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Neurobiology

Complement Plays an Important Role in Spinal Cord Injury and Represents a Therapeutic Target for Improving Recovery following Trauma

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Initiation of an inflammatory cascade following traumatic spinal cord injury (SCI) is thought to cause secondary injury and to adversely impact functional recovery, although the mechanisms involved are not well defined. We report on the dynamics of complement activation and deposition in the mouse spinal cord following traumatic injury, the role of complement in the development of SCI, and the characterization of a novel targeted complement inhibitor. Following traumatic injury, mice deficient in C3 had a significantly improved locomotor score when compared with wild-type controls, and analysis of their spinal cords revealed significantly more tissue sparing, with significantly less necrosis, demyelination, and neutrophil infiltration. Wild-type mice were also treated with CR2-Crry, a novel inhibitor of complement activation that targets to sites of C3 deposition. A single intravenous injection of CR2-Crry 1 hour after traumatic injury improved functional outcome and pathology to an extent similar to that seen in C3-deficient animals. CR2-Crry specifically targeted to the injured spinal cord in a distribution pattern corresponding to that seen for deposited C3. As immunosuppression is undesirable in patients following SCI, targeted CR2-Crry may provide appropriate bioavailability to treat SCI at a dose that does not significantly affect systemic levels of serum complement activity. ***(Am J Pathol 2006, 169:1039–1047; DOI: 10.2353/ajpath.2006.060248)***

Spinal cord injury (SCI) is characterized by an initial traumatic injury phase, followed closely by secondary events that result in edema, ischemia, excitotoxicity, and inflammation.[1] The mechanisms of secondary injury are not well defined, but it is clear that inflammatory processes play a significant role in functional recovery.[1,2] While the initial traumatic injury is difficult to guard against, the subsequent inflammatory cascade represents a therapeutic target for SCI. The only clinical therapy accepted currently for acute SCI is methylprednisolone, a therapy that has yielded disappointing results, with the data from clinical trails being contradictory and inconclusive.[3–5]

Complement activation, by any one of three pathways (classical, alternative, or lectin), converges at the cleavage of C3 by C3 convertases and then proceeds in a common pathway to form the terminal proinflammatory and cytolytic membrane attack complex (MAC). Other biologically active by-products of this process are C3 opsonins that bind receptors on immune effector cells and the soluble chemotactic and proinflammatory mediators C3a and C5a. Little is known about the role of complement in inflammation and secondary injury following traumatic SCI. Recent studies by Anderson et al[6] demonstrated that complement proteins are deposited at sites of SCI on neurons and oligodendrocytes for a sustained period following injury in rats. In a subsequent study, it was shown that the complement inhibitory proteins factor H and clusterin were present at increased levels among neurons and oligodendrocytes after SCI in rats, and it was suggested that these complement inhibitors function to limit the inflammatory reaction in the injured spinal cord.[7] Other data implicating complement in the pathogenesis of SCI come from therapeutic studies, also performed in rats, showing that intraparenchymal infusion of vaccinia virus complement control protein (VCP)[8,9] improves cord integrity and motor function.

The objectives of the current study were twofold. First, to investigate the dynamics of complement activation and its

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role in the development of SCI in mice, a more versatile model than the rat for studying disease processes. To address this we used a contusion-induced model of SCI in normal mice and in mice deficient in the third component of complement (C3). The second objective was to investigate the neuroprotective effect of a novel targeted complement inhibitor that is administered intravenously. For the therapeutic studies we used mouse CR2-Crry, a complement inhibitory fusion protein that functions at the C3 level. The complement inhibitor, Crry, is targeted specifically to sites of complement activation by means of the complement receptor 2 (CR2)-targeting moiety.[10] CR2 is a member of the C3-binding protein family, and its natural ligands are cleavage fragments of C3 that become deposited at sites of complement activation. Targeted complement inhibition has been shown to provide significant benefits over systemic (untargeted) inhibition in terms of efficacy and host susceptibility to infection.[11] The use of mouse Crry is appropriate when studying the effects of complement inhibition in mice, because complement inhibitors display different degrees of species selectivity. Crry is a structural and direct functional analog of human complement receptor 1 (CR1), and data obtained with Crry in rodents will likely translate in functional terms to the use of CR1 in humans.

Materials and Methods

CR2-Crry Complement Inhibitor

The fusion protein CR2-Crry was produced and purified as described previously.[10] In brief, a cDNA construct of the recombinant fusion protein was prepared by joining the mouse CR2 sequence encoding the four N-terminal short consensus repeat (SCR) units (residues 1–257 of mature protein, National Center for Biotechnology Information GenBank, accession number M35684) to sequences encoding extracellular regions of mouse Crry. The Crry sequence used encoded residues 1–319 of the mature protein (National Center for Biotechnology Information GenBank, accession number NM013499). To join CR2 to Crry, linking sequences encoding $(GGGGS)_2$ were used. The recombinant protein was expressed in NSO cells and purified by anti-Crry affinity chromatography as described.[10] CR2-Crry has a circulatory half-life in C57BL/6 mice of ~8 hours.[10]

Animals and SCI Model

Female wild-type C57Bl/6 and C57Bl/6 C3-deficient mice (Jackson Laboratories, Bar Harbor, ME), weighing 16 to 20 g and between 6 and 8 weeks old were used in this study. C57Bl/6 wild type were randomized into sham (laminectomy, no SCI damage), vehicle control (phosphate-buffered saline [PBS]), and CR2-Crry treatment groups. Another group using C3-deficient animals was also included. Each animal was anesthetized with 10 mg/kg ketamine and 6 mg/kg xylazine by intraperitoneally injection. Animals were breathing spontaneously, and body temperature was maintained using a heat mat for the duration of the experiment. A laminectomy at T12 was performed, and the dura was exposed using aseptic technique. A weight-drop contusion injury was performed (5 g, 3 cm) using the procedure previously described.[11] An initial study assessed the presence, time course, and activation of complement after SCI in C57Bl/6 mice. Animals ($n = 3$) underwent SCI and were sacrificed at 1-, 2-, 4-, 24-, and 48-hours after injury, and complement activation was assessed by the presence of deposited C3. On completion of these initial experiments four groups of animals (sham, vehicle control, CR2-Crry-treated, and C3-deficient mice) were subjected to SCI. Following injury, animals were allowed to recovery for 1 hour at which point animals randomized to CR2-Crry treatment were administered a single dose of 0.25 mg of CR2-Crry by tail vein injection. All other animals received intravenous injections of phosphate buffered saline. Following injury animals were monitored for locomotor recovery and animals in different groups sacrificed at 24 hours, 72 hours, 7 days, and 21 days. All procedures were approved by the Medical University of South Carolina's committee for animal research, in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Locomotor Recovery

Locomotor recovery was assessed using the Basso, Beattie, and Bresnahan (BBB) rating scale developed for rats,[12] but later adapted by others for mice.[13–16] The BBB locomotor rating scale is an open-field 21 point evaluation and is rated according to categories describing the quality of joint movements, the trunk, abdomen, and paw placement, stepping, trunk stability, and tail position. Animals were assessed preoperatively, on the day of surgery, and then daily postoperatively by an observer blinded to animal treatment. Changes in BBB score during spinal cord injury between vehicle control, C3-deficient, CR2-Crry-treated, and control animals were determined by analysis of variance with repeated measures using Scheff's test for posthoc comparisons. A P value of less than 0.05 was considered statistically significant. All data were subjected to statistical analysis using Statview Analysis Software (version 5; SAS Institute Inc., Cary, NC).

Histology

Animals were transcardially perfused with 4% paraformaldehyde at 24 hours, 72 hours, 7 days, and 21 days after injury, and spinal cords removed. Spinal cords were either frozen for cryosectioning or processed in paraffin using standard techniques.[17] Morphometric analyses to determine the degree of tissue damage following injury were conducted using transverse sections of spinal cord stained with a standard hematoxylin and eosin (H&E) stain. The cross-sectioned area of spinal cord was measured at 100-μm increments extending 2 mm either side of the injury epicenter, and averaged for animals in each group and time point as previously described.[18] The presence of myelin damage was assessed by staining transverse sections of the spinal cord at the epicenter of injury with the Luxol fast blue stain.[17] All assessments were performed by a pathologist in a blinded fashion.

Immunofluorescence Staining for Complement Component C3, CR2-Crry, and Neutrophils

Cryosections were fixed in cold acetone for 5 minutes and then washed in running water followed by PBS. Sections were then incubated for 1 hour at room temperature with anti-mouse C3 fluorescein isothiocyanate (FITC), anti-mouse neutrophil antibody clone number (GR) 1 FITC (BD Pharmingen, San Diego, CA), or anti-mouse CR2 (Santa Cruz Biotechnology, Santa Cruz, CA) and then washed in three changes of PBS. C3- and GR1-stained sections were coverslipped with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and analyzed using a Zeiss LSM5 Confocal microscope (Carl Zeiss, Oberkochen, Germany). CR2-stained sections were then incubated with rabbit anti-goat biotinylated antibody (Vecta Laboratories, Burlingame, CA) and then washed before being incubated with a streptavidin FITC (Vecta Laboratories). Sections were coverslipped and analyzed as described above. The presence of complement component C3 was assessed at the injury site and caudal and rostral to injury, in transverse sections of spinal cord harvested at all time points. Neutrophils were quantified at the epicenter, defined as the section exhibiting maximal tissue damage. All counts were performed with the observer blinded to the experimental group. The total number of neutrophils/mm^2 was determined at the epicenter. No attempt was made to differentiate between neutrophil infiltration into white or gray matter because of the early dissolution of tissue at the injury site at early time points. Neutrophils were assessed in spinal cords from all treatment groups at 24 hours, 72 hours, and 7 days after injury. CR2 staining was used to identify the presence of CR2-Crry protein at the site of injury in the treatment group and control animals 12 hours after injury.

^{125}I Radiolabeling and Biodistribution

Radiolabeling was conducted using ^{125}I (Amersham Biosciences, Pittsburgh, PA) by the Iodo-Gen method (Pierce Chemical, Rockford, IL), with 5 mCi used to label 100 μg of CR2-Crry protein. Free iodine was removed from the mixture after labeling by anion-exchange resin. Iodine incorporation was in the 50 to 80% range. Radiolabeled protein was injected intravenously for 1 hour after SCI to C57Bl/6 mice as outlined above. For control purposes, age-matched C57Bl/6 mice that did not undergo any surgical procedure were injected intravenously with ^{125}I-labeled CR2-Crry to confirm the specificity of CR2 targeting. After 12 hours of recovery, mice were sacrificed ($n = 3$ per group) and blood removed by cardiac puncture. Following blood removal, animals were perfused with PBS before removal of the heart, lung, liver, kidney, brain, and spinal cord. Tissues were rinsed in RPMI (Gibco, Carlsbad, CA) and counted with a Packard 5780 gamma counter at the ^{125}I window with appropriate corrections for count decay.

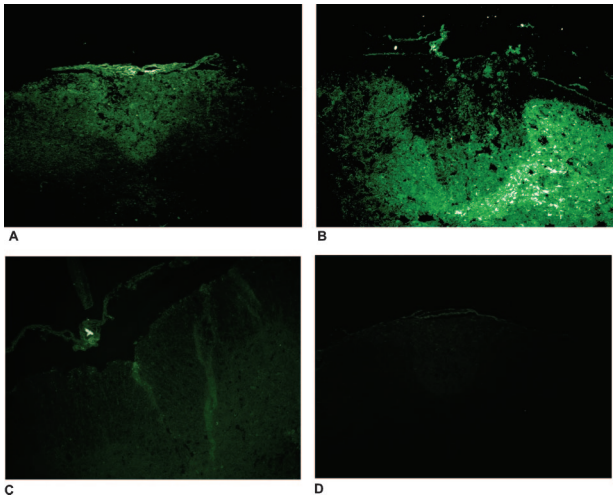

Figure 1. Complement activation as shown by C3 deposition at the site of spinal cord injury. 1 hour (**A**), 24 hours (**B**), and 72 hours (**C**). C3 deposition is marked by green fluorescent staining of the polyclonal anti-mouse C3 antibody in spinal cords harvested at different time points after injury ($n = 3$). In contrast laminectomy sham controls lack immunoreactivity for C3 (**D**).

Results

Time Course of Complement Activation after SCI

The presence of C3, deposition of which marks a site of complement activation by any pathway, was assessed in mice that had undergone SCI and in sham laminectomy controls. No staining for C3 was observed in sham operated-animals in any compartments of the spinal cord. In contrast, C3 deposition was evident following SCI in spinal cords harvested at 1 hour, 2 hours, 4 hours, 12 hours, and 24 hours after injury. At 1 hour, 2 hours, and 4 hours post-SCI, C3 deposition was centered to the white matter of the injury site and within the ventral horns of the gray matter (Figure 1A). At later time points of 12 hours and 24 hours, C3 staining was evident in surviving white matter, with staining also present throughout the gray matter and extending into the ventral and dorsal horns (Figure 1B). By day 3 after injury, complement deposition was almost undetectable, with no C3 staining evident at the injury site (Figure 1, C and D). This result is different from that reported for complement deposition in the rat spinal cord following injury, in which complement deposition was evident for up to 42 days after injury.[6] An additional apparent difference in the mouse model was that, at all time points, spinal cord sections 10 mm rostral and caudal to the injury site showed a much reduced C3 staining pattern compared to sections taken from the injury site. Complement deposition was seen up to 20 mm from the injury site in rats, with no apparent decrease in immunoreactivity and with increasing distance from the site of injury.[7] However, this apparent difference is likely a consequence of animal size and differences in size of impact injury required to produce an equivalent condition.

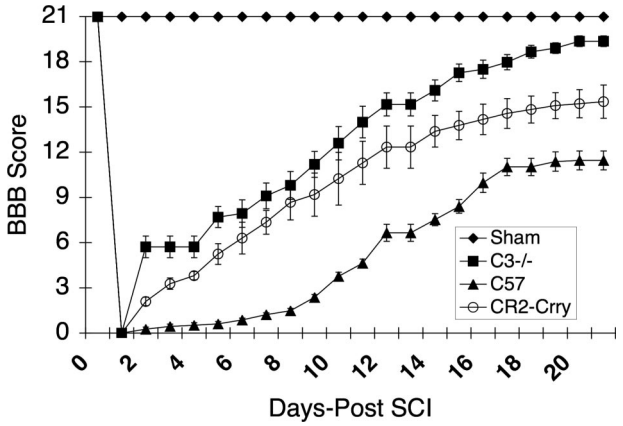

Figure 2. Combined BBB locomotor scores post-SCI within sham, vehicle control, C3-deficient, and CR2-Crry groups. Note significant improvement in BBB score at day 3, 7, and 21 in both the C3-deficient and CR2-Crry groups when compared to vehicle controls ($P = 0.001$) ($n = 12$). The values are expressed as mean ± SE.

Effect of C3 Deficiency and of Complement Inhibition on Locomotor Recovery following SCI

To investigate the role of complement in SCI, we induced contusion injury to the spinal cord in wild-type mice and in mice deficient in C3, a central protein of the complement system and common for all pathways of activation. Following injury, locomotor recovery was assessed using the modification of the BBB rating scale.[13] All animals had a BBB score of 21 pre-injury and a score of 0 immediately after injury, with bilateral hindlimb paralysis (Figure 2). Two days after injury, and every day thereafter through the termination of the study at day 21, the C3-deficient mice had a significantly improved BBB score compared to the wild-type controls ($P < 0.001$) (Figure 2). By day 21 after injury, the C3-deficient mice showed a near normal BBB score of 19.6 ± 1.2 ($P < 0.001$), whereas the BBB score for wild-type mice was only 11.5 ± 2.14 which was significantly lower than that of C3-deficient mice ($P < 0.001$). These data indicate that C3 plays an important role in the posttraumatic events that affect functional recovery. Next, we determined whether C3 blockade, using an intravenously administered inhibitor previously shown to target to sites of complement activation, is a feasible posttraumatic therapeutic approach for improving functional recovery.

Using the same spinal cord paradigm, a group of mice were treated with a single intravenous injection of 0.25 mg CR2-Crry at 1 hour after SCI. As with the C3-deficient mice, the CR2-Crry-treated mice had a significantly improved BBB score compared to sham-operated controls at all time points from day 2 following traumatic injury ($P < 0.001$) (Figure 2). The C3-deficient mice appeared to have a better outcome than the CR2-Crry-treated mice, but the difference was not significant.

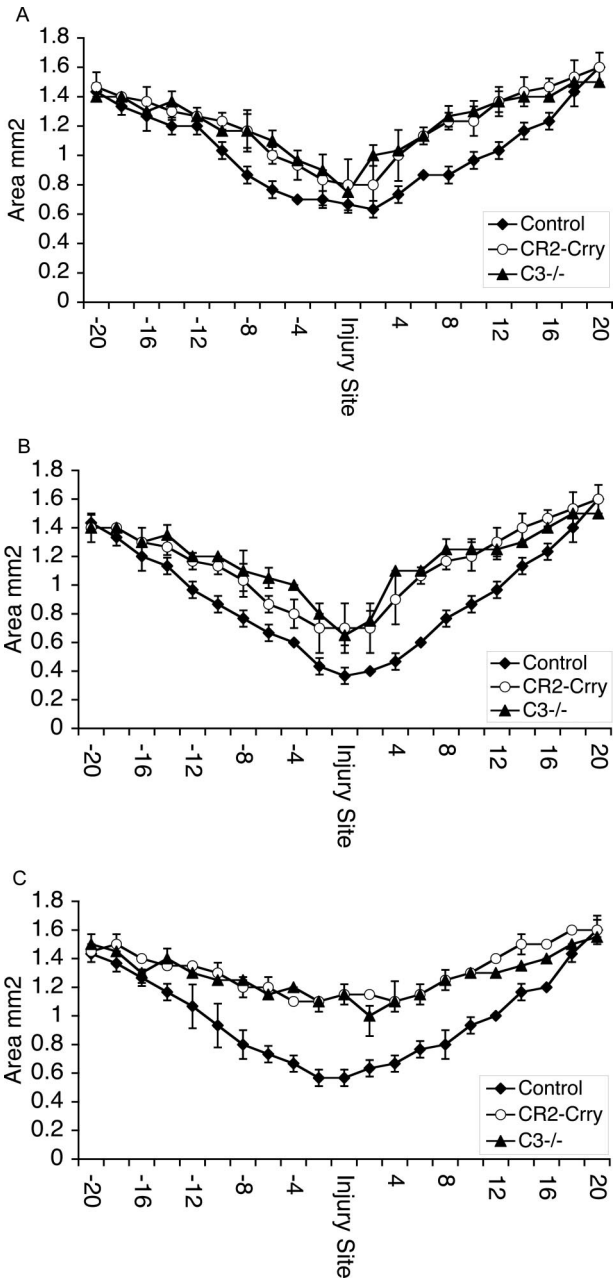

Figure 3. Tissue sparing as assessed by analyzing the cross-sectional area of spinal cords removed from vehicle controls, C3-deficient, and CR2-Crry-treated animals at 24 hours (**upper panel**), 72 hours (**middle panel**), and 7 days after injury (**lower panel**). Measurements were made from histological sections taken at 100-μm increments extending 2 mm either side of the injury site. No significant difference in tissue sparing was evident at 24 and 72 hours. Significant tissue sparing was noted in CR2-Crry and C3-deficient animals compared to vehicle controls at day 7 ($P = 0.002$). Mean ± SD, $n = 4$.

Effect of C3 Deficiency and of Complement Inhibition on the Extent of Tissue Destruction and Demyelination following SCI

To determine whether C3 deficiency or complement inhibition with CR2-Crry attenuated overall spinal cord tissue damage, we determined the cross-sectioned area of spinal cords at 100-μm increments extending 2 mm ei-

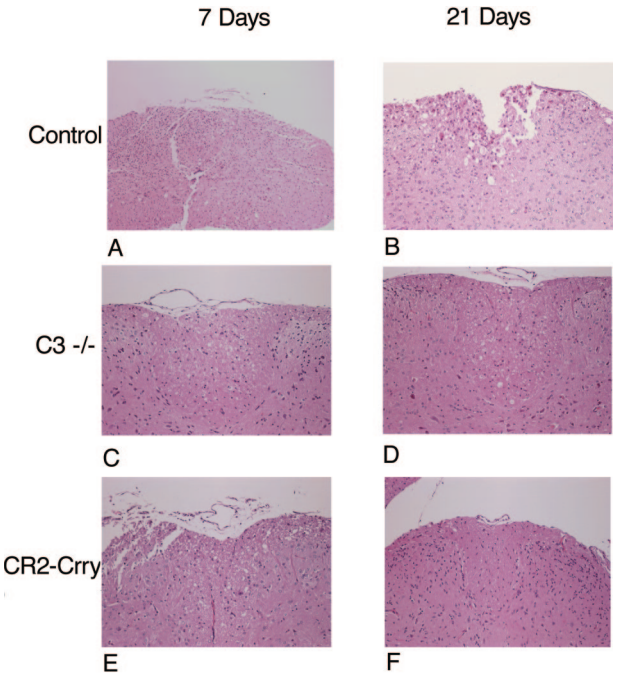

Figure 4. H&E-stained sections of spinal cord centered on the injury site at days 7 and 21 after injury. **A–B:** vehicle control. **C–D:** C3-deficient animals. **E–F:** CR2-Crry-treated animals. Original magnification, ×100.

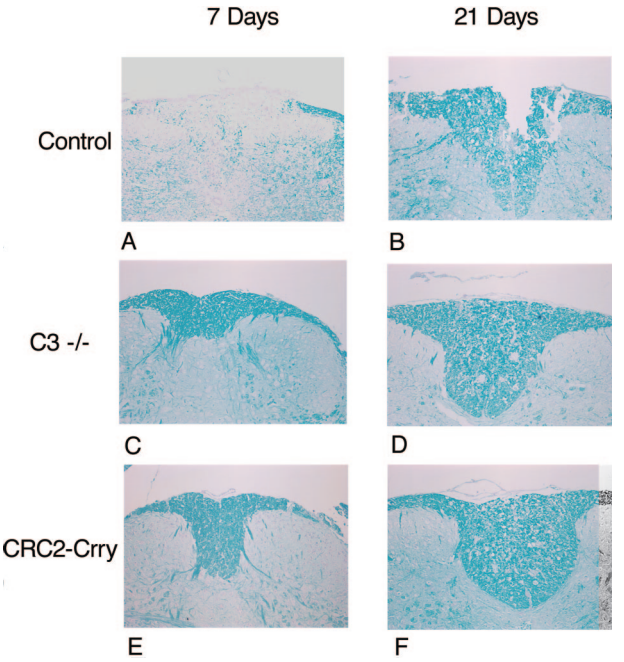

Figure 5. Luxol fast blue stained section of spinal cord centered on the injury site at days 7 and 21 after injury. **A–B:** vehicle control. **C–D:** C3-deficient animals. **E–F:** CR2-Crry-treated animals. Original magnification, ×100.

ther side of the initial injury impact site. Measurements were made using spinal cords isolated from C3-deficient mice and mice treated with CR2-Crry or vehicle control (PBS). At 24 hours after injury, the profile of tissue damage was similar in both C3-deficient and CR2-Crry-treated groups (Figure 3A). In the control group, there was a clear trend toward increased injury compared to the C3-deficient/inhibited groups, but by 24 hours after SCI the difference did not reach statistical significance at the injury site or on either side of the injury site. Comparable relative profiles were obtained for the three groups of animals at 72 hours after SCI (Figure 3B). Seven days after injury, however, there was significantly more tissue sparing at and around the injury site in C3-deficient mice and in mice treated with CR2-Crry compared to vehicle control mice (Figure 3C). There was no difference in tissue sparing between C3-deficient and CR2-Crry-treated mice at 7 days after SCI.

We also analyzed the extent of necrosis and demyelination in cords isolated from the different groups of animals 7 days and 21 days after SCI. In the vehicle control group, H&E staining of cord sections (centered around the injury site) revealed marked areas of necrosis with vacuolization of cells at day 7, with necrosis being somewhat less evident at day 21 (Figure 4, A and B). In contrast, the white matter beneath the injury site in cords isolated from C3-deficient mice appeared grossly intact at days 7 and 21 (Figure 4, C and D). Cords from CR2-Crry-treated mice also exhibited significant attenuation of injury when compared with vehicle controls, although there appeared to be more vacuolization in the cells within the white matter compared to the C3-deficient animals (Figure 4, E and F). Luxol fast blue staining of cord sections from the control group revealed obvious demylineation in the central core of the white matter beneath the impact site (Figure 5, A and B). By comparison, there was markedly less demyelination in cords from C3-deficient mice and CR2-Crry-treated mice at 7 and 21 days after SCI (Figure 5, C–F). There was no apparent difference in the extent of demyelination between the C3-deficient and complement-inhibited groups of animals.

Neutrophil Infiltration

Infiltration of neutrophils is thought to be a significant factor in the development of secondary injury in spinal cords following traumatic injury, and complement activation products can provide activating and chemotactic signals and up-regulate expression of adhesion molecules. We therefore investigated the presence of neutrophils at the site of injury in animals from all three groups at 24 hours, 72 hours, and 7 days after injury. Neutrophil infiltration was most pronounced within the first 24 hours after injury in all groups, with declining numbers present at 72 hours and 7 days (Figure 6). At all time points, however, neutrophil infiltration was significantly inhibited in both C3-deficient and CR2-Crry-treated mice ($P < 0.001$). There was no significant difference between neutrophil numbers in the C3-deficient and the complement inhibited mice at any time point. In this experiment, the total number of neutrophils present on each section was counted. No distinction was made between white and gray matter, due to the extensive damage noted at 24 and 72 hours after injury and because sections were analyzed using immunofluorescence, which did not permit morphological evaluation.

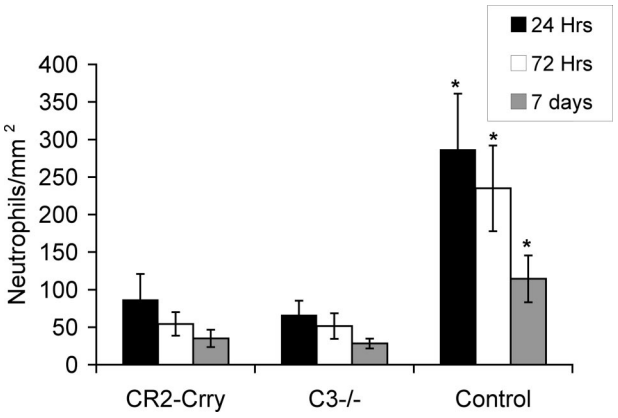

Figure 6. Neutrophil infiltration as assessed by immunofluorescent staining with anti-mouse GR1-fluorescein labeled antibody. The total number of neutrophils per section was counted. The number of neutrophils is significantly lower in C3-deficient and CR2-Crry-treated animals across all time points when compared with vehicle control (* $P = 0.001$). Mean ± SD, $n = 5$ per group.

Targeting and Biodistribution of Therapeutically Administered CR2-Crry

We recently demonstrated that intravenously administered CR2-Crry targets sites of complement activation in a mouse model of complement-dependent intestine ischemia and reperfusion injury.[11] The microenvironment of the spinal cord is different, and access of macromolecules and inflammatory cells is restricted by the blood-spinal cord barrier. On damage, however, the blood-spinal cord barrier becomes temporarily more permeable, and this may account for the access of intravenously administered CR2-Crry to its targeting ligand (C3) within the spinal cord. To support the concept that CR2-Crry functions by targeting specifically to the spinal cord following injury, we assessed the tissue distribution of ^{125}I-labeled CR2-Crry in normal control mice and in mice subjected to SCI. ^{125}I-labeled CR2-Crry was injected intravenously 1 hour after SCI, as per therapeutic protocol, and biodistribution determined 12 hours later. In control mice (no SCI), CR2-Crry was distributed primarily in the blood, with tissue localization restricted to the liver and kidney and, to a lesser extent, the heart (Figure 7A). The location of CR2-Crry within the kidney and liver is likely associated with the nonspecific clearance of the protein. Of note, no ^{125}I-labeled CR2-Crry was detected in the CNS tissues of the spinal cord or brain, indicating that under normal physiological conditions CR2-Crry cannot pass the blood-brain/spinal cord barrier. In contrast, there was significant localization of ^{125}I-labeled CR2-Crry within the spinal cord of injured mice. In the spinal cord, levels of CR2-Crry was highest at the site of injury but was also detected at sites rostral and caudal to the site of injury (Figure 7A). The absence of ^{125}I-labeled CR2-Crry in brain tissue of injured mice is also supportive of the specific targeting of CR2-Crry. To further investigate the specific targeting of CR2-Crry to the injured spinal cord, we performed immunofluorescent staining with an anti-CR2 antibody. CR2 is expressed conservatively and found primarily on B cells and dendritic cells. Therefore,

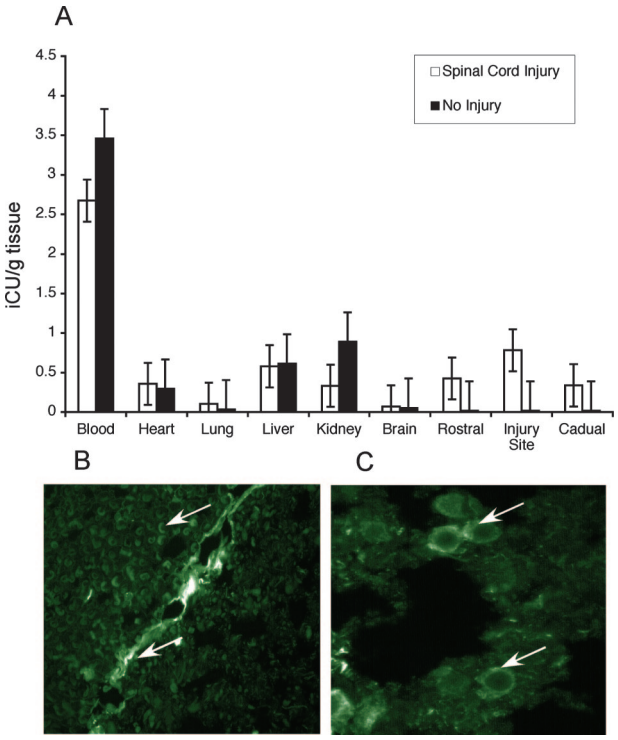

Figure 7. A: Biodistribution of CR2-Crry following spinal cord injury. ^{125}I CR2-Crry was injected into normal C57Bl/6 mice (no injury) and C57Bl/6 animals 1 hour following spinal cord injury. Tissue distribution was assessed 12 hours after initial injury in major organs and tissues of the central nervous system. **B:** Immunolocalization of CR2-Crry binding with an anti-CR2 Ab. Positive immunoflourescence is noted in the white matter beneath the injury site with a pattern similar to that observed in C3 stained sections (**arrows**). **C:** CR2 immunoreactivity seen in neuronal cells within the dorsal horn of the gray matter (**arrows**). Images are taken under oil immersion using ×63 objective and are representative of $n = 4$.

the presence of CR2 immunoreactivity within the spinal cord was deemed to be indicative of localization of recombinant CR2-Crry protein, and indeed no immunoreactivity for CR2 antibody could be detected in sham controls or SCI control (untreated) animals (not shown). CR2 immunoreactivity was, however, seen in CR2-Crry-treated animals when sections of spinal cord were assessed 12 hours after SCI. Staining was seen primarily within the white matter around the injury site and morphologically appeared to stain oligodendrocytes, fiber tracts, and vascular structures (Figure 7B). This distribution of CR2 corresponds to the distribution of deposited C3 in injured spinal cords reported above (Figure 1), and is a further indication of the C3-targeting specificity of CR2-Crry. Staining was also noted, to a lesser degree, in the dorsal horns of the gray matter with immunolocalization seen in cells with neuronal morphology (Figure 7C).

Discussion

There are approximately 10,000 new serious spinal cord injuries each year, with a disproportionate number of injuries occurring in young people. Following the initial trauma, there is a secondary injury cascade that results in increased cell death, cavitation, and demyelination that is detrimental to functional recovery. Disruption of the

blood-brain barrier and the triggering of an inflammatory response are important components of secondary SCI injury, and while the initial trauma is difficult to guard against, secondary inflammatory events represent potential therapeutic targets.

Although the damaging effects of inflammation in SCI are well documented, there is a paucity of information concerning the mechanisms that control this process. Recent studies using rat models of SCI have highlighted a potential role for the complement system in the development of spinal cord damage. Anderson et al[7] have described the deposition of complement components C1q, factor B, C4, and C5b-9, as well as complement inhibitors factor H and clusterin within neurological tissue of rats following SCI.[7] These data implicate complement in the development of secondary injury, but whether complement is an effector or a bystander in this inflammatory milieu is unclear from these studies. In the current study, the functional significance of complement deposition was addressed using C3-deficient animals and a targeted complement inhibitory protein, CR2-Crry. We first confirmed that complement activation occurred within injured spinal cords of mice, as has been shown for rats,[6] by immunostaining for C3, a protein common to all complement activation pathways. Levels of deposited C3 peaked at 24 hours after injury, which was similar to that reported in the rat, but a notable difference was that whereas complement could be detected 42 days post-SCI in rats, C3 deposition was undetectable 3 days post-SCI in mice. These differences are probably due to species differences, but we cannot exclude other factors such as antibody affinity or antibody detection methods. The cell types associated with C3 deposition within spinal cord lesions were not specifically assessed in this study, although Anderson et al[6] have shown that complement is deposited on oligodendrocytes, neurons, and axons in injured rat spinal cords.

In a previous study on the use of a complement inhibitor in a rat model of SCI, vaccinia virus complement control protein (VCP) was infused directly into the spinal cord over a 10-minute period following contusion induced SCI. Vaccinia virus complement control protein is a functional analog of CR1. It was shown that VCP reduced leukocyte infiltration, qualitatively reduced cord destruction and had some effect on locomotor recovery.[8,9] The authors observed an improvement in recovery within the first week and an overall improvement over the 6-week duration of the experiment. While these observations did not reach significance, they highlighted a potential for the use of exogenous complement regulatory proteins in spinal cord injury therapy. In another study, rats were treated with sCR1 following SCI, and treated rats had less leukocyte infiltration and improved motor function.[19] In this previous study, motor function was assessed using a nonstandard scale, rendering cross-comparison difficult, but functional improvements in inhibitor-treated rats were not great. The rats received an injection of sCR1 1 hour after injury and daily thereafter. In the current study, mice were treated with a single intravenous injection of a targeted complement inhibitor, CR2-Crry, at 1 hour after traumatic injury, and this was sufficient to protect the spinal cord to a statistically similar level to that seen in C3-deficient animals. In the CR2-Crry-treated mice, improvements in neurological recovery were observed early after injury and continued throughout the duration of the experiment, with the difference between controls and treated animals being marked and statistically significant. CR2-Crry biodistribution and C3 staining, together with CR2 immunolocalization studies confirm the presence of the CR2-binding ligand (C3 breakdown fragments) in cell types associated with neurological injury, and demonstrate that CR2-Crry traffics across the blood-spinal cord barrier to sites of complement activation. Since sCR1 and VCP have a similar activity spectrum to Crry, it is likely that untargeted Crry would have some similar therapeutic effect in this model. However, the efficacy of untargeted Crry depends on systemic complement inhibition and a significantly higher dose of inhibitor, and this would render the host susceptible to infection, a relevant and important issue for SCI.

While the data presented show that complement deficiency or inhibition provides protection from the secondary effects of initial spinal cord contusion injury, the mechanism(s) of complement activation and complement-dependent injury are incompletely characterized. The inflammatory response following SCI is marked by the infiltration of monocytes/macrophages and neutrophils, and data from studies showing that adhesion molecule blockade or neutrophil ablation is protective, indicate that these cells play a key role in secondary destructive processes.[17,18,20,21] Thus, our data suggest that the protective effect of C3 deficiency/inhibition is due, at least in part, to its effect on neutrophil trafficking. Two complement activation products downstream of C3 cleavage are C3a and C5a, both of which have leukocyte chemotactic and activating properties. C5a can also upregulate expression of the adhesion molecules P- and E-selectin, intercellular adhesion molecule-1 and vascular adhesion molecule-1 on endothelial cells.[22–25] Of note, C5a is implicated in brain cryoinjury, because injury and neutrophil extravasation is reduced in C5-deficient mice and C5a receptor antagonist-treated mice.[26]

However, we cannot exclude an important role for the terminal product of the complement cascade, the membrane attack complex (MAC). The MAC can be directly cytolytic and may contribute directly to neuronal injury and demyelination but can also induce the production of pro-inflammatory mediators, including adhesion molecules on endothelial cells, when deposited at sublytic concentrations. Indeed, studies indicate an important role for the MAC in rodent models of experimental allergic encephalomyelitis (EAE), a demyelinating disease that mimics multiple sclerosis. In rodent models of EAE, injury and myelin loss is attenuated in animals deficient in C5 and C6 and increased in animals deficient in CD59a, an inhibitor of MAC formation.[27–31] A contradictory finding was reported, however, in a rat model of immunological demyelination in which intraspinal infusions of IgG antibodies were administered in the presence of serum deficient in either C3, C4, C5, C6, or factor B.[32] Deficiency in serum C3 and C4, but not C5, C6, or factor B was

protective against demylineation, suggesting a role for the classic pathway of complement activation and no role for the MAC (C4 is a classic pathway protein, factor B is an alternative pathway protein, and C6 is a terminal pathway protein involved in MAC formation[32]).

In the context of relating our findings to human therapy, there are three human membrane inhibitors of complement activation that function at the C3 level; decay accelerating factor, membrane cofactor protein, and CR1. In rodents, Crry appears to function as the principal membrane inhibitor of C3 activation, and soluble (untargeted) Crry has been used widely for studying complement inhibition in rodent models of disease. Because complement inhibitors display different degrees of species selectivity, the use of Crry is appropriate when studying the effects of complement inhibition in rodents. Crry is a structural and direct functional analog of human CR1, and data obtained with Crry in rodents can be expected to translate to CR1 in humans. Nevertheless, soluble forms of CR1 have been investigated in the clinic, but with generally disappointing results. However, a similar targeting strategy to that described here could be applied to CR1 or other human complement inhibitors with the goal of increasing efficacy. Effective therapy with a single intravenous injection, as demonstrated in the current model, would allow for early inhibitor delivery on patient presentation. Complement provides an important host defense mechanism, and an additional advantage of CR2-mediated targeting of complement inhibitors is a reduced level of systemic immunosuppression at therapeutically effective doses. Patients with spinal cord injuries are at increased risk of infection, in particular urinary tract infection. We have previously shown that the dose of CR2-Crry that was used in the experiments reported here (0.25 mg), minimally effects systemic complement levels and does not increase host resistance to infection.[10]

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