Supplemental Materials and Methods

Experimental design and treatment

The experiment was performed in three parts. In Part A, mice were randomly assigned to either single- (ISO) or pair-housed conditions with an ovariectomized female mouse (PH: pair housing) with daily compatibility monitoring (e.g., weight gain and absence of fight wounds) for 14 consecutive days before being subjected to MCAO.[1] Immediately after surgery, we placed the mice back into their original cages, and the assigned housing conditions were maintained until the mice were euthanized. For mice tested on Day 1 after MCAO induction, RhANP (GL Biochem Ltd., Shanghai, China) at doses of 1.0, 0.5 and 0.1 μg/g or 0.9% saline (10 ml/kg) was injected intravenously into mice at the end of MCAO surgery. For mice tested on Day 3 post-MCAO, different doses of RhANP were administered intravenously on Day 0 (right after MCAO surgery), Day 1 and Day 2 post-MCAO. Similarly, mice received intraperitoneal injection of NPR-A selective antagonist A71915 (0.5 mg/g; Bachem, Torrance, CA) or 0.9% saline (10 ml/kg) at the end of MCAO surgery when tested on Day 1, or mice received an additional two injections on Day 1 and Day 2 post-MCAO when tested on Day 3 post-MCAO. In Part B, ISO mice were randomly assigned to groups and were either intravenous injection of anti-γδ TCR monoclonal antibody (mAb; 500 μg/mice every other day for 3 days, starting 3 days before MCAO, eBioscience, Vienna, Austria) or were sham-treated with normal hamster serum IgG. PH mice were also similarly intravenously injected daily with normal hamster serum IgG. In Part C, mice were randomly assigned to bilateral SDV or sham operation, which were performed 28 days prior to MCAO induction. After 14 days of recovery, mice were randomly allocated to single- or pair-housed environments for 14 consecutive days before being subjected to MCAO.

Photoconversion and cell migration analysis

After completion of MCAO or sham surgery, Kaede-Tg mice were placed on their backs, and the abdominal skin was prepped by hair shaving followed by disinfection with 70% alcohol. Then, a 1-cm midline abdominal incision was made. The small intestine was pulled out of the abdominal cavity with an aluminum

foil blanket covering all but the small intestine. After selective exposure of the small intestine to a 405-nm laser for 10 minutes, the bowel was then returned to the abdominal cavity, and the abdominal wall was closed. For the sham operation of intestinal photoconversion, the small intestine was pulled out of the abdominal cavity but without UV irradiation. Kaede-photoconversion was specific to cells in the small intestine. Thus, Kaede-Red$^+$ cells in other organs must have originate from the small intestine. We employed the migration index to reflect the degree of migration of γδ T cells to other sites, which was calculated as Kaede Red$^+$ CD3$^+$ γδ TCR$^+$/ Kaede Green$^+$ CD3$^+$ γδ TCR$^+$.

Lung wet/dry (W/D) weight ratio

The right lungs were excised and immediately weighed to record the wet weight. Subsequently, the lung samples were placed in an incubator at 60 ℃ for 48 hours and the dry weight was measured (lung wet/dry ratio = wet weight/dry weight).

2,3,5-Triphenyl-tetrazolium chloride (TTC) staining

Brain sections (2 mm) were incubated with prewarmed 2% TTC at 37 °C for 30 minutes and then fixed with 10% formalin. The images of stained slices were photographed. Calculation of the infarct volume was performed in an unbiased, blinded fashion.

Neurological deficit scores

Neurological deficit scores were assessed at 1 day and 3 days after MCAO as follows: 0, no neurologic deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side and rotating immediately when placed on a bench; 4, no spontaneous locomotor activity or barrel rolling. Scores were accessed by researchers who were blinded to the groups.

Open field test

The open field test was performed to assess motor function and exploratory locomotion. Before testing, mice were acclimated to the testing room for at least 30 minutes. Mice were placed separately in the center of an open field chamber (40 cm × 40 cm) with 16 infrared beam emitting LEDs on each side for 20 minutes. The total number of beam breaks, which reflects interference with the light source by animals, was automatically recorded by a computer-operated PAS Open Field system (San Diego Instruments, San Diego, CA) and was analyzed as a measure of spontaneous locomotor activity.

Cylinder test

The cylinder test was used to assess asymmetrical forelimb use after stroke. Mice were placed in the center of a glass cylinder with a diameter of 9 cm and a height of 15 cm for 5 minutes. Two mirrors were placed behind the cylinder to ensure that the forelimbs on either side were visible to the observer or camera at all times. A total of 20 vertical movements were recorded for each mouse. Each time the mouse stood upright and placed its paw on the cylinder wall, whether the left or the right paw first touched the cylinder was recorded, and simultaneous paw touches were excluded. If two forelimbs were placed on the wall in such quick succession that slow-motion analysis could not determine which paw was placed first, then the placement was recorded as "simultaneously". The data were expressed as a percentage of impaired forelimb relative to the total contacts [left/(left + right + simultaneously) × 100%].

Enzyme-linked immunosorbent assay (ELISA)

The mice were anesthetized with isoflurane and euthanized at Day 1 or Day 3 post-MCAO, and blood was collected via cardiac puncture and centrifuged (3000 × g, 15 min) to obtain serum. The brain and lung were collected and frozen in liquid nitrogen immediately after removal and kept at -80℃. The brain tissues were

homogenized in ice-cold 0.01 M PBS containing protease inhibitors (KeyGen Biotech, Nanjing, China) and then centrifuged at 3000 × g for 10 minutes at 4 °C to obtain the supernatants. The supernatants were collected and stored at −80 °C for ELISA detection. The serum expression levels of ANP (R&D Systems, Minneapolis, MN) and brain and lung concentrations of IL-6 (NeoBioscience, Shenzhen, China), IL-17A (NeoBioscience, Shenzhen, China), IL-22 (NeoBioscience, Shenzhen, China) and interferon-gamma (IFN-γ) (NeoBioscience, Shenzhen, China) were measured using commercial ELISA kits according to the manufacturer's instructions.

Hematoxylin-eosin (HE) staining

Lung tissues were collected and immediately fixed in 4% paraformaldehyde (pH 7.4) at 4 ℃ for approximately 48 hours and then embedded in paraffin for sectioning at 5 μm. Paraffin-embedded lung sections were subsequently deparaffinized and rehydrated with xylene and alcohol and counterstained with hematoxylin and eosin to label the nucleus and cytoplasm, respectively. Images of stained samples were obtained under light microscopy (BX51, Olympus, Japan).

Morphological changes were scored as nil (0), mild (1), moderate (2), or severe (3) injury based on the following five pathological features: (i) the presence of exudates, (ii) hyperemia/congestion, (iii) neutrophil infiltration, (iv) intra-alveolar hemorrhage/debris, and (v) cellular hyperplasia. The sums of scores of different mice in each group were averaged. The scoring was performed in an unbiased, blinded fashion.

Bacterial counting

Mice were euthanized under xylazine-ketamine anesthesia. Lungs were aseptically collected after thoracotomy, weighed and homogenized in 1 ml of sterile PBS. For determination of colony-forming units (CFU), 10 μl lung homogenates were serially diluted (1:10) in sterile PBS, and 20 μl of tissue homogenates were plated onto LB broth agar plates, incubated at 37 ℃ for 24 hours and counted to quantify the number of bacterial

colonies. Bacterial loads were expressed as CFU per ml.

Evans blue dye permeability assay

Twenty-four or 72 hours after reperfusion, 4 ml/kg 2% Evans blue (Sigma, St Louis, MO, USA) in saline was injected into the tail vein of post-stroke mice. Three hours after Evans blue injection, mice were sacrificed and transcardially perfused with PBS thoroughly to confirm that the blood that contained intravascular Evans blue dye was flushed out of the pulmonary and systemic circulations. The ischemic hemispheres of the brain, lung, jejunum and ileum were then incised, precisely weighed, homogenized in 1 ml of N,N-dimethylformamide (Sigma, St Louis, MO, USA), incubated overnight at 55 ℃ and centrifuged at 12000 × g for 20 minutes. The OD of the supernatants was measured at 620 nm by spectrophotometry.

Myeloperoxidase (MPO) activity determination

To quantify neutrophil infiltration, we determined MPO activity using an MPO kit (Nanjing Jiancheng, China) according to the manufacturer's protocols. Briefly, the brain and lung tissues were excised and precisely weighed, followed by homogenization with MPO assay buffer (1:19, wt/vol). The homogenates were then added to the reaction system. MPO activity of the sample was determined by measuring absorbance at 460 nm and presented as units per gram of total protein (U/g).

16S rRNA analysis

Fresh stool pellets of mice were collected, placed in 1.5-mL tubes, snap-frozen on dry ice and stored at −80 °C until use. The 16S rRNA analysis of fecal samples was performed by GENEWIZ Biotech Co., Ltd. (Suzhou, China). Briefly, total genomic DNA was extracted from the samples by using a Soil DNA kit and subsequently amplified in 25-μL triplicate reactions with bacterial 16S rRNA gene (V3–V4 region)-specific forward primers of the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers of the

sequence “GGACTACNVGGGTWTCTAATCC”. Validation and quantification of the amplified DNA libraries were performed on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA). After quality filtering, the effective sequences were grouped into operational taxonomic units.

Flow cytometric analysis

At 24 or 72 hours after MCAO, mice were euthanized under xylazine-ketamine anesthesia. Blood was collected via cardiac puncture in heparinized tubes, and the ischemic brain hemisphere, lung, and small intestine were collected. The spleen and mesenteric lymph nodes were gently homogenized with the end of a 1 ml syringe plunger, and the cells were eluted from the strainer with 5 ml PBS. The small intestine was carefully cleaned and opened longitudinally, washed with PBS, cut into 1.0–1.5 cm pieces and incubated in 20 ml of HBSS/10 mM HEPES, 8% FBS, 4 mM EDTA, and 0.5 mM dithiothreitol at 37 ℃ with constant agitation for 15 minutes twice. After washing with $Ca2^{+}$/$Mg2^{+}$-PBS to remove EDTA, the remaining pieces of small intestine and lung were then cut into small pieces and digested in RPMI-1640 medium containing fetal bovine serum (FBS), collagenase IV (0.1 mg/mL) and DNase I (0.1 mg/mL) at 37 ℃ for 40 minutes. The digested tissues were prepared into a single cell suspension by vigorously shaking and passing through a 40 μm cell strainer after the removal of the red blood cells. All of the suspensions were then collected, washed once in cold flow cytometry buffer and separated by a Percoll gradient (Sigma, St Louis, MO, USA). Lymphocytes were harvested from the interphase of the Percoll gradient, washed three times and resuspended in FACS buffer. We isolated brain leukocytes as described by previous studies.[2,3] The ischemic brain hemispheres were gently homogenized with 7 ml of RPMI-1640, followed by the addition of 3 ml of 100% Percoll (final concentration 30%); then, samples were and overlaid over 2 ml of 70% Percoll. Gradients were centrifuged at 500 × g for 20 minutes at 20 ℃ to obtain lymphocytes.

Cell surface molecular staining of the obtained cells was performed with optimal concentrations of BV510-conjugated fixable viability dye (Biolegend, San Diego, CA), PerCP/cyanine5.5-conjugated anti-CD45 (BD

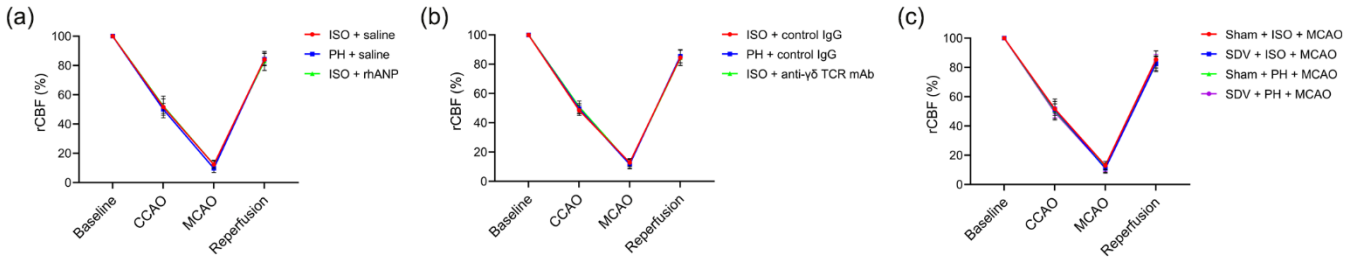
Biosciences, San Jose, CA), PECy7-conjugated anti-CD3 (BD Biosciences, San Jose, CA), APC-conjugated anti-γδ TCR (eBioscience, San Diego, CA) and PECy7-conjugated anti-CD4 (eBioscience, San Diego, CA). IL-17 production in activated cells was detected by intracellular cytokine staining with BV421-conjugated anti-IL-17 (Biolegend, San Diego, CA). The fluorescence of the photoconvertable Kaede protein was monitored in the green (FITC) and red (PE) channels. For experiment Part C, the absolute count of Kaede-red γδ T cells was measured by adding counting beads (Invitrogen, Carlsbad, CA)) and calculated as follows: absolute cell count (cells/μl) = (cell count×bead volume)/(bead count×cell volume)×bead concentration. All samples were analyzed on a BD LSRFortessa X-20 cytometer (BD Biosciences, San Jose, CA).

Western blotting

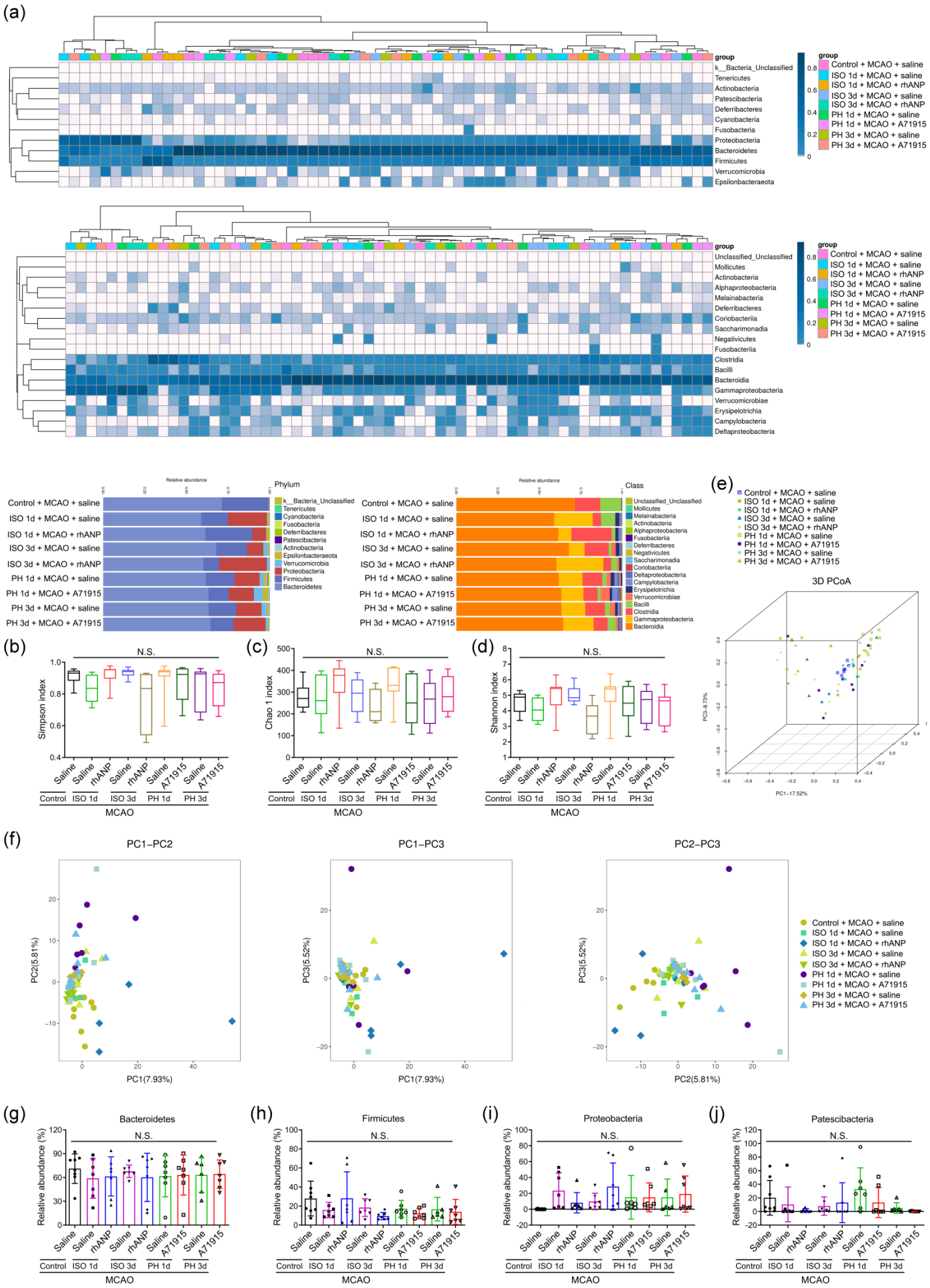
Tissue samples from the brain and lung were homogenized in ice-cold RIPA lysis buffer containing protease inhibitors (KeyGen Biotech, Nanjing, China) and then centrifuged at 3000 × g for 10 minutes at 4 ℃ to obtain the supernatants. Protein concentrations were measured using the BCA method. Proteins from each sample (40 μg) were separated via 10% SDS–PAGE gels (Epizyme Biomedical Technology, Shanghai, China) and electrophoretically transferred to PVDF membranes (Millipore; Merck KGaA) using an electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with 5% skimmed milk for 2 hours at room temperature, the membranes were incubated overnight at 4 ℃ with primary antibodies targeted against anti-IL-17A (Santa Cruz, CA, USA) and anti-β-actin (Proteintech, Wuhan, China). Subsequently, the membranes were incubated with anti-mouse IgG HRP-conjugated antibody. Protein bands were visualized using ECL Western Blotting Detection Reagents (Beyotime, China) and captured using a UVP gel documentation system (UVP, LLC, Phoenix, AZ, USA). Band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

References:

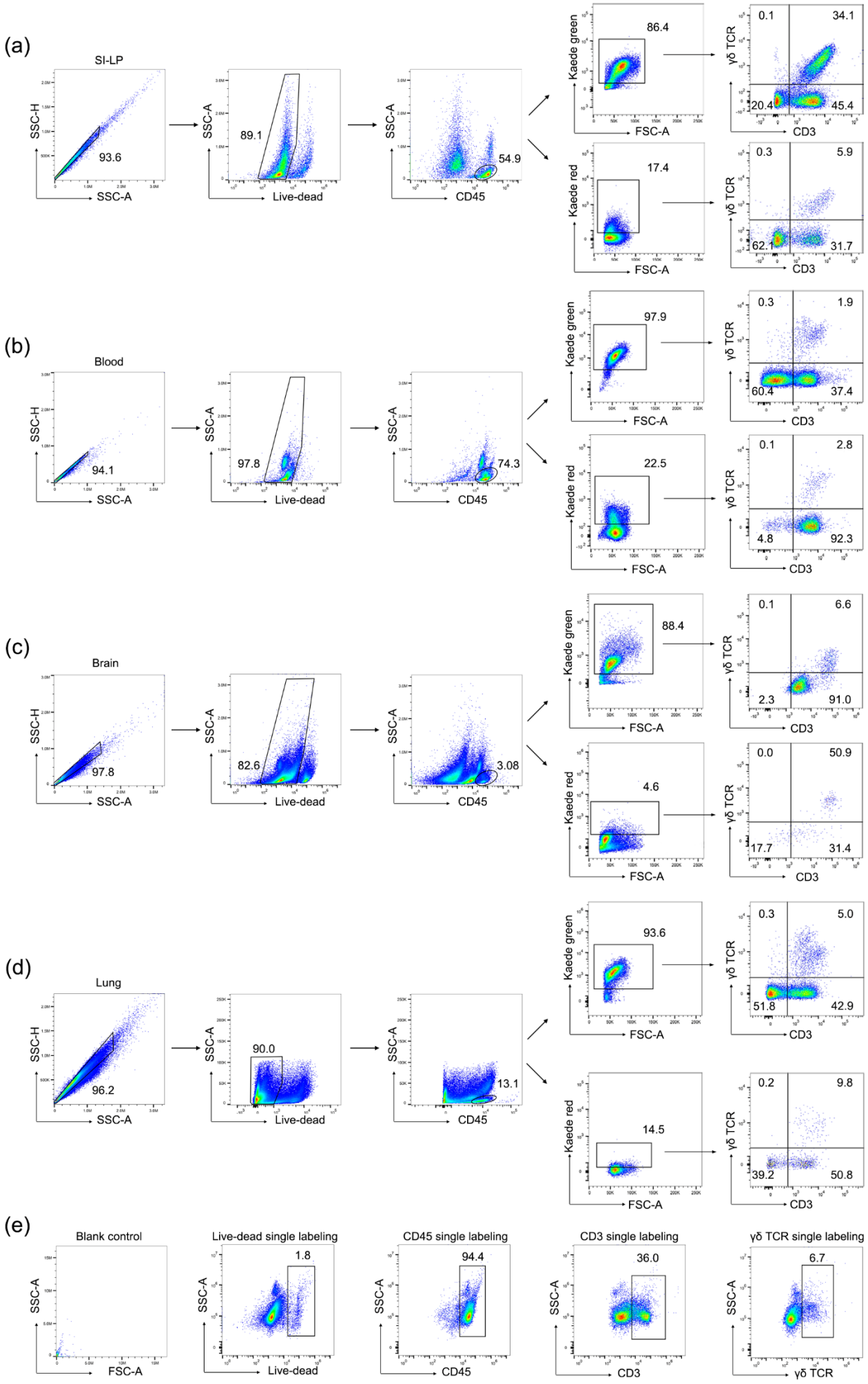
1. Meng C, Zhang JC, Shi RL, et al. **Inhibition of interleukin-6 abolishes the promoting effects of pair housing on post-stroke neurogenesis**. *Neuroscience* 2015;**307**:160–170.
2. Pino PA, Cardona AE. **Isolation of brain and spinal cord mononuclear cells using percoll gradients**. *J Vis Exp* 2011;(48):2348.
3. Benakis C, Brea D, Caballero S, et al. **Commensal microbiota affects ischemic stroke outcome by regulating intestinal γδ T cells**. *Nat Med* 2016;**22**:516–523.



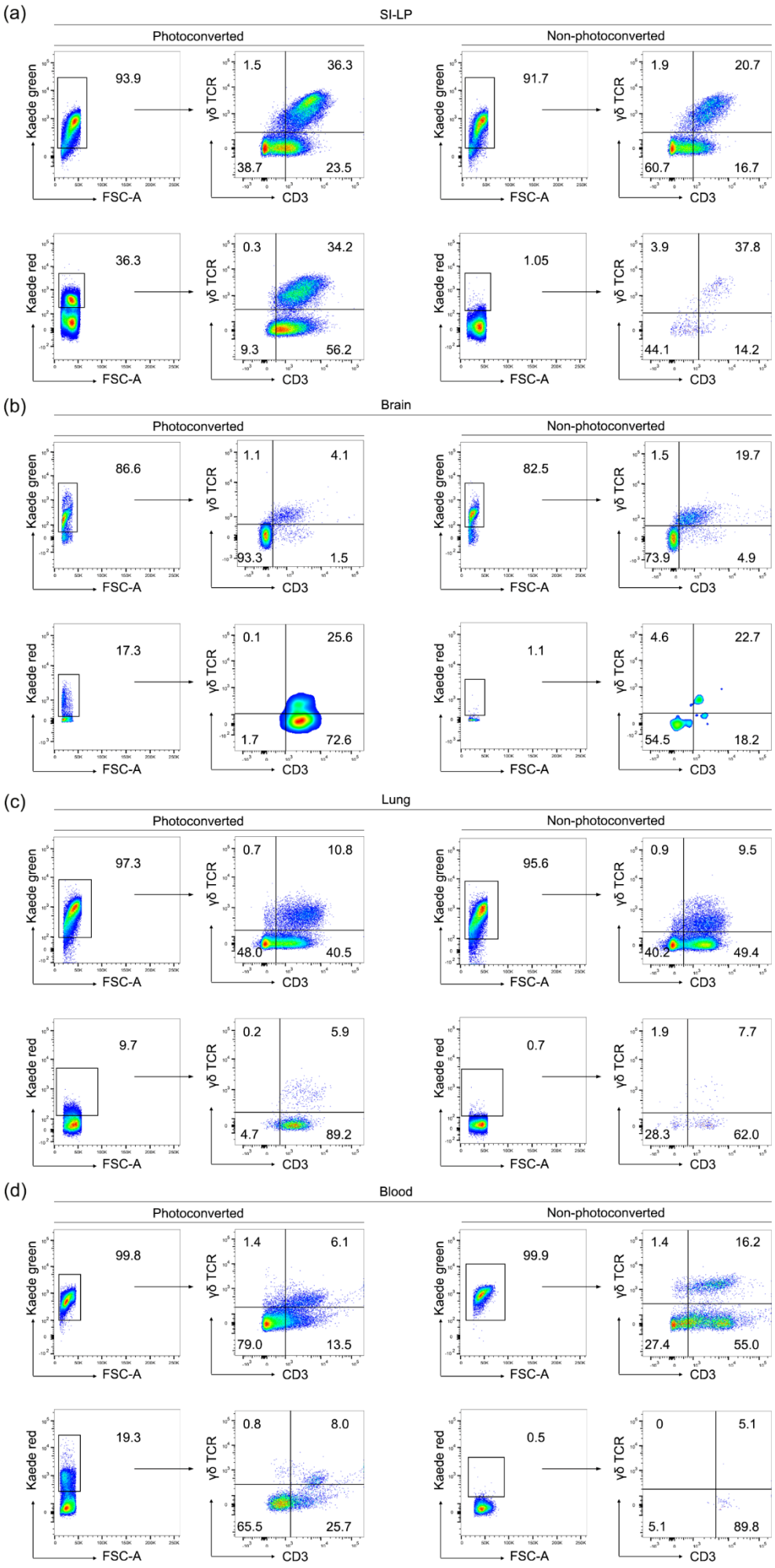
Supplemental Figure 1. Changes in regional cerebral blood flow (rCBF) during middle cerebral artery occlusion (MCAO) and reperfusion. (a) Comparison of rCBF among ISO + saline, PH + saline and ISO + rhANP. (b) Comparison of rCBF among ISO + control IgG, PH + control IgG and ISO + anti-γδ TCR mAb. (c) Comparison of rCBF among Sham + ISO + MCAO, SDV + ISO + MCAO, Sham + PH + MCAO and SDV + PH + MCAO. CCAO, common carotid artery occlusion; MCAO, middle cerebral artery occlusion; ISO, isolated housing; mAb, monoclonal antibody; PH, pair housing; rhANP, recombinant human atrial natriuretic peptide; SDV, subdiaphragmatic vagotomy.


(a)
group
k__Bacteria_Unclassified
Tenericutes
Actinobacteria
Patescibacteria
Deferribacteres
Cyanobacteria
Fusobacteria
Proteobacteria
Bacteroidetes
Firmicutes
Verrucomicrobia
Epsilonbacteraeota
Control + MCAO + saline
ISO 1d + MCAO + saline
ISO 1d + MCAO + rhANP
ISO 3d + MCAO + saline
ISO 3d + MCAO + rhANP
PH 1d + MCAO + saline
PH 1d + MCAO + A71915
PH 3d + MCAO + saline
PH 3d + MCAO + A71915
Unclassified_Unclassified
Mollicutes
Actinobacteria
Alphaproteobacteria
Melainabacteria
Deferribacteres
Coriobacteriia
Saccharimonadia
Negativicutes
Fusobacteriia
Clostridia
Bacilli
Bacteroidia
Gammaproteobacteria
Verrucomicrobiae
Erysipelotrichia
Campylobacteria
Deltaproteobacteria
Relative abundance
Phylum
Class
(b)
N.S.
Simpson index
(c)
Chao 1 index
(d)
Shannon index
Saline
rhANP
A71915
Control
ISO 1d
ISO 3d
PH 1d
PH 3d
MCAO
(e)
3D PCoA
PC1−17.52%
PC3−8.73%
(f)
PC1−PC2
PC1−PC3
PC2−PC3
PC1(7.93%)
PC2(5.81%)
PC3(5.52%)
(g)
Bacteroidetes
(h)
Firmicutes
(i)
Proteobacteria
(j)
Patescibacteria
Relative abundance (%)

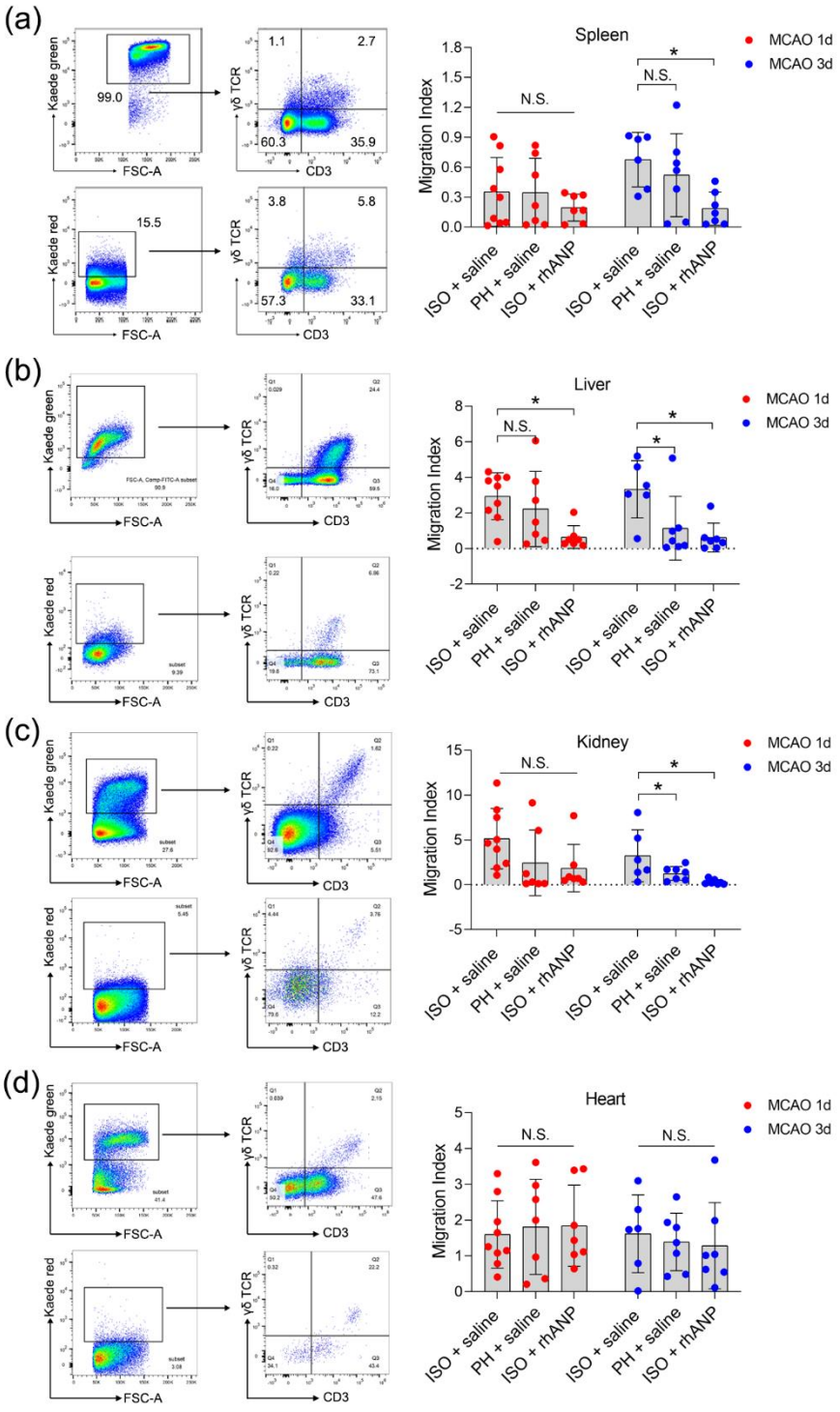
Supplemental Figure 2. Altered gut bacterial composition. (a) Heatmap of the fecal bacterial abundances and relative abundance at the phylum and class levels in the different groups. (b) Chao1 index. (c) Shannon index. (d) ACE index. (e) Principal coordinate analysis (PCoA) of gut bacteria (Bray–Curtis dissimilarity). (f) Principal component analysis (PCA) of gut bacteria data. The abundance of *Bacteroidetes* (g), *Firmicutes* (h), *Proteobacteria* (i), and *Patescibacteria* (j) in the different groups. Data are represented as individual values plus the means ± SDs (n = 6–8 mice/group). Comparisons were determined by one-way ANOVA followed by post hoc Newman–Keuls multiple comparison tests. N.S. not significant. MCAO, middle cerebral artery occlusion; ISO, isolated housing; PH, pair housing; rhANP, recombinant human atrial natriuretic peptide.


(a)
SI-LP
SSC-H
SSC-A
93.6
SSC-A
Live-dead
89.1
SSC-A
CD45
54.9
Kaede green
FSC-A
86.4
γδ TCR
CD3
0.1
34.1
20.4
45.4
Kaede red
FSC-A
17.4
γδ TCR
CD3
0.3
5.9
62.1
31.7
(b)
Blood
SSC-H
SSC-A
94.1
SSC-A
Live-dead
97.8
SSC-A
CD45
74.3
Kaede green
FSC-A
97.9
γδ TCR
CD3
0.3
1.9
60.4
37.4
Kaede red
FSC-A
22.5
γδ TCR
CD3
0.1
2.8
4.8
92.3
(c)
Brain
SSC-H
SSC-A
97.8
SSC-A
Live-dead
82.6
SSC-A
CD45
3.08
Kaede green
FSC-A
88.4
γδ TCR
CD3
0.1
6.6
2.3
91.0
Kaede red
FSC-A
4.6
γδ TCR
CD3
0.0
50.9
17.7
31.4
(d)
Lung
SSC-H
SSC-A
96.2
SSC-A
Live-dead
90.0
SSC-A
CD45
13.1
Kaede green
FSC-A
93.6
γδ TCR
CD3
0.3
5.0
51.8
42.9
Kaede red
FSC-A
14.5
γδ TCR
CD3
0.2
9.8
39.2
50.8
(e)
Blank control
SSC-A
FSC-A
Live-dead single labeling
1.8
SSC-A
Live-dead
CD45 single labeling
94.4
SSC-A
CD45
CD3 single labeling
36.0
SSC-A
CD3
γδ TCR single labeling
6.7
SSC-A
γδ TCR

Supplemental Figure 3. Flow cytometric gating strategies of migratory gamma-delta T (γδ) T cells in the main organs. Representative flow cytometric plots show the gating strategy of migratory γδ T cells of the lamina propria of the small intestine (a), blood (b), brain (c), and lung (d). (e) Representative flow cytometric plots show blank and single staining of spleen cells, including live-dead, CD45, CD3 and γδ TCR. SI-LP, lamina propria of the small intestine.

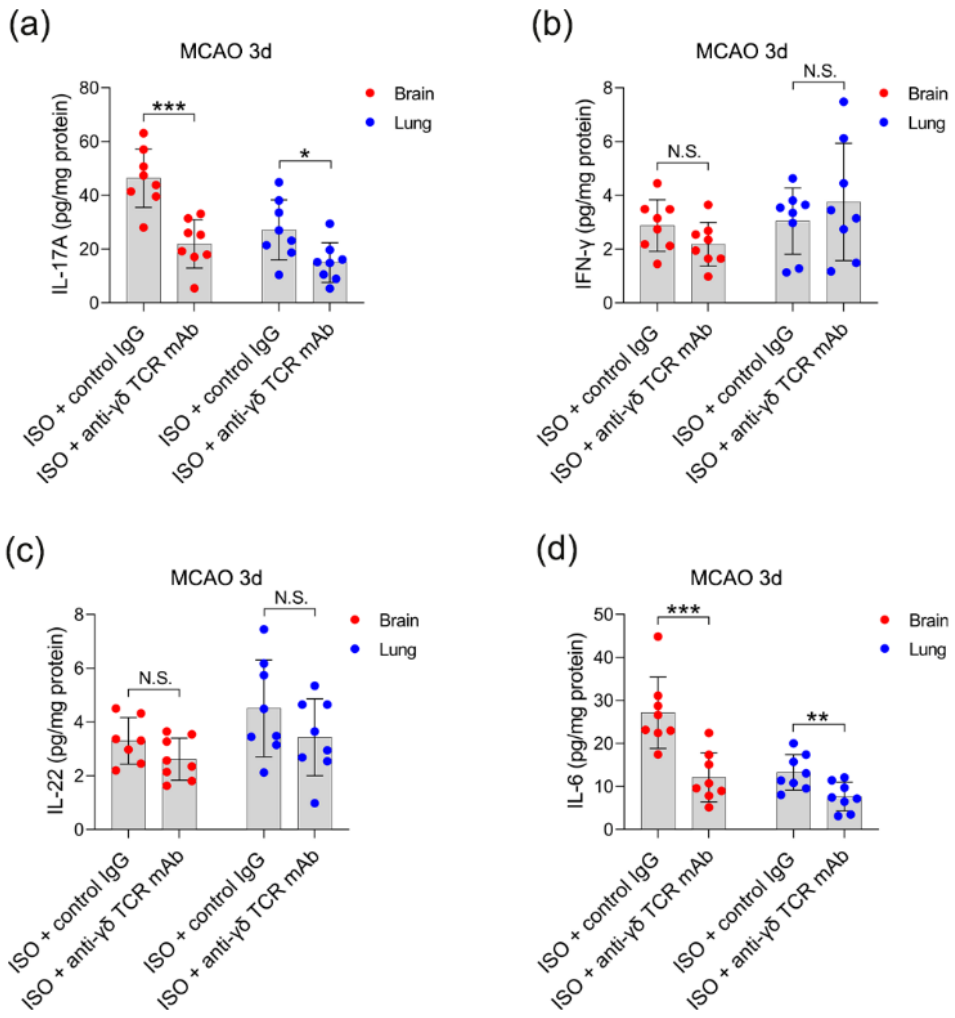

(a)
SI-LP
Photoconverted
Non-photoconverted
Kaede green
Kaede red
FSC-A
γδ TCR
CD3
93.9
1.5
36.3
38.7
23.5
91.7
1.9
20.7
60.7
16.7
36.3
0.3
34.2
9.3
56.2
1.05
3.9
37.8
44.1
14.2
(b)
Brain
86.6
1.1
4.1
93.3
1.5
82.5
1.5
19.7
73.9
4.9
17.3
0.1
25.6
1.7
72.6
1.1
4.6
22.7
54.5
18.2
(c)
Lung
97.3
0.7
10.8
48.0
40.5
95.6
0.9
9.5
40.2
49.4
9.7
0.2
5.9
4.7
89.2
0.7
1.9
7.7
28.3
62.0
(d)
Blood
99.8
1.4
6.1
79.0
13.5
99.9
1.4
16.2
27.4
55.0
19.3
0.8
8.0
65.5
25.7
0.5
0
5.1
5.1
89.8

Supplemental Figure 4. Representative flow cytometric plot of γδ T cells in the main organs of non-photoconverted and photoconverted Kaede-Tg mice. Representative flow cytometric plots show the green-to-red fluorescence conversion and expression of Kaede Red^+ γδ T cells and Kaede $Green^+$ γδ T cells in the small intestine of non-photoconverted and photoconverted Kaede-Tg mice 24 hours post photoconversion or sham surgery (a). Representative flow cytometric plots show the migration of red fluorescence-expressing cells into the main organs and the expression of Kaede Red^+ γδ T cells and Kaede $Green^+$ γδ T cells in the brain (b), lung (c), and blood (d) of non-photoconverted and photoconverted Kaede-Tg mice 24 hours post photoconversion or sham surgery mice. SI-LP, lamina propria of the small intestine.

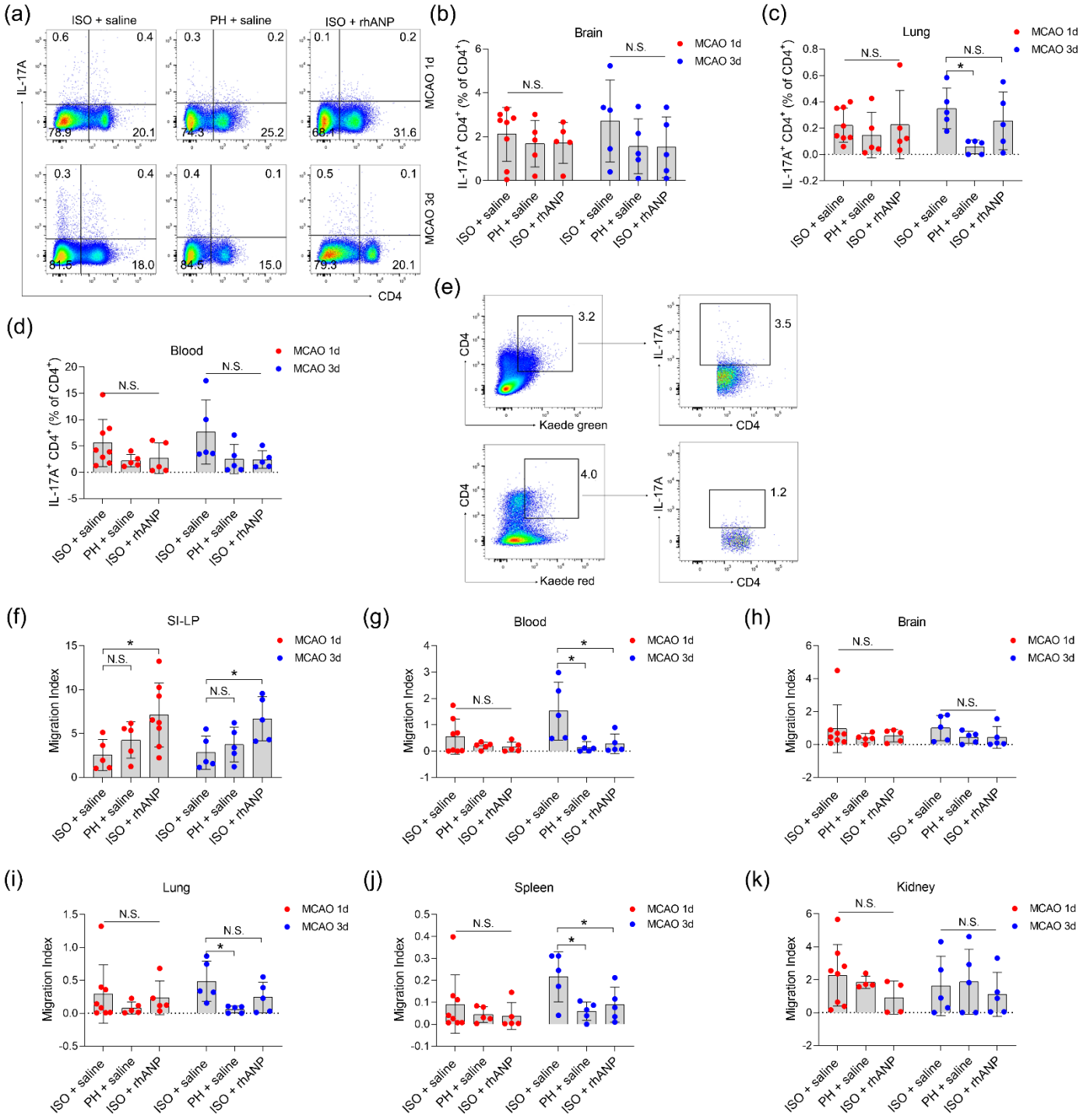


Supplemental Figure 5. Tracking γδ T-cell migration from the small intestine to peripheral organs in Kaede-Tg mice after stroke. (a–d) Representative flow cytometric plots show the migratory γδ T cells of the spleen, liver, kidney and heart (left side), and the graphs show the extent of migration of photoconverted γδ T cells to the liver, kidney and heart (right side). Data are represented as individual values plus the means ± SDs (n = 6–8/group). Comparisons were determined by one-way ANOVA followed by post hoc Newman–

Keuls multiple comparison tests. N.S., not significant, $^{*}P < 0.05$. MCAO, middle cerebral artery occlusion; ISO, isolated housing; PH, pair housing; rhANP, recombinant human atrial natriuretic peptide.



Supplemental Figure 6. The effect of γδ T-cell depletion on cytokines in the brain and lung in ISO stroke mice. Concentrations of IL-17A (a), IFN-γ (b), IL-22 (c) and IL-6 (d) in the brain and lung of ISO mice 72 hours post-stroke. Data are represented as individual values plus the means ± SDs (n = 7–8/group). Comparisons were determined by unpaired Student's t test. N.S., not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ISO, isolated housing; MCAO, middle cerebral artery occlusion; mAb, monoclonal antibody.



Supplemental Figure 7. Tracking Th17 cell migration from the small intestine to peripheral organs in Kaede-Tg mice after stroke. (a) Flow cytometric gating strategy of Th17 cells ($CD4^+$ $IL17A^+$) in the lungs of post stroke mice. (b–d) Flow cytometry analysis of Th17 cells in the brains, lungs and blood of post stroke mice subjected to ISO or PH 24 and 72 hours after MCAO. (e) Representative flow cytometric plots show the migratory Th17 cells in the lungs. (f) The graph shows the extent of migration of photoconverted Th17 cells from the SI-LP. The migration index was calculated as Kaede Red^+ $CD4^+$ $IL17A^+$/Kaede $Green^+$ $CD4^+$ $IL17A^+$, and represented the migration of Th17 cells to other sites. Graphs (g–k) show the extent of

migration of photoconverted Th17 cells to the blood, brain, lung, spleen and kidney. Data are represented as individual values plus the means ± SDs (n = 6–8/group). Comparisons were determined by one-way ANOVA followed by post hoc Newman–Keuls multiple comparison tests. N.S., not significant, $^{*}P < 0.05$. MCAO, middle cerebral artery occlusion; ISO, isolated housing; PH, pair housing; rhANP, recombinant human atrial natriuretic peptide; SI-LP, lamina propria of small intestine.