Published in final edited form as:

Brain Res. 2009 October 28; 1298C: 171–177. doi:10.1016/j.brainres.2009.04.047.

Synergistic neuroprotective effects of C3a and C5a receptor blockade following intracerebral hemorrhage

Matthew C. Garrett, Marc L. Otten^{*}, Robert M. Starke, Ricardo J. Komotar, Paola Magotti, John D. Lambris, Michal A. Rynkowski, and E. Sander Connolly

Department of Neurological Surgery, Columbia University, New York, NY, USA

Abstract

Background—Intracerebral hemorrhage (ICH) is associated with neurological injury that may be ameliorated by a neuroprotective strategy targeting the complement cascade. We investigated the role of C5a-receptor antagonist (C5aRA) solely and in combination with C3a-receptor antagonist (C3aRA) following ICH in mice.

Methods—Adult male C57BL/6J mice were randomized to receive vehicle, C5aRA alone or C3aRA and C5aRA 6 and 12 h after ICH, and every 12 h thereafter. A double injection technique was used to infuse 30 μ L of autologous whole blood into the right striatum. A final group of mice received a sham procedure consisting only of needle insertion followed by vehicle injections. Brain water content and flow cytometry analysis for leukocyte and microglia infiltration and activation in both hemispheres were measured on day 3 post ICH. Neurological dysfunction was assessed using a Morris water-maze (MWM), a 28-point scale, and a corner test at 6, 12, 24, 48 and 72 h after ICH induction.

Results—Neurological deficits were present and comparable in all three cohorts 6 h after ICH. Animals treated with C5aRA and animals treated with combined C3aRA/C5aRA demonstrated significant improvements in neurological function assessed by both the corner turn test and a 28-point neurological scale at 24, 48 and 72 h relative to vehicle-treated animals. Similarly, C5aRA and C3aRA/C5aRA-treated mice demonstrated better spatial memory retention in the Morris water-maze test compared with vehicle-treated animals (C3aRA/C5aRA: 23.4 ± 2.0 s $p \leq 0.0001$ versus vehicle: 10.0 ± 1.7 s). Relative to vehicle-treated mice, the brain water content in C3aRA/C5aRA-treated mice was significantly decreased in the ipsilateral cortex and ipsilateral striatum (ipsilateral cortex: C3aRA/C5aRA: 0.755403 ± 0.008 versus 0.773327 ± 0.003 $p = 0.01$ striatum: 0.752273 ± 0.007 versus 0.771163 ± 0.0036 $p = 0.02$). C5aRA-treated mice and C3aRA/C5aRA-treated mice had a decreased ratio of granulocytes (CD45⁺/CD11b⁺/Ly-6G⁺) in the hemorrhagic versus non-hemorrhagic hemispheres relative to vehicle-treated animals (C5aRA: 1.78 ± 0.36 $p = 0.02$ C3aRA/C5aRA: 1.59 ± 0.22 $p = 0.005$ versus vehicle: 3.01).

Conclusions—While administration of C5aRA alone provided neuroprotection, combined C3aRA/C5aRA therapy led to synergistic improvements in neurofunctional outcome while reducing inflammatory cell infiltration and brain edema. The results of this study indicate that simultaneous blockade of the C3a and C5a receptors represents a promising neuroprotective strategy in hemorrhagic stroke.

Keywords

Complement; C3aRA; C5aRA; Murine ICH; Intracerebral hemorrhage

^{*}Corresponding author. Department of Neurosurgery, Columbia University, 710 West 168th Street, Room 431, New York, NY 10032, USA. Fax: +1 212 305 2026. E-mail address: mo2289@columbia.edu (M.L. Otten).

1. Introduction

Hemorrhagic stroke accounts for approximately 15% of all strokes but is associated with a disproportionate degree of morbidity. Studies have indicated that inflammatory processes and complement activation in particular may be involved in exacerbating brain injury after the hemorrhagic event (Hua et al., 2000). Early attempts at complement inhibition using cobra venom factor, C1-esterase inhibitor, and soluble complement receptor-1 revealed reduced edema formation in hemorrhagic models (Xi et al., 2001; Xi et al., 2002) and decreased infarct volumes in animal stroke models (Figuerola et al., 2005; De Simoni et al., 2003; Vasthare et al., 1998). However, the lack of specificity of these agents left it unclear as to which complement components are most relevant in the pathogenesis of cerebral injury.

C3a and C5a, collectively known as the anaphylotoxins, induce a wide variety of detrimental biochemical responses, including neutrophil recruitment, formation of free radicals, and increased permeability of the blood–brain barrier (Elsner et al., 1994). Recent studies have revealed that functional inhibitors, as well as genetic knockouts of C3, were protective against ICH-induced edema (Yang et al., 2006) and cerebral ischemic/reperfusion injury (Mocco et al., 2006). However, attempts to inhibit C5 have met with conflicting results. Genetic knockouts of C5 have shown increased vulnerability to ICH (Nakamura et al., 2004), ischemic stroke (Mocco et al., 2006), and excito-toxic injury (Pasinetti et al., 1996). In contrast, functional inhibition of C5 and the C5a receptor have shown neuroprotection against ischemia–reperfusion injury in MCAO models (Costa et al., 2006; Kim et al., in press) as well as visceral (Fleming et al., 2003) and renal models (de Vries et al., 2003). Recently, our group showed that blocking C3a using a receptor antagonist was protective in a murine model of ICH (Rynkowski et al., 2008). Given that C5a presented a similarly promising target with overlapping and possibly compensatory properties to C3a, we examined the effects of C5a receptor blockade alone and in combination with C3a receptor blockade to determine if dual therapy had synergistic benefits.

2. Results

2.1. Neurological function and behavioral tests

At 6 h after ICH, when the first drug injection was given, there were significant behavioral deficits in each cohort over sham ($n = 11$) as assessed using the 28-point scoring system and corner turn test (Fig. 1). All tests demonstrated a gradual recovery of function in C5aRA-treated ($n = 13$) and C3aRA/C5aRA-treated mice ($n = 16$) over 72 h after ICH. Compared with vehicle-treated animals ($n = 17$), C5aRA-treated mice, and C3aRA/C5aRA-treated mice showed significant improvement at 24, 48 h in the turn test (Fig. 1) and significant improvement at 48 and 72 h in the 28 point test (Fig. 2).

2.2. Morris water-maze (MWM) test

C5aRA-treated mice demonstrated a slightly better performance in the Morris water-maze but only the C3aRA/C5aRA-treated mice ($n = 9$) reached statistical significance over vehicle (23.4 ± 2.0 versus 10.0 ± 1.7 s respectively). Additionally, when the C3aRA/C5aRA-treated mice were compared with sham animals ($n = 11$), there were no statistical differences in the time spent in the target quadrant (sham = 0.4 ± 0.9 s) (Fig. 3).

2.3. Brain water content

C5aRA-treated ($n = 8$) mice had mildly decreased brain water content over vehicle but only C3aRA/C5aRa-treated ($n = 10$) mice had edema reduction that reached statistical significance over vehicle-treated mice ($n = 12$) (ipsilateral striatum: 0.752273 ± 0.007 versus 0.771163 ± 0.004 respectively $p = 0.007$) (Fig. 4).

Water contents in the ipsilateral cortex and in the ipsilateral basal ganglia were not different in C3aRA/C5aRA-treated and sham mice ($n = 6$) (ipsilateral striatum: 0.76717 ± 0.005). Water content in the contralateral cortex, contralateral striatum, and cerebellum was not statistically different between any of the groups (data not shown).

2.4. Granulocytes infiltration and activation

Flow cytometric analysis revealed that granulocyte infiltration, expressed as the ratio of cell infiltration in hemorrhagic/non-hemorrhagic hemispheres at 3 days after ICH, was less in C5aRA-treated ($n = 8$) and C3aRA/C5aRA-treated mice ($n = 8$) than in vehicle-treated mice ($n = 7$) (1.7793 ± 0.36 , 1.58534 ± 0.22 , and 3.010886 ± 0.36 respectively) (Fig. 5). Lymphocytes and microglia failed to reach statistical significance in any drug group. Sham operated animals ($n = 7$) showed no significant differences in CD45 positive cell populations between hemispheres (1.28462 ± 0.12).

3. Discussion

The complement cascade has a key role regulating the inflammatory process through its influence on neutrophil recruitment, disruption of the blood–brain barrier with subsequent edema formation (Murphy et al., 1992; Peerschke et al., 1993). The development of clinically useful therapies targeting this cascade has been hindered, however, by insufficient understanding of which complement subcomponents contribute to post-hemorrhagic injury. Given the wide range of detrimental cellular responses associated with C3a and C5a activation, this was considered a highly promising target of inhibition.

This investigation used complement receptor inhibitors to evaluate the relative contributions of specific components of the complement cascade to post-hemorrhagic neurological injury. We demonstrated that dual blockade of C3a receptor and C5a receptor had a significant neuroprotective effect.

Neurological deficits are an important component of ICH studies to prove clinical relevance. The corner test and 28-point neurological scoring system have been extensively used in ICH models and have proven to be useful for evaluating the extent of brain injury (Belayev et al., 2003; Clark et al., 1998). The Morris water-maze test is a frequently used test that evaluates sensorimotor functioning and cognitive–spatial learning, and has been linked to evaluating damage in the striatum and hippocampus (Dunnett and Iversen, 1982; Gibson and Murphy, 2004). Secondary brain edema is one of the key causative factors of the high morbidity and mortality associated with ICH (Xi et al., 1998). Although complement activation has been linked to edema formation, the exact mechanism has not been thoroughly elucidated. C5b and the subsequent formation of the membrane attack complex, as well as C5a-mediated permeability of the blood–brain barrier are both proposed mechanisms of edema formation (Xi et al., 1998). Yet, genetic knockouts of C5 undergoing simulated intracerebral hemorrhage paradoxically had increased edema, suggesting a build-up of upstream factors may be more important (Nakamura et al., 2004) or perhaps C5 is protective. In the present study, we demonstrated that while C5aRA-treated mice had mildly decreased brain water content compared to vehicle-treated mice, the combination of C3aRA and C5aRA led to a statistically significant reduction in brain water content compared to vehicle-treated mice. This suggests that both C3a and C5a have roles in edema formation and dual blockade may be more clinically effective than either inhibitor individually.

Recent studies have shown that the recruitment of neutrophils with the concomitant release of cytotoxic mediators is a secondary contributor to neurologic injury following stroke (Aronowski and Hall, 2005; Yang et al., 2006; Xi et al., 2006). C3a and C5a are among the most potent chemotactic molecules known to man, active at pico-molar concentrations

(D'Ambrosio et al., 2001). The C3a receptor has been shown to be expressed and upregulated all CNS cell types following neurological injury (Ames et al., 1996; Gasque et al., 1998; Nataf et al., 1999). In a rabbit model, intra-cisternal injection of C5a induced a chemical meningitis with massive recruitment of inflammatory cells and cytokines (Stahel et al., 1998). In contrast, work with complement inhibitors such as sCR1 (Huang et al., 1999) and C1-INH (De Simoni et al., 2004; De Simoni et al., 2003; Akita et al., 2003) showed decreased neutrophil accumulation and a commensurate improvement in neurological performance in models of focal ischemia.

Consistent with this work we have shown that C5aRA-treated and C3aRA/C5aRA-treated mice demonstrated significantly reduced ratios of granulocyte infiltration in hemorrhagic hemisphere versus non-hemorrhagic hemisphere compared to vehicle-treated mice. These data suggest that C5aRA has a strong effect on inflammatory cell migration following experimental ICH.

In summary, the results of this study suggest that C3a and C5a are involved in complement-mediated cerebral injury following ICH induction. In a previous study (Rynkowski et al., 2008) using this same model our group showed that delayed dosing of C3aRA alone was significantly neuroprotective and resulted in improved behavioral scores and non-significantly improved levels of edema and neutrophil infiltration. While each of these inhibitors was protective in isolation when used together the protective effects were frequently more than additive.

While selective inhibition of C3 and C3a has consistently demonstrated a beneficial neuroprotective effect, studies involving C5 inhibition have been mixed. Our results are consistent with prior studies using functional inhibition of C5 (Costa et al., 2006) and C5a (Kim et al., in press) and contrast those using genetic C5 knockouts (Nakamura et al., 2004; Mocco et al., 2006). It is possible that selective deletion of the C5 gene may have unforeseen effects on multiple cellular processes leading to a model that is artifactually more vulnerable to injury (Pasinetti et al., 1996). It is also possible that the effect of the complement system may depend on the absolute level of activity; that is, partial inhibition may have different effects from total deficiency. These results would be similar to those examining heme oxygenase I which found that the protective versus detrimental effect was dependent upon level of upregulation (Suttner and Dennery, 1999). Further work is needed to investigate these possibilities.

4. Experimental procedures

4.1. Animal preparation and intracerebral infusion

All procedures were approved by the Columbia University Institutional Animal Care and Use Committee. Adult male C57BL/6J mice weighing 23–30 g were randomized to receive intraperitoneal injection of either C5aRA (1 mg/kg) (hexapeptide-derived macrocycle AcF [OPdChaWR]), combined C3aRA (SB290157, Calbiochem, Darmstadt, Germany) and C5aRA (1 mg/kg of each drug) or an equal volume of DMSO. Each drug diluted in DMSO and PBS (1.16% v/v) was given 6 and 12 h after ICH induction, followed by twice daily doses for 72 h. The sham group of animals was given PBS and DMSO (1.16% v/v) in the same manner. All drug, vehicle and sham cohorts were tested concurrently by one operator in identical conditions. The results of the vehicle control group have been published previously to validate the efficacy of C3aRA (Rynkowski et al., 2008).

Mice were anesthetized with a single intraperitoneal dose of ketamine (90 mg/kg) and xylazine (5 mg/kg). Next, the mice were placed in a stereotactic frame (ASI Instruments, Inc., Warren, MI, USA) and subjected to ICH using autologous blood infusion as previously described

(Rynkowski et al., 2008). Briefly, a 1-mm burr hole was drilled 2.3 mm lateral to the midline and 0.2 mm anterior to the bregma. Thirty μL of autologous whole blood was drawn from the tail artery into a capillary tube. A 30-gauge needle was then advanced 3.5 mm through the burr hole into the right striatum (coordinates: 0.2 mm anterior, 2.3 mm lateral to the bregma, 3.5 mm ventral). A total of 30 μL of autologous whole blood was injected via the double injection technique using a microinfusion pump (KDS220, KD Scientific Inc., Holliston, MA, USA) (Rynkowski et al., 2008). An initial amount of 5 μL was delivered at a rate of 1.5 $\mu\text{L}/\text{min}$. Following a 7 minute period without injection, an additional 25 μL was delivered at a constant rate of 1.5 $\mu\text{L}/\text{min}$. Ten minutes after the end of the second injection, the needle was slowly removed, the burr hole was occluded with bone wax and the skin incision was closed with Nexaband® (Abbott Laboratories, North Chicago, IL). Sham animals received only needle insertion. Rectal temperature was maintained at 37.0 °C using a feedback-controlled heating lamp, and the animals recovered in a temperature-controlled incubator for 45 min post-procedure.

4.2. Neurofunction and behavioral testing

Acute neurological deficits were assessed using a previously described 28-point and corner turn test at 6, 12, 24, 48 and 72 h post-injury (vehicle $n = 17$, C5aRA $n = 13$, C3aRA/C5aRA $n = 16$, sham $n = 11$) (Clark et al., 1997; Bouet et al., 2007). The 28-point scale measures body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each point was graded from 0 to 4. The maximum deficit score was 28. For the corner turn test, the mouse was allowed to walk down a corridor into a 30° corner. To exit the corner, the animal could turn either to the right or left. The mouse's choice of turn direction was noted. The number of right and left turns out of 10 total attempts was recorded. The laterality index (LI) was calculated for each mouse, according to the formula: $\text{LI} = (\text{Number of right turns} - \text{Number of left turns}) / (\text{Total number of turns})$ (Bouet et al., 2007).

4.3. Morris water-maze test (MWM)

Navigational memory was evaluated by means of a Morris water-maze (MWM) as previously described (vehicle $n = 10$ C5aRA $n = 8$ C3aRA/C5aRA = 9 Sham $n = 11$) (Ten et al., 2003). Testing consisted of five days of pre-operative training followed by one testing period 72 h post ICH. For the first three days, mice were placed in an 80 cm-diameter pool filled with opaque water and were given three attempts to find a flag-marked, partially submerged platform within 120 s. On day 4, the flag was removed from the platform and the mouse was given three attempts to locate the platform using only peripheral navigational cues. On day 5, the landing platform was removed and each mouse was placed only one time in the pool for 60 s. On postoperative day 3, mice were placed in the same swimming pool without the landing platform for 60 s. The time spent in the quadrant where the landing had been situated before was recorded in a blinded fashion. Only one attempt was given to each animal.

4.4. Determination of brain water content

Mice were reanesthetized with ketamine (100 mg/kg i.p.), xylazine (10 mg/kg i.p.) and sacrificed 3 days post-hemorrhage to determine brain water content (vehicle $n = 12$, C5aRA $n = 8$, C3aRA/C5aRA $n = 10$, and sham $n = 6$). Brains were removed immediately en bloc and divided into two hemispheres along the midline. The cortex of each hemisphere was then carefully dissected from the striatum. The cerebellum was separated and retained as a control. Each of the components was then weighed on an electronic analytical balance (Model AG 104, Mettler-Toledo, Inc., Columbus, OH, USA) to determine the wet weight. The sections were then placed onto pre-weighed cover slips and dried overnight in a vacuum oven for 24 h to obtain the dry weight. Brain water content (%) was calculated as: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100$.

4.5. Preparation of brains and flow cytometry analysis (FACS)

Both cerebral hemispheres were analyzed for infiltrating inflammatory cells using flow cytometry. Mice were euthanized 72 h following hemorrhagic stroke onset (vehicle $n = 7$, C5aRA $n = 8$, C3aRA/C5aRA $n = 8$, and sham $n = 7$). Following transcardiac perfusion with phosphate buffered saline (PBS), brains were harvested, divided into ipsilateral and contralateral hemispheres, and minced in RPMI (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen; Carlsbad, CA). The resulting suspension was passed through a microfilter (70 μm), pelleted, resuspended in 30% Percoll (Amersham, Piscataway, NJ, USA) and centrifuged at 27,000 g for 30 min. Following centrifugation, the myelin layer was discarded and the remaining suspension was washed with Dulbecco's phosphate buffered saline containing 1% FBS.

4.6. Flow cytometric analysis

Granulocytes were isolated and identified using a previously described antibody-based system (Stevens et al., 2002). All antibodies used for flow cytometry were rat anti-mouse monoclonal antibodies (BD Pharmingen, Franklin Lakes, NJ, USA): fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Ly-6G monoclonal antibody, R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD45 monoclonal antibody, and PerCP-Cy5.5-conjugated rat anti-mouse CD11b monoclonal antibody. Antibodies were diluted in D-PBS containing 1% FBS. Cell extracts were incubated simultaneously with the three antibodies and Fc Block (Anti-CD16/CD32) for 30 min. Flow cytometric analysis was carried out using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed using FlowJo software (Tree Star, Ash-land, OR, USA). An antibody to CD45, a cell-surface marker expressed by all leukocytes as well as microglia, was used to exclude all other cell types. The CD11b marker, expressed by all non-lymphocyte leukocytes, was used to distinguish leukocytes from other cell types. Finally, the Ly-6G marker, expressed primarily by granulocytes and lymphocytes (Nagendra and Schlueter, 2004), was used to identify granulocytes. Using these three markers, we were able to separate the CD45 positive cells into three general subcategories: (1) lymphocytes (CD45 positive, CD11b negative, Ly-6G positive), (2) microglia (CD45 positive, CD11b positive, Ly-6G negative) and (3) granulocytes (CD45 positive, CD11b positive, Ly-6G positive). The number of cells that expressed each leukocyte marker was compared to the number of total CD45 positive cells to obtain a percent concentration of each cell subtype. This percentage was compared to the non-hemorrhagic hemisphere and thus yielded a hemorrhagic/non-hemorrhagic ratio.

4.7. Statistical analysis

All data are presented as mean $\pm$ standard error of the mean. Comparisons between groups were made using Kruskal–Wallis ANOVA on ranks and compared to control. A value of $p < 0.05$ was considered statistically significant.

Acknowledgments

This work was supported by NIH grants AI068730, GM069736 and NS040409.

References

- Akita N, Nakase H, Kaido T, Kanemoto Y, Sakaki T. Protective effect of c1 esterase inhibitor on reperfusion injury in the rat middle cerebral artery occlusion model. *Neurosurgery* 2003;52:395–400. [PubMed: 12535370]discussion 400–391
- Ames RS, Li Y, Sarau HM, Nuthulaganti P, Foley JJ, Ellis C, Zeng Z, Su K, Jurewicz AJ, Hertzberg RP, Bergsma DJ, Kumar C. Molecular cloning and characterization of the human anaphylatoxin c3a receptor. *J Biol Chem* 1996;271:20231–20234. [PubMed: 8702752]

- Aronowski J, Hall CE. New horizons for primary intracerebral hemorrhage treatment: experience from preclinical studies. *Neurol Res* 2005;27:268–279. [PubMed: 15845210]
- Belayev L, Saul I, Curbelo K, Busto R, Belayev A, Zhang Y, Riyamongkol P, Zhao W, Ginsberg MD. Experimental intracerebral hemorrhage in the mouse: histological, behavioral, and hemodynamic characterization of a double-injection model. *Stroke* 2003;34:2221–2227. [PubMed: 12920264]
- Bouet V, Freret T, Toutain J, Divoux D, Boulouard M, Schumann-Bard P. Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. *Exp Neurol* 2007;203:555–567. [PubMed: 17067578]
- Clark WM, Lessov NS, Dixon MP, Eckenstein F. Monofilament intraluminal middle cerebral artery occlusion in the mouse. *Neurol Res* 1997;19:641–648. [PubMed: 9427967]
- Clark W, Gunion-Rinker L, Lessov N, Hazel K. Citicoline treatment for experimental intracerebral hemorrhage in mice. *Stroke* 1998;29:2136–2140. [PubMed: 9756595]
- Costa C, Zhao L, Shen Y, Su X, Hao L, Colgan SP, Stahl GL, Zhou T, Wang Y. Role of complement component c5 in cerebral ischemia/reperfusion injury. *Brain Res* 2006;1100:142–151. [PubMed: 16780818]
- D'Ambrosio AL, Pinsky DJ, Connolly ES. The role of the complement cascade in ischemia/reperfusion injury: implications for neuroprotection. *Mol Med* 2001;7:367–382. [PubMed: 11474130]
- De Simoni MG, Storini C, Barba M, Catapano L, Arabia AM, Rossi E, Bergamaschini L. Neuroprotection by complement (c1) inhibitor in mouse transient brain ischemia. *J Cereb Blood Flow Metab* 2003;23:232–239. [PubMed: 12571454]
- De Simoni MG, Rossi E, Storini C, Pizzimenti S, Echard C, Bergamaschini L. The powerful neuroprotective action of c1-inhibitor on brain ischemia–reperfusion injury does not require c1q. *Am J Pathol* 2004;164:1857–1863. [PubMed: 15111332]
- de Vries B, Kohl J, Leclercq WK, Wolfs TG, van Bijnen AA, Heeringa P, Buurman WA. Complement factor c5a mediates renal ischemia–reperfusion injury independent from neutrophils. *J Immunol* 2003;170:3883–3889. [PubMed: 12646657]
- Dunnett SB, Iversen SD. Sensorimotor impairments following localized kainic acid and 6-hydroxydopamine lesions of the neostriatum. *Brain Res* 1982;248:121–127. [PubMed: 6812858]
- Elsner J, Oppermann M, Czech W, Kapp A. C3a activates the respiratory burst in human polymorphonuclear neutrophilic leukocytes via pertussis toxin-sensitive g-proteins. *Blood* 1994;83:3324–3331. [PubMed: 8193368]
- Figuerola E, Gordon LE, Feldhoff PW, Lassiter HA. The administration of cobra venom factor reduces post-ischemic cerebral injury in adult and neonatal rats. *Neurosci Lett* 2005;380:48–53. [PubMed: 15854749]
- Fleming SD, Mastellos D, Karpel-Massler G, Shea-Donohue T, Lambris JD, Tsokos GC. C5a causes limited, polymorphonuclear cell-independent, mesenteric ischemia/reperfusion-induced injury. *Clin Immunol* 2003;108:263–273. [PubMed: 14499250]
- Gasque P, Singhrao SK, Neal JW, Wang P, Sayah S, Fontaine M, Morgan BP. The receptor for complement anaphylatoxin c3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. *J Immunol* 1998;160:3543–3554. [PubMed: 9531317]
- Gibson CL, Murphy SP. Progesterone enhances functional recovery after middle cerebral artery occlusion in male mice. *J Cereb Blood Flow Metab* 2004;24:805–813. [PubMed: 15241189]
- Huang J, Kim LJ, Mealey R, Marsh HC Jr, Zhang Y, Tenner AJ, Connolly ES Jr, Pinsky DJ. Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. *Science* 1999;285:595–599. [PubMed: 10417391]
- Hua Y, Xi G, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg* 2000;92:1016–1022. [PubMed: 10839264]
- Kim GHM, Arias J, Sosunov E, Rynkowski S, Hahn M, Kellner D, Komotar CP, Otten RJ, Ducruet ML, Mack AF, Connolly WJ, Dummy ES. Protective effect of c5a receptor inhibition following murine reperfused stroke. *Neurosurgery*. in press
- Mocco J, Mack WJ, Ducruet AF, Sosunov SA, Sughrue ME, Hassid BG, Nair MN, Laufer I, Komotar RJ, Claire M, Holland H, Pinsky DJ, Connolly ES Jr. Complement component c3 mediates

- inflammatory injury following focal cerebral ischemia. *Circ Res* 2006;99:209–217. [PubMed: 16778128]
- Murphy HS, Shayman JA, Till GO, Mahrougui M, Owens CB, Ryan US, Ward PA. Superoxide responses of endothelial cells to c5a and TNF-alpha: divergent signal transduction pathways. *Am J Physiol* 1992;263:L51–59. [PubMed: 1322051]
- Nagendra S, Schlueter AJ. Absence of cross-reactivity between murine ly-6c and ly-6g. *Cytometry A* 2004;58:195–200. [PubMed: 15057973]
- Nakamura T, Xi G, Hua Y, Schallert T, Hoff JT, Keep RF. Intracerebral hemorrhage in mice: model characterization and application for genetically modified mice. *J Cereb Blood Flow Metab* 2004;24:487–494. [PubMed: 15129180]
- Nataf S, Stahel PF, Davoust N, Barnum SR. Complement anaphylatoxin receptors on neurons: new tricks for old receptors? *Trends Neurosci* 1999;22:397–402. [PubMed: 10441300]
- Pasinetti GM, Tocco G, Sakhi S, Musleh WD, DeSimoni MG, Mascarucci P, Schreiber S, Baudry M, Finch CE. Hereditary deficiencies in complement c5 are associated with intensified neurodegenerative responses that implicate new roles for the c-system in neuronal and astrocytic functions. *Neurobiol Dis* 1996;3:197–204. [PubMed: 8980020]
- Peerschke EI, Reid KB, Ghebrehiwet B. Platelet activation by c1q results in the induction of alpha IIb/beta 3 integrins (GPIIb-IIIa) and the expression of P-selectin and procoagulant activity. *J Exp Med* 1993;178:579–587. [PubMed: 7688027]
- Rynkowski MA, Kim G, Garrett MC, Zacharia BE, Otten ML, Sosunov SA, Komotar RJ, Hassid BG, Ducruet AF, Lambris JD, Connolly ES. C3a receptor antagonist attenuates brain injury after intracerebral hemorrhage. *J Cereb Blood Flow Metab*. 2008a
- Rynkowski MA, Kim GH, Komotar RJ, Otten ML, Ducruet AF, Zacharia BE, Kellner CP, Hahn DK, Merkow MB, Garrett MC, Starke RM, Cho BM, Sosunov SA, Connolly ES. A mouse model of intracerebral hemorrhage using autologous blood infusion. *Nat Protoc* 2008b;3:122–128. [PubMed: 18193028]
- Stahel PF, Morganti-Kossmann MC, Kossmann T. The role of the complement system in traumatic brain injury. *Brain Res Brain Res Rev* 1998;27:243–256. [PubMed: 9729408]
- Stevens SL, Bao J, Hollis J, Lessov NS, Clark WM, Stenzel-Poore MP. The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. *Brain Res* 2002;932:110–119. [PubMed: 11911867]
- Suttner DM, Dennery PA. Reversal of ho-1 related cytoprotection with increased expression is due to reactive iron. *Faseb J* 1999;13:1800–1809. [PubMed: 10506583]
- Ten VS, Bradley-Moore M, Gingrich JA, Stark RI, Pinsky DJ. Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. *Behav Brain Res* 2003;145:209–219. [PubMed: 14529818]
- Vasthare US, Barone FC, Sarau HM, Rosenwasser RH, DiMartino M, Young WF, Tuma RF. Complement depletion improves neurological function in cerebral ischemia. *Brain Res Bull* 1998;45:413–419. [PubMed: 9527016]
- Xi G, Keep RF, Hoff JT. Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J Neurosurg* 1998;89:991–996. [PubMed: 9833826]
- Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematoma brain edema in rats. *Stroke* 2001;32:162–167. [PubMed: 11136932]
- Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Brain edema after intracerebral hemorrhage: the effects of systemic complement depletion. *Acta Neurochir* 2002;(Suppl 81):253–256.
- Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol* 2006;5:53–63. [PubMed: 16361023]
- Yang S, Nakamura T, Hua Y, Keep RF, Younger JG, He Y, Hoff JT, Xi G. The role of complement c3 in intracerebral hemorrhage-induced brain injury. *J Cereb Blood Flow Metab* 2006;26:1490–1495. [PubMed: 16552422]

Fig. 1.

Corner test performance expressed by the laterality index in vehicle-treated ($n = 17$), C5aRA-treated ($n = 13$), C3aRA/C5aRA-treated ($n = 16$), and sham ($n = 11$) mice at 6, 12, 24, 48 and 72 h after intraatrial infusion of 30 μ L autologous blood. First injection was given at 6 h after hemorrhage. Values are expressed as mean $\pm$ SEM. *Significantly different compared to vehicle by Kruskal-Wallis ANOVA on ranks ($p < 0.05$).

Fig. 2. 28-point neurological score in vehicle-treated ($n = 17$), C5aRA-treated ($n = 13$), C3aRA/C5aRA-treated ($n = 16$) and sham mice ($n = 11$) at 6, 12, 24, 48 and 72 h after intrastriatal infusion of 30 μ L autologous blood. First injection was given at 6 h after hemorrhage. Values are expressed as mean $\pm$ SEM. *Significantly different compared to vehicle by Kruskal–Wallis ANOVA on ranks ($p < 0.05$).

Fig. 3.

Morris water-maze performance in vehicle-treated ($n = 10$), C5aRA-treated ($n = 8$), C3aRA/C5aRA-treated ($n = 9$), and sham mice ($n = 11$) expressed as the time spent in the target quadrant 72 h after ICH. Values are expressed as mean $\pm$ SEM. *Significantly different from vehicle by Kruskal–Wallis ANOVA on ranks ($p < 0.05$).

Fig. 4.

Brain water content at 72 h after ICH induction in vehicle-treated ($n = 12$), C5aRA-treated ($n = 8$), C3aRA/C5aRA-treated mice ($n = 10$) and sham ($n = 6$) mice. *Significantly different from vehicle by Kruskal–Wallis ANOVA on ranks ($p < 0.05$).

Fig. 5. Hemorrhagic versus non-hemorrhagic hemisphere ratio of activated granulocytes in vehicle-treated ($n = 7$), C5aRA-treated ($n = 8$), C3aRA/C5aRA-treated ($n = 8$) and sham ($n = 7$) mice at 72 h after ICH. Values are expressed as mean $\pm$ SEM. *Significantly different from vehicle by Kruskal-Wallis ANOVA on ranks ($p < 0.05$).