HYALURONAN AND ITS DEGRADATION PRODUCTS HAVE OPPOSING EFFECTS ON EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS PATHOGENESIS THAT ARE DEPENDENT AND INDEPENDENT OF CD44

by

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A DISSERTATION

Presented to Neuroscience Graduate Program
School of Medicine
Oregon Health & Science University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

September 2012

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APCs- antigen presenting cells

BBB- blood-brain barrier

CD44- cluster determinant 44

CDI- cumulative disease index

CNS- central nervous system

CSF- cerebral spinal fluid

DMEM- Dulbecco's Modified Eagle's medium

EAE- experimental autoimmune encephalomyelitis

EBV- Epstein Barr Virus

EC-endothelial cell

ECM- extracellular matrix

ELISA- enzyme-linked immunosorbent assay

ERM- Ezrin/Radixin/Moezin

HA- Hyaluronan

HA4- HA tetrasaccharide

HA12- HA dodecasaccharide

HABP- HA binding protein

HARE- HA receptor for endocytosis

HAS- HA synthases

HBSS- Hank’s balanced salt solution

HEV- high endothelial venule

HLA- human leukocyte antigen

HMW- high molecular weight

HYAL- hyaluronidase

IL- interleukin

INF- interferon

merlin- moesin-ezrin-radixin-like protein

MHC- major histocompatibility complex

MOG- myelin oligodendrocyte glycoprotein

MMPs- matrix metalloproteases

MS- multiple sclerosis

NF- neurofilament light chain

NO- nitric oxide

PDGF- plasma derived growth factor

PEG- polyethylene glycol

PLP- proteolipid protein

PSGL-1- P-selectin glycoprotein ligand 1

qRT-PCR- quantitative real-time polymerase chain reaction

RHAMM- receptor for HA mediated motility

RTKs- receptor tyrosine kinases

SAS- subarachnoid space

TCR- T-cell receptor

TGFβ- transforming growth factor β

TLR- Toll- like receptor

T_H1- $CD4^+$ T helper 1

T_H2- $CD4^+$ T helper 2

TNF- tumor necrosis factor

TSG-6- Tumor necrosis factor stimulated gene-6

VEGF - vascular endothelial growth factor

WT- wild type

ACKNOWLEDGEMENTS

Many people have provided me support, advice and help during the process of this thesis and throughout my life. As the majority of them will never look upon these pages, it behooves me to thank them in person. However, a certain “core” group of individuals deserve indelible recognition for their efforts. These people include:

My parents: Jim and Lucinda for their total and unconditional love and support. I can never thank either of you enough for what you have provided me and made possible.

My sister: Kristi, who has always had to be the trail-blazer and has deceptively large feet despite her size. Thank you for showing the way.

My mentor: Larry Sherman for his encouragement, support, enthusiasm and most importantly, his patience. Even when the chips were down, he always managed to pull me through or pick me up. I have learned a tremendous amount under his tutelage that will no doubt serve me well in all my future endeavors.

My second mentor: Steve Matsumoto who has continually helped me keep “it” all in perspective. His hand or probing intellect has touched nearly every experiment described in this thesis and he is also one of the best barbecue cooks I know.

My third mentor: Sue Aicher, who gave me the push. Her love of life and science is infectious. Without her support, guidance and faith, I would probably be swinging a hammer.

My lab mates, past and present: Marnie Preston, Kerstin Feistel, Ellen Witkowski, Jaime Struve, Rubing Xing, Fatima Banine, Weiping Su and especially Scott Foster, the EAE man. A more talented, generous group there is not.

My thesis committee: Phillip Copenhaver, Mike Forte, Dennis Bourdette and Owen McCarty whose ideas contributed significantly to this thesis and constructive feedback pushed me to be a better scientist.

My “flow” collaborators: Michelle Berny-Lang and Asako Itakura, who drove while I shifted gears.

I dedicate this thesis to my wife and children. You all are my reason for being and my inspiration. I could never have done this without you. I look forward to our next amazing adventure.

ABSTRACT

Demyelination and axonopathy characterize the central nervous system (CNS) inflammatory disease multiple sclerosis (MS). While the etiology remains unknown, extensive evidence suggests encephalitogenic mononuclear cells mediate disease pathology. A key step in disease onset involves the activation of $CD4^+$ T-cells that recognize myelin antigens and their extravasation across the blood-brain-barrier (BBB). These cells mediate disease progression by recruiting components of the adaptive immune system that lead to the damage of myelin and axons. These events are recapitulated in a murine model of MS, experimental autoimmune encephalomyelitis (EAE). Therapeutically impairing extravasation of myelin-reactive T-cells in EAE and MS reduces disease severity and recurrence. However, the mechanisms involved in extravasation into the CNS are not completely understood. The extra-cellular matrix (ECM) glycosaminoglycan hyaluronan (HA) and its primary receptor CD44 have been implicated in this process; however, their respective roles remain unclear. Furthermore, HA has diverse, size-specific biological activity; the function of which has not been studied in regard to CNS demyelinating disease.

The studies presented in this dissertation examine the contribution of HA and CD44 to activated T-cell capture and rolling on CNS endothelial cells (ECs). This process is known to facilitate extravasation and therefore has therapeutic potential. Using an *in vitro* rolling assay, it was determined that CD44 on CNS ECs, but not activated lymphocytes, is necessary for capture and rolling. Further analysis of $CD44^{-/-}$ CNS EC cultures demonstrated that CD44 is required to tether HMW HA to the cell surface, suggesting the glycosaminoglycan is also critical to lymphocyte rolling. This idea was confirmed by the finding that degradation of HMW HA from the surface of CNS ECs with

a pegylated PH20 hyaluronidase (PEG-PH20) impaired lymphocyte rolling. Furthermore, EAE onset was delayed in $CD44^{-/-}$ and PEG-PH20 treated animals and hyaluronidase treated animals had fewer infiltrating $CD4^{+}$ T-cells two days following disease onset in controls.

Long-term treatment of EAE animals with PEG-PH20; however, worsened symptoms and promoted $CD4^{+}$ cell infiltration later in disease. This suggested HA fragments produced by PEG-PH20 may exacerbate disease, possibly through TLR4 signaling. To test this hypothesis, HA dodecasaccharides (HA12) were administered to EAE animals. Surprisingly, disease onset was delayed, demyelination was decreased and overall disease severity was less suggesting HA12 may impair lymphocyte extravasation. This idea was tested in the *in vitro* rolling assay and HA12 treatment of activated lymphocytes, but not CNS ECs was found to impair lymphocyte capture and rolling. These findings suggesting a mechanism by which HA oligosacchdarides modulate inflammatory demyelinating disease. However, the effect of HA12 on lymphocytes was not dependent on CD44 or TLR4 implying another HA receptor may be involved. Collectively, these data indicate HMW HA tethered to CD44 on CNS ECs and HA oligosaccharides may represent potential therapeutics to reduce immune cell infiltration into the CNS during inflammatory demyelinating disease. Furthermore, this work highlights the importance of the effects of HA synthesis and catabolism on inflammatory processes.

Chapter 1: Background and Introduction

Multiple Sclerosis: past and present

The disease multiple sclerosis (MS) has likely afflicted humanity for at least hundreds of years. Symptomatic descriptions in the biographical case history of the Dutch Saint Lidwina of Schiedam (1380-1433) are consistent (if somewhat exaggerated) with the disease we know today (Medaer, 1979). However, the most widely recognized first recorded case of MS is from the personal diary of the English nobleman Sir Augustus d'Esté (1794-1848). Excerpts describe a disease spanning 26 years, beginning with a bout of optic neuritis at 28 years of age that quickly resolved. Over subsequent years, d'Esté suffered from various neurological symptoms ranging from weakness and numbness to paralysis and incontinence. Two years prior to his death, clinicians delivered a diagnosis of paraplegia (Landtblom *et al.*, 2010). Convention at the time based diagnosis on clusters of symptoms, and vague, inaccurate diagnoses were common. It was not until 1868 when seminal work by Jean Martin Charcot defined the disorder as 'la sclérose en plaques disséminées' based on correlated symptomological and histological criteria (Charcot, 1868; see Talley, 2003 for review). This characterization provided a nosological framework that has been refined with improved histological methods and diagnostic tools.

Today, MS is the most prevalent neurological disease in young (20-40 years old) adults, with an estimated 2.5 million cases diagnosed worldwide (Miller, 2012). Approximately 85% of these cases are clinically categorized as having the relapsing-remitting form of MS. These patients have periods of clinical stability, punctuated by attacks that completely, or as the disease progresses, partially resolve. Following multiple attacks, typically over many years of disease, ~50% of relapsing-remitting MS patients develop

secondary progressive MS at which point, neurological deficit accumulates without obvious attacks. A small percentage of patients, 10% or less, present with primary progressive MS characterized by rapid, unremitting neurological deficits (Nylander & Hafler, 2012). Currently, clinical diagnosis requires attacks be spaced out through time and correlate to T2-hyperintense and T1-gadolinium-enhancing lesions on magnetic resonance imagining (Nylander & Hafler, 2012). Gadolinium-enhanced lesions are reflective of local inflammation, a hallmark of active disease. Although more than a century has passed since MS was first described, the etiology of the disease remains unknown. Furthermore, MS continues to be one of the most difficult central nervous system (CNS) diseases to diagnose and treat, due to its unpredictable presentation and progression.

The importance of histological observations to the understanding of MS pathology

Charcot's characterization of MS was preceded by reports by Robert Carswell (1838) and Jean Cruveilhier (1829-1842), documenting cases of paralysis associated with gross histological evidence of numerous focal lesions throughout the brain and spinal cord (Hickey, 1999b). These analyses provided little microscopic detail, yet both commented on discoloration and shrinkage of the neural tissue, now known to be caused by demyelination (loss of myelin); axonal loss and inflammation characteristic of active MS lesions (Popescu & Lucchinetti, 2012). Both reports influenced Charcot's work, as did Eduard Rindfleisch's description of perivascular 'round cells' within a CNS lesion that contained denuded nerve fibers (Lassmann, 2005). This was likely the first description of infiltrating immune cells into a MS lesion. Charcot's synthesis of this and his own work histologically defined MS lesions by the presence of demyelination, immune cell infiltration and axonal sparing. These criteria are still generally accepted today. Charcot's

immediate successors, including Joseph Babinski and Otto Marburg, broadened the criteria for diagnosis by demonstrating that inflammatory cells were directly involved in demyelination (Babinski, 1885), and that some axons degenerate in lesioned areas (Marburg 1906). The former observation is still generally accepted; however, debate over the later point has consumed much of the last century (Kornek & Lassmann, 1999). The prevailing notion had been that axons are generally spared until the patient develops secondary progressive MS. However, Trapp et al observed significant axonal damage and degeneration within two weeks of diagnosis (Trapp *et al.*, 1998). Interestingly, these authors observed a correlation with increasing local inflammation and axon damage, suggesting the two phenomena are linked. Collectively these findings demonstrate a role for immune cells in the two major MS pathologies. Amazingly, these conclusions were reached primarily through simple, diligent histological techniques.

The discovery and function of CNS myelin

It is important to note that the significance of the early MS histopathological observations was unclear, because myelin and its function were only just being discovered. The year prior to Carswell's study, the German anatomist Christian Ehrenberg described some nerve fibers in longitudinal section as having 'four parallel lines', the outer two forming the 'external boundary' (myelin) of the fiber and the inner two indicating the "boundaries of the inner cavity" (axon) (cited in Rosenbluth, 1999). Myelin was initially thought to be a component of the nerve cell fiber. Rudolf Virchow likened myelinated nerve fibers to long bones. He used the term "myelos" (Greek for marrow) to describe these fibers, and though it was later determined myelin was produced by oligodendrocytes, the name was retained (Virchow, 1858). Virchow was the first to hypothesize that myelin acts as an insulator of charge along the nerve fiber, allowing current to pass only at non-myelinated sites (Virchow 1854). In 1871, Louis Antoine Ranvier demonstrated that myelin sheaths

along nerve fibers are punctuated with non-myelinated sites or “nodes”. These so-called “nodes of Ranvier” contain concentrations of voltage-gated ion channels that propagate action potentials by passing current (reviewed by Rosenbluth, 2009). As Virchow proposed, myelin between the nodes serves to increase membrane resistance and reduce capacitance along the axonal length, allowing current to “jump” from node to node in what is termed saltatory conduction (Huxley & Stampfli, 1949). It is now known that the saltatory conduction of action potentials is critical for normal CNS function and is impaired when myelin is damaged or eliminated from the axon in MS.

Physiologic consequence of immune-mediated demyelination and axonal damage

The most defining feature of MS pathology is demyelination of CNS axons by immune cells. It is also apparent that the majority of these axons initially survive this insult. While some axons remyelinate (Hanafy & Sloane, 2011) and recover near-normal conduction velocities (Felts & Smith, 1992; Honmou *et al.*, 1996), many are chronically demyelinated and suffer conduction block. Initially, chronically-demyelinated axons can recover function by redistributing and increasing expression of voltage gated Na^+ channels along the demyelinated length of the axon (England *et al.*, 1990; Novakovic *et al.*, 1998). However, channel redistribution results in slow and inefficient continuous conduction of action potentials (Bostock & Sears, 1978). Furthermore, upregulation of Na^+ channels in chronically demyelinated axons increases metabolic demand to maintain ion concentration gradients, causing failure of the Na^+/K^+ ATPase pump. As a consequence, Ca^{2+} levels within the cell rise, causing mitochondrial dysfunction and eventual axonal damage, Wallerian degeneration and finally neuronal death (Waxman, 2006).

In addition to demyelination, CNS infiltrating immune cells and their products mediate axonal damage. T-cells, for example, are capable of neuronal cytotoxicity by a contact-dependent mechanism (Giuliani *et al.*, 2003; Hoftberger *et al.*, 2004). Additionally, macrophages respond to pro-inflammatory signals from T-cells to produce cytotoxic nitric oxide (NO) (Bogdan, 2001). NO induces conduction block in axons by interfering with ATP synthesis (Su *et al.*, 2009). This energy deficit results in increased intracellular Ca^{2+} and cell death, as described above. Demyelinated axons are particularly susceptible to NO-induced conduction block (Redford *et al.*, 1997). In addition to NO, macrophages in MS lesions release high levels of the neurotransmitter glutamate which is known to cause excitotoxcity of neurons (Werner *et al.*, 2001; Groom *et al.*, 2003; Gonsette, 2008). Interestingly, prolonged exposure of myelinated CNS axons to glutamate receptor agonists results in demyelinating lesions, oligodendrocyte death, and axonal damage, suggesting excitotoxicity secondary to inflammation can account for much of the pathology observed in MS (Matute *et al.*, 2001). Collectively, these findings demonstrate infiltrating immune cells cause demyelination, resulting in neuronal dysfunction, and can mediate axonal damage and neuronal death both directly and indirectly.

T-cells are essential components of the MS lesions and likely promote demyelination

Modern histological and immunohistological techniques have revealed much about the immune cell contingent associated with active demyelinating MS lesions. The predominant immune cells within active lesions are “foamy” (myelin-laden) macrophages and microglia. This has led to the idea that macrophages/microglia are major effectors of demyelination. Macrophages arise from infiltrating monocytes while microglia are native to the CNS (Charo & Ransohoff, 2006). During disease, macrophages/microglia are

activated to secrete multiple cellular substances that cause injury to oligodendrocytes and to ingest myelin debris (Rostasy, 2005). Various components of the adaptive immune systems, including antibodies, complement and $CD4^+$ T-cells have been implicated in macrophages/microglia activation (Prineas & Graham, 1981; Rotshenker, 2003). All of these macrophage-activating factors are represented to some degree within classified subtypes of active MS lesions (Lucchinetti *et al.*, 2000; Nylander & Hafler, 2012). Among them; however, $CD4^+$ T-cells are the only ones present in all lesion types (Traugott *et al.*, 1983; Chitnis, 2007; Popescu & Lucchinetti, 2012). This finding implies that $CD4^+$ T-cells have a primary role in promoting demyelination.

Modeling MS: The usefulness of experimental autoimmune encephalomyelitis (EAE)

The most compelling evidence that $CD4^+$ T-cells may be mediating MS pathology comes from studies of experimental autoimmune encephalomyelitis (EAE). This model was unintentionally identified in 1933 by Thomas Rivers, who observed acute disseminating encephalomyelitis in monkeys injected with rabbit brain extracts as a control (Rivers *et al.*, 1933). Today, EAE is typically induced by subcutaneous injection of a myelin antigen, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) emulsified in Complete Freund's Adjuvant. Often pertussis toxin is co-administered to induce more severe and reliable disease. Many species are susceptible to EAE, but it is typically induced in inbred rodent strains for ease of genetic manipulation, or in non-human primates due to their phylogenetic relevance to humans. Different inbred mouse strains immunized with different myelin peptides exhibit markedly different disease courses (Batoulis *et al.*, 2011). For example, SJL/J mice immunized with $PLP_{139-151}$ have a relapsing/remitting disease, while C57/BL6 immunized with MOG_{35-55} have chronic symptoms. This variability is convenient for

model-specific disease types or phases, and they are similar to varied disease courses observed in MS. Histologically, EAE lesions exhibit demyelination, coincident with macrophage, T-cell, or (in some models), B-cell infiltration (Pachner, 2011). Due to their striking pathological similarity, this model initiated the hypothesis that MS may be an autoimmune disease (Baxter, 2007). Experimental evidence for this hypothesis is derived from adoptive transfer studies in EAE, where myelin-activated lymphocytes from a donor are injected into a host to induce disease (Paterson, 1960). It was determined that $CD4^+$ T-cells specifically are required to induce disease in naïve animals (Pettinelli & McFarlin, 1981). These findings prompted researchers to focus on all aspects of $CD4^+$ T-cell biology and its contribution to MS.

Genetic susceptibility in MS is related to genes involving CD4+ T-cell immunity

It is clear there is genetic susceptibility in MS, particularly related to genes involving $CD4^+$ T-cell activation. Among female monozygotic twins, there is a concordance rate of ~25% for developing MS, compared to 5% among dizygotic twins (Willer *et al.*, 2003). Both rates are higher than the ~1 in 1000 prevalence in the general northern-European population. Linkage studies indicate that susceptibility is primarily related to alleles of the human leukocyte antigen (HLA)- DR and DQ genes (Oksenberg & Barcellos, 2005; Sospedra & Martin, 2005). The HLA locus encodes the human MHC class II proteins that are typically expressed by antigen-presenting cells (APCs). CD4+ T-cell activation requires stimulation of the T-cell receptor (TCR) by a cognate antigen bound to MHC II on an APC. Polymorphisms in HLA genes are estimated to account for 17-60% of the genetic susceptibility in MS (Hauser & Oksenberg, 2006). Furthermore, single nucleotide polymorphisms in other genes important for T-cell activation and survival; such as interleukin 2 (IL-2) and 7 (IL-7) receptors, have also been reported to increase susceptibility, although to a lesser degree (Gregory *et al.*, 2007; International Multiple

Sclerosis Genetics *et al.*, 2007). These findings support a role for CD4+ immunity in mediating MS pathology; however, the incomplete penetrance of these susceptibility loci suggest a role for non-genetic factors in disease initiation.

Epidemiology and molecular mimicry in T-cell activation: Clues to MS etiology

Because genetic linkage studies have provided only modest correlations, many researchers have proposed an environmental trigger of MS immune pathology. Proposed candidates include viral and bacterial infection, solar radiation, nutrition, and exposure to chemical agents. Among these, viral pathogens are the best studied and may be the most biologically plausible. A possible example of viral causation comes from studies of MS "outbreaks" among natives on Faroe and Shetland-Orkney Islands, following prolonged exposure to non-native soldiers post-World War II (Fujinami, 2001). Numbers of cases increased dramatically in the ten years following the end of the war, suggesting a critical window of exposure. Although, over 24 different viruses have been linked to MS (Chastain & Miller, 2012), the strongest evidence supports the ubiquitous herpesvirus Epstein Barr (EBV) playing a role. Virtually all MS patients are seropositive for EBV, while individuals who have had infectious mononucleosis, the disease caused by uncontrolled EBV infection, are more than two times as likely to develop MS (Ascherio *et al.*, 2001; Nielsen *et al.*, 2007). Conversely, individuals never infected by EBV are at low risk of developing MS (Hauser & Oksenberg, 2006). It is unknown how EBV or other viruses might cause MS pathogenesis; however, the leading theory involves ectopic activation of T-cells.

T-cells reactive to myelin components (such as myelin basic protein (MBP) and PLP) are present in the blood of both normal individuals and MS patients (Valli *et al.*, 1993; Wucherpfennig *et al.*, 1994; Steinman, 1996). Both myelin proteins are expressed in the

thymus, which should result in reactive T-cell death or anergy (Pribyl *et al.*, 1993; 1996), but T-cells with low affinity for myelin antigens persist (Targoni & Lehmann, 1998). This escape from negative selection offers a mechanistic hypothesis for MS initiation termed molecular mimicry. According to this hypothesis, pathogen-derived peptides that mimic myelin peptides are expressed by MHC class II molecules on APCs in the peripheral blood and lymphatics. Myelin-reactive T-cells encounter these mimics and become autosensitized, initiating an autoimmune response against CNS antigens (Chastain & Miller, 2012). Interestingly, peptides derived from the expression of DNA polymerase of EBV robustly stimulate several MBP-reactive MS T-cell clones (Wucherpfennig & Strominger, 1995). Furthermore, these EBV peptides are recognized by reactive T-cells in the context of HLA class II DR2a protein (DRB5*0101 allele), which has one of the strongest genetic associations with MS predisposition (Lang *et al.*, 2002). These findings collectively provide indirect evidence for CD4+ T-cell mediated molecular mimicry as an etiological basis for MS pathology. Coupled to histological and genetic linkage studies, this evidence suggests $CD4^+$ T-cells are the primary initiators of MS pathology. Evolving research is focused on the multiple roles that CD4+ cells play in this process.

$CD4^+$ T-cell biology and the MS autoimmune hypothesis

Adoptive transfer studies in EAE further suggest that MS is primarily a $CD4^+$ T-cell-driven autoimmune disease. Upon stimulation of their TCRs via a myelin antigen, $CD4^+$ T-cells differentiate into distinct subsets. These include CD4+ T helper 1 (T_H1), helper 2, (T_H2) and T_H17 cells, each with unique effector functions (See Figure 1.1 for schematic). Evidence suggests these subsets coordinate to mediate the pathology of EAE and MS (Steinman, 2007).

T_H1 cells, for example, are induced by IL-12 signaling to produce the pro-inflammatory cytokines interferon-γ (INFγ), IL-12 and tumor necrosis factor alpha (TNFα). These signals activate macrophages/microglia which mediate CNS pathogenesis (see above), suggesting T_H1 cells drive disease. This idea is supported by adoptive transfer experiments, showing T_H1 cells alone are sufficient to induce EAE (Bennett & Stuve, 2009). Furthermore, T_H1 associated cytokines INFγ and IL-12 are detected in the CNS and cerebral spinal fluid (CSF) during EAE, and MS (Segal & Shevach, 1996; Gutcher & Becher, 2007). Furthermore, MS symptoms are worsened by INFγ administration (Panitch *et al.*, 1987). Additionally, INFγ produced by T_H1 cells induces MHC II expression in the CNS and triggers release of chemokines that attract macrophages (Goverman, 2009). Conversely, T_H2 cells stimulated by IL-4, produce anti-inflammatory factors such as IL-4, IL-10 and transforming growth factor β (TGFβ) that suppress T_H1 inflammation by activation of FOXp3$^+$ T-regulatory cells (Bennett & Stuve, 2009). T_H2 suppression may account for inactivity of disease between attacks. These findings suggest that T_H1 mediated inflammation is sufficient to establish MS-like pathology, which can be tempered by T_H2.

However, contrary to the T_H1 CNS autoimmune-mediated hypothesis, IL-12, INFγ and TNFα-deficient mice develop severe EAE (Ferber *et al.*, 1996; Liu *et al.*, 1998). Conversely, IL-23 null mice are completely resistant to EAE (Cua *et al.*, 2003). IL-23 maintains the survival of T_H17 cells, which are induced by TGFβ and IL-6 to produce pro-inflammatory IL-17 (Hu *et al.*, 2011). These findings imply activated T_H17 rather than T_H1 cells are essential to CNS autoimmunity. Moreover, classic T_H1 and T_H2 cytokines (INFγ and IL-4 respectively) collectively suppress IL-17 *in vitro* and mitigate IL-17 dependent tissue damage, suggesting they may regulate T_H17 inflammation (Park *et al.*, 2005).

Furthermore, IL-17 transcripts have been isolated from MS lesions (Lock *et al.*, 2002). However, IL-17-deficient mice are still susceptible to EAE, and IL-17 overexpression in $CD4^+$ T-cells does not exacerbate disease, calling into question the extent of T_H17 involvement in CNS autoimmunity (Haak *et al.*, 2009). Interestingly, the T_H17 phenotype is unstable and can convert into a T_H17/T_H1 phenotype, characterized by production of both INFγ and IL-17. Additionally, if treated with IL-12, T_H17 cells can re-acquire a T_H1 phenotype (Batoulis *et al.*, 2011). This phenotypic plasticity has led some to question the purity of T_H17 cells in earlier adoptive transfer experiments and to suggest "contaminating" INFγ-producing T_H1 cells were also present and may have been driving disease (Hofstetter *et al.*, 2005; Langrish *et al.*, 2005). Evidence that T_H1 cells enter the non-inflamed CNS prior to T_H17 cells further supports this idea (O'Connor *et al.*, 2008). Conflicting data and a lack of consensus on the involvement of individual $CD4^+$ T-cell subsets suggests other players remain to be identified. However, therapies directed at T_H1/T_H17 CD4+ T-cells will likely moderate autoimmune inflammatory CNS disease.

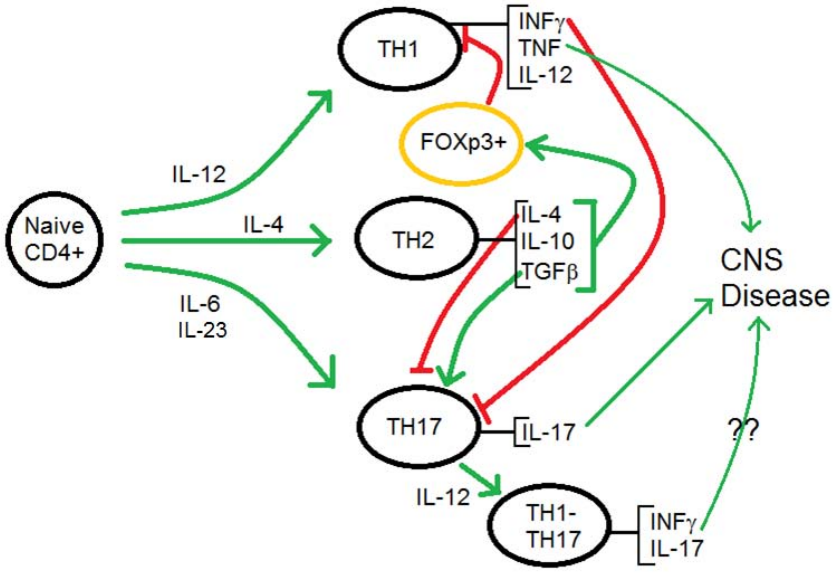

Figure 1.1. T_H cell differentiation, regulation and contribution to CNS disease. Green arrows represent facilitation and red arrows inhibition. ?? indicates assumed function without direct experimental verification. This diagram is updated from that presented in (Steinman, 2007) to include data discussed in (Bennett & Stuve, 2009; Goverman, 2009; Batoulis *et al.*, 2011).

Translating animal models to MS

While the exact mechanisms mediating EAE pathogenesis and their analogy to MS are questioned, EAE has clearly proven itself a useful testing ground for MS therapeutics. Currently, eight medications are FDA approved for the treatment of MS. Four of these are re-formulations of INF-β, which has mixed results in EAE (Yu *et al.*, 1996; Luca *et al.*, 1999). Of the remaining four, all were pursued after showing promise in the EAE model, and two (glatiramer acetate and natalizumab) were developed as a direct result of proof-of-principle experiments in the EAE model (Steinman & Zamvil, 2006). Two of these drugs (natalizumab and fingolimod) act to suppress disease by impairing the movement of lymphocytes from the periphery into the CNS. In the case of fingolimod, lymphocytes are retained within lymphatic system. By contrast, natalizumab allows lymphocytes to access the blood (and presumably other organs and tissues), but selectively impairs $CD4^+$ T-cell entry to the CNS (Yednock *et al.*, 1992; Baron *et al.*, 1993). With a 67% reduction in incidence of relapse and a 40% reduced risk of sustain progression of disability, natalizumab is clearly effective in slowing disease (Polman *et al.*, 2006). However, complications from opportunistic infection in patients not observed in the EAE model have marred this drug's success. A number of cases of progressive multifocal leukoencephalopathy resulting from John Cunningham polyomavirus infection highlight the necessity of immune surveillance within the CNS. *Nonetheless, blocking T-cell entry to the CNS is an effective means of slowing MS progression and EAE is an ideal model to test reagents aimed at this goal.*

The blood-CNS interface is an obstacle to infiltrating T-cells

Under normal and disease conditions, T-cell infiltration, or extravasation into the brain parenchyma is actively regulated. Cells enter either through the CSF by crossing the choroid plexus epithelium fed by leptomeningeal capillaries, or directly through

capillaries of the CNS vasculature (Neuwelt *et al.*, 2011). Choroid plexus epithelial and CNS endothelial cells (ECs) possess tight-junctions that form the anatomical basis of the so called blood-CSF barrier and the blood-brain-barrier (BBB), respectively. Tight junctions are macromolecular complexes formed of the transmembrane proteins claudin, occludin and junctional adhesion molecule that link adjacent cells, preventing circulating blood cell access to the CNS. In non-disease conditions, resting T-cells do not cross the BBB (Engelhardt, 2006). However, there is a large pool of CD4+/ CD69+, central memory T-cells in the subarachnoid space (SAS) that may be competent to enter the CNS parenchyma (Kivisakk *et al.*, 2003). This has led to the idea that CSF T-cells are predominately responsible for normal CNS immune surveillance. Because CNS inflammation requires T-cell re-activation by APCs in the CNS, T-cells within the SAS are ideally positioned to initiate disease (Goverman, 2009). This idea is supported by findings that EAE-associated CNS inflammation is initially detected in the SAS (Lassmann & Wisniewski, 1978) and myelin-reactive T-cells contact MHC class II+ APCs in the SAS, resulting in their proliferation prior to T-cell extravasation in the CNS parenchyma (Kivisakk *et al.*, 2009). These studies have led to the notion that T-cell re-activation in the SAS produces cytokine signaling that activates remote CNS ECs of the BBB, where the majority of T-cell extravasation occurs in both EAE and MS (Brown & Sawchenko, 2007).

In contrast to the blood-CSF barrier, the BBB allows minimal extravasation of memory or activated T-cells into the perivascular space (the space between the basement membrane of the endothelium and the glial limitans) in the absence of endothelial inflammation (Engelhardt & Ransohoff, 2005). The small number of T-cells that do cross the BBB, persist in the perivascular space for a short time without re-activation (Hickey, 1999a). However, peripheral APCs present in the perivascular space (Serafini *et al.*,

2000) of meningeal and CNS parenchymal blood vessels alone are sufficient to stimulate myelin-reactive T-cells and promote EAE (Greter *et al.*, 2005). Therefore, a small number of myelin-reactive T-cells extravasating into the perivascular space may be sufficient to induce disease. Furthermore, TNFα and INFγ produced by the T_H1 and T_H17 T-cells in the periphery induce CNS EC expression of cell adhesion molecules and cytokines implicated in T-cell extravasation (Piccio *et al.*, 2002; Larochelle *et al.*, 2011). Therefore, while SAS may be an initiation site of CNS inflammation, the perivascular space throughout the CNS parenchyma is also permissive to T-cell re-activation. *Because the majority of T-cell extravasation related to disease pathogenesis occurs through EC of the BBB, understanding the molecular mechanisms involved in this process is imperative and may aid in identifying therapeutic targets.*

T-cells extravasation is a coordinated multi-step molecular process

T-cell extravasation is mediated by sequential interactions of signaling and cell adhesion molecules on T-cells and ECs lining the vessel. Studies of T-cell recruitment into various organs including the lymph, gut, skin and CNS have revealed some commonalities in the process, but not necessarily in their molecular trafficking determinants (Ransohoff *et al.*, 2003). The classic model of extravasation is derived from extensive studies of lymphocytes moving from the blood stream to the lymph across the high endothelial venule (HEV). This is an essential step in the adaptive immune process. Extravasation at this site is generally considered a four-step process, where each step is associated with unique molecular players (Figure 1.2A). In the first step, T-cell, EC carbohydrate ligands, and selectin adhesion molecules initiate tethering and rolling along the endothelial surface (Step1). Rolling decreases the velocity of T-cells, allowing them to monitor their environment for ‘activating factors’ such as chemokines that signal through Gαi-protein coupled receptors (Step 2). T-cell ‘activation’ results in the conformational

change and clustering of constitutively expressed integrins on the cell surface which, in turn induces binding to integrin receptors on the endothelial surface that mediate firm adhesion (Step 3). Following adhesion, extravasation is culminated in the diapedesis of the T-cell into the perivascular space (Step 4) (For complete review see (Ransohoff *et al.*, 2003; Engelhardt, 2006; Greenwood *et al.*, 2011).

The molecular mechanism of T-cell extravasation into the CNS is less well understood. In general, the four steps hold true; however, a major point of controversy is the role of selectins and their ligands (Step 1) in the initial capture and rolling of T-cells on CNS ECs and their requirement for subsequent extravasation (Figure 1.2B). During EAE or following TNFα stimulation of CNS ECs, T-cell capture and rolling (Step 1) is observed *in vivo* by intravital microscopy and can be impaired by blocking antibodies against P- and E-selectin on ECs, suggesting they mediate this process (Kerfoot & Kubes, 2002; Piccio *et al.*, 2002). However, repeated administration of P- and E-selectin blocking antibodies does not prevent overall T-cell extravasation in EAE or reduce disease severity, but rather delays disease onset, suggesting capture and rolling is not necessary for disease pathogenesis (Engelhardt *et al.*, 1997). Furthermore, blocking P-selectin glycoprotein ligand 1 (PSGL-1) on T-cells or deleting its expression does not prevent extravasation or disease (Engelhardt & Ransohoff, 2005). However, when blocking antibodies against P-selectin and α4-intergrin (a mediator of T-cell firm adhesion and the molecular target of natalizumab) are co-injected in EAE animals, they synergize to allow less T-cell extravasation and result in better disease outcome than α4 integrin antibodies alone (Kerfoot *et al.*, 2006). *Collectively, these findings imply that T-cell capture and rolling is not obligatory, but does facilitate disease pathogenesis and is therefore a relevant therapeutic target.* Furthermore, blocking both P-selectin and α4-intergrin does not

abolish EAE symptoms, while targeting the P-selectin/PSGL-1 pathway using knockout animals does not entirely prevent T-cell capture and rolling (Kerfoot *et al.*, 2006). *This implies other adhesion molecules are involved in T-cell capture and rolling and contribute to disease pathogenesis.* I hypothesize that the transmembrane receptor cluster determinant 44 (CD44) and its primary ligand, the extracellular matrix (ECM) molecule hyaluronan (HA), contribute to this process (Figure 1.2B, Step 1, ?).

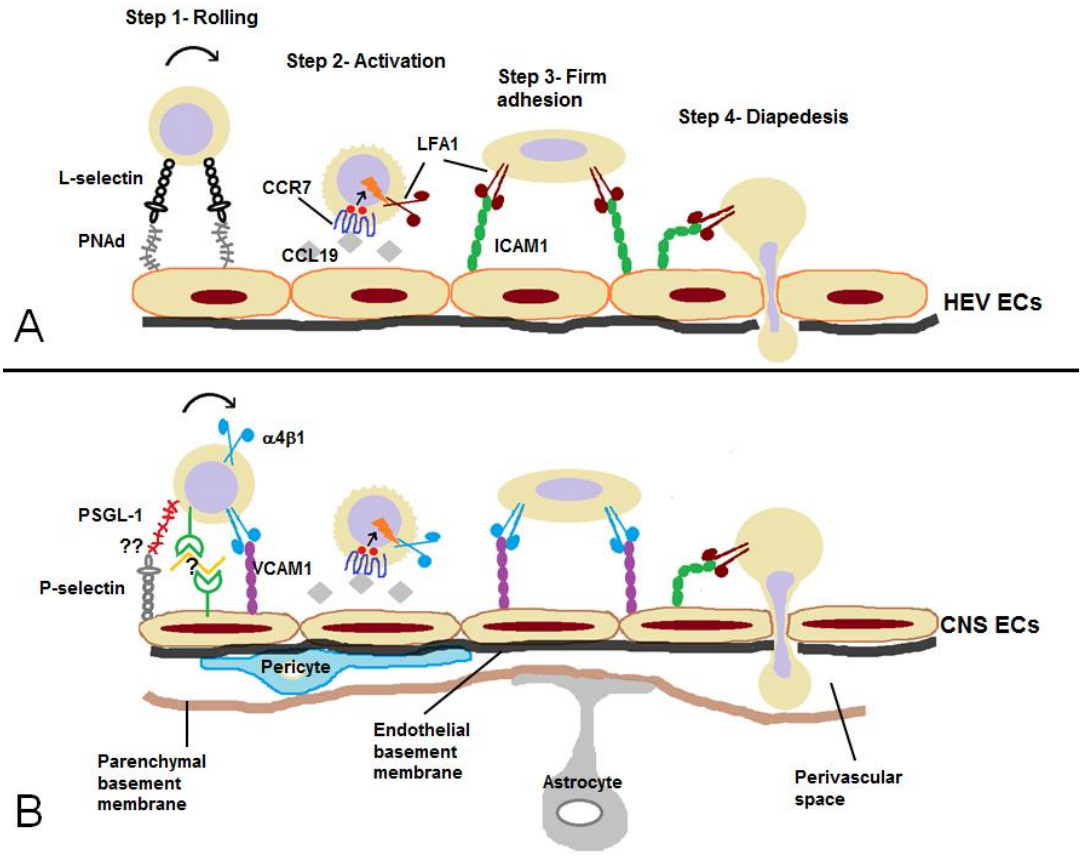

Figure 1.2. The multistep process of T-cell extravasation. A) The molecular mechanisms of T-cell recruitment to the endothelium of the HEV. B) The proposed molecular mechanisms of T-cell recruitment to the CNS endothelium during EAE and MS updated from that proposed by (Greenwood *et al.*, 2011). ?? indicates the speculative involvement of P-selectin and PSGL-1 ligand in this process. ? denotes that CD44 (green) and HA (yellow) may be involved in T-cell recruitment to CNS endothelium, but has not been verified.

CD44 and HA mediate T-cell rolling during inflammation

CD44 glycoproteins are a family of single-pass transmembrane receptors that are present on a number of cell types, including T-cells and ECs where it is constitutively

expressed (Johnson *et al.*, 2000). Through alternative splicing of 10 variant exons and post-translational modification including N- and O-linked glycosylation, the highly conserved CD44 gene produces distinct proteins, ranging in size from 80-250 kDa (Ponta *et al.*, 2003). The most commonly expressed form is hematopoietic or standard CD44 (CD44s), which contains no variant (CD44v) exon encoded sequences and ranges from 80-90 kDa in size. All isoforms of CD44 express an N-terminal domain homologous to the HA binding extracellular Link protein (Lesley & Hyman, 1998). This domain enables CD44 to bind HA and other glycosaminoglycans; however, its binding activity is tightly regulated and requires conformational activation (Johnson *et al.*, 2000). The membrane-proximal region is less conserved and contains the insertion site for the variant exons and multiple glycosylation sites that influence HA binding. The C-terminal cytoplasmic domain contains phosphorylation sites that regulate interactions with signaling molecules and the cytoskeleton (Ponta *et al.*, 2003). CD44 has been shown to participate in numerous cellular behaviors, including growth, survival and migration, primarily through interactions with HA. It facilitates these behaviors via direct cell-cell adhesion interactions and through interactions with signaling molecules. Specifically, CD44 has been shown to promote the activation of receptor tyrosine kinases (RTKs), (Sherman *et al.*, 2000) and promote signaling through Src kinases and Rho GTPases (Johnson *et al.*, 2000). It also interacts with proteins that regulated cytoskeletal organization, such as moesin-ezrin-radixin-like (merlin) and the Ezrin/Radixin/Moesin (ERM) family (see Ponta *et al.*, 2003 for review).

HA is secreted as an un-branched glycosaminoglycan, composed of repeating disaccharide units of glucuronic acid and N-acetyl-glucosamine (Fraser *et al.*, 1997). It is a ubiquitous molecule found in the ECM of all tissues, where it influences hydration, provides a back-bone for large proteoglycan complexes in the ECM, and mediates

cellular migration and adherence. HA is a simple molecule that can be composed of 2,000 to >25,000 disaccharide units, with molecular weights in the 10^7 Da range. Unlike other glycosaminoglycans, which are modified in the Golgi, HA is synthesized and secreted at the inner leaflet of the cell membrane as a linear molecule. Mammals express three HA synthases (HAS1-3) that produce distinct sizes of HA. HAS1 and 2 produce high molecular weight (HMW) forms (≥2.0-4.0x10^6 Da), while HAS3 generates intermediate sizes of lower molecular weight forms (≤2.5x10^5 Da) (Itano & Kimata, 2002). Preferential HA size and expression patterns of the various HASs suggest that different HA products influence different cellular processes. HASs produce linear molecules that can incorporate into the surrounding cellular matrix or be retained at the cell surface via HA binding receptors such as CD44 and receptor for HA mediated motility (RHAMM) or by interactions with the HAS proteins themselves (Toole, 2004). Localization of HA at the cell surface facilitates cell-cell adhesion interactions, promotes receptor clustering and enhances cell signaling. Likewise, release of HA into the surrounding matrix can influence these processes in neighboring and migrating cells.

Both CD44 and HA are clearly involved in inflammation. Extensive experimental evidence from CD44 null animals and manipulations with CD44 modifying antibodies in a variety of inflammatory models, demonstrate that CD44-HA interactions play multiple roles in both pro-inflammatory and anti-inflammatory events (Johnson & Ruffell, 2009). CD44 expression and HA binding activity are up-regulated on T-cells in response to TCR activation and pro-inflammatory cytokine signaling (Lesley *et al.*, 1994; DeGrendele *et al.*, 1997b). Moreover, T-cell activation results in receptor clustering, which facilitates cell-cell adhesion (Pure *et al.*, 1995). Therefore, CD44 on myelin-reactive T-cells in MS and EAE is primed to interact with HA. HA becomes enriched in the ECM of many cells in response to inflammation (Fraser *et al.*, 1997). HAS transcription is regulated by

various inflammatory factors, including growth factors and cytokines (Sugiyama *et al.*, 1998; Kennedy *et al.*, 2000; Ijuin *et al.*, 2001). Of particular relevance to T-cell extravasation, TNFα stimulation of microvascular ECs increases HA synthesis and activates CD44 to bind and tether HA on the cell surface (Mohamadzadeh *et al.*, 1998; Nandi *et al.*, 2000). *Thus, CD44 and HA are bi-directionally positioned to influence the adhesion of T-cells to the CNS endothelium.*

The involvement of CD44 in lymphocyte extravasation was first proposed when *in vitro* antibody screens designed to identify lymphocyte homing molecules demonstrated that an anti-CD44 antibody blocked lymphocyte adhesion to lymph and synovial ECs (Jalkanen *et al.*, 1987). However, since the antibody did not inhibit HA binding to CD44, this observation was not pursued. Later, antibodies that induce CD44 shedding from the cell surface were shown to delay lymphocyte recruitment into skin (but not recirculation back to the lymph) in a model of hapten-induced delayed-type hypersensitivity (Camp *et al.*, 1993). This observation suggested that CD44 played a role in facilitating extravasation at sites away from the lymph. DeGrendele et al were the first to demonstrate that antibodies blocking CD44 binding to HA could prevent lymphocyte cell line rolling on immobilized HA or cultured ECs *in vitro* (DeGrendele *et al.*, 1996). Furthermore, *in vivo*, they demonstrated that T-cell homing and subsequent extravasation into sites of super-antigen induced inflammation requires CD44-HA-dependent T-cell rolling on ECs (DeGrendele *et al.*, 1997a). More recently, it has been shown *in vivo* that ovalbumin-activated T_H1 and T_H2 cell rolling on TNFα stimulated intestinal microvascular ECs is CD44-HA dependent (Bonder *et al.*, 2006). Collectively, these data suggest a “sandwich model” of CD44-HA mediated rolling, whereby activated CD44 on T-cells binds to HA expressed by activated CD44 on inflamed ECs to initiate

rolling (Figure 1.3). *This evidence demonstrates CD44-HA interactions mediate T-cell rolling in peripheral vessels; however their involvement in CNS T-cell rolling is unknown.* Interestingly, in the short-term T-cell homing model, CD44 association with α4 integrin via its cytoplasmic domain in T-cells is required to mediate firm adhesion. This suggests that primary and secondary adhesion molecules directly interact to mediate extravasation (Siegelman *et al.*, 2000; Nandi *et al.*, 2004). *Due to α4 intergrin involvement in EAE and MS pathogenesis, it is reasonable to consider CD44 and its ligands may have redundant function at the level of the CNS.*

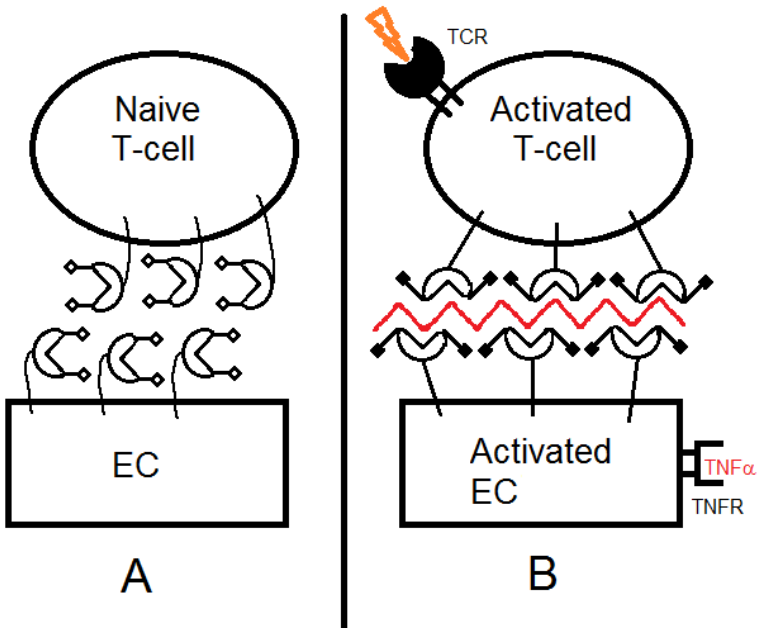

Figure 1.3. The 'sandwich model' of CD44-HA dependent rolling of activated T-cell on activated ECs. A) CD44 on naïve T-cells and unstimulated ECs is inactive to HA (red line) binding via steric hindrance from N-linked glycosylations. B) TCR activation in T-cells and TNFα activation of ECs triggers conformational changes in N-linked glycosylations, activating HA binding and triggering HA synthesis in ECs (not shown). TNFR (tumor necrosis factor receptor).

Manipulation of CD44 influences EAE onset and progression

Several lines of evidence suggest that CD44 is involved in EAE pathogenesis. Two studies have demonstrated that EAE disease symptoms and immune cell infiltrates can be abrogated by injection of CD44s blocking antibody (IM7) (Brennan *et al.*, 1999;

Brocke *et al.*, 1999); perhaps by impairing CD44-dependent extravasation. Brennan et al propose, that CD44 shedding from the surface of the myelin-active T-cell in response to antibody treatment as a potential mechanism (Brennan *et al.*, 1999). However, shedding induces a $CD44^{low}$ state attributed to naïve T-cells still capable of interacting with lymphatic ECs, suggesting in this case, myelin-reactive cells may have preferentially regressed to the lymph node rather than extravasate into the brain (Bazil & Horejsi, 1992; Stoop *et al.*, 2002). In contrast, a third study determined that blocking antibodies against CD44v6, 7 and 10, but not CD44s, impaired T-cell extravasation (Laman *et al.*, 1998). However, in this study, IM7 administration was halted 4 days post immunization, which is generally considered prior to myelin-reactive T-cell egress from the lymph node (Kuerten & Lehmann, 2011). It is therefore possible that the effects of antibody treatment are attributable to deficits in T-cell activation rather than extravasation. Interestingly, a recent study found that EAE induced in $CD44^{-/-}$ mice is significantly attenuated and correlated with a T-cell phenotypic shift from pro-inflammatoryT_H1/T_H17 to anti-inflammatory T_H2 (Guan *et al.*, 2011). However, the number of infiltrating T-cells was also reduced in $CD44^{-/-}$ mice, suggesting extravasation may also be impaired. It is therefore unclear whether the contribution of CD44 to CNS inflammation described in these studies is due to alterations in myelin-reactive T-cell phenotype or impaired extravasation into the CNS. Moreover, the role of HA in EAE pathogenesis remains unclear. *Given the conflicting and inconclusive results of these studies, a direct assessment of CD44-HA interactions in T-cell rolling during CNS inflammation is needed.*

Hyaluronidases and their products could modulate T-cell rolling in the CNS

HA synthesis must be balanced with HA catabolism. An imbalance in this process has been noted in several pathological conditions, including rheumatoid arthritis, periodontal

disease and scleroderma, suggesting HA metabolism is a finely tuned system (Jiang *et al.*, 2011). While HASs regulate HA synthesis, hyaluronidases (HYALs) are primarily responsible for its degradation. HYALs were first identified as 'spreading factors' because they increased the diffusion of dyes, drugs and viral particles via their degradation of HA (reviewed in (Girish & Kemparaju, 2007). Mammals have six known genes that code for HYALs, termed HYAL1-5 and PH20 (SPAM1). Humans lack HYAL5, but have a pseudogene, pHYAL1, which is not translated (Stern *et al.*, 2006). HYALs generally fall into two distinct categories designated by their pH-dependent activity. In humans, HYAL1 and 2 are considered acid active (pH 3-4), and PH20 is neutrally active (pH 4-7.5) (Girish & Kemparaju, 2007). The role of HYAL3 as a functional hyaluronidase remains unclear, although evidence suggests it may be active at pH 3-4 (Hemming *et al.*, 2008; Reese *et al.*, 2010) while HYAL4 has no predicted activity (Csoka *et al.*, 2001). Therefore, PH20 appears unique among human HYALs in that it is active across a broad spectrum of pH levels. *This finding implies that PH20 would be an ideal molecular tool to test the involvement of HA expressed by CNS ECs to T-cell rolling because it will retain its enzymatic function at the pH of blood (7.35-7.45)*. HYAL degradation of EC HA has been previously shown to impair T-cell rolling and extravasation *in vitro* and *in vivo* (DeGrendele *et al.*, 1996; DeGrendele *et al.*, 1997a); however, its efficacy has not been demonstrated in CNS inflammation.

HA degradation via HYALs results in the formation of low molecular weight and oligosaccharide HA fragments. Consistent with the hypothesis that HA synthesis and catabolism are tightly regulated, HYAL expression is induced by the same pro-inflammatory cytokines that promote HASs (Sampson *et al.*, 1992; Girish & Kemparaju, 2007). Furthermore, inflammatory products such as reactive oxygen species fragment HA at glycoside linkages, increasing HA oligosaccharide concentrations beyond

physiologic levels at sites of inflammation (Noble, 2002). As a consequence, low molecular weight and HA fragments are more abundant at sites of injury and inflammation than in the physiologic state. The presence of HA fragments can interfere with HMW HA interactions with membrane HA receptors. For example, HA oligosaccharides (6-40mers) enhance CD44 cleavage from the surface of tumor cells, while HMW forms inhibit cleavage (Sugahara *et al.*, 2003; Sugahara *et al.*, 2006). Furthermore, HA oligosaccharides (as small as decasaccharides) can displace CD44-bound HMW HA on the surface of keratinocytes (Tammi *et al.*, 1998). HA oligosaccharides as small as 6mers also competitively bind CD44 with increasing concentrations (Teriete *et al.*, 2004). Due to its less elaborate structure, low molecular weight HA bound to membrane receptors diminishes receptor aggregations resulting in less intracellular signaling and weakened cell-cell adhesion interactions. *Therefore HA oligosaccharides may serve to impair T-cell extravasation by binding up available HA receptors on activated T-cells or displacing HMW HA bound to CD44 on the surface of activated CNS ECs*.

Accumulating evidence suggests HA fragments engage in cell signaling (see (Jiang *et al.*, 2007; 2011) reviews). This observation has led to the idea they act as sensors of tissue damage and inflammation and are therefore dangerous in that they promote pathology. HA oligosaccharides in particular signal through Toll- like receptors (TLRs) during inflammation (Taylor *et al.*, 2004; Jiang *et al.*, 2005). TLRs are a family of pattern-recognition receptors that are activated by molecular patterns associated with pathogens. Activation of TLRs initiate MyD88 mediated signaling pathways leading to the production of pro-inflammatory cytokine and chemokines (Aderem & Ulevitch, 2000; Takeda *et al.*, 2003). HA is a component of *Streptococcus* and *P. Multocida* bacterial cell coat and is thus proposed to signal through TLR2 and 4, which are classically activated

by mycobacterium and LPS respectively. HA-mediated TLR pro-inflammatory signaling has been observed in macrophages (McKee *et al.*, 1997; Jiang *et al.*, 2005; Ernens *et al.*, 2010), dendritic cells (Termeer *et al.*, 2000; Termeer *et al.*, 2002), B lymphocytes (Iwata *et al.*, 2009) and ECs (Taylor *et al.*, 2004). TLR signaling in ECs induces expression of pro-inflammatory cytokines that can increase expression of HASs and other cell adhesion molecules that facilitate T-cell extravasation (Estess *et al.*, 1999; Taylor *et al.*, 2004). Conversely, TLR4 signaling in T-cells has an anti-inflammatory effect, the removal of which exacerbates an experimental model of colitis (Gonzalez-Navajas *et al.*, 2010)*. Taken together, these data suggest that HA oligosaccharides may have multiple and possibly opposing influences on T-cell extravasation and CNS inflammation in general. These effects are likely to be timing and cell-type dependent, however understanding the molecular mechanism behind their action will provide much needed insight into CNS inflammatory disease pathogenesis.*

Hypothesis and Rationale

MS and EAE pathogenesis are driven, at least in part, by the inflammatory actions of T-cells within the CNS. Impairing T-cell extravasation during both of these diseases has a beneficial effect on disease initiation, progression and severity. However, the process of T-cell extravasation in the CNS is not fully understood and cannot be completely abrogated therapeutically. CD44-HA adhesive interactions are involved in T-cell rolling and extravasation in peripheral tissues; however, their involvement in these processes within the CNS has not been examined. Furthermore, studies using $CD44^{-/-}$ animals or antibodies that block HA binding to the receptor suggest these molecules are important to EAE pathogenesis, but the mechanism remains to be identified. *These findings led to the hypothesis that interfering with CD44-HA interactions at the level of the CNS endothelium would impair myelin-reactive T-cell extravasation and decrease disease severity*. To test this hypothesis, I utilized an *in vitro* model of activated lymphocyte rolling on CNS ECs and an *in vivo* model of CNS inflammation (EAE). My findings demonstrate that HA tethered to CD44 on CNS endothelial cells is involved in activated T-cell rolling and extravasation in CNS inflammation, but that CD44 on activated lymphocytes is dispensable to this process. However, chronic degradation of HMW HA from CNS ECs increases HAS1, and HA expression on the endothelium and promotes T-cell extravasation in late stage EAE. Surprisingly, HMW HA degradation products (oligosaccharides) delay EAE onset, reduce demyelination, and impair lymphocyte rolling. HA oligosaccharides mediate this effect through interactions with lymphocytes that is independent of CD44 and TLR4. Overall, these findings support a role for CD44-HA adhesion interactions in myelin-reactive T-cell extravasation in demyelinating CNS disease. They also suggest HYAL degradation products may have opposing effects on disease pathogenesis and progresion.

Chapter 2: Experimental Procedures

Induction of EAE– EAE in WT (wild-type) C57BL/6 or BL/6S129 and CD44$^{-/-}$ mice was induced using mouse myelin oligodendrocyte glycoprotein, peptides 35-55 (MOG_{35-55}), synthesized artificially by Peptides International. MOG_{35-55} was combined with complete Freund's adjuvant containing heat-inactivated mycobacterium tuberculosis as previously described (Tuohy *et al.*, 2004).

EAE scoring– Beginning the day following EAE induction, an experimenter, blinded to genotype or treatment condition, assigned a clinical disease score daily until day 13 or day 21. The following clinical disease scoring scale was used: 0, no symptoms; 1, tail-weakness (completely flaccid); 2, hindlimb weakness (animal can be easily flipped radially onto its back when grasped at base of tail); 3, animal walks with hind limbs splayed outwards; 4, one hindlimb partially or substantially paralyzed; 5, both hindlimbs completely paralyzed, no spastic movement; 6, moribund (animal is euthanized immediately). Increments of 0.5 were used for disease severity between indicated scores.

Hyaluronidase and HA4 or HA12 oligosaccharide administration in mice–PEG-PH20 and HA12 was provided by Halyozyme Therapeutics Inc. An aliquot of a PEG-PH20 or HA4 or HA12 stock solution was prepared in advance for each day that injections were to take place. Aliquots were diluted in PBS and passed through a 0.22 μm low protein binding syringe filter to sterilize the solution. Mice were randomly assigned to two groups to receive injections every other day of either 50 μl subcutaneous sterile PBS (vehicle control) or 50 μl subcutaneous PEG-PH20 (50 U/kg)

or HA12 (50 mg/kg) into hind flanks. For PEG-PH20 experiments, injections continued until the experiment was terminated on day 13 or day 21 post-induction of EAE or 6 days after beginning of injections in the case of naïve animals. For HA12 experiments, injections continued until termination of the experiment (day 20).

Splenocyte culture and isolation–Splenocytes from WT, $CD44^{-/-}$ or $TLR4^{-/-}$ C57BL/6 mice were cultured in T75 flasks coated with anti-CD3 and anti-CD28 (eBioscience) antibodies for 72 hr to induce T-cell-specific activation and clonal expansion as previously described (Sugie *et al.*, 2004). Cultures were harvested using a Lympholyte® (Sigma) gradient according to the manufacturer's protocol. The lymphocyte layer was removed using a sterile Pasteur pipette and pelleted by centrifugation. The resulting pellet was washed and suspended in RPMI medium supplemented with 1% FBS, 2mM L-Glutamine, 50μM 2-ME and 1mM sodium pyruvate at a concentration of $1x10^7$ splenocytes/ml. Cultures were maintained in a humidified 5% CO_2-95% air atmosphere at 37°C.

Murine primary cortical endothelial cell culture–Primary critical endothelial cells from WT and $CD44^{-/-}$ mice were isolated and grown as previously described (Deli *et al.*, 2000). Briefly, forebrains from 8-week-old WT or $CD44^{-/-}$ mice were isolated, minced, then digested with 1 mg/ml collagenase CLS2 (Worthington Biochemical) in Dulbecco's Modified Eagle's medium (DMEM; Sigma) containing 50 μg/ ml gentamycin and 2 mM glutamine in a shaker for 1 hr at 37°C. The cell pellet was separated from white matter and other cellular debris by centrifugation in DMEM with 20% bovine serum albumin (1000x g, 20 min). The microvessels obtained in the pellet were further digested with 1 mg/ml collagenase/dispase (Roche) in DMEM for 45 min at 37°C. Microvessel fragments

were separated on a 33% continuous Percoll gradient (1000x g, 10 min), collected, and washed twice in DMEM before plating on 24 well plates, 35 mm plastic dishes or glass coverlips coated with rat tail collagen and human fibronectin (Sigma) for static adhesion, parallel plate assays or immunocytochemistry respectively. Cultures were maintained in DMEM supplemented with 20% plasma-derived bovine serum (Atlas Biologicals), 1 ng/ml fibroblast growth factor-2 (R&D Systems) and 4 μg/ml puromycin (Sigma) in a humidified 5% CO_2-95% air atmosphere at 37°C. Four days following plating, EC cultures visually contained continuous monolayers of adherent spindle shaped cells consistent with previously published findings (Deli *et al.*, 2000) (Figure 3.4A). EC cultures contained strong CD31 (an endothelial marker) labeling that clearly delimited the cell membrane of most $Hoechst^+$ nuclei (Figure 3.4B). No $GFAP^+$ astrocyte contamination was observed in these cultures (Figure 3.4B, bottom panel).

Static adhesion assay– CNS EC cell monolayers in 24 well plates were stimulated with TNFα (10 ng/mL) 4hr prior to co-culture**.** For individual well co-cultures, $1x10^4$ WT CD3/CD28 stimulated LCs were loaded with calcein-AM dye (10 mM, Molecular Probes) for 15 min, followed by a spin, wash with fresh medium and a 30min incubation according to the manufacturer's protocol. During LC incubation either activated CNS ECs or activated LCs were treated with varying concentration of HA12 (50, 10 and 1 ug/mL) or PBS. Following HA12 treatment, activated LCs were added to ECs in the 24 well plates and allowed to co-culture for 1 hr. Cultures were washed twice with co-culture medium and fluorescence was measured at 538 nm by a FlexStation plate reader (Molecular Devices). Relative fluorescence of HA12 treated wells was compared as percent of PBS treated controls. All treatments were performed in triplicate in a minimum of two experiments.

Parallel plate assay–Lymphocyte adhesion and rolling along brain ECs was quantified under flow conditions using a parallel-plate flow chamber. A 35mm dish with an EC monolayer was assembled to a flow chamber (150-μm channel depth, 1.26-mm channel width) and mounted on the stage of a Zeiss Axiovert 200M microscope (Zeiss) and maintained at 37°C in an air curtain incubator. The exit port was connected to an infuse and withdraw syringe pump (Harvard Apparatus) to control flow rate through the chamber. The microscope was equipped with a CCD camera (Axiocam MRm, Zeiss) and imaging software (Stallion SlideBook v5.0.0.10, Intelligent Imaging Innovations, Inc.) for monitoring cell movement.

EC monolayers were stimulated with 10 ng/ml TNFα for 4 hr prior to flow chamber assembly to increase CD44 surface expression and HA binding activity (e.g. Figure 3.3B). In selected experiments, EC monolayers were incubated with either sterile PBS as vehicle control or 100 U/ml PEG-PH20 (Halozyme, Inc.) in PBS for 1 hr at 37°C prior to use in the flow chamber assembly. For experiments examining the effect of HA12 on LCs, LCs were pre-treated with either PBS, TLR4 blocking antibody (Clone MTS510, BD Pharmigen, 100 ng/mL) or isotype control antibody (Clone R35-95, BD Pharmigen, 100 ng/mL) for 10min prior to treatment with PBS or HA12 (10 ug/mL) for 30min. Additionally, $CD44^{-/-}$ and $TLR4^{-/-}$ LC were treated with HA12 (10 ug/mL) for 30 min prior to use in the parallel plate. After EC monolayers were washed with Hanks balanced salt solution (HBSS), 0.6 mL of CD3/CD28 stimulated lymphocytes (at $1x10^6$ total cells/mL in HBSS) were superfused through the chamber for 7 minutes at 0.5 dyn/cm^2 thereby mimicking the fluid mechanical environment of CNS postcapillary venules.

Interactions between lymphocytes and brain ECs were visualized in real-time by phase-contrast digital videomicroscopy. A single field of view (10x; 0.55 mm^2) was monitored during each trial. The number of total interacting cells and the average rolling velocity of each interacting cell were analyzed for each experiment. Interacting lymphocytes were defined as those that interacted with the EC monolayer for at least 1 second. Average rolling speed was determined using the particle tracking features of the Stallion imaging software. Criteria were set with the automated particle tracking function to exclude mask objects that were 1) not phase bright 2) less than 8 μm in diameter and 3) were moving faster than 100μm/sec. Each particle path was manually examined and in some cases, manual particle tracking was used to correct inaccuracies in the automated path.

CD44 exon specific RT-PCR analysis– Total RNA was isolated from WT and $CD44^{-/-}$ EC cultures using TRIzol Reagent (Invitrogen). cDNAs were synthesized using MultiScribe™ reverse transcriptase (Applied Biosystems) according to the manufacturer's protocol and a CD44 primer from the 3' non-variant portion of mouse CD44 (exon 19, 5' tag gca cta cac ccc aat ctt ca 3'). cDNA products were amplified using Phusion® Hot Start II DNA Polymerase (Finnzymes) and a primer from the 5' non-variant region of mouse CD44 (exon 1, 5' tcc ctc cgt ttc atc cag cac 3') and another primer straddling two non-variant 3' exons (exon 16-17, 5' ggt tcg cac ttg agt gtc ca 3'). The PCR reaction was performed using a Mastercycler thermocycler (Eppendorf) with the following protocol: 40 cycles of 10 sec at 98 °C, 30 sec at 64 °C and 1.5 min at 72 °C, followed by incubation at 72 °C for 5 min. This reaction was stopped at the end of the 25^{th} cycle and 2 μl was removed for CD44 variant specific nested primer analysis. The thermocycler protocol was subsequently re-started at the 25^{th} cycle and the reaction was continued to completion.

The 2 μl from the above reaction was amplified using the same reagents and protocols listed above with the exception that variant exon 6 (5' tgg ttt cag aac gga tgg cag g 3'), 7 (5' cca caa caa cca tcc aag tca aa 3'), and 10 (5' tct tcc cac aga tac aac tac tt 3') specific nested 5' primers were added. All reaction products were analyzed by electrophoresis in 1.5% agarose and visualized by ethidium bromide staining.

Sybr green I and TaqMan Assay real-time RT-PCR analysis (qRT-PCR)– Total RNA from WT and CD44$^{-/-}$ EC cultures was obtained as above for HAS1-3 analysis. RNA from WT 0hr and 72hr CD3/CD28 stimulated lymphocyte cultures was obtained for TLR4 analysis. Single stranded cDNAs were synthesized using the ImProm-II Reverse Transcriptase synthesis kit (Promega Corporation) according to the manufacturer's protocol. The following primer sets were designed using Primer Express® Software v3.0 (Applied Biosystems) and synthesized by Integrated DNA Technologies. The primer sequences were: HAS1 forward, 5′-gcg agc act cag gat cat ctt-3′ and reverse, 5′-cca gga gtc cat agc gat ctg-3'; HAS2 forward, 5′-aaa ggg acc tgg tga gac aga a-3′ and reverse, 5′-ccc att ttt gca tga tgc aa-3′; HAS3 forward, 5′-gcg cat tgc ctt tcc aaa-3′ and reverse, 5′-tgc cac cca gca cct cat-3'. For TLR4 analysis, specific TaqMan primers and probes (Mm00445274_m1) were obtained from Applied Biosystems. The 18s ribosomal RNA was used as a normalizing unit for each reaction. Primer sets were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit; Applied Biosystems). The qPCR assays were carried out with Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) in a 7500 Fast TaqMan instrument (Applied Biosystems) using a default thermo-cycling program. Or in the case of TLR4, cDNA was amplified in 1× Universal Master Mix (Applied Biosystems) with the following thermal cycler protocol: 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Assays

were performed in triplicate. The normalized expression of the target gene with respect to 18S was computed for all samples using the ΔΔCT method in Microsoft Excel.

HA quantification–HA was quantified from culture medium supernatant or cellular lysates of WT and CD44$^{-/-}$ ECs cultured to confluence in 35mm dishes. Cell lysates were obtained by incubation of EC monolayers in 200ul of buffer containing 20mM Tris-HCL, 150mM NaCl, 1mM EDTA and 1% TritionX-100. Samples were applied to an enzyme-linked immunosorbent assay (ELISA)-based assay (Echelon Biosciences) according to the manufacturer's instructions. Medium supernatant samples were diluted 1:4 and cell lysates 1:2 to a final volume of 350 μl in the kit diluent buffer. Triplicate 100 μL fractions were transferred into the ELISA plate. At the end of the assay, absorbances were read at 450 nm on a 96-well plate reader (Molecular Devices).

Immunohistochemistry–At appropriate EAE time points, mice were euthanized using isoflurane (NovaPlus) and perfused transcardially with heparin saline followed by 4% paraformaldehyde. Lumbar spinal cords were removed, freeze-embedded and serially-sectioned at a thickness of 10 μm on a cryostat (Leica) and placed on glass slides. Sections were washed with PBS 3 x 5 min and blocked in PBS with 5% BSA with 0.05% Triton X-100 (blocking buffer) for 1 hr at room temperature. Sections were then incubated with primary antibodies against CD3 (1:150, AbD Serotec), CD4 (1:300, BD Pharmigen), CD31 (1:50, Ab cam), neurofilament-L (1:1000, Millipore) and/or HAS1 (1:25, Santa Cruz Biotech) in PBS overnight at 4°C. Biotinylated HA binding protein (bHABP, 1:250, Calbiochem) was used in place of a primary antibody to visualize HA. Negative controls included omitting the primary antibody. Sections were washed with PBS 3 x 5 min the next day and then incubated with the appropriate secondary antibody

(goat anti-rat Alexa 488 and goat anti-rabbit Alexa 633, 1:1000, Molecular Probes) or Cy3 streptavidin (1:2000, Jackson Labs) in place of secondary to visualize HA for 2 hr at room temperature. Sections were washed in PBS, and then incubated in FluoroMyelin (1:300, Invitrogen) for 20 min at room temperature to visualize myelin and Hoechst 33342 (1:5000, Invitrogen) for 10 min at room temperature to label nuclei. Sections were washed in PBS and mounted with Prolong Gold mounting media (Invitrogen) then imaged using a Zeiss Axioskop 40 fluorescence microscope (Zeiss) or an inverted Leica SP5 AOBS spectral confocal system (Leica).

Immunocytochemistry–EC cultures on coverslips were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, rinsed with PBS, and treated for 1 hr with blocking buffer (see above). Cultures were incubated overnight at 4°C with the primary antibody diluted in PBS. Cells were stained with antibodies against CD44 (IM7 hybridoma, 1:40, ATCC), CD31 (see above) and glial fibrillary acidic protein (GFAP 1:500, Dako). bHABP (see above) was used to visualize HA. Subsequently, cells were rinsed 3 x 5 min in PBS and incubated with the relevant secondary antibodies or Cy3 streptavidin as above for 2 hr at room temperature. Cultures were rinsed 3 x 5 min in PBS and incubated in Hoechst for 10 min, mounted and examined as above.

Stereology–Following immunohisto-chemical labeling, 12 sections of the lumbar spinal cord were analyzed from each animal (n=8 animals per group). Digital photomicroscopic images were captured on a Zeiss Axiovert 200M (Zeiss) fluorescence microscope interfaced with a Marianas Digital Microscopy Workstation (Intelligent Imaging Innovation Inc). Montages of the entire lumbar cord were created and analyzed using SlideBook™ software. Using FluoroMyelin labeling, a mask delimiting the spinal cord white matter was created for each section and a grid was generated within the mask area for

analysis. The grid consisted of 10 μm boxes spaced 50 μm apart in the xy axis with each box representing a 2500 μm square area. Based on FluoroMyelin labeling, boxes that fell entirely within lesioned areas were counted and divided by the total number of boxes within the grid and multiplied by 100 to determine a percent lesion area.

Infiltrating T-cell counts were performed by setting a threshold value for CD3 or CD4 immunolabeling in the green channel that resolved single cells. Subsequently, a mask was applied within the green channel to record each cell's position in the section. The software was then used to overlay the green channel mask onto the mask generated for the lesion analysis and to record the number of $CD3^+$ or $CD4^+$ cells within the white matter in each section.

Statistical Analysis–Differences between treatment groups in HA ELISA and parallel plate assays were analyzed by a Student's *t*-test. Differences in mean EAE disease score between groups were analyzed by a repeated Measures ANOVA. Statistical significance was defined as $p<0.05$ for all analyses.

Chapter 3: Hyaluronan Anchored to Activated CD44 on CNS Vascular Endothelial Cells Promotes Lymphocyte Extravasation in Experimental Autoimmune Encephalomyelitis[1]

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Abstract

The extravasation of lymphocytes across central nervous system (CNS) vascular endothelium is a key step in inflammatory demyelinating diseases including multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). The glycosaminoglycan hyaluronan (HA) and its receptor, CD44, have been implicated in this process but their precise roles are unclear. We find that $CD44^{-/-}$ mice have a delayed onset of EAE compared to wild type animals. Using an *in vitro* lymphocyte rolling assay, we find that fewer slow rolling (<1 μm/sec) wild type (WT) activated lymphocytes interact with $CD44^{-/-}$ brain vascular endothelial cells (ECs) than with WT ECs. We also find that

[1] Winkler CW et al. Hyaluronan Anchored to Activated CD44 on CNS Vascular Endothelial Cells Promotes Lymphocyte Extravasation in Experimental Autoimmune Encephalomyelitis. J.Biol Chem. 2012, Aug 3.

$CD44^{-/-}$ ECs fail to anchor HA to their surfaces, and that slow rolling lymphocyte interactions with WT ECs are inhibited when the ECs are treated with a pegylated form of the PH20 hyaluronidase (PEG-PH20). Subcutaneous injection of PEG-PH20 delays the onset of EAE symptoms by ~1 day and transiently ameliorates symptoms for 2 days following disease onset. These improved symptoms correspond histologically to degradation of HA in the lumen of CNS blood vessels, decreased demyelination, and impaired $CD4^+$ T-cell extravasation. Collectively these data suggest that HA tethered to CD44 on CNS ECs is critical for the extravasation of activated T cells into the CNS providing new insight into the mechanisms promoting inflammatory demyelinating disease.

Introduction

Multiple Sclerosis (MS) is a central nervous system (CNS) disorder characterized by the extravasation of pathogenic lymphocytes into the brain and spinal cord. Lymphocyte recruitment into the CNS during MS attacks results in inflammation, demyelination and axonopathy, leading to neurological disability (Holmoy, 2007; McFarland & Martin, 2007). Experimental autoimmune encephalomyelitis (EAE) is an animal model of inflammatory demyelinating disease that recapitulates many of the pathological and clinical traits of MS. The molecular events that contribute to lymphocyte extravasation in MS and EAE include interactions between adhesion molecules on the surface of both activated lymphocytes and CNS vascular endothelial cells (ECs). These interactions lead to intracellular signaling events that enhance cell-cell adhesion and promote the crossing of lymphocytes across CNS vascular endothelium (Greenwood *et al.*, 2011). In most tissues, this process is initiated by L-selectin on lymphocytes and P- and E-selectins on ECs binding to their transmembrane glycoprotein ligands (Ley *et al.*, 2007). These transient interactions result in lymphocyte rolling along the endothelial cell

surface, enabling signaling that induces expression and activation of integrins that mediate firm adhesion (Kinashi, 2005). However, the involvement of selectins in EAE and MS pathogenesis is a contentious issue. While there is evidence that P- and E-selectins are expressed by superficial blood vessels of the brain and that they can mediate rolling (Kerfoot & Kubes, 2002; Piccio *et al.*, 2002; Kerfoot *et al.*, 2006), evidence from both antibody blocking experiments and from experiments with knock-out and transgenic mouse models suggest selectins and their ligands are not essential for the development of EAE (Engelhardt *et al.*, 1997; Doring *et al.*, 2007). These findings indicate the involvement of other adhesive molecules in lymphocyte rolling on vessels within the CNS.

CD44 is a single-pass transmembrane glycoprotein widely expressed on a number of cell types including lymphocytes and ECs (Siegelman *et al.*, 1999; Pure & Cuff, 2001; Jong *et al.*, 2008; Rampon *et al.*, 2008). CD44 functions as a receptor for hyaluronan (HA), a glycosaminoglycan that is synthesized in a wide range of sizes up to $\sim 10\times10^7$ Da. HA is composed of repetitive disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid. It is synthesized at the inner plasma membrane by one of three HA synthases (HAS1-3) that extrude HA into the extracellular matrix of many cell types including ECs (Mohamadzadeh *et al.*, 1998; Girish & Kemparaju, 2007). Once extruded, HA can be tethered to the surface of ECs by HA synthases or by CD44 (Nandi *et al.*, 2000). CD44 and HA have been implicated in regulating cell-cell adhesion, proliferation, migration and differentiation (Ponta *et al.*, 2003).

CD44 expression and HA synthesis are increased in response to pro-inflammatory signals and numerous studies have implicated CD44 and HA in lymphocyte-endothelial cell interactions as well as the regulation of inflammatory responses (Johnson & Ruffell,

2009). Pro-inflammatory stimulation of lymphocytes and ECs facilitates post-translation modification of CD44 inducing HA binding activity (Lesley *et al.*, 1994; Nandi *et al.*, 2000; Ponta *et al.*, 2003; Johnson & Ruffell, 2009). Disruption of HA-CD44 interactions by anti-CD44 antibodies is sufficient to impair activated T-cell adhesion to endothelial cells *in vitro* (DeGrendele *et al.*, 1996). Additionally, CD44-HA interactions are required for super-antigen stimulated T-cells to efficiently home to sites of inflammation in the peritoneal cavity (DeGrendele *et al.*, 1997a).

In the context of inflammatory CNS disease, blocking antibodies against CD44 delay EAE onset and decrease disease severity coincident with fewer lymphocytes present in the CNS (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999). Similarly, one report found that EAE induced in $CD44^{-/-}$ mice is significantly attenuated (Guan *et al.*, 2011). However, in contrast to the studies utilizing CD44 blocking antibodies, this study attributed the decrease in EAE disease severity to a phenotypic shift in the activated lymphocyte population through an HA-independent mechanism (Guan *et al.*, 2011). It is unclear, therefore, whether the contribution of CD44 to EAE and MS disease progression is linked to lymphocyte extravasation or alterations in lymphocyte phenotypes. The requirement for HA in EAE onset and progression is also not clear.

To elucidate the role of CD44 and HA in lymphocyte-EC interactions during EAE pathogenesis, we utilized $CD44^{-/-}$ mice and a pegylated form of recombinant human PH20 (PEG-PH20) to degrade HA in the lumen of CNS blood vessels. We find that HA is tethered by CD44 to the luminal surface of TNFα stimulated ECs isolated from the brain and that slow rolling lymphocyte interactions are disrupted on ECs lacking CD44. In contrast, CD3/CD28 stimulated $CD44^{-/-}$ lymphocytes interact normally with wild type brain ECs. Removal of HA from ECs with PEG-PH20 treatment also results in impaired

lymphocyte rolling. *In vivo* PEG-PH20 treatment delays the onset of EAE and reduces the number of T-cell infiltrates early in disease. These data indicate that HA tethered to CD44 on ECs promotes lymphocyte rolling during EAE pathogenesis.

Results

EAE onset is delayed in CD44$^{-/-}$ mice

Inhibition of CD44 activity using neutralizing antibodies (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999) or deletion of the *CD44* gene (Guan *et al.*, 2011) are reported to attenuate EAE onset and progression. To confirm that genetic ablation of *CD44* is sufficient to ameliorate EAE symptoms, we tested how EAE progresses in CD44$^{-/-}$ mice that develop severe disease. Clinical disease signs were observed over a 25-day period in 8-week old female WT and CD44$^{-/-}$ mice on a C57B6;129 background, actively immunized with MOG_{35-55}. Disease was evident by day 10 post-inoculation in WT animals with peak scores occurring by day 14 (Figure 3.1A). Consistent with the findings of Guan and co-workers (Guan *et al.*, 2011), CD44$^{-/-}$ mice manifested disease with a delayed onset, no earlier than day 14 post-inoculation and as late as day 18 with an average peak disease on day 16 (Figure 3.1B). However, in contrast to the previous study (Guan *et al.*, 2011), we found that CD44$^{-/-}$ mice developed similar levels of disability. For both groups, scores remained elevated to at least a score of 2.5 after 20 days post-inoculation. These data demonstrate that CD44 contributes to the initiation of EAE onset but is not necessary for disease progression.

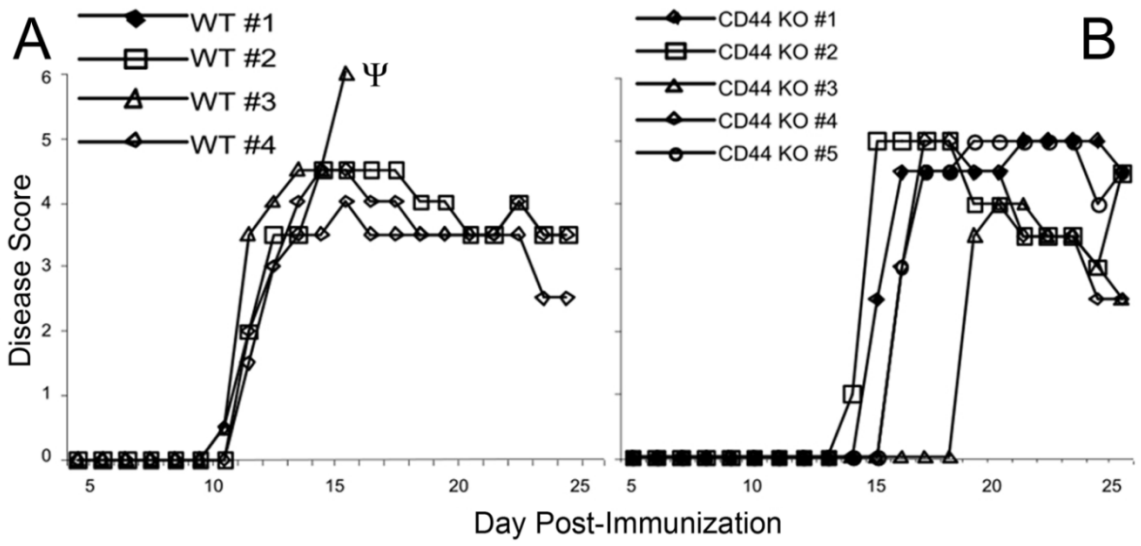

Figure 3.1. EAE disease onset is delayed in CD44$^{-/-}$animals. Disease was induced by inoculation of female WT (A) or CD44$^{-/-}$ (B) C57/B6/S129 background mice with MOG_{35-55} peptide emulsified in Complete Fruend's Adjuvant. Disease symptoms manifest on or near day 10 post inoculation in WT animals but 3-8 days later in CD44$^{-/-}$. Ψ represents animal being euthanized due to severe disease.

CD44 on brain ECs but not lymphocytes is critical for lymphocyte recruitment and rolling

Previous studies utilizing CD44 neutralizing antibodies suggest that CD44 contributes to EAE onset by promoting lymphocyte extravasation (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999). A second study suggests that CD44 promotes EAE pathogenesis by regulating lymphocyte phenotypes (Guan *et al.*, 2011). The exact role of CD44 in EAE pathogenesis remains unclear.

We used an *in vitro* parallel plate assay to determine if CD44 on the lymphocytes and/or the EC cells affects adhesion and rolling during early stages of lymphocyte extravasation. We examined WT and CD44$^{-/-}$ lymphocyte adhesion and rolling on WT and CD44$^{-/-}$ brain ECs. Fewer WT lymphocytes (44.6% less, *$p<0.005$) interacted with CD44$^{-/-}$ ECs (Figure 3.2C, D) compared to WT EC controls (Figure. 3.2A). The presence

of CD44 on the lymphocytes, however, was not critical. Thus there was no difference in the number of interacting cells between WT and CD44$^{-/-}$ lymphocytes when superfused across WT ECs (Figure 3.2B, D).

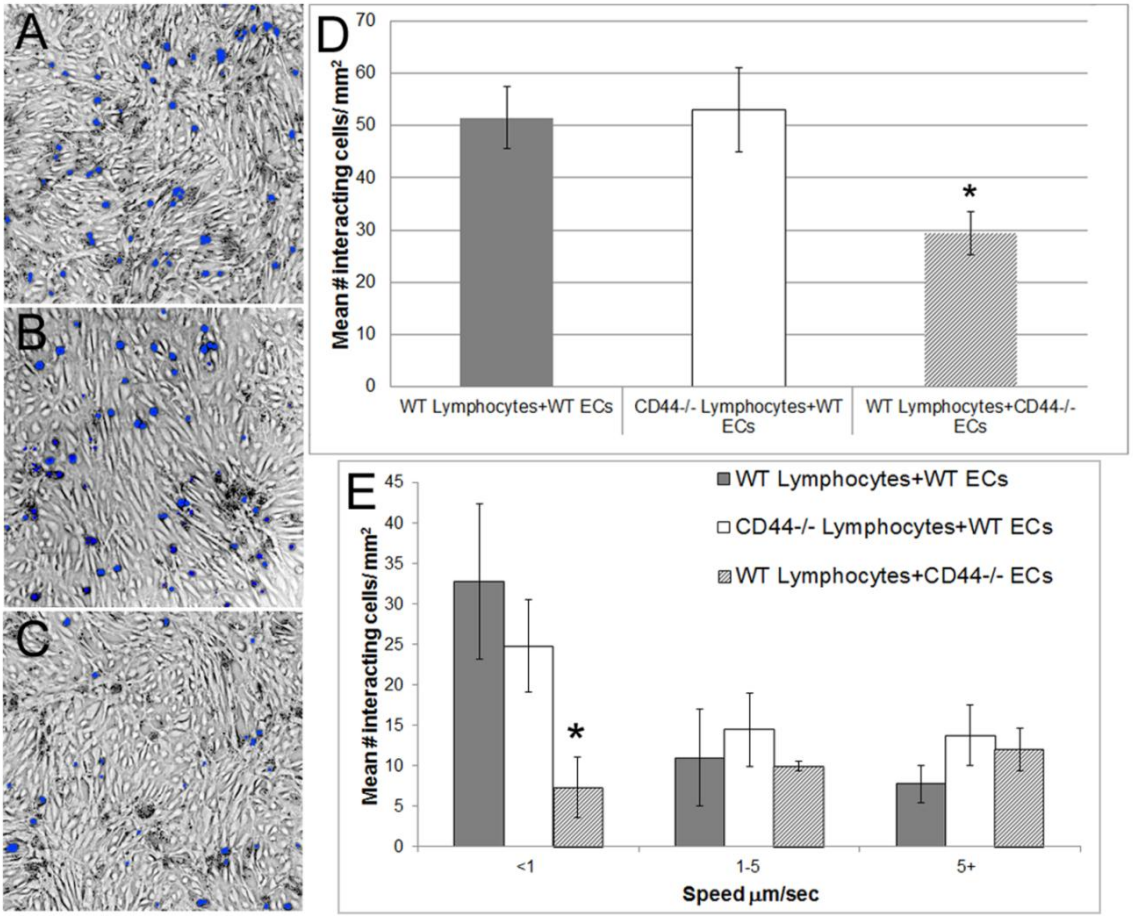

Figure 3.2. CD44 on activated brain ECs but not lymphocytes is critical for slow rolling adhesion interactions of lymphocytes to brain ECs. A-C) Representative brightfield images of psuedo colored (blue) WT lymphocytes bound to WT ECs (A), CD44$^{-/-}$ lymphocytes bound to WT ECs (B) or WT lymphocytes bound to CD44$^{-/-}$ ECs (C) at the end of a 7 minute experiment. (D) The number of lymphocytes interacting with the endothelial surface was significantly reduced in the absence of endothelial but not lymphocytic CD44. (E) CD44$^{-/-}$ ECs interacted with significantly fewer slow rolling (<1μm/sec) WT lymphocytes than WT ECs. The number of CD44$^{-/-}$ lymphocytes interacting with WT ECs in all three speed bins was not significantly different from control. Data are from three independent experiments performed in triplicate. *p<0.005, t- test compared to WT Lymphocytes+WT ECs.

Using particle-tracking techniques, the average rolling speed of all interacting cells was determined. Bins for slow (0-1μm/sec), medium (1-5μm/sec) and fast (>5μm/sec) rolling cells are plotted against the mean number of interacting cells for each group (Figure 3.2D). Within the slow rolling population, significantly fewer (*p<0.05) interacting cells

were observed when WT lymphocytes were superfused across CD44$^{-/-}$ ECs, but not when CD44$^{-/-}$ lymphocytes were superfused across WT ECs (Figure 3.2D). Meanwhile, the mean number of medium and fast rolling interacting lymphocytes in the experimental groups was not significantly different from controls. These results indicate that CD44 on CNS ECs, but not on lymphocytes, contributes to the capture and slowing of activated lymphocytes along the endothelial surface.

Standard CD44 tethers HA to the surface of brain ECs

Inflammatory mediators trigger HA synthesis and surface expression of CD44 by ECs (Johnson & Ruffell, 2009). CD44 binds HA as it is extruded through the cell membrane by HA synthases and can tether it to the cell surface (Nandi *et al.*, 2000). We therefore tested if HA is similarly anchored to CD44 on the surface of brain ECs. Brain EC cultures from WT (Figure 3.3A,B) and CD44$^{-/-}$ (Figure 3.3C,D) mice were isolated, stimulated with TNFα and assayed for expression of CD31, HA, and CD44. Both WT and CD44$^{-/-}$ cultures expressed high levels of the tight junctional marker CD31 as expected (Figure 3.3A,C and Figure 3.4B). WT cultures also displayed high levels of membrane CD44 that co-localized with areas of HA labeling (Figure 3.3B, arrows). In contrast, CD44 and HA were undetectable in CD44$^{-/-}$ brain EC cultures (Figure 3.3D).

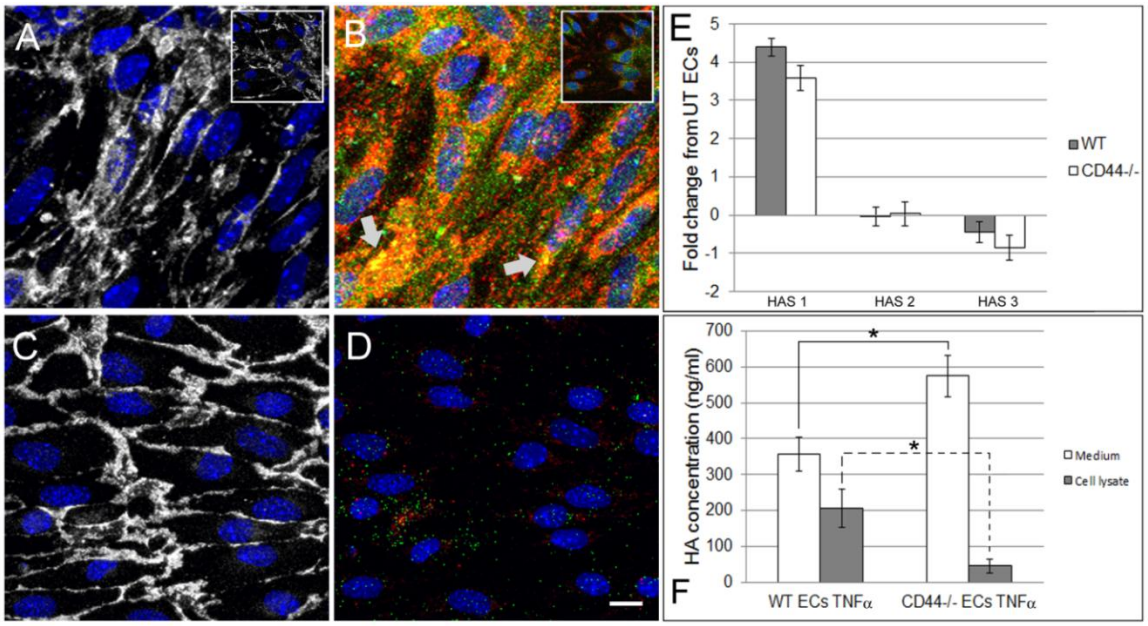

Figure 3.3. CD44 anchors HA to the surface of CNS endothelial cells. (A,B) WT EC cultures express high levels of CD31 (white) in addition to high levels of CD44 (green) and HA (red). Areas of CD44-HA colocalization are indicated (B, arrows). (C,D) CD44$^{-/-}$ EC cultures also express high levels of CD31, but CD44 and HA were undetectable. All cultures were stimulated with TNFα (5ng/ml) for 4 hr. Unstimulated WT cultures expressed little CD44 or HA (insets A,B). Scale bar is 10μm. All images are 6μm z-projections. (E) qRT analysis of HAS mRNA in WT and CD44$^{-/-}$ EC cultures show similar levels of induction when stimulated with TNFα for 4 hr. (F) Significantly more HA was found in the supernatant of CD44$^{-/-}$ EC cultures compared to WT ECs in response 4 hr TNFα stimulation. Conversely, significantly less HA was found in the cell lysis fraction of CD44-/- ECs compared to WT ECs. *p<0.005, t- test compared to WT ECs.

CD44 splice variants 6, 7 and 10 have been implicated in the early initiation of EAE (Laman *et al*., 1998). To determine if these variants could play a role in anchoring HA to ECs, we assayed ECs with and without TNFα stimulation for CD44 variant 6, 7 and 10 mRNA expression. CD3/CD28 stimulated lymphocytes, which transiently express CD44 variant 6 were used as a positive control (Arch *et al.*, 1992). Using CD44-specific primers corresponding to the 5' and 3' non-variant exons, we determined that WT ECs, regardless of TNFα stimulation, express predominantly standard CD44 (Figure 3.4C). Stimulated lymphocytes express predominately standard CD44, but a weaker band corresponding to CD44 variant 6 at the predicted size of 906bp is present (Figure 3.4C, red arrow). These results were confirmed using nested CD44 variant 6, 7 and 10 specific

primers to re-amplify EC and lymphocyte CD44 non-variant exon PCR products. A band corresponding to the predicted size product for CD44 variant 6 but not 7 and 10 was observed in lymphocytes and no predicted variant bands were observed in ECs (data not shown). These data indicate that standard CD44 is the predominant form of CD44 expressed by CNS ECs.

Our finding that HA is absent from the surface of CD44-/- ECs indicates that either CD44 anchors HA to brain EC cells or that HA synthesis in impaired in brain ECs lacking CD44. We examined HAS1-3 mRNA expression from WT and $CD44^{-/-}$ EC cultures via qRT-PCR. In WT cultures, mRNA expression of HAS1 increased ~4 fold, HAS 2 was unchanged and HAS3 decreased ~0.5 fold in response to TNFα stimulation (Figure 3.3E).

The same trend of mRNA expression for all the HAS genes was observed in $CD44^{-/-}$ cultures following TNFα stimulation (Figure 3.3E). Furthermore, an ELISA assay was used to measure the amount of HA produced by WT and $CD44^{-/-}$ EC cultures in response to TNFα stimulation. $CD44^{-/-}$ cultures contained significantly more HA in the culture supernatant and less on the cell surface than WT controls. (Figure 3.3F, *$p<0.05$). All together, these findings indicate that loss of CD44 does not affect HA synthesis by brain vascular ECs and that standard CD44 is required to anchor HA to the surface of these cells.

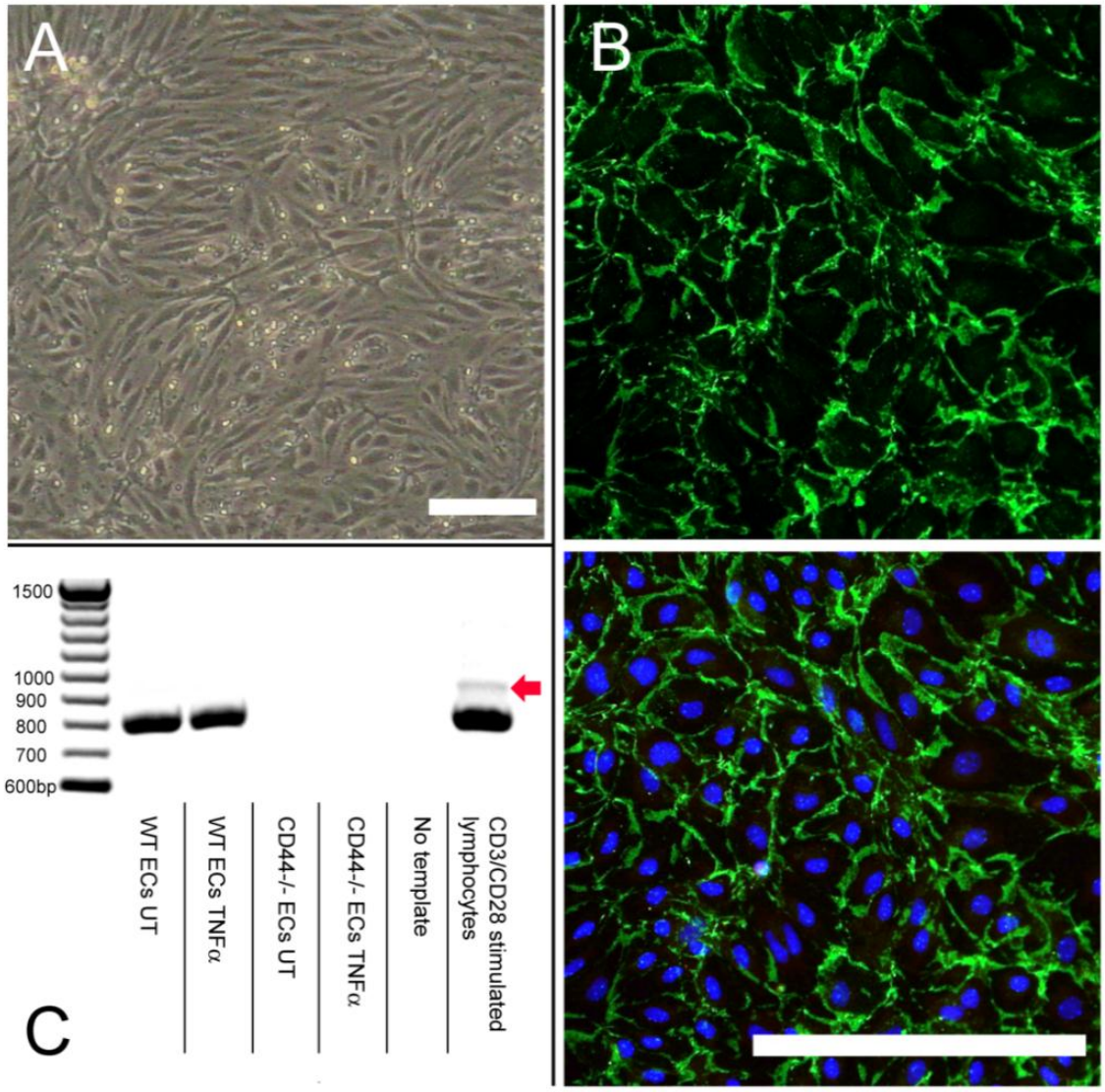

Figure 3.4. Primary EC culture characterization. (A) Bright-field image of EC culture 4 days post-plating. Visually the culture contains a continuous monolayers of adherent spindle shaped cells consistent with previous descriptions of cultured CNS ECs. (B) top panel, CD31 (green) labeling in an EC culture 4 days post-plating clearly delimits the cell membrane and demonstrates the tight-junction formation between adjacent cells. (B) bottom panel, overlay of CD31 staining in the top panel with nuclear stain (Hoechst, blue) and GFAP (red). No $GFAP^{+}$ astrocyte contamination was observed in these cultures. Scale bars represent 100 μm. C) RT-PCR characterization of CD44 variants in primary EC cultures and CD3/CD28 stimulated lymphocytes. The strong bands at 786bp represents standard CD44 which is the predominate species in in stimulated and unstimulated WT ECs and lymphocytes. Stimulated lymphocytes express a faint band at 906bp corresponding to the predicted CD44 variant 6 product (red arrow). $CD44^{-/-}$ ECs do not express CD44 mRNA.

Degrading HA at the surface of brain ECs impairs lymphocyte recruitment and rolling

Given that CD44 anchors HA to brain vascular ECs, we tested if HA on the surface of brain ECs is required for lymphocyte recruitment and rolling. WT and $CD44^{-/-}$ brain EC cultures were treated with a pegylated form of a hyaluronidase that functions at neutral pH (PEG-PH20) or vehicle prior to analysis in the parallel plate assay. PEG-PH20 treatment resulted in a loss of HA binding protein (HABP) staining; indicating that HA had been effectively degraded in these cultures (data not shown). Compared to vehicle controls, there was a significant decrease in the number of lymphocytes interacting with WT ECs pre-treated with PEG-PH20 (47.5%, *$p<0.05$; Figure. 3.5A). PEG-PH20 did not further decrease lymphocyte binding to $CD44^{-/-}$ ECs (Figure 3.5A, NS). Similar to our findings with $CD44^{-/-}$ brain ECs (Figure. 3.2C), PEG-PH20 treatment of WT ECs significantly (*$p<0.05$) reduced the number of slow rolling cells to a similar extent as observed in both the vehicle and PEG-PH20-treated $CD44^{-/-}$ ECs (Figure 3.5B). No significant differences were observed between groups at the medium and fast rolling speeds. Taken together, these results demonstrate that HA tethered to CD44 on the surface of brain ECs is involved in the recruitment and rolling of lymphocytes.

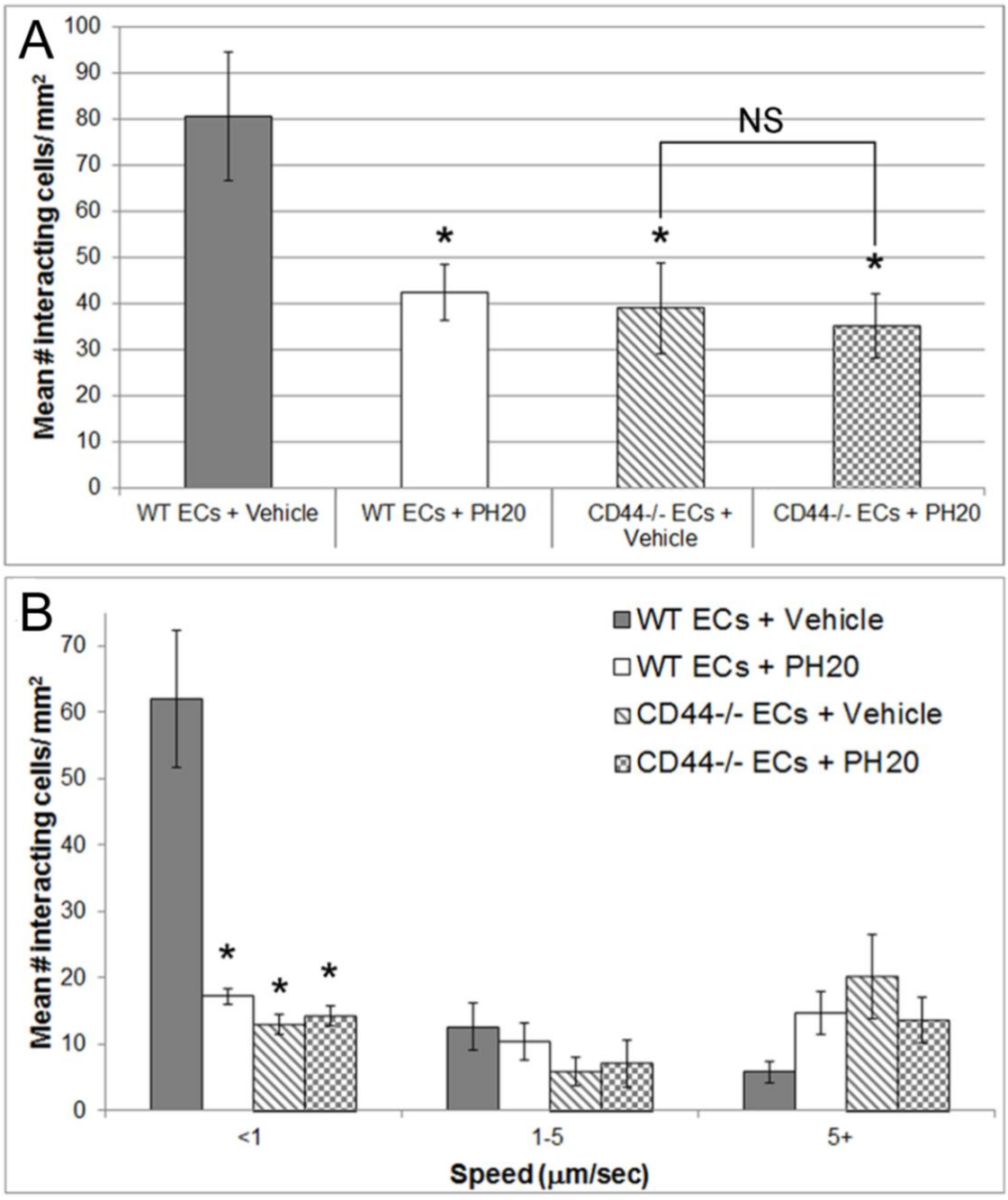

Figure 3.5. Treating WT CNS ECs with PEG-PH20 impairs lymphocyte interactions with the endothelial surface and alters rolling to a similar extent as on CD44$^{-/-}$ ECs. A) PEG-PH20 treated (1 hr, 100 U/ml) WT ECs had significantly fewer interacting lymphocytes compared to control. (NS) CD44$^{-/-}$ EC cultures treated with PEG-PH20 were not significantly different in the number of interacting lymphocytes compared to vehicle treated CD44$^{-/-}$ ECs. B) Treatment of WT ECs with PEG-PH20 significantly decreased the number of slow rolling (<1μm/sec) cells to a similar extent as observed with CD44$^{-/-}$ ECs. Data from all experimental groups represent four independent experiments performed in duplicate *p<0.005, t- test compared to WT ECs+Vehicle.

Subcutaneous treatment with PEG-PH20 delays the onset of EAE but does not ameliorate later disease

Because degradation of HA by PEG-PH20 leads to decreased lymphocyte binding and rolling on brain ECs *in vitro*, we hypothesized that degradation of HA by PEG-PH20 would ameliorate EAE onset and severity *in vivo*. To test the ability of PEG-PH20 to remove HA from the luminal surface of CNS vessels, naïve animals were subcutaneously injected every other day with PEG-PH20 or vehicle for 6 days. Spinal tissue was harvested and labeled for CD31 and HA (Figure 3.6).

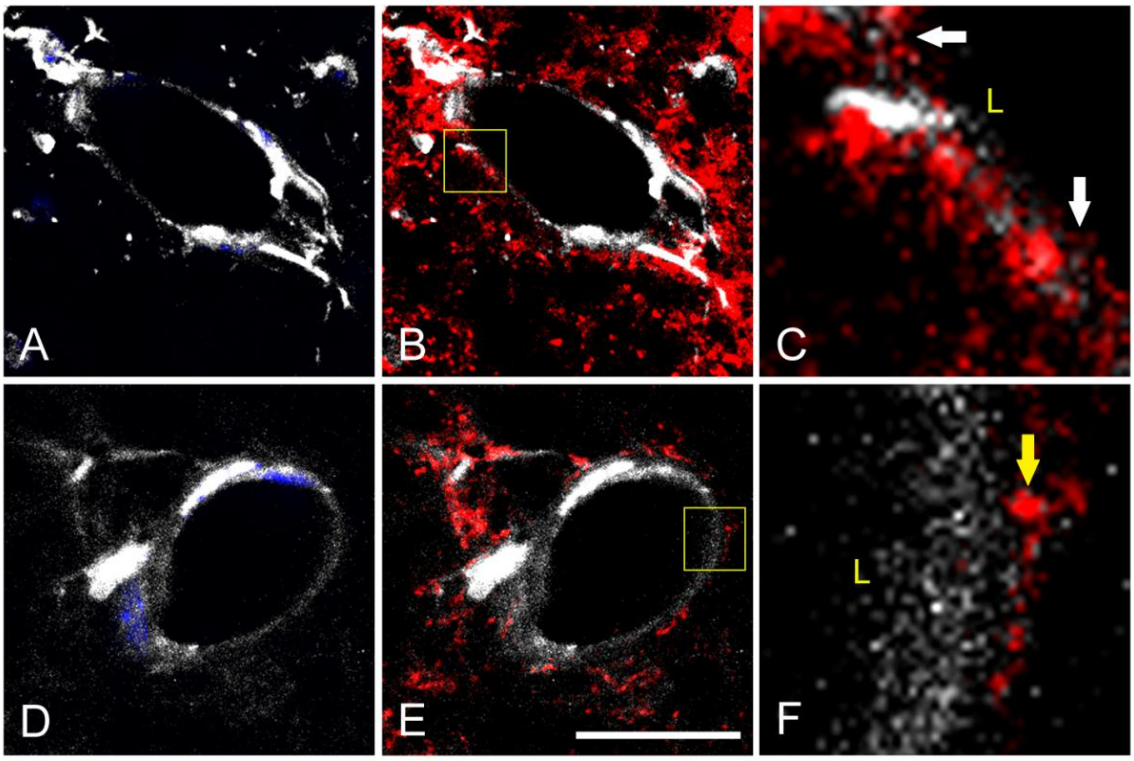

Figure 3.6. Subcutaneously injected PEG-PH20 degrades and removes HA from the lumen of naïve CNS blood vessels. (A, B) CD31 (white) positive CNS blood vessels from naïve animals treated with PBS express HA (red, in B) on the luminal surface and in the perivascular area. (C) Enlargement of boxed area in (B) demonstrates the HA (red) signal along the luminal surface of the vessel (white arrows). (D, E) CD31+ Vessels (white) treated with PEG-PH20 have virtually no HA signal (red) evident on the luminal surface. (F) Enlargement of the boxed are in (E) demonstrates the absence of HA signal on the luminal side of the vessel while some HA signal (red) is evident in the perivascular area (yellow arrow). The amount of perivascular HA signal in PEG-PH20 treated animals is reduced compared to PBS controls. The blue label in A and D represents cell nuclei (Hoechst stain). Yellow "L" indicates the luminal side of the vessel wall. Scale bar is 25μm. All images are 6 μm z-projections

Vehicle-treated vessels displayed a thin band of HABP label along the luminal surface (Figure 3.6A-C) while PEG-PH20-treated vessels were devoid of HABP within the vessel lumen (Supplemental Figure 3.6D-F). These results demonstrate that subcutaneous PEG-PH20 administration removes HA from the lumen of CNS blood vessels.

Mice with EAE were injected with either vehicle (PBS) or PEG-PH20 every other day beginning 7 days post-MOG inoculation. Similar to our observations in $CD44^{-/-}$ mice, clinical disease signs of EAE were delayed in animals treated with PEG-PH20 compared to animals treated with vehicle (Figure. 3.7). Mean clinical disease scores were also significantly lower on both day 11 and 12 in PEG-PH20-treated animals as compared to controls (* $p<0.05$; Figure 3.7). Clinical scores of PEG-PH20 treated animals lagged behind vehicle-injected animals until day 19 post-inoculation. At later times in disease progression, PEG-PH20 treated animals demonstrated more severe symptoms, starting at day 19 and became significant by day 21 (* $p<0.05$; Figure 3.7).

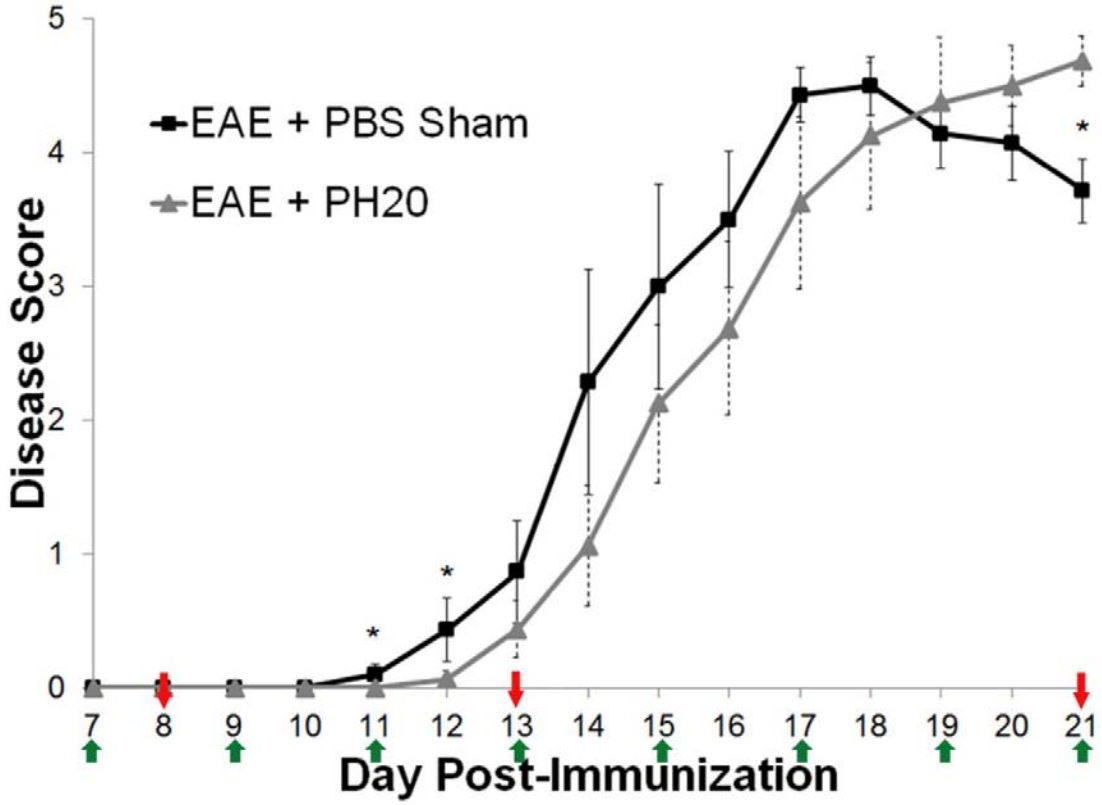

Figure 3.7. Subcutaneously injected PEG-PH20 delays the clinical onset of active EAE but does not ameliorate disease severity later in disease. EAE was induced in 48 C57BL/6 female mice. Half (24) of the animals were randomly selected to receive subcutaneous PBS (vehicle) injections (50 ul) and the other half, PEG-PH20 (1000 U in 50 ul PBS). Injections were given in the hind flank every other day beginning 7 days post inoculation (green arrows). Mice were scored by a blinded experimenter. PEG-PH20 treated animals had delayed onset of disease by two days, but by day 21 had higher disease scores than controls (*). n=8 animals were randomly selected from each group and euthanized 8, 13 and 21 days post inoculation for histology (red arrows). *p<0.05, repeated measures ANOVA.

At 8 days post-inoculation, lumbar spinal tissues from control and PEG-PH20 treated animals contained no histological evidence of $CD4^+$ cell extravasation within the spinal cord (data not shown). By day 13 post-inoculation, demyelinated lesions in vehicle-treated mice, visualized by staining with FluoroMyelin, contained numerous $CD4^+$ cells (Figure 3.8A). In addition to having lower clinical scores, there was a significant reduction in the number of infiltrating $CD4^+$ cells (2.3 fold fewer cells; $p<0.005$; Figure 3.8B) and less demyelination (69.1% less than controls; $p<0.005$; Figures 3.8C, D) in the spinal cords of PEG-PH20 treated mice.

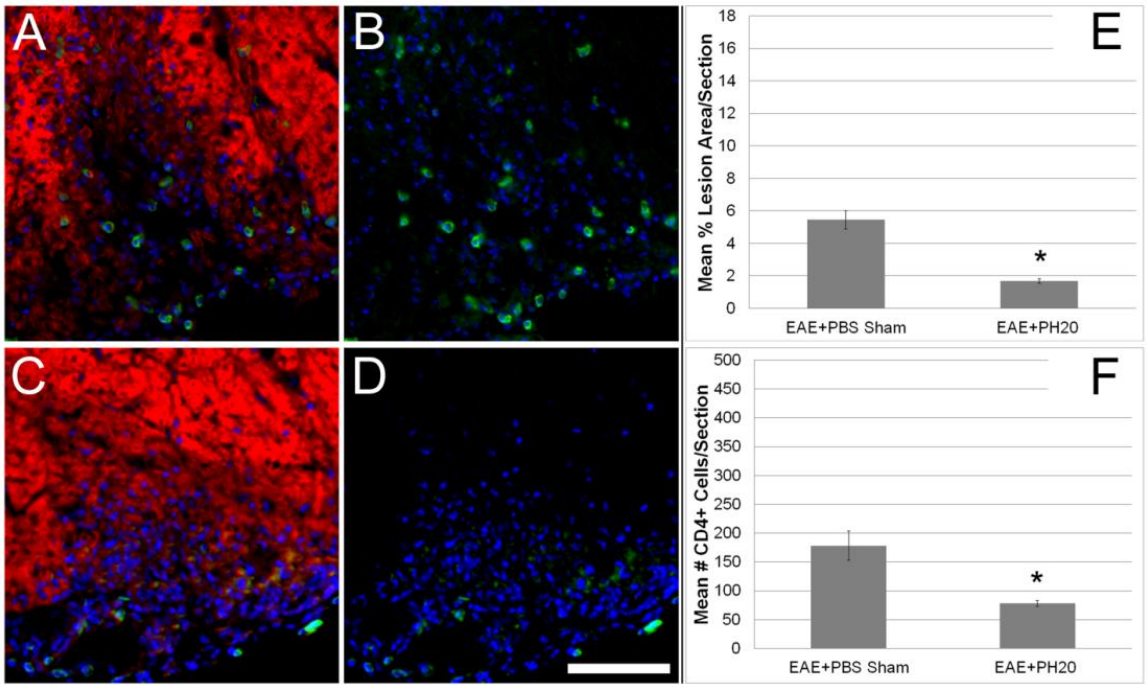

Figure 3.8. PEG-PH20 treated EAE animals have less demyelination and fewer CD4$^+$ infiltrating T-cells than PBS sham controls 13 days post inoculation. Representative ventral funicular lesions of lumbar spinal cord from PBS (vehicle, A) and PEG-PH20 treated (B) EAE animals taken on day 13 are illustrated. Myelin FluoroMyelin) is shown as red, CD4+ T-cells are green and cell nuclei are stained blue (Hoechst). Vehicle-treated animals have more demyelination than PEG-PH20 treated animals as quantified by stereology in (C). Also, fewer CD4$^+$ cells are present in PEG-PH20 treated sections (D) by stereologic quantification demonstrating delayed infiltration. Scale bar=100μm.* p<0.005, t- test.

To address the possibility that delayed disease onset in PEG-PH20 treated animals is related to reduced axonopathy, spinal cords from naïve, day 13 control and PEG-PH20-treated animals were labeled with neurofilament-L. Representative, high magnification images of similar sized lesion areas are illustrated in Figure 3.9. Axons from PEG-PH20-treated animals demonstrated fewer varicosities and swellings than controls (Figure 3.9, F vs. E). Additionally, more denuded axons were evident in PEG-PH20 treated lesions than controls (Figure 3.9 H and I arrows).

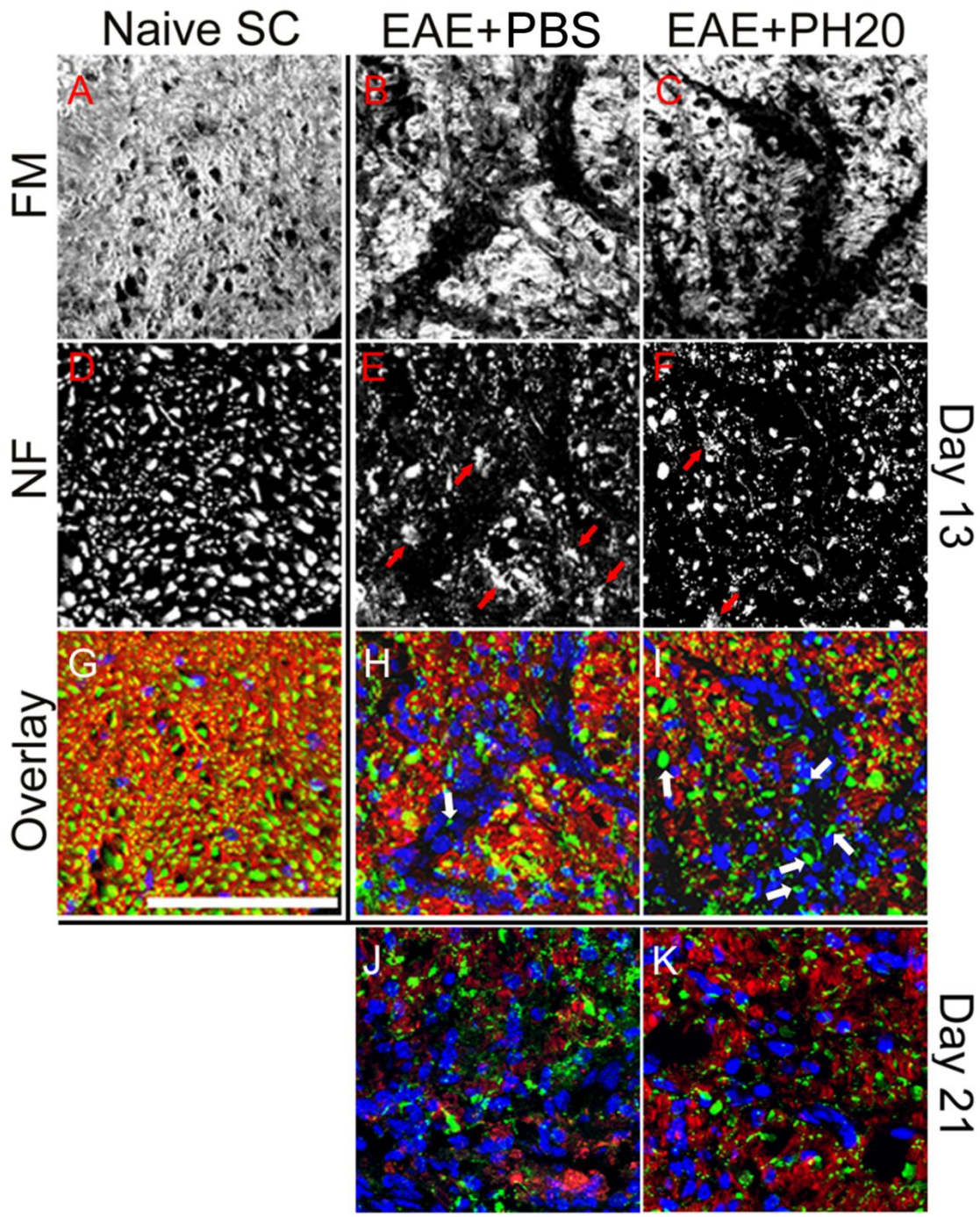

Figure 3.9. Some axons in demyelinated lesions are spared at EAE day 13 but not day 21 by PEG-PH20 treatment. Representative ventral lumbar spinal cord sections from a naïve animal (left column), an EAE PBS vehicle control (middle column) and a PEG-PH20 treated EAE animal (right column) on day 13 (top) and 21 (bottom) are shown. Demyelinating lesions, identified by the lack of FluoroMyelin label (FM row and red in overlay) in EAE day 13 PEG-PH20 treated animal contained more neurofilament (NF row and green in overlay) positive axons (arrows in overlays) compared to PBS vehicle control. Demyelinated areas from both treatment groups contain few axons relative to naïve control indicating PEG-PH20 mediated axonal sparing was not extensive at the EAE day 13 time point. By EAE day 21, there was no visual difference between groups in the number of axons in demyelinated areas (bottom two overlays). Scale bar= 100μm.

Stereologic analysis of spinal cords from mice with EAE 21 days post-inoculation revealed that PEG-PH20 treatment continued to significantly reduce the degree of demyelination albeit to a lesser extent than at day 13 (24.4% smaller lesion volume than controls; Figure 3.10A-C). However, consistent with the elevated disease scores at this time point, these animals demonstrated significantly increased numbers of infiltrating T-cells (1.9 fold more than controls; Figure 3.10D). Additionally, the number of neurofilament-L positive axons proximal to and within areas of demyelination was visually indistinguishable between treatment groups (Figure 3.9J, K).

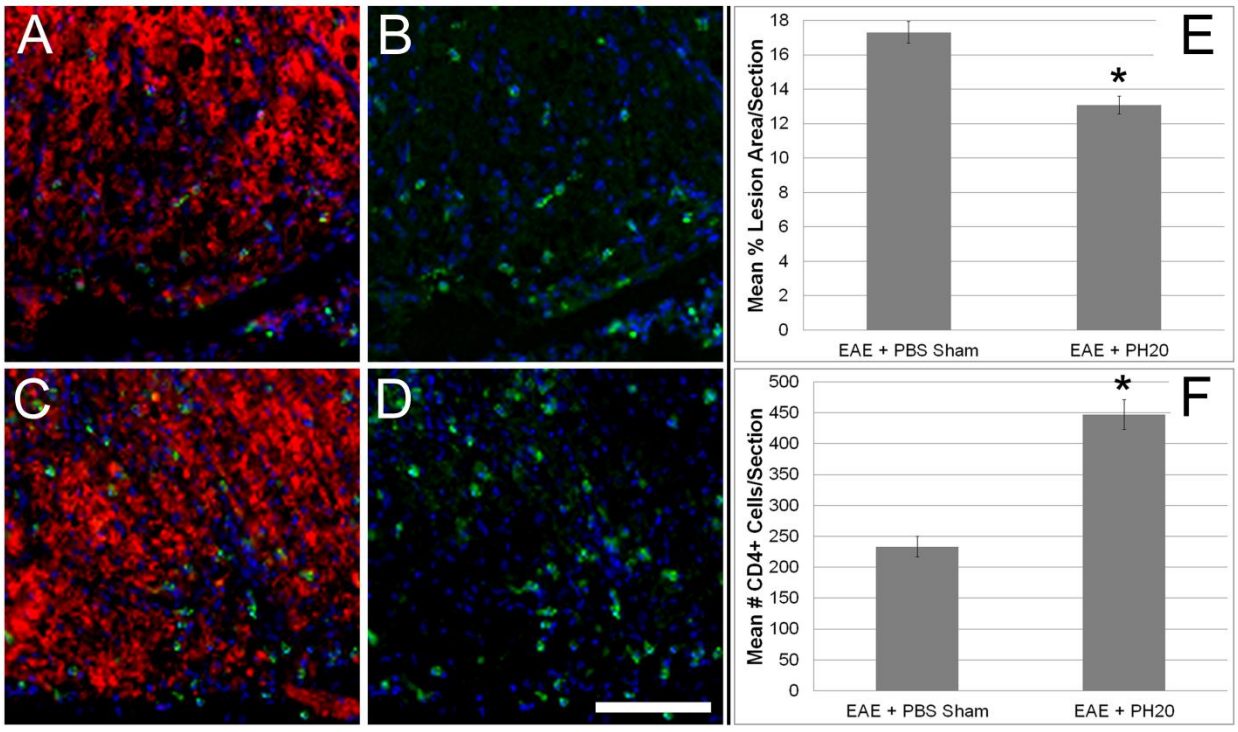

Figure 3.10. PEG-PH20 treated EAE animals have less demyelination and more CD4$^+$ T-cells 21 days post inoculation. Representative ventral funicular lesions of lumbar spinal cord from PBS (vehicle, A) and PEG-PH20-treated (B) EAE animals taken on day 21 are illustrated. Myelin FluoroMyelin) is shown as red, CD4+ T-cells are green and cell nuclei are stained blue (Hoechst). Vehicle-treated animals have more demyelination than PEG-PH20 treated animals as quantified by stereology in (C). Also, more CD4$^+$ cells are present in PEG-PH20 treated sections (D) by stereologic quantification suggesting elevated CNS inflammation relative to control at this time-point. Scale bar=100μm. * $p<0.005$, t- test.

Overall, these data suggest that PEG-PH20 treatment effectively delays disease onset by impairing T-cell extravasation resulting in less CNS inflammation, demyelination and

axonopathy early in disease. However, prolonged administration of PEG-PH20 increases CNS inflammation resulting in more severe clinical disease and negates/diminishes respectively its benefit to axon survival/demyelination observed in early disease.

HA is removed from CNS bloods vessels at EAE day 13 by PEG-PH20 but is re-expressed at EAE day 21

To confirm that PEG-PH20 treatment leads to chronic reductions in HA in CNS blood vessels, lumbar spinal tissue from animals with EAE was harvested from PEG-PH20-treated and vehicle-treated mice at 13-days post-inoculation and labeled for ECs, T-cells and HA (Figure 3.11A, B).

A representative image of a blood vessel from a vehicle-treated animal shows HA labeling within the EC lumen adjacent to CD31 positive tight-junctions (Figure 3.11A). Additionally, $CD4^+$ cells were often observed (18/32 vessels) co-localizing with HA within the lumen of the vessel (arrow and arrowhead). $CD4^+$ cells are also observed in the perivascular space co-localized with high levels of HA, as is typical of active EAE lesions (curved arrows, Figure 3.11A). In contrast, HA is undetectable within the luminal area of blood vessels from PEG-PH20 treated animals. No $CD4^+$ cells are observed within the lumen of PEG-PH20 treated vessels (0/26 vessels), however, some $CD4^+$ cells are present in HA-rich areas within the perivascular area (block arrows, Figure 3.11B).

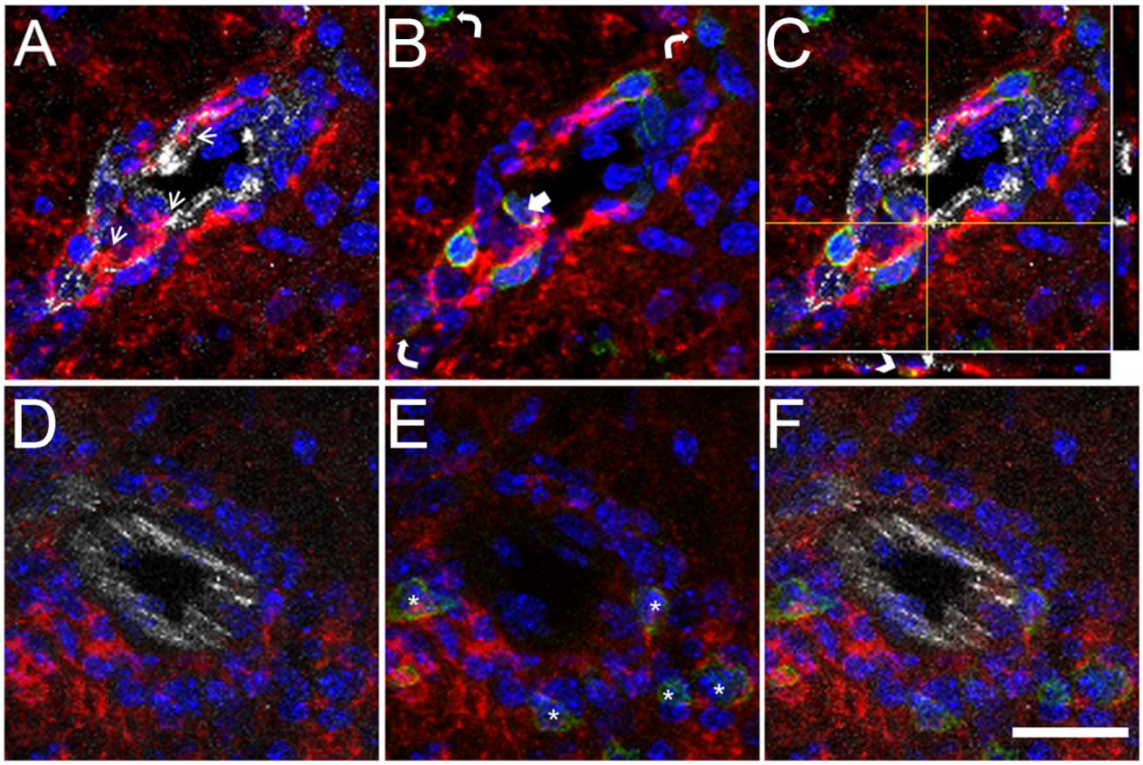

Figure 3.11. Subcutaneously injected PEG-PH20 degrades and removes HA from CNS blood vessels of day 13 EAE animals. Representative images of CNS blood vessels (CD31, white) from EAE animals treated with PBS (vehicle) (A) or PEG-PH20 (B) are shown. A) In vehicle-treated EAE vessels, HA (red) is present in the vessel lumen (arrows) and frequently co-localizes with $CD4^+$ (green) T-cells (18/32 vessels, block arrow). The orthogonal view (far right A) demonstrates $CD4^+$ T-cell contacting HA tethered to the endothelial lumen proximal to a CD31 positive tight junction (arrow head). B) In PEG-PH20 treated vessels, HA is absent from the vessel lumen. $CD4^+$ T-cells were never visualized contacting the vessel lumen (0/26 vessels). $CD4^+$ cells were only found in the immediate perivascular area (asterisk) and not widespread throughout the demyelinating lesion as in vehicle-treated animals (curved arrows). Scale bar is 25μm. Images are 8μm z-projections.

A possible explanation for increased T-cell infiltration into the CNS of mice with EAE following prolonged PEG-PH20 treatment is that HA is re-expressed in the lumen of CNS vessels at later times of disease progression. To address this possibility, CNS vessels from day 21 EAE animals treated with vehicle or PEG-PH20 were examined for HA expression. We find that HA is present along the lumen surface (arrows) in both vehicle- and PEG-PH20 treated EAE day 21 vessels (Figure 3.12A,B), but that the intensity of staining in both cases is significantly less than is observed in vessels from day 13 vehicle-treated EAE animals. Consistent with the low intensity HA staining, few

vessels in vehicle-treated day 21 EAE mice contained $CD4^+$ cells interacting with the luminal surface (2/34 vessels). However, despite the relatively weak HA label, vessels from PEG-PH20 treated EAE day 21 mice often had $CD4^+$ cells interacting with the luminal surface (14/27 vessels, Figure 3.12B, block arrow and arrow heads).

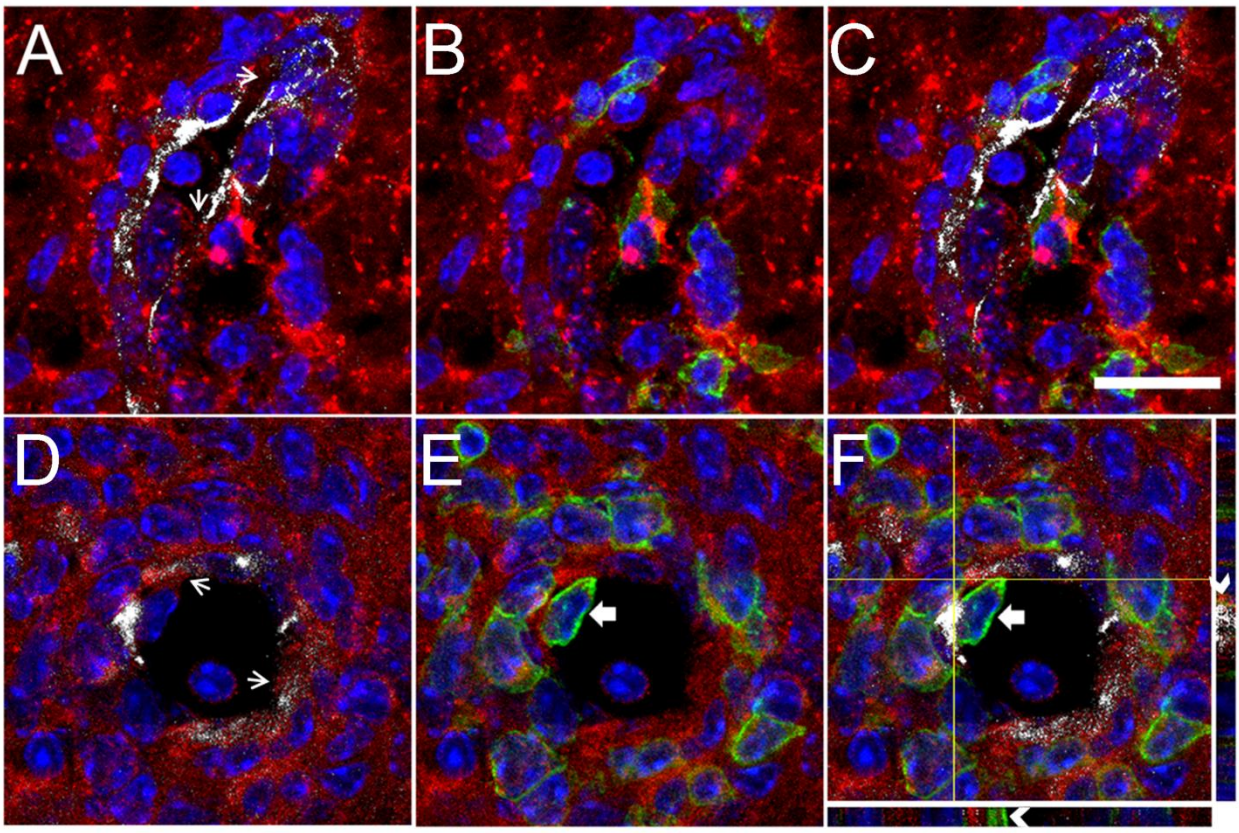

Figure 3.12. CNS blood vessels of PEG-PH20 treated EAE animals re-express HA 21 days post-inoculation. Representative images of CNS blood vessels (CD31, white) from EAE animals treated with PBS (vehicle, A) or PEG-PH20 (B) are shown. A) In vehicle-treated EAE vessels, HA (red) is present in the vessel lumen (arrows) but with less intensity than was observed in EAE day 13 vehicle treated animals (Figure 6A) Few $CD4^+$cells are observed contacting the luminal surface (2/34 vessels). B) Within PEG-PH20 treated vessels, HA is observed with similar intensity of signal as in PBS treated vessels of the same time point (arrows). Many $CD4^+$ cells are observed contacting the vessel lumen (14/27 vessels, block arrow). The orthogonal view (far right B) demonstrates a $CD4^+$ T-cell (block arrow) within the vessel lumen in close apposition to HA present in the luminal area proximal to a CD31 positive tight junction (arrow head in right orthogonal view). The same cell is also in direct apposition to luminal HA in an area distal from intense CD31 staining (arrow head in lower orthogonal view). Scale bar is 25μm. Images are 6μm z-projections.

Given that CNS ECs increase expression of HAS1 mRNA in response to pro-inflammatory stimulation (Figure 3.3E), we reasoned that HAS1 expression may be elevated in EAE day 21 PEG-PH20 treated vessels relative to PBS EAE controls. We

examined HAS1 expression in EAE day 21 vessels via immunohistochemistry and found HAS1 expression was higher in PH20 treated vessels relative to PBS controls (Figure 3.13B vs A, left panels). Also, HAS1 labeling co-localized with HA labeling within the luminal area of PEG-PH20 treated vessels suggesting the HA may be newly synthesized (Figure 3.13B, middle panel, white arrows).

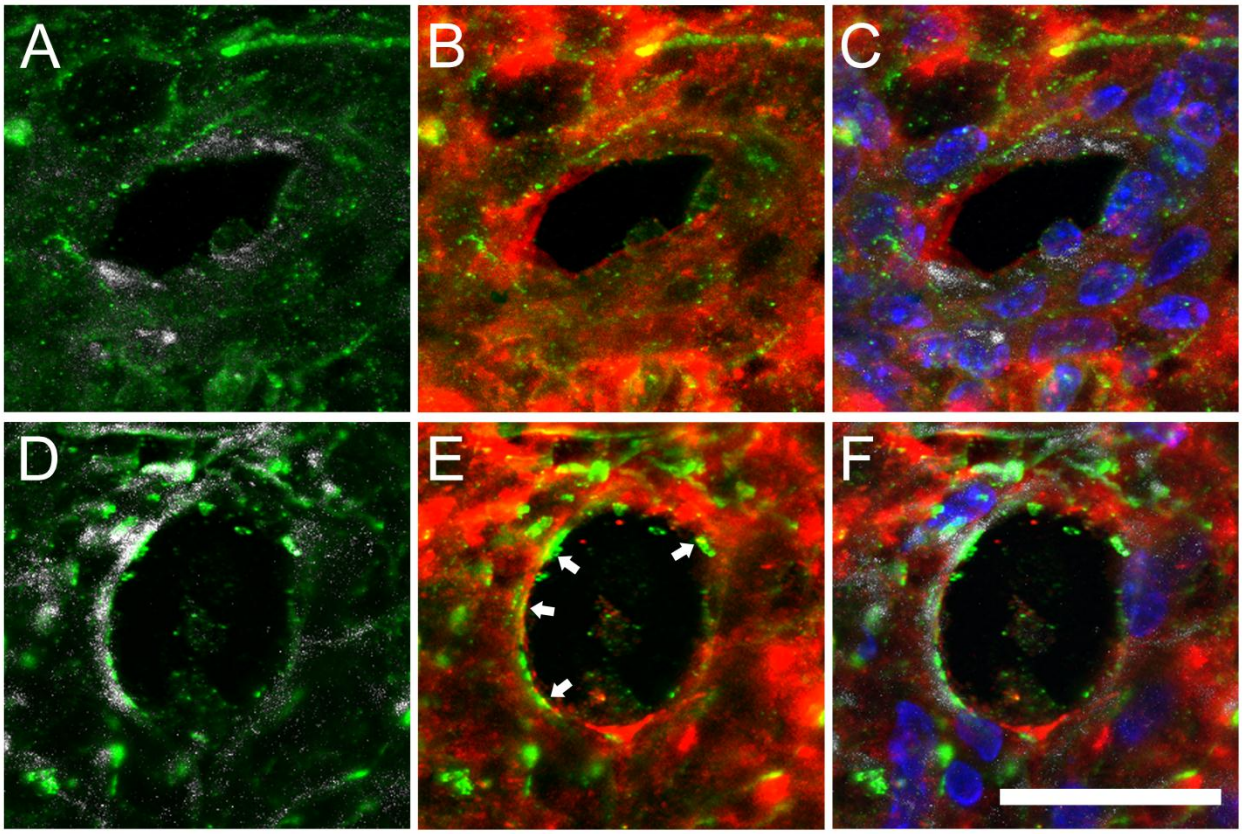

Figure 3.13. CNS blood vessels of PEG-PH20 treated EAE animals express more HAS1 21 days post-inoculation than PBS controls. Representative images of CNS blood vessels (CD31, white) from EAE animals treated with PBS (vehicle, A) or PEG-PH20 (B) are shown. A) In PBS treated EAE vessels, HAS1 (green) and HA (red) are present in the vessel lumen, but no obvious co-localization is observed. B) HAS1 staining in PEG-PH20 treated vessels is brighter than in PBS controls and areas of co-localization with HA are observed. Co-localization may indicate that HA within this vessel is newly synthesized. Scale bar is 25μm. Images are 6μm z-projections.

These results are consistent with the hypothesis that PEG-PH20 degrades HA from the vessel lumen in early disease, impairing T-cell extravasation. However, HA is re-expressed within the lumen of PEG-PH20-treated vessels at EAE day 21 due to

increased HAS1 expression. HA re-expression may therefore account for the elevated disease score and the increased number of T-cells observed within the spinal cord.

Discussion

We have demonstrated that HA and the standard form of CD44 on the luminal surface of CNS ECs contribute significantly to the initial capture and subsequent rolling of encephalitogenic lymphocytes in an inflammatory demyelinating disease model. We found that both $CD44^{-/-}$ animals and animals treated with a hyaluronidase experience delayed onset of EAE that could be accounted for by reduced numbers of encephalitogenic lymphocytes in demyelinating lesions. Our *in vitro* data indicate that CD44 expressed by stimulated brain ECs, but not by stimulated lymphocytes, contributes to lymphocyte rolling on ECs, and that this effect depends on HA being anchored to CD44 on the ECs. While this finding is novel in regard to CNS disease, a role for endothelial HA tethered to CD44 in lymphocyte extravasation has also been observed in a model of arthritis (Mikecz *et al.*, 1995). Together, these data support the idea that HA anchored to the CNS endothelial surface is a critical mediator of encephalitogenic immune cell trafficking in multiple organs and may represent a therapeutic target to treat inflammatory disease.

The finding that CD44 expressed by activated lymphocytes was dispensable for lymphocyte-EC interactions implies that lymphocytes possess an alternative mechanism for HA binding. One candidate HA binding protein is the receptor for hyaluronan mediated motility (RHAMM), which is expressed by developing CD3+ thymocytes although is reported to be largely absent from mature lymphocytes (Pilarski *et al.*, 1993; Pilarski *et al.*, 1994; Gares & Pilarski, 2000). Like CD44, RHAMM can be expressed at the cell surface where it can bind and tether HA and is capable of signaling through Src

kinase to influence cytoskeletal rearrangement (Turley *et al.*, 2002). If RHAMM expression was retained beyond thymic development in $CD44^{-/-}$ lymphocytes, it could account for the lack of phenotype observed in our flow model. Another candidate is the HA receptor for endocytosis (HARE). It can be expressed on the cell surface, but it predominately localizes within sinusodial endothelia and its expression by lymphocytes has not been reported (Zhou *et al.*, 2000; Falkowski *et al.*, 2003). Interestingly, antibody blockage of HA binding to HARE impairs tumor cell metastasis, a process likened to active lymphocyte extravasation (Bockhorn *et al.*, 2007; Simpson *et al.*, 2012). HARE is also capable of initiating intracellular signaling via MAP kinases (Kyosseva *et al.*, 2008). If a redundant or compensatory mechanism for HA binding on activated lymphocytes exists, it may function beyond the initial capture and rolling of lymphocytes to influence cellular rearrangement in preparation for extravasation.

PEG-PH20 treatment of mice with EAE results in the degradation and removal of HA from the luminal surface of spinal cord vessels after 6 days of treatment (EAE day 13). However, after 14 days of PEG-PH20 treatment (EAE day 21), HAS1 protein expression was elevated in the vessel lumen relative to PBS controls and HA was re-expressed in the same area. Coincident with HA re-expression, the number of $CD4^{+}$ infiltrates within the spinal cord of PEG-PH20 treated animals was elevated relative to controls. The mechanism underlying this reversal in disease progression is unknown. It is possible that degradation products of the injected enzyme may be signaling back onto ECs to enhance HAS1 expression. A likely candidate receptor is Toll-like receptor (TLR) 4 which induces NF-κB dependent signaling in response to HA fragments resulting in pro-inflammatory cytokine production (Shimada *et al.*, 2008). Indeed, cytokines such as TFNα have been shown to increase HAS expression and HA synthesis on human ECs

resulting in enhanced monocyte adhesion (Vigetti *et al.*, 2010). Additionally, HA signaling through TLR4 on ECs increases the expression of other molecules involved in immune cell extravasation. For example, in dermal ECs, TLR4 signal induced by HA fragments increases expression of the cell adhesion molecule ICAM-1 and the chemokine MIP-2 which are involved in the recruitment of neutrophils (Taylor *et al.*, 2004). CD44/HA interactions mediate neutrophil and lymphocyte adhesion and migration across endothelium (Khan *et al.*, 2004). Additionally, siRNA inhibition of endogenous hyaluronidases in fibroblasts subjected to collagen-induced arthritis decreases TLR4 expression, NF-κB activation and IL-1β production elicited by TNFα stimulation (Campo *et al.*, 2012).

An alternative explanation for enhanced T-cell infiltration in PEG-PH20-treated EAE animals at day 21 may be that the injected enzyme is being inactivated by the endogenous humoral response. Both non-human primates and mice have been shown to generate anti-PH20 specific antibodies against injected recombinant PH20 protein (Deng *et al.*, 2002; Hardy *et al.*, 2004). Interestingly, co-injection of recombinant protein with an adjuvant results in higher anti-PH20 titers. Because our subcutaneous PEG-PH20 injections were made coincident with adjuvant injections, the humoral response against PEG-PH20 may have been enhanced.

Using *in vitro* and *in vivo* approaches, we determined that HA anchored to standard CD44 on CNS endothelial cells contributes to the adhesion and subsequent extravasation of lymphocytes. However, approximately 50% of activated lymphocytes are still capable of slow rolling in our blood flow model and PEG-PH20 treated animals still develop EAE. The question remains what other cell adhesion molecules contribute to the capture and rolling of encephalitogenic T-cells? Endothelial P-selectin and its T-cell ligand, PSGL-1 have been implicated in the development of EAE but this is a

contentious issue. Several studies, using P-selectin$^{-/-}$ animals and blocking antibodies down-play the importance of these molecules in EAE development (Engelhardt *et al.*, 1997; Doring *et al.*, 2007), while others contend they are critical mediators of this process (Kerfoot & Kubes, 2002; Piccio *et al.*, 2002). A recent study claims that P-selectin facilitates the ability of T-cell α4 integrin to induce firm adhesion of T-cells to CNS endothelium, but is not itself critical for the induction of EAE (Kerfoot *et al.*, 2006). These conclusions stem from the finding that P-selectin$^{-/-}$ animals have delayed EAE onset but eventually develop severe disease. Additionally, if blocking antibodies to P-selectin and α4 integrin are co- injected, animals have delayed onset and quicker recovery than α4 integrin antibodies alone. The authors of this study speculate P-selectin facilitates firm adhesion by mediating capture and rolling of T-cells to the endothelial surface but that α4 integrin mediates encephalitogenic T-cell extravasation into CNS tissue. These results mirror closely our own findings with regard to EAE in CD44$^{-/-}$ animals and suggest that HA tethered to CD44$^{-/-}$ ECs may have redundant function with P-selectin in EAE development. The fact that a common signaling pathway activated by both CD44 and PSGL-1 mediates slow-rolling in neutrophils could explain why manipulating either pathway alone fails to completely abrogate T-cell rolling *in vitro* (Yago *et al.*, 2010).

T-cell recruitment into the CNS depends on α4 integrins (Yednock *et al.*, 1992). However α4 integrins have also been implicated in mediating T-cell capture (Vajkoczy *et al.*, 2001). Low affinity α4 integrin interacting with endothelial VCAM-1 allows for the transient capture of encephalitogenic T-cells at the CNS endothelial luminal surface. Following capture, G-protein-coupled signaling induces high affinity α4 integrin permanent adherence that is pertussis toxin sensitive. Therefore, α4 integrin could

represent another adhesion molecule contributing to T-cell adhesion in our experimental conditions. Indeed HA tethered to CD44 on endothelial cells may be functioning to capture T-cells and facilitate signaling to induce high affinity α4 integrin expression in inflammatory disease. Due to the complex nature of T-cell capture, rolling and adherence to the CNS endothelium, a potential therapeutic strategy may include the blockage of a combination of cell adhesion molecules so far discussed. Further experiments may be required to reveal additional mechanisms involved in this process.

Axonal degeneration is responsible for chronic disability in MS and EAE (Wujek *et al.*, 2002). We attempted to quantify neurofilament-L labeling but due to the extensive and highly variable axonal damage were unable to produce reliable counts. We determined that the quality and number of axons in and around lesions was increased with PEG-PH20 treatment on day 13 post-inoculation. However the causative relationship between PEG-PH20 treatment and reduced axonal damage is unclear due to fewer $CD4^+$ cells in the spinal cord at this time point. A recent EAE study demonstrates foci of infiltrating immune cells correlate with locally impaired axonal transport and markers of axonal damage at disease onset (Soulika *et al.*, 2009). It is likely the increase in spared axons is related to lower levels of inflammation within demyelinating lesions and not directly attributable to the activity of PEG-PH20. This idea is supported by the fact that the quality and number of axons in and around lesions was indistinguishable between treatment groups at 21 days post-inoculation. These findings are reminiscent of other studies inhibiting immune cell infiltration where axonopathy continues despite decreased CNS inflammation (Trapp & Nave, 2008).

Our experiments showed that both $CD44^{-/-}$ and PEG-PH20 treated mice eventually became symptomatic and attained disease scores equivalent to controls. A recent study

(Guan *et al.*, 2011) reported a delay in onset of disease, which the authors did not discuss further, coupled with prolonged attenuation of symptoms when EAE was induced in $CD44^{-/-}$ animals. The authors conclude that signaling through CD44 in encephalitogenic $CD4^{+}$ T-cells results in epigenetic changes that drive differentiation of the cell toward a Th1/Th17 pro-inflammatory phenotype. Although such phenotypic shifts may contribute to EAE progression, results from our group and others (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999) indicate that CD44 also plays a significant role in the initiation of T cell rolling on ECs and, therefore, the extravasation of encephalitogenic lymphocytes into the CNS.

It is possible that the difference between our studies is related to differences in the severity of EAE that is being induced. Guan et al reported a maximal mean disease score of ~3.25 in WT controls and ~1.15 for $CD44^{-/-}$ while our animals reached a maximal mean score of 4.33 for controls and 4.40 for $CD44^{-/-}$. Recently it was shown that the monosaccharide N-Acetylglucosamine, a component of HA, effectively inhibited T-cell activation and ameliorated MOG_{35-55} induced C57BL/6 EAE. However, treatment is clinically ineffective if EAE is induced in 2D2 T cell receptor transgenic mice, which display more robust disease (Grigorian *et al.*, 2011). The severity of EAE disease is known to vary depending on the population of cellular infiltrates. Other studies have shown that adoptive transfer of myelin reactive $CD8^{+}$ T-cells induces more severe disease than active disease induction alone (Sun *et al.*, 2001). Although we did not directly assay $CD8^{+}$ cells, our ability to induce more severe disease implies a greater contribution of the $CD8^{+}$ phenotype to disease pathogenicity in our model. As such, the contribution of deficient CD44 signaling to $CD4^{+}$ encephalitogenic T-cell phenotype described by Guan et al may be masked in our findings. Because myelin reactive $CD8^{+}$ cells are more prevalent within MS lesions than $CD4^{+}$ cells (Hauser *et al.*, 1986), our

findings may better reflect the potential therapeutic outcomes of manipulating CD44 or HA.

In conclusion we have demonstrated that standard CD44 on CNS ECs but not activated lymphocytes contributes to lymphocyte rolling on the endothelial surface. These findings expand the knowledge of mechanisms promoting inflammatory demyelinating CNS disease and suggest that HA anchored to EC CD44 represents a therapeutic target to reduce immune cell infiltration into the brain. Future studies aimed at testing whether transient HA degradation during early stage disease might have the potential to enhance other therapeutic agents that limit MS attacks will reveal the potential of targeting HA as a means of limiting disease severity.

Chapter 4: Hyaluronan oligosaccharides act on activated lymphocytes to delay the onset of experimental autoimmune encephalomyelitis[2]

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Abstract

Multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) are inflammatory demyelinating diseases characterized by mononuclear cell infiltration into the central nervous system (CNS). The glycosaminoglycan hyaluronan (HA) and HA receptors, including CD44 and particular Toll-like receptors (TLRs), have been implicated in MS and EAE pathogenesis. We previously found that digestion of high molecular weight HA tethered to brain vascular endothelial cells using a hyaluronidase delayed EAE onset. It is unclear if these effects were due to the removal of HA or the generation of HA digestion products that acted on mononuclear cell signaling. Here, we find that subcutaneously injected HA dodecasaccharides (HA12) delay the onset and

[2] Submitted to the Journal of Leukocyte Biology prior to defense.

reduce the severity of EAE. Although HA12 treatment had no effect on the infiltration of $CD3^+$ cells into the CNS, it leads to less severe demyelination. HA12 impaired activated lymphocyte rolling on CNS vascular endothelium when applied to lymphocytes but not CNS endothelial cells. The effects of HA12 on lymphocyte rolling were not abrogated in CD44-null or TLR4-null lymphocytes, or in wild type lymphocytes treated with a TLR4 neutralizing antibody. These data support the hypothesis that HA oligosaccharides can modulate inflammatory demyelinating disease in the CNS by acting directly on lymphocytes.

Introduction

Multiple Sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) are inflammatory diseases characterized by the infiltration or extravasation of myelin-reactive lymphocytes into the central nervous system (CNS) (Kuerten & Lehmann, 2011; Nylander & Hafler, 2012). These encephalitogenic cells, particularly $CD4^+$ T-cells, cause demyelination, axonal damage and both glial cell and neuronal death (Goverman, 2009). Impairing lymphocyte extravasation across CNS vascular endothelium delays disease progression and reduces disease severity (Yednock *et al.*, 1992; Baron *et al.*, 1993; Polman *et al.*, 2006). However, our understanding of the molecular mechanisms underlying this process is incomplete and the molecules involved have been disputed (Kerfoot *et al.*, 2006; Doring *et al.*, 2007).

Several studies, including the work present in Chapter 3, have implicated a role for the transmembrane receptor and adhesion molecule CD44 in promoting EAE pathogenesis by facilitating lymphocyte extravasation (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999, Guan *et al*., 2011). This role depends at least in part on lymphocyte interactions with high molecular weight (HMW) forms of the CD44 ligand hyaluronan

(HA) bound to CNS vascular endothelial cells (see Chapter 3). HA is a large (up to 10^7 Da) glycosaminoglycan composed of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine. In mammals, it is synthesized at the inner leaflet of the cell membrane by one of three synthases (HAS1-3) that extrude HA into the extracellular matrix as a linear, non-sulfated molecule. During inflammation, HAS expression increases, resulting in local accumulation of HMW HA (Kennedy *et al.*, 2000; Tammi *et al.*, 2005). Concomitant induction of HYAL expression results in accumulation of HA fragments as small as oligosaccharides (Sampson *et al.*, 1992; Girish & Kemparaju, 2007). Furthermore, inflammatory products such as reactive oxygen species fragment HA at glycoside linkages increasing HA oligosaccharide concentrations beyond physiologic levels at sites of inflammation (Noble, 2002).

There are conflicting data regarding the roles of CD44 in HA oligosaccharide signaling. CD44 is widely expressed, including by lymphocytes and CNS endothelial cells (EC) and is capable of both cell adhesion and signaling interactions (Lokeshwar *et al.*, 1996; Pure & Cuff, 2001; Ponta *et al.*, 2003). CD44 on lymphocytes has been shown to interact with HA tethered to CD44 on ECs to mediate capture, rolling and adhesion (DeGrendele *et al.*, 1996; DeGrendele *et al.*, 1997a; Nandi *et al.*, 2004). Our own findings support a role for CD44-HA interactions in lymphocyte extravasation into the CNS and suggest that disrupting these interactions has a beneficial outcome for inflammatory demyelinating diseases (see Chapter 3). HA oligosaccharides are capable of interfering with CD44-HA interactions via several mechanisms. These include CD44 receptor cleavage (Sugahara *et al.*, 2003), displacement of bound HMW HA (Tammi *et al.*, 1998) or competitive binding (Teriete *et al.*, 2004). Conceivably, loss of CD44 or displacement of HWM HA would result in decreased receptor aggregation, diminishing cell-cell adhesion

interactions and intracellular signaling. These effects, singly or in combination may interrupt activated lymphocyte rolling and adhesion in the CNS.

During inflammation, HA fragments, including oligosaccharides may signal through a number of receptors other than CD44, including Toll-like receptor-2 (TLR2) and -4 (TLR4) (Taylor *et al.*, 2004; Jiang *et al.*, 2005; Scheibner *et al.*, 2006). TLRs are a family of pattern-recognition receptors involved in microbial recognition by the immune system (Barton & Medzhitov, 2003). HA oligosaccharide-mediated signaling through TLRs is generally considered to be pro-inflammatory (Stern *et al.*, 2006; Jiang *et al.*, 2007); however their roles in HA-mediated CNS inflammation and lymphocyte extravasation have not been addressed. Naïve lymphatic-derived mouse $CD4^+$ T cells express transcripts for TLR4 but not TLR2 (Gelman *et al.*, 2004). Furthermore, TLR4 signaling inhibits T cell activation (Gonzalez-Navajas *et al.*, 2010) and HA oligosacchardies can induce elevated CD44 and TLR4 expression (Campo *et al.*, 2010; Campo *et al.*, 2012). It is possible; therefore, that HA oligosaccharide-induced TLR4 signaling may influence T-cell mediated inflammation during EAE progression leading to reduced extravasation of encephalitogenic cells.

In this study, we tested the ability of HA oligosaccharides to inhibit the onset and progression of EAE. We show that dodecasaccharides of HA (HA12) delay the onset of EAE and reduce disease burden. Treating lymphocytes with HA12 impairs their interaction with CNS ECs, however the candidate receptors CD44 and TLR4 do not mediate this effect. These findings suggest another HA receptor or receptors on lymphocytes signals in response to HA12 and can modulate the onset and course of inflammatory demyelinating disease.

Results

HA oligosaccharides delay the onset of EAE

We previously determined that HA tethered to CD44 on the surface of CNS ECs facilitates lymphocyte rolling that contributes to the onset of EAE (described in Chapter 3). Degradation of HA by a hyaluronidase delayed EAE onset. These effects may have resulted from the removal of HMW HA from the lumen of CNS blood vessels or the generation of low molecular weight forms of HA that have distinct biological activities on ECs or lymphocytes. HA oligosaccharides, including those generated by HMW HA digestion, can prevent CD44 binding to HMW HA through various means, including ligand displacement and receptor cleavage (Tammi *et al.*, 1998; Sugahara *et al.*, 2003), but can also have distinct biological activities that influence inflammatory responses (Genasetti *et al.*, 2008; Campo *et al.*, 2010; Campo *et al.*, 2012). We therefore tested if two different oligosaccharides of HA, HA12 and HA4 influenced EAE onset and progression. HA4, HA12 or PBS alone (vehicle control) were subcutaneously administered to mice every 2 days starting 7 days after the induction of chronic EAE. Clinical scores were rated over a 20-day period in 8 week old WT C57/B6 mice immunized with MOG_{35-55} to induce active EAE. Symptoms were evident in PBS-treated animals by 12 days post-inoculation with a mean onset on day 13 (Figure 4.1A,C). In contrast animals treated with HA4 (Figure 4.1A) or HA12 (Figure 4.1C) on average had a 2 day delay in disease onset, with a mean onset on day 15 (*$p<0.05$ repeated measures ANOVA). Although not statistically significant, mean daily EAE scores of HA12 treated animals after day 15 remained lower than PBS treated controls through the end of the experiment while animals treated with HA4 had disease scores that actually exceeded control by day 20 post-inoculation. Reinforcing this observation, the mean cumulative disease index (CDI) score of HA12 treated animals was significantly less than PBS controls (*$p<0.05$ t-test, Figure 4.1D) but there was no significant difference in the CDIs

of animals treated with HA4 (Figure 4.1 B). Thus, both HA12 and HA4 delay the onset of EAE, but only HA12 results in an improved CDI score.

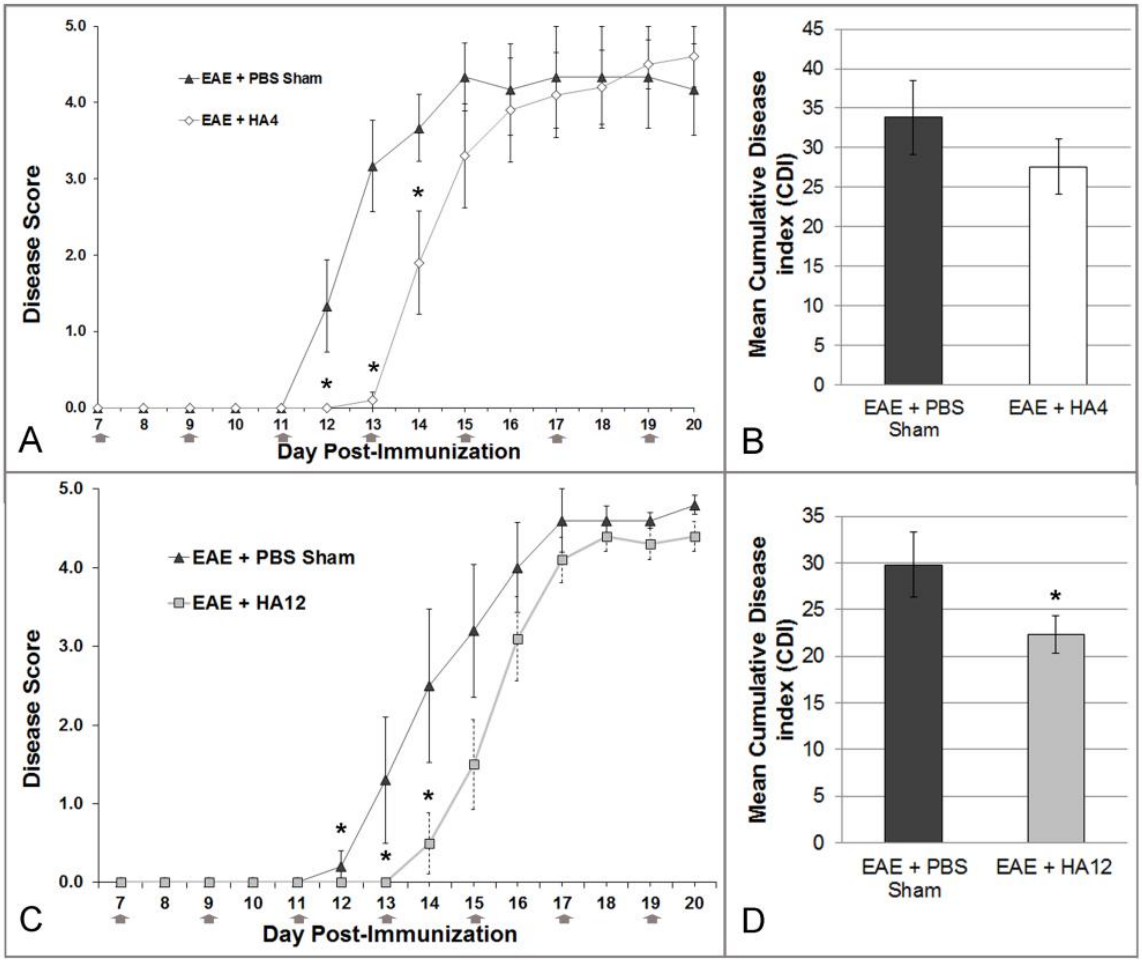

Figure 4.1. Subcutaneously injected HA 4 and HA12 delays the clinical onset of active EAE and ameliorates chronic disease burden. A-D) EAE was induced in 10 C57BL/6 female mice in two different experiments. For each experiment, half (n=5) of the animals were randomly selected to receive subcutaneous PBS (vehicle) injections (50 ul) and the other half, HA4 (A, B) or HA12 (C, D) (50 mg/kg, in 50 ul PBS). Injections were given in the hind flank every other day beginning 7 days post inoculation (gray arrows). Mice were injected and scored by a blinded experimenter. HA4 (A) and HA12 (C) treated animals had delayed onset of disease by two days (*).Animals were perfused for histology 20 days post inoculation. *p<0.05, repeated measures ANOVA. B and D) Mean cumulative disease score was determined by summing individual daily scores for each animal and averaging across the group. HA4 (B) CDI scores were not significantly different than PBS control. However HA12 (D) treated animals had significantly lower CDI than control (*). *p<0.05 t-test.

HA oligosaccharides improve EAE histological outcome

Our finding that HA12 treated animals have delayed disease onset and less chronic disease burden suggests that CNS inflammation was reduced by this treatment. Therefore, EAE lumbar spinal cord sections were histologically assayed for myelin and infiltrating $CD3^+$ T-cells. PBS treated controls exhibited diffuse and robust demyelination as visualized by FluoroMyelin stain (Figure 4.2A, B red). In contrast, HA12-treated animals had significantly less demyelination as determined by stereologic quantification (Figure 4.2D, E and G). $CD3^+$ T-cells were distributed fairly evenly across demyelinated areas in PBS controls (Figure 4.2B, C). However, $CD3^+$ T-cells were concentrated toward a presumably perivascular focal point within the lesions of HA12-treated animals (Figure 4.2E, F). Interestingly, there was no significant difference in the mean number of $CD3^+$ T-cells between groups via stereology (Figure 4.2H).

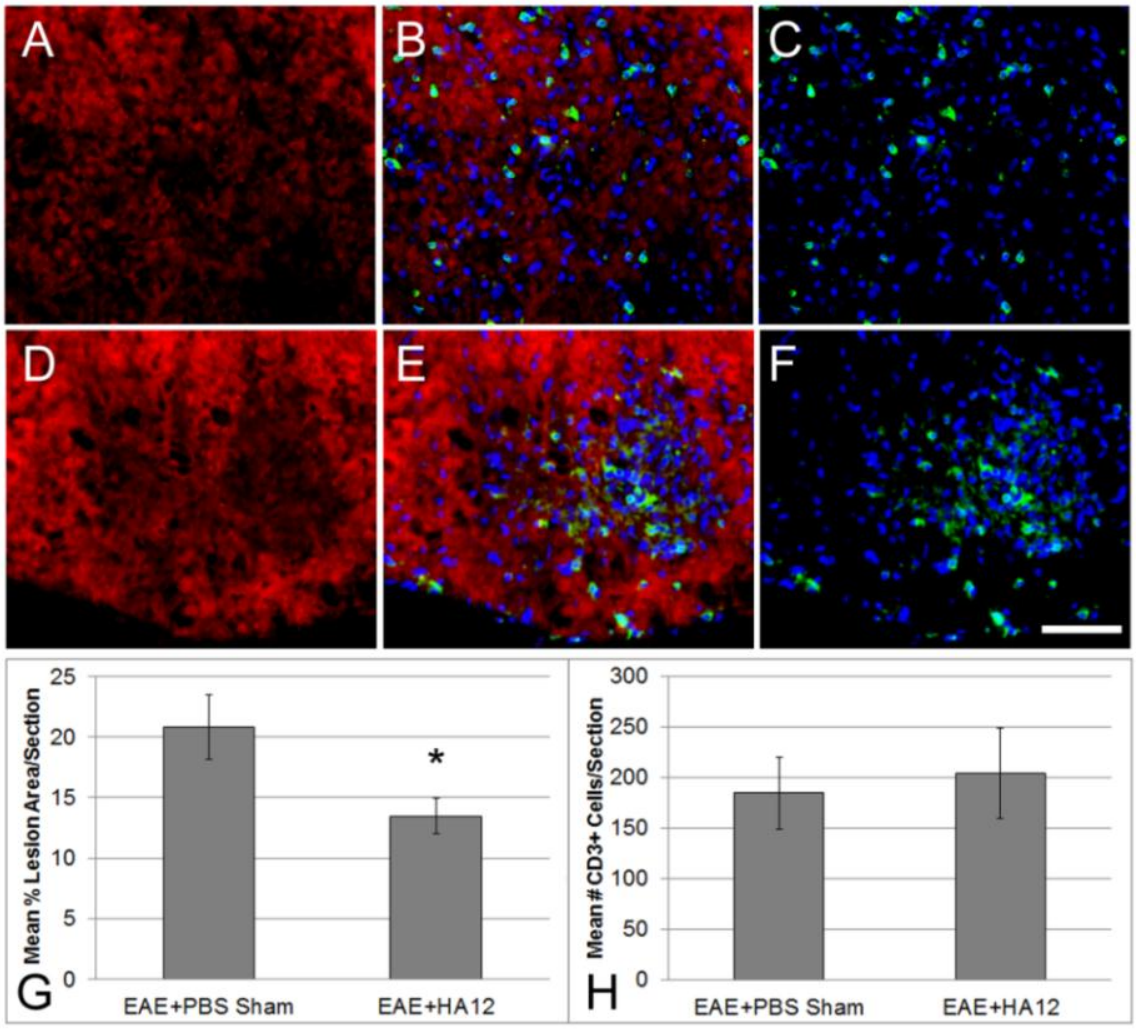

Figure 4.2. HA12 treated EAE animals have less diffuse demyelination and more concentrated sites of infiltration than PBS sham controls 20 days post inoculation. Representative ventral funicular lesions of lumbar spinal cord from PBS (vehicle, A-C) and HA12 treated (D-F) EAE animals taken on EAE day 20. Myelin FluoroMyelin) is shown as red, $CD3^+$ T-cells are green and cell nuclei are stained blue (Hoechst). PBS-treated animals have more demyelination than PEG-PH20 treated animals as quantified by stereology in (G). No significant difference in $CD3^+$ cell number is was quantified (H), however infiltrates are concentrated at presumably perivascular sites consistent with recent infiltration. Scale bar=100μm.* $p<0.05$, t- test.

HA oligosaccharides impair activated lymphocyte rolling on CNS ECs

Our previous study indicated that digestion of HA by a hyaluronidase inhibited T cell rolling on CNS vascular ECs. To test if HA12 similarly inhibited T cell rolling on ECs, we utilized an *in vitro* static adhesion assay where calcein-AM loaded WT lymphocytes or CNS WT ECs grown in a mono-layer were pre-treated with HA12 prior to interaction in a

co-culture for 1 hour. Following two washes with culture medium, fluorescent intensity emitted by lymphocytes was decreased in wells where lymphocytes had been treated with 50 ug/mL and 10 ug/mL HA12 (Figure 4.3A). No change in fluorescent intensity was observed in co-cultures where ECs had been treated with HA12 (Figure 4.3B). These findings demonstrate HA12 impairs activated lymphocyte adherence through a lymphocyte-dependent mechanism.

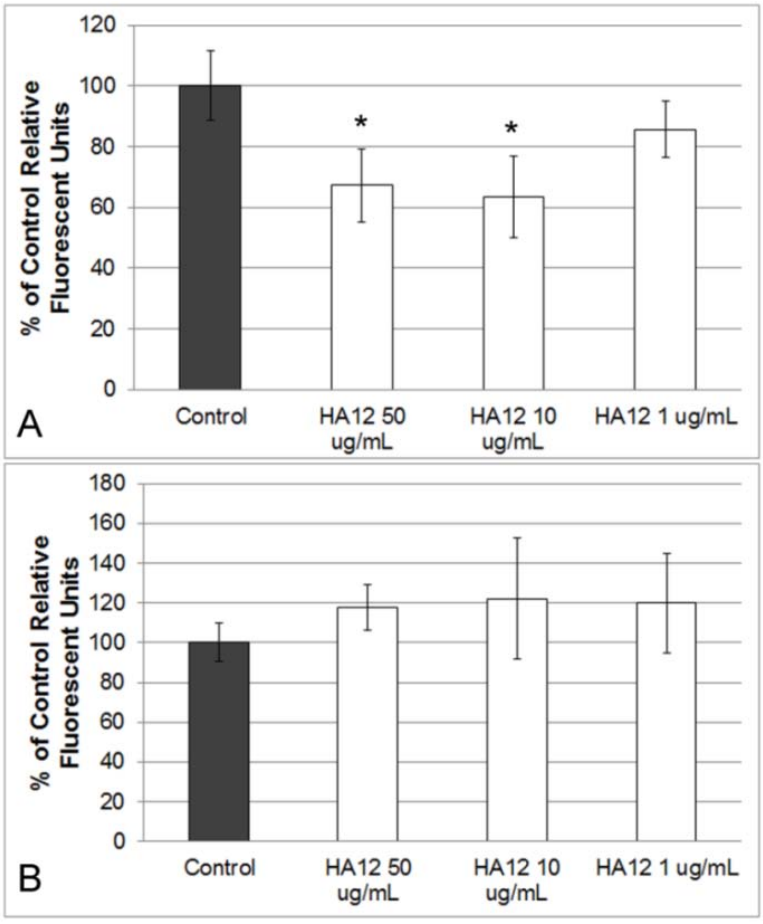

Figure 4.3. Pre-treatment of activated LCs, not CNS ECs with HA12 disrupts LC adhesion. CD3/CD28 stimulated LCs were loaded with calcein-AM dye for 15min. Activated LCs (A) or CNS ECs (B) were pre-incubated with various concentration of HA12 (50, 10 and 1 ug/mL) or PBS 30 minutes prior to a 1 hour co-culture. Fluorescence was measured at 538 nm after 2 washes with culture medium. * $p<0.05$, t- test.

Because CD44-HA interactions are proposed to be critical for lymphocyte rolling on ECs (DeGrendele *et al.*, 1996), we utilized an *in vitro* parallel-plate physiologic flow assay to better model this process. Confirming our static adhesion assay results, HA12 treatment

of lymphocytes prior to use in the flow assay reduced the number of interacting cells compared to PBS controls (Figure 4.4B vs. A).

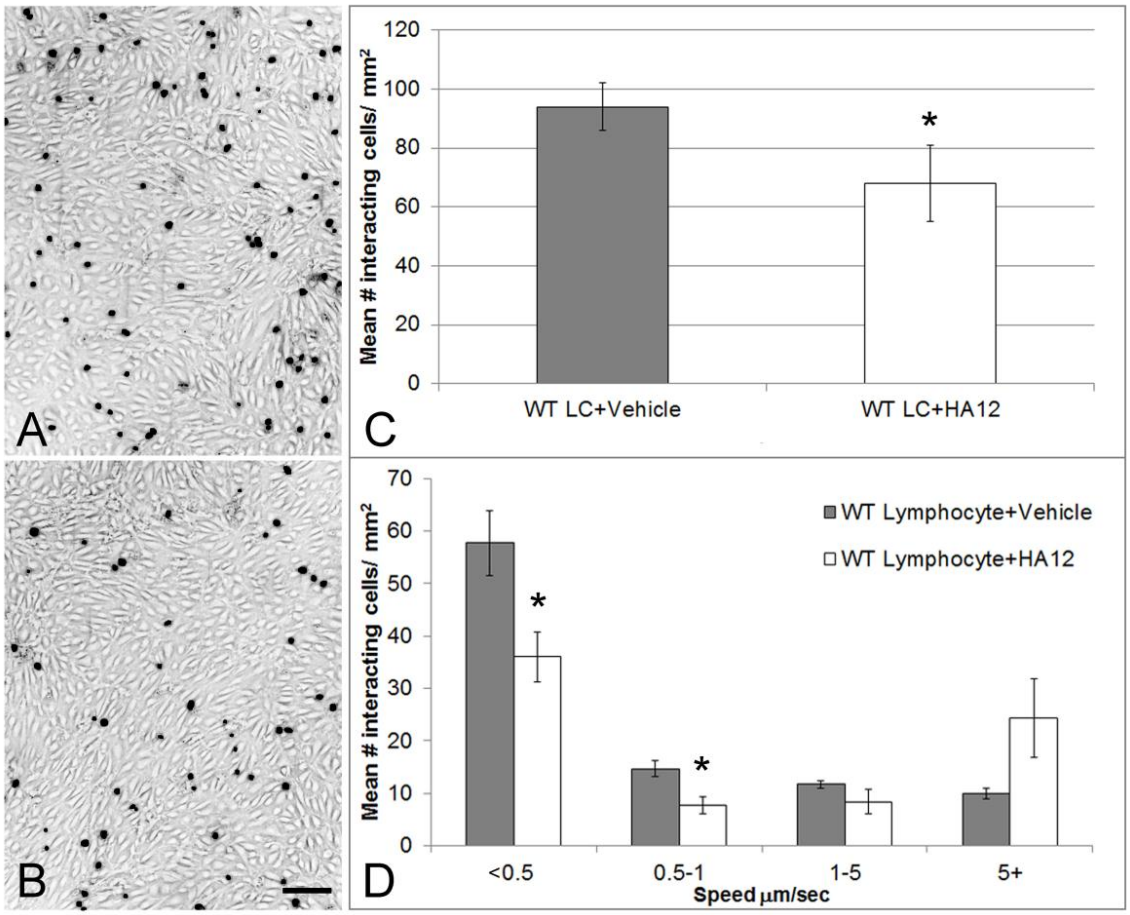

Figure 4.4. Treating WT LCs with HA12 impairs activated LC rolling on CNS ECs. Representative bright-field images of psuedo colored (black) of PBS treated WT LCs (A) and HA12 treated WT LCs bound to WT ECs at the end of a 7 minute experiment. (C) HA12 treated (30min, 10 ug/mL) WT LCs had significantly fewer interactions with CNS endothelial cell than PBS treated controls. D) Treatment of WT LCs with HA12 significantly decreased the number of slowest (<0.5 μm/sec) and slow (0.5-1 μm/sec) rolling cells compared to PBS control. Data from all experimental groups represent two independent experiments performed in triplicate. *p<0.05, t- test compared to WT LCs+Vehicle.

Quantification of the parallel plate assays revealed a 27.9.% decrease in the number of interacting cells (Figure 4.4C, *p<0.05 t-test). Using particle tracking software, the average rolling speed of all cells was determined. Bins for slowest (<0.5 μm/sec), slow (0.5-1 μm/sec), medium (1-5 μm/sec) and fast (5+ μm/sec) rolling cells were generated and plotted against mean number of interacting cells for both treatment groups (Figure

4.4D). Within the slowest and slow groups, HA12 treatment significantly decreased the number of rolling lymphocytes (Figure 4.4D, *p<0.05, t-test). HA12 treatment also trended toward increasing the number of fast rolling interacting cells although not significantly. Collectively, these data indicate that HA12 treatment interferes with the capture and slow rolling of lymphocytes on CNS ECs, decreasing the opportunity for subsequent lymphocyte extravasation.

The effect of HA12 on activated lymphocyte rolling is independent of CD44

We previously demonstrated that CD44 on lymphocytes is dispensable for the adhesive interactions with HA tethered to CNS endothelial cells (Figure 3.2). However, CD44 also functions as a signaling molecule that can influence lymphocyte migration and cell-cell adhesion (Ponta *et al.*, 2003). CD44 may therefore mediate other aspects of lymphocyte interactions with ECs that could be altered by HA12. To address this possibility, $CD44^{-/-}$ lymphocytes were pretreated with HA12 or PBS prior to use in the parallel plate assay. Similar to WT lymphocytes, HA12 treatment of $CD44^{-/-}$ lymphocytes significantly reduced the number of interacting cells compared to control (28.1%, Figure 4.5A, *p<0.05 t-test). Furthermore, like WT cells, HA12 treatment significantly decreased the number of $CD44^{-/-}$ lymphocytes in the slowest rolling bin and trended toward an increase in the number of fast rolling cells (Figure 4.5B, *p,0.05 t-test). Interestingly, the number of slow rolling cells was not significantly different between the groups; however, this may be accounted for by mild deficits in lymphocyte extravasation previously observed in the $CD44^{-/-}$ phenotype (Protin *et al.*, 1999). Overall, these data imply that the impaired slowest/slow rolling phenotype generated by HA12 treatment is not mediated by CD44.

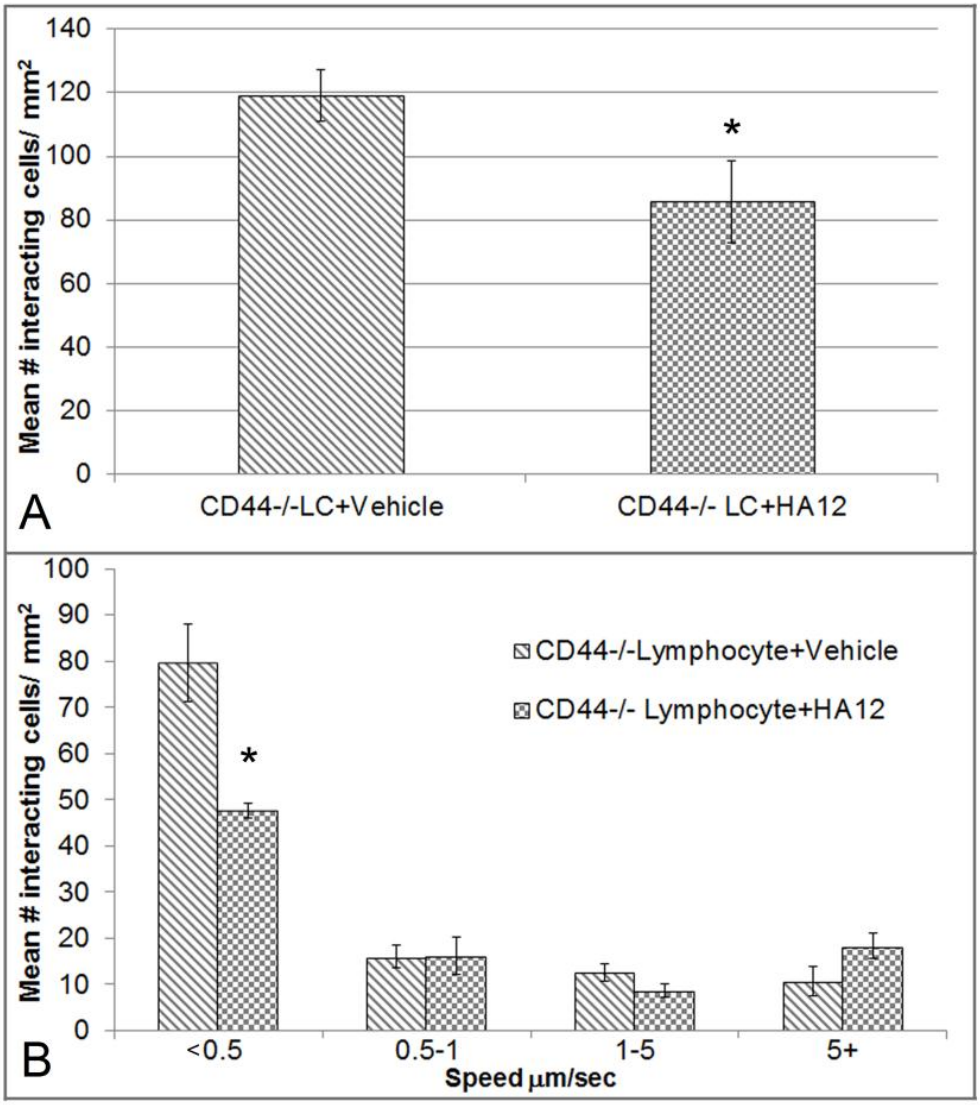

Figure 4.5. HA12 impaired LC rolling phenotype is not altered in CD44$^{-/-}$ LCs. (A) HA12 treated (30min, 10 ug/mL) CD44$^{-/-}$ LCs had significantly fewer interactions with activated CNS ECs than PBS treated CD44$^{-/-}$ LC controls. D) Treatment of CD44$^{-/-}$ LCs with HA12 significantly decreased the number of slowest (<0.5 μm/sec) rolling cells compared to PBS control. Data from all experimental groups represent two independent experiments performed in triplicate. *p<0.05, t- test compared to CD44$^{-/-}$ LCs+Vehicle.

The effect of HA12 on activated lymphocyte rolling is independent of TLR4

HA oligosaccharides can signal through TLR4, typically inducing expression of pro-inflammatory cytokines (Taylor *et al.*, 2004; Shimada *et al.*, 2008). However, TLR4 signaling has been shown to have an inhibitory role in T-cell mediated inflammation (Gonzalez-Navajas *et al.*, 2010). Previously, TLR4 mRNA transcripts in T-cells have

been reported to decrease with CD3/CD28 activation out to 24 hr (Gelman *et al.*, 2004; Gonzalez-Navajas *et al.*, 2010). Consistent with these findings, at the 72 hr post-activation time-point we utilized for our experiments, we found a modest induction of TLR4 transcripts in activated lymphocytes (Figure. 4.6).

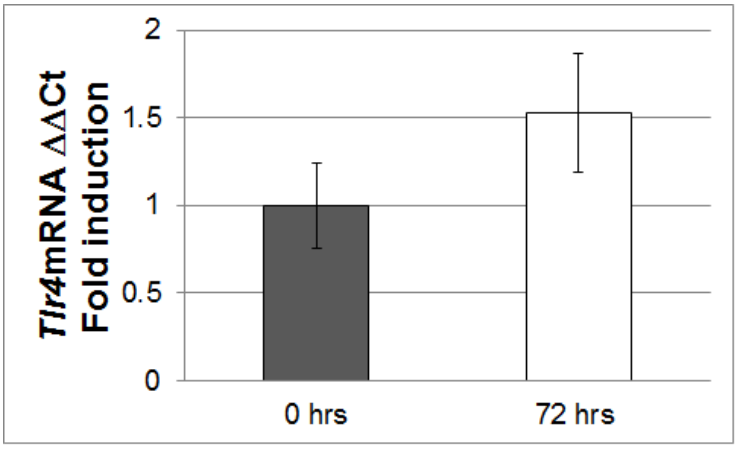

Figure 4.6. TLR4 mRNA transcript is modestly increased at 72hr of activation in LC. qRT analysis of *Tlr4* mRNA in WT LCs stimulated with anti-CD3/CD28 for 0 and 72hr. There is a modest, but statistically insignificant increase in transcript at this time point.

To test if the effects of HA12 on lymphocyte rolling are regulated by TLR4, we assayed activated lymphocyte recruitment and rolling in WT lymphocytes pre-treated with HA12 and a TLR4 blocking antibody (anti-TLR4) or an isotype control (anti-Iso). Additionally, we assayed TLR4$^{-/-}$ lymphocytes pretreated with HA12. HA12 treatment alone decreased the number of interacting lymphocytes as expected (45%, Figure 4.7A). This effect was not altered by the TLR4 blocking antibody or an isotype control antibody (49% and 52% respectively Figure 4.7A). Furthermore, activated TLR4$^{-/-}$ lymphocytes treated with HA12 had a 49% decrease in interacting cells relative to PBS controls (Figure 4.7A). No significant difference in number of interacting lymphocytes was observed in any of the HA12- treated groups (Figure 4.7A). Similarly, no significant difference was observed in the number of interacting cells within speed bins between the HA12 only, HA12 + TLR4 blocking antibody, HA12 + isotype control or HA12 + TLR4$^{-/-}$ lymphocyte

treatment groups (Figure 4.7B). These data indicate that TLR4 signaling is not required to mediate the effects of HA12 on lymphocyte rolling.

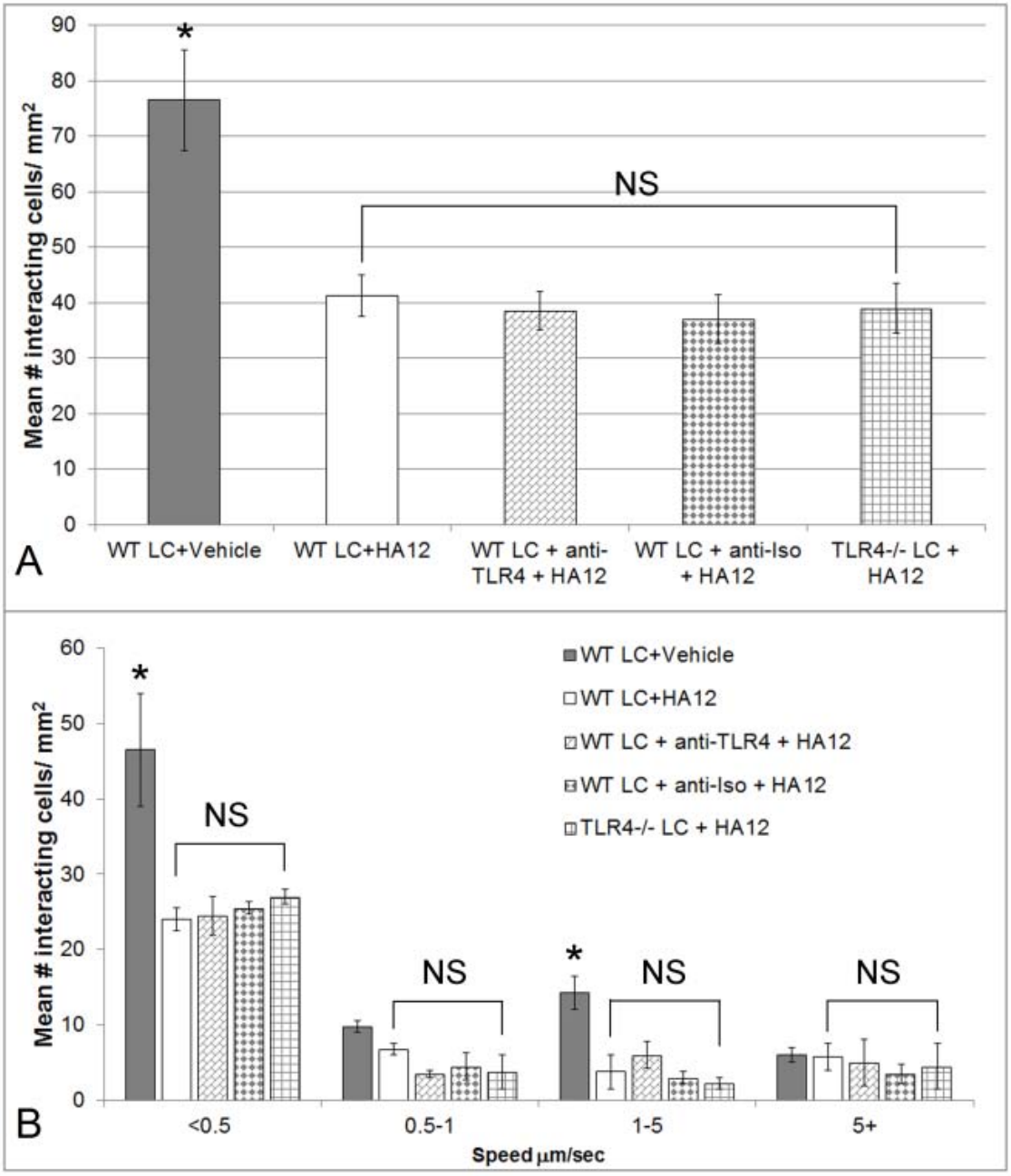

Figure 4.7. Blockage or knockout of TLR4 signaling does not alter HA12 mediated LC rolling impairment. A) WT LCs treated with PBS (vehicle) interacted more frequently with activated CNS ECs than did WT LCs treated with HA12 (10 ug/mL, 30 min) only, HA12+TLR4 blocking antibody (anti-TLR4, clone MTS510, 100 ng/mL, 10min prior to HA12), HA12+Isotype antibody (anti-Iso, Rat IgG2a,κ, 100ng/mL, 10min prior to HA12) or HA12 treated TLR4$^{-/-}$ LCs. B) PBS treated LCs has significantly more rolling cells in the slowest (<0.5 mm/sec) speed bin and the medium (1-5 mm/sec). However, no significance (NS) was observed in the number of rolling cells between any of the HA12 treated LC + treatment conditions in any speed bin. Data from all experimental groups represent one experiment performed in triplicate. *p<0.05, t-test compared to WT LCs+HA12.

Discussion

Digestion of HMW HA tethered to CNS ECs in mice with EAE is sufficient to delay the onset of disease and impair lymphocyte rolling (Figure 3.6 and 3.5). HWM HA digestion produces HA fragments and oligosaccharides that contribute to an already increased pool of HA products resulting from inflammatory processes. We find that subcutaneously injected HA12 delays EAE onset and reduces disease burden. Additionally, histological analysis reveals less demyelination in discrete spinal cord EAE lesions in HA12-treated animals 20 days post-immunization, consistent with the idea that HA12 treatment delays demyelinating disease. These findings are consistent with an impaired extravasation phenotype; however, we did not find a significant difference in number of infiltrating $CD3^+$ T-cells at the day 20 time point. Our previous study indicated that manipulations of HA resulting in impaired extravasation have quantifiable differences in infiltrating T-cells at disease onset and not later (described in Chapter 3). Therefore, the observation of similar numbers of infiltrating cells in the treatment group compared to controls could be accounted for by perivascular proliferation of T-cells consistent with newly active lesions (Serafini *et al.*, 2006; Liu *et al.*, 2007).

Our findings also demonstrate that HA12 treatment of activated lymphocytes but not CNS ECs is sufficient to impair lymphocyte interactions with CNS endothelia. This implies an HA receptor competent to interact with HA oligosaccharides is mediating this process. CD44 and TLR4 are known HA oligosaccharide receptors expressed by lymphocytes; however our results indicate they are not involved in lymphocyte rolling and capture. Therefore the question remains: how is HA12 treatment of activated lymphocytes mediating this effect? It is likely that another HA receptor, such as RHAMM interacts with HA oligosaccharides to impair lymphocyte-CNS endothelial interactions.

RHAMM is known to mediate cell adhesion and migration in both normal and pathological states (Sherman *et al.*, 1994) and has been implicated in T-cell development and migration (Pilarski *et al.*, 1993). Furthermore, RHAMM is expressed at the cell surface of lymphocytes and facilitates cytoskeletal re-arrangements that could influence T-cell extravasation (Turley *et al.*, 2002). However, HA and HA oligosaccharides interact with RHAMM to promote leukocyte infiltration in an arthritis model (Nedvetzki *et al.*, 2004) and macrophage recruitment during lung injury (Zaman *et al.*, 2005). Collectively these data imply that RHAMM-HA interactions in immune cells are generally pro-inflammatory and would likely promote lymphocyte extravasation at the CNS endothelium, contrary to our observed phenotype.

Our finding that TLR4 signaling is not required for the effects of HA12 on lymphocyte rolling was surprising in light of several studies implicating TLR4 in the activities of HA oligosaccharides. TLR4 has been implicated in inhibiting activation of naïve T-cells and may be functionally expressed on circulating T-cells (Komai-Koma *et al.*, 2004; Gonzalez-Navajas *et al.*, 2010). HA oligosaccharides also signal through TLR2 which has been shown to have some over-lapping function with TLR4 (Jiang *et al.*, 2005; Shimada *et al.*, 2008), although in other studies, HA oligosaccharides functioned through TLR4 and not TLR2 (Termeer *et al.*, 2002). Impairing signaling of both receptors can also be required to completely abolish HA oligosaccharide-induced activity (Jiang *et al.*, 2005; Shimada *et al.*, 2008). Therefore, if TLR signaling were involved in lymphocyte rolling at the CNS endothelium we would expect a modest phenotype when TLR4 signaling is impaired that is magnified when TLR2 signaling is abolished. Nonetheless, experiments impairing signaling through TLR2 either alone or in combination with TLR4 could further establish if TLR signaling is involved in the effects of HA12 on lymphocyte rolling.

Tumor necrosis factor stimulated gene-6 (TSG-6) could also influence the effects of HA oligosaccharides on lymphocyte rolling. TSG-6 is a secreted protein whose expression is up-regulated in many cell types including peripheral blood mononuclear cells in response to cytokines and growth factors (Milner & Day, 2003). Its expression by lymphocytes is unknown; however a gene with significant sequence homology to TSG-6 was identified in lymphocytes in lampreys (Mayer *et al.*, 2002). TSG-6 is principally known to rearrange components of the ECM through catalytic transfer with serine proteases (Jiang *et al.*, 2011), but can also facilitate lymphocyte capture and rolling on ECs by promoting HA adhesion to the cell surface *in vitro* (Lesley *et al.*, 2004). Furthermore, HA oligosaccharides (8-10mers) can competitively bind to TSG-6 displacing HMW species (Heng *et al.*, 2008). This suggests that TSG-6 could function to support HA mediated adhesion with lymphocytes at the CNS vascular endothelium. Conversely, TSG-6 has been shown to prevent neutrophil infiltration into sites of inflammation during arthritis presumably by interfering with HA-CD44 interactions at the synovial endothelium (Szanto *et al.*, 2004). Therefore HA oligosaccharide-induced expression of TSG-6 at sites of inflammation may also prevent immune cell extravasation.

Collectively, our data support the notion that digestion products of HWM HA species can delay the onset of CNS inflammatory disease and impair the rolling and subsequent extravasation of activated lymphocytes. These products likely mediate this effect through HA receptor interactions on lymphocytes, although the molecular mechanism remains unknown. We propose that HA degradation products produced during CNS inflammation, through the activities of hyaluronidases or other mechanisms, may function as a molecular brake on lymphocyte extravasation as local concentrations of HA

fragments increases throughout disease. Moreover, HA oligosaccharides are not overtly pro-inflammatory as has been previously suggested. As such, HA oligosaccharides represent a potential therapeutic tool aimed at slowing inflammatory demyelinating disease progression and reducing severity.

Chapter 5: Conclusions and Future Directions

Much effort has been expended in understanding the etiology of MS (see Chapter 1), but the precise mechanisms underlying disease onset and progression remain unknown. Lacking this knowledge, attention has focused on treating disease symptoms. Evidence suggests that immune cells, particularly CD4+ T-cells are mediating MS pathology and symptomatology. Great strides have occurred in understanding the basic biology of T-cell activation and trafficking during autoimmune CNS inflammation. Much of this work has taken place in the animal model EAE which has also proven itself an effective therapeutic screen. Most notably the development of the α4-integrin blocking antibody natalizumab, demonstrates the therapeutic validity of impairing T-cell extravasation. Adhesion molecules on lymphocytes and ECs that mediate T-cell capture and rolling are also therapeutic targets as they facilitate this process (Kerfoot & Kubes, 2002; Piccio *et al.*, 2002; Kerfoot *et al.*, 2006). Studies have suggested a role for the transmembrane signaling receptor and adhesion molecule CD44 and by association, its primary ligand HA, in promoting EAE pathogenesis by facilitating lymphocyte extravasation (Laman *et al.*, 1998; Brennan *et al.*, 1999; Brocke *et al.*, 1999).

A role for CD44-HA interactions in CD4+ T-cell extravasation in CNS demyelinating disease

The work presented in this thesis expands upon these observations by experimentally demonstrating HMW HA tethered to CD44 on CNS ECs facilitates activated T-cell capture and rolling. Surprisingly, CD44 on T-cells is dispensable for this process. These results are confirmed by PEG-PH20 hyaluronidase degradation of HMW HA on ECs, which impairs T-cell rolling, delays EAE onset and reduces demyelination. Interestingly, prolonged treatment of CNS ECs with PEG-PH20 results in HA re-expression *in vivo* during late-stage EAE. HA re-expression is coincident with increased HAS1 expression

and suggests that T-cell capture and rolling may be re-established at this time point. Indeed, increased numbers of $CD4^+$ T-cells in the CNS were observed later in EAE (Day 21) and disease score was elevated. This finding implies the removal of HMW HA from CD44 on CNS endothelial cells induces pro-inflammatory signaling. Likely mediators of this effect are degradation and fragmentation products of HWM HA which signal through CD44 and TLRs on ECs (Savani *et al.*, 2001; Taylor *et al.*, 2004; Slevin *et al.*, 2007). Therefore, I investigated the role of HA oligosaccharides in EAE pathogenesis and activated T-cell rolling. Surprisingly, treatment of EAE animals with oligosaccharides delays disease onset and reduces demyelination and CDI scores. Furthermore, HA oligosaccharides impair T-cell capture and rolling through a lymphocyte dependent mechanism that does not involve CD44 or TLR4. Collectively, these data support a model where HMW HA bound to CD44 on ECs promotes T-cell extravasation early in disease (Figure 5.1, A), while HA fragments produced by HYALs and reactive oxygen species during chronic inflammation may have opposing effects on disease pathogenesis that are independent of CD44 or TLR4 on lymphocytes (Figure 5.1, B). These findings highlight the importance of HA synthesis and catabolism during inflammatory CNS disease and suggest manipulations of this process may have therapeutic potential.

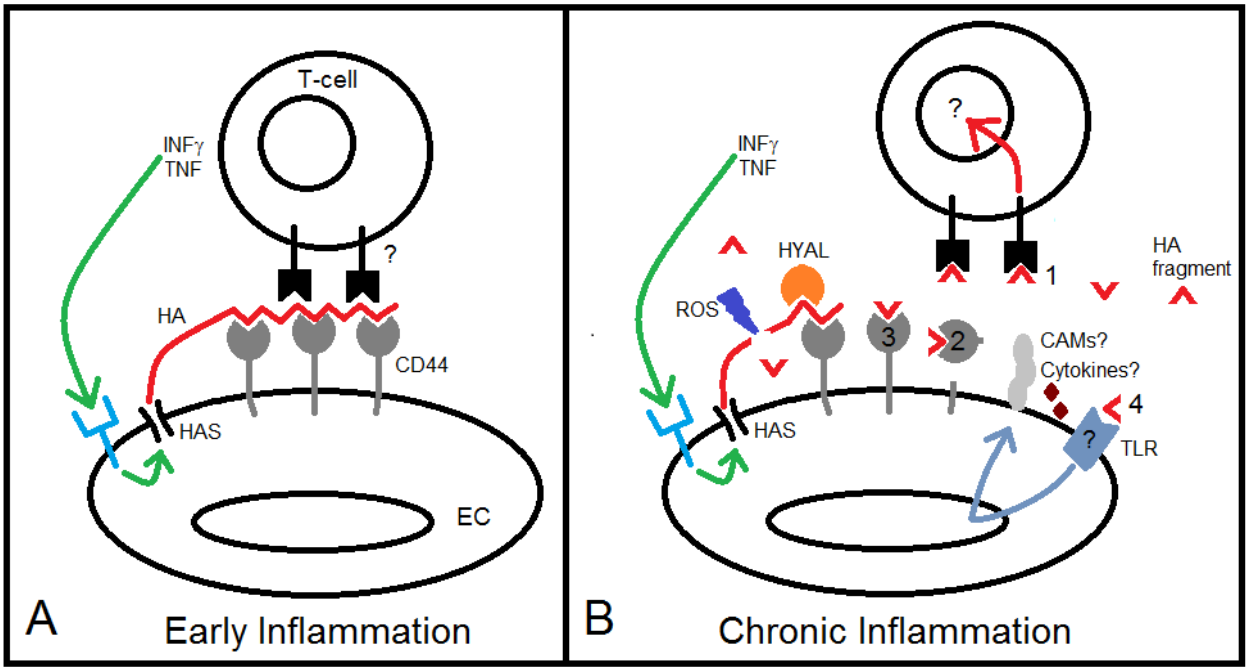

Figure 5.1. A Model of how HA and its degradation products could influence activated T-cell capture and rolling on CNS ECs. A) During early inflammation, HMW HA synthesis is induced by pro-inflammatory cytokines and is tethered to the surface of the EC cell via CD44. The T-cell initiates capture and rolling via an unknown cell surface receptor to initiate the process of extravasation. B) During chronic inflammation HYALs and reactive oxygen species degrade and fragment HMW HA. HA fragments 1) interact with HA receptor(s) on lymphocytes and impair capture and rolling, potentially via intracellular signaling, 2) possibly induce shedding of CD44 on the surface of ECs, 3) exclude HMW HA from the surface of ECs and 4) possibly signal through TLR to induce expression of cell adhesion molecules and cytokines. ? denotes lack of experimental data.

CD44 on WT T-cells may be involved in capture and rolling

The finding that HMW HA tethered to EC CD44 facilitates T-cell rolling supports the endothelial component of the 'sandwich model' hypothesis presented in Chapter 1. It is surprising, however, that T-cell CD44 is not necessary for this process. Alternative HA receptors have been discussed in Chapters 3 and 4; however, the work in this thesis does not exclude the possibility that CD44 on WT T-cells is sufficient to mediate T-cell rolling in the CNS. We and others have demonstrated that $CD44^{-/-}$ mice develop chronic EAE symptoms following a delay in disease onset (Guan *et al.*, 2011; Winkler *et al.*, 2012). This modest phenotype could be accounted for by our finding that T-cell capture and rolling decreases ~50% in the absence of CD44 on ECs. Additionally, HA receptors

such as RHAMM have been shown to compensate for the loss of lymphocyte CD44 during inflammation (Nedvetzki *et al.*, 2004). However, CD44 blocking antibody (IM7) injection into WT EAE mice completely prevents disease and T-cell extravasation (Brennan *et al.*, 1999; Brocke *et al.*, 1999). It has been proposed that IM7 induces CD44 shedding from T-cells, preventing capture and rolling during inflammatory disease; however this mechanism has not been directly confirmed in a rolling assay (Mikecz *et al.*, 1995; Brennan *et al.*, 1999). Alternatively, IM7 has been shown to directly block CD44-HA binding (Lesley *et al.*, 1990) and is the proposed mechanism by which IM7 treatment of WT T-cells completely blocks capture and rolling on ECs *in vitro* (DeGrendele et al., 1996). *Overall, these findings suggest acute removal or inactivation of CD44's HA binding activity on WT T-cells is necessary to demonstrate the receptor's contribution to capture and rolling.* The most direct way to determine if CD44 on WT T-cells is sufficient to facilitate this process is to intravenously inject activated, fluorescently labeled T-cells that have been treated with IM7 into EAE mice at the time of disease onset and perform intravital microscopy as described by Kerfoot et al (Kerfoot & Kubes, 2002; Kerfoot *et al.*, 2006). The advantages of this technique over *in vitro* parallel plate is that it will conclusively determine the contribution of CD44 on WT T-cells to capture and rolling in the context of inflammatory CNS disease and CD44's effect on extravasation can be directly measured by histology.

Degradation of HMW HA from CD44 on ECs may promote CNS inflammation

A fundamental, yet unexplained finding in this thesis is that degradation of HMW HA from CNS ECs during chronic EAE worsens disease and increased $CD4^+$ infiltrates. A possible explanation for this observation is that because onset of CNS inflammation was delayed, so is its remission. To test this hypothesis, a long-term (to day 35 post-

immunization) EAE experiment was performed where PEG-PH20 was subcutaneously administered over the course of disease using the same protocol described for Figure 3.7 (Figure 5.2). Interestingly, disease scores, demyelination and $CD4^+$ infiltrates were higher in PEG-PH20 animals at day 35 relative to controls (Figure 5.2 A, B and C respectively). This finding established the pro-inflammatory effect of PEG-PH20 treatment is long-lasting and is therefore related to treatment. As previously discussed, HA degradation products signaling through TLR2 and/or TLR4 could be mediating this effect by increasing expression of inflammatory signals and cell adhesion molecules on ECs. However, alternative mechanisms may account for these observations.

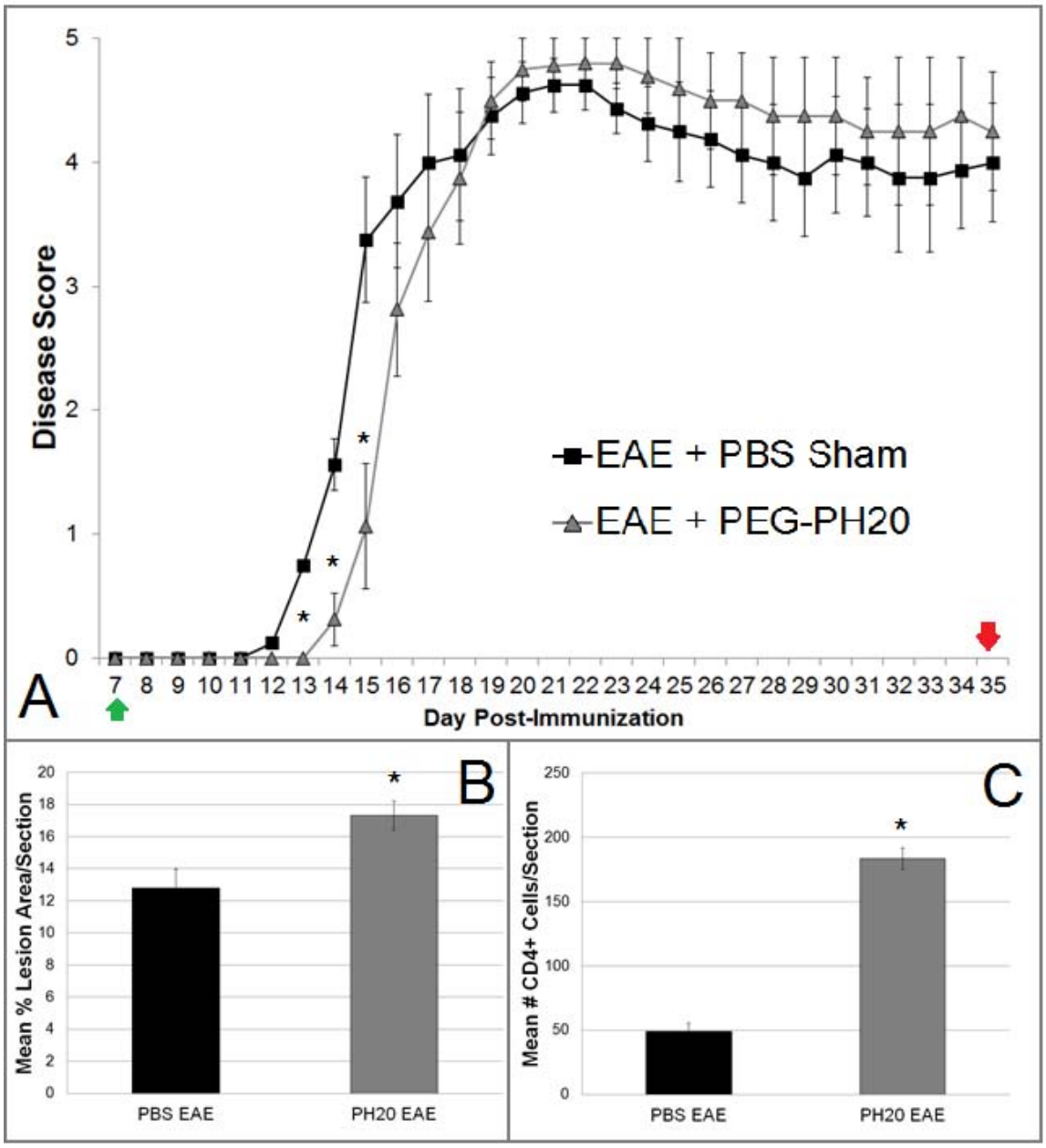

Figure 5.2. Subcutaneously injected PEG-PH20 delays the clinical onset of EAE, but exacerbates long-term chronic disease. A) EAE was induced in 16 C57BL/6 female mice. Half (8) of the animals were randomly selected to receive subcutaneous PBS (vehicle) injections (50 ul) and the other half, PEG-PH20 (1000 U in 50 ul PBS). Injections were given in the hind flank every other day beginning 7 days post inoculation (green arrow) and ending 35 days post inoculation (red arrow). Mice were scored by a blinded experimenter. PEG-PH20 treated animals had delayed onset of disease by three days, but by day 20 had higher disease scores than controls *p<0.05, repeated measures ANOVA. Stereological analysis (as described in Chapter 2) determined that by day 35, PH20 treated mice had more demyelination (B) and $CD4^+$ T-cell infiltrates (C). *p<0.05, t-test.

It is possible that removing HMW HA from CD44 on ECs alters the cytoskeletal dynamics of the endothelium to favor T-cell extravasation. Cytoskeletal reorganization in

ECs is critical for this process (Etienne-Manneville *et al.*, 2000). EC cytoskeletal rearrangement is increased during angiogenesis (the growth of new vessels), which often occurs as a result of inflammation (Bryan & D'Amore, 2007). Interestingly, HMW HA is inhibitory to angiogenesis (Slevin *et al.*, 2007); however, the mechanism is unknown. I propose that CD44's ability to facilitate activation of receptor tyrosine kinases (RTKs) may be involved (see Chapter 1). When HMW HA is bound to CD44, the receptor is unable to promote RTK activation, possibly via steric exclusion or the presence of an as yet unidentified 'growth inhibitory complex' (Ponta *et al.*, 2003). In this state, dephosphorylated merlin is bound to CD44 (Morrison *et al.*, 2001), suppressing its interaction with ERMs that control actin cytoskeletal dynamics and promote T-cell extravasation (Figure 5.3, A). During inflammation however, HMW HA degradation is increased, allowing CD44 to facilitate RTK dimerization and activation. Subsequent RTK activation by inflammatory, angiogenic growth factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (Croll *et al.*, 2004; Hassoun *et al.*, 2009) inactivates merlin by phosphorylation and induces its dissociation from CD44 thus allowing CD44 to interact with ERMs (Figure 5.3 B). Concomitantly, RTK phosphorylation of ERM proteins promotes their binding to CD44 and links the RTK-CD44-ERM complex to the actin cytoskeleton (Orian-Rousseau *et al.*, 2002). In this activated state, CD44 could directly influence actin cytoskeletal organization. This idea is supported by the finding that PDGF receptor mediated cytoskeletal rearrangement in fibroblasts is restored following HMW HA treatment with a phosphatase inhibitor, which promotes phospho-merlin (Li *et al.*, 2006). Collectively, this hypothesis explains how removal of HMW HA bound to CD44 could promote angiogenesis and actin cytoskeletal dynamics to facilitate T-cell extravasation into the CNS (Figure 5.3). A test of this hypothesis requires identification of a candidate CD44-interacting RTK in endothelial

cells. The VEGF and PDGF receptors are likely candidates as their ligands are produced during inflammation.

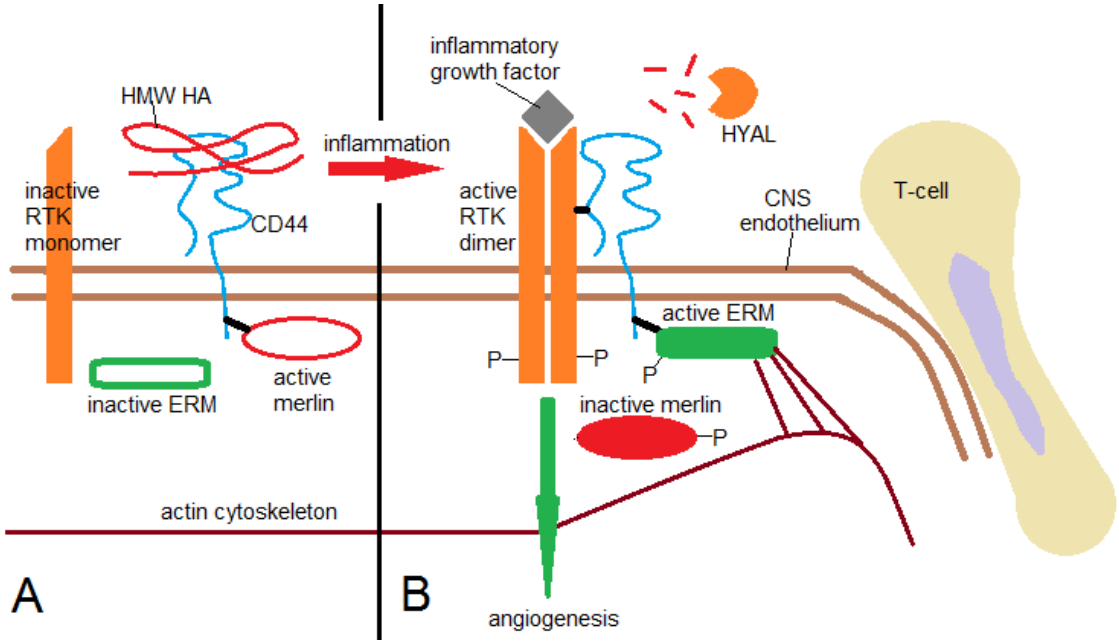

Figure 5.3. A model of how degradation of HMW HA from CD44 on CNS ECs during inflammation can promote T-cell extravasation. A) CD44's interaction with RTKs is impaired by its binding to HMW HA. Active phospho-merlin is bound to CD44 impair interactions with ERM cytoskeletal organizer proteins. B) During inflammation, HMW HA is degraded from the surface of ECs, allow CD44 to interact with RTKs and induce their activation. Inflammatory growth factors (VEGF, PDGF) produced during inflammation, stimulate RTKs and induce angiogenesis. Activated RTKs phosphorylate merlin and ERM proteins, allow CD44 to be linked to the cytoskeleton and promote T-cell extravasation.

It is equally possible that increased angiogenesis is mediating the long-term enhanced disease severity observed in PEG-PH20 treated EAE animals. Aside from pro-inflammatory signaling through TLRs, HA oligosaccharides are capable of inducing angiogenesis by signaling through CD44 and RHAMM (Savani *et al.*, 2001; Cao *et al.*, 2006; Slevin *et al.*, 2007; Jiang *et al.*, 2011). Angiogenesis involves not only cytoskeletal alterations, but increased vascular permeability that facilitates influx of inflammatory factors into the CNS and the extravasation of immune cells during disease (Pan *et al.*, 1997; Graesser *et al.*, 2002; Dvorak, 2005). Primary effectors of vascular permeability related to angiogenesis are matrix metalloproteases (MMPs). MMPs breakdown the EC basement membrane, allowing growth factor diffusion and new vessel formation

(Pepper, 2001; Bryan & D'Amore, 2007). HA oligosaccharides signaling through CD44 increase expression of MMP-9 by ECs during angiogenesis (Matou-Nasri *et al.*, 2009). CD44 also docks MMP-9 on the surface of ECs, which is required for EC migration associated with angiogenesis (Matou-Nasri *et al.*, 2009). Interestingly, MMP-9 is also produced by myelin-reactive T-cells and has been implicated in promoting disruption of the BBB (Leppert *et al.*, 1998; Leppert *et al.*, 2001; Shinto *et al.*, 2011). While it has not been experimentally determined, MMP-9 produced by ECs and T-cells during MS could be 'paving the way' for angiogenic growth into lesions as extravasation occurs. Other pro-angiogenic factors are also present within active CNS lesions. These include VEGF, INFγ, TNFα and HA (Van Meir, 1995; Proescholdt *et al.*, 2002; Back *et al.*, 2005). Among these, VEGF is the most potent angiogenic factor (Bryan & D'Amore, 2007). Its production is stimulated in many pathological conditions where cellular hypoxia and inflammation are present (Jackson *et al.*, 1997). Interestingly, VEGF and HA oligosaccharides synergize to facilitate more rapid and sustained angiogenesis than when applied individually (Montesano *et al.*, 1996).

Despite these observations, very few studies have addressed the idea that angiogenesis could contribute to MS pathogenesis. A handful of histological studies suggest that vessel density and endothelial cell proliferation are increased in EAE and MS lesions (Proescholdt *et al.*, 2002; Kirk *et al.*, 2004; Holley *et al.*, 2010). Furthermore, MRI studies have demonstrated evidence of increased cerebral perfusion during relapsing-remitting and secondary progressive MS suggesting there may be increased number of vessels in inflammatory lesions (Rashid *et al.*, 2004). Additionally, increased permeability of vessels in normal appearing white matter during MS could also be interpreted as new vessels expansion into the region (Plumb *et al.*, 2002). Overall, these findings suggest that the environment in and around the MS lesion is permissive to vessel growth and the

addition of HA oligosaccharides would likely facilitate the process. This could be easily tested by probing PBS and PH20 treated EAE tissue from the experiments described in this thesis with mitotic markers (e.g. Ki67) and vessel specific antibodies (e.g. CD31). It would also be interesting to generate high-resolution (12T) magnetic resonance images of PH20 treated and control brains to determine alterations in CNS vascular density.

Are HA fragments truly 'danger signals'?

A surprising observation from the work in this dissertation is that HA oligosaccharides suppress CNS inflammation when chronically administered in EAE. They appear to mediate this effect through signaling in activated lymphocytes. This finding is novel, in that in all previous studies of interactions between HA oligosaccharides and immune cells, inflammation is induced (McKee *et al.*, 1997; Termeer *et al.*, 2000; Termeer *et al.*, 2002; Jiang *et al.*, 2005; Scheibner *et al.*, 2006). Moreover, HA oligosaccharides have garnered a reputation for inducing pro-inflammatory signaling in multiple cell types through TLRs (Fieber *et al.*, 2004; Taylor *et al.*, 2004; Voelcker *et al.*, 2008; Campo *et al.*, 2012). As such, they have been termed 'dangerous'. However, in all of these studies, pro-inflammatory responses to HA oligosaccharides were elicited and measured from *in vitro* systems. Therefore the effects of HA oligosaccharides promoting inflammation may be over-stated.

The effect of HA oligosaccharides *in vivo* has not been well characterized, however, several studies have examined their ability to affect and modify various pathologies. Injection of HA fragments as small as tetrasaccharides was found to prevent synovial degeneration by up-regulation of heat shock proteins and suppress prostaglandin E_2 mediated inflammation in a model of arthritis (Asari *et al.*, 1998; Xu *et al.*, 2002). This is particularly interesting in respect to the studies of Campo et al (Campo *et al.*, 2010;

Campo *et al.*, 2012), who demonstrated tetrasaccharides promote inflammation via TLR4 in chondrocytes. Other, recent *in vivo* data demonstrates orally administered HA oligosaccharides 35 kD in weight signal through TLR4 on gut epithelial cells to promote expression β-Defensin 2, which is critical for intestinal epithelial integrity (Hill *et al.*, 2012). Furthermore, fragments as small as tetrasaccharides were tested in this model and did not illicit overt inflammation or tissue damage. Finally, a mixture of di- to decasaccharides injected into mice with malignant peripheral nerve sheath tumors enhanced chemotherapy treatment and was found to be non-immunogenic (Slomiany *et al.*, 2009). Collectively, these studies demonstrate HA oligosaccharides in sufficient quantity to influence cellular interactions are not sufficient to induce inflammation *in vivo*.

It is fascinating that the administration of potent, pro-inflammatory signaling molecules such as HA oligosaccharides does not appear to induce inflammation *in vivo*. Because we know HA synthesis and catabolism are inextricably linked, we must also consider the effect of HMW HA on inflammation. Aside from involvement in recruitment and adhesion of immune cells to inflamed endothelium (Khan *et al.*, 2004; Kessler *et al.*, 2008; Winkler *et al.*, 2012), HMW HA is generally anti-inflammatory in terms of its influence on cell signaling (Stern *et al.*, 2006; Bollyky *et al.*, 2007). As discussed in Chapter 1 HMW HA is degraded by HYALs and fragmented by reactive oxygen species during inflammation. Presumably, these catabolic processes occur more rapidly than does HA synthesis. Therefore, based on the findings of this thesis and the literature cited above, I propose a model whereby the loss of HMW HA *in vivo* more robustly promotes inflammation than the does the accumulation of HA fragments (Figure 5.3). Furthermore, a mechanism exists such that accumulating HA fragments can function as a brake on inflammation which promotes resolution and allows for restored equilibrium of HA synthesis and catabolism. This makes evolutionary sense in that chronic inflammation is typically

detrimental. However, it is intriguing to wonder if such a brake mechanism would exist in the opposing direction, such that excess accumulation of HMW species is not overly immunosuppressive. Based on the model presented here, a small excess of HMW HA could dramatically increase the likelihood of immune-suppression which also carries negative evolutionary consequence. Overall, the work presented and discussed here stresses the exquisite balance required to maintain the physiologic state.

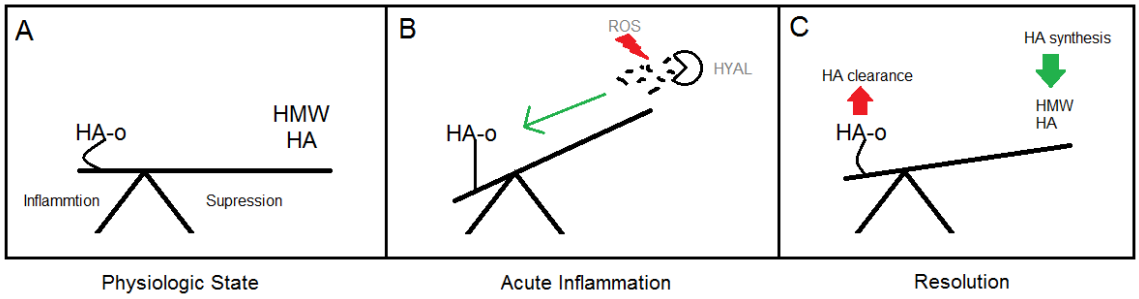

Figure 5.4. Proposed model of how products of HA synthesis and catabolism influence inflammation *in vivo.* A) During physiologic state HA synthesis and catabolism are in homeostasis. B) Loss of HMW HA by degradation and fragmentation quickly promotes inflammation (or possibly loss of suppression), while accumulating HA fragments apply a molecular break. C) Resolution of inflammation begins as HA synthesis produces HMW HA *de novo* (green arrow) and HA fragments are cleared from the system (red arrow). Note the emphasis on the fulcrum being oriented toward HA-o, as work discussed in this thesis demonstrates adding super-physiologic concentrations *in vivo* is not overtly inflammatory. HA-o= HA oligosaccharides.

Our knowledge of the mechanisms mediating demyelinating CNS inflammatory disease is growing. As this thesis exemplifies, they are rarely straight-forward and often have location, concentration and timing specific effects. Nonetheless, the work presented here posits that manipulations of the synthesis and catabolism of HA may have therapeutic benefit. An emerging trend in the field of medicine is the utilization of combinatorial or adjuvant therapies. The reagents described here could be used in in this fashion to promote the efficacy of existing disease modifying drugs. Both hyaluronidases and HA oligosaccharides have been show to effectively modify pathological states such as cancer and diabetes. However, further studies are required to determine additionally

mechanisms of action that will influence their feasibility and efficacy within the unique environment of the CNS compartment.

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