CHARACTERIZATION OF THE PRESENCE AND DISTRIBUTION OF CD1D IN THE CENTRAL NERVOUS SYSTEM OF MULTIPLE SCLEROSIS

by

Fraser Muir

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

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submitted by Fraser Muir in partial fulfilment of the requirements for

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Examining Committee:

Dr. Wayne Moore

Co-supervisor

Dr. Jacqueline Quandt, PhD

Co-supervisor

Dr. Cornelia Laule, PhD

Supervisory Committee Member

Dr. Katerina Dorovini-Zis, Dr. Lorne F. Kastrukoff

Additional Examiner

Additional Supervisory Committee Members:

Dr. Wolfram Tetzlaff, PhD

Supervisory Committee Member

Supervisory Committee Member

ABSTRACT

Background: Research into diffusely abnormal white matter (DAWM) in archival MS brain tissue has shown that there is a lipid-specific depletion with preservation of myelin proteins within DAWM, implicating a response against myelin lipids in multiple sclerosis (MS). CD1, the Class I MHC-like protein family, present lipid antigens to the immune system. CD1a, b, and d have been observed in the central nervous system (CNS) of MS patients, yet no studies have quantified the presence of CD1d in the CNS. **Methods:** Archival formalin-fixed, paraffin-embedded MS and control brain tissues were sectioned and stained with luxol fast blue (LFB) for myelin, and HLA-DR (class II MHC). Lesions were categorized as active, or chronic active, based upon HLA-DR and LFB staining characteristics. Sections were stained for CD1d, ionized calcium-binding adapter molecule 1 (Iba-1, microglia), glial fibrillary acidic protein (GFAP, astrocytes), DAPI (nuclei), and Sudan Black B (myelin). Tissues were imaged using an epifluorescent microscope and lesions were outlined based on absence of myelin staining. CD1d-positive cells were quantified per mm^2 and the number of cells double labeling with Iba-1 or GFAP were noted. **Results:** CD1d immunoreactivity was significantly increased in MS compared to control tissue. CD1d-positive cells were more prevalent in areas of active demyelination in MS lesions, and colocalized primarily with GFAP-positive reactive astrocytes. The edges of lesions contained CD1d-positive cells in similar numbers to active lesions but had significantly more than quiescent lesion centers. CD1d was found occasionally within Iba-1-positive cells. **Conclusions:** Our findings show increased CD1d in the CNS of MS patients, and demonstrate positivity being greatest in areas of active demyelination which is novel and supports a lipid-targeted autoimmune process contributing to the pathogenesis of MS. CD1d is primarily localized to GFAP-positive astrocytes and highlights a role for these cells compared to the rare CD1d-positive microglia.

LAY SUMMARY

In multiple sclerosis (MS) the insulating sheath of the neurons is comprised of myelin which is considered to be the target of attack by the immune system. This sheath is primarily composed of lipids, which are fat molecules. A family of immune molecules, known as CD1, direct the immune system to target lipids by “presenting” them to the immune system for destruction and removal. We set out to investigate whether CD1 molecules are contributing to the disease process in MS. In this project, we investigated if a member of the CD1 molecular family, CD1d, is present in the brains of people with MS and where it is found. We found that CD1d was present in higher amounts within MS brains than in control brains, and that this presence was highest in areas where the immune system was still prominent and potentially still involved in damaging brain tissue.

PREFACE

The experiments conducted were approved by the Clinical Research Ethics Board at the University of British Columbia (certificate number: H01-70430).

The images used in Figure 1.2 were contributed by Dr. Cornelia Laule.

The project outline in this thesis was designed in collaboration with Dr. Wayne Moore and Dr. Jacqueline Quandt. Dr. Wayne Moore also validated lesion classification, and patient information. Dr. Farah Samadi and Vlady Pavlova assisted with the processing and staining of some tissues. Otherwise, this represents the original, unpublished work of Fraser Muir. Findings from this thesis have been incorporated into a manuscript prepared for submission to an academic journal in June 2018.

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LIST OF ABBREVIATIONS

α-GalCer – alpha-galactosylceramide

ALDHL1 – aldehyde dehydrogenase 1 family member L1

CD – cluster of differentiation

CFA – complete Freund's adjuvant

CNS – central nervous system

CSF – cerebrospinal fluid

CSPG – chondroitin sulfate proteoglycans

DAPI – 4',6-diamidino-2-phenylindole

DAWM – diffusely abnormal white matter

DMF – dimethyl fumarate

DMT – disease modifying therapy

EAE – experimental autoimmune encephalomyelitis

EBV – Epstein Barr virus

ER – endoplasmic reticulum

EtOH – ethanol

FGF2 – basic fibroblast growth factor

FoxP3 – forkhead box P3

GFAP – glial fibrillary acidic protein

HLA – human leukocyte antigen

Iba-1 – ionized calcium-binding adapter molecule 1

IFN – interferon

Ii – invariant chain

IL – interleukin

iNKT cell – invariant natural killer T cell

LFB – luxol fast blue

MAG – myelin-associated glycoprotein

MBP – myelin basic protein

MHC – major histocompatibility complex

MOG – myelin oligodendrocyte glycoprotein

MRI – magnetic resonance imaging

MS – multiple sclerosis

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

NKT cell – natural killer T cell

Nrf2 – nuclear factor erythroid 2-related factor 2

OPC – oligodendrocyte precursor cell

PBS – phosphate buffered saline

PLP – myelin proteolipid protein

PPMS – primary-progressive multiple sclerosis

RPMS – relapsing-progressive multiple sclerosis

RRMS – relapsing-remitting multiple sclerosis

SPMS – secondary-progressive multiple sclerosis

TBS – tris buffered saline

TCR – T cell receptor

TGF-β – transforming growth factor beta

TMEV – Theiler's murine encephalomyelitis virus

TNF-α – tumor necrosis factor-alpha

Treg – T regulatory cell

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DEDICATION

For my parents, Neil and Laurel.

Chapter 1: INTRODUCTION

1.1 Cells of the central nervous system

1.1.1 Neurons

The functional unit of the nervous system, the neuron, is a cell specialized for the receipt of electrical signals and transmission of those signals to other neurons through the release of neurotransmitters. Neurons may generally be characterized as having highly branched dendrites taking inputs from other neurons, with a specialized projection known as the axon responsible for transmitting signals to further targets. Axons can range from microns up to a meter in length in the human spinal cord (Purves et al., 2008).

1.1.2 Oligodendrocytes and myelin

Oligodendrocytes are the most common glial cell in the central nervous system (CNS), comprising approximately 75% of glial cells in the neocortex (Pelvig, Pakkenberg, Stark, & Pakkenberg, 2008). The oligodendrocyte is responsible for myelinating the axons within the CNS and is capable of myelinating multiple axons, unlike the myelinating cell of the peripheral nervous system, the Schwann cell (Purves et al., 2008).

Myelin is the multi-layered insulating sheath surrounding the axon in the white matter of the CNS composed of layers of tightly wrapped oligodendrocyte plasma membrane (Miron, Kuhlmann, & Antel, 2011). It has a characteristic, high ratio of lipid to protein composition of 70-85% lipid and 15-40% protein (Morell & Quarles, 1999). The primary proteins, in order of abundance, are proteolipid protein (PLP), myelin basic protein (MBP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), myelin associated glycoprotein (MAG), and myelin oligodendrocyte protein (MOG) (Cuzner & Norton, 1996). Myelin was first shown to be a process of the oligodendrocyte in 1962, and it has several functions; most importantly myelin speeds up the conduction of the action potential down the

length of the axon (Bunge, Bunge, & Pappas, 1962). The method by which myelin regulates the conduction speed in axons is through saltatory conduction, whereby the action potential jumps from one node of Ranvier (a small gap in the myelin sheath where ion channels and proteins cluster) to another (Hartline, 2008; Lillie, 1925). This lowers the resistance of the axon and increases the speed of the action potential by 20 – 100 times (Nave & Werner, 2014). Myelin also functions to provide trophic support to the neuron (Miron et al., 2011).

1.1.3 Microglia

Microglia are the resident immune cell of the CNS, similar to tissue macrophages, and constitute 5-10% of all brain cells (Aguzzi, Barres, & Bennett, 2013). These cells are responsible for the surveillance of the CNS for insults or injury and are rapidly mobilized in response to any threat to the brain or spinal cord. Though similar to macrophages, microglia arise from a different population than circulating monocytes, being derived from the myeloid precursors of the embryonic yolk sack, and thereafter maintaining their numbers in the CNS through self-renewal (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Ginhoux et al., 2010; Hashimoto et al., 2013). In addition to their important role as guardians of the CNS through phagocytosis, antigen presentation, and modulation of inflammation, microglia also play important roles in synaptic pruning and modulation, neuronal proliferation, and apoptosis (Q. Li & Barres, 2018).

Microglia can be readily identified in histologic and immunofluorescent images of the CNS by their characteristic morphology. Mature microglia present with a highly branched, ramified appearance, and are highly motile as they constantly survey the surrounding environment (Nimmerjahn, 2005; Zusso et al., 2012). Upon exposure to stressors, or as they sense environmental change, microglia become activated and undergo morphological change, becoming more amoeboid with short, thick processes, and express the ionized calcium-binding adaptor molecule 1 (Iba-1) (Ito et al., 1998). This

change is often associated with increased phagocytic activity, as well as the release of various chemokines and cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin 1-beta (IL-1β) (Ransohoff, 2016). While classically activated microglia were historically classified as either type M1 or M2 microglia (characterized by cell surface marker or protein expression as pathogenic or protective, respectively), recent work has shown that microglial responses are much more nuanced than this, with microglial activation not being so one-dimensional (Ransohoff, 2016).

1.1.4 Astrocytes

Astrocytes are one of the most populous glial cell in the CNS, making up between 17 and 20% of all glial cells in the human neocortex, second only to oligodendrocytes (Pelvig et al., 2008). Astrocytes tile the entirety of the CNS and play a critical role in both the healthy and unhealthy CNS through actions as diverse as maintenance of neuronal synapse homeostasis to gliosis secondary to damage and attempts to repair. In the white matter astrocytes are referred to as fibrous, while in the grey matter they are referred to as protoplasmic due to their different appearances in these two regions (Peters, Palay, & Webster, 1976).

The prototypical marker for astrocytes over the past several decades has been glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament expressed by all, or almost all, astrocytes following CNS insults (Pekny et al., 1995; Pekny & Pekna, 2004; Voskuhl et al., 2009). Studies using cell injection and immunohistochemistry have shown that GFAP-staining severely underestimates the size and volume of astrocyte cells, with only roughly 15% of the cytoplasm being labelled in a rodent study (Bushong, Martone, Jones, & Ellisman, 2002). Newer markers have been developed more recently, including glutamine synthetase, S100β, and Aldehyde Dehydrogenase 1 Family Member L1

(aldhL1), though these still may not label all astrocytes, or exclusively astrocytes, in the CNS (Cahoy et al., 2008; Gonçalves, Concli Leite, & Nardin, 2008; Norenberg, 1979; Yang et al., 2011).

During development astrocytes work together with microglia to prune synapses. Astrocytes induce the production by synapses of C1q, an immune protein that "tags" cells for identification by immune cells, thus targeting the synapses for destruction by microglia (Stevens et al., 2007).

In the healthy CNS astrocytes act as house-keeping cells, maintaining favorable conditions throughout the CNS for the function of neurons and other glial cells. They regulate blood flow through the vessels of the brain via the production of and release of prostaglandins, nitric oxide species, and arachidonic acid, with evidence suggesting this is done based on the levels of neuronal activity at the time (Gordon, Mulligan, & MacVicar, 2007; Iadecola & Nedergaard, 2007; Koehler, Roman, & Harder, 2009; Schummers, Yu, & Sur, 2008). This is possible as astrocytes maintain connections with both blood vessels, as well as neurons. For neurons, astrocytes are also critical for maintaining the homeostasis of synaptic interstitial fluid conditions such as pH and ion balance (Brown & Ransom, 2007), as well as for clearing neurotransmitters from the synaptic cleft (Sattler & Rothstein, 2006; Seifert, Schilling, & Steinhäuser, 2006). Via the aquaporin 4 channels along their processes lining blood vessels, astrocytes are also able to regulate and maintain fluid levels within the CNS (Simard & Nedergaard, 2004). Astrocytes are further able to support neurons during periods of sustained activity due to their ability to store glycogen, which they are capable of breaking down into lactate, which can then be transferred to neurons for use (Brown & Ransom, 2007; Pellerin et al., 2007; Voutsinos-Porche et al., 2003).

1.2 MS background and pathogenesis

1.2.1 Epidemiology

Multiple sclerosis (MS) is a leading cause of neurological disability in young adults, with over 2.5 million individuals affected globally (Milo & Kahana, 2010). Canada has the highest prevalence of MS globally with 55 to 248 individuals per 100 000 being affected. MS is a predominantly female disease with more than three times as many women affected than men (Orton et al., 2006), although men are more likely to have a more aggressive disease course (Ribbons et al., 2015).

MS symptoms are highly variable, and may include weakness in limbs, sensory and motor dysfunction, cognitive and visual disturbances, and ataxia. These symptoms are related to the location of the lesions in the CNS, where lesions found within the spinal cord are most often associated with motor deficits. There are several common disease courses that can unfold following initial disease onset. Relapsing-remitting MS (RRMS) is the most common, being between 85-90% of diagnoses (Fred D Lublin, 2005). It is characterized by periods of disability (relapses) followed by either complete or partial recovery during phases of remission. After ten to twenty years, between 60-70% of RRMS patients will experience a change to a progressive course, known as secondary-progressive MS (SPMS) (Fred D Lublin et al., 2014; Wingerchuk & Carter, 2014). Primary progressive MS (PPMS) has a steady, insidious progression of disease symptoms which accumulate over time with no periods of recovery or relapse and is less common with approximately 10% of diagnoses. There also exists a form of the progressive disease in which patients experience acute relapses on top of the steady disease accumulation; this had been termed progressive-relapsing MS (PRMS) but this characterization is now rarely used (F D Lublin & Reingold, 1996; Fred D Lublin et al., 2014).

1.2.2 Etiology

The etiology of MS remains unclear. However, multiple risk factors have been found to affect the odds of developing MS. Prevalence of MS has been associated with living at higher latitudes, though this affect appears to have somewhat attenuated after 1980 (Alonso & Hernán, 2008). This has been suggested to be due to changes in sun exposure, and consequently vitamin D levels, as vitamin D has been inversely associated with a risk of developing MS (K. L. Munger et al., 2004). Cigarette smoking is also a risk factor for MS, with studies showing a relative risk of developing MS in smokers as compared to never smokers of up to 1.7 (Hernán, Olek, & Ascherio, 2001). Smoking has also been associated with an accelerated transition from RRMS to SPMS (Hernán et al., 2005). Obesity in childhood and adolescence was found to greatly increase the relative risk of developing MS, though this was much less in males than females (Kassandra L. Munger et al., 2013; Kassandra L. Munger, Chitnis, & Ascherio, 2009). MS has also been associated with viral infections, particularly Epstein Barr virus (EBV). It was found that the risk of MS in individuals seronegative for EBV was very low, and that the risk of MS increased greatly amongst individuals infected with EBV (Levin, Munger, O'Reilly, Falk, & Ascherio, 2010). There are also a variety of genetic risk factors that contribute to the risk of acquiring the disease, most notably the human leukocyte antigen (HLA)-DRB1 variants, while some variants of HLA-DRB5 seem to modify disease progression (Caillier et al., 2008). Genome wide association studies have been conducted and found that genes related to immunological functions were significantly overrepresented (Sawcer et al., 2011).

The role the microbiome may play in MS has also recently become a topic of discussion. It is understood that there is a gut-brain connection, and the flora of the gut play an important role in homeostasis, as well as a role in regulating the immune system. In MS patients there is a change in the balance of the microbiome present in gut, with a decrease in some microbes while others are enhanced (Chen et al., 2016; Jangi et al., 2016). There is also evidence for the microbiome of MS

patients possibly contributing to the disease, by way of a transgenic mouse model of MS developing spontaneous disease at a higher rate following colonization of their guts with microbes from MS patients than the microbes from the unaffected twin (Berer et al., 2017).

1.2.3 Pathology

The initial events leading to the development of MS have yet to be elucidated and there is much debate over where the disease first starts. The evidence gathered from models of MS have revealed the critical role the adaptive immune system plays in the pathogenesis of MS, though this must take into account the large differences between the pathology of animal models and human MS (Constantinescu, Farooqi, O'Brien, & Gran, 2011).

MS has a very characteristic pathology, with the primary pathological hallmark being the multiple lesions, also known as plaques, that can be found throughout the CNS. These lesions are focal areas of demyelination characterized by inflammation, gliosis, and axonal loss (Popescu & Lucchinetti, 2012). Though historically characterized as a white matter disease, it has become increasingly evident that MS lesions can often be found within the grey matter of the CNS (Calabrese, Filippi, & Gallo, 2010).

1.2.3.1 Inflammation

1.2.3.1.1 Blood-brain barrier alterations and immune cell recruitment

The first noticeable signs of inflammation in the CNS is permeability of the blood brain barrier (BBB) and infiltration of lymphocytes, which form a characteristic structure of vessels ringed with a perivascular cuff of infiltrating lymphocytes (Frohman, Racke, & Raine, 2006). The type, and ratio of lymphocytes can vary depending on the age of the lesion, with active, early lesions having a larger proportion of T cells than B cells. These T cells within the perivascular cuff also tend to be $CD4^+$,

while the T cells that infiltrate into the surrounding parenchyma are more often $CD8^+$ (Babbe et al 2000, Serafini et al 2004, Ozawa et al 1994).

Cytokines and chemokines expressed within the lesion cause upregulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the endothelial cells of vessels, allowing for the recruitment and ingress of lymphocytes into the perivascular space (Shukaliak & Dorovini-Zis, 2000; D Wong & Dorovini-Zis, 1995; Donald Wong & Dorovini-Zis, 1992). Permeability of the BBB also allows for transmission of serum components into the parenchyma that normally would not be capable of transiting past the BBB (Gay & Esiri, 1991; Plumb, McQuaid, Mirakhur, & Kirk, 2006).

The perivascular space is populated with a variety of antigen presenting cells including microglia, macrophages, and dendritic cells – making the perivascular space the staging point for activation of lymphocytes and the continuation of the inflammatory response (Fabriek et al., 2005; Serafini et al., 2006).

1.2.3.1.2 T cells

Staining for CD3 (a pan-T cell marker) it can be seen that there are a large population of T cells within MS lesions, with populations of $CD4^+$ and $CD8^+$ T cells both present (Traugott, Reinherz, & Raine, 1983). $CD4^+$ T cells, also known as T helper cells, can be further divided into subtypes, the major ones being Type 1 (Th1) and Type 2 (Th2), with smaller subtypes like Type 17 (Th17). Th1 T cells are proinflammatory, secreting a variety of factors that enhance the inflammatory response such as interleukin (IL) 1, IL-2, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) (Codarri, Fontana, & Becher, 2010). Th2 T cells somewhat oppose the effect of Th1 T cells, in that they serve to dampen the inflammatory immune response, decreasing macrophage activity and instead promoting antibody production by B cells. This is done by producing factors such as IL-4, IL-

10, and transforming growth factor beta 1 (TGF-β) (Codarri et al., 2010). There also exists a subset of $CD4^+$ T cells with a regulatory function ($CD4^+$ Tregs), the most well characterized of these being $CD4^+$ Forkhead box P3(FoxP3)$^+$ $CD25^+$. These T cells are normally required for maintaining systemic tolerance of self-antigens and downregulating the activity of other T cells populations through the secretion of various factors such as IL-10, IL-35, and TGF-β, as well as through interactions of cell surface molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)(Collison et al., 2010; Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009; Takahashi et al., 2000; Vieira et al., 2004). Disruptions in the function of $CD4^+$ Treg cells in MS have been described; most notably, in some subsets of $CD4^+$ Tregs there is a loss of IL-10 secretion (Astier & Hafler, 2007). In MS patients $CD4^+$ Tregs were found to have less of a capacity to suppress the immune response – partly through reductions in expression of FoxP3 (Huan et al., 2005; Viglietta, Baecher-Allan, Weiner, & Hafler, 2004).

$CD8^+$ T cells are known as cytotoxic T cells and are the most abundant T cell in MS lesions (Booss, Esiri, Tourtellotte, & Mason, 1983). In MS lesions, blood, and cerebrospinal fluid (CSF) $CD8^+$ T cells undergo clonal expansion (Babbe et al., 2000; Jacobsen et al., 2002; Skulina et al., 2003). They may act as mediators in the pathogenesis of MS, with evidence for them playing either a pathogenic or regulatory role in an animal model of MS (Friese et al., 2008; Friese & Fugger, 2005; Goverman, Perchellet, & Huseby, 2005). In MS it is challenging to provide direct evidence for the role they play in pathogenesis, however there exists suggestive evidence that they are involved. In the peripheral blood of MS patients it has been found that $CD8^+$ cells can respond to CNS antigens by producing IFN-γ and TNF-α and $CD8^+$ T cells secreting IL-17 and IFN-γ were significantly increased (Crawford et al., 2004; Dressel et al., 1997; Tsuchida et al., 1994). In MS lesions the amount of $CD8^+$ T cells and macrophages were proportional to the amount of axon damage present, and in acute lesions IL-17 producing $CD8^+$ T cells were found to be significantly increased in number (Bitsch, Schuchardt,

Bunkowski, Kuhlmann, & Brück, 2000; Tzartos et al., 2008). During relapses MS patients showed an especially large increase in the number of $CD8^+$ T cells expressing cytotoxic molecules, such as perforin, though this number was still elevated outside of relapses when compared to non-MS individuals (Frisullo et al., 2011). There is a growing body of evidence to support a regulatory role for $CD8^+$ T cells ($CD8^+$ Tregs) in MS. Though there are many types of $CD8^+$ Tregs, evidence points to their numbers being significantly reduced in the periphery and CSF of MS patients during relapses and periods of acute disease activity relative to healthy patients or those patients in remission (Correale & Villa, 2010; Frisullo et al., 2010; Mikulkova et al., 2010). These $CD8^+$ Tregs demonstrate regulatory activity, and $CD8^+$ Tregs specific to CNS antigens such as myelin proteins (MBP, MAG, and PLP for example) have been shown to repress myelin specific $CD4^+$ T cells in an IFN-γ-, perforin-dependent manner (Baughman et al., 2011).

1.2.3.1.3 B cells

B cells, though present early on in the pathology of MS lesions, are not as numerous as T cells (S L Hauser et al., 1986). However, they are capable of forming follicles in the leptomeninges where they undergo clonal expansion. When stimulated by the appropriate antigen B cells mature to plasma cells and begin to secrete immunoglobulins (Ig), which accumulate in the CSF giving rise to the oligoclonal bands characteristic of MS CSF (Burgoon, Gilden, & Owens, 2004; Hemmer, Archelos, & Hartung, 2002). The primary product of these plasma cells is IgG (Esiri, 1977). It is also possible that B cells may play a regulatory role in the immune response by mediating production of IL-10, and TGF-β through interactions with T cells (Boppana, Huang, Ito, & Dhib-Jalbut, 2011).

1.2.3.1.4 Microglia and macrophages

In addition to resident microglial cells, macrophages in the MS lesion are also derived from recruited circulating monocytes, which are attracted and infiltrate into the perivascular space (Simpson et al.,

2000). These macrophages are the primary effector cells for demyelination within the lesion by stripping and phagocytosing myelin via interactions with Fc and complement receptors (Prineas, Kwon, Sternberger, & Lennon, 1984; Smith, 2001). Once phagocytosed the myelin debris is digested within the lysosome and may be presented on the cell surface, fulfilling the role of the macrophage as one of the major antigen presenting cells in the MS lesion (Ousman & Kubes, 2012; Ulvestad et al., 1994). Interestingly, macrophages filled with lipid/myelin debris within the MS lesion appear to have a more immunomodulatory profile, while those actively engaged in demyelination show these markers to a lesser degree (Boven et al., 2006). Once activated by factors within the lesion milieu such as macrophage colony stimulating factor and insulin-like growth factors macrophages also upregulate the production of factors such as inducible nitric oxide synthase (iNOS) and nitric oxide (NO) - major players in the damage of myelin and oligodendrocytes (Gveric, Cuzner, & Newcombe, 1999; Hill, Zollinger, Watt, Carlson, & Rose, 2004; Raivich & Banati, 2004).

1.2.3.1.5 Astrocytes

In addition to their role in the formation of the glial scar, astrocytes also play a role in the inflammatory response. The astrocytes within the lesion are capable of responding to, and producing, free radical species such as NO, and modifying macrophage activity in, and recruitment to the lesion (J. S.-H. Liu, Zhao, Brosnan, & Lee, 2001; Min, Yang, Kim, Jou, & Joe, 2006; Van Der Voorn et al., 1999). Reactive astrocytes are also capable of phagocytosing debris within the lesion, including debris of myelin and axons, and are capable of presenting myelin peptides, though their role as antigen presenting cells remains not fully elucidated (Constantinescu et al., 2005; Dong & Benveniste, 2001; Kort, Kawamura, Fugger, Weissert, & Forsthuber, 2006; Krogsgaard et al., 2000; Lee, Moore, Golenwsky, & Raine, 1990; Morcos, Lee, & Levin, 2003; Ulvestad et al., 1994)

1.2.3.2 Demyelination

The primary effector cell of demyelination and the physical removal of the myelin sheath is the macrophage. The macrophage also retains other means by which it can cause damage to myelin and oligodendrocytes; as previously noted in Section 1.2.3.1 activated macrophages are capable of producing free radical species such as NO. In addition to these free radicals they also produce a multitude of other cytokines and enzymes which may, alone or in concert with others, cause damage to myelin, oligodendrocytes, and other surrounding cells. This could mean they contribute to MS pathology not only through their effects on myelin and oligodendrocytes, but also the damage they cause to surrounding cells in the CNS (Cross, Manning, Keeling, Schmidt, & Misko, 1998; Hill et al., 2004; Nakanishi, 2003; Van Der Goes et al., 1998).

Anti-myelin antibodies are an obvious candidate for a cause of demyelination. MS patients have been shown to have increases in serum antibodies specific to myelin components, and immunoglobulins have been shown to accumulate in MS lesions, CSF, and peripheral blood (Blauth, Owens, & Bennett, 2015; Esiri, 1977; Pröbstel, Sanderson, & Derfuss, 2015). These anti-myelin antibodies may support demyelination in conjunction with activated complement via interactions with macrophage Fc and complement receptors (Smith, 1999). However, evidence *in vivo* for immunoglobulin binding myelin sheaths in MS lesions has not been conclusive (Barnett, Parratt, Cho, & Prineas, 2009). Thus, while the fact that there is little doubt that B cells and antibodies play a role in the pathology of MS, as supported by the success of B cell-targeted therapies, whether the primary target of these antibodies is myelin itself or any of the many other CNS component targets that antibodies exist for in MS lesions remains unsolved (Chiba et al., 2006; Gorczyca et al., 2004; Zhang et al., 2005).

Though not responsible for the direct removal of myelin or the myelin sheath, the large number of T cells present in the MS lesion would suggest that there may be targeting of oligodendrocytes for destruction, with consequent loss of the myelin sheath. Due to the expression of HLA-E molecules on oligodendrocytes it is possible they are targeted for destruction by $CD4^+$ T cell subtypes expressing CD56 and NKG2C which can recognize HLA-E (Zaguia et al., 2013). Other T cell subtypes may also be able to interact with, and target oligodendrocytes for destruction. δγ T cells are capable of recognition of the heat shock proteins that oligodendrocytes express, and oligodendrocytes may express major histocompatibility complex (MHC) class I meaning they can be recognized by antigen-specific $CD8^+$ T cells (Höftberger et al., 2004; Pouly & Antel, 1999).

1.2.3.3 Remyelination

Following demyelination oligodendrocyte precursor cells (OPCs) are recruited to the site of damage, where they undergo differentiation to become oligodendrocytes and then begin the process of enveloping and depositing myelin on the denuded axons (A. H. Crawford, Chambers, & Franklin, 2013; S. H. Kang, Fukaya, Yang, Rothstein, & Bergles, 2010). However, this newly deposited myelin fails to fully replicate the structure of the original myelin sheath – frequently these new myelin sheaths are thinner, and have a shorter internodal distance – though it does restore saltatory conduction (Blakemore, 1973; Prineas & Connell, 1979). This remyelination is spontaneous following demyelination, though it can be highly variable in terms of its extent and success. For example, remyelination appears to fail completely in the center of chronic active lesions, though some remyelination may still be evident at the active edges of the lesion (Raine & Wu, 1993; Wolswijk, 2000). The ability for the CNS to remyelinate is affected by age, as with increasing age the efficiency of myelination decreases (Shields, Gilson, Blakemore, & Franklin, 1999; Sim, Zhao, Penderis, & Franklin, 2002). Remyelination may also be taking place concurrently with still active inflammation and demyelination in the MS lesion, which may further impact the efficiency of remyelination or

cause immediate demyelination (Lau et al., 2012). In fact, inflammation may actually promote remyelination, and *in vitro* experiments found that remyelination was decreased following removal of macrophages or T cells (Kotter, Setzu, Sim, Van Rooijen, & Franklin, 2001; Kotter, Stadelmann, & Hartung, 2011; Miron & Franklin, 2014).

1.2.3.4 Axonal loss

Despite much attention in MS being given over to the inflammatory response and demyelination, these pathological features do not act as good predictors for disease progression (Stys, Zamponi, van Minnen, & Geurts, 2012; Trapp & Nave, 2008). Instead, it has been found that axonal loss is the best correlate for disease progression and accumulation of disability (De Stefano et al., 2001; Petzold et al., 2005; Tallantyre et al., 2010). In MS lesions it is common to see transected axons. This is due to demyelination and results in Wallerian degeneration where the axon distal from the transection degenerates, as well as retrograde degeneration where the axon dies back to the neuron cell body (Trapp et al., 1998).

In demyelinated axons there is an increase in the number of mitochondria and respiratory chain enzymes, potentially reflecting the increased metabolic needs of demyelinated axons following demyelination and loss of trophic support from the oligodendrocyte (Mahad et al., 2009; Witte et al., 2009). This increase in metabolic requirements, or mitochondrial dysfunction, can lead to a case where the axon is no longer able to meet its energy requirements, leading to what is termed “virtual hypoxia” and eventual axonal death (Trapp & Stys, 2009). This metabolic stress is especially acute in thin-caliber axons and may explain the preferential loss of small-caliber axons in MS (Evangelou et al., 2001).

In addition to the metabolic disturbances potentially causing axonal death, the axons may be a target of the inflammatory response within the MS lesion as well. The free radicals, cytokines,

chemokines, and enzymes produced and released into the inflammatory milieu of the lesion by macrophages and infiltrating lymphocytes may cause cellular damage to the axons in addition to the damage to the myelin sheath itself (Dutta & Trapp, 2010; Haider et al., 2011; Hendriks, Teunissen, de Vries, & Dijkstra, 2005). The infiltrating lymphocytes are also capable of causing targeted damage on top of the rather non-specific release of factors, with $CD4^+$ and $CD8^+$ T cells able to target neurons for destruction in manner independent of MHC class I or antigen recognition (Giuliani, Goodyer, Antel, & Yong, 2003). Antibodies may also play a role in the destruction of axons in MS lesions, as there is evidence for them accumulating on axons, as well as antibodies from MS patients having been shown capable of destroying axons *in vitro* (DeVries, 2004; Kwon & Prineas, 1994; Zhang et al., 2005).

1.2.3.5 Gliosis

In response to insults to the CNS, astrocytes undergo astrogliosis, or the formation of a glial scar. Broadly, astrogliosis is a wide range of morphological, cellular, and functional changes that astrocytes undergo in response to CNS changes, pathologies, or insults. These changes are not a simply all-or-none response, but demonstrate a wide spectrum of potential responses, dictated by the scale/severity of the stimulus or insult (Sofroniew, 2009). In MS lesions it is also possible to observe reactive, gemistocytic astrocytes with large, "filled" cytoplasm and few processes, which are associated with reactive gliosis during inflammation (Tihan, Vohra, Berger, & Keles, 2006).

Following insults to the CNS there is an upregulation of GFAP in astrocytes, with increases in the size of both the cell body and processes, though in mild cases of astrogliosis there is no observable astrocyte proliferation (Wilhelmsson et al., 2006). In severe astrogliosis there is astrocytic proliferation, as well as rearrangement of the tissue architecture, with astrocytes beginning to overlap their domains, whereas in healthy tissue this is not observed (Bushong et al., 2002; Halassa,

Fellin, Takano, Dong, & Haydon, 2007). It is by this process that the glial scar is formed, which acts as a barrier protecting the rest of the CNS from the initial site of the threat, thus preserving function (Faulkner et al., 2004).

While the purpose of the glial scar is to protect the surrounding CNS tissue, the glial scar can prove pathogenic as well. The glial scar inhibits cell migration across its length, as well as prevents axonal growth, which can prove detrimental for functional recovery (Cregg et al., 2014; Silver & Miller, 2004; Voskuhl et al., 2009). One of the means by which reactive astrocytes may prevent axon regrowth is through the upregulation and release of chondroitin sulfate proteoglycans (CSPGs), some of which *in vitro* have been shown to inhibit axon growth (Johnson-Green, Dow, & Riopelle, 1991; Silver & Miller, 2004). Astrogliosis may also be detrimental for remyelination by the secretion of factors which inhibit the maturation of OPCs, such as basic fibroblast growth factor (FGF2) and hyaluronan (Back et al., 2005; Goddard, Berry, & Butt, 1999).

1.2.4 Treatments

Currently there is no cure for MS, and contemporary treatments are focussed on slowing progression and decreasing the rate of relapses experienced by patients, though the majority focus on the latter. There are currently a number of disease modifying therapies (DMTs) available for RRMS patients which are effective in reducing the relapse rate, and one, ocrelizumab, was recently found to be effective in PPMS patients (Montalban et al., 2017). Due to the presumed autoimmune nature of MS these DMTs all target the immune system directly or target the immune system's ability to access the CNS.

DMTs are tiered by their efficacy and risk which must be weighed against the characteristics of disease activity experienced by the patient, and how responsive they have been to prior DMTs. There are two treatment strategies for MS with the goal of minimizing disease progression: (1) the

escalation strategy, and (2) the induction strategy (Comi, 2008). The escalation strategy starts patients on low risk, first tier, DMTs, and escalates to more effective, but risky second and third tier treatments should the result of the treatment be suboptimal. In the induction strategy there is an initial, aggressive treatment using higher risk DMTs followed by a maintenance regimen of a lower risk DMT (Comi, 2008).

The first tier of DMTs available in British Columbia consists of some of the first ones developed for the treatment of MS, including interferon-β 1a/1b and glatiramer acetate, which are some of the most well tolerated therapies available (Comi, Radaelli, & Sørensen, 2017). Additionally, dimethyl fumarate (DMF) is included in the first DMT tier. Interferon-β 1a/1b is thought to ameliorate disease progression in MS primarily through reducing the production of pro-inflammatory cytokines, while concurrently increasing the expression of anti-inflammatory cytokines (Kozovska et al., 1999; Z. Liu, Pelfrey, Cotleur, Lee, & Rudick, 2001). Glatiramer acetate is a heterogeneous mixture of four peptides similar to myelin basic protein, and though many mechanisms of action have been proposed, including immunomodulation, it is effective in reducing relapse rate and disease progression in RRMS patients (Bell et al., 2017; Johnson et al., 1995). DMF is an oral DMT which modifies the inflammatory response by suppressing inflammatory cytokine production through the inhibition of NFκB, and activation of the antioxidant Nrf2 pathway (Albrecht et al., 2012; Linker et al., 2011).

In British Columbia the second tier DMT is fingolimod. Fingolimod's primary mechanism of action is as an agonist to sphingosine-1-phosphatase receptors, interfering with key mechanisms used by leukocytes to traffic out of lymph nodes, sequestering them there, thus preventing leukocytes from accessing the CNS (Cohen & Chun, 2011).

The third tier DMT in British Columbia is the monoclonal antibody natalizumab. Monoclonal antibodies are capable of targeting specific cells or checkpoints that influence disease progression. Natalizumab is a humanized monoclonal antibody specific to the α4-integrin, which, when blocked, inhibits diapedesis of leukocytes into the CNS, and reduces the number of relapses experienced by RRMS patients (Baron, Madri, Ruddle, Hashim, & Janeway Jr., 1993; Miller et al., 2003; Rudick et al., 2006). Ocrelizumab is another humanized monoclonal antibody, but it is specific to CD20. By targeting CD20, B cell levels are depleted for several months following treatment, with an effect on relapse rate and MRI progression (Hauser et al., 2017). Ocrelizumab has also been associated with lower rates of progression in PPMS patients, and is currently the only FDA-approved treatment for PPMS though it has not yet been approved for PPMS in Canada (Montalban et al., 2017).

Finally, there are some DMTs that, while not approved for treatment of MS, have been used off-label for the treatment of MS. One such DMT is mitoxantrone. Mitoxantrone is a general immunosuppressive which inhibits T-cell activation, and proliferation of B and T cells, which is effective in MS, though comes at increased risk of cardiotoxity (Marriott, Miyasaki, Gronseth, & O'Connor, 2010).

Ultimately, options for treatment of MS patients that have shown suboptimal responses to all other treatments include intense immunosuppression, and autologous hematopoietic stem cell transplantation (Atkins et al., 2016). Autologous hematopoietic stem cell transplantation has been shown to be most effective in cases of MS where the disease was particularly aggressive, and treatment could be initiated prior to accumulation of a high level of disability (Sormani et al., 2017).

1.3 Animal models of multiple sclerosis

Due to practical and ethical challenges posed by performing experimentation in humans, scientists make use of models of diseases in animals. These models enable replication of the complex

interplay of biological systems at play during pathology *in vivo.* Research into the pathology of MS is no different, and I will briefly introduce two of the most popular MS animal models currently in use. A detailed overview of these models is beyond the scope of this thesis; refer to the reviews by Glatigny and Bettelli., Procaccini *et al.*, and Tsunoda and Fujinami (Glatigny & Bettelli, 2018; Procaccini, De Rosa, Pucino, Formisano, & Matarese, 2015; Tsunoda & Fujinami, 2010).

1.3.1 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) arises following the exposure of susceptible animals to CNS antigens. Though the disease courses are highly variable depending on a multitude of factors, this disease model is still the most widely used for studying the autoimmune inflammatory and demyelinating responses which closely model aspects presented in MS and are thought to underlie MS disease pathogenesis.

The predecessors to EAE were performed by repeated injections of brain matter into monkeys where it was noticed that the majority developed an encephalomyelitis accompanied by myelin destruction (Rivers & Schwentker, 1935). The protocol was later further refined by adding complete Freund's adjuvant (CFA), a mixture of heat-killed mycobacterium in mineral oil (Kabat, Wolf, & Bezer, 1946). CFA not only hastened the onset of disease, but also reduced the number of injections required to initiate symptoms to one.

EAE can be induced by either passive or active means. In active induction animals are injected with myelin antigens [such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or myelin proteolipid protein (PLP)] in conjunction with CFA (Stromnes & Goverman, 2006a). In passive induction T cells specific for myelin antigens are transferred to naïve animals (Stromnes & Goverman, 2006b). In both methods the mechanism for action is the activation of myelin-specific CD4+ T cells (Vandenbark, Gill, & Offner, 1985; Zamvil et al., 1985). These activated CD4+ cells are

then capable of crossing the BBB, where they will present myelin antigens resulting in the pathogenic inflammatory response.

1.3.2 Theiler's murine encephalomyelitis virus

Another animal model of MS is Theiler's murine encephalomyelitis virus (TMEV), a single-stranded *Picornaviridae* virus whose natural host is mice and was first described in 1937 (Theiler, 1937). There are two main groups of TMEV, distinguished primarily by their virulence and disease pathogenesis. The group induced by inoculation with the GDVII strain of virus demonstrates an acute encephalitis that rapidly progresses and is highly virulent. In the second group, known as Theiler's Original, the disease induced is biphasic with an acute stage followed by a chronic, demyelinating stage with the virus persisting within the CNS (Lipton, 1975; Tsunoda, Iwasaki, Terunuma, Sako, & Ohara, 1996). The presence of an acute inflammatory response, followed by a chronic, persisting, demyelinating phase allowed TMEV to be used as a model for MS.

1.4 Lipid antigen presentation

1.4.1 Mechanisms

Unlike proteins, lipids are unable to be presented to the immune system by MHC molecules; instead, they use a family of MHC-like molecules known as CD1 for presentation to immune effector cells (Sieling et al., 1995). The CD1 molecular family was first discovered in the 1980s, and by the late 1990s it became clear that their role was to bind and present lipids to T cells (Beckman et al., 1994; Zeng et al., 1997). The CD1 group of molecules is composed of five members, CD1a-e. These could be grouped based on sequence homologies: group 1 composed of CD1a-c, group 2 which consisted of solely CD1d, and CD1e – often classified separately as it has somewhat intermediate homology (Calabi, Jarvis, Martin, & Milstein, 1989). These molecules bind lipids through the

interactions between their hydrophobic antigen-binding pockets and the hydrophobic moieties on the target lipids with their headgroups protruding outwards for interaction with the T cell receptor (Borg et al., 2007). The complexity, size, and number of hydrophobic binding pockets vary for each CD1 molecule, which accounts for the wide variety of lipids capable of being presented by the various CD1 molecules and their specificity for different antigens (Vartabedian, Savage, & Teyton, 2016).

In synthesis and trafficking both group 1 and group 2 CD1 molecules behave in similar manners. Following synthesis the CD1 molecules traffic to the endoplasmic reticulum (ER) where they are associated with β2 microglobulin, similar to MHC I molecules, which allows the CD1 molecules to traffic from the ER to the cell surface via the secretory pathway (Fig. 1.1 A) (H. S. Kim et al., 1999; Park et al., 2004). In the ER the antigen binding groove of the CD1 molecules are loaded with an endogenous lipid, with evidence suggesting this loading on CD1d is done by microsomal triglyceride transfer protein (Brozovic et al., 2004; Stephanie K Dougan et al., 2005). In the ER CD1a and CD1d have also been shown to be capable of associating with the invariant chain (Ii), which is normally the targeting molecule of MHC class II (Jayawardena-Wolf, Benlagha, Chiu, Mehr, & Bendelac, 2001; S. J. Kang & Cresswell, 2002; Sloma et al., 2008). In these cases it has been shown that this can allow CD1d to traffic directly to the late endolysosomal pathway without first being directed to the plasma membrane, while CD1a is still directed to the cell surface (Fig 1.1 D). CD1e is unique to the CD1 family of molecules in that it does not traffic to the cell surface, rather it accumulates within the Golgi network due to the ubiquitination of its cytoplasmic tail before trafficking to the endolysosomal pathway (Angénieux et al., 2005).

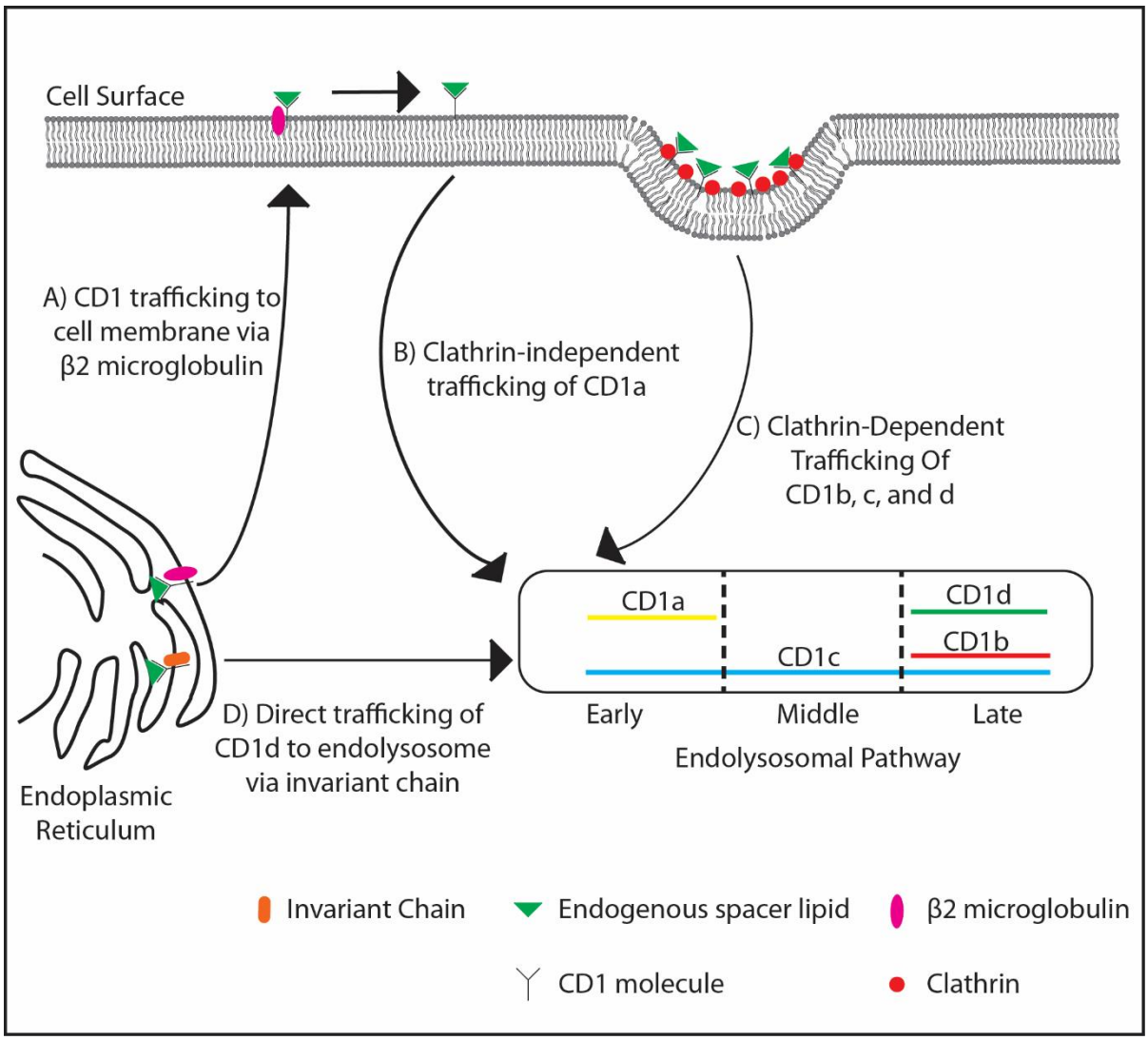

Figure 1.1 CD1 trafficking and distribution in humans. A) CD1 molecules associate with β2 microglobulin and traffic to the cell surface loaded with an endogenous spacer lipid. B) CD1a is internalized in a clathrin-independent manner where it enters and remains in the early endolysosomal pathway. C) CD1b, c, and d are internalized via clathrin-coated pits. Once internalized CD1c localizes throughout the entire endolysosomal pathway, while CD1b and d are localized solely to the late endolysosome. D) CD1d is capable of directly trafficking to the endolysosomal pathway through associating with the invariant chain.

Once CD1 molecules have reached the surface they are then trafficked back to the endolysosomal compartments. For CD1a this occurs in a clathrin/dynamin-independent manner, as it lacks the tyrosine-based internalization motif found on the cytoplasmic tails of CD1b-d (Fig 1.1 B). CD1a

accumulates within the early, recycling compartment of the endolysosomal pathway. The tyrosine-based motif on CD1b-d interacts with adaptor protein complex 2, internalizing the clathrin-coated pits on which they accumulate (Fig 1.1 C) (Sugita et al., 1999). Adaptor protein complex 3 is then responsible for targeting CD1b and CD1d to the late endolysosomal compartments (Briken, Jackman, Watts, Rogers, & Porcelli, 2000; Sugita et al., 2002). The tyrosine-based motif on CD1c allows it to be targeted to the early, medial, as well as late endolysosomal pathway giving it the widest distribution of all CD1 molecules (Sugita, van Der Wel, Rogers, Peters, & Brenner, 2000). This differential targeting optimizes the surveillance of each CD1 molecule within endolysosomal pathway in humans, where they will have access to both endogenous and exogenous antigens.

The later compartments of the endolysosomal pathway have a lower pH which, for lipids with longer alkyl chains, has been shown to create more favorable conditions for lipid loading onto CD1 molecules, specifically CD1b and CD1d. The low pH causes conformational changes in the CD1 molecules, relaxing the structure allowing for easier lipid loading (Relloso et al., 2008). Furthermore, the specificity of lipids presented by the different CD1 molecules is most likely not only due to the physical properties of the lipid binding pockets, but also a product of the presence or absence of required lipid transfer proteins, as well as the distance a given lipid might manage to travel through the endolysosomal pathway (Moody et al., 2002). In order to load lipids onto the CD1 molecules they utilize the molecules used for lipid and glycolipid metabolism already present within the endolysosome, such as saposins, as lipid transport proteins (Kang & Cresswell, 2004; Winau et al., 2004; Zhou et al., 2004). The different members of the saposin family work in different ways, for example: saposin B forms soluble complexes with lipids which can load onto CD1 molecules, while saposin C associates with the lipid membrane allowing for loading CD1c (Leon et al., 2012). In addition to the saposin family of lipid transport proteins there are also the Niemann-Pick C1 and C2 proteins which have been shown to be capable of impacting lipid loading onto CD1d (Sagiv et al.,

2006; Schrantz et al., 2007). There is also evidence for lipid transport proteins having the ability to remove lipids from CD1 molecules, suggesting there may be some competition between antigens based on the affinity of both the lipid transport protein and the CD1 molecule for the antigen (Zhou et al., 2004). After being loaded with antigen the CD1 molecules traffic back to the plasma membrane where they can display the antigen to CD1-restricted T cells.

Once on display on the cell surface the CD1-antigen complex may interact with the T cell receptors (TCRs) of nearby CD1-restricted T cells. The majority of the research on CD1-TCR interactions comes from studying the interactions of CD1d-antigen complexes with the semi-invariant TCR of invariant natural killer T cells (iNKT) cells. In order to recognize and bind the wide range of antigens presented by CD1d despite the rigid, semi-invariant nature of the TCR, it is capable of inducing conformational changes on the CD1d-antigen complex to create a conserved binding conformation (Girardi et al., 2011; Y. Li et al., 2010; E. D. Yu, Girardi, Wang, & Zajonc, 2011). This induced conformational change is not unique to CD1d-iNKT interactions, as MHC-TCR interactions have been previously shown to have induced conformation changes as well (Tynan et al., 2007).

The potency of the antigen can vary based on a number of factors, but it appears that one of the largest influences is that of the affinity of the TCR for the presented antigen and thus the length of association, as well as orientation of the lipids in the binding groove (Girardi et al., 2011; Wang et al., 2010). Another factor influencing antigen potency is the type of antigen-presenting cell presenting the antigen to the TCR; in fact iNKT cells activated by the same antigen may express a different cytokine profile based on the antigen presenting cell that presented the antigen (Bai, Constantinides, et al., 2012).

1.4.2 Lipid antigen presentation in animals

CD1 molecules can be found in all mammalian species, though the number of CD1 genes, and group of CD1 molecules present in each species can range widely. In guinea pigs both group 1 and group 2 CD1 molecules are present, while rats and mice have only the group 2 CD1d molecule (Bradbury, Belt, Neri, Milstein, & Calabi, 1988; Dascher et al., 1999; Ichimiya, Kikuchi, & Matsuura, 1994). In mice the processing of CD1d is similar to what occurs in humans, though due to the lack of other CD1 molecules CD1d is much more widely distributed throughout the endolysosomal pathway than in humans.

1.4.3 CD1d-expressing antigen presenting cells

In humans CD1 molecules are expressed on a wide variety of cells. CD1d can be found on B cells, activated T cells, cortical thymocytes, some monocyte-derived macrophages and dendritic cells, and dendritic cells of the epithelium and lymph node paracortex (Exley et al., 2000; Gerlini et al., 2001; Spada et al., 2000). Canchis *et al.* also found that vascular smooth muscle cells were stained positively for CD1d (Canchis et al., 1993).

1.5 Role of CD1d-restricted T cells

1.5.1 Natural killer T (NKT) cells

NKT cells are a group of T cells restricted to antigens presented by the MHC Class I-like molecule CD1d and are considered part of the innate immune system (Bendelac, Savage, & Teyton, 2007). NKT cells can be further subdivided into type 1 or type 2 NKT cells. Type 1 are known as invariant NKT cells, due to their expression of a semi-invariant TCR which is reactive to the marine sponge derived glycolipid α-galactosylceramide (α-GalCer) (Kawano et al., 1997). Type 2 NKT cells express a more variant TCR and can thus recognize a wider range of antigens presented by CD1d.

1.5.1.1 Role in disease: a dual role

NKT cells have been implicated in a wide variety of diseases and conditions, with decreased circulating numbers being associated with a wide variety of autoimmune disorders, as well as tumor immunity, asthma, and antimicrobial responses (Dougan, Kaser, & Blumberg, 2007; Van Der Vliet et al., 2001). The role of NKT cells in various diseases is not a simple one, as it has been implicated as both a driver and inhibitor of pathogenesis, sometimes in the same disease, two examples of which will be discussed.

In **rheumatoid arthritis**, an autoimmune disorder of the joints, patients show a decrease in iNKT cell numbers circulating throughout the blood (Linsen et al., 2005; Yanagihara, Shiozawa, Takai, Kyogoku, & Shiozawa, 1999). The iNKT cells remaining in the blood display a Th0 profile and accumulate within the synovia of joints, which is favorable as this means that they are not activated. Further suggesting some positive effect of iNKT cells in rheumatoid arthritis is that following treatment with anti-CD20 antibodies there is an observed increase in iNKT numbers (Parietti, Chifflot, Sibilia, Muller, & Monneaux, 2010). However, in mouse models of antibody-induced arthritis, activated iNKT cells promote the development of disease by inhibiting production of TGF-β through expression of IL-4 and IFN-γ (H. Y. Kim et al., 2005).

In a **mouse model of type 1 diabetes** $CD4^+$ iNKT cells have been shown to have a protective effect against the disease and is further supported by evidence that in CD1d-deficient mice the disease onset was hastened and incidence was increased (Magalhaes, Kiaf, & Lehuen, 2015; Shi et al., 2001). These iNKT cells have this effect through multiple mechanisms including Th2 skewing, as well as increasing the proliferation and activity of T regulatory cells (Forestier et al., 2007; Ly, Mi, Hussain, & Delovitch, 2006). In these same models $CD4^-CD8^-$ double negative iNKT cells have been shown to be pathogenic, and promote the development of type 1 diabetes, which demonstrates that even within

iNKT cells the different groups can have different effects on disease progression (Simoni, Diana, Ghazarian, Beaudoin, & Lehuen, 2013).

1.5.1.2 In animal models of multiple sclerosis

Activated NKT cells were found to be capable of protecting animals from EAE (Maricic, Halder, Bischof, & Kumar, 2014; Singh et al., 2001). Activation of NKT cells was lost in CD1d knockout (KO) mice, as well as when β-galactosylceramide was presented on CD1d. This showed the necessity for both CD1d presentation, as well as an antigen recognition by the NKT cell TCR, for NKT cell activation (Singh et al., 2001). CD1d expression in the CNS was not critical for NKT cells to abrogate EAE in mice, rather, it was critical only in the thymus, where CD1d is expressed on $CD4^+CD8^+$ double positive thymocytes which are required for NKT cell maturation. This suggested that the effects of NKT cells is independent of CD1d-antigen presentation, and that other factors causing activation of iNKT cells are at play (Mars et al., 2008). Activated NKT cells were capable of infiltrating the CNS of EAE mice and promoted a shift to a Th2 response through the production of IL-4 and IL-10, ameliorating disease activity by inhibiting Th1 and Th17 responses (Jahng et al., 2001; Singh et al., 2001). Following adoptive transfer of iNKT cells producing IL-4 and IL-10, but lacking IFN-γ expression the Th1 response was inhibited in the CNS of EAE mice, with no change in the Th17 response, while IFN-γ producing iNKT cells had minimal effects on either the Th1 or Th17 responses. This suggested that the modulation of Th1 responses in the CNS of EAE mice due to iNKT cells is due to IL-4 and IL-10, but not IFN-γ, while the mechanisms of modulating the Th17 response have yet to be elucidated (Oh & Chung, 2011).

In TMEV the presence of iNKT cells was associated with increased mortality. In transgenic mice deficient for iNKT $CD8^+$ T cells were able to rapidly clear the viral infection from the CNS, while in mouse lines enriched for iNKT cells the viral infection was found to persist. This was found to be due

to the inhibitory effect iNKT cells have upon $CD8^+$ T cell anti-viral activity within both the spleen, as well as in the cervical lymph nodes (Mars et al., 2014). It was found that during the acute phase of infection that CD1d-restricted cells were likely to be protective, as CD1 knockout mice showed lower lymphoproliferation than wild-type mice. This role appeared to change later once the infection became chronic, with CD1 knockout mice showing a higher lymphoproliferation than in the wild-type mice, suggesting that the CD1-restricted cells were then playing an immunosuppressive role in the CNS (Tsunoda, Tanaka, & Fujinami, 2008). It was also found that when the NKT cells were depleted with antibodies specific to their T cell receptor that demyelination was increased, further suggesting a regulatory role for NKT cells in demyelinating diseases.

1.5.1.3 In multiple sclerosis

In MS patients the overall numbers of circulating iNKT cells are reduced. However, when patients are in remission the number of $CD4^+$ iNKT cells are not highly impacted. These $CD4^+$ iNKT cells release higher levels of IL-4 and are skewed to a Th2 profile, which suggests they may contribute to the modulation of disease activity, similar to what is observed in murine models of MS (Araki et al., 2003). This decrease in iNKT cells is also carried over into the CNS of MS patients, with very few iNKT cells observed in lesions (Illes et al., 2000). Additionally, IFN-γ is produced by CD1b-restricted T cells, which may influence disease pathogenesis, and there are more glycolipid recognizing T cells in the periphery of MS patients than in controls (Shamshiev et al., 1999).

1.5.2 Gamma Delta (γδ) T cells

γδ T cells are a subset of T cells distinguished by the light and heavy chains of their TCRs, composed of γ and δ chains, respectively. This differs from more common T cells whose TCRs are composed of α and β chains. γδ T cells compose between 0.5-16% of all T cells in both the circulating blood as well as lymphoid tissue in humans, while having a very heterogenous distribution in the tissues of mice making up 1-4% of all T cells in lymphoid tissues, but being much higher in mucosa and

epithelial tissues (Goodman & Lefrancois, 1989; Groh et al., 1989; Parker et al., 1990). The γδ T cell is one of the first responders at the site of pathology, and are critical to the development of the immune response through their production of cytokines such as IL-17, IFN-γ, and IL-4 in mouse models (Cai et al., 2011; Ferrick et al., 1995; Jensen et al., 2008; Roark et al., 2007). It has been shown that large portions of all T cells in acute lesions of MS patients are of the γδ type, and that these were primarily constrained to the leading edge of chronic active lesions – sometimes extending into the surrounding white matter (Selmaj, Cannella, Brosnan, & Raine, 1990; Wucherpfennig et al., 1992).

The γδ TCR is capable of recognizing a wide range of antigens. Of particular interest is its capability to bind lipids and glycosphingolipids; the Vδ1 TCR was shown to be capable of binding cardiolipin, α-GalCer, and sulfatide (a component of myelin) in the contexts of CD1d (Levine et al., 2011; Luoma et al., 2013). Of note, the majority of T cells reactive to sulfatide in the context of CD1d express the Vδ1 TCR (Bai, Picard, et al., 2012). Though the γδ TCR is capable of binding and recognizing CD1d-antigen complexes, the manner in which it does differs from that of the iNKT TCR as it binds the complex with a very different geometry (Uldrich et al., 2013).

1.6 Rational for lipid antigen presentation in the pathogenesis of MS

1.6.1 Lipid-specific depletion in diffusely abnormal white matter

Following the development of magnetic resonance imaging (MRI) as a means to image the CNS of MS patients there soon became evident a phenomena in approximately 25% of RRMS patients, which was called diffusely abnormal white matter (DAWM) (Laule et al., 2017). This abnormality was found to be a region of intermediate signal intensity between that of the lesion, and that of the normal appearing white matter (Fig. 1.2). Correlational studies between MRI and histological methods found that DAWM was associated specifically with a reduction of luxol fast blue (LFB) and

Weil's staining, indicating a loss of myelin phospholipids, and a relative preservation of myelin proteins (Laule et al., 2011, 2013). This loss of myelin phospholipids was associated with a change of the myelin water fraction, an indicator of myelin loss. These findings suggest that there may be lipid-specific destruction, autoimmune in nature, occurring in the DAWM.

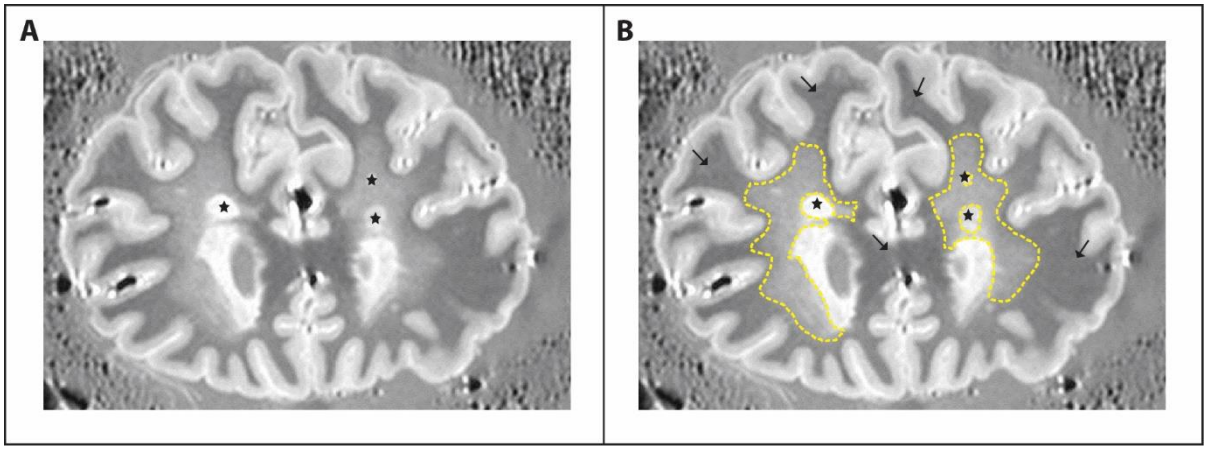

Figure 1.2 Diffusely abnormal white matter in MR imaging. A) A proton density scan of formalin-fixed paraffin-embedded brain tissue, showing distinct lesions (stars). B) The diffusely abnormal white matter (outlined with yellow dashed lines) surrounding the lesions is a region of intermediate intensity when compared to the lesion and the distant normal appearing white matter (arrows). Images courtesy of Dr. Cornelia Laule.

1.6.2 CD1d in animal models of MS

In both of the primary animal models of MS, EAE and TMEV, CD1d deficient mice show changes in disease outcomes, and it is clear that CD1d-restricted T cells play an important role in the pathogenesis of animal models of MS, primarily through their ability to regulate the immune response (Jahng et al., 2001; Maricic et al., 2014; Singh et al., 2001; Tsunoda et al., 2008).

1.6.3 CD1 in human multiple sclerosis

When compared to animal models of MS, there is a dearth of publications looking at CD1 molecules in human MS, especially within the CNS. In 1996 a short communication published by Battistini *et al.* found evidence for an increase in the expression of CD1b in MS lesions (Battistini, Fischer, Raine, &

Brosnan, 1996). They found that this increase was restricted primarily to astrocytes within areas of active demyelination, with minimal reactivity found in chronic silent lesions, areas of normal white matter adjacent to lesions, and in control white matter. CD1b was expressed primarily in hypertrophic astrocytes and could be seen in perivascular inflammatory cells both within the lesion and surrounding it. Interestingly, they failed to find significant CD1a or CD1c expression within the same MS lesions.

CD1a expression was also described in the parenchyma of a single brain out of the ten examined by Serafini *et al.* (Serafini et al., 2006). $CD1a^{+}$ dendritic cells were found in the perivascular space of two early active, subcortical lesions, as well as a chronic active lesion, though none were detected in a chronic inactive lesion from this same patient. Finally, CD1d was found to be in reactive astrocytes, and occasional microglia in the MS lesions of one case of acute MS in a study by Höftberger *et al.* (Höftberger et al., 2004).

When exposed to MS patient sera it was found that dendritic cells differentially regulate the expression of CD1 molecules, having increased expression of group 1 CD1 while group 2 (CD1d) expression remained unaffected (Bine et al., 2012). Bine *et al.* found that in treated MS patients the number of circulating monocytes expressing group 1 and group 2 CD1 molecules was significantly higher relative to untreated patients and controls. In untreated patients they did still find a small portion of circulating monocytes were positive for group 2 CD1d, while none were found to express any group 1 molecules.

1.7 Hypothesis, rational, and experimental aims

We hypothesized that a lipid-specific autoimmune response may contribute to MS disease pathogenesis, and this process may be attributable to the presence of CD1d. To test our hypothesis we addressed the following aims:

1. To develop a method of defining specific regions of MS lesions based on the presence or absence of myelin.
2. To establish whether CD1d was present in MS lesions, if there was an increase in CD1d in MS lesions, and to characterize the cell types and locations where CD1d localized in the brain of MS patients in comparison to CD1d in the brain of controls.

Chapter 2: MATERIALS AND METHODS

2.1 Patient selection

This study was approved by the Clinical Research Ethics Board of the University of British Columbia. The study was conducted on archival, paraffin-embedded, formalin fixed brain tissue samples obtained from 11 MS cases (mean age 46.8 years), and four control cases without CNS disease (mean age 62.5 years) which exhibited no other neurodegenerative condition, see Tables 2.1 and 2.2 for stage of disease, disease duration, and other patient data. Diagnosis of MS was confirmed by a neuropathologist.

MS Case	Age (years)	Disease Duration	PMI (hours)	MS Type	Number of Lesions	Lesion Type	
						Active	Chronic Active
1	35/M	20yr	17	RPMS	1	1	
2	44/F	> 15yr	48	PPMS	1		1
3	32/F	nd	< 24	MS	1		1
4	53/F	nd	nd	MS	2		2
5	57/F	> 13yr	nd	MS	1	1	
6	40/M	< 1	nd	MS	1	1	
7	54/F	14yr	96	MS	1		1
8	48/F	16yr	nd	MS	4	2	2
9	49/F	11yr	48	MS	1		1
10	45/F	>25yr	nd	MS	2		2
11	58/F	30yr	72	MS	1		1

Table 2.1 MS patient information. Post mortem interval (PMI) was time between time of death and autopsy. Multiple sclerosis (MS), was occasionally classed in charts as primary progressive (PPMS) or relapsing-progressive (RPMS). Hospital records were incomplete in some cases (nd = no data).

Normal Case	Age (years)	PMI (hours)	Cause of Death	Number of Blocks
1	54/M	72	Pulmonary emboli, metastatic adeno carcinoma	1

2	71/F	72	Myocardial Infarct, Atheroclerosis	1
3	71/F	72	Cardiac Arrhythmia	2
4	54/F	48	Bronchopneumonia, renal failure, portal vein thrombosis	1

Table 2.2 Control patient information. Post mortem interval (PMI) was time between time of death and autopsy.

2.2 Tissue handling and processing

Brain tissue was removed at autopsy and underwent tissue processing and embedding at the Vancouver General Hospital Department of Pathology and Laboratory Medicine according to the protocol outlined in Table 2.3. The archival, formalin-fixed paraffin-embedded blocks were sectioned on a Leitz 1512 rotary microtome at 5 micrometers in thickness. Sections were mounted on Superfrost Plus microscope slides (Fisherbrand, Pittsburgh, PA) and allowed to dry overnight in a 30^{o}C oven.

Step	Solution	Time (hours)	Temperature (^{o}C)
1	Formalin	1	45
2	70% Ethanol	0.5	50
3	70% Ethanol	0.5	50
4	99% Ethanol	0	50
5	99% Ethanol	1	50
6	99% Ethanol	1	50
7	99% Ethanol	1	50
8	99% Ethanol	1	50
9	Xylene	1.5	50
10	Xylene	1.5	50
11	Paraffin	1	60
12	Paraffin	1	60
13	Paraffin	1	60

14	Paraffin	1	60

Table 2.3 Tissue processing protocol used by the Vancouver General Hospital Department of Pathology and Laboratory Medicine.

2.3 Histochemistry, immunohistochemistry and immunofluorescence

2.3.1 Histochemistry

LFB staining was conducted as outlined in (Kluver, H., and Barrera, 1953).

2.3.2 Immunohistochemistry

For immunohistochemical staining, slides were first warmed in a 60°C oven for 30 minutes, allowed to cool, then deparaffinized through a series of xylene washes followed by washes in 100% ethanol (EtOH) three times and 90% EtOH once for five minutes before being put in dH_20. Slides then underwent antigen retrieval in citrate buffer (pH 6.0) in a microwave for 2 minutes at 90% power, 2 minutes at 70% power, and 6 minutes at 50% power. After cooling the slides were washed in tris-buffered saline (TBS, pH 7.60) for 5 minutes before endogenous peroxidases in the tissue were removed by incubating samples with 3% hydrogen peroxide in 50% methanol for twenty minutes. Slides were washed three more times for 5 minutes in TBS before Fc-receptor binding was blocked by incubation with 5% normal horse serum for thirty minutes prior to primary antibody application. HLA-DR (Dako, Glostrup, Denmark) primary antibody was then applied in TBS and allowed to incubate overnight at room temperature. Negative controls were serial sections incubated with non-specific, isotype-matched immunoglobulins. Slides were then washed three times for 5 minutes in TBS with 0.017% Tween 20 (Fisher Scientific, Fair Lawn, NJ). Staining was then done using the ImPRESS HRP Anti-Mouse IgG Polymer Detection Kit (Vector Laboratories, Burlingame, CA). The slides were then counter stained with hematoxylin (Leica Biosystems, Richmond, IL) for 1 minute, followed by a brief immersion in 0.5% HCl, and 10 seconds in 1.5% Na_2CO_3, washing in dH_20 between each step. Slides were then dehydrated in 95% and 100% EtOH before going through three

100% xylene baths, and were then coverslipped using Vectamount (Vector Laboratories, Burlingame, CA). For full information on antibodies used, concentrations, and isotype controls see Appendix A.

2.3.3 Immunofluorescence

Immunofluorescent staining was performed on serial sections of the selected blocks, and human lymph node (as a positive control for CD1d and Iba-1) using a multi-step staining protocol. Staining specificity of the CD1d and Iba-1 antibodies were confirmed in human, formalin-fixed paraffin-embedded lymph node for each staining run. In addition, the CD1d antibody was further validated using a rabbit polyclonal CD1d antibody (AbCam, Cambridge, UK) to confirm the staining characteristics. Deparaffinization was performed following 30 minutes in a 60^{o}C oven using three five-minute washes in 100% Xylene (Leica Biosystems, Richmond, IL). This was followed by three washes five-minute washes in 100% ethanol (Leica Biosystems, Richmond, IL), and one in 90% ethanol before being rinsed in distilled water. Antigen retrieval was performed in a microwave using citrate buffer (pH 6.0) for 2 minutes at 90% power, 2 minutes at 70% power, and 6 minutes at 50% power. The slides were then allowed to cool for one hour. Once cooled, slides were rinsed with Robertson's PBS (pH 7.50) and blocked using 5% normal donkey serum (Sigma-Aldrich, St. Louis, MO) for 30 minutes before applying the primary mouse antibody against CD1d and allowing to incubate at room temperature overnight. Primary antibodies were washed off using PBS (pH 7.50) with 0.017% Tween 20 (Fisher Scientific, Fair Lawn, NJ). Donkey anti-mouse, AlexaFluor 647 (Molecular Probes, Eugene, OR) was applied at 1:300 for three hours before being washed with PBS/tween20. The second round of primaries was a cocktail of goat anti-Iba-1, and rabbit anti-GFAP which incubated overnight at room temperature, followed by washing, and application of a cocktail of donkey anti-goat and anti-rabbit AlexaFluor 568 and 488 secondary antibodies Molecular Probes, Eugene, OR) for 3 hours. After washing off the secondary antibodies 4',6-Diamidino-2-Phenylindole

(DAPI, Molecular Probes, Eugene, OR) was applied at 1:300 for 15 minutes followed by three more 5-minute washes. Sudan Black B (SBB) (Fisher Scientific, Fair Lawn, NJ) was then applied (0.015% Sudan black in 70% ethanol) for three minutes in order to minimize autofluorescence, this protocol being adapted from (Baschong, Suetterlin, & Laeng, 2001; Romijn et al., 1999; Schnell, Staines, & Wessendorf, 1999). Slides were vigorously washed in PBS/tween20 to remove excess Sudan Black before being coverslipped using Prolong Gold (Invitrogen, Eugene, OR) anti-fade mounting media. All negative control slides were stained using the appropriate isotype control antibodies at matched concentrations. For complete information on antibodies used see Appendix B.

To investigate the specificity of Sudan Black B to stain myelin a rabbit antibody against myelin basic protein (MBP, Dako, Glostrup, Denmark) was applied to slides at 1:800 for three hours, after deparaffinization and antigen retrieval as described above. This was followed by an anti-rabbit AlexaFluor 488 secondary antibody (Molecular Probes, Eugene, OR) at 1:300 overnight at room temperature. Sudan Black B application and coverslipping was performed as described above, and negative control slides were stained using a concentration matched rabbit IgG (Dako, Glostrup, Denmark). For complete antibody information see Appendix B.

2.4 Lesion classification

Lesion classification was done using characteristics previously established based upon the presence, absence, and/or distribution of both LFB (myelin), as well as HLA-DR (microglia and macrophages)(Bö et al., 1994; Raine, 1997). Chronic active lesions were characterized by a prominent ring of HLA-DR positivity (Fig. 2.1 F), with definitively less positivity in the center, and a loss of LFB positivity within the lesion (Fig. 2.1 C). Active lesions showed uniform HLA-DR positivity throughout (Fig. 2.1 E), with variable retention of LFB positivity - evidence of an ongoing,

inflammatory demyelinating response (Fig. 2.1 B). Normal white matter was defined as minimal HLA-DR positivity with full retention of LFB staining (Fig. 2.1 A, D).

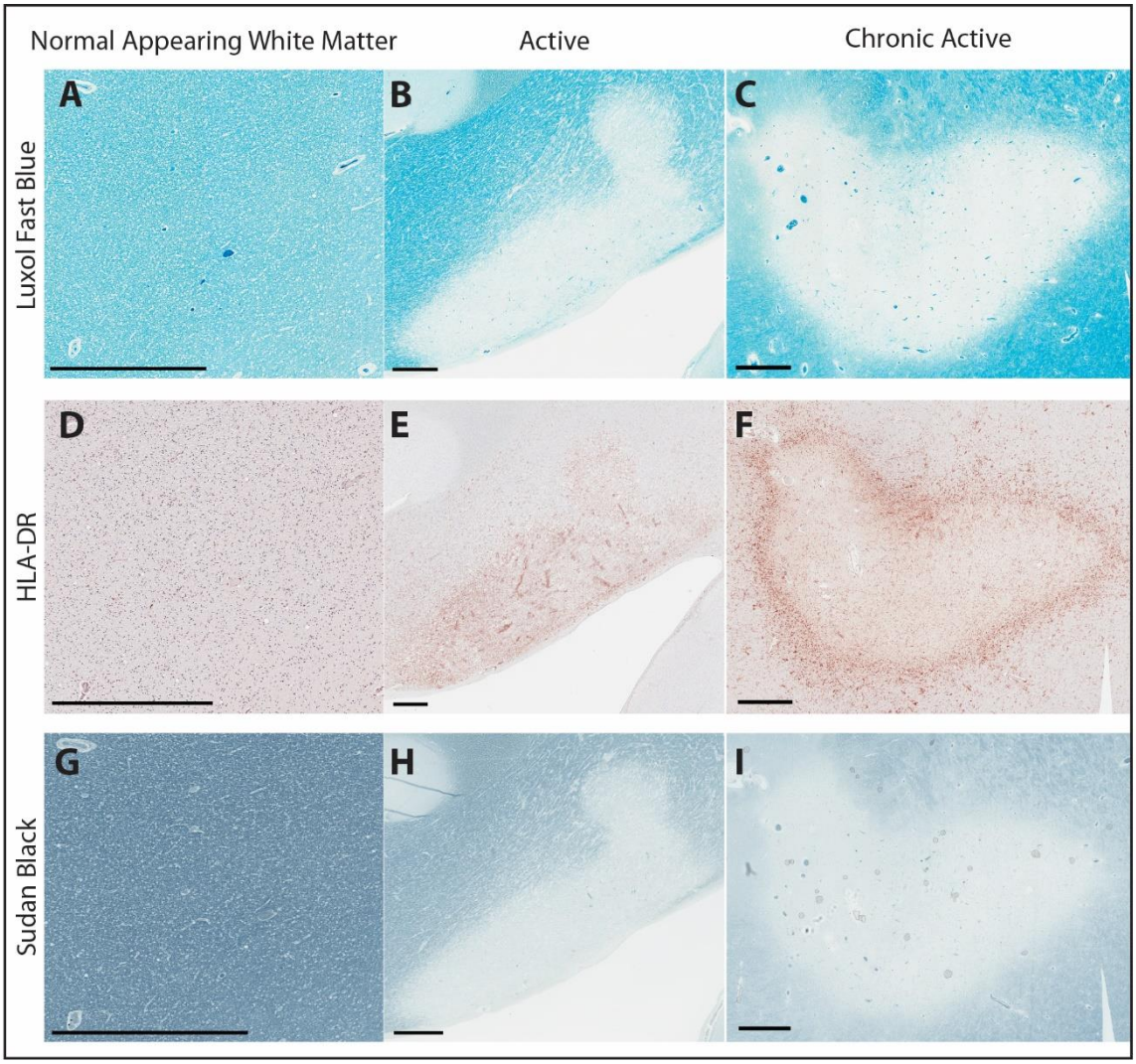

Figure 2.1 Classification of MS lesions based on histochemical and immunohistochemical staining characteristics. Lesions were classified as either active, or chronic active, based on the presence or absence of myelin (LFB staining, A, B, and C), and microglia/macrophages (HLA-DR, D, E, and F). Chronic active lesions lacked myelin (LFB staining) throughout the center, with HLA-DR positivity around the periphery of the lesion. Active lesions had variable amounts of myelin throughout while also having HLA-DR positivity throughout the lesion; normal appearing white matter exhibited no loss of myelin, with limited HLA-DR positivity. Sudan Black B (G, H, and I) staining for myelin showing same pattern as LFB in A, B, and C. Scale bar = 1mm.

2.5 Imaging

All imaging for quantification was performed in ZEN 2.0 (Carl Zeiss GmbH) on a Zeiss Axio Observer Z1 with attached digital camera. The colour camera used for brightfield was a Zeiss Axiocam 105, and the black and white camera used for immunofluorescence was a Zeiss Axiocam 506 monochrome.

All slides were fully imaged first using the 2.5x objective in brightfield after having calibrated the slide holder, this was used to set the tissue area for defining the tiling area for both the brightfield and immunofluorescent acquisitions. The tissue area was outlined in the preview, and then imaged with the 10x objective to acquire a brightfield image to be used later for lesion outlining. This area was then subsequently imaged in fluorescence using the 20x objective with 2x2 binning, and 6% overlap between tiles. Autofocus was run every third tile, using GFAP as the reference channel, and DAPI as a reference channel in the negative controls. All fluorescent images were stitched using DAPI as the reference channel.

Brightfield images of LFB and HLA-DR slides were acquired using an Aperio CS2 Digital Pathology Scanner (Leica Biosystems) using the 20x objective.

2.6 Quantification

Lesions were first outlined using the acquired brightfield images. This was done by outlining the extent of compete demyelination based on the absence of Sudan Black B staining, which was found to associate with other myelin stains. In the case of chronic active lesions a region was required to be defined as the lesion edge. Curiously, while studies in the literature refer to a lesion “edge” or lesion “border” these were defined subjectively, and, to our knowledge, no specific measurements

have been employed to precisely delineate this region (Barnett et al., 2009; Lucchinetti et al., 2000; Prineas et al., 1984). We defined a region extending 500 micrometers out from the region of complete demyelination as the lesion edge. This was based on observations of HLA-DR staining which indicated that 500 micrometers would be sufficient to capture the border of microglial/macrophage activity in the majority of lesions. Thus, the lesion edge was defined as the region between a line plotted along the demyelinated and myelinated white matter interface, and a line outlined 500 micrometers outside this first line. These lines were drawn and measured in ZEN 2.0. The outline along the demyelinated and myelinated white matter interface was obtained on the brightfield images of Sudan Black B staining and was transferred to the immunofluorescent images in ZEN 2.0, where the outer line was then measured and drawn (Fig. 2.2 B, C, and D). The alignment and sizes of the outlines were confirmed by measurement and comparison of image landmarks between the Sudan Black image and the immunofluorescent image.

Thresholding for each fluorescent channel was performed using values obtained from positive control tissues to control for autofluorescence and non-specific staining. The threshold was the level at which positive cells were clearly imaged with minimal background in the control lymph node tissue containing clearly positive and negative populations. Negative controls were checked to ensure there was no staining present other than non-specific autofluorescence.

Cell counting was performed using a 100 by 100 micrometer grid overlaid on the images using Zen 2.0 (Fig. 2.2 E) and using an unbiased counting frame. Quantification was performed in a systematic random manner, a method whereby sampling is done in a systematic fashion from a randomized starting location. The first grid square to be sampled was selected using random.org to generate two numbers between one and three, which were then used as the X and Y coordinates of the first grid square to count. The coordinates 1,1 were assigned to the first grid square that was fully

encompassed by the outline of the lesion in the top left corner (Fig. 2.2 F). From this randomized starting location every third grid square was then quantified, in both the horizontal and vertical axes, resulting in $1/9^{th}$ of all grid squares being quantified.

In the sampled grid squares the number of nuclei were counted, followed by assessing which nuclei were associated with CD1d-positivity. Next, these CD1d-positive cells were examined for labelling with anti-GFAP or anti-Iba-1 antibodies. Due to the interest in assessing CD1d-positive cell density and distribution specifically within the parenchyma, nuclei associated with blood vessels were not included in the quantification.

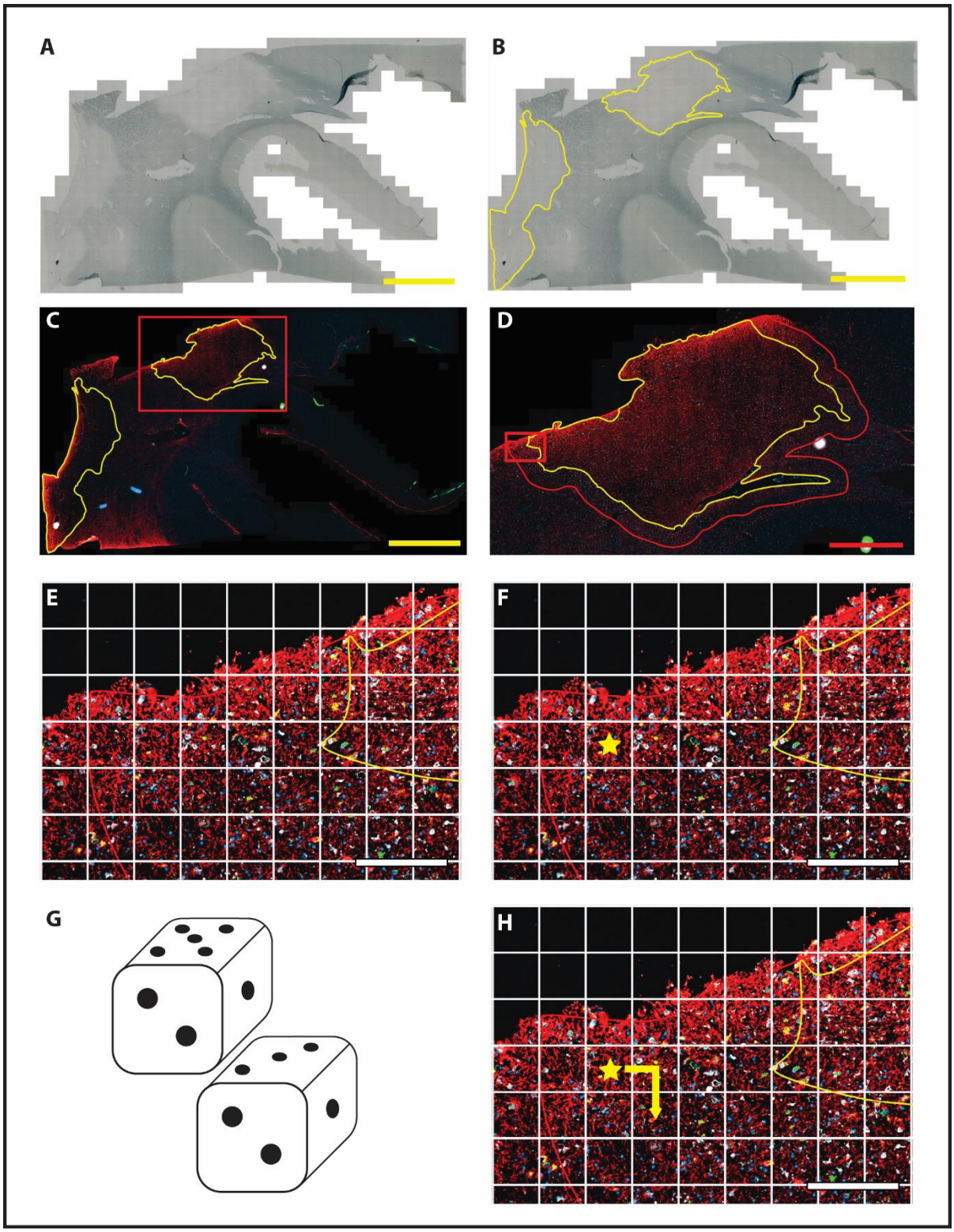

Figure 2.2 Imaging and sampling workflow. A) The slide is first acquired in brightfield with the 10x objective. B) The extent of demyelination is outlined (yellow) based on the absence of Sudan Black B. C) These outlines are transferred to

the 20x immunofluorescence image. D) A line (red) is drawn 500µm outside the extent of demyelination. E) A 100x100µm grid is laid over the image. F) The first grid square fitting fully within the outlined area is identified (yellow star). G) Two numbers between one and three are randomly generated. H) The numbers generated in G are used as grid references from the grid square identified in F to determine the starting grid for quantification. Every third grid square in the horizontal and vertical axes are then quantified, such that 1/9th of all grid squares are quantified. Yellow scale bar = 5000µm, red scale bar = 2000µm, white scale bar = 200µm.

2.7 Statistics

In all analyses each lesion was treated as a separate n, with no pooling between lesions taken from the same patient or other patients. Data were entered in Excel, then transferred to Prism 6 (GraphPad Software, Inc) for analyses and graphing. Non-parametric tests were used to analyze our data which were not normally distributed, as determined by a Shapiro-Wilk test. When comparing more than two groups Kruskal-Wallis test with Dunn's multiple comparison test was used. The Mann-Whitney test was used for determining the difference between two groups, while for paired measures Wilcoxon matched-pairs rank test was used. In all cases a p value of less than 0.05 was considered significant, and reported results used two-tailed analyses.

In analyses where a percentage of cells was calculated, results with no positive cells were not included as a percentage of no value cannot be calculated.

Chapter 3: RESULTS

3.1 Method development

3.1.1 Lesion characterization

To investigate the possibility of lipid antigen presentation occurring in the CNS of MS patients we first had to establish a methodology to differentiate the different areas of lesion, and non-lesion tissue. First, LFB was utilized as a screening stain to determine presence or absence of myelin and to establish the outline of the lesion. Sudan Black B, which was used to minimized autofluorescence in the tissue, also stains myelin lipids; this was illustrated by similar staining pattern of regions with Sudan Black B and MBP as illustrated in Fig. 3.1. The area outlined by the extent of complete demyelination – as evidenced by lack of Sudan Black B staining – was considered the lesion center, while, as noted previously in Material and Methods section 2.6, the lesion edge was defined as an area extending 500µm out from the interface between myelinated and demyelinated white matter. 500µm was chosen as the extent of the lesion edge following discussions with a neuropathologist (Dr. Wayne Moore) and based on experience that this area would capture a large portion of edge activity in chronic active lesion edges without incorporating the surrounding normal appearing white matter. As seen in Fig. 3.2 this outlining based on Sudan Black B corresponded well with where demyelinated white matter met myelinated white matter as shown by LFB positivity; this area also corresponded well with where increased HLA-DR positive cell staining began.

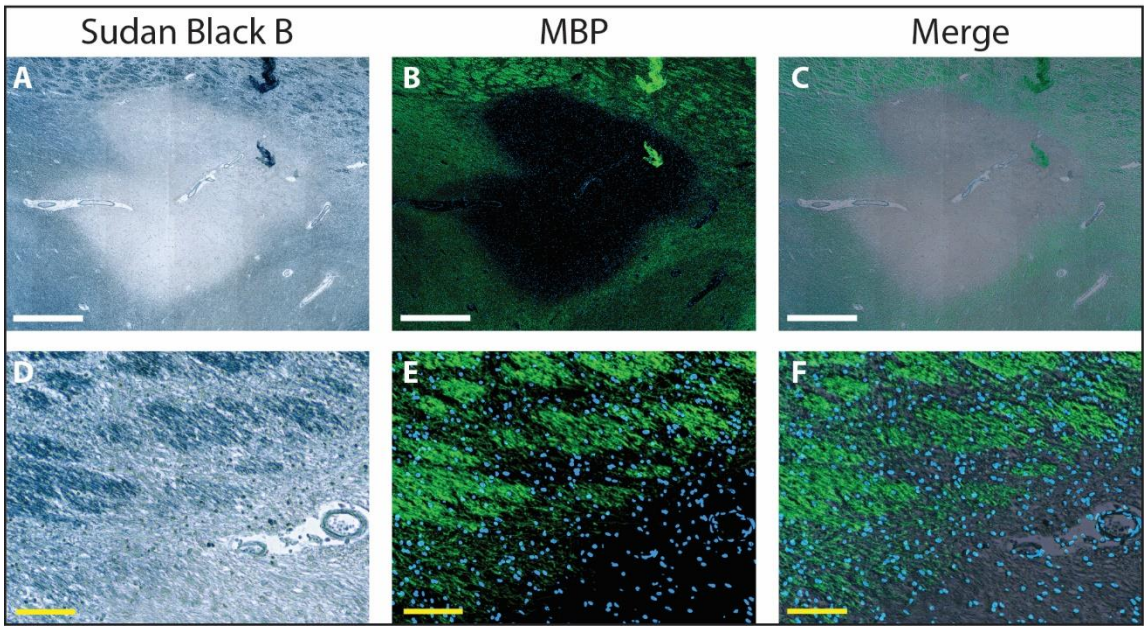

Figure 3.1 Association of Sudan Black B and MBP. Brightfield of a MS lesion showing Sudan Black B staining (A, D), with corresponding fields imaged in immunofluorescence showing MBP positivity in green (B, E). An overlay of both Sudan Black B and MBP is provided in C and F. White scale bars = 1000µm, yellow scale bars = 100µm.

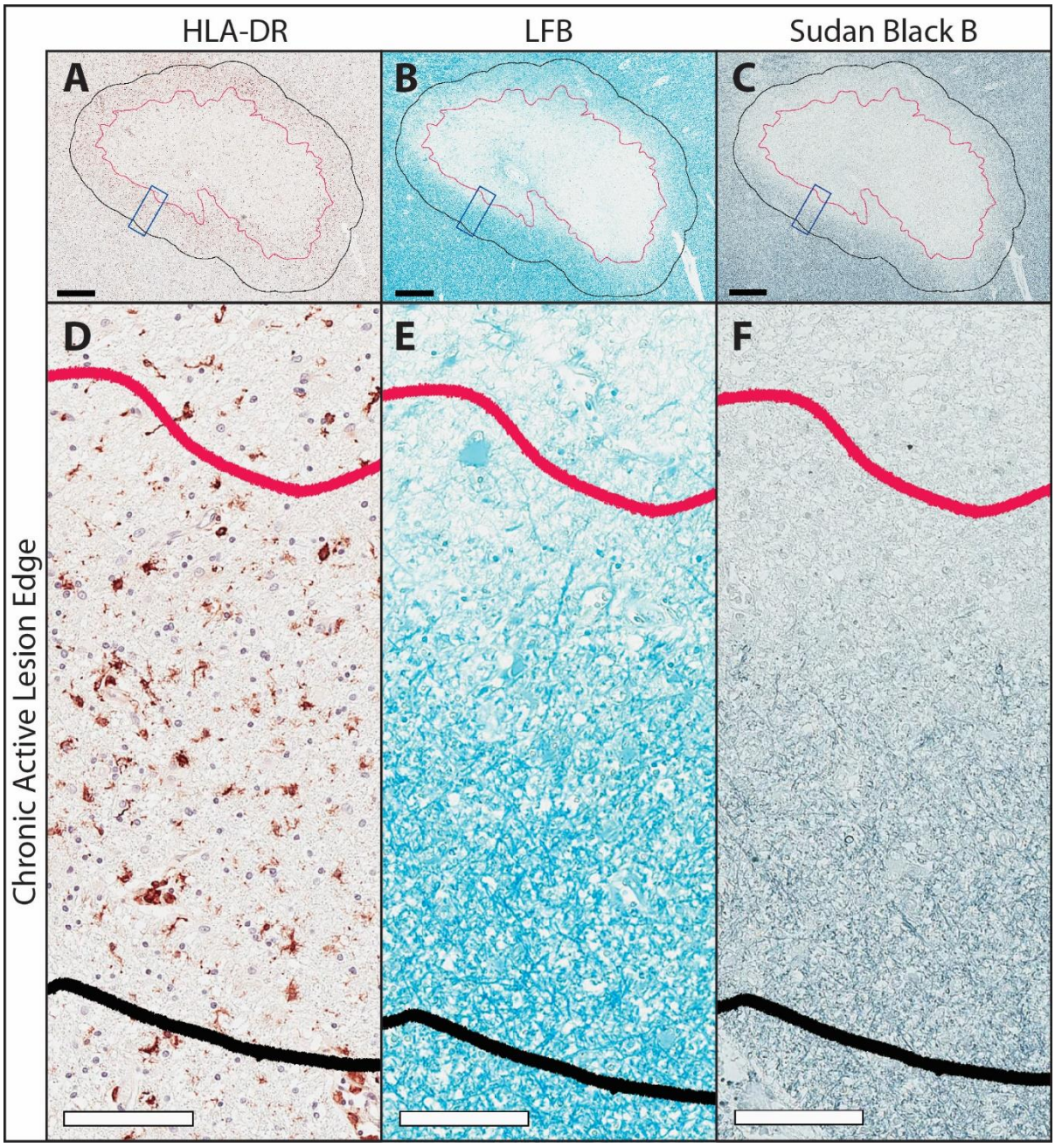

Figure 3.2 Defining lesion edge and histologic characteristics of lesion edge. In chronic active lesions an active edge of 500µm was defined extending beyond the edge of complete demyelination as defined by Sudan Black B positivity as can be seen in C and F. This edge was characterized by an increase in HLA-DR staining density (A, D), as well as positive myelin staining (LFB, B, E). Black scale bar = 500µm, white scale bars = 100µm. D, E, and F are higher magnifications of the regions outlined by blue rectangles in A, B, and C, respectively.

3.2 Lipid antigen presentation in multiple sclerosis

3.2.1 CD1d-positive cell density is increased in MS lesions

Given the findings of Battistini *et al.* of increased CD1b in MS lesions, the occasional CD1a+ dendritic cell in Serafini *et al.*, and Höftberger *et al.'s* finding of CD1d expression in reactive astrocytes in one MS patient using immunofluorescence, we wished to investigate and quantify the CD1d lipid antigen presentation occurring in the CNS of MS patients. The number of nuclei with associated cytoplasm positive for CD1d were counted to give a density per mm^2 in the lesion center, edge, or region of control tissue. When comparing all MS regions together against control tissue it was found that the density of CD1d-positive cells was significantly increased in MS regions (mean = 109.5 ± 102.3 cells/mm^2 vs mean = 13.70 ± 9.776 cells/mm^2. Fig. 3.3 A, p = 0.0184). When the MS tissue region subtypes were compared against each other, and to control tissue, active lesions were found to have a significantly higher density of CD1d-positive cells than control tissue (mean = 247.9 ± 98.43 cells/mm^2 vs mean = 13.70 ± 9.776 cells/mm^2. Fig. 3.3 B, p = 0.0028) and chronic active lesion centers (mean = 247.9 ± 98.43 cells/mm^2 vs mean = 40.10 ± 38.59 cells/mm^2. Fig. 3.3 B, p = 0.0076). The density of CD1d-positive cells in chronic active lesion edges also approached a statistically significant higher density than controls (mean = 115.9 ± 83.24 cells/mm^2 vs mean = 13.70 ± 9.776 cells/mm^2. Fig. 3.3 B, p = 0.1095). Taken together, CD1d was detected more frequently in MS tissues than in control tissue, and there also is a difference in the density of CD1d within different MS lesion types.

Paired analyses of chronic active lesion centers and chronic active lesion edges revealed that CD1d-positive cell density was significantly greater at the legion edge than at the center of the lesion (Fig. 3.4, p = 0.001). The higher density of CD1d-positive cells at the legion edge, where HLA-DR-positivity is higher, associates higher CD1d-positivity with regions of greater HLA-DR-positivity.

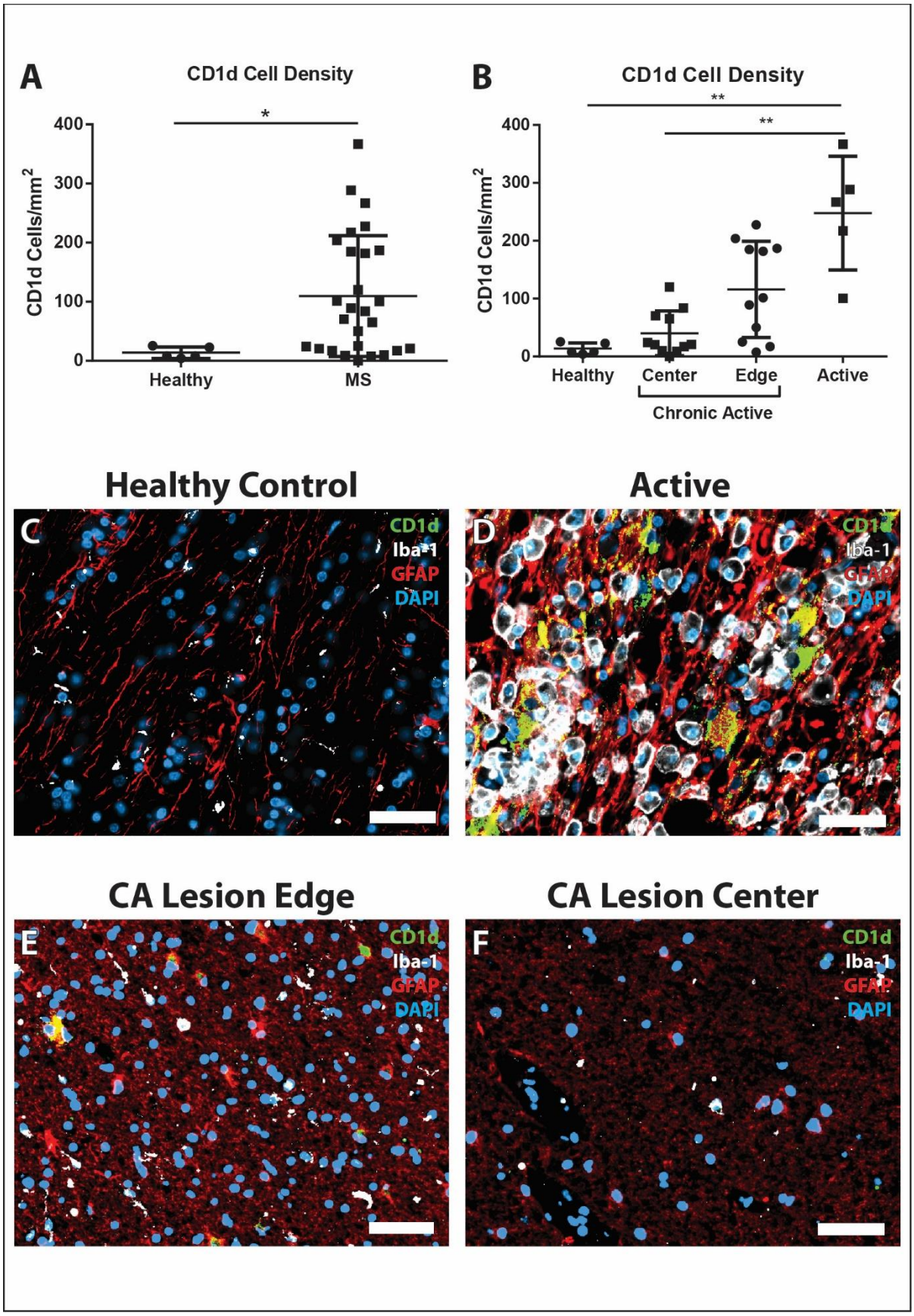
A
CD1d Cell Density
CD1d Cells/mm2
400
300
200
100
0
*
Healthy
MS
B
CD1d Cell Density
CD1d Cells/mm2
400
300
200
100
0
**
**
Healthy
Center
Edge
Active
Chronic Active
Healthy Control
Active
C
CD1d
Iba-1
GFAP
DAPI
D
CD1d
Iba-1
GFAP
DAPI
CA Lesion Edge
CA Lesion Center
E
CD1d
Iba-1
GFAP
DAPI
F
CD1d
Iba-1
GFAP
DAPI

Figure 3.3 Changes in the density of CD1d in MS patients. (A) Multiple sclerosis tissue showed a significant increase in the density of CD1d-positive cells when compared to controls (n = 5, and 27, respectively). (B) The increase in CD1d-postive cell density was significantly different in different lesion types, and different areas within those lesions. Active lesions had a much greater density than both chronic active lesion centers and controls (n = 5, 11, and 5, respectively). The edges of lesions had a higher density of CD1d-positive cells than controls, though did not achieve significance (n = 11, and 5, respectively). Immunofluorescence shows highly reactive astrocytes (red, GFAP), but not microglia (white, Iba-1), immunoreactive for large amounts of CD1d (green) in the active lesion (D) with double-labelling appearing as yellow, while no CD1d is evident in the control (C). The chronic active lesion edge (E) and chronic active lesion center (F) demonstrate a more intermediate astrocyte phenotype (less hyperplastic and more fibrillary), though there are appreciable levels of CD1d in the reactive astrocytes within the chronic active lesion edge, which is not present in the chronic active lesion center. DAPI (blue) as nuclear stain; scale bars = 50µm. **$p \leq 0.01$, *$p \leq 0.05$. Bars represent the mean, and error bars the standard deviation. (A) Mann-Whitney, (B) Kruskal-Wallis with Dunn's multiple comparison test.

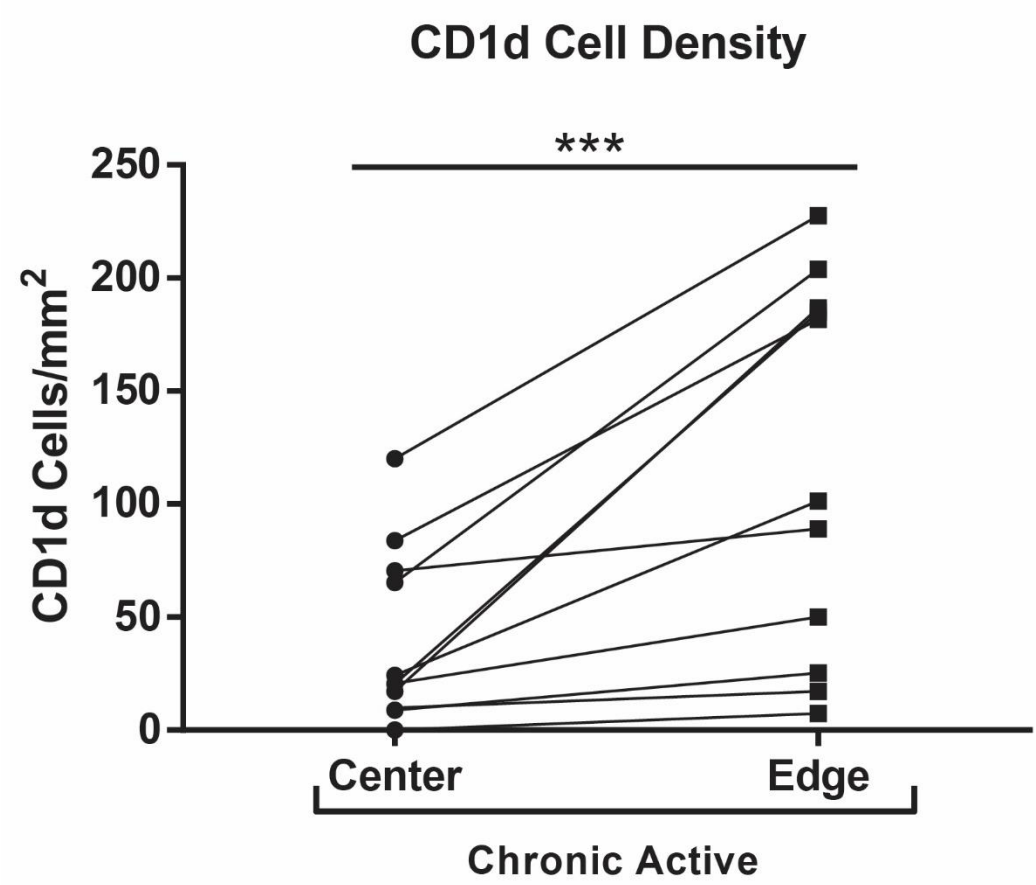

Figure 3.4 CD1d density is greatest in areas of active demyelination. Density of CD1d was significantly greater in the chronic active lesion edge than in the paired chronic active lesion center (*p = 0.001, n = 11 per group, Wilcoxon matched-pairs signed rank test).**

3.2.2 Proportion of CD1d-positive cells

The inflammatory process brings many infiltrating cells into the MS lesion and possibly also the surrounding area; there was a possibility that the increase in CD1d-positive cell density shown in Fig. 3.3 was simply due to hypercellularity in lesion and peri-lesional areas. To exclude this possibility the total cell density was calculated in MS, healthy, as well as in the individual MS tissue regions as seen in Fig. 3.5. There was no significant difference in the total cell densities between MS and the control tissue (Fig. 3.5A), and when individual MS tissue regions were compared the only region showing an increase in overall cell density was the acute lesions when compared against the chronic active

lesion center tissue (mean = 1641 ± 366.4 cells/mm^2 vs mean = 839.7 ± 563.3 cells/mm^2. Fig. 3.5 B, p = 0.0181).

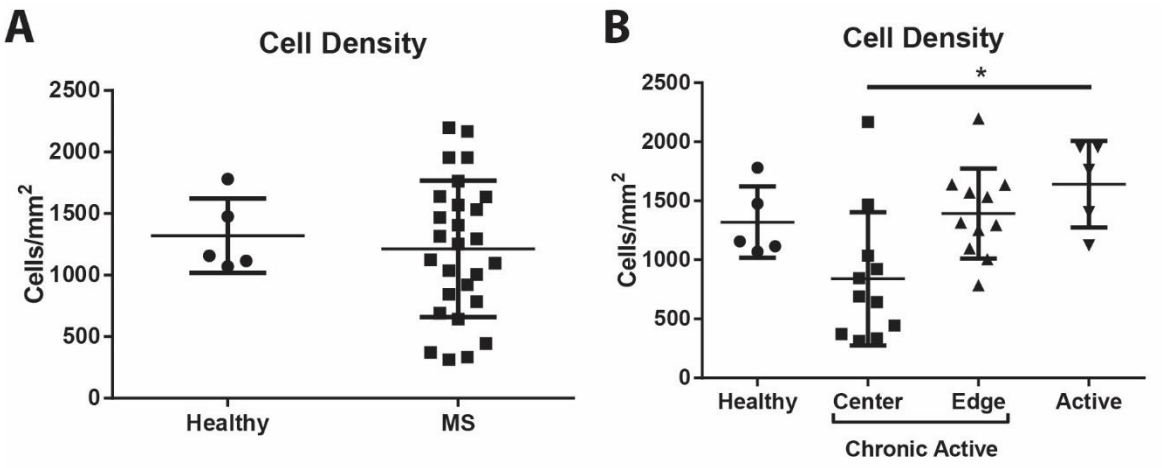

Figure 3.5 Total cell density is only increased in active MS lesions. (A) Total cell density was not increased when all MS regions were compared against control samples (n = 27 and 5, respectively, Mann-Whitney test). (B) Active lesions showed a significant increase in the total cell density when compared against chronic active lesion centers (n = 5, and 11, respectively, Kruskal-Wallis with Dunn's multiple comparison test). *p ≤ 0.05. Bars represent the mean, and error bars the standard deviation.

The percentage of cells positive for CD1d was also investigated and was compared between MS tissue and control tissue. Similar to the results of CD1d-positive cell density in Fig. 3.3A there was a significant increase in the percentage of cells staining positive for CD1d in MS tissue relative to control tissue (mean = 9.860 ± 8.655% vs mean = 1.078 ± 0.8249%. Fig. 3.6 A, p = 0.0007). In addition, the percent of CD1d-positive cells was significantly higher in active lesions than in controls (mean = 16.04 ± 7.750% vs mean = 1.078 ± 0.8249%. Fig. 3.6 B, p = 0.0035). No other significant differences were observed.

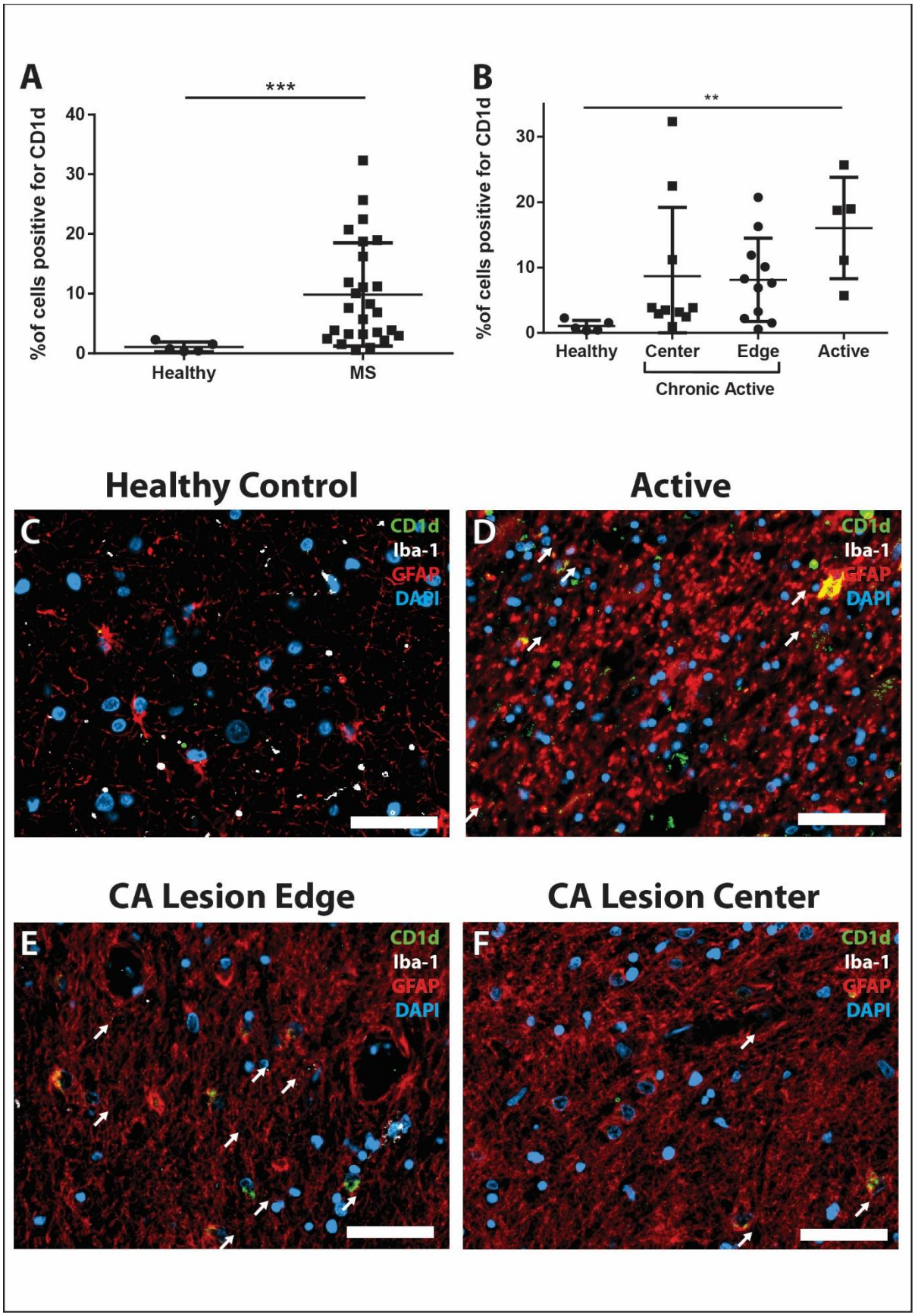
A
***
%of cells positive for CD1d
40
30
20
10
0
Healthy
MS
B
**
%of cells positive for CD1d
30
20
10
0
Healthy
Center
Edge
Active
Chronic Active
Healthy Control
Active
C
CD1d
Iba-1
GFAP
DAPI
D
CD1d
Iba-1
GFAP
DAPI
CA Lesion Edge
CA Lesion Center
E
CD1d
Iba-1
GFAP
DAPI
F
CD1d
Iba-1
GFAP
DAPI

Figure 3.6 The percentage of cells positive for CD1d in MS and control tissue. (A) A significantly higher proportion of the cells in MS tissues were positive for CD1d than in those of control samples (n = 26, and 5, respectively, Mann-Whitney test). (B) There was also a significant increase in the percentage of cells staining in active MS lesions when compared to control, though there was no difference found between areas of chronic active lesions and control tissue (n = 5 control, 5 active, 10 chronic active lesion center, and 11 chronic active lesion edge, Kruskal-Wallis with Dunn's multiple comparison test). Immunofluorescence shows increased numbers of CD1d-positive cells in an active lesion (D) and a chronic active lesion edge (E), with noticeable CD1d positivity throughout multiple cells (arrows), while a chronic active lesion center (F) shows occasional CD1d-positive cells. Control (C) shows minimal CD1d staining. DAPI (blue) as nuclear stain, CD1d in green, GFAP in red, Iba-1 in white; scale bars = 50μm. ***p ≤ 0.001, **p ≤ 0.01. Bars represent the mean, and error bars the standard deviation.

3.2.3 CD1d-positive cells were primarily GFAP-positive in MS tissue

The majority of cells in MS tissue which were positive for CD1d staining were also positive for GFAP, and the percentage of CD1d-positive cells also labelling with GFAP was significantly higher than in the control tissue (mean = 72.57 ± 14.45% CD1d+GFAP+ vs mean = 30.33 ± 20.22% CD1d+GFAP+. Fig. 3.7 A. $p \leq 0.0001$). The percentage of CD1d-positive also labelling with GFAP was significantly higher in chronic active lesion edge and chronic active lesion centers than in control tissue when MS regions were compared separately against control tissue (mean = 71.95 ± 13.41% CD1d+GFAP+ and mean = 74.97 ± 18.38% CD1d+GFAP+ vs mean = 30.33 ± 20.22% CD1d+GFAP+. Fig. 3.7 B, $p = 0.0232$, and $p = 0.0066$, respectively). Quantifying Iba-1+ CD1d+ cells proved challenging as they were rare, and, as such, it was not possible to get a large enough sample to make any inferences from the data collected. A good example of a rare Iba-1+, CD1d+ cell can be seen in the inset of Fig. 3.7 E. There were a sizeable percentage of CD1d-positive cells that co-labelled with neither GFAP or Iba-1, with this being greatest in control tissue. Notably, the CD1d-positive cells not labelling with GFAP were not Iba-1-positive in the vast majority of cases.

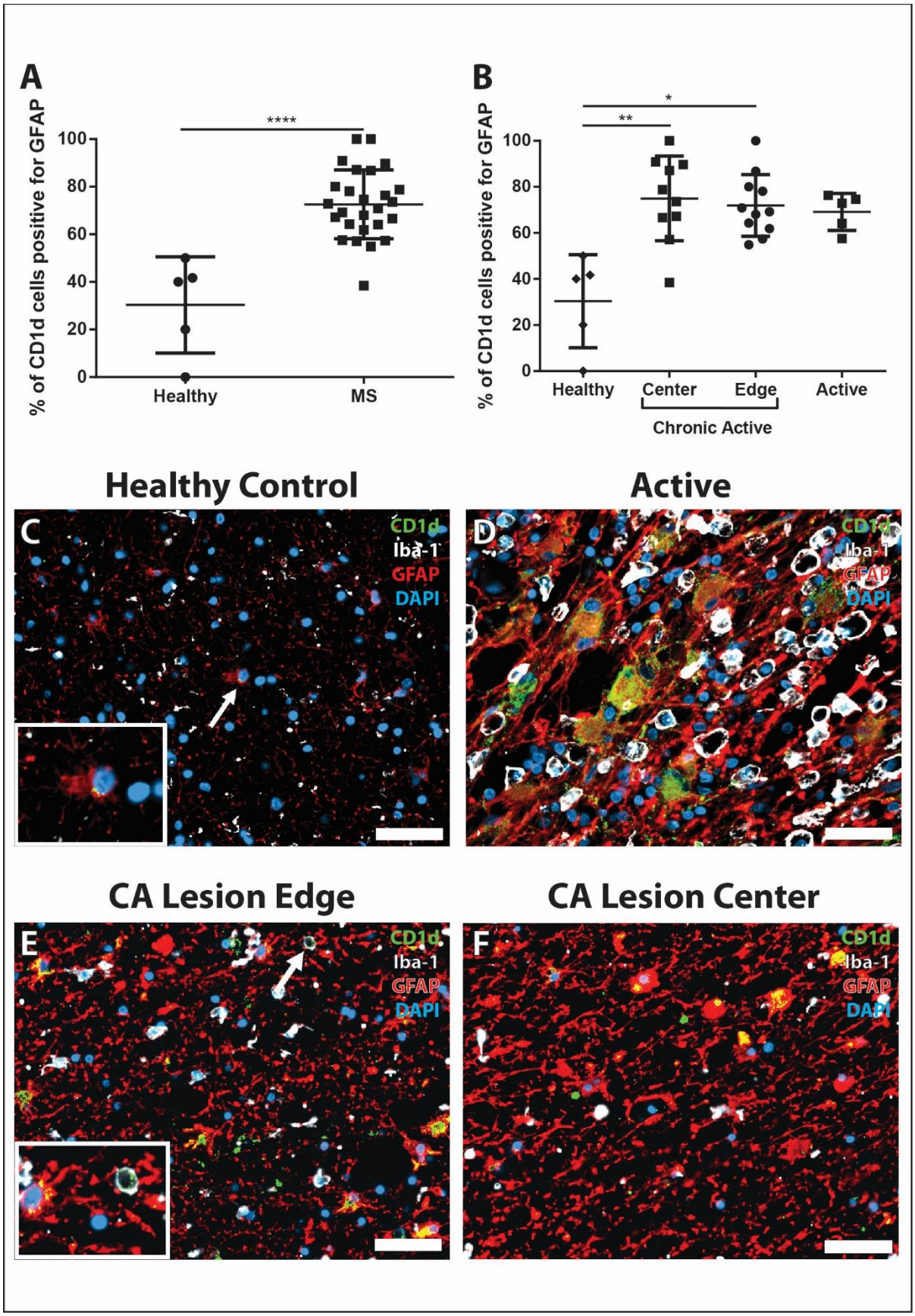
A
% of CD1d cells positive for GFAP
****
100
80
60
40
20
0
Healthy
MS
B
% of CD1d cells positive for GFAP
*
**
100
80
60
40
20
0
Healthy
Center
Edge
Active
Chronic Active
Healthy Control
Active
C
D
CD1d
Iba-1
GFAP
DAPI
CA Lesion Edge
CA Lesion Center
E
F

Figure 3.7 Percentage of CD1d-positive cells also positive for GFAP is higher in MS lesion tissues. (A) In MS tissues CD1d-positive cells were significantly more likely to be GFAP-positive than in control tissue (n = 26, and 5, respectively, Mann-Whitney test). (B) The percentage of CD1d-postive cells also positive for GFAP was significantly higher in chronic active lesion centers and chronic active lesion edges when compared to control tissue (n = 10, 11, and 5, respectively. Kruskal-Wallis with Dunn's multiple comparison test). (C) A rare CD1d-positive astrocyte in control tissue showing minimal cytoplasmic positivity, with staining restricted to the perinuclear region (arrow, inset). (D, E, F) Large, reactive astrocytes in MS lesions showing cytoplasmic CD1d-positivity. The occasional CD1d-, Iba-1-positive cell was seen in MS tissues, though they were rare (E, arrow, inset). DAPI as nuclear stain, CD1d in green, GFAP in red, Iba-1 in white; scale bars = 50µm. ****p ≤ 0.0001, *p ≤ 0.01, *p ≤ 0.05. Bars represent the mean, and error bars the standard deviation.

3.2.4 Qualitative observations

Qualitatively we saw large differences in cell morphology of CD1d-positive cells between the different MS lesion types. We observed a pattern in the morphology with lesions with higher immune cell activity / HLA-DR positivity (i.e. active lesions, and chronic active lesion edges) showing CD1d-positive cells that appeared much larger, hypertrophic, and had CD1d-positivity throughout a larger portion of the cytoplasm. As the data showed in Fig. 3.7A and B, these CD1d-positive cells were almost always astrocytes, and tended to have a more reactive, hypertrophic phenotype, with increased GFAP content and cell size, and occasionally multiple nuclei. It was noted that in regions with demyelinating activity the CD1d-content tended to be higher, both in immunofluorescent intensity and the portion of cytoplasm staining positive, as the phenotype of the astrocytes became more reactive. This is illustrated by the micrographs in Fig. 3.3D, E; Fig. 3.6D; and Fig. 3.7D, E, and F where the increase in both the number of CD1d-positive cells, as well as CD1d content is evident. These astrocytes, being associated with more active areas of demyelination, were primarily found in the regions defined as active or chronic active lesions. There were still rare CD1d-positive astrocytes present within chronic silent lesions, and observable outside of the region we defined as the lesion

edge, but these were generally more fibrillary rather than hyperplastic and showed less CD1d positivity.

CD1d-positive cells and the degree of CD1d positivity in each cell appeared to decrease at the peripheral boundary of the lesion, most notably in the case of active lesions. Within and around MS lesions perivascular cell staining of CD1d was often observed. Often the lumen of these vessels were filled with a diffuse positivity in the CD1d channel (FITC), as well as distinctly CD1d-positive cells. This diffuse staining was not associated with any nuclei and was interpreted as being most likely due to artifacts from tissue fixation.

Chapter 4: DISCUSSION

4.1 Overview of experimental findings

In our study we showed that CD1d is present and increased in MS tissue compared to controls, and also that CD1d is primarily localized to astrocytes within the CNS of MS patients. Furthermore, we have established a strong methodology for defining lesion regions for quantification in MS tissues which will be an asset going forward in the investigation of MS pathology.

Prior to our study, there were only three reports investigating the possible presence of the lipid-presenting CD1 molecular family in the CNS of MS patients: one study found evidence for the expression of CD1b in reactive astrocytes and microglia in the lesions, another showing very limited CD1a expression on perivascular dendritic cells in a single case, while the final study showed CD1d expression within reactive astrocytes and some microglia in one acute MS case (Battistini et al., 1996; Höftberger et al., 2004; Serafini et al., 2006). Our work set out to expand upon this work by assessing the levels of CD1d within different classes of MS lesions from formalin-fixed paraffin-embedded archival tissue in a semi-quantitative manner, as well as determining the major cell types CD1d localized to.

To quantify CD1d within MS lesions we first developed a robust, repeatable method to define the lesion area, and to differentiate between the active and quiescent regions of chronic active MS lesions. We showed that staining characteristics of the dye Sudan Black B stained myelin with good agreement with myelin protein staining in multiple samples observed, and that lesions outlined based on the presence or absence of Sudan Black B agreed well qualitatively with other methods used to examine MS lesions using LFB and HLA-DR staining. We found that the area extending 500µm out from the edge of complete demyelination appeared to contain the majority of immune activity based on HLA-DR immunohistochemical staining.

We showed that CD1d immunoreactivity was significantly higher in MS tissue than in control tissue, and that this varied within MS lesion areas, with active lesions having a significantly higher density of CD1d-positive cells than controls or chronic active lesion center. The centers of chronic active lesions showed significantly less CD1d-positive cell density than the chronic active lesion edges, where immune activity was still taking place. This increase in CD1d-positive cell density did not appear to be driven by the total increase in cell density due to inflammatory infiltrates entering the lesion, as the percentage of total cells staining positive for CD1d also was significantly increased in MS tissues compared to healthy. Finally, we demonstrated that the primary CD1d-positive cell in MS lesional tissue was the GFAP-positive astrocyte.

We report a number of novel findings: the finding that CD1d immunoreactivity is increased in a larger cohort of MS samples compared to control tissue, that CD1d immunoreactivity is greatest in areas of immune activity (defined by increased HLA-DR staining), and that CD1d positivity is predominantly confined to GFAP-positive astrocytes with a reactive morphology. This work adds to the body of literature from both human and animal models that CD1d is likely involved in the pathogenesis of MS, and may reflect a regulatory role of astrocytes based on lipid antigen presentation within MS.

4.2 CD1d immunoreactivity is increased in multiple sclerosis lesions and is greatest in areas of immune activity

The finding of CD1d in MS lesions fit well with the previous literature which had found that CD1b was upregulated in active lesions, and that CD1d could be expressed in acute MS lesions (Battistini et al., 1996; Höftberger et al., 2004). By establishing a repeatable method of establishing different regions of MS lesions based on the presence or absence of myelin it was possible to quantify the presence of CD1d within areas of active demyelination (active lesions, and the edge of chronic active

lesions), as well as in areas of complete demyelination (chronic active lesion centers). This careful regional analysis enabled our study to be the first to establish that not only is CD1d present in MS lesions, but that this presence varies significantly in regions of different immune activity.

The presence of CD1d in the areas of active demyelination and lesion expansion in MS fits well with it having a role in these processes. The distribution of γδ T cells being greatest at the active periphery of MS lesions, and extending outwards occasionally into the surrounding white matter and accumulating in the early stages of MS lesion development (Wucherpfennig et al., 1992) is very similar to what we observed for CD1d. This strongly suggests the possibility for lipid antigen presentation to γδ T cells during the active phases of demyelination in MS lesions, which could have a very important role in the expansion of the lesion, as well as the continued immune response, given the ability for γδ T cells to release large quantities of IL-17. Further support for this possible role of γδ T cells is their previously demonstrated ability to respond to sulfatide, a major component of myelin. The lack of iNKT cells in MS lesions (Illes et al., 2000) does not necessarily suggest that they are not effector cells in human MS, rather, this may simply reflect the challenge of capturing a population of effector cells that act over a very brief window of time in a disease as chronic as MS. Here we may make inferences from the data collected in mouse models of MS that NKT-cells are capable of infiltrating the CNS where they could interact with lipid-antigen presenting cells, and may have protective effect (Jahng et al., 2001; Singh et al., 2001).

The data from mouse models on the effect and distribution of NKT cells, and the presence of CD1d-reactive γδ T cells – capable of propagating the immune response – in MS lesions suggests that lipid antigen presentation in the context of CD1d may be a key player in the regulation of the inflammatory response in MS.

4.3 CD1d in MS lesions is primarily confined to GFAP-positive reactive astrocytes

CD1d is expressed by astrocytes and in view of the fact that this marker is expressed on lipid antigen presenting cells, our observation raises the possibility that astrocytes are lipid antigen presenting cells in the MS lesion. Though astrocytes are a non-traditional antigen presenting cell this adds to the body of literature demonstrating their ability to regulate the immune response in the CNS through various means, such as cytokine release or management of the BBB. This supports the previous findings from Battistini *et al.* and Höftberger *et al.* who found CD1b and CD1d expression primarily on reactive astrocytes (Battistini et al., 1996; Höftberger et al., 2004). However, unlike these previous works, our study did not find many instances of CD1d localizing to microglia or macrophages (Iba-1 positive), though this could be due in part to differences in the CD1d antibody sensitivity and tissue preparation. When quantified, the number of CD1d-positive Iba-1-positive cells were negligible, especially when considering there was a reasonable portion of CD1d-positive cells which did not co-label with either Iba-1 or GFAP, suggesting that there is another cell type in the CNS expressing CD1d. In light of the findings by Serafini *et al.* that there may be occasional CD1a expressing dendritic cells in MS lesions it is possible that these CD1d-positive cells could be dendritic cells, and this is given further credibility by the findings that some dendritic cells are capable of expressing CD1d (Gerlini et al., 2001; Spada et al., 2000). Another possibility is that these cells may be of a subpopulation of astrocytes which do not express GFAP, though this could be solved by future studies using other markers for astrocytes such as ALDHL1.

4.4 CD1d and immune cell recruitment

Observations of CD1d positive cells around the vasculature suggest that CD1d may play a role in recruiting circulating immune cells into the CNS. This is consistent with the observation that endothelial cells are capable of expressing CD1d in the skin, and together with the upregulation of

adhesion molecules known to occur in MS lesions, may aid in the recruitment of CD1d-restricted immune cells (Bonish et al., 2000). Due to the localization of CD1d to astrocytes that was observed the probability of CD1d expression at the glia limitans would also allow for exposure of lipid antigens to the variety of circulating immune cells that enter into the perivascular space following the upregulation of adhesion molecules, and expression of cytokines and chemokines driving the recruitment of immune cells to the lesion site. The localization of CD1d to locations key to the attraction, activation, and infiltration of immune cells into the lesion site suggests CD1d may aid in driving this activity in addition to more traditional molecules such as MHC class I or class II. Conversely, should cells recognize lipid antigens presented in the context of CD1d on the endothelium or at the glia limitans, they may be able to traffic back to the lymph nodes allowing for expansion of the immune response to lipids from the CNS to the periphery.

4.5 Limitations

There are several limitations to this study, largely as a result of working with archival formalin- fixed human tissue. This study was primarily conducted using immunofluorescence and immunohistochemistry techniques, the results of which can be sensitive to a multitude of factors resulting from the method in which the tissue was handled, fixed, and prepared. Following death, a variety of autolytic cellular processes commence, and these are only stopped with fixation of the tissue. Hence, the varying post-mortem interval of the tissues we used in this study may contribute to some variability in the results. Furthermore, using immunohistochemistry and immunofluorescence on post-mortem tissue only captures a “snapshot” of the processes going on at any point during the pathology of this disease. This single moment of time may not be reflective of the full pathology, and as such the images collected must be viewed with a mind to trying to understand the processes that may be occurring both before and after.

Immunofluorescence and immunohistochemistry both rely on the property of antibodies to specifically bind protein epitopes; this property can be affected by tissue fixation and antigen-retrieval techniques. It is possible for the epitopes to be masked, and thus not recognized by antibodies. Once excised, human tissue is frequently fixed in formalin as large samples, which means that formalin penetration speed is not equal throughout the tissue and may result in heterogenous fixation through the sample. Due to this, it is necessary to optimize the antibodies used, the method of antigen retrieval, as well as antibody and tissue controls.

In addition, it was brought to our attention that recent lots (2017 and later) of the goat Iba-1 antibody used in this study have not consistently demonstrated the ability to stain Iba-1 in human or mouse tissues. We used a much earlier lot of the antibody (from 2014) which in our hands successfully stained positive controls of formalin-fixed, paraffin-embedded human lymph node tissue as well as CNS tissues. The antibody also stained frozen formalin-fixed human brain tissue, frozen mouse brain and spinal cord tissue, as well as mouse microglia generated from embryonic cortical cultures (Ravikumar et al., 2014; Sheean et al., 2015; Surgucheva, Newell, Burns, & Surguchov, 2014; A. C. Yu, Neil, & Quandt, 2017). Given that some investigators have reported little success in staining with the Abcam Iba-1 antibody, we have not ruled out in this study that reduced binding of the antibody in formalin-fixed paraffin-embedded human tissue may in part, or in whole, explain the CD1d-positive Iba-1-negative GFAP-negative cells. To fully determine whether CD1d may also be localized to Iba-1 expressing cells in MS lesions or in tissues from patients presenting with other disorders, additional testing will be required with well characterized antibodies that have been determined to effectively stain Iba-1 expressing cells in human FFPE. To address the issue of whether or not these cells are indeed microglia, future studies will need to employ additional markers such as CD68 that can rule out macrophage populations, or additional markers which have recently been identified such as TMEM119 (Satoh et al., 2016), or others currently under

investigation (Marco Prinz; Freiburg University, Germany; unpublished communications) in laboratories looking to identify markers that are unique to human microglia both in health and disease.

In immunofluorescent microscopy of human brain tissue autofluorescence is a major hurdle to overcome (Romijn et al., 1999). One of the major sources of autofluorescence is the naturally occurring pigment lipofuscin, which accumulates within the CNS as mammals age (Brizzee, Ordy, & Kaack, 1974). After staining tissues with fluorescent probes, autofluorescence can be diminished by staining the tissue with the lipid dye Sudan Black B (Baschong et al., 2001; Romijn et al., 1999; Schnell et al., 1999). Following the application of Sudan Black B much of the autofluorescence in the tissue is greatly reduced, though careful use of positive and negative controls, as well as thresholding during image acquisition is still required.

Finally, using immunofluorescence we are only able to characterize the presence of the CD1d protein. Further research using in-situ hybridization to localize RNA or expression to a particular cell type would increase the specificity of our study and enable inferences to be made about CD1d expression levels.

4.6 Conclusion

The presence of CD1d is significant in the CNS lesions of MS patients, and this varies between lesion types and areas within the lesions themselves. CD1d immunoreactivity is confined primarily to regions of immune activity, consistent with the localization of CD1d-restricted T cells described in other studies. Notably, CD1d is localized primarily to reactive astrocytes, and while consistent with prior studies, there remains a large portion of CD1d-positive cells that failed to co-label with either GFAP or Iba-1 that require further characterization. Taken together this provides further evidence

for the relevance and potential importance of CD1d and associated lipid antigen presentation in the pathogenesis of MS.

4.7 Future Directions

Our study is an excellent starting point from which to continue investigating the role that CD1d and CD1d-restricted T cells have in the pathology of MS and other CNS disorders. An important next step is to demonstrate evidence of CD1d-TCR interactions, by utilizing antibodies against the most relevant CD1d partners, Vδ1 chain (γδ T cells) and the Vα24-Jα18 or Vβ11 chains (iNKT cells), in conjunction with CD1d antibodies. We attempted to investigate this with several different antibodies, though none were found to be effective in formalin-fixed paraffin-embedded positive control tissue and the tissue that was used in our study. As such, future studies would be best performed in frozen tissue samples to minimize the risk of epitope masking and reduce other challenges that occur working with formalin-fixed, paraffin-embedded tissues. Investigations could focus primarily on active regions of lesions as the CD1d-restricted T cells, for the most part, are very rapid effector cells. This would be of special interest in analyzing what may be suspected to be pre-active MS plaques, clusters of activated microglia in the absence of leukocyte infiltration or demyelination, as it may give some evidence for whether CD1d antigen presentation is secondary to another process in the MS plaque establishment, or if it is a primary factor.

As our study only looked at the presence of CD1d protein using immunofluorescence, future studies could also look at CD1d RNA to quantify CD1d expression in specific cell types, unequivocally. This could be done through the use of fluorescent in-situ hybridization and quantification of fluorescent intensity, or through microdissection and single cell analysis followed by PCR.

During our study, there were an appreciable number of CD1d-positive cells that did not label with either GFAP or Iba-1 within lesion areas. Determining the cellular identity of these cells would be of

great interest, and they could potentially be identified by staining these same tissues with markers for dendritic cells, pericytes, B cells or astrocytic markers other than GFAP such as ALDHL1 or S100β.

Another area of interest would be to investigate the different expression pattern or role, if any, CD1d may play in cortical or gray matter lesions. Much of the research in MS and animal models of MS focus on the white matter of the CNS, however, the role of cortical lesions in the pathogenesis of MS is well supported in the literature. In conducting our study we found evidence within one cortical lesion for a significant upregulation of CD1d. Though CD1d+, Iba-1+ cells were rare in the white matter MS lesions observed, in the single cortical lesion examined there was a higher number of CD1d-positive cells that also showed immunoreactivity for Iba-1 (Fig. 4.1). This co-localization was also on occasion found in close proximity to GFAP-positivity. Since our study was focused on white matter lesions, this lesion was not included in our analysis. However, the profile of the cells expressing CD1d were very different from what we observed in the white matter; in the cortical plaque there were some CD1d-expressing GFAP-positive cells, with a large number of CD1d-, Iba-1-positive; however, the majority stained only with CD1d (Fig. 4.1 B). These CD1d-positive cells were intensely stained for CD1d, with expression generally only on one side of the nucleus, suggesting an acentric nucleus or polarized expression. These cells tended to be approximately 15 to 20 microns in diameter, and the density of positive cells in the cortical lesion was extremely high. This suggests that there may be a distinct role for CD1d and CD1d-expressing cells in the pathogenesis of cortical lesions which would be of interest to investigate.

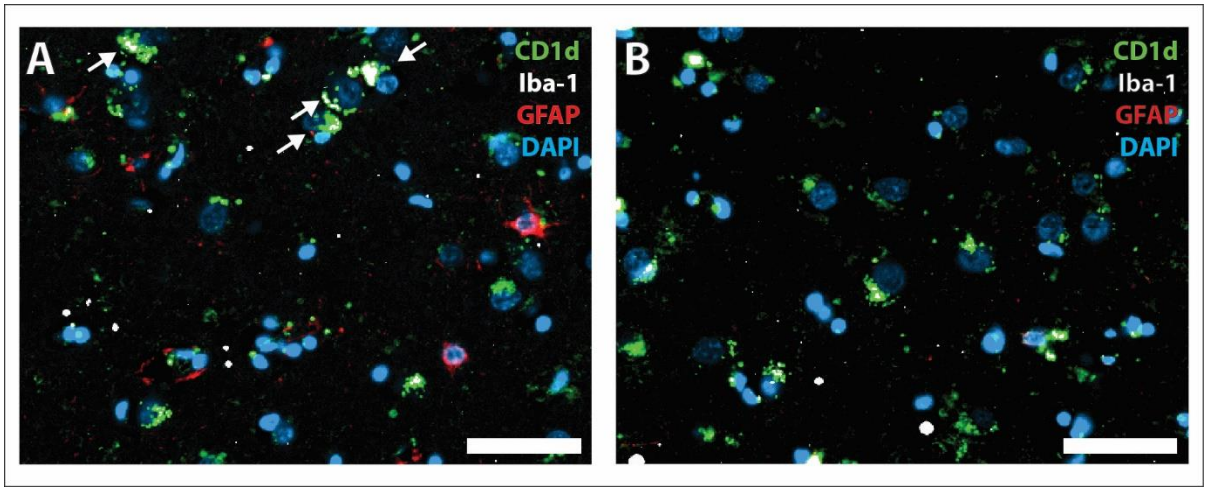

Figure 4.1 CD1d positivity within a cortical lesion. (A) Within a cortical lesion we observed a large number of CD1d-positive cells; a significant portion of these cells also appeared to be Iba-1-positive (arrows). (B) Another image from the same cortical lesion in which the majority of the CD1d immunoreactive cells showed no associated Iba-1 or GFAP staining. DAPI as nuclear stain, CD1d in green, GFAP in red, Iba-1 in white; scale bars = 50µm.

During our study we also acquired some preliminary data examining CD1d expression in other pathologies of the CNS such as: acute (< 1 week) and chronic (> 1 month) infarcts, acute (< 1 week) and chronic (> 1 month) contusions, hemorrhage, and fungal infarcts. Similar to trends we noted in MS lesions, CD1d protein was detected most frequently in the acute pathologies (acute infarct and acute contusion, hemorrhage, and fungal infarct), and primarily localized to reactive astrocytes. CD1d expression decreased in more chronic pathologies (chronic infarct and chronic contusion). This suggests that not only is CD1d involved in the pathogenesis of MS, but that it may be an early, fundamental response to insults of the CNS. Further studies would be able to expand on this preliminary data using the methodologies we developed in our study, and would give us a better understanding of the basic mechanisms by which the CNS responds to insults.

Given that astrocytes have been the primary cell observed expressing CD1 molecules both in MS, as well as animal models of MS, it would be of interest to elucidate their role in the effects ascribed to

the actions of CD1-antigen presentation. This could be done using a $CD1d^{fl/fl}$ mouse crossed with an astrocyte targeted expression system, such as the Glast-CreER and Rosa26-Green Fluorescent Protein reporter mouse, as described by Slezak *et al.* (Slezak et al., 2007). Outcomes with respect to the role of astrocyte-specific CD1d could then be assessed in different models of MS or other CNS conditions.

Finally, given the presence of lipid antigen presentation in the CNS during MS it would be of interest to investigate if there is a correlation between the presence of DAWM and the presence of CD1d. This analysis would be possible using the MR-histology correlation methodology previously described in Laule *et al.* (Laule et al., 2013).

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APPENDICES

Appendix A: Antibodies used for immunohistochemistry

Primary Antibody	**Species/Isotype**	**Working Concentration**	**Manufacturer**	**Catalogue number**
HLA-DR	Mouse IgG1	0.77mg/mL	Dako	M0775
Normal IgG	**Species/Isotype**	**Working Concentration**	**Manufacturer**	**Category number**
Mouse IgG		0.01mg/mL	Sigma	9269

Appendix B: Antibodies used for immunofluorescence

Primary Antibody	**Species/Isotype**	**Working Concentration**	**Manufacturer**	**Catalogue number**
CD1d	Mouse IgG1	0.01mg/mL	AbD Serotec	MCA982G
Iba-1	Goat	0.004mg/mL	AbCam	Ab107159
GFAP	Rabbit	0.0058g/L	Dako	Z0334
MBP	Rabbit	0.014g/L	Dako	A0623
CD1d	Rabbit	0.001mg/mL	AbCam	Ab151768
Secondary Antibody	**Wavelength (nm)**	**Working Concentration**	**Manufacturer**	**Catalogue number**
Donkey anti-mouse	647	0.0067mg/mL	Molecular Probes	A21571
Donkey anti-goat	568	0.0067mg/mL	Molecular Probes	A11058
Donkey anti-rabbit	488	0.0067mg/mL	Molecular Probes	A21206
Normal IgG	**Working Concentration**	**Manufacturer**	**Catalogue number**	

Mouse IgG	0.01mg/mL	Sigma	9269
Goat IgG	0.004mg/mL	Sigma	I5256
Rabbit IgG	0.0058g/L (GFAP)	Dako	X0936
	0.014g/L (MBP)		
	0.001mg/mL (CD1d)		