Selective NLRP3 (Pyrin Domain–Containing Protein 3) Inflammasome Inhibitor Reduces Brain Injury After Intracerebral Hemorrhage

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Background and Purpose—Intracerebral hemorrhage (ICH) is a devastating disease without effective treatment. As a key component of the innate immune system, the NOD-like receptor (NLR) family, NLRP3 (pyrin domain–containing protein 3) inflammasome, when activated after ICH, promotes neuroinflammation and brain edema. MCC950 is a potent, selective, small-molecule NLRP3 inhibitor that blocks NLRP3 activation at nanomolar concentrations. Here, we examined the effect of MCC950 on brain injury and inflammation in 2 models of ICH in mice.

Methods—In mice with ICH induced by injection of autologous blood or bacterial collagenase, we determined the therapeutic potential of MCC950 and its mechanisms of neuroprotection.

Results—MCC950 reduced IL-1β (interleukin-1β) production and attenuated neurodeficits and perihematomal brain edema after ICH induction by injection of either autologous blood or collagenase. In mice with autologous blood-induced ICH, the protection of MCC950 was associated with reduced leukocyte infiltration into the brain and microglial production of IL-6. MCC950 improved blood–brain barrier integrity and diminished cell death. Notably, the protective effect of MCC950 was abolished in mice depleted of either microglia or Gr-1+ myeloid cells.

Conclusions—These results indicate that the NLRP3 inflammasome inhibitor, MCC950, attenuates brain injury and inflammation after ICH. Hence, NLRP3 inflammasome inhibition is a potential therapy for ICH that warrants further investigation.

Visual Overview—An online visual overview is available for this article. (Stroke. 2018;49:184-192. DOI: 10.1161/STROKEAHA.117.018904.)

Key Words: brain edema • cell death • inflammation • neuroprotection

Intracerebral hemorrhage (ICH) is a devastating, often fatal aftermath of stroke, but not generally amenable to treatment.1–4 Emerging evidence has demonstrated that a stroke-initiated inflammatory cascade accelerates the formation of edema that surrounds hematomas, exacerbates the mass effect, and amplifies cell death.4–6 After the ictus of ICH, blood components, including leukocytes, enter the brain and activate resident immune cells such as microglia. Subsequently, the infiltrating leukocytes and activated microglia boost the local production of proinflammatory factors. Together with cell death products, these catastrophic events amplify blood–brain barrier (BBB) disruption and destroy surrounding tissues, contributing to the development of perihematomal edema and the aggravated mass effect.4–4 Therefore, brain inflammation has emerged as a major modifiable determinant now targeted for the development of novel ICH treatment.7

As a critical component of the innate immune response to tissue injury, activation of the NOD-like receptor (NLR) NLRP3 (pyrin domain–containing protein 3) inflammasome leads to the cleavage of pro–IL (interleukin)-1β and pro–IL-18, as well as the subsequent release of biologically active IL-1β, IL-18, and other soluble mediators of inflammation. Recent evidence indicates that the ICH-induced activation of NLRP3 inflammasome can amplify the inflammatory response by releasing IL-1β and promoting neutrophil infiltration, thereby exacerbating brain edema.8–11 Although previous studies provide mechanistic insights into NLRP3 as a promising target for restricting neuroinflammation and brain edema after ICH, they do not reflect clinical application because they involve either knockout models or use of pharmacological agents with limited potency and nonspecificity preceding ICH induction.9–11 For clinical translation of prior experimental
outcomes, preclinical testing in relevant models is mandatory. However, this option has been precluded by the lack of selective, potent NLRP3 inflammasome inhibitors.

Now, we have devised a means of clinically relevant testing in an experimental ICH model by using MCC950, a selective, small-molecule NLRP3 inflammasome inhibitor with proven high potency in vitro and in vivo. We hypothesized that the pharmacological inhibition of NLRP3 inflammasome by administration of MCC950 would reduce inflammation and brain edema after ICH induced by the injection of autologous blood or collagenase.

Materials and Methods
Details of materials and experimental procedures are available from the online-only Data Supplement. This article adheres to the AHA Journals implementation of the Transparency and Openness Promotion Guidelines.

Animals
All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Barrow Neurological Institute (Phoenix, AZ). All experiments were conducted in accordance with the National Institutes of Health (Bethesda, MD) Guide for the Care and Use of Laboratory Animals, and experiments were designed, performed, and reported according to the Animal Research: Reporting In Vivo Experiments guidelines (https://www.nc3rs.org.uk/arrive-guidelines). Male C57BL/6 mice, 8 to 10 weeks old (20–25 g), were purchased from the Charles River Laboratories (Wilmington, MA). Female mice were not used to avoid any influences of sex steroids. All mice were housed in animal facilities under a standardized light–dark cycle and had free access to food and water. All animal surgeries were performed under anesthesia. Animals were randomly divided and assigned to experimental groups using a random number list generated by Microsoft Excel 2013.

Induction of ICH in Mice
ICH was induced in mice by injection of autologous blood or bacterial collagenase, as we previously described.12–16 Details of ICH induction are given in the online-only Data Supplement.

Study Design and Drug Administration
A total of 302 male C57BL/6 mice (Charles River Laboratories), 8 to 10 weeks old, were used in this study. In autologous blood model, the mortality rate was 8.5% (20 of total 236) and exclusion rate was 16.7% (36 of total 220). In the collagenase model, the mortality rate was 13.3% (8 of total 60) and exclusion rate was 20% (12 of total 60). Six wild-type mice were used for in vitro experiments. MCC950 was dissolved in phosphate-buffered saline, as previously described.12 Mice were given MCC950 at a dose of 10 mg/kg by intraperitoneal injection at indicated time points after ICH. Mice that received an equal volume of vehicle (phosphate-buffered saline) only were designated as controls. For the microglia depletion experiment, PLX3397 (Selleckchem, Houston, TX) was given by oral gavage at 40 mg/kg for 21 days before ICH induction, as previously described.27 Drug treatment was continued until the end of experiments. Subsequently, anti-mouse Gr-1 monoclonal antibody (MAB-RB6-3C5; BioXcell, West Lebanon, NH) was given by intraperitoneal injection 1 day before and 1 day after ICH surgery at 250 µg per mouse to deplete Gr-1+ myeloid cells in vivo.9,10 In mice subjected to microglia depletion, no mice were excluded. More than 90% of microglia were depleted in mice receiving PLX3397. In mice subjected to Gr-1+ cell depletion, the exclusion rate was 5.9% (1 of total 17 because of death after surgery). The depletion of Gr-1+ cells was verified by sampling circulating blood, and ≥90% of Gr-1+ cells were depleted in all mice included.

Behavioral Assessment
Behavioral assessment was conducted at days 1 and 3 after ICH using the modified Neurological Severity Score together with corner-turning test to comprehensively evaluate motor, sensory, reflex, and balance functions, as previously described.21–24 The range of scores for modified Neurological Severity Score is from 0 to 18, defined as follows: a score of 13 to 18 indicates severe injury, 7 to 12 indicates moderate injury, and 1 to 6 indicates mild injury. Mice were given one point if they failed to perform a task. The corner-turning test assesses sensorimotor and postural asymmetries. In this process, each mouse is allowed to proceed into a corner with an angle of 30° and then must turn right or left. Each mouse repeated this procedure for 10x, with at least 30 s between trials. The percentage of right turns is then calculated.

Magnetic Resonance Imaging
Lesion volume and perihematomal edema were quantified at day 3 after ICH using a 7-T small animal MRI, as previously described.13,16 T2-weighted image sequence and susceptibility-weighted image sequence were scanned to detect lesion volume and hematoma volume, respectively. Details are described in the online-only Data Supplement.

Brain Water Content Measurement
Water contents were measured at day 3 after ICH, as previously described.25 In brief, without perfusion, brains were removed from mice and divided into 3 parts: the ipsilateral, the contralateral, and the cerebellum. The brain samples were weighed immediately on an electronic balance to obtain wet weights, then dried for 24 hours at 100°C, and weighed again to obtain dry weights. The following formula was used to calculate the brain water content: (wt weight–dry weight)/wt weight×100%.

Flow Cytometry
To quantify cells expressing NLRP3, microglia (CD11b+CD45lowLy6G+), neutrophils (CD11b+CD45+Ly6G+), monocyte/macrophages (CD11b+CD45+), CD4+ T cells (CD45+CD3+CD4+), CD8+ T cells (CD45+CD3+CD8+), B cells (CD45+CD3+CD19+), NK cells (CD45+CD3+CD56+CD16+CD57+), and cytokines produced by microglia, and cell apoptosis (Annexin V+) in the brain, we isolated cellular components from brain tissue to perform flow cytometry analysis as we previously described.25,26 Details appear in the online-only Data Supplement.

Real-Time Polymerase Chain Reaction
At day 3 after ICH, total RNA was extracted from brain tissue in the ipsilateral hemisphere for real-time polymerase chain reaction analysis as described in the online-only Data Supplement.

Assessment of BBB Permeability
Evans Blue dye (Sigma, St. Louis, MO) extravasation assays were conducted at day 3 after ICH as a tracer to measure BBB permeability, as previously described.16,27 Details are given in the online-only Data Supplement.

Western Blots
At day 3 after ICH, Western blots analyzed for NLRP3 inflammasome components and tight junction protein expression in the ipsilateral cerebral hemisphere, as we previously described.16 Details are given in the online-only Data Supplement.

Statistical Analysis
We determined each sample size by power analysis using a significance level of α=0.05 with 80% power to detect statistical differences. SAS 9.1 software (SAS Institute Inc, Cary, NC) was used for power analysis and sample size calculations. Data were analyzed by investigators blinded to experimental treatments. Data are shown as means±SD. SPSS 19.0 software was used to compare differences between 2 groups where appropriate. One-way ANOVA followed by...
the Tukey post hoc test or 2-way ANOVA with multiple comparisons followed by Bonferroni post hoc test was used for comparison of multigroup data. $P$ values <0.05 are considered significant.

Results

MCC950 Attenuates Brain Injury and Improves Long-Term Outcome After ICH

To determine whether the NLRP3 inflammasome inhibitor, MCC950, affects brain injury after ICH, we examined neurodeficits, lesion volume, and perihematomal edema in ICH mice receiving MCC950 or a phosphate-buffered saline vehicle. ICH was induced by injection of autologous blood or collagenase. Mice received MCC950 (10 mg/kg) or vehicle for 3 consecutive days starting immediately after ICH induction (Figure 1A). Neurological function was evaluated by using modified Neurological Severity Score and corner-turning tests at days 1 and 3 after ICH. Lesion volume, perihematomal edema, and brain water content were measured at day 3 after ICH. Compared with vehicle recipients, we found that MCC950-treated mice had significantly reduced neurodeficits, lesion volumes, and perihematomal edema after ICH (Figure 1B and 1C). MCC950 reduced brain water content after ICH (Figure I in the online-only Data Supplement). In addition, MCC950 reduces neurodeficits until day 28 after ICH induction (Figure 1D), suggesting that NLRP3 inflammasome inhibition can provide long-term benefit after ICH. Of note, the benefit of MCC950 to

Figure 1. MCC950 attenuates brain injury and improves long-term outcome after intracerebral hemorrhage (ICH). ICH was induced in C57BL/6 mice by injection of autologous blood or collagenase. A, Flow chart illustrates MCC950 administration and experimental design. Mice received daily intraperitoneal (IP) injections of MCC950 (10 mg/kg) or an equal volume of phosphate-buffered saline (PBS) vehicle for 3 consecutive days starting immediately after ICH induction. B, Neurological tests were performed to evaluate the motor, sensory, and balance functions in mice receiving vehicle or MCC950 at days 1 and 3 after injection of autologous blood (left) or collagenase (right). C, T2-weighted image (T2WI) sequences were scanned to assess lesion volume at day 3 after ICH induced by injection of autologous blood (left) or collagenase (right), as outlined in red. Susceptibility-weighted sequences were assessed for hematoma lesion volume, visible in yellow regions. Quantification of lesion volume and perihematomal edema in mice receiving MCC950 or vehicle at day 3 after ICH induced by injection of autologous blood (left) or collagenase (right). n=8 mice per group. D, Mice received vehicle or MCC950 at a dose of 10 mg/kg by intraperitoneal injection. The assessments of modified Neurological Severity Score (mNSS) score and corner test were performed at days 7, 14, and 28 after ICH induced by injection of collagenase. n=10 per group. Data are presented as mean±SD. *P<0.05, **P<0.01.
reduce neurodeficits and brain edema was restricted to within 24 hours after ICH (Figure II in the online-only Data Supplement).

**MCC950 Inhibits the Activation of NLRP3 Inflammasome Components and IL-1β Production After ICH**

The effect of MCC950 on NLRP3 inflammasome activation and IL-1β production was examined in brain tissues of ICH mice. At day 3 after ICH, we found that MCC950 reduced the mRNA expression of NLRP3 inflammasome components (NLRP3/Caspase-1/ASC) and IL-1β (Figure 2A). In addition, the protein expression of NLRP3, caspase-1, and IL-1β in the brain was suppressed by MCC950 treatment (Figure 2B and 2C). Of interest, MCC950 does not affect lipopolysaccharides-induced production IL-1β and TNF-α (tumor necrosis factor-α) from splenocytes (Figure III in the online-only Data Supplement). These results demonstrate that MCC950 effectively inhibits the activation of NLRP3 inflammasome components and IL-1β production in the brain after ICH.

**MCC950 Reduces Leukocyte Infiltration and Production of Proinflammatory Factors by Microglia After ICH**

Next, we determined the impact of MCC950 on brain inflammation after ICH. Using flow cytometry, we examined such cellular components as brain-infiltrating leukocytes and microglia in the brains of ICH mice (Figure 3A). At day 3 post-ICH, the numbers of brain-infiltrating leukocytes, neutrophils (CD11b+CD45+Ly6G⁺), monocyte/macrophages (CD11b+CD45+Ly6C⁺), CD4⁺ T cells (CD45+CD3⁺CD4+), and CD8⁺ T cells (CD45+CD3⁺CD8⁺) were reduced in ICH mice receiving MCC950 compared with those in vehicle-treated controls (Figure 3B and 3C). Of note, MCC950 reduced the infiltration of CD4⁺ T cells, CD8⁺ T cells and neutrophils until day 7 after ICH (Figure IV in the online-only Data Supplement). Further, microglia cell numbers decreased as did microglial expression of factor IL-6 (Figure 3D). In contrast, the microglial expression of IL-10 and TGF-β (transforming growth factor-β) was increased in ICH mice receiving MCC950 (Figure 3D). These results indicate that MCC950 can reduce brain inflammation and cause a shift in microglia phenotype toward an anti-inflammatory status.

**MCC950 Preserves BBB Integrity and Reduces Cell Death After ICH**

BBB disruption after ICH contributes to vasogenic edema and the expansion of perihematomal edema. To measure the effect of MCC950 on BBB disruption after ICH, we examined Evans Blue dye extravasation and the expression of

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**Figure 2.** MCC950 inhibits NOD-like receptor (NLR) family, NLRP3 (pyrin domain-containing protein 3) inflammasome activation and IL (interleukin)-1β expression. Intracerebral hemorrhage (ICH) was induced in C57BL/6 mice by injection of autologous blood. Mice received daily intraperitoneal (IP) injections of MCC950 (10 mg/kg) or an equal volume of vehicle for 3 consecutive days starting immediately after ICH induction. At day 3 after ICH or sham surgery, brain tissues were harvested for the extraction of mRNA and protein. A. The mRNA expression levels of the NLRP3 inflammasome components and IL-1β after ICH. B. Western blot images show the expression of NLRP3, IL-1β, and caspase-1 in the brains of indicated groups of mice receiving sham plus vehicle, ICH plus vehicle, or ICH plus MCC950. C. Data points show the protein expression levels of NLRP3, IL-1β, and caspase-1 in the brains of indicated groups of mice receiving sham plus vehicle, ICH plus vehicle, or ICH plus MCC950. n=6 mice per group. Data are presented as mean±SD. *P<0.05, **P<0.01.
tight junction proteins. At day 3 after ICH, MCC950 significantly ameliorated leakage of the dye (Figure 4A), denoting a reduction of BBB permeability. MCC950 also preserved expression of the tight junction proteins (claudin-5 and ZO-1; Figure 4B). In addition, MCC950 reduced numbers of Annexin V–expressing cells in the brain at day 3 after ICH, suggesting a decrease in cell death (Figure 4C). Together, these results demonstrate that MCC950 preserves BBB integrity and reduces cell death after ICH.

Benefit of MCC950 Against ICH Involves Microglia and Gr-1+ Myeloid Cells

Because microglia are the predominant cell subset expressing NLRP3 inflammasome after ICH (Figure V in the online-only Data Supplement), we sought to understand to what extent microglia may contribute to the protective effect of MCC950. The survival of microglia depends on signaling through CSF1R (colony-stimulating factor 1 receptor); therefore, we used a CSF1R inhibitor, PLX3397, to deplete microglia...
before ICH induction (Figure 5A). Consistent with our previously described PLX3397 treatment, the present result was a loss of >90% microglia (CD11b+CD45int) from mice undergoing ICH. Of interest, we found that the benefit of MCC950 treatment was abolished in ICH mice receiving PLX3397 (Figure 5B and 5C), suggesting that microglia participate in the beneficial effect of MCC950 after ICH.

Because NLRP3 inflammasome was clearly expressed in Gr-1+ myeloid cells, consisting mainly of neutrophils and monocytes that are known contributors to BBB disruption and brain inflammation, we therefore determined whether Gr-1+ myeloid cells also add to the benefit of MCC950. An anti–Gr-1+ mAb (RB6-8C5) was used to deplete Gr-1+ myeloid cells from mice before ICH induction (Figure 6A). We monitored the counts of Gr-1+ cells in circulating blood at 24 hours before the first injection of anti–Gr-1 mAb and 48 hours after the second injection of anti–Gr-1 mAb (Figure 6B). Consistent with a previous report, anti–Gr-1+ mAb treatment depleted >90% of Gr-1+ myeloid cells (Figure 6B), that is, mainly neutrophils and monocytes. Of note, the depletion of Gr-1+ myeloid cells diminished the beneficial effects of MCC950 after ICH (Figure 6C and 6D), suggesting that the protection of MCC950 also involves Gr-1+ myeloid cells.

**Figure 4.** MCC950 treatment preserves blood–brain barrier (BBB) integrity and reduces cell death after intracerebral hemorrhage (ICH). ICH was induced in C57BL/6 mice by injection of autologous blood. Mice received daily intraperitoneal (IP) injections of MCC950 (10 mg/kg) or an equal volume of vehicle for 3 consecutive days starting immediately after ICH induction. At day 3 after ICH, brain tissues were prepared for measurements of BBB integrity and cell death. A, Histology images show that MCC950 decreased Evans Blue dye leakage compared with vehicle (phosphate-buffered saline [PBS]) treatment. B, Western blot images show expression of the tight junction proteins, claudin-5, and ZO-1, in the brains of ICH mice receiving MCC950 or vehicle. C, Flow cytometry plots and summarized results show percentages of Annexin V+ cells in the brains of ICH mice receiving MCC950 or vehicle. n=6 mice per group. Data are presented as mean±SD. *P<0.05, **P<0.01.

**Figure 5.** Microglia contribute to the benefit of MCC950 after intracerebral hemorrhage (ICH). A, Schematic diagram illustrates drug administration and experimental design. C57BL/6 mice received oral gavage of PLX3397 (40 mg/kg) for 21 days and continuously until the experiment ended. ICH was induced in PLX3397-treated mice by injection of autologous blood. Thereafter, these mice received daily injections of MCC950 (10 mg/kg, intraperitoneal [IP]) or an equal volume of vehicle for 3 consecutive days starting immediately after ICH induction. At day 3 after ICH, neurodeficits and brain water content were assessed. B, Quantification of modified Neurological Severity Score (mNSS) and corner-turning test scores in indicated groups of ICH mice. C, Quantification of brain water content in indicated groups of ICH mice. n=6 per group. Data are presented as means±SD.
Discussion

This study provides the first evidence that the NLRP3 inflammasome inhibitor, MCC950, attenuates hemorrhagic brain injury. MCC950 significantly reduced neurodeficits and perihematomal edema in 2 mouse models of ICH induced by injection of autologous blood or bacterial collagenase. MCC950 treatment was sufficient to reduce the resulting leukocyte infiltration, microglial production of proinflammatory factors, BBB disruption, and cell death after ICH. In addition, the benefit of MCC950 protection from ICH was diminished in mice subjected to depletion of microglia or Gr-1+ myeloid cells (neutrophils and monocytes). Together, these results suggest that MCC950 has potential therapeutic value for reducing ICH injury.

Mechanistic studies have shown an essential role for the NLRP3 inflammasome in brain injury and neuroinflammation after ICH.9–11 The activation of NLRP3 inflammasome facilitates caspase-1 activation and IL-1β processing, leading to the amplification of the inflammatory response, which culminates in the expansion of perihematomal edema, and thereby exacerbating hemorrhagic brain injury.2–11 As a small molecule, the NLRP3 inflammasome inhibitor, MCC950, was recently found to selectively inhibit NLRP3 inflammasome formation and reduce pyroptosis and IL-1β signaling.17 In the present study, our results confirmed the ability of MCC950 to effectively inhibit the activation of NLRP3 inflammasome components and IL-1β production in experimental ICH. In advancing previous findings,9–11 our study for the first time provides efficacy data depicting pharmacological inhibition by NLRP3 inflammasome in the setting of ICH in vivo.

Other than MCC950, several small molecules inhibit the NLRP3 inflammasome. For example, glyburide inhibited IL-1β production at micromolar concentrations in response to the activation of NLRP3 and was effective in reducing edema formation.33 Purinergic 2X7 receptor antagonist (blue brilliant G) also diminished NLRP3 inflammasome activation and lessened hemorrhagic brain injury.34 However, because of the limited potency and nonspecific nature of those agents, the extent to which pharmacologically selective targeting by NLRP3 inflammasome can impact ICH injury is still unclear. However, results from this study provide new evidence that selective NLRP3 inflammasome inhibition offers the benefit of reducing ICH injury and improving long-term outcome, which is an essential step for bringing NLRP3 inflammasome-targeted therapies from the bench into clinical application.

Reportedly, NLRP3 might present certain advantages over the use of biological inhibitors of IL-1β.17 Because MCC950 does not block the major antimicrobial inflammasome NLRC4 or NLRP1, specific targeting of NLRP3 will not result in the complete blockade of IL-1β in vivo, and antimicrobial responses may remain intact. In support of this view, we found that MCC950 does not affect lipopolysaccharides-induced production IL-1β and TNF-α from splenocytes. Considering the immunosuppression that follows stroke,35–37 MCC950 may...
have the advantage of less immunosuppressive effects than biologics such as canakinumab (anti-IL-1β monoclonal antibody), which increased the risk of these infections in a clinical setting.\textsuperscript{28,39} Inflammation of the brain and BBB dysfunction are acknowledged as key contributors to the expansion of perihematoma edema after ICH onset.\textsuperscript{4,25,4,39} In that context, we postulate that MCC950 may mitigate the brain’s inflammatory milieu to confer protection from the effects of ICH. In support of this view, we found that the benefit of MCC950 treatment was abolished in mice depleted of microglia via a CSF1R inhibitor. That outcome, together with the finding that NLRP3 inflammasome is expressed primarily in microglia,\textsuperscript{38,39} supports this view, we found that the benefit of MCC950 treatment after ICH. Nevertheless, the precise operating mechanisms by which MCC950 restricts brain inflammation after ICH remains uncertain and requires future investigation.

**Conclusions**

We have demonstrated that the NLRP3 inflammasome inhibitor, MCC950, attenuates brain injury and inflammation after ICH. Therefore, MCC950 may serve as a promising candidate for further investigation in advanced preclinical ICH studies.

**Disclosures**

None.

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Selective NLRP3 (Pyrin Domain–Containing Protein 3) Inflammasome Inhibitor Reduces Brain Injury After Intracerebral Hemorrhage
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A selective NLRP3 inflammasome inhibitor reduces brain injury after intracerebral hemorrhage

Cover title: NLRP3 inhibitor reduces hemorrhagic brain injury

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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=32x32; 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), interleukin-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^{-ΔΔCt} 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).
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## 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 Figure IV. 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-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.