

The role of complement in spinal cord injury

PhD dissertation

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2011, Budapest

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List of abbreviations

SCI, spinal cord injury

CP, classical pathway

AP, alternative pathway

MBL, mannose-binding lectin

MAP, membrane attack pathway

CNS, central nervous system

MAC, membrane attack complex

fB, factor B

CR1, complement receptor 1

DAF, decay-accelerating factor

MCP, membrane cofactor protein

TBI, traumatic brain injury

AD, Alzheimer's disease

Wt, Wild-type

C3^{-/-}, C3-deficient

fB^{-/-}, fB-deficient

CD59^{-/-}, CD59-deficient

PBS, phosphatebuffered saline

AfB, anti-fB antibody

SCR, short consensus repeat

BBB, Basso, Beattie, Bresnahan rating scale

BMS, Basso Mouse Scale

1. INTRODUCTION

1.1. Background and significance

1.1.1. Spinal cord injury

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(2, 3). 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(4-6).

1.1.2. Complement system and neuroinflammation

Complement is important for host defense against pathogens and is an effector mechanism for both innate and adaptive immune responses. Complement is also involved in immune homeostasis mechanisms including the catabolism of immune complexes and clearance of apoptotic cells. Under certain disease conditions in which inappropriate or excessive complement activation occurs, complement control mechanisms breakdown or are overcome and complement causes tissue injury. Complement play a key role in the pathogenesis of many inflammatory and ischemic disease conditions. Following primary injury to the spinal cord, a complex cascade of pathophysiologic processes occur that result in secondary injury and progressive degenerative effects that can determine the extent of recovery. The mechanisms involved in secondary tissue injury are not well defined, but inflammation and ischemia are considered to be key components. Evidence indicates an important, albeit not well defined, role for complement in the

pathophysiological processes that occur following both traumatic brain and spinal cord injury (2, 6-10)(2, 6-10)(2, 6-10)[2, 6-10].

1.2. Overview of the complement system and effector mechanisms

In order to appreciate the role of complement in spinal cord injury, it is essential to first understand the basics of the system. The complement system (Fig. 1), a major component of the innate immune response, is activated in many neurodegenerative diseases (11, 12). The complement system is a self amplifying cascade of proteases(13). Activation results in 1) attraction and activation of phagocytes(14, 15); 2) opsonisation; and 3) formation of the membrane attack complex (MAC or C5b-9)(16). The complement system consists of nearly 30 proteins involved in non-specific immune response and the role of various complement components in the pathophysiology of several disorders has been well studied in the past two decades. The complement system is activated in several disorders, particularly in response to foreign proteins, polysaccharides, cell surface components of microbial origin, or toxic proteins associated with the pathogenesis of several disorders. These foreign proteins activate the complement system by various pathways. The complement system is activated by the three major pathways, the classical pathway (CP), alternative pathway (AP), and lectin-mediated pathway. Activation of one of the pathways may lead to activation of the other pathway. In the central nervous system (CNS), the two main pathways found to be activated are the classical and the alternative pathway(17, 18). Some complement factors, including **C1q (initiator of the classical complement pathway)**, **C1r**, **C1s**, **C3** and **C4** are locally produced in the CNS (19-22), whereas others derive from the blood. Activated complement can trigger an adaptive immune response, involving antigen presenting cells, T-cells and B-cells. The classical pathway is normally antibody dependent whereas the alternative pathway is usually antibody independent. Activation of complement by any pathway leads to the formation of a C3 convertase that cleaves C3 into C3a and C3b, with the latter binding covalently to the activating surface and participating in the formation of additional C3 convertase complexes (amplification loop). C3 convertases also participate in the formation of C5 convertase, which cleaves C5 to yield C5a and C5b. Formation of C5b initiates the terminal complement pathway resulting in the sequential assembly of complement

proteins C6, C7, C8 and (C9)_n to form the cytolytic membrane attack complex (MAC or C5b-9). Cleavage fragments of C3 covalently bound to activating surfaces act as ligands for receptors on immune effector cells. C3a and C5a are chemotactic and can activate leukocytes. Formation of the MAC on an activating cell surface can cause direct cell lysis. Host cells are normally protected from complement by membrane-bound inhibitors of the complement activation (inhibit C3 convertase formation) and of the terminal complement pathway (inhibit MAC formation). Membrane inhibitors of complement activation are complement receptor 1 (CR1), decay-accelerating factor (DAF) and membrane cofactor protein (MCP). Rodents express an additional inhibitor of complement activation termed Crry that is a structural and functional homolog of human CR1 (23, 24). Control of the terminal pathway and MAC formation in cell membranes occurs through the activity of CD59 that binds to C8 and C9 in the assembling MAC. The complement anaphylatoxins are responsible for the various proinflammatory events in the CNS and chemotaxis of the immunocompetent cells (25).

1.3. The complement system in neurodegeneration

The complement system is known to perform a wide range of functions in the human body. It forms an essential component of the host immune system and is associated with the clearance of molecules of foreign origin as well as the elimination of invading pathogens from the body. It plays an important role in adaptive immunity(26). However, it is nonspecific in action and unable to distinguish between self and non-self. Under normal conditions, it is strictly regulated by complement regulatory molecules. However, in neuroinflammatory disorders, the complement regulatory molecules fail to control the activated complement components. These activated complement components then act as a double-edged sword and are responsible for the degeneration of neurons(27). The devastating roles of the complement components in neurodegenerative disorders are well documented. Spinal cord injury (SCI)(6), traumatic brain injury (TBI)(28), Alzheimer's disease (AD)(29), multiple sclerosis (MS)(30, 31), myasthenia gravis(32) and Parkinson's disease (PD)(33) are examples of a few disorders in which activated complement components play an important role. Not only do these disorders arise out of

dysfunction in the normal metabolic machinery, but also neuroinflammatory disorders associated with microbial infections show involvement of complement components.

1.4. Why it is important to study the role of complement in spinal cord injury?

An impact to the spinal cord results in a primary injury, but the impact also triggers a series of downstream events that lead to secondary injury of tissue and the progressive degeneration of the spinal cord. Therapeutic interventions that minimize secondary injury will improve functional recovery after traumatic injury. Inflammation plays a key role in secondary injury following spinal cord injury (SCI). Inflammation is a rapid immune response that can be initiated by infection or tissue damage. An important component of an inflammatory response is the complement system, a collection of blood proteins that form part of the immune system and that can be activated by injured cells and tissues. Activation of complement amplifies the inflammatory response and produces molecules that can be directly toxic or that can recruit and activate cells of the immune system to produce toxic molecules. Inhibiting the complement system has been shown to be an effective therapy for inflammatory disease in various animal models, and some complement inhibitors are in clinical trials.

We have shown that complement deficient mice and normal mice treated with a complement inhibitor are protected from neuronal injury following SCI, and that they have significantly improved functional scores over time compared to control mice. These data indicate an important role for complement in secondary injury, and indicate complement inhibition will reduce inflammation and provide neuroprotection following spinal cord injury. However, complement-dependent mechanisms involved in secondary injury following SCI are not known, and there remain concerns regarding the clinical application of the currently available systemic (body-wide) complement inhibitors with regard to their safety and efficacy. Complement activation products are important for host defense and immune maintenance mechanisms, and systemic complement inhibition can compromise the protective and beneficial roles of complement. This will not be optimal

in patients at risk of infection, and urinary tract infection is a frequent complication during initial and ongoing medical rehabilitation after SCI.

We propose to develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI using a novel and validated approach. The strategy involves the targeting of complement inhibitors to sites of complement activation and injury, and we have shown the approach to be highly effective in vitro and in mouse models of SCI and other inflammatory disease conditions. The targeting strategy enhanced the activity and protective effect of complement inhibitors by 10-20 fold compared to untargeted counterparts. Furthermore, untargeted, but not targeted complement inhibitors systemically inhibited complement and increased susceptibility to infection in a mouse model.

We propose to fully characterize our targeted complement inhibitors in a mouse model of SCI. In addition to therapeutic endpoints, we will use different types of complement inhibitor to investigate complement-dependent disease mechanisms. We will determine relationships between the generation of different complement activation products and other molecules associated with inflammation in order to gain a better understanding of the mechanisms of secondary tissue injury following SCI. These preclinical studies will establish the guidelines necessary for the translation of this therapy to the clinic using recombinant human proteins.

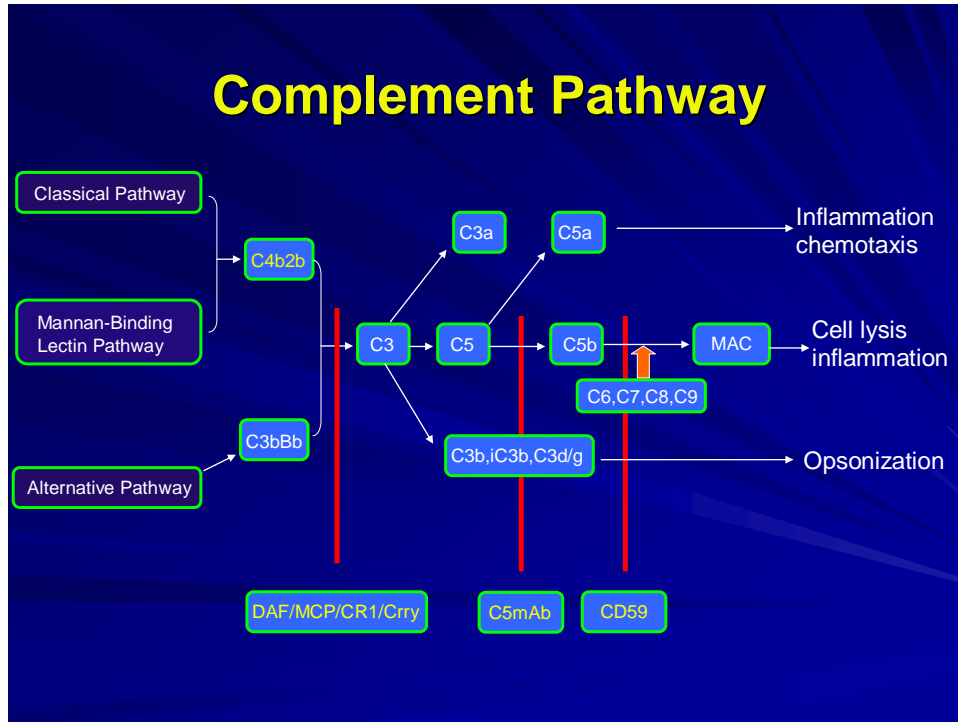


Fig. 1. The complement system The constituent pathways of the Complement system and the component proteins are shown.

1.4.1. Complement activation in the classical pathway and the alternative pathway

The classical pathway of complement activation is found to be a major factor in the etiology of these disorders(33-37). Recent evidence indicates the involvement of the alternate pathway of complement activation in the disorders of the central nervous system(38). This emphasizes the importance of both pathways in the pathogenesis of neurodegenerative disorders. Classical pathway activation is usually antibody-dependent and is initiated when C1q binds to an immune complex. Activated C1 cleaves both C4 and C2 to generate C4a and C2a fragments, which combine to form the classical pathway C3 convertase, involved in the cleavage of component C3 to form C3a and C3b. C3a is an anaphylatoxin and acts via various receptors present on the cellular surfaces. C3b

combines with C4a and C2a to form C5 convertase, which leads to the formation of C5a and C5b. The former is an anaphylatoxin and the latter is involved in the production of terminal complement components, C6 to C9. C5b, with these terminal complement components, results in the formation of MAC(39, 40). The alternate pathway also results in the formation of complement anaphylatoxins and MAC, but activated through C3 component, requires factor B to form C3 convertase and C5 convertase(40).

The alternative pathway is activated on surfaces of pathogens that have neutral or positive charge characteristics and do not express or contain complement inhibitors. This is due to a process termed “tickover” of C3 that occurs spontaneously, involves the interaction of conformationally altered C3 with factor B, and results in the fixation of active C3b on pathogens or other surfaces reviewed in(38, 39), and activated by spontaneous hydrolysis of C3 to a cleavage product (C3b analog) that binds factor B (fB), leading to formation of the alternative pathway C3 convertase. The alternative pathway also provides an amplification loop for the classical and lectin pathways. The alternative pathway does not utilize C1q or MBL as the molecules that recognize antibody-marked targets, but relies on a postulated “tick-over” deposition of activated C3b on all cell membranes. What determines whether complement is activated or not on a particular cell surface, are the perturbations in the interaction between the deposited C3b and regulatory molecules. C3b in the circulation undergoes conversion, at a slow rate, to an active but uncleaved C3b-like form, C3i or C3(H₂O). This C3b can bind to nucleophilic targets on cell surfaces and form a complex with plasma factor B that is then cleaved by factor D to form the C3 convertase, C3bBb (see fig. 1). In addition, properdin binds to and stabilizes the alternative pathway C3 convertase, extending the lifetime of the active convertase three- or four- fold(41). The C3 convertase catalyzes further cleavage of C3 into C3b. The fact that each newly produced C3b molecule, even those that arise from the classical pathway, has the potential to form the convertase and thus to produce more C3b provides a means of amplification.

Both the classical path way and the alternative pathway C3 convertases participate in the formation of the C5 convertase. In the classical pathway, one C3 molecule can

covalently bind C4b in the classical pathway C3 convertase, C4b2a, to form C4b2a3b, the classical pathway C5 convertase. Similarly, the addition of activated C3b to the alternative pathway C3 convertase, C3bBb, forms C3bBbC3b, the alternative pathway C5 convertase. Both C5 convertases can cleave C5 to yield C5a and C5b. C5a has powerful proinflammatory and chemotactic properties(42).

1.4.2. Complement activation in the lectin pathway

The lectin pathway is activated when mannose binding protein (MBL) or ficolins bind to conserved carbohydrate structures.

1.4.3. The membrane attack pathway (MAP), the terminal pathway

The terminal complement components formed by the cleavage of C5 either by the classical or the alternate pathway result in the formation of membrane attack complex (MAC). The MAC formation is regulated by complement defense protein CD59, deficiency of which results in neurodegenerative diseases(43). The neurotoxic effects of MAC on neuronal cells are time and concentration dependent. Mechanisms like free radical generation, cytokines, eicosanoids, and increased permeability to Na⁺ and Ca²⁺ ions induced by MAC might be responsible for the observed effect(44). Apart from their lytic properties, recent findings suggest that both cytolytically active and inactive forms of terminal complement complexes are involved in the accumulation of leukocytes into the cerebrospinal fluid and endothelial cell activation. At sublytic concentration, it not only protects host cells but also stimulates protein biosynthesis. The role of MAC, at sublytic and lytic concentration, is outlined by Wurznner(45). This explains the concentration-dependent dual role of MAC. At lytic concentration, it acts as a neurodegenerative agent and at sublytic concentration it acts as a neuroprotective agent. Marked neurodegeneration in CNS disorders, even in the presence of the MAC, indicates the failure of regulation of MAC components and the need to restrict its activity to

sublytic concentration by proper regulation of the pathways leading to MAC formation. Thus, regulating the complement pathways leading to its generation can control formation of MAC(46).

All pathways converge at C3 activation with the subsequent cleavage of C5. During this process, the anaphylatoxins C3a and C5a are generated, and C5 cleavage initiates the terminal complement pathway that culminates in the formation of the membrane attack complex (MAC). The MAC can be directly cytolytic and can stimulate the production of proinflammatory molecules when deposited in cell membranes at sublytic concentrations (for a review of the complement system, see Ref (47)). Cleavage of C5 is the final enzymatic step of the complement system. C5b bound to the convertase sequentially binds C6 and C7. The C5b67 complex is released from the convertase and associates with adjacent membrane via a labile hydrophobic binding site in the C6 component. The C5b67 complex stably associates with the membrane, then binds C8, and finally, multiple copies (up to 12) of C9. MAC induces concentration-dependent neuronal cell death and changes in membrane permeability to Na⁺, K⁺ and Ca²⁺, release of cytokines, eicosanoids, and reactive-free radicals. These changes occur at sublytic concentrations of MAC(44). MAC is also responsible for the demyelination of neurons in demyelinated forms of certain disorders(48). Upon binding to C5b-8, C9 unfolds and inserts in the membrane. The recruitment of additional C9 molecules, membrane attack complexes, MACs, form a functional pore in the cell membrane through which ions and small molecules can pass, bringing about osmotic lysis of the cell.

1.5. Complement inhibitors as therapeutic agents

Although, inflammation plays an important role in CNS disorders, no currently available anti-inflammatory agent offers significant neuroprotection in such disorders(40). Various types of complement inhibitory proteins are currently under investigation for therapy of inflammatory and ischemic disease (reviewed in (49-52)(49-52)(49-52)[49-52]). Interventional studies with soluble complement inhibitors in rodent models of traumatic brain injury, and studies using transgenic mice with astrocyte-targeted expression soluble Crry indicate complement plays an important role in inflammation and secondary tissue

damage (28, 53, 54)(28, 53, 54)(28, 53, 54)[28, 53, 54]. Clinical studies have shown elevated levels of complement proteins in cerebrospinal fluid from patients with traumatic brain injury, and a recent study demonstrated that a viral complement control protein modulates inflammation following spinal cord injury in rats (2, 55, 56)(2, 55, 56)(2, 55, 56)[2, 55, 56].

Virtually all complement inhibitory strategies reported to date depend on systemic complement inhibition. We have shown that the targeting of complement inhibitors to sites of complement activation and disease significantly improves their efficacy and obviates the need for systemic inhibition. One strategy to target complement inhibitors is to link them to antibody fragments, and we have shown that antibody-linked complement inhibitors (DAF, Crry and CD59) are significantly more effective than untargeted counterparts in vitro and in an animal model of tubulointerstitial disease(57-59)(57-59)(57-59)[57-59]. Significantly, for CD59 to function effectively, targeting to the site of complement activation is a requirement (57)(57)(57)[57]. Due to considerations of species selective activity, Crry is a relevant C3 inhibitor for studies in mice.

In this study we propose to use a targeting strategy based on a fragment of complement receptor 2 (CR2) to target complement inhibitors specifically to sites of complement activation. Natural ligands for CR2 are iC3b, C3dg and C3d, cell-bound breakdown fragments of C3 that are deposited on activating surfaces(60, 61)(60, 61)(60, 61)[60, 61]. These C3 ligands are relatively long lived and are present in high concentrations at sites of complement activation. We have shown that human CR2-DAF and CR2-CD59 bind to C3-coated targets and are at least 20-fold more potent than their untargeted counterparts at providing protection from complement (58)(58)(58)[58] (see attached manuscript). We recently characterized CR2-Crry in a mouse model of intestinal ischemia/reperfusion injury (IRI) and demonstrated that CR2-Crry was 10-fold more effective than Crry-Ig, an untargeted systemic counterpart. Furthermore, CR2-Crry, unlike Crry-Ig, was therapeutically effective at a dose that did not result in systemic complement inhibition and that did not increase susceptibility to infection.

2. Objectives

Traumatic spinal cord injury initiates a cascade of pathophysiological events that cause secondary injury and determine the extent of functional recovery. Although the processes that occur following SCI are complex, inflammation is considered to play a key role in the progressive degenerative events that take place, especially within the lesion penumbra. The complement system plays a key role in the pathogenesis of many inflammatory and ischemic conditions, and recent evidence indicates it also plays an important role in secondary SCI. Various systemic complement inhibitors are currently under therapeutic investigation as anti-inflammatory agents, but there remain concerns regarding their efficacy and safety. Complement activation products are important for host defense and immune homeostasis mechanisms, and systemic complement inhibition can compromise the protective and immunomodulatory roles of complement. In this context, CNS injury has been shown to be immunosuppressive, and further immune suppression by systemic complement inhibition may not be optimal in patients at risk of infection (urinary tract infection is a frequent complication during initial and ongoing medical rehabilitation after SCI).

The goals of the present study were (1) To investigate the dynamics of complement activation and its role in the development of SCI in mice. (2) To determine in vivo relationships between the generation of different complement activation products with cytokine production, adhesion molecule expression, leukocyte infiltration and activation, and injury. (3) **To investigate the neuroprotective effect of a novel targeted complement inhibitor and** develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI.

2.1. To develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI

We have developed a strategy to target complement inhibitors to sites of complement activation and injury, and have shown that targeted complement inhibitors are 10-20 fold more effective in vitro and in an experimental models of ischemia and reperfusion injury compared to conventional systemic approaches to inhibit complement. We hypothesize that our novel strategy to target complement inhibitors to the site of SCI will improve bioavailability, obviate the need to systemically inhibit complement, and provide a safe and highly efficacious therapy. We propose to investigate our hypothesis in a mouse model of SCI. Targeted complement inhibition will be achieved by the use of soluble recombinant chimeric molecules consisting of a targeting moiety linked to a complement inhibitor. Complement inhibitors will be mouse Crry (inhibits early in the complement pathway) or CD59 (inhibits late in pathway). The targeting moiety will be a fragment of complement receptor 2 (CR2) that binds to long lived degradation products of C3 that are deposited at sites of complement activation. In addition to therapeutic endpoints, we will utilize the targeted complement inhibitors that function at different points in the complement cascade to investigate disease mechanisms in a clinical setting.

2.2. To investigate the neuroprotective effect of a novel targeted complement inhibitor.

To investigate the neuroprotective effect of two complement inhibitory strategies, a novel targeted complement inhibitor (CR2-Crry) which targets specifically to sites of complement activation and does not require systemic complement inhibition and an anti-fB antibody that suppresses the alternative pathway of complement activation. 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(62). 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(63). 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.

As discussed in section 5.2, in the present study, we show that fB-deficient mice have an improved outcome after SCI. The level of protection afforded to fB-deficient mice was similar to that seen in our previous study using C3-deficient mice(64). These data indicate a dependence on the alternative pathway for complement-mediated SCI. Together with the previous findings described above using B cell and C1q-deficient mice, it is likely that the alternative pathway plays a critical role in amplifying antibody-mediated classical pathway initiated complement activation. In this context, reduced pathology and improved locomotor recovery in fB-deficient mice was associated with significantly reduced C3 and MAC deposition compared to wt control mice, even though the classical (and lectin) pathway is intact in fB-deficient mice. A similar dependence on both antibody-mediated lectin pathway activation and alternative pathway activation has been described in models of intestine ischemia and reperfusion injury(65-67). In a more clinically relevant paradigm, we demonstrated that specific inhibition of the alternative pathway with anti-fB mAb was also protective against SCI, and to a similar level as fB deficiency.

3. METHODS AND MATERIALS

3.1. Mice

Female wild-type C57BL/6 and C57Bl/6 C3-deficient (C3^{-/-}) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of fB-deficient (fB^{-/-}) mice on C57BL/6 background were generated as described (68) and provided by Dr. J. Thurman (University of Colorado Health Sciences Center, Denver, CO) and a breeding colony established. In mice, there are two genes encoding CD59; CD59a is widely expressed and is the primary regulator of the MAC in mice, whereas CD59b expression is limited to the testis and, at very low levels, bone marrow (69). In this study, CD59a-deficient mice on C57BL/6 background were used and were generated as described (70). fB and CD59 deficiency was confirmed by genotyping. Mice weighing between 18–22 g (6–8 weeks old) were used in experiments. All mice were fed on standard laboratory food and given tap water ad libitum with a light–dark cycle of 12 hours.

3.2. Complement Inhibitor-CR2-Crry

The fusion protein CR2-Crry was produced and purified as described previously (71). 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 Gen-Bank, 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 (71). CR2-Crry has a circulatory half-life in C57BL/6 mice of ~ 8 hours (71).

3.3. Anti-fB anti-body

The isolation and characterization of anti-fB mAb 1379 used in these studies was described previously, and the mAb effectively inhibits the mouse alternative pathway(72). The mAb was generously provided by Drs. V. M. Holers, J. M. Thurman (University of Colorado Health Sciences Center, Denver, CO), and G. S. Gilkeson (Medical University of South Carolina).

3.4. Spinal cord injury surgery and antibody treatment

3.4.1. Treatment

Wild-type (wt) mice were randomized into sham (laminectomy, no SCI damage), vehicle control (phosphatebuffered saline [PBS]), and CR2-Crry treatment and anti-fB mAb treatment groups. Other groups consisted of C3-deficient (C3^{-/-}), fB-deficient (fB^{-/-}) and CD59a-deficient (CD59^{-/-}) mice. For 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. For anti-fB mAb treatment, wt mice were randomized into four groups, with mice in each group receiving an intravenous (tail vein) injection of 100 µl PBS vehicle control or 2 mg anti-fB mAb in 100µl PBS at 1 and 12 hours after surgery, 12 and 24 hours after surgery, or 24 and 36 hours after surgery. The 2mg dose used was based on previous studies characterizing the therapeutic effect of this Ab in mouse models of inflammation (72-74).

3.4.2. SCI model

3.4.2a. Instruments



Fig. 2. Instruments of the spinal cord injury surgery.

3.4.2b. Anaesthesia

Mice were anesthetized with ketamine/xylazine anesthesia (80 mg/kg/10 mg/kg, i.p.) Animals were breathing spontaneously, and body temperature was maintained using a heat mat for the duration of the experiment.

3.4.2c. SCI Surgery

Mice subjected to laminectomy at the level of the 12th thoracic vertebra (T12), followed by contusion-induced SCI using the NYU weight drop impactor as described previously(64). After injury, the muscles and the subcutaneous tissue were closed in layers, the skin was closed with metal wound clips (World precision instruments), and mice were given 1 ml of saline per day for 3 days to compensate for loss of blood and dehydration. Bladders were expressed before testing, and each mouse was evaluated for 4 5 minutes on the day before surgery, immediately after surgery, and then manual bladder expression was performed twice daily until full bladder function was observed. Sham mice received a dorsal laminectomy without impact injury. There was less than 5% surgical mortality, and zero mortality of mice that recovered from surgery. Groups of mice were sacrificed at 1, 3, 7 and 21 days after injury, and spinal cords were isolated for analysis.

3.5. Locomotor Function Analysis

Open-field observations of locomotor function recovery were independently scored by two observers blinded to experimental groups using the Basso, Beattie and Bresnahan (BBB) rating scale developed for rats(75), but later adapted by others for mice(76-79) or Basso Mouse Scale (BMS)(80).

3.5.1. *The Basso, Beattie and Bresnahan (BBB) rating scale***TABLE 1**

Basso, Beattie, and Bresnahan Locomotor Rating Scale

0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL–HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL–HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL–HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL–HL coordination
14	Consistent weight-supported plantar steps, consistent FL–HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping

- 15 Consistent plantar stepping and consistent FL–HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
 - 16 Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
 - 17 Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
 - 18 Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
 - 19 Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift off, and tail is down part or all of the time
 - 20 Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
 - 21 Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up
-

Note.

Slight: Partial joint movement through less than half the range of joint motion.

Extensive: Movement through more than half of the range of joint motion.

Sweeping: Rhythmic movement of HL in which all three joints are extended and then fully flex and extend again; animal is usually sidelying and plantar surface of paw may or may not contact the ground; no weight support across the HL is evident.

No weight support: No contraction of the extensor muscles of the HL during plantar placement of the paw; or no elevation of the hindquarter.

Weight support: Contraction of the extensor muscles of the HL during plantar placement of the paw; or, elevation of the hindquarter.

Plantar stepping: The paw is in plantar contact with weight support and then the HL is advanced forward and plantar contact with weight support is reestablished.

Dorsal stepping: Weight is supported through the dorsal surface of the paw at some point in the step cycle.

FL–HL coordination: For every FL step a HL step is taken and the HLs alternate.

Occasional: Less than or equal to half; #50%.

Frequent: More than half but not always; 51–94%. **Consistent:** Nearly always or always; 95–100%. **Trunk instability:** Lateral weight shifts which cause waddling from side to side or a partial collapse of the trunk.

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).

3.5.2. *The Basso Mouse Scale (BMS)***TABLE 2****BASSO MOUSE SCALE (BMS) [80]**

0	No ankle movement
1	Slight ankle movement
2	Extensive ankle movement
3	Plantar placing of the paw with or without weight support -OR Occasional, frequent or consistent dorsal stepping but no plantar stepping
4	Occasional plantar stepping
5	Frequent or consistent plantar stepping, no coordination -OR Frequent or consistent plantar stepping, some coordination, paws rotated at initial contact and lift off (R/R)
6	Frequent or consistent plantar stepping, some coordination, paws parallel at initial contact (P/R, P/P) -OR Frequent or consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift off (R/R)
7	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift off (P/R) -OR Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and severe trunk instability
8	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and mild trunk instability -OR Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and normal trunk stability and tail down or up & down

- 9 Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off (P/P), and normal trunk stability and tail always up.
-

Slight: Moves less than half of the ankle joint excursion.

Extensive: Moves more than half of the ankle joint excursion.

Plantar placing: Paw is actively placed with both the thumb and the last toe of the paw touching the ground.

Weight support: (dorsal or plantar): The hindquarters must be elevated enough that the hind end near the base of the tail is raised off of the surface and the knees do not touch the ground during the step cycle.

Stepping: (dorsal or plantar): Weight support at lift off, forward limb advancement and re-establishment of weight support at initial contact.

Occasional: Stepping less than or equal to half of the time moving forward.

Frequent: Stepping more than half the time moving forward.

Consistent: Plantar stepping all of the time moving forward with less than 5 missed steps (due to medial placement at initial contact, butt down, knee down, skiing, scoliosis, spasms or dragging) or dorsal steps.

Coordination: For every forelimb step a hindlimb step is taken and the hindlimbs alternate during an assessable pass. For a pass to be assessable, a mouse must move at a consistent speed and a distance of at least 3 body lengths. Short or halting bouts are not assessable for coordination. At least 3 assessable passes must occur in order to evaluate coordination. If less than 3 passes occur then the mouse is scored as having no coordination.

Some coordination: Of all assessable passes (a minimum of 3), most of them are not coordinated.

Most coordination: Of all assessable passes (a minimum of 3), most of them are coordinated.

Paw position: Digits of the paw are parallel to the body (P), turned out away from the body (external rotation: E) or turned inward toward midline (internal rotation; I).

Severe trunk instability: Severe trunk instability occurs in two ways.

- (1) The hindquarters show severe postural deficits such as extreme lean, pronounced waddle and/or near collapse of the hindquarters predominantly during the test. Or
- (2) Five or more of any of the following events stop stepping of one or both hindlimbs
- Haunch hit: the side of hindquarters rapidly contacts the ground
 - Spasms: sustained muscle contraction of the hindlimb which appears to immobilize the limb in a flexed or extended position
 - Scoliosis: lateral deviation of the spinal column to appear “C” shaped instead of straight

Mild trunk instability: Less than 5 events listed above and some sway in the hindquarters. Mild trunk instability is scored when the pelvis and haunches predominantly dip, rock, or tilt from side-to-side (tilt). If the tail is up, the swaying of the pelvis and/or haunches produces side-to-side movements of the distal third of the tail which also indicates mild trunk instability (side tail).

Normal trunk stability: No lean or sway of the trunk, and the distal third of the tail is steady and unwavering during locomotion. No severe postural deficits or events and less than 5 instances of mild instability.

3.6. Histopathological Analysis

Spinal cords were removed at 1, 3, 7 and 21 days postinjury for histological analysis. Immediately after sacrifice, mice were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Spinal cords were then removed, placed into 4% paraformaldehyde/PBS, and either cryoprotected in 30% sucrose for 48 hours before storing at -80°C or placed in formalin and processed to paraffin for histological analysis. For histological assessment, sections of spinal cord were stained with hematoxylin and eosin (H&E) or luxol fast blue (LFB) as previously described (Clark G: Staining Procedures. Baltimore, MD: Williams and Wilkins, 1981, pp 111–129) and (81, 82) Histopathological damage was assessed quantitatively by an independent reviewer blinded to the experimental groups. H&E and LFB sections were scored from 0 to 3 for the presence and intensity of inflammatory cell infiltration, neuronal vacuolation, and hemorrhage (where 0, is no evidence and 3 severe). Scores were then expressed as a

cumulative score of 0–9. When assessing recovery at 21 days after injury, the extent of demyelination, graded between 0–3 (with 0 being no evidence of demyelination) was added for a cumulative score of 0–12. To further quantify spinal cord injury, morphometric analyses was conducted to determine the degree of tissue sparing after injury. Transverse sections of spinal cord were stained with H&E, and the cross-sectioned area of spinal cord was measured at 150 μm increments extending 2mm either side of the injury epicenter using Zeiss Axiovision image analysis software (Carl Zeiss, Oberkochen, Germany). Measurements were averaged for animals in each group at each time point as previously described (83). All assessments were performed in a blinded fashion.

3.7. Neutrophil and Macrophage Infiltration

The presence of infiltrating neutrophils and macrophages was assessed using immunohistochemistry on frozen spinal cord sections. Standard immunohistochemical methods were used as previously described(71). Neutrophils and macrophages were identified by anti-mouse Gr-1 and Mac-3 (BD Biosciences), respectively. Neutrophils and macrophages were quantified at the spinal cord injury epicenter, defined as the section exhibiting maximal tissue damage. The total number of neutrophils and macrophages were quantified using computerized image analysis methods, as previously described(84, 85). Results are expressed as the number of neutrophils/macrophages per mm^2 . Specificity of immunostaining was confirmed by both the use of isotype control antibody and by the omission of primary antibody.

3.8. Complement Deposition

Spinal cord 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 either anti-mouse C3 fluorescein isothiocyanate (FITC) (Dako, Ely, UK), mouse anti-mouse fB mAb 1379 (see above), rabbit anti rat C9 that is cross reactive with mouse C9(86), or rat anti-mouse CD59 mAb 7A6(87). For C9 and CD59 visualization, donkey anti-rabbit FITC and donkey anti-rat FITC antibodies were used.

For fB visualization, we used a mouse on mouse staining kit and protocol from Vector laboratories (Burlingame, CA). Sections were then counterstained with DAPI (Thermo Scientific, Rockford, IL) for nuclear detail. Sections were then coverslipped and analyzed for fluorescence intensity using a Zeiss LSM5 Confocal microscope. Fluorescence intensity for each complement component was scored on a scale of 0–3, where 0 is no staining, 1, mild, 2, moderate, and 3, intense. All observations were made by an observer blinded to group identities.

3.9. Statistical Analysis

All data are presented as Mean \pm SEM or mean \pm SD as indicated. Analyses were performed using Statview Analysis Software (version 5; SAS Institute Inc., Cary, NC) or SPSS 13.0 for Windows. Statistical significance between groups was determined by two-way analysis of variance with Bonferroni/ Dunn's corrected post hoc t-tests. For Locomotor functional analysis, repeated measures of analysis of variance was used to determine differences between the groups. $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. Recovery of Locomotor Function Post-Traumatic Injury

4.1.1 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 wt 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(75). All animals had a BBB score of 21 pre-injury and a score of 0 immediately after injury, with bilateral hindlimb paralysis (Fig. 3). 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 ($P < 0.001$) (Fig. 3). 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 shamoperated controls at all time points from day 2 following traumatic injury ($P < 0.001$) (Figure 3). The C3-deficient mice appeared to have a better outcome than the CR2-Crry-treated mice, but the difference was not significant.

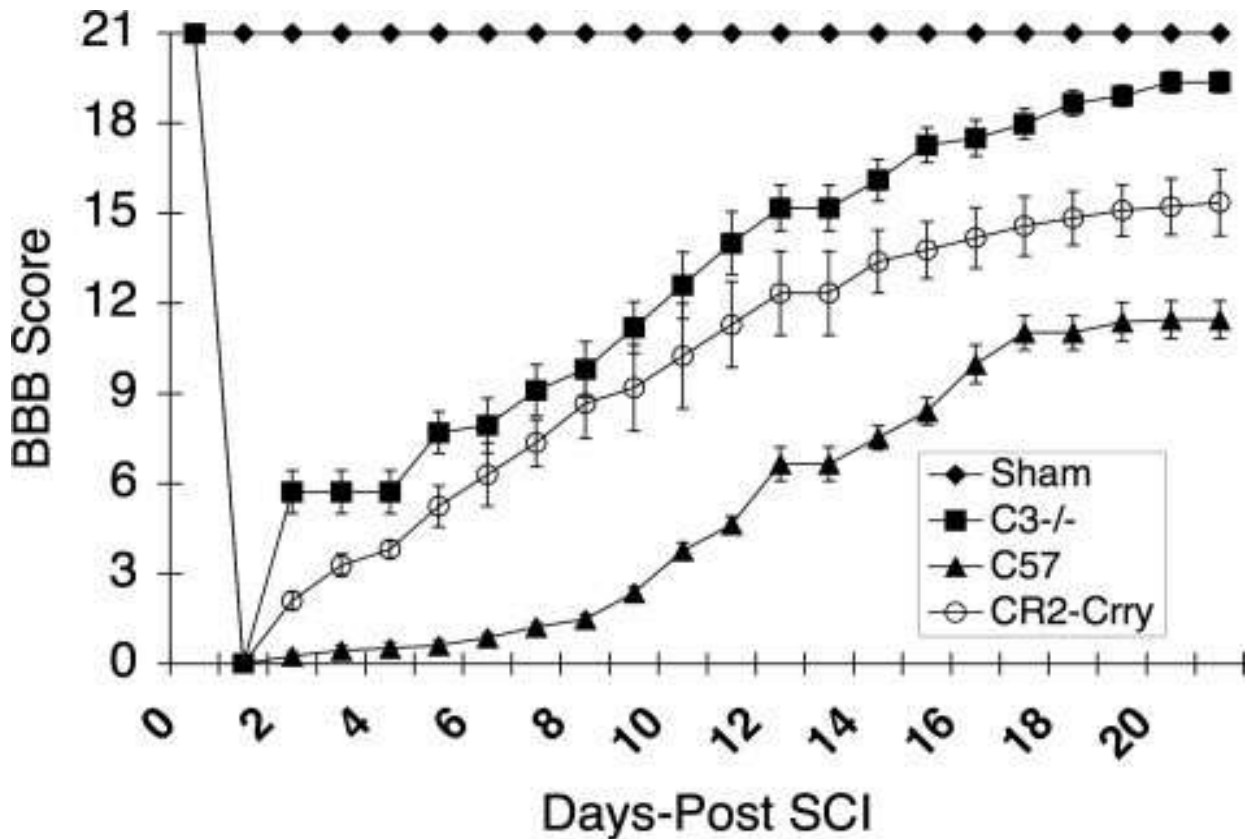


Fig. 3 Combined BBB locomotor scores post-SCI within sham, vehicle control, C3-deficient, and CR2-Crry groups. Note significant improvement in BBB score at day 3, 7, and 21 in both the C3-deficient and CR2-Crry groups when compared to vehicle controls ($P = 0.001$) ($n = 12$). The values are expressed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

4.1.2 Effect of fB and CD59 Deficiency and of alternative complement pathway inhibition on Locomotor Recovery following SCI

Contusion injury to the spinal cord was induced in mice deficient in fB or CD59, in wt untreated mice, and in wt mice subsequently treated with anti-fB mAb or PBS (vehicle). Locomotor function was assessed by the BMS scale, and all mice subjected to contusion injury exhibited a BMS score of 0 immediately after injury. Over the course of 21 days, locomotor function significantly improved in fB^{-/-} mice and wt mice treated with anti-fB mAb at 1 and 12 hours after SCI compared to wt and PBS treated controls (Fig. 4). There were no significant differences in BMS scores between fB^{-/-} mice and anti-fB-treated (1/12 hours) mice over the course of the experiment. When anti-fB mAb was administered at 12 and 24 hours after SCI, there was a trend toward improved locomotor function when compared to controls, but the difference did not reach significance (Fig. 4). We also administered anti-fB mAb at 24 and 36 hours post-SCI, but there was no difference in functional recovery compared to PBS treated mice (data not shown). Unless otherwise stated, all data shown below for anti-fB treated mice was obtained using a 1 and 12 hour post-SCI treatment schedule. In contrast to the improvement seen in fB^{-/-} mice and anti-fB (1/12 hours) treated mice, recovery of locomotor function was significantly impaired in CD59^{-/-} mice compared to wt and vehicle treated controls. There was no difference in functional recovery between PBS (vehicle) treated mice and untreated wt mice after SCI.

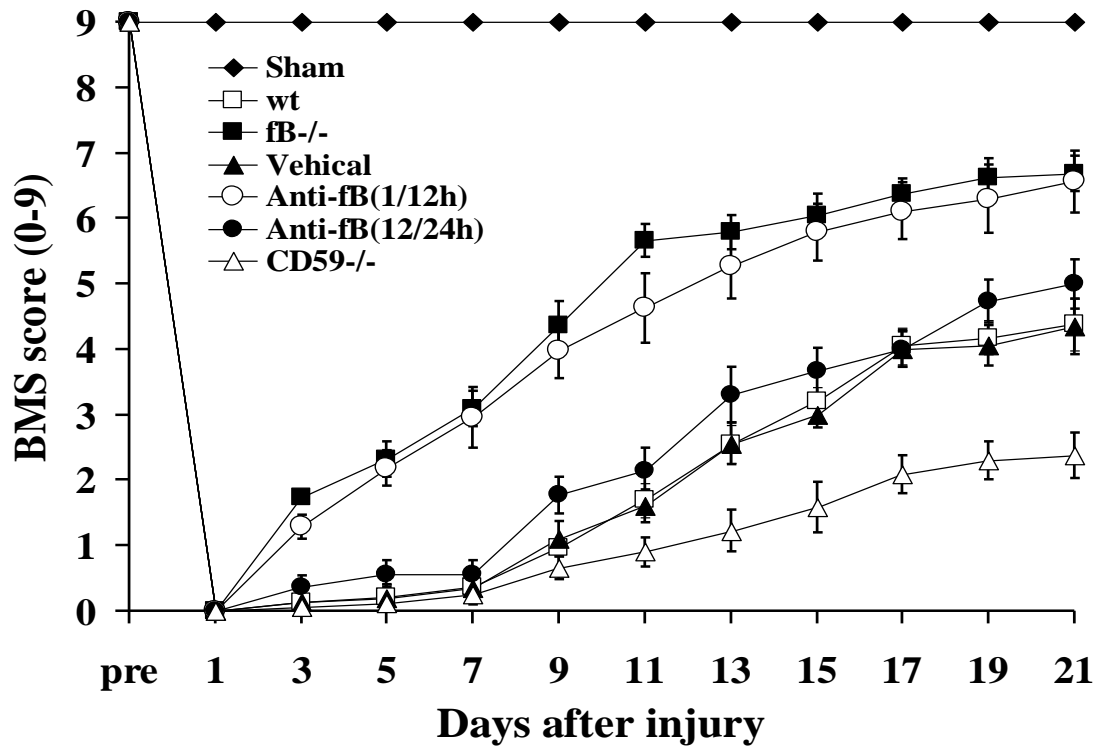


Fig. 4. Locomotor recovery after SCI in complement deficient and inhibited mice. Open-field 10-point BMS scores were recorded for 21 days after contusion-induced SCI in the indicated groups of mice. Anti-fB mAb treatment group received an i.v. injection of 2 mg at 1 and 12 or 12 and 24 hours after injury. BMS scores are significantly higher for fB^{-/-} mice and anti-fB mAb (1/12h)-treated mice compared to wt or vehicle (PBS)-treated mice from day 3 postinjury. No significant difference between vehicle controls and anti-fB mAb (12/24 hours) treated mice. BMS scores are significantly lower in CD59^{-/-} mice compared to wt and vehicle control mice from day 11 after injury ($P < 0.01$). Mean \pm SE, n = 8 - 10.

4.2. Effect of Complement Deficiency and Complement Inhibition on Tissue Damage And Demyelination after SCI

4.2.1. Macroscopic images of spinal cords isolated at 72 hours postinjury.

Spinal cord contusion results in a primary hemorrhage, inflammation, and loss/damage of neurons. We determined the effect of fB deficiency, fB inhibition (1/12 hours), or CD59 deficiency on spinal cord tissue injury by macroscopic examination of spinal cords and by assessment of histological changes within the spinal cords postinjury. At 72 hours after injury, macroscopic examination of control spinal cords demonstrated marked indentation at the injury site with evidence of hemorrhage. These features were markedly reduced in fB^{-/-} and anti-fB mAb treated animals, but appeared exacerbated in spinal cords from CD59^{-/-} mice (Fig. 5).

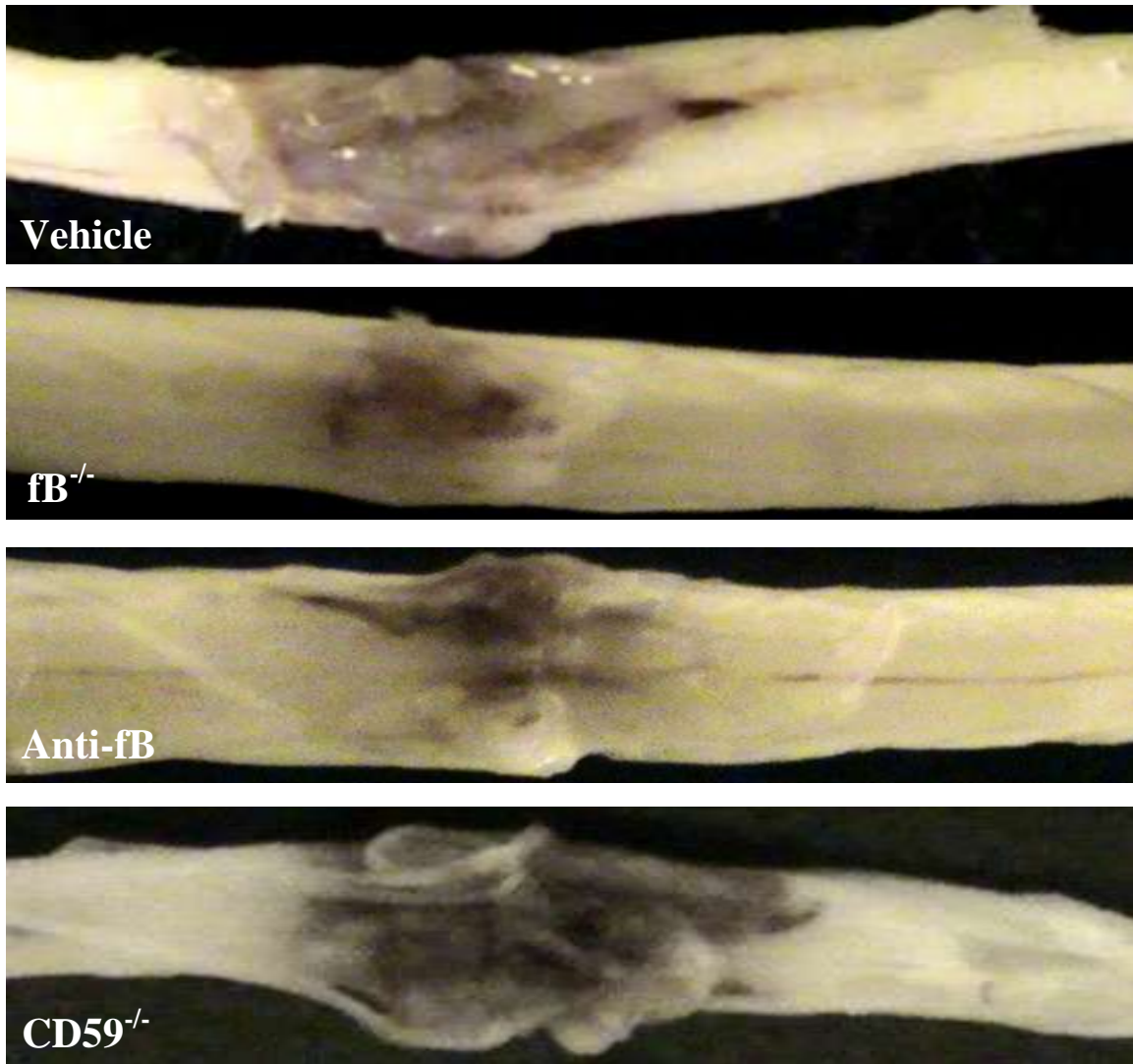


Fig. 5. Macroscopic images of spinal cords isolated at 72 hours postinjury. Mice received anti-fB mAb or PBS (vehicle) at 1 and 12 hours after injury. Representative images shown, n = 6–8 per group.

4.2.2 Effect of C3 Deficiency and of Complement Inhibition on the Extent of Tissue Destruction 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 either 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 (Fig. 6A). 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 (Fig. 6B). 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 (Fig. 6C). There was no difference in tissue sparing between C3-deficient and CR2-Crry-treated mice at 7 days after SCI.

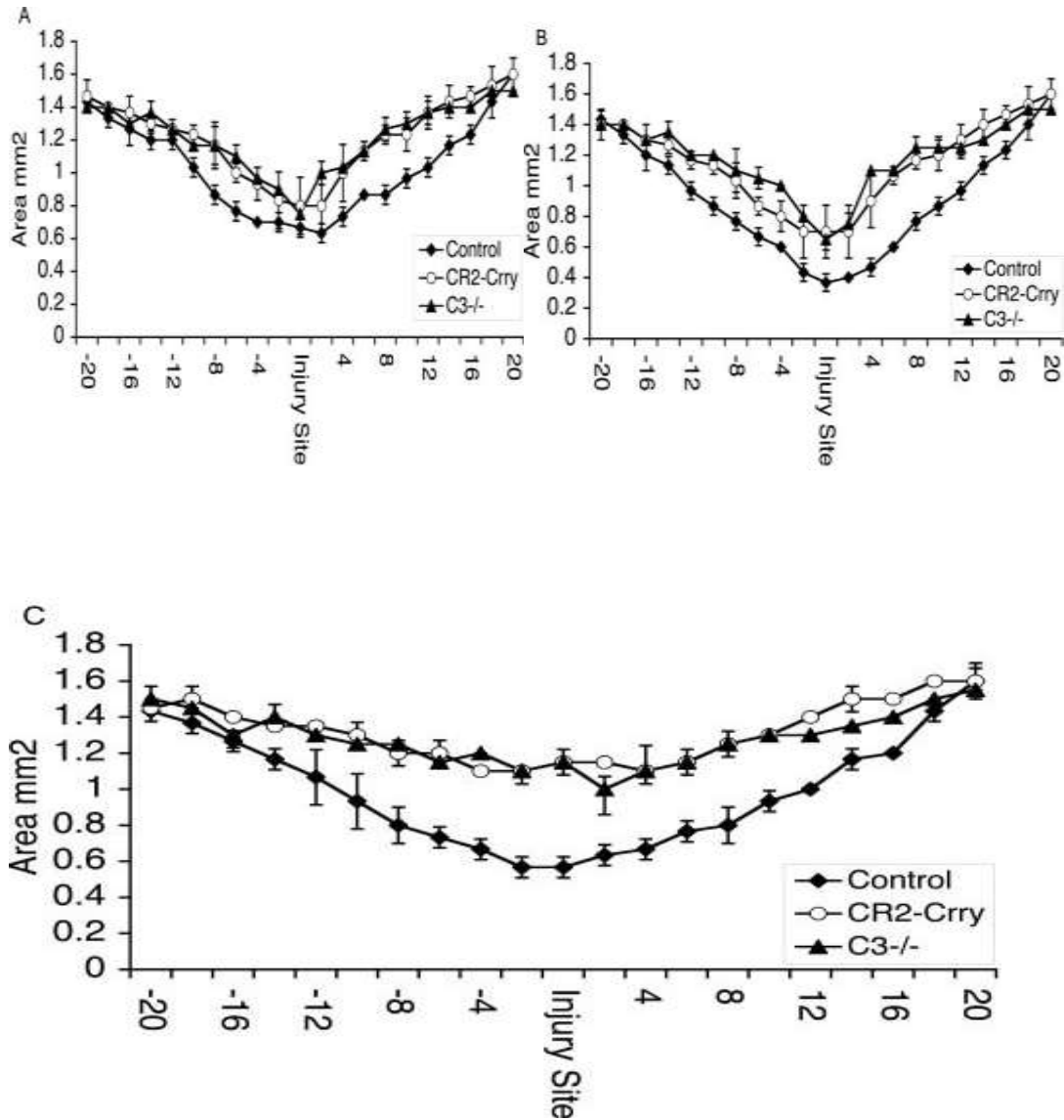


Fig. 6. Tissue sparing as assessed by analyzing the cross-sectional area of spinal cords removed from vehicle controls, C3-deficient, and CR2-Crry treated animals at 24 hours (A), 72 hours (B), and 7 days after injury (C). Measurements were made from histological sections taken at 100- μ m increments extending 2 mm either side of the injury site. No significant difference in tissue sparing was evident at 24 and 72 hrs. Significant tissue sparing was noted in CR2-Crry and C3-/- animals compared to vehicle controls at day 7 ($P = 0.002$). Mean \pm SD, $n = 4$.

4.2.3. Effect of C3 deficiency and of complement inhibition on the extent of tissue demyelination following 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 (Fig. 7a, 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 (Fig. 7a, 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 (Fig. 7a, E and F).

Luxol fast blue staining of cord sections from the control group revealed obvious demyelination in the central core of the white matter beneath the impact site (Fig. 7b, 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 (Fig. 7b, C–F). There was no apparent difference in the extent of demyelination between the C3-deficient and complement-inhibited groups of animals.

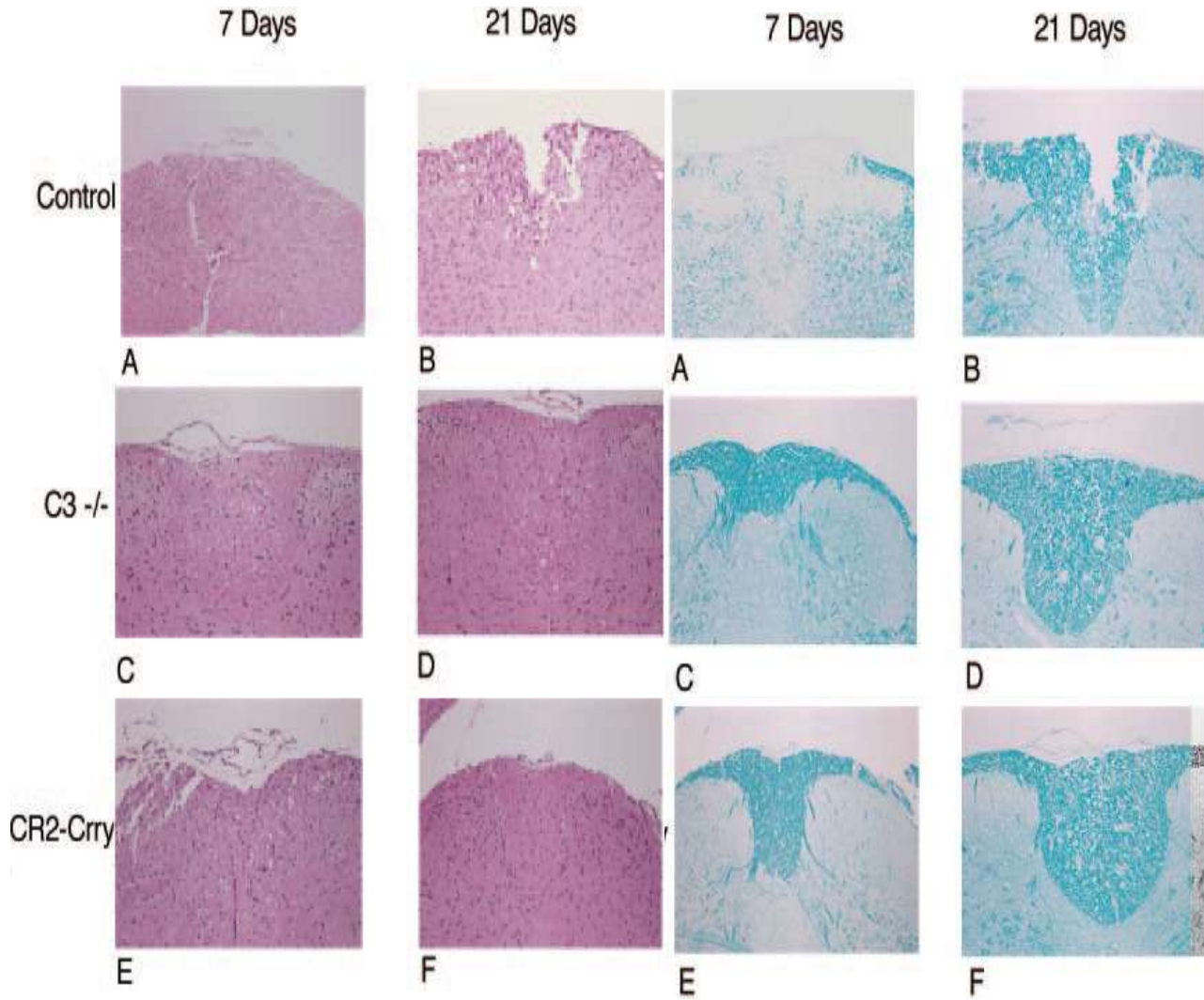


Fig. 7a. H&E-stained sections of spinal cord centered on the injury site at days 7 and 21 after injury. **A–B:** vehicle control. **C–D:** C3-deficient animals. **E–F:** CR2-Crry-treated animals. Original magnification, $\times 100$.

Fig. 7b. 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, $\times 100$.

4.2.4. *Histopathology of spinal cord sections after SCI*

We next analyzed spinal cords microscopically at the injury epicenter using H&E stain to assess the extent of inflammatory cell infiltrate, neuronal vacuolation and hemorrhage. Sections were prepared from cords isolated at 24 and 72 hours postinjury, and the sections were graded for a total cumulative score of 0–9 (see Materials and Methods). Injury to spinal cords was evident in all groups at both 24 and 72 hours (Fig. 8).

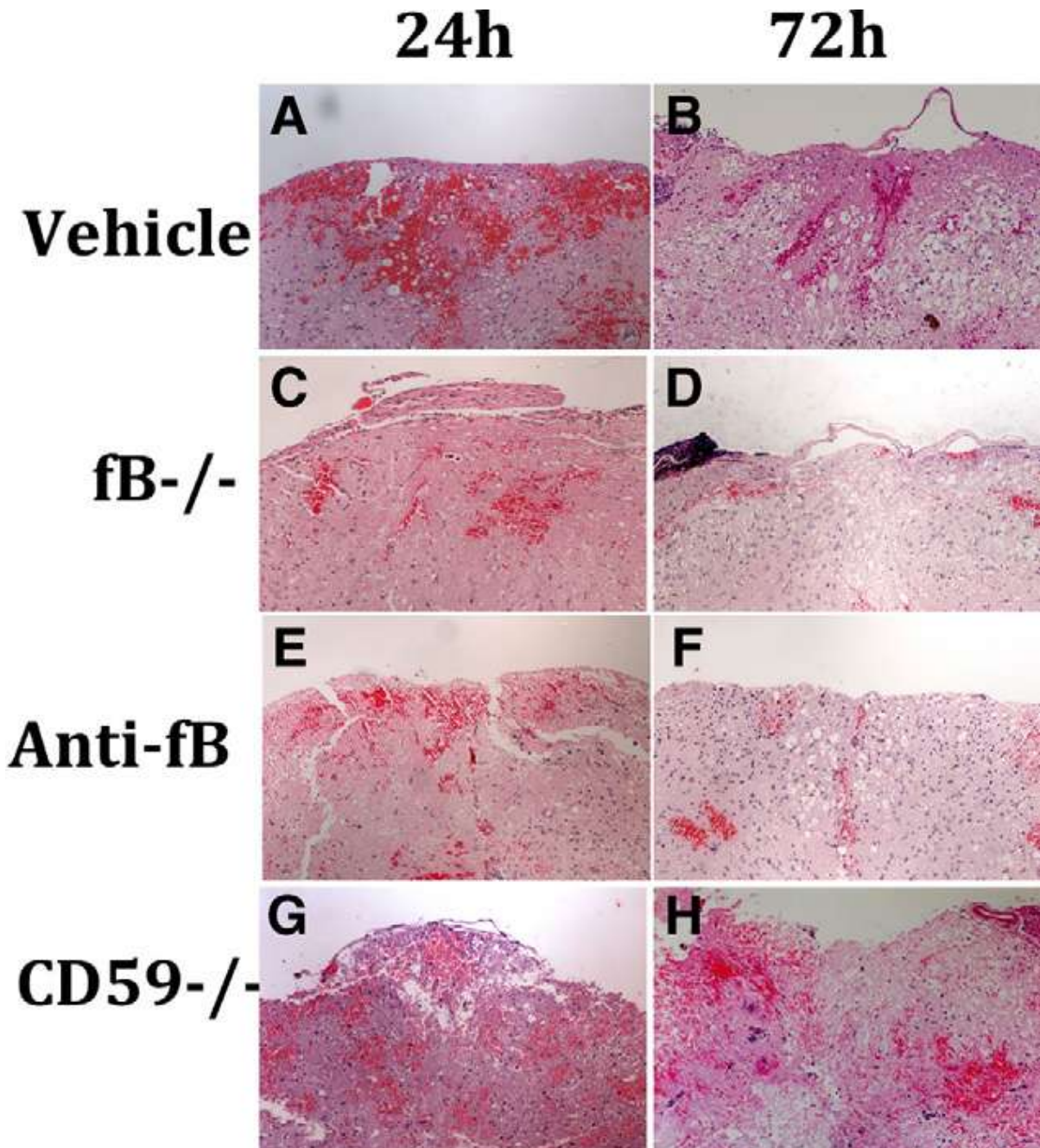


Fig. 8. Histopathology of spinal cord sections after SCI. A-H: Transverse sections from the epicenter of injury were prepared from spinal cords isolated at 24 and 72 hours after injury and stained with H&E. Mice received anti-fB mAb or PBS (vehicle) at 1 and 12 hours after injury. Representative images shown, n = 6 per group.

No significant differences were noted at 24 hours postinjury, but at 72 hours there was significantly less damage seen in fB^{-/-} mice and anti-fB mAb - treated mice compared to control mice. In contrast, damage was significantly exacerbated in CD59^{-/-} mice compared to controls 72 hours after injury.

4.2.5. Quantitative assessment of histopathological inflammation and injury

Representative images of spinal cord sections postinjury are shown in Fig. 8, with quantification of data shown in Fig. 9. Histologically, control and CD59^{-/-} spinal cord sections demonstrated evidence of hemorrhage, pronounced inflammation, neuronal cell vacuolation, and demyelination, and while these features existed in fB^{-/-} mice and anti-fB mAb - treated mice, they were markedly reduced.

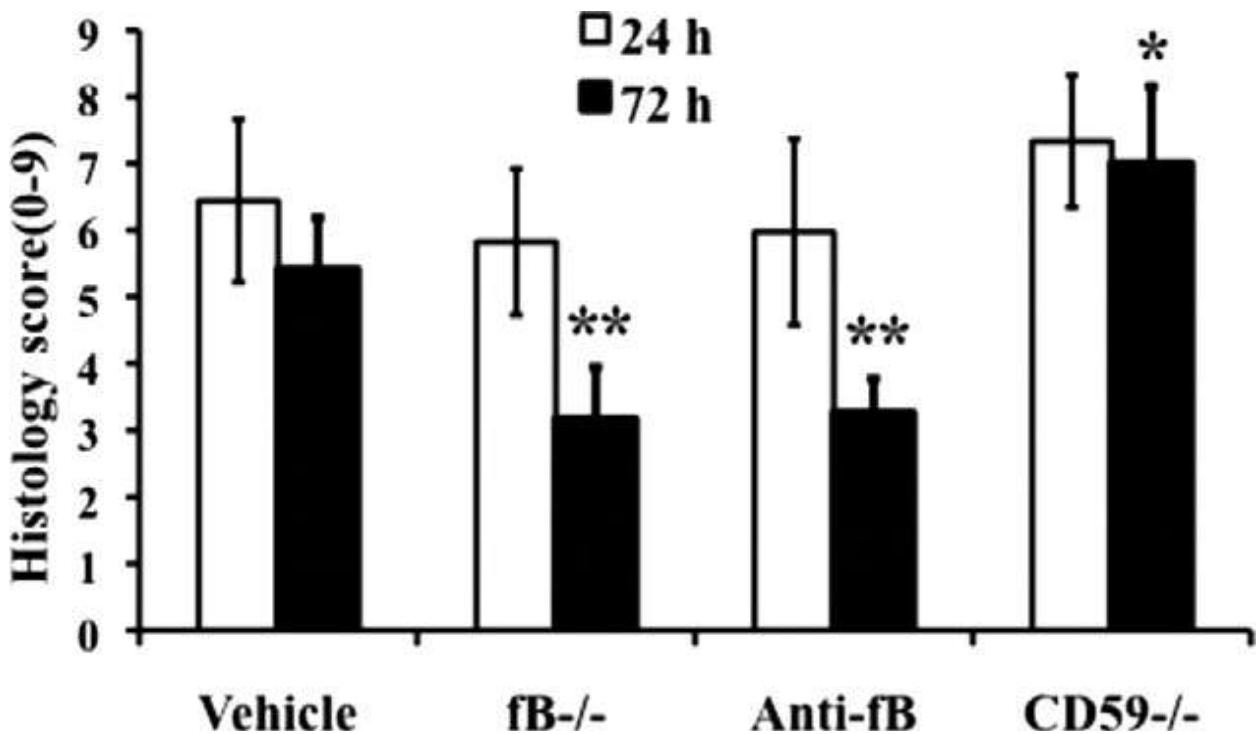


Fig. 9. Quantitative assessment of histopathological inflammation and injury. H&E-stained sections from the epicenter of injury were scored from 0 to 3 for the presence and intensity of inflammatory cell infiltration, neuronal vacuolation, and hemorrhage (0 = no evidence, 3 = severe). Scores were then expressed as a cumulative score of 0-9. Assessments were made from spinal cords isolated at 24 and 72 hours postinjury. Mice received anti-fB

To further quantify the impact of complement deficiency or inhibition on SCI, we analyzed tissue destruction by determining the cross-sectional area of spinal cords at 150 μ m increments extending 2 mm either side of the initial injury impact site. In accord with our subjective histological assessments, there was significantly increased tissue sparing in fB-deficient and fB-inhibited mice, and significantly less tissue sparing in CD59^{-/-} mice, when compared to control mice (Fig. 10).

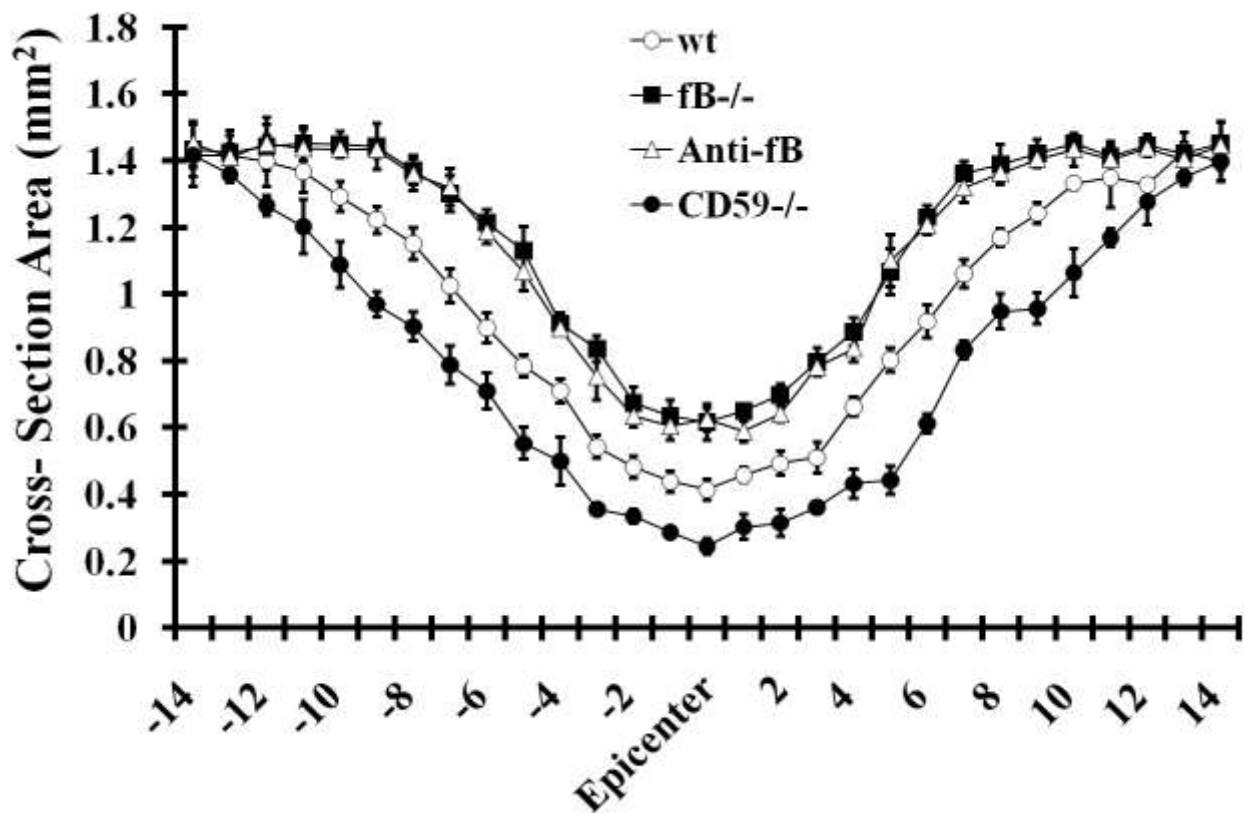


Fig. 10. Morphometric analysis of tissue sparing 3 days after injury. The cross-sectional area of H&E stained spinal cord sections was measured at 150 μ m increments extending 2 mm either side of the injury site. There was a significant level of tissue sparing in fB^{-/-} mice and anti-fB-treated mice compared with vehicle control (PBS)-treated mice, and a significant decrease in tissue sparing in CD59^{-/-} mice compared with wt (at injury site and out to 1.2 mm each side of the injury site, $P < 0.01$). Mice received anti-fB mAb or PBS at 1 and 12 hours after injury. Mean \pm SD n = 6 per group.

4.3. Expression of Neutrophils and Macrophage Infiltration after Spinal Cord Injury

After SCI, the infiltration and activation of neutrophils and macrophages is considered to play an important role in the propagation of inflammation and tissue damage. Neutrophils are typically the first leukocytes to arrive at the injury site, while macrophages/microglia appear later but, unlike neutrophils, persist in the spinal cord(88). Complement activation products provide chemotactic and activating signals for neutrophils and macrophages, as well as induce the expression of inflammatory cytokines/chemokines and adhesion molecules. Using complement deficient and inhibited mice, we investigated the influence of the alternative and terminal complement pathways in neutrophil and macrophage infiltration after SCI. Inflammatory cell influx was evaluated by immunohistochemical analysis using Gr-1, a marker antibody for neutrophils, and Mac-3, a marker antibody for microglia/ macrophages. Neutrophils and macrophages were quantified at the site of injury in mice from all groups at 1 and 3 or 7 days post-SCI.

4.3.1a. Effect of C3 deficiency and of complement inhibition on the Neutrophil Infiltration following SCI

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 (Fig. 11). 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.

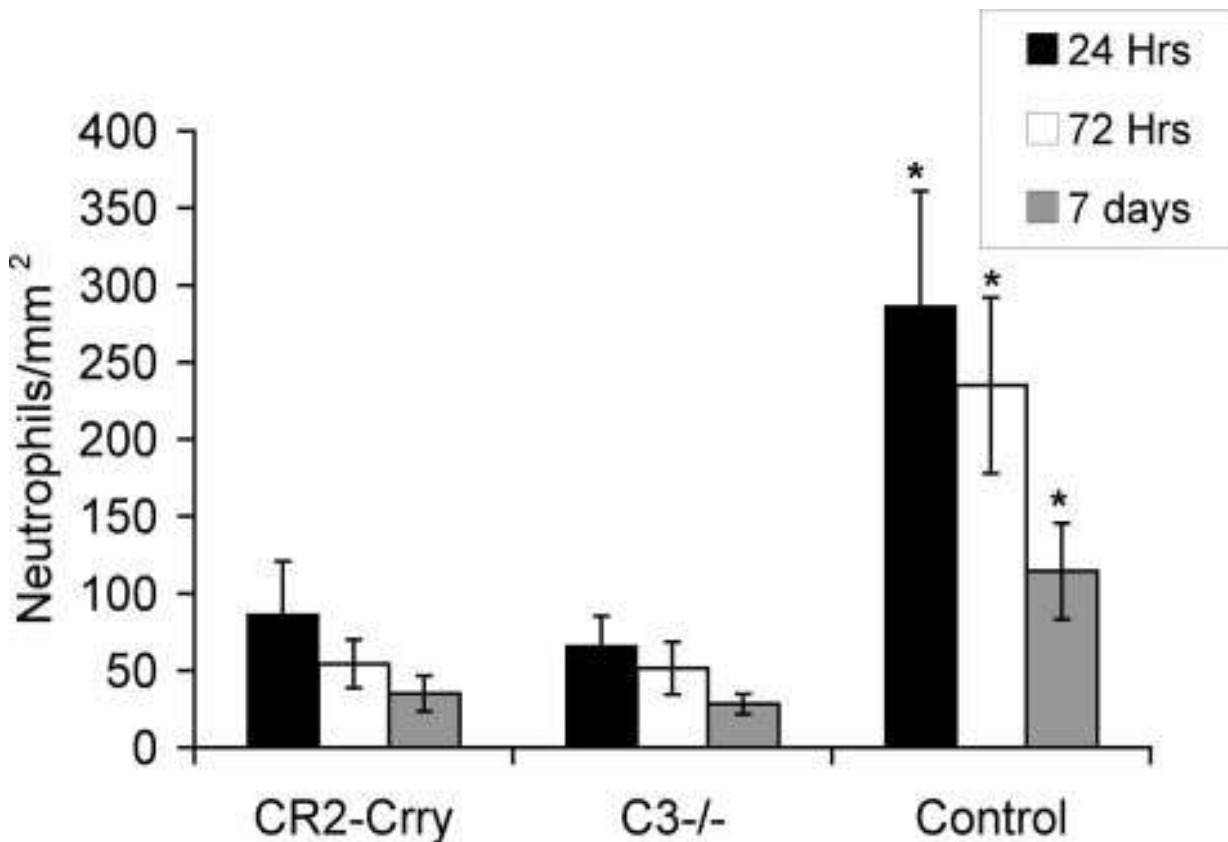


Fig. 11. Neutrophil infiltration as assessed by immunofluorescent staining with anti-mouse GR1-fluorescein labeled antibody. The total number of neutrophils per section was counted. The number of neutrophils is significantly lower in C3-deficient and CR2-Crry-treated animals across all time points when compared with vehicle control (* $P = 0.001$). Mean \pm SD, $n = 5$ per group.

4.3.1b . Effect of fB and CD59 deficiency and of alternative pathway complement inhibition on the neutrophil infiltration following SCI

Neutrophil infiltration was pronounced at 24 hours after injury in all groups, and as expected, numbers declined by 72 hours post-SCI. However, at both 24 and 72 hours post-SCI, neutrophil numbers were significantly reduced in spinal cords from fB-deficient and fB-inhibited mice compared to control mice (Fig. 12). At 24 hours post-SCI, there was no significant difference in neutrophil numbers between CD59^{-/-} and control mice, but neutrophil numbers were significantly higher in CD59^{-/-} mice at 72 hours post-SCI.

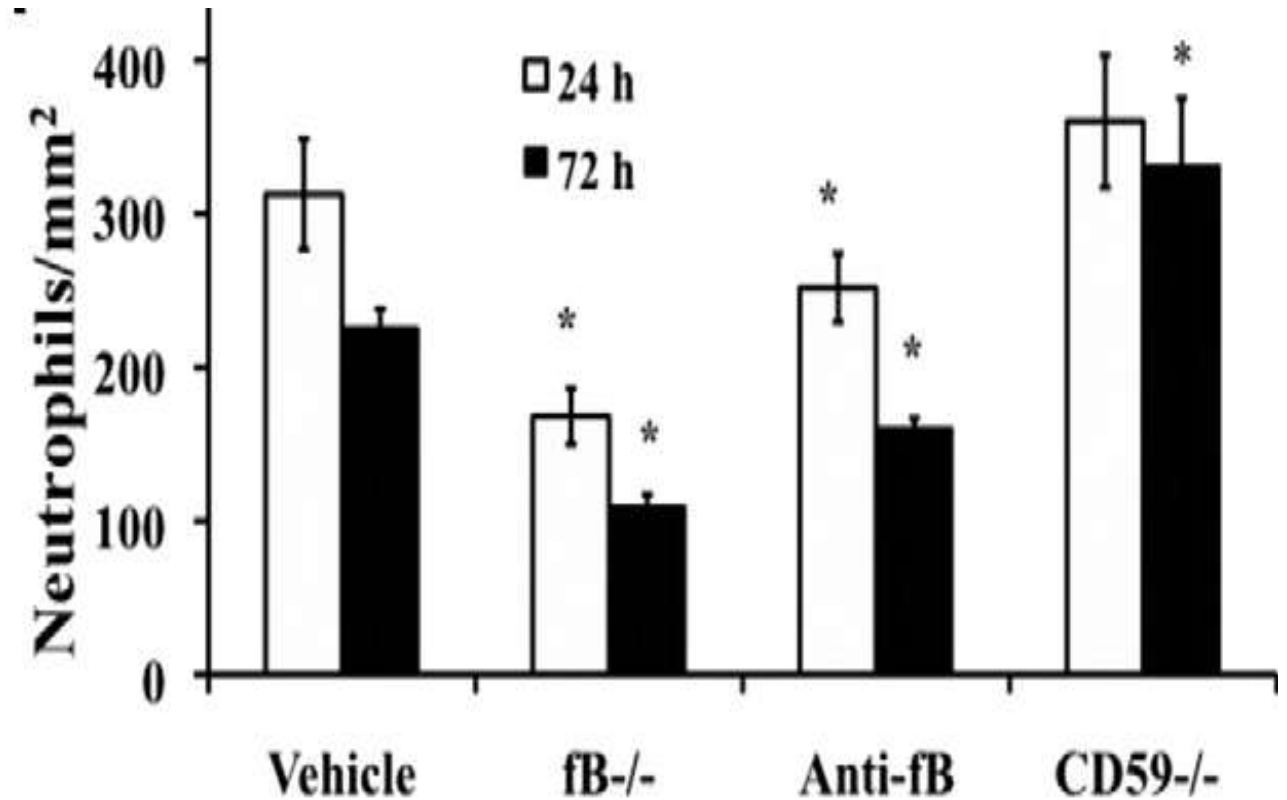


Fig. 12. Neutrophil infiltration after SCI. Sections from the epicenter of injury were prepared from spinal cords isolated at 24 and 72 hours after injury. Mice received anti-fB mAb or PBS (vehicle) at 1 and 12 hours after injury. Neutrophils were identified and counted by immunohistochemistry using anti-mouse Gr-1 antibody. Results are expressed as the number of neutrophils per mm². Mean \pm SD n = 6 per group. *P < 0.01 vs. vehicle control.

4.3.2. Expression of Macrophage Infiltration after Spinal Cord Injury

Not surprisingly, there was no difference in the number of macrophages between any of the groups at 24 hours post-SCI, in accord with previous data on the dynamics of macrophage infiltration following SCI. At 72 hours post-SCI, macrophage numbers were increased in all groups, but numbers were significantly lower in fB-deficient and fB-inhibited mice compared to control mice (Fig. 13). In CD59^{-/-} mice at 72 hours post-SCI, macrophage numbers were the same as in control mice, and this is in contrast to the increased neutrophil infiltration seen in CD59^{-/-} mice.

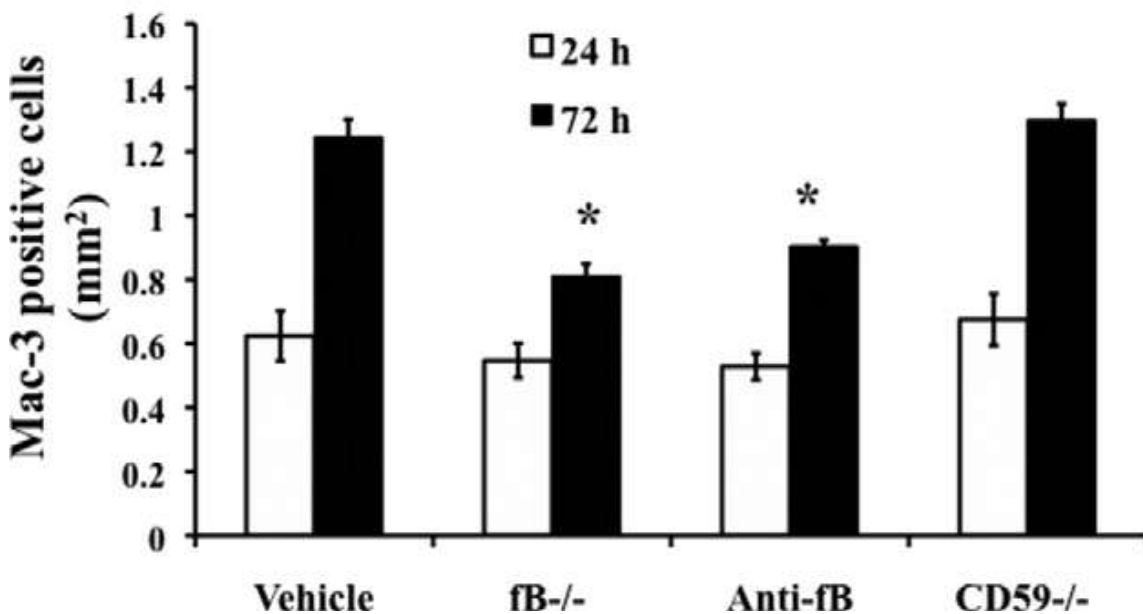


Fig. 13. Macrophage infiltration after SCI. Sections from the epicenter of injury were prepared from spinal cords isolated at 24 and 72 hours after injury. Mice received anti-fB mAb or PBS (vehicle) at 1 and 12 hours after injury. Macrophages were identified and counted by immunohistochemistry using anti-mouse Mac-3 antibody. Results are expressed as the number of macrophages per mm². Mean \pm SD n = 6 per group. * $P < 0.01$ vs. vehicle control.

These data indicate an important role for the alternative pathway in neutrophil and macrophage infiltration after SCI, and together with the above data demonstrate that the infiltration of these leukocytes is associated with tissue injury.

In this study, the total number of neutrophils and macrophages 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 postinjury in some groups and because sections were analyzed using immunohistochemistry, which did not permit morphological evaluation.

4.4. 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(63). The microenvironment of the spinal cord is different, and access of macromolecules and inflammatory cells is restricted by the bloodspinal cord barrier. On damage, however, the bloodspinal 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 ¹²⁵I-labeled CR2-Crry in normal control mice and in mice subjected to SCI. ¹²⁵I-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 (Fig. 14). The location of CR2-Crry within the kidney and liver is likely associated with the nonspecific clearance of the protein. Of note, no ¹²⁵I-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 ¹²⁵I-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 (Fig. 14A). The absence of ¹²⁵I-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, 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 (Fig. 14B). 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 (Fig. 14C).

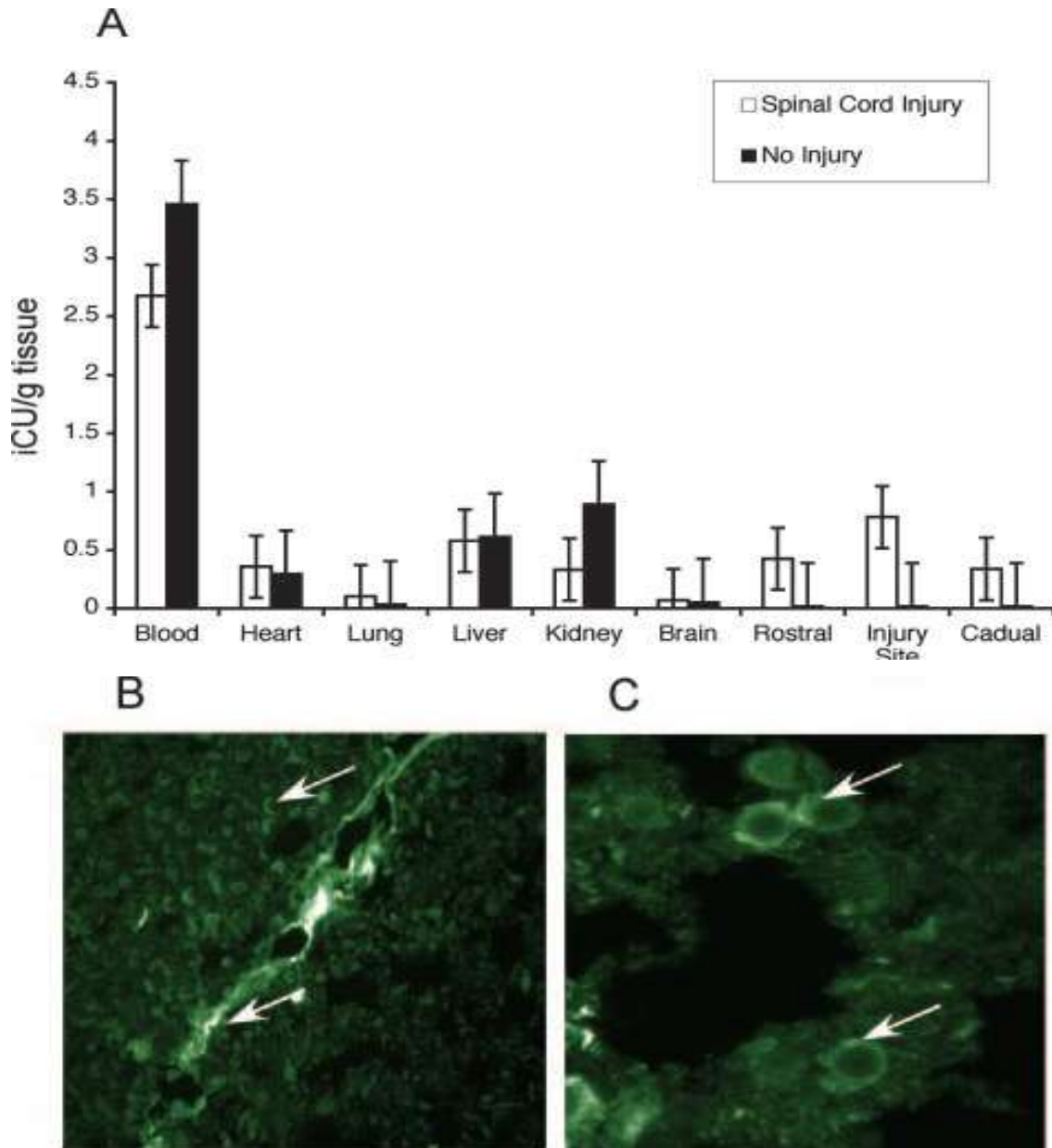


Fig. 14. A: Biodistribution of CR2-Crry following spinal cord injury. ^{125}I CR2-Crry was injected into normal C57Bl/6 mice (no injury) and C57Bl/6 animals 1 hour following spinal cord injury. Tissue distribution was assessed 12 hours after initial injury in major organs and tissues of the central nervous system. **B: Immunolocalization of CR2-Crry binding with an anti-CR2 Ab.** Positive immunofluorescence 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 $\times 63$ objective and are representative of $n = 4$.

4.5. Deposition of Complement Activation

To correlate post-traumatic inflammation and injury with complement activation, we examined spinal cord sections for deposited C3, fB, and C9 (MAC) by immunofluorescence microscopy.

4.5.1. Time Course of Complement Activation 1, 24 and 72 hours 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 (Fig. 15 A). 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 (Fig. 15 B). By day 3 after injury, complement deposition was almost undetectable, with no C3 staining evident at the injury site (Fig. 15 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(10). 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(6). However, this apparent difference is likely a consequence of animal size and differences in size of impact injury required to produce an equivalent condition.

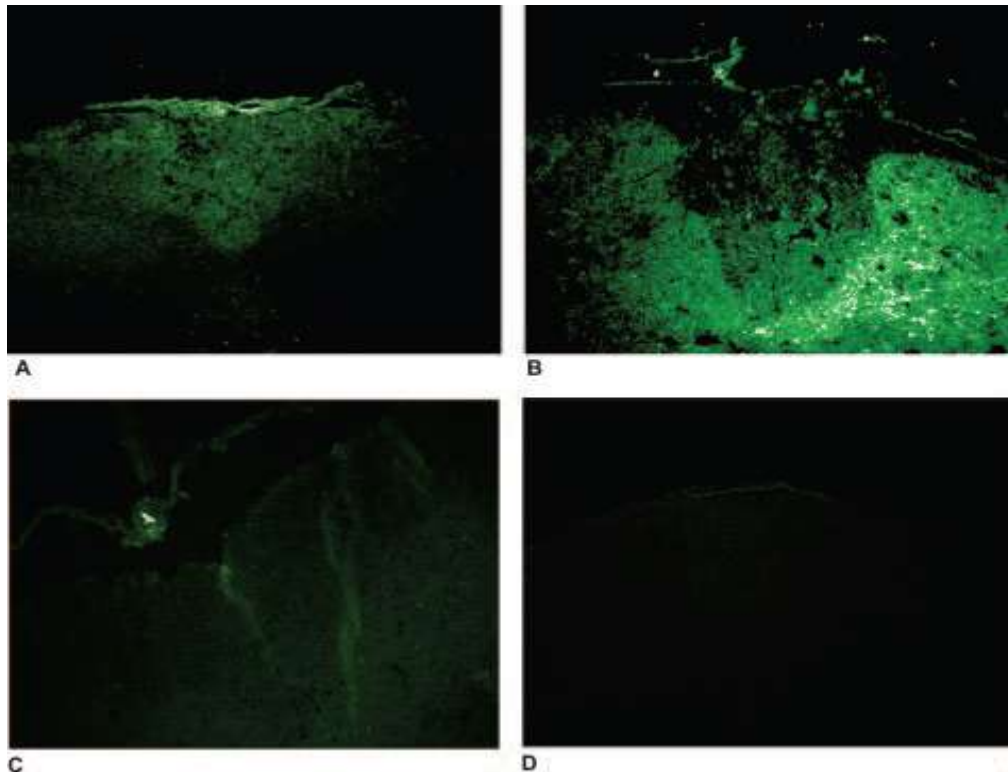


Fig. 15. Complement activation as shown by C3 deposition at the site of spinal cord injury. 1 hour (**A**), 24 hours (**B**), and 72 hours (**C**). C3 deposition is marked by green fluorescent staining of the polyclonal anti-mouse C3 antibody in spinal cords harvested at different time points after injury (n= 3). In contrast laminectomy sham controls lack immunoreactivity for C3 (**D**).

4.5.2. Complement deposition at epicenter of injury 24 h post-SCI

Representative immunofluorescence images showing complement deposition, as well as CD59 expression, at 24 hours after injury are shown in Fig. 16. There was no evidence of C3, fB, or C9 staining in spinal cords from sham operated mice, and expression of CD59 was similar in wt control treated and fB^{-/-} mice and absent in CD59^{-/-} mice (Fig. 16).

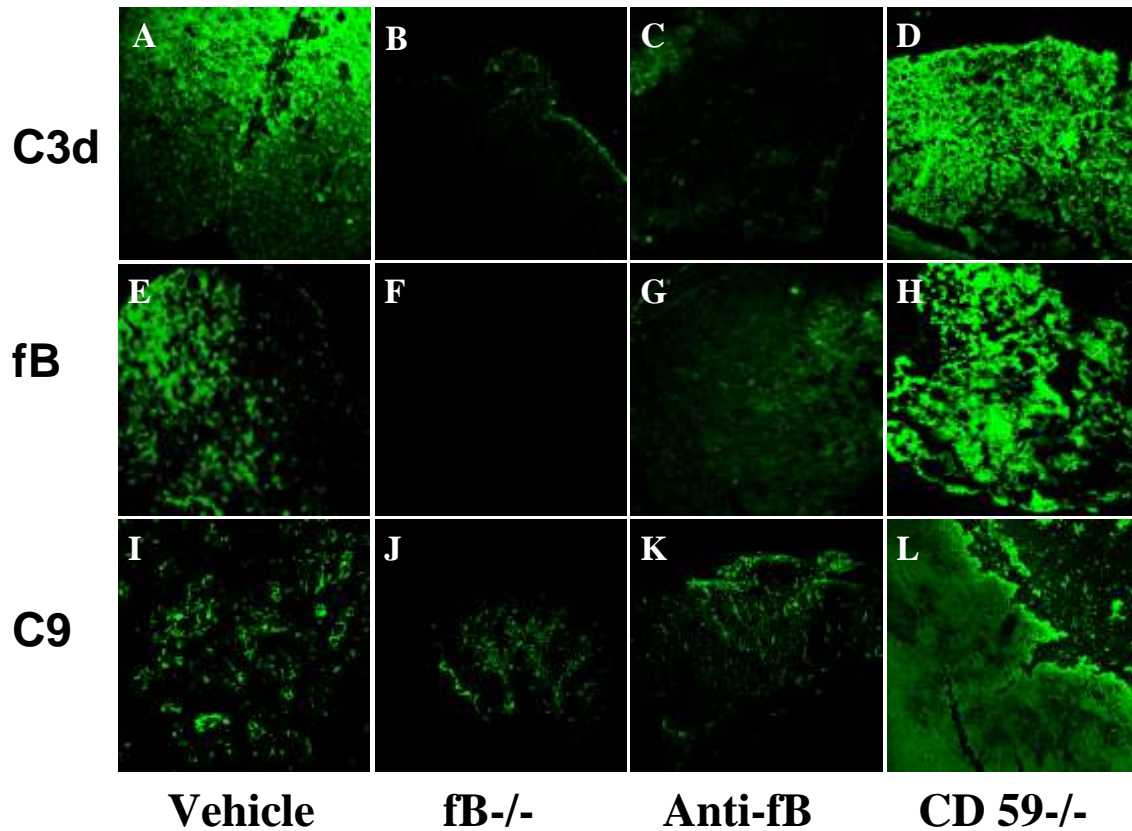


Fig. 16.. Complement deposition at epicenter of injury 24 h post-SCI. To correlate post-traumatic inflammation and injury with complement activation, we examined sections for deposited C3d, fB, and C9 (MAC). There was pronounced deposition of all 3 complement proteins in the white and gray matter of vehicle control mice. C3d and C9 deposition was significantly lower in fB^{-/-} and anti-fB Ab treated mice compared to vehicle control mice. As expected, fB was undetectable in fB deficient mice, and was detected at only very low levels in anti-fB Ab treated mice. Deficiency of CD59 resulted in significantly increased levels of C9 deposition compared to all groups (including vehicle controls), but did not effect levels of C3 or fB deposition. Sections were examined at 200x magnification. n = 6 per group.

4.5.3. Quantitative assessment of complement deposition post-SCI

In agreement with previous data obtained using mice(64) (for C3 deposition) and rats(6) (for fB and C9 deposition), there was pronounced deposition of all three complement proteins in the white and gray matter of control mice at 24 hours postinjury. C3 and C9 deposition was significantly lower in fB-deficient and fB inhibited mice compared to control mice. As expected, fB was undetectable in fB-deficient mice and was detected at only very low levels in fB-inhibited mice. Deficiency of CD59 resulted in significantly increased levels of C9 deposition compared to all groups (including controls) but did not effect levels of C3 or fB deposition (Fig. 17A).

Similar relative profiles for C3, fB, and C9 deposition were seen at 72 hours post-SCI in all groups, although C3 and fB levels were lower than at 24 hours after SCI (Fig. 17B). C9 levels were similar at 24 and 72 hours after SCI (Fig. 17 A and B).

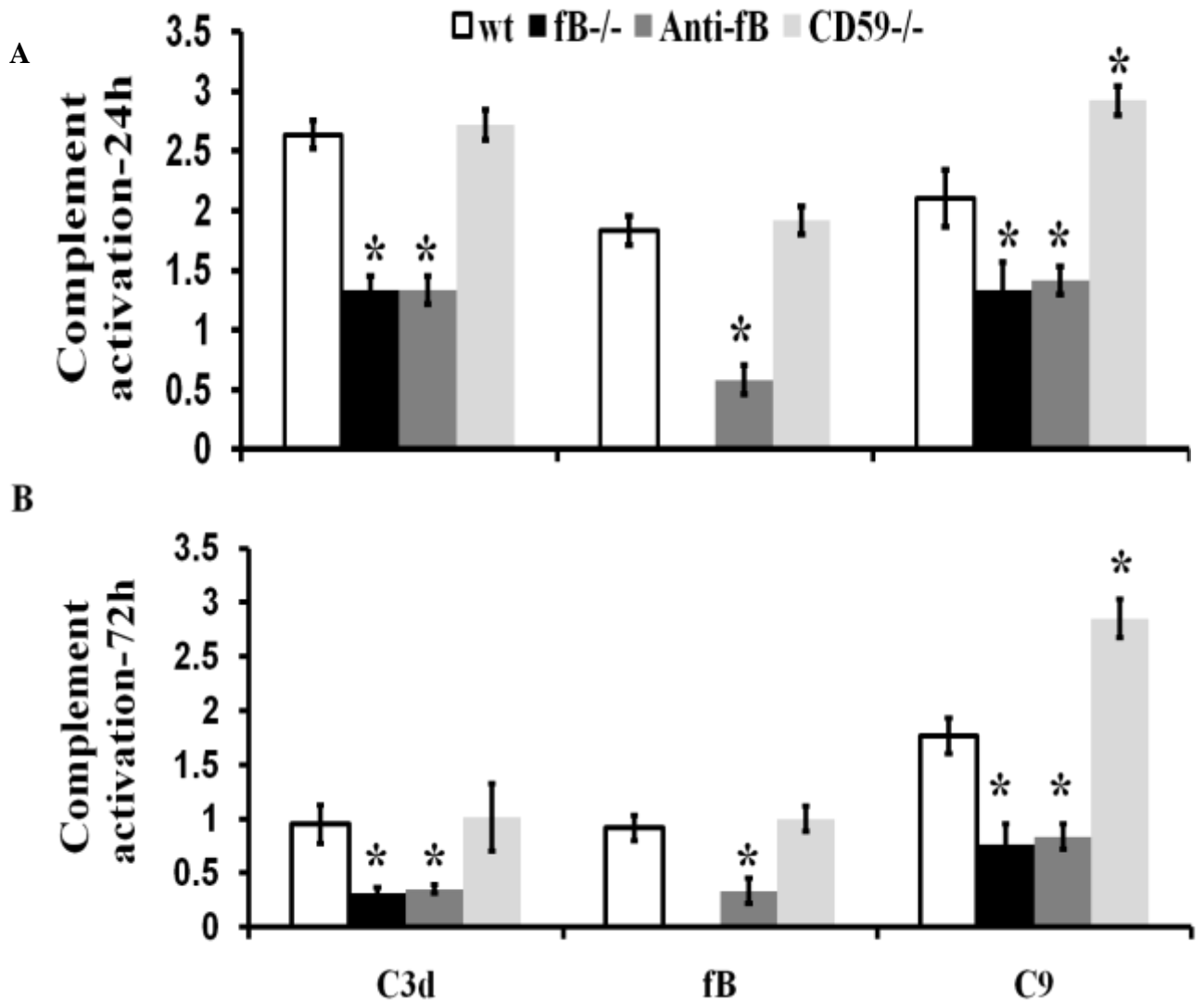
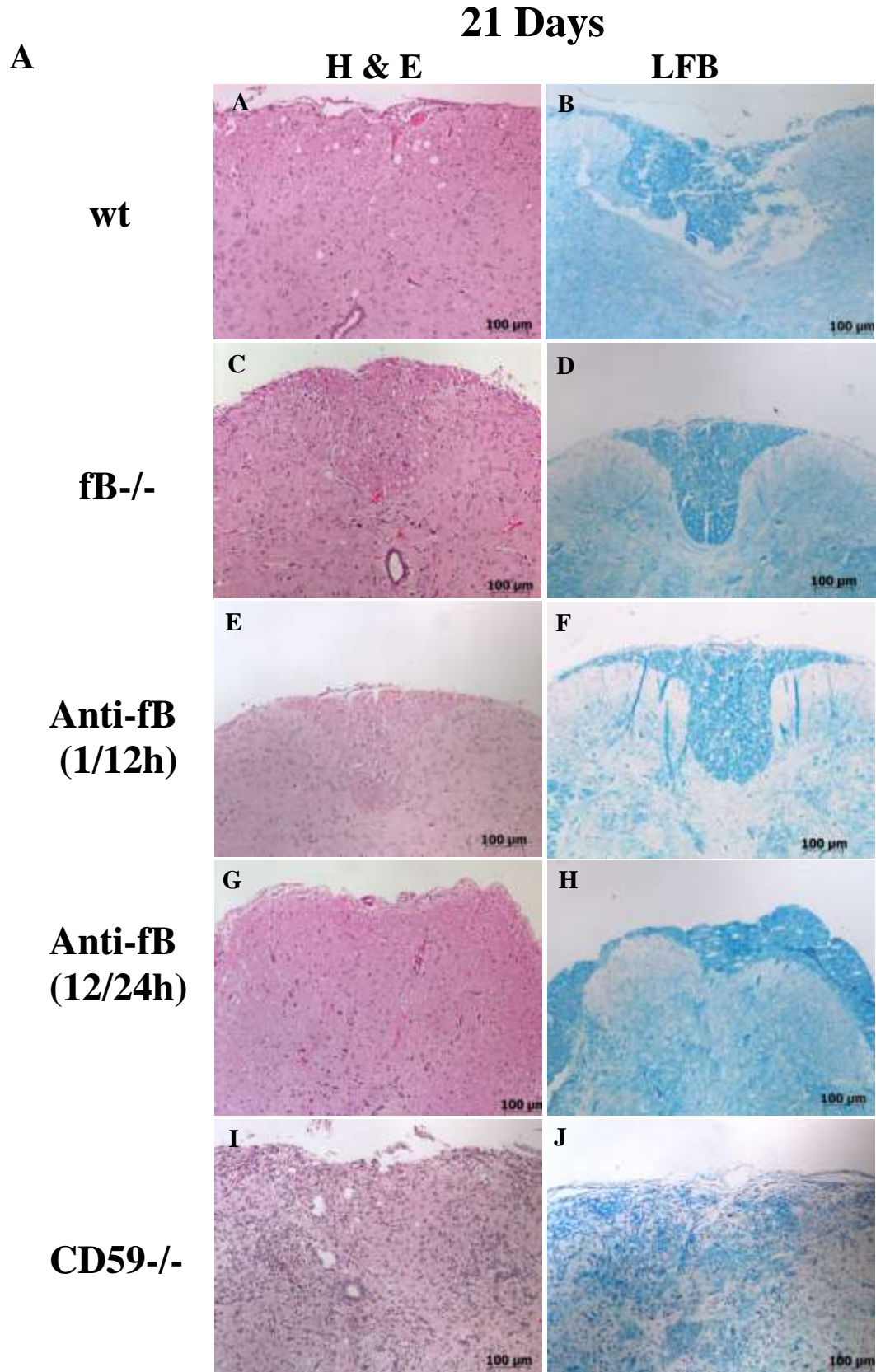


Fig. 17. Quantitative assessment of complement deposition post-SCI. The deposition of C3 (C3d), fB, and C9 (MAC) was analyzed at 24 hours (A) and 72 hours (B) after injury in spinal cord sections from the epicenter of injury. Mice received anti-fB mAb or PBS (vehicle) at 1 and 12 hours after injury. Analysis was performed using immunofluorescence microscopy, and sections scored for fluorescence intensity on a 4 point scale (see Materials and Methods). Mean \pm SD, n = 6. * P < 0.01.

In this study, we detected C3 deposition at 72 hours post-SCI, although at low levels. This is in contrast to our previous report that C3 was undetectable in mouse spinal cords 72 hours after injury, and we attribute this difference to the use of a different detection antibody (see Materials and Methods).

4.6. Histological Analysis of Recovery

Data presented above show that after SCI, fB-deficient mice and fB-inhibited mice (when treated at 1 and 12 hours after SCI) have a significantly improved outcome compared with controls in terms of inflammation, tissue injury, and functional locomotor recovery. To determine the extent of histological recovery, we analyzed spinal cords 21 days after the initial impact injury. H&E and LFB staining of cord sections from wt and PBS treated controls revealed demyelination in the central core of the white matter beneath the impact site and evidence of vacuolation with some necrosis with inflammatory cells still present (Fig. 18 A and B.). By comparison, at 21 days after SCI, there was markedly less demyelination and inflammation and no evidence of necrosis in cords from fB deficient mice and mice treated with anti-fB mAb at 1 and 12 hours after SCI. There was no apparent difference in the extent of demyelination between the fB-deficient and fB-inhibited (1/12 hours) groups. In contrast, CD59^{-/-} mice exhibited a serious lack of structural organization, abundant inflammatory cell infiltration, scarring, vacuolation, and no obvious myelin structure. Also, in agreement with locomotor recovery data, when anti-fB mAb was administered 12 and 24 hours after SCI (or 24 and 36 hours, not shown), there was no significant improvement in histological evidence of recovery when compared to wt and PBS treated controls.



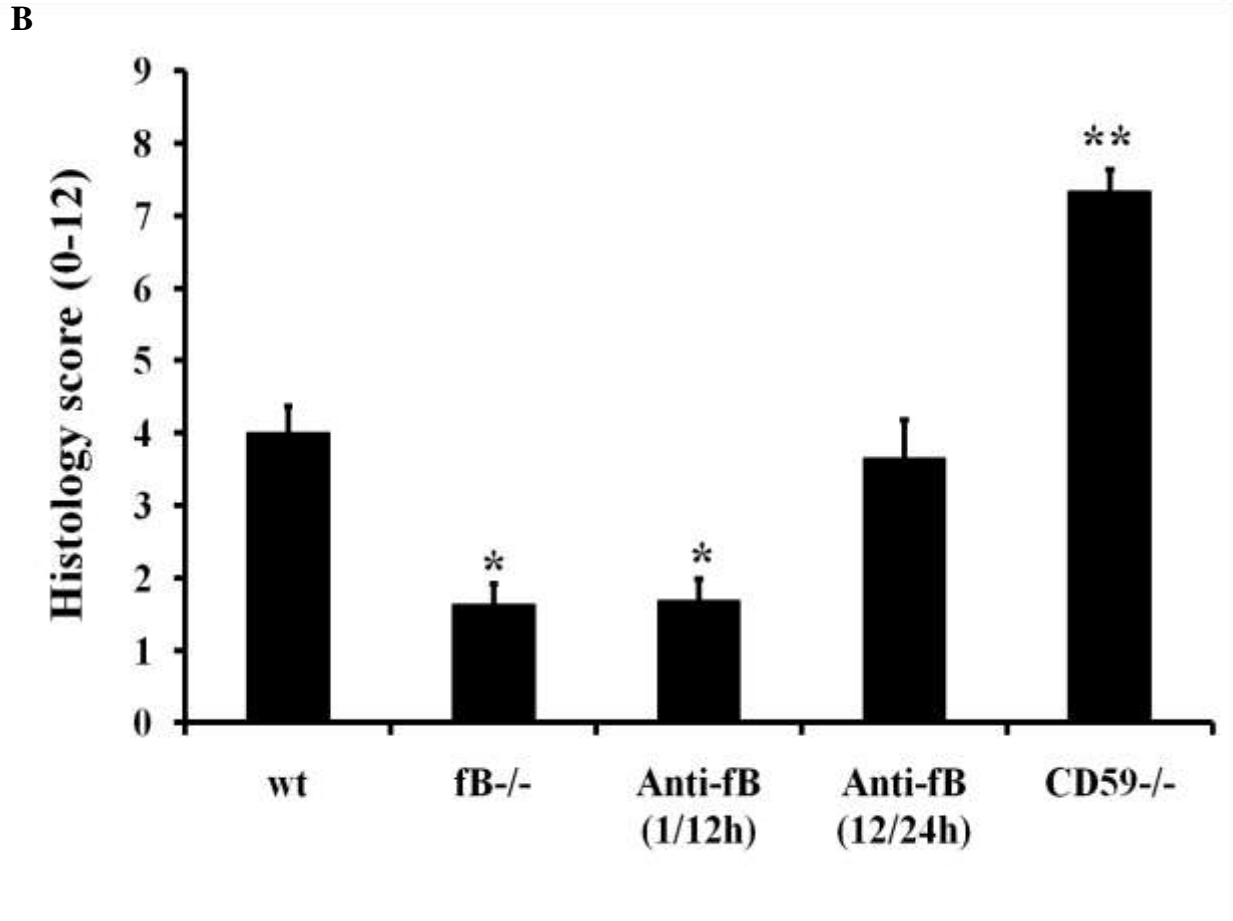


Fig. 18. Histopathology of spinal cord sections 21 days after injury. **A:** Images of transverse sections from spinal cords at the epicenter of injury. Sections stained with either H&E or LFB. Representative images, n = 6 per group. **B:** Quantitative assessment of histopathological inflammation, injury, and demyelination. H&E-stained sections were scored on a 0–3 scale for the presence and intensity of inflammatory cell infiltration, neuronal vacuolation, and hemorrhage. LFB-stained sections scored for demyelination (0 = no evidence, 3 = severe). Anti-fB mAb treatment groups received injections at 1 and 12, or 12 and 24 hours after injury. Vehicle control group (PBS) received injections at 1 and 12 hours after injury. Scores were then expressed as a cumulative score of 0–12. Mean \pm SD, n = 6 per group. *P < 0.001 and **P < 0.0001 vs. vehicle control and wt.

5. 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.

Post-traumatic inflammation and secondary tissue damage after SCI is characterized by, among other things, complement activation, expression of inflammatory cytokines and chemokines, the up-regulation of adhesion molecules, and the infiltration of neutrophils and macrophages. Complement activation products play known roles in promoting the expression of these inflammatory molecules and the recruitment and activation of leukocytes.

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. (6) 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(6). 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.

Complement is an important component of host defense, and the classical and lectin pathways provide important contributions to defense against some pathogens. Thus, compared with C3 inhibition, alternative pathway inhibition would be less generally immunosuppressive and may have less potential for increasing the incidence of sepsis or infection. Complement is also implicated in neuroprotective and regenerative mechanisms. Activated microglia and macrophages have been shown to be important for neuron protection and axon growth in animal models(89), and complement activation

products, in particular C5a, can recruit and activate these cells. A recent study demonstrated that the treatment of rats 14 days post-SCI with a C5a receptor antagonist reduced locomotor recovery and myelination(90). Complement also participates in phagocytic clearance of apoptotic cells and cellular debris via their opsonization with C1q and C3 activation products, and interfering with this clearance pathway can impair axon growth (89, 91). Thus, compared with total complement blockade, allowing activation of the classical (or lectin) pathway while inhibiting alternative pathway amplification could feasibly improve the balance between the neurodegenerative and neuroprotective effects of complement inhibition.

5.1 Role of C3 and complement inhibitory protein CR2-Crry during SCI

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(10), 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 al6 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(56, 92). 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(93). 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 (81, 83, 94, 95). 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 (96-99). 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(100).

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(101).

5.2 Role of fB through alternative pathway during SCI

In the present study, we show that fB-deficient mice have an improved outcome after SCI. The level of protection afforded to fB-deficient mice was similar to that seen in our study using C3-deficient mice. These data indicate a dependence on the alternative pathway for complement-mediated SCI. Together with the previous findings described above using B cell and C1q-deficient mice, it is likely that the alternative pathway plays a critical role in amplifying antibody-mediated classical pathway initiated complement activation. In this context, reduced pathology and improved locomotor recovery in fB-deficient mice was associated with significantly reduced C3 and MAC deposition compared to wt control mice, even though the classical (and lectin) pathway is intact in fB-deficient mice. A similar dependence on both antibody-mediated lectin pathway activation and alternative pathway activation has been described in models of intestine ischemia and reperfusion injury(65-67). In a more clinically relevant paradigm, we demonstrated that specific inhibition of the alternative pathway with anti-fB mAb was also protective against SCI, and to a similar level as fB deficiency. Selective inhibition of the alternative pathway while leaving the classical and lectin pathways intact has potential benefits over inhibiting all pathways at the C3 activation step.

In the current study, we show that compared with control treated mice, there are significantly reduced neutrophil numbers in fB-deficient and fB-inhibited mice at both time points post-SCI. We similarly showed reduced macrophage/ microglia numbers in fB-deficient or fB-inhibited mice compared with controls at 72 hours postinjury, whereas it was previously shown that C1q deficiency was (interestingly) associated with increased macrophage infiltration(102). Together, these data indicate a key role for the alternative pathway in neutrophil and macrophage infiltration after SCI.

As discussed above, for reasons relating to the protective 3068 Qiao et al AJP December 2010, Vol. 177, No. 6 roles of complement that are relevant for SCI patients, there are potential benefits of selectively inhibiting the alternative pathway of complement to reduce secondary damage and improve functional recovery after SCI.

5.3 Role of CD59 through terminal complement pathway during SCI

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(28, 48, 103-106). 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(107). Deficiency in serum C3 and C4, but not C5, C6, or factor B was protective against demyelination, 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(107)).

The exacerbated injury and impaired recovery of locomotor function in CD59a-deficient mice, together with increased deposits of the MAC (C9) in these mice compared to wt control treated mice, indicate an important contribution of the terminal pathway and the MAC to secondary neuronal injury after trauma. Furthermore, significant functional deficit persisted in CD59-deficient mice at 21 days after injury compared with controls. It is possible that neoepitope expression and cell lysis resulting from enhanced MAC formation in CD59-deficient mice could result in enhanced complement activation.

However, while there were significantly increased deposits of C9 in CD59-deficient mice at both 24 and 72 hours post-SCI compared with wt treated control mice, CD59 deficiency had little effect on C3 or fB deposition. Previous studies using CD59a-deficient mice have similarly implicated the MAC in spinal cord demyelination and axonal injury in murine acute experimental allergic encephalomyelitis(106), as well as in axonal damage during Wallerian degeneration (peripheral nerve injury)(108). The alternative and terminal complement pathways have also been implicated in causing

neuropathology in a different model of traumatic neuroinflammation, traumatic brain injury. In these previous studies, CD59a deficiency impaired neurological outcome and increased neuronal cell death and brain tissue destruction compared to wt mice(109),

It is interesting that unlike fB deficiency, CD59 deficiency did not significantly effect the number of macrophages at 72 hours post injury. Neutrophils, on the other hand, were present in significantly higher numbers in CD59-deficient mice compared to wt treated controls at 72 hours after injury. The MAC can influence cellular infiltration when deposited in sublethal quantities in cell membranes by inducing the expression of adhesion molecules, cytokines, and chemokines, including IL-8(98, 110-114). Why there is an apparent difference in CD59-deficient mice on neutrophil and macrophage infiltration at 72 hours post injury (compared to controls) is not clear, but a consequence could be the protraction of neutrophil mediated inflammatory events and a reduction in macrophage- mediated protective events. In addition to showing that the complement inhibitor CD59 plays an important role in providing protection against SCI, the data have potential clinical implications.

However, the MAC plays a very limited known role in host defense and immune homeostatic/repair mechanisms, and because our data indicate that the MAC is a significant contributor to pathology in SCI, selective inhibition of the terminal pathway could potentially have even greater benefit over blocking complement at an early step in any pathway.

6. Summary and conclusions

An impact to the spinal cord results in a primary injury, but the impact also triggers a series of downstream events that lead to secondary injury of tissue and the progressive degeneration of the spinal cord. Therapeutic interventions that minimize secondary injury will improve functional recovery after traumatic injury. Inflammation plays a key role in secondary injury following spinal cord injury (SCI). Inflammation is a rapid immune response that can be initiated by infection or tissue damage. An important component of an inflammatory response is the complement system, a collection of blood proteins that form part of the immune system and that can be activated by injured cells and tissues. Activation of complement amplifies the inflammatory response and produces molecules that can be directly toxic or that can recruit and activate cells of the immune system to produce toxic molecules. Inhibiting the complement system has been shown to be an effective therapy for inflammatory disease in various animal models, and some complement inhibitors are in clinical trials.

We have shown that complement deficient mice and normal mice treated with a complement inhibitor are protected from neuronal injury following SCI, and that they have significantly improved functional scores over time compared to control mice. These data indicate an important role for complement in secondary injury, and indicate complement inhibition will reduce inflammation and provide neuroprotection following spinal cord injury. However, complement-dependent mechanisms involved in secondary injury following SCI are not known, and there remain concerns regarding the clinical application of the currently available systemic (body-wide) complement inhibitors with regard to their safety and efficacy. Complement activation products are important for host defense and immune maintenance mechanisms, and systemic complement inhibition can compromise the protective and beneficial roles of complement. This will not be optimal in patients at risk of infection, and urinary tract infection is a frequent complication during initial and ongoing medical rehabilitation after SCI.

We propose to develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI using a novel and validated approach. The strategy involves the targeting of complement inhibitors to sites of complement activation and injury, and we have shown the approach to be highly effective

in vitro and in mouse models of SCI and other inflammatory disease conditions. The targeting strategy enhanced the activity and protective effect of complement inhibitors by 10-20 fold compared to untargeted counterparts. Furthermore, untargeted, but not targeted complement inhibitors systemically inhibited complement and increased susceptibility to infection in a mouse model.

We propose to fully characterize our targeted complement inhibitors in a mouse model of SCI. In addition to therapeutic endpoints, we will use different types of complement inhibitor to investigate complement-dependent disease mechanisms. We will determine relationships between the generation of different complement activation products and other molecules associated with inflammation in order to gain a better understanding of the mechanisms of secondary tissue injury following SCI. These preclinical studies will establish the guidelines necessary for the translation of this therapy to the clinic using recombinant human proteins.

7. Abstract

An inflammatory cascade that is initiated following traumatic spinal cord injury (SCI) is thought to cause secondary injury and adversely impact functional recovery. Complement is implicated in the inflammatory response and the secondary neuronal damage that occurs after traumatic spinal cord injury (SCI). Complement can be activated by the classical, lectin, or alternative pathways, all of which share a common terminal pathway that culminates in formation of the cytolytic membrane attack complex (MAC).

Here, we investigated the role of the alternative and terminal complement pathways and role of complement in the secondary injury process occurring following acute contusion SCI in mice.

The dynamics of complement activation post-SCI were investigated by assessing the deposition of C3 in spinal cords. C3 deposition occurred within 1 h post-SCI at the injury site, with C3 staining extending into the ventral and dorsal horns by 12 h. C3 deposition was almost undetectable at 3 days after SCI. Mice deficient in C3 had a significantly improved locomotor score compared to wild type controls and regained almost full locomotor function by day 21 after injury.

To further investigate the role of complement in SCI, we determined the effect of fB and CD59 deficiency on SCI. Mice deficient in fB had significantly improved locomotor scores compared to wt controls, although recovery was not as complete as in C3 deficient mice (difference was significant, $p < 0.01$). CD59 deficient mice had significantly worse locomotor scores compared to wt controls.

Wild type mice were also treated with CR2-Crry, an inhibitor of mouse complement activation that targets to sites of complement activation. A single intravenous injection of CR2-Crry 1 h post-injury significantly improved functional recovery and reduced tissue injury and demyelination compared to PBS treated controls and to a similar extent 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.

Together, these data indicate an important role for complement in the secondary injury process following SCI, with a significant role for both the alternative pathway and

the membrane attack complex in causing tissue injury. The feasibility of targeted complement inhibition as a therapeutic strategy for improving post-traumatic recovery.

8. ABSTRACT IN HUNGARIAN (ÖSSZEFOGLALÁS)

A traumát követő gerincvelő sérülés (spinal cord injury - SCI) során az aktiválódó gyulladási kaszkád másodlagos károsodást okoz és hátrányosan befolyásolja a gyógyulást. Ismert, hogy a komplement rendszer befolyásolja a gyulladási választ és a neuronális károsodást, amely a trauma kapcsán kialakult gerincvelő-sérülésnél jelentkezik. A komplement rendszer három útvonalon keresztül aktiválódhat (klasszikus, lektin ill. alternatív út); az aktiváció egy közös terminális útvonalon keresztül a membránkárosító-komplex (membrane attack complex - MAC) kialakulásához vezet.

Jelen munkánkban egerekben vizsgáltuk a komplement kaszkád alternatív- és terminális útvonalának jelentőségét az akut kontúziót követő másodlagos gerincvelő-károsodásban. A SCI utáni komplement-aktiváció dinamikáját a gerincvelőben lévő C3 depozitumok meghatározásával követtük. A depozitumok már a sérülést követő 1 órán belül kimutathatóak voltak, 12 óra után az elülső és hátsó szarv felé terjedtek, végül 3 nap múlva már nem voltak jelen. Megfigyeltük, hogy a C3 deficiens egereknek jelentősen jobb volt mozgásszervi státusza a vad típusú kontroll egerekhez képest és 21 nappal a sérülés után teljesen visszanyerték mozgásszervi funkcióikat.

A komplement rendszer szerepét tovább vizsgálva SCI-ben, meghatároztuk a fB és CD59 deficiencia hatását. Az fB hiányos egerek mozgásszervi score-ja szignifikánsan jobb volt a vad típushoz képest, bár a gyógyulás nem volt olyan mértékű, mint C3 deficiencia esetében ($p < 0,01$). A CD59 hiányos egerek jelentősen rosszabb mozgásszervi score-ral rendelkeztek a vad típushoz képest. A vad típusú egereket a komplementrendszer aktivációját lokálisan gátló CR2-Crry-vel kezeltük. A C3-deficiens állatoknál tapasztaltakhoz hasonlóan, a CR2-Crry egyszeri intravénás alkalmazása 1 órával a sérülést követően jelentős mértékben javította a funkcionális gyógyulást, csökkentette a szöveti sérülést és a demyelinizációt a PBS-sel kezelt kontroll csoporthoz képest. CR2-Crry kifejezetten a sérült gerincvelőt célozza meg a C3 depozitumoknak megfelelően.

Ezek az adatok együttesen a komplement rendszer kiemelt szerepét mutatják a gerincvelő sérülés másodlagos károsodásában. Ebben meghatározó szerepe van mind az alternatív útvonalnak, mind pedig a MAC kialakulásának. Terápiás lehetőségként felmerül a komplement rendszer gátlása a poszt-traumás gyógyulás javításában.

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10. PUBLICATIONS OF PH.D. CANDIDATE

1. List of publication related to the thesis

Peer reviewed articles

- 1) Atkinson C, Song H, Lu B, **Qiao F**, Burns TA, Holers VM, Tsokos GC, Tomlinson S: Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *The Journal of clinical investigation* 2005, 115(9):2444-2453.
- 2) **Qiao F**, Atkinson C, Song H, Pannu R, Singh I, Tomlinson S: Complement plays an important role in spinal cord injury and represents a therapeutic target for improving recovery following trauma. *The American journal of pathology* 2006, 169(3):1039-1047.
- 3) Huang Y, **Qiao F**, Atkinson C, Holers VM, Tomlinson S: A novel targeted inhibitor of the alternative pathway of complement and its therapeutic application in ischemia/reperfusion injury. *J Immunol* 2008, 181(11):8068-8076.
- 4) He S, Atkinson C, **Qiao F**, Cianflone K, Chen X, Tomlinson S: A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice. *The Journal of clinical investigation* 2009, 119(8):2304-2316.
- 5) **Qiao F**, Atkinson C, Kindy MS, Shunmugavel A, Morgan BP, Song H, Tomlinson S: The alternative and terminal pathways of complement mediate post-traumatic spinal cord inflammation and injury. *The American journal of pathology*, (2010) 177(6):3061-3070.

2. Other publications

Peer reviewed articles

1) Ishibashi Y, Takahashi M, Isomatsu Y, **Qiao F**, Iijima Y, Shiraishi H, Simsic JM, Baicu CF, Robbins J, Zile MR, and Cooper G 4th. Role of microtubules versus myosin heavy chain isoforms in contractile dysfunction of hypertrophied murine cardiocytes. *Am J Physiol Heart Circ Physiol.* (2003) Sep; 285(3): H 1270- 85 .

2) Cheng GM, **Qiao F**, Thomas N Gallien, Dhandapani Kuppuswamy and George Cooper,IV. Inhibition of Beta-Adrenergic Receptor Trafficking in Adult Cardiocytes by MAP4 Decoration of Microtubules. *Am J Physiol Heart Circ Physiol.* 2005 Mar; 288(3):H1193-202. Epub (2004) Nov 4.

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complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2009 Jul; 50(7):3056-64.

8) He S, Atkinson C, **Qiao F**, Chen X, Tomlinson S: Ketamine-xylazine-acepromazine compared with isoflurane for anesthesia during liver transplantation in rodents. *J Am Assoc Lab Anim Sci*, (2010) Jan;49(1):45-51.

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15) Elvington M, Huang Y, Morgan BP, **Qiao F**, van Rooijen N, Atkinson C, Tomlinson S: A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer. *Blood*, 119(25):6043-6051.

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11. Acknowledgement

I would like to thank from the bottom of heart:

Dr. Stephen Tomlinson, PhD., Professor and Vice Chair of the Department of Microbiology/Immunology at the Medical University of South Carolina, USA. My mentor, who helped and supported my research work with great enthusiasm.

Dr. Carl Atkinson, PhD, Associate professorprofessor of the Department of Microbiology/Immunology at the Medical University of South Carolina, USA, who supported my research work with great enthusiasm.

I would like to thank **Dr. Béla Merkely**, MD, PhD, DSc, Professor and director of the Department of Heart Center at the Semmelweis University, Budapest, Hungary, for helping me continuously along my career.

I am grateful to **Dr. Gábor Széplaki**, MD, PhD, Professor at Department of Heart Center at the Semmelweis University, Budapest, Hungary for a lot of helpful discussion about our research.

I would like to thank my colleagues at the Medical University of South Carolina for their help and the friendly atmosphere.

Finally, I would like to dedicate my work to my Mom, my husband and my daughter,. Without their encouragement I wouldn't have finished this work.