LOW MOLECULAR WEIGHT HYALURONIC ACID INCREASES CD44 EXPRESSION BY OLIGODENDROCYTES AND ENHANCES INFLAMMATION

by

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Chapter 1: Introduction and Background

Section 1: Multiple sclerosis: an autoimmune disorder affecting the nervous system

Multiple sclerosis (MS) is an autoimmune disease named for the characteristic multiple scars (sclera) caused by destruction of nerve-insulating myelin in the central nervous system. The loss of myelin around neuronal axons interrupts the saltatory conduction of the electrical signal necessary for normal neurological function. The eventual failure to replace damaged myelin coupled with inflammation-related axonal pathology leads to accumulated neurological disability (Hogan et al. 1995; Sim et al. 2002; Wujek et al. 2002). Symptoms can include vision problems, pain, numbness, loss of balance, bladder and bowel dysfunction, muscle weakness, spasticity and paralysis.

MS is one of the most common neurological diseases. An estimated 400,000 persons in the United States are living with MS, and approximately 2 million are living with the disease worldwide (National MS Society Sourcebook Online). Prevalence is much greater in areas above the 40th parallel than in lower latitudes, leading to the hypothesis that lower exposure to sunlight and lower vitamin D synthesis results in higher incidence of MS (Brown 2006). More Caucasians, particularly those of northern European ancestry, suffer from MS than other ethnic groups.

MS is two to three times more common in women than in men, similar to the gender bias of other autoimmune diseases. Most persons diagnosed with MS are between the ages of 20 and 50, although some cases are diagnosed in young children and in older adults. An

individual's genetic background is thought to affect whether he or she will be susceptible

to MS. Genes influencing susceptibility are believed to be linked to those of the major

histocompatibility complex, which control antigen presentation to T cells (Gran and

Rostami 2001). But the fact that the monozygotic twin of an MS patient has only a 1 in 4

chance of developing the disease shows that genetic background is probably not the only

factor influencing susceptibility (National MS Society Sourcebook Online).

Etiology of MS

The cause of MS remains a mystery. There is ongoing debate as to whether the

neuroinflammatory reaction is necessarily in response to antigen recognition in the

immune system or in some cases could be initiated by events within the CNS itself. The

immune-initiated disease hypothesis postulates that autoreactive T cells generated in the

systemic compartment invade the CNS and induce an inflammatory cascade that injures

previously normal neural tissues. This explanation has long been favored, and the

induction of disease in the animal model for MS, experimental autoimmune

encephalomyelitis (EAE), supports this hypothesis. EAE can be induced in naïve animals

by systemic immunization with neural auto-antigens or by transfer of CD4+ T cells

reactive to neural antigens into a naïve host (Zamvil et al. 1985; Owens et al. 2001).

In contrast, the neural-initiated disease hypothesis postulates that lethal changes in neural

cells, specifically the myelin-producing oligodendrocytes, could be the starting point of

MS disease development. According to this model, in response to as-yet-unidentified

insults, such as a virus, reactivated exogenous retrovirus, or intrinsic abnormalities,

oligodendrocytes undergo apoptosis and over time fail to be replaced. A precipitating oligodendrocyte injury could result in recruitment of an innate (microglia) and then adaptive (lymphocyte) immune response (Barnett and Prineas 2004; Prat and Antel 2005). An example of disease possibly caused by this sequence of events is Theiler murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), a chronic inflammatory demyelinating disease caused by a virus of the same name. Still, progression of this disease requires activation of T cells to endogenous myelin epitopes (McMahon et al. 2005).

It is generally hypothesized that an environmental trigger coupled with genetic susceptibility determines who falls prey to MS or similiar autoimmune diseases such as type I diabetes. The environmental trigger may be a viral infection, which could sensitize the immune system by inducing cross-reactivity to a virus-derived peptide(s) bearing similarity to myelin epitopes, a phenomenon known as molecular mimicry. The precipitating infection could occur years or even decades before the onset of autoimmune disease. Substantiating the argument that molecular mimicry occurs *in vivo*, Hemmer and co-workers showed, using synthetic peptide combinatorial libraries, that there is considerable flexibility and degeneracy in antigen recognition by the T cell receptor (Hemmer et al. 1997). They identified foreign antigen-derived ligands that can activate myelin-reactive T cells with much higher potency than myelin peptides themselves. If molecular mimicry is a common occurrence, as these results suggest, it appears that other factors, such as decreased costimulation requirements of autoreactive T cells, must be present for the induction of autoimmune disease (Gran et al. 1999; Hemmer et al. 1999).

Somewhat reminiscent of molecular mimicry, epitope spreading occurs when T cells become reactive to epitopes distinct from and non-crossreactive with the disease-inducing epitope (Vanderlugt and Miller 2002). This diversification of T cell reactivity has been observed in relapsing forms of EAE (R-EAE) (Lehmann et al. 1992; McRae et al. 1995; Yu et al. 1996; Vanderlugt et al. 2000) and in TMEV-IDD (Miller et al. 1997) as well as in individuals with MS (Tuohy et al. 1999). Indeed, T cells that have become reactive through epitope spreading have been shown to be the major functional cause of disease progression in R-EAE and TMEV-IDD (McRae et al. 1995; Tuohy et al. 1999; Vanderlugt et al. 2000; Neville et al. 2002).

T cells which have become reactive to myelin via molecular mimicry, epitope spreading or an unidentified mechanism circulate through the CNS, and, finding their cognate antigen, proliferate and cause an autoimmune inflammatory response. While myelin-reactive T cells are found at similar frequencies in the peripheral blood of MS patients and healthy individuals, myelin-reactive T cells from MS patients exhibit a memory or activated phenotype, while in healthy individuals they display a naïve phenotype (Zhang et al. 1994; Lovett-Racke et al. 1998; Scholz et al. 1998). There are substantial differences in the cytokines secreted and the specific chemokine receptors expressed by cells from the two groups, indicating that myelin-reactive T cells from MS patients are relatively more inflammatory (Crawford et al. 2004; Kivisakk et al. 2004).

Section 2: Infiltration of CD4+ T cells and the Production of Proinflammatory Cytokines are Important Early Events in MS Pathology

The commonly held view of the progression of MS begins with one or more CD4+ T cells recognizing a myelin epitope as a foreign, rather than self, antigen and becoming activated, leading to clonal expansion of T cells ready to mount an attack against myelin (Martin and McFarland 1995; Frohman et al. 2006). This population of T cells upregulates their expression of cell-surface adhesion molecules, which allows them more efficient passage out of the vasculature through the endothelial layer into the CNS parenchyma (Gran and Rostami 2001). Once inside the CNS, the T cells must re-encounter their cognate antigen in order to recognize their target. The myelin antigen is presented to the primed T cells via CNS-resident microglia, astrocytes, or by vessel-associated CD209+ dendritic cells, as shown by in vivo studies in mice (Gran and Rostami 2001; Greter et al. 2005; Heppner et al. 2005; McMahon et al. 2005).

Following CNS infilitration and reactivation, CD4+ T cells flood the microenvironment with increased levels of proinflammatory cytokines and chemokines. These soluble mediators recruit other effector cells such as lymphocytes and monocytes/macrophages in addition to doing direct damage to myelin. Reactive T cells also cause myelin damage by production of reactive oxygen species and nitric oxide (Gran and Rostami 2001). Other effector cells include CD8+ T cells and B cells which increase their production of proinflammatory cytokines in response to activation. Proliferating B cells produce

myelin-specific antibodies, augmenting myelin destruction. (Genain et al. 1999; Raine et al. 1999).

CD4+ T cells are considered to be the primary mediators of MS and EAE. Experiments in EAE mice have shown that when encephalitogenic CD4+ T cells were blocked from entering the CNS the disease was suppressed (Yan et al. 2003). CD4+ T cells comprise two major functional subsets, T-helper type 1 (Th1) and T-helper type 2 (Th2). Th1 cells are distinguished by their production of proinflammatory cytokines such as interleukin (IL)-2, osteopontin, interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) (Mosmann et al. 1986; Coffman et al. 1991; Weber et al. 1996; Denhardt et al. 2001) (see Table 1). They mediate delayed type hypersensitivity reactions and macrophage activation (Murphy and Reiner 2002). Th2 cells produce anti-inflammatory, or immunoregulatory, cytokines such as IL-4, IL-5, and IL-13 and induce the production of noncomplement-fixing antibodies as well as contribute to eosinophilic inflammation and allergic reactions. IL-10 is produced by Th2 cells in mice and by both Th1 and Th2 cells in humans (Farrar et al. 2002).

Table 1: Major Actions of Selected Proinflammatory Cytokines

Cytokine	Source	Effects
IL-2	Naïve Th1cells some CD8+ T cells	Stimulates proliferation of T and B cells Activates natural killer (NK) cells
TNFα	Th1 cells (some Th2 and cytotoxic T lymphocytes) Neutrophils B cells Macrophage Dendritic cells NK cells Astrocytes Endothelial cells	Local inflammation Endothelial cell activation Activates macrophages, induces NO production Chemotaxis Induces oligodendrocyte apoptosis
IFNγ	Th1 cells CTL	Cell-mediated immune responses to intracellular pathogens Activates of macrophages Induces nitric oxide synthase Terminal differentiation of Th1 cells
osteopontin	Th1 cells Macrophages	Costimulates T cell proliferation Enhances 1L-12 and IFNg promotes Th1 differentiation
IL-12	Macrophages Dendritic cells	Drives Th1 differentiation T cell expansion and proliferation IFNγ production Activates NK cells
IL-17	Th$_{IL-17}$ cells (CD4+ memory cells)	T cell costimulation Proliferation, maturation, chemotaxis of neutrophils Induction of proinflammatory cytokines
IL-23	Dendritic cells Macrophages T cells B cells Endothelial cells	Proliferation of memory T cells IFNγ production Induction of Th$_{IL-17}$ cells
IL-27	Monocytes Macrophages Dendritic cells	T cell expansion and proliferation Induces IL-12R IFNγ production

A new subset of Th cells named Th$_{IL-17}$ cells, possibly distinct from either Th1 or Th2

subsets, was recently described by Langrish *et al* (2005). The origin of these CD4+ T

cells is unclear, but they can be generated by isolating T cells from EAE-susceptible SJL

mice immunized with an encephalitogenic peptide and culturing them with IL-23. The

authors of the study consider them to be a unique Th subset due to their production of IL-

17, IL-17F, IL-6, TNFα and low levels of IFNγ (Langrish et al. 2005). It should be

noted, however, that these cells continue to produce low levels of IFNγ, a hallmark

cytokine of the Th1 subset.

The cytokine profile of myelin-specific T cells determines the ability of these cells to

cause inflammation. Naïve T cells differentiate into Th1 or Th2 effector cells upon

recognition of antigen, and the relative size of the Th1 population versus the Th2

population is a critical modulator of the immune response (Romagnani 1996; D'Elios and

Del Prete 1998). The factors which drive naïve T cells towards either Th1- or Th2-

dominated populations include the type and amount of antigen, as well as the density and

affinity of the peptide ligand, the nature of the antigen-presenting cells and their

costimulants, the circulating hormones in the microenvironment, and, most importantly,

the cytokines present in the cellular milieu at the time the naïve T cell encounters its

antigen (Romagnani 1996; D'Elios and Del Prete 1998; O'Garra 1998; O'Garra et al.

1998; Kang and Kim 2006). IL-12 is known to be critical for differentiation of cells

toward the Th1 subtype and the initiation of cell-mediated responses (Decken et al.

1998). The more recently-discovered cytokines IL-23 and IL-27 are closely structurally

related to IL-12 and share its functions of IFNγ production and promotion of T cell

expansion (Oppmann et al. 2000; Pflanz et al. 2002).

The Proinflammatory Cytokines TNFα and IFNγ Play Major Roles in MS and EAE

Multiple sclerosis, like other organ-specific autoimmune diseases, is thought to be

mediated by Th1 cells (Ando et al. 1989; Simon et al. 1994; Steinman 2001; Pedotti et al.

2003; Steinman and Zamvil 2003), which produce IFNγ and TNFα. There is convincing

evidence of the involvement of these proinflammatory cytokines in the pathology of MS

and EAE. TNFα expression promotes T cell adhesion to the vascular endothelium and

subsequent extravasation, as well as activation of macrophages and production of another

proinflammatory cytokine, IL-1 (Kieseier et al. 2005). TNFα has been shown to be

present in MS lesions and to cause damage *in vitro* to myelin and oligodendrocytes

(Selmaj and Raine 1988; Selmaj and Raine 1988; Cannella and Raine 1995; Akassoglou

et al. 1998; Bitsch et al. 1998; Raine et al. 1998). Patients with active MS show a

correlation in TNFα levels and blood-brain barrier damage (Sharief et al. 1993).

Transgenic TNFα-overexpressing mice display spontaneous inflammatory demyelination

and increased severity of EAE induced by immunization (Gran and Rostami 2001).

Knockout models have shown that TNF-receptor I (TNFR1) signaling is critical for

demyelination, although CNS inflammation can develop without TNFR1 expression

(Probert et al. 2000).

In contrast, some protective effects of TNFα have been observed. TNFα and TNF receptor knockout mice show increased susceptibility to CNS inflammation and tissue injury. TNFRI knockout mice displayed a very severe form of MOG-induced EAE (Probert et al. 2000). Disease resolution involving TNFα and its receptors (TNFR1 and TNFRII) is likely due to the induction of activation-induced cell death (AICD) in infiltrating T cells when TNFα binds to either of these receptors (Segal and Cross 2000; Zipp 2000), thereby reducing inflammation.

The pathogenic effect of IFNγ is largely due to its role in driving naïve T cells toward Th1 differentiation by signaling through signal transducer of activated T cells (STAT-1), which in turn activates the T-box transcription factor T-bet, the key inducer of IFNγ and Th1 cell differentiation (Afkarian et al. 2002; Neurath et al. 2002). IFNγ is also a powerful inducer of nitric oxide synthase 2 (NOS2) expression, resulting in high levels of nitric oxide production by macrophages, astrocytes, and other cell types. Expression of NOS2 correlates with EAE disease activity and severity (Cross et al. 1994; Okuda et al. 1995). In addition, both IFNγ and TNFα have been shown to cause cell death in human oligodendrocyte cell lines by altering their gene expression (Selmaj and Raine 1988). Blood levels of IFNγ are elevated in MS patients (Balashov et al. 1997; Becher et al. 1999) and T cell clones from the cerebral spinal fluid of MS patients express higher levels of IFNγ than controls (Wolinsky et al. 2003). Treatment of relapsing-remitting MS patients with IFNγ led to disease exacerbation, significant increases in the proliferative responses of peripheral blood leukocytes and in natural killer cell activity against myelin. The conclusion from that study was that IFNγ was unsuitable for use as a therapeutic

agent in MS and agents that specifically inhibit IFNγ should be investigated (Panitch et al. 1987).

On the other hand, Chu and coworkers found that IFNγ-deficient mice were unable to suppress the expansion of the activated CD4+ T cell population and therefore had more severe EAE (Chu et al. 2000). Lack of IFNγ not only worsened disease but permitted disease development in otherwise resistant mouse strains (Billiau et al. 1988; Willenborg et al. 1999). IL-12 is considered a critical cytokine for induction of IFNγ and Th1 differentiation, yet IL-12 knockout mice are unusually susceptible to EAE (Becher et al. 2003; Cua et al. 2003; Zhang et al. 2003). These data may suggest that a moderate level of IFNγ is protective in some way, or it may lend credence to the idea that the Th1/Th2 paradigm does not neatly fit EAE and that the Th_{IL-17} population mentioned above is more important for the development of EAE. It may also highlight differences in EAE models and the human disease, MS.

Demyelinated plaques develop as a result of CNS inflammation, leading to axon loss and irreversible disability

The intense perivascular infiltration of the CNS by activated T and B cells, neutrophils, monocytes and macrophages and their high levels of production of inflammatory mediators result in the demyelinated plaques characteristic of MS and EAE. Acute lesions show indistinct margins, indicating ongoing demyelination and the presence of many lymphocytes. Hypercellularity, myelin-laden macrophages, parenchymal edema,

degenerating axons and loss of myelin and oligodendrocytes are noted (Traugott et al. 1981). Remyelination is seen occasionally (Prineas et al. 1993). Astrocytes become hypertropic but at this point there is little or no astroglial scarring (Frohman et al. 2006) (Figure 1a).

Lesions of chronic active multiple sclerosis display hypocellular centers with naked (demyelinated) axons embedded in a matrix of fibrotic astrocytes known as the "glial scar." There are a few infiltrating leukocytes and virtually no oligodendrocytes. Inflammatory mediators, particularly TNFα, concentrated in MS and EAE lesions are toxic to oligodendrocytes and cause them to undergo apoptosis (Selmaj and Raine 1988; Selmaj and Raine 1988). Numerous macrophages are present, filled with lipids derived from phagocytosed myelin (Prineas and Graham 1981) (Figure 1b).
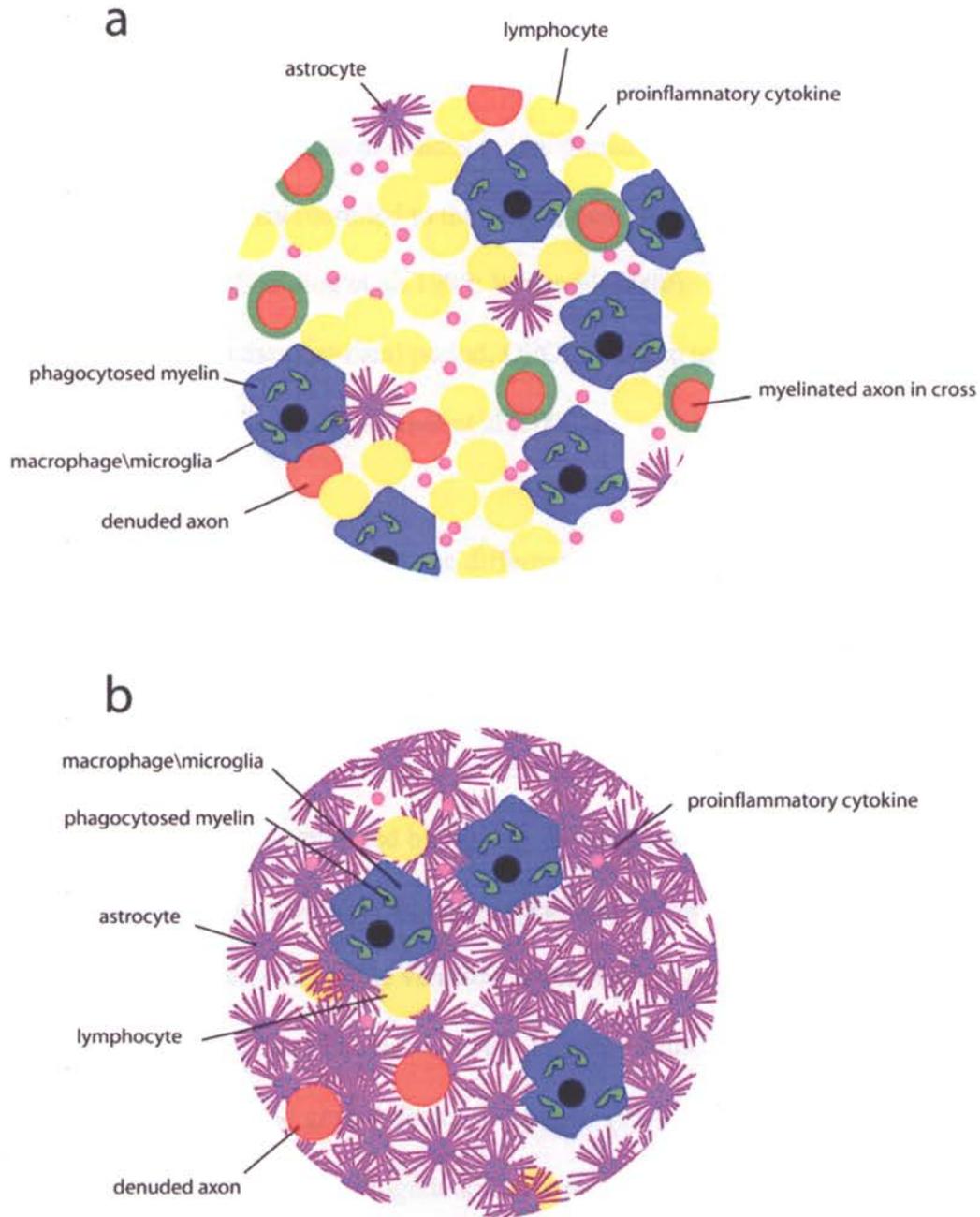
Figure 1: Acute and chronic EAE or MS lesions. The acute lesion (a) shows numerous infiltrating lymphocytes (CD4+ T cells, B cells, NK cells). Some axons (shown in cross-section) have lost their myelin sheath. Macrophages show phagocytosed myelin. Reactive astrocytes are hypertrophic. The chronic lesion (b) shows only a few infiltrating lymphocytes, all surviving axons have lost their myelin sheaths, and phagocytosed myelin is seen inside macrophages. Proliferative, hypertrophic astrocytes have formed a dense glial scar.

Remyelination, while not as apparent as in acute MS and EAE lesions, is sometimes seen at the edges of chronic lesions, in areas termed "shadow plaques" (Frohman et al 2006). Remyelination in both types of lesions is likely mediated by oligodendrocyte precursor cells (OPCs) which are recruited to the lesion, mature and replace apoptotic oligodendrocytes (Scolding et al. 1998; Wolswijk 2000). While oligodendrocyte birth is nearly complete in the post-natal period, OPCs continue to be born throughout life and proliferate after CNS injury (Horner et al. 2000).

Cells of the oligodendrocytes lineage differentiate through distinct phenotypic stages, which have been identified both *in vivo* and *in vitro* based on the expression of specific antigenic markers and the ability or inability to proliferate and migrate. The terms designating the early stages are used somewhat interchangeably, making it more precise to refer to the markers espressed by these cells (only a few selected markers will be discussed here). Early oligodendrocyte precursors derive from nestin-positive neuroepithelial stem cells of the ventricular zones during development and persist into adulthood (Baumann and Pham-Dinh 2001). These cells are migratory and mitotic, and express platelet-derived growth factor receptor-α (PDGFR-α), in addition to nestin and other earlier-stage markers. Oligodendrocyte progenitors continue to produce nestin and can be identified using the monoclonal antibody A2B5, which recognizes several gangliosides. They begin to extend processes and assume a bipolar morphology. Preoligodendrocytes show immunoreactivity to the monoclonal antibody O4, as well as retaining expression of earlier markers, with the exception of nestin (McDonald and Belegu 2006). They are more arborized than cells in the progenitor stage. Immature,

premyelinating oligodendrocytes are no longer mitotic or migratory, and begin to express

RIP antigen, an unknown cytosolic epitope on oligodendrocytes. They are also positive

for staining with O1, a monoclonal antibody that recognizes galactocerebrosides. While

they are nearly fully arborized, they do not yet myelinate nearby axons. Mature,

myelinating oligodendrocytes are commonly identified using antibodies to myelin

component proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), and

myelin-associated glycoprotein (MAG) (Baumann and Pham-Dinh 2001, McDonald and

Belegu 2006) and have many processes which myelinate nearby axons (Figure 2).
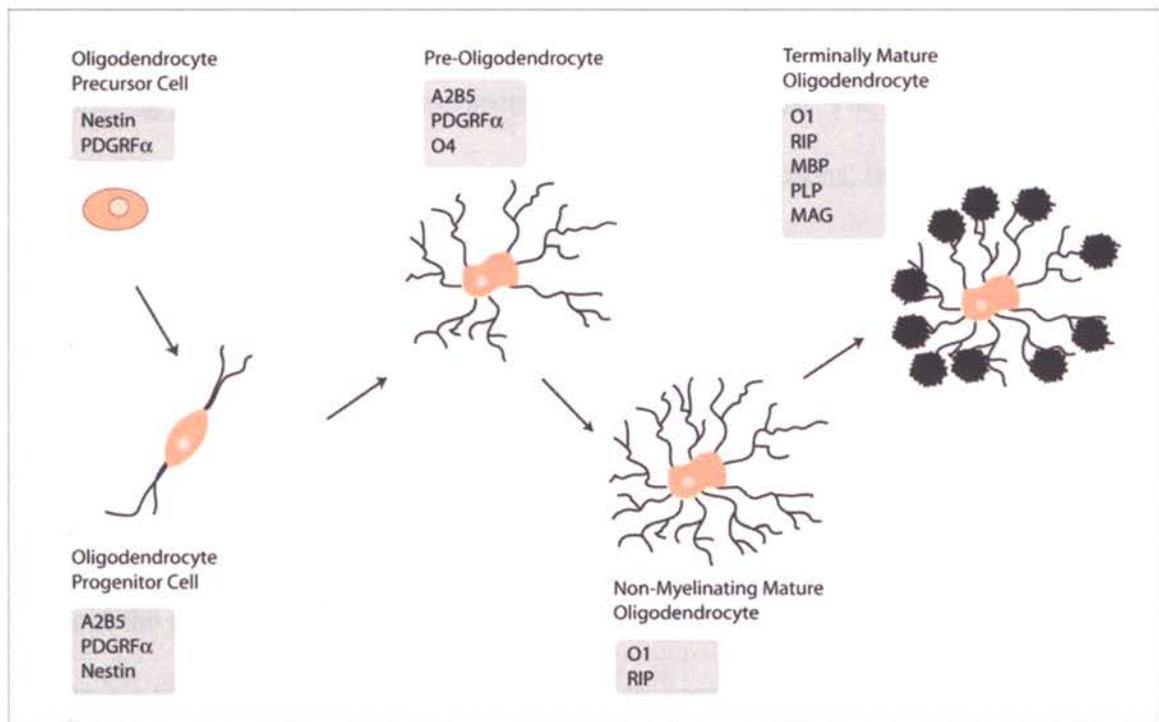

Figure 2. Morphologic changes and differential expression of antigenic markers characterize progression of the oligodendrocyte lineage from oligodendrocyte precursor cells to terminally differentiated, myelinating oligodendrocytes. Only a few selected antigenic markers are shown (adapted from McDonald and Belegu, 2006).0
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While remyelination continues for a time after onset of disease or injury, it eventually fails, possibly due to the inhibition of oliogodendrocyte precursor maturation into myelinating cells (Back et al. 2005; Frohman et al. 2006). A number of anti-differentiation molecules are contained within the gliotic scar, including Nogo receptor-interacting protein 1 (Mi et al. 2005), and high molecular weight hyaluronic acid produced by reactive astrocytes (Back et al. 2005).

The loss of myelin and myelinating oligodendrocytes disrupts the functional relationship between the glial cell and the axons associated with it. Each oligodendrocyte sends out myelinating processes, which wrap sections of multiple axons. The length of the axon is wrapped in sections with small gaps between myelinated sections, termed nodes of Ranvier. The electrical signal from the neuronal cell body travels rapidly from node to node (saltatory conduction) along the axon. The myelin sheath also promotes neuronal survival and growth by protecting the axon from injury and contributing trophic factors (Wilkins et al. 2003).

Without the protective wrapping of myelin, the axon, and eventually the neuron itself, is vulnerable to damage from cytokines, nitric oxide, proteases, superoxides, CD8+ T cells, and glutamate excitotoxicity (Pitt et al. 2000). Remyelination, even if it were to occur, is obviously futile once the axon is compromised. Cumulative loss of axons, as a result of inflammatory demyelination and eventual transection, correlates with irreversible disability in MS patients. The number of axons dwindles as the disease progresses, with

old lesions sometimes showing an axonal loss of more than 80 percent (Bjartmar C 2003).

The progression of MS from its unknown starting point within the body involves many cells types, cytokines, and mediators of inflammation. One particular molecular that warrants discussion is CD44, a cell-surface receptor that plays a role in multiple physiological phenomena, including inflammation.

Section 3: CD44, A Transmembrane Receptor Implicated in Inflammation

The family of class I transmembrane glycoproteins designated CD44 is thought to mediate many cellular processes including the regulation of growth, survival, differentiation and motility (Ponta et al. 2003). The interactions between CD44 and its principal ligand, hyaluronic acid (HA) play a role in cancer, specifically in tumorigenesis and tumor metastasis. There is a wealth of evidence that CD44 is involved in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (Girgrah et al. 1991; Haegel et al. 1993; Estess et al. 1998; Brocke et al. 1999; Fujii et al. 1999; Mikecz et al. 1999; Back et al. 2005). CD44's specific contributions to inflammation include leukocyte recruitment, and its interactions with and metabolism of HA.

An early step in the inflammatory cascade is the recruitment of inflammatory cells such as leukocytes. Leukocyte recruitment is mediated not only by local production of chemoattractants but also by cell-adhesion molecules including integrins, selectins, members of the immunoglobulin superfamily, and CD44. CD44 is constitutively

expressed on leukocytes and parenchymal cells including endothelial, epithelial and smooth muscle cells. CD44 expression is upregulated on activated T cells and assists in their extravasation through the epithelial wall of the blood vessel and into the parenchyma (Lesley et al. 1993; McHeyzer-Williams and Davis 1995; DeGrendele et al. 1997; Estess et al. 1998).

Studies in which anti-CD44 antibodies inhibited inflammation in EAE demonstrated a crucial role for CD44 in the pathogenesis of this disease. The onset of EAE was delayed and the severity of disease was decreased due to the inhibition of trafficking of activated leukocytes to sites of inflammation (Brocke et al. 1999). Similar studies in mouse models of inflammatory bowel disease (Wittig et al. 2000), collagen- and proteoglycan-induced arthritis (Zeidler et al. 1995), cutaneous inflammation (Schall et al. 1993) and IL-2 induced vascular leak syndrome (Rafi-Janajreh et al. 1999; Mustafa et al. 2002) add to the evidence of CD44's importance in leukocyte recruitment.

Structure of CD44

CD44 family proteins represent alternatively spliced mRNA species and post-translationally modified products of a single gene. They range from 80 to 250 kDa and can be extensively glycosylated, sulfated, and modified by the attachment of glycosaminoglycans. They are expressed on the surface of most vertebrate cells, with the most commonly expressed form being hematopoietic CD44 (CD44H), also referred to as standard CD44 (CD44s). This is the smallest CD44 isoform as it lacks the alternatively

spliced variant exons, which in other isoforms make up part of the stem structure of the molecule (Ponta et al. 2003)

The molecule is composed of an extracellullar, HA-binding, amino-terminal domain, a membrane-proximal stem structure, a transmembrane region and a cytoplasmic tail region. The amino-terminal domain binds HA via two clusters of conserved basic residues, the BX7B motif, which is found in other HA-binding proteins. Two pairs of intra-molecular disulfide bonds are also essential for HA binding. This binding region is present in all CD44 isoforms, including the soluble, intracellular form of CD44. Soluble CD44 is formed mainly by proteolytic cleavage at the plasma membrane and possibly by differential splicing. (Peach et al. 1993; Bajorath et al. 1998). It is upregulated in the serum of individuals with malignant tumors (Cichy and Pure 2003). The membrane-proximal stem region, containing the insertion site for the variant exons, is less well conserved, whereas the C-terminal transmembrane and cytoplasmic domains of CD44 are highly conserved, with the exception of soluble CD44. Several phosphorylation sites that regulate interaction of CD44 with the cytoskeleton are contained in the cytoplasmic domain (Peach et al. 1993; Lesley et al. 2000) (Figure 3).
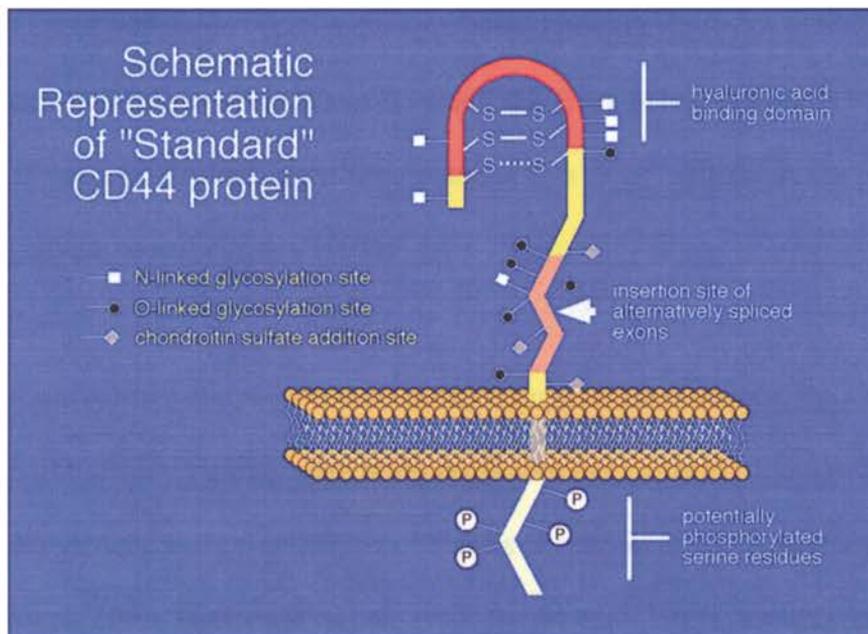
Figure 3. Structural domains and HA binding sites of the CD44 receptor. The N-terminal HA binding sites are shown, along with intramolecular disulfide bonds. 10 variable exons can be inserted in the extracellular domain. N- and O-linked glycosylation occurs in the extracellular domain, along with chondroitin sulfate addition. Cytoplasmic phosphorylation sites, important for signaling through the cytoskeleton, are also shown. (Diagram provided by Larry Sherman)

CD44-HA Binding Affinity

The binding affinity of CD44 for HA is regulated. Most naïve cells, which express low levels of CD44, do not bind HA, but can be induced to bind HA when these cells are activated with inflammatory stimuli, including proinflammatory cytokines. Binding requires the transition of CD44 from a low- to high-affinity binding state. In T cells, this transition can be induced by several stimuli: activation of the T cell receptor, the cytokines interleukin 2 (IL-2) and tumor necrosis factor (TNF), and chemokines including MIP-1β, IL-8, and RANTES (DeGrendele et al. 1997; Levesque and Haynes 1997). Primarily via a TNFα-dependent mechanism, interleukin 1α (IL-1α), IL-1β, IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), IFNγ, and

lipopolysaccharide each induce peripheral blood monocytes to bind HA via CD44. The anti-inflammatory cytokines IL-4 and IL-13 are potent inhibitors of binding, whereas IL-10 induces HA binding when alone, but acts as an inhibitor in the presence of IL-1 (Levesque and Haynes 1997).

Rather than a simple conformational change in the molecule, the change from a low- to high- affinity binding state relies on slower mechanisms. Increased expression of CD44, which occurs upon activation of T cells (Lesley et al. 1993; McHeyzer-Williams and Davis 1995; DeGrendele et al. 1997; Estess et al. 1998), variable glycosylation (Katoh et al. 1995; Lesley et al. 1995; English et al. 1998; Skelton et al. 1998), receptor clustering (Lesley et al. 1993; Lesley et al. 2000) glycosaminoglycan attachment (Sleeman et al. 1997; Cichy and Pure 2000), phosphorylation (Pure et al. 1995), inclusion of variant exons (Sleeman et al. 1997), and sulfation (Maiti et al. 1998) can all enhance the binding of CD44 to HA.

In light of the pleotropic effects of CD44, it is surprising that CD44 knock-out (CD44KO) mice display an almost normal phenotype (Schmits et al. 1997; Protin et al. 1999; Stoop et al. 2001). One of their few defects is impaired lymphocyte homing to the lymph nodes and thymus (Protin et al. 1999). An experiment by Nedvetzki *et al* supported the hypothesis that other molecules can compensate for the absence of CD44. CD44KO mice were administered a single dose of type II collagen and developed collagen-induced arthritis (CIA) more quickly and more severely than WT mice. In WT

mice anti-CD44 antibodies ameliorated the disease, indicating the involvement of CD44 when it is present (Nedvetzki et al. 2004).

One other possible explanation for the increase in disease severity in CD44KO mice is the fact that following binding of cell surface CD44 to HA, HA is internalized and digested by the cell (Culty et al. 1992). In the absence of CD44, HA accumulates and could thereby intensify the inflammatory cascade. In the above-mentioned experiment, histochemical examination of HA in the joint tissues of WT and CD44KO mice revealed fourfold higher accumulation of HA in the CD44KO mice, which may be in part responsible for their more severe disease.

Even when CD44 is present and able to bind HA, an overabundance of HA can overwhelm CD44's ability to bind and internalize HA, leading to HA accumulation. Demyelinated CNS lesions from animals with EAE and human MS patients show high levels of both CD44 and HA (Figure 4). This indicates the importance of each of these molecules and their interaction in the pathogenesis of EAE and MS.
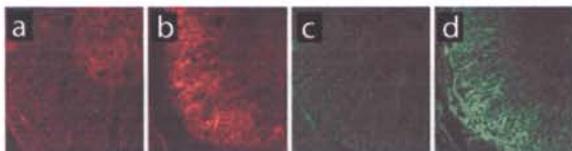

Figure 4. (Back et al. 2005) CD44 and HA are upregulated in demyelinated lesions from EAE mice. HA levels are higher in spinal tissues from mice with active EAE disease (b) compared to control mice (a). CD44 expression is also higher in EAE mice (d) compared to controls (c).

Section 4: Hyaluronic Acid is the Principle Ligand for CD44

While CD44 binds to other components of the extracellular matrix (ECM) such as

collagens (Turley and Moore 1984), fibronectin (Jalkanen and Jalkanen 1992), and

chondroitin sulfate (Naujokas et al. 1993), its major ligand is HA. This nonsulfated,

linear glycosaminoglycan is composed of alternating subunits of D-glucuronic acid and

D-*N*-acetylglucosamine. The polymer ranges in molecular mass from roughly 200kDa to

4000 kDa and beyond (Fraser et al. 1997).

HA is synthesized as an unmodified polysaccharide by one of three related hyaluronan

synthases, HAS1, HAS2, and HAS3. These three enzymes produce different lengths of

HA molecules; HAS1 generates medium to longer chains of up to 2,000 kDa; HAS2

generates molecules $\geq$ 4000 kDa , and HAS3 synthesizes the shorter forms of $\leq$250 kDa

(Fraser et al. 1997; Spicer and Nguyen 1999). As it is synthesized, the HA polymer is

extruded through the plasma membrane onto the cell surface or into the ECM (Fraser et

al. 1997; Weigel et al. 1997; Spicer and Nguyen 1999; Rilla et al. 2002; Toole 2004).

HA is degraded by hyaluronidases, which maintain appropriate levels of HA in the body

by regulating its turnover. The half-life of HA varies from minutes to weeks depending

on the tissue (Fraser et al. 1997). These degradative enzymes are encoded by six

hyaluronidase genes and one psuedogene (Csoka et al. 1999; Sherman et al. 2002). They

are expressed differentially in various adult tissues, and their expression levels change

following injury or inflammation (Csoka et al. 1999; Sherman et al. 2002).

HA performs a number of physiological functions and is expressed in nearly all extracellular matrices (Fraser et al. 1997; Toole 2004). It possesses a remarkable viscosity and ability to retain water and therefore is important in tissue homeostasis and biomechanical integrity. As it binds many proteoglycan molecules, which are highly negatively charged and therefore repel each other, the HA backbone and its attached proteoglycans form a "bottlebrush" configuration (Figure 5). HA lubricates joints and stabilizes gel-like connective tissues by forming a kind of scaffold for proteoglycans and other extracellular macromolecules. It provides environmental cues to cells during development, tissue remodeling and cancer progression, and provides an adhesive substrate for migrating cells. Some forms of HA promote angiogenesis (West et al. 1985). More recently, it has been shown to play a number of roles in the immune response, which are discussed below.
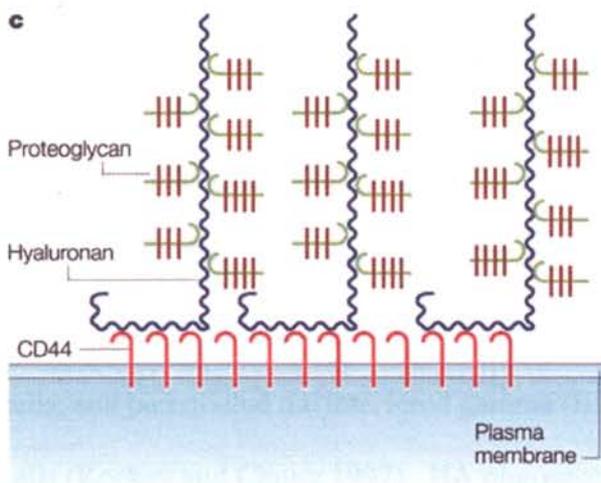

Figure 5. HA interacts with extracellular proteoglycans and CD44 on the cell surface, forming a pericellular coat. HA is tethered to the cell surface by CD44 (and/or other receptors, not shown). Many proteoglycans attach to the HA backbone, forming a bottlebrush configuration due to mutual repulsion between the negatively-charged molecules. (from Toole, BP 2004)

HA Molecules of Various Sizes Have Different Effects on Cells

While high molecular weight forms of HA (1000 kDa and above) are found in nearly all healthy tissues, low molecular weight HA (LMWHA) accumulates at sites of injury and inflammation (Balazs et al. 1967; Bjermer et al. 1989; Gazzinelli et al. 1992; Noble et al. 1993; McKee et al. 1996; McKee et al. 1997; Horton et al. 1998). At these sites, HA is degraded by hyaluronidases to low molecular weight forms as well as synthesized *de novo* in response to proinflammatory cytokines (Sampson et al. 1992; Mohamadzadeh et al. 1998).

Degradation products of HA ranging from 1.5 kDa (hexasaccharides) to 500 kDa have been shown to trigger the secretion by microglia and macrophages of various cytokines and chemokines, important mediators of tissue injury and repair (McKee et al. 1996; Hodge-Dufour et al. 1997; Horton et al. 1998) primarily via activation of NF-κB (McKee et al. 1997). In cultured macrophages, proinflammatory mediators including TNFα, IL-1β, chemokines, and inducible nitric oxide sythase (Noble et al. 1993; Boyce et al. 1997; McKee et al. 1997) were upregulated in response to LMWHA. In primary rat liver cell cultures, LMWHA directly stimulated the expression of iNOS in endothelial and Kupffer cells, and potentiated the interferon gamma (IFNγ)-stimulated formation of iNOS in those cells (Rockey and Chung 1997). HA oligosaccharides are able to activate dendritic cells (Termeer et al. 2000; Termeer et al. 2002) and stimulate endothelial cell recognition of injury (Taylor et al. 2004). Through binding to CD44, HA delivers costimulatory signals to T cells and increases T cell proliferation (Lesley et al. 1993; Galandrini et al. 1994).

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In contrast to LMWHA, high molecular weight HA (HMWHA) has a distinctive set of biological effects. It appears to be protective against chronic inflammation in its use as a therapeutic treatment for human inflammatory diseases such as osteoarthritis and rheumatoid arthritis (Goa and Benfield 1994; Brandt et al. 2000). HMWHA has been shown to inhibit NF-kB activation and subsequent cytokine expression (Neumann et al. 1999) and was protective against T cell-mediated liver injury in mice by reducing proinflammatory cytokine levels (Nakamura et al. 2004). Overexpression of HMWHA protected against acute lung injury in mice (Jiang et al. 2005).

As with the inflammatory response in general, within the context of central nervous system (CNS) inflammation and demyelination, the effects of HA depend on its molecular weight. Activated T cells infiltrating the CNS produce HA species ranging from 200-400 kDa, while CNS resident microglia produce similar species in response to inflammation (Back et al. 2005). This low molecular weight form of HA, as discussed above, potentiates the expression of proinflammatory mediators in and around EAE and multiple sclerosis lesions.

Astrocytes are the main source of HA in the CNS, although microglia also contribute (Bignami et al. 1991; Asher and Bignami 1992; Bignami et al. 1992; Eggli et al. 1992; Marret et al. 1994; Back et al. 2005). Under physiological conditions, the HA produced by astrocytes is a high molecular weight form (about 1000 kDa) which has been shown to keep oligodendrocyte precursors in a premature, pre-myelinating state (Marret et al.

1994; Back et al. 2005). Overproliferation of astrocytes in response to injury and inflammation (reactive gliosis) leads to a fibrotic scar and high accumulations of HMWHA, and the eventual failure of remyelination.

HA Binds to Other Receptors in Addition to CD44

In addition to CD44, there are a number of other proteins that bind HA on the cell surface, as well as some intracellular and extracellular proteins that contain HA-binding motifs (Hofmann et al. 1998; Huang et al. 2000). Proteins which bind extracellular HA include LYVE-1 (for lymphatic vessel endothelial hyaluronan 1) (Banerji et al. 1999), layilin (Borowsky and Hynes 1998; Bono et al. 2001), HARE (hyaluronan receptor for endocytosis (Zhou et al. 2000), and RHAMM (receptor for HA-mediated motility), the cell-surface form of which is designated CD168 (Hoare et al. 1993; Assmann et al. 1998; Cheung et al. 1999).

The Toll-like receptors 2 and 4 (TLR2, TLR4), receptor complexes associated with innate immunity and host defense against bacterial infection, are activated by HA oligosaccharides (Termeer et al. 2002; Taylor et al. 2004). Because HA is a repeating disaccharide structure with features of pathogen-associated molecular patterns, it is not surprising that some forms of HA interact with the Toll-like receptors. The study by Termeer and coworkers found that small HA fragmentation products (sHA) produced by enzymatic digestion of HMWHA induce phenotypic and functional maturation of dendritic cells. Mice carrying mutant TLR-4 alleles were non-responsive to sHA. The

addition of anti-TL4 mAb to human monocyte-derived DCs blocked sHA-induced production of TNFα. This suggests that the size of the HA molecule may determine the receptor it binds to, and adds to the evidence that lower molecular weight forms of HA can mediate inflammation.

The study by Nedvetzki and colleagues discussed earlier (Nedvetzki et al. 2004) showed that CD44-knockout mice displayed more severe joint inflammation due to collagen-induced arthritis than wild-type mice. By blocking RHAMM with anti-RHAMM mAb or using soluble peptide competition, they demonstrated that RHAMM acted as the major receptor mediating the effects of HA and promoting inflammation in the knockout mice. They hypothesized that joint inflammation in the CD44KO mice was exacerbated due the greater accumulation of HA in these mice. The HA accumulation was attributed to the inability of RHAMM to promote endocytosis of HA and subsequent lysosomal digestion (Naor et al. 1997). They concluded that RHAMM compensated for CD44 deficiency and, in fact, potentiated inflammation in the absence of CD44.

The above discussion of CD44, HA, and their interaction has focused on the inflammatory aspect of MS pathology. I now review the data pertaining to their role in demyelination and remyelination.

Section 5: A Possible Role for CD44 and HA in Demyelination and Remyelination

Following demyelination in MS and EAE, substantial remyelination occurs, but the eventual failure of remyelination has long been an enigma. Most remyelination occurs in lesions that develop early in the disease process (Prineas et al. 1993) and in satellite areas of large lesions. Even in these lesions, remyelination appears to be transient, as there is little remyelination observed as the lesion itself grows older (Frohman et al. 2006). Remyelination is attributed to the recruitment and maturation of oligodendrocyte progenitor cells (OPCs) (Scolding et al. 1998; Wolswijk 2000). While recruitment of OPCs to the lesion appears to be normal in MS, their differentiation into mature myelin-producing oligodendrocytes is insufficient to allow remyelination to continue as the disease progresses. Premyelinating OPCs have been shown to accumulate in demyelinated MS and EAE lesions (Scolding et al. 1998; Chang et al. 2000; Wolswijk 2000; Peterson et al. 2002). Their differentiation may be impeded by molecules expressed by cells within these lesions, including CD44 and HA.

In the CNS, CD44 is expressed by astrocytes, radial glia and microglia (Girgrah et al. 1991; Akiyama et al. 1993; Moretto et al. 1993; Ikeda et al. 1996; Sherman et al. 2000; Liu et al. 2002). The observation that CD44 expression is highly and persistently upregulated in glial cells (microglia, astrocytes, and oligodendrocytes) following CNS or traumatic peripheral nerve injury, ischemic disease, and inflammatory demyelinating conditions such as MS and EAE raises questions regarding a role for elevated CD44

expression in demyelination (Haegel et al. 1993; Brennan et al. 1999; Brocke et al. 1999;
Jones et al. 2000).

Under physiological conditions, cells of the oligodendrocyte lineage express little or no
CD44 (Moretto et al. 1993; Bouvier-Labit et al. 2002; Liu et al. 2002; Liu et al. 2004).
As mentioned above, they can be induced to express significant levels of CD44 *in vitro* or
as a result of pathology (Moretto et al. 1993; Ito et al. 2001; Bouvier-Labit et al. 2002).
Mice that chronically overexpressed CD44 in myelinating cells of the CNS and
peripheral nervous system showed CNS dysmyelination and progressive demyelination in
the absence of inflammation (Tuohy et al. 2004). This study also showed extensive
reactive astrogliosis, suggesting not only that aberrant CD44 expression by OPCs and
oligodendrocytes can cause demyelination, but that it can potentiate gliosis independent
of an inflammatory response.

Immature, premyelinating OPCs failed to mature when transfected with CD44 *in vitro*
(Liu et al. 2004) suggesting involvement of CD44 in preventing remyelination. In a
study by Back *et al* (2005), HA was found to accumulate around CD44-transfected OPCs
and oligodendrocytes, as it does in early inflammatory demyelinating lesions. Similarly,
HA accumulation was found around the CD44-overexpressing oligodendrocytes and
OPCs in the transgenic mice used in the study by Tuohy and coworkers (2004). Although
these cell types do not synthesize HA, they bound significant amounts of HA when
induced to express CD44. The Back *et al* study also demonstrated that a high molecular
weight form of HA is produced by reactive astrocytes and, in concert with chronically

elevated CD44 expression, inhibited OPC maturation. These data suggest that elevated expression of CD44 may alter the interaction with HA in and around demyelinating lesions and contribute to the failure of remyelination.

What was not clear from these studies is how the LMWHA produced by infiltrating T cells in EAE and MS lesions might contribute to demyelination and remyelination. The following chapter will address this question.

Summary

Autoimmunity in MS is thought to be initiated by T cell recognition of the body's myelin as a foreign, rather than self, antigen. CD4+ cells of the Th1 subset invade the CNS, proliferate in response to antigen recognition, and begin a cascade of inflammatory events. Inflammatory mediators attack myelin and oligodendrocytes, resulting in demyelinated plaques. These eventually fail to remyelinate, and permanent neurological disability results.

CD44 and its principal ligand, HA, have been implicated in both the inflammatory and demyelinating components of MS and its animal model, EAE. Via CD44's enhancement of leukocyte recruitment and its binding (or binding by other HA receptors) of both low and high molecular weight forms of HA, as well as the proinflammatory effects of LMWHA itself, CNS inflammation is exacerbated and perpetuated. The accumulation of HMWHA in chronic lesions, combined with the increased levels of CD44 expression in

those lesions, impede the maturation of OPCs into myelinating oligodendrocytes and prevent remyelination.

The present study was designed to test the hypothesis that LMWHA increases proinflammatory cytokine production by immune cells in a CD44-dependent manner and upregulates CD44 expression by oligodendrocytes or oligodendrocyte progenitors, thus compounding the effects of HMWHA on remyelination. Project aims were to 1) test whether LMWHA increases splenocyte proliferation, 2) test whether LMWHA increases proinflammatory cytokine production by immune cells, 3) determine whether CD44 is the required receptor for increased cytokine production, and 4) assess whether LMWHA or conditioned media containing proinflammatory cytokines increase CD44 expression in oligodendrocytes and oligodendrocyte progenitors.

Chapter 2: Materials and Methods

Animals

Different strains of mice were used for different experiments. Male (2-6 month-old) B10.PL Vβ8.2 MBP Ac$_{1-11}$ TCR transgenic mice, which carry a transgene specific for recognition of the acetylated N-terminal peptide of myelin basic protein (amino acids 1-11) were utilized in the initial splenocyte culture studies outlined below. The transgene in these mice is inserted into the variable (V) region of the T cell receptor (TCR) β chain. Controls for these mice were B10.Pl wild-type (WT) littermates. Male (3-6 months old) CD44-null C57BL/6:129-CD44^{tm1Hbg}/J (CD44KO) mice, in which transcription from the CD44 gene has been completely ablated via a neomycin resistance/lac Z cassette inserted in exon 1, were used with age-matched C57/BL6129SF2/J WT control mice. Female (3-6 month-old) C57/BL6 mice were used for myelin oligodendrocyte glycoprotein (MOG)-induced EAE splenocyte cultures (see below). All mice were from Jackson Labs.

Flow cytometry

We analyzed splenocytes from naïve MBP Ac$_{1-11}$-specific TCR transgenic male mice after dissociation (resting cells), or after 72-hr stimulation at 4 x 10^6 cells/ml in RPMI stimulation media (RPMI 1640 (Mediatech) with L-glutamine 4 mM, HEPES 25 mM, NA pyruvate 1mM, fetal bovine serum (FBS, Summit Biotechnology) 1%, and 2-mercaptoethanol (Sigma) 5 x 10^{-5} M) with 2 μg/ml MBP Ac $_{1-11}$ antigenic peptide. Culture conditions were 37 °C, 5% CO$_2$ in air. Cells were incubated with anti-CD69 and anti-CD25 antibodies to confirm activation of T cells and anti-CD4, anti-CD44, and

biotinylated HA binding protein (HABP) for 30 minutes at 4 °C (all antibodies from BD

Biosciences Pharmingen). Detection of HABP was by Cy5-conjugated streptavidin.

Staining was assessed by 3-color flow cytometry, using a FACScan instrument (Becton-

Dickenson) and analyzed by CellQuest software (Becton-Dickenson). Specificity of

HABP binding was verified by pretreating cells with 20 U/ml hyaluronidase at 37 °C for

2 hours prior to HABP and Cy5-conjugated streptavidin staining. For each experiment,

cells were stained with isotype control antibodies to determine levels of non-specific

staining and establish quadrant settings prior to calculating the percent positive cells.

Intracellular Cytokine Staining

Single cell suspensions from naïve TCR transgenic mouse spleens were prepared and

cultured at 10 x 10^6 cells/ml in RPMI stimulation media containing 0.2 µg/ml MBP Ac_{1-11} peptide and 100 ug/ml HA or vehicle for 24 hours at 37 °C in 5% CO_2. The last 5

hours of culture were in the presence of Brefeldin A, to retain proteins in the endoplasmic

reticulum. The cells were then stained for 30 minutes at 4 °C with anti-Vβ8.2 mAb (BD

Biosciences Pharmingen) to insure the presence of the TCR transgene and anti-CD4 PE-

Cy5 (BD Biosciences Pharmingen). Cells were fixed and permeabilized with

Cytofix/Cytoperm solution (Pharmingen) and stained with anti-IFNγ, anti-TNFα or IgG1

isotype control antibodies labeled with phycoerythrin (Pharmingen) for 30 minutes at 4

°C. Cells were washed twice in Perm/Wash buffer (Pharmingen) and once in FACS

staining buffer (PBS 1% BSA, 0.05% NaN_3) prior to three-color flow cytometry analysis

as described above.

Splenocyte cultures

Spleens were harvested from naive TCR transgenic mice with WT controls from B10.PL background, or C57/BL6129SF2/J CD44KO mice with WT controls from C57/BL6129SF2/J background. Spleens were crushed, cells dissocciated and passed thru a 70 µm filter. Cells were then centrifuged at room temperature for 10 minutes at 500 x g. Red blood cells were lysed by the addition of 5 ml ACK buffer (0.15 M NH_4Cl, 1mM $KHCO_3$, 1.1mM EDTA) for 5 minutes. Lysing action was quenched with RPMI 1640 media (Mediatech) and cells were centrifuged as above. Cells were resuspended and again passed through a 70 µm filter, resuspended and counted. After a final centrifugation, resuspension at 4 x 10^6 cells/ml in RPMI stimulation media, and addition of either 0.2 µg/ml MBP Ac_{1-11} peptide for TCR transgenic cultures or 1.0 anti-CD3 mAb (BD Biosciences Pharmingen) for CD44KO or WT C57/BL6129SF2/J cultures, cells were plated and cultured for 3 days. HA or vehicle was added as described below.

Hyaluronate suspensions

High molecular weight HA of approximately 1600 kDa was purchased from Sigma. Medium molecular weight HA (~900 kDa) and low molecular weight HA (~400 kDa) was purchased from Hyaluron, Inc. HA suspensions were prepared by dissolving the lyophilized sodium salts in 10% ethanol in water (vehicle) at a concentration of 8 mg/ml, followed by sterilization using a 0.2 µm syringe filter. 12.5 µl of HA suspension or vehicle was used per 1 ml cell suspension for a final concentration of 100 µg/ml HA.

³H-Thymidine Incorporation Assays

Aliquots of the spleen cultures described above were cultured in 96-well plates with the same concentration of MBP Ac $_{1-11}$ antigenic peptide or anti-CD3 mAb and HA or vehicle as the aliquot cultured for analysis by ELISA (below). Splenocytes were plated at 2 x 10^6 cells/ml, and cultures were grown for 72 hours at 37 °C with 5% CO_2 in air. The last 18 hours in culture were in the presence of 0.5 µCi/well of ^{3}H-thymidine. Plates were harvested on to 96-well glass-fiber filter sheets and DNA incorporation of radioactivity was measured by liquid scintillation using a Wallac β-counter.

ELISAs

Cytokine levels from splenocyte cultures were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (BD Biosciences Pharmingen). The limit of detection by ELISA for IFNγ was 31.3 pg/ml, 15.6 pg/ml for TNFα, 31.3 pg/ml for IL-10, and 7.8 pg/ml for IL-4. Supernatants were cleared by centrifugation for 10 minutes at 2000 x g before loading into prepared wells. Four dilutions of each supernatant were assayed, with triplicate wells for each dilution. Absorbances were read at 450 nm with a BioRad microplate reader and, after subtraction of background values, the readings were used to construct a standard curve. Cytokine concentrations were calculated using only dilutions that fell within the standard curve, on Excel software.

IM7 Antibody Purification

The IM7.8.1 hybridoma (IgG$_{2b}$κ) was a gift from Dr. Jonathan Sleeman, Forschungszentrum Karlsruhe, Germany. Cultures were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. Supernatants were collected and cleared by centrifugation at 20,000 x g for 30 min. Saturated ammonium sulfate solution was added until supernatant turned cloudy, stirred overnight at 4 °C to precipitate proteins, and centrifuged for 1 hr at 20,000 x g. The precipitate was dissolved in a small volume of PBS and dialyzed against > 20 volumes PBS for 48 hours total at 4°C, with four changes of PBS. The supernatant was then diluted with an equal volume of binding buffer from the Immunopure (G) IgG Purification kit (Pierce Biotechnology) and loaded onto the equilibrated Protein G column. Samples were run through the column using a BioRad BioLogic low pressure liquid chromatography apparatus. After the sample had flowed through the column, the IM7 antibody was eluted with the kit's elution buffer (pH 2.8). Eluted fractions were neutralized, absorbances read on a Beckman spectrophotometer, and fractions with high absorbances were combined. Pooled fractions were dialyzed overnight in PBS and concentrations calculated from final 280 nm absorbance.

Immunofluorescence Studies of Oligodendrocyte and Oligodendrocyte Progenitor Cells

Rat oligodendrocytes and oligodendrocyte progenitor cells (OPCs) were generously provided by Dr. Stephen Back and his lab. Cells were cultured on coverslips at 37 °C in BDM/B104 media in 7% CO_2 in air. OPCs and mature oligodendrocytes were

differentiated using thyroxine and ciliary neurotrophic factor as previously described

(Back et al. 2005). Cells were treated with 1:1 conditioned media from MOG-induced

EAE splenocytes (see below), BDM/B104 media alone, or 1:1 BDM/B104: IL-2

containing RPMI stimulation media for 24 hours. Cells were then washed twice with

Hanks buffered saline solution, fixed in 4% paraformaldehyde for 10 minutes, washed

three times in PBS and soaked overnight at 4 °C. Primary antibodies used were anti-

CD44 mouse hybridoma 5G8 supernatant (hybridoma provided by Dr. Jonathan

Sleeman) at 1:10 dilution, and the OPC marker O1 at 1:100 dilution, or MBP at 1:50

dilution to stain mature oligodendrocytes. The anti-CD44 secondary antibody used was

Alexa 488 anti-mouse IgG (1:500), the secondary antibody for O1 was Alexa 546 anti-

mouse IgM (1:500), and the secondary for MBP was Alexa 546 anti-rabbit (1:500), all

from Molecular Probes. Primary antibody incubation was overnight at 4 °C; secondary

antibody incubation was for 1 hour at room temperature. Cells were then washed and

coverslips mounted on slides using Fluoromount mounting media. Images were

captured using a Zeiss epifluorescent upright microscope (Carl Zeiss MicroImaging, Inc.)

with a digital CCD camera and AxioVision Software.

Conditioned media from MOG-induced EAE splenocytes

Four female C57/BL6 mice were immunized with 200 µg per mouse MOG_{35-55} peptide

and 400 µg per mouse complete Freund's adjuvant (CFA). On day 10 post-injection,

animals were positive for EAE disease and were euthanized by CO_2 inhalation prior to

splenectomy. Splenocytes were recovered and an aliquot was used in a proliferation

assay to determine whether the cells responded to the MOG antigen. The remainder of the cells was cultured at 4×10^6 cells per ml in RPMI stimulation media with 20 µg/ml MOG$_{35\text{-}55}$ peptide for 72 hours. Media was then changed to RPMI growth media (contains IL-2 to select for cells expressing the IL-2 receptor) and cultured for 5 days. Cells were then restimulated with anti-CD3 and anti-CD28 plate-bound antibodies (BD Biosciences Pharmingen). Conditioned media was collected from the cultures after 72 hours.

Western Blot

Splenocytes from naive TCR transgenic mice were cultured 72 hours in the presence of cognate antigen and 100 ug/ml LMWHA or vehicle. Supernatants were collected after 72 hours and added to OPC cultures at a ratio of 1:1 with the OPC culture media (BDM/B104). OPCs were also treated with 1:1 RPMI stimulation media used for splenocyte culture or with 100 ug/ml LMWHA. After 24 hours, cells were lysed using RIPA buffer (10 mM Tris, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EGTA, 1 mM PMSF) in the presence of protease inhibitors leupeptin, aprotinin, pepstatin, and NaV. Lysates were centrifuged for 10 minutes at 1000 rpm, and Laemmli buffer added to supernatant. Samples were boiled 10 minutes before adding to 7% SDS polyacrylamide gel. Gel was electrophoresed at 25 mA until dye marker ran off gel, then transferred to nitrocellulose membrane (Trans-Blot, BioRad) at 25 volts for 25 minutes. Membrane was blocked for 30 minutes in 5% blocking buffer (5% dry milk in PBS with 0.1% Tween) and incubated overnight at 4 °C with primary antibodies 5G8 (anti-rat CD44, 1:100) and anti-goat β-actin (1:400) to determine equal

loading of lanes. Following three washes in blocking buffer, anti-mouse IgG horseradish

peroxidase conjugate (BioRad) at 1:2000 dilution, and anti-goat IgG horseradish

peroxidase conjugate (Santa Cruz Biotech) at 1:1000 were added. After a 1-hour

incubation and three washes in blocking buffer, ECL Plus solution (Amersham

Biosciences) was added for five minutes. Blots were developed on a Kodak film

developer and bands were quantitated using NIH Image J.

Chapter 3: Experimental Results

Activated but not resting T cells accumulate pericellular HA

I began by testing whether activated T cells accumulate pericellular coats of HA, which, in conjunction with upregulated CD44 expression, may assist their extravasation through the blood-brain barrier. I harvested cultured splenocytes from a myelin basic protein Ac_{1-11} T cell receptor transgenic mouse (MBP Ac_{1-11} TCR tg) on a B10.PL background. Approximately 90% of T cells from these mice are specific for the Ac_{1-11} epitope of myelin basic protein (MBP), making it possible to specifically activate T cells using the cognate antigen. CD4+ T cells within activated splenocyte cultures were identified by incubation with anti-CD4-FITC conjugated mAb, and their activation was confirmed by high levels of CD44 expression, detected by anti-CD44-PE-conjugated mAb. Biotinylated HA binding protein (HABP) with Avidin-Cy5 as a detection molecule was used to assess the level of pericellular HA accumulation, with Avidin-Cy5 used alone as a negative control. Flow cytometry data showed approximately 85% of CD4+/CD44 high cells were positive for HA accumulation (Figure 6a). Hyaluronidase, which degrades pericellular HA, demonstrated the specificity of HA staining. While the same percentage (frequency) of CD4+/CD44 high T cells were HABP positive following brief exposure to hyaluronidase, the mean fluorescence intensity of treated cells was roughly half that of untreated, confirming the specificity of the HABP binding (Figure 6b).
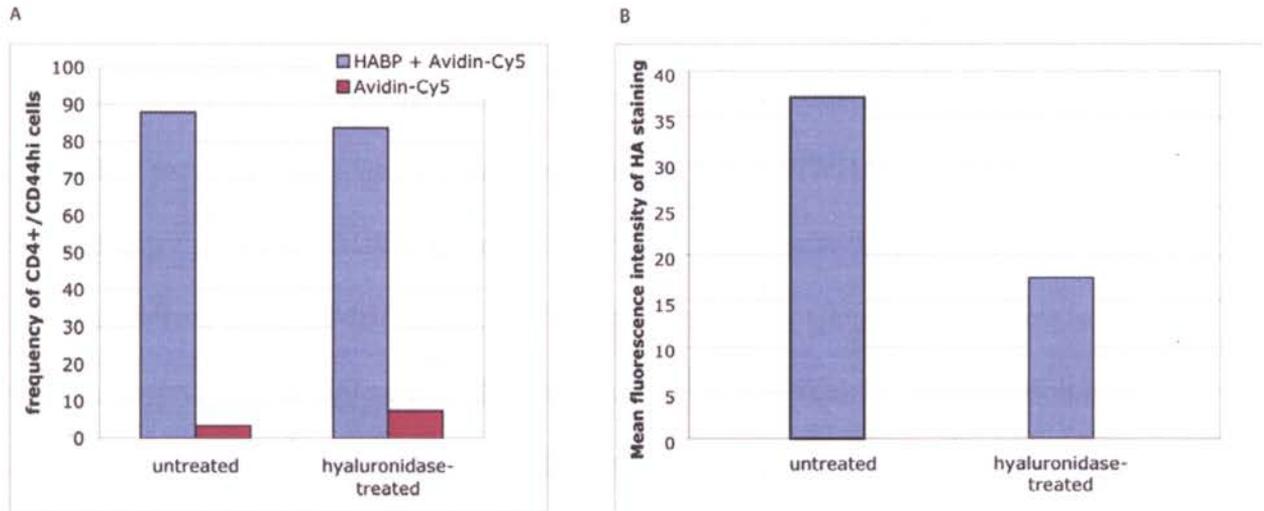
Figure 6. Activated CD4+ T cells produce hyaluronic acid. CD4+ T cells were isolated from MBP Ac 1-11 specific TCR transgenic mice and cultured 4 days in the presence of antigen. Cells were then stained with CD44-PE and hyaluronic acid binding protein (HABP) with avidin-Cy5, or CD44-PE and avidin-Cy5 alone. The frequency of positive cells and their mean fluorescence intensity was determined by 3-color flow cytometry.

As previously published in Back *et al* (2005), I examined the same cells as described in

Figure 6 using confocal microscopy. This revealed resting CD4+ cells with low levels

of CD44 staining and undetectable levels of HA staining (Figure 7a) and activated

CD4+/CD44 high cells with prominent HA staining (Figure 7b). This corroborated my

flow cytometry results and others' published reports that activated T cells express
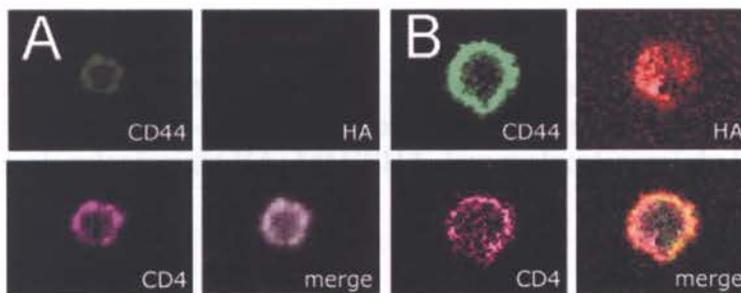
elevated CD44 and produce HA.

Figure 7. Activated T cells express high levels of CD44 and accumulate pericellular HA. Confocal microscopy of fluorophore-conjugated antibodies reveal a representative CD4+ resting T cell with low levels of CD44 immunofluorescence and undetectable HA (a), and a representative CD4+ activated T cell with high CD44 immunofluorescence and a pericellular coat of HA (b). The merged images show the combined immunofluorescence from labeled CD4, CD44, and HA binding protein.

HA does not increase T cell proliferation in the presence of antigen

I next tested the ability of exogenous HA to increase proliferation in splenocyte cultures.

I used HA preps of ~400 kDa (low molecular weight HA; LMWHA), ~900 kDa (medium

molecular weight HA; MMWHA) and ~1.6 kDa (high molecular weight HA; HMWHA)

in cultures from B10.PL MBP Ac1-11 TCR transgenic mice. Cultures were stimulated 3

days in the presence of 100 ug/ml HA or an equal volume of vehicle. Proliferation was

assessed at 72 hours following 18 hrs of ^{3}H-thymidine treatment. None of the treatments

increased proliferation compared to vehicle (Figure 8). This experiment was repeated

numerous times, in this mouse strain (B10.PL) and in cells from B6129SF2/J mice. No

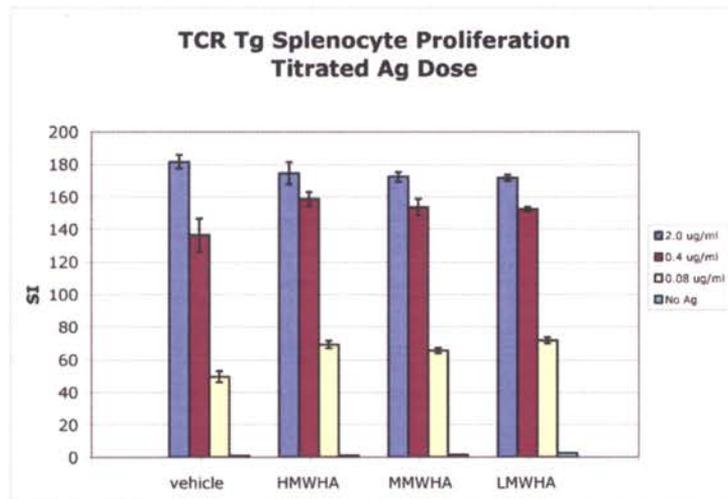significant differences in proliferation were observed.

Figure 8. Different-sized forms of HA do not increase proliferation in the presence of antigen. MBP Ac1-11 -specific TCR transgenic splenocytes were cultured with cognate antigen for 48 hours, then for 18 hours in the presence of ^{3}H-thymidine. Proliferation was measured by β-scintillation counting. (High molecular weight HA: HMWHA, medium molecular weight HA: MMWHA, low molecular weight HA: LMWHA.)

In contrast to its inability to affect proliferation, HA showed a marked effect on levels of

proinflammatory cytokines in the same MBP Ac1-11 TCR Tg spleen cells tested for

proliferation. LMWHA, but not HMWHA, consistently increased IFNγ and TNFα levels, in some cases dramatically. As expected, splenocyte cultures from different animals responded more or less robustly to antigen stimulation and HA treatment. ELISA measurement of IFNγ concentrations in cell culture supernatants indicated between 200 and 10,000 percent increase in response to LMWHA over vehicle treatment (Figure 9a). TNFα concentrations ranged from 160 to 1200 percent increases over vehicle (Figure 9b). The increase in LMWHA-treated cultures was not due to overall greater numbers of splenocytes in LMWHA-treated cultures, as proliferation assays showed no variation (see figure 8 above). I did not observe increases in the regulatory cytokines IL-10, IL-4 or TGFb (data not shown).

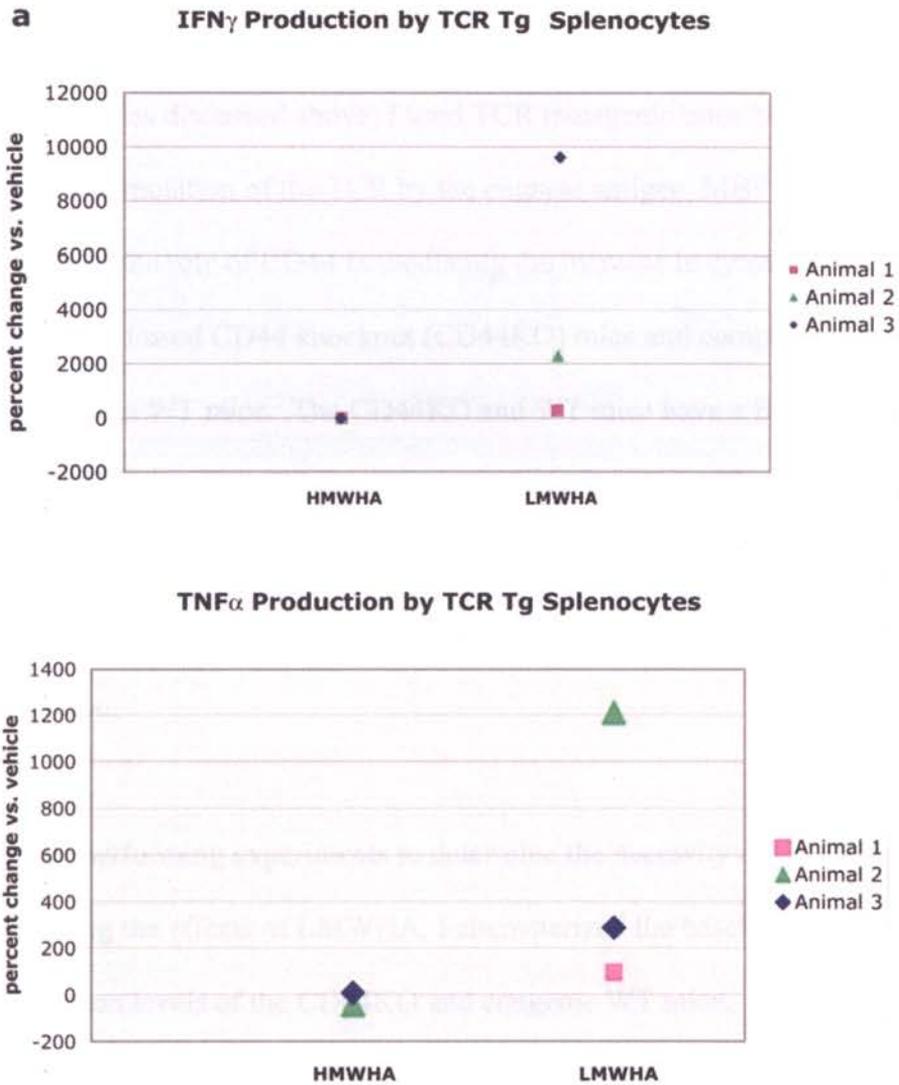**IFNγ Production by TCR Tg Splenocytes**

TNFα Production by TCR Tg Splenocytes

Figure 9. Low molecular weight HA increases splenocyte production of TNFα and IFNγ. Primary splenocytes from B10.PL MBP Ac1-11 TCR transgenic mouse spleens were treated with MBP Ac1-11 peptide and HA or vehicle and cultured for 3 days. Supernatant levels of IFNγ and TNFα were analyzed by ELISA.

CD44KO splenocytes proliferate more rapidly and produce higher levels of cytokines than WT splenocytes

In the studies discussed above, I used TCR transgenic mice because of the ease of specific stimulation of the TCR by the cognate antigen, MBP Ac1-11. In order to determine the role of CD44 in mediating the increase in cytokine levels in response to LMWHA, I used CD44 knockout (CD44KO) mice and compared those results with results from WT mice. The CD44KO and WT mice have a B6129SF2/J background, and their T cells possess wild-type receptors that are not specific for a particular antigen. In order to activate T cells within splenocyte cultures from these animals, I used soluble anti-CD3 monoclonal antibodies (mAbs) to cause cross-linking of the TCR and induce activation.

Before performing experiments to determine the necessity of CD44 expression in mediating the effects of LMWHA, I characterized the baseline proliferation and cytokine production levels of the CD44KO and congenic WT mice. I noted two differences in the response of CD44KO splenocytes compared to WT: 1) CD44KO cells proliferate more rapidly than WT, and 2) cytokine levels were higher in CD44KO culture supernants.

CD44KO cultures were more proliferative in response to non-specific stimulation of the T cell receptor (TCR) with anti-CD3 mAb. Proliferation levels, measured in counts per minute (cpm), were consistently higher in CD44KO cultures compared to WT (Figure 10). CD44KO splenocytes also displayed wider variation in proliferation levels per animal. This enhanced proliferation may be due to more robust activation or due to

increased resistance of CD44KO T cells to activation-induced cell death (McKallip et al. 2002)

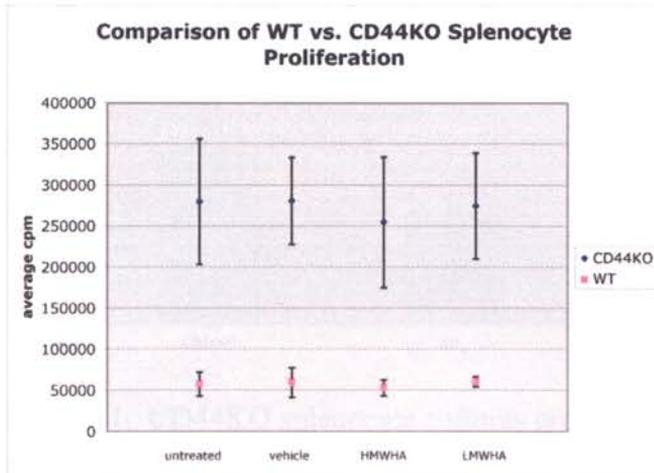**Comparison of WT vs. CD44KO Splenocyte Proliferation**

Figure 10. Activated CD44KO splenocytes are more proliferative than WT splenocytes. The average of 5 separate experiments shows CD44KO proliferation is approximately 260,000 cpm in all treatment categories, whereas WT splenocytes showed an average 55,000 cpm for all treatments. Proliferation was assessed after 72 hrs in culture with 1.0 ug/ml anti-CD3 mAb, with the final 18 hrs in the presence of ^{3}H-thymidine. DNA incorporation of ^{3}H-thymidine was assessed by β-scintillation counting.

As observed using splenocytes from TCR transgenic mice, neither HMWHA nor LMWHA increased proliferation in CD44KO or WT splenocyte cultures. The result was the same when CD4+ T cells from either genotype were cultured alone and activated by plate-bound anti-CD3 and anti-CD28 mAbs (data not shown).

Activated CD44KO splenocyte cultures produced higher overall levels of proinflammatory cytokines than activated WT splenocytes, based on the calculated mean of 5 separate experiments (Figure 11a,b). However, the wide variation in absolute cytokine levels between spleen cultures from individual animals obscured any statistical significance. Higher cytokine levels may be due to the higher proliferation of CD44KO cultures resulting in more cytokine-producing cells. The increased responsiveness of

CD44KO splenocytes to non-specific T cell stimulation suggests that the absence of
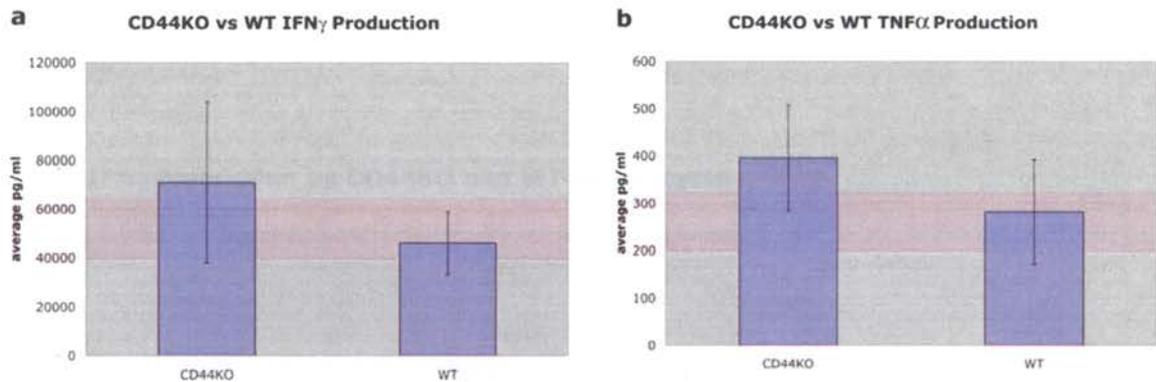
CD44 may potentiate inflammation.

Figure 11. CD44KO splenocyte cultures produce higher levels of proinflammatory cytokines than WT splenocytes. CD44KO and WT splenocytes were cultured in the presence of 1.0 ug/ml anti-CD3 antibody to activate T cells within the cultures. Supernatants were collected after 72 hrs and analyzed by ELISA. Results were calculated as the mean of 5 separate experiments.

CD44 is not required for proinflammatory responses of immune cells to LMWHA

To test if CD44 is required for the effects of LMWHA on cytokine production, I treated

activated CD44KO and WT cells with LMWHA (various species ranging from

approximately 400 to 900 kDa) and degraded HA (various species less than 400 kDa)

(Figure 12). Both CD44KO and WT cultures showed an increased IFNγ and TNFα

cytokine levels in response to LMWHA but not degraded HA This implies that CD44 is

not required for the observed LMWHA-induced immune cell response, and that HA of

molecular weight between 400 and 900 kDa is capable of inducing proinflammatory

cytokines, while lower molecular weight species of HA are not. I also observed that the

average cytokine increase in LMWHA-treated cultures, calculated as the percent change

compared to vehicle-treated cultures from the same animal, was greater in CD44KO than

WT. Together with my data on proliferation and absolute cytokine levels (Figures 10 and 11), this demonstrates the enhanced responsiveness of immune cells in the absence of CD44.
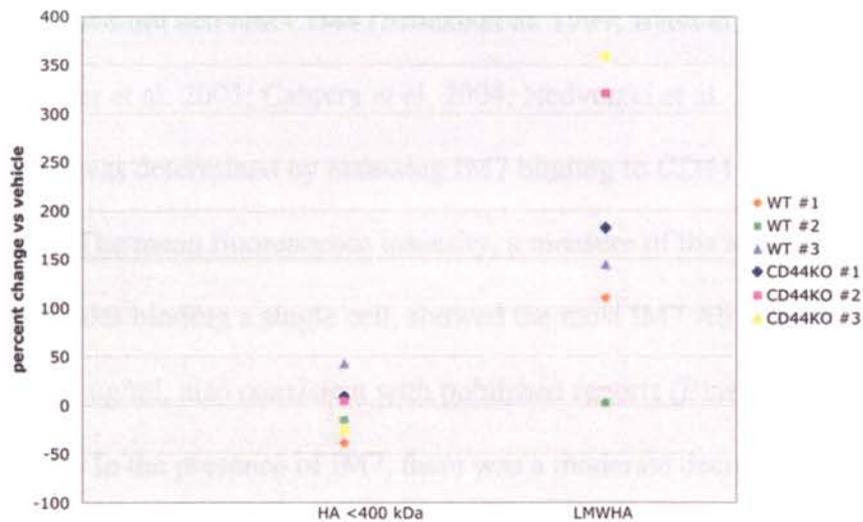
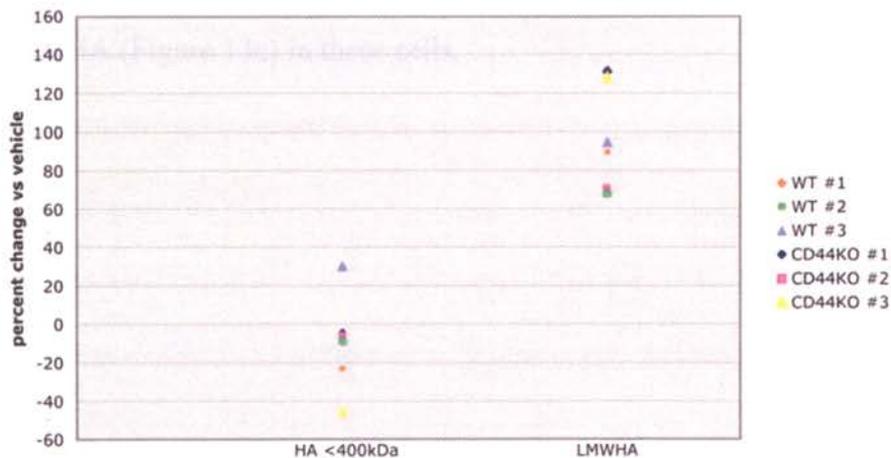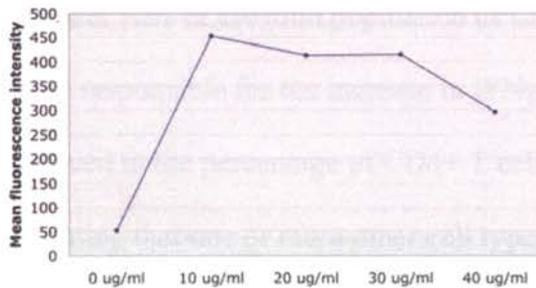Figure 12. CD44 is not required for cytokine response to LMWHA. Three CD44KO and three WT spleens were harvested and cultured. Following 72 hrs non-specific TCR stimulation with 1.0 ug/ml anti-CD3 antibody and treatment with vehicle, 100 ug/ml LMWHA (including species 400-900 kDa) or HA including species < 400kDa, culture supernatants were collected and analyzed for IFNγ (a) and TNFα (b) levels by ELISA.

To rule out the possibility that my results in the CD44KO animals were misleading due to compensation for the loss of CD44 by another HA receptor during the knock-out animals' development, I used the CD44 blocking antibody IM7 to inhibit CD44's ability to bind HA. This antibody prevents HA from binding to the CD44 receptor presumably by steric hindrance and, consistent with previous reports, has been shown to effectively block and not activate CD44 (Brocke et al. 1999; Blass et al. 2001; McKallip et al. 2002; Termeer et al. 2003; Cabrera et al. 2004; Nedvetzki et al. 2004). A saturating dose of 30 ug/ml was determined by assessing IM7 binding to CD44 via flow cytometry (Figure 13a). The mean fluorescence intensity, a measure of the average number of IM7 molecules binding a single cell, showed the most IM7 Ab bound at a dose of between 10 and 30 ug/ml, also consistent with published reports (Blass et al. 2001; McKallip et al. 2002). In the presence of IM7, there was a moderate decrease in LMWHA-induced IFNγ levels which was not significant due to standard error (figure 13b), and no decrease in LMWHA-induced TNFα levels, suggesting that CD44 is not mediating the response to LMWHA (Figure 13c) in these cells.

a **Saturation Curve of IM7 Ab staining of CD44+ splenocytes**

Mean fluorescence intensity

500
450
400
350
300
250
200
150
100
50
0

0 ug/ml   10 ug/ml   20 ug/ml   30 ug/ml   40 ug/ml

b **Effect of CD44 Blocking Abs on IFNγ Levels**

avg % change vs vehicle

140
120
100
80
60
40
20
0
-20
-40
-60

HA low   HA low + IM7   IM7

c **Effect of CD44 Blocking Abs on TNFα Production**

Avg % change vs vehicle

180
160
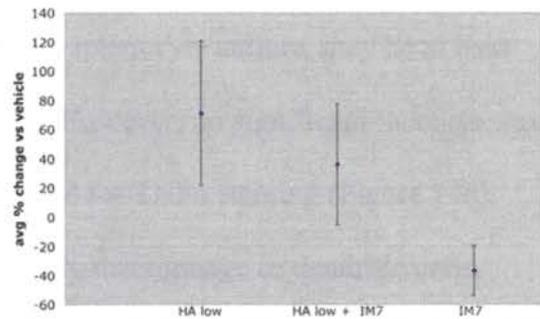140
120
100
80
60
40
20
0
-20

HA low   HA low + IM7   IM7 only

Figure 13. CD44 blocking antibodies do not significantly reduce LMWHA-induced increases in IFNγ (b) or TNFα levels (c). WT splenocyte cultures were treated with 30 ug/ml IM7 antibody for 90 minutes before the addition of anti-CD3 mAb and LMWHA. Flow cytometry was performed on activated splenocytes to determine the saturating dose of blocking antibody. Mean fluorescence intensity of IM7+ cells shows the saturating dose of IM7 antibody is between 10 and 30 ug/ml (a).
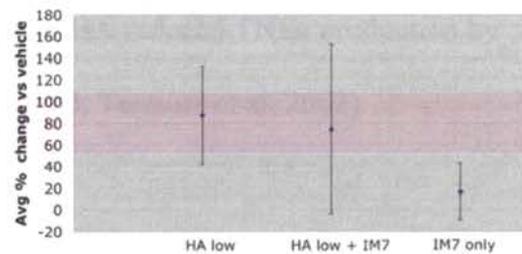
T cells increase IFNγ production in response to LMWHA

The rise in cytokine levels observed with LMWHA treatment could be due to a rise in production by the CD4+ T cells believed to mediate inflammation in MS and EAE, or to cytokine production by another cell type within the splenocyte culture. To test whether CD4+ cells were responsible for the increase, I performed intracellular staining of CD4+ T cells for IFNγ and TNFα and analyzed the results by flow cytometry. The percentage of CD4+ T cells positive for IFNγ immunostaining increased from 8% with vehicle treatment to 24% with LMWHA treatment (figure 14a). The mean fluorescence intensity per CD4+ T cell was also increased in response to LMWHA, indicating increased

expression of IFNγ on a per-cell basis (data not shown). HMWHA and MMWHA showed no change compared to vehicle. This result suggests CD4+ T cells, which make up about 10% of the total population of cells in the splenocyte culture, may be at least partly responsible for the increase in IFNγ levels. However, no significant increase was observed in the percentage of CD4+ T cells positive for TNFα staining (Figure 14b), indicating that one or more other cell types, possibly macrophage or dendritic cells, increase their expression of TNFα in response to LMWHA. This notion is consistent with studies that showed LMWHA increases TNFα levels in cultured macrophage (Noble et al. 1993) and very low molecular weight forms of HA induced TNFα production by dendritic cells (Noble et al. 1993; Termeer et al. 2000; Termeer et al. 2002).

a Percent of CD4+ T cells producing IFNγ

b Percent of CD4+ T cells producing TNFα
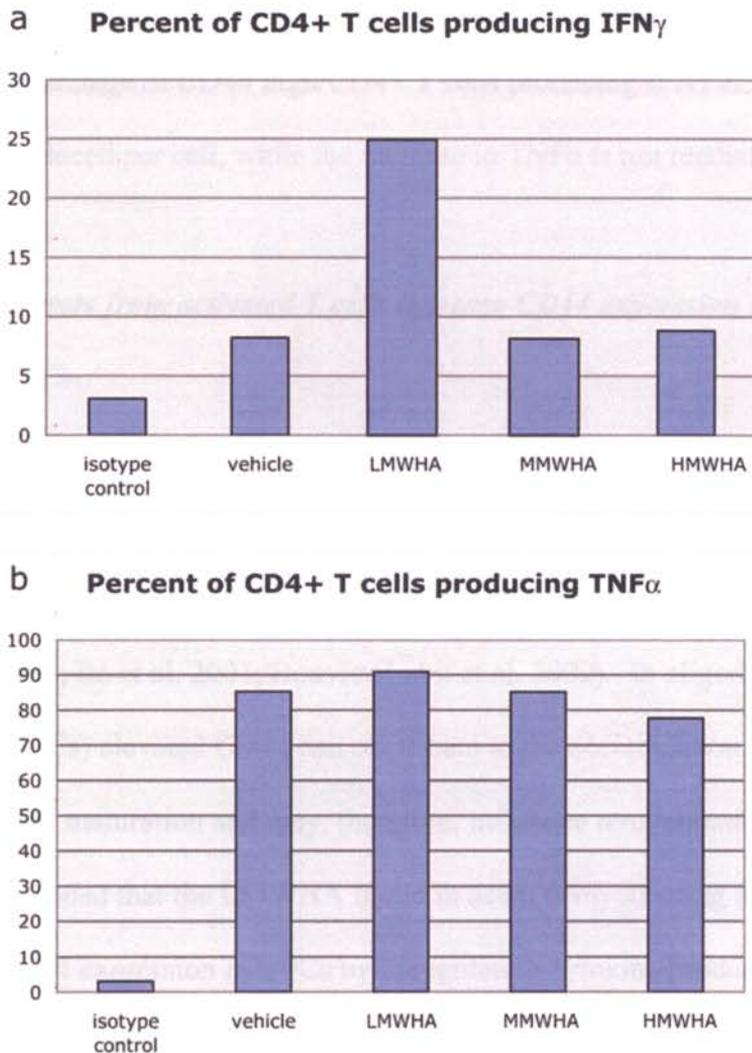
Figure 14. LMWHA increases the percentage of CD4+ T cells expressing IFNγ (a) but not TNFα (b). TCR transgenic splenocyte cultures were grown 72 hours in the presence of 1.0 ug/ml anti-CD3 antibody and various molecular weights of HA or vehicle. Intracellular cytokine staining was performed after 5 hours of Brefeldin A treatment, and cytokine expression was analyzed by flow cytometry.

In summary, I found that splenocytes from two different genetic backgrounds increase

proinflammatory cytokine production, but not proliferation, when treated with LMWHA.

T cells within these cultures express high levels of CD44, a major receptor for HA

binding. However, I determined that CD44 is not required for the proinflammatory

response to LMWHA, as shown by my data in CD44KO cell cultures and by blocking antibodies to CD44. The increase in IFNγ is due at least in part to an increase in the percentage of CD44 high/CD4+ T cells producing IFNγ and a greater amount of IFNγ produced per cell, while the increase in TNFα is not mediated by CD4+ T cells.

Factors from activated T cells increase CD44 expression by oligodendrocytes and OPCs

CD44 expression is known to be upregulated on glial cells during inflammatory demyelination such as occurs during EAE and MS (Haegel et al. 1993; Moretto et al. 1993; Ito et al. 2001; Bouvier-Labit et al. 2002). In oligodendrocyte progenitor cells (OPCs) elevated CD44 can contribute to the accumulation of HMWHA, which inhibits OPC maturation and may, therefore, influence remyelination failure (Back et al. 2005). I reasoned that the LMWHA found in acute demyelinating lesions may indirectly induce CD44 expression in OPCs by upregulating cytokine production by activated T cells. To test this hypothesis, I examined the effects of conditioned media from activated T cells on oligodendrocytes and OPCs. Oligodendrocytes were treated for 24 hours with conditioned media (CM) from activated T cell cultures isolated from EAE mice (see Materials and Methods). Anti-CD44 immunofluorescence revealed a marked increase in CD44 in CM-treated oligodendrocyte cultures compared to cultures treated with media only (Figure 15, a and b). I confirmed that cultures contained mature, myelinating oligodendrocytes by immunolabeling with an anti-myelin basic protein antibody (Figure

15, c, d, and e).
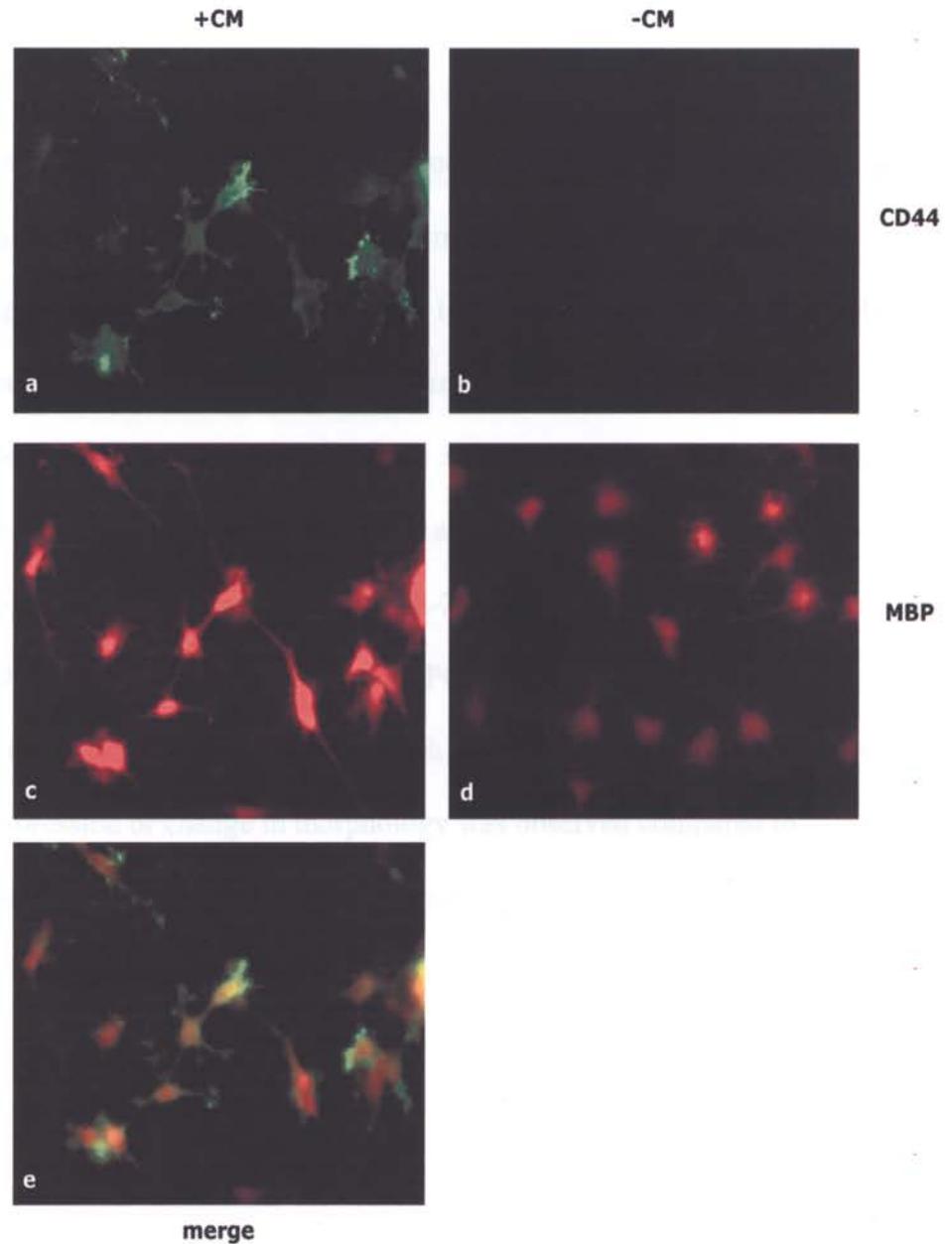
Figure 15. Factors from activated splenocytes increase CD44 expression by oligodendrocytes. Mature oligodendrocytes were treated for 24 hours with conditioned media (CM) from activated T cells. Immunofluorescence detection of CD44 increased in response to conditioned media (a and b). Oligodendrocyte myelin marker MBP immunofluorescence levels were similar in both conditioned media-treated (c) and IL-2 media-treated (d) cultures. The merged image (e) shows coexpression of CD44 and MBP.

Next, I investigated the effect of conditioned media on OPCs. These cells have been shown to accumulate around MS and EAE lesions and fail to differentiate into myelinating oligodendrocytes (Engel and Wolswijk 1996; Scolding et al. 1998; Maeda et al. 2001). I found that OPCs, like mature oligodendrocytes, increase CD44 expression in response to some factor or factors in the conditioned media from activated T cells. Untreated OPCs express low levels of CD44 (Figure 16a) and show an immature, bipolar morphology as detected by the OPC marker O1 (Figure 16b). OPCs treated 24 hours with conditioned media increase expression of CD44 and appear to have more elaborate processes and some membrane ruffling (Figure 16, c and d), however, this is still an immature morphology. To determine whether the IL-2 contained in the culture media had an effect on CD44 expression or morphology, OPCs were treated with the same concentration (1:1) of IL-2 media as cells treated with conditioned media (e) and (f). No increase in CD44 expression or change in morphology was observed compared to untreated preoligodendrocytes (Figure 16, a and b), indicating that these effects were due to factors produced by the T cells themselves.
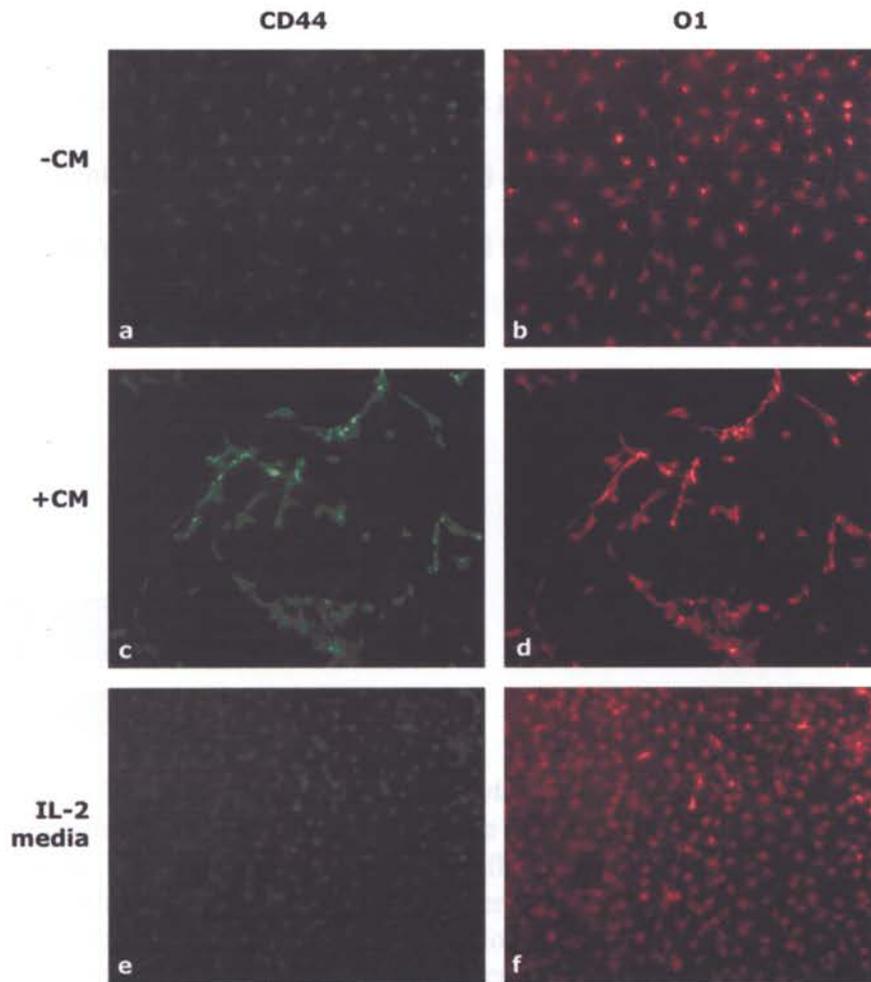
Figure 16. OPCs increase CD44 expression in response to factors from activated T cells. Untreated OPCs (a) express low levels of CD44 and show an immature, bipolar morphology as shown by the OPC marker O1 (b). OPCs treated for 24 hr with conditioned media from activated T cells increase expression of CD44, as shown by anti-CD44 immunofluorescence (c), and undergo morphological changes (d). No increase in CD44 expression or change in morphology was observed in OPCs treated with IL-2-containing media compared to untreated OPCs (compare (e) and (f) with (a) and (b).

To test if LMWHA further promoted the induction of CD44 in OPCs, I cultured OPCs with LMWHA, conditioned media (supernatant) from activated splenocyte cultures treated with LMWHA, or vehicle-treated conditioned media and performed Western blots on the OPC cell lysates as a means of quantitating changes in CD44 expression. As shown in Figure 17, lane 2, supernatants from activated splenocytes grown in the

presence of LMWHA increased CD44 expression by OPCs to a greater degree (3.1 fold) than vehicle-treated supernatant (lane 1), which increased CD44 levels 1.9 fold over treatment with T cell media ("vehicle") alone (lane 3). OPCs treated with 100 ug/ml LMWHA alone did not show enhanced CD44 expression compared to controls (lane 4).
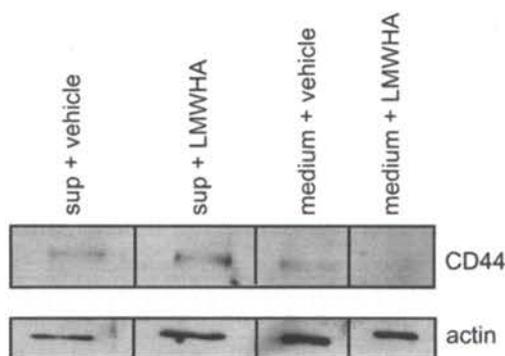
Figure 17. Supernatants from splenocyte cultures treated with LMWHA increase CD44 expression by OPCs. Splenocytes were cultured with antigen and 100 ug/ml LMWHA or vehicle. Supernatants were collected after 72 hours and added to OPC cultures in a 1:1 ratio with the OPC culture media. After 24 hours, cells were lysed and Western blotting was performed on the lysate. As additional controls, OPCs were treated with vehicle (RPMI Stim media used in splenocyte culture) or 100 ug/ml LMWHA. Bands were measured by by NIH Image J.

The conditioned media from splenocyte cultures that had been grown in the presence of LMWHA was assayed by ELISA and found to contain high levels of IFNγ and TNFα ; 1.52 ug/ml and 0.186 ug/ml, respectively. The upregulation of CD44 expression may be due to the presence of these proinflammatory cytokines, however, in a single attempt to determine this, antibody inhibition of IFNγ and TNFα did not have an effect. This experiment should be repeated for confirmation. The question remains whether other cytokines may be more important in inducing CD44 expression in cells of the oligodendroycte lineage.

Chapter 4: Discussion

The results presented in this thesis indicate that exogenous LMWHA increases the

production of proinflammatory cytokines IFNγ and TNFα by activated T cells and other

cells within splenocyte cultures. Activated T cells, expressing high levels of CD44 and

pericellular LMWHA, infiltrate the CNS during inflammatory demyelinating diseases

such as MS and EAE. The increase in LMWHA production by T cells may occur due to

signals from receptors on the activated T cells themselves, or signals from APCs to T

cells, which upregulate *de novo* production of LMWHA via HA synthases HAS1 and

HAS 3 (Spicer and Nguyen 1999). Alternatively, or in addition, high or medium

molecular weight HA may undergo degradation by hyaluronidases, which are

constitutively expressed by T cells but are also known to be more highly expressed in

pathological states (Sampson et al. 1992; Mohamadzadeh et al. 1998; Csoka et al. 1999).

The increased LMWHA made by CNS-infiltrating cells could then induce the production

of factors that elevate CD44 expression by oligodendrocytes and OPCs, making them

more susceptible to damage and cell death. A diagram outlining these ideas and how

LMWHA may contribute to inflammatory demyelinating diseases, including MS, is

shown in Figure 18.
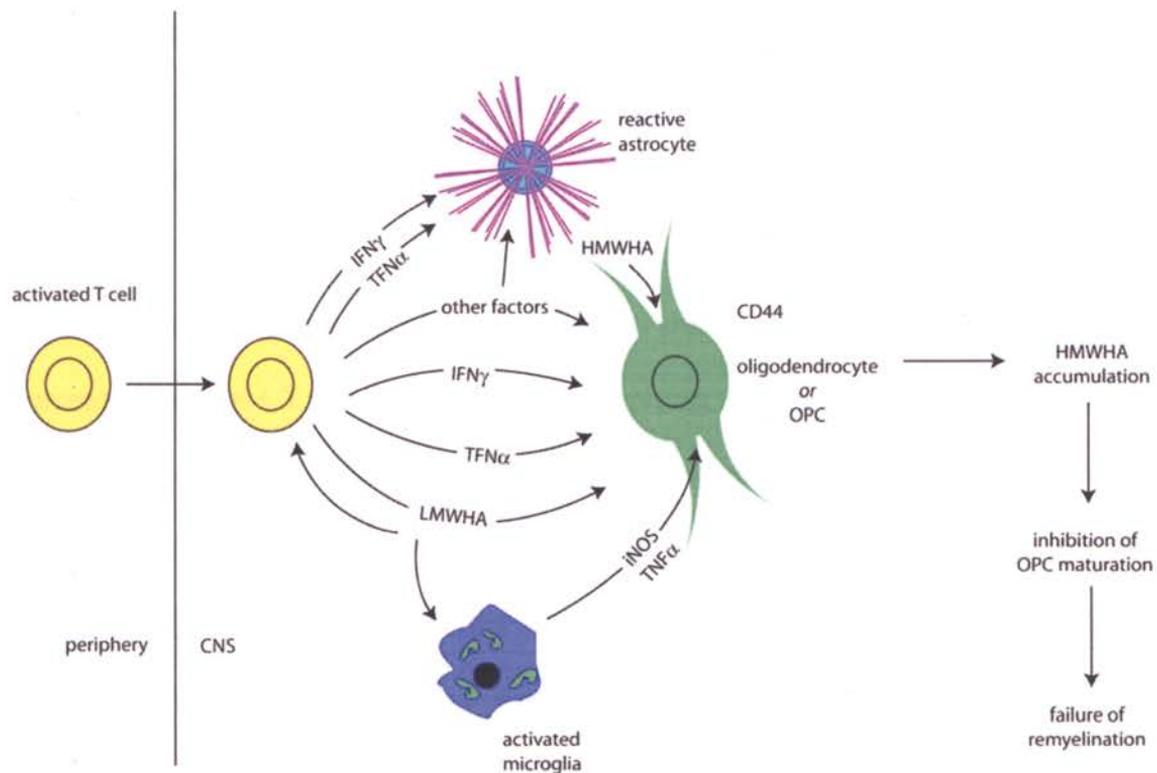
Figure 17. Proposed model for the sequence of events leading to failure of remyelination in MS and EAE. Activated T cells enter the CNS and produce proinflammatory mediators: IFNγ, TNFα, LMWHA, and other factors. These mediators activate microglia and increase HMWHA production by astrocytes, as well as upregulating CD44 expression in oligodendrocytes and OPCs. The interaction of CD44 and HMWHA inhibits OPC maturation, contributing to the failure of remyelination.

The LMWHA used in this study is similar to that produced by infilitrating T cells in MS and EAE lesions. I did not observe an increase in proinflammatory cytokines with forms of HA substantially higher than 800 kDa or lower than 300 kDa, indicating that only HA molecules in this specific size range are proinflammatory under the conditions tested. The increase in cytokines is not due to higher proliferation in cultures treated with LMWHA as none of the forms of HA tested enhanced proliferation. However, a study by Galandrini *et al* (2002) found that plate-immobilized HA of undefined molecular weight was costimulatory for anti-CD3 mAb-triggered peripheral blood T cell proliferation. This discrepancy could be explained by the difference in the action of soluble HA versus

plate-bound HA molecules, in which multimeric HA binding may be able to induce greater CD44 clustering and thereby enhance the proliferative signal. It is also possible that different sizes of HA can induce T cell proliferation under certain conditions.

My intracellular staining data for IFNγ and TNFα shows a significant increase in the percentage of CD4+ T cells producing IFNγ, implying that CD4+ T cells could be primarily responsible for the increase in IFNγ. The other cell type within the splenocyte culture that expresses IFNγ is the natural killer T cell (Sague et al. 2004), which may also increase IFNγ production in the presence of LMWHA. In the study by Sague et al, LMWHA (molecular weight range not specified) did not increase IFNγ in primary NK cell cultures except when IL-2, IL-12, or IL-18 was added. These cytokines are almost certainly present in activated splenocyte cultures, making it possible for NK cells to contribute to IFNγ levels under the conditions tested in my experiments.

The percentage of CD4+ T cells producing TNFα did not change with LMWHA treatment, although TNFα levels in the culture increased. This could indicate that the production of TNFα per CD4+ T cell increases with LMWHA treatment, or it could suggest that TNFα production is upregulated by another cell type within the culture. The most likely cell types would be macrophages and dendritic cells, as LMWHA with an average molecular weight of 250 kDa has been shown to activate monocytes and macrophages, which then produce TNFα (Noble et al. 1993; Hodge-Dufour et al. 1997). Also, HA oligosaccharides have been shown to induce maturation of human monocyte-

derived dendritic cells, with TNFα production being a hallmark of dendritic cell maturation (Termeer et al. 2000; Termeer et al. 2002).

My results suggest CD44 is not required to mediate the proinflammatory effects of LMWHA. Both CD44KO and WT splenocytes responded to treatment with LMWHA by increasing levels of IFNγ and TNFα. In CD44KO cultures, however, proliferation was roughly 5 times that of WT splenocytes, and overall levels of cytokines were higher in cultures from CD44KO mice, possibly due to greater proliferation. The increased responsiveness of CD44KO splenocytes to non-specific T cell stimulation suggests that the absence of CD44 may enhance inflammation. This may be due to the role of CD44 as a positive regulator of activation-induced cell death (McKallip et al. 2002), or to overcompensation by another receptor, whose expression is upregulated or whose signaling is potentiated in the absence of CD44 (Nedvetzki et al. 2004).

I also used CD44 blocking antibodies to determine the requirement for CD44 in the proinflammatory response to LMWHA. Using this approach, I found that blocking the CD44 receptor did not significantly inhibit LMWHA's ability to stimulate cytokine production, strengthening my conclusion that signaling through CD44 is not essential for LMWHA to increase the production of proinflammatory cytokines.

That upregulation of TNFα and IFNγ expression persists even in the absence of CD44 suggests signaling through another HA receptor may be involved. In the case of IFNγ, it may be that RHAMM compensates for the loss of CD44, as suggested by a study of

collagen-induced arthritis (CIA) in CD44KO mice. In this study, inflammation was exacerbated in the knock-out animals, although IFNγ levels were not tested (Nedvetzki et al. 2004). Other HA receptors expressed by T cells may also potentiate IFNγ production. Lyve-1, TLR2 and TLR4 are not expressed by T cells, and therefore would not be candidates for HA signaling in splenocyte cultures. It is unclear whether layilin is expressed by T cells (Bono et al. 2001).

The increase in TNFα levels observed in splenocyte cultures, but not in CD4+ T cell cultures (data not shown), could be due to HA binding and signaling through TLR4 or TLR2 on macrophage or dendritic cells. Earlier reports showed LMWHA can induce TNFα production by macrophages and dendritic cells (Hodge-Dufour et al. 1997; Termeer et al. 2000; Termeer et al. 2002) and a recent study showed LMWHA of approximately 200 kDa induced macrophage activation and TNFα production via a combination of TLR4 and TLR2 signaling (Jiang et al. 2005). In order to determine which cell type is responsible for the increase in TNFα seen in my splenocyte cultures, it should be tested whether LMWHA species of 400 kDa average molecular weight are capable of activating macrophages and dendritic cells, and assessing the relative contribution by these cell types to TNFα levels by intracellular staining and flow cytometry analysis. Blocking antibodies to TLR2 and TLR4, siRNA, and/or knockout mice could be used to determine whether these receptors are involved.

Cytokine-containing supernatants from splenocyte cultures induced CD44 expression in cells of the oligodendrocyte lineage, which normally express very little CD44 *in situ*. My

immunofluorescence studies demonstrated an increase in CD44 expression by oligodendrocytes and their progenitor cells when treated with supernatants from activated T cells isolated from EAE mice. These T cells (as a component of splenocyte cultures) had been further stimulated with anti-CD3 and anti-CD28 antibodies to enhance levels of IFNγ and TNFα in the supernatants. Western blot analysis of oligodendrocyte progenitor cells treated with supernatants from splenocyte cultures grown in the presence of LMWHA increased CD44 expression compared to treatment with supernatants from splenocytes grown in the presence of vehicle. The supernatants were assayed by ELISA and shown to contain high levels of IFNγ and TNFα. It is possible that these cytokines have a role in increasing CD44 expression in oligodendrocytes and OPCs, given that they are present in EAE and MS lesions in higher-than-normal concentrations (Cannella and Raine 1995; Akassoglou et al. 1998; Bitsch et al. 1998; Raine et al. 1998) and that CD44 expression is induced in glial cells in EAE and MS (Girgrah et al. 1991; Haegel et al. 1993) (Girgrah N 1991, Haegel H 1993). This possibility should be explored in future experiments, both *in vitro* and by use of siRNA inhibition of IFNγ and TNFα *in vivo*.

Upregulation of CD44 in oligodendrocytes and their precursors would likely enhance binding of HA and therefore the effects of its accumulation in the CNS in MS, EAE, and other neuroinflammatory states. CD44 expression may alter the uptake and degradation of HA in the lesion microenvironment. HMWHA has been shown to keep OPCs in an immature, premyelinating state (Back et al. 2005) by an as-yet unidentified mechanism, and the effect of LMWHA on this cell type has not been fully explored. My treatment of OPCs with LMWHA did not increase CD44 expression, indicating that other mechanisms

induce CD44 in OPCs. These would be interesting and important questions for additional research.

Taken together, these results imply that inhibiting the synthesis of LMWHA or degradation of HMWHA to LMWHA could reduce inflammation and the subsequent upregulation of CD44 expression on oligodendrocytes and OPCs, making them less susceptible to injury and apoptosis. HA synthases and hyaluronidases may be appropriate therapeutic targets in the treatment of MS and related diseases.

Summary and Conclusions

The major findings of this study include the following:

1) Activated T cells accumulate pericellular coats of HA coincident with elevated levels of CD44.

2) Neither LMWHA nor HMWHA increase proliferation of activated T cells.

3) LMWHA enhances the expression of proinflammatory cytokines by splenocytes.

4) Both CD44KO and WT splenocytes increase levels of IFNγ and TNFα in response to LMWHA.

5) Blocking antibodies to CD44 do not inhibit the proinflammatory effects of LMWHA.

6) CD44KO T cells proliferate more rapidly in response to non-specific stimulation of the T cell receptor than WT T cells.

7) CD44KO splenocytes show a more pronounced increase in proinflammatory cytokines in response to LMWHA than WT splenocytes.

8) Factors from activated T cells and other immune cells increase CD44 expression by oligodendrocytes and OPCs, an effect that is enhanced when T cells are grown in the presence of LMWHA.

This study has demonstrated a role for LMWHA in the production of proinflammatory cytokines IFNγ and TNFα, and shown that the CD44 receptor is not required to mediate the effects of LMWHA on cytokine production by immune cells. These data also suggest the inflammatory response is potentiated in the absence of CD44 on immune cells. In regards to the effects of immune cell activation on CNS-resident cells, I conclude that

factors expressed by activated splenocytes in response to LMWHA upregulate CD44 expression in oligodendrocytes and OPCs. Increased CD44 levels may increase binding by HMWHA, shown to be abundant in the lesions of MS and EAE, making OPCs more responsive to the maturation-inhibiting effects of CD44-HA interactions and impeding remyelination.

References

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* **3**:549-57.

Akassoglou, K., Bauer, J., Kassiotis, G., Pasparakis, M., Lassmann, H., Kollias, G. and Probert, L. (1998). Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. *Am J Pathol* **153**:801-13.

Akiyama, H., Tooyama, I., Kawamata, T., Ikeda, K. and McGeer, P. L. (1993). Morphological diversities of CD44 positive astrocytes in the cerebral cortex of normal subjects and patients with Alzheimer's disease. *Brain Res* **632**:249-59.

Ando, D. G., Clayton, J., Kono, D., Urban, J. L. and Sercarz, E. E. (1989). Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell Immunol* **124**:132-43.

Asher, R. and Bignami, A. (1992). Hyaluronate binding and CD44 expression in human glioblastoma cells and astrocytes. *Exp Cell Res* **203**:80-90.

Assmann, V., Marshall, J. F., Fieber, C., Hofmann, M. and Hart, I. R. (1998). The human hyaluronan receptor RHAMM is expressed as an intracellular protein in breast cancer cells. *J Cell Sci* **111** (Pt 12):1685-94.

Back, S. A., Tuohy, T. M., Chen, H., Wallingford, N., Craig, A., Struve, J., Luo, N. L., Banine, F., Liu, Y., Chang, A., Trapp, B. D., Bebo, B. F., Jr., Rao, M. S. and Sherman, L. S. (2005). Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nat Med* **11**:966-72.

Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J. and Aruffo, A. (1998). Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. *J Biol Chem* **273**:338-43.

Balashov, K. E., Smith, D. R., Khoury, S. J., Hafler, D. A. and Weiner, H. L. (1997). Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand. *Proc Natl Acad Sci U S A* **94**:599-603.

Balazs, E. A., Watson, D., Duff, I. F. and Roseman, S. (1967). Hyaluronic acid in synovial fluid. I. Molecular parameters of hyaluronic acid in normal and arthritis human fluids. *Arthritis Rheum* **10**:357-76.

Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M. and Jackson, D. G. (1999). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* **144**:789-801.

Barnett, M. H. and Prineas, J. W. (2004). Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol* **55**:458-68.

Baumann, N. and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev* **81**:871-927.

Becher, B., Durell, B. G. and Noelle, R. J. (2003). IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. *J Clin Invest* **112**:1186-91.

Becher, B., Giacomini, P. S., Pelletier, D., McCrea, E., Prat, A. and Antel, J. P. (1999). Interferon-gamma secretion by peripheral blood T-cell subsets in multiple sclerosis: correlation with disease phase and interferon-beta therapy. *Ann Neurol* **45**:247-50.

Bignami, A., Asher, R. and Perides, G. (1991). Brain extracellular matrix and nerve regeneration. *Adv Exp Med Biol* **296**:197-206.

Bignami, A., Perides, G., Asher, R. and Dahl, D. (1992). The astrocyte--extracellular matrix complex in CNS myelinated tracts: a comparative study on the distribution of hyaluronate in rat, goldfish and lamprey. *J Neurocytol* **21**:604-13.

Billiau, A., Heremans, H., Vandekerckhove, F., Dijkmans, R., Sobis, H., Meulepas, E. and Carton, H. (1988). Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* **140**:1506-10.

Bitsch, A., da Costa, C., Bunkowski, S., Weber, F., Rieckmann, P. and Bruck, W. (1998). Identification of macrophage populations expressing tumor necrosis factor-alpha mRNA in acute multiple sclerosis. *Acta Neuropathol (Berl)* **95**:373-7.

Bjermer, L., Lundgren, R. and Hallgren, R. (1989). Hyaluronan and type III procollagen peptide concentrations in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis. *Thorax* **44**:126-31.

Blass, S. L., Pure, E. and Hunter, C. A. (2001). A role for CD44 in the production of IFN-gamma and immunopathology during infection with Toxoplasma gondii. *J Immunol* **166**:5726-32.

Bono, P., Rubin, K., Higgins, J. M. and Hynes, R. O. (2001). Layilin, a novel integral membrane protein, is a hyaluronan receptor. *Mol Biol Cell* **12**:891-900.

Borowsky, M. L. and Hynes, R. O. (1998). Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. *J Cell Biol* **143**:429-42.

Bouvier-Labit, C., Liprandi, A., Monti, G., Pellissier, J. F. and Figarella-Branger, D. (2002). CD44H is expressed by cells of the oligodendrocyte lineage and by oligodendrogliomas in humans. *J Neurooncol* **60**:127-34.

Boyce, D. E., Thomas, A., Hart, J., Moore, K. and Harding, K. (1997). Hyaluronic acid induces tumour necrosis factor-alpha production by human macrophages in vitro. *Br J Plast Surg* **50**:362-8.

Brandt, K. D., Smith, G. N., Jr. and Simon, L. S. (2000). Intraarticular injection of hyaluronan as treatment for knee osteoarthritis: what is the evidence? *Arthritis Rheum* **43**:1192-203.

Brennan, F. R., O'Neill, J. K., Allen, S. J., Butter, C., Nuki, G. and Baker, D. (1999). CD44 is involved in selective leucocyte extravasation during inflammatory central nervous system disease. *Immunology* **98**:427-35.

Brocke, S., Piercy, C., Steinman, L., Weissman, I. L. and Veromaa, T. (1999). Antibodies to CD44 and integrin alpha4, but not L-selectin, prevent central nervous system inflammation and experimental encephalomyelitis by blocking secondary leukocyte recruitment. *Proc Natl Acad Sci U S A* **96**:6896-901.

Brown, S. J. (2006). The role of vitamin D in multiple sclerosis. *Ann Pharmacother* **40**:1158-61.

Cabrera, P. V., Blanco, G., Alaniz, L., Greczanik, S., Garcia, M., Alvarez, E. and Hajos, S. E. (2004). CD44 and hyaluronic acid regulate in vivo iNOS expression and

metalloproteinase activity in murine air-pouch inflammation. *Inflamm Res* **53**:556-66.

Cannella, B. and Raine, C. S. (1995). The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* **37**:424-35.

Chang, A., Nishiyama, A., Peterson, J., Prineas, J. and Trapp, B. D. (2000). NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci* **20**:6404-12.

Cheung, W. F., Cruz, T. F. and Turley, E. A. (1999). Receptor for hyaluronan-mediated motility (RHAMM), a hyaladherin that regulates cell responses to growth factors. *Biochem Soc Trans* **27**:135-42.

Chu, C. Q., Wittmer, S. and Dalton, D. K. (2000). Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med* **192**:123-8.

Cichy, J. and Pure, E. (2000). Oncostatin M and transforming growth factor-beta 1 induce post-translational modification and hyaluronan binding to CD44 in lung-derived epithelial tumor cells. *J Biol Chem* **275**:18061-9.

Cichy, J. and Pure, E. (2003). The liberation of CD44. *J Cell Biol* **161**:839-43.

Coffman, R. L., Varkila, K., Scott, P. and Chatelain, R. (1991). Role of cytokines in the differentiation of CD4+ T-cell subsets in vivo. *Immunol Rev* **123**:189-207.

Crawford, M. P., Yan, S. X., Ortega, S. B., Mehta, R. S., Hewitt, R. E., Price, D. A., Stastny, P., Douek, D. C., Koup, R. A., Racke, M. K. and Karandikar, N. J. (2004). High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood* **103**:4222-31.

Cross, A. H., Misko, T. P., Lin, R. F., Hickey, W. F., Trotter, J. L. and Tilton, R. G. (1994). Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J Clin Invest* **93**:2684-90.

Csoka, A. B., Scherer, S. W. and Stern, R. (1999). Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics* **60**:356-61.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A. and Sedgwick, J. D. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**:744-8.

Culty, M., Nguyen, H. A. and Underhill, C. B. (1992). The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. *J Cell Biol* **116**:1055-62.

D'Elios, M. and Del Prete, G. (1998). Th1/Th2 balance in human disease. *Transplant Proc* **30**:2373-7.

Decken, K., Kohler, G., Palmer-Lehmann, K., Wunderlin, A., Mattner, F., Magram, J., Gately, M. K. and Alber, G. (1998). Interleukin-12 is essential for a protective Th1 response in mice infected with Cryptococcus neoformans. *Infect Immun* **66**:4994-5000.

DeGrendele, H. C., Estess, P. and Siegelman, M. H. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* **278**:672-5.

Denhardt, D. T., Noda, M., O'Regan, A. W., Pavlin, D. and Berman, J. S. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* **107**:1055-61.

Eggli, P. S., Lucocq, J., Ott, P., Graber, W. and van der Zypen, E. (1992). Ultrastructural localization of hyaluronan in myelin sheaths of the rat central and rat and human peripheral nervous systems using hyaluronan-binding protein-gold and link protein-gold. *Neuroscience* **48**:737-44.

Engel, U. and Wolswijk, G. (1996). Oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from adult rat spinal cord: in vitro characteristics and response to PDGF, bFGF and NT-3. *Glia* **16**:16-26.

English, N. M., Lesley, J. F. and Hyman, R. (1998). Site-specific de-N-glycosylation of CD44 can activate hyaluronan binding, and CD44 activation states show distinct threshold densities for hyaluronan binding. *Cancer Res* **58**:3736-42.

Estess, P., DeGrendele, H. C., Pascual, V. and Siegelman, M. H. (1998). Functional activation of lymphocyte CD44 in peripheral blood is a marker of autoimmune disease activity. *J Clin Invest* **102**:1173-82.

Farrar, J. D., Asnagli, H. and Murphy, K. M. (2002). T helper subset development: roles of instruction, selection, and transcription. *J Clin Invest* **109**:431-5.

Fraser, J. R., Laurent, T. C. and Laurent, U. B. (1997). Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* **242**:27-33.

Frohman, E. M., Racke, M. K. and Raine, C. S. (2006). Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* **354**:942-55.

Fujii, K., Tanaka, Y., Hubscher, S., Saito, K., Ota, T. and Eto, S. (1999). Crosslinking of CD44 on rheumatoid synovial cells augment interleukin 6 production. *Lab Invest* **79**:1439-46.

Galandrini, R., Galluzzo, E., Albi, N., Grossi, C. E. and Velardi, A. (1994). Hyaluronate is costimulatory for human T cell effector functions and binds to CD44 on activated T cells. *J Immunol* **153**:21-31.

Gazzinelli, R. T., Hartley, J. W., Fredrickson, T. N., Chattopadhyay, S. K., Sher, A. and Morse, H. C., 3rd (1992). Opportunistic infections and retrovirus-induced immunodeficiency: studies of acute and chronic infections with Toxoplasma gondii in mice infected with LP-BM5 murine leukemia viruses. *Infect Immun* **60**:4394-401.

Genain, C. P., Cannella, B., Hauser, S. L. and Raine, C. S. (1999). Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* **5**:170-5.

Girgrah, N., Letarte, M., Becker, L. E., Cruz, T. F., Theriault, E. and Moscarello, M. A. (1991). Localization of the CD44 glycoprotein to fibrous astrocytes in normal white matter and to reactive astrocytes in active lesions in multiple sclerosis. *J Neuropathol Exp Neurol* **50**:779-92.

Goa, K. L. and Benfield, P. (1994). Hyaluronic acid. A review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing. *Drugs* **47**:536-66.

Gran, B., Hemmer, B. and Martin, R. (1999). Molecular mimicry and multiple sclerosis--a possible role for degenerate T cell recognition in the induction of autoimmune responses. *J Neural Transm Suppl* **55**:19-31.

Gran, B. and Rostami, A. (2001). T cells, cytokines, and autoantigens in multiple sclerosis. *Curr Neurol Neurosci Rep* **1**:263-70.

Greter, M., Heppner, F. L., Lemos, M. P., Odermatt, B. M., Goebels, N., Laufer, T., Noelle, R. J. and Becher, B. (2005). Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat Med* **11**:328-34.

Haegel, H., Tolg, C., Hofmann, M. and Ceredig, R. (1993). Activated mouse astrocytes and T cells express similar CD44 variants. Role of CD44 in astrocyte/T cell binding. *J Cell Biol* **122**:1067-77.

Hemmer, B., Fleckenstein, B. T., Vergelli, M., Jung, G., McFarland, H., Martin, R. and Wiesmuller, K. H. (1997). Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J Exp Med* **185**:1651-9.

Hemmer, B., Gran, B., Zhao, Y., Marques, A., Pascal, J., Tzou, A., Kondo, T., Cortese, I., Bielekova, B., Straus, S. E., McFarland, H. F., Houghten, R., Simon, R., Pinilla, C. and Martin, R. (1999). Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. *Nat Med* **5**:1375-82.

Heppner, F. L., Greter, M., Marino, D., Falsig, J., Raivich, G., Hovelmeyer, N., Waisman, A., Rulicke, T., Prinz, M., Priller, J., Becher, B. and Aguzzi, A. (2005). Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med* **11**:146-52.

Hoare, K., Savani, R. C., Wang, C., Yang, B. and Turley, E. A. (1993). Identification of hyaluronan binding proteins using a biotinylated hyaluronan probe. *Connect Tissue Res* **30**:117-26.

Hodge-Dufour, J., Noble, P. W., Horton, M. R., Bao, C., Wysoka, M., Burdick, M. D., Strieter, R. M., Trinchieri, G. and Pure, E. (1997). Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. *J Immunol* **159**:2492-500.

Hofmann, M., Fieber, C., Assmann, V., Gottlicher, M., Sleeman, J., Plug, R., Howells, N., von Stein, O., Ponta, H. and Herrlich, P. (1998). Identification of IHABP, a 95 kDa intracellular hyaluronate binding protein. *J Cell Sci* **111 (Pt 12)**:1673-84.

Hogan, M. V., Pawlowska, Z., Yang, H. A., Kornecki, E. and Ehrlich, Y. H. (1995). Surface phosphorylation by ecto-protein kinase C in brain neurons: a target for Alzheimer's beta-amyloid peptides. *J Neurochem* **65**:2022-30.

Horner, P. J., Power, A. E., Kempermann, G., Kuhn, H. G., Palmer, T. D., Winkler, J., Thal, L. J. and Gage, F. H. (2000). Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J Neurosci* **20**:2218-28.

Horton, M. R., Burdick, M. D., Strieter, R. M., Bao, C. and Noble, P. W. (1998). Regulation of hyaluronan-induced chemokine gene expression by IL-10 and IFN-gamma in mouse macrophages. *J Immunol* **160**:3023-30.

Huang, L., Grammatikakis, N., Yoneda, M., Banerjee, S. D. and Toole, B. P. (2000). Molecular characterization of a novel intracellular hyaluronan-binding protein. *J Biol Chem* **275**:29829-39.

Ikeda, K., Nakao, J., Asou, H., Toya, S., Shinoda, J. and Uyemura, K. (1996). Expression of CD44H in the cells of neural crest origin in peripheral nervous system. *Neuroreport* **7**:1713-6.

Ito, M., Blumberg, B. M., Mock, D. J., Goodman, A. D., Moser, A. B., Moser, H. W., Smith, K. D. and Powers, J. M. (2001). Potential environmental and host participants in the early white matter lesion of adreno-leukodystrophy: morphologic evidence for CD8 cytotoxic T cells, cytolysis of oligodendrocytes, and CD1-mediated lipid antigen presentation. *J Neuropathol Exp Neurol* **60**:1004-19.

Jalkanen, S. and Jalkanen, M. (1992). Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol* **116**:817-25.

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G. D., Mascarenhas, M. M., Garg, H. G., Quinn, D. A., Homer, R. J., Goldstein, D. R., Bucala, R., Lee, P. J., Medzhitov, R. and Noble, P. W. (2005). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* **11**:1173-9.

Jones, L. L., Liu, Z., Shen, J., Werner, A., Kreutzberg, G. W. and Raivich, G. (2000). Regulation of the cell adhesion molecule CD44 after nerve transection and direct trauma to the mouse brain. *J Comp Neurol* **426**:468-92.

Kang, B. Y. and Kim, T. S. (2006). Targeting cytokines of the interleukin-12 family in autoimmunity. *Curr Med Chem* **13**:1149-56.

Katoh, S., Zheng, Z., Oritani, K., Shimozato, T. and Kincade, P. W. (1995). Glycosylation of CD44 negatively regulates its recognition of hyaluronan. *J Exp Med* **182**:419-29.

Kieseier, B. C., Hemmer, B. and Hartung, H. P. (2005). Multiple sclerosis--novel insights and new therapeutic strategies. *Curr Opin Neurol* **18**:211-20.

Kivisakk, P., Mahad, D. J., Callahan, M. K., Sikora, K., Trebst, C., Tucky, B., Wujek, J., Ravid, R., Staugaitis, S. M., Lassmann, H. and Ransohoff, R. M. (2004). Expression of CCR7 in multiple sclerosis: implications for CNS immunity. *Ann Neurol* **55**:627-38.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. and Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* **201**:233-40.

Lehmann, P. V., Forsthuber, T., Miller, A. and Sercarz, E. E. (1992). Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **358**:155-7.

Lesley, J., English, N., Charles, C. and Hyman, R. (2000). The role of the CD44 cytoplasmic and transmembrane domains in constitutive and inducible hyaluronan binding. *Eur J Immunol* **30**:245-53.

Lesley, J., English, N., Perschl, A., Gregoroff, J. and Hyman, R. (1995). Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. *J Exp Med* **182**:431-7.

Lesley, J., Hyman, R. and Kincade, P. W. (1993). CD44 and its interaction with extracellular matrix. *Adv Immunol* **54**:271-335.

Levesque, M. C. and Haynes, B. F. (1997). Cytokine induction of the ability of human monocyte CD44 to bind hyaluronan is mediated primarily by TNF-alpha and is inhibited by IL-4 and IL-13. *J Immunol* **159**:6184-94.

Liu, Y., Han, S. S., Wu, Y., Tuohy, T. M., Xue, H., Cai, J., Back, S. A., Sherman, L. S., Fischer, I. and Rao, M. S. (2004). CD44 expression identifies astrocyte-restricted precursor cells. *Dev Biol* **276**:31-46.

Liu, Y., Wu, Y., Lee, J. C., Xue, H., Pevny, L. H., Kaprielian, Z. and Rao, M. S. (2002). Oligodendrocyte and astrocyte development in rodents: an in situ and immunohistological analysis during embryonic development. *Glia* **40**:25-43.

Lovett-Racke, A. E., Trotter, J. L., Lauber, J., Perrin, P. J., June, C. H. and Racke, M. K. (1998). Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest* **101**:725-30.

Maeda, Y., Solanky, M., Menonna, J., Chapin, J., Li, W. and Dowling, P. (2001). Platelet-derived growth factor-alpha receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. *Ann Neurol* **49**:776-85.

Maiti, A., Maki, G. and Johnson, P. (1998). TNF-alpha induction of CD44-mediated leukocyte adhesion by sulfation. *Science* **282**:941-3.

Marret, S., Delpech, B., Delpech, A., Asou, H., Girard, N., Courel, M. N., Chauzy, C., Maingonnat, C. and Fessard, C. (1994). Expression and effects of hyaluronan and of the hyaluronan-binding protein hyaluronectin in newborn rat brain glial cell cultures. *J Neurochem* **62**:1285-95.

Martin, R. and McFarland, H. F. (1995). Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit Rev Clin Lab Sci* **32**:121-82.

McDonald, J. W. and Belegu, V. (2006). Demyelination and remyelination after spinal cord injury. *J Neurotrauma* **23**:345-59.

McHeyzer-Williams, M. G. and Davis, M. M. (1995). Antigen-specific development of primary and memory T cells in vivo. *Science* **268**:106-11.

McKallip, R. J., Do, Y., Fisher, M. T., Robertson, J. L., Nagarkatti, P. S. and Nagarkatti, M. (2002). Role of CD44 in activation-induced cell death: CD44-deficient mice exhibit enhanced T cell response to conventional and superantigens. *Int Immunol* **14**:1015-26.

McKee, C. M., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., Choi, A. M. and Noble, P. W. (1997). Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. *J Biol Chem* **272**:8013-8.

McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C. and Noble, P. W. (1996). Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest* **98**:2403-13.

McMahon, E. J., Bailey, S. L., Castenada, C. V., Waldner, H. and Miller, S. D. (2005). Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med* **11**:335-9.

McRae, B. L., Vanderlugt, C. L., Dal Canto, M. C. and Miller, S. D. (1995). Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med* **182**:75-85.

Mi, S., Miller, R. H., Lee, X., Scott, M. L., Shulag-Morskaya, S., Shao, Z., Chang, J., Thill, G., Levesque, M., Zhang, M., Hession, C., Sah, D., Trapp, B., He, Z., Jung, V., McCoy, J. M. and Pepinsky, R. B. (2005). LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat Neurosci* **8**:745-51.

Mikecz, K., Dennis, K., Shi, M. and Kim, J. H. (1999). Modulation of hyaluronan receptor (CD44) function in vivo in a murine model of rheumatoid arthritis. *Arthritis Rheum* **42**:659-68.

Miller, S. D., Vanderlugt, C. L., Begolka, W. S., Pao, W., Neville, K. L., Yauch, R. L. and Kim, B. S. (1997). Epitope spreading leads to myelin-specific autoimmune responses in SJL mice chronically infected with Theiler's virus. *J Neurovirol* **3 Suppl 1**:S62-5.

Mohamadzadeh, M., DeGrendele, H., Arizpe, H., Estess, P. and Siegelman, M. (1998). Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J Clin Invest* **101**:97-108.

Moretto, G., Xu, R. Y. and Kim, S. U. (1993). CD44 expression in human astrocytes and oligodendrocytes in culture. *J Neuropathol Exp Neurol* **52**:419-23.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**:2348-57.

Murphy, K. M. and Reiner, S. L. (2002). The lineage decisions of helper T cells. *Nat Rev Immunol* **2**:933-44.

Mustafa, A., McKallip, R. J., Fisher, M., Duncan, R., Nagarkatti, P. S. and Nagarkatti, M. (2002). Regulation of interleukin-2-induced vascular leak syndrome by targeting CD44 using hyaluronic acid and anti-CD44 antibodies. *J Immunother* **25**:476-88.

Nakamura, K., Yokohama, S., Yoneda, M., Okamoto, S., Tamaki, Y., Ito, T., Okada, M., Aso, K. and Makino, I. (2004). High, but not low, molecular weight hyaluronan prevents T-cell-mediated liver injury by reducing proinflammatory cytokines in mice. *J Gastroenterol* **39**:346-54.

Naor, D., Sionov, R. V. and Ish-Shalom, D. (1997). CD44: structure, function, and association with the malignant process. *Adv Cancer Res* **71**:241-319.

Naujokas, M. F., Morin, M., Anderson, M. S., Peterson, M. and Miller, J. (1993). The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* **74**:257-68.

Nedvetzki, S., Gonen, E., Assayag, N., Reich, R., Williams, R. O., Thurmond, R. L., Huang, J. F., Neudecker, B. A., Wang, F. S., Turley, E. A. and Naor, D. (2004). RHAMM, a receptor for hyaluronan-mediated motility, compensates for CD44 in inflamed CD44-knockout mice: a different interpretation of redundancy. *Proc Natl Acad Sci U S A* **101**:18081-6.

Neumann, A., Schinzel, R., Palm, D., Riederer, P. and Munch, G. (1999). High molecular weight hyaluronic acid inhibits advanced glycation endproduct-induced NF-kappaB activation and cytokine expression. *FEBS Lett* **453**:283-7.

Neurath, M. F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A., Mizoguchi, E., Mudter, J., Galle, P. R., Bhan, A., Autschbach, F., Sullivan, B. M., Szabo, S. J., Glimcher, L. H. and Blumberg, R. S. (2002). The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* **195**:1129-43.

Neville, K. L., Padilla, J. and Miller, S. D. (2002). Myelin-specific tolerance attenuates the progression of a virus-induced demyelinating disease: implications for the treatment of MS. *J Neuroimmunol* **123**:18-29.

Noble, P. W., Lake, F. R., Henson, P. M. and Riches, D. W. (1993). Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. *J Clin Invest* **91**:2368-77.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**:275-83.

O'Garra, A., McEvoy, L. M. and Zlotnik, A. (1998). T-cell subsets: chemokine receptors guide the way. *Curr Biol* **8**:R646-9.

Okuda, Y., Nakatsuji, Y., Fujimura, H., Esumi, H., Ogura, T., Yanagihara, T. and Sakoda, S. (1995). Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J Neuroimmunol* **62**:103-12.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F. and Kastelein, R. A. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**:715-25.

Owens, T., Wekerle, H. and Antel, J. (2001). Genetic models for CNS inflammation. *Nat Med* **7**:161-6.

Panitch, H. S., Hirsch, R. L., Schindler, J. and Johnson, K. P. (1987). Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. *Neurology* **37**:1097-102.

Peach, R. J., Hollenbaugh, D., Stamenkovic, I. and Aruffo, A. (1993). Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J Cell Biol* **122**:257-64.

Pedotti, R., DeVoss, J. J., Youssef, S., Mitchell, D., Wedemeyer, J., Madanat, R., Garren, H., Fontoura, P., Tsai, M., Galli, S. J., Sobel, R. A. and Steinman, L. (2003). Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination. *Proc Natl Acad Sci U S A* **100**:1867-72.

Peterson, J. W., Bo, L., Mork, S., Chang, A., Ransohoff, R. M. and Trapp, B. D. (2002). VCAM-1-positive microglia target oligodendrocytes at the border of multiple sclerosis lesions. *J Neuropathol Exp Neurol* **61**:539-46.

Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., de Waal Malefyt, R., Rennick, D. and Kastelein, R. A. (2002). IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* **16**:779-90.

Pitt, D., Werner, P. and Raine, C. S. (2000). Glutamate excitotoxicity in a model of multiple sclerosis. *Nat Med* **6**:67-70.

Ponta, H., Sherman, L. and Herrlich, P. A. (2003). CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* **4**:33-45.

Prat, A. and Antel, J. (2005). Pathogenesis of multiple sclerosis. *Curr Opin Neurol* **18**:225-30.

Prineas, J. W., Barnard, R. O., Kwon, E. E., Sharer, L. R. and Cho, E. S. (1993). Multiple sclerosis: remyelination of nascent lesions. *Ann Neurol* **33**:137-51.

Prineas, J. W. and Graham, J. S. (1981). Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* **10**:149-58.

Probert, L., Eugster, H. P., Akassoglou, K., Bauer, J., Frei, K., Lassmann, H. and Fontana, A. (2000). TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease. *Brain* **123 (Pt 10)**:2005-19.

Protin, U., Schweighoffer, T., Jochum, W. and Hilberg, F. (1999). CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *J Immunol* **163**:4917-23.

Pure, E., Camp, R. L., Peritt, D., Panettieri, R. A., Jr., Lazaar, A. L. and Nayak, S. (1995). Defective phosphorylation and hyaluronate binding of CD44 with point mutations in the cytoplasmic domain. *J Exp Med* **181**:55-62.

Rafi-Janajreh, A. Q., Chen, D., Schmits, R., Mak, T. W., Grayson, R. L., Sponenberg, D. P., Nagarkatti, M. and Nagarkatti, P. S. (1999). Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J Immunol* **163**:1619-27.

Raine, C. S., Bonetti, B. and Cannella, B. (1998). Multiple sclerosis: expression of molecules of the tumor necrosis factor ligand and receptor families in relationship to the demyelinated plaque. *Rev Neurol (Paris)* **154**:577-85.

Raine, C. S., Cannella, B., Hauser, S. L. and Genain, C. P. (1999). Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann Neurol* **46**:144-60.

Rilla, K., Lammi, M. J., Sironen, R., Torronen, K., Luukkonen, M., Hascall, V. C., Midura, R. J., Hyttinen, M., Pelkonen, J., Tammi, M. and Tammi, R. (2002). Changed lamellipodial extension, adhesion plaques and migration in epidermal keratinocytes containing constitutively expressed sense and antisense hyaluronan synthase 2 (Has2) genes. *J Cell Sci* **115**:3633-43.

Rockey, D. C. and Chung, J. J. (1997). Regulation of inducible nitric oxide synthase and nitric oxide during hepatic injury and fibrogenesis. *Am J Physiol* **273**:G124-30.

Romagnani, S. (1996). Development of Th 1- or Th 2-dominated immune responses: what about the polarizing signals? *Int J Clin Lab Res* **26**:83-98.

Sague, S. L., Tato, C., Pure, E. and Hunter, C. A. (2004). The regulation and activation of CD44 by natural killer (NK) cells and its role in the production of IFN-gamma. *J Interferon Cytokine Res* **24**:301-9.

Sampson, P. M., Rochester, C. L., Freundlich, B. and Elias, J. A. (1992). Cytokine regulation of human lung fibroblast hyaluronan (hyaluronic acid) production. Evidence for cytokine-regulated hyaluronan (hyaluronic acid) degradation and human lung fibroblast-derived hyaluronidase. *J Clin Invest* **90**:1492-503.

Schall, T. J., Bacon, K., Camp, R. D., Kaspari, J. W. and Goeddel, D. V. (1993). Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta chemokines attract distinct populations of lymphocytes. *J Exp Med* **177**:1821-6.

Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., Furlonger, C., Zakarian, A., Simard, J. J.,

Ohashi, P. S., Paige, C. J., Gutierrez-Ramos, J. C. and Mak, T. W. (1997). CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* **90**:2217-33.

Scholz, C., Patton, K. T., Anderson, D. E., Freeman, G. J. and Hafler, D. A. (1998). Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J Immunol* **160**:1532-8.

Scolding, N., Franklin, R., Stevens, S., Heldin, C. H., Compston, A. and Newcombe, J. (1998). Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. *Brain* **121** (Pt 12):2221-8.

Segal, B. M. and Cross, A. H. (2000). Fas(t) track to apoptosis in MS: TNF receptors may suppress or potentiate CNS demyelination. *Neurology* **55**:906-7.

Selmaj, K. and Raine, C. S. (1988). Tumor necrosis factor mediates myelin damage in organotypic cultures of nervous tissue. *Ann N Y Acad Sci* **540**:568-70.

Selmaj, K. W. and Raine, C. S. (1988). Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol* **23**:339-46.

Sharief, M. K., McLean, B. and Thompson, E. J. (1993). Elevated serum levels of tumor necrosis factor-alpha in Guillain-Barre syndrome. *Ann Neurol* **33**:591-6.

Sherman, L. S., Rizvi, T. A., Karyala, S. and Ratner, N. (2000). CD44 enhances neuregulin signaling by Schwann cells. *J Cell Biol* **150**:1071-84.

Sherman, L. S., Struve, J. N., Rangwala, R., Wallingford, N. M., Tuohy, T. M. and Kuntz, C. t. (2002). Hyaluronate-based extracellular matrix: keeping glia in their place. *Glia* **38**:93-102.

Sim, F. J., Zhao, C., Penderis, J. and Franklin, R. J. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J Neurosci* **22**:2451-9.

Simon, A. K., Seipelt, E. and Sieper, J. (1994). Divergent T-cell cytokine patterns in inflammatory arthritis. *Proc Natl Acad Sci U S A* **91**:8562-6.

Skelton, T. P., Zeng, C., Nocks, A. and Stamenkovic, I. (1998). Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. *J Cell Biol* **140**:431-46.

Sleeman, J. P., Kondo, K., Moll, J., Ponta, H. and Herrlich, P. (1997). Variant exons v6 and v7 together expand the repertoire of glycosaminoglycans bound by CD44. *J Biol Chem* **272**:31837-44.

Spicer, A. P. and Nguyen, T. K. (1999). Mammalian hyaluronan synthases: investigation of functional relationships in vivo. *Biochem Soc Trans* **27**:109-15.

Steinman, L. (2001). Multiple sclerosis: a two-stage disease. *Nat Immunol* **2**:762-4.

Steinman, L. and Zamvil, S. (2003). Transcriptional analysis of targets in multiple sclerosis. *Nat Rev Immunol* **3**:483-92.

Stoop, R., Kotani, H., McNeish, J. D., Otterness, I. G. and Mikecz, K. (2001). Increased resistance to collagen-induced arthritis in CD44-deficient DBA/1 mice. *Arthritis Rheum* **44**:2922-31.

Taylor, K. R., Trowbridge, J. M., Rudisill, J. A., Termeer, C. C., Simon, J. C. and Gallo, R. L. (2004). Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* **279**:17079-84.

Termeer, C., Averbeck, M., Hara, H., Eibel, H., Herrlich, P., Sleeman, J. and Simon, J. C. (2003). Targeting dendritic cells with CD44 monoclonal antibodies selectively

inhibits the proliferation of naive CD4+ T-helper cells by induction of FAS-independent T-cell apoptosis. *Immunology* **109**:32-40.

Termeer, C., Benedix, F., Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Freudenberg, M., Galanos, C. and Simon, J. C. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* **195**:99-111.

Termeer, C. C., Hennies, J., Voith, U., Ahrens, T., Weiss, J. M., Prehm, P. and Simon, J. C. (2000). Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J Immunol* **165**:1863-70.

Toole, B. P. (2004). Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* **4**:528-39.

Traugott, U., Scheinberg, L. C. and Raine, C. S. (1981). Lymphocyte responsiveness to oligodendrocytes in multiple sclerosis. *J Neuroimmunol* **1**:41-51.

Tuohy, T. M., Wallingford, N., Liu, Y., Chan, F. H., Rizvi, T., Xing, R., Bebo, B., Rao, M. S. and Sherman, L. S. (2004). CD44 overexpression by oligodendrocytes: a novel mouse model of inflammation-independent demyelination and dysmyelination. *Glia* **47**:335-45.

Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A. and Kinkel, P. R. (1999). Regression and spreading of self-recognition during the development of autoimmune demyelinating disease. *J Autoimmun* **13**:11-20.

Turley, E. and Moore, D. (1984). Hyaluronate binding proteins also bind to fibronectin, laminin and collagen. *Biochem Biophys Res Commun* **121**:808-14.

Vanderlugt, C. L. and Miller, S. D. (2002). Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* **2**:85-95.

Vanderlugt, C. L., Rahbe, S. M., Elliott, P. J., Dal Canto, M. C. and Miller, S. D. (2000). Treatment of established relapsing experimental autoimmune encephalomyelitis with the proteasome inhibitor PS-519. *J Autoimmun* **14**:205-11.

Weber, G. F., Ashkar, S., Glimcher, M. J. and Cantor, H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* **271**:509-12.

Weigel, P. H., Hascall, V. C. and Tammi, M. (1997). Hyaluronan synthases. *J Biol Chem* **272**:13997-4000.

West, D. C., Hampson, I. N., Arnold, F. and Kumar, S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science* **228**:1324-6.

Wilkins, A., Majed, H., Layfield, R., Compston, A. and Chandran, S. (2003). Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. *J Neurosci* **23**:4967-74.

Willenborg, D. O., Fordham, S. A., Staykova, M. A., Ramshaw, I. A. and Cowden, W. B. (1999). IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol* **163**:5278-86.

Wittig, B. M., Johansson, B., Zoller, M., Schwarzler, C. and Gunthert, U. (2000). Abrogation of experimental colitis correlates with increased apoptosis in mice deficient for CD44 variant exon 7 (CD44v7). *J Exp Med* **191**:2053-64.

Wolinsky, J. S., Toyka, K. V., Kappos, L. and Grossberg, S. E. (2003). Interferon-beta antibodies: implications for the treatment of MS. *Lancet Neurol* **2**:528.

Wolswijk, G. (2000). Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis. *Brain* **123 (Pt 1)**:105-15.

Wujek, J. R., Bjartmar, C., Richer, E., Ransohoff, R. M., Yu, M., Tuohy, V. K. and Trapp, B. D. (2002). Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J Neuropathol Exp Neurol* **61**:23-32.

Yan, S. S., Wu, Z. Y., Zhang, H. P., Furtado, G., Chen, X., Yan, S. F., Schmidt, A. M., Brown, C., Stern, A., LaFaille, J., Chess, L., Stern, D. M. and Jiang, H. (2003). Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system. *Nat Med* **9**:287-93.

Yu, M., Johnson, J. M. and Tuohy, V. K. (1996). Generation of autonomously pathogenic neo-autoreactive Th1 cells during the development of the determinant spreading cascade in murine autoimmune encephalomyelitis. *J Neurosci Res* **45**:463-70.

Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R. and Steinman, L. (1985). T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature* **317**:355-8.

Zeidler, A., Brauer, R., Thoss, K., Bahnsen, J., Heinrichs, V., Jablonski-Westrich, D., Wroblewski, M., Rebstock, S. and Hamann, A. (1995). Therapeutic effects of antibodies against adhesion molecules in murine collagen type II-induced arthritis. *Autoimmunity* **21**:245-52.

Zhang, G. X., Gran, B., Yu, S., Li, J., Siglienti, I., Chen, X., Kamoun, M. and Rostami, A. (2003). Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* **170**:2153-60.

Zhang, J., Markovic-Plese, S., Lacet, B., Raus, J., Weiner, H. L. and Hafler, D. A. (1994). Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **179**:973-84.

Zhou, B., Weigel, J. A., Fauss, L. and Weigel, P. H. (2000). Identification of the hyaluronan receptor for endocytosis (HARE). *J Biol Chem* **275**:37733-41.

Zipp, F. (2000). Apoptosis in multiple sclerosis. *Cell Tissue Res* **301**:163-71.