SUPPLEMENTAL MATERIAL

A selective NLRP3 inflammasome inhibitor reduces brain injury after intracerebral hemorrhage

Cover title: NLRP3 inhibitor reduces hemorrhagic brain injury

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ONLINE SUPPLEMENT

Supplemental Methods

Intracerebral Hemorrhage Model

ICH was induced by injection of either autologous blood or bacterial collagenase. Mice were first anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) by i.p. injection. Thereafter, mice were fixed on a stereotactic frame. A hole was drilled on the skull's right side of (2.3 mm lateral to midline, 0.5 mm anterior to the bregma). For the autologous blood model, a double-injection method was used as in previous publications.[1, 2] 30 μl non-heparinized autologous blood was withdrawn from the angular vein and infused as described. The first 5 μl was injected at a rate of 1 μl/min at a depth of 3 mm beneath the hole to generate a clot, after which the needle was moved to a depth of 3.7 mm and paused for 5min. The remaining 25 μl was injected at the same rate of 1 μl/min. In some experiments, ICH was also induced by injection of bacterial collagenase. 0.0375U bacterial collagenase dissolved in 0.5 μl saline was infused at a rate of 0.5 μl/min through the infusion pump at the caudate nucleus (0.5 mm anterior, 2.3 mm left lateral and 3.5 mm deep relative to bregma). Sham controls were injected with an equal volume of saline. After surgery, animals remained under observation with free access to food and water.

Magnetic Resonance Imaging (MRI)

Lesion volume and hematoma volume were quantified at day 3 after ICH using a 7T small-animal MRI. T2-weighted images (turbo RARE pulse sequence, repetition time/echo time=4500/65.5 ms) and susceptibility-weighted image (SWI) sequences were used to measure lesion volume and hematoma volume, respectively. The setup parameters are as follows: For T2W sequence scan, the field of view was 28×28 mm, and the matrix was 256×256 mm. Twenty coronal slices (0.5-mm thick) were acquired from the frontal pole to the brain stem. SWI sequence scans represent susceptibility-weighted images (repetition time=30ms; echo time=10 ms; field of view=32×32; image matrix=256×256; 0.3 mm slice thickness). The volumes were manually outlined and calculated by multiplying the sum of the volume by the distance between sections (0.5mm in T2WI) using MIPAV software. Two investigators blinded as to protocol calculated the lesion and hematoma volumes.

Flow Cytometry

At day 3 after ICH and perfusion with cold PBS, the cerebral tissues were removed and mechanically homogenized through 40 um nylon cell strainers (Becton Dickinson, Franklin Lakes, NJ, USA) in PBS on ice. After centrifugation, the cell pellets were resuspended in 5 ml of 30% Percoll (GE Healthcare Bio Science AB, Uppsala, Sweden) and centrifuged at 700×g for 10 min. Cell pellets were harvested on the bottom of the tube and washed once with 5 ml 1% BSA solution for staining. All antibodies were purchased from Biolegend (San Diego, CA, USA), unless otherwise indicated. The protocol of cell staining followed the manual's instructions. The following antibodies were used: GFAP (2E1.E9), CD45 (30-F11), CD11b (M1/70), CD3 (145-2C11), CD4 (GK1.4), CD8 (53-6.7), NK1.1 (PK136), CD19 (1D3), Ly6C (HK1.4), Ly6G (1A8), Gr-1 (NIMP-R14), interlukin-6 (IL-6) (MP5-20F3), IL-10 (JES5-16E3), TNF-α (MP6-XT22), TGF-β (TW7-20B9), and anti-NLRP3 antibody (Ab4207, Abcam, Cambridge, MA, USA); Alexa Fluor®488-conjugated donkey anti-goat IgG (H+L) was the secondary antibody (Invitrogen, Carlsbad, CA, USA). Fluorescence minus one (FMO) controls were stained, respectively, at the same time. Flow cytometry was also used to evaluate cell apoptosis in the brain tissue with indicated groups using the Annexin V detection kit (eBioscience, San Diego, CA, USA), as detailed in the manual's protocol. Flow cytometry was performed using a FACS Aria III (BD Bioscience, San Jose, CA, USA), and data were analyzed by Flow Jo version 7.6.1 (flowjo.com).

Real-time Quantitative RT-PCR

At day 3 after ICH, total RNA was extracted from the ipsilateral hemisphere of brain tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manual's instructions. The concentration of RNA

was quantified by ultraviolet spectrophotometry at 260/280 nm. Total RNA was reverse transcribed into cDNA using TransScript First-Strand cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China). All procedures were performed strictly as per instructions. PCR was performed on an Opticon 2 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) with the appropriate primers and SYBR green PCR Master Mix (Roche Diagnostics, Basel, Switzerland). The primers used to measure gene expression are listed as follows: NLRP3 (forward, TGG TCA AGG AGC ATC CAA GCA; AAG TGT TCA TCC TCA GGC TCA AA), caspase-1 (forward, CCG AAG GTG ATC ATC ATC CA; reverse, ATA GCA TCA TCC TCA AAC TCT TCT G), ASC (forward, GTG GGT GGC TTT CCT TGA TT; reverse, TTG TCT TGG CTG GTG GTC TCT), IL-1β (forward, ACG CTT ACC ATG TGA GCT G; reverse, GCC ACA GGG ATT TTG TCG TT); and β-actin (forward, CCG TCT TCC CCT CCA TCG T; reverse, ATC GTC CCA GTT GGT TAC AAT GC). Samples were performed in duplicate and normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method. The expression levels of mRNAs were calculated as fold changes vs. control. Melting curves were routinely performed to determine the specificity of the PCR reaction.

Western Blots

Mice were sacrificed at day 3 after ICH, and cerebral tissues from ipsilateral hemispheres were harvested to extract proteins. Proteins were electrophoresed and transferred onto a PVDF membrane (Merck KGaA, Darmstadt, Germany). After being blocked, membranes were incubated with primary antibodies: anti-NLRP3 (1:1000, AdipoGen, San Diego, CA, USA); anti-caspase-1 p20 (1:1000, Millipore, Billerica, MA, USA); anti-IL-1β (1:1000, Cell Signaling Technology, Danvers, MA, USA); anti-zonula occluden-1 (ZO-1) (1:1000, Invitrogen, Carlsbad, CA, USA); anti-claudin-5 (1:1000, Invitrogen, Carlsbad, CA, USA) and anti-β-actin (1:1000, Cell Signaling Technology, Danvers, MA, USA). After storage at 4°C overnight, membranes were incubated for 1 h at room temperature with the species-appropriate horseradish peroxidase (HRP) labeled secondary antibody (1:5000, Transgen Biotech, Beijing, China). The protein-specific signals were detected using a Bio-Rad 721BR08844 Gel Doc Imager (Bio-Rad, Hercules, CA, USA).

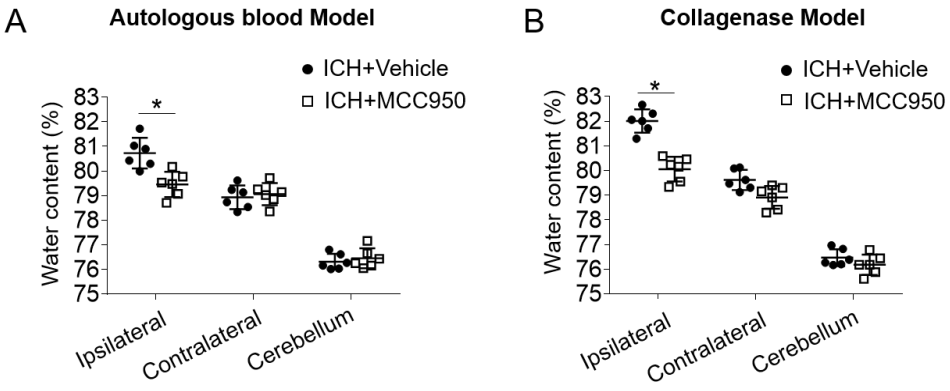
References

1. Li M, Li Z, Ren H, Jin WN, Wood K, Liu Q, et al. Colony stimulating factor 1 receptor inhibition eliminates microglia and attenuates brain injury after intracerebral hemorrhage. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2016
2. Li M, Ren H, Sheth KN, Shi FD, Liu Q. A tspo ligand attenuates brain injury after intracerebral hemorrhage. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2017;31:3278-3287

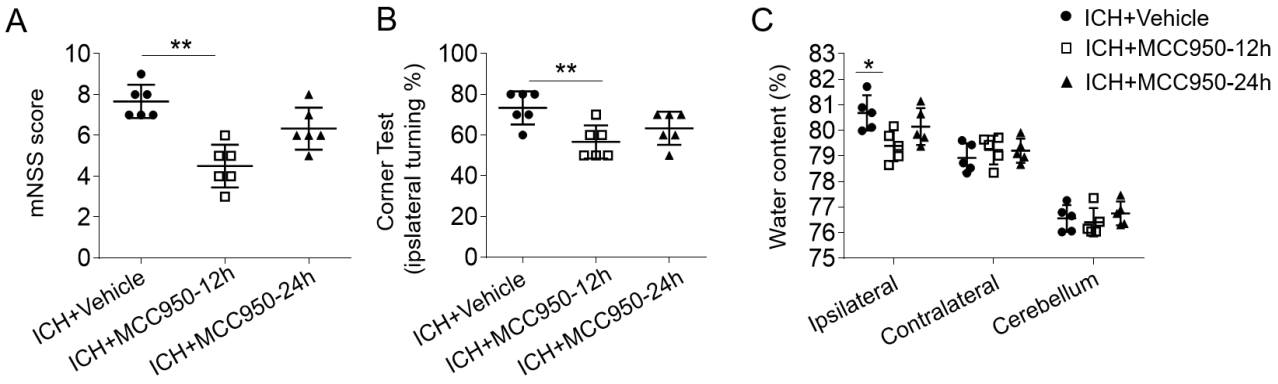
Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

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.

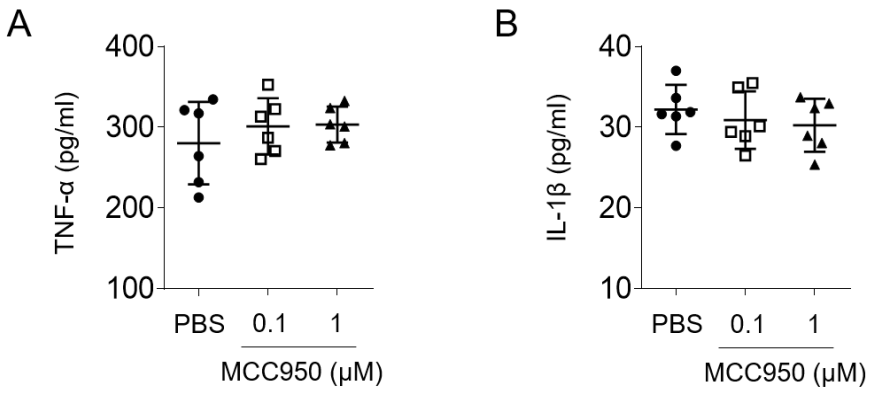
Supplemental Figures and Figure Legends



Supplemental Figure I. Effect of MCC950 on water content in brains after ICH.
ICH was induced in C57BL/6 mice by injection of autologous blood or collagenase. Mice received daily injections of MCC950 (10 mg/kg, i.p.) or an equal volume of vehicle for three consecutive days starting immediately after ICH induction. **A-B.** Brain water content in the ipsilateral brain hemisphere from ICH mice receiving MCC950 or vehicle at day 3 after onset. *P<0.05, n = 6 mice per group. Data are presented as mean ± SD.

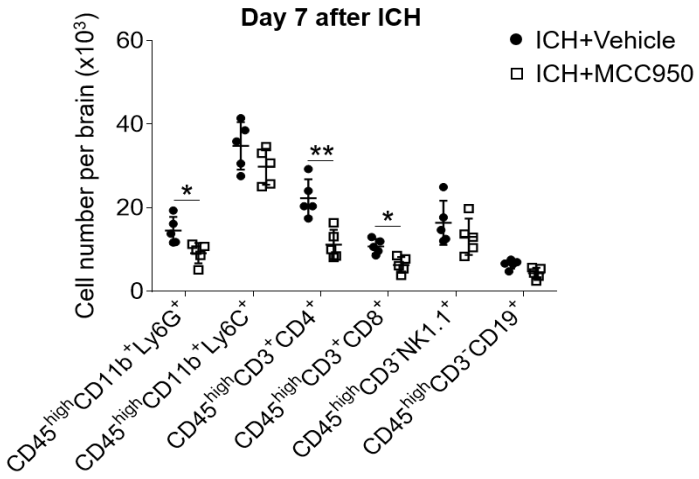


Supplemental Figure II. The protective effect of MCC950 is limited to within 24 h after ICH.
ICH was induced by injection of autologous blood. Groups of ICH mice were given MCC950 (10 mg/kg) or vehicle by daily i.p. injection for three consecutive days, starting from 12 h or 24 h after ICH. **A-B.** The assessments of mNSS score and corner test were performed at day 3 after ICH. **C.** Brain water content was measured in the ipsilateral hemisphere at day 3 after ICH. n = 6 per group. Data were presented as mean ± SD. *P<0.05, **P<0.01.

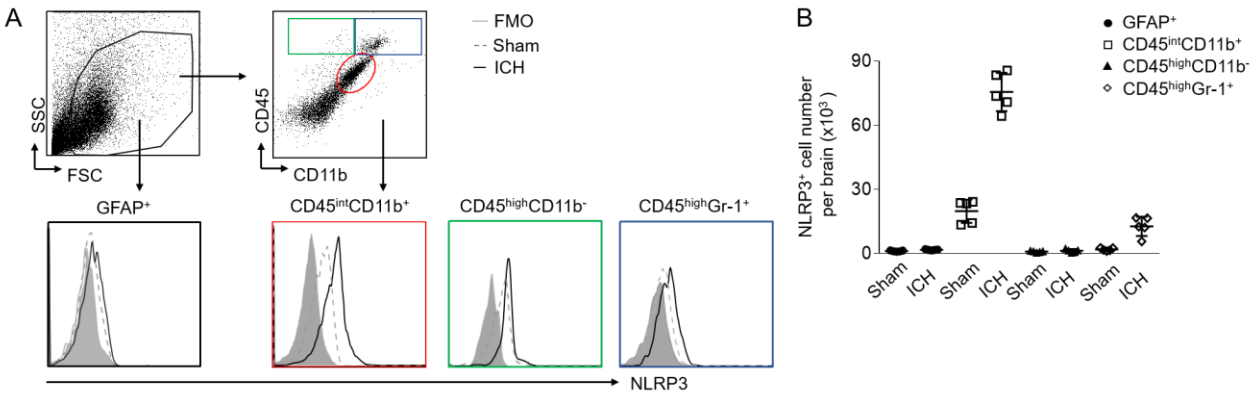


Supplemental Figure III. MCC950 does not affect LPS-induced production of IL-1β and TNF-α from splenocytes.

Splenocytes were harvested from spleen tissues of wild type mice and stimulated with 0.2 μM LPS in presence or absence of MCC950. **A-B.** Production of TNF-α (**A**) and IL-1β (**B**) from splenocytes stimulated with LPS and treated with MCC950 (0.1 and 1 μM) was measured by ELISA. n = 6 per group. Data were presented as mean ±SD.



Supplemental <u>Figure IV.</u> Infiltration of immune cell subsets at day 7 after ICH.
ICH was induced in C57BL/6 mice by injection of autologous blood. Mice received vehicle or MCC950 at a dose of 10 mg/kg by intraperitoneal injection. Immune cells were isolated from brain tissues of ICH mice receiving MCC950 or vehicle at indicated time. Data points show counts of brain-infiltrating leukocytes in the brains of ICH mice receiving indicated treatment at day 7 after ICH. n = 5 per group. Data were presented as mean ±SD. *P<0.05, **P<0.01.



Supplemental Figure V. NLRP3 expression is upregulated predominantly by microglia in mice after ICH.

ICH was induced in C57BL/6 mice by injection of autologous blood. Cell suspensions were prepared from brains of mice at day 3 after ICH onset. Flow cytometry analysis was performed to measure NLRP3 expression. **A.** Gating strategy of $GFAP^+$, $CD45^{int}CD11b^+$, and $CD45^{high}$ $CD11b^-$, $CD45^{high}$ $Gr\text{-}1^+$ cells expressing NLRP3. **B.** Data plots show the expression of NLRP3 in indicated cell subsets. n =5 mice per group. Data are presented as mean ± SD.