SUPPLEMENTAL MATERIAL

Corticosterone-mediated body weight loss is an important catabolic process for post-stroke immunity and survival

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Materials and Methods

Animals

Experiments were performed in 12-week-old C57BL/6 mice (Jackson Lab, Bar Harbor, ME). Only male mice were used in this proof of concept study due to recognized sex differences in stroke-induced injury size and body fat/muscle composition. A total of 132 mice were used; 60 mice to assess the association between weight loss and injury severity (Figure 1), 34 mice for acute immune cell deployment (Figures 2, 3, and 4), and 51 mice for adrenal corticosterone study (Figure 5). A total of 13 animals were excluded for MCAO surgical failure (6), not meeting MCAO criteria (3), or premature mortality before experimental endpoint (4). Animals were randomly assigned for the surgery. All animals received unique codes and the codes were revealed after the final analysis. The mice were housed at the institute's animal facility, which monitors and maintains temperature, humidity, and 12-hour light/dark cycles. A maximum of five mice was housed in a cage with an individual ventilating system and irradiated bedding (1/800 Bed o'Cobs, The Anderson, Maumee, OH). Sterilized food (PicoLab Rodent diet 5053, LabDiet, St. Louise, MO) and water were freely accessible in their cage.

Transient Middle Cerebral Artery Occlusion and Post-surgical Care

Briefly, mice were anesthetized with isoflurane. A fiber optic probe was glued to the parietal bone (2 mm posterior and 5 mm lateral to the bregma) and connected to a Laser-Doppler Flowmeter (Periflux System 5010; Perimed, Järfälla, Sweden) for continuous monitoring of cerebral blood flow (CBF) in the ischemic territory. For MCAO, a 6-0 Teflon-coated black monofilament surgical suture (Doccol, Redland, CA) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the cerebral arterial circle to obstruct the origin of the middle cerebral artery. After 30 min of occlusion, the filament was withdrawn to allow reperfusion. Mice were then placed in a recovery cage until the animal regained consciousness and resumed activity. Using a rectal probe controlled by a Masterflex pump and Thermistor temperature controller (Cole-Parmer, Vernon Hills, IL), animal's body temperature was maintained at 37ºC±0.5ºC during MCAO and recovery after surgery.

To prevent post-surgery dehydration, hydrogel (ClearH_2O, Portland, ME) was provided and sterile saline was injected intraperitoneally twice daily. Moistened food was also placed in the cage after surgery. Only animals that exhibited >80% reduction of pre-stroke baseline in CBF during MCAO and >80% reperfusion of baseline at 10 min were included in the study. Buprenorphine (0.5 mg/kg, every 12 h for minimum first 24 h after surgery, subcutaneous) and bupivacaine (0.1 ml of 0.25-0.5% on incision site before injection) were administered as analgesics. Mortality rate was 7%. Body weight was measured before surgery and daily up to 7 days (D) post-stroke. Percent body weight reduction (%BW_{red}) was calculated as follows: [(body

weight before surgery) - (body weight at 1D, 3D, or 7D post-surgery)]/(body weight before surgery) × 100.

Infarct Volume and Swelling Measurement

One day, 3D, and 7D after MCAO, brains were excised, frozen, and serial sections spanning ~7.2 mm rostrocaudal (roughly +3.1 mm and extending to -4.1 mm from bregma) were collected. The entire infarct region was cryosectioned for injury size measurement (20 μm thickness) and collected serially at 600 μm intervals. A total of 13 brain sections were used for injury size measurement. Infarct volume and hemispheric swelling were measured using Axiovision software (Zeiss, Germany). Infarct volume was calculated by subtracting the hemispheric difference from the injured area to correct edema and expressed as IV_{Ind}. To calculate percent hemispheric swelling (%SW), the difference between ipsilateral and contralateral hemispheric volume was divided by contralateral hemisphere volume and multiplied by 100.

Single Cell Preparation and Flow Cytometry Analysis

The spleen was harvested and dissociated mechanically. The spleen single cell suspensions were obtained by filtering through a 70 μm cell strainer followed by red blood cell lysis using a Red Blood Cell Lysing Buffer Hybri-Max™ (Millipore Sigma). The blood was collected via cardiac-perfusion using heparinized phosphate buffered saline. The red blood cells were lysed as mentioned above and cells were filtered through a 70 μm cell strainer. The spleen and blood single cells were fixed with 4% paraformaldehyde and incubated with 10% fetal bovine serum in phosphate-buffered saline at 4ºC for 1 hour. Then the single cells from the spleen and blood were incubated with (i) a cocktail of phycoerythrin-conjugated antibodies (PE-Lin) against T cells (PE rat anti-mouse CD90.2, Clone 53-2.1, 553005), B cells (PE rat anti-mouse CD45R/B220, Clone RA3-6B2, 553089), natural killer cells (PE mouse anti-mouse NK-1.1, Clone PK136, 557391; and PE rat anti-mouse CD49b, Clone DX5, 553858) and granulocytes (PE rat anti-mouse Ly-6G, Clone 1A8, 551461); (ii) phycoerythrin and cyanine 7-conjugated CD11b (PE-Cy7 rat anti-mouse CD11b, Clone M1/70, 552850); and (iii) fluorescein isothiocyanate-conjugated Ly-6C (FITC rat anti-mouse Ly6C, Clone AL-21, 553104). For assessing each immune cell type, PE-CD90.2, PE-CD45R/B220, PE-NK-1.1 or PE-CD49b single antibody was used. Monocyte/macrophage (MM) subset was gated as PE-Lin negative and PE-Cy7-CD11b positive (Lin-/CD11b+), and the selected MM subset was further analyzed for the distribution of Ly-$6C^{high}$ and Ly-$6C^{low}$ MM subsets using FITC-Ly-6C.

Brain single cells from contralateral and ipsilateral hemispheres were prepared using Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, San Diego, CA) with gentleMACS Octo Dissociator (Miltenyi Biotec) according to manufacturer's instructions. To obtain mononuclear cells from brain single cell suspension, discontinuous Percoll gradient method (30%, 37%, and 70%; GE Healthcare, Pittsburgh, PA) was used.[1] To detect myeloid lineage cells in the central nervous system, PE-conjugated CD45 (PE rat anti-mouse CD45, Clone 30-F11, 553081 or PE-Vio770-CD11b, M1/70.15.11.5, 130-099-704) and PE-Cy7-CD11b antibodies were used to identify CD11b positive and CD45 low expressed population (CD11b+/$CD45^{low}$) and CD11b positive and CD45 high expressed population (CD11b+/$CD45^{high}$) in the brain, which were further analyzed with FITC-Ly6C antibody. The stained single cells from the spleen, the blood, and the brain were washed with phosphate-buffered saline and passed through a 70 μm cell strainer before flow cytometer analyses (Accuri C6, BD Biosciences, San Jose, CA or MACSQuant VYB, Miltenyi Biotec). A total of 100,000 cells were counted per sample. All antibodies used for flow

cytometry were purchased from BD Biosciences unless noted otherwise. For multicolor compensation, Igκ/Negative Control Compensation Particles Set (BD Biosciences, 552845 for anti-rat and -hamster and 552843 for anti-mouse) and MACS Comp Bead Kit (130-107-755, Miltenyi Biotec) were used.

Supplementary figures

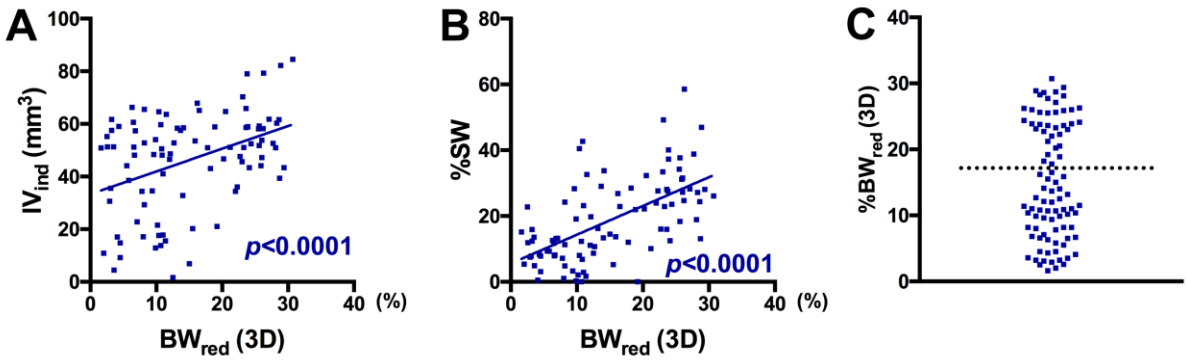

Figure I. 3D post-stroke BW loss as a good index to gauge stroke severity. ***A, B,*** Retrospective correlation analysis between weight reduction and infarct size (A) and swelling (B) at 3D post-stroke. ***C,*** Distribution of percentile body weight loss at 3D of total 91 C57BL/6 mice. The dotted line indicates 18% of $%BW_{red}$. *n*=91.

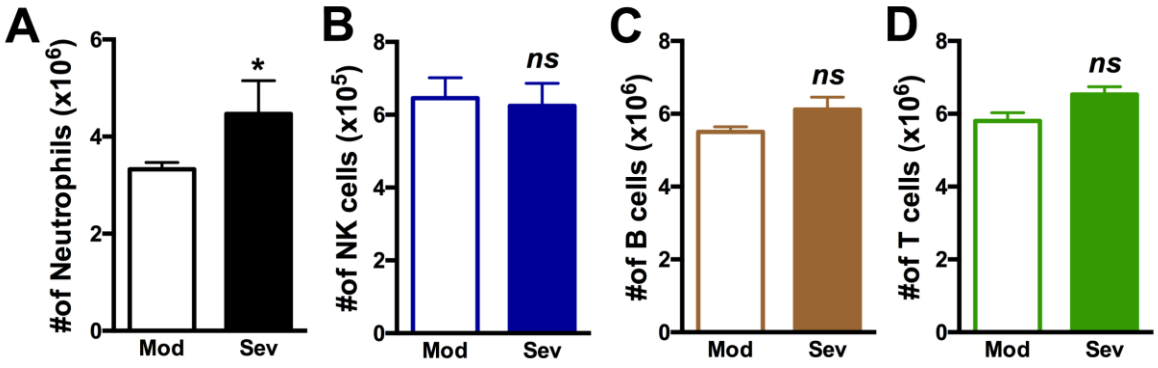

Figure II. Effect of body weight loss on spleen neutrophil, NK, B and T cell population at 3D post-stroke. A number of neutrophils (A), NK cells (B), B cells (C) and T cells (D) in the spleen of the mice with moderate (Mod) and severe (Sev) BW loss 3D after stroke. **p*<0.05. *n*=6-11/group.

Supplementary references

1. Woo M, Yang J, Beltran C, Cho S. Cell Surface CD36 Protein in Monocyte/Macrophage Contributes to Phagocytosis during the Resolution Phase of Ischemic Stroke in Mice. Journal of biological chemistry. 2016;291:23654-23661

*** 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:	Yes

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:	Yes
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:	N/A

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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