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Brain ischemia induces diversified neuroantigen-specific T cell responses that exacerbate brain injury

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Supplemental Methods

Human brain sections.

Human brain sections were obtained from the Department of Pathology of the Ohio State University (Columbus, OH) and Banner Boswell Medical Center (Sun City, AZ). The ethics consent was waived by the Institutional Review Board because autopsy tissues were used. Brain tissue was collected within 4 h after death. Among the 10 cases studied, 5 were from patients with ischemic stroke who died within 7-10 days after stroke onset (three males and two females). The locations of stroke lesions were within the cortical areas supplied by the middle cerebral artery. The tissue was collected from the middle cerebral artery field of perfusion. 5 control cases were from individuals without history of neurological or neuropsychiatric diseases who died from non-neurological disease (three males and two females), and the location of selected tissue sections was matched with stroke patients. All included patients have no acute myocardial infarction, heart failure, autoimmune disease, hematological system disease or any infection before stroke at the time of death. Stroke patients and control subjects did not differ significantly in terms of age at death (stroke: 76.6 ± 8.3 years of age; control: 70.8 ± 7.2 years of age; mean ± s. e. m.; $p > 0.05$; unpaired t test).

Mice.

We purchased male C57BL/6 (B6, H-2^b) and Rag$2^{-/-}$ mice from Taconic (Santa Maria, CA). OVA and 2D2 transgenic mice were purchased from The Jackson laboratory (Bar Harbor, ME). All mutant mice were backcrossed to the B6 background for at least 12 generations and were housed in pathogen-free conditions at the animal facilities. For all experiments, 10- to 12-week-old, age-matched male littermates were used between experimental groups. Female mice were not used to avoid any influences of sex steroids. All mice were randomly assigned to each experiment. Mice were housed no more than 5 animals per cage under standardized light-dark cycle conditions with access to food and water ad libitum. All animal experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of Barrow Neurological Institute (Phoenix, AZ), Tianjin Neurological Institute (Tianjin, China) and Beijing Tiantan Hospital (Beijing, China).

Drug administration.

PLX3397 (Selleck Chemicals, Houston, Texas) was prepared and given as we previously described. PLX3397 was dissolved in dimethyl sulfoxide followed by 1:50 dilution with phosphate-buffered saline (PBS). Mice received vehicle (2% DMSO in PBS) or PLX3397 (40 mg/kg) by oral gavage for 21 days prior to MCAO induction. The treatment was continued until the end of experiments.

Middle cerebral artery occlusion procedure.

Adult male 10- to 12-week-old mice were subjected to 60 min focal cerebral ischemia produced by transient intraluminal occlusion of the middle cerebral artery (MCA) using a filament as described previously. MCA occlusion (MCAO) was performed under anesthesia induced by inhalation of 3.5% isoflurane and maintained by inhalation of 1.0–2.0% isoflurane in 70% N_2O and 30% O_2. Body temperature was monitored throughout surgery with a rectal probe and maintained at 37.0 ± 0.5 ℃ using a heating pad (Sunbeam, Neosho, MO, USA). Regional cerebral blood flow (CBF) was monitored for 5 min both before and after MCAO, and immediately before and after reperfusion with a laser Doppler probe (model P10, Moor Instruments, Wilmington, DE, USA). Baseline CBF was measured as relative perfusion units and defined as 100% flow. A monofilament made of 6-0 nylon with rounded tip was used to induce focal cerebral ischemia for 60 min by occlusion of the right MCA. After 60 min of MCAO, the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion. Mice were excluded upon death or non-satisfactory cerebral blood flow (CBF) during occlusion or 10 min after reperfusion. We only included mice that had a residual CBF < 15% throughout the

ischemic period and CBF recovery >80% within 10 min of reperfusion. Thereafter, cerebral blood flow was monitored for a further 10 min before the wound was sutured, and mice were allowed to recover from anesthesia. Sham-operated mice were subjected to the same surgical procedure, but the filament was not advanced far enough to occlude the middle cerebral artery. 7T MRI scanning was employed to measure infarct volume after MCAO (see Neuroimaging). Among the total of 732 mice used in this study, 46 mice were excluded due to death after MCAO surgery, and 39 mice were excluded due to inadequate reperfusion. The total mortality rate of mice subjected to MCAO was ~7% (46 in 614).

Photothrombotic stroke procedure.

Photothrombotic occlusion was performed as previously described. Mice subjected to photothrombotic surgery were anaesthetized by inhalation of 3.5% isoflurane and maintained by inhalation of 1.0–2.0% isoflurane in 70% N_2O and 30% O_2.Body temperature was maintained during surgery at 37 ±0.5 ℃ using a heating pad controlled by a rectal probe. Mice were injected intraperitoneally with rose bengal at a dose of 150 mg/kg (Sigma Aldrich) 5 min prior to illumination. The bregma was then identified and the end of a fiber optic cable with a diameter of 4 mm was placed over the top of the skull rostrocaudally centered on and approximately 2 mm lateral to the bregma. 5 minutes after injection of the Rose Bengal solution a cold KL 1600 LED light source (Schott, Elmsford, NY, USA) with a green bandpass filter (Thor Labs Inc., Newton, NJ, USA) was turned on and the skull was illuminated for 20 min. For the sham group, similar procedure was applied except that rose bengal was replaced with 0.2 ml of 0.9% saline. Among the total of 732 mice used in this study, 4 mice were excluded due to death after photothrombosis surgery. The total mortality rate of mice subjected to photothrombosis was ~5.5% (4 in 72).

Neurological Assessment

Neurological deficit assessment was performed by experimenters blinded to the sham and MCAO groups as we previously described. The modified Neurological Severity Score (mNSS) consisted of motor, sensory, reflex, and balance assessments with the highest possible score being 18. The rating scale was as follows: A score of 13–18 indicates severe injury, 7–12 indicates moderate injury, and 1–6 indicates mild injury. Following surgery, each mouse was assessed on a scale from 0 to 18 after recovery from the MCAO surgical procedure. Mice with score <6 or above a score of 13 at 24 h post MCAO (prior to treatment) were not included in the study.

Neuroimaging.

Infarct size was assessed using a 7T small animal MRI (Bruker Daltonics Inc., Billerica, MA, USA) with a 30-cm horizontal-bore magnet and BioSpecAvance III spectrometer with a 72mm linear transmitter coil and a mouse surface receiver coil for mouse brain imaging, as we described previously. During scans, mice were placed on a regulated-heated blanket (Bruker Daltonics Inc., Billerica, MA, USA) to maintain body temperature at 37.0 ± 0.5 °C. Axial 2D multi-slice T2-weighted images of brain with fat-suppressed Rapid Acquisition with Relaxation Enhancement (RARE) sequence (TR = 4000 ms, effective TE = 60ms, number of average = 4, FOV = 19.2 mm×19.2 mm, matrix size = 192×192).

In order to quantify MIRB signal, we scanned Multislice Gradient Echo (MGE) sequence and acquired T2*-map in the brain. For in vivo T2*-mapping, 10 gradient echoes were acquired (MGE: TR=120 ms, echo time=3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0, 27.0, 30.0 ms, field of view=35.0mm×35.0mm, matrix =128×128, slice thickness=0.5 mm, flip angle 80°). T2* maps were generated by Bruker's built-in software, by fitting the T2* decayed signal curve along echo time series. R2* (R2*=1/T2*) was used to quantify the signal intensity, and relative R2* values were used for comparison among different groups. The mean R2* values were measured within a region of interest (ROI) drawn by a blinded, experienced MR specialist. In the brain, ROIs were drawn around the individual dark spots in five consecutive slices on T2* map with the fixed size of 300 pixels per slice. The MRI data were analyzed with Image J software (National Institutes of Health) as we previously reported.

Cell isolation and passive transfer.

Naïve-like $CD4^+$ T cells ($CD4^+CD44^{low}CD62L^{high}$) were isolated from pooled splenocytes of WT, OVA or 2D2

mice. All mice from which $CD4^+$ T cells were purified had been backcrossed to C57BL/6 background for at least 12 generations. The spleen tissues were separated and placed on a pre-moistened 70-μm cell strainer, and were then gently homogenized with the end of a 1-ml syringe plunger. The strainer was washed with 10 ml erythrocyte lysis buffer (349202, BD FACS™, San Jose, CA, USA), and the eluted cells were incubated for 5 min at room temperature and washed with 40 ml PBS. $CD4^+$ T cells were purified via two rounds of cell sorting selection with the high-speed sort of FACSAria flow cytometer (purity>99%). Purity of sorted naïve 2D2 $CD4^+$ T cells was verified by flow cytometry before transfer. Highly purified (> 99%) $CD4^+$ T cells (1×10^7) were injected via tail veil into $Rag2^{-/-}$ recipient mice at 24 h prior to MCAO or sham operations.

For SPIO-Molday ION Rhodamine-B (MIRB) labeling assay, sorted $CD4^+$ T cells were cultured in RPMI culture medium with 10% FBS (Invitrogen, Grand Island, NY, USA), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (0.1 mg/ml), as well as MIRB (BioPhysics Assay Laboratory, Inc, Worcester, MA, USA) at a concentration of 12.5 μg/ml ex vivo at 5% CO_2/37° C. After MIRB incubation for 24 h, $CD4^+$ T cells were centrifuged (400g for 5 min) and washed twice with PBS to remove extracellular MIRB. MIRB-labeled $CD4^+$ T cells were then purified using FACSAria. 1×10^7 MIRB-labeled $CD4^+$ T cells (> 99% purity) were injected via the tail vein into $Rag2^{-/-}$ recipient mice as we previously described.

CFSE assay.

To test 2D2 $CD4^+$ T cell reactivity to antigen challenge, single cell suspensions were prepared and labeled with 0.5 μM CFSE. Cells labeled with or without CFSE were incubated at 37 ℃ for 3 d in round-bottom plates (2×10^6 cells/well) with or without antigen stimulation (10 μg/ml MOG_{35-55},MOG_{91-108}, $MOG_{103-125}$; OVA was used as a non-brain antigen control). After antigen stimulation, cells were stained with fluorochrome-conjugated anti–CD3-AF647 (145-2C11, 553066, BD Biosciences, San Jose, CA, USA) and anti–CD4-PE/Cy7 (RM4-5, 552775, BD Biosciences, San Jose, CA, USA), and followed by flow cytometry analysis. To test 2D2 $CD4^+$ T cell expansion *in vivo*, naïve 2D2 $CD4^+$ T cells were labeled with 4 μM CFSE and then injected via the tail vein into $Rag2^{-/-}$ recipient mice followed by MCAO. After reperfusion, 2D2 $CD4^+$ T cells were isolated from $Rag2^{-/-}$ recipients for CFSE assay to measure CFSE intensity as we previously described.

For brain APC assay, microglia-like cells ($CD11b^+CD45^{int}$), macrophages ($CD11b^+CD45^{hi}F4/80^+$) and dendritic cells ($CD11c^+CD45^{hi}F4/80^-$) were sorted by FACS from single cell suspension prepared from brain tissues pooled from $Rag2^{-/-}$ mice at day 4 after 60 min MCAO and reperfusion. 5×10^5 individual populations of FACS-sorted APCs were co-cultured in 96-well plates with 5×10^5 FACS-sorted, CFSE-labeled 2D2 $CD4^+$ T cells ($CD4^+CD44^{low}CD62L^{high}$) in either the presence or absence of MOG_{35-55}. At 72 h after coculture, CFSE intensity in 2D2 $CD4^+$ T cells was measured as we previously described.

Tetramer preparation and tetramer staining.

Customized PE-labeled tetramers (Class II MHC Monomers, ProImmune, Sarasota, FL) containing the specific epitopes were used to evaluate surface expression of the antigen-specific TCRs in autoreactive $CD4^+$ T cells. MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) were used to generate the DRB1*1501/MOG_{35-55}or I-A(b)/MOG_{35-55}. MOG_{91-108} (SDEGGYTCFFRDHSYQEE) and $MOG_{103-125}$ (HSYQEEAAVELKVEDPFYWINPG) were used to generate the DRB1*0401 or I-A(b)/MOG_{91-108} and DRB1*0401 or I-A(b)/$MOG_{103-125}$ tetramers. Phycoerythrin–conjugated biotin was used for cross-linking of peptide–MHC II monomers. The splenic or CNS-infiltrated cells were washed with phosphate-buffered saline and then stained with individual tetramers (0.1mg/ml, 5 μL/test) (MOG_{35-55}, MOG_{91-108} and $MOG_{103-125}$ tetramers) at 37 ℃ for 2 hours followed by staining with other antibodies on ice, including antibodies to CD3 (145-2C11, 553066, BD Biosciences, San Jose, CA, USA), CD4 (RM4-5, 552775, BD Biosciences, San Jose, CA, USA), CD8 (53-6.7, 557654, BD Biosciences, San Jose, CA, USA).The subsequent washing and cell sorting were then performed at 4 ℃.

Flow cytometry.

Quantitative analysis of immune cell subsets and functional marker expressions were prepared from spleen or brain tissue and stained with fluorochrome-conjugated antibodies as described. All antibodies were purchased from BD or eBioscience, Inc. unless otherwise indicated. Antibodies were labeled with one of the following

fluorescent tags: fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCP-Cy5.5, or allophycocyanin (APC). The following antibodies were used in this study: CD3 (145-2C11, 553066, BD Biosciences, San Jose, CA, USA), CD4 (RM4-5, 552775, BD Biosciences, San Jose, CA, USA), CD8 (53-6.7, 557654, BD Biosciences, San Jose, CA, USA), IFN-γ (XMG1.2, 557649, BD Biosciences, San Jose, CA, USA), CD69 (H1.2F3, 561932, BD Biosciences, San Jose, CA, USA), CD25 (OX-39, 554866, BD Biosciences, San Jose, CA, USA), CD45 (30-F11, 12-0451-83, eBioscience, San Diego, CA, USA), CD11b (M1/70, 25-0112-82, eBioscience, San Diego, CA, USA), F4/80 (BM8, 123119, Biolegend, San Diego, CA, USA), GFAP (GA5, 50-9892-82, eBioscience, San Diego, CA, USA), Vα 3.2 (RR3-16, 11-5799-82, eBioscience, San Diego, CA, USA), Vβ 11 (RR3-15, 139004, Biolegend, San Diego, CA, USA), MHC II (I-A/I-E, M5/114.15.2, 562564, BD Biosciences, San Jose, CA, USA). Flow cytometric measurements were performed on a FACSAria (BD Biosciences, San Jose, CA, USA) and analyzed using FACSDiva and Flowjo 7.6 software (Informer Technologies, Ashland, OR, USA).

To determine TCR Vβ usage, we used a mouse VβTCR screening panel (BD Pharmingen, San Jose, CA, USA), according to the manufacturer's instructions. Briefly, after collecting blood and tissue leukocytes, each sample was divided into15 separate FACS tubes and stained for the Ab mixture, including 1 of 15 respective TCR VβFITC-conjugated mAbs. Flow cytometric data were collected on a FACSAria flow cytometer and analyzed with Diva software.

ELISA.

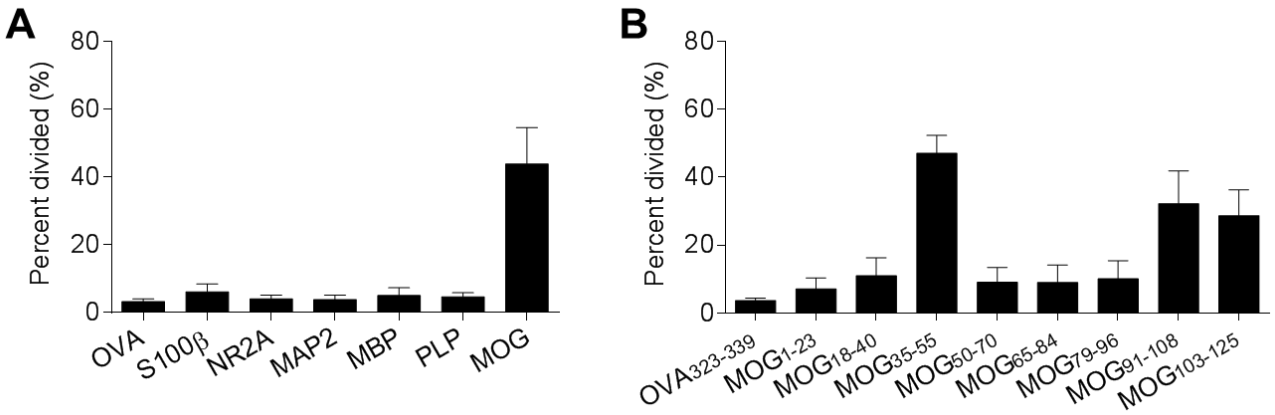
Cytokines in brain tissue homogenates were measured using a Multi-Analyte ELISArray kit (SA Biosciences, Valencia, CA, USA), IL-2 was detected using mouse IL-2 ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA). Protein homogenates were extracted from mouse brains using the Halt Protease Inhibitor Cocktail kit (Thermo Fisher Scientific, Fremont, CA, USA) and centrifuged at 13,000 rpm for 20 min at 4 ℃. Thereafter, supernatants were collected and stored at −80 ℃ until the assay. The levels of cytokines were detected simultaneously at 1:20 dilutions according to the manufacturer's instructions, and reactions analyzed at a wavelength of 450 nm using a 96-well microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry.

For the human tissues, sections were incubated with primary antibodies against human CD4 (1F6, ab846, Abcam, Cambridge, MA, USA), βIII Tubulin (2G10, ab78078, Abcam, Cambridge, MA, USA), CD69 (CH11, Abcam, Cambridge, MA, USA), IFN-γ (H-145, Santa Cruz, Santa Cruz Biotechnology, CA, USA) or IL-17 (ab79056, Abcam, Cambridge, MA, USA) at 4°C overnight, and then incubated with appropriate fluorochrome-conjugated secondary antibodies. For mouse tissues, incubated with primary antibodies against mouse CD4 (GK1.5, 14-0041-85, eBioscience, San Diego, CA, USA), Iba1 (ab15690, Abcam, Cambridge, MA, USA), MOG_{35-55} (PA5-19602, Invitrogen, Grand Island, NY, USA), at 4°C overnight, and then incubated with appropriate fluorochrome-conjugated secondary antibodies for 2 h at room temperature. Images were acquired on a fluorescence microscope (Olympus, model BX-61, Center Valley, PA, USA).

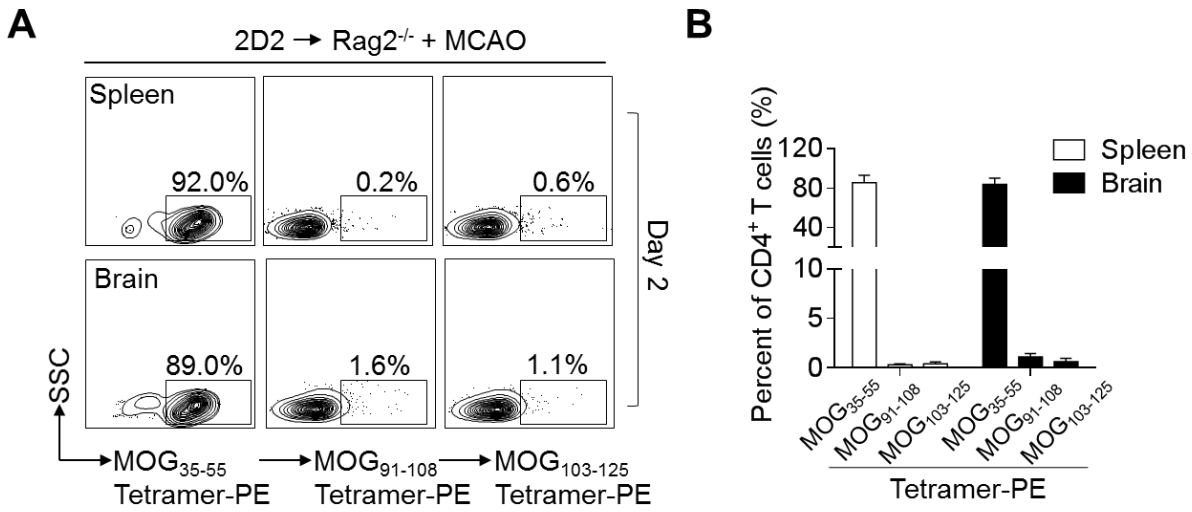
Statistical analyses.

The sample size was determined by power analysis using a significance level of $\alpha = 0.05$ with 80% power to detect statistical differences. Power analysis and sample size calculation were performed using SAS 9.1 software (SAS Institute Inc. Cary, NC). The experimental design was based on previous publications with similar mechanistic studies. The exclusion criteria are described in the individual method section. Animals were randomly assigned to treatment condition. Randomization was based on the random number generator function in Microsoft Excel software. All results were analyzed by investigators blinded to the treatment. Data are presented as the means ±s.e.m. Statistical significance was determined by the two-tailed unpaired Student's *t*-test for two groups, one-way analysis of variance (ANOVA) followed by Tukey post-hoc test for three or more groups, or two-way ANOVA accompanied by Bonferroni post hoc test for multiple comparisons. Values of $p < 0.05$ were considered significant. All statistical analyses were performed using Prism 6.0 software (GraphPad, San Diego, CA).

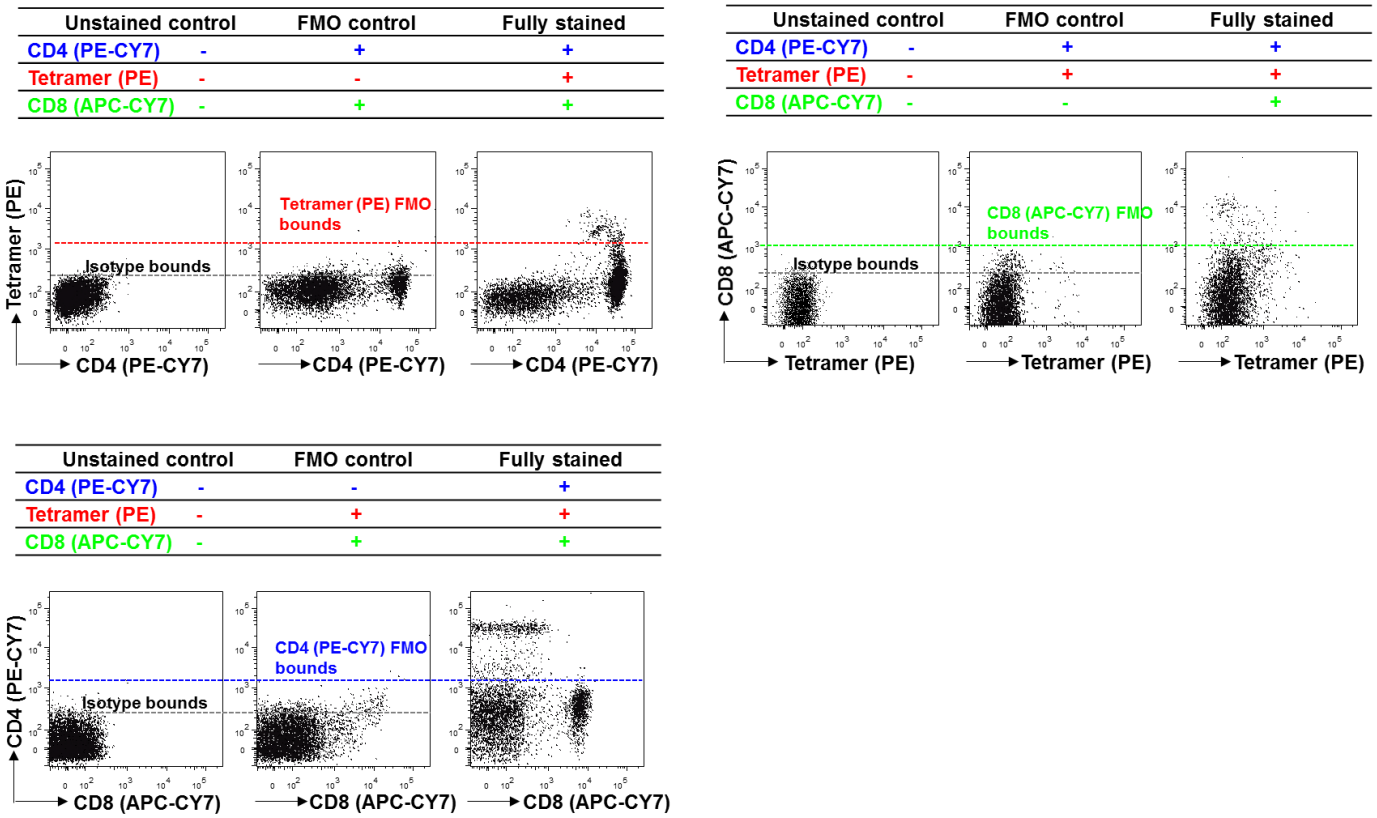


Supplemental Figure I. Responses of brain-infiltrating 2D2 T cells to brain antigen proteins or peptides after ischemia.

2D2 $CD4^+$ T cells were sorted from naïve 2D2 mice without signs of CNS autoimmune disease, and i.v. transferred into $Rag2^{-/-}$ mice followed by 60 min MCAO and reperfusion. At day 4 after MCAO, 2D2 $CD4^+$ T cells were re-isolated from the brain of $Rag2^{-/-}$ recipients. **A.** Bar graph shows CFSE dilution of 2D2 $CD4^+$ T cells in response to *in vitro* stimulation with mouse brain antigen proteins (OVA, S100β, NR2A, MAP2, MBP, PLP and MOG) after co-cultured with APCs for 72 h. **B.** Bar graph shows CFSE dilution of 2D2 $CD4^+$ T cells in response to *in vitro* stimulation with mouse OVA or MOG peptides ($OVA_{323-339}$, MOG_{1-23}, MOG_{18-40}, MOG_{35-55}, MOG_{50-70}, MOG_{65-84}, MOG_{79-96}, MOG_{91-108}, $MOG_{103-125}$) after co-cultured with APCs for 72 h. $n = 9$ per group.

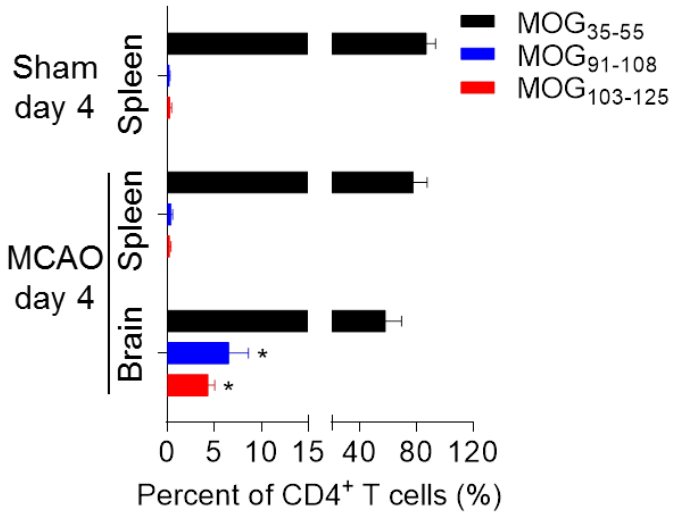


Supplemental Figure II. 2D2 T cell reactivity against neuroantigen epitopes at day 2 after brain ischemia. 2D2 $CD4^+$ T cells ($CD4^+CD44^{low}CD62L^{high}V\alpha3.2^+V\beta11^+$) were sorted from naïve 2D2 mice without any signs of CNS autoimmune disease, and transferred i.v. into $Rag2^{-/-}$ mice before sham or 60 min MCAO surgery. At day 2 after surgery, 2D2 $CD4^+$ T cells were re-isolated from spleen and brain of $Rag2^{-/-}$ recipients, and stained with PE-labeled MHC class II tetramers containing MOG_{35-55}, MOG_{91-108}, or $MOG_{103-125}$. Representative flow cytometry plots (**A**) and bar graph (**B**) show MOG epitope-specific T cells. *n* = 8 per group.



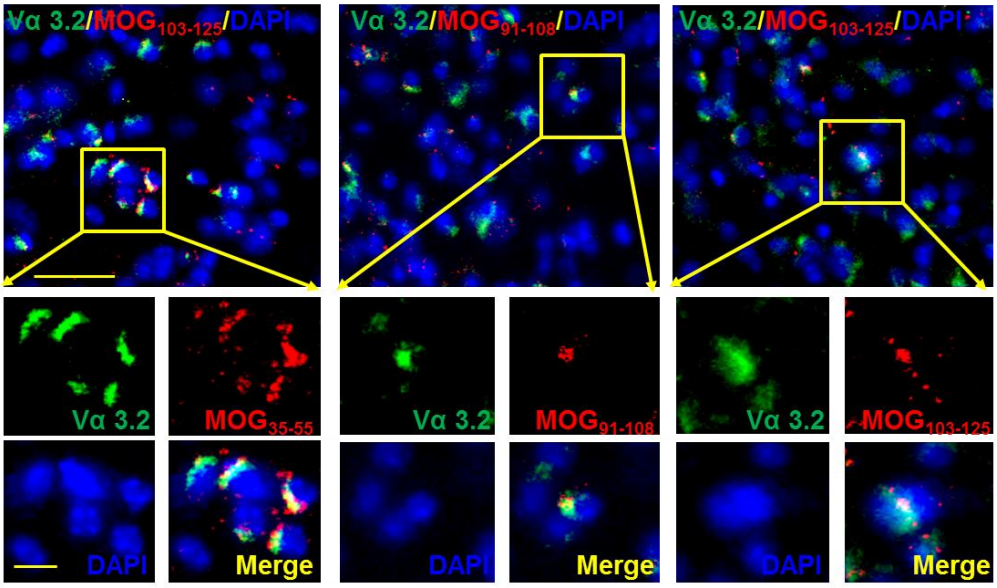
Supplemental Figure III. Fluorescence minus one (FMO) controls for the identification of tetramer-PE in CD4$^+$ T cells.

Single cell suspensions were prepared and sorted for CD3$^+$ cell population from spleen tissues of naïve 2D2 mice, and stained with fluorochrome-conjugated antibodies. Representative flow cytometry dot plots show the identification of gating boundaries of CD4 (PE-CY7), CD8 (APC-CY7) and Tetramer (PE) with corresponding FMO controls.

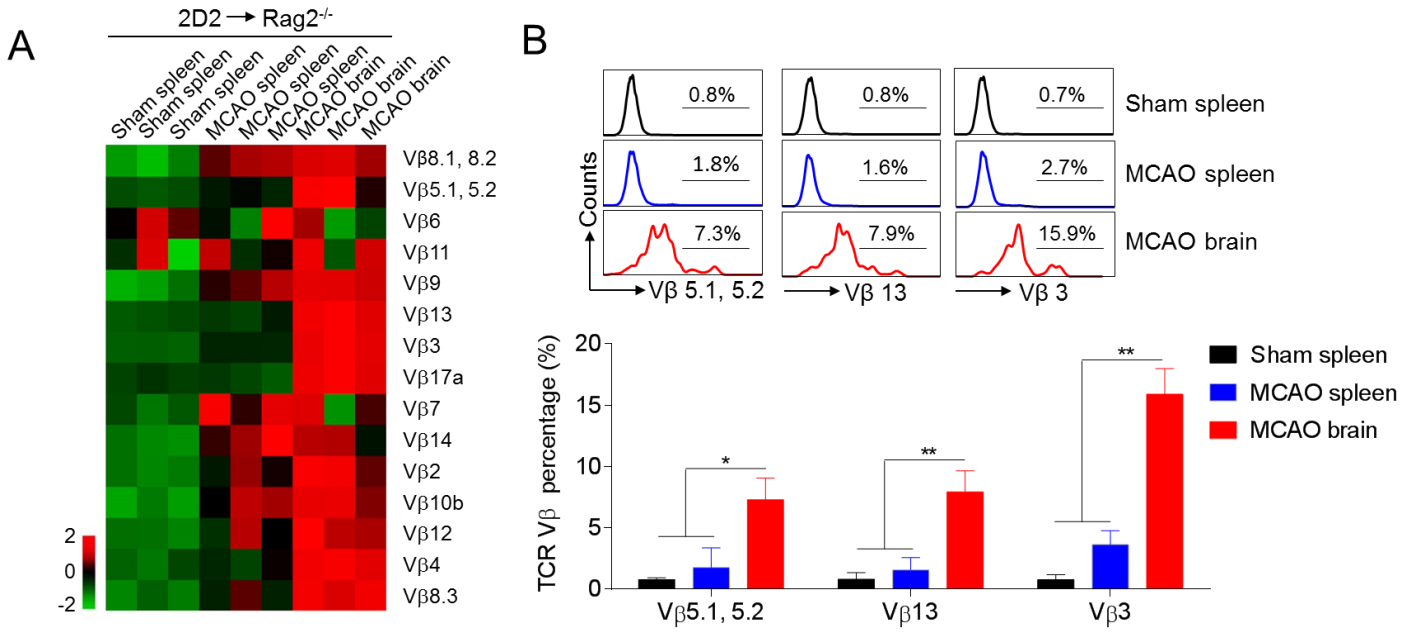


Supplemental Figure IV. The impact of Rag2-/- mice in the effect of brain ischemia on 2D2 T cell response in vivo.

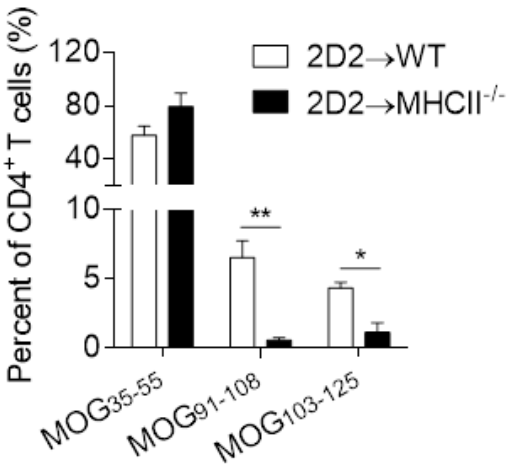
2D2 $CD4^+$ T cells were sorted from naïve 2D2 mice without any sign of CNS autoimmune disease, and transferred i.v. into wild type (C57) mice following sham or 60 min MCAO surgery. After re-isolation at day 4 after surgery, 2D2 $CD4^+$ T cells were stained with PE-labeled MHC class II tetramers containing MOG_{35-55}, MOG_{91-108}, or $MOG_{103-125}$. In sham group, 2D2 $CD4^+$ T cells were re-isolated at day 4 after operation. The bar graph showed MOG epitope-specific T cells by flow cytometry. Error bars represent s.e.m. *$p< 0.05$.



Supplemental Figure V. MOG epitope-specific 2D2 CD4$^+$ T cells in the brain after cerebral ischemia. 2D2 CD4$^+$ T cells were isolated from splenocytes of naïve 2D2 mice without sign of CNS autoimmune disease, then were adoptively transferred into Rag2$^{-/-}$ mice followed by 60 min MCAO. At day 4 after MCAO and reperfusion, Rag2$^{-/-}$ mice were sacrificed and brain sections were stained with antibody against Vα 3.2 and PE-labeled MHC II tetramers containing MOG_{35-55}, MOG_{91-108}, or $MOG_{103-125}$ epitope. Representative images show indicated MOG epitope-specific CD4$^+$ T cells in brain sections. Vα 3.2$^+$ 2D2 T cells (Green, Vα 3.2$^+$) were seen co-localized with MHC II tetramers (Red, MOG_{35-55}-Tetramer-PE, MOG_{91-108}-Tetramer-PE, or $MOG_{103-125}$-Tetramer-PE) in the peri-infarct area. Scale bars: 20 μm, 10 μm (inset).

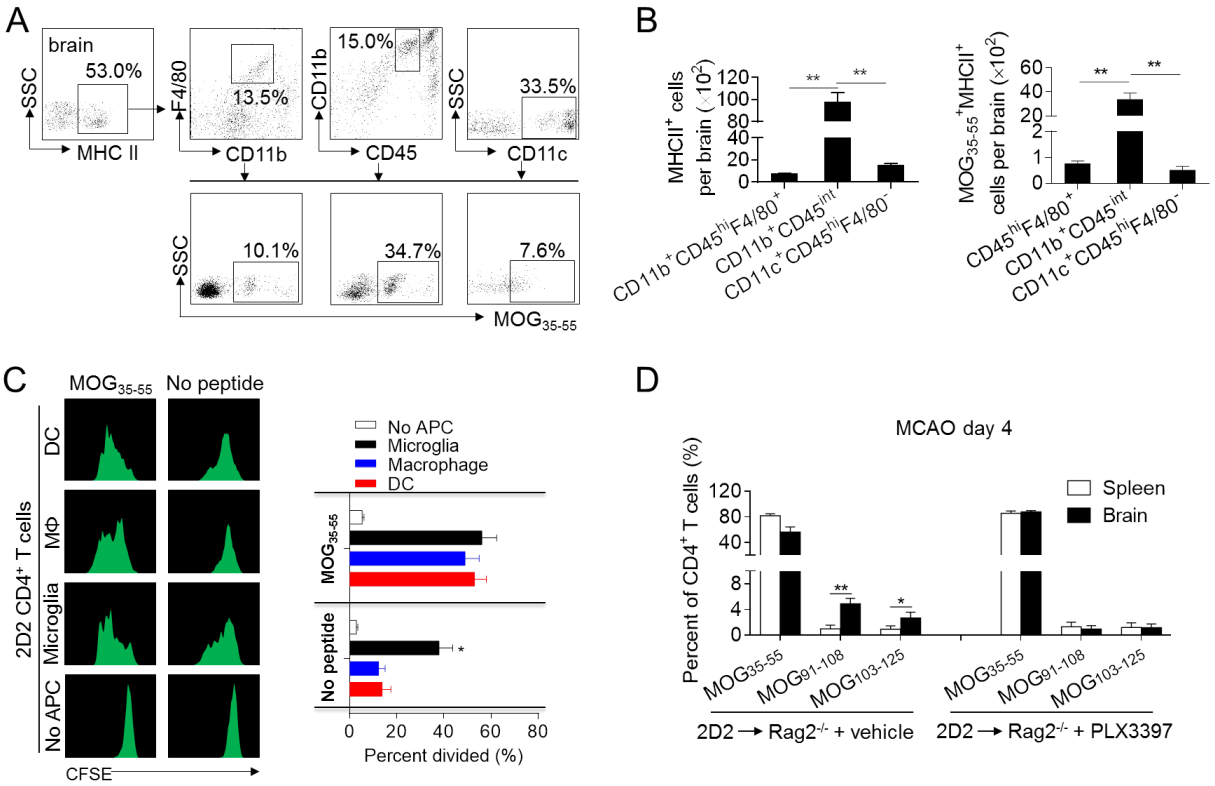


Supplemental Figure VI. TCRvβ usage in brain-infiltrating CD4$^+$ T cells from 2D2 mice after MCAO. 2D2 CD4$^+$ T cells were isolated from splenocytes of 2D2 mice without any sign of CNS autoimmune disease and adoptively transferred into Rag2$^{-/-}$ mice followed by sham or 60 min MCAO procedures. At day 4 after sham or MCAO, 2D2 CD4$^+$ T cells were re-isolated from spleen or brain of Rag2$^{-/-}$ recipients. **A.** A mouse Vβ TCR screening panel containing 15 FITC-conjugated mAbs was used to assess TCR Vβ usage in 2D2 CD4$^+$T cells from spleens and brains of MCAO or Rag2$^{-/-}$ mice adoptively transferred with 2D2 CD4$^+$ T cells. Heat map of the Vβ TCR panel from 2D2 CD4$^+$ T cells after resorting from MCAO or control Rag2$^{-/-}$ mice. Heat maps were generated using Z-score transformed within each data set. Red shades represent increased expression of Vβ TCR whereas green shades represent decreased expression. Results were from three independent experiments. **B.** Representative histograms and bar graph display percentages of each Vβ expression level with significant changes within the 15 subfamilies as compared to sham spleen as control. All gates were set using FMO controls. n = 10 per group. Error bars represent s.e.m. *p< 0.05, **p< 0.01.

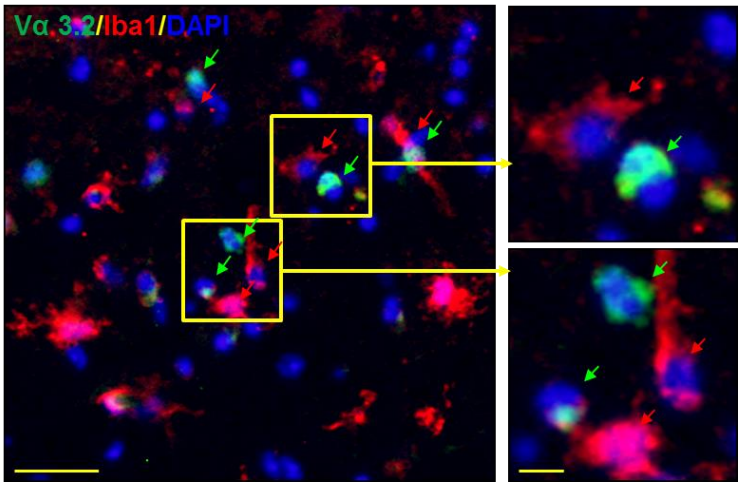


Supplemental Figure VII. The reactivity of 2D2 CD4$^+$ T cells after transfer into wild type mice as compared to MHCII$^{-/-}$ recipient mice followed by brain ischemia.

2D2 CD4$^+$ T cells were sorted from spleens of 2D2 mice, and transferred i.v. into wild type or MHCII$^{-/-}$ recipient mice before 60 min MCAO surgery. Bar graph shows quantification of surface expression of antigen-specific TCRs at day 4 after reperfusion. Error bars represent s.e.m. *p< 0.05, **p< 0.01.

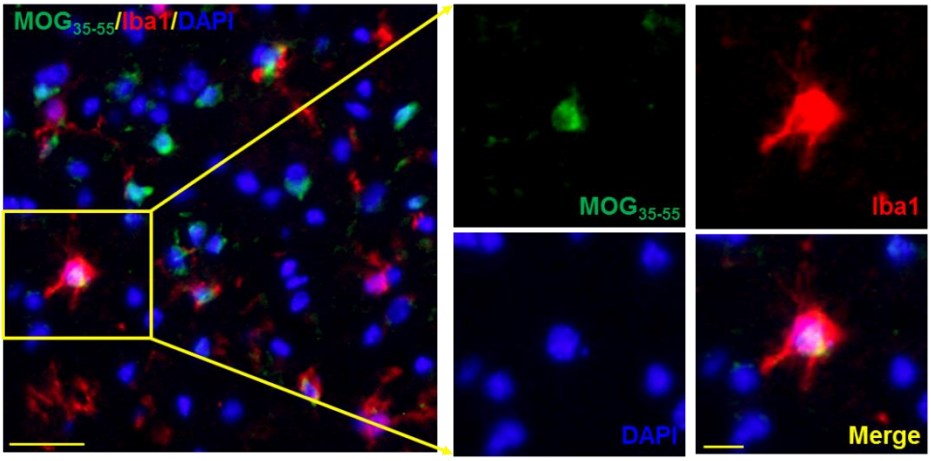


Supplemental Figure VIII. Microglia-like cells are APCs expressing MHCII after brain ischemia.
A. Brain cell suspensions were prepared from Rag2$^{-/-}$ mice at day 4 after MCAO. Gating strategy shows macrophages (CD11b^{+}CD45^{hi}F4/80^{+}), microglia-like cells (CD11b^{+}CD45^{int}) and dendritic cells (DCs; CD11c^{+}CD45^{hi}F4/80^{-}). Representative flow cytometry plots show expression of MHCII and MOG_{35-55} in gated APC populations. **B.** Bar graph shows quantification of MHC class II or MOG_{35-55} expression in gated APC populations. *n* = 8 per group. **C.** FACS-purified macrophages, microglia-like cells, and DCs were obtained from brains pooled from Rag2$^{-/-}$ mice at day 4 after MCAO per group. Thereafter, individual populations of APCs were co-cultured with FACS-sorted, CFSE-labeled 2D2 CD4^{+} T cells (1:1 ratio) in either the presence or absence of MOG_{35-55}. At 72 h after co-culture, CFSE dilution was measured to assess the expansion of 2D2 CD4^{+} T cells. Flow cytometry plot and cumulative bar graph showing CFSE dilution. *n* = 8 per group. **D.** PLX3397 was used to deplete microglia. Rag2$^{-/-}$ mice received PLX3397 via oral gavage for 21 days, and then 2D2 CD4^{+} T cells from 2D2 mice were transferred i.v. into Rag2$^{-/-}$ mice prior to MCAO surgery. The mice continued to receive PLX3397 until sacrifice at day 4 after reperfusion. At that time, 2D2 CD4^{+} T cells were re-isolated from brain or spleen of Rag2$^{-/-}$ mice and stained with PE-labeled tetramers containing MOG_{35-55}, MOG_{91-108}, or $MOG_{103-125}$ epitope. Flow cytometry assessment shows quantifications of MOG epitope-specific 2D2 CD4^{+} T cells. *n* = 10 per group. Error bars represent s.e.m. *p< 0.05, **p< 0.01.

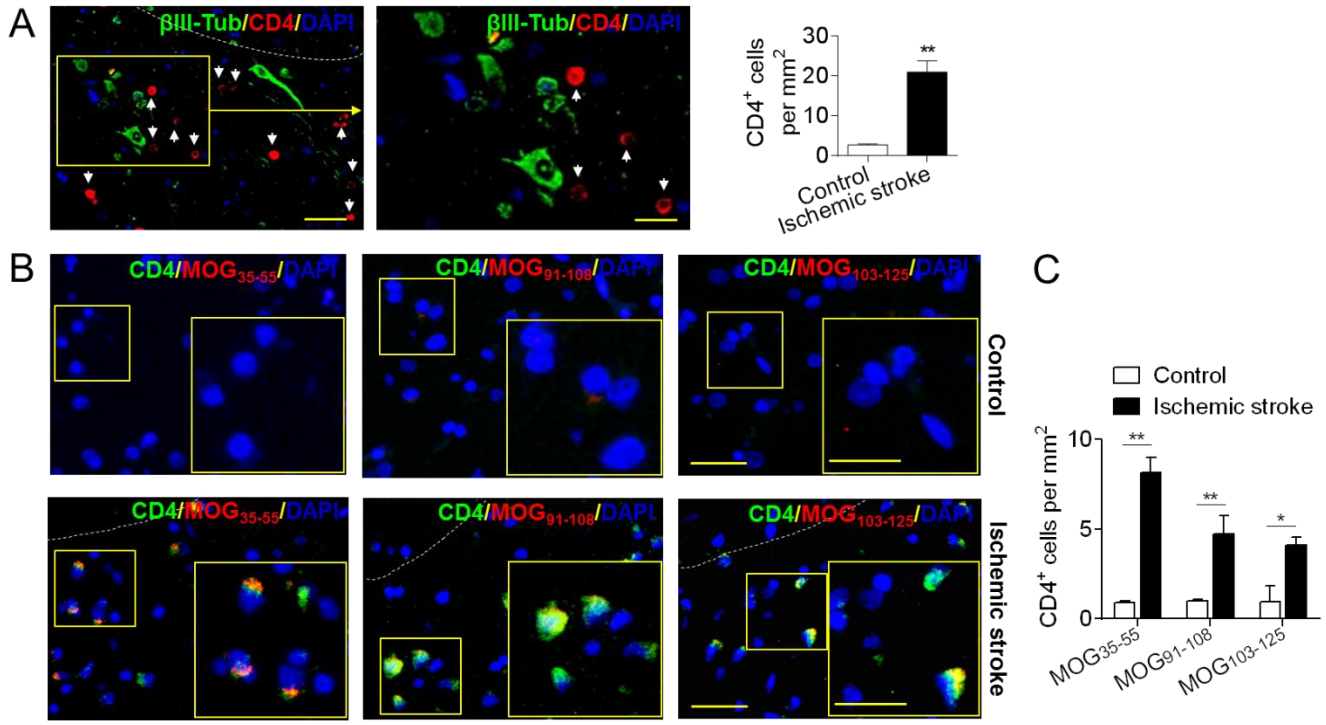


Supplemental Figure IX. Co-localization of 2D2 CD4$^+$ T cells with Iba1$^+$ cells in the brain after cerebral ischemia.

2D2 CD4$^+$ T cells were isolated from splenocytes of naïve 2D2 mice without sign of CNS autoimmune disease, then were adoptively transferred into Rag2$^{-/-}$ mice followed by 60 min MCAO. At day 4 after MCAO and reperfusion, Rag2$^{-/-}$ mice were sacrificed and brain sections were stained with antibodies against Vα 3.2 and Iba1. Representative images show some of 2D2 CD4$^+$ T cells (Green, Vα 3.2$^+$) resided in close proximity to Iba1$^+$ cells (Red, Iba1$^+$) in the peri-infarct area. Scale bars: 20 μm, 10 μm (inset).



Supplemental Figure X. Co-localization of MOG_{35-55} with $Iba1^+$ cells in the brain after cerebral ischemia. 2D2 $CD4^+$ T cells were isolated from splenocytes of naïve 2D2 mice without sign of CNS autoimmune disease, then were adoptively transferred into $Rag2^{-/-}$ mice followed by 60 min MCAO. At day 4 after MCAO and reperfusion, $Rag2^{-/-}$ mice were sacrificed and brain sections were stained with antibodies against MOG_{35-55} and Iba1. Representative images show MOG_{35-55}^+ cells (Green, MOG_{35-55}^+) co-localized with $Iba1^+$ cells (Red, $Iba1^+$) in the peri-infarct area of 2D2 mice 4 days after MCAO. Scale bars: 20 μm, 10 μm (inset).



Supplemental Figure XI. Presence of MOG-reactive T cells in patients with ischemic stroke.

A. Immunostained images show $CD4^+$ T cells (red, $CD4^+$) in close proximity of ischemic neurons (green, βIII-tubulin$^+$) in the peri-infarct in brain sections from an ischemic stroke patient (at day 7 after onset). Bar graph shows quantification of $CD4^+$ T cells from ischemic stroke patients (at day 7-10 after onset). **B-C.** Brain sections from patients with ischemic stroke or non-neurological controls were stained with anti-$CD4^+$ mAb and PE-labeled MHC class II tetramers containing MOG_{35-55}, MOG_{91-108}, or $MOG_{103-125}$ epitope. Immunostaining images show $CD4^+$ T cells (green, $CD4^+$) colocalizing with MHC class II tetramers (red, tetramer-PE) in the peri-infarct area of the brain of ischemic stroke patients (at day 7-10 after onset) (**B**). Bar graph shows quantification of MHC class II tetramer$^+CD4^+$ T cells in the peri-infarct area of the the brain of ischemic stroke patients (at day 7-10 after onset) (**C**). Scale bars: (**A**) left: 50 μm, right: 20 μm; (**B**) 40 μm, inset 20 μm. In **A**-C, n = 10 sections from 5 patients per group. Error bars represent s.e.m. *p< 0.05, **p< 0.01.

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	▣ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ▣ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ▣ An overall study timeline is provided.
Inclusion and exclusion criteria	▣ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	▣ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ▣ Type and methods of randomization have been described. ▣ Methods used for allocation concealment have been reported.
Blinding	▣ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ▣ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	▣ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	▣ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ▣ Baseline data on assessed outcome(s) for all experimental groups have been reported. ▣ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ▣ Statistical methods used have been reported. ▣ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	▣ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ▣ Different sex animals have been used. If not, the reason/justification is provided. ▣ Statements on approval by ethics boards and ethical conduct of studies have been provided. ▣ Statements on funding and conflicts of interests have been provided.