Role of Erythrocyte CD47 in Intracerebral Hematoma Clearance

Wei Ni, MD; Shanshan Mao, MD, PhD; Guohua Xi, MD; Richard F. Keep, PhD; Ya Hua, MD

Background and Purpose—Enhancing hematoma clearance through phagocytosis may reduce brain injury after intracerebral hemorrhage. In the current study, we investigated the role of cluster of differentiation 47 (CD47) in regulating erythrophagocytosis and brain injury after intracerebral hemorrhage in nude mice.

Methods—This study was in 2 parts. First, male adult nude mice had an intracaudate injection of 30 μL saline, blood from male adult wild-type (WT) mice, or blood from CD47 knockout mice. Second, mice had an intracaudate injection of 30 μL CD47 knockout blood with clodronate or control liposomes. Clodronate liposomes were also tested in saline-injected mice. All mice then had magnetic resonance imaging to measure hematoma size and brain swelling. Brains were used for immunohistochemistry and Western blot.

Results—Erythrophagocytosis occurred in and around the hematoma. Injection of CD47 knockout blood resulted in quicker clot resolution, less brain swelling, and less neurological deficits compared with wild-type blood. Higher brain hemoglobin-1 levels and more microglial activation (mostly M2 polarized microglia) at day 3 were found after CD47 knockout blood injection. Co-injection of clodronate liposomes, to deplete phagocytes, caused more severe brain swelling and less clot resolution.

Conclusions—These results indicated that CD47 has a key role in hematoma clearance after intracerebral hemorrhage.

Key Words: brain injury • CD47 • erythrophagocytosis • intracerebral hemorrhage • magnetic resonance imaging • nude mice

Hematoma clearance occurs after intracerebral hemorrhage (ICH) with erythrocytes in the clot being phagocytized by microglia/macrophages.1–3 Enhancing such phagocytosis results in less brain injury after ICH.2,4 Although many factors may affect the initiation of phagocytosis, one factor is cluster of differentiation 47 (CD47).5,6 CD47, an integrin-associated protein, is expressed on erythrocytes and other cells, and it regulates target cell phagocytosis.3,7 CD47 on erythrocytes blocks phagocytosis through interaction with an inhibitory receptor, signal-regulatory protein-α (SIRPα), expressed by microglia/macrophages.7–9 CD47-deficient erythrocytes are more prone to phagocytosis than wild-type (WT) cells,10,11 and it is suggested that CD47 down-regulation might lead to clearance of erythrocytes as they age.12

Macrophages have an important role in preserving tissue integrity and function by engulfing old and damaged cells, including erythrocytes.13,14 Macrophage/microglia have been classified as classically activated (M1 phenotype) and alternatively activated (M2 phenotype).15,16 In this study, we investigated the role of erythrocyte CD47 in hematoma clearance and brain injury in an ICH model in nude mice. Brain swelling and hematoma clearance were also examined after depleting macrophages/microglia with clodronate liposomes.

Materials and Methods

Animal Preparation and Intracerebral Injection

All animal procedures were approved by the University Committee on Use and Care of Animals, University of Michigan, and were conducted in accordance with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. Ten adult male C57BL/6 mice (WT; Charles River Laboratories) and 14 male CD47 knockout mice (CD47 KO; University of Michigan Breeding Core) were used as blood donors. Blood from those animals or saline was then injected into a total of 63 male nude mice (aged 3–5 months; Charles River Laboratories). Nude mice were chosen to facilitate inter-animal injections.

The ICH model was produced as previously described.17,18 Briefly, animals were anesthetized with ketamine (90 mg/kg IP; Abbott Laboratories) and xylazine (5 mg/kg IP; Lloyd Laboratories). Body temperature was maintained at 37.5°C by a feedback-controlled heating pad. Donor blood was collected from WT or CD47 KO mice by femoral artery catheterization by PE10 tube. Nude mice were positioned in a stereotaxic frame and a cranial burr hole (1 mm) was drilled near the right coronal suture 2.5 mm lateral to the midline. A 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 3.5 mm ventral, and 2.5 mm lateral to the bregma). Either 30 μL of donated blood or saline was infused at a rate of 2 μL/min using a microinfusion pump. The needle remained in position for a further 10 minutes and then was gently removed. The burr hole was filled with bone wax, and the skin incision sutured closed.

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Experimental Groups
In the first part, nude mice had an intracaudate injection of 30 μL saline (n=14) or blood donated from WT (n=14) or CD47 KO (n=14) mice. Nude mice underwent magnetic resonance imaging (MRI) and behavioral testing and were euthanized at 3 days. In the second part, nude mice had an injection of a mixture of 30 μL CD47 KO blood with 5 μL of clodronate liposome or control liposome (FormuMax Scientific, Palo Alto, CA). Control mice received 30 μL saline plus 5 μL of clodronate liposome. Mice were euthanized at 3 days after MRI (n=7 per group). Brains were used for histology or Western blot assays. Death did not occur in all experimental groups.

Magnetic Resonance Imaging and Measurement
Nude mice had a preoperative MRI as baseline and postoperative scans at days 1 and 3. Mice were anesthetized with 2% isoflurane/air mixture throughout MRI examination. MRI scanning was performed in a 7.0-T Varian MR scanner (183-mm horizontal bore; Varian, Palo Alto, CA) at the Center for Molecular Imaging of the University of Michigan. The imaging protocol for all the mice included a T2 fast spin-echo (repetition time/echo time=4000/60 ms) and T2* gradient-echo sequences (repetition time/echo time=250/5 ms). The field of view was 20x20 mm, and the matrix was 256x256 mm. Twenty-five coronal slices (0.5-mm thick) were acquired from the frontal pole to the brain stem, and the images were preserved as 256x256 pixels pictures. Afterwards, 2 parameters (T2* lesion volume and brain swelling ratio) were calculated in National Institutes of Health (NIH) Image J. The T2* lesion was outlined along the border of the hyperintense area over all slices and multiplied by section thickness. The ratio was calculated as (ipsilateral−contralateral hemispheric volume)/contralateral hemispheric volume×100%. All measurements were performed by a blinded observer.

Behavioral Tests
Forelimb use asymmetry and corner turn tests were used to assess behavioral deficits in all the nude mice models. For forelimb use asymmetry measurement, behavior score was recorded by determining the number of times the ipsilateral (unimpaired) forelimb (I), contralateral forelimb (C), and both forelimbs (B) used as a percentage of total number of limb usage. A single, overall limb use asymmetry score was calculated as follows: limb use asymmetry score=(I−C)/(I+C+B). Corner turn test was performed as follows. The mouse was allowed to proceed into a corner with a 30° angle. When the mouse turned, its choice of direction (left or right) was recorded. Each mouse repeated this procedure for 20 times. The percentage of right turns was calculated. The neurological scores were evaluated by a blinded observer.

**Immunohistochemistry Staining**
Immunohistochemistry staining was performed as described previously. Briefly, mice were euthanized (ketamine 120 mg/kg and xylazine 5 mg/kg IP) and perfused with 4% parafomaldehyde in 0.1 mM phosphate-buffered saline (pH 7.4). Brain samples were embedded in a mixture of 30% sucrose, and optimal cutting temperature compound (Sakura Finetek, Inc) and 18 μm slices were sectioned on a cryostat. Hematoxylin and eosin staining was used for erythrocyte phagocytosis observation. Immunohistochemistry studies were performed with avidin–biotin complex technique as previously described. The primary antibodies were polyclonal rabbit anti-heme oxygenase-1 (HO-1; Abcam; 1:400 dilution), polyclonal rabbit anti-β1b (Wako; 1:400 dilution), polyclonal rat anti-CD86 (Abcam; 1:100 dilution), and polyclonal rabbit anti-CD206 (Abcam; 1:500 dilution).

**Western Blotting**
Western blot analysis was performed as previously described. Briefly, brain tissue was perfused with 0.1 mM phosphate-buffered saline (pH 7.4) after euthanasia, and bilateral basal ganglia sampled. Then, each sample was immersed in Western sample buffer and sonicated. Protein concentration was determined by Bio-Rad protein assay kit, and 50 μg protein from each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). The primary antibodies were polyclonal rabbit anti-heme oxygenase-1 (HO-1; Abcam; 1:2000 dilution), polyclonal rabbit anti-Iba1 (Wako; 1:1000 dilution), polyclonal rabbit anti-CD86 (Abcam; 1:100 dilution), and polyclonal rabbit anti-CD206 (Abcam; 1:500 dilution).
system (Amersham) and exposed to Kodak X-OMAT film. Relative band densities were analyzed with NIH Image J.

**Statistical Analysis**

All the data in this study are presented as mean±SD. Data were analyzed by Student t test or analysis of variance with Student–Newman–Keuls post hoc test. P<0.05 was considered statistically significant.

**Results**

Erythrophagocytosis was examined on coronal sections of mice brains after hematoxylin and eosin staining. In mice that received either WT blood or CD47 KO blood, erythrocytes were phagocytized by macrophages and microglia in the perihematomal area at day 3 (Figure 1A). Hematoma volume (assessed by T2* lesion volume) showed no difference between 2 groups at day 1 (14.3±2.9 versus 14.4±3.2 mm³, P>0.05; Figure 1B and 1C). However, when ICH was induced with CD47 KO blood, T2* volumes were significantly smaller compared with WT blood at day 3 (7.8±1.7 versus11.8±4.3 mm³, P<0.01; Figure 1B and 1C).

ICH caused brain swelling in the ipsilateral hemisphere. At day 3, ICH-induced brain swelling was much less in the CD47 KO blood group (1.5±1.5% versus 8.6±4.1%, P<0.01; Figure 2A). Neurological deficits were assessed by forelimb use asymmetry and corner turn tests before ICH and then at day 1 and day 3 after ICH. WT blood and CD47 KO blood resulted in same behavioral deficits at day 1. However, the neurological deficits were less in CD47 KO blood–injected group at day 3 (forelimb use asymmetry, 48±5% versus 61±3% in the WT blood–injected group, P<0.01; Figure 2B;
corner turn score, 79±8% versus 87±8% in the WT blood–injected group, P<0.05; Figure 2C).

There was almost no immunoreactivity for HO-1 in the ipsilateral basal ganglia after saline injection at day 3 but a marked increase after injection of WT or CD47 KO blood (Figure 3A). Quantification of HO-1 levels by Western blot indicated that brain HO-1 levels were greater in mice injected with CD47 KO blood compared with WT blood (1.79±0.3 versus 1.2±0.21, P<0.01; Figure 3B).

Intracerebral injection of blood caused significant microglia activation at either day 1 or day 3. There was massive increase in ameboid-shaped Iba-1-positive cells in the ipsilateral basal ganglia after injection of WT blood, and this was even greater in the CD47 KO blood–injected group (Figure 3C). These results were confirmed by Western blot. Iba-1 protein levels were upregulated in ipsilateral basal ganglia after injection of WT blood (P<0.05 versus saline; Figure 3D), and there was a further increase in mice with a CD47 KO blood injection (1.46±0.3 versus 0.75±0.25, P<0.01; Figure 3D).

Microglia/macrophage polarization is categorized into classical (M1) and alternative (M2) activation. In the present study, both CD86 (an M1 cell surface marker) and CD206 (an M2 cell surface marker) were expressed in the ipsilateral basal ganglia 3 days after CD47 KO or WT blood injection (Figure 4A and 4B). Only a few cells expressed both CD86 and CD206 (Figure 1 in the online-only Data Supplement). Three days after WT blood injection, CD86 levels in the ipsilateral basal ganglia were higher than those with CD47 KO blood (CD86/β-actin: 1.16±0.29 versus 0.68±0.19 with CD47 KO blood injection; P<0.05), but CD206 levels were lower (CD206/β-actin: 0.61±0.16 versus 1.53±0.42 in CD47 KO blood injection group; P<0.05; Figure 4C).

To elucidate the role of microglia/macrophages in erythrophagocytosis and brain injury after ICH, clodronate liposomes were used to selectively deplete phagocytic macrophages.24 Co-injection of clodronate liposome or control liposome with CD47 KO blood resulted in similar T2* lesions at day

Figure 4. Immunoreactivity of CD86 (A) and CD206 (B) in the perihematomal area at day 3 after injection of 30 μL blood from wild-type (WT) or cluster of differentiation 47 (CD47) knockout (KO) mice. Scale bar=20 μm. C, CD86 and CD206 protein levels in the ipsilateral basal ganglia 3 days after injection of WT or CD47 KO blood. Values are means±SD; n=4 for each group, *P<0.05 vs the WT group.

Figure 5. A, T2*-weighted magnetic resonance imaging (MRI) showing the lesions in the ipsilateral hemisphere at days 1 and 3 after injection of 30 μL blood from cluster of differentiation 47 (CD47) knockout (KO) mice mixed with 5 μL clodronate liposome or control liposome into the right basal ganglia. Bar graphs show quantification of lesion volumes. Values are means±SD; n=7 for each group, *P<0.05 vs control group. B, T2-weighted MRI showing brain swelling at day 1 after injection of 30 μL blood from CD47 KO mice mixed with 5 μL clodronate liposome or control liposome or saline with clodronate. Bar graphs show quantification of brain swelling ([ipsilateral−contralateral hemisphere]/contralateral hemisphere)×100%) at days 0, 1, and 3. Values are means±SD; n=7 for each group, #P<0.01 vs the other groups.
1 (14.9±2.3 versus 15.1±2.2 mm³ in control group; *P*<0.05). However, T2* lesions were larger in the clodronate-treated group at day 3 (10.9±2.3 versus 7.7±2.0 mm³ in control liposome group, *p*<0.05; Figure 5A). Clodronate liposome co-injection also resulted in more severe brain swelling than control liposomes at both day 1 (11.2±3.6% versus 5.2±2.9%, *P*<0.01; Figure 5B) and day 3 (7.1±1.1% versus 2.0±2.0%, *P*<0.05; Figure 5B). Clodronate liposomes injected with saline rather than blood did not cause brain swelling (Figure 5B). In addition, we found that clodronate liposome itself has no effects on microglia activation after intracerebral injection (Figure II in the online-only Data Supplement).

Whether clodronate liposome treatment differentially affects M1 or M2 microglia/macrophages was examined using CD206 and arginase-1 as markers of the M2 phenotype and CD86 and CD16 as markers of the M1 phenotype. Clodronate liposome co-injection reduced CD206 immunoreactivity and protein levels (*P*<0.05; Figure 6) after ICH. It also reduced arginase-1 protein levels (*P*<0.05; Figure 6). In control brains, CD206 immunoreactivity was low, and injection clodronate liposome did not markedly change brain CD206 immunoreactivity (Figure III in the online-only Data Supplement). In contrast, clodronate liposomes did not change CD86 protein levels in the ipsilateral basal ganglia after ICH (CD86/β-actin: 1.01±0.23 versus 1.03±0.18 in the control group; *P*>0.05; Figure 6), nor did it affect CD16 protein levels (Figure 6). These data suggest a specific effect of the clodronate liposome on the M2 phenotype.

### Discussion

There were 4 major findings in the current study: (1) hematoma clearance was faster when the ICH was formed using CD47 KO blood; (2) the injection of CD47 KO blood caused less brain swelling and neurological deficits; (3) CD47 KO blood induced greater HO-1 upregulation, microglial activation, and M2 phenotype polarization; and (4) co-injection of clodronate liposomes depleted M2 microglia/macrophages and caused more brain swelling and less hematoma clearance. We hypothesize that erythrocyte CD47 inhibits microglia/macrophage M2 polarization, erythrophagocytosis, and thus hematoma clearance and that the effect of those changes is to enhance ICH-induced brain injury. That hypothesis is supported by the finding that depleting M2 microglia/macrophages with clodronate liposomes also enhanced ICH-induced brain injury.

Microglia are cells within the brain that are activated in response to injury. In normal brain, microglia are in a quiescent state, but in the event of injury, they become highly phagocytic and are involved in clearing debris, damaged cells, and clot from the site of damage. Activation of microglia occurs after ICH and is enhanced in aged rats. Macrophages clear damaged or aged cells via phagocytosis. Erythrocytes are eliminated by phagocytosis after accumulating of eat me signals on the cell surface. Recent studies have demonstrated that activating microglia/macrophages enhances hematoma resolution and improves functional outcome in a rat model of ICH.

![Figure 6. Immunoreactivity of CD86 and CD206 (A and B), protein levels of CD86 and CD16 (C), protein levels of CD206 and Arg1 (D) in the ipsilateral basal ganglia at day 3 after injection of 30 μL blood from cluster of differentiation 47 (CD47) knockout (KO) mice with 5 μL clodronate liposome or control liposome into the right basal ganglia, scale bar=20 μm. Values are mean±SD; n=4 for each group, *P*<0.05, #P<0.01 vs the vehicle group.](http://stroke.ahajournals.org/DownloadedFrom/10.1161/STROKEAHA.115.012650)
CD47 has an important role in erythrophagocytosis. CD47 expression was increased in the perihematoma white and gray matter, and iron played role in CD47 expression in pig ICH. CD47 downregulation might lead to clearance of erythrocytes as they age. In the present study, injection of CD47-deficient blood resulted in a stronger response of microglia/macrophages and faster hematoma clearance. CD47 inhibition could be a target for enhancing hematoma removal and reducing ICH-induced brain injury. Recently, there has been considerable interest in the cancer field in targeting CD47 with blocking antibodies to enhance phagocytosis, and that approach is currently in clinical trial (NCT02216409, NCT02367196).

Macrophages are professional phagocytes that have an important role in preserving tissue integrity and function by engulfing old and damaged cells, including erythrocytes. Two different forms of macrophage/microglia have been identified; classically activated (M1 phenotype, cytotoxic macrophages) and alternatively activated (M2 phenotype, neuroprotective macrophage). M1 activation results in a decreased ability to phagocyte myelin, whereas M2 activation increases phagocytic activity. In cerebral ischemia, M2 phenotype macrophage/microglia populate the ischemic core aiding in resolving inflammation and promoting wound healing at 24 hours after insult. In spinal cord injury, tumor necrosis factor-α in combination with iron results in M1 polarization and plays detrimental role in recovery. We found that thrombin stimulates microglia to release the pro-inflammatory cytokines, tumor necrosis factor-α and interleukin-1β, and microglial inhibition with minocycline reduces brain injury after ICH. Minocycline selectively inhibits M1 polarization of microglia in an amyotrophic lateral sclerosis mouse model. In the current study, we found that clodronate liposome can selectively impact M2 polarization in the ICH model. The mechanism of clodronate liposome in macrophage depletion has been described. Liposomes are engulfed by macrophages with phagocytic function. Then, clodronate, which is released after the phospholipid bilayers of the liposomes are disrupted by lysosomal phospholipases, induces macrophage apoptosis. Here, we demonstrate that CD47 KO blood injection enhances M2 microglia/macrophage activation and that depletion of M2 macrophages causes more brain swelling and slower hematoma clearance after ICH. Together, these results indicate that erythrophagocytosis has a role in the mechanism of brain injury after ICH.

HO-1 is markedly upregulated after ICH primarily in microglia, and it is a key enzyme in degrading heme released from hemoglobin. Increased brain HO-1 levels may accelerate heme degradation and result in brain damage after ICH and SAH. However, some literature report a protective function, and in the current study, CD47 KO blood increased HO-1 levels and was associated with reduced ICH-induced brain injury. The role of HO-1 in ICH-induced brain injury may depend on cell location and degree of activity.

We focused on acute phase in this proof-of-concept study because significant reductions of brain swelling (>80%) and T2* lesion (>30%) were found in CD47 KO blood group compared with the WT blood group at day 3. A long-term study is needed in the future to determine the effects of erythrocyte CD47 on brain atrophy and long-term functional outcome following ICH.

In conclusion, compared to WT blood, intracerebral injection of CD47 KO blood resulted in quicker hematoma clearance and less brain injury in nude mice. Co-injection of clodronate liposomes with blood depleted M2 microglia/macrophages, reduced hematoma clearance, and caused more severe brain swelling. These results suggest a significant role of CD47 in hematoma removal after ICH.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIALS

Title: The role of erythrocyte CD47 in intracerebral hematoma clearance

Supplemental Figures

**Supplemental Figure I**: Double labeling of CD86 and CD206 in the peri-hematomal area at day-3 after 30µl blood injected into right basal ganglia, scale bar = 50µm.

The primary antibodies were: polyclonal rabbit anti-CD206 IgG (Abcam, USA, 1:200 dilution) and monoclonal rat anti-CD86 IgG (Thermo Scientific, USA; 1:100 dilution). The secondary antibodies were Alexa Fluro 594-conjugated donkey anti-rabbit mAb and Alexa Fluro 488-conjugated donkey anti-rat mAb (Invitrogen, Grand Island, NY, USA; both at a 1:500 dilution).

**Supplemental Figure II**: Immunoreactivity of Iba-1 in the ipsilateral basal ganglia at day-3 after injection of 30µl saline with or without 5µl clodronate liposome into the right basal ganglia, scale bar = 50µm.
Supplemental Figure III: Immunoreactivity of Iba-1 in the ipsilateral basal ganglia at day-3 after injection of 30µl saline with or without 5µl clodronate liposome into the right basal ganglia, scale bar = 20µm.
脳内血腫の除去における赤血球上のCD47 の役割
Role of Erythrocyte CD47 in Intracerebral Hematoma Clearance
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背景および目的：脳腫瘍による血腫の除去を促進することにより脳内出血後の脳損傷を軽減する可能性がある。本研究では、脳腫瘍を有する大脳動脈の脳内出血後の脳損傷におけるCD47の役割を検討した。

方法：本研究は2部よりなり。第1部では、成体の雄マウスの尾内に生理食塩水30μL、成体の野生型（WT）雄マウスの血液、CD47 ノックアウトマウスの血液のいずれかを注射投与した。第2部では、CD47 ノックアウト血液30μLとクロドネートリボソーム、または対照のリボソームを尾内に注射投与した。生理食塩水を注射したマウスでもクロドネートリボソームの試験を実施した。全マウスに磁気共鳴画像法（MRI）を施行し、血腫サイズおよび脳の腫脹を測定した。脳を用いて免疫組織化学法およびウェスタンブロット法を行った。

結果：血腫の内部および周辺で赤血球貪食が起こった。CD47 ノックアウト血液を注射投与すると、野生型血液に比べ速やかに血腫が消減した。脳の腫脹および神経脱落症状の程度も軽減した。CD47 ノックアウト血液の注射投与後3日目に脳のヘマトキシリンとエオジン染色1価の上昇とミクログリアの活性化亢進（ほとんどのミクログリアがM2に志向）が認められた。食細胞除去のため同時投与したクロドネートリボソームは脳内出血を抑制し、血腫の消失が減少した。

結論：本研究の結果から、CD47 は脳内出血後の脳腫瘍除去において重要な役割を果たしていることが示された。

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