SC Spinal cord injury research fund

Final Report, August 25th, 2014

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Title: Inhibition of the alternative complement pathway to treat spinal cord injury

The overall aim of the project is to characterize and compare the effect of 2 types of targeted complement inhibitor on complement activation, inflammation and injury in a mouse model of spinal cord injury (SCI). The 2 inhibitors are CR2-fH and B4scFv-fH, both of which inhibit the alternative pathway of complement, but contain different targeting vehicles: CR2, that targets the C3d complement activation product, and B4scFv, a single chain antibody that targets epitopes that become exposed in the injured spinal cord. The epitopes that become exposed are recognized by natural pathogenic IgM and trigger complement activation.

The first 6 months were focused on characterizing our new B4scFv targeting vehicle (the CR2-fH construct is better characterized, and has been therapeutically applied in other models of injury). We have demonstrated that the B4 targeting vehicle recognizes both mouse and human hypoxic, but not normoxic healthy endothelial cells, and that B4scFv linked to complement inhibitors block the binding of pathogenic IgM and inhibit complement activation. Furthermore, B4scFv-mediated targeting of complement inhibition significantly improves locomotor activity and reduces tissue injury after SCI, and also reduces injury and improves outcome after ischemic stroke, another model involving CNS injury.

However, in trying to prepare the fusion protein B4scFv-fH, we encountered technical difficulties. We therefore turned our attention to investigating CR2-fH (part A), and also the role of B1 cells in SCI. B1 cells are the source of natural antibodies, and B4 mAb from which our scFv is derived is a representative natural Ab that is involved in activating complement and promoting SCI (part B).

Part A. CR2-fH and SCI

Locomotor recovery following CR2-fH treatment: CR2-fH administered 30 min after SCI significantly improved locomotor recovery as measured by BMS scores (Fig 1).

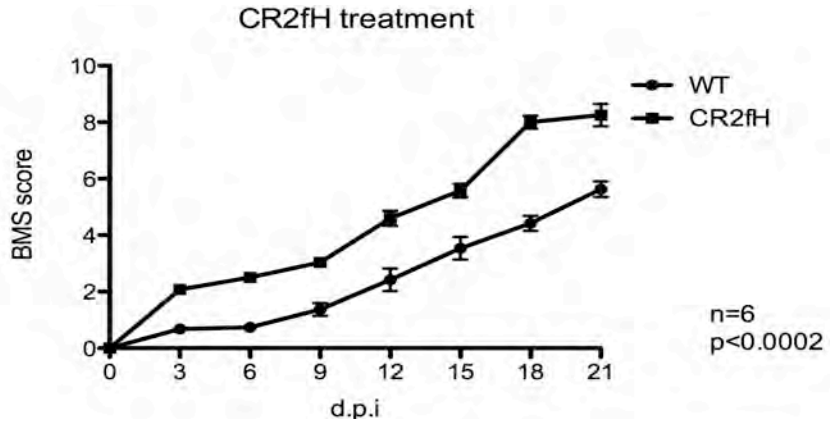

Fig. 1. Locomotor activity (BMS) after SCI. Mice were treated with PBS or single injection of CR2-fH (0.25 mg), 30 min after SCI. Mean +/- SEM, n = 7-10

Reduced complement deposition at the site of injury following CR2-fH treatment: Animals were sacrificed at 24 hours after SCIO and CR2-fH treatment. Spinal cords were harvested, fixed and embedded in paraffin. Six microns sections were prepared, deparaffinized and stained for complement deposition by anti-C3d immunohistochemistry. The results shown in Fig 2 indicate a decrease in C3d in CR2-fH treated groups, demonstrating effectiveness of CR2-fH at reducing complement activation.

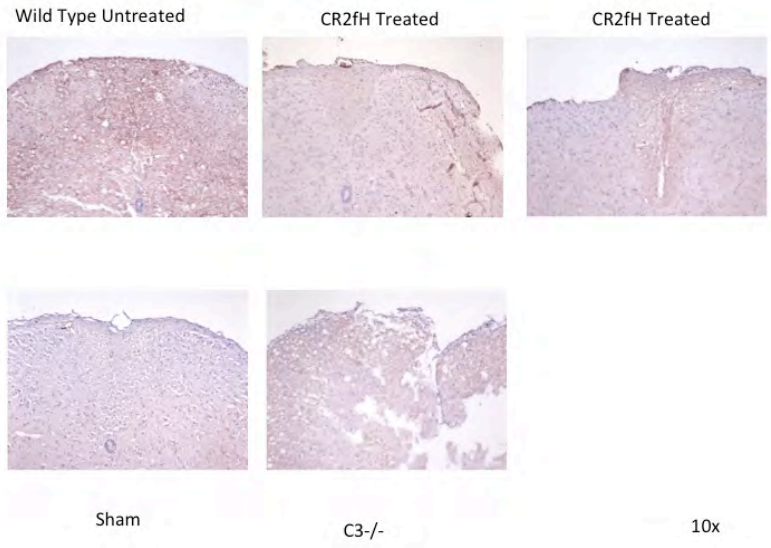

Fig. 2 anti-C3d immunohistochemistry. Spinal cord sections from CR2-fH treated mice revealed reduced deposition of C3d, a complement activation product. Representative images (n= 5-6)

In vivo live animal imaging: We attempted to demonstrate targeting of CR2-fH to the injured spinal cord by labeling CR2-fH with a fluorescent probe, and measuring localization of fluorescence in the whole animal after SCI and injection of fluorescently labeled CR2-fH. Fig. 3 shows representative image. We also labeled fH (no CR2 targeting vehicle), and also used sham operated mice. Although targeting was enhanced by addition of CR2 moiety in comparison to fH alone, we observed targeting of CR2-fH in sham animals to some extent. This is likely due to the fact that the surgery alone, without SCI, is probably activating complement, and thus the surgical site is being targeting. We are continuing with this approach, except that we plan to sacrifice the animals and perform quantitative fluorescence analysis of extracted spinal cords, along with confocal analysis.

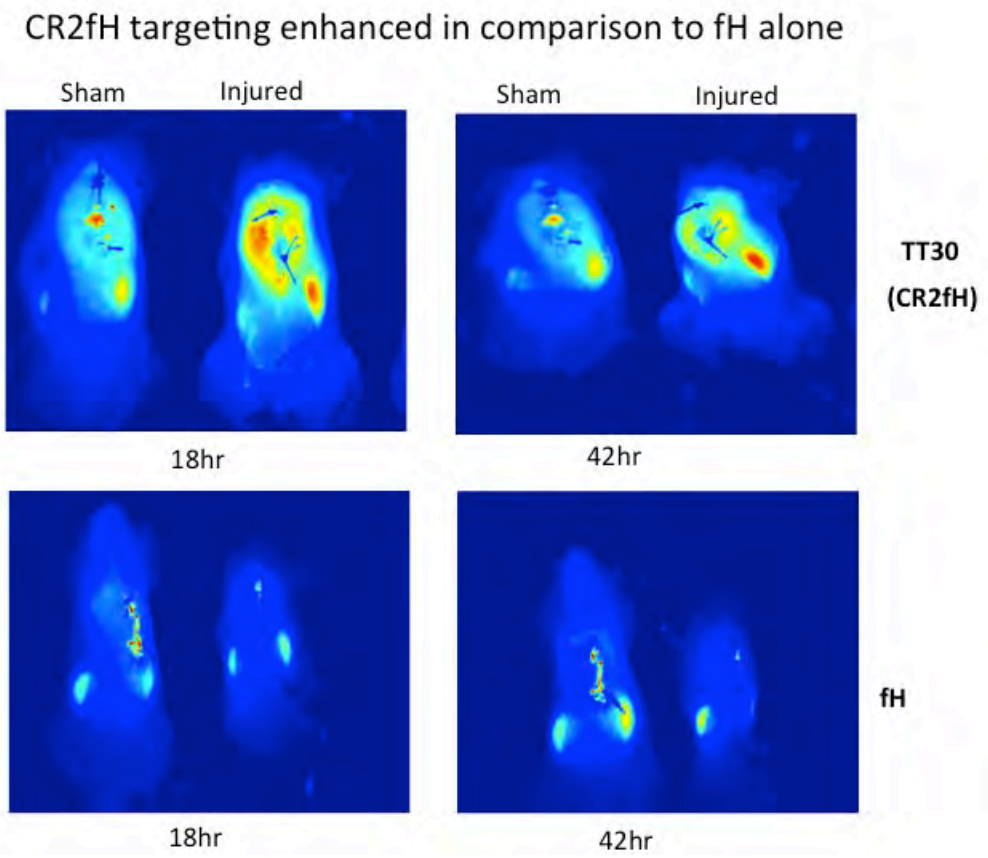

Fig. 3. Live animal fluorescence tomography. Localization of fluorescently labeled CR2-fH after sham operation of SCI.

Analysis of macrophage subsets following treatment with CR2fH: It has been shown previously that M2 macrophages promote remyelination and accelerate locomotor recovery after spinal cord injury. We attempted to measure the macrophage polarization 3 days following CR2-fH treatment. We measured mRNA levels using NanoString that utilizes labeled capture probes to accurately measure gene expression. A trend toward a decrease in M1 genes and an increase in M2 genes was observed, but the results did not reach significance (Fig. 4). An additional study is underway to repeat this investigation at later time points after SCI

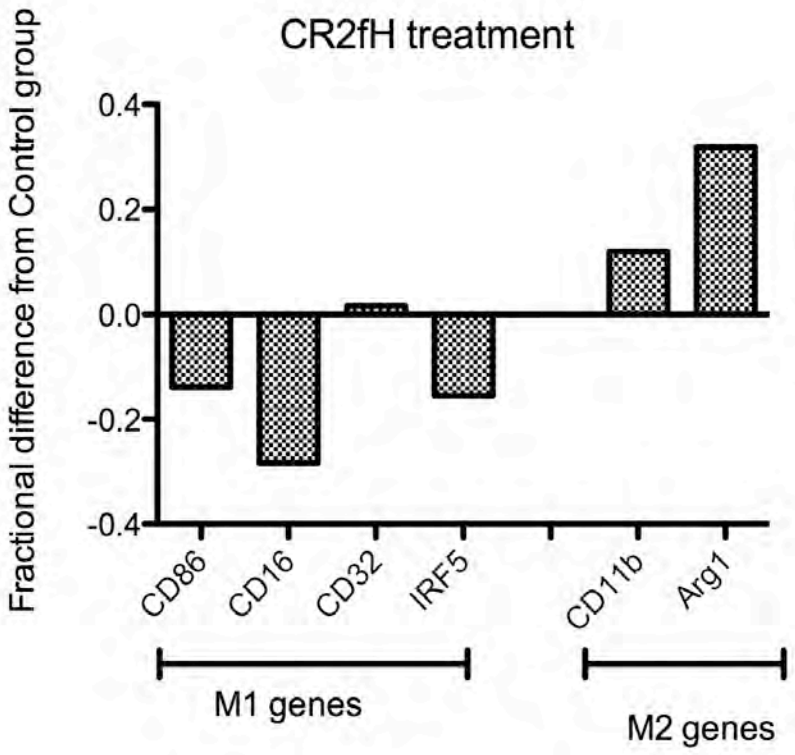

Fig. 4. Nanostring analysis of M1 and M2 macrophage genes 3 days after SCI and CR2-fH treatment.

Gait measurements: BMS score (Fig 1) employs cues like trunk stability, ability for plantar stepping and motor coordination to provide a score as a standard measure of locomotor recovery. Recent studies have utilized automated gait measurements in order to avoid inter-observer variation and make more accurate measurements of gait symmetry and hence locomotor recovery. To establish a role for complement prior to testing with CR2-fH, we performed a pilot study to measure gait in C3 deficient and wild type mice. Data shown in Fig 5 demonstrate improved gait symmetry in C3 deficient mice compared to controls, a result validating role of complement and establishing feasibility of technique for studying effect of CR2-fH.

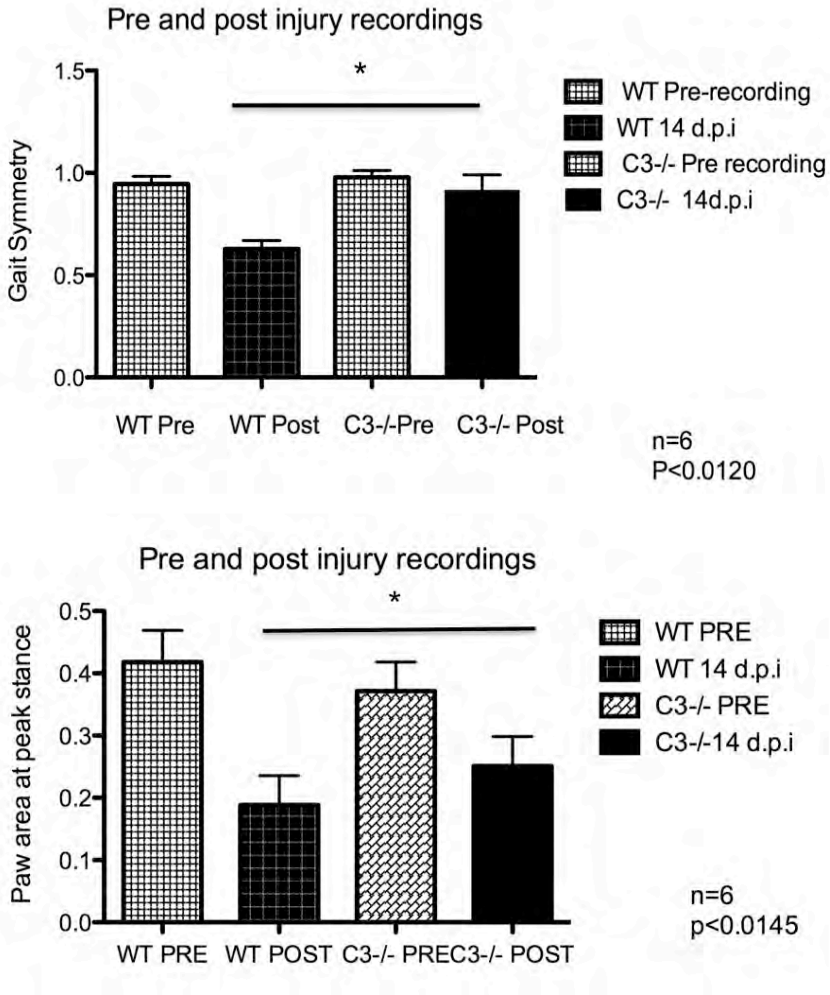

Fig. 5 Gait measurements in wt mice and C3 deficient mice pre and post (14 days) injury.

Part B. Natural antibodies in SCI and B4 scFv

IgM antibodies worsen outcomes after injury in Rag-/- mice: Rag 1-/- mice lack mature B and T cells and these mice had improved recovery following SCI compared to WT mice (Fig. 6). Reconstitution of Rag1-/- mice with either B4 mAb (anti-annexin IV) or C2 mAb (anti-phospholipid) restored SCI in Rag 1-/- mice. These 2 mAbs are representative of natural IgM Abs found in mouse and human serum. Control IgM mAb, F632, and no effect, demonstrating specificity.

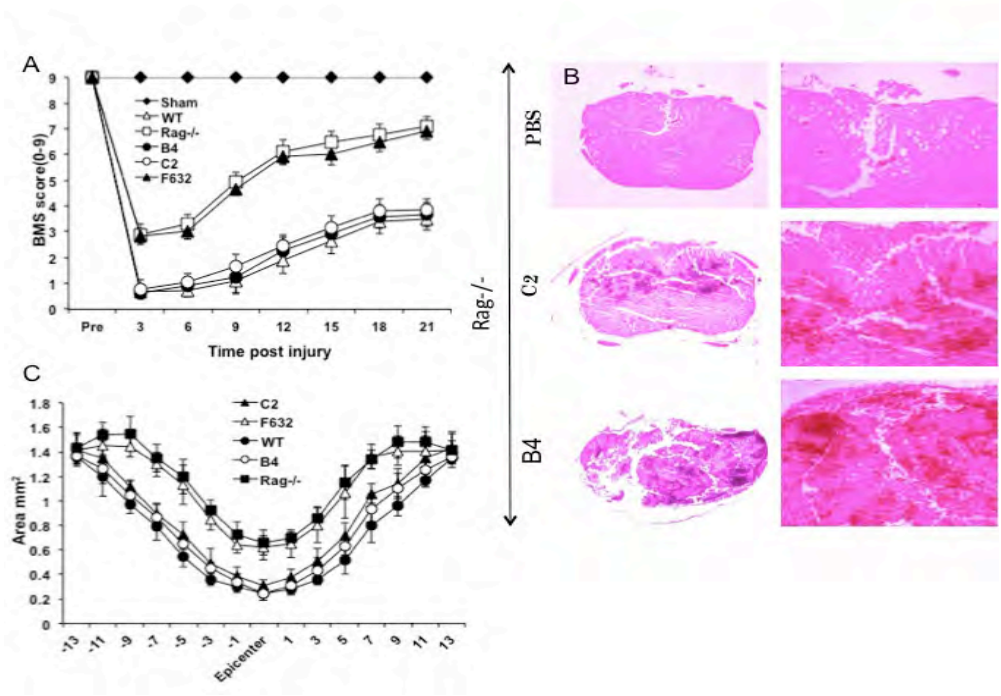

Fig. 1: Rag-/- mice are protected from injury which is reconstituted by C2 and B4 mAb
A. BMS score showing improved locomotor function in Rag1-/- mice. Reconstitution with either B4 or C2 mAbs lead to decreased recovery. F632 antibody acts as isotype control and did not affect locomotor recovery.
B. Rag1-/- mice showing improved histology at 72hrs in comparison to Rag1-/- mice with B4 and C2 mAb reconstitution.
C. Morphometric analysis revealed least lesion size in Rag1-/- mice and F632 treated mice. Administration of both B4 and C2 IgM worsened myelin sparing indicative increased damage.

Lack of IgMs due to B1 depletion confers protection from injury: Natural IgM Abs are produced by B1 cells, which are found predominantly in the peritoneal cavity. To

investigate the role of B1 cells in SCI, we depleted B1 cells by injection of water i.p. over a 6 week period. Flow cytometry confirmed B1 cell depletion. There was an overall decrease in CD19+ population and B1 cells were defined by CD5 hi and B220lo markers (Fig 7).

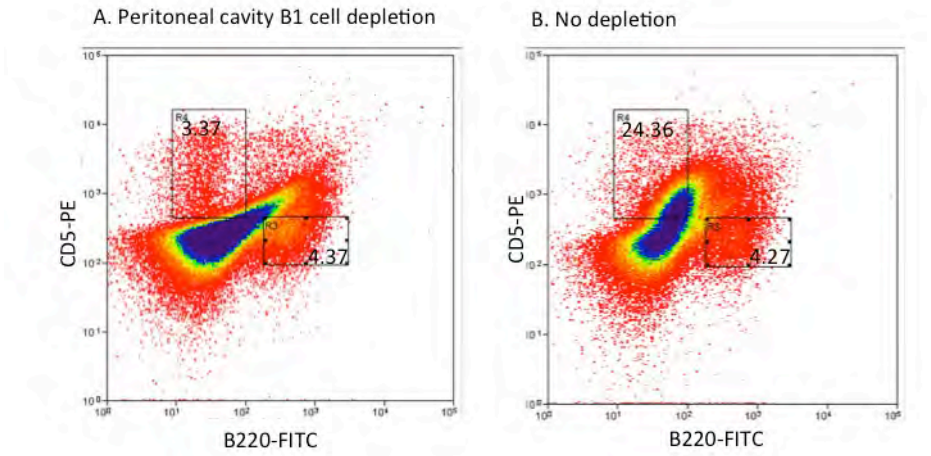

Fig. 7. Flow analysis showing B1 cells depletion after ip injection of water over 6 week period.

Levels of serum C2 IgM are decreased after B1 cell depletion: Data show that a natural Ab of specificity shown to be involved in complement activation and causing SCI (see above) is depleted from serum subsequent to B1 cell depletion (Fig 9)

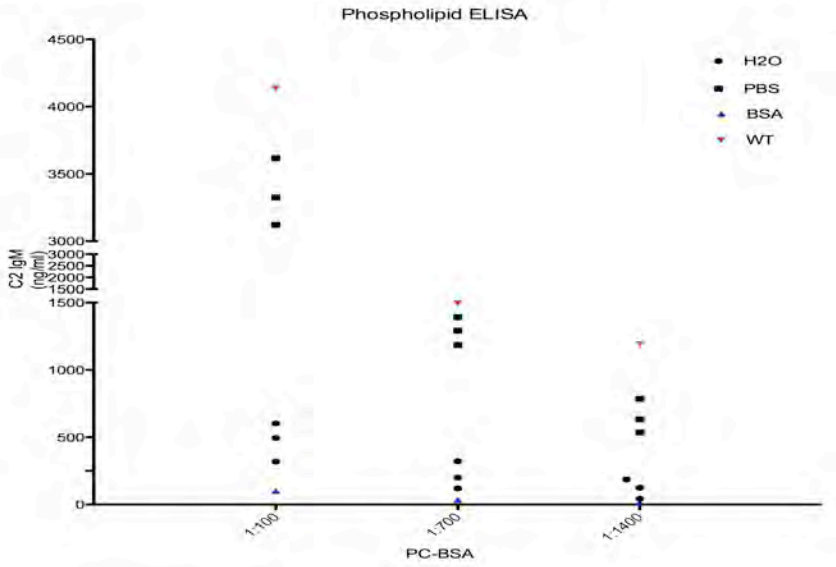

Fig. 9. Anti-PC-BSA IgM reactivity in serum from B1 cell depleted (H2O) and control (PBS) mice. ELISA analysis using PC-BSA coated plates and anti-IgM detection antibody.

IgM and C3 deposition after SCI in B1 cells depleted mice and controls: Spinal cord sections were prepared from mice 3 days after SCI from either B1 cell depleted (water treated) mice or control mice (PBS treated). Confocal analysis revealed significantly less staining of both IgM and complement (C3d) in sections from B1 cell depleted mice. Furthermore, IgM and C3d colocalized, indicating IgM-mediated complement activation (Fig 9)

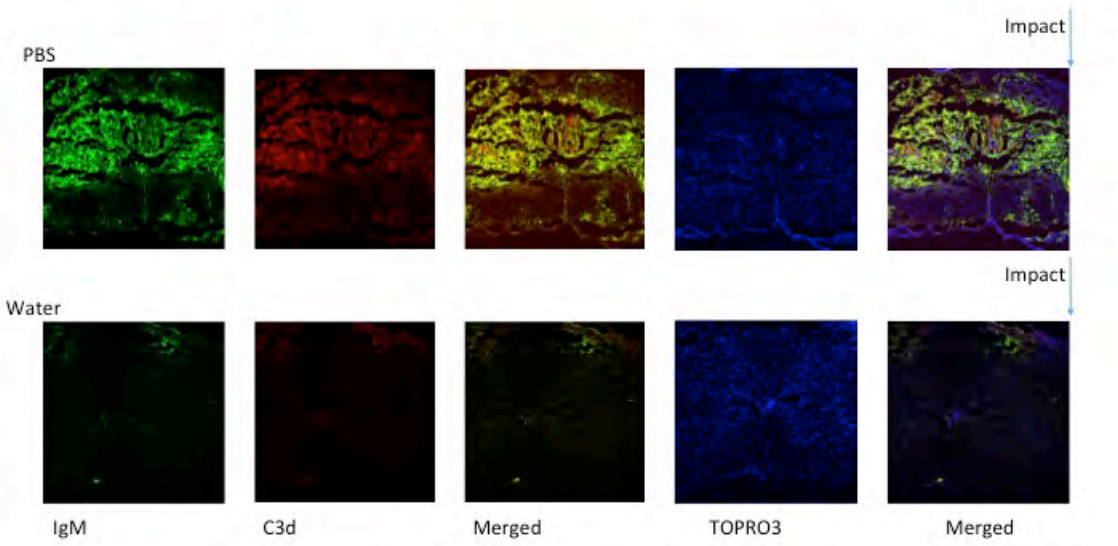

Fig. 9. IgM and C3d staining in spinal cords. Section prepared from B1 cell depleted mice (water treated) or controls (PBS treated). Representative images 3 days after SCI.

Depletion of B1 cells protects against SCI: The extent of spinal cord injury was measured using hematoxylin and eosin staining and complement deposition was measured using Immuno histochemistry. Since B1 cells are responsible for producing natural IgMs that recognize the neoepitopes that are exposed at injury site, their depletion conferred protection from injury. The depleted mice have reduced extent of injury as shown by H&E and measurement of lesion size rostral and caudal to the site of injury (Fig 10 A,B). Immunohistochemistry for complement fragment C3d was done to assess the activation of complement at the site of injury (Fig 10 C). Reduced complement activation was seen in B1 depleted mice possibly due to the lack of IgMs. This indicates importance of IgM mediated complement activation as a major contributory mechanism for secondary injury.

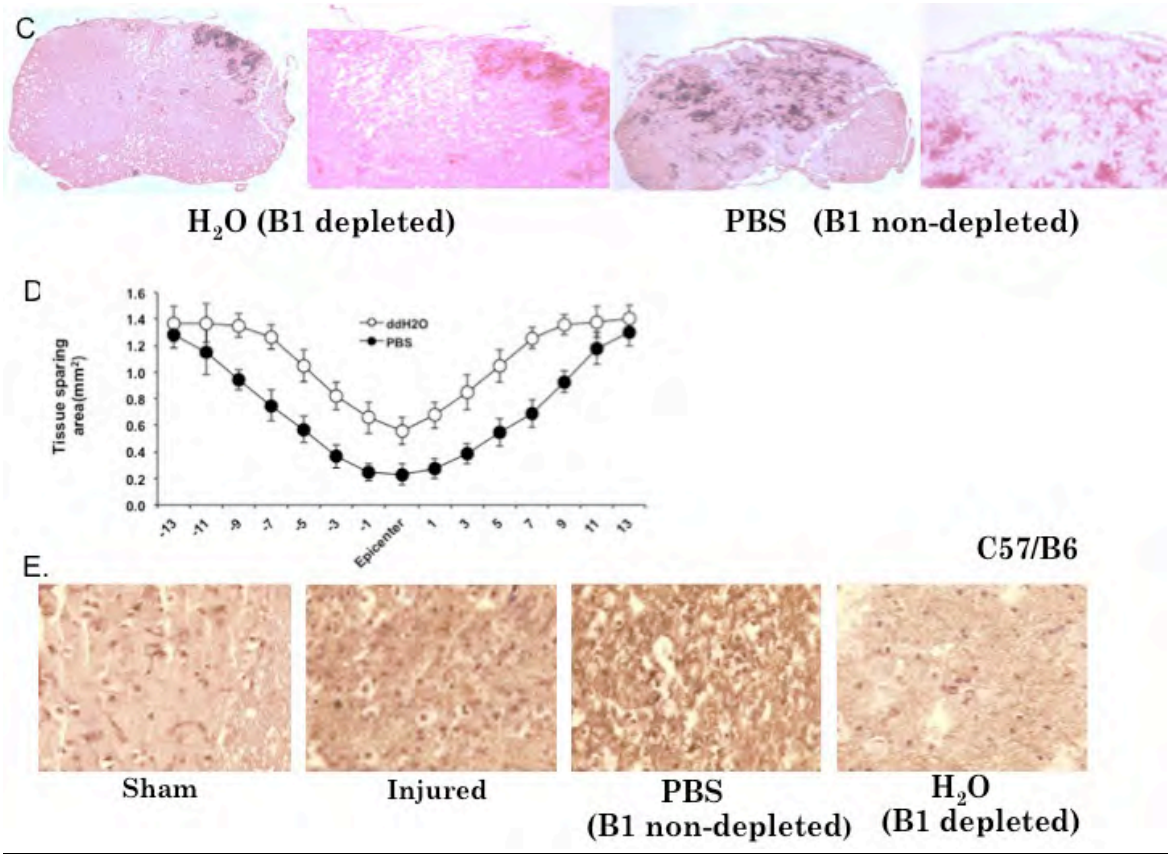

Fig 10: B1 depletion confers protection from injury.
A. Reduced hemorrhaging at injury site 72 hrs after injury in B1 depleted mice
B. Morphometric analysis showing reduced lesion size in B1 depleted mice.
C. Reduced complement deposition due to lack of IgMs in B1 depleted mice

In conclusion, we have demonstrated an important role for the alternative pathway of complement in promoting SCI under clinically relevant conditions of pharmacological complement inhibition with CR2-fH. We have additionally demonstrated a key role for natural antibodies in promoting SCI, and have shown that B1 cells are a primary source of these pathogenic self-reactive antibodies that activate complement. The antibody data validate the use of scFv's directed at post-ischemic neoepitopes as targeting vehicles for

delivery of therapeutics to treat SCI. Although we encountered technical difficulty in preparing an scFv-fH construct, we are having success with alternative scFv's linked to other inhibitors, and in due course we will test these inhibitors in our model of SCI.