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The Alternative Complement Pathway Propagates Inflammation and Injury in Murine Ischemic Stroke

Andrew Elvington,* Carl Atkinson,* Hong Zhu,† Jin Yu,‡ Kazue Takahashi,‡ Gregory L. Stahl,§ Mark S. Kindy,‖ and Stephen Tomlinson*¶

There is mounting evidence indicating an important role for complement in the pathogenesis of cerebral ischemia-reperfusion injury, or ischemic stroke. The role of the alternative complement pathway in ischemic stroke has not been investigated, and there is conflicting data on the role of the terminal pathway. In this study, we show that compared with wild-type mice, mice deficient in the alternative pathway protein factor B or mice treated with the alternative pathway inhibitor CR2-fH have improved outcomes after 60-min middle cerebral artery occlusion and 24-h reperfusion. Factor B-deficient or CR2-fH–treated mice were protected in terms of improved neurologic function and reduced cerebral infarct, demyelination, P-selectin expression, neutrophil infiltration, and microthrombi formation. Mice deficient in both the classical and lectin pathways (C1q/MBL deficient) were also protected from cerebral ischemia-reperfusion injury, and there was no detectable C3d deposition in the ipsilateral brain of these mice. These data demonstrate that the alternative pathway is not alone sufficient to initiate complement activation and indicate that the alternative pathway propagates cerebral injury via amplification of the cascade. Deficiency of C6, a component of the terminal cytolytic membrane attack complex, had no effect on outcome after ischemic stroke, indicating that the membrane attack complex is not involved in mediating injury in this model. We additionally show that the protective effect of factor B deficiency and CR2-fH treatment is sustained in the subacute stage of infarct development, adding to the clinical relevance of these findings. The Journal of Immunology, 2012, 189: 4640–4647.

Transient ischemic stroke is a pathological process in which a cerebral vessel is occluded creating an ischemic core, and once the blockage is resolved, either naturally or through pharmaceutical intervention, the returned blood flow creates a paradoxical secondary injury (reviewed in Refs. 1, 2). Stroke is a leading cause of death and disability, and the role of complement in cerebral ischemia-reperfusion injury (IRI) has received much attention recently.

Complement can be activated by either the classical, lectin, or alternative pathways. The classical and lectin pathways can be activated by Abs, certain polysaccharides, and apoptotic cells. The alternative pathway can be activated spontaneously but also functions to amplify the other pathways. All pathways converge with the formation of a C3 convertase and the cleavage of C3 to produce C3a and C3b. C3a is a soluble bioactive peptide, and C3b becomes covalently bound to the activating surface, where it is further cleaved to produce iC3b and C3d opsonins. C3 cleavage also leads to C5 cleavage to yield C5a and C5b. C5a has multiple inflammatory activities, including the recruitment and activation of leukocytes, whereas C5b initiates the terminal pathway and formation of the cytolytic membrane attack complex (MAC).

Although there is good clinical evidence for a role of complement in ischemic stroke (3), our current understanding of the complement-dependent mechanisms involved in ischemic stroke derives mostly from murine studies and the use of complement-deficient and complement-inhibited mice. In a seminal study, it was shown that after middle cerebral artery occlusion (MCAO), ischemic neurons upregulate the classical complement pathway molecule C1q and that a soluble complement inhibitor, sCR1, is protective (4). Furthermore, an sCR1 derivative with sialyl LewisX glycosylation inhibited both complement activation and selectin-mediated adhesion and demonstrated enhanced therapeutic activity. Subsequent studies demonstrated that genetic deficiency of the central complement protein C3 (5, 6), as well as inhibition of C3 convertases (5), reduced infarct volumes and inflammation and improved neurologic deficit after MCAO. As noted above, the principle complement effector molecules that are produced after activation of any pathway are C3a, C5a, C3 opsonins, and the MAC. In the context of rodent ischemic stroke, both C3a and C5a signaling have been shown to contribute to ischemia-related inflammation and injury, as both C3a receptor (6, 7) and C5a receptor (8, 9) antagonism reduced cerebral inflammation, infarct volume, and neurologic deficit. It should be noted, however, that C5 deficiency is not protective after focal cerebral ischemia, a finding that is not consistent with a role for C5a (6).

A recent study demonstrated an important role for natural self-reactive IgM in activating complement after ischemic stroke (10). Complement activation by natural IgM has also been shown to occur in other organ models of IRI, with IgM-mediated activation occurring via the lectin pathway (11–13). A role for the lectin pathway is also indicated in cerebral IRI, as lectin pathway deficiency is protective in murine models (14, 15). Clinical data also

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Abbreviations used in this article: ECA, external carotid artery; (b) factor B; GFAP, glial fibrillary acidic protein; IRI, ischemia-reperfusion injury; MAC, membrane attack complex; MCAO, middle cerebral artery occlusion; tPA, tissue-type plasminogen activator; wt, wild-type.
suggest a role for the lectin pathway in human ischemic stroke (14, 16). And although IgM also binds C1q and is a powerful activator of the classical pathway, C1q deficiency is not protective after murine focal cerebral ischemia (6, 17). The role of the alternative pathway of complement activation in ischemic stroke has not been investigated, although this pathway is important for propagating inflammation and injury in multiple pathological conditions (18, 19).

A role of the terminal pathway in ischemic cerebral injury has been investigated using neonatal rats that are developmentally deficient in C9. Reconstitution of neonatal rats with C9 exacerbated cerebral injury after carotid artery ligation with hypoxia, indicating an important role for the MAC (20). In contrast, in a mouse model of neonatal hypoxic-ischemic brain injury involving permanent ischemia, C6 deficiency was not protective, indicating that the MAC does not play a role in cerebral injury in this model (21). In a model of MCAO and transient ischemia, as used in the current study, the effect of CD59a deficiency, a membrane-bound inhibitor of MAC formation, was dependent on gender and the period of ischemia (22).

In the current study, we investigated the role of the alternative pathway in propagating secondary inflammation and injury after transient focal cerebral ischemia and reperfusion. In addition, we further investigated the role of the terminal pathway in transient ischemic stroke after MCAO by a direct approach by using mice that are deficient in C6 and that cannot form the MAC. We also investigated a therapeutic strategy for limiting cerebral inflammation and injury using CR2-fH, which targets sites of complement activation via CR2-mediated recognition of C3 opsonins (23). It has been shown previously that CR2-fH specifically inhibits the alternative pathway (23, 24). A human counterpart of CR2-fH termed TT30, also shown to be specific for the alternative pathway (25), is currently in clinical trials.

Materials and Methods

Mice

All mice used in these studies, including complement-deficient mice, were on the C57BL/6 genetic background, male, and 8–9 wk old at the time of study. Wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The following complement-deficient strains of mice were used: B6/J (lack alternative pathway) (26); C1q/MBL−/− (lack classical and lectin pathway) (27); C6−/− (lack ability to form the MAC) (28, 29); CD59a−/− (lack membrane inhibitor of MAC formation) (30). The C6−/− and CD59a−/− mice were kindly provided by Dr. B. Paul Morgan (University of Wales, Cardiff, U.K.). Deficient mice were bred and housed at the Medical University of South Carolina. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Middle cerebral artery occlusion

Mice were subjected to 60-min MCAO, followed by a reperfusion period of 24 h. Mice were sacrificed and brains isolated for analysis, as previously described (5, 10). Briefly, mice were anesthetized, the external carotid artery (ECA) exposed, and the blunted tip of a 6-0 nylon suture inserted through the ECA into the internal carotid artery. The suture was advanced so as to block the middle cerebral artery. After 60 min of ischemia, the suture was withdrawn, the ECA ligated, and the incision closed. Laser Doppler flowmetry was used to assess cerebral blood flow before, during, and after ischemia; mice not achieving reduction in blood flow to 20% of pre-ischemia levels were excluded from analysis. Temperature, blood pressure, and heart rate were also monitored before, during, and 10 min after ischemia.

CR2-fH and CR2-Crry preparation and treatment

The recombinant fusion protein CR2-fH, a site-targeted inhibitor of the alternative pathway (24), was prepared as previously described (23). CR2-Crry, an inhibitor of all complement pathways at the C3 activation step, was prepared as previously described (31). Protein purity was assessed by SDS gel electrophoresis, and complement inhibitory activity was evaluated by zymosan assay (23). For complement inhibitor treatments, randomized wild-type (wt) C57BL/6 mice received 0.4 mg CR2-fH, 0.25 mg CR2-Crry, or 100 μl vehicle control (PBS) intravenously via tail vein 30 min post-reperfusion (5). Inhibitor dosing was based on previously published dose-response data for CR2-fH and CR2-Crry in a model of intestinal IRI (23).

Twenty-four hour survival

Survival to 24 h after 60 min of MCAO was compared among groups. Survival was compared using Fisher’s exact 2 × 2 test.

Evaluation of infarct volume

After sacrifice of mice, brains were perfused with PBS, isolated, and placed in a rodent brain matrix (EMS, Hatfield, PA). Two-millimeter slices were prepared and stained with 2% triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO). Total infarct area was assessed using ImageJ Analysis Software (National Institutes of Health) and calculated by summation of infarcted areas of all slices for each hemisphere. Animals that did not survive to the established endpoint (24 h, 72 h, or 7 d) were excluded from the determination of infarct volumes.

Neurologic deficit scoring

Neurologic deficits were determined as described (32) according to the following scoring system: 0, normal function; 1, flexion of torso and contralateral forelimb when lifted; 2, normal posture at rest with circling to contralateral side when held by tail on flat surface; 3, leaning to contralateral side when at rest; 4, no spontaneous movement. Scoring was performed by an observer blinded to experimental groups.

Histopathology

At sacrifice, mice were perfused with PBS. Brains were then removed, fixed overnight in 4% paraformaldehyde in PBS, and either immersed in 20% sucrose for cryosectioning or processed to paraffin for Luxol fast blue/Nissl (American MasterTech, Lodi, CA) or H&E staining for morphological analysis.

Immunohistochemistry

Paraffin processed sections cut at 8 μm were subjected to Ag retrieval by citrate steam for 30 min (ICH World, Elylicot City, MD), and primary Abs were then applied in PBS for 2 h at room temperature. Primary Abs used were goat anti-mouse C3d (1:20; R&D Systems, Minneapolis, MN), goat anti-mouse IgM (1:50; Sigma-Aldrich), and rat anti-mouse Ly6G+Ly6C (Gr-1, 1:20; BD Biosciences, San Jose, CA). Brain sections from mice sacrificed at 72 h were stained with rabbit anti-mouse glial fibrillary acidic protein (GFAP) (1:1000; Dako, Carpinteria, CA) in PBS for 2 h at room temperature. Immunohistochemistry to detect P-selectin expression was done on 8-μm cyosections stained with rat anti-mouse CD62P (1:10; BD Biosciences) overnight at 4°C. Primary Abs were detected using the appropriate ImmPact kit (Vector Laboratories, Burlingame, CA), developed using NovaRed (Vector Laboratories), and sealed with CryoSeal-60 (Richard-Allan Scientific, Kalamazoo, MI). Primary Abs were omitted for negative controls. A blinded observer imaged the slides by light microscopy (Nikon Eclipse E600).

Assessment of complement deposition, microthrombi formation, and neutrophil infiltration

Brain tissue sampled was located between +2 mm and −2 mm relative to the bregma, with injury confirmed with Luxol fast blue/Nissl or counterstaining. C3d deposition within the penumbra was scored as described previously (33): 0, no observable staining; 1, mild staining; 2, moderate staining; 3, severe staining. To assess microthrombi formation, H&E sections were scored as described previously (5): 0, no observable thrombi; 1, modest RBC attachment; 2, focal RBC attachment and fibrin; 3, 50% occlusion by RBC and fibrin; 4, total occlusion. To quantify neutrophil infiltration in the penumbra, 10 random fields, at ×40 magnification, from Gr-1–stained sections were counted. All assessments were performed by an observer blinded to experimental groups.

Evaluation of apoptosis

Paraffin brain sections from mice sacrificed at 72 h were evaluated for apoptosis by TACS 2 TdT-DAB in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). The staining protocol was followed according to the manufacturer’s instructions. Negative and positive controls were included with each staining batch. Apoptotic cells in the peri-infarct region of the
penumbra were counted from 10 random fields at ×40 magnification by light microscopy. The observer was blinded to the experimental groups.

Statistical analysis

Statistical analyses were performed using Prism 4 software (GraphPad, La Jolla, CA). Parametric data (infarct volumes) were analyzed for statistical significance with one-way ANOVA test. Non-parametric data (neurologic deficits, C3d deposition scores, microthrombi scores, and cell counts) were analyzed for statistical significance with Kruskal–Wallis test. Comparisons between two groups were conducted using Student t test (parametric) or Mann–Whitney U test (non-parametric). Differences were considered significant when p < 0.05.

Results

Alternative pathway deficiency and inhibition protects against cerebral IRI

Neurologic deficit was assessed 24 h after 60 min of cerebral ischemia. Compared to wt controls, mice deficient in either factor B (fB; no alternative pathway) or C1q/MBL (only alternative pathway) displayed significant improvements in median neurologic deficit (Fig. 1A). There was no significant difference in neurologic deficits between fB- and C1q/MBL-deficient mice. To investigate the role of the alternative pathway in a more clinically relevant paradigm, wt mice were treated with either CR2-fH, a targeted inhibitor of the alternative pathway, or CR2-Crry, a similarly targeted inhibitor that blocks all complement activation pathways. Treatments were given at 30 min after reperfusion. Both inhibitors significantly improved median neurologic deficit compared with wt control mice, and there was no significant difference either between CR2-fH– and CR2-Crry–treated mice or between inhibited mice and fB- or C1q/MBL-deficient mice (Fig. 1A). Focal cerebral infarct volumes were also analyzed 24 h after reperfusion. Compared to wt controls, mean infarct volumes were significantly decreased in both fB- and C1q/MBL-deficient mice, although infarct volumes were significantly smaller in C1q/MBL-deficient mice compared with fB-deficient mice (Fig. 1B). Furthermore, consistent with the effect of CR2-fH and CR2-Crry on neurologic deficit, both inhibitors significantly reduced mean infarct volumes compared with wt controls, and although there was a trend toward improved outcome with CR2-Crry compared with CR2-fH, the difference was not significant (Fig. 1B).

The terminal complement pathway does not play a role in cerebral injury after 60-min transient ischemia

The terminal complement pathway and MAC formation is common to all activation pathways and has been implicated as a key mediator of IRI in various organs. However, a previous study demonstrated that deficiency of CD59a, an endogenous membrane-bound inhibitor of MAC formation, exacerbated cerebral IRI after a 30-min but not after a 60-min ischemic period (22). To investigate more directly the role of the terminal pathway and the MAC in cerebral IRI, we determined the effect of C6 deficiency, a component protein of the MAC, in the more severe model of cerebral IRI incorporating 60 min of MCAO. C6 deficiency was not protective in terms of either neurologic function or infarct volume compared with wt control mice (Fig. 2), indicating that the terminal pathway does not contribute to IRI in this model. We also confirmed the previous data demonstrating that CD59a deficiency was without effect on cerebral injury after 60 min of MCAO (Fig. 2).

**FIGURE 1.** Effect of complement deficiency and CR2-fH or CR2-Crry treatment on neurologic deficit and infarct volume after 60-min MCAO and 24-h reperfusion. (A and B) Neurologic deficit (A) and infarct volume (B) in C1q/MBL−/− mice, fB−/− mice, and wt mice treated with CR2-fH or CR2-Crry 30 min after reperfusion. Horizontal bar indicates median neurologic score and mean infarct volume, n = 12–15 for neurologic deficit and n = 8–11 for infarct volume. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** Effect of terminal pathway deficiency and terminal pathway inhibitor deficiency on neurologic deficit and infarct volume after 60-min MCAO and 24-h reperfusion. (A) Neurologic deficit in C6−/− and CD59a−/− mice. Horizontal bar represents median; n = 10–11. No statistical difference between groups. (B) Infarct volume in C6−/− and CD59a−/− mice. Horizontal bar indicates mean, n = 6–8. No statistical difference between groups.
2). Subsequent studies focused on the role of the alternative pathway in this model of ischemic stroke.

Neuronal tissue integrity

Neuronal integrity, as demonstrated by myelin staining with Luxol fast blue/Nissl staining, was improved in C1q/MBL−/− mice, fB−/− mice, and CR2-fH–treated mice compared with wt controls. There was no apparent improvement in C6−/− mice (Fig. 3). Thus, improved neuronal integrity correlates with improvements in infarct volumes and neurologic deficits for the different groups of mice shown in Fig. 1. Taken together, these data indicate that the alternative pathway alone is not sufficient to cause injury after cerebral ischemia-reperfusion but that it plays a key role in propagating injury after complement activation by either the classical or lectin pathway, and published data have shown dependence on the lectin pathway (14, 15).

Twenty-four hour survival and physiological measurements

C1q/MBL deficiency significantly improved the survival to 24 h (18 of 19) compared with wt (12 of 18, p = 0.03 by Fisher’s exact 2 × 2 test). Mice deficient in fB or treated with CR2-fH appeared to have increased survival rates to 24 h compared with wt mice (15 of 18 and 17 of 20, respectively), but the difference did not reach significance. There was also no significant difference between 24-h survival rates of wt mice and mice deficient in C6 or CD59α (11 of 16 and 8 of 11, respectively).

To confirm that cerebral blood flow was interrupted by the MCAO procedure, blood flow was measured by laser Doppler, and there were no significant differences in cerebral blood flow between any of the groups before, during, or 10 min after ischemia (data not shown). Changes in blood pressure and body temperature can significantly influence the outcome after stroke, and we therefore measured blood pressure, heart rate, and temperature before, during, and after ischemia. There were no differences between the groups (data not shown). Also, as expected, there was no damage observed in the contralateral hemisphere in any group.

Complement deposition is reduced in complement-deficient and -inhibited mice after MCAO

Penumbral deposition of the C3 activation product, C3d, was examined on sections of brain isolated 24 h after reperfusion, at which time complement deposition is maximal in the 60-min MCAO model (34). C3d staining was seen in the penumbra throughout the ipsilateral hemisphere in wt mice but was virtually absent in C1q/MBL mice (Fig. 4). Mice deficient in fB or treated with CR2-fH had significantly reduced C3d staining compared with wt mice, and the staining that was observed was associated predominantly with the vasculature in the penumbra (Fig. 4). Brain sections from C6-deficient mice and wt mice had similar levels of C3d staining, and contralateral tissue did not show any C3d staining for any of the experimental groups (data not shown). These data support the above outcome measurements in terms of the conclusion that the alternative pathway amplifies complement activation and drives cerebral injury subsequent to classical/lectin pathway initiation.

Reduced complement deposition is not due to reduced IgM binding in ischemic brain

Natural self-reactive IgM Abs that recognize neoepitopes exposed on ischemic tissue have been shown to play an important role in activating complement and propagating IRI, including cerebral IRI (10). We therefore wanted to confirm that the effect of the different complement deficiencies on cerebral IRI was not due to differences in the binding of IgM after reperfusion. Immunohistochemical analysis of brain sections isolated 24 h after reperfusion revealed similar levels of IgM staining in all groups, and IgM staining was absent in sections isolated from the contralateral hemisphere (Fig. 5).

Microthrombi formation is reduced in alternative pathway-deficient and -inhibited mice

Secondary thrombus formation in the penumbra can perpetuate further ischemic injury and is a significant complication after ischemic stroke. We previously demonstrated fibrinogen deposition

![FIGURE 3](image-url)

**FIGURE 3.** Neuronal tissue integrity in complement-deficient and CR2-fH–treated mice after 60-min MCAO and 24-h reperfusion. Brain sections from the ipsilateral hemisphere were stained with Luxol fast blue/Nissl. Section from contralateral hemisphere shown as undamaged control. Representative images shown, n = 4/group. Original magnification ×10; scale bar, 200 μm.

![FIGURE 4](image-url)

**FIGURE 4.** C3d deposition in penumbral tissue in complement-deficient and CR2-fH–treated mice after 60-min MCAO and 24-h reperfusion. (A) Representative immunohistochemistry images taken at original magnification ×40. Scale bar, 50 μm. There was no observable C3d staining in the contralateral hemispheres in any group. (B) Quantification of C3d staining. Horizontal bar represents median, n = 5. *p < 0.05, **p < 0.01.
and microthrombi formation in the cerebrovasculature after MCAO, and we therefore investigated whether the alternative pathway contributes to this postischemic complication. Brain sections were analyzed for the presence of RBC accumulation and fibrin deposition as previously described (5). There were significantly lower numbers of microthrombi and partially occluded vessels in fB-deficient and CR2-fH–treated mice compared with wt mice (Fig. 6). Microthrombi formation appeared to be even lower in C1q/MBL-deficient mice compared with fB-deficient and CR2-fH–treated mice, although the difference did not reach significance.

**P-selectin expression and neutrophil infiltration is reduced in alternative pathway-deficient and -inhibited mice**

Infiltrating leukocytes are important mediators of postischemic inflammation and injury, and complement activation products are involved in their recruitment, either directly or indirectly via upregulation of inflammatory mediators such as the adhesion molecule P-selectin. The expression of P-selectin and the infiltration of neutrophils are directly linked to cerebral inflammation and injury after ischemic stroke (4, 35), and we therefore examined how modulation of the alternative pathway affects these two parameters. Compared to wt mice, fB-deficient and CR2-fH–treated mice both had significantly reduced P-selectin expression and neutrophil infiltration in the cerebral vasculature 24 h after MCAO (Fig. 7). Furthermore, P-selectin expression was undetectable in C1q/MBL-deficient mice, and neutrophil infiltration was significantly lower in C1q/MBL-deficient mice compared with fB-deficient and CR2-fH–treated mice. There was no detectable expression of P-selectin in the contralateral hemisphere from any experimental group. Thus, blocking or inhibiting the alternative pathway reduces cerebral inflammation, and this correlates with the above data showing reduced cerebral injury, reduced complement deposition, and reduced microthrombi formation. The enhanced effect of C1q/MBL deficiency on the above measured parameters compared with fB deficiency is indicative of the alternative pathway exerting its effect in propagating pathogenesis after ischemic stroke via its function in the complement cascade amplification loop.

**CR2-fH treatment is protective at 72 h after MCAO**

To address better the clinical relevance of our findings, we investigated the effect of a single post-reperfusion injection of CR2-fH, as well as fB deficiency, on infarct maturation and longer-term outcome after MCAO. Compared to wt mice, CR2-fH–treated or fB-deficient mice had significantly reduced median neurologic deficits at 3 d after reperfusion (Fig. 8A). Furthermore, the median

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**FIGURE 5.** IgM deposition in penumbral tissue in complement-deficient and CR2-fH–treated mice after 60-min MCAO and 24-h reperfusion. There were no discernible differences in the levels or distribution of IgM staining between any groups. No IgM staining was observed in the contralateral hemispheres in any group. Representative images, \( n = 3 \). Original magnification \( \times 40 \); scale bar, 50 \( \mu m \).

**FIGURE 6.** Microthrombi formation in penumbral tissue in complement-deficient and CR2-fH–treated mice after 60-min MCAO and 24-h reperfusion. Microthrombi formation was assessed in H&E-stained brain sections. Horizontal bars represent median microthrombi scores, \( n = 5–7 \). *p < 0.05.

**FIGURE 7.** P-selectin expression and neutrophil infiltration in penumbral tissue in complement-deficient and CR2-fH–treated mice after 60-min MCAO and 24-h reperfusion. (A) P-selectin expression as detected by immunohistochemistry. Expression is associated with the vasculature. Representative images, \( n = 3 \). Original magnification \( \times 10 \); scale bar, 200 \( \mu m \). No staining was detected in contralateral hemisphere of any group. (B) Neutrophil infiltration as detected by immunohistochemical staining of Gr-1+ cells. Horizontal bar represents median, \( n = 4–5 \). **p < 0.01, ***p < 0.001.
neurologic deficit in CR2-fH–treated and fB-deficient mice was lower at 72 h after reperfusion than it was at 24 h after reperfusion. Infarct volumes also remained significantly smaller at 72 h after reperfusion in CR2-fH–treated and fB-deficient mice compared with wt mice (Fig. 8B). Because astrocyte responses can be modulated by complement activation products (36), we also determined how alternative pathway inhibition with CR2-fH effects astrogliosis, a hallmark of brain injury (37). As determined by GFAP expression, CR2-fH treatment dampened the reactive astrocyte presence associated with the injury border seen in wt mice at 3 d after reperfusion (Fig. 8C). Reduction in the GFAP reactivity was also observed in fB-deficient mice. There were no differences between groups in GFAP reactivity in the corresponding contralateral sections (data not shown). To determine if CR2-fH treatment affects delayed cell death, we determined apoptosis in the peri-infarct region of the penumbra at 72 h after MCAO. As shown in Fig. 8D, there were fewer apoptotic cells in the penumbral region of brains from mice treated with CR2-fH compared with wt control mice. The number of apoptotic cells in brains from fB-deficient mice was similar to that seen in CR2-fH–treated mice. There were no observed differences in the contralateral hemispheres between groups (data not shown).

CR2-fH–mediated protection from cerebral injury persists to 7 d after reperfusion

Whereas C1-INH (38) and C3aR antagonist (39) have been shown to be neuroprotective in stroke models, with improved outcome maintained for 7 d after ischemia, lectin pathway deficiency has been shown to provide only acute (24 h) protection after MCAO (40). To determine if CR2-fH–mediated protection persists into the subacute phase of stroke, neurologic deficit and infarct volume was assessed at 7 d after MCAO. Compared to vehicle-treated controls, mice receiving a single dose of CR2-fH at 30 min after reperfusion had significantly reduced mean infarct volumes and displayed a strong trend toward neurologic improvement (p = 0.07) 7 d after MCAO (Fig. 9). These data indicate that CR2-fH treatment provides sustained neuroprotection, rather than just delaying cerebral ischemic injury.
Discussion
This study demonstrates an important role for the alternative complement pathway in the pathogenesis of murine ischemic stroke. Although the alternative pathway can be spontaneously activated, the current data demonstrate that a functioning alternative pathway is not alone sufficient to initiate complement activation and propagate murine cerebral IRI. Rather, the alternative pathway serves to amplify complement activation initiated by the classical and/or lectin pathway. Previous data demonstrating that lectin pathway-deficient (14, 15) but not classical pathway-deficient (6, 17) mice are protected from cerebral IRI, indicating that it is alternative pathway-mediated amplification of the lectin pathway that drives injury in this model of ischemic stroke. Further, we have previously shown an important role for self-reactive natural IgM in propagating cerebral IRI (10), and others have shown that IgM mediates IRI via activation of the lectin pathway, at least after ischemia and reperfusion of the intestine and heart (11–13).

In this study, we also show that mice deficient in C6, a component protein of the MAC, are not protected from cerebral injury after 60-min MCAO and 24-h reperfusion. This finding indicates that the terminal complement pathway and the MAC do not play a role in murine cerebral IRI after 60 min MCAO, and is in accord with a previous study that demonstrated mice deficient in CD59a, an inhibitor of the MAC, do not display exacerbated cerebral injury in the same model (22). It is interesting that in contrast to this finding, the MAC is implicated as an important mediator of IRI in various other organs and tissues (41, 42), as well as in other neuroinflammatory conditions such as spinal cord injury (33) and traumatic brain injury (43). Nevertheless, it should be noted that C6 deficiency is protective in a neonatal model of hypoxic-ischemic brain injury involving permanent ischemia, implicating a role for the MAC in a different stroke model (21). Furthermore, CD59a-deficient mice show worse outcomes after 30-min MCAO, as opposed to 60-min MCAO, indicating that the terminal pathway may contribute to cerebral IRI under conditions of less severe injury/ischemia (22). Also, although outcomes were not different between wt and CD59a-deficient mice after 60-min MCAO, the level of apoptosis was increased in CD59a-deficient mice, and the authors suggested that MAC formation after severe ischemic stroke may be redundant and secondary to injury propagated by resident and infiltrating inflammatory cells. In this context, although our data indicate that the MAC is not essential for the progression of complement-dependent cerebral injury after 60-min MCAO, we demonstrated that the protective effect of upstream blockade of the alternative pathway was associated with reduced levels of apoptosis and inflammation (discussed later). Further studies are needed fully to address the role of the MAC in ischemic stroke, which in experimental models appears to be dependent on the model and/or severity of injury.

The alternative pathway plays an important role in driving inflammation and propagating injury in many pathological conditions (reviewed in Ref. 18), including neuroinflammatory conditions such as traumatic spinal cord injury (33), traumatic brain injury (44), and laser-induced choroidal neovascularization (45). Hallmarks of cerebral IRI (and inflammation in general) are the expression of endothelial adhesion molecules and the infiltration of leukocytes (35, 46, 47), both of which can be directly and indirectly modulated by complement activation products such as C3a and C5a. We demonstrated previously that C3 deficiency or CR2-Cry treatment (inhibits all complement pathways at C3 activation) protects against cerebral IRI and decreases the level of P-selectin expression and neutrophil infiltration and microthrombi formation after 60-min MCAO (5). In this study, we demonstrate that fB deficiency or specific inhibition of the alternative pathway has a similar effect, and we show no significant difference between CR2-H and CR2-Cry treatment outcomes in terms of neurologic deficit and infarct volume at 24 h after reperfusion. In addition, the reduction in infarct volume and improvement in neurologic score seen in CR2-H–treated mice at 24 h after reperfusion was further improved at 72 h, with improvements persisting for 7 d after reperfusion. Because complement has many important physiological functions, specific inhibition of the alternative pathway will likely be a beneficial therapeutic approach compared with inhibition of all complement pathways and is less likely to have undesirable side effects. In this regard, complement activation products have been shown to have protective roles against certain types neuronal injury (reviewed in Ref. 3), and the classical and lectin pathways are known to play important roles in host defense against some pathogens, as well as provide some important regulatory and homeostatic functions (48, 49). For example, the classical and lectin pathways play a role in the removal of apoptotic and injured cells, important for the resolution of inflammation and tissue repair (50).

Recombinant tissue-type plasminogen activator (tPA) is the only approved pharmacological agent for the treatment of ischemic stroke, and in general it must be administered within 3 h of symptom onset. tPA promotes reperfusion by enhancing the dissolution of blood clots, but there is the risk of uncontrollable intracranial hemorrhage, and for this reason physicians are often reluctant to use this drug. There is currently no intervention that can reverse the effects of tPA on acute hemorrhage. It is noteworthy that thrombolytic therapy has been shown to activate complement (51), and complement inhibition may thus have the potential to synergize with thrombolytic therapy, possibly even extending the window for tPA treatment. In this context, a human counterpart of CR2-H, named TT30, is currently in phase 1 clinical trial for nocturnal paroxysmal hemoglobinuria (http://clinicaltrials.gov/ct2/results?term=tt30). TT30 has demonstrated neuroprotection in murine laser-induced choroidal neovascularization (a model of age-related macular degeneration) (52) and has been shown to prevent alternative pathway activity, C3 opsonin accumulation, and MAC formation on RBCs, without interfering with the classical and lectin activation pathways (53).

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