POLY I:C INDUCED GENE ABLATION NEGATES POTENTIALLY BENEFICIAL EFFECTS OF A4-INTEGRIN DEPLETION ON EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

APPROVED BY SUPERVISORY COMMITTEE

Olaf Stüve, M.D., Ph.D.

Nancy E. Street, Ph.D.

Ann Stowe, Ph.D.

Chandrashekhar Pasare, Ph.D.

DEDICATION

This humble work is dedicated to my beloved parents, José L. Castro Echevarría and Carmen Rojas Gómez, for the unconditional love, guidance, and unending faith in me. And to the most amazing sister there is, Debbie A. Castro Rojas, for being my inspiration.

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by

CYD MARIE CASTRO ROJAS

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Publication No. _____

Cyd Marie Castro Rojas, Ph.D.

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Supervising Professor: Olaf Stüve, M.D., Ph.D.

In the experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis, leukocytes transmigrate through the blood brain barrier into the perivascular space and enter the parenchyma of the central nervous system (CNS). This entry is facilitated by the interaction of very late activation antigen-4 (α4β1-integrin) on activated T cells with vascular cell adhesion molecule-1 that is expressed by inflamed endothelium. It has been demonstrated that α4-integrin antagonism in multiple sclerosis patients can be significantly beneficial, but it can also lead to detrimental CNS infections due to impairing of immune surveillance. In this work, we seek to understand cell type specificity of α4-integrin

antagonism in order to improve immune competence in the CNS. We generated the conditional knock out mouse strain Mx1.Cre^{+} α4-integrin$^{fl/fl}$ on the C57BL/6 background that enables α4-integrin gene ablation by the Cre-lox recombination system upon poly I:C treatment. We hypothesize that antagonism of α4-integrin diminishes immunocompetence within the CNS by differentially affecting leukocyte subsets. In order to test this hypothesis, we actively and passively induced EAE on Mx1.Cre^{+} α4-integrin$^{fl/fl}$. We observed that disease susceptibility and severity in poly I:C treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice were similar to that of wild type (WT), but the disease onset was significantly delayed. Additionally, while there was decreased migratory capabilities of CD45^{+} splenocytes from Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, the absolute number and composition of leukocytes in the CNS of EAE mice was similar in WT and poly I:C treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice. The presence of Evans Blue dye in the parenchyma of poly I:C treated mice suggest a role of poly I:C in this migratory effect. On the other hand, adoptively transferred cells from poly I:C treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ mice did not transfer EAE disease into WT. Based on these data, we concluded that Mx1.Cre^{+} α4-integrin$^{fl/fl}$ strain is limited to study the role of α4-integrin ablation on immunocompetence, however it is successful for the generation of α4-integrin deficient leukocytes. Finally, we determined optimization techniques for CNS leukocyte isolation that can be of benefit for future analyses.

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LIST OF ABBREVIATIONS

Ac – Accutase

APCs – Antigen presenting cells

BBB – Blood brain barrier

CNS – Central nervous system

CPVS – Cerebral perivascular spaces

CFA – Complete Freund's adjuvant

DCs – Dendritic cells

DEPC – Diethylpyrocarbonate

EBD – Evans blue dye

EDSS – Expanded disability status scale

EAE – Experimental autoimmune encephalomyelitis

ECM – Extracellular matrix

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

Fc receptors – Fragment crystallizable receptors

GPCRs – G protein-coupled receptors

H&E – Hematoxylin and eosin

IFN – Interferon

I.p. – Intraperitoneal

IACUC – Institutional Animal Care and Use Committee

I.v. – Intravenous

LAL – Limulus amebocyte lysate

LN – Lymph nodes

LFA-1 – Lymphocyte function-associated antigen 1

Mac-1 – Macrophage 1 antigen

MHC – Major histocompatibility complex

mAb – Monoclonal antibody

MS – Multiple Sclerosis

MBP – Myelin basic protein

MOG – Myelin oligodendrocyte glycoprotein

MOG_{35-55} – Myelin oligodendrocyte glycoprotein peptide 35-55

Pc – Percoll

PBS – Phosphate-buffered saline

Ptx – Pertussis toxin

Poly I:C – Polyinosinic-polycytidylic acid

Pp – Papain

PPMS – Primary progressive multiple sclerosis

PLP – Proteolipid protein

QPCR – Quantitative polymerase reaction

RBC – Red blood cells

RRMS – Relapsing-remitting MS

RSD – Relative standard deviation

SPMS – Secondary progressive multiple sclerosis

SC – Spinal cord

Spl – Splenocytes

Th_1 – T helper type 1

Th_{17} – T helper type 17

UV – Ultraviolet

WT – Wild type

VCAM-1 – Vascular cell adhesion molecule-1

VLA – Very late antigen

CHAPTER ONE
Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease characterized by the migration of lymphocytes into the central nervous system (CNS) (Lopes Pinheiro et al., 2016; Prat et al., 2002). This leads to inflammation, demyelination, and axonal damage that underlie patient's neurological symptoms and permanent deficits (Haines et al., 2011; Zhang et al., 2011). Cell adhesion molecules on the surface of leukocytes and endothelial cells have been shown to be involved in leukocyte recruitment into the CNS during inflammation (Butcher, 1991; B Engelhardt et al., 1995; B Engelhardt et al., 1998; Yusuf-Makagiansar et al., 2002). The blockade of these adhesion molecules, specifically α4-integrin, reduces lymphocyte migration and relapse rate substantially (Correale et al., 2007; D. H. Miller et al., 2003; Yednock et al., 1992). However, long-term use of anti- α4-integrin monoclonal antibodies leads to opportunistic infections, suggesting that normal immune surveillance is crucial to protect the CNS (Bloomgren et al., 2012; Dubey et al., 2016; Outteryck, 2016; Williamson et al., 2015).

In the experimental autoimmune encephalomyelitis (EAE) murine model of MS, leukocyte entry into the CNS is facilitated by the interaction of very late antigen 4 (VLA-4 or α4β1-integrin) on activated T cells with vascular cell adhesion molecule-1 (VCAM-1) on inflamed endothelium (Cannella et al., 1990; B. Engelhardt et al., 1998; Yednock et al., 1992). By utilizing this model, the present work seeks to understand cell type specificity of α4-integrin antagonism in order to improve immunocompetence in the CNS.

We hypothesized that antagonism of α4-integrin diminishes immunocompetence within the CNS by deferentially affecting leukocyte subsets. In order to analyze the effect of adhesion molecule blockade, we generated the conditional knockout mouse strain by crossing Mx1.Cre^{+}α4-integrin$^{fl/fl}$ on the C57BL/6 background. In these mice, α4-integrin ablation on leukocytes can be achieved by treating the mice with the synthetic TLR-3 agonist poly I:C. Utilizing standard EAE induction methods, we observed that disease susceptibility and severity in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice were similar to that of wild type (WT), however, the disease onset was delayed. By *in vitro* migration assays we observed decreased migratory capabilities of CD45^{+} splenocytes from Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice. In contrast, the absolute number and composition of leukocytes in the CNS of EAE mice was similar in poly I:C treated WT and Mx1.Cre^{+}α4-integrin$^{fl/fl}$. The presence of Evans Blue dye in the parenchyma of poly I:C treated mice suggested an effect on migratory behavior. We performed adoptive transfer experiments showing that lymph node cells from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice does not transfer disease into WT recipients, further supporting a role of poly I:C on migration during active induction.

Based on these data, we concluded that TLR-3 agonist is critical in affecting migration pattern of leukocytes and the EAE disease course in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice. We also concluded that the Mx1.Cre^{+}α4-integrin$^{fl/fl}$ strain is a suitable inducible model useful to reduce expression of α4-integrin in leukocytes. Finally, we focused on optimizing methods that allowed for a more efficient way to isolate immune cells from CNS tissues.

CHAPTER TWO
Review of the Literature

2.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that remains incurable. The disease manifests as a wide range of neurological deficits, including cognitive impairment, impaired coordination, visual disturbances, and extremity numbness (Weinshenker et al., 1989). MS affects approximately 400,000 people in the US and 2.5 million worldwide and it is the most common inflammatory neurological disease among young and middle age adults. MS is prevalent in females (Harbo et al., 2013) with reports suggesting sex-linked genetic traits and hormonal impact as plausible causes for this predominance (O. L. Quintero et al., 2012) . MS-related health care costs are estimated to be more than $10 billion annually in the United States (Adelman et al., 2013).

Approaches to accurately diagnose MS has historically progressed from basic guidelines of observing CNS lesion dissemination in space and time, as proposed by Schumacher (Schumacher et al., 1965), to the inclusion of laboratory results based on finding oligoclonal bands or an elevated IgG index in the cerebrospinal fluid (CSF) as proposed by the Poser committee (Poser et al., 1983). In 2001, the McDonald international panel emphasized the inclusion of magnetic resonance imaging (MRI) and gadolinium in establishing the diagnosis (Polman et al., 2011). Typically MRI lesions in the periventricular and subcortical regions, as well as the brainstem, cerebellum, and spinal cord, can raise the suspicion of MS. Clinicians created the expanded disability status scale (EDSS), which

utilizes a numerical 0 to 10 rating, to quantify patient impairment and examine the efficacy of therapies (Kurtzke, 1983).

MS is characterized by the destruction of the myelin sheath that surrounds axons. This destruction, and subsequent disruption of neuronal signaling leads to relapsing-remitting MS (RRMS) symptoms (Lucchinetti et al., 2000). Oligodendrocytes produce myelin sheath in the CNS, a process called myelination. The myelin sheath serves to facilitate the transmission of nerve impulses. The sheath is composed of a complex mixture of 70% lipid and 30% protein in humans (Quarles, 2007). Myelin proteolipid protein (PLP) accounts for ~50% of the protein fraction; while ~30% is myelin basic protein (MBP) (Greer, 2013). PLP and MBP are embedded under the surface of intact myelin. The myelin olilgodendrocyte glycoprotein (MOG), which comprises ~0.05% of the myelin sheath, is localized in the exposed region of the myelin (Kroepfl et al., 1996).

2.1.1 Multiple Sclerosis Classifications

There are several empirical classifications of MS based on clinical characteristics. The most common classification is the relapsing-remitting MS (RRMS) that accounts for approximately 80-85 percent of all MS cases at the time of diagnosis (Weinshenker et al., 1989). RRMS is characterized by acute periods where symptoms and disability are exacerbated followed by remission periods where symptoms and disability resolve to varying degrees. Neurologic dysfunction and disability accumulates over many years and patients often develop secondary progressive MS (SPMS) where relapses are uncommon as the disease progresses (Loleit et al., 2014). Another form of MS is the primary progressive MS

(PPMS), in which accumulation of disability happens independently of exacerbations. There are many immunomodulatory therapies to treat RRMS with many patients having a positive prognosis, however PPMS patients do not have the same responses (Feinstein et al., 2015).

2.1.2 Etiology of Multiple Sclerosis

MS is considered an immune-mediated disease, but it's etiology remains unknown. It is thought that immunologic, genetic, environmental, and viral causes play a factor in the disease.

The specific major histocompatibility complex (MHC) II is associated with the risk of developing RRMS. The MHC II allele *HLA-DRB1*15:01* is strongly associated with MS patients (Matis et al., 1983; Weissert et al., 2009). MHC II molecules are required in antigen presentation to $CD4^+$ T cells, resulting in their activation and proliferation. These associations strongly suggest that MS is a T cell-mediated autoimmune disease. Furthermore, current immunomodulatory therapies utilized to treat MS are aiming to target the re-activation of auto-reactive immune cells against myelin antigens in the CNS.

MS is two to three times more common in women than in men (Dunn et al., 2015; Kalincik et al., 2013). Reasons to explain female dominance include hormonal impact (Airas, 2015; Shuster, 2008) and sex-linked genetic traits (Olga L Quintero et al., 2012).

The most consistent evidence for an environmental MS risk is the geographical latitude (Simpson et al., 2011) and it is believed that exposure to sunlight may be the greatest factor. Higher latitude correlates with increased prevalence, incidence and mortalities. More recently, vitamin D3 supplementation has been found to improve MS symptoms (Sotirchos et

al., 2016), thus, suggesting that ultraviolet (UV) exposure might be key. Higher latitudes experience less seasonal sunlight, which is required for Vitamin D synthesis.

Viral, bacterial or fungal infections have also been considered to contribute to MS. It has been suggested that these agents could be compromise the BBB, therefore facilitating leukocyte infiltration in CNS (Kirk et al., 1996), or by causing antigen-specific responses due to molecular mimicry of pathogen components and CNS tissues (Cusick et al., 2012).

Today, there is no single identifiable factor that can be attributed to cause MS. However, it is likely that several factors interplay in concert to cause the disease.

2.1.3 Antigen presentation in Multiple Sclerosis

In MS pathogenesis, activated $CD4^+$ T cells that recognize CNS auto-antigens, or structurally similar foreign antigens, have an important role (Riedhammer et al., 2015). Microscopically, MS lesions are mostly confined to CNS white matter and are found most frequently in periventricular areas. Venules or capillaries center the acute MS lesions. This association of acute lesions with blood vessels suggests that extravasating immunocompetent cells participate in plaque formation and that this is the area where antigen presentation occurs (Larochelle et al., 2011). $CD4^+$ and $CD8^+$ T cells, B cells, plasma cells, macrophages, and dendritic cells (DCs) comprise the inflammatory infiltrates. The majority of myeloid and lymphoid cells express adhesion molecules that likely play a role in extravasation from the blood into cerebral perivascular spaces (CPVS) (Holman et al., 2011).

2.2 CNS Autoimmunity

2.2.1 Experimental Autoimmune Encephalomyelitis

Experimental Autoimmune Encephalomyelitis (EAE) is the animal model for CNS inflammation. Presentation of myelin protein antigen in secondary lymphoid organs is the first step in EAE pathogenesis (Rodríguez-Rodríguez et al., 2003). Once activated in the periphery, leukocytes adhere to the endothelium of blood vessel walls and migrate to the CNS. However, these cells need to be re-activated in the CNS with CNS auto-antigen in the context of MHC (Schmidt, 1999). During inflammation, antigen-specific cells do persist for long periods of time and may play an important role in the amplification of the immune response. EAE lesions in the brain and spinal cord show inflammatory infiltrated of monocytes and lymphocytes (W F Hickey et al., 1983; Serafini et al., 2000). Within the CNS, the most potent antigen presenting cells (APCs) are microglial cells (Heppner et al., 2005).

There is evidence that hematopoietically-derived APCs, including macrophages and DCs, reside and present antigen in CPVS and that this is essential in the initiation and perpetuation of CNS inflammation (Bechmann et al., 2001). B cells are also competent APCs that are abundantly present in CPVS during inflammation (E. R. Pierson et al., 2014).

2.2.2 Role of integrins in leukocyte migration into the CNS

Cell adhesion molecules, like selectins and integrins, play a key role in leukocyte extravasation. Leukocytes and endothelial cells differentially express selectins, which are a family of three members (E-, P-, and L-selectin) and are involved in early stages of extravasation (Gonzalez-Amaro et al., 1999). Integrins are heterodimeric transmembrane

glycoproteins that attach cells to extracellular matrix proteins of the basement membrane or to ligands on other cells (Campbell et al., 2011).

Migration of leukocytes from the periphery into the CNS involves multiple steps (Wilson, 2010). First, slow rolling on endothelial walls allows activated leukocytes to identify proper arrays of chemoattractants and integrin ligands. Prolonged selectin-mediated rolling of neutrophils and lymphocytes may also lead to integrin activation. Once firmly arrested, integrins serve for leukocytes to bind to other blood-borne leukocytes and platelets (Ala et al., 2003). Activated T cells and B cell blasts express high levels of adherence integrins (B. Engelhardt, 2006). All other leukocytes maintain their integrins in mostly inactive states and must undergo in situ modulation to develop high avidity for their special ligands. Following rolling, the arrest of lymphocytes and myeloid cells in venules is mediated by the *in situ* activation of at least one of four main integrins: α_4 β_7, LFA-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), or VLA-4 (α4β1) (Arroyo et al., 1992; Y Shimizu et al., 1990). Integrins of the β1 subfamily, specifically VLA-1, VLA-2, VLA-4, VLA-5 and VLA-6, have been shown to facilitate leukocyte migration across extracellular matrix (de Sousa, 1994). It is important to note that there is considerable redundancy: multiple ligands have been identified for a single receptor, and multiple receptors bind a single ligand (Lowell et al., 2012). The pro-adhesive properties of integrins are overlapping and additive and depend on specific cytoskeletal and transmembrane associations with cytoskeletal adaptor molecules. Integrins are activated bidirectionally: 1) cytoplasmic rearrangements of their subunit tails and 2) extracellular binding by their ligands. A specific combination of chemokines and G protein-coupled receptors (GPCRs) is required for

activation of integrin-dependent arrest. Antagonism of VLA-4 was shown to dramatically reduce leukocyte trafficking into the CNS during inflammation and diminish the progression of disease in preclinical studies on rodent models of MS (Yednock et al., 1992).

2.2.3 Role of VLA-4 in Costimulation

Integrin receptor/ligand interactions provide a potent costimulatory signal to CD3-mediated T cell activation (Davis et al., 1990; Nguyen et al., 2008). Specifically, the VLA-4 mediated interaction of resting human $CD4^+$ T lymphocytes with FN (fibronectin) has been shown to promote CD3-mediated T cell proliferation (Y. Shimizu et al., 1990). Co-immobilization with monoclonal antibodies mAb to CD3 and FN consistently resulted in strong T cell proliferation. Other investigators showed that immobilized FN enhances anti-CD3 induced proliferation of both $CD45RA^{dim}$ (memory) and $CD45RA^{HI}$ (naïve) subsets of $CD4^+$ and $CD8^+$ T cells, and that this effect was inhibited with a mAb against the β1 subunit of VLA-4 (Kitani et al., 1998). Additionally, Nojima et al. showed that the A and B epitopes of VLA-4 play a key role in VLA-4 mediated T cell costimulation (Nojima et al., 1990).

2.3 Role of α4-integrin in CNS immunocompetence

2.3.1 α4-integrin Antagonism in EAE

As previously described, leukocytes activated in the periphery are able to adhere to the endothelium of blood vessel walls in order to traffic into the CNS, where re-activation by antigen presentation from perivascular APCs leads to inflammation. In 1992, Yednock et al., who would later develop a successful therapy for RRMS patients, made a revolutionary

finding. They reported that lymphocytes and monocytes bound selectively to inflamed EAE brain vessels and that this could be reversed with antibodies against VLA-4 (Yednock et al., 1992). When tested *in vivo,* it was clear that anti-α4-integrin effectively prevented the development of EAE and the accumulation of leukocytes in the CNS. Later, Theien et al. compared the ability of anti-VLA-4 to regulate PLP $_{139\text{-}151}$-induced relapsing EAE when administered either before or after disease onset (Theien et al., 2001a). This experimental set up allows testing the differential contribution of VLA-4 during the different stages of the disease. They reported that anti-VLA-4 inhibited the onset and reduced the severity of EAE disease (Theien et al., 2001a). In contrast, treatment either at the peak of acute disease or during remission exacerbated disease relapses and increased the accumulation of $CD4^+$ T cells in the CNS. Interestingly, anti-VLA-4 treatment before or during the disease enhanced T helper type 1 $CD4^+$ T cell (Th_1) responses to both the priming peptide and endogenous myelin epitopes. This group also investigated the ability of small-molecule VLA-4 antagonist to regulate $PLP_{139\text{-}151}$-induced EAE and showed that this agent was able to delay EAE onset but led to severe disease exacerbation upon termination of treatment (Theien et al., 2003). The murine anti- α4-integrin antibody, PS/2, was also used in EAE (Theien et al., 2001b) and the reported confounding results suggest that this treatment might have multiple effects on the immune system. Another group reported high anaphylaxis and mortality in EAE mice treated with PS/2 (Ji et al., 2011) even with reported low concentrations of endotoxin in their PS/2 protocol.

Today, despite all the described efforts, the effects of *in vivo* α4-integrin antagonism on cellular immune responses in secondary lymphoid organs, the migration of APCs into the CNS, and the re-activation of T cells in the brain are still not completely elucidated.

2.3.2 α4-integrin Antagonism in Multiple Sclerosis

Based on the findings made by Yednock et al., a humanized recombinant monoclonal IgG4 (which does not activate complement) antagonist called natalizumab (Tysabri®) was marketed to treat MS patients (Rice et al., 2005). Natalizumab blocks α4-integrin on the surface of mononuclear immune cells, preventing their migration from the periphery into the CNS. Natalizumab is administered parenterally as an intravenous infusion. The reported biological half-life of natalizumab at doses used in a large phase II trial and two phase III trials of approximately 11 days (Yaldizli et al., 2009). However, individuals who received natalizumab at dose of 3 mg/kg per month had detectable serum levels of antibody for 3 to 8 weeks (Warnke et al., 2010).

Based on the clinical effectiveness of natalizumab in MS, Crohn's disease, and rheumatoid arthritis, the Food and Drug administration (FDA) approved natalizumab for the treatment of RRMS in 2004 (Richard A Rudick et al., 2004). Currently, natalizumab is the most successful second-line disease modifying treatment for relapsing forms of MS. However, after long-term treatment with natalizumab (~ 2 years) 1 in 200 patients developed a fatal demyelinating disease called progressive multifocal leukoencephalopathy (PML) (Yousry et al., 2006). Patients that had previous immunosuppression or have detectable

humoral responses to the John Cunningham virus have a higher risk to develop PML when treated with natalizumab but it does not appear to exacerbate the risk of other infections. These observations taken together suggest that natalizumab impairs surveillance of the CNS but the mechanism is currently unknown. Niino et al. have shown that natalizumab treatment significantly decreased the migratory capacity of white blood cells *in vitro* (Niino et al., 2006) while Krumbholz et al. showed an increase of immature and mature $CD19^+$ B cells in circulation (Krumbholz et al., 2008). The implications of circulating cell sequestration on the immunocompetence of the CNS remains to be analyzed.

CHAPTER THREE
Materials and Methods

3.1 Mice

C57BL/6J mice and *Mx1.Cre* $^{+}$ (B6.Cg-Tg(Mx1-cre)1Cgn/J) were purchased from The Jackson Laboratory, Bar Harbor, ME, USA (Kuhn et al., 1995). $\alpha 4^{fl/fl}$ mice were generated, described, and obtained from Dr. Thalia Papayannopoulou, University of Washington (Scott et al., 2003). Briefly, a targeting vector was constructed including the promoter and first two exons of α4 integrin gene, a PGK-neo-p(A) cassette flanked by *lox*P elements, with an additional *lox*P inserted distal to the second exon. AK7 cells were electroporated with linearized vector and floxed clones resulted from homologous recombination. $\alpha 4^{floxed}$ clones were identified with specific primers. Clones with normal XY karyotype were injected into C57BL/6 blastocysts and transferred into pseudo pregnant females. Resulting male chimeras were then bred to C57BL/6 females. Offspring were genotyped and animals heterozygous for the floxed *α4* allele were crossed to generate floxed homozygotes.

$\alpha 4^{fl/fl}$ females were bred to *Mx1.Cre*$^{+}$ males. Progeny were genotyped for the *cre* transgene by PCR utilizing generic *cre* primers (5' –GTGAAACAGCATTGCTGTCACTT-3' and 5' –GCGGTCTGCCAGTAAAAATATC- 3'). *Mx1.Cre*$^{+}$ $\alpha 4^{fl/+}$ mice were intercrossed, and *Mx1.Cre*$^{+}$ progeny were genotyped for the *α4* allele (5'-GTCCACTGTTGGGCAAGTCC-3' and 5'-AAACTTGTCTCCTCTGCCGTC3'). Eight to twelve week old, both female and male mice were used for all experiments. *Mx1.Cre*$^{+}$ $\alpha 4^{fl/fl}$

mice received 3 intra peritoneal injections of 300 μg poly(I)-poly(C) (poly I:C; Sigma Chemical Company, St. Louis, Mo.) given at 2 day intervals on order to activate the Cre recombinase. This was followed by a "wash-out" period of three weeks in which mice were then analyzed or immunized for EAE.

CD11c.Cre^{+} were described by and obtained from Dr. Boris Reizis, Columbia University Medical Center (Caton et al., 2007). Briefly, a clone was generated containing the *Itgax (CD11c)* gene but lacking the 5' end of the adjacent *Itgam (CD11b)* gene. The recombination cassette containing the Cre recombinase open reading frame replaced the coding part of the first *CD11c* exon. The clone insert was released from the targeting vector backbone by restriction enzymes and fertilized in oocytes. The founder line was analyzed and genotyped utilizing generic and specific Cre primers. *CD11c.Cre*$^{+}$ were bred with $\alpha 4^{fl/fl}$ mice and progeny were genotyped for the *cre* gene with generic Cre primers and for $\alpha 4^{fl/fl}$ as described above.

All mice described in this work were crossed and maintained at the UT Southwestern Medical Center Animal Resource Center in a pathogen-free animal facility. All animal procedures were performed in accordance with protocols approved by the UT Southwestern Institutional Animal Care and Use Committee (IACUC).

3.2 Active Induction of EAE

Mice were anesthetized with 200 mg/kg tribromomethanol (1.5% Avertin) injected i.p. Active EAE was induced by s.c. injections into the flanks with 200 μg of mouse myelin oligodendrocyte glycoprotein$_{35–55}$ (MOG_{p35-55}) (MEVGWYRSPFSRVVHLYRNGK; CS Bio

Menlo Park, CA, USA) emulsified in complete Freund's adjuvant (CFA) (DIFCO Laboratories, Detroit, MI, USA) containing 400 µg of heat inactivated *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). Mice also received i.p. injections of 200 ng pertussis toxin on days 0 and 2 (List Biological Laboratories Inc., Campbell, CA, USA). Clinical signs of EAE were assessed daily and reported following the classical criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and uni-lateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund (Cravens et al., 2013). At least three independent experiments were conducted with a minimum of five mice per group.

3.3 Passive Induction of EAE

For passive induction of EAE by adoptive transfer of myelin-specific T cell, single cell suspensions were prepared from splenocytes isolated from actively immunized mice. Cells were activated for 72 hours with MOG_{35-55} and IL-12 *in vitro* (S. D. Miller et al., 2007). After incubation, 5 million cells were injected i.p. into C57BL/6 recipients. Clinical signs of EAE were assessed daily and reported following the classical criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and uni-lateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund.

3.4 Isolation of cells

Splenocytes and lymph node cells were isolated by pressing through a 70µM nylon mesh cell strainer. Cells were treated with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO,

USA), washed twice with cold PBS, and re-suspended in EAE media or PBS for counting with hemocytometer.

For CNS isolation, mice were perfused via the left ventricle with cold PBS. Brain and spinal cord were harvested and pressed through a 70 μM nylon mesh cell strainer, washed with cold PBS and processed with Percoll (Pc) gradients or enzymatic digestions as specified.

3.4.1 Percoll™ gradients

Pellets were resuspended in 37% Pc and centrifuged. Cell pellets were resuspended in 30% Pc and centrifuged against 70% Pc. The cell monolayer between the 30%-70% Pc interface was collected and washed with PBS. Single cells suspensions were counted and stained.

3.4.2 Enzymatic digestions

Brains and spinal cords processed using enzymatic dissociations were first finely minced using a sterile scalpel, washed with cold PBS and then processed based on the specific enzymes used. The commercially available neural tissue dissociation kit (Kitani et al.) was used following the manufacturer's protocol (Neural Tissue Dissociation Kit (P), Miltenyi Biotec). Ac (Accutase®, Global Cell Solutions) was added to the tissues at 1mL per tissue and incubated at room temperature for 15, 30, or 60 minutes. 20U of papain (Pp, Sigma-Aldrich) was added per tissue and then incubated for 15, 30, or 60 minutes at 37°C; immediately following the incubation, Pp was quenched using 150μg ovomucoid (Sigma-

Aldrich). For the combination of Ac + Pp, 20U of Pp was first added to the brain isolates and incubated for 30 minutes at 37°C. After 30 minutes, the Pp was quenched using 1.5µg ovomucoid. The brains were then washed with cold PBS and 1ml of Ac was added for 15 minutes at room temperature. Following all enzymatic dissociation methods, brains were washed with cold PBS and then subjected to one wash with 37% Pc to remove remaining myelin. The myelin-free single cell suspensions were then counted using a hemocytometer.

3.5 Immunophenotyping

$1x10^6$ cells from spleens, lymph nodes, bone marrow, and CNS were resuspended in FACS buffer (5% Fetal Bovine Serum (FBS) in PBS) and Fc receptors were blocked with anti-CD16/32 (Tonbo Biosciences) for 10 minutes at 4°C. For blood analysis, 60 µl of blood were also treated with 1µg anti-CD16/32 but incubated and stained at room temperature. Cells were then stained for surface markers with fluorochrome-conjugated mAbs: Integrin α4-FITC from Santa Cruz Biotechnology, Inc.; CD3e-Pacific Blue, CD19-Alexa Fluor 700, CD11c-PE, CD11b-APC, GR-1-APC-Cy7 all from BD Biosciences (San Jose, CA, USA); CD45-PE-Cy7 from eBioscience (San Diego, CA, USA); CD4-PE-Texas Red, CD8-Pacific Orange both from Invitrogen (Grand Island, NY, USA); CD11a-PE and CD49e-PE from BioLegend (San Diego, CA, USA); biotinylated PDCA-1 from Miltenyi (Auburn, CA, USA) which was revealed with SA-Q Dot 655 from Invitrogen (Grand Island, NY, USA). Cells were then washed, resuspended in staining buffer, and fixed in 0.5% paraformaldehyde. For enzymatic dissociation comparison experiments, $1x10^6$ cells from individual brains and spinal cords were stained with 1 µL Zombie Aqua™ Fixable Viability Dye (BioLegend) and

incubated for 15-30 minutes at room temperature, following manufacturer's recommendations. Cells were then washed using FACs Buffer at 1500rpm for 5 minutes, followed by incubation with 1µg anti-CD16/32 (Tonbo Biosciences) for 15 minutes at 4°C. Cells were then stained with CD45-PE-Cy7 from eBioscience (San Diego, CA, USA) and CD11b-APC from BD Biosciences (San Jose, CA, USA) for 35-40 minutes at 4°C.

Fluorescence minus one (FMO) controls were prepared by adding all antibodies but one, for each parameter to discriminate positive staining from non-specific background (**Figure 3.4 J**). Up to 500,000 events were acquired on a BD FACS LSR II at UT Southwestern Flow Core or FACS LSRFortessa SORP at The Moody Foundation Flow Cytometry Facility, Children's Medical Center Research Institute at UT Southwestern. Data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

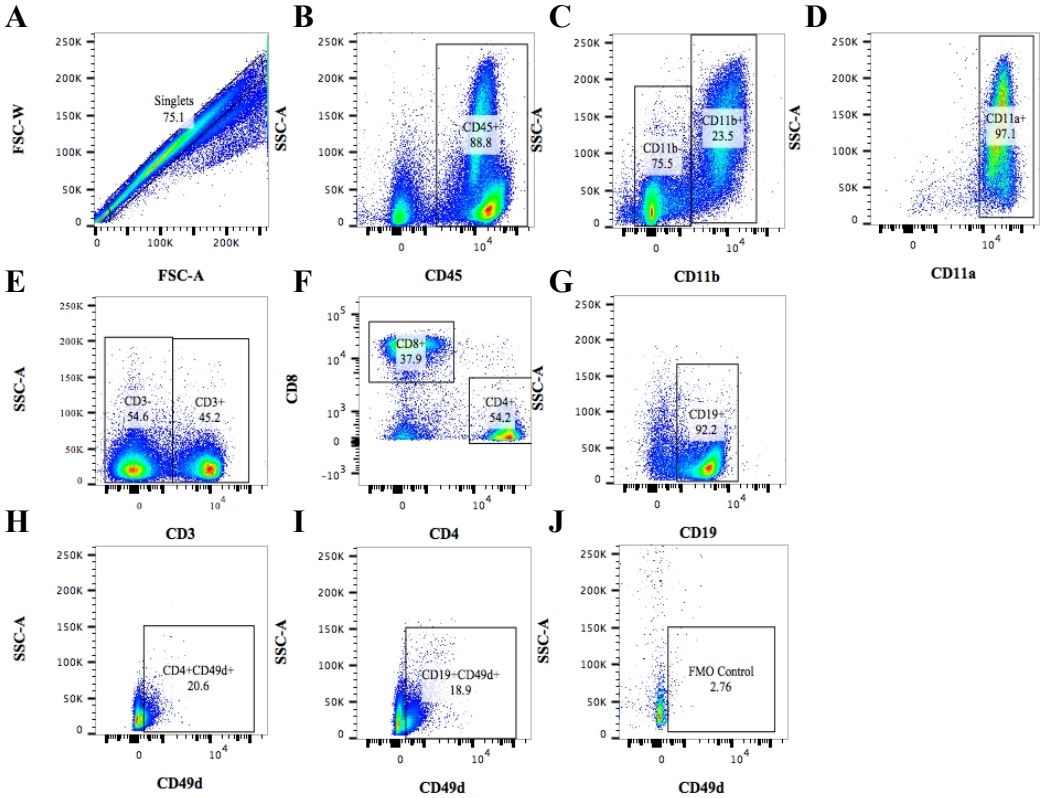

Figure 3.1. Gating strategy for identifying differences in populations and expression of CD49d (α4-integrin). To remove false-positive events, single cells were selected (**A**) and CD45 positive were designated as all leukocytes (**B**). CD11b negative lymphocytes (**C**) were further analyze with CD3 positivity (**E**) into CD4 or CD8 positive T cells (**F**). B cells were analyzed based on CD3 negativity and CD19 positivity (**G**). Integrin expression was gated for each population of interest (**D** and **H-I** representations) and fluorescence minus one (FMO) samples were utilized as controls (**J**).

3.6 Proliferation assay

15 days post immunization, single cell suspensions were generated by isolating the LNs of the immunized mice. Utilizing the CellTrace™ CFSE (5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester) Cell Proliferation kit (Life Technologies, Carlsbad, CA), $CD4^+$ T cell proliferation against antigens was determined. Briefly, isolated $20x10^6$ LN cells were incubated for 5 minutes at room temperature with 1μM CFSE. After incubation, cells were washed with RPMI media twice, then incubated in a 96-well-round bottom plate at $1x10^6$ cells per well with specified antigen for 96 hours. Post incubation, cells were washed with staining FACS buffer two times, then the Fc receptors were blocked with anti-CD16/32 (BD Biosciences, Franklin Lakes, NJ,) for 15 minutes at 4°C before staining with mAbs for 30 minutes at 4°C. Cells were stained utilizing the following monoclonal antibodies: CD3e-Pacific Blue, CD45-PE-Cy7 and CD4-PE-Texas Red. Cells were analyzed with a LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

3.7 RNA isolation and quantitative real-time PCR

TRI Reagent® Solution was utilized for RNA extraction of freshly isolated tissues of mice sacrificed on day 15 post immunization. Mice were overdosed with 400 mg/kg tribromomethanol and transcardially perfused with 20 mL ice cold PBS. Spleen, brain and spinal cord tissues were placed in 10-20 volumes of TRI Reagent solution after dissection Tissues were homogenized in a glass homogenizer, transferred into a new tube and allowed to rest for 5 minutes at RT. 200 μl chlorophorm was added to each sample, mixed vigorously for 15 seconds, and centrifuged at 12000g for 15 minutes at 4°C. After centrifugation, the

upper aqueous phase was transferred into a new tube. An equal amount of isopropanol was added and incubated on ice for 15 minutes. Samples were centrifuged at 12000g for 15 minutes at 4°C. Supernatant was decanted and the pellet was washed twice with 75% ethanol, dried and resuspended with 100 µl DEPC (diethylpyrocarbonate)-treated H_2O. RNA concentration was measured with a NanoDrop (Thermo Scientific NanoDrop™ 1000 Spectrophotometer). Taqman gene expression assays and the Step One Plus (Applied Biosystems. Foster City, CA) were utilized to detect IFNγ, IL-17a, IL-12a, Csf2 (GM-CSF), IL-23a, IL-6. Fold change in expression relative to untreated group was determined using the ddCt algorithm method described by the seller. The dCt was normalized to the housekeeping gene *GAPDH*.

3.8 In vitro migration assay

We performed an *in vitro* migration assay by Boyden Chamber as described before (Stuve et al., 1996). Briefly, we utilized a Boyden chamber containing a polycarbonate membrane filter (Transwell® Permeable Supports, Corning Inc., Corning NY) precoated on it's upper surface with 20 µg/ml FN. A total of $6x10^5$ splenocytes, suspended in EAE media, were added to the upper chamber. Chambers were then incubated at 37°C for 6 to 8 hours. Following incubation, the content of the lower chamber was collected and the number of cells were counted with a hemocytometer and the phenotype of the cells determined by flow cytometry.

3.9 Evaluation of BBB permeability

Mice were injected intravenously (i.v.) with 200 μL of 3% (weigth/volume) Evans Blue dye and perfused with 4% paraformaldehyde after ~3 hrs. Brains and spinal cords were fixed in 4% paraformaldehyde and photographed with a dissecting microscope. For quantification of Evans Blue dye, tissues were dried at 56°C overnight, then incubated with 8 mL/g *N, N*-dimethylformamide at 56°C for 48 hours. Evans Blue dye is soluble in *N, N*-dimethylformamide, therefore we prepared exponential dilutions for a standard curve and measured absorbance with spectrophotometer at 650nm (Ibla et al., 2006; Xu et al., 2001).

3.10 Histology

Brains were perfused and isolated as described above and fixed in 10% formalin. Brains were then coronally sectioned, embedded in Tissue-tek O.C.T. Compound, and snap frozen in liquid nitrogen. 6 μm thick section were cut utilizing a freezing microtome and mounted on Fisher Brand Superfrost Plus glass slides. Samples were stained with hemotoxylin and eosin (H&R (Fisher Scientific, Pittsburgh, PA)) and prepared for light microscopy examination.

3.11 Endotoxin Detection Assay

ToxinSensor™ Single Test Kit (GenScript, Piscataway, NJ, USA) was utilized per manufacturer's recommendations by diluting Pc using Limulus amebocyte lysate (LAL) Reagent Water, adding 200μL of the diluted sample to the provided LAL vials, and then

incubating vials for 60 minutes at 37°C and testing for the formation of a firm gel after the incubation.

3.12 Statistical analysis

All experiments were repeated at least twice and at least 5 mice were utilized per treatment group. The means of samples were compared using an unpaired Student's t-test. Mean clinical scores significance between groups was analyzed by Mann-Whitney *U* test. The criterion for significance (alpha) has been set at *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data are given as mean ± standard error. All analyses were performed with Prism 6 for Windows (GraphPad Software, La Jolla, CA, USA).

3.13 Media and reagents

Table 3.1 Media and reagents		
Reagent	**Contents**	**Preparation and storage**
EAE media	90% Roswell Park Memorial Institute media (RPMI 1640 medium; Cellgro Mediatech, Manssas, VA, USA), 10% fetal calf serum, 20 mM L-glutamine, 10 mM HEPES, 100 IU/mL penicillin, 1% non-essential amino acids, 0.1 mg/mL streptomycin, 1mM sodium pyruvate, $5.5x10^{-5}$ M 2-mercaptoethanol	RPMI, HEPES, sodium pyruvate, non-essential amino acids and 2-mercaptoethanol were stored at 4°C. Rest of reagents were stored at -20°C. All reagents were combined and media was filter sterilized with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). EAE media was stored at 4°C and discarded if phenol red pH indicator was not optimal.
1.5% Avertin	2.5 mg Tribromoethanol, 300µL 2-methyl-2-butanol, 50 mLs distilled hot water	Tribromoethanol and 2-methyl-2-butanol were mixed in a 50 mL falcon tube and hot water was added until all reagents were dissolved. Solution was sterilized with 0.22 µm syringe filter unit and kept in a 50 mL falcon tube wrapped in foil at 4°C. Reagent was used within two weeks.
FACS Buffer	5% FBS PBS	Store at 4°C

CHAPTER FOUR
Poly I:C-induced α4-integrin ablation effects on experimental autoimmune encephalomyelitis

4.1 Introduction

Antagonism of α4β1 integrin (also known as very late antigen-4; VLA-4) was shown to dramatically reduce leukocyte trafficking into the CNS during inflammation and diminish progression of EAE in preclinical studies on rodent models of MS (Yednock et al., 1992). Based on these findings, a humanized monoclonal IgG4 antagonist called natalizumab (Tysabri®) was marketed to treat MS patients (Rice et al., 2005). Natalizumab blocks α4-integrin on the surface of mononuclear immune cells, preventing their migration from the periphery into the CNS. Currently, natalizumab is the most successful second-line disease modifying treatment for relapsing forms of MS (Hutchinson, 2007; Polman et al., 2006; R. A. Rudick et al., 2008). However, after long-term treatment with natalizumab, 1 in 200 patients might developed a fatal demyelinating disease called progressive multifocal leukoencephalopathy (PML) (Ransohoff, 2005; Vermeer et al., 2015). These facts, taken together, suggest that natalizumab impairs surveillance of the CNS, but the mechanism is currently unknown.

Previously, Stuve, et al. demonstrated that continuous natalizumab administration to patients caused a prolonged reduction of lymphocyte subsets present in cerebrospinal fluid (Stüve et al., 2006). Another study done by del Pilar Martin, et al. shows that natalizumab specifically reduced the number of antigen presenting cells associated with CPVS (del Pilar Martin et al., 2008) thus suggesting that leukocytes present in these

spaces might be important for appropriate immuno competence of the CNS. But, the requirement of α4-integrin expressing leukocytes in CPVS for effective immuno competence in the CNS has not been experimentally established.

The objective of this study was to analyze the effects of leukocyte α4-integrin ablation on immunocompetence of the CNS. We hypothesize that antagonism of α4-integrin diminishes immune competence within the CNS by differentially affecting leukocyte subsets.

To address this hypothesis, our lab generated the Mx1.Cre^{+}α4-integrin^{flfl} mouse strain, in which α4-integrin can be conditionally deleted on interferon-induced GTP-binding protein Mx1 expressing cells through the Cre-loxP system. First, we analyzed the effect of α4-integrin deletion on leukocytes *in vivo* by active induction of EAE on poly I:C treated Mx1.Cre^{+}α4-integrin^{flfl} and C57BL/6 mice and immunophenotyping of secondary lymphoid tissue and CNS. Next, we analyzed the effect of α4-integrin deletion on leukocytes *in vitro* by migration and proliferation assays.

4.2 Results

4.2.1 Expression of α4-integrin is reduced in leukocytes of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice while EAE disease course is delayed when compared to poly I:C treated C57BL/6.

In the Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mouse model, the Cre recombinase is under the control of the *Mx1* promoter which can be induced to high levels by admininstration of synthetic double stranded RNA (poly I:C). Poly I:C antagonizes TLR3 in endosomes

leading to production of type I interferon that is recognized by IFN receptors in cells. This activates downstream transcription factors that can translocate to the nucleus and start transcription of antiviral genes including *Mx1*. In Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, Cre recombinase targets loxP sites flanking the *Itga4* (α4-integrin) gene, causing its deletion (**Figure 4.1**). With this system we are able to conditionally delete α4-integrin on all IFN-receptor expressing cells, which includes leukocytes. Eight-to-ten-week old Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and C57BL/6 mice received 3 i.p. injections of 300 µg poly I:C given at 2 day intervals. Followed by a wash-out period of three weeks, mice were immunized with 200 µg MOG_{35-55} in CFA. To determine the degree of deletion of α4-integrin on leukocytes, we did phenotypic characterization of spleen and lymph nodes from poly I:C treated C57BL/6 and Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice before (naïve) and 15 days post immunization (**Figure 4.2 A-D**). We observed 2x or greater reduction in CD49d (α4-integrin) expressing CD45^{+} leukocytes in naïve lymph nodes (32%) and spleens (22%) from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice when compared to C57BL/6 wild type (76% in lymph nodes and 71% in spleen) (**Figure 4.2A and C**). Similar results were observed for lymphocyte and granulocyte populations in naïve lymph nodes and spleens. Data from 15 days post-immunization demonstrated an increase in CD45^{+}CD11b^{+} CD49d expressing cells in lymph nodes when compared to naïve. However, there were 3x less CD45^{+}CD11b^{+} CD49d expressing cells in lymph

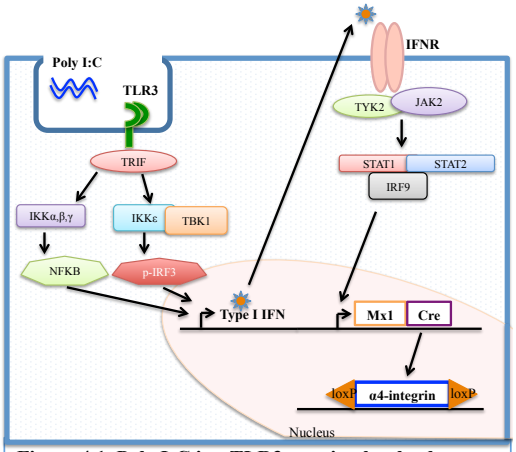

Figure 4.1. Poly I:C is a TLR3 agonist that leads to activation of Mx1 antiviral gene leading trascription of Cre recombinase and downstream deletion of α4-integrin.

nodes and $CD45^+$ CD49d expressing cells in spleens poly I:C treated $Mx1.Cre^+\alpha4\text{-integrin}^{fl/fl}$ mice when compared to C57BL/6 wild type (**Figure 4.2B and D**).

We hypothesized that due to the observed reduction of α4-integrin expression on leukocytes from secondary lymphoid organs, the migration of cells, specifically $CD4^+$ cells, into the CNS would also be reduced causing amelioration of EAE disease course. To test this, we followed the clinical disease course for 30 days. Unexpectedly, the EAE disease course of poly I:C treated $Mx1.Cre^+\alpha4\text{-integrin}^{fl/fl}$ mice remained comparable to that of C57BL/6 (**Figure 4.2E**), albeit there is a significant delay of disease onset from day 7 for poly I:C treated C57BL/6 to day 10 for poly I:C treated $Mx1.Cre^+\alpha4\text{-integrin}^{fl/fl}$ mice. Cells from poly I:C treated $Mx1.Cre^+\alpha4\text{-integrin}^{fl/fl}$ mice were capable of proliferating *in vitro* when presented with Con A and MOG_{35-55} similarly to cells from poly I:C treated C57BL/6 (**Figure 4.2F**). This demonstrated that cells with reduced expression of CD49d are capable of responding to antigen and expanding.

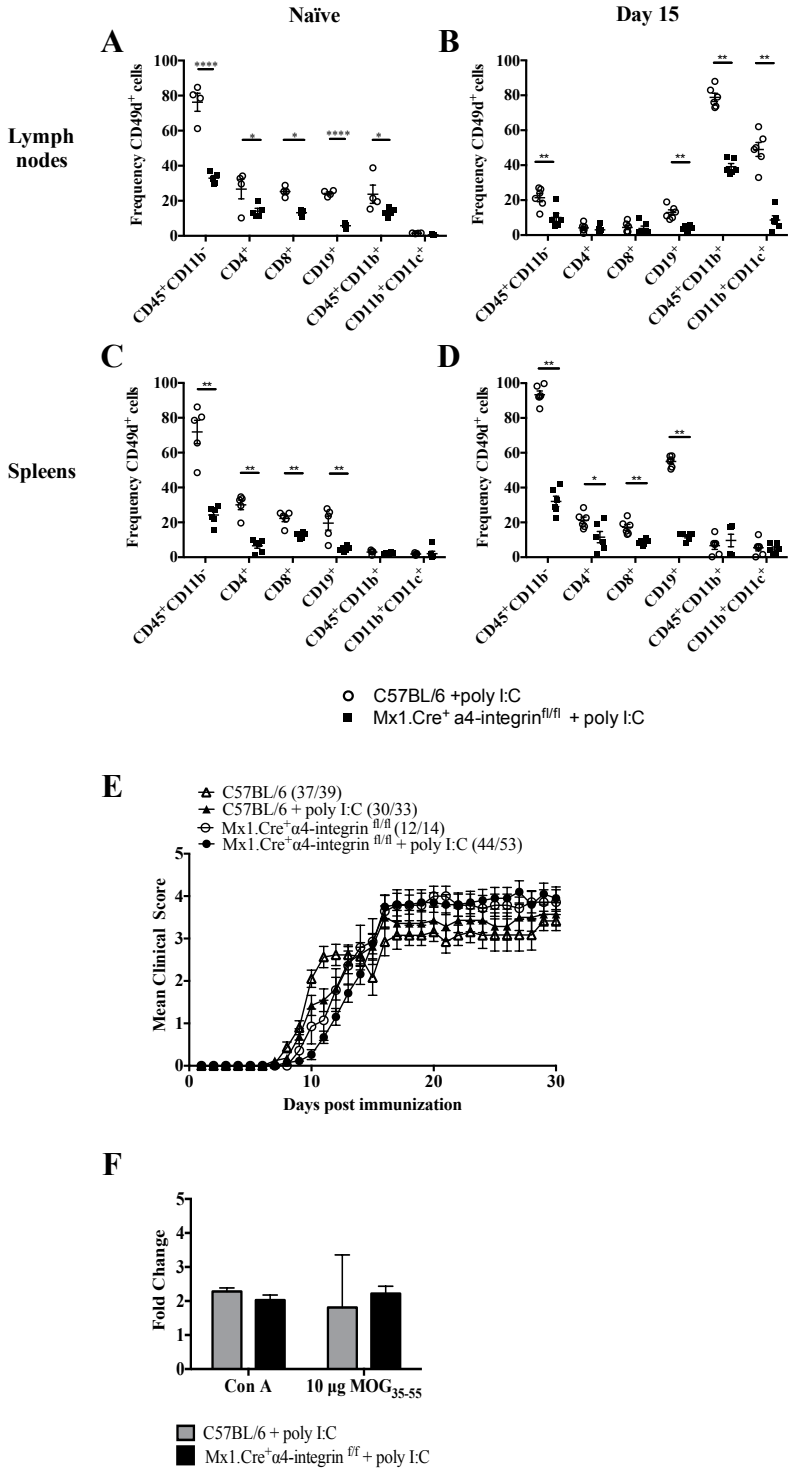

Figure 4.2: Expression of α4-integrin is reduced in leukocytes of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice while EAE disease course is delayed when compared to C57BL/6. α4-integrin expression was analyzed by CD49d expression **(A through D)**. Single live cells from lymph nodes **(A and B)** and spleens **(C and D)** were analyzed for CD45 and CD11b surface expression. CD45^{+} CD11b^{-} T cells were gated on CD3 positivity and divided into CD4^{+} and CD8^{+} populations. B cells were gated on CD3

negativity and analyzed for CD19 expression. Granulocytes were analyzed by $CD45^+$ $CD11b^+$ surface expression and dendritic cells were further interrogated for CD11c expression. Clinical signs of EAE **(E)** were assessed daily and reported with the following criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and uni-lateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund. Cell proliferation of lymph node cells isolated 15 days post immunization is not affected by α4-integrin decreased expression **(F)**. Fold change was calculated as the ratio of proliferating/$CFSE^{lo}$ cells in Con A and MOG_{35-55} versus media.

4.2.2 Splenocytes from poly I:C Mx1.Cre$^+$α4-integrin$^{fl/fl}$ show reduced migratory capabilities in vitro

To test the effect of α4-integrin deletion after poly I:C treatment on migratory competence of cells, we performed an *in vitro* migration assay by Boyden Chamber as described before (Stuve et al., 1996). A total of 5 x 10^5 splenocytes in complete media were added to the upper part of the Boyden Chamber pre-coated with fibronectin. Six hours after incubation, cells in the bottom chamber were collected and analyzed by flow cytometry. We observed a significant reduction in the migration of $CD45^+$ splenocytes from poly I:C Mx1.Cre$^+$α4-integrin$^{fl/fl}$ when compared to C57BL/6 (**Figure 4.3A**). Further characterization of the splenocytes revealed that $CD45^+$ $CD3^-$ cells (**Figure 4.3B**), specifically granulocytes ($GR1^+$) and plasmacytoid dendritic cells ($PDCA1^+$) (**Figure 4.3C**) migrated less. When comparing the lymphocyte compartment, we observed significant reduction in $CD19^+$ B cells from poly I:C Mx1.Cre$^+$α4-integrin$^{fl/fl}$ mice (**Figure 4.3B**).

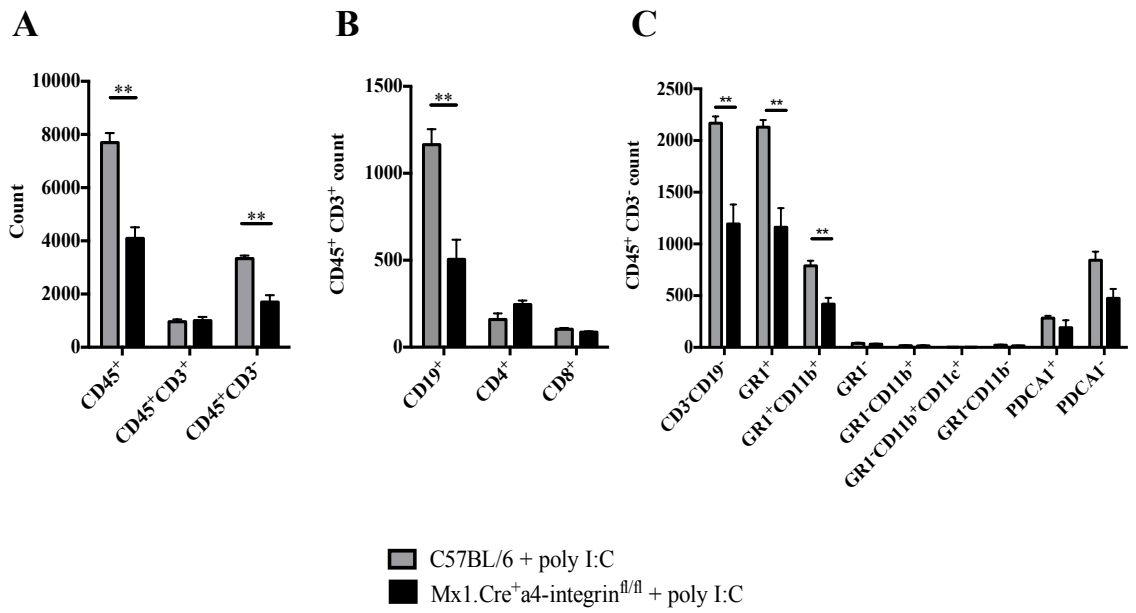

Figure 4.3: Splenocytes from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice show reduced migratory capabilities *in vitro*. A Boyden chamber was utilized to study cell migration in vitro. The transwell was coated with fibronectin and a total of 6x10^{5} splenocytes, suspended in EAE media, was added to the upper chamber. Bottom chambers were then incubated at 37°C for 6 hours. Leukocytes that were able to utilize α4-integrin to cross the coated transwell were collected and analyzed by fluorescence-activated cell sorting and counts are displayed **(A)**. CD45 positive cells were gated out of singlets and live cells **(A, B and C)**. *p<0.05, **p<0.01.

4.2.3 α4-integrin surface expression is significantly reduced but not depleted in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice cell type isolated from the CNS

To analyze the CNS of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 mice, mononuclear cells were isolated from brains and spinal cords by Percoll™ (Pc) gradient. We observed a significant reduction in the percentage of cells expressing CD49d in the CNS of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice at day 15 post immunization when compared to C57BL/6 (**Figure 4.4A**). As stated before, there was not a complete depletion of CD49d^{+} in cells with our inducible model. Therefore, there was a low number of CD49d^{+} lymphocytes (18%) and myeloid cells (20%) infiltrating the CNS.

These cells, however, do not show altered surface expression of the CD49d protein when their mean flourescence intensity is compared to wild type cells (**Figure 4.5A and D**). To visualize the level of infiltration in the CNS, we counted and compared absolute numbers of live mononuclear cells from C57BL/6 mice and Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice that were naive and 15 days post immunization. There was 2.62 fold increase in the number of cells from C57BL/6 mice after immunization and 3.43 fold increse for Mx1.Cre^{+}α4-integrin$^{fl/fl}$ immunized mice (**Figure 4.4C**), as expected. We then compared the same mouse strains after poly I:C treatment. We observed a 7.17 fold increase in the number of cells isolated from CNS of poly I:C treated C57BL/6 mice and a 2.61 fold increase in the number of cells of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$.

These data suggested a few possibilities: 1) poly I:C could have compromised the BBB allowing for higher transmigration, 2) there were cells infiltrating into the CNS of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ because there is no absolute requirement of α4-integrin for their migration, 3) incomplete α4-integrin deletion resulted in entry and expansion of α4-integrin positive populations. Further analysis of the CNS subpopulations revealed a higher infiltration of CD45^{+} CD3^{+} cells, more specifically CD4^{+} T cells, and CD11b^{+}CD11c^{+} dendritic cells in the poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ (**Figure 4.4 D-F**).

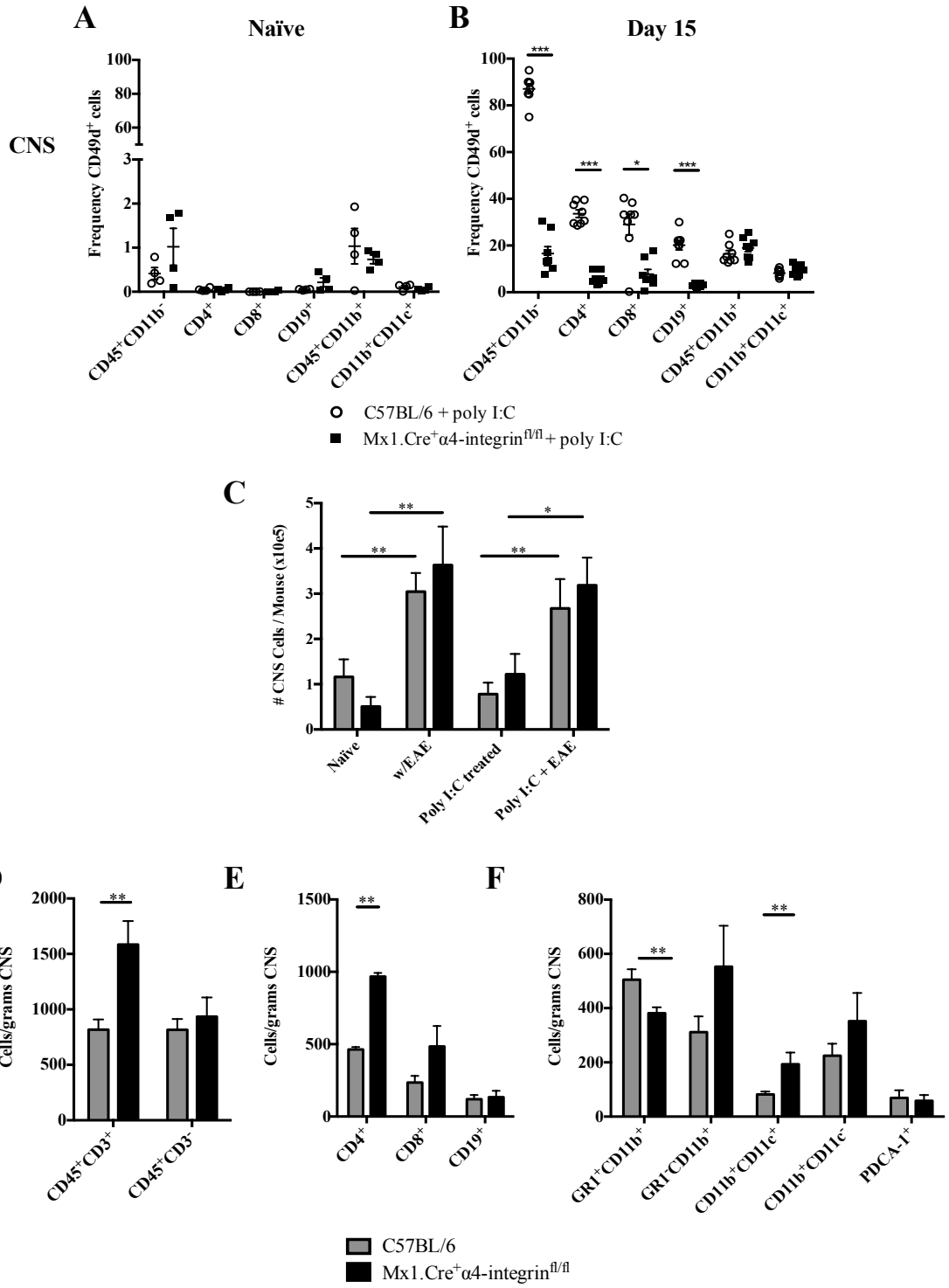
A
Naïve
B
Day 15
CNS
Frequency CD49d+ cells
CD45+CD11b-
CD4+
CD8+
CD19+
CD45+CD11b+
CD11b+CD11c+
C57BL/6 + poly I:C
Mx1.Cre+α4-integrinfl/fl + poly I:C
C
CNS Cells / Mouse (x10e5)
Naïve
w/EAE
Poly I:C treated
Poly I:C + EAE
D
E
F
Cells/grams CNS
CD45+CD3+
CD45+CD3-
GR1+CD11b+
GR1-CD11b+
CD11b+CD11c+
CD11b+CD11c-
PDCA-1+
C57BL/6
Mx1.Cre+α4-integrinfl/fl

Figure 4.4: α4-integrin surface expression is significantly reduced but not depleted in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice cell type isolated from the CNS. Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice that are poly I:C treated show reduced frequency of α4-integrin (CD49d) expressing cells in CNS 15 days post immunization **(A, B)** as analyzed by immunophenotyping. However, there is a similar number of infiltrating cells in the CNS as counted 15 days post immunization with MOG_{35-55} **(C).** Number of CNS cells/mouse was determined by visually counting number of cells in a hemocytometer after Percoll gradient isolation. There were 3 independent isolation experiments with 3 brains per group **(C)**. CNS isolated cells were analyze by immunophenotyping, **(D-F)** showing a significant increase on lymphocyte **(D)** and CD11b^{+}CD11c^{+} dendritic cell populations infiltration. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are given as mean ± standard error.

4.2.4 Poly I:C affects permeability of BBB in Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice

To directly test if poly I:C could have compromised the BBB thus increasing infiltration on brain or spinal cord, we performed an Evans Blue Dye (EBD) permeability assay. EBD has high affinity for serum albumin. Once the BBB is compromised the serum-dye complex can penetrate the parenchyma of the CNS allowing for visualization and quantification by spectrophotometry (650nm). For this experiment we injected EBD at day 14 after immunization, when mice presented a clinical score of 3 or 4. Brains and spinal cords were collected 3 hrs after injeccion and EBD was quantified (**Figure 4.5A**). We did not observe a difference in the amount of dye infiltrating into the CNS between C57BL/6 and Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice, however we did observe a distinct pattern of infiltration in the spinal cord tissue (**Figure 4.5B**). These results were supported by histological analysis results of spinal cord from C57BL/6 and Mx1.Cre^{+}α4-integrin$^{fl/fl}$ immunized mice, with and without poly I:C treatment (**Table 3.1**). A high increase of infiltration in the spinal cord of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice was observed (**Figure 4.5C**).

In summary, this *in vivo* BBB permeability assay demonstrates anatomical differences of infiltration between poly I:C treated Mx1.Cre^{+} α4-integrin$^{fl/fl}$ and poly I:C treated C57BL/6 mice. It is important to note that different cells might have different patterns of migration depending on the type of integrin being expressed (Rothhammer et al., 2011). We compared by flow cytometry the expression of α5-integrin (CD49e, **Figure 4.6B and E**), which has been reported to bind VCAM-1 (Weber et al., 1996), and the expression of the LFA-1 alpha L chain, CD11a (**Figure 4.6C and F**), which has been reported to be preferentially expressed by T helper type 17 CD4^{+} T cells (Th_{17}) in the trafficking to CNS during EAE (Rothhammer et al., 2011). We did not observe a significant up-regulation or down-regulation of these molecules on cells infiltrated into the CNS or cells from lymph nodes. (**Figure 4.6**).

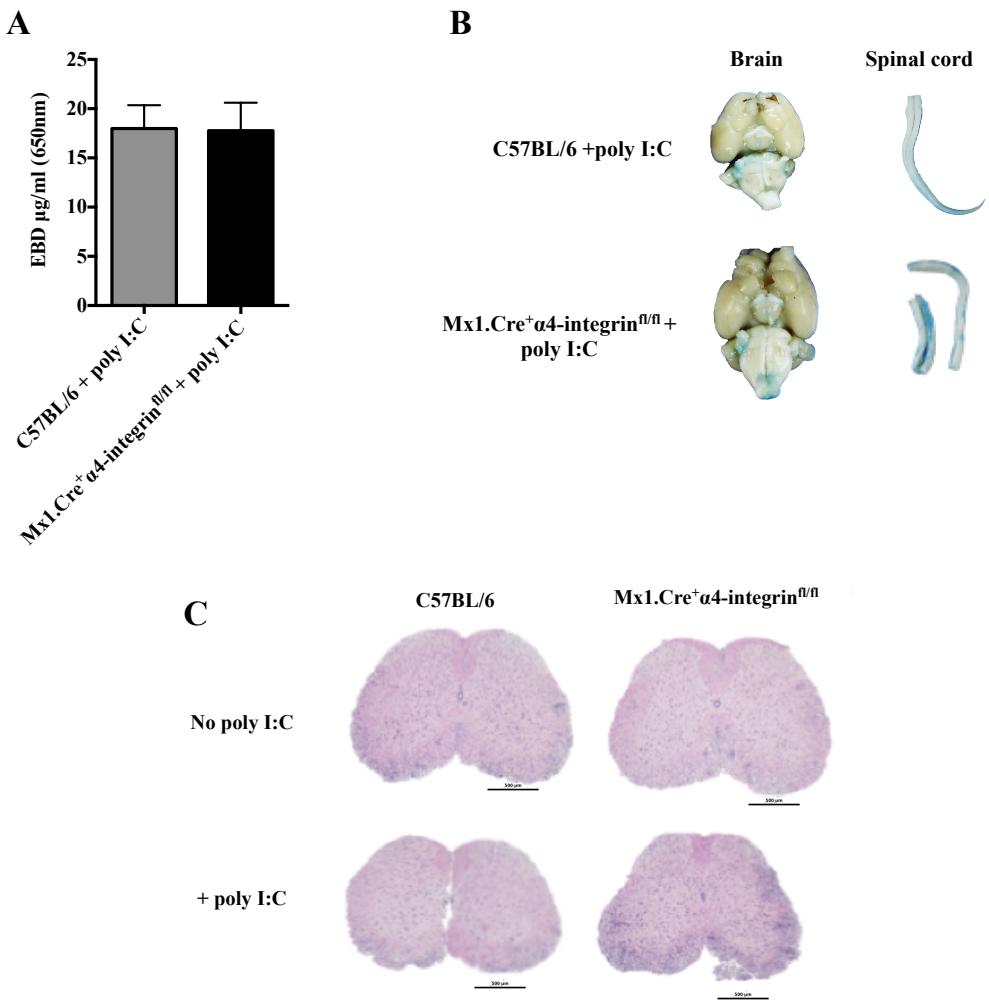

Figure 4.5. Poly I:C affects patter of infiltration in brain and spinal cord tissue of EAE mice. Evans Blue Dye (EBD) was utilized to assess the permeability of the blood brain barrier. We observed no significant difference in the amount of EBD in tissue **(A)** but a dissimilar patter of infiltration is observed **(B).** H&E stain analysis of spinal cord tissue shows location of inflammation **(C, Table 3.1)** Scale bar is 500µm.

Table 4.1. Variation on the location of inflammation in CNS of poly I:C treated C57BL/6 and Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice.

Mouse	Treatment	Onset of EAE	Specimen	Location of inflammation
C57BL/6	Poly I:C + EAE	D9	Brain	• Corticospinal tracts • Sensory trigeminal tract • Spinal trigeminal tract
			Spinal cord	• Upper cervical spinal cord • Spinal cord white matter
Mx1.Cre^{+}α4-integrin$^{fl/fl}$	Poly I:C + EAE	D11	Brain	• Ventral anterior cochlear nucleus • Middle and inferior cerebellar peduncles
			Spinal cord	• Spinal cord white matter

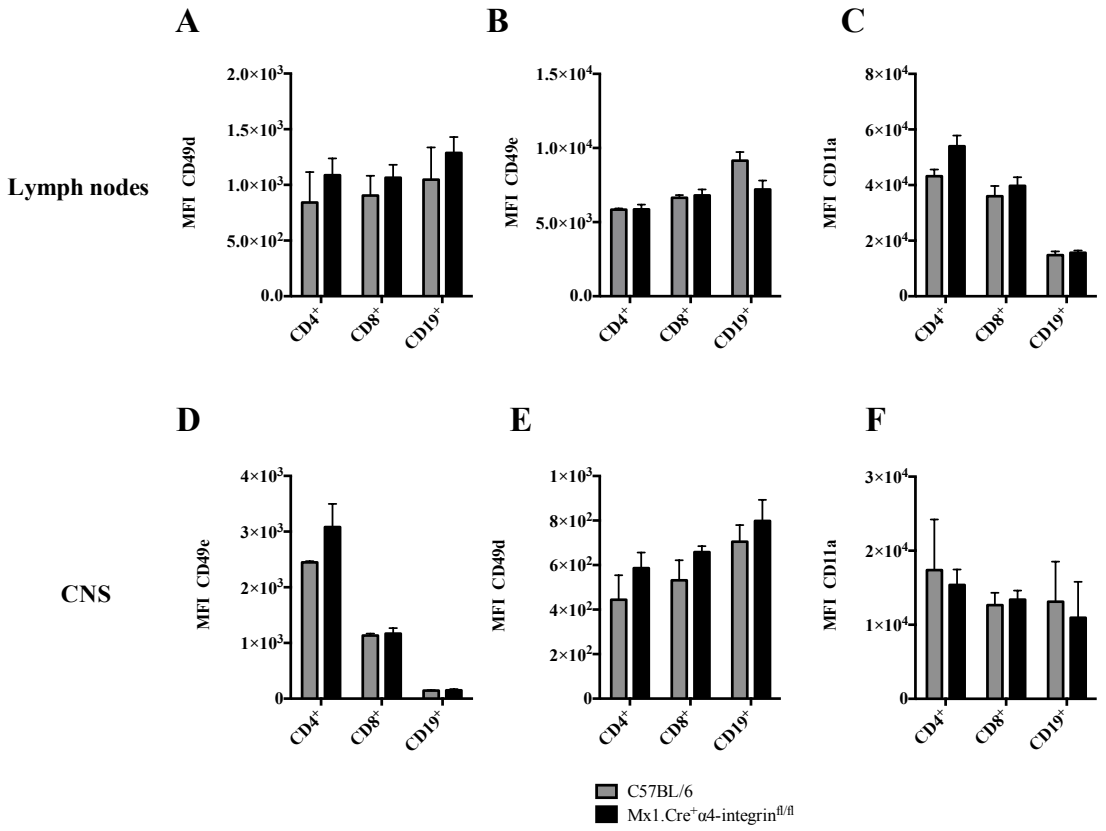

Figure 4.6: Poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 mice express similar α4-integrin , α5-integrin, and LFA-1α surface expression. Mean fluorescence intensity (MFI) of α4-integrin (CD49d), α5-integrin (CD49e), and LFA-1α (CD11a) surface expression of cells isolated with Percoll gradients15 days post immunization from CNS.

4.2.5 Poly I:C treatment increases pro-inflammatory cytokine levels on CNS during EAE

To further analyze the cytokine profile of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 and try to elucidate additional effects of poly I:C, we performed quantitative real-time PCR (qPCR) from whole brains 15 days post immunization. At the transcription level, we observed a significant increase of IFNγ, IL-17A, GM-CSF, and IL-6 cytokines when mice are treated with poly I:C (**Figure 4.7A, B, D, and F**). Additionally, we observed an increase of IL-12A and decrease of IL-23A in poly I:C treated C56BL/6 mice only (**Figure 4.7C and E**).

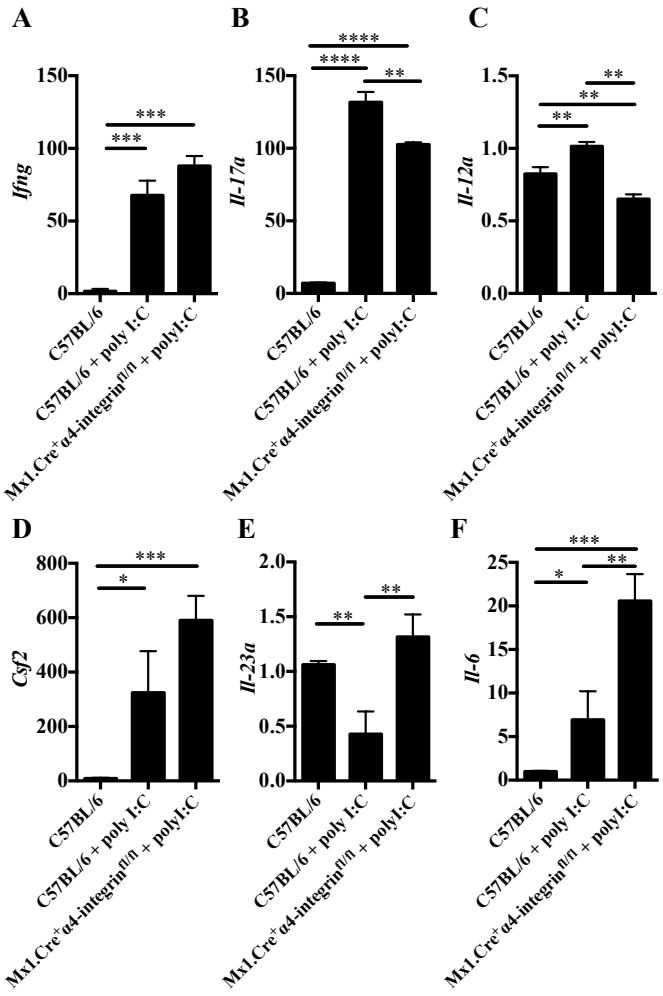

Figure 4.7. Poly I:C treatment increases pro-inflammatory cytokine levels on CNS during EAE. Fold change relative to untreated. *GAPDH* was utilized as housekeeping gene. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.6 Adoptively transferred lymph node cells from Poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ does not transfer EAE disease in C57BL/6 mice

In order to exclude the systemic effect of poly I:C on the BBB during EAE, we tested the role of α4-integrin ablation by passively transfering activated cells from

immunized poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice or C57BL/6 mice into naïve C57BL/6 mice. Lymph nodes from immunized poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 were collected and single cell suspensions were activated *in vitro* with MOG_{35-55} and IL-12. Following 72 hrs, 10 million cells were transfered into naïve C57BL/6 mice and clinical scores were recorded for 30 days. Our results demonstrated that both recipient groups have disease onset at day 7 (**Figure 4.8**). However, 75% of the poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ cells recipients get a mild disease and are able to recover by day 15 post immunization. In contrast, 100% of the poly I:C treated C57BL/6 cells recipients get typical EAE disease course and are able to recover by day 30.

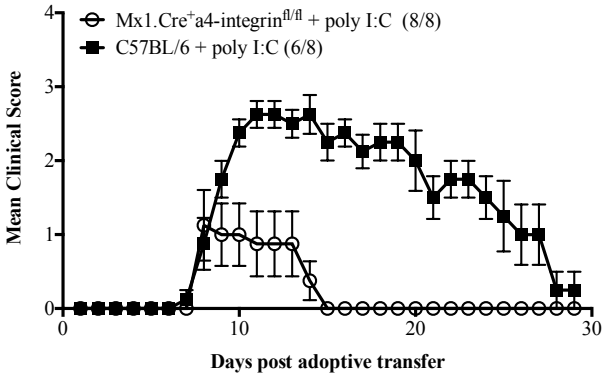

Figure 4.8. Adoptively transferred lymph node cells from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ does not transfer EAE disease in C57BL/6 mice. Lymph nodes from poly I:C treated immunized mice were isolated 10 days pot immunization. Cells were activated *in vitro* with MOG_{35-55} and IL-12 for 72 hours. $10x10^{6}$ cells activated cells were then transferred into naïve C57BL/6 mice. Clinical signs of EAE were assessed daily and reported with the following criteria: 0 = no clinical disease, 1 = limp tail, 2 = partial hind leg paralysis, 3 = complete hind leg and uni-lateral paralysis, 4 = complete hind leg and partial front leg paralysis, 5 = moribund.

CHAPTER FIVE
Enzymatic dissociation improves the isolation of mononuclear cells from individual brains and spinal cords of experimental autoimmune encephalomyelitis mice.

5.1 **Introduction**

Leukocyte migration into the brain and spinal cord is crucial for the initiation and perpetuation of central nervous system (CNS) autoimmunity. Immunophenotyping of CNS infiltrating leukocyte subsets in the animal model experimental autoimmune encephalomyelitis (EAE) allows for the identification of relevant pathogenic cells and potential cellular targets for immunotherapy in multiple sclerosis (MS) and possibly other inflammatory neurological diseases of the CNS.

Pc gradients are commonly utilized to isolate mononuclear cells from the lipid-enriched environment of the CNS as it allows for a separation of myelin from cells by density centrifugation (Lelios et al., 2014; Mufazalov et al., 2016; Pino et al., 2011). Pc is a colloidal silica coated in polyvinylpyrrolidone and is stated to be non-toxic to cells (Pertoft et al., 1978). However; the Pc gradient method does not provide a high yield of cells, has low reproducibility, and requires a large number of mice to perform analytical assays with the isolated cells; all of these characteristics are counter to what is expected from a standard laboratory technique or assay. A standard assay should be reliable, inert, non-toxic, efficient, effective, readily available, affordable, and should allow for further applications with minimal artificial manipulation of the isolated cells.

Using the aforementioned criteria, we decided to compare Pc to various methods of enzymatic tissue dissociation. Previous reports have shown that enzymatic digestions offer an improved efficacy as shown by a greatly increased cell yield (Legroux et al., 2015) . A distinction not separated in these reports is that EAE is characterized by the inflammation of both spinal cord and brain, as well as the infiltration of spinal cord and brain by immune cells. The inflammation and infiltration of immune cells is not identical between spinal cord and brain, and varies during classical versus non-classical EAE. These differences have been attributed to the distinct microenvironments of the spinal cord and brain (E. Pierson et al., 2012)

The differences in spinal cord and brain, therefore, require separate analysis to fully understand the effects of the distinct microenvironments on infiltrating immune cells. However, due to low cell yield using the Pc method, brains and spinal cords were typically processed together.

To improve the isolation of cells from the brain and spinal cord, we chose the enzymes Accutase® (Ac) and papain (Pp), as they were readily available, affordable, inert, and non-toxic, as well as a commercially available dissociation kit existed. We then tested these for reliability, efficiency, and efficacy, as compared to Pc.

Here we identify enzymatic methods that allow for brains and spinal cords to be processed individually and provide sufficient cells for downstream applications including, but not limited to, flow cytometry, ELISpot, and qPCR assays.

5.2 Results

5.2.1 Enzymatic dissociation of brains improves the efficacy of cell isolation

Brains were isolated from mice immunized for experimental autoimmune encephalomyelitis (EAE), as described in the Methods sections, with a minimum disease score of 3 and subsequently processed using various methods to recover neural cells and leukocytes. The methods tested include: Percoll™ (Pc), commercial neural dissociation kit (Kitani et al.), Accutase® 15minutes (Ac15), Accutase® 30 minutes (Ac30), Accutase® 60 minutes (Ac60), papain 15 minutes (Pp15), papain 30 minutes (Pp30), papain 60 minutes (Pp60), and Accutase® 15 minutes + papain 30 minutes (Ac15+Pp30). The total cell counts of individual brains and spinal cords were calculated to determine the efficacy of each method. All enzymatic digestions provided a significantly increased cell yield from brain tissue, in comparison to Pc (**Figure 5.1**). Individual brains digested with the Kit averaged the highest cell yield, providing over 46 times the cell yield averaged from individual brains using the Pc method, and about 2.5 times the cell yield of the average of all other enzymatic methods (**Table 5.1**).

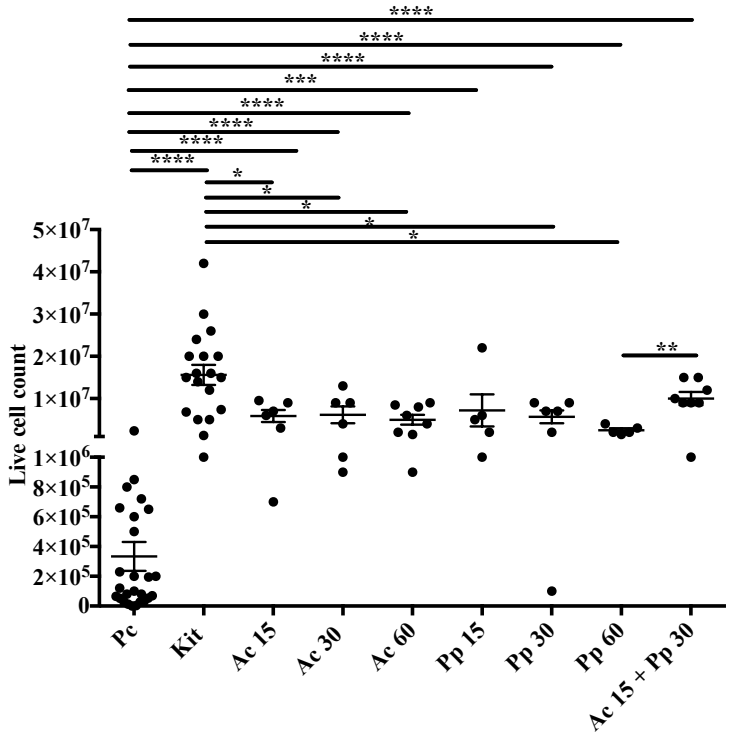

Figure 5.1. **Enzymatic dissociation of individual mouse brains improves the efficacy of cell isolation.** Brain tissues from individual mice with EAE scores >3 were passed through a 70 µn strainer and processed with Percoll™ gradients or were digested for 15 min, 30 min, or 60 min with Accutase and/or papain as indicated. Cells from enzyme digested tissues were isolated using a one step 37% Percoll™ and passed through a 70 µn strainer. Cells were resuspended in PBS and live cells were counted using a hemocytometer. *$\rho < 0.05$, **$\rho < 0.01$, ****$\rho < 0.0001$.

Table 5.1 Enzymatic dissociation improves the efficacy of cell isolation in brain

Method: Brain Tissue	Average	Standard Deviation	Relative Standard Deviation (%)	Efficiency
Pc	3.33E+05	±4.95E+05	148.67	2.78E+03
Kit	1.56E+07	±1.04E+07	66.62	4.46E+05
Ac 15	5.87E+06	±3.44E+06	58.67	3.91E+05
Ac 30	6.15E+06	±4.94E+06	80.28	2.05E+05
Ac 60	4.99E+06	±3.32E+06	66.63	8.31E+04
Pp 15	7.20E+06	±8.53E+06	118.42	4.80E+05
Pp 30	5.68E+06	±3.75E+06	65.93	1.89E+05
Pp 60	2.50E+06	±1.00E+06	40.00	4.17E+04
Ac 15 + Pp 30	1.00E+07	±4.44E+06	44.40	2.22E+05
All enzymatic digestions	6.06E+06	±2.27E+06	37.44	2.30E+05

5.2.2 Enzymatic dissociation of spinal cords improves the efficacy of cell isolation and allows for the complete analysis of individual EAE mice

From spinal cords, only the enzymatic digestions Ac60, Pp15, Pp30, and Ac15+Pp30 had significantly increased cell yields as compared to Pc (**Figure 5.2**). The average cell yield from all spinal cord enzymatic digestions is over 48 times higher than that of Pc (**Table 5.2**). Unexpectedly, the average cell yield of all enzymatic digestions for the brain was only 1.01 times higher than that of the spinal cord. However, histology of spinal cord of EAE mice shows a large number of infiltrating leukocytes and inflammation that correlates with clinical disease severity (Recks et al., 2011). The

enzymatic methods allow for the independent analysis of cells from individual brains and spinal cords, something that is not possible when using Pc gradients.

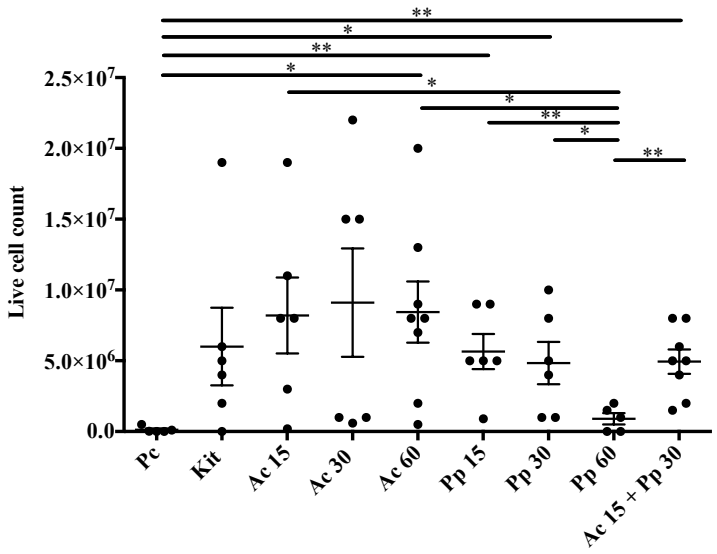

Figure 5.2. Enzymatic dissociation of individual mouse spinal cords improves the efficacy of cell isolation. Spinal cord tissues from individual mice with EAE scores >3 were passed through a 70 μn strainer and processed with Percoll™ gradients or were digested for 15 min, 30 min, or 60 min with Accutase and/or papain as indicated. Cells from enzyme digested tissues were isolated using a one step 37% Percoll™ and passed through a 70 μn strainer. Cells were resuspended in PBS and live cells were counted using a hemocytometer. *$\rho < 0.05$, **$\rho < 0.01$, ****$\rho < 0.0001$.

Table 5.2 Enzymatic dissociation improves the efficacy of cell isolation in spinal cord				
Method: Spinal Cord Tissue	**Average**	**Standard Deviation**	**Relative Standard Deviation (%)**	**Efficiency**
Pc	1.24E+05	±2.14E+05	172.74	1.03E+03
Kit	6.00E+06	±6.72E+06	112.05	1.71E+05
Ac 15	8.20E+06	±6.57E+06	80.08	5.47E+05
Ac 30	9.10E+06	±9.38E+06	103.03	3.03E+05
Ac 60	8.44E+06	±6.11E+06	72.47	1.41E+05
Pp 15	5.65E+06	±3.04E+06	53.84	3.77E+05
Pp 30	4.83E+06	±3.66E+06	75.64	1.61E+05
Pp 60	9.00E+05	±8.94E+05	99.38	1.50E+04
Ac 15 + Pp 30	4.94E+06	±2.43E+06	49.15	1.10E+05
All enzymatic digestions	6.01E+06	±2.86E+06	47.57	2.36E+05

5.2.3 Reliability and efficiency are improved using enzymatic dissociations

When studying CNS cells during EAE disease, reliability is absolutely required. To quantify reliability, we determined the relative standard deviation (Becker et al.) for each method used for CNS cell isolation using the ratio of the standard deviation (σ) to the mean (μ).

$$RSD = \frac{\sigma}{\mu} \times 100$$

For example, the RSD for Pp60 means that the standard deviation of cell yield is within 40% of the mean. Although 40% may still seem high, it is limited by the number of tissues collected and could potentially be reduced by increasing the number of replicate mice used. Pc showed the lowest reliability with an RSD of 148.67% for cells isolated from the brain and an RSD of 172.74% for cells isolated from the spinal cord. The most reliable method for the brain was Pp60 (40%) and for the spinal cord was Ac15+Pp30 (49.15%) (**Table 5.1** and **Table 5.2** respectively).

Efficiency was determined using the ratio of total cell yield divided by the time required by the method. The time required was determined based off of the incubation time for each method, as the exact processing time may differ between users. Pp15 and the Kit were the most efficient methods for isolating cells from the brain, while Ac 15 was the most efficient method for isolating cells from the spinal cord.

5.2.4 Percoll™ isolation method causes an increased cell death

After collecting cells using each isolation method, we stained the cells with Zombie Aqua™ dye to analyze the effect of each method on cell viability. The Pc method was associated with the lowest total cell viability. We observed an increased viability in the enzymatic dissociation methods, as compared to Pc, with the Pp60 minutes providing the best viability for brains with an average viability of 91%, and the Kit providing the highest viability at 93% for spinal cord (**Figure 5.3 A and B**). This was surprising due to the believed innocuous nature of Pc. A literature review revealed, however, that while the beads which make-up Pc may not affect cell viability or differentiation, the Pc product may contain significant levels of endotoxins (Soderlund et al., 2000). The exact concentration of endotoxin varies by lot, which makes it even less likely to obtain reproducible results with regard to cell numbers and phenotypes in separate experiments. The Pc utilized in this study contains more than 5 EU/mL of endotoxin. Endotoxin has pleiotropic effects on leukocyte activation and differentiation. In fact, incubation of splenocytes with Pc causes an increase in the production of inflammatory cytokines, including IFNγ and IL-17A, as assessed by ELISAs (data not

shown). The field of assisted reproductive technology has known for over 20 years that Pc can contain high levels of endotoxin and has, thus, already found suitable replacements for Pc with little to no endotoxin contamination (Henkel et al., 2003). These replacement products include: Percoll Plus™ (GE Healthcare), OptiPrep (Nycomed), PureSperm (NidaCon Laboratories), and Isolate (Irvine Scientific).

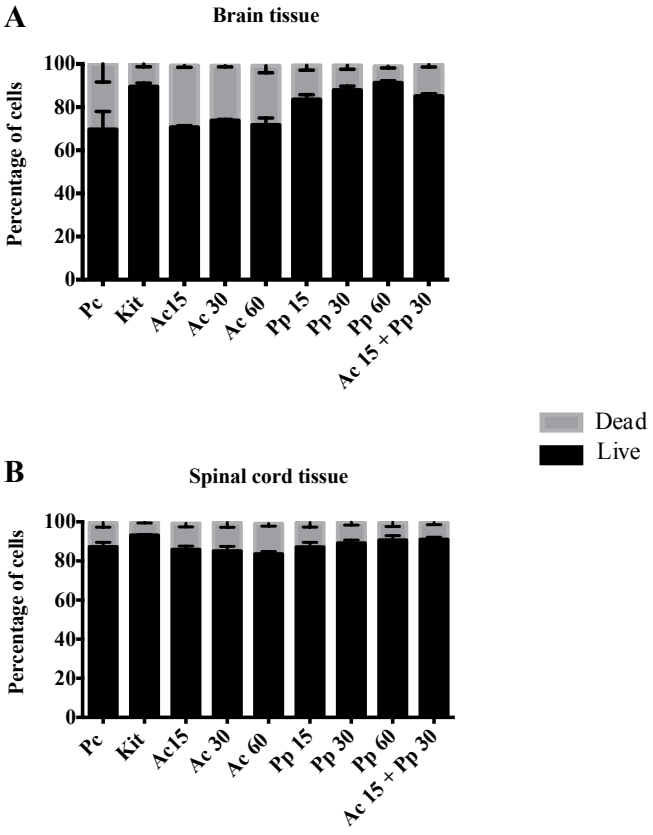

Figure 5.3. Enzymatic dissociation methods increases cell viability.
Cells from brain and spinal cord tissues from individual mice with EAE scores >3 were isolated with the specified methods and $1x10^6$ cells were stained with Zombie Aqua™ Fixable Viability Dye for the discrimination of live and dead cells.

5.2.5 Leukocyte populations are decreased using the Percoll™ isolation method

$CD45^+$ leukocytes and their infiltration into brain and spinal cord play an important role in the initiation and progression of EAE. We determined the proportions of lymphocytes ($CD45^+CD11b^-$), granulocytes ($CD45^+CD11b^+$), activated microglia/macrophages ($CD45^{INT}CD11b^+$), parenchymal microglia ($CD45^-CD11b^+$), and other cell types ($CD45^-CD11b^-$) isolated using Pc versus enzymatic digestions, by immunophenotyping. In the brain, enzymatic digestion using Ac15 and Ac60 gave the highest percentage of lymphocytes at 12%, with Ac30 and Pp30 giving the next highest percentage at 10.5%. Pp30 provided the highest granulocyte yield at 13% while Ac60 provided the next highest yield at 12%. Activated microglia/macrophages were best isolated with Pp 30, at 14%. The highest percentage of parenchymal microglia from live cells came from Pc at 9% (**Figure 5.4A-F**).

Analyzing the spinal cord, the Kit provided the highest percentage of lymphocytes at 10%, and granulocytes at 33%. Activated microglia/macrophages were at 13% with the Kit. Parenchymal microglia were highest in the Pc isolation method, with 9% (**Figure 5.5 A-F**)

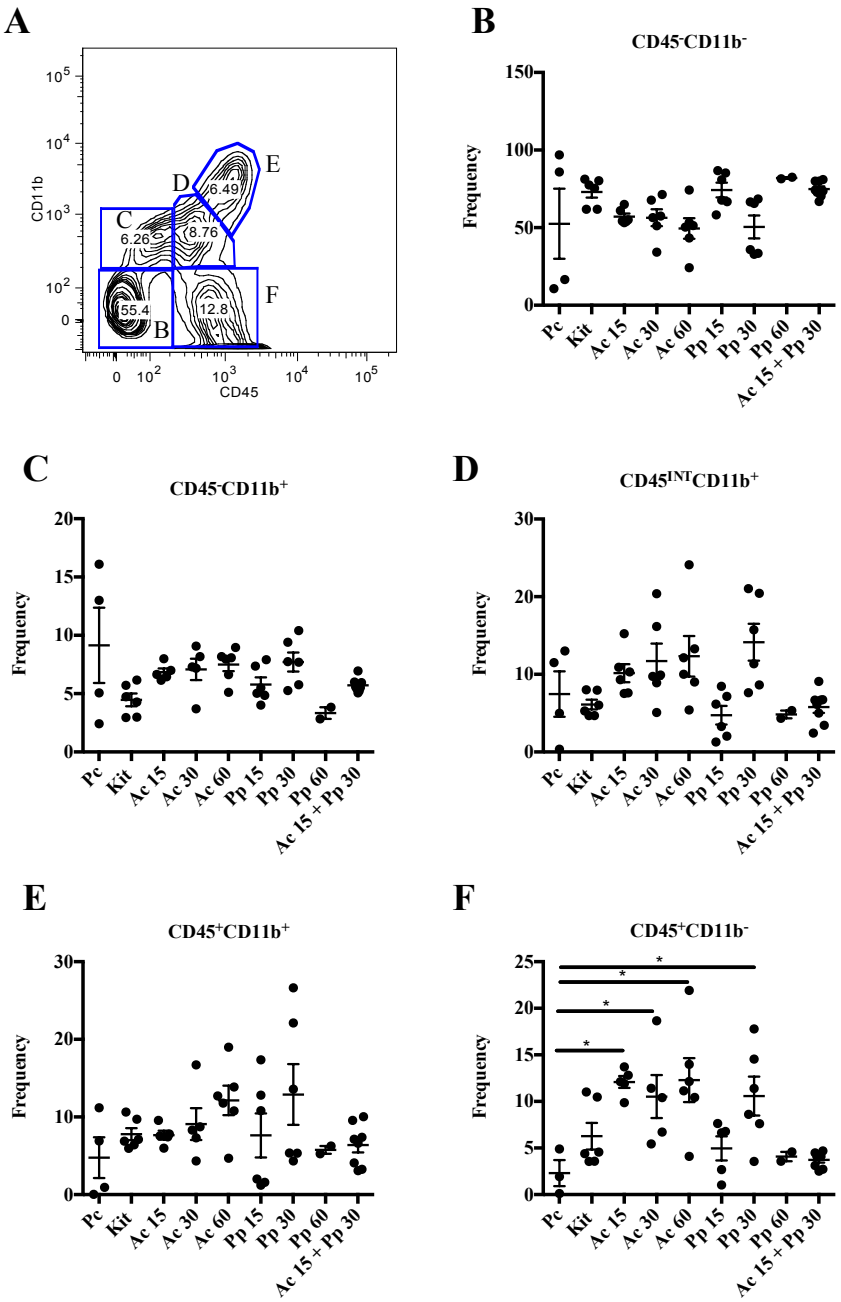

Figure 5.4 Proportion of leukocytes and other cell type populations for individual brain tissue. Percentages of lymphocytes ($CD45^{+}CD11b^{-}$, **F**), granulocytes ($CD45^{+}CD11b^{+}$, **E**), activated microglia/macrophages ($CD45^{INT}CD11b^{+}$, **D**), parenchymal microglia ($CD45^{-}CD11b^{+}$ **C**), and other cell types ($CD45^{-}CD11b^{-}$, **B**) isolated using Percoll™ versus enzymatic digestions, by FACS analysis. Representation gates from live single cells (**A**)

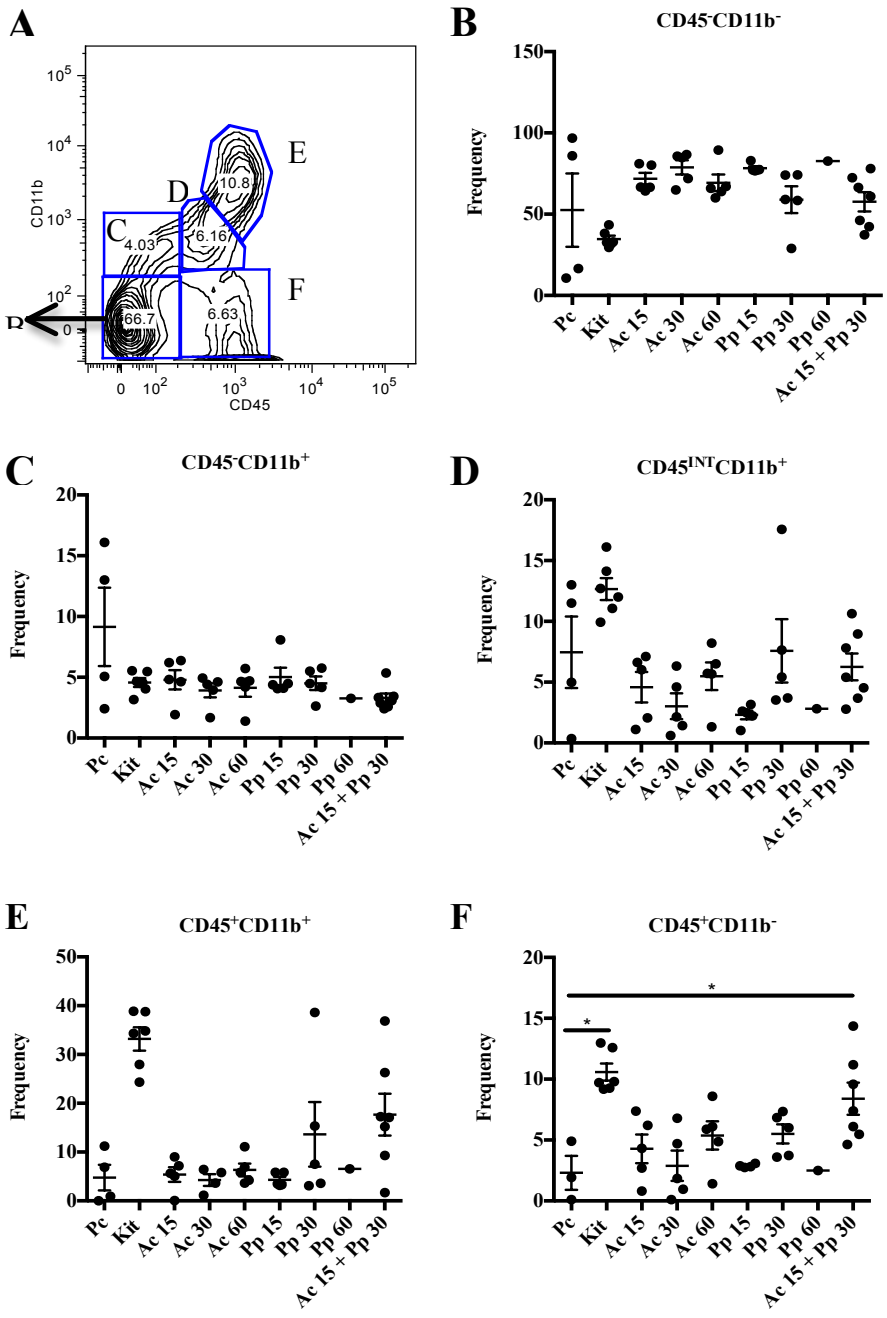

Figure 5.5. Proportion of leukocytes and other cell type populations for individual spinal cord tissue. Percentages of lymphocytes ($CD45^{+}CD11b^{-}$, **F**), granulocytes ($CD45^{+}CD11b^{+}$, **E**), activated microglia/macrophages ($CD45^{INT}CD11b^{+}$, **D**), parenchymal microglia ($CD45^{-}CD11b^{+}$ **C**), and other cell types ($CD45^{-}CD11b^{-}$, **B**) isolated using Percoll™ versus enzymatic digestions, by FACS analysis. Representation gates from live single cells (**A**).

CHAPTER SIX
Conclusions and Future Work

6.1 Observations made in these studies

In order to study any differential effect of α4-integrin antagonism on leukocyte subsets that are required for immunocompetence of the CNS, we utilized the Mx1.Cre^{+}α4-integrin^{flfl} mouse strain, in which α4-integrin can be conditionally deleted on interferon-induced GTP-binding protein Mx1 expressing cells through the Cre-loxP system. We found that this mouse model worked as expected in generating functional leukocytes in secondary lymphoid organs with reduced α4-integrin surface expression. However, in spite of a delay in disease onset, the EAE disease course of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice remained comparable to that of C57BL/6. Splenocytes isolated from poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice did show a significant migration reduction *in vitro.* When we analyzed the cells that infiltrated the CNS during EAE, we observed significant reduction of α4-integrin surface expression, but a similar level of infiltration when compared to C57BL/6. Despite these confounding results, we determined that there was no significant up-regulation of LFA-1 or α5-integrins. Additionally, we demonstrated that poly I:C causes anatomical differences of infiltration in the spinal cord of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice and an increase in transcription of pro-inflammatory cytokines in the CNS of EAE mice. We also demonstrated that adoptive transfer experiments can exclude the effects of poly I:C during EAE. Taken together, this data demonstrate that this mouse model could be useful for generating α4-integrin deficient cells that can be further utilized on adoptive transfer

models. Finally, we demonstrate that enzymatic dissociation methods improve the isolation of mononuclear cells from individual brains and spinal cords of EAE mice.

6.2 EAE disease course in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 mice

Leukocyte migration from the periphery into the CNS involves multiple steps (Holman et al., 2011; Luster et al., 2005; Ransohoff et al., 2003). Integrins such as VLA-4 (α4β1- integrin) have been shown to facilitate leukocyte migration across the basement membrane of blood vessels and across the extracellular matrix (Bauer et al., 2009; Kunkel et al., 2000). In EAE, an early event is the presentation of antigen in secondary lymphoid organs. This antigen presentation causes activation of leukocytes that are then capable of adhering to the endothelium of blood vessel walls and migrating into the CNS. Once in the CNS, antigen-specific T cells are re-activated through the presentation of an identical or similar antigen by perivascular APCs, including hematopoietic macrophages (W. F. Hickey et al., 1988) and DCs (Greter et al., 2005). Yednock et al. reported that lymphocytes and monocytes bound selectively to inflamed EAE brain vessels, and that antibodies against α4 integrin can inhibit this effect (Yednock et al., 1992). When tested *in vivo*, anti-α4 integrin effectively prevented the infiltration of leukocytes in the CNS and prevented the development of EAE (Yednock et al., 1992). It has been shown that monoclonal antibodies against VLA-4 inhibit EAE onset and reduced the severity of clinical course (Theien et al., 2001a). Based on these data, we hypothesized that deletion of α4 integrin by poly I:C treatment in the Mx1.Cre^{+}α4-integrin$^{fl/fl}$ model would result in

prevention of EAE or less severity in the clinical course. Unexpectedly, the EAE disease course of poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice remained comparable to that of C57BL/6. This result suggested a few possibilities to explore: 1) incomplete α4-integrin deletion resulted in entry and expansion of α4-integrin positive populations, 2) there might have been overcompensating expression of integrin(s) with redundant roles in CNS extravasation during EAE, and 3) poly I:C effects on the innate immune system were altering EAE outcome.

6.3 Infiltration of leukocytes in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ and C57BL/6 mice

In this study we have shown a high level of infiltrating cells in poly I:C treated Mx1.Cre^{+}α4-integrin$^{fl/fl}$ mice with EAE that it is similar to C57BL/6 mice with EAE. We utilized Pc gradients to isolate leukocytes from CNS. Pc gradients have the limitation of requiring a high number of CNS tissues to obtain significant results. In EAE mice we obtained an average of 400,000 leukocytes (Figure 4.4 C). This factor might have limited or obscured the actual number of CD49d expressing infiltrates in the CNS.

Rothhammer et al. reported that Th17 lymphocytes did not require α4-integrin for extravasation into the CNS, but rather LFA-1 (Rothhammer et al., 2011). The early studies of VLA-4 on EAE suggested that only VLA-4 expressing CNS antigen-specific T cell lines were able to induce the disease. Encephalitogenic T cells produce high levels of IFNγ, thus, having a strong Th_1 cytokine signature that correlates with VLA-4 expression (Kuchroo et al., 1993). However, it was also demonstrated that Th_{17} are also able to

induce EAE (Domingues et al., 2010; Jager et al., 2009) and that antibodies against α4-integrin do not block Th_{17} extravasation into the CNS (Rothhammer et al., 2011). Although we did not observe an up-regulation of LFA-1 in CNS infiltrates of poly I:C treated Mx1.Cre^{+}α4-$integrin^{fl/fl}$, further studies are needed to elucidate any role Th_{17} lymphocytes might have in this model. It would also be useful to analyze the cell and cytokine profile of cells isolated for adoptive transfer experiments since, in EAE, adoptive transfer of Th_1 or Th_{17} cell populations drive the disease.

6.4 Effect of poly I:C in EAE

Poly I:C is a synthetic analogue of viral double stranded RNA, and an agonist for TLR3 and RIG-I (Li et al., 2012; Matsumoto et al., 2008). It is a strong inducer of innate immune responses against infectious agents (Alexopoulou et al., 2001) It has been demonstrated that systemic challenge with poly I:C induces production of type I IFN and IL-1β (Field et al., 2010). Preliminary EAE induction of C57BL/6 performed previously, aimed to analyze the effect of poly I:C treatment one week, two weeks, and three weeks before immunization. PBS was used as control. We observed no significant difference in EAE severity between the treatment groups (**Figure 6.1)**. Previous reports have shown that poly I:C treatment or induction of type I IFN prior to EAE induction has a beneficial effect on disease course. These beneficial effects of type I IFN on EAE, required their expression within the CNS (Khorooshi et al., 2015). It is also potentially relevant that most of these studies were performed in the SJL/J EAE model, and there may be strain specific effects (Touil et al., 2006).

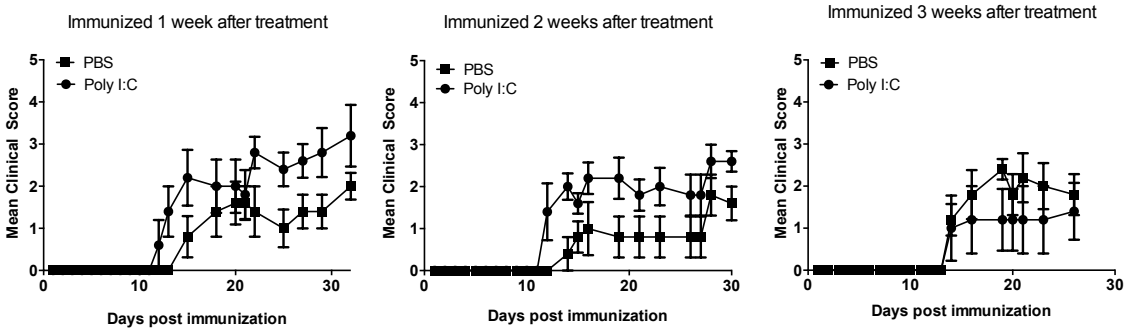

Figure 6.1: EAE disease course of C57BL/6 mice treated with 3 doses of poly I:C or PBS as a control and later immunized after 1, 2, or 3 weeks of treatment. 5 mice per group were treated with 300μg of poly I:C or 100μL PBS.

6.5 The role of APCs on immunocompetence

Another area of future interest is to elucidate the role of APCs, specifically DCs, in CPVS and the implications for immunocompetence of the CNS. As previously mentioned, Del Pilar et al reported specifically that natalizumab treatment causes a significant reduction in the number of $CD209^{+}$ DCs in CPVS when compared to control brain tissue (del Pilar Martin et al., 2008). It is currently incompletely understood: 1) to what extent α4-integrin antagonism decreases the migration of myeloid APCs into perivascular spaces of the CNS, 2) how depletion of myeloid APCs subsets within the CNS affects re-activation and retention of T cells within the brain, and 3) how the reduction of specific myeloid APCs populations in perivascular spaces of the CNS affects CNS immunocompetence.

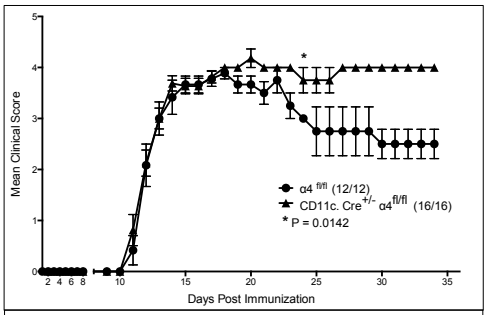

Figure 6.2: Characterization of EAE disease course on CD11c.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ to elucidate role of APC on immunocompetence.

To address these questions, we generated a CD11c.$Cre^{+/-}$ α4-$integrin^{fl/fl}$ crossed on C57BL/6 background to study the role of α4-integrin on myeloid cells. In these mice,

$CD11c^{+}$ DCs are targeted to constitutively delete α4-integrin. Preliminary clinical scores show that CD11c.$Cre^{+/-}$ α4-$integrin^{fl/fl}$ have a similar disease onset as C57BL/6 but are not able to recover as well at day 23 (**Figure 6.3**). Syngeneic bone marrow chimera with CD45.1 congenic wild type mice and CD45.2 congenic $CX_3CR1^{+/GFP}$ Tg mice could be utilized to assess the role of hematopoietically-derived myeloid cells in the CNS.

6.6 Improvement in CNS leukocyte isolation methods for future experiments

Based on the limited leukocyte yield observed after EAE induction in poly I:C treated Mx1.Cre^{+}α4-$integrin^{fl/fl}$ and C57BL/6 mice, we sought to establish optimized methods for CNS leukocyte isolation. In this study we identified and analyzed enzymatic dissociation methods that were readily available, affordable, inert, and non-toxic, for the isolation of mononuclear cells from individual brains and spinal cords. Specifically, we tested dissociation methods for reliability, efficiency, and efficacy. We determined reliability using the relative standard deviation of the mean total cell yield of each method. The most reliable method for the isolation of leukocytes from the brain was Pp60 (40%) and for the spinal cord was Ac15+P30 (49.15%) (**Table 5.1** and **Table 5.2** respectively). Efficiency was determined by calculating the ratio of total cell yield divided by the time required by each method. The most efficient method was Pp15 for the brain and Ac15 for the spinal cord. We additionally observed that the Kit was the most effective, in terms of producing a higher cell yield, at isolating cells from the brain, and Ac30 at isolating cells from the spinal cord.

The Pc method was consistently the worst performing method with regard to reliability, efficiency, and efficacy. Surprisingly, Pc had the lowest viability, potentially due to a high concentration of endotoxin (>5 EU). Less than 1 EU of endotoxin has been shown to cause pleiotropic effects due to general activation via innate immune receptor signaling (Flak et al., 2000; Morris et al., 2014). Overall, enzymatic dissociation provides a more reliable, efficient, and effective method for isolating mononuclear cells from individual EAE brains and spinal cords as compared to Pc. There are many other enzymes that can be used and have been shown to be successful in the isolation of mononuclear cells from the CNS and we recommend evaluation of priorities in determining the enzymatic method of preference (Legroux et al., 2015; Robinson et al., 2014).

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