**SUPPLEMENTAl MATERIAL**

**Neuronal CD200 Signaling is Protective in the Acute Phase of Ischemic Stroke.**

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## Supplemental Methods

### Ischemic stroke model

All mice were anesthetized with isoflurane (4% for induction and 1.5–2% for maintenance). A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a 6.0 mm monofilament (Doccol Corp, Redlands, CA, USA) into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Reperfusion was performed by withdrawing the suture 60 min after the occlusion. All the mice were sacrificed at 3 and 7 days of reperfusion. Sham-operated animals underwent the same surgical procedure, but the suture was not advanced to the middle cerebral artery. Rectal temperatures were maintained at approximately 37 °C during surgery and ischemia with an automated temperature control feedback system (TC 1000 mouse, CWE Inc., USA).

### CD200fc fusion protein administration

CD200Fc fusion protein (CD200 Fc) and control mouse IgG2a (IgG control) were administered in C57BL6 (The Jackson Laboratory) stroke mice at a dose of 100 µg/100 µL subcutaneously every 12h interval starting from 24h prior stroke surgery and continued for 3 days after reperfusion as described previously.[61] To investigate the stroke outcomes, mice were sacrificed at 3 days of reperfusion.

### Behavior testing

<u>Neurological deficit scores</u>

Neurological deficit scores (NDS) were recorded by a 4-point scale: 0-no deficit; 1-forelimb weakness, torso turning to the ipsilateral side when held by the tail; 2-circling to the affected side; 3-unable to bear weight on affected side and 4-no spontaneous activity or barrel rolling.

<u>Corner test</u>

Sensorimotor activity was measured by the corner test as described previously.[1, 2] Briefly, the mouse entered a corner that was made by moving two card board pieces at an angle of 30 degrees in front of the nose. Contact with the vibrissae led to a rear and the direction in which the mouse turned was recorded. Normal mice do not exhibit a turning preference, but after ischemia, mice have a turning preference to the non-impaired side. The percentage of right turns was calculated for twenty trials in each sitting. The corner test has been used to detect both sensory and motor abnormalities in the stroke model in young animals.

<u>Y-maze test</u>

Spontaneous alternation using a Y-maze is a test for habituation and spatial working memory[62]. The symmetrical Y-maze consists of three white opaque plastic arms at a 120° angle from each other. After placing pups in the center, the animal is allowed to freely explore the three arms. Over the course of multiple arm entries, the subject should show a tendency to enter a less recently

visited arm. The test consists of a single 5 min trial; spontaneous Alternation (%) is defined as consecutive entries in 3 different arms (arm A,B,C), divided by the number of total alternations (total arm entries minus 2).[62] Mice with less than 8 arm entries during the 5-min trial were excluded from the analysis. An entry occurs when all four limbs are within the arm. All the arms were cleaned between each trial.

**Tissue harvesting and flow cytometry (FC)**

The ipsilateral hemispheres were placed in complete Roswell Park Memorial Institute (RPMI) 1640 (Lonza) medium, followed by mechanical and enzymatical digestion with 150 µL collagenase/dispase (1 mg/mL) and 300 µL DNAse (10 mg/mL; both Roche Diagnostics) for 45 minutes at 37 °C with mild agitation. The cell suspension was filtered through a 70µm filter. Leukocytes were harvested from the interphase of a 70%/30% Percoll gradient. Cells were washed and blocked with mouse Fc Block (eBioscience) prior to staining with primary antibody conjugated fluorophores: CD45-eF450 (# 48-0451-82, eBioscience), CD11b-AF700 (# 101222, Biolegend), Ly6C-APC-eF780 (#47-5932-82, eBioscience), Ly6G-PE (#127608, Biolegend), CD68-APC (#107614, Biolegend) and CD206-PE-cy5.5(#141720, Biolegend). For each surface marker, 0.25 µg (1:100) of antibody was used to stain 1× 106 cells. All the antibodies were commercially purchased from Biolegend/eBioscience. For live/dead cell discrimination, a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was used according to manufacture instruction (Thermo Fisher Scientific, MA, USA). Cells were briefly fixed in 2% paraformaldehyde (PFA). Data were acquired on a CytoFLEX S (Beckman Coulter) and analyzed using FlowJo (Treestar Inc.). No less than 100,000 events were recorded for each sample. Cell type-matched fluorescence minus one (FMO) controls were used to determine the positivity of each antibody.

**Intracellular cytokine staining**

For intracellular cytokine staining, an ex vivo Brefeldin A protocol was followed. Prior to staining, brain leukocytes were incubated with BFA (10 µg/mL, Sigma) in 1 mL complete RPMI for 2 h at 37 °C (5% CO2). Afterward, cells were resuspended in Fc Block, stained for surface antigens and washed. The cells were incubated in 100 µL of fixation/permeabilization solution (BD Biosciences) for 20 min, then washed twice in 300 µL 1x permeabilization/wash buffer (BD Biosciences), resuspended in an intracellular antibody cocktail (0.25 µg for each antibody, 1:100 dilution) containing TNFα-PE-Cy7 (# 25-7321-82, eBioscience) and IL-1β-PE (# 12-7114-82, eBioscience), IL-10-APC (# 506904, BioLegend) and IL-4-PerCP-Cy5.5 (# 504124, BioLegend) and subsequently fixed with 2% PFA.

**Phagocytosis assay**

Prior to blocking in FC procedure (above), *Escherichia coli* (K-12 strain) BioParticles™, fluorescein conjugate (# E2861, Sigma-Aldrich) were added to the isolated microglia ex *vivo* in a final concentration of 0.05mg/mL as described previously.[10] After 1 h incubation at 37 °C with periodic agitation, the cells were washed three times with FACS buffer, re-suspended in FACS buffer, stained for microglia surface markers, and fixed in PFA.

**Immunohistochemistry**

On the day of tissue collection, all the mice were anesthetized by Avertin® and then transcardially perfused with 0.1M sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde for post-fixation of the brains. Immunohistochemical staining of fixed-frozen sections (30µm-thickness) was performed as described previously.[13] Briefly, the brains were cut and mounted onto gelatin-coated slides and allowed to air dry. The sections were then blocked in 3% BSA-0.1M PBS with 10% donkey serum (Sigma) for 2 hours and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-NeuN (1:500, R&D Systems, MN, USA), rat anti-CD200 (1:300, abcam, USA), anti-von Willebrand Factor (VWF, sc-365712, Santa Cruz biotechnology Inc, TX, USA). After being washed in TBS+0.025% Triton X-100, the sections were incubated with the indicated secondary antibodies for 1 h. The following secondary antibodies were used: donkey anti-rabbit IgG Alexa Fluor 488 conjugate (R37118; 1: 500, Invitrogen, USA), donkey anti-mouse IgG Alexa Fluor 488 conjugate (A21202; 1: 500, Invitrogen), and donkey anti-rat IgG Alexa Fluor 594 conjugate (A21209; 1: 500, Invitrogen). The nuclei were stained with DAPI (S36939, Invitrogen). Brain images were obtained on Leica DMi8 confocal microscope.

**Assessment of brain tissue infarct by cresyl violet (CV) staining**

At day 3 after reperfusion, all the animals were anesthetized with tribromoethanol (Avertin® ip injection at a dose of 0.25mg/g body weight). Animals were perfused transcardially with ice cold 0.1M sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde (PFA); the brain was removed from the skull and post-fixed for O/N in 4% PFA and subsequently placed in cryoprotectant solution (30% sucrose). The brain was cut into 30-µm free-floating coronal sections on a freezing microtome and the sections were put into a 96 well plate with anti-freezing media. Eight brain sections were chosen from the middle row of the plate (row D and well #1, 3, 4, 5, 6, 7, 9, and 11) and were mounted on glass slide and stained by CV for evaluation of ischemic damage. CV staining of fixed-frozen sections and brain infarct analysis were performed as described previously.[14, 15]

**TUNEL staining**

To assay apoptotic cell death, cells were stained with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay kit (*In Situ* Cell Death Detection Kit, Fluorescein, Millipore Sigma) according to manufacture instruction and counterstained using 4',6-diamidino-2-phenylindole (DAPI). TUNEL-positive cells in in the outer boundary zone of the peri-infarct area were counted by an observer who was blinded to the treatment group.

**Primary Cortical Neuron Culture**

Cultures of cortical neurons from E15–16 embryonic C57BL/6 mouse (Jackson Laboratory) were prepared as described previously.[19, 20] Briefly, cortical tissue isolated from 8-10 E15-16 mice were dissected into small pieces and incubated at 37 °C with 5% trypsin/EDTA followed by trituration with siliconized Pasteur pipettes. Trypsin activity was stopped by adding 1 mL of FBS. The cells were then passed through a 40µm filter to ensure single cell population. The cells were counted and plated on poly-d-lysine (50 µg/ml; Sigma)-coated round glass slide in 12W culture plates with 500mL of DMEM medium without FBS. After 24h, culture medium was replenished with Neurobasal media supplemented with 1% B27 (ThermoFisher Scientific) with L-glutamate and 1% PS. The microtubule-associated protein 2 (MAP2; Santa Cruz Biotechnology, USA)-

immunocytochemical staining showed that the purity of primary neurons was over 97%. Half of the medium was replaced every 3 days. The primary neurons were used at 10 days of culture.

**Splenic CD3$^+$ T cells isolation**

CD3+ T cells were isolated from a 7-week old naïve male C57BL/6 mouse using the MojoSort™ Mouse CD3 T Cell Isolation Kit (#480024 BioLegend) according to manufacture instruction. Briefly, the C57BL/6 mouse was anesthesized, and the spleen was harvested into cold 1x PBS. After a wash in PBS (x3) the spleen was diced into 20-30 slices. The slices were pooled into a Eppendorf tube containing combined enzyme mix 1 and 2 (1237.5 µL). The suspended slices in the enzyme mix were triturate (x15) and incubate at 37 ºC for 15 mins, and then triturated (x20) and incubated one more time. The suspension was diluted with 5 mL 1x cold PBS and centrifuged at 300 x g for 10 mins at 4 ºC. The pellets were resuspended in cold PB buffer (0.5% BSA in PBS, 1 mL) and passed through a moistened 70 µm cell strainer to get single cells. Abiotin-antibody cocktail (20 µL) was added to the cell suspension (with $\leq 10^7$ cells) in a sterile polypropylene tube followed by trituration (10x) and incubation for 15 minutes on ice. Next streptavidin Nanobeads (20 µL) was added and the cells were incubated again on ice for 15 minutes. The tube containing the cell suspension was placed in a QuadroMACS sepparator magnet (# 130-090-976, Miltenyi Biotec) for 10 minutes and liquid (containg CD3 T cells) was decanted into a sterile collecting tube. The pellet-Streptavidin Nanobeads were washed in PB buffer (2.5 mL) and the tube was subjected to the QuadroMACS sepparator magnet for 10 mins, followed by centrifuging at 300×g for 10 mins at RT. Pellets (CD3 T cells) were resuspended in 1 mL RPMI 1640 containing 1% P/S, and 10% FBS (10%). After counting, the cells were plated at 1 x $10^6$ cell per T-75 flask, and rested for 5-7 days before co-culture with cortical neurons. The purity of T cells was confirmed by flow cytometry, and CD45$^+$CD3$^+$ cells were considered as splenic T cells.

**Primary neuron-CD3$^+$ T cells co-culture and ICC**

Splenic CD3$^+$ T cells were added to cultured neurons at division (DIV) 10 -15, in 8-well poly-D-lysine (PDL) coated chamber slides. The medium for the co-culture was RPMI 1640 medium_302001 (American Type Culture Collection ATCC, Manassas) supplemented with FBS (10%), P/S (1%) and IL-2 (25 ng/mL). The co-culture was allowed to interact for 24 h followed by a wash with 1x PBS and fixed with 4% PFA, permeabilized with triton X-100 (0.3%) and then stained with primary antibodies. The following primary antibodies were used at 1:100: mouse anti-CD200R1_SC-53101 (Santa Cruz Biotechnology Inc), mouse anti CD3 (# NBP2-43674 Novus Biologicals), rabbit anti-CD200 (# 6s-6030R, ThermoFisher Scientific), rabbit anti MAP2 (# PA5-17646, Fisher Scientific), and goat anti-CD200R1_AF2554 (R&D Systems). The following fluorophore-conjugated secondary antibodies were used at 1:400: Donkey anti-mouse Alexa Fluor 488 (# A-32766, Invitrogen) , donkey anti-goat Alexa Fluor 488 (# A-11055, Invitrogen), Donkey anti-mouse Alexa Fluor 594 (# A32744, Invitrogen), Donkey anti-Rabbit Alexa Fluor 594 (# A-21207, Invitrogen) and Donkey anti-Rabbit Alexa Fluor 488 (# R37118, ThermoFisher scientific). Images were captured in Leica DMi8 Confocal Microscope at 20x magnification.

**Oxygen-Glucose Deprivation (OGD)**

To model ischemia/reperfusion conditions *in vitro*, the neuronal cultures were exposed to OGD as described previously.[21] The culture medium was replaced with serum-free, glucose-free Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES and 5 mg/ml gentamicin, pH 7.2), and the cultures were incubated in an experimental hypoxia chamber in a saturated atmosphere of 95% $N_2$ and 5% $CO_2$ for 2 hours. The control (normoxia) cells were cultured in the presence of normal levels of glucose and were incubated in a humidified atmosphere of 95% air and 5% $CO_2$. After 1 hour of OGD, cells were then supplemented with 10 mM glucose and incubated for 24 h at 37 °C.

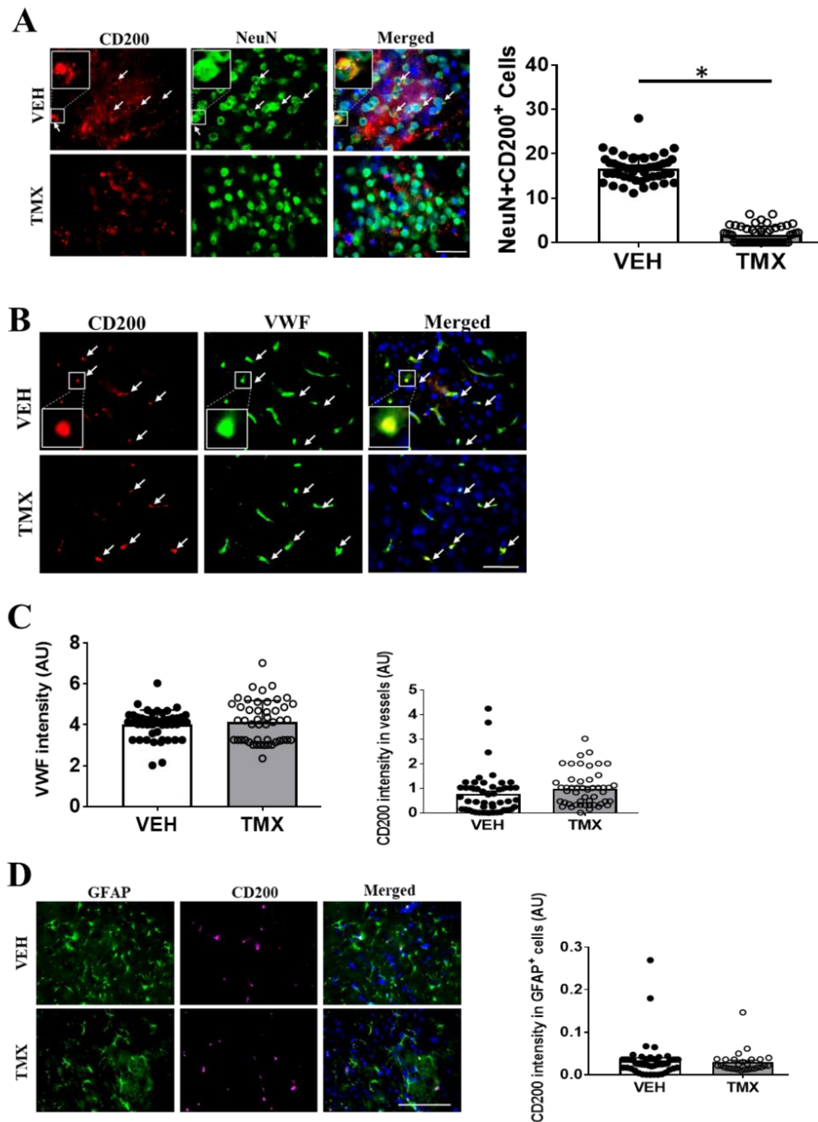
**Brain CD200 levels by ELISA**

Brain was homogenized using Dounce Homogenizer in 10 volumes of NP40 cell lysis buffer (FNN0021; Thermo Fisher Scientific) supplemented with 1 mm phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma-Aldrich). All steps were carried out at 4 °C. The homogenate was centrifuged initially at 700 g for 5 min to eliminate unruptured cells and debris and then further centrifuged at 12,500 g for 20 min, and the supernatant was used to measure CD200 levels by ELISA. The cytokine levels were normalized by total brain protein.

**CD200-CD200R1 binding assay**

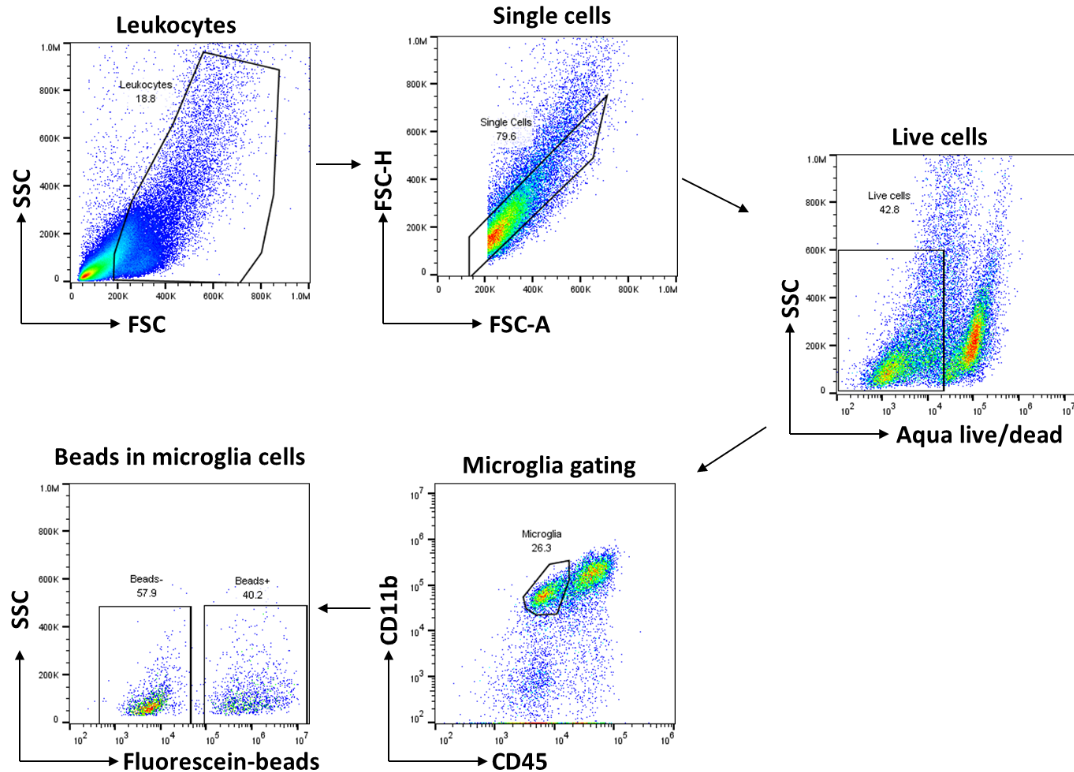
The ligand-receptor binding assay was performed by using recombinant mouse CD200R1 Fc chimera peptide_2554-CD (R&D Systems) and biotinylated recombinant mouse CD200-Fc Chimera (carrier-free)_786004 (BioLegend). Briefly we first coated Nunc™ MaxiSorp™ ELISA plate with Goat anti-CD200R1_AF2554 (R&D Systems) at 4 µg/mL (100 µL per well) overnight and at 4 ºC. After washing the plate (x3) with wash buffer (PBS + 0.05% Tween-20, PBST), the plate was blocked for 1 h using 1x assay diluent A_430904 (BioLegend) and washed one more time. Recombinant mouse CD200R1 Fc chimera peptide_2554-CD was then added at eight different concentrations (64, 96, 128, 192, 256, 512, 1024, 2048ng/mL; 100 µL per well) and the plate was incubated for 2 h, RT, and with gentle shaking. The plate was washed (x3) and absorbent to dry. Biotinylated recombinant mouse CD200 Fc chimera (carrier free)_786004 was added (1000 ng/mL, 100 µL per well) and incubated for 1 hr, RT, and with gentle shaking. Following 4 washes with PBST, Avidin-HRP_430904 diluted 1000x was added and incubated for 30 min, RT, and with gentle shaking. The plate was washed (x5) and TMB substrate solution (100 µL per well) was added and incubated for 20-30 min at RT, and in the dark. The blue chromophore formed was stopped by addition of 1M $H_2SO_4$ (100 µL) and the absorbance was read at 450 nm within 20-30 mins using EnSpire™ Multimode Plate Reader (PerkinElmer, Inc.).

**Supplemental Figure I**



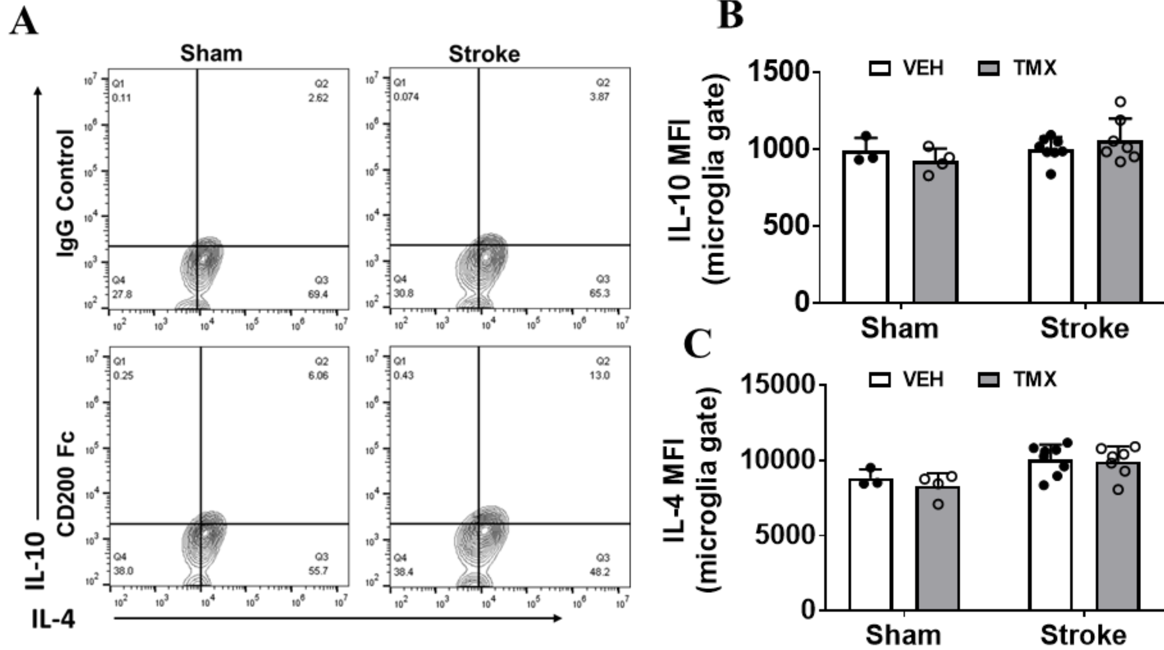

**Sup. Fig. I.** Neuronal, endothelial and astrocytic CD200 expression in neuronal CD200 CKO and control mice. (**A**) Representative images of co-expression of CD200 (red) with NeuN (green) in neurons. $NeuN^+CD200^+$ double positive cells were only seen in VEH treated brain but not in TMX treated mice as indicated by arrows. $NeuN^+CD200^+$ double positive cells in (A) were quantified. (**B**) Representative images of co-expression of CD200 (red) with VWF (endothelial cell marker) in endothelial cells. $VWF^+CD200^+$ double positive cells were seen in both VEH and TMX treated mice as indicated by arrows. (**C**) Quantification of VWF immunofluorescence intensity (left) and quantification of CD200 immunofluorescence intensity in $VWF^+$ vessels in the brain (right). (**D**) Representative images of co-expression of CD200 (red) with GFAP in astrocytes. All quantitative analyses were performed with eight 63× fields/animal in the peri-infarct area at the inner boundary zone of the infarct. Scale bar = 50µm. N = 6 animals/group; $*P < 0.05$.

**Supplemental Figure II**




**Sup. Fig. II: Gating strategy of microglia phagocytosis assay.** All leukocytes were first gated as singlet events (FSC-A vs FSC-H), and then gated as live cell population (SSC-A vs. aqua live/dead). Fluorescein conjugated *Escherichia coli* (K-12 strain) bioparticles (Beads) were incubated with isolated leukocytes at 37°C for 1h. Among the live cells, microglia cells were differentiated from other leukocytes based on cell surface marker CD45 and CD11b gating ($CD45^{intermediate}CD11b^{+}$). Fluorescein signal was measured by flow cytometry in the FITC channel. Microglia gated fluorescein-positive were counted as phagocytosing events.

**Supplemental Figure III**




**Sup. Fig. III: Microglial expression of intracellular anti-inflammatory markers in CD200 Fc treated mice. (A)** Representative flow plots showing IL-10 and IL-4 expression in gated microglia. **(B&C)** Quantification of MFI of IL-10/IL-4 in (A). N = 7-8 stroke/3-4 sham animals per group.

*** Preclinical Checklist**
*Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research.* ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See ["Reporting Standard for Preclinical Studies of Stroke Therapy"] and ["Good Laboratory Practice: Preventing Introduction of Bias at the Bench"] for more information.*

*This study invovles animal models:*
Yes

**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: | Yes |
| 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: | Yes |
| An overall study timeline is provided: | Yes |

**Inclusion and exclusion criteria**

| | |
|---|---|
| A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article: | Yes |

**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: | Yes |
| Type and methods of randomization have been described: | Yes |
| Methods used for allocation concealment have been reported: | N/A |

**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: | Yes |
| Blinding procedures have been described with regard to masking of group assignment during outcome assessment: | Yes |

**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: | Yes |

**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: | Yes |
| Baseline data on assessed outcome(s) for all experimental groups have been reported: | Yes |
| Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms: | N/A |
| Statistical methods used have been reported: | Yes |
| 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: | Yes |

**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: | Yes |
| Different sex animals have been used. If not, the reason/justification is provided: | Yes |

| | |
|---|---|
| Statements on approval by ethics boards and ethical conduct of studies have been provided: | Yes |
| Statements on funding and conflicts of interests have been provided: | Yes |

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