supplemental Figure 4. Saturated doses of ILY ablate more than 95% T cells in peripheral blood but only partially abate T cells in the spleen and do not effect T cells in the thymus. *LckCre*$^{+}$*ihCD59*$^{+}$ mice were injected with saturated doses of ILY (150 and 300ng/g, i.v. n=3). Circulating T cells, splenic and thymic T cells were analyzed 2 h post injection.

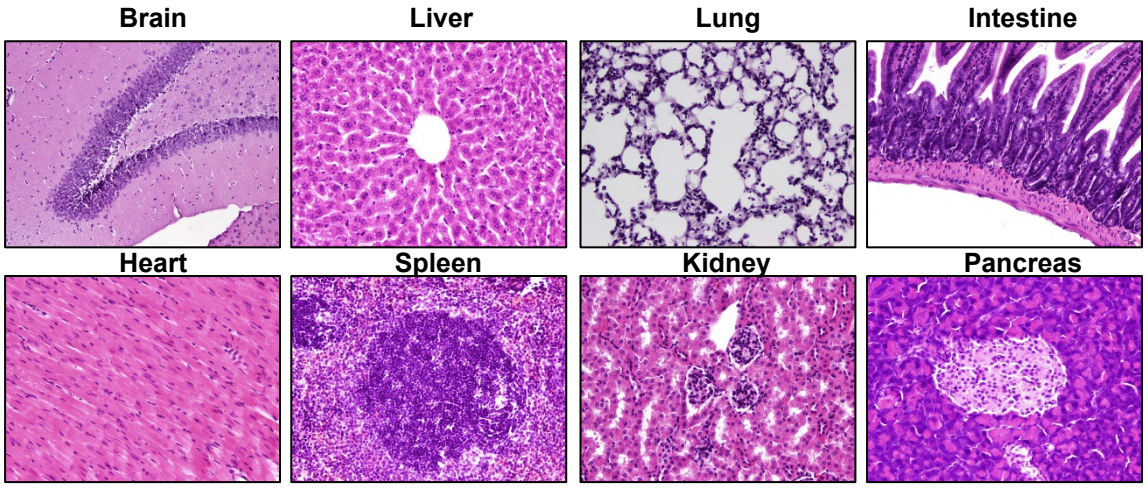
Feng et al. Supplemental Figure 5A-B



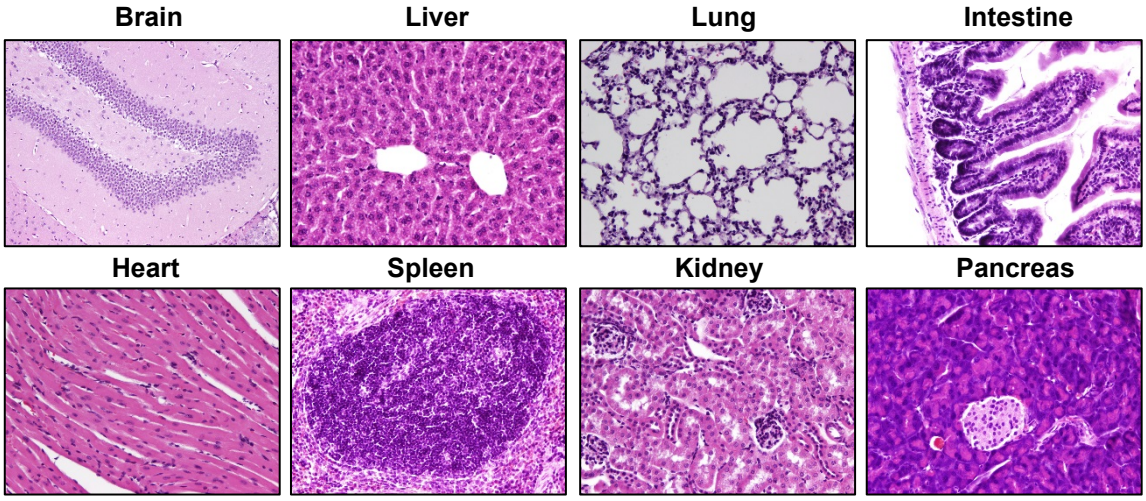
Feng et al. Supplemental Figure 5C

C

***LckCre*$^{+}$*ihCD59*$^{+}$ mice with 150ng/g ILY**

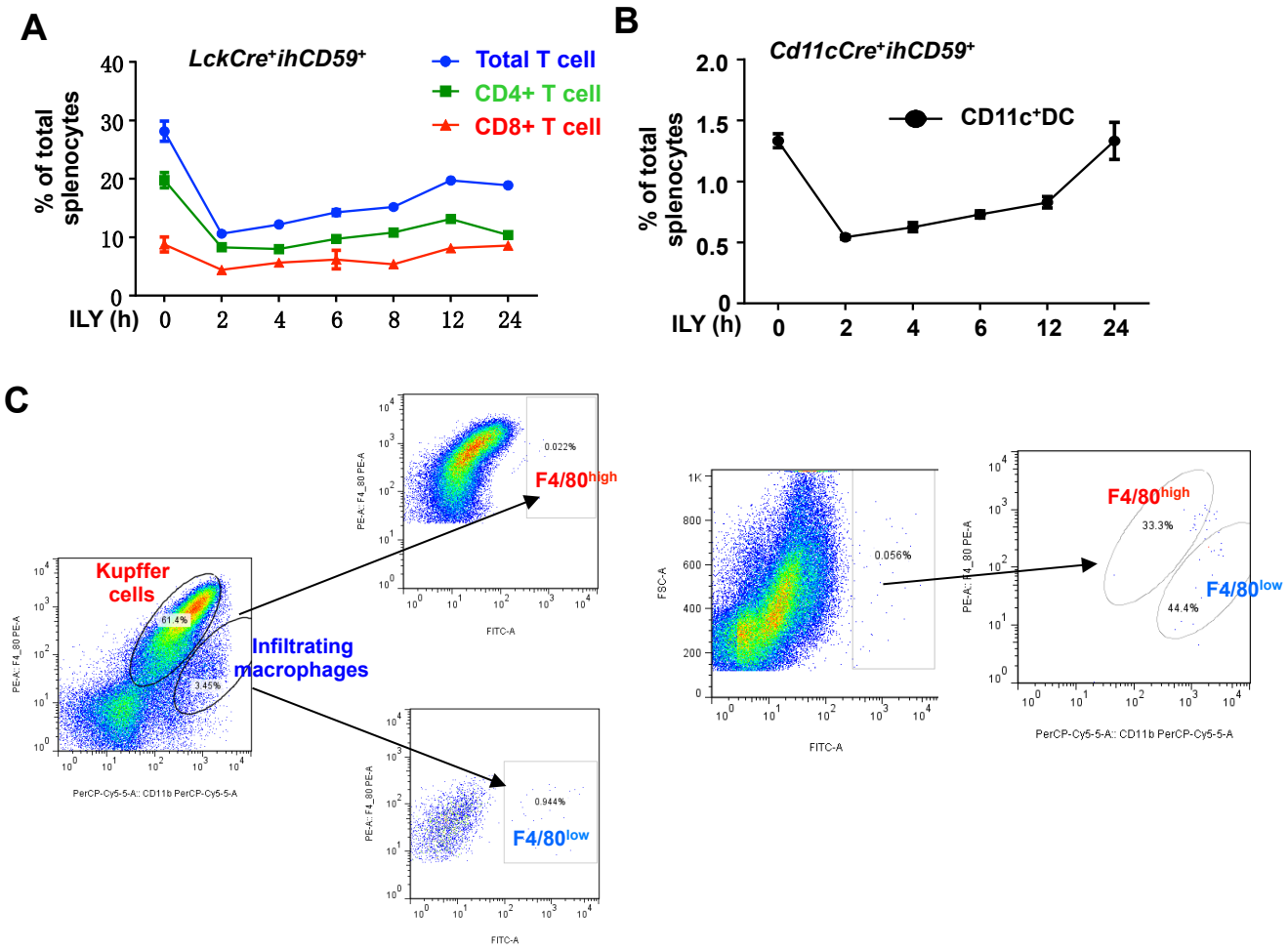


***LckCre*$^{+}$*ihCD59*$^{+}$ mice with 300ng/g ILY**



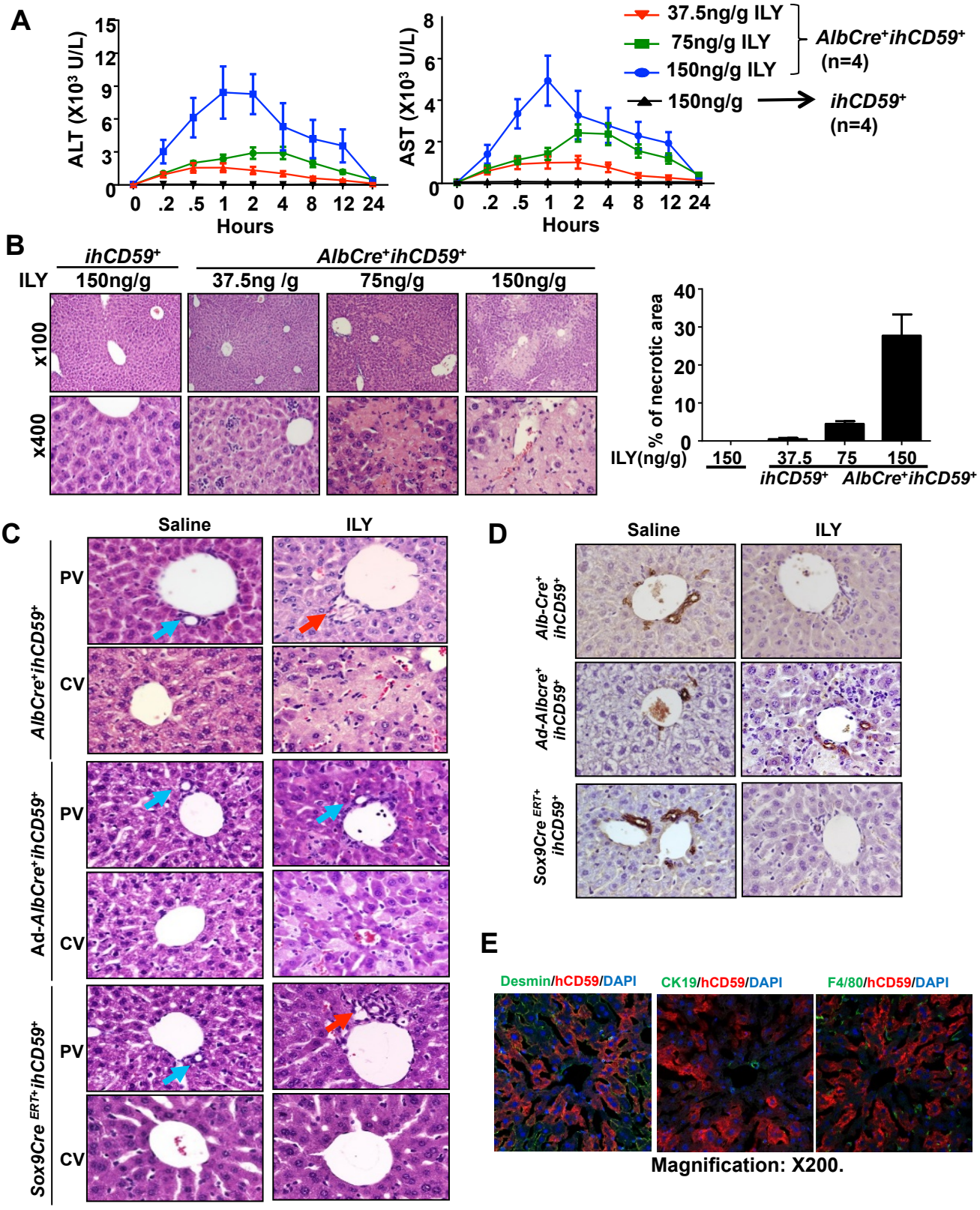
Supplemental Figure 5: Saturated doses of ILY cause minimal side effect on other organs in *LckCre*$^{+}$*ihCD59*$^{+}$ mice. *LckCre*$^{+}$*ihCD59* mice were injected with saturated doses of ILY (150 or 300ng/g, i.v.). Blood and serum were collected 24 hours after ILY injection for (A) complete blood count test and (B) Biochemical assays (The values represent the means ± SD, n=3-4, *P ≤ 0.05, ***P ≤ 0.001, as determined by 2-tailed Student's *t* test).). (C) Major organs were obtained for H&E staining. Representative images from 3 or 4 mice are shown. Magnification: Brain, X100. All other organs, X200. WBC: While blood cell; RBC: red blood cell; BUN: blood urea nitrogen; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AMYL: amylase; CK: creatine kinase; LD: lactate dehydrogenase.

Feng et al. Supplemental Figure 6



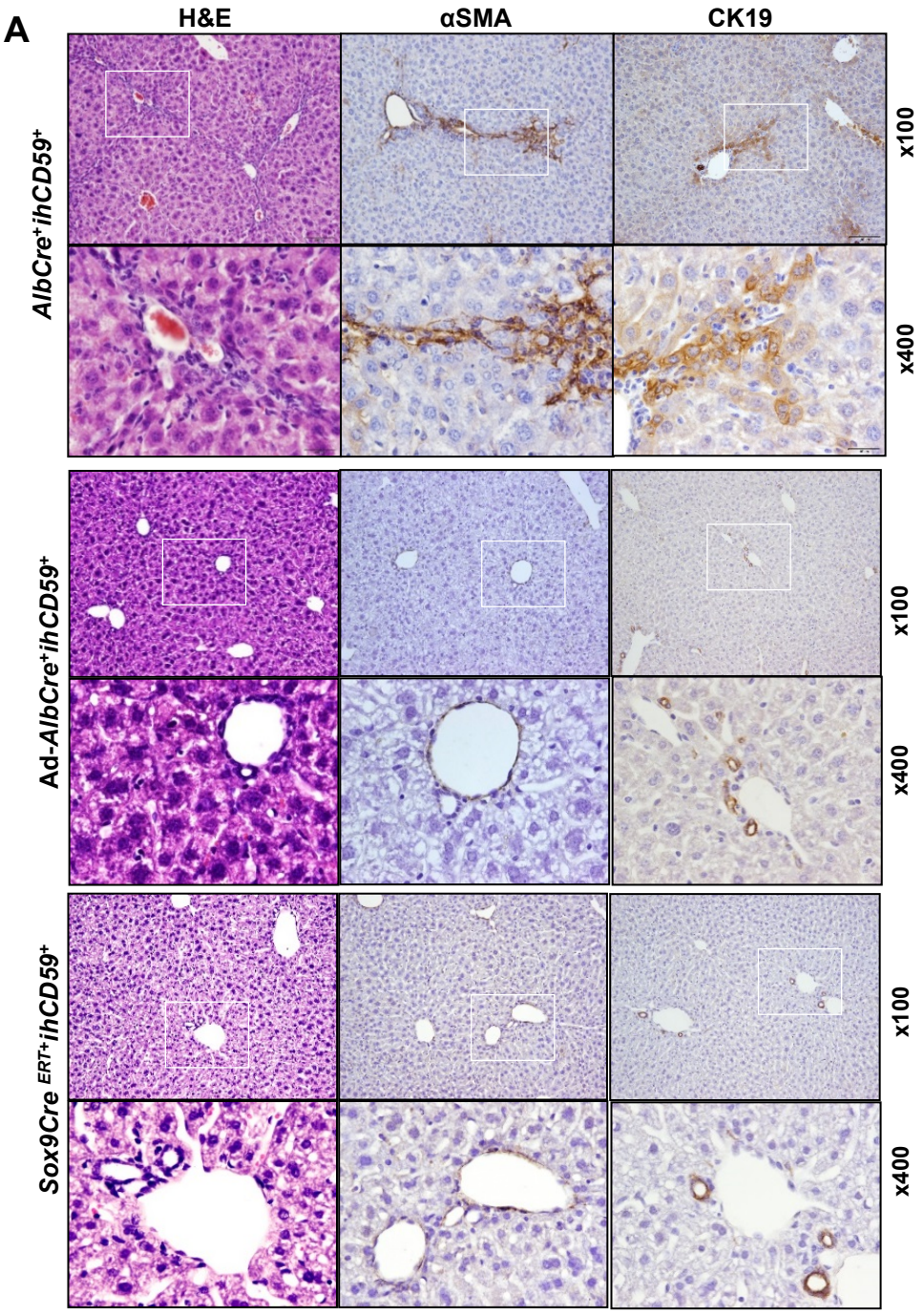
Supplemental Figure 6. Injection of ILY ablates T cells and DCs in Cre+ihCD59+ mice (A, B), and adoptive transfer GFP+ monocytes into wild-type C57BL6 mice (C). (A, B) Treatment of ILY ablates T cells and DCs in the spleens of *LckCre+ihCD59+* and *Cd11cCre+ihCD59+* mice, respectively (The values represent the means ± SD, n=3-6 for each time point). (C) 7.5X10^6 GFP+ monocytes from ROSA26-EGFP transgenic mice were adoptively transferred into C57BL6 mice, in which Kupffer cells were not depleted. Three days later, liver macrophages were isolated and subjected to FACS analyses. Few Kupffer cell-like GFP +CD11b^{low} F4/80^{high} cells were identified (Representative graphs from 4 animals).

Feng et al. Supplemental Figure 7

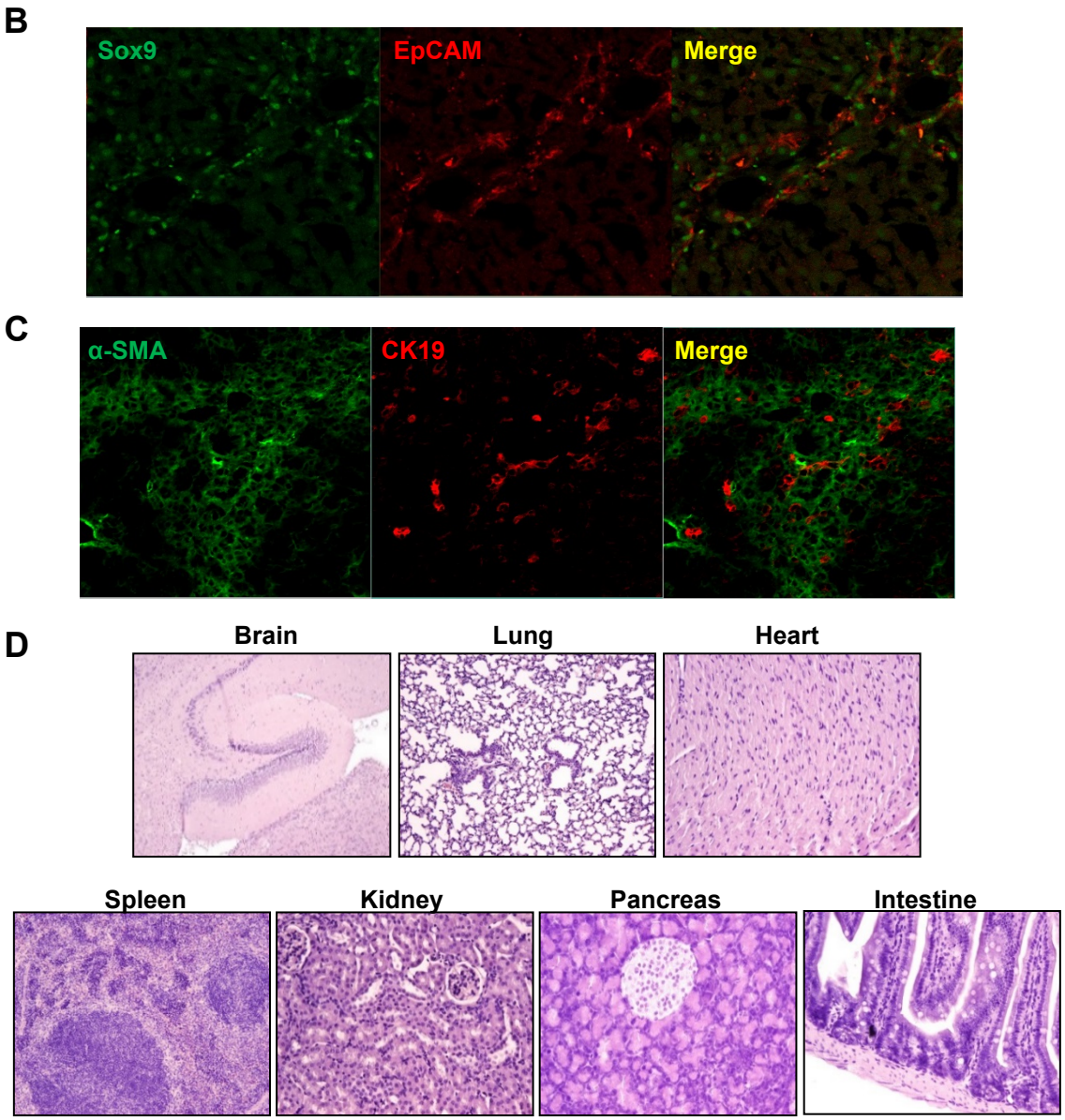


Supplemental Figure 7. ILY specifically induces hepatocyte and/or BEC injury in three different models of *Cre*$^+$*ihCD59*$^+$ mice. (A) Serum ALT and AST levels after ILY treatment (The values represent the means ± SD, n=4-6 for each time point). (B) H&E staining of *ihC59*$^+$ or *AlbCre*$^+$*ihCD59*$^+$ mouse liver after ILY injection. The percentage of necrotic area is shown (The values represent the means ± SD, n=6). (C) H&E staining of livers from various lines of *Cre*$^+$*ihCD59*$^+$ mice 12h after ILY injection. Blue arrows indicate healthy bile duct. Red arrows indicate destructed bile ducts. Magnification: X400. PV: portal vein, CV: central vein. (D) CK19 staining of livers 12h post ILY injection (n=6). (E). Infection of Ad-*AlbCre* induces expression of hCD59 in hepatocytes but not in bile duct cells or F4/80$^+$ Kupffer cells in *ihCD59*$^+$ mice. Mice were injected with Ad-*AlbCre* for 7 days, livers were collected for staining with hCD59 (red), CK19 (green), and F4/80 (green). There is no overlap between hCD59 (red) and CK19 or F4/80. Representative images in panels B-E from 6 mice are shown.

Feng et al. Supplemental Figure 8A

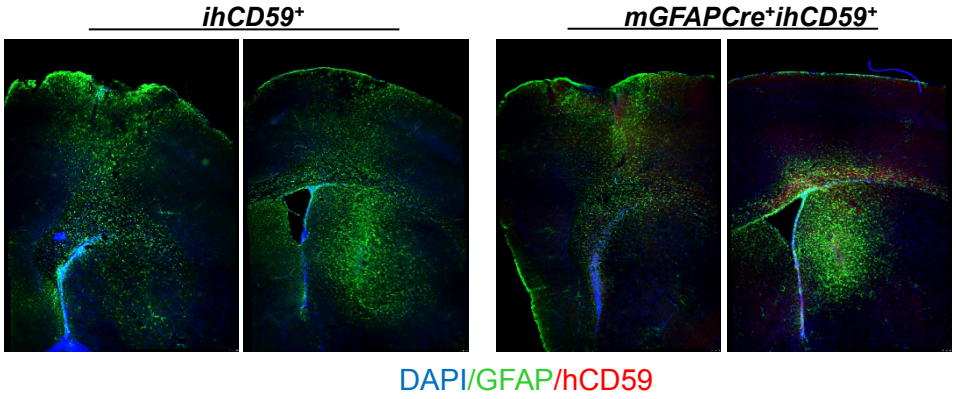


Feng et al. Supplemental Figure 8B-D



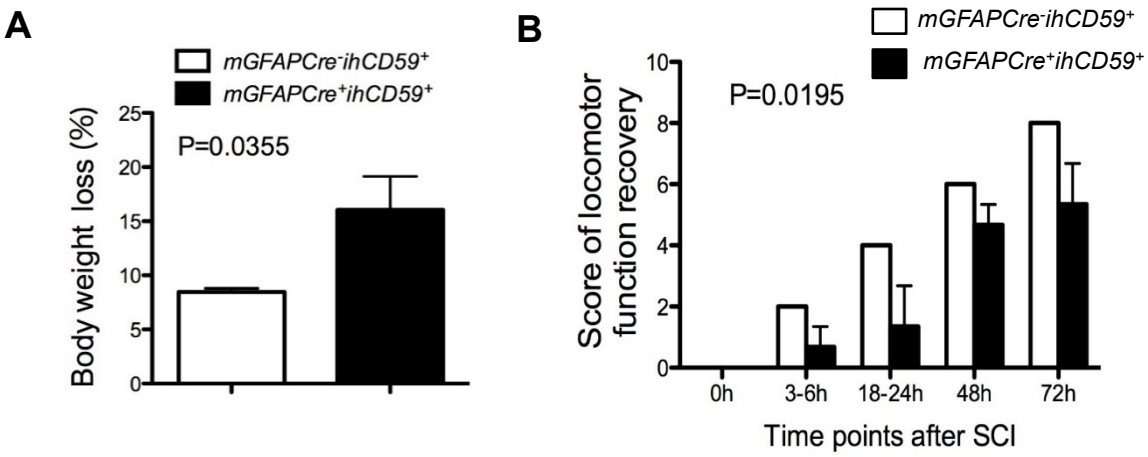
Supplemental Figure 8. Multiple ILY injections induce obvious fibrosis and expansion of LPCs in *AlbCre^{+}ihCD59^{+}* mice but not in *Ad-AlbCre^{+}ihCD59^{+}* and *Sox9Cre^{ERT+}ihCD59^{+}* mice. Cre^{+}ihCD59^{+} mice were received three ILY injections (150ng/g, every 3d). Liver sections were subjected to H&E staining, immunohistochemical staining for αSMA or CK19 (A) or immunofluorescence method for double staining of EpCAM (red) and Sox9 (green) (B), or of CK19 (Red) and αSMA (green) (C). (D) Various organs from ILY-treated *AlbCre^{+}ihCD59^{+}* mice were subjected to H&E staining. Representative images from 6 mice in each group are shown. Magnification: Brain, X100. All other organs, X200.

Feng et al. Supplemental Figure 9



Supplemental Figure 9. Traumatic brain injury model and ILY's systemic (i.v.) injection. ILY (50ng/g, i.v., every other day for 7 days) was administered to the mice after the CCI injury were bilaterally performed. Two representative photographs from each genotype of three different mice are shown.

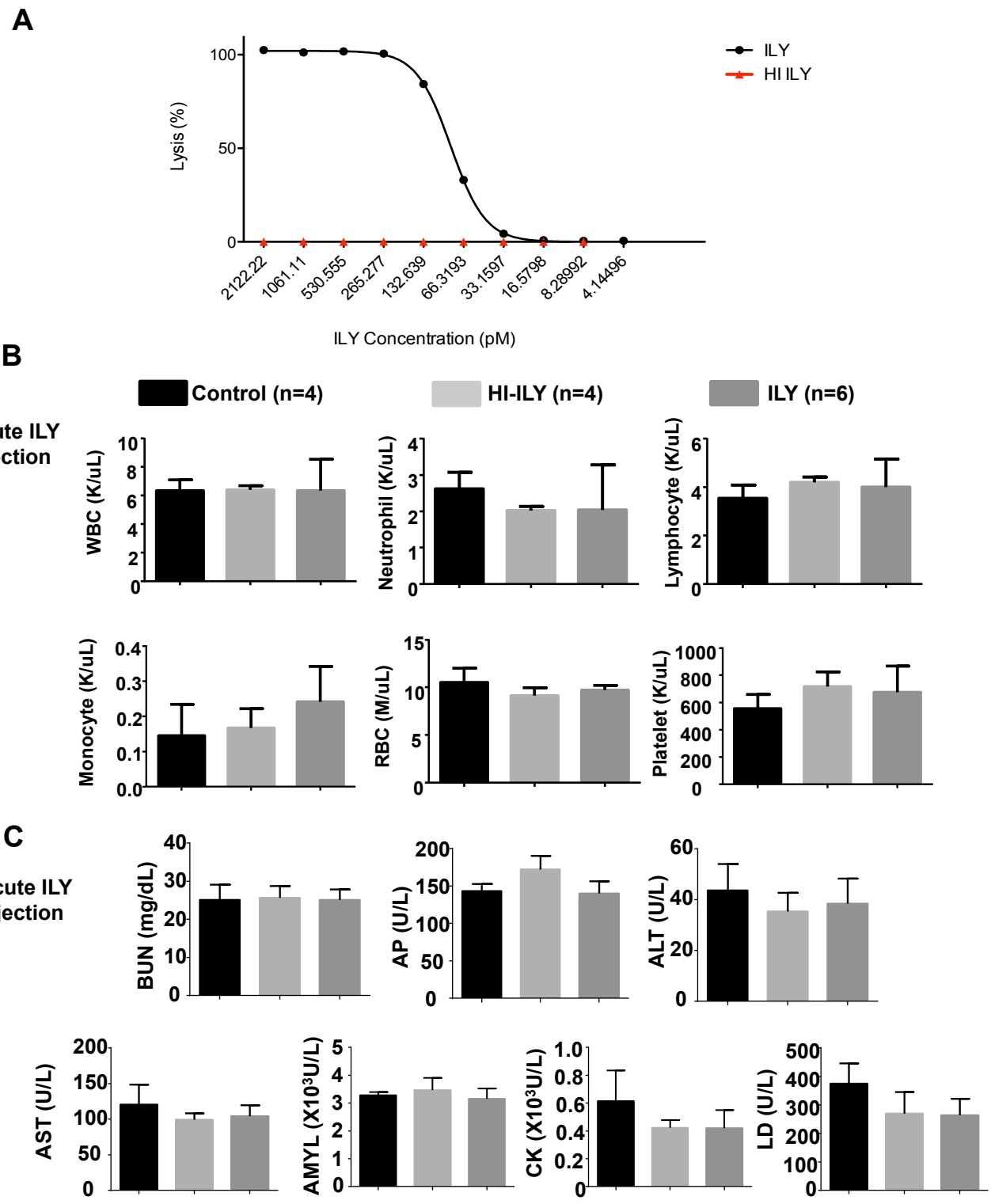
Feng et al. Supplemental Figure 10



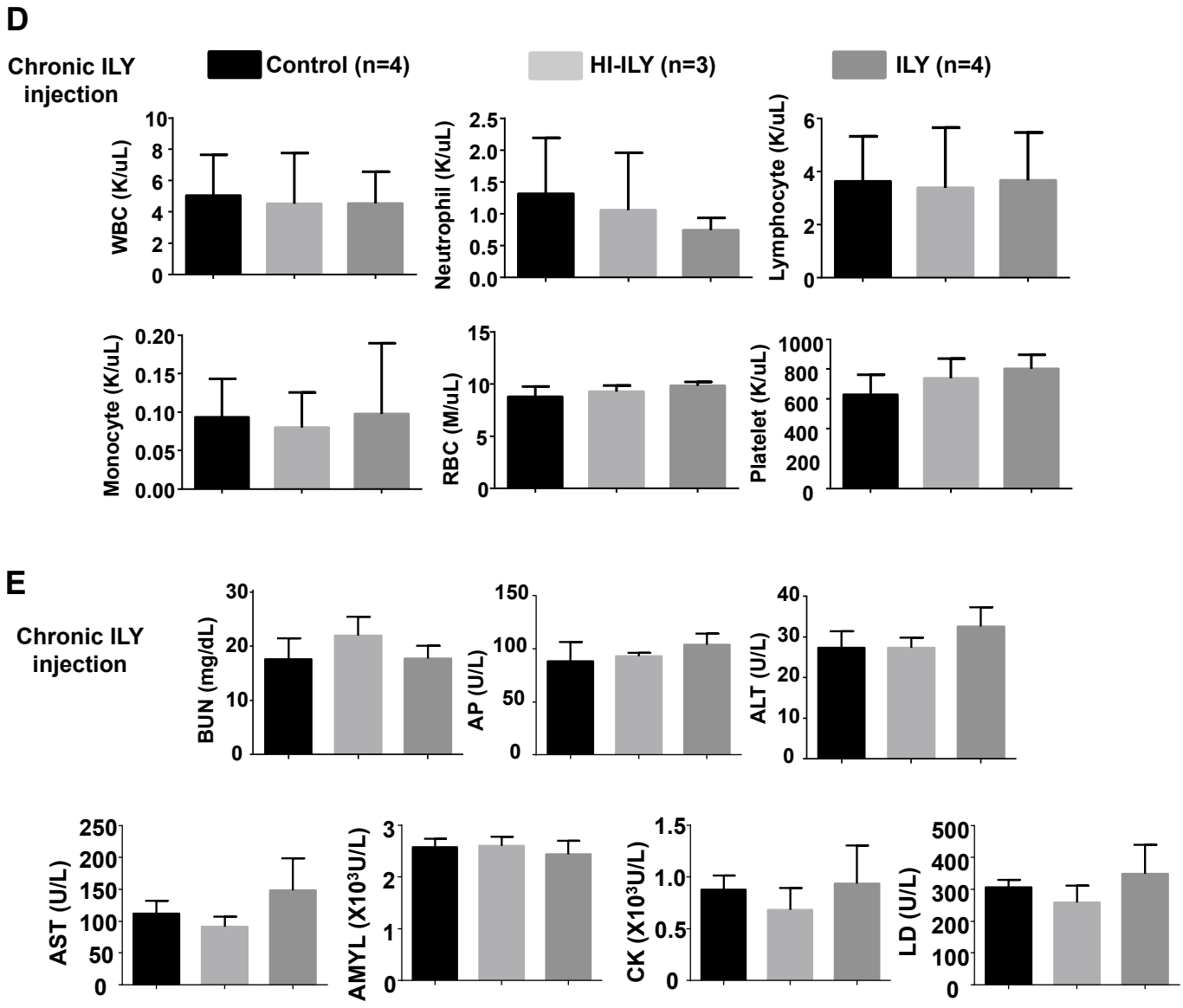
Supplemental Figure 10. ILY-mediated astrocyte ablation results in loss of body weight (A) and delayed recovery of locomotor function (B) in mice with spinal cord injury (SCI). (A) The body weight of the mice was measured before spinal cord injury and at the time of euthanasia after the surgery. The values represent the means ± SD, n=5 mice. P was determined by two-tailed Student's *t* test. (B) The locomotor activity was measured by the following arbitrary score. P was determined by 2 way ANOVA. The values represent the means ± SD, n=5 mice.

Note: Experiment were performed with 5 mice for per group at two different times. The phenotypic difference between two groups in the first experiment after SCI prompted us to measure the body weight and locomotor activity of the mice (n=5) in the following experiments. Locomotor activity was monitored by the following scores: 0: Limited/no mobility, 2: Mobile but with forelimbs only, 4: Mobile with a little support from hind limbs, 6: Walking with good support from all four limbs, 8: Able to stand on hind legs and climb up.

Feng et al. Supplemental Figure 11A-C

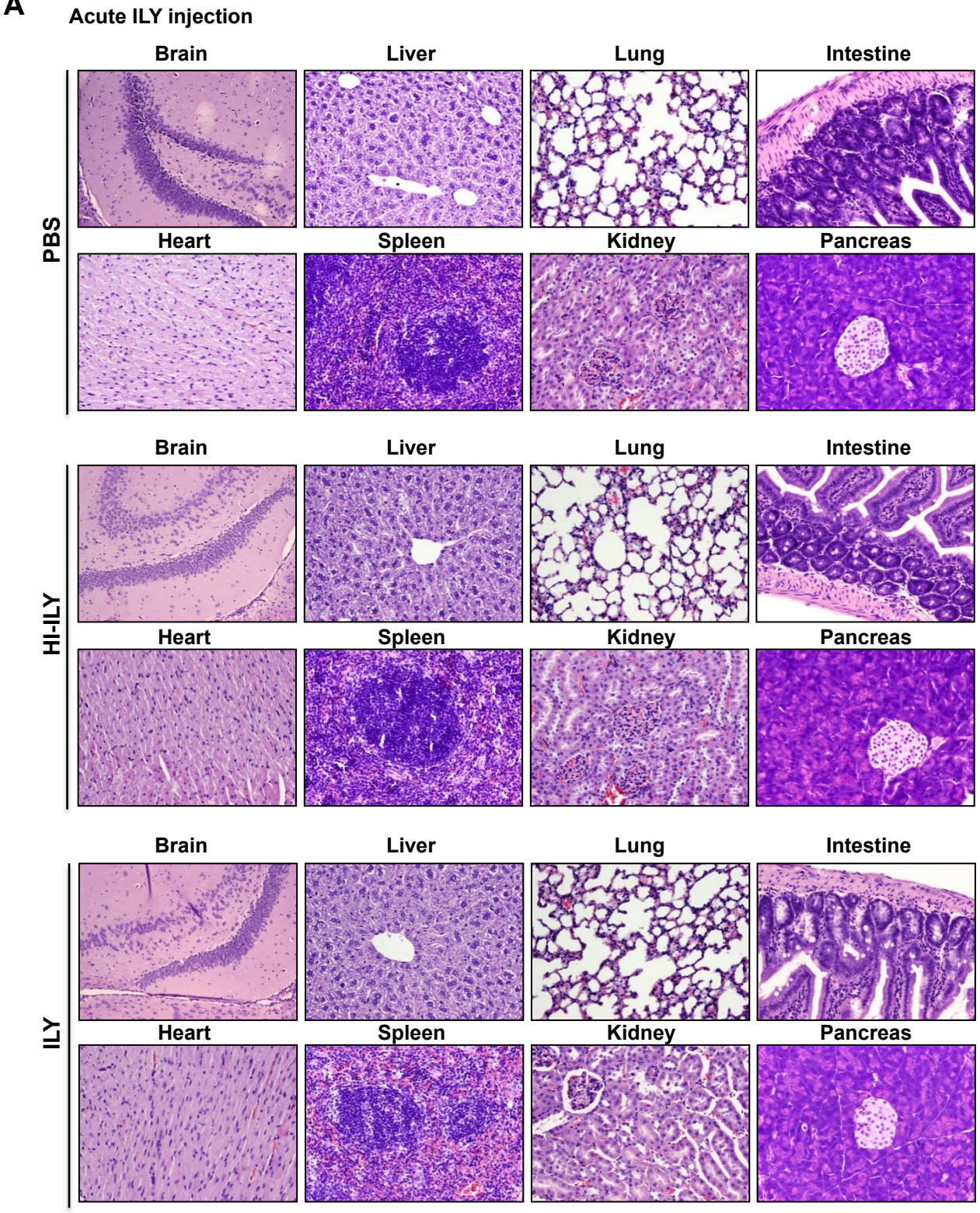


Feng et al. Supplemental Figure 11D-E

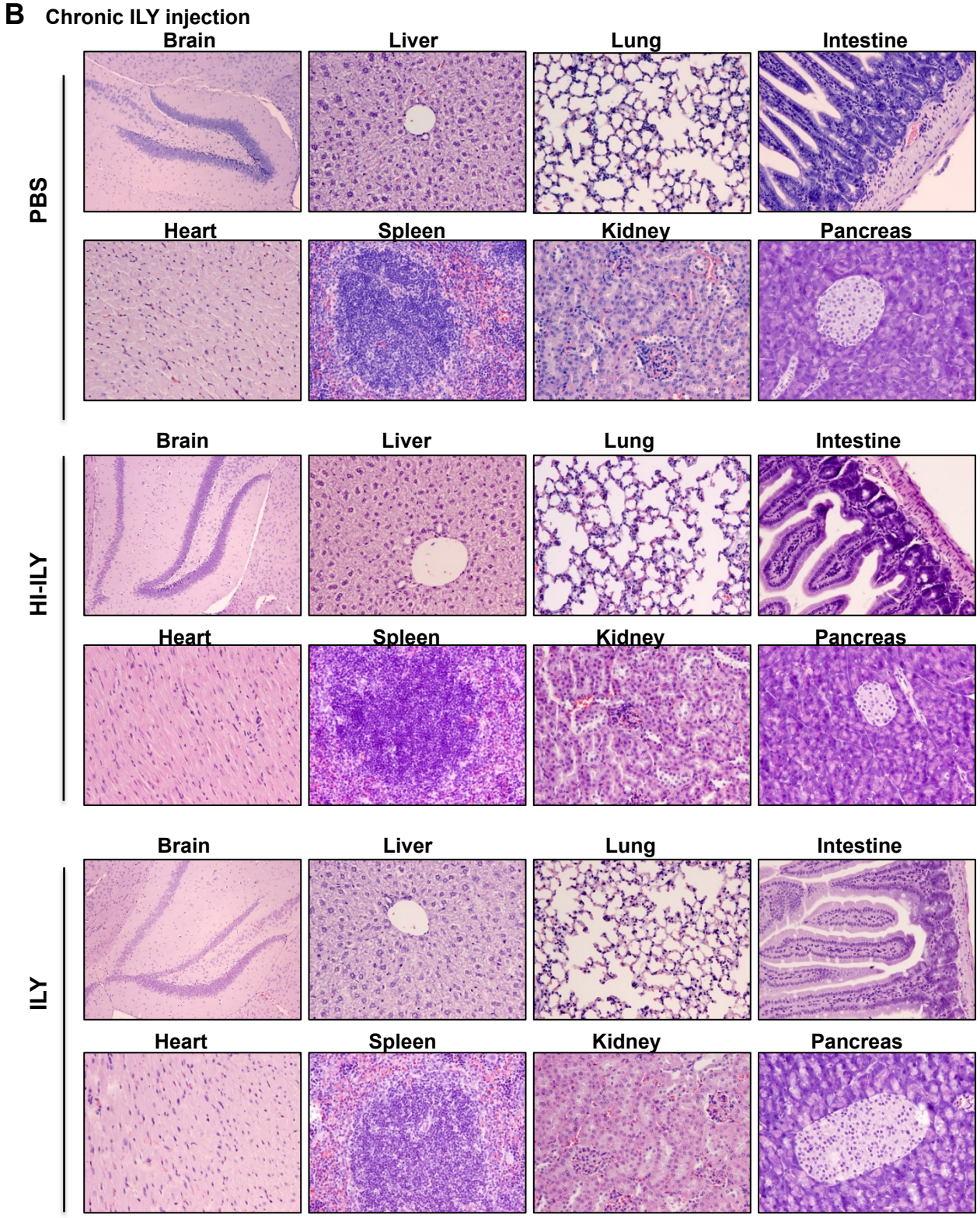


Supplemental Figure 11: Acute and chronic toxicity study of ILY. Related to all figures. (A) Heat inactivated ILY (HI) (ILY was incubated at 100^0 C for 10 min) lost the activity to lyse human red cells as compared to ILY (The values represent the means ± SD, n=3 for each concentration). (B, C) Acute toxicity Study of ILY. *ihCD59*$^+$ mice were treated with PBS, 1500ng/g ILY or 1500ng/g heat inactivated ILY by i.v. injection of a single dose. Blood and serum were collected 24h after ILY injection for (B) complete blood count test and (C) Biochemical assays (The values represent the means ± SD, n=3-6). (D,E) Chronic toxicity Study of ILY. *ihCD59*$^+$ mice were treated with PBS (Control), 1500ng/g ILY or 1500ng/g heat inactivated (HI)-ILY by daily i.p injection for 15d. Blood and serum were collected 24h after the last ILY injection for (D) complete blood count test and (E) Biochemical assays (The values represent the means ± SD, n=3-4). WBC: While blood cell; RBC: red blood cell; BUN: blood urea nitrogen; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AMYL: amylase; CK: creatine kinase; LD: lactate dehydrogenase.

Feng et al. Supplemental Figure 12A

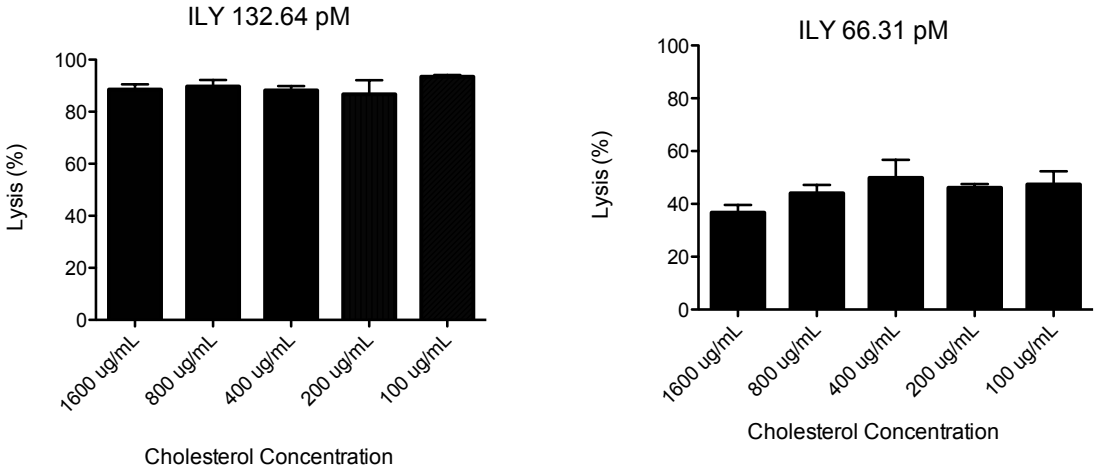


Feng et al. Supplemental Figure 12B



Supplemental Figure 12: No acute and no chronic toxicity effect of ILY on major organs. *ihCD59*$^{+}$ mice were treated with PBS, 1500ng/g ILY or 1500ng/g heat inactivated (HI)-ILY of single injection (panel A) or by daily i.v. injection for 15d (panel B). Major organs were obtained for H&E staining. Representative images from 3 to 6 mice are shown. Magnification: Brain, X100. All other organs, X200.

Feng et al. Supplemental Figure 13



Supplemental Figure 13. Exogenous cholesterol does not influence the ILY-mediated hemolysis. The different concentrations (from 100 ug/ml to 1600 ug/ml) of the cholesterol used in ILY-mediated hemolytic assay were based on its solubility and its background lysis in the assay. The cholesterol and ILY were sequentially added to the hemolytic assay. Results represent mean $\pm$ SD (n=3). $P > 0.05$ for ILY 132.64 pM groups and $P > 0.05$ for ILY 66.31pM groups were determined by one-way ANOVA.