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*J Immunol* 2006; 177:7266-7274; doi: 10.4049/jimmunol.177.10.7266

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Complement-Dependent P-Selectin Expression and Injury following Ischemic Stroke

Carl Atkinson,* Hong Zhu,† Fei Qiao,* Juan Carlos Varela,* Jin Yu,† Hongbin Song,* Mark S. Kindy,‡‡ and Stephen Tomlinson2*

The mechanisms that contribute to inflammatory damage following ischemic stroke are poorly characterized, but studies indicate a role for both complement and P-selectin. In this study, we show that compared with wild-type mice, C3-deficient mice showed significant improvement in survival, neurological deficit, and infarct size at 24 h after middle cerebral artery occlusion and reperfusion. Furthermore, P-selectin protein expression was undetectable in the cerebral microvasculature of C3-deficient mice following reperfusion, and there was reduced neutrophil influx, reduced microthrombus formation, and increased blood flow postreperfusion in C3-deficient mice. We further investigated the use of a novel complement inhibitory protein in a therapeutic paradigm. Complement receptor 2 (CR2)-Crry inhibits complement activation at the C3 stage and targets to sites of complement activation. Treatment of normal mice with CR2-Crry at 30 min postreperfusion resulted in a similar level of protection to that seen in C3-deficient mice in all of the above-measured parameters. The data demonstrate an important role for complement in cerebrovascular thrombosis, inflammation, and injury following ischemic stroke. P-selectin expression in the cerebrovasculature, which is also implicated in cerebral ischemia and reperfusion injury, was shown to be distal to and dependent on complement activation. Data also show that a CR2-targeted approach of complement inhibition provides appropriate bioavailability in cerebral injury to enable complement inhibition at a dose that does not significantly affect systemic levels of serum complement activity, a potential benefit for stroke patients where immunosuppression would be undesirable due to significantly increased susceptibility to lung infection. The Journal of Immunology, 2006, 177: 7266–7274.

Cerebral ischemia initiates a cascade of events that can lead to secondary neuronal damage with increased extent of infarct and poorer clinical outcome. The restoration of blood flow to the area surrounding the infarct elicits an inflammatory response that plays a significant role in secondary injury (1, 2). The mechanisms associated with the initiation and perpetuation of inflammation are incompletely understood, but evidence from clinical and animal studies has implicated the complement system in postischemic cerebral injury (3, 4). Mice deficient in C1q (blocked in the classical pathway of complement activation) have improved neurological outcome following the induction of cerebral ischemia, although the data proved difficult to interpret because C1q deficiency conferred significant protection only in neonatal mice (5, 6). C1 inhibitor, however, was shown to be effective at reducing cerebral infarct volume in mature rodents (6–8). Soluble complement receptor CR2 (sCR2) has also been shown to be protective in a mouse model of ischemic stroke (9), although it was without significant effect in a neonatal rat model (10).

P-selectin is an important mediator of platelet accumulation and neutrophil recruitment, and P-selectin expression is up-regulated in vitro by complement activation products (11, 12). Furthermore, P-selectin-deficient mice and mice treated with blocking P-selectin Abs are protected from the effects of cerebral ischemia and reperfusion (13–16). Huang et al. (9) described a hybrid molecule consisting of sCR1 that was modified with a sialyl Lewis&superscript; α glycosylation, and which had both complement inhibitory and selectin antagonist activity. Administration of this hybrid inhibitor, sCR1sLex, resulted in a significant reduction in neurological deficit, neutrophil accumulation, and infarct volume (9). The exact mechanism for its protective effect was not clear, but of note, it has been shown in other models of inflammation that the therapeutic efficacy of sCR1sLex is dependent on systemic complement inhibition, even though sCR1sLex localizes to the vasculature (9, 17–21). Systemic complement inhibition can alter the host’s ability to mount an effective immune response (22, 23), a factor of clinical relevance to stroke where patients often succumb to lung infections (24).

In this study, we assess the neuroprotective effect of C3 deficiency and C3 inhibition with CR2-Crry, a novel targeted inhibitor. We have shown previously that appropriate targeting of complement inhibition can improve efficacy without hindering host immunity (23). CR2-Crry consists of a targeting domain, CR2, linked to a complement inhibitory protein, mouse Crry (23). Because complement inhibitors display different levels of species selectivity, the use of Crry in a mouse model is appropriate. CR2 binds to long-lived C3 cleavage fragments, iC3b, C3dg, and C3b, which are deposited on complement-activating surfaces such as inflamed endothelium. We also investigate the role of complement activation on cerebrovascular expression of...
P-selectin. Complement activation products C5a and the cyto-
lytic membrane attack complex (MAC) have been shown to
induce P-selectin expression in vitro (25, 26), but there is little
information available on how these complement activation
products affect P-selectin expression in vivo.

Materials and Methods

Middle cerebral artery occlusion (MCAO) and reperfusion

Eight-week-old male C57BL/6 and C57BL/6 C3-deficient mice (The Jack-
son Laboratory) were used in experiments. Mice were anesthetized with
chloral hydrate (350 mg/kg) and xylazine (4 mg/kg) i.p., and the left com-
mon carotid artery (ECA). Each mouse was exposed through a midline incision
in the neck. The superior thyroid and occipital arteries were divided and
a microsurgical clip was placed around the origin of the external carotid
artery (ECA). The distal end of the ECA was ligated with 6-0 silk and
transected, and 6-0 silk was tied loosely around the ECA stump. The clip
was then removed, and the blunted tip of a 4-0 nylon suture was inserted
into the ECA stump. The loop of the 6-0 silk was tightened around the
stump, and the ECA incision was advanced into and through the internal
carotid artery until it rested in the anterior cerebral artery. After the nylon
suture had been placed for 60 min, it was pulled back into the ECA, and
the incision was closed. Body temperature was monitored using a rectal ther-
ometer and body temperatures were maintained at 37°C. Cerebral blood
flow and blood pressure were measured before, during, and after ischemia
using previously described methods (27, 28). For therapeutic studies,
C57BL/6 mice were randomized into CR2-Cry-treated and PBS-treated
groups. Thirty minutes postreperfusion, PBS vehicle or CR2-Cry at a dose
of 0.25 mg was administered i.v. by tail vein injection. Mice were allowed
to recover, and were monitored over a 24-h period for neurological deficit.
All procedures were approved by the Medical University of South Carolina
on Animal Research, in accordance with the National Institutes of Health
Guidance for Care and Use of Laboratory Animals.

CR2-Cry complement inhibitor

The recombinant fusion protein, CR2-Cry, was prepared by joining mouse
CR2 sequence encoding the four N-terminal short consensus repeat units
(residues 1–257 of mature protein, National Center for Biotechnology In-
formation (NCBI) GenBank accession no. M35684) to sequences encoding
extracellular regions of mouse Cry. The Cry sequence used encoded resi-
dues 1–257 of mature protein (National Center for Biotechnology
Information (NCBI) GenBank accession no. NM013499). To join CR2 to Cry, a linking sequence encoding (GGGGS),
was used. CR2-Cry was expressed and purified as described previously
(23).

Measurement of infarct volume

Mice were anesthetized with an i.p. injection of sodium pentobarbital at
24 h postreperfusion. The brains were transected perfused with 10%
PBS, removed, and placed in a Rodent Brain Matrix (EMS). Coronal sec-
tions were prepared and subjected to 2% trisbuffered formaldehyde
chloride (TTC) staining (29). The infarct area in each section was determined
using NIH Image Analysis Software. Total infarct volume for each brain was
calculated by summation of the infarct areas of all brain slices for each
hemisphere. Animals not surviving to 24 h were not analyzed for infarct
volume due to difficulties in interpretation of postmortem changes.

Behavioral analysis

Behavioral analysis was determined before ischemia and in mice surviving
24 h postreperfusion. Behavioral/neurological deficit was scored as fol-
lows: 0, normal motor function; 1, flexion of torso and contralateral fore-
limb when the mouse is lifted by the tail; 2, circling to the contralateral side
when held by tail on flat surface, but normal posture at rest; 3, leaning
to the contralateral side at rest; 4, no spontaneous motor activity (30).

Histopathology

Mice were sacrificed and perfused with PBS and brains then carefully
removed and placed into 4% paraformaldehyde for 24 h at 4°C. Following
fixation, brains were either immersed in 4% paraformaldehyde in 20% sucrose
solution and then embedded in OCT medium for cryosectioning, or
processed to paraffin and stained with H&E and Nissl stains as previously
described (31) for morphological analysis.

Immunofluorescence double staining for C3, fibrinogen,
neutrophils, CR2, P-selectin, and endothelial cells

Cryosections were cut at 8 μm, fixed in cold acetone for 5 min, and then
washed in running water followed by PBS. Primary Abs and reagents used
were all commercially available. Double immunofluorescent staining was
performed using the FITC-labeled Abs directed against C3 (MP Biome-
cals), CR2, and fibrinogen (BD Pharmingen), and P-selectin and CR2 were
localized by a rat anti-mouse P-selectin Ab (BD Pharmingen) and a polyclonal goat anti-mouse CR2 Ab (Santa Cruz Bio-
technology), and then subsequently visualized with either rabbit anti-rat
Alexa 488 or 555 for P-selectin or rabbit anti-goat Alexa 555 secondary Ab
(Molecular Probes). The presence of endothelial cells in tissue sections was
confirmed by the broad spectrum endothelial marker CD31 (BD Pharmin-
gen) and visualized by incubation with anti-rat Alexa 488 or 555 (Molec-
ular Probes). Nuclear staining was obtained by incubation with TO-PRO-3
(Molecular Probes). Following staining sections were coversoned using
Vexta fluorescent hard mount (Vector Laboratories). Ab binding was as-
sessed in ipsilateral and contralateral sections of the brain using a Leica
tCS-SP2 confocal microscope. Neutrophils were quantified in both sec-
tions within all groups with the observer blinded to the experimental group.
Specificity of staining was assessed by omission of primary Abs, the use of
isotype controls, and, where available, blocking peptides were used to con-
firm that binding and staining could be abolished.

Assessment of apoptosis

DNA fragmentation was detected in situ by TUNEL labeling with the apo-
ptosis fluorescein detection system (Promega). Apoptosis was detected on
frozen sections in line with the manufacturer’s recommendations. Sections
were examined using a Leica fluorescent microscope, and the number of
TUNEL-positive apoptotic cells was counted and averaged from six ran-
donately selected fields in the ischemic penumbra. The penumbra was se-
lected to avoid confusion and identification of necrotic cells in the necrotic
core in infarct. For negative controls, TdT enzyme was omitted from the
reaction mix.

Assessment of microthrombi

Cryosections were cut at 8 μm and then fixed in cold acetone for 5 min.
Sections were then either stained with a conventional H&E stain, or stained
for the presence of fibrinogen deposition by immunofluorescence as de-
scribed above. Cryosections stained for fibrinogen were assessed by two
blinded observers and scored from 0 to 5 for the presence of intravascular
and extravascular fibrinogen within the damaged cerebral hemisphere us-
using previously described methodology (32). Briefly, scoring was denoted
as: 0, no staining observed; 1, staining limited to intravascular space; 2,
deposition within the lumen and perivascular space; and 3, vascular, extra-
vascular, and or parenchymal tissue staining (32). The presence of mi-
icrothrombi was further quantified using H&E-stained sections, and the
extent of microthrombi formation was assessed in a blinded fashion by
independent investigators and scored as follows: 0: no thrombi, 1: some
RBC attachment, 2: focus of RBC and fibrin, 3: RBC and fibrin 50% occlusion,
and 4: total occlusion.

Real-time RT-PCR

Total RNA was extracted from brains using guanidine isothiocyanate and
phenol-chloroform by standard methods (33). CDNA was made from 1 μg
of total RNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time
RT-PCR analysis was subsequently performed using the IQ SYBR Green
Supermix kit (Bio-Rad) following the manufacturer’s protocols. The anal-
ysis was performed using a My IQ Real-Time detection system (Bio-Rad)
using previously described (33) intron-spanning primers specific for P-
selectin forward 5′-ATGCCGCTGCTACTGGACACT-3′, reverse 5′-CTT
CATGGCAATGGAAGC-3′. All reactions were performed in triplicate, and
the GAPDH gene was used as an internal control. In each PCR (RNA
extraction, cDNA synthesis, and RT-PCR), negative controls were in-
ccluded. Results of quantitative real-time PCR (qRT-PCR) are presented as
told increases compared with sham controls.

Statistical analysis

Physiological variables and infarct volumes were compared between
groups by Student’s t test and ANOVA. Nonparametric data (neurological
outcome, fibrinogen deposition, and neutrophil quantification) were sub-
jected to Kruskal-Wallis test. A value of $p<0.05$ was considered statis-
tically significant.
Complement deficiency and inhibition protects against focal ischemic stroke

Three groups of mice were subjected to 60 min of MCAO-induced ischemia and 24 h of reperfusion: normal C57BL/6 mice, C3-deficient mice, and C57BL/6 mice treated with 0.25 mg of CR2-Crry 30 min postreperfusion. All C3-deficient mice survived to the primary end point of 24 h postreperfusion (n = 14/14). CR2-Crry-treated mice had an 80% survival (n = 16/20), whereas control C57BL/6 mice had only a 62% survival rate (n = 16/25). The improvement in survival of both C3-deficient and complement-inhibited mice compared with control mice was significant (p < 0.002).

Results

Complement deficiency or inhibition improves survival postischemic stroke

The extent of cerebral infarct was analyzed in animals surviving to 24 h posts ischemia. Wild-type (wt) controls showed marked areas of infarct involving the lateral, ventromedial, and dorsolateral cortex and the lateral medial striatum with an average infarct volume of 26 ± 12.8%. In contrast, C3-deficient mice and CR2-Crry-treated mice had limited injury which was localized to areas close to the site of occlusion with average infarct volumes of 3.25 ± 3.1% and 2.7 ± 3.2, respectively. The infarct volumes of C3-deficient and CR2-Crry-treated mice was significantly different to control mice (p < 0.001) (Fig. 1, A and B), with no significant difference between C3-deficient and CR2-Crry-treated mice (p = 0.3) (Fig. 1, A, C, and D).

For further validation of the neuroprotective effect of C3 deficiency and complement inhibition, we performed histochemical analysis of brain sections with the tinctorial stains H&E and Nissl stain. As with TTC staining, both H&E and Nissl staining revealed a significant increase in infarct size in sections from control animals compared with C3-deficient and CR2-Crry-treated animals, with the frontoparietal cortex and caudate putamen of the ipsilateral hemisphere appearing pale compared with the contralateral hemisphere (Fig. 2). Control animals showed evidence of degenerated neurons as evidenced by diffuse eosinophilia, and there was infiltration of inflammatory cells within the penumbra area (Fig. 2). The intensity and severity of these features was markedly reduced in C3-deficient and CR2-Crry-treated animals (Fig. 2). Nissl staining further demonstrated widespread pyknosis and karyorrhexis within the infarct site (Fig. 2). There was no damage evident in the contralateral hemisphere of any group.

Complement deficiency and inhibition improves neurological function

Animals surviving to 24 h postischemia were assessed for neurological function. C3-deficient mice and mice receiving CR2-Crry showed significant neurological improvement when compared with wt controls (p < 0.01) (Fig. 3). As for infarct volume, there...
was no significant difference between C3-deficient mice and CR2-Crry-treated mice ($p = 0.6$). Thus, the observed improvement in neurological recovery correlates with the reduction seen in infarct volume and implicates complement in the development of cerebral injury postischemia.

**Cerebral blood flow and physiological measurements**

Measurements were taken to confirm that cerebral blood flow was interrupted by the MCAO procedure and that the observed effects were not due to physiological differences between each group. Cerebral blood flow was measured by laser Doppler at three specific time points: before ischemia, during ischemia, and 10 min postreperfusion. No significant difference in cerebral blood flow was noted between any of the groups preischemia and during ischemia. However, C3-deficient animals had a significantly increased blood flow postreperfusion when compared with wt controls ($p = 0.005$), perhaps suggesting that complement activation exerts an early effect postischemia (Fig. 4). As expected, there was no increase in postreperfusion blood flow in CR2-Crry-treated mice compared with control-treated mice, because the inhibitor was administered after the 10 min postischemic time point that blood flow measurements were taken. Changes in blood pressure and body temperature have been shown to significantly influence the outcome poststroke and therefore we determined whether there were any differences between each group. Blood pressure, heart rate, and body temperature were monitored preischemia, during ischemia, and postischemia. No significant difference was seen for any of the parameters at any of the time points (Table I).

**Analysis of complement deposition, CR2-Crry targeting, and P-selectin**

Complement deficiency and inhibition significantly improved neurological outcome as assessed by survival, infarct volume, and neurological score. To confirm that CR2-Crry effectively inhibits local complement activation, we examined brain sections for C3 deposition by immunofluorescence microscopy. It has been reported previously that maximal deposition of complement occurs at 24 h postischemia (34, 35), and we assayed for C3 deposition at this time point. C3 deposition was seen in ipsilateral sections from control mice with staining concentrated to infarcted areas (Fig. 5A). No C3 was detected in contralateral brain sections (data not shown). As expected, complement deposition could not be demonstrated in C3-deficient mice (data not shown). There was also no appreciable staining for C3 in the brains of CR2-Crry-treated mice (Fig. 5D), indicating that targeted complement inhibition effectively inhibits complement deposition in the cerebral vasculature. To confirm our assumption that C3 staining was localized to endothelial surfaces, we performed double immunofluorescent staining for C3 and CD31, a broad-spectrum mouse endothelial marker (Fig. 5). Colocalization of both Abs was seen within ipsilateral sections of brains from control mice (Fig. 5C). C3 deposition was localized predominantly to endothelial cells lining the brain vasculature, but with some deposition on neuronal cells. There was also some C3 staining on what appeared to be cellular debris in the infarct zone of control and CR2-Crry-treated animals.

We investigated CR2-Crry targeting specificity by anti-CR2 immunofluorescence microscopy, and colocalized CR2 staining with either CD31 or C3 (the CR2 targeting ligand). Endogenous CR2 is expressed predominantly on B cells and dendritic cells, and immunofluorescence detection within the brain should be relatively specific for CR2-Crry. As confirmation, CR2-positive staining was seen only within the ipsilateral hemisphere of CR2-Crry-treated mice. The distribution of CR2 within ipsilateral brain sections mirrored the vascular expression of C3 seen in control mice, supporting its targeting to deposited C3, but we were unable to demonstrate colocalization of C3 and CR2 (Fig. 6, A–C). This is not a particularly surprising result because we have shown that CR2-Crry effectively inhibits complement activation and C3 deposition, and the C3 present before administration of CR2-Crry that serves as targeting ligand may be masked from the anti-C3 Ab by CR2-Crry.

P-selectin deficiency and P-selectin antagonism confer protection from cerebral injury in models of MCAO (13, 16, 36, 37), and

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Neurological deficit at 24 h postischemia and reperfusion. Neurological score was assessed on a scale of 0–4 as described previously (30). There was a significant improvement in neurological outcome postischemia in C3-deficient ($n = 14$) and CR2-Crry-treated ($n = 16$) animals compared with wt controls ($n = 16$) ($*, p < 0.01$). No significant difference was observed between C3-deficient and complement-inhibited animals.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Cerebral blood flow preischemia, during ischemia, and 10 min postreperfusion. There were no significant differences between the groups at the preischemic and ischemic time points. C3-deficient animals had a significantly improved postischemia perfusion rate compared with wt and CR2-Crry-treated animals ($*, p = 0.005$).

|                  | Before Ischemia | Ischemia | After Reperfusion |
|------------------|----------------|----------|-------------------|
|                  | MAP  | HR  | Temp | MAP  | HR  | Temp | MAP  | HR  | Temp |
| Control          | 98.1 ± 22.1 | 397.1 ± 49.7 | 35.4 ± 0.5 | 91.1 ± 17.3 | 399.3 ± 46.3 | 35.1 ± 0.4 | 95.6 ± 16.4 | 403.8 ± 41.0 | 35.4 ± 0.53 |
| C3⁻/⁻            | 101.6 ± 14.4 | 406.9 ± 64.74 | 35.5 ± 0.47 | 100.2 ± 17.3 | 390.6 ± 41.0 | 34.8 ± 0.6 | 100.9 ± 21.4 | 431.4 ± 47.2 | 34.7 ± 0.99 |
| CR2-Crry         | 93 ± 26.5 | 366.6 ± 42.0 | 36.0 ± 0.28 | 99.1 ± 11.1 | 399.2 ± 46.3 | 35.8 ± 0.2 | 97.5 ± 14.1 | 401.6 ± 47.4 | 35.7 ± 0.2 |

*MAP, Mean arterial (blood) pressure; HR, heart rate; Temp, body temperature.*
in vitro studies have implicated complement activation products C3a, C5a, and MAC in the expression of P-selectin (25, 26). To determine whether complement activation products are involved in P-selectin expression in the cerebral vasculature, we investigated expression of P-selectin by immunofluorescence in C3-deficient mice (24, 26) and control mice. In CR2-Crry-treated mice, C3 appears to present only on necrotic debris (F). Scale bars: 28.8 μm (A–C), 27.4 μm (D–F). Representative images (n = 5).

Analysis of apoptosis
Ischemic damage to the brain has been shown to induce two distinct forms of neuronal cell loss: immediate necrotic cell death and delayed programmed cell death or apoptosis. Ischemic damage mediated by complement is likely to play a role in both types of cell death, and we quantified apoptotic cell death by performing TUNEL staining in coronal sections from animals in the different groups. TUNEL-positive cells were present within peri-infarct areas in the penumbra in all groups. TUNEL-positive cells in this area had distinct apoptotic nuclear morphology as revealed by propidium iodide staining and as shown previously in infarct penumbra (38, 39). Cells within the main body of the infarct were, not surprisingly, mostly necrotic, and for this reason we only quantified the number of apoptotic cells in the penumbra area. At 24 h postischemic stroke, C3-deficient mice had significantly fewer apoptotic cells than either control mice or CR2-Crry-treated mice (p = 0.002). The average number of apoptotic cells per high-powered field was 7 ± 3 (C3-deficient), 11 ± 3 (CR2-Crry treated), and 14 ± 4 (control) (n = 5 mice). The difference between CR2-Crry-treated mice and control mice was not significant.

Fibrinogen deposition and microthrombi formation
Fibrinogen deposition and microthrombi were observed in the cerebral vasculature of control mice after 24 h of reperfusion. Immunofluorescence assessment of fibrinogen deposition together with anti-CD31 endothelial localization revealed marked differences in presence and distribution of fibrinogen between control and CR2-Crry-treated groups. In control mice, significant fibrinogen staining was seen in small arterioles, venules, and capillaries, with deposits present on the endothelial surface and within the
perivascular space (Fig. 9, A–C). Fibrinogen deposits in CR2-Crry-treated mice were noted but at significantly reduced intensity, frequency, and distribution (Fig. 9, D–F). Quantification of fibrinogen deposits using previously described scoring methods highlighted a significant reduction in fibrinogen deposits associated with complement deficiency and inhibition (Fig. 9G) \((p < 0.02)\). Secondary thrombus formation in the penumbra area is a significant complication postischemic stroke, which perpetuates the development of further ischemic damage and neurological dysfunction. P-selectin and fibrinogen deposition are thought to, at least in part, mediate the development of platelet aggregation and thrombus formation. Given the effect of complement on P-selectin expression and fibrinogen deposition following ischemic stroke, we directly quantified thrombus formation in C3-deficient and CR2-Crry-treated mice by assessing microthrombi in the penumbra area of brain. Brain sections were analyzed for the presence of RBC accumulation, fibrin deposition, and thrombus formation following ischemia and reperfusion as previously described (36).

Morphologically, vessels in wt controls frequently showed thrombus, with thrombus formation associated with neutrophils in intravascular spaces (Fig. 10A). Vessels in C3-deficient and CR2-Crry-treated animals were largely free of organized thrombus with only a limited number of RBC attached to the vessel walls (Fig. 10, B and C).

There was a significantly higher number of microthrombi and partially occluded vessels in wt control mice compared with C3-deficient and CR2-Crry-treated animals \((p = 0.01\) and \(p = 0.02\), respectively) (Fig. 10D). Furthermore, there was no significant difference between C3-deficient and CR2-Crry-treated animals.

**Effect of C3 deficiency and complement inhibition on neutrophil infiltration**

The role of neutrophils in the induction of postischemic injury is well-documented, with neutrophil depletion studies demonstrating a mechanistic role (40–43). Because complement activation products (C3a and C5a) and P-selectin are known to play important

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**FIGURE 7.** P-selectin expression and colocalization with endothelium marker in ipsilateral hemisphere 24 h postischemia and reperfusion. Images show immunofluorescence staining for P-selectin (green), endothelial cells (red), and nuclei (blue), with P-selectin and endothelial cell colocalization indicated by yellow (composite). P-selectin is present in control animals following ischemic stroke, with expression restricted to endothelial cells lining vessels in the penumbra area \((A–C)\). P-selectin was not detected in C3-deficient mice \((D)\). The presence of an intact endothelium is confirmed by endothelial staining \((E)\). P-selectin was detected in CR2-Crry-treated animals \((G)\), but the intensity and frequency was significantly reduced. Representative images \((n = 5)\). Scale bars: 32 μm \((A–C)\), 34.4 μm \((D–F)\), 36.8 μm \((G–I)\).

**FIGURE 8.** mRNA expression of P-selectin in the brain. mRNA was assessed by qRT-PCR at 24 h postischemia and reperfusion in wt, C3-deficient, and CR2-Crry-treated mice. Results are expressed as fold change compared with sham-operated animals \((n = 4\) each group). There are significantly lower levels of P-selectin mRNA in C3-deficient mice and CR2-Crry-treated mice compared with wt control mice \((*, p = 0.001; ***, p = 0.005)\). Compared with sham controls, there was no significant increase in P-selectin mRNA in C3-deficient mice \((p = 0.6)\).

**FIGURE 9.** Fibrinogen deposition and endothelial localization in ipsilateral hemisphere 24 h postischemia and reperfusion. Images show immunofluorescence staining for fibrinogen (green), endothelial cells (red), and nuclei (blue), with fibrinogen and endothelial cell colocalization indicated by yellow (composite). Fibrinogen deposition was evident in both control and CR2-Crry-treated mice, but whereas deposits were present in the intra- and extravascular compartments in control mice, fibrinogen was restricted to intravascular endothelial surfaces in CR2-Crry-treated animals. \(G\). Quantification of total fibrinogen deposition \((32)\). There was a significant decrease in fibrinogen deposition in C3-deficient mice and CR2-Crry-treated mice compared with controls \((*, p < 0.02)\). Representative images \((n = 5\) brains). Scale bars: 37.2 μm \((A–C)\), 27.8 μm \((D–F)\).
roles in leukocyte chemotaxis and vascular adherence, we anticipated complement deficiency or inhibition would result in a reduction of neutrophil migration into the ischemic brain. Neutrophils were present in all three experimental groups (C3-deficient, CR2-Crry treated, and wt control), and with similar tissue distribution. However, the number of neutrophils present in wt controls was significantly higher than in C3-deficient and CR2-Crry-treated animals (p < 0.002) (Fig. 11).

### Discussion

Complement activation can occur via one of three pathways: the classical, alternative, or lectin pathway (44). Previous studies on the role of complement in cerebral injury following ischemia have focused on the role of the classical pathway (5, 35, 45). Studies in a mouse model have shown that C1q, the initiating molecule of the classical pathway, begins to accumulate on neurons between 3 and 6 h after the onset of cerebral ischemia (35), and the administration of C1 inhibitor has been shown to reduce cerebral infarct volume and improve neurological outcome (albeit not always significantly) in mouse and rat models (6, 8, 46). However, although C1 inhibitor therapy showed marked improvement, it did not preclude the deposition of complement activation products. Furthermore, deficiency of C1q was shown to be neuroprotective only in neonatal mice, and not mature mice (5). C3 was detected within the brains of C1q-deficient mice, inferring activation of nonclassical pathways of complement. Thus, blockade of C3 activation, an event common to all pathways, may provide better protection.

The current data show that C3 deficiency or treatment of wt mice with a single i.v. dose of the targeted complement inhibitor, CR2-Crry, significantly improves neurological outcome as measured by several parameters poststroke. Significant potential advantages of targeted complement inhibition are increased bioavailability and efficacy, and decreased immunosuppression. We have shown previously that at the dose used in the current study, CR2-Crry does not significantly affect serum complement activity or host immunity to infection (23). This is a potentially desirable characteristic because stroke patients frequently succumb to dysphagia and pneumonia (47). The targeting of CR2-Crry to areas of complement activation in the brain following i.v. injection was demonstrated by the localization of CR2-Crry to the microvasculature of ipsilateral sections of brain following ischemia and reperfusion. There was no CR2-Crry deposition in contralateral sections and in sections from control animals, and the localization of CR2 was consistent with the pattern of C3 deposition seen in untreated wt animals. Together, the data show that a CR2-targeted approach of complement inhibition provides appropriate bioavailability in cerebral injury to enable complement inhibition at a dose that will not significantly affect systemic levels of serum complement activity. Previously published studies investigating complement inhibitory strategies used systemic inhibitors (9, 48).

The complement inhibitor sCR1sLex localizes to sites of P- and E-selectin expression and has been shown to be more effective at protecting against ischemic stroke than its unmodified form, sCR1 (9, 48). Unlike CR2-Crry, however, both inhibitors are dependent on systemic complement inhibition for efficacy (19, 20, 49). It was hypothesized that the enhanced efficacy of sCR1sLex in ischemic stroke was due to its ability to inhibit selectin-mediated adhesive events and coagulant reactions in addition to inhibiting complement. We show here that complement inhibition alone, when appropriately targeted, provides effective protection from injury and coagulopathy. It is perhaps also worth pointing out that in the current study CR2-Crry was administered i.v. at 90 min postischemia and 30 min postreperfusion, whereas in the previous study with sCR1 and sCR1sLex the inhibitors were administered at 45 min postischemia and at the start of reperfusion.

The concept of endothelial activation postischemia is well documented (50–52). P- and E-selectin up-regulation has been demonstrated in a number of rodent models, and the importance of selectin expression in the propagation of inflammation and damage in the brain is supported by rodent experiments with selectin-deficient animals and P-selectin antagonists (13, 36). A function blocking mAb recognizing both P- and E-selectin has also been shown to reduce infarct volume and improve neurological scores in nonhuman (baboon) stroke, without evidence of systemic complement activation or immune suppression (36). P-selectin expression on the endothelial surface promotes leukocyte adhesion and migration, and also promotes platelet aggregation and pathological coagulopathy, all factors that have been associated with the enhancement of ischemic cerebral injury. P-selectin expression may also increase complement activation, because a recent in vitro study showed that human P-selectin can directly activate complement and bind C3b (53). Given that the complement activation fragments C3a, C5a, and MAC can induce endothelial P-selectin expression, and that P-selectin inhibition is associated with improved neurological function, we sought to investigate the in vivo effect of complement activation on P-selectin expression in the cerebrovasculature following stroke. There was strong expression of P-selectin in wt control mice following ischemic stroke, but we were unable to demonstrate expression in C3-deficient animals. Furthermore, the expression of P-selectin in CR2-Crry-treated mice was absent or markedly reduced compared with expression in untreated mice. These data indicate that P-selectin-mediated effects of cerebral injury are dependent on and secondary to complement activation. In contrast to these findings, data from a model of renal ischemia and reperfusion injury show that P-selectin expression is independent of complement activation status, indicating independent pathways of renal injury (54). The authors concluded that the in vivo interaction between complement and P-selectin...
was limited because of time and spatial considerations. Renal isch-
ephil infiltration (CR2-Crry treatment was associated with a significant reduction in neutro-
were quantified by immunofluorescence microscopy. C3 deficiency and
a result of reduced damage to the endothelial surface. Platelet cap-
development of microvascular thrombi (55). In this study, we show
ischemia, and is associated with platelet accumulation and the de-
formed in a variety of ischemic models, including cerebral
emia (2, 24, 32). Indeed, the accumulation of fibrinogen has been
inhibition with CR2-Crry did not reduce apoptotic cell burden
whether it is associated with an overall reduction in necrotic cell
complement deficiency reduces apoptotic cell death compared with control mice. Whether this inhibition is related to an interac-
tion of the complement system with the apoptotic machinery, or
whether it is associated with an overall reduction in necrotic cell
burden, a reduction in inflammatory cell infiltration, apoptotic cell
clearance or a combination of these factors is unclear and may
warrant further investigation. However, we found that complement
inhibition with CR2-Crry did not reduce apoptotic cell burden
compared with controls, even though it was protective, and we do
not have an explanation for this observation.
Neutrophils have been identified as key effector cells for post-
ischemic damage in the brain following stroke, and strategies that
limit or inhibit neutrophil infiltration have proven effective in re-
ducing postischemic damage. In this study, we demonstrated a
significant reduction in neutrophil infiltration associated with com-
plement deficiency and CR2-Crry treatment. The mechanism(s)
associated with this decrease in neutrophils cannot be inferred
from the current data, but the inhibition of complement-dependent
P-selectin expression likely plays a significant role considering the
therapeutic effects of P-selectin blockade and deficiency previ-
ously reported.
Postischemic injury can be perpetuated by the development of
secondary ischemia caused by platelet and neutrophil accumula-
tion and aggregation within the microvasculature of the brain,
which may lead to the formation of microthrombi and further isch-
emia (2, 24, 32). Indeed, the accumulation of fibrinogen has been
demonstrated in a variety of ischemic models, including cerebral
ischemia, and is associated with platelet accumulation and the de-
velopment of microvascular thrombi (55). In this study, we show
that C3-deficient and complement-inhibited animals have reduced
cerebrovascular fibrinogen deposition with a correlated reduction in
microthrombi formation. The observed reduction in fibrinogen
could be related to a decrease in P-selectin expression or may be
a result of reduced damage to the endothelial surface. Platelet cap-
turing or intermittent platelet adhesion can be mediated via P-
selectin and is a more efficient mechanism of capture than fibrin-
gen-ogen-GPIIb/IIIa interaction at high shear rates (55). Once platelets
become captured and activated there is an enhancement of the
fibrinogen-GPIIb/IIIa capture mechanism and microthrombi can
result. Deposition of fibrinogen may also be exacerbated when
endothelial denudation occurs, although in this study there was no
evidence of denudation based on the presence of strong endothelial
P-selectin expression in control animals following stroke. Of note,
C3-deficient mice had a significant increase in postperfusion ce-
bral blood flow compared with control animals. This improved
circulatory response may be a result of reduced platelet accumu-
lation, which is an early event postperfusion and is associated
with continued ischemia due to microvascular plugging.

Disclosures
S. Tomlinson has stock in Taligen Therapeutics, which has licensed a
patent for developing CR2-targeted complement inhibitors.

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