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Alternative Activation-Skewed Microglia/Macrophages Promote Hematoma Resolution in Experimental Intracerebral Hemorrhage

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Abstract

Microglia/macrophages (MMΦ) are highly plastic phagocytes that can promote both injury and repair in diseased brain through the distinct function of classically activated and alternatively activated subsets. The role of MMΦ polarization in intracerebral hemorrhage (ICH) is unknown. Herein, we comprehensively characterized MMΦ dynamics after ICH in mice and evaluated the relevance of MMΦ polarity to hematoma resolution. MMΦ accumulated within the hematoma territory until at least 14 days after ICH induction. Microglia rapidly reacted to the hemorrhagic insult as early as 1–1.5 h after ICH and specifically presented a “protective” alternatively activated phenotype. Substantial numbers of activated microglia and newly recruited monocytes also assumed an early alternatively activated phenotype, but the phenotype gradually shifted to a mixed spectrum over time. Ultimately, markers of MMΦ classic activation dominated at the chronic stage of ICH. We enhanced MMΦ alternative activation by administering intraperitoneal injections of rosiglitazone, and subsequently observed elevations in CD206 expression on brain-isolated CD11b⁺ cells and increases in IL-10 levels in serum and perihematoma tissue. Enhancement of MMΦ alternative activation correlated with hematoma volume reduction and improvement in neurologic deficits. Intraventricular injection of alternative activation signature cytokine IL-10 accelerated hematoma resolution, whereas microglial phagocytic ability was abolished by IL-10 receptor neutralization. Our results suggest that MMΦ respond dynamically to brain hemorrhage by exhibiting diverse phenotypic changes at different stages of ICH. Alternative activation-skewed

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Conflict of Interest

The authors declare no competing financial interests.

Author contributions

C.-F.C., N.H., and J.W. conceived the study and wrote the paper; C.-F.C., J.R.W., Q.L., and S.C.R. performed research; C.-F.C., Q.L., N.H. and J.W. analyzed and interpreted data.

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MM Φ aid in hematoma resolution, and IL-10 signaling might contribute to regulation of MM Φ phagocytosis and hematoma clearance in ICH.

Keywords

intracerebral hemorrhage; hematoma resolution; microglia/macrophage polarization; phagocytosis; interleukin-10

Introduction

Intracerebral hemorrhage (ICH) accounts for the highest mortality among all strokes (van Asch et al., 2010), and no therapeutic strategy has been approved for its treatment (Keep et al., 2012). After an intraparenchymal bleed, primary injury develops within the first few hours as a result of hematoma formation, expansion, and the mass effect. Subsequently, secondary damage activates cytotoxic and inflammatory cascades (Wang, 2010; Zhou et al., 2014) that contribute substantially to neurologic deterioration in patients with ICH (Wu et al., 2010; Ziai, 2013). Although preclinical studies have tried to identify potential neuroprotectants that target secondary injury (Kellner and Connolly, 2010), mortality and morbidity of ICH have not declined (Xi et al., 2014). As targeting secondary injury does not appear to provide adequate intervention for ICH patients (Toyoda and Grotta, 2015), an alternative strategy might be to remove the hematoma before it can release the toxic hemoglobin degradation products that elicit secondary injury cascades (Ni et al., 2016; Sonni et al., 2014). Clinical evidence shows that hematoma size and expansion are the major determinants of ICH outcomes (Brott et al., 1997; LoPresti et al., 2014). Until now, however, little preclinical work has evaluated potential strategies to limit hematoma expansion or accelerate hematoma resolution after ICH (Flores et al., 2016; King et al., 2011; Wu et al., 2016; Zhao et al., 2015b; Zhao et al., 2007b).

Microglia/macrophages (MM Φ) are the major phagocytes in the innate immune system. Under in vitro conditions, MM Φ can be polarized into distinct classically and alternatively activated phenotypes; however, in response to microenvironmental signals in vivo, MM Φ dynamically switch their phenotype, both temporally during an immune response and based on the local stimuli (Hu et al., 2015; Mosser and Edwards, 2008). Accumulating evidence indicates that classically activated and alternatively activated MM Φ within the lesion area can individually contribute to tissue damage or repair in the brain under conditions of spinal cord injury (Kigerl et al., 2009; Kroner et al., 2014), traumatic brain injury (Kumar et al., 2013; Wang et al., 2013), ischemic stroke (Hu et al., 2012), multiple sclerosis (Mikita et al., 2011), and Parkinson's disease (Pisanu et al., 2014). To date, no study has comprehensively characterized MM Φ dynamics or delineated the functional significance of MM Φ phenotype on hematoma resolution in vivo (Wan et al., 2016; Zhang et al., 2016; Zhao et al., 2015a).

Under physiologic conditions, macrophages have the ability to clear senescent erythrocytes and regulate iron homeostasis by metabolizing hemoglobin and its metabolic byproducts (Mosser and Edwards, 2008). Microglia, the brain's resident macrophages, are capable of clearing erythrocytes after ICH (Egashira et al., 2015), and pharmacologic enhancement of microglial phagocytosis has been shown to reduce hematoma volume in a mouse blood-

injection model of ICH (Zhao et al., 2007b). Modulation of MM Φ phenotype might offer a therapeutic strategy for treating ICH by limiting hematoma expansion (Wang et al., 2003; Zhao et al., 2015a; Zhao et al., 2009). However, the relationship between MM Φ phenotype, phagocytosis, and hematoma resolution after ICH is still unknown (Zhao et al., 2015a). One subset of alternatively activated MM Φ that might potentially be beneficial for the process of hematoma clearance is interleukin (IL)-10-regulated MM Φ (Mosser and Edwards, 2008; Naito et al., 2014). IL-10/heme oxygenase (HO)-1 signaling not only regulates microglial erythrocyte clearance in subarachnoid hemorrhage (Schallner et al., 2015), but also promotes resolution of inflammation by skewing alternative activation of macrophages in a mouse model of peripheral nerve injury (Siqueira Mietto et al., 2015).

In the present study, we sought to gain an understanding of the kinetics of MM Φ polarization after ICH and how MM Φ polarization affects hematoma clearance.

Materials and Methods

Animals

All experimental protocols were conducted in accordance with the National Institutes of Health guidelines and were approved by the Johns Hopkins University and Yale University Animal Care and Use Committee. The experiments were conducted in C57BL/6 male mice (Charles River Laboratories; Frederick, MD) and Cx3cr1^{GFP/+} mice (C57BL/6 background; kind gift from Dr. Jonathan Bromberg, University of Maryland, Baltimore, MD). The sham experiments for Figures 1, 2, 4, and 6, and the Rosiglitazone versus GW9662 experiments for Figure 8E and F and Supplementary Fig 3 were conducted in C57BL/6 male mice (The Jackson Laboratory) at Yale University. A total of two hundred and sixty-three mice were subjected to this study. Group size calculation was based on our previous experience of the variability, reproducibility and statistical analyses of the outcome measures in this model. To enhance the clinical relevance of the study, we used mice that were 10–12 months old because ICH occurs more often in middle-aged and elderly individuals. All efforts were made to minimize the numbers of animals used and ensure minimal suffering.

ICH model

After anesthetizing mice with 1–3% isoflurane inhalation and ventilating them with oxygen-enriched air (20%:80%), we injected a total of 0.5 μ L of 0.1 U collagenase VII-S (Sigma, St. Louis, MO) at 0.1 μ L/min into the left basal ganglion at the following coordinates relative to bregma: 0.8 mm anterior, 2 mm lateral, and 2.8 mm deep, as described previously (Chang et al., 2014). The craniotomy was sealed with bone wax, and the scalp was closed with 4-0 silk sutures. Rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ throughout the experimental and recovery periods (DC Temperature Controller 40-90-8D; FHC Inc., ME). Sham-operated mice received the same treatment, including needle insertion, but collagenase was not injected.

Rosiglitazone and GW9662 treatment

Mice were randomly assigned (<http://www.randomization.com>) to receive either 0.5 mg/kg rosiglitazone (potassium salt, a peroxisome proliferator-activated receptor γ [PPAR γ]

agonist; Cayman Chemical, Ann Arbor, MI) and/or 5 mg/kg GW9662 (a potent PPAR γ antagonist; Cayman Chemical) or vehicle (sterilized water; Hospira, USA) by intraperitoneal injection. Mice were injected with these treatments immediately after collagenase injection and then once daily until euthanasia. The delivery route and dosing regimens were based on previous work (Allahtavakoli et al., 2006; Burton et al., 2008; Cuartero et al., 2013; Hyong et al., 2008; Luo et al., 2006; Shimada et al., 2015; Zhao et al., 2009) and our own preliminary tests.

Fresh ICH brain section preparation and hematoma volume assessment

Mice were anesthetized with isoflurane and euthanized at various time points after ICH by transcardial perfusion with phosphate-buffered saline (PBS). Fresh 1-mm brain slices were prepared based on established standard procedure (Chang et al., 2015). Images of the brain slices were digitalized by scanner (600 dpi; Epson Perfection V750 PRO) and analyzed with Image J software (NIH, Bethesda, MD). The hematoma volume in cubic millimeters was calculated as the summation of the hematoma areas multiplied by the interslice distance (1 mm) (Foerch et al., 2008; Wu et al., 2012).

Neurologic function evaluations

On days 1, 4, and 7 post-ICH, mice were evaluated with neurologic severity scores, the forelimb placing test, the hindlimb adduction test, and the corner turn test as described previously (Chang et al., 2014; De Ryck et al., 1989; Zhu et al., 2014). Briefly, for neurologic severity score assessment, mice were evaluated for body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling. Each test was graded from 0 to 4, establishing a maximum deficit score of 24. Forelimb and hindlimb placing were assessed with a vibrissae-elicited forelimb placing test and a hindlimb adduction test, respectively. For the forelimb placing test, the mouse was placed on the edge of a benchtop and the vibrissae on one side were brushed. Intact animals placed the ipsilateral forelimb quickly onto the tabletop. During the hindlimb adduction test, the mouse was placed on the edge of a platform, and one side of the hind paw was gently pulled down away from the edge. Upon sudden release, intact animals quickly retrieved and replaced the ipsilateral hindlimb. Both tests were quantified as a percentage of successful placing responses in 10 trials. For the corner turn test, the mouse was allowed to proceed freely into a 30° corner and choose either left or right to exit the corner. The choice of direction during 10 repeats was recorded, and the percentage of ipsilateral turns was calculated.

Immunofluorescence

Animals were euthanized at various time points after ICH by isoflurane overdose and then perfused transcardially with ice-cold 0.1 M PBS (pH 7.4) and 4% paraformaldehyde in 0.1 M PBS. Brains were removed, post-fixed overnight in 4% paraformaldehyde, and then placed in 30% sucrose for 2 days. For single and double immunofluorescence labeling, brain sections were incubated overnight at 4°C with the following primary antibodies: anti-ionized calcium binding adaptor molecule 1 (Iba1, microglia/macrophage marker), anti-glial fibrillary acidic protein (GFAP, astrocyte marker), anti-CD206 (alternative activation marker), anti-CD16/CD32 (classic activation marker), and anti-NeuN (neuron marker; Table 1). After being washed three times, the sections were incubated with Alexa Fluor 488-

and/or Alexa Fluor 594-conjugated secondary antibody (1:1000; Molecular Probes, Eugene, OR) for 2 h at 37°C. For each mouse, we acquired images from at least three randomly selected fields per section and chose at least two randomly selected sections from the major hemorrhagic territory using a fluorescence microscope (Nikon TE 2000-E) (Zhao et al., 2015c). NeuN-positive cells were quantified and expressed as cells/mm² (Chang et al., 2014). To avoid antibody interaction, we incubated the brain sections with primary and secondary antibodies separately and sequentially for the time periods specified above. Negative controls that were performed in parallel without primary antibodies showed very low levels of nonspecific signal.

Colocalization analysis and quantification

Colocalization of MMΦ marker (Iba1) and classic activation marker (CD16/32) or alternative activation marker (CD206) was analyzed and quantified as previously described (Li et al., 2004). Briefly, the MMΦ marker (in red) and the individual classic/alternative activation markers (in green) are deemed to be colocalized in areas stained with the combined color (in yellow) when the two markers are overlaid. The colocalization signal was simultaneously detected and turned into gray scale image by Image J software. The degree of colocalization was expressed as arbitrary gray pixels. No colocalization signal was detected in the contralateral hemisphere.

Isolation of brain-infiltrating CD11b-positive cells after ICH

Mice were euthanized and perfused with 50 mL of ice-cold PBS on day 3 after ICH. Brains were removed, and 4-mm fresh coronal perihematomal tissue sections were collected and processed in ice-cold PBS according to our established method (Chang et al., 2015). CD11b-positive cells were isolated and purified according to published protocol (Bedi et al., 2013; Garcia et al., 2014; Taetzsch et al., 2015), and cell sorting was carried out with cells pooled from three mice per experiment. Briefly, three coronal hemorrhagic (ipsilateral) or uninjured (contralateral) hemispheres were collected into a GentleMacs C-tube (Miltenyi Biotec, Auburn, CA) and digested with papain (Neural Tissue Dissociation Kit; Miltenyi Biotec). The gentleMACS Dissociator (Miltenyi Biotec) was used for mechanical dissociation according to the manufacturer's directions. After three rounds of dissociation, each followed by a period of incubation at 37°C, contaminating erythrocytes were lysed with RBC lysis buffer (Sigma). Single-cell suspensions were rinsed in Hanks' balanced salt solution (HBSS) containing Ca²⁺ and Mg²⁺, passed over a 40-μm filter (BD Falcon, Franklin Lakes, NJ), and centrifuged at 300×g for 10 min. The cell pellets were resuspended in PBS containing 5% fetal bovine serum and incubated with anti-myelin immunoglobulin-conjugated magnetic microbeads (Myelin Removal Kit; Miltenyi Biotec) to remove myelin. CD11b-positive cells were then enriched by subsequent positive-selection steps with CD11b antibody-coated magnetic microbeads and a MACS magnetic separator according to the manufacturer's instructions (Miltenyi Biotec). The CD11b-positive cells were then stained for CD11b, CD45, and CD206 for use in flow cytometry or stored at -80°C for whole cell protein extraction and Western blot analysis.

Flow cytometry

After being washed with cold wash buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS), 1×10^5 cells per sample were incubated for 10 min with FcR blocking reagent (Miltenyi Biotec) at 4°C. All samples were then incubated for 30 min with conjugated antibodies against cell-surface markers CD11b-FITC and CD45-PE in the dark. After surface staining, cells underwent fixation with Fix/Perm Buffer (BD Biosciences) for 30 min at 4°C followed by permeabilization (BD Biosciences) for 5 min. Intracellular staining was performed with CD206-APC and isotype-matched control for 30 min at 4°C (Cuartero et al., 2013) (Table 1). Stained samples were washed, and the whole cell suspension was acquired by FACSCalibur flow cytometry with CellQuest software (BD Pharmingen, San Jose, CA). We used single-stained positive controls for cytometric compensation and unstained negative controls and isotype controls (Table 1) to determine fluorescence due to background and non-specific binding (Hammond et al., 2014a). Results were analyzed with FlowJo software (Tree Star, Ashland, OR).

Western blot analysis

Perihematomal tissue-isolated CD11b-positive cells were collected as described above. Cells from three separate animals were pooled and lysed in M-PER (Mammalian Protein Extraction Reagent; ThermoFisher Scientific, Rockford, IL) containing Complete Mini Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Germany). Total protein was quantified by the Bradford protein assay (Bio-Rad, USA). Western blotting was carried out as previously described (Chang et al., 2011). Briefly, equal amounts of protein (30 µg protein per sample lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45 µm polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk in PBS-T (PBS containing 0.1% Tween-20) and probed with primary antibodies against HO-1 and cyclophilin A (Table 1) at 4°C overnight. The membranes were washed three times in PBS-T and then incubated with HRP-linked anti-rabbit secondary antibody (Cell Signaling, Danvers, MA) for 1 h. Membranes were washed again, and proteins were visualized with ECL Plus (Amersham, Piscataway, NJ). Images were digitized by ImageQuant LAS 4000 (GE, Piscataway, NJ). The relative intensity of HO-1 signal was normalized to the corresponding cyclophilin A (loading control) intensity. Expression was quantified as densitometric units with ImageQuant software.

ELISA

At 1–1.5 h after ICH, we collected 4-mm, equal-weight coronal sections encompassing the major hemorrhagic territory from sham and ICH mice. We extracted protein with T-PER reagent (ThermoFisher) containing protease inhibitors (Roche) and quantified it with the Bradford protein assay. We then quantified IL-10 in equal amounts of protein (4 mg in a total volume of 100 µL) with the IL-10 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Tissue IL-10 concentrations were analyzed from samples and standards in duplicates and expressed as picograms per milliliter as previously described (Chang et al., 2014).

Organotypic hippocampal slice cultures (OHSCs)

OHSCs were prepared from postnatal day 5–7 Cx3cr1^{GFP/+} neonates according to a previously described method with slight modification (De Simoni and Yu, 2006; Gogolla et al., 2006). The neonates were decapitated, and the brains were rapidly removed and placed in ice-cold HBSS. The hippocampus was then isolated and sectioned into 350- μ m coronal slices with a McIlwain tissue chopper (Ted Pella, Redding, CA). The hippocampal slices were carefully separated. Four slices from two neonates were transferred onto one hydrophilic PTFE cell culture insert (pore size: 0.4 μ m; Millicell-CM, Millipore) in a 6-well culture plate. We incubated the slices in 1200 μ L of freshly prepared culture medium consisting of DMEM, 25% HBSS, 35 mM glucose, 25 mM HEPES, 25% heat-inactivated horse serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Grand Island, NY) at 37°C in a humidified incubator enriched with 5% CO₂. The low-serum (5% heat-inactivated horse serum) culture medium was changed the next day and then replaced twice a week. OHSCs were incubated with or without 10 μ M hemoglobin (Sigma), 0.5 μ M rosiglitazone (Cayman), and 20 μ g/mL mouse IL-10 receptor alpha antibody (R&D Systems) on day 14 of culture.

Phagocytosis

To test the phagocytic ability of Cx3cr1^{GFP/+} OHSCs, we examined the uptake of 1 μ m-diameter fluorescent latex beads (2.5% solids in water; Fluoresbrite polychromatic red, Polysciences, Warrington, PA) 1 day after the different treatments. The fluorescent beads in PBS containing 50% heat-inactivated horse serum were added onto the OHSCs in one cell culture insert of a 6-well culture plate at a final concentration of 4.55×10^8 beads/5 mL. OHSCs were incubated at 37°C in 5% CO₂ for 4 h, and the beads were resuspended by gentle shaking every 30 min. In parallel, we incubated some OHSCs at 4°C to inhibit phagocytosis. These slices served as negative controls. After incubation, we washed the OHSCs three times in ice-cold PBS for 5 min each to remove excess beads, and then fixed the tissue with 4% paraformaldehyde overnight at 4°C. Using a fluorescence microscope, an investigator blind to treatment counted the number of latex beads ingested by Cx3cr1^{GFP/+} microglia in three randomly chosen fields (0.08 mm²)/slice/condition (4 slices per condition). The experiments were run in triplicate, and the results are presented as the number of ingested beads/mm² (Ajmone-Cat et al., 2013).

Reverse transcription real-time PCR

We extracted total RNA either from a 2-mm coronal brain section containing the major hemorrhagic territory (~50 mg) collected at 1–1.5 h, 6 h, 1 day, 3 days, 7 days, and 14 days after ICH, or from hemoglobin- or rosiglitazone-treated OHSCs, using QIAzol Lysis Reagent (miRNeasy Mini Kit; QIAGEN, Valencia, CA). We transcribed 1 μ g of RNA from each sample into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and then performed real-time PCR on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix II with UNG (uracil N-glycosylase, Applied Biosystems). The following TaqMan Gene Expression Assay Mixes (Applied Biosystems) were used: *Mrc1* (CD206; Mm00485148_m1), *Chi3l3* (YM1/2; Mm00485148_m1), *Arg1* (arginase-1; Mm00475988_m1), *IL4* (IL-4; Mm00445259_m1),

IL10 (IL-10; Mm00439614_m1), *Tgfr1* (TGF- β ; Mm01227699_m1), *Fcgr2b* (CD32; Mm00438875_m1), *Fcgr3* (CD16; Mm00438882_m1), *Itgam* (CD11b; Mm00434455_m1), *Cd86* (CD86; Mm00444543_m1), *Ifng* (IFN- γ ; Mm01168134_m1), and *cd74* (MHCII; Mm00658576_m1). The primer and probe sequences used in these commercially available assay mixes are available online. The endogenous control was *Gapdh* (GAPDH; Mm99999915_g1). The cycle time values of candidate genes were normalized to *Gapdh* in the same sample. The expression levels of mRNA are presented as fold change versus sham control in the animal study (Chang et al., 2014) and fold change versus unstimulated control in the OHSCs (Ajmone-Cat et al., 2013).

IL-10 administration

Mice were anesthetized with 1–3% isoflurane in oxygen-enriched air (20%:80%) and immobilized in a stereotaxic frame (Stoelting, Wood Dale, IL). After making a midline incision in the skin, we used a 1- μ L Hamilton syringe to administer 200 ng of recombinant mouse IL-10 (1 μ L; R&D Systems) or vehicle (1 μ L saline) by intracerebroventricular injection (Liesz et al., 2009) at 2 h and 1 day after ICH induction. The injections were delivered at the following coordinates: 1.0 mm lateral, 0.5 mm posterior, and 2.5 mm deep relative to the bregma.

Statistical analysis

Data are presented as mean $\pm$ SD. In histological and biochemical studies, one-way or two-way repeated measure ANOVA was used for comparisons among multiple groups. For behavioral tests and body weight measurement, two-way ANOVA was used to detect significant differences between and among treatment groups. Bonferroni post hoc analysis was used to examine significant differences. Student's t-test was used to detect differences between two groups (SigmaStat 3.5; Systat Software Inc., San Jose, CA). Statistical significance was set at $P < 0.05$.

Results

MM Φ are clustered mainly within the hematoma core and at the adjacent hematoma border after ICH

We used the collagenase-induced ICH model to evaluate hematoma resolution in the mouse brain. The collagenase-induced hematoma was consistently located in the striatum and gradually resolved over the course of 14 days (Fig. 1A). Because MM Φ and astrocytes are the major cell types that respond to hematoma formation after ICH (Wang, 2010; Zhao et al., 2009), we examined the temporal and spatial profile of Iba1⁺ (MM Φ) and GFAP⁺ (astrocytes) cells. At 1 and 3 days post-ICH, activated Iba1⁺ MM Φ and GFAP⁺ astrocytes had accumulated at the inner and outer hematoma borders, respectively. Interestingly, at later time points (7 and 14 days), Iba1⁺ MM Φ were largely clustered and stacked within the hematoma core, whereas GFAP⁺ astrocytes remained around the outer boundary of the hematoma (Fig. 1B). The amount of GFAP⁺ signal was largely increased in the hemorrhagic brains from day 1 to day 14 post-ICH, whereas Iba1⁺ signal was decreased from day 7 post-ICH (Supplementary Fig. 1). A slight increase of Iba1⁺ and GFAP⁺ immunoreactivity was only observed along with the needle track in the sham-operated brains (Fig. 2), consistent

with a minimal response after the small physical disruption of the brain (Chang et al., 2011). On days 7 and 14, intact nuclei were observed in the Iba1⁺ MMΦ, indicating that the Iba1⁺ signal did not come from the autofluorescence of red blood cells in the hematoma core (Fig. 1B, inset). The results suggest that MMΦ could be the major cell type involved in natural clearance of hematoma in the ICH brain.

Polarization dynamics of MMΦ after ICH

To examine the profile of MMΦ phenotype during ICH progression, we first characterized polarized brain cells by surface marker expression and cytokine production using real-time PCR. We found that the mRNA expression of classic activation markers (CD32, CD16, CD11b, CD86, IFN γ , and MHCII) peaked from days 3 to 7 post-ICH and remained elevated for at least 14 days (Fig. 3A). However, real-time PCR reflects the changes of mRNA in brain tissue containing a mixture of cell types; MMΦ (Hu et al., 2015), neutrophils (Cuartero et al., 2013), and astrocytes (Jang et al., 2013) have also been demonstrated to have different polarization states. Accordingly, we further performed double-immunofluorescence staining with the MMΦ marker Iba1 and with markers of classic activation (CD16/32) or alternative activation (CD206) proteins to evaluate the polarization of MMΦ after ICH. When we examined Iba1 and CD16/32 double-labeling in ICH brain sections (Supplementary Fig. 2), CD16/32 immunoreactivity was difficult to detect at 1 day post-ICH, but activated Iba1⁺ cells appeared at the hemorrhagic territory. At 3 days post-ICH, Iba1 and CD16/32 colocalized to the hematoma core region, peaking at day 7 and persisting until day 14 (Fig. 3B, C). Most of the CD16/32 signal was derived from Iba1⁺ cells (Fig. 3B, bottom panel and Fig. 3C), suggesting that the polarization signals come mainly from MMΦ rather than other types of cells in the brain. This finding also supports the reliability of the post-ICH polarization profile at the protein level. Notably, the sham surgery did not induce CD16/32 expression, while some activated Iba1⁺ MMΦ were observed along with the needle track (Fig. 4).

In contrast to the expression pattern of classic activation markers, alternative activation marker expression (IL-10, TGF- β , and IL-4) increased as early as 1–1.5 h after ICH, as detected by real-time PCR (Fig. 5A). Interestingly, expression of both IL-4 and IL-10 declined at 6 h after ICH, and then increased again. Following the rapid induction of the first wave of M2-polarizing cytokines, expression of other markers of alternative activation peaked at day 3 (YM1/2 and CD206) or day 7 (TGF- β and Arg1). At 14 days post-ICH, only CD206 mRNA expression was consistently higher than that in the sham group (Fig. 5A). Consistent with the mRNA results, we observed increased CD206 immunoreactivity and CD206-Iba1 double-positive signal around the hematoma border (Supplementary Fig. 2) on days 1 and 3 post-ICH. On days 7 and 14, CD206 and Iba1 colocalized mainly within the hematoma core, peaking at day 7, and the inner boundary of the hematoma (Fig. 5B, C). The sham operation induced Iba1⁺ cells activation and these cells continuously expressed CD206 after surgery through day 14 (Fig. 6).

An elevation of alternative activation markers at the very early stage of ICH might imply that resident microglia transform into a “protective” phenotype in response to the onset of ICH. To test this possibility, we measured the IL-10 levels in perihematomal tissue and HO-1

expression in brain-isolated CD11b⁺ cells, because IL-10/HO-1 signaling is relevant to hematoma clearance and promotes MMΦ alternative activation (Avdic et al., 2013; Weis et al., 2009). We found that IL-10 was increased in the hemorrhagic brain tissue compared with that in the contralateral hemisphere at 1–1.5 h post-ICH (19.5 ± 0.5 pg/mL vs. 1.9 ± 0.9 pg/mL; $P < 0.05$; Fig. 5D). HO-1 was detected specifically in the CD11b⁺ cells from hemorrhagic brain tissue. HO-1 protein expression was ~1.4-fold higher in the hemorrhagic hemisphere than in the contralateral hemisphere (Fig. 5E; $P < 0.05$). Taken together, these findings reveal a temporal shift in MMΦ phenotype after ICH from alternative activation-dominant at the acute phase (from onset to day 1) to broad spectrum mixed phenotype at the subacute phase (days 3 to 7) to predominantly classically activated at the chronic stage (day 14).

Repeated treatment of ICH mice with rosiglitazone induces MMΦ alternative activation-skewed polarization, accelerates hematoma resolution, and reduces neurologic deficits

Previous work showed that the PPAR γ agonist rosiglitazone can promote MMΦ alternative activation in vivo (Pisanu et al., 2014) and in vitro (Ballesteros et al., 2014) as well as a clinical trial (SHRINC) regarding the effect of PPAR γ agonist on hematoma resolution is on-going (Gonzales et al., 2013). Therefore, we used rosiglitazone as a pharmacologic tool to study the relationship between MMΦ phenotype, hematoma resolution, and ICH outcome in mice. In addition, because leukocyte infiltration peaks from day 3 to day 5 after ICH (Hammond et al., 2014b; Loftspring et al., 2009; Mracsko et al., 2014), and the hematoma was largely resolved after day 7 post-ICH in our model, we chose days 4 and 7 post-ICH to evaluate the maximum effects of MMΦ on hematoma resolution. Rosiglitazone administration reduced hematoma volume compared to that of vehicle-treated mice on day 4 (5.4 ± 1.3 mm³ vs. 9.1 ± 1.7 mm³; $P < 0.05$) and day 7 (1.0 ± 0.2 mm³ vs. 1.9 ± 0.4 mm³; $P < 0.05$; Fig. 7A, B) post-ICH. Body weight was significantly greater in the rosiglitazone-treated group than in the vehicle-treated group on day 1 ($P < 0.05$) but not on days 4 and 7 (Fig. 7C). Additionally, compared with the vehicle-treated group, rosiglitazone-treated mice exhibited better performance in neurologic deficit testing, the corner turn test, the hindlimb adduction test, and the forelimb placing test (Fig. 7D, Table 2).

Because alternatively activated MMΦ can clear debris and support tissue repair through their phagocytic ability (Arnold et al., 2007), we further investigated whether the acceleration of hematoma resolution correlates with the boost in MMΦ alternative activation after rosiglitazone treatment. Using flow cytometric analysis, we were able to distinguish between activated microglia (CD11b⁺CD45^{low}) and infiltrating macrophages (CD11b⁺CD45^{high}) (Mracsko and Velkamp, 2014) and detect changes in alternative activation marker CD206 expression in specific cell populations (Fig. 8A–C). In mice that had been treated with rosiglitazone after ICH induction, the expression of CD206 was increased on both CD11b⁺CD45^{low} microglia and CD11b⁺CD45^{high} macrophages (Fig. 8C, D) on day 3 post-ICH. Among CD11b⁺CD45^{low} microglia, the number of cells expressing CD206 increased from $6.3 \pm 1.2\%$ (vehicle) to $8.7 \pm 0.3\%$ ($P = 0.029$), and among CD11b⁺CD45^{high} macrophages, the number increased from $9.0 \pm 0.9\%$ (vehicle) to $11.9 \pm 0.8\%$ ($P = 0.02$). Immunofluorescence for CD206 was elevated in ipsilateral brain sections from rosiglitazone-treated mice compared to that in the vehicle-treated mice (fluorescence

intensity: 9.224 ± 2.140 vs. 5.613 ± 2.753 ; $P = 0.049$; Fig. 9); however, CD206 immunoreactivity was not detectable in contralateral brain sections from rosiglitazone-treated mice.

To confirm the actions of rosiglitazone on PPAR γ activation and the importance of MM Φ alternative phenotype skewing on hematoma resolution, we utilized the PPAR γ antagonist GW9662 to block the PPAR γ effects on alternative polarization of MM Φ . We also injected IL-10 to induce alternative activation of MM Φ independently of PPAR γ . Rosiglitazone treatment of mice significantly increased the mRNA expression of alternative activation markers YM1/2 (~45%; $P < 0.001$), Arg1 (~43%; $P = 0.008$), and IL-10 (~29%; $P < 0.001$) in perihematoma tissue. Mice treated with PPAR γ antagonist GW9662 did not show increased expression of YM1/2, Arg1 and IL-10 (Fig. 8E, F). Notably, co-treatment of rosiglitazone and GW9662 in ICH mice significantly reduced rosiglitazone-induced IL-10 expression (~40%; $P = 0.002$), while YM1/2 and IL-10 expression in the rosiglitazone and GW9662 co-treated group were higher than that in the GW9662-treated group (Fig. 8E, F). GW9662 treatment of the rosiglitazone-treated ICH mice abolished rosiglitazone-induced hematoma resolution in the hemorrhagic brain on day 3 post-ICH (Supplementary Fig. 3). The modulatory effect of rosiglitazone on MM Φ alternative activation was further evident by serum IL-10 (Fig. 8F). Rosiglitazone increased IL-10 in the serum of ICH mice compared to that in the serum of vehicle-treated ICH mice on day 2 (37.5 ± 10.5 vs. 18.4 ± 0.2 pg/mL; $P = 0.011$) and day 3 (33.3 ± 13.3 vs. 16.5 ± 2.9 pg/mL) post-ICH but not on days 1 (12.9 ± 3.4 vs. 11.5 ± 3.3 pg/mL; $P = 0.575$), 4 (12.2 ± 2.9 vs. 23.1 ± 12.4 pg/mL; $P = 0.062$), or 7 (17.7 ± 4.3 vs. 23.2 ± 9.9 pg/mL; $P = 0.238$). IL-10 was undetectable in the serum of sham-operated mice (data not shown). Compared with that in the vehicle-treated group, intraventricular IL-10 administered at 2 h and 1 day post-ICH reduced hematoma volume (9.1 ± 2.0 mm³ vs. 4.8 ± 1.1 mm³; $P < 0.05$) on day 3 after ICH-induction (Fig. 8G).

IL-10 receptor neutralization diminishes phagocytic ability of microglia in OHSCs

Next we tested potential regulatory mechanisms that might mediate microglial polarization and phagocytosis. We mimicked ICH stimulation in OHSCs by applying hemoglobin because this ex vivo system maintains the cell-cell interactions of the brain and hemoglobin is the main product of erythrocyte lysis. We first assessed OHSC responsiveness to hemoglobin over time by using qPCR. The pattern of expression of mRNA for IL-10 mirrored the biphasic pattern we observed in vivo, peaking within 3 h, declining, and then increasing again at days 1 and 3. Expression of Arg1 peaked at 1 day and classic activation markers CD11b and CD86 at 3 days after hemoglobin stimulation (Fig. 10A). This early and sustained increase of IL-10 mRNA expression in both perihematoma brain and hemoglobin-stimulated OHSCs supports the importance of IL-10 signaling in regulating microglial response to hemoglobin. In the fluorescent bead ingestion assay, exposure of Cx3Cr1^{GFP/+} OHSCs to hemoglobin or rosiglitazone for 24 h enhanced microglial phagocytosis of beads (Fig. 10B). In the unstimulated control group, microglia ingested 3.0 ± 0.5 fluorescent beads/cell, but in the hemoglobin-exposed group, that number rose to 20.6 ± 3.4 fluorescent beads/cell ($P < 0.05$), and in the rosiglitazone-treated group, it rose to 9.9 ± 3.7 fluorescent beads/cell ($P < 0.05$; Fig. 10C). Neutralizing the IL-10 receptor by co-incubation with IL-10 receptor alpha antibody for 24 h decreased the number of fluorescent bead-containing

microglia in the hemoglobin-treated group (12.0 ± 1.8 fluorescent beads/cell; $P < 0.05$) and in the rosiglitazone-treated group (5.2 ± 1.5 fluorescent beads/cell; $P < 0.05$). These findings indicate that IL-10 signaling is involved in enhancement of microglial phagocytic function after ICH. In addition, rosiglitazone increased the number of neurons (686.7 ± 174.8 cells/mm²) in the perihematoma area compared with that in the vehicle-treated group (538.3 ± 206.5 cells/mm²) on day 4 post-ICH ($P = 0.043$; Fig. 11).

Discussion

Recent studies in stroke and other neurologic disorders suggest a dual role for MMΦ in brain injury and recovery (Hu et al., 2012; Kigerl et al., 2009; Kroner et al., 2014; Kumar et al., 2013; Mikita et al., 2011; Pisanu et al., 2014; Wang et al., 2013). To our knowledge, this is the first study that comprehensively characterize the dynamic of MMΦ response to ICH and examine the relation of MMΦ phenotype to hematoma resolution. Although we reported in 2003 that tissue plasminogen activator assists in the clearance of hematoma, presumably by modulating MMΦ activation or function (Wang et al., 2003), microglial activation and macrophage infiltration are generally believed to play a deleterious role after ICH insult (Hammond et al., 2014b). MMΦ aggravate ICH-induced brain injury from acute to chronic pathologic stages through mechanisms that include excessive production of reactive oxygen species (Aronowski and Hall, 2005; Katsuki, 2010; Wang and Dore, 2007), release of proinflammatory mediators (cytokines and chemokines) (Mayne et al., 2001; Wang and Tsirka, 2005b), and damage to blood-brain barrier integrity (Power et al., 2003; Wang and Tsirka, 2005a). Because of their ability to clear cell debris and phagocytize blood components, MMΦ might also be beneficial to ICH outcomes (Fang et al., 2014; Ni et al., 2016; Zhao et al., 2009; Zhao et al., 2015b; Zhao et al., 2007a). Therefore, a better understanding of the dynamic equilibrium between harmful and helpful MMΦ and the function of specific MMΦ phenotypes after ICH will advance our knowledge of post-ICH recovery and guide ICH treatment in the future (Zhao et al., 2015a).

In this study, we show that striatal hematoma resolves spontaneously through the body's own defense mechanism in about 14 days after collagenase injection. In the acute phase of ICH, most MMΦ accumulate within the hemorrhagic territory. Then, in the chronic stage, they enter the hematoma core. This evidence supports the notion that MMΦ are main players in the progress of hematoma resolution (Egashira et al., 2015; Wang et al., 2003; Zhao et al., 2009). MMΦ become polarized toward classically activated or alternatively activated phenotypes at different stages after various central nervous system (CNS) injuries (Zhang et al., 2016). We and others have reported that microglia are rapidly activated within 1 h after the onset of ICH (Aronowski and Hall, 2005; Wang and Dore, 2007), suggesting that microglia could be the first non-neuronal cells to react to ICH. Most published studies have chosen day 1 as the earliest time point to study MMΦ polarity after disease onset (Hu et al., 2012; Kigerl et al., 2009; Kumar et al., 2013; Pisanu et al., 2014). In fact, leukocytes infiltrate as early as 5 h after ICH onset (Wang and Dore, 2007). To understand how microglia respond to hemorrhagic insult before they interact with leukocytes, we monitored microglial phenotype profiles at the ultra-early phase of ICH. Consistent with our previous finding (Wang and Dore, 2007), we observed a rapid phenotypic change in CD11b-positive microglial cells with specific upregulation of alternative signatures (Shouval et al., 2014;

Weis et al., 2009) in the hemorrhagic milieu at 1–1.5 h post-ICH. Alternatively activated MMΦ have increased phagocytic activity and provide neuroprotection by reducing production of inflammatory mediators and downregulating neurotoxic pathways (Hu et al., 2015). Adoptive transfer of alternatively activated macrophages improves functional recovery in spinal cord injury (Ma et al., 2015). This ultra-early alternative activation of resident microglia before the first wave of peripheral leukocyte infiltration (Hammond et al., 2012; Wang and Dore, 2007) suggests that microglia react quickly to ICH insult. In fact, microglia showed a prominent tendency to maintain the status of alternative activation in an ex vivo model of organotypic brain slices subjected to oxygen-glucose deprivation (Girard et al., 2013) and in an in vitro model of human adult microglia and blood-derived macrophages treated with lipopolysaccharide or myelin (Durafour et al., 2012). These results support our findings that microglia, the first line of CNS innate immune cells, tend to perform an initial restorative function after ICH. Interestingly, this ultra-early upregulation of some alternative activation markers (IL-4 and IL-10) declined at 6 h, but increased again at 1 day post-ICH. The reasons for this early burst of expression of IL-4/-10 mRNA is not clear. Recently, a study using autologous blood ICH model showed that proinflammatory marker CD16/32 peaks as early as 4 h after ICH induction (Wan et al., 2016); however, we could not detect the increase of CD16/32 expression before day 1 post-ICH in our collagenase model. MMΦ polarization at the ultra-early stages of ICH is still largely unknown. Until now, the role of immune system activity have rarely been evaluated before 1 day post-ICH in preclinical studies (Hammond et al., 2012; Wang and Dore, 2007; Zhao et al., 2014). A better understanding of the molecular and cellular mechanisms that mediate the loss of alternative activation cues in the hemorrhagic milieu during this ultra-acute period may improve our approach for treating ICH.

Studies of cerebral ischemia and traumatic brain injury show that newly recruited MMΦ at the site of injury express signature markers of alternative activation at day 1, whereas classic activation markers predominate on day 7 after insult (Hu et al., 2012; Wang et al., 2013). Our findings reveal that microglia undergo a similar phenotype shift, from alternative activation-dominant to classic activation-dominant, in the progression of ICH. Because of technical limitations, we cannot exclude the possibility that CD11b-positive neutrophils may contribute to fluctuations in the polarization gene profile seen in this study. Studies have proposed that neutrophils may participate in blood component elimination within brain parenchyma (Zhao et al., 2014) and express polarization markers in the ischemic brain (Cuartero et al., 2013); however, the polarization markers largely colocalized with MMΦ markers in our histological study. Moreover, the tissue level measurements in this study might reflect microenvironmental circumstances in the perihematomal area. The local stimuli are able to trigger MMΦ dynamically toward certain phenotype (Mosser and Edwards, 2008). Therefore, most of the phenotypic change that we observed should reflect the action of MMΦ after ICH. In future studies, we will focus on transcriptome profiling of ICH brain by using cell sorting to differentiate the functional dynamic changes of microglia, macrophages, and neutrophils at the single cell level.

Notably, unlike in previous studies, we found that among the alternative activation markers, mannose receptor (CD206), responsible for MMΦ phagocytosis (Regnier-Vigouroux, 2003), was highly expressed during the chronic phase of ICH. Moreover, our histologic results

showed that MMΦ accumulated in the hematoma area. These findings indicate that hematoma clearance by MMΦ continues for at least 14 days after ICH. Interestingly, in the sham-operated brains, we observed that MMΦ expressed the marker CD206, rather than the proinflammatory marker CD16/32, showing a reparative mechanism of action on microglia in the injured brain. The microglia in the contralateral hemisphere did not express CD206. This result implies that even a small disruption of brain integrity can trigger the MMΦ reparative phenotype which aids in clearance of cell debris and brain repair. However, after ICH, iron overload continues for 4 weeks (Wu et al., 2003), and macrophages can be induced to adopt the harmful proinflammatory phenotype in an iron-enriched environment (Kroner et al., 2014; Papatriantafyllou, 2011). Thus, additional studies are needed to gain a better understanding of the pathophysiology in which MMΦ phenotypes overlap in the chronic stage of ICH.

Hematoma size determines ICH outcomes in humans (Broderick et al., 1993) and rodents (MacLellan et al., 2006). Researchers have documented that resolving hematoma before MMΦ can trigger the detrimental secondary inflammatory cascade could provide a therapeutic strategy for ICH treatment (Mracsko and Veltkamp, 2014; Zhao et al., 2009). Our findings that PPARγ agonist rosiglitazone increases phagocytosis of erythrocytes by microglia in OHSCs and promotes hematoma resolution in vivo support this premise. This is further supported by our observation that inhibiting PPARγ activation with GW9662 treatment decreased rosiglitazone-induced expression of alternative activation markers (especially IL-10) and abolished PPARγ-mediated hematoma resolution. Similarly, PPARγ activation increases MMΦ alternative activation in animal models of Parkinson's disease (Pisanu et al., 2014) and peripheral pain (Hasegawa-Moriyama et al., 2012). However, the link between MMΦ alternative activation and hematoma resolution after ICH is still missing, as is the molecular mechanism that underlies the regulation of alternatively activated microglia-mediated phagocytosis. In agreement with a previous study that used the blood ICH model (Zhao et al., 2007b), our work shows that rosiglitazone treatment accelerates hematoma clearance and improves functional outcomes in the collagenase-induced ICH model. Furthermore, we provide novel evidence that this beneficial effect of rosiglitazone is associated with an increase in CD206 expression on MMΦ, a favorable local microenvironment for alternative activation (increased IL-10, arginase-1, and YM1/2 expression) in the hemorrhagic brain, and an elevation of IL-10 levels in serum. A clinical trial regarding the safety of pioglitazone on hematoma resolution in human is still ongoing (Gonzales et al., 2013). Future work would evaluating the efficacy and safety of different PPARγ agonists on improvement of mass effect and neurological outcomes in different preclinical ICH models.

Previous studies have reported that serum IL-10 levels are elevated in ICH patients (Dziedzic et al., 2002) and that IL-10-secreting cells are elevated in blood after cerebral ischemia (Pelidou et al., 1999). In fact, IL-10 is able to polarize monocytes toward a protective phenotype (Avdic et al., 2013; Koscsó et al., 2013) and reduce infarct volume in the ischemic brain by modulating lymphocyte function (Liesz et al., 2009). Although the protective function of alternatively activated MMΦ has been appreciated in several CNS diseases, the role of diverse alternatively activated phenotype in various CNS injuries has not been reported. In particular, the role of IL-10 and IL-10 signaling in ICH is largely unclear.

Although we cannot formally rule out the effect of rosiglitazone on regulatory T cell-derived IL-10 in our study, previous studies from our lab have shown that IL-10 released from regulatory T cell can regulate microglia alternative phenotype (Zhou et al., 2016). When we injected IL-10, a cytokine which has shown to polarize MMΦ toward alternative activation, intraventricularly into the ICH mice, we found a reduction of hematoma volume at day 3 post-ICH. These results indicates that other inducers of alternative activation of MMΦ, independent of PPAR γ , can also promote resolution and repair in the ICH brain. Furthermore, blocking IL-10 receptor prevented rosiglitazone-enhanced microglial phagocytosis in vitro. Taken together, our results indicate that IL-10 signaling and IL-10-induced MMΦ alternative activation are crucial protective mechanisms that modulate MMΦ phagocytosis and hematoma clearance.

Interestingly, the fact that IL-10 expression over time (e.g. a decrease at 6 h and 1 day) did not correlate with the enlargement of hematoma volume in vivo or with the time of hemoglobin stimulation ex vivo, suggests that an unknown mechanism may underlie IL-10-induced MMΦ alternative activation under ICH conditions. Further loss-of-function and gain-of-function studies will establish an understanding of how IL-10 signaling regulates MMΦ function and ICH outcomes.

Conclusions

Our study provides comprehensive data regarding the temporal and spatial pattern of MMΦ polarization after ICH. The ultra-early and transient alternative activation shifts to a mixed broad spectrum of phenotypes at the subacute stage and finally to a classic activation-dominant response during the chronic stage after ICH. Chronic classical activation of MMΦ may hinder neurologic recovery from ICH. Enhancing MMΦ alternative activation early might accelerate hematoma resolution by promoting MMΦ-mediated phagocytosis, which might be modulated by IL-10 receptor signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ajmone-Cat MA, et al. Microglial polarization and plasticity: evidence from organotypic hippocampal slice cultures. *Glia*. 2013; 61:1698–711. [PubMed: 23918452]
- Allahtavakoli M, et al. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma ligand, reduces infarction volume and neurological deficits in an embolic model of stroke. *Clin Exp Pharmacol Physiol*. 2006; 33:1052–8. [PubMed: 17042914]

- Arnold L, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med*. 2007; 204:1057–69. [PubMed: 17485518]
- Aronowski J, Hall CE. New horizons for primary intracerebral hemorrhage treatment: experience from preclinical studies. *Neurol Res*. 2005; 27:268–79. [PubMed: 15845210]
- Avdic S, et al. Human cytomegalovirus interleukin-10 polarizes monocytes toward a deactivated M2c phenotype to repress host immune responses. *J Virol*. 2013; 87:10273–82. [PubMed: 23864618]
- Ballesteros I, et al. Rosiglitazone-induced CD36 up-regulation resolves inflammation by PPARgamma and 5-LO-dependent pathways. *J Leukoc Biol*. 2014; 95:587–98. [PubMed: 24338629]
- Bedi SS, et al. Immunomagnetic enrichment and flow cytometric characterization of mouse microglia. *J Neurosci Methods*. 2013; 219:176–82. [PubMed: 23928152]
- Broderick JP, et al. Volume of intracerebral hemorrhage. A powerful and easy-to-use predictor of 30-day mortality. *Stroke*. 1993; 24:987–93. [PubMed: 8322400]
- Brott T, et al. Early hemorrhage growth in patients with intracerebral hemorrhage. *Stroke*. 1997; 28:1–5. [PubMed: 8996478]
- Burton JD, et al. Potential of peroxisome proliferator-activated receptor gamma antagonist compounds as therapeutic agents for a wide range of cancer types. *PPAR Res*. 2008; 2008:494161. [PubMed: 18779871]
- Chang CF, et al. Translational intracerebral hemorrhage: a need for transparent descriptions of fresh tissue sampling and preclinical model quality. *Transl Stroke Res*. 2015; 6:384–9. [PubMed: 25907620]
- Chang CF, et al. Caveolin-1 deletion reduces early brain injury after experimental intracerebral hemorrhage. *Am J Pathol*. 2011; 178:1749–61. [PubMed: 21435456]
- Chang CF, et al. (-)-Epicatechin protects hemorrhagic brain via synergistic Nrf2 pathways. *Ann Clin Transl Neurol*. 2014; 1:258–271. [PubMed: 24741667]
- Cuartero MI, et al. N2 neutrophils, novel players in brain inflammation after stroke: modulation by the PPARgamma agonist rosiglitazone. *Stroke*. 2013; 44:3498–508. [PubMed: 24135932]
- De Ryck M, et al. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats. *Stroke*. 1989; 20:1383–90. [PubMed: 2799870]
- De Simoni A, Yu LM. Preparation of organotypic hippocampal slice cultures: interface method. *Nat Protoc*. 2006; 1:1439–45. [PubMed: 17406432]
- Durafour BA, et al. Comparison of polarization properties of human adult microglia and blood-derived macrophages. *Glia*. 2012; 60:717–27. [PubMed: 22290798]
- Dziedzic T, et al. Intracerebral hemorrhage triggers interleukin-6 and interleukin-10 release in blood. *Stroke*. 2002; 33:2334–5. [PubMed: 12215608]
- Egashira Y, et al. Intercellular cross-talk in intracerebral hemorrhage. *Brain Res*. 2015; 1623:97–109. [PubMed: 25863131]
- Fang H, et al. CD36-mediated hematoma absorption following intracerebral hemorrhage: negative regulation by TLR4 signaling. *J Immunol*. 2014; 192:5984–92. [PubMed: 24808360]
- Flores JJ, et al. PPARgamma-induced upregulation of CD36 enhances hematoma resolution and attenuates long-term neurological deficits after germinal matrix hemorrhage in neonatal rats. *Neurobiol Dis*. 2016; 87:124–33. [PubMed: 26739391]
- Foerch C, et al. Experimental model of warfarin-associated intracerebral hemorrhage. *Stroke*. 2008; 39:3397–404. [PubMed: 18772448]
- Garcia JA, et al. Isolation and analysis of mouse microglial cells. *Curr Protoc Immunol*. 2014; 104(Unit 14):35. [PubMed: 24510618]
- Girard S, et al. Microglia and macrophages differentially modulate cell death after brain injury caused by oxygen-glucose deprivation in organotypic brain slices. *Glia*. 2013; 61:813–24. [PubMed: 23404620]
- Gogolla N, et al. Preparation of organotypic hippocampal slice cultures for long-term live imaging. *Nat Protoc*. 2006; 1:1165–71. [PubMed: 17406399]

- Gonzales NR, et al. Design of a prospective, dose-escalation study evaluating the Safety of Pioglitazone for Hematoma Resolution in Intracerebral Hemorrhage (SHRINC). *Int J Stroke*. 2013; 8:388–96. [PubMed: 22340518]
- Hammond MD, et al. Gr1+ Macrophages and Dendritic Cells Dominate the Inflammatory Infiltrate 12 Hours After Experimental Intracerebral Hemorrhage. *Transl Stroke Res*. 2012; 3:s125–s131. [PubMed: 23259009]
- Hammond MD, et al. alpha4 integrin is a regulator of leukocyte recruitment after experimental intracerebral hemorrhage. *Stroke*. 2014a; 45:2485–7. [PubMed: 25013026]
- Hammond MD, et al. CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage. *J Neurosci*. 2014b; 34:3901–9. [PubMed: 24623768]
- Hasegawa-Moriyama M, et al. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuates postincisional pain by regulating macrophage polarization. *Biochem Biophys Res Commun*. 2012; 426:76–82. [PubMed: 22910418]
- Hu X, et al. Microglial and macrophage polarization-new prospects for brain repair. *Nat Rev Neurol*. 2015; 11:56–64. [PubMed: 25385337]
- Hu X, et al. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke*. 2012; 43:3063–70. [PubMed: 22933588]
- Hyoung A, et al. Rosiglitazone, a PPAR gamma agonist, attenuates inflammation after surgical brain injury in rodents. *Brain Res*. 2008; 1215:218–24. [PubMed: 18479673]
- Jang E, et al. Phenotypic polarization of activated astrocytes: the critical role of lipocalin-2 in the classical inflammatory activation of astrocytes. *J Immunol*. 2013; 191:5204–19. [PubMed: 24089194]
- Katsuki H. Exploring neuroprotective drug therapies for intracerebral hemorrhage. *J Pharmacol Sci*. 2010; 114:366–78. [PubMed: 21081835]
- Keep RF, et al. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. *Lancet Neurol*. 2012; 11:720–31. [PubMed: 22698888]
- Kellner CP, Connolly ES Jr. Neuroprotective strategies for intracerebral hemorrhage: trials and translation. *Stroke*. 2010; 41:S99–102. [PubMed: 20876519]
- Kigerl KA, et al. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J Neurosci*. 2009; 29:13435–44. [PubMed: 19864556]
- King MD, et al. Attenuation of hematoma size and neurological injury with curcumin following intracerebral hemorrhage in mice. *J Neurosurg*. 2011; 115:116–23. [PubMed: 21417704]
- Kosco B, et al. Adenosine augments IL-10-induced STAT3 signaling in M2c macrophages. *J Leukoc Biol*. 2013; 94:1309–15. [PubMed: 23922379]
- Kroner A, et al. TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord. *Neuron*. 2014; 83:1098–116. [PubMed: 25132469]
- Kumar A, et al. Traumatic brain injury in aged animals increases lesion size and chronically alters microglial/macrophage classical and alternative activation states. *Neurobiol Aging*. 2013; 34:1397–411. [PubMed: 23273602]
- Li Q, et al. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J Neurosci*. 2004; 24:4070–81. [PubMed: 15102922]
- Liesz A, et al. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med*. 2009; 15:192–9. [PubMed: 19169263]
- Loftspring MC, et al. Intracerebral hemorrhage leads to infiltration of several leukocyte populations with concomitant pathophysiological changes. *J Cereb Blood Flow Metab*. 2009; 29:137–43. [PubMed: 18827833]
- LoPresti MA, et al. Hematoma volume as the major determinant of outcomes after intracerebral hemorrhage. *J Neurol Sci*. 2014; 345:3–7. [PubMed: 25034055]
- Luo Y, et al. Neuroprotection against focal ischemic brain injury by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. *J Neurochem*. 2006; 97:435–48. [PubMed: 16539667]

- Ma SF, et al. Adoptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury. *Brain Behav Immun*. 2015; 45:157–70. [PubMed: 25476600]
- MacLellan CL, et al. Gauging recovery after hemorrhagic stroke in rats: implications for cytoprotection studies. *J Cereb Blood Flow Metab*. 2006; 26:1031–42. [PubMed: 16395282]
- Mayne M, et al. Adenosine A2A receptor activation reduces proinflammatory events and decreases cell death following intracerebral hemorrhage. *Ann Neurol*. 2001; 49:727–35. [PubMed: 11409424]
- Mikita J, et al. Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult Scler*. 2011; 17:2–15. [PubMed: 20813772]
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008; 8:958–69. [PubMed: 19029990]
- Mracsko E, et al. Leukocyte invasion of the brain after experimental intracerebral hemorrhage in mice. *Stroke*. 2014; 45:2107–14. [PubMed: 24916913]
- Mracsko E, Veltkamp R. Neuroinflammation after intracerebral hemorrhage. *Front Cell Neurosci*. 2014; 8:388. [PubMed: 25477782]
- Naito Y, et al. Heme oxygenase-1 and anti-inflammatory M2 macrophages. *Arch Biochem Biophys*. 2014; 564:83–8. [PubMed: 25241054]
- Ni W, et al. Role of Erythrocyte CD47 in Intracerebral Hematoma Clearance. *Stroke*. 2016; 47:505–11. [PubMed: 26732568]
- Papatriantafyllou M. Macrophages: Iron macrophages. *Nat Rev Immunol*. 2011; 11:158.
- Pelidou SH, et al. High levels of IL-10 secreting cells are present in blood in cerebrovascular diseases. *Eur J Neurol*. 1999; 6:437–42. [PubMed: 10362896]
- Pisanu A, et al. Dynamic changes in pro- and anti-inflammatory cytokines in microglia after PPAR-gamma agonist neuroprotective treatment in the MPTP mouse model of progressive Parkinson's disease. *Neurobiol Dis*. 2014; 71:280–91. [PubMed: 25134730]
- Power C, et al. Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. *Ann Neurol*. 2003; 53:731–42. [PubMed: 12783419]
- Regnier-Vigouroux A. The mannose receptor in the brain. *Int Rev Cytol*. 2003; 226:321–42. [PubMed: 12921240]
- Schallner N, et al. Microglia regulate blood clearance in subarachnoid hemorrhage by heme oxygenase-1. *J Clin Invest*. 2015; 125:2609–25. [PubMed: 26011640]
- Shimada K, et al. Protective Role of Peroxisome Proliferator-Activated Receptor-gamma in the Development of Intracranial Aneurysm Rupture. *Stroke*. 2015; 46:1664–72. [PubMed: 25931465]
- Shouval DS, et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity*. 2014; 40:706–19. [PubMed: 24792912]
- Siqueira Mietto B, et al. Role of IL-10 in Resolution of Inflammation and Functional Recovery after Peripheral Nerve Injury. *J Neurosci*. 2015; 35:16431–42. [PubMed: 26674868]
- Sonni S, et al. New avenues for treatment of intracranial hemorrhage. *Curr Treat Options Cardiovasc Med*. 2014; 16:277. [PubMed: 24366522]
- Taetzsch T, et al. Redox regulation of NF-kappaB p50 and M1 polarization in microglia. *Glia*. 2015; 63:423–40. [PubMed: 25331559]
- Toyoda K, Grotta JC. Seeking best medical treatment for hyperacute intracerebral hemorrhage. *Neurology*. 2015; 84:444–5. [PubMed: 25552577]
- van Asch CJ, et al. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. *Lancet Neurol*. 2010; 9:167–76. [PubMed: 20056489]
- Wan S, et al. Microglia Activation and Polarization After Intracerebral Hemorrhage in Mice: the Role of Protease-Activated Receptor-1. *Transl Stroke Res*. 2016; 7:478–487. [PubMed: 27206851]
- Wang G, et al. Microglia/macrophage polarization dynamics in white matter after traumatic brain injury. *J Cereb Blood Flow Metab*. 2013; 33:1864–74. [PubMed: 23942366]
- Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. *Prog Neurobiol*. 2010; 92:463–77. [PubMed: 20713126]

- Wang J, Dore S. Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage. *Brain*. 2007; 130:1643–52. [PubMed: 17525142]
- Wang J, et al. Protective role of tuftsin fragment 1–3 in an animal model of intracerebral hemorrhage. *Ann Neurol*. 2003; 54:655–64. [PubMed: 14595655]
- Wang J, Tsirka SE. Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage. *Brain*. 2005a; 128:1622–33. [PubMed: 15800021]
- Wang J, Tsirka SE. Tuftsin fragment 1–3 is beneficial when delivered after the induction of intracerebral hemorrhage. *Stroke*. 2005b; 36:613–8. [PubMed: 15692122]
- Weis N, et al. Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. *Mol Biol Cell*. 2009; 20:1280–8. [PubMed: 19129475]
- Wu G, et al. Minimally invasive surgery for ICH evacuation followed by rosiglitazone infusion therapy increased perihematomal PPARgamma expression and improved neurological outcomes in rabbits. *Neurol Res*. 2016; 38:261–8. [PubMed: 27082035]
- Wu H, et al. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. *Neurobiol Dis*. 2012; 45:388–94. [PubMed: 21930208]
- Wu H, et al. Dynamic changes of inflammatory markers in brain after hemorrhagic stroke in humans: a postmortem study. *Brain Res*. 2010; 1342:111–7. [PubMed: 20420814]
- Wu J, et al. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. *Stroke*. 2003; 34:2964–9. [PubMed: 14615611]
- Xi G, et al. Progress in translational research on intracerebral hemorrhage: is there an end in sight? *Prog Neurobiol*. 2014; 115:45–63. [PubMed: 24139872]
- Zhang Z, et al. Microglial Polarization and Inflammatory Mediators After Intracerebral Hemorrhage. *Mol Neurobiol*. 2016; doi: 10.1007/s12035-016-9785-6
- Zhao H, et al. Microglia/Macrophage Polarization After Experimental Intracerebral Hemorrhage. *Transl Stroke Res*. 2015a; 6:407–9. [PubMed: 26446073]
- Zhao X, et al. Hematoma resolution as a therapeutic target: the role of microglia/macrophages. *Stroke*. 2009; 40:S92–4. [PubMed: 19064796]
- Zhao X, et al. Cleaning up after ICH: the role of Nrf2 in modulating microglia function and hematoma clearance. *J Neurochem*. 2015b; 133:144–52. [PubMed: 25328080]
- Zhao X, et al. Polymorphonuclear neutrophil in brain parenchyma after experimental intracerebral hemorrhage. *Transl Stroke Res*. 2014; 5:554–61. [PubMed: 24696130]
- Zhao X, et al. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. *Stroke*. 2007a; 38:3280–6. [PubMed: 17962605]
- Zhao X, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. *Ann Neurol*. 2007b; 61:352–62. [PubMed: 17457822]
- Zhao X, et al. Toxic role of prostaglandin E2 receptor EP1 after intracerebral hemorrhage in mice. *Brain Behav Immun*. 2015c; 46:293–310. [PubMed: 25697396]
- Zhou K, et al. Regulatory T cells ameliorate intracerebral hemorrhage-induced inflammatory injury by modulating microglia/macrophage polarization through the IL-10/GSK3beta/PTEN axis. *J Cereb Blood Flow Metab*. 2016
- Zhou Y, et al. Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation. *Prog Neurobiol*. 2014; 115:25–44. [PubMed: 24291544]
- Zhu W, et al. Mouse models of intracerebral hemorrhage in ventricle, cortex, and hippocampus by injections of autologous blood or collagenase. *PLoS One*. 2014; 9:e97423. [PubMed: 24831292]
- Ziai WC. Hematology and inflammatory signaling of intracerebral hemorrhage. *Stroke*. 2013; 44:S74–8. [PubMed: 23709738]

Highlights

- After ICH, microglia/macrophages (MM Φ) undergo phenotypic changes.
- Rosiglitazone induces MM Φ alternative activation in ICH.
- Promotion of MM Φ alternative activation enhances hematoma resolution in ICH.
- IL-10 signaling involves in regulation of MM Φ phagocytosis and hematoma clearance in ICH.

Figure 1.

Microglia/macrophages (MMΦ) accumulate within the hematoma territory until 14 days after ICH. **(A)** Representative coronal brain sections show the temporal changes in hematoma resolution after ICH. The boxed area indicates the location of representative fluorescence images in the hemorrhagic brain. Scale bar: 1 cm. $n = 3$ mice per time point. **(B)** Identification of Iba1- and GFAP-positive cells in brain sections at 1, 3, 7, and 14 days post-ICH. Immunoreactivity of Iba1 (ionized calcium binding adaptor molecule 1; MMΦ marker) and GFAP (glial fibrillary acidic protein; astrocyte marker) are shown in green and red, respectively. The inset column represents higher magnification of the boxed area in the corresponding merged images. Nuclei were stained with DAPI (blue). Scale bars: 200 μm (merged column); 20 μm (inset column). $n = 3$ mice per time point.

Figure 2.

Identification of Iba1- and GFAP-positive cells in brain sections at 1, 3, 7, and 14 days after sham operation. Immunoreactivity of Iba1 (ionized calcium binding adaptor molecule 1; MMΦ marker) and GFAP (glial fibrillary acidic protein; astrocyte marker) are shown in green and red, respectively. Nuclei were stained with DAPI (blue). Scale bars: 100 μm.

Figure 3.

Markers of classically activated microglia/macrophages (MM Φ) increase over time after ICH. **(A)** Temporal profile of mRNA expression for each marker of classically activated MM Φ after ICH. $n = 4$ to 6 per time point. **(B)** Representative double-immunofluorescence staining of Iba1 (ionized calcium binding adaptor molecule 1; MM Φ marker; red) and CD16/32 (classic activation marker; green) on hemorrhagic brain sections at 1, 3, 7, and 14 days post-ICH. Dotted lines mark the hematoma boundary in the Iba1-fluorescent images. Arrows indicate the cells and area of colocalization. The inset images represent higher magnification of the boxed area in the corresponding merged images. Nuclei were stained with DAPI (blue). Scale bars: 100 μ m (merged row), 10 μ m (inset row). **(C)** Bar graph shows the degree of MM Φ and CD16/32 colocalization in gray pixel intensity at 1, 3, 7, and 14 days post-ICH. $n = 4$ mice at 1 and 7 days; $n = 7$ mice at 3 and 14 days. Values are mean $\pm$ SD; * $P < 0.05$ vs. sham or contralateral side; # $P < 0.05$ vs. previous time point. S, sham; Contra, contralateral; Ipsi, ipsilateral.

CD16/32/Iba1/DAPI

Figure 4.

Markers of classically activated microglia/macrophages ($\text{MM}\Phi$) over time after sham operation. Representative double-immunofluorescence staining of Iba1 (ionized calcium binding adaptor molecule 1; $\text{MM}\Phi$ marker; red) and CD16/32 (classic activation marker; green) on sham-operated brain sections at 1, 3, 7, and 14 days post-surgery. Dotted lines mark the needle track. Nuclei were stained with DAPI (blue). Scale bars: 100 μm (low-magnification image); 10 μm (high-magnification image).

Figure 5.

Markers of alternatively activated microglia/macrophages (MMΦ) increase rapidly after ICH and subside with time. (A) Gene expression for markers of alternatively activated MMΦ at various time points after ICH determined by qPCR. $n = 4$ to 6 mice per time point. (B) Representative double-labeling of Iba1 (ionized calcium binding adaptor molecule 1; MMΦ marker; red) and CD206 (mannose receptor; alternative activation marker; green) on hemorragic brain sections at 1, 3, 7, and 14 days post-ICH. Dotted lines mark the hematoma boundary in the representative Iba1 images. Arrows indicate the cells and area of colocalization. The inset images represent higher magnification of the boxed area in the corresponding merged images. Nuclei were stained with DAPI (blue). Scale bars: 100 μm (merged row); 10 μm (inset row). (C) Bar graph shows the degree of MMΦ and CD16/32 colocalization in gray pixel intensity at 1, 3, 7, and 14 days post-ICH. $n = 4$ mice at 1 and 7 days; $n = 7$ mice at 3 and 14 days. (D) Bar graph shows IL-10 protein concentration in the sham (S) group and the contralateral (Contra) and ipsilateral (Ipsi) hemispheres of the ICH group at 1–1.5 h after ICH. $n = 4$ mice per group. (E) Top: Representative immunoblot of HO-1 protein expression in CD11b-positive cells isolated from the sham mice and contralateral and ipsilateral hemispheres of the ICH mice. Bottom: Densitometric analysis shows significantly higher HO-1 protein expression in CD11b-positive cells isolated from

the ipsilateral hemisphere of ICH mice than in those isolated from the contralateral hemisphere at 1–1.5 h after ICH. $n = 3$ mice per group. Values are mean $\pm$ SD; * $P < 0.05$ vs. sham or contralateral group; # $P < 0.05$ vs. previous time point.

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Figure 6.

Markers of alternatively activated microglia/macrophages (MM Φ) over time after sham operation. Representative double-immunofluorescence staining of Iba1 (ionized calcium binding adaptor molecule 1; MM Φ marker; red) and CD206 (alternative activation marker; green) on sham-operated brain sections at 1, 3, 7, and 14 days post-surgery. Dotted lines mark the needle track. Nuclei were stained with DAPI (blue). Scale bars: 100 μ m (low-magnification image); 10 μ m (high-magnification image).

Figure 7.

Administration of rosiglitazone accelerates hematoma resolution and improves neurologic function in 12-month-old mice subjected to ICH. **(A)** Representative serial coronal sections show the hematoma territory at 4 days (top) and 7 days (bottom) post-ICH in mice administered rosiglitazone (Rosi) or vehicle (Veh). **(B)** Quantification of the hematoma volume in rosiglitazone- and vehicle-treated groups at 4 and 7 days post-ICH. $n = 8$ mice per group. **(C)** Changes in body weight of rosiglitazone- and vehicle-treated mice. $n = 9$ to 14 mice per group. **(D)** Neurologic function of ICH mice was assessed by the neurologic deficit score (top left), corner turn test (top right), hindlimb placing test (bottom left), and forelimb placing test (bottom right). $n = 9$ to 14 mice per group. Values are mean $\pm$ SD; $*P < 0.05$ vs. vehicle group.

Figure 8.

Administration of rosiglitazone promotes microglia/macrophage (MMΦ) alternative activation in 12-month-old mice subjected to ICH. (A) Representative dot-plot scatter analysis of CD11b-positive cells isolated from the brains of control and ICH animals after vehicle (Veh) or rosiglitazone (Rosi) treatment. A gate was drawn to exclude cellular debris from dead cells for further analysis. (B) Gated cells from (A) were analyzed for the expression of CD11b and CD45 to identify CD11b⁺CD45^{high} (macrophage) and CD11b⁺CD45^{low} (microglia) populations in control (left dot plots) and ICH (right dot plots) groups treated with or without rosiglitazone. CD45^{low} cells and CD45^{high} cells were classified as CD11b⁺ microglia and macrophages, respectively. (C) Representative dot plots showing expression of CD206-positive subsets of CD11b⁺CD45^{high} and CD11b⁺CD45^{low} brain macrophages and microglia in control and ICH groups at 3 days post-ICH. (D) Representative histograms show that CD206 staining of CD11b⁺CD45^{high} (bottom left) and CD11b⁺CD45^{low} (top left) cells was brighter in brains of rosiglitazone-treated mice (blue trace) than in brains of vehicle-treated mice (yellow trace). Isotype background staining is shown as a red trace. Quantification shows that rosiglitazone treatment increased the percentage of CD11b⁺CD45^{high}CD206⁺ and CD11b⁺CD45^{low}CD206⁺ cells in the ICH brains (right). Cells were pooled from three mice per group for a total of three separate experiments. (E) Bar graphs represent mRNA expression of alternative activation markers

YM1/2 and Arg1 in brains of vehicle-, rosiglitazone-, GW9662-, and rosiglitazone +GW9662-treated ICH mice. n = 3 to 5 mice per group. (F) Bar graphs show IL-10 mRNA expression in rosiglitazone-, vehicle-, GW9662-, and rosiglitazone+GW9662-treated mice at 3 days after ICH. Compared with vehicle treatment, rosiglitazone significantly increased IL-10 mRNA expression at 3 days in the ipsilateral hemisphere. IL-10 mRNA expression significantly decreased in mice that cotreatment of GW9662 with rosiglitazone, compared with rosiglitazone-treated group (top). n = 3 to 5 mice per group. Serum IL-10 protein levels were significantly higher in the rosiglitazone-treated group than in the vehicle-treated group at 2 and 3 days post-ICH (bottom). n = 4 to 6 mice per group. (G) Representative coronal brain sections show hematoma in vehicle- and IL-10-injected ICH brains at 3 days post-ICH (top). Hematoma volume of IL-10-injected ICH brains was significantly smaller than that of vehicle-injected ICH brains on day 3 post-ICH (bottom). Scale bar: 1 cm. Values are mean $\pm$ SD; * P < 0.05 vs. vehicle or control group; # P < 0.05 vs. rosiglitazone group; & P < 0.05 vs. GW9662 group. Con, contralateral; Ipsi, ipsilateral; n.d., not detectable; Veh, vehicle; Rosi, rosiglitazone; GW, GW9662.

Figure 9.

Effect of repeated rosiglitazone treatment on CD206 expression in the ICH brain.

Representative low-magnification images show CD206 immunoreactivity (red) within and around the hematoma on brain sections from vehicle (Veh)- and rosiglitazone (Rosi)-treated Cx3cr1^{GFP/+} mice at 4 days post-ICH. Immunofluorescent signal of CD206 was barely detectable in the contralateral hemisphere (right). I, II, III, IV, and V indicate the images from five independent animals, and the numbers represent raw fluorescence intensity for each image. Scale bars: 100 μm . Quantification analysis of fluorescence intensity indicates that rosiglitazone administration significantly increased CD206 immunoreactivity in the ICH brain at 4 days post-ICH (left). $n = 5$ mice per group. Values are mean $\pm$ SD; * $P < 0.05$ vs. vehicle group. Contra, contralateral.

Figure 10.

IL-10 receptor blockade inhibits phagocytosis by Cx3cr1^{GFP/+} microglia in organotypic hippocampal slice cultures (OHSCs). **(A)** Stimulation of OHSCs with hemoglobin induced changes in mRNA expression of classic and alternative activation markers. RT-PCR was carried out with total RNA extracted from hemoglobin-treated or untreated OHSCs at 3 h, 6 h, 12 h, 1 day, and 3 days after stimulation. *n* = 3 independent experiments. **(B)** Representative images show Cx3cr1^{GFP/+} microglia ingesting 1 μm-diameter fluorescent latex beads in unstimulated OHSCs (Con), OHSCs stimulated with hemoglobin (Hb), and OHSCs stimulated with rosiglitazone (Rosi). These groups were treated with either PBS (top row) or 20 μg/mL IL-10 receptor alpha antibody (RA; bottom row). The inset column represents higher magnification of the boxed area in each image. Scale bars: 10 μm. **(C)** Quantification of ingested fluorescent beads/cell in OHSCs after 24 h of treatment. *n* = 3 independent experiments. Values are mean ± SD; **P* < 0.05 vs. control group; #*P* < 0.05 vs. PBS group. Con, control.

Figure 11.

Effect of repeated rosiglitazone treatment on neuronal survival in the ICH brain.

Representative images show NeuN immunoreactivity (red) in the perihematoma of brain sections from vehicle- and rosiglitazone-treated Cx3cr1^{GFP/+} mice at 4 days post-ICH (top). Quantification analysis of NeuN-positive cells indicates that rosiglitazone administration significantly increased the number of surviving neurons in the ICH brain at 4 days post-ICH (bottom). Nuclei were stained with DAPI (blue). Scale bars: 50 μ m. n = 5 mice per group. Values are mean $\pm$ SD; **P* < 0.05 vs. vehicle group. Veh, vehicle; Rosi, rosiglitazone.

Table 1

Antibodies used in immunofluorescence Western blotting and flow cytometry

Primary antibody	Commercial source	Catalog number	Species	Antibody type	Working concentration
Iba1	Wako	019-19741	Rabbit	Polyclonal	IF 1:1000
GFAP	Invitrogen	13-0300	Rat	Monoclonal, IgG2a, κ	IF 1:500
CD206	abcam	ab8918	Mouse	Monoclonal, IgG1	IF 1:50
CD16/CD32	BD	553142	Mouse	Monoclonal, IgG2b, κ	IF 1:50
HO-1	Enzo	ADI-SPA-895-D	Rabbit	Polyclonal	WB 1:500
Cyclophilin A	Millipore	07-313	Rabbit	Polyclonal	WB 1:1000
NeuN	Cell Signaling	12943S	Rabbit	Polyclonal	IF 1:500
CD11b-FITC	Miltenyi	130-081-201	Rat	Monoclonal, IgG2b, κ	FC 1:50
CD45-PE	Miltenyi	130-102-596	Rat	Monoclonal, IgG2b, κ	FC 1:100
CD206-APC	Biolegend	141708	Rat	Monoclonal, IgG2a, κ	FC 1:100
Isotype-FITC	eBioscience	11-4031-85	Rat	Monoclonal, IgG2b, κ	FC 1:50
Isotype-PE	BD	555848	Rat	Monoclonal, IgG2b, κ	FC 1:100
Isotype-APC	eBioscience	17-4321-81	Rat	Monoclonal, IgG2a, κ	FC 1:100

Abbreviations: Iba1, ionized calcium binding adaptor molecule 1 (microglia/macrophages marker); GFAP, glial fibrillary acidic protein (astrocyte marker); CD206, mannose receptor; HO-1, heme-oxygenase-1; WB, Western blotting; NeuN, neuronal nuclei antigen (neuron marker); FC, flow cytometry

Neurobehavioral outcomes in vehicle- and rosiglitazone-treated mice at days 1, 4, and 7 after intracerebral hemorrhage

Table 2

Test	Day 1		Day 4		Day 7	
	Vehicle	Rosiglitazone	Vehicle	Rosiglitazone	Vehicle	Rosiglitazone
Neurologic deficit score	12.1 ± 1.5	9.2 ± 1.8	9.3 ± 1.3	6.7 ± 1.7 ^a	7.6 ± 1.4	5.4 ± 1.3 ^b
Corner turn test (%)	92.7 ± 11.9	99.1 ± 3.0	86.4 ± 12.1	71.8 ± 14.7 ^c	82.2 ± 18.6	64.3 ± 11.3 ^d
Hindlimb adduction test (%)	10.9 ± 11.2	16.9 ± 11.1	49.2 ± 21.4	61.5 ± 15.7	67.5 ± 11.7	80.0 ± 12.2 ^e
Forelimb placing test (%)	12.3 ± 15.9	35.4 ± 16.1 ^f	49.2 ± 21.4	61.5 ± 15.7 ^g	67.5 ± 11.7	80.0 ± 12.2 ^h

^a $p < 0.05$;
^b $p = 0.003$;
^c $p = 0.101$;
^d $p = 0.042$;
^e $p = 0.048$;
^f $p < 0.05$;
^g $p < 0.05$;
^h $p < 0.05$