Examining the Regulation of Inflammation through CD200 and CD200R Following Spinal Cord Injury

Thesis

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Abstract

Spinal cord injury (SCI) is a debilitating condition resulting in permanent loss of sensation and paralysis with a lifetime estimated health cost ranging from one million to four million dollars. After SCI, a complex cellular response exacerbates the initial damage and the spinal cord fails to regenerate damaged neurons. Various immune cells are recruited to the site of injury, including resident microglia and macrophages, which are known for their dual and conflicting role of facilitating neural growth/regeneration and pathogenesis. Targeting the inflammatory immune response could serve to reduce secondary damage and create a permissive environment for axonal regeneration.

One mechanism for regulating myeloid cells is through the neuroimmune regulatory proteins, CD200 and CD200R, which contribute to neuron-mediated inhibition of myeloid cell activation and pro-inflammatory signaling cascades. CD200 is expressed on a wide variety of cells throughout the body, including neurons, while CD200R is primarily expressed on microglia and macrophages. While a regulatory role of CD200/CD200R is evident in many central nervous system (CNS) disease models, it is not known if this mechanism contributes to neuroprotection following trauma to the CNS. By understanding how CD200 regulates myeloid cells after SCI, we hope to reduce inflammation with the goal of creating an environment conducive for axonal regeneration.

The overall hypothesis is that CD200 is downregulated after SCI resulting in decreased CD200R activation on macrophages and microglia causing increased inflammation. The goal is to determine if delivery of a soluble CD200R agonist after SCI
will reduce inflammation and secondary damage. We determined that CD200 is highly expressed within the gray matter and to some extent within the white matter, while CD200R is expressed at extremely low levels on resting microglia. After lesioning the dorsal roots, sciatic nerve and corticospinal tract, we determined that CD200 expression is maintained after injury or not expressed by these specific neurons. Injection of CD200R antagonist into the naïve spinal cord resulted in microglial activation.

Following contusion SCI, CD200 expression is decreased within the lesion, while CD200R increases with the time course of macrophage migration into the injured spinal cord. In the crush injury model, CD200 expression is maintained in the spared gray matter rostral and caudal to the lesion and within the lesion, while the glial scar does not express CD200. Administration of CD20Fc to bone marrow derived macrophages does not reduce LPS induced increases in nitric oxide production.

Based on our results, CD200 does not appear to play a significant role in reversing myeloid cell activation under the specific conditions tested. While antagonizing CD200R in the spinal cord resulted in microglial activation, the presence of CD200 or CD200R agonists was not sufficient to overcome activation in the presence of activating stimuli. We believe that CD200R agonists would not be sufficient to overcome the excessive number of inflammatory stimuli present after SCI, therefore targeting CD200R for treatment in locations with fewer inflammatory stimuli or before macrophage activation may prove successful.
Dedication

Dedicated to my wonderful wife, Megan.
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Introduction

Spinal cord injury (SCI) is a devastating condition affecting as many as 1.3 million individuals in the United States with a lifetime estimated healthcare cost ranging from one million to four million dollars, depending on age of the individual and location of injury (Christopher and Dana Reeve Foundation, 2009; National Spinal Cord Injury Statistical Center, 2013). Previously, the only treatment for SCI was the anti-inflammatory steroid, methylprednisolone, but a recent study has shown that there is a lack of evidence to support its continued use (Hurlbert et al., 2013), emphasizing the dire need for new therapies to help SCI patients.

Anti-inflammatory therapies have been a therapeutic target after SCI due to influx of peripheral immune cells and high degree of inflammation present after injury. Immediately after injury, neurons and glial cells become activated and secrete inflammatory cytokines and chemokines resulting in further activation of resident glial cells and recruitment of peripheral immune cells (Bartholdi and Schwab, 1997; McTigue et al., 1998). Trafficking immune cells, specifically macrophages, persist in the lesion indefinitely, perpetuating further inflammation and secondary damage (Fleming et al., 2006; Kigerl et al., 2009).

Neurons express neuroimmune regulatory elements (NIRegs) that are responsible for maintaining myeloid cells in a quiescent state. One example is CD200, which activates CD200R on macrophages and microglia resulting in inhibition of proinflammatory signaling cascades (Wright et al., 2000). CD200 knock out mice show increased signs of microglial activation and quicker response following insult to the CNS.
CD200/CD200R signaling has been shown to be important for its immunosuppressive actions in multiple immune and neurological disorders such as aging (Cox et al., 2012), allograft rejection (Gorczynski, 2001), Alzheimer's Disease (Walker et al., 2009), facial nerve axotomy and experimental autoimmune encephalopathy (EAE), a research model of multiple sclerosis (Hoek et al., 2000). Administration of function blocking CD200R antibodies have revealed exacerbation of neuroinflammation and neurodegeneration, while activation of CD200R with solublized CD200 results in neuroprotection in EAE (Meuth et al., 2008; Liu et al., 2010b).

While a neuroimmune regulatory role of CD200/CD200R is evident in many CNS disease models, it is currently unknown if this mechanism contributes to neuroprotection following traumatic injury to the central nervous system. We hypothesize that after SCI, CD200 expression is reduced due to neuronal death, resulting in reduced activation of CD200R on myeloid cells, which contributes to excessive inflammation. The goal of this project has been to establish whether or not interaction between CD200-CD200R is sufficient to regulate inflammation and immune cell activation following spinal cord injury.

Researching the interaction between CD200 and CD200R in the context of SCI is significant because no targeted therapies exist for spinal cord injury, and modulating the activation of CD200R would create a highly specific therapy for targeting the inflammatory immune response after SCI. By activating CD200R on macrophages, we hope to decrease proinflammatory signaling cascades in order to create an environment that is beneficial for axonal regeneration.
Chapter 1: Regulation of Microglia and Macrophages with CD200

In 1918, Pío del Río-Hortega developed a silver carbonate stain leading to visualization of cells with cytoplasmic extensions which he termed “microglia” (Gill and Binder, 2007). Today, microglia are known as the resident immune cells of the central nervous system, taking on many roles to maintain homeostasis and protect the CNS from exogenous materials. Neurons take an active role in maintaining microglia in a quiescent state with neuroimmune regulatory elements.

1.1: Microglia Actively Survey and Protect the Central Nervous System

Under normal conditions, microglia are classically referred to as “resting.” However, recent experiments show that microglia maintain an active role in the CNS during normal conditions. Two-photon imaging shows that microglial processes actively survey their environment with estimates that the entire volume of the brain is surveyed in four to five hours (Davalos et al., 2005; Nimmerjahn et al., 2005). As result of this discovery, the term “resting” microglia is not necessarily applicable; rather the term “surveying” is used to describe microglial activity in the normal functioning CNS. However, these observations are only made based on the outermost layers of the cortex, begging the question as to what activity microglia have deeper within the CNS parenchyma. In addition, microglia are not simply surveying the CNS, but rather they are active in modulating neuronal function. One such example has shown that microglia phagocytose inactive synapses on developing neurons (Schafer et al., 2012). These
experiments show that microglia perform an active role maintaining normal function within the CNS.

Microglia are activated when they come into contact with material that is foreign to the normal CNS, whether it is pathogenic or non-pathogenic. “Activated” or “effector” microglia are characterized by retraction of processes, enlargement of soma and upregulation of specific markers such as ionized calcium-binding adapter molecule 1 (Iba-1) (Ito et al., 1998; Schwab et al., 2001; Stence et al., 2001). Microglia respond by releasing cytokines, chemokines and phagocytosing foreign material to protect the CNS. If the blood brain barrier (BBB) breaks down, such as after an ischemic event (Denes et al., 2007), microglia respond to plasma components. For example, fibrinogen induces microglia phagocytic activity (Adams et al., 2007), thrombin induces nitric oxide production and release of IFNγ and other various inflammatory cytokines (Möller et al., 2000), and albumin increases superoxide production from microglia (Si et al., 1997). Microglia express a variety of toll-like receptors (TLRs), leading to NFκB activation and induction of inflammatory cytokines in order to clear bacterial or viral infections (Qureshi et al., 1999; Alexopoulou et al., 2001; Olson and Miller, 2004).

1.2: Neuroimmune Regulatory Elements

One of the side effects of the microglial inflammatory response is that neurons can be unintentionally damaged, therefore under normal conditions, neurons must play an active role in suppressing microglial activation. Neuroimmune regulatory elements (NIRegs) are proteins expressed by neurons within the CNS that maintain microglia in a quiescent state. These factors regulate microglia through cell surface interactions or at a distance through release of soluble ligands. CD22-CD45, CD172A-CD47, CX3CL1-CX3CR1 and CD200-CD200R are examples of currently identified NIReg pairs (Wright
et al., 2000; Mott et al., 2004; Cardona et al., 2006; Gitik et al., 2011). It has been hypothesized that microglial activation occurs in part due to removal of this inhibitory signal; either through natural down-regulation by an unhealthy neuron or after injury to the CNS. For example, CX3CL1 (fractalkine) expression is reduced in aged animals which contributes to observed increased microglial activation (Wynne et al., 2010). Both CD47 and CD200 were found to be down-regulated within multiple sclerosis lesions, suggesting that local microglia are predisposed toward an inflammatory phenotype (Koning et al., 2007). Neurons express CD22 on their surface and release a soluble form which regulates microglial proinflammatory cytokine production (Mott et al., 2004). These NIRegs also regulate leukocytes during times of peripheral cell recruitment (Griffiths et al., 2007).

NIRegs clearly show strong regulatory role in limiting microglia activation and protecting neurons from inflammatory damage. Manipulating these regulatory elements may prove useful in management of certain inflammatory diseases. CD200 is of particular interest due to its wide expression, allowing for application to many diseases within the CNS and throughout the entire body.

1.3: CD200-CD200R

CD200, originally known as OX-2, is a 41-47 kDa immunoglobulin-like glycoprotein (Clark et al., 1985; Barclay et al., 1986) that was originally discovered in 1979 in the rat thymus and spleen (McMaster and Williams, 1979). McMaster et. al. isolated various glycoproteins from rat thymus and spleen and then created antibodies for these proteins. In addition to rat thymus, the MRC OX-2 monoclonal antibody also bound to brain and thymus homogenate and all lymphocytes (McMaster and Williams, 1979). The localization of the MRC OX-2 monoclonal antibody was continued by Neil
Barclay, who in 1981, showed the presence of OX-2 on dendritic cells, endothelial cells, B cells, T cells, and within the spleen and lymph nodes, on peripheral nerves, brain, spinal cord and some smooth muscle cells (Barclay, 1981; Barclay and Ward, 1982). OX-2 expression in the brain was further characterized and was found on blood vessels and neurons in the gray and white matter (Webb and Barclay, 1984). Within the CNS, CD200 is expressed on neurons and, in certain cases, on astrocytes and oligodendrocytes (Hoek et al., 2000; Chitnis et al., 2007; Koning et al., 2009; Costello et al., 2011).

Up to this point, a potential cell-to-cell regulatory role for CD200 had been proposed, but no confirmed function had been shown. Through Reginald Gorczynski’s work studying allograft rejection, an immune regulatory role for CD200 was established. It was discovered that animals who had higher levels of CD200+ dendritic cells had reduced incidence of allograft rejection and decreased levels of inflammatory cytokines (Gorczynski et al., 1998; 1999a). The regulatory role for CD200 was further established in vitro when administration of a function blocking CD200 monoclonal antibody resulted in an increase in inflammatory cytokines from mixed leukocyte cultures (Ragheb et al., 1999). To prove that CD200 was the protein responsible for decreased allograft rejection, a soluble CD200 protein was created by fusing the extracellular portion of CD200 to the Fc region of IgG2a. The CD200 fusion protein (CD200Fc) successfully reduced skin allograft and renal xenograft rejections and the effect was reversed when a CD200 antibody was administered. Further in vitro experiments showed that CD200Fc decreased the proinflammatory cytokines IL-2 and IFNγ while simultaneously increasing the anti-inflammatory cytokines IL-4 and IL-10 from mixed leukocyte cultures (Gorczynski et al., 1999b).

While an immunoregulatory role for CD200 had been established, the mechanism of how it promoted these effects and on what cell type the effects were
induced was unclear. In order to determine which cell types CD200 acts on, various groups created fluorescently labeled soluble CD200 proteins. The first group found that CD200 binds to macrophages, but did not establish whether or not CD200 had an effect on these cells (Preston et al., 1997). By raising monoclonal antibodies against macrophages that also blocked the binding of CD200Fc, it was determined that microglia and macrophages express a receptor for CD200 (Preston et al., 1997; Gorczynski et al., 2000; Wright et al., 2000). Today, it is accepted that myeloid cells are the primary source of CD200R. However, there have been mixed reports of CD200R expression on B cell and T cells (Wright et al., 2000; Jenmalm et al., 2006; Rijkers et al., 2008), which is likely dependent on the certain cellular conditions or specific subsets of these different cell types. Astrocytes have also been shown to express CD200R and respond to CD200Fc (Liu et al., 2010b).

Activation of CD200R by CD200 induces an intracellular signal transduction pathway that results in modulation of cytokine expression. When CD200R is activated, Dok1 and Dok2 are recruited to the intracellular portion where they bind and are phosphorylated. Dok1 and Dok2 then bind to RasGTP and SHIP which results in inhibition of ERK and Ras. JNK, MAPK and p38 are also inhibited after CD200R activation, however the exact mechanism by which this occurs is unknown (Zhang et al., 2004; Zhang and Phillips, 2006; Mihrshahi and Brown, 2010). CD200R activation reduced phosphorylation of ERK, p38 and JNK, as observed by western blot, resulting in reduction of IL-13 and TNF (Zhang et al., 2004). Others have shown that activation of JNK is linked to secretion of TNFα (Ishizuka et al., 1999) and p38 inhibitors are used as an anti-inflammatory therapy (Kumar et al., 2003). CD200Fc mediated inhibition of ERK may serve a different role than modifying cytokine production as activation of CD200R resulted in apoptosis of microglia treated with IFNγ, but not resting microglia,
in vitro (Liu et al., 2010b). Recruitment of Dok2 to CD200R has also been shown to occur after engagement with CD200Fc giving further evidence that CD200Fc successfully mimics endogenous CD200 protein (Lyons et al., 2012). Creation of CD200 and CD200R knock out mice further characterized the immunoregulatory role of CD200 on macrophages and microglia. Microglia from CD200−/− mice showed signs of activation such as shorter glial processes, increased CD11b and CD45 expression and aggregation in the spinal cord. In a facial nerve transection model, activated microglia are normally detected at 4 days post injury (dpi) in wild type mice, but activated microglia were detected at 2 dpi in CD200−/− mice. Envelopment of damaged neurons was well advanced in knockout animals by day four while there was little evidence of envelopment in wild type (WT) mice, showing a faster and more exacerbated microglial response in CD200 knockout mice. CD200−/− mice had earlier onset and exacerbation of symptoms following induction of experimental autoimmune encephalomyelitis (EAE), compared to wild type mice. Additionally, lymph nodes in the periphery were larger in size and macrophages showed signs of activation (Hoek et al., 2000). Mixed glial cultures prepared from CD200−/− mice, containing 70% astrocytes and 30% microglia, showed increased expression of IL-1β, IL-6 and TNFα when stimulated with LPS or Pam3CSK4 compared to mixed glia prepared from WT mice (Costello et al., 2011). Spleen cells isolated from CD200R knock out mice showed increased TNFα and IL-12 secretions compared to controls. Importantly, this effect could not be reversed after administration of CD200Fc (Boudakov et al., 2007). These data show that when CD200R is not activated in knock out mice, macrophages and microglia are primed toward activation and secrete increased pro-inflammatory cytokines.
Deficits in CD200-CD200R signaling have been observed in various diseases associated with neuroinflammation. Alzheimer’s disease patients have reduced CD200 mRNA in the hippocampus (Walker et al., 2009). Amyloid beta decreases CD200 expression from astrocytes in vitro (Lyons et al., 2012) and from neurons in vitro and in vivo (Lyons et al., 2007). Treating mixed glia cultures with CD200Fc reduced amyloid beta induced increases in IL-1β and TNFα (Lyons et al., 2012). Importantly, CD200Fc was able to reverse a reduction in long term potentiation (LTP) induced by amyloid beta in hippocampal slice culture (Lyons et al., 2012), showing the potential therapeutic ability of CD200Fc for Alzheimer’s patients.

In addition to Alzheimer’s disease, CD200 and CD200R have been found to be down-regulated in multiple sclerosis lesions, suggesting that CD200 may play a role in modulating disease severity (Koning et al., 2007). Administration of a function blocking CD200R antibody via intravenous injection on day 3 and day 5 post MOG immunization resulted in an aggravated clinical course as seen by increased severity of symptoms associated with an increase in the number of T cells and macrophages within EAE lesions (Meuth et al., 2008). On the other hand, administration of the CD200R agonist, CD200Fc, resulted in a decrease in severity of symptoms when the treatment was delivered at the onset of clinical symptoms. Additionally, administration of CD200Fc ten days after manifestation of symptoms, also resulted in reduction of EAE clinical symptoms (Liu et al., 2010b) showing the strong functional effect CD200 agonists have on modulating symptom severity in neuroinflammatory diseases.

The beneficial effects of CD200 have been shown through various in vitro experiments. When CD200R is blocked by antagonists, IFNγ induced release of IL-6 is increased resulting in increased neuronal cell death in vitro (Meuth et al., 2008). This experiment suggests that the reported reduction of CD200 expression in EAE and
Alzheimer’s disease could be causing increased loss of neurons due to high levels of inflammation.

While the functional ability of CD200R antagonists is interesting, finding a way to reduce inflammation shows greater clinical relevance. *In vitro* studies have shown that administration of CD200R agonists successfully reduced pro-inflammatory cytokine release from macrophages after stimulation with IFN-γ and IL-17, but not LPS (Jenmalm et al., 2006). A similar effect was observed with astrocytes, where CD200Fc was able to decrease IFNγ and TNFα induced secretions of IL-6, NO and CCL2 (Liu et al., 2010b). High concentrations of CD200Fc were able to overcome LPS and IFNγ induced deficits in microglial migration in retinal explant cultures (Carter and Dick, 2004), showing that CD200R activation has additional effects other than limiting inflammation.

Based on *in vitro* and *in vivo* experiments manipulating CD200R, CD200Fc is a potent anti-inflammatory stimulus. Manipulation of this system should be further explored as a treatment for neuroinflammatory diseases involving local microglia and trafficking macrophages.
Chapter 2: Spinal Cord Injury

After traumatic injury, the spinal cord fails to regenerate damaged neurons, which can lead to permanent loss of sensation and paralysis. The extent of damage and functional deficits depend upon the location of injury along the spinal cord and the severity of the initial insult. The failure of repair is due to various reasons: 1. Primary mechanical injury results in direct cell damage and acute hemorrhagic necrosis (Ducker et al., 1971; Grossman et al., 2001; Beattie et al., 2002); 2. Resulting inflammation and immune system activation following injury, specifically recruitment of neutrophils, macrophages, microglia, B and T cells, promotes tissue repair as well as secondary damage and neurotoxicity (Perry et al., 1987; Popovich et al., 1999; Kigerl et al., 2006; Gensel et al., 2009); and 3. The glial scar, consisting of reactive astrocytes and proteoglycans, physically and chemically protects spared tissue from the lesion site while simultaneously blocking regenerating axons (Faulkner et al., 2004).

2.1: Injury Models

Several spinal cord injury models are employed to study different aspects of spinal cord injury (SCI). Each human injury is heterogeneous, requiring that researchers use various research models to address different questions (Bunge et al., 1993; Norenberg et al., 2004).

For animal models of SCI, a laminectomy at the site of proposed injury is performed to reveal the spinal cord. In contusion, crush and compression models, the dura remains intact at the time of injury while in axotomy models, the dura is cut.
2.1.1: Contusion Injury Models

The contusion injury model creates a bruise on the spinal cord and is the most widely used model for SCI research because it is the most commonly observed injury in humans (Norenberg et al., 2004). Alfred Allen created the first contusion injury device in 1911. Allen’s weight drop device dropped an object onto the exposed spinal cord, allowing for the force generated to be calculated as gram-centimeters, a product of the weight and the distance from which the object was dropped (Allen, 1911). Since 1911, various modifications have been made to the contusion injury device in order to provide additional information to the researcher in an effort improve reproducibility. Today, the New York University (NYU) Impactor Device (Young, 2002), OSU ESCID Device (Stokes, 1992; Stokes et al., 1992; Jakeman et al., 2000) and the Infinite Horizons Impactor Device (Scheff et al., 2003) are used in SCI research to produce contusion injuries.

2.1.2: Crush and Compression Injury Models

Crush or compression models maintain pressure on the spinal cord for a given amount of time using clips, forceps or balloon catheters. These models of spinal cord injury were preferred over early contusion injury models due to increased reliability and reproducibility, especially in smaller vertebrate animals such as mice and rats (Khan and Griebel, 1983). Previous work has shown that the time of compression with an aneurysm clip directly related to functional severity allowing for experiments to be easily reproduced (Rivlin and Tator, 1978). However, there is still some variability depending on the model used. The hemorrhagic lesion is most consistent in clip injury models and most variable in balloon compression and early contusion models (Khan et al., 1985). Utilizing the forceps crush injury model is often used to study regeneration across the
resulting lesion because a complete spinal cord crush will completely “destroy all neural tissues at the injury site and is considered to be an extraordinary barrier for regeneration” (Liu et al., 2010a).

### 2.1.3: Transection and Hemisection Injury Models

A transection injury is a complete transverse cut through the entire cross section of the spinal cord, whereas a hemisection injury is only a partial lesion. Complete transection injuries are preferred for studying functional aspects after injury, such as autonomic dysreflexia (Rabchevsky et al., 2012; Zhang et al., 2013). Transections allow for complete separation of the cord, therefore hemisections are preferred for regeneration studies (Borgens et al., 1986). Hemisections can be used to cut specific tracts, such as the corticospinal tract, in order to study various functions and regenerative capabilities. These models are often used for testing cell or tissue guidance channel transplantation studies (Blight, 2000). One such example is the work performed by Mary Bunge with her work transplanting Schwann cells into the injured spinal cord (Bunge, 1994; Xu et al., 1999).

### 2.2: Primary Damage and Cell Death

The primary lesion is a result of mechanical damage which shears neurons and blood vessels, resulting in massive cell death by both necrosis and apoptosis (Grossman et al., 2001; Beattie et al., 2002). Damage to neurons results in lack of innervation to the caudal spinal cord, while damage to blood vessels results in hemorrhagic necrosis and interruption of blood flow resulting in ischemia (Ducker and Perot, 1971; Ducker et al., 1971). The necrotic lesion spreads in the rostral and caudal direction. In the contusion injury model, the spreading lesion creates a non-uniform, oblong shaped lesion, which
increases in length over time (Grossman et al., 2001). Hemorrhagic damage occurs in the gray matter to a greater extent than the white matter as the gray matter is more highly vascularized (Ducker et al., 1971; Ganong, 2001). When axons are severed, the distal portions undergo Wallerian degeneration which further contributes to demyelination, tissue loss and activation of microglia in regions distal to the lesion (Popovich et al., 1997). After injury, the cross section of the spinal cord shrinks due to extensive loss of tissue, which is directly related to the severity of injury (Noble and Wrathall, 1985).

While it was understood that SCI results in massive cellular death, two competing theories, the neuronal theory and the vascular theory, attempted to explain what caused functional deficits following injury (Nelson et al., 1977). The neuronal theory states that the loss of function is due to neurological dysfunction (Kobrine, 1975). Kobrine argues that after experimental spinal cord injury, there is immediate loss of sensation and ability to propagate action potentials, which is explained by disruption in neuronal communication (Kobrine, 1975). The vascular theory states that due to mechanical injury, blood vessels are damaged resulting in ischemia which induces cellular necrosis, explaining the loss of sensation and paralysis (Balentine, 1978). Balentine performed an extensive spinal cord injury time course in rats in order to document early necrotic damage seen in SCI. He documented hemorrhage and necrosis as early as 3-5 minutes post injury, giving support to the vascular theory. However, he concluded that even though “the altered necrotic zone is attributable to vascular injury, [this] does not exclude the possibility that cellular changes were induced by the physical force of the trauma” (Balentine, 1978). Clearly, both mechanical damage and ischemia are to blame for the expanding necrotic lesion observed after SCI.
2.3: Secondary Inflammatory Damage

Immediately after injury, various inflammatory cytokines and chemokines are upregulated within the spinal cord. Alterations in TNFα, IL-1β, IL-6, macrophage colony stimulating factor (MCSF), CCL2, CCL3, CXCL10, CXCL1 are detected by RT-PCR or in situ hybridization studies as early as 1 hour post injury and return to baseline within 1 day to 1 week post injury (Bartholdi and Schwab, 1997; McTigue et al., 1998; Streit et al., 1998; Lee et al., 2000a; 2000b). At these early time points, resident CNS cells such as neurons, astrocytes and microglia are responsible for cytokine and chemokine secretion (Bartholdi and Schwab, 1997). Importantly, this early induction of chemokines is required for recruitment of peripheral immune cells such as neutrophils, B cells, T cells, and macrophages (McTigue et al., 1998).

2.3.1 Neutrophils

The primary role of neutrophils is to serve as the body’s first line of defense after injury. Neutrophils traffic to the lesion site to phagocytose cellular debris and to release cytotoxic molecules to kill pathogens, however, neutrophils are not very accurate and damage intact tissue in the process (Weiss, 1989). Neutrophils begin arriving within 6 hours post injury, peak at 24 hours, and then decrease from 24-48 hours (Carlson et al., 1998; Streit et al., 1998; Guth et al., 1999; Kigerl et al., 2006). After SCI, neutrophils are generally restricted to the necrotic zone within the gray matter suggesting that their primary role is removal of necrotic cells (Carlson et al., 1998; Streit et al., 1998). While they serve this beneficial role, neutrophils also expand the necrotic zone through the release of reactive oxygen species and inflammatory cytokines (Dusart and Schwab, 1994; Carlson et al., 1998). Activation by proinflammatory cytokines and cellular debris induces NADPH oxidase activity, facilitating the release of oxygen metabolites which
further damages the surrounding tissue, resulting in the spread of the necrotic lesion (Weiss, 1989; Vaziri et al., 2004; Parham, 2009).

TNFα is created by neurons after SCI and is released into the CSF and interstitial fluid, which likely serves to recruit neutrophils (Harrington et al., 2005). Administration of the TNFα antagonist, etanercept, resulted in reduced neutrophil migration to the injured spinal cord (Genovese et al., 2006). Along with recruitment, TNFα activates NFκB in neutrophils which increases release of inflammatory mediators, thus promoting secondary tissue damage (McDonald and Cassatella, 1997).

After SCI, endothelial cells upregulate ICAM-1, P-selectin and factors to recruit neutrophils into the lesion (Lorant et al., 1993; Hamada et al., 1996; Bethea et al., 1998; Isaksson et al., 1999). Various techniques have shown that blocking neutrophil recruitment after SCI increases recovery. For example, treatment with anti-P-selectin antibody reduced the number of neutrophils within the lesion and increased functional recovery of the hindlimbs. A reduction in intramedullary hemorrhages was also observed suggesting that neutrophils damage endothelial cells resulting in increased hemorrhagic necrosis following SCI (Taoka et al., 1997). Immediately after injury, ICAM-1 is upregulated on endothelial cells with peak expression at 6 hours post injury, coinciding with neutrophil migration (Hamada et al., 1996). ICAM-1 KO had fewer neutrophils at 24 hours post injury in the white matter, but there was no difference in the gray matter likely due to loss of blood vessel integrity allowing neutrophils to freely enter the parenchyma (Isaksson et al., 2000). Additionally, administration of anti-CD11d antibody blocked neutrophil entry into the spinal cord resulting in decreased leukocyte number and reduced oxidative damage (Ditor et al., 2006).
2.3.2: B and T cells

Break down of the blood spinal cord barrier (BSCB) and secretion of chemokines recruit the adaptive immune system to the injured spinal cord. T cells begin to traffic to the lesion and peak at 14 dpi in mice. T cell numbers then decline, until reaching a second peak at 42 dpi (Kigerl et al., 2006). B cell numbers peak at 3 hours and 24 hours post injury and remain at low levels until at least 5 weeks post injury (Schnell et al., 1997). Once in the spinal cord, B and T cells associate with MHCII+ microglia and macrophages and clusters of B and T cells resemble germinal centers of secondary lymphoid tissue, suggesting that lymphocytes continue to be activated and reactivated within the lesioned spinal cord (Ankeny et al., 2006).

Induction of CXCL10 and CCL5 precede the arrival of T cells to the injured spinal cord (Jones et al., 2005). Antibody treatment to deplete CXCL10, results in reduced T cell recruitment and increased tissue sparing, suggesting that T cells have a largely pathogenic effect after SCI (Gonzalez et al., 2003). However, T cells may also have the capacity to secrete various neurotrophic factors (Moalem et al., 2000).

Further investigation into different T cell populations after injury has shown that only certain subpopulations are harmful. Whether T cells promote repair or induce additional damage is largely determined by whether the T cell responds to CNS antigens. Experiments comparing wild type and transgenic mice with T cells specifically reactive toward myelin basic protein (MBP) have shown that MBP reactive T cells are especially pathogenic after SCI. Transgenic mice were found to have reduced white matter sparing and increased lesion spread in the rostral and caudal directions compared to wild type (Jones et al., 2002). Adoptive transfer of T cells from 1 week post injury SCI rats into naïve animals resulted in induction of EAE like symptoms, indicating that T cells become autoreactive to CNS proteins after SCI (Popovich et al., 2001).
The presence of autoreactive T cells (Popovich et al., 1996) and elevation of CNS specific autoantibodies (Ankeny et al., 2006) suggests the potential for autoimmunity after SCI. The induction of CNS autoimmunity could be due to polyspecific T cells that become autoreactive due to molecular mimicry or through peripheral priming and activation. Autoimmunity by molecular mimicry is the idea that the immune system has had prior exposure to non-CNS antigen that resembles proteins in the CNS resulting in the creation of polyspecific T cells that are reactive against CNS proteins (Wucherpfennig et al., 2007). Current theories speculate that certain viral proteins resemble CNS antigens and thus induce polyspecificity (Miller et al., 1997). It is hypothesized that prior viral exposure is responsible for increased lymphocyte recruitment observed after SCI in rats raised in conventional housing compared to those raised in a specific pathogen free environment (Schnell et al., 1997). Peripheral priming could be induced by neuroantigen traveling to secondary lymphoid tissue after being phagocytosed by antigen presenting cells (APCs) or through drainage to the blood or lymphatics (Ling et al., 2003; Karman et al., 2004). Peripheral priming is a plausible theory as activated B and T cells increase in the spleen and bone marrow as early as 1 day post SCI (Ankeny et al., 2006).

The presence of autoantibodies after SCI results in exacerbated pathology. After SCI, B cell knock out (BCKO) mice exhibited greater locomotor recovery, decreased lesion size and increased gray and white matter sparing compared to WT controls, which is explained by a reduction in antibody production. Indeed, injection of antibodies collected from mice post-SCI into the uninjured spinal cord resulted in damage to the spinal cord and paralysis (Ankeny et al., 2009). It is important to note, that antibodies do not act autonomously, rather accumulation of complement and activation of Fc receptors on immune cells induces an inflammatory response (Anderson et al., 2004;
Ankeny et al., 2006). Animals deficient in complement or Fc receptor had reduced lesion size after injection of purified antibody from post-SCI mice (Ankeny et al., 2009).

In contrast, some groups have been able to create antibodies against specific growth inhibitory components, such as myelin, to induce recovery after SCI. Transplantation of tumor cells that create myelin specific antibodies into the injured spinal cord, resulted in axonal sprouting and recovery of motor function after SCI (Schnell and Schwab, 1990; Bregman et al., 1995; Merkler et al., 2001). Importantly, administration of these antibodies did not result in induction of EAE symptoms (Merkler et al., 2003). Direct intrathecal delivery of Nogo-A antibody immediately after injury resulted in improved motor behavior in conjunction with increased regeneration of corticospinal axons (Liebscher et al., 2005). The possible success of antibody treatment could be due adjuvant used during antibody treatment. Using Complete Freud’s Adjuvant (CFA) results in a Th1 response, and is associated with induction of EAE, while incomplete Freud’s adjuvant or alum leads to a Th2 response (Falcone and Bloom, 1997; Yip et al., 1999). This shift in cytokine profiles to a Th2 response caused by alum or incomplete Freud’s adjuvant mediates the protective effect associated with antibody treatments after SCI (Sicotte et al., 2003).

2.3.3: Microglia and Macrophages

Microglia are the first responders after any insult to the CNS, with spinal cord injury being no exception. Within the first hour after injury, microglia are activated and begin retracting their processes, taking on a round morphology (Dusart and Schwab, 1994; Kreutzberg, 1996), and begin to secrete pro-inflammatory cytokines (TNFα and IL-1) and chemokines (MIP-1α and MIP-1β) (Bartholdi and Schwab, 1997).
breakdown results in influx of plasma proteins, such as fibrinogen, which activates microglia and induces phagocytic activity (Adams et al., 2007).

Initially, microglia derived macrophages are the primary phagocytes occupying the lesion until monocyte derived macrophages begin to arrive at around 3 days post injury and then predominate in the lesion by 7 dpi (Popovich and Hickey, 2001). Release of pro-inflammatory cytokines from microglia causes activation and trafficking of peripheral monocytes to the lesion (Bartholdi and Schwab, 1997). Macrophage activation peaks at 7-14 dpi but remains elevated for at least 6 weeks post injury (Kigerl et al., 2006). These activated macrophages secrete pro-inflammatory cytokines such as TNFα, IL-1, IL-6 and iNOS (Leskovar et al., 2000) and persist for extended periods of time due to pro-inflammatory signals in the lesion environment (Kigerl et al., 2009). Once activated, microglia and macrophages are indistinguishable by morphology or antigen expression (Davoust et al., 2008; David and Kroner, 2011), but work using chimera rats and mice has shown that microglia derived macrophages tend to occupy the edges of the lesion while monocyte derived macrophages prefer to occupy the central lesion (Popovich and Hickey, 2001; Donnelly et al., 2011; Mawhinney et al., 2012).

Macrophages generally adopt one of at least two different phenotypes: the M1 phenotype is classified as pro-inflammatory and associated with pathogen clearance, while the M2 phenotype is classified as anti-inflammatory and associated with tissue repair. These phenotypes are distinguishable by cytokine release, morphology, function and cellular markers. Inflammatory cytokines, such as IFNγ, promote M1 macrophage differentiation, identified by upregulation of CD16/32, CD86 and iNOS. While M2 macrophages are induced by IL-4 and IL-13, which cause upregulation of arginase 1 and CD206 (Ding et al., 1988; Gordon, 2003; Gordon and Taylor, 2005; Sica et al., 2006). Macrophage conditioned media (MCM) from M1 macrophages is neurotoxic, while MCM
from M2 macrophages promote axon growth, *in vitro* (Kigerl et al., 2009). These experiments support a beneficial effect of anti-inflammatory macrophages compared to pro-inflammatory macrophages.

At early time points, there is a mixture of M1 and M2 macrophages within the lesion, but by 14 dpi, M1 macrophages constitute about 75% of the macrophages within lesion and around 95% by 28 dpi (Kigerl et al., 2009). The conflicting role of macrophages after SCI is likely due to the presence of these heterogeneous phenotypes.

Due to macrophage detrimental effects, many therapeutic strategies have been aimed at reducing macrophage recruitment after SCI in order to enhance recovery. Depletion of hematogenous macrophages resulted in improved hind-limb functional recovery in rats, in conjunction with increased white matter sparing and increased sprouting from spared axons (Popovich et al., 1999). Administration of a CD11d antibody during the first 48 hours after SCI successfully reduced macrophage recruitment to the spinal cord after a compression injury resulting in improved locomotor behavior and increased white matter sparing (Gris et al., 2004). CX3CR1 KO mice had greater functional recovery after SCI due to reduced recruitment of a specific macrophage subset, suggesting that only certain types of macrophages are detrimental after injury (Donnelly et al., 2011).

Despite the negative effects caused by inflammatory damage, macrophages and microglia are important for recovery due to their secretion of neurotrophic factors and phagocytosis of cellular debris (Blight, 1985; Elkabes et al., 1996; Zeev Brann et al., 1998). After SCI, microglia are known to secrete BDNF, a neurotrophic factor associated with axonal regeneration (Dougherty et al., 2000). Transplantion of gelfoam seeded with microglia into the injured spinal cord resulted in increased neurite outgrowth (Rabchevsky and Streit, 1997). Microglia promoted the growth of sensory axons from
dorsal root ganglion cells when transplanted in a nitrocellulose membrane (Prewitt et al., 1997). While these microglia are likely not activated in the same way as seen after SCI, these cells have the potential to promote recovery after injury given the proper conditions. Depletion of macrophages using thymidine kinase under a CD11b promoter resulted in a reduction in myelin clearance, neurotrophin synthesis, and angiogenesis after a sciatic nerve injury, which reduced the capacity of the sciatic nerve to regenerate (Barrette et al., 2008). Transplantation of peripheral nerve grafts into the spinal cord after a hemisection injury resulted in significantly fewer axons growing into the graft when CD11b+ cells were depleted (Barrette et al., 2008). Inhibition of macrophage recruitment after SCI, by depleting CCR2, resulted in impaired degradation of myelin within the injury epicenter (Ma et al., 2002), which is inhibitory to axon regeneration (Bähr and Przyrembel, 1995).

In order to inhibit the negative effects, without completely inhibiting macrophage recruitment, therapies to reduce inflammation have been employed. Attempts to reduce inflammation through the use of minocycline treatment has proven successful in both mice (Wells et al., 2003) and rats (Lee et al., 2003) resulting in improved hind limb motor function. Specifically, minocycline reduced lesion size, number of apoptotic cells, and TNFα secretions, while increasing IL-10 resulting in increased white matter sparing (Lee et al., 2003; Wells et al., 2003; Stirling et al., 2004).

2.4: Astrocytes and The Glial Scar

Relative to the macrophage/microglial response, astrocyte proliferation and scar formation is seen as a secondary response initiated by primary damage after injury and through macrophage/microglial mediated secretions. Astrocyte proliferation is the first step in initiation of scar formation, which is caused by inflammatory cytokines from
macrophages/microglia such as IL-1β, IL-6, IFNγ, and TNFα (Giulian, 1987; Balasingam et al., 1994; Klein et al., 1997; Herx and Yong, 2001). The glial scar serves as a physical and chemical barrier in order to protect spared tissue from the neurotoxic lesion (Faulkner et al., 2004). While the scar has this largely beneficial function, it can also prevent axons from regenerating to their target across the lesion. Adding to the conundrum, astrocytes secrete both growth promoting and growth inhibiting molecules (Fehlings and Hawryluk, 2010). There is a growing body of evidence that macrophages and microglia may bias astrocytes and the formation of the glial scar to alter the beneficial or detrimental effects.

2.4.1 Beneficial Aspects of the Glial Scar

Historically, the glial scar has been viewed negatively after SCI, which drove various experiments to knock out or inhibit large components of astrocyte function or completely ablate astrocytes. These data generally lead to the consensus that the glial scar serves some beneficial purpose after SCI as removal of astrocytes generally resulted in negative outcomes. In one such case, Faulkner and colleagues conditionally ablated astrocytes after SCI by administering ganciclovir to mice expressing herpes simplex virus-thymidine kinase transgene under a GFAP promoter. This ablation of astrocytes resulted in severe motor deficits which were attributed to increased leukocyte infiltration, widespread inflammation, dysmelination, neuronal and oligodendrocyte death along with a failure to restore the BBB (Faulkner et al., 2004). This study shows that the astrocyte response, at least in some degree, is protective to the surrounding spared tissue after SCI.

Additional studies using GFAP and vimentin double knock out mice helped to clarify that the glial scar has beneficial as well as detrimental functions. Knockout of
both GFAP and vimentin resulted in abnormal scar formation, while knockout of one or the other did not alter the formation of the glial scar (Pekny et al., 1999; Menet et al., 2003). In one case, a positive role for glial scar formation was suggested as double knockout animals had higher levels of cellular debris, erythrocytes and bleeding within the lesion (Pekny et al., 1999). These data suggest that astrocytes are involved in clearing debris and necessary for reestablishing the BBB after SCI. This study did not comment on behavioral changes between the different knockout mice. In a separate study, double knockout mice had greater locomotor recovery thought to be due to a greater amount of 5HT reactivity in the caudal spinal cord after SCI (Menet et al., 2003). However, knockout of either GFAP or vimentin did not result in significant increase of 5HT in the caudal spinal cord compared to wild type, in the same study. Since glial scars formed properly in GFAP or vimentin knockout mice, but not in the double knockout mice, these data suggest that the glial scar is inhibitory to sprouting 5HT fibers caudal to SCI and negatively affects locomotor recovery after SCI.

2.4.2 Negative Aspects of the Glial Scar

One of the major inhibitory factors to axonal growth within the glial scar is chondroitan sulfate proteoglycans (CSPGs). CSPGs, such as neurocan, brevican and NG2, are upregulated in and around the lesion epicenter after spinal cord injury (Andrews et al., 2012) and are inhibitory to axonal sprouting by initiating growth cone collapse (Tom et al., 2004), by binding to PTPσ on neurite growth cones (Shen et al., 2009). With the discovery of the strong inhibitory nature of CSPGs, much of glial scar research has shifted toward modifying the inhibitory side chains or ablating CSPGs completely. However, it is important to realize that CSPGs evolved for beneficial functions as well: to protect regenerating spared neurons from the neurotoxic lesion.
CSPGs at early time points protect neurons from the inhibitory and neurotoxic environment of the lesion, thereby serving a beneficial function. Administration of xyloside, an inhibitor to CSPG formation, results in completely different functional outcomes depending on when the treatment is delivered post SCI. When xyloside treatment was delivered immediately after injury, mice had reduced locomotor recovery compared to controls. At two days post injury, mice had improved locomotor recovery while administration at seven days post injury resulted in no changes in locomotor recovery compared to controls. When comparing lesion size, the immediate treatment group had the largest lesion size, but greatest reduction in CSPGs; the two day post injury treatment group had smallest lesion with similar CSPG reduction as seen in the immediate treatment group, while the seven day post injury group had a slightly reduced lesion compared to control, but showed no difference in CSPG content (Rolls et al., 2008). These data show that inhibition of CSPGs serve beneficial functions depending on the timing, therefore treatments to target the inhibitory aspects of the glial scar, such as CSPGs, should be administered at certain time points, rather than given as a blanket treatment for SCI.

2.4.3 Macrophages Modify the Glial Scar

In order to modulate the glial scar to become more beneficial, one potential therapeutic route is to modulate the macrophage mediated inflammatory response because higher levels of inflammation have been linked to increased CSPG production within the glial scar (Fitch and Silver, 1997). Various in vitro experiments have shown the effect macrophage secretory products can have on CSPG production. Rat astrocyte cultures secreted the highest number of CSPGs in the presence of IL-1β, EGF, TGFβ and TGFβ+EGF (Smith and Strunz, 2005). The CSPG neurocan was increased in astrocyte
cultures after treatment with TGFβ and EGF, with EGF showing the greatest increase, while PDGF, IFNγ and IL-1β decreased neurocan (Asher et al., 2000). These results show that different cytokines can have differential effects on astrocytes and CSPG production, and therefore macrophages may be a target to alter CSPG production.

Macrophage conditioned media (MCM) from zymosan activated macrophages increase tenasin production in vitro. This increase was determined to be caused by bFGF and TGFβ, but the effect of bFGF and TGFβ was reduced when IFNγ was added to the astrocyte cultures (Smith and Hale, 1997). In an example of spinal cord injury, Fujiyoshi and colleagues administered IFNγ via intraperitoneal injection to mice immediately after injury daily for 14 days. IFNγ treatment reduced proliferation of astrocytes at the epicenter, however, a greater amount of GFAP reactivity was seen rostral and caudal to the injury site. Because astrocyte proliferation was decreased, CSPG production around the epicenter was also reduced. However, because additional astrocyte proliferation was found rostral and caudal to the injury epicenter, CSPGs were expressed diffusely throughout the entire cord (Fujiyoshi et al., 2010). The researchers found greater functional recovery with the IFNγ treatment, likely due to reduced concentration of astrocyte proliferation and CSPG production directly around the lesion epicenter. In an in vitro example, DiProspero et. al. showed that IFNγ resulted in reduction of tenascin, laminin and fibronectin (DiProspero et al., 1997). Taken together, IFNγ may have a beneficial role in reducing CSPG production by blocking the effects of pro-CSPG forming cytokines such as bFGF and TGFβ.

Macrophages can also directly affect neuron outgrowth in permissive and inhibitory environments. Dorsal root ganglion (DRG) neurons grown on growth permissive laminin have longer processes when grown in the presence of M2 macrophage conditioned media (MCM), when compared to M1 MCM. M2 MCM was
able to promote additional DRG growth on myelin-associated glycoprotein (MAG), a growth inhibitory substrate, when compared to M1 MCM. DRGs were able to grow across a CSPG barrier slightly better than DRGs grown in M1 MCM (Kigerl et al., 2009).

Future therapies that target the inflammatory response could decrease further neuron loss and enhance neuron regenerative potential by reducing the negative effects associated with the glial scar.
Chapter 3: Hypothesis

In the central nervous system, microglia are the primary resident immune cell, with the purpose of maintaining homeostasis and protecting neurons from foreign material. After trauma or infection, microglia become activated and mount an inflammatory response to remove the insult and reestablish homeostasis. However, the increase in inflammation can inadvertently damage neurons, terming the microglial response as a “double-edged sword” (Griffiths et al., 2007).

After SCI, microglia activate, secrete high levels of inflammatory cytokines, and differentiate into macrophages (Dusart and Schwab, 1994; Kreutzberg, 1996; Bartholdi and Schwab, 1997). At early time points, there is a mixture of M1 and M2 macrophages, but at later time points, M1 macrophages dominated the lesion (Kigerl et al., 2009). High levels of pro-inflammatory cytokines create a neurotoxic lesion that is damaging to axons, resulting in further cell death. In response to damage and inflammatory cytokines, astrocytes proliferate and form a glial scar around the edges of the lesion to protect the spared tissue; responding to high levels of pro-inflammatory cytokines and increasing production of molecules that are inhibitory to neurite growth (Yong et al., 1991; Fitch and Silver, 1997). While at times the inflammatory response is beneficial, it does not resolve after SCI resulting in lasting functional deficits.

One mechanism for regulating macrophages and microglia is through neuroimmune regulatory proteins (NIReg), which contribute to neuron-mediated inhibition of myeloid cell activation. CD200 and CD200R are NIReg pairs that have been recently studied for their anti-inflammatory effects in various disease models.
CD200 is expressed on a wide variety of cells throughout the entire body, including neurons, and in select cases on astrocytes and oligodendrocytes within the CNS (Hoek et al., 2000; Koning et al., 2009; Costello et al., 2011). CD200R expression is restricted to myeloid cells, such as microglia and macrophages, where activation results in inhibition of proinflammatory signaling cascades (Wright et al., 2000). Deficits in CD200R signaling have been implicated in multiple sclerosis, Alzheimer’s disease, and Parkinson’s Disease (Koning et al., 2009; Walker et al., 2009; Zhang et al., 2011).

While a regulatory role of CD200/CD200R is evident in many CNS neuroinflammatory diseases, it is not known if this mechanism regulates inflammation following traumatic injury to the central nervous system. We hypothesize that CD200 expression is reduced following SCI, resulting in diminished activation of CD200R on myeloid cells contributing to high levels of inflammation. Therefore administration of CD200R agonists will decrease inflammatory cytokine release from myeloid cells. By reducing inflammation, we hope to reduce secondary damage and create a beneficial environment for axonal regeneration in order to improve locomotor recovery and reduce pathology after SCI.
Chapter 4: Materials and Methods

4.1: Surgical Procedures and Animal Care

Experiments and post-operative animal care procedures were performed according to The Ohio State University Institutional Laboratory Animal Care and Use Committee. For all subsequent surgeries, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After all operations, mice received subcutaneous injections of gentacin (5mg/kg) and saline for 5 days post injury. Bladders of spinal cord injured animals were voided twice daily for the duration of the study and mice were monitored for signs of infection and other post-surgical complications.

4.1.1: CD200 and CD200R Distribution Study

For the spinal cord injury immunohistochemistry tissue bank, 40 adult C57Bl/6 mice were divided into naïve (uninjured) (n=5), laminectomy (n=5), or injured mice broken into 1, 4, 7, 14, 21, 28 days post injury (dpi) survival times (n=5/group). A laminectomy at the T9 vertebral level was performed and mice were given a 75 kDyne force hit with a 1.25mm probe using the Infinite Horizons Impactor Device (IH Device) (Precision Systems and Instrumentation) to produce the contusion injury (Scheff et al., 2003; Hansen et al., 2013). For the crush injuries, a T9 laminectomy was performed and the spinal cord was crushed extradurally on the lateral sides for two seconds using #5 Dumont jeweler forceps with a modified blunt tip, as described previously (Zhang et al., 1996).
4.1.2: Altered Expression of CD200 and CD200R Following Select Lesions of the Spinal Cord

Select nervous system injuries were performed on mice to study altered expression of CD200. For the following studies, the injuries were all performed on the right side, allowing for the contralateral side to be used as an internal control. A laminectomy at the L4/L5 vertebral level was performed and the dorsal root was transected to produce the dorsal rhizotomy injuries (n=3; 7 dpi). Sciatic nerve injuries were produced by axotomizing the sciatic nerve along the hind limb (n=3/group; 7 and 14 dpi). A dorsal hemisection was performed using microscissors at the T9 vertebral level (n=3; 7 dpi).

4.1.3: CD200R Antagonist Intraspinal Microinjection

CD200R antagonists were delivered to the ventral horn white matter of the naïve spinal cord via intraspinal microinjection (ISM) using glass pipettes. Glass pipettes were pulled to create a fine tip and beveled to approximately 30µm in outer diameter. A total of 24 adult C57Bl/6 mice were divided into 3 treatment groups: CD200R antagonist (AbD Serotec MCA2281EL, 1 mg/ml), IgG2a isotype control (AbD Serotec MCA1212EL, 1 mg/ml) dissolved in 0.1 M phosphate buffered saline (PBS) and PBS control. The treatment groups were further divided into 1 and 3 days post injection survival times (n=4/group). Mice received a laminectomy at the T9 vertebral level. The spinal cord was stabilized using a stereotaxic frame and approximately 1 µl of treatment was injected into two separate locations within the cord using calibrated pressure injection over the course of 45 minutes. This procedure is reported to be non-traumatic as microglia are not activated, myelin is not damaged and behavioral deficits were not evident (Popovich
et al., 2002). Hind limb motor function was assessed 1 day post surgical procedure using the Basso Mouse Scale (BMS) (Basso et al., 2006).

4.2: Tissue Collection and Processing

Mice were anesthetized with ketamine and xylazine at various times post injury as described above and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) in order to collect spinal cords for histology. Tissues were post-fixed for two hours in 4% paraformaldehyde solution, rinsed in 0.2M phosphate buffer (PB) and then stored in 30% sucrose. Spinal cords were blocked in Tissue-Tek OCT Compound (Sakura 4583), sectioned using a cryostat and collected on Superfrost Plus slides (VWR) and stored at -20°C until immunohistochemistry was performed. The block of spinal cord specimens with crush injuries were sectioned in the sagittal plane at 15µm while all other spinal cord blocks were sectioned in the transverse plane at 10µm. Eriochrome cyanine stain followed by cresyl violet counter stain was performed to determine the injury epicenter in the spinal cord injury studies and the specific spinal level in the dorsal rhizotomy, sciatic nerve and hemisection injuries.

4.3: Immunohistochemistry

For immunohistochemistry, slides were dried at 37°C for a minimum of one hour before being rehydrated with 0.1M PBS. After blocking with a solution of 1% Blokhen (Aves BH-1001), 0.1% fish gelatin (Sigma G7765) and 0.1% Triton X-100 (Sigma T9284) in 0.1M PBS, slides were incubated with primary antibody overnight at 4°C. Standard fluorescent and DAB immunohistochemistry techniques were used unless otherwise specified. Primary and secondary antibodies are listed in Table 1 and 2, respectively and were diluted into the blocking solution described above. Peroxidase labeling was
performed using Vector ABC Elite Kit (Vector PK-6100), and DAB staining was performed using DAB Peroxidase Kit (Vector SK-4100).

Labeling of CD200R in spinal cord sections required the use of a tyramide signal amplification (TSA) kit (Perkin Elmer SAT700001EA). After the primary antibody was applied overnight, a biotinylated secondary was added, followed by addition of a peroxidase to the complex using Elite-ABC Kit. The peroxidase catalyzes the tyramide on the biotinyl tyramide complex causing the tyramide to quickly react with electron rich amino acids. This creates many biotin-binding sites for detection of CD200R using a fluorescent streptavidin.

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<td>1:1,500</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Rabbit</td>
<td>Wako 019-19741</td>
<td>1:1,000</td>
</tr>
<tr>
<td>NeuN</td>
<td>Mouse</td>
<td>Chemicon MAB377</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 1. List of primary antibodies used for immunohistochemistry in vitro and in vivo.
Table 2: Secondary Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Vendor</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Goat anti-Rabbit</td>
<td>Vector BA-1000</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Fluorescein Streptavidin</td>
<td>Vector SA-5001</td>
<td>1:500</td>
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<tr>
<td>AlexaFluor 488 Donkey anti-Goat</td>
<td>Mol. Probes A11055</td>
<td>1:200</td>
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<tr>
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<td>Mol. Probes A11034</td>
<td>1:200</td>
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<tr>
<td>AlexaFluor 488 Goat anti-Mouse</td>
<td>Mol. Probes A11029</td>
<td>1:200</td>
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<tr>
<td>AlexaFluor 546 Donkey anti-Goat</td>
<td>Mol. Probes A11056</td>
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<tr>
<td>AlexaFluor 546 Goat anti-Rabbit</td>
<td>Mol. Probes A11035</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2. List of secondary antibodies used for immunohistochemistry in vitro and in vivo.

4.4: Intraspinal Microinjection Analysis

Microglia activation was determined by Iba-1 reactivity. Injected CD200R and IgG antibodies were detected with Alexa Fluor 488 goat anti-rat secondary antibody. Microglia activation was quantified on every section that antibody was detected by digital image analysis as described previously (Donnelly et al., 2009). The scan area was defined by spread of injected antibody and degree of Iba-1 reactivity was quantified using proportional area analysis. All measurements were performed on MCID Elite Analysis Software (mcid.co.uk).

4.5: Bone Marrow Derived Macrophage Isolation

Bone marrow derived macrophages (BMDMs) were isolated from bone marrow as described previously (Longbrake et al., 2007). 8-10 week old adult C57Bl/6 mice were deeply anesthetized with ketamine and xylazine followed by cervical dislocation. Bone marrow was aseptically isolated bilaterally from femurs and tibias by flushing with RPMI media. Bone marrow was dissociated into a single cell suspension and red blood cells
were lysed using a lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₄EDTA). Cells were then plated in RPMI (Fisher 15-040-CV) with 10% fetal bovine serum (FBS) (Sigma F2442), 1% Penicillin/Streptomycin (Life Technologies 15140-148), 1% HEPES (Fisher NC0229916), 1% Glutamax (Life Technologies 35050-061), 0.001% B-Mercaptoethanol (Fisher O34461100) and 20% supernatant from sL929 cells. Supernatant from sL929 cells, containing colony stimulating factor, are used to stimulate bone marrow cells to macrophages (Burgess et al., 1985). Cells were allowed to grow for 7 days in L929 supplemented media to allow full differentiation into macrophages.

For immunocytochemistry, BMDMs were replated onto cover slips in 24 well plates at approximately 50,000 cells/well in plating media (10% FBS, 1% Penicillin/Streptomycin and 1% Glutamax in RPMI) and allowed to adhere to the bottom of the plate overnight. BMDMs were differentiated to M1 or M2 phenotypes by stimulation for 24 hours with IFNγ (20ng/ml; eBioscience 14-8311-63) and LPS (100ng/ml; Sigma L2880) or IL-4 (20ng/ml; eBioscience 14-8041-80), respectively. To collect cells for histology, 500ul 4% paraformaldehyde was added to the 500µl media on the cover slips for 30 minutes. After the paraformaldehyde and media was aspirated, wells were rinsed with 0.1M PBS and then stored in 0.1M PBS at 4°C until histology was performed. BMDMs were stained with Iba-1 (1:500; Wako) and CD200R (1:500; R&D Systems) primary antibodies overnight at 4°C, followed by one hour at room temperature in the appropriate secondary antibody.

CD200R immunoreactivity was quantified on BMDMs by measuring the staining intensity. Three random images were taken for each BMDM activation phenotype (M0, M1, or M2) and the intensity of CD200R and Iba-1 was measured using MCID Elite Analysis Software (mcid.co.uk).
4.6: Griess Assay

BMDM nitric oxide production after activation of CD200R was measured using the Griess Assay. BMDMs were plated in 96-well plates at 100,000 cells/well and allowed to adhere overnight. Cells were pre-treated for 30 minutes with the soluble CD200 fusion protein, CD200Fc (R&D Systems 3355-CD-050), or IgG1 isotype control (R&D Systems 110-HG-100) at 1, 2.5, 5, 10, 20 and 100 µg/ml. After pre-treatment, cells were stimulated for 24 hours with LPS (100ng/ml; Sigma L2880), IFNγ (20ng/ml; eBioscience 14-8311-63) or unstimulated in conjunction with CD200Fc or IgG treatments. Media was transferred to a clean 96-well plate and 20µl Griess Reagent and 130µl of dH2O was added to each well, according to the Griess Reagent Kit for Nitrite Determination (Invitrogen G-7921). Reagent and media were allowed to incubate for 30 minutes at room temperature and the plate was read at 546nm. Nitric oxide concentration was calculated based on a standard curve. All conditions were run in triplicate.

4.7: Statistical Analysis

Nitric oxide production, as measured by the Griess assay, was analyzed using Two-way ANOVA followed by Bonferroni’s post-hoc comparisons. Differences in Iba-1+ proportional area after CD200 antibody ISM were analyzed using an unpaired t-test. Differences in CD200R staining intensity on BMDMs were analyzed using One-way ANOVA. Results are reported as group mean ± standard error of the mean (SEM) and were considered statistically significant at p<0.05. All statistical analysis were performed using Graph Pad Prism 5 software.
Chapter 5: Results

5.1: Distribution of CD200 in the Naïve Spinal Cord

We hypothesized that a reduction in CD200 after spinal cord injury would lead to excessive macrophage and microglia activation. First, we determined the distribution of CD200 and CD200R in the uninjured spinal cord. Using immunohistochemistry, we stained naïve tissue sections from mid-thoracic spinal cord and observed preferential expression of CD200 within the gray matter (Figure 1), which is consistent with previous literature (Koning et al., 2009; Shrivastava et al., 2012). However, limited CD200 expression within the white matter was observed. Specifically, CD200 is expressed highly within the superficial dorsal horn (Figure 1d), intermediolateral cell column (Figure 1b), and lamina X of the gray matter (Figure 1b), and within the fiber bundles of the ventromedial white matter (Figure 1c), the corticospinal tract (Figure 1d) and in the radial projections in the dorsolateral white matter. CD200 expression is restricted to the nodes of Ranvier of myelinated axons (Chang et al., 2011), which explains why expression is lower in the white matter.

Several reports have indicated that astrocytes express CD200 (Costello et al., 2011), therefore to determine if astrocytes express CD200 in the naïve spinal cord, sections were counterstained with antiserum against glial fibrillary acidic protein (GFAP). In contrast, our data shows that CD200 does not appear to co-localize with GFAP immunolabeled fibrous astrocytes within the white matter (Figure 1a,c), except possibly in the superficial dorsal horn (Figure 1d). GFAP preferentially labels fibrous astrocytes within the white matter while protoplasmic astrocytes in the gray matter.
express little to no GFAP. Therefore, it is possible that astrocytes that do not highly express GFAP could express CD200. The use of additional immunohistochemical markers for astrocytes, such as glutamate aspartate transporter (GLAST) (Costello et al., 2011) or ALDH1L1 (Cahoy et al., 2008), should be used to determine if astrocytes express CD200 in the resting CNS.

To confirm that the pattern of staining was specific for CD200, we compared the staining of a rat monoclonal antibody (Figure 1a-e) and goat polyclonal antibody (Figure 1f) in the naïve spinal cord. As these antibodies are raised against different epitopes within CD200, it would be highly unlikely for their staining patterns to overlap if they were not specific (Rhodes, 2006). While the goat polyclonal antibody had greater background than the rat monoclonal antibody, the pattern of staining with the two different antibodies are highly similar allowing us to conclude that our antibody is specific to CD200. Non-specific binding of secondary antibodies was not detected on slides incubated without primary antibody.

As seen in figure 1b, CD200 brightly labels the gray matter; however there are gaps in the staining which we hypothesized were neuronal cell bodies. To confirm, we counterstained with anti-NeuN antibody. As seen in figure 1e, the gaps in CD200 staining are filled in by NeuN positive label, confirming the presence of neuronal cell bodies. CD200 is not expressed on the cell bodies themselves (Shrivastava et al., 2012). However, because the expression of NeuN is restricted to neuronal cell bodies (Mullen et al., 1992), we would not expect CD200 to directly co-localize with NeuN.

In summary, CD200 immunoreactivity is preferentially expressed within the spinal cord gray matter but does not co-localize with NeuN positive profiles. Instead, CD200 appears to localize to regions enriched in small diameter myelinated and unmyelinated axons within the spinal cord.
Using immunohistochemistry, CD200R was detected at extremely low levels in naïve tissue sections (Figure 2), which is consistent with previous reports that CD200R is only detectable by flow cytometry on resting microglia (Koning et al., 2009). It is unlikely that our technique or antibody did not label properly as the antibody has been used previously (Shrivastava et al., 2012) and the same procedure was performed on injured tissue, resulting in successful detection of CD200R. CD200R appears to be expressed on microglia that express higher levels of Iba-1, as denoted by arrows in figure 2. This is consistent with previous reports in a rat model of glaucoma where increased expression of OX-42 was correlated with increased expression of CD200R on microglia (Taylor et al., 2011).
Figure 1. Distribution of CD200 in cross section of mid-thoracic naive mouse spinal cord. CD200 is highly expressed in the gray matter, including lamina X (Lam X) and in select locations of the white matter, such as the intermediolateral cell column (IML) (A-B). CD200 is expressed in the ventral horn (VH) and to a lesser extent in the ventral white matter (VWM). Fibrous astrocytes in the ventral white matter, labeled with GFAP, do not express CD200 (C). CD200 is expressed in the corticospinal tract (CST) and highly expressed in the dorsal horn (DH) (D). NeuN positive cell bodies in the gray matter surrounded by CD200 (E). Specificity of CD200 antibody is confirmed by comparing staining patterns from a rat monoclonal (A-E) and goat polyclonal (F) antibody. Scale bar = 200µm (A), 100µm (B-F).
Figure 2. CD200R is expressed at extremely low levels within the naïve spinal cord. Arrows mark sites of low CD200R expression colocalizing with microglia expressing higher levels of Iba-1 compared to surrounding cells. White lines outline the gray and white matter borders. Merge with DAPI (A), Merge (B), Iba-1 (C), CD200R (D). Scale bar = 100 µm.
5.2: Alteration of CD200 Expression After Select Nervous System Injuries

Based on the pattern of CD200 expression seen in Figure 1, we hypothesized that CD200 was expressed on specific axonal populations, including small diameter sensory axons in the dorsal roots and dorsal horn and descending corticospinal axons. This pattern of expression appears similar to other cell adhesion molecules in the CNS, such as L1 CAM and GAP43 (Bartolome et al., 2002; Runyan et al., 2005). L1 CAM has been shown to be located on small diameter primary afferents, and decreases in a time-dependent manner after dorsal rhizotomy injury (Runyan et al., 2005). We hypothesized that after dorsal rhizotomy, CD200 would also decrease in the affected superficial dorsal horn as Wallerian degeneration would lead to eventual loss of sensory afferents (Aldskogius and Kozlova, 1998).

After dorsal rhizotomy, the expression of CGRP, a marker of small diameter nociceptor afferents (Chung et al., 1988), is decreased in the dorsal horn confirming successful injury to the dorsal roots (Figure 3b). Adjacent sections were stained with eriochrome cyananine and cresyl violet (EC/CV) to determine the approximate spinal level of the denervated dorsal horn (Figure 3a). At seven days post injury, we observed increased expression of Iba-1 and GFAP within the dorsal horn on the injured side (Figure 3d+e). Unexpectedly, CD200 expression was slightly increased compared to the contralateral side (Figure 3c).

Next, a sciatic nerve injury was performed to determine if CD200 expression is changed following a distal axotomy of sensory axons and motor neurons. The sciatic nerve was transected in the gluteal region, resulting in a glial reaction in both the dorsal and ventral horns. The motor neurons within the ventral horn react to the injury due to retrograde signaling to the cell bodies in the ventral horn while distal axons underwent Wallerian degeneration. The number of sensory fibers in the dorsal horn decrease after
sciatic nerve transection (Himes and Tessler, 1989), but those that remain make additional synaptic connections (Molander et al., 1988).

The sciatic nerve enters the spinal cord at the L4-6 spinal level and EC/CV staining was used to identify sections near the L5 spinal level (Figure 4a + 5a). At 7 dpi, we observed an increase in Iba-1 and GFAP immunoreactivity in both the dorsal and ventral horns (Figure 4b,d). By 14dpi, Iba-1 appeared to have increased while GFAP reactivity remained at similar levels (Figure 5b,d). CD200 expression did not change at 7 or 14 dpi compared to contralateral side (Figure 4c + 5c).

Based on the axon-like staining in the base of the dorsal horn illustrated in Figure 1d, we hypothesized that CD200 was present on axons within the dorsal corticospinal tract (CST). Therefore, a T9 hemisection injury was performed to determine if CD200 expression was altered by damage to the CST at 7dpi. The spinal cord was examined caudal to the injury site at the L5 spinal level (Figure 6a). To determine if hemisection successfully transected the corticospinal tract, immunohistochemistry for protein kinase C gamma (PKCγ) was performed. PKCγ expression is restricted to the superficial dorsal horn and corticospinal tract (Mori et al., 1990). PKCγ labels the intact CST brightly in dorsal rhizotomy mice (Figure 6c), but expression was noticeably absent after hemisection injury (Figure 6b), confirming successful transection of the corticospinal tract. We observed a slight increase in Iba-1 immunoreactivity at 7dpi (Figure 6f), compared to dorsal rhizotomy animals (Figure 6g). However, we did not observe increased GFAP after hemisection (Figure 6d) compared to control (Figure 6e). We saw no change in CD200 expression after hemisection (Figure 6d) when compared to dorsal rhizotomy (Figure 6e).

In summary, glial reactivity was observed in all three models. After dorsal rhizotomy and sciatic nerve injury, Iba-1 and GFAP immunoreactivity were increased in
the areas corresponding to degenerating axons or retrograde cell body reaction. After hemisection, increases in Iba-1, but not GFAP were observed. Alterations in CD200 immunoreactivity varied based on the lesion. Sciatic nerve injury and hemisection resulted in no observed difference in CD200 expression while unexpectedly, CD200 appeared to be increased after dorsal rhizotomy. This suggests that CD200 is either not expressed on these axons as hypothesized, or that the expression of CD200 is maintained after injury. Additionally, the presence of CD200 alone is not sufficient to inhibit microglial activation after injury to these specific axons.
Figure 3. Spinal cord sections 7 days post dorsal rhizotomy. EC/CV stain suggests sections are from the sacral spinal level (A). CGRP immunolabeling confirmed injury to dorsal afferents as visualized by a reduction in DAB label (B). CD200 expression was slightly increased following dorsal rhizotomy (C). A slight increase in GFAP (D) and Iba-1 (E) was observed on the injury side compared to contralateral side. Scale bar = 300µm.
Figure 4. Spinal cord sections 7 days post sciatic nerve transection. Spinal cord sections from the L5 spinal level as seen by EC/CV (A). Iba-1 immunoreactivity is increased in the dorsal and ventral horns (B). CD200 expression is similar when compared to contralateral side (C). Asterisk denotes section artifact (C). GFAP reactivity is increased in the ventral horn, and slightly in the dorsal horn (D). Scale bar = 300µm.
Figure 5. Spinal cord sections 14 days post sciatic nerve transection. Spinal cords were examined at the L5 spinal level as visualized by EC/CV (A). Iba-1 reactivity is slightly greater compared to 7dpi (B). CD200 reactivity is not altered at this time point (C). GFAP is increased in both the dorsal and ventral horns (D). Scale bar = 300µm.
Figure 6. Spinal cord lumbar sections 7 days post T9 hemisection. EC/CV stain shows a representative section at the L5 level (A). B-G denote high power images from the region outlined by the black box in A. Reduction in PKCγ label in hemisection (B) compared to control (C) animal confirms successful damage to the corticospinal tract. CD200 expression does not appear to be altered after hemisection (D) compared to control (E). Iba-1, but not GFAP, immunoreactivity appears to be slightly increased after hemisection (F and D, respectively) compared to control (G and E, respectively). Lines denote gray-white matter border. Scale bar = 300µm (A), 100µm (B-G).
**5.3: Intraspinal Microinjection of CD200R Antagonist Results in Microglia Activation**

Previous studies have shown that microglia in the naïve spinal cord of adult CD200KO mice show signs of increased activation (Hoek et al., 2000), however, because CD200 is expressed throughout the body there is speculation that complete knock out of CD200 may have various unknown effects resulting in increased levels of activation. We hypothesized that the high expression of CD200 in the gray matter of the spinal cord serves to activate CD200R and maintains the resting state of microglia. Therefore, in order to determine if preventing the interaction between CD200 and CD200R on microglia in the uninjured spinal cord is sufficient to induce activation, a CD200R monoclonal antibody, known to function as a receptor antagonist, was delivered to the naïve spinal cord via intraspinal microinjection (ISM). Previous studies have shown that this antibody successfully inhibits the interaction between CD200 and CD200R in vitro (Wright et al., 2000) and in vivo (Meuth et al., 2008; Wang et al., 2011; Zhang et al., 2011). Injection of 5 µl of 1 mg/ml CD200R antibody was sufficient to induce microglia activation within the striatum, compared to control antibody (Zhang et al., 2011).

Because the spinal cord has a smaller volume than the brain, we injected 1 µl volume of the same concentration used previously. Low endotoxin CD200R monoclonal antibody and low endotoxin IgG1 control antibody were used directly from manufacturer’s stock. Sterile, cell culture grade PBS was used as a control for the IgG. The IgGs or PBS were injected into the ventral horn of naïve mice using non-traumatic microinjection techniques (Popovich et al., 2002). Tissue was collected at 1 or 3 days post injection (dpi), which reflects the time of peak microglial activation after damage (n= 4 per group). Mice did not exhibit any locomotor behavioral deficit after injection as assessed by the Basso mouse scale (BMS).
Tissues from the spinal cord region containing the injection sites were sectioned and immunohistochemistry was performed. To maintain blinding, the analysis was done by the investigator after assuring that the tissue sections were de-identified with respect to treatment group. Injection sites were first identified by staining a series of sections with anti-Iba-1 using DAB (Figure 7). Higher levels of Iba-1 label were seen within the presumptive injection sites (Figure 7c) compared to the contralateral side (Figure 7b). After completion of the analysis, the code was revealed to confirm that all surgical injection sites had been identified and were included in the analysis.

To determine the extent of antibody spread to the spinal cord, fluorescent immunohistochemistry was performed to detect IgG present in the tissue and sections were co-labeled with Iba-1 (Figure 8). The majority of CD200R (Figure 8a) or IgG (Figure 8b) control antibody was localized to the ventral horn of antibody-injected animals confirming proper delivery to the intended location. Note higher levels of Iba-1 detected within these regions. PBS injected animals did not show detectable IgG reactivity within the spinal cord (Figure 8c).

Microglia activation was quantified as proportional area of the IgG spread occupied by Iba-1 positive cells and processes (Donnelly et al., 2009). Iba-1 proportional area was not significantly different at 1 day post injection (Figure 9a). At 3 day post injection, however, the mice injected with CD200R antagonist had significantly more Iba-1 proportional area (p=0.0035) (Figure 9b). Further, the scan area between IgG injected groups were not statistically significant (Figure 9c,d). These results show that blocking CD200R in vivo results in microglia activation.

PBS injected animals were not included in the results or analysis as these animals had extremely high levels of microglia activation. Prior studies in our lab and collaborator labs have not found similar responses to PBS, so we tentatively suspect that
the PBS had become contaminated, although the samples were not retained for later testing. Notably, the antagonist and control antibodies were obtained commercially and used at stock concentrations (undiluted), therefore the antibody treatment groups did not contain the PBS solution used for the third group.
Figure 7. Microglial reactivity following spinal cord microinjection. Iba-1 labeled with DAB in spinal cord cross section one day after antibody injection of IgG (A). Note increased Iba-1 reactivity (C) compared to the contralateral side (B). Scale bar = 50µm.
Figure 8. Detection of IgG spread and Iba-1 immunoreactivity following spinal cord microinjection. CD200R (A, A’) or IgG (B) monoclonal antibody within the ventral horn spinal cord. Note PBS injected mice do not show IgG reactivity (C). A’ shows higher power inset of boxed region in A with DAPI nuclei shown in blue. Scale bars = 100µm.
Figure 9. Proportional area and scan area of Iba-1 positive label in CD200R antibody and IgG antibody injected mice 1 and 3 days post injection. Proportional area of Iba-1 immunoreactivity was not statistically significant between groups at 1 dpi (p=0.5281) (A). At 3 dpi, Iba-1 proportional area was significantly higher in mice injected with CD200R antibody (p=0.0035) (B). Scan area used for proportional area analysis was not different between treatment groups (C, D).
5.4: Distribution of CD200 and CD200R after Spinal Cord Injury

We have established that CD200 is highly expressed relative to very low levels of CD200R in the naïve spinal cord (Figure 1 + 2). Based on our results from the dorsal rhizotomy, sciatic nerve and hemisection injuries, CD200 is not sufficient to limit microglia activation after minor insult to the nervous system (Figure 3-6). The ability for CD200R ligation to regulate inflammation is directly related to its level of expression (Jenmalm et al., 2006). Therefore, since macrophages express CD200R at higher levels compared to microglia (Walker et al., 2009), we hypothesized that CD200 and CD200R may play a greater role after spinal cord injury when high numbers of peripheral monocytes are recruited to the lesion.

Mouse spinal cord contusion injury tissue bank was created using the Infinite Horizons Impactor Device. Spinal cords were collected at 1, 4, 7, 14, 21 and 28 days post injury. Tissue sections were stained with eriochrome cyanine and counter stained with cresyl violet (EC/CV) in order to determine the injury epicenter (Figure 10). Sections adjacent to the injury epicenter were immunolabeled for CD200 or CD200R.

Overall, CD200 expression at the epicenter decreases after SCI (Figure 11). CD200 immunoreactivity was present within the lesion at 1 dpi and 4 dpi (Figure 11 a, b), but the expression pattern does not have the clear gray-white matter borders as seen in the naïve spinal cord (Figure 1). The maintained expression seen at these time points is likely due to cellular debris within the lesion, as demonstrated in EC/CV stained tissue in an adjacent section (Figure 10). At 7, 14, 21 and 28dpi (Figure 11c-f), CD200 expression is almost completely absent with a small amount of expression maintained in the dorsal horn. High power images at 14dpi (Figure 12) show minimal CD200 expression within the dorsal horn (Figure 12b), and no expression within the glial scar (Figure 12c) or within the central lesion (Figure 12d). There is occasional overlap of GFAP and CD200
as observed in Figure 12, which could be astrocytic processes. These data show that CD200 is not expressed within the lesion epicenter at chronic time points post injury.
Figure 10. Eriochrome cyanine and cresyl violet labeled spinal cord cross sections at the injury epicenter. The injury epicenter was identified by the section that had the least amount of eriochrome cyanine stain (blue). Scale bar = 300µm.
Figure 11. Transverse spinal cord sections reveal CD200 expression decreases after contusion spinal cord injury. At 1 and 4 dpi, CD200 immunoreactivity is observed within the lesion (A, B). By 7 dpi, CD200 immunoreactivity is almost completely absent (C). At 14, 21 and 28 dpi, CD200 reactivity is observed minimally in the central lesion and at higher levels on the dorsal part of the section. Note CD200 does not appear to co-localize with GFAP from 14-28dpi when the glial scar is present. (D-F). Scale bars = 200µm.
Figure 12. CD200 and GFAP expression at 14 days post injury (A). Slight CD200 expression appears to be maintained in the areas of the dorsal horn (B). CD200 expression does not co-localize with GFAP, but is observed in the dorsal roots (C). The lesion epicenter does not appear to have remaining CD200 reactivity (D). Occasional co-localization of GFAP and CD200 could be seen on small astrocytic processes. Scale bar = 100µm.
As stated previously, we hypothesized that after a larger insult to the spinal cord, we may see greater expression of CD200R on infiltrating macrophages compared to microglia. Transverse spinal cord sections at the injury epicenter were labeled with CD68, a phagocytic macrophage marker, and CD200R. Expression of CD200R within the lesion epicenter is minimal at 1 dpi, but expression gradually increases from 4 to 28 dpi (Figure 13), coinciding with the time course of macrophage infiltration (Kigerl et al., 2006). From 7 to 28 dpi, CD200R expression is throughout the entire cross section of the injury epicenter (Figure 13c-f), which would coincide with the spared white matter rim and glial scar. As the glial scar forms from 14 to 28dpi (Figure 10), CD200R expression appears concentrated within the central lesion, while expression is markedly reduced within locations that would correspond with the glial scar (Figure 14).
Figure 13. Transverse sections at the injury epicenter of CD200R and CD68 expression after contusion spinal cord injury. CD68 labels phagocytic macrophages and highly co-localizes with CD200R at later time points. CD200R expression is very low at 1 dpi, but is highly expressed at later time points post injury correlating with influx of trafficking macrophages. Scale bars = 200 μm.
Figure 14. Expression of CD200R 28 days post injury at the epicenter after contusion spinal cord injury. There is a high concentration of CD200R and CD68 positive cells within the center of the lesion, while expression is less dense outside of the central lesion. Scale bar = 200µm (A) and 100µm (B-D).
The contusion injury model creates a large lesion that extends in both the rostral and caudal directions. Due to the size of the injury in the contusion model, it can be difficult to visualize the extent of the entire lesion. The crush injury model was employed because the lesion is smaller allowing for visualization of the rostral/caudal borders and spared tissue. CD200 expression is maintained within the spared gray matter both rostral and caudal to the crush injury at 28 dpi (Figure 15, arrows), but is notably absent within the lesion borders (Figure 15). CD200 expression is observed within the lesion, which is not observed in the contusion injury model. It is possible that CD200+ B cells, T cells or Schwann cells (Rijkers et al., 2008; Chang et al., 2011) may be present after crush injury, but not contusion injury. Future experiments should be pursued to identify the unknown cell type expressing CD200 within the crush lesion. High expression of GFAP surrounds the central lesion indicative of the glial scar. CD200 appears to co-localize with the edges of the GFAP positive glial scar bordering the lesion, however the bulk of the glial scar does not express CD200 (Figure 15, asterisk). CD200R expression within the lesion epicenter in the crush injury model was similar to the contusion injury model (Figure 16).
Figure 15. Expression of CD200 in a sagittal section at 28 days post crush spinal cord injury, merge (A), GFAP (B), CD200 (C). Note high expression of CD200 in the spared gray matter (arrows) and within the central lesion, while expression does not co-localize with the GFAP positive glial scar (asterisks). Scale bar = 200µm.
Figure 16. CD200R and Iba-1 expression in crush lesion epicenter at 7 days post injury. The majority of cells are both CD200R and Iba-1 positive confirming the presence of macrophages. Scale bar A = 100µm, B,C,D = 50µm.
5.5: Effect of CD200Fc on LPS or IFNγ Stimulated Bone Marrow Derived Macrophages

We have established that CD200 is expressed in the uninjured spinal cord (Figure 1), but does not decrease following injury to specific axons or motorneurons (Figure 3-6). After contusion injury, CD200 expression is drastically decreased within the lesion starting at 7 dpi and remains low within the glial borders surrounding the lesion at 28 dpi (Figure 11). We have also shown that antagonizing CD200R in the naïve spinal cord is sufficient to activate microglia (Figure 9). Therefore, we next hypothesized that due to a lack of normal levels of CD200 in and surrounding the site of a SCI, the surrounding microglia and/or trafficking macrophages are not receiving this inhibitory signal resulting in persistent inflammation and enhanced neuronal death (Meuth et al., 2008). If this is the case, then we propose that activating CD200R after SCI will help reduce inflammation. Before testing this in the complex in vivo microenvironment, we wished to next determine whether the anti-inflammatory effects of CD200Fc are indeed sufficient to alter the function of isolated macrophages in vitro. We explored the effect on macrophages, rather than microglia, because CD200R appears to be more highly expressed on macrophages compared to microglia (Figure 13+14).

To test this hypothesis, we first established that isolated bone marrow derived macrophages (BMDMs) express CD200R and Iba-1 in our in vitro assay (Figure 17). M1 BMDMs appear round and phagocytic while M0 and M2 BMDMs extend long thin filopodia, as described previously (Longbrake et al., 2007). It appeared that CD200R expression was slightly lower on M1 BMDMs compared to M0 or M2 BMDMs. Color intensity analysis was performed on three random images for each macrophage phenotype to determine if there were differences in expression (Figure 18). Differences in Iba-1 intensity were not statistically significant between phenotypes, indicating...
consistent labeling (Figure 18a). Differences in CD200R intensity were also not statistically significant between macrophage phenotypes (Figure 18b), which is consistent with previous reports (Copland et al., 2007).

We then administered increasing doses of CD200Fc, a soluble CD200-IgG fusion protein and potent CD200 agonist, to IFN\(\gamma\) or LPS stimulated or control BMDMs and measured the resulting nitric oxide produced using a Griess assay (Liu et al., 2010b).

Unstimulated (M0) macrophages did not produce detectable levels of nitric oxide, as predicted since this is considered an unactivated macrophage phenotype (Longbrake et al., 2007) (Figure 19a). As expected, activation of CD200Fc had no effect on unstimulated BMDMs. Unexpectedly, IFN\(\gamma\) stimulated macrophages also did not produce detectable levels of nitric oxide in this assay, in contrast to other published results (Blanchette et al., 2003; Herbst et al., 2011). This may indicate that the dose of INF\(\gamma\) used was insufficient to generate a full pro-inflammatory phenotype in BMDMs under the conditions used here. Thus, as with the M0 macrophages, adding CD200Fc had no effect on INF\(\gamma\) stimulated BMDMs (Figure 19b). In contrast, robust nitric oxide production was observed from LPS stimulated BMDMs. However, CD200Fc pre-treatment at any concentration used was not sufficient to reduce nitric oxide production compared to IgG control in response to LPS (Figure 19c). While we cannot rule out the possibility that CD200R activation was suboptimal in this assay, the results suggest that activation of CD200R alone is insufficient to prevent pro-inflammatory signaling in LPS activated macrophages.

It should be noted that the presence of negative nitric oxide concentrations are likely due to bubbles present in the solutions when read by the plate reader, which would alter absorbance values. Every effort was made to remove remaining bubbles, however some remained when the plate was read.
Figure 17. Bone marrow derived macrophages express CD200R. Unstimulated (M0) (A), IFNγ + LPS stimulated (M1) (B) and IL-4 stimulated (M2) (C) BMDMs highly express CD200R. Scale bar = 50µm.
Figure 18. Iba-1 (A) and CD200R (B) intensity is not different between bone marrow derived macrophage phenotypes. Color intensity analysis was performed on three random images containing multiple cells from each phenotype. One-way ANOVA revealed no differences in expression of Iba-1 (p = 0.3262) or CD200R (p = 0.1169) between phenotypes.
Figure 19. CD200Fc does not alter nitric oxide production from LPS or IFN\(\gamma\) stimulated BMDMs. M0 BMDMs did not produce nitric oxide under normal conditions and CD200Fc had no effect (A). IFN\(\gamma\) did not induce nitric oxide production (B). Robust nitric oxide production was observed from LPS stimulated BMDMs, however CD200Fc had no effect (C).
Chapter 6: Discussion

After spinal cord injury, mechanical damage severs axons and sheers blood vessels, initiating an inflammatory cascade ultimately resulting in permanent paralysis and loss of function below the injury site. The inflammatory response does not resolve; with upregulation of proinflammatory genes and evidence of immune cells present for months to years post injury (Fleming et al., 2006; Byrnes et al., 2011; Dulin et al., 2013). Macrophages hold promise as a targeted therapy due to their beneficial and detrimental effects following injury. Depending on the phenotype, macrophages have the ability to encourage or inhibit axon regeneration (Kigerl et al., 2009). Altering the macrophage response to reduce inflammation could reduce the inhibitory nature of the glial scar and reduce the toxicity of the lesion allowing for axons to reach the caudal spinal cord.

Macrophages and microglia both express CD200R, which is important for regulating inflammatory cytokine release (Wright et al., 2000). Based on the targeted, anti-inflammatory nature of CD200, examining its role after trauma could prove to be a useful therapy and our lab is the first to study the regulation of CD200 and CD200R as a potential therapeutic target to reduce inflammation after SCI.

CD200 appears to be expressed by axons within the spinal cord (Webb and Barclay, 1984), however, after injury to specific axon populations, CD200 expression was not altered. This leads us to conclude that CD200 is not expressed on the axons previously hypothesized or expression is maintained after injury to those fibers. It is possible that oligodendrocytes or myelin could be maintaining diffuse CD200 expression following injury (Chitnis et al., 2007; Koning et al., 2009). Schwann cells express
CD200 in the PNS before and after peripheral nerve injury (Chang et al., 2011). After peripheral nerve injury, Schwann cells down regulate CD200 at the distal stump to signal for phagocytosis while CD200 is upregulated at the proximal stump to protect the spared axon (Chang et al., 2011), therefore it is possible that oligodendrocytes in the spinal cord could maintain expression of CD200 in an effort to help regulate microglia activation and protect other neurons in the vicinity.

After sciatic nerve injury, there is a retrograde cellular reaction in the ventral horn and some sensory fibers in the dorsal horn die (Himes and Tessler, 1989), while those that remain undergo additional sprouting (Molander et al., 1988). GAP43 and CD200 have been shown to have similar expression patterns in the developing cochlea and it is hypothesized they are coexpressed by small diameter fibers and may be involved in axonal guidance (Barclay and Ward, 1982; Webb and Barclay, 1984; Bartolome et al., 2002). It is possible that at 7 dpi, degenerating axons in the dorsal horn have reduced CD200 expression however as remaining afferents sprout and make new connections, they upregulate CD200 to aid in axon guidance. We observed maintained CGRP immunoreactivity in sciatic nerve injured animals (data not shown), further suggesting that despite axonal degeneration, protein expression remains constant in the dorsal horn. In result, it appears as though CD200 expression is not altered following injury.

It is also possible that the presence of CD200 inhibits microglia phagocytosis resulting in the appearance of maintained expression. After peripheral nerve injury, while evidence of astrocyte and microglia activation in the dorsal horn occurred, Castro-Lopes et al. observed no evidence of phagocytosis (Castro-Lopes et al., 1990). CD200 has been referred to as a “don’t eat me signal” displayed by neurons in order to signal to microglia that they are healthy (Neher et al., 2012). CD200 is decreased in brains of Alzheimer’s disease patients suggesting that neurons down regulate CD200 as a distress
signal to microglia (Walker et al., 2009). Further, it is possible that CD200 may regulate microglia in the process of synaptic pruning, as CD200 is expressed on boutons and spines of neurons and this expression is decreased with age (Ojo et al., 2011; Schafer et al., 2012).

Because CD200 is maintained after dorsal rhizotomy and hemisection injuries in the present study, signals for microglia to phagocytose cellular debris may be inhibited due to remaining CD200, resulting in the appearance of maintained CD200 expression. In fact, Wallerian degeneration is much slower in the Wld<sup>e</sup> mouse (Perry et al., 1991; Mack et al., 2001), which may be explained by higher levels of CD200 expression present in this mutant mouse strain (Chitnis et al., 2007). If this maintained CD200 expression is inhibiting phagocytosis of CD200 positive cellular debris, delivery of a CD200R antagonist to the dorsal horn should result in increased clearance of cellular debris, resulting in a reduction of CD200 immunoreactivity following dorsal rhizotomy. While presence of CD200 may inhibit phagocytosis after dorsal rhizotomy, due to the high number of inflammatory stimuli present after SCI, the presence of CD200 within the lesion at 4 and 7 dpi will likely not be sufficient to inhibit phagocytosis of cellular debris.

Future experiments should be performed to determine if CD200 expression is reduced at later time points, to allow more time for potential reduction in CD200. If CD200 is not expressed by the axons injured in this study, additional work should be performed to determine which cell types express CD200 using immunohistochemistry. However, immunohistochemistry may not provide the resolution necessary to establish differences between cell types, therefore techniques such as flow cytometry or electron microscopy should be employed.

Despite maintained expression of CD200, microglial activation still occurred following injury, suggesting that CD200 is not able to inhibit all aspects of microglial
activation. Therefore, we conclude that the presence of CD200 is not sufficient to inhibit microglia activation after these injuries. Due to low levels of CD200R expression on microglia, CD200 may not be a potent signal to overcome activation after these types of injuries, i.e. the activation signals due to neuronal damage may be too strong of a stimulus for CD200 to overcome.

It has been shown that microglia express CD200R at a significantly lower level compared to macrophages (Walker et al., 2009). Cross linking CD200R on monocytes was required to reduce tetanus toxin induced secretions of IL-5 and IL-13 (Jenmalm et al., 2006). Based on these data, because microglia express low levels of CD200R, they may not be as responsive to CD200 regulation. We hypothesized that the regulatory role of CD200 may exert a greater effect on macrophages recruited to the CNS after spinal cord injury.

Previous work with chimera mice rats has shown that through 3 dpi most of the macrophages within the lesion are microglial derived while starting at 7 dpi, the majority of macrophages are monocyte derived. Further, at these later time points microglia-derived macrophages tend to occupy the spared white matter rim while monocyte-derived macrophages are more likely to be found in the gray matter and lesion epicenter following spinal cord injury (Popovich and Hickey, 2001; Donnelly et al., 2011; Mawhinney et al., 2012). At 1 and 4 dpi, when the majority of the cells within the lesion are likely microglia derived, CD200R expression is lower compared to later time points. Starting at 7 dpi, we see a large increase in CD200R expression throughout the entire cross section suggesting that both monocyte derived and microglia derived macrophages highly express CD200R at this time point. As the glial scar forms from 14 to 28dpi, we see a reduction in CD200R expression within the outer rim of tissue where microglia derived macrophages are more likely to be present. At these time points CD200R
expression is maintained at high levels within the central lesion on monocyte derived macrophages. Based on these data, it appears as though monocyte derived macrophages express CD200R at higher levels compared to microglia derived macrophages after spinal cord injury.

CD200R agonists have been shown to be beneficial in other CNS inflammatory diseases such as EAE, Alzheimer’s Disease and aging (Lyons et al., 2007; Liu et al., 2010b; Cox et al., 2012). Thus, manipulation of CD200R may serve to reduce inflammation following SCI. However, based on the present data, it is likely that treatment with CD200R agonists will only be effective if administered by an appropriate delivery method. For example, delivery of CD200Fc directly into the spinal cord lesion is not predicted to be a beneficial treatment following SCI due to the overwhelming number of inflammatory stimuli present within the lesion. The in vitro experiments showed that CD200Fc was unable to reduce nitric oxide production from LPS stimulated BMDMs. LPS is a potent TLR4 agonist (Poltorak et al., 1998), therefore, this suggests that CD200R activation may not be sufficient to overcome TLR4 stimulation. Recent work with TLR knock out mice has revealed that EAE susceptibility is not altered in TLR4 knock out mice (Miranda-Hernandez et al., 2011). This suggests that TLR4 is not activated during the course of EAE, which may explain the use of CD200Fc to successfully reduce EAE symptom severity (Liu et al., 2010b).

After spinal cord injury, there is a plethora of inflammatory stimuli such as cellular debris, necrotic and apoptotic cells. It is likely that TLR4 agonists, such as heat shock proteins and fibronectin (Ohashi et al., 2000; Okamura et al., 2001), are present after SCI as they are present in other CNS pathologies (Bell et al., 2013; Noelker et al., 2013). Future experiments should identify the TLR4 agonists present after SCI and test the ability of CD200Fc to reverse BMDM activation after treatment with these specific
agonists. Assuming these stimuli are as potent as LPS, CD200Fc may not be able to overcome this level of activation, suggesting that treatment of macrophages within the central lesion may not be beneficial. Rather, delivery of CD200Fc through different methods to target specific macrophage populations may result in favorable outcomes.

As stated previously, CD200Fc treatment in EAE resulted in reduced symptom severity when CD200Fc was delivered intravenously. Further study into the mechanism of CD200Fc revealed that CD11b+ cells within the spinal cord expressed lower levels of LFA-1 and VLA-4 resulting in fewer macrophages trafficking into EAE lesions (Liu et al., 2010b). Previously, delivery of anti-VLA-4 antibodies after SCI resulted in a reduction of macrophage recruitment and improvement of locomotor function (Fleming et al., 2008). Based on these experiments, we propose that delivery of CD200Fc intravenously will reduce macrophage recruitment to the lesion following spinal cord injury. Further, activation of CD200R on monocytes before they enter the lesion may serve to alter the inflammatory response of macrophages once they are activated within the spinal cord.

We observed minimal expression of CD200 within the glial scar and the edges of the spared tissue. Microglia and macrophages within this region are not receiving the inhibitory signal from CD200 due to loss of neurons within this region. Targeting microglia and macrophages with CD200R agonists within the glial scar may be advantageous as increased levels of inflammation are associated with higher levels of CSPG production (Fitch and Silver, 1997). Some reports have shown that astrocytes upregulate CD200 in EAE lesions, suggesting that given the right conditions, astrocytes will express CD200 (Chitnis et al., 2007; Koning et al., 2009; Costello et al., 2011). Genetic based therapies or viral vectors could target the glial scar to increase CD200 expression which may reduce inflammation from microglia associated with the scar and
around the edges of the central lesion. This could further protect spared tissue from inflammatory damage and decrease the inhibitory nature of the glial scar.

As the classification implies, neuroimmune regulatory elements are responsible for regulating myeloid cell activation. Based on our data, it does not appear that activation of CD200R is sufficient to overcome potent inflammatory stimuli. While CD200 may play a role in maintaining myeloid cells in a quiescent state, it does not appear to be strong enough to reverse activation once it has been initiated. Administration of CD200Fc directly into the injured spinal cord lesion will likely not successfully reduce inflammation given the number of inflammatory stimuli that have already activated macrophages. However, administration of CD200R agonists systemically to activated macrophages before they enter the spinal cord or within the glial scar may serve of benefit following spinal cord injury. While this thesis leaves many questions unanswered, there are many future avenues of research that can assess the treatment potential of CD200R agonists after spinal cord injury.
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