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Analysis of 4BL B cells in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

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This thesis is dedicated to my family; without them I would not be where I am.

Abstrakt

Die Ätiologie der Multiplen Sklerose (MS) ist und bleibt umstritten. Die Pathologie der MS wird durch eine Entzündungsreaktion im Zentralnervensystem (ZNS) angetrieben, die schließlich Demyelinisierung und Neurodegeneration verursacht. Histopathologische Studien haben Infiltrationen von T- und B-Zellen sowie Plasmazellen, aktivierten Makrophagen und Mikroglia im MS-Gehirn gezeigt. Die Zahl der infiltrierenden B-Zellen im ZNS von Patienten mit MS ist höher als bei anderen entzündlichen Hirnerkrankungen, ihre Rolle bleibt jedoch rätselhaft. Kürzlich wurde eine neue B-Zellen-Untergruppe, 4-1BBL+ B-Zellen (auch 4BL-Zellen genannt), vermehrt bei älteren Menschen gefunden, die starke Induktoren von Granzyme B-produzierenden CD8+ T-Zellen aufweisen dürfte (Lee Chang et al., 2016; Lee Chang et al., 2014). Hier stellten wir die Hypothese auf, dass 4BL-Zellen potenzielle neue Entzündungsmediatoren bei chronischer MS sein könnten. Um unsere Hypothese zu testen, führten wir immunhistochemische (IHC) Färbungen an mehreren MS-Gehirnautopsien durch, um herauszufinden, ob 4BL-Zellen in das ZNS eindringen. Parallel dazu untersuchten wir die Pathogenität von induzierten 4BL-Zellen im Tiermodell, das üblicherweise für MS verwendet wird, der experimentellen autoimmunen Enzephalomyelitis (EAE). Unsere Ergebnisse zeigten 4BL-Zellen nur in zwei von 24 MS-Fällen. Andererseits zeigen unsere Ergebnisse aus EAE-Experimenten, dass induzierte 4BL-Zellen die CD4+-T-Zellantwort verstärken. Weitere Studien sind daher erforderlich, um das Vorhandensein und die Rolle von 4BL-Zellen bei MS eindeutig zu bestimmen.

Abstract

The etiology of multiple sclerosis (MS) remains a matter of debate. The pathology of MS is driven by an inflammatory response in the central nervous system (CNS), which eventually causes demyelination and neurodegeneration. Histopathological studies have shown infiltrations of T- and B cells, as well as plasma cells, activated macrophages and microglia in the MS brain. The numbers of infiltrating B cells in the CNS of patients with MS are higher than in other inflammatory brain diseases, however their role remains enigmatic. Recently, a novel B cell subset, 4-1BBL$^+$ B cells (also named 4BL cells), have been found increased in elderly and seem to be strong inducers of Granzyme B-producing CD8$^+$ T cells (Lee Chang et al., 2016; Lee Chang et al., 2014). Here we hypothesized that 4BL cells might be potential novel mediators of inflammation in chronic MS. To test our hypothesis, we performed immunohistochemistry (IHC) stainings on several MS brain autopsies to find out if 4BL cells infiltrate into the CNS. In parallel, we studied the pathogenicity of induced 4BL cells in the animal model commonly used for MS, experimental autoimmune encephalomyelitis (EAE). Our results revealed 4BL cells only in two MS cases out of 24. On the other hand, our results from EAE experiments show that induced 4BL cells enhanced the CD4$^+$ T cell response. Further studies will be needed to determine the presence and role of 4BL cells in MS.

Contents

1 INTRODUCTION

The following introduction aims to help the reader to understand the most relevant features of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), thus facilitating the understanding of the results presented on this thesis. To this end, basic concepts of immunology and neurology are briefly explained, followed by a more detailed explanation of MS and EAE.

1.1 THE IMMUNE SYSTEM

In our environment there are many pathogenic microbes and toxic substances that are trying to penetrate our skin/mucosa continuously. Our immune system has several mechanisms to detect them and destroy them, as well as mechanisms to discriminate them from self-molecules. These mechanisms are driven by a complex network of lymphoid organs, cells, and soluble factors. The primary lymphoid organs are those where generation and maturation of lymphocytes takes place. The primary lymphatic organs are the red bone marrow and the thymus gland. In the secondary lymphoid organs, the immune cells are activated and perform their functions. These organs include the lymph nodes, the spleen, tonsils and mucosa associated lymphoid tissue (MALT). The cells of the immune system are white blood cells and they all derive from hematopoietic stem cells in the bone marrow. They include: B and T cells, natural killer (NK) cells, neutrophils, eosinophils, basophils, monocytes/macrophages and dendritic cells (DC) (Charles A Janeway et al., 2001). Their functions are briefly described in figure 1.

The immune system is actually composed of two interconnected systems: the innate immune system and the adaptive immune system. The first one provides a primary and fast response against pathogens and also influences the cells from the adaptive immune response. When the innate host defense is not enough for pathogen clearance, the adaptive immune response is needed. As we will see later, the adaptive response is slower but antigen specific (Punt et al., 2019). Moreover, the immune response mediators can be classified into cell-mediated immunity (CMI) and humoral immunity. As the name indicates, CMI is mediated by cells from the immune system (Figure 1). Humoral immunity is mediated by complement, antibodies and other soluble factors transported by blood. Antibodies are secreted by plasma cells, and they have three main functions: (1) Avoid pathogens to enter inside the host cells (neutralization), (2) facilitate phagocytosis of pathogens by immune cells, (3) activation of the complement system. The complement system involves a variety of proteins that can opsonize pathogens as well as induce inflammation (Charles A Janeway et al., 2001).

1.1.1 The innate immune system

A wide range of microorganisms that can cause pathology (pathogens) can easily enter our body. The innate immune system is found in all vertebrates and provides the first line of host defense against these pathogens. The components of the innate immune system are the epithelial barriers, leukocytes, and circulating effector proteins such as complement and cytokines. First, in order to enter our body pathogens must penetrate the epithelial barrier. Epithelial cells are held together by tight junctions, creating a physical barrier between the skin and the external environment. Besides to be present in the skin, epithelial barriers are also found in our gastrointestinal, respiratory, and urogenital tracts. In addition, epithelial cells can secrete chemical substances that are microbicide or inhibit microbial growth (Alberts et al., 2002). If a pathogen manages to cross the epithelial barrier, usually it is immediately recognized by macrophages that reside in the tissues. Macrophages are continuously maturating from blood circulating monocytes that migrate into tissues. These cells can recognize, phagocyte and destroy many pathogens without help from the adaptive immune system. However, pathogens have developed several mechanisms to avoid being destroyed. As a result, infection can be established and once this happens the inflammatory response is initiated by macrophages (Charles A Janeway et al., 2001).

Inflammation is a protective response that is induced when our immune system senses pathogens, external injuries, chemicals or disease. This response is characterized by vasodilatation which allows more immune cells to reach fast the inflammation site. The goal of the inflammatory response is to fight the harmful factor and to heal the affected site, however inflammation can become chronic, eventually causing tissue injury, organ dysfunction, and even death (Sherwood & Toliver-Kinsky, 2004). Chronic inflammation can occur for many reasons such as a failure in eliminating the agent causing inflammation or due to an autoimmune disorder (Pahwa et al., 2022).

1.1.2 The adaptive immune system

The innate immune response mentioned before is a prerequisite for the adaptive response to take place. There are three major features of the adaptive immune response: specificity, memory and self-tolerance. (1) The specificity is achieved because individual lymphocytes have receptors which allows them to distinguish small differences between antigens. (2) Once we have been exposed to a specific antigen, our immune system has the ability to respond more efficiently to that antigen if we ever get exposed to it again. This is called 'immunological memory' and it is achieved because during the first antigen encounter specific memory cells are generated towards this antigen. (3) Self-tolerance is essential in order to avoid the immune system attacking our own cells and proteins (Abbas et al., 2017) .

Lymphocytes are the main players of the adaptive immunity; They possess a variety of receptors that allows them to recognize a wide range of antigens. There are two major types of lymphocytes: T lymphocytes and B lymphocytes (also called T cells and B cells). On T cells, the antigen receptors are called T cell receptors (TCR) (Charles A Janeway et al., 2001). T cells only recognize antigens when they are displayed by host proteins called major histocompatibility complex (MHC) molecules. (Abbas et al., 2017). The MHC class I molecules are expressed on all nucleated cells and are recognized by CD8+ cytotoxic T cells (CTLs). MHC class II are expressed by antigen-presenting cells (APCs) and are recognized by CD4+ T-helper cells (Rich et al., 2018). The term CD stands for 'cluster of differentiation' which is a designation for proteins expressed on the cells surface. There are many different CDs, each of them is assigned with a number. CDs are very useful for characterization of cell phenotypes (Actor, 2019). When CD4 T cells recognize antigens displayed by the MHC class II molecules, they get activated and induce B cells to secrete antigen-specific antibodies. In contrast, when CD8 T cells recognize antigens presented on MHC class I molecules, they become cytotoxic and can kill cells presenting specific peptides (Rich et al., 2018)

The structures that recognize the antigens on B cells are called immunoglobulins (Ig). Immunoglobulins that are expressed on the B cell surface act as receptors for the antigens and are also known as B cell receptors (BCR). In the lymphoid tissues, B cells interact with CD4 T cells which induces B cells to switch their receptors increasing their affinity towards a specific antigen. B cells can differentiate into plasma cells and migrate to the inflammation site where they secrete antibodies. Moreover, B cells express antigen on the cell surface through MHC class II molecules, which will be presented to naive T cells (Abbas et al., 2017).

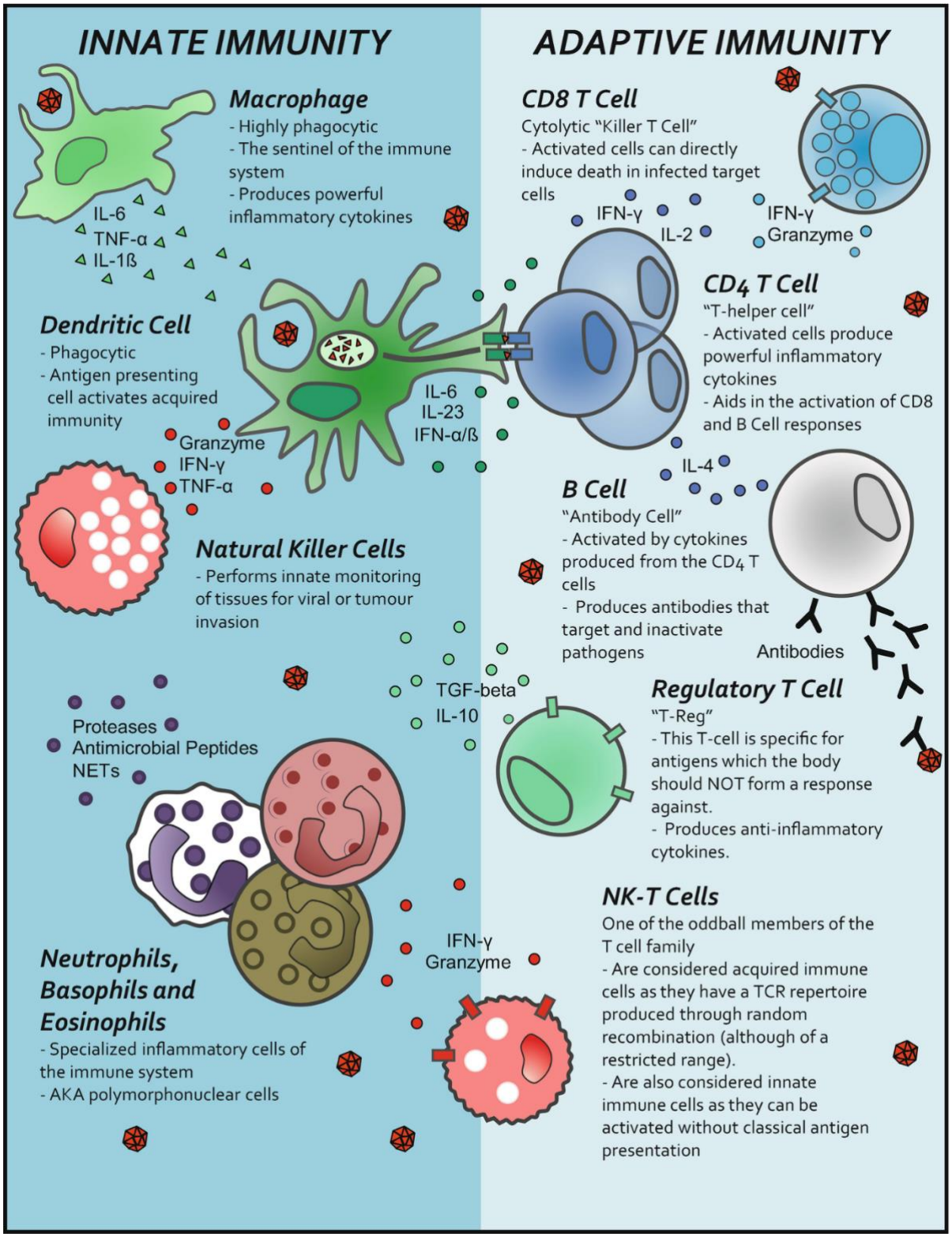

Figure 1. Innate and adaptive immunity. *Cells, cytokines and proteins involved in the innate and adaptive immune responses. IL: interleukin, TNF-a: tumor necrosis factor alpha, IFN: interferon, TGF: tumor growth factor; NETs: neutrophil extracellular traps; NK: natural killer; TCR: T cell receptor (McComb et al., 2019). Permission conveyed through Copyright Clearance center, Inc.*

1.2 THE CENTRAL NERVOUS SYSTEM (CNS)

The nervous system is divided into peripheral nervous system (PNS) and central nervous system (CNS). The CNS is comprised by the brain and spinal cord which are surrounded by three layers: dura mater, arachnoid mater and pia mater; Together are known as meninges and they contain the cerebrospinal fluid (CSF) in which the CNS organs are soaked. Moreover, the CNS is protected by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCB); These two barriers regulate the cellular and molecular exchange between the blood vessels and the brain, therefore, they are essential for the CNS homeostasis (Furtado et al., 2018).

The cellular components of the CNS are neurons, glial cells (astrocytes, oligodendrocytes macrophages, microglia and ependymal cells), and choroid plexus cells:

- **Neurons** are responsible for process and transfer information that they receive through their axons, dendrites and synapses (Kovacs, 2018). Axons are like electricity wires and they are surrounded by structures called myelin sheaths. Myelin's function is to increase the speed at which the electrical impulse is transmitted along the axon. Two areas can be differentiated within the brain and the spinal cord, which are the white and the gray matter. In the white matter the axons are heavily myelinated and this is what makes them look white. On the other hand, in the gray matter we find high amounts of neuronal cell bodies which are not myelinated (Mercadante & Tadi, 2022).
- **Astrocytes** have several functions such as helping neurons to set up a synapsis, neuronal migration, support the BBB and regulation of local blood flow. Usually, after CNS injury, astrocytes upregulate glial fibrillary acidic protein (GFAP) which is known as astrogliosis and it is observed often in the MS brain. GFAP is a filament that interacts with the cell cytoskeleton, adhesion molecules and other proteins, giving mechanical support to astrocytes and neighboring neurons (Kovacs, 2018).
- **Oligodendrocytes** produce the myelin sheaths that cover the axons (Kovacs, 2018).
- **Macrophages and microglia** are the brain's tissue resident macrophages and they are activated after CNS injury. They provide the first line of defense against pathogens invading the CNS. Moreover, both microglia and macrophages can act as antigen presenting cells (APCs). Nonetheless, microglia are inefficient T cell activators while perivascular, meningeal and choroid plexus macrophages are the APCs that reactivate T cells when these infiltrate into the CNS (Goverman, 2009; Schetters et al., 2018).
- **Ependymal cells** form the ventricular system's lining. They are in contact with the CSF and are important for fluid homeostasis (Kovacs, 2018).

- **Choroid plexus cells** are cuboidal epithelial cells with tight junctions between them, forming the BBB (Kovacs, 2018).

The CNS has traditionally been considered inaccessible for immune cells under physiological conditions. Now it is recognized that T cells are actively patrolling the CNS. Therefore, one can say that the CNS is not an immune privileged site but its immunological features are different from the rest of the body; The BBB and the BCB strictly regulate the cellular and molecular entrance. Moreover, in the CNS there are no lymphatic vessels and dendritic cells (DC). However, DCs and macrophages are strategically located in the meninges, perivascular space and choroid plexus where they can promote antigen presentation (Ousman & Kubes, 2012).

Immune surveillance in the CNS is essential to avoid cerebral infections. Immune cells can enter into the healthy CNS at least via 3 different paths: (1) through the blood-CSF barrier, (2) through perivascular space, (3) through post-capillary venules that communicate with the brain parenchyma (Figure 2). Extravasation through the third pathway only occurs under inflammatory conditions (Ousman & Kubes, 2012; Pedemonte et al., 2006)

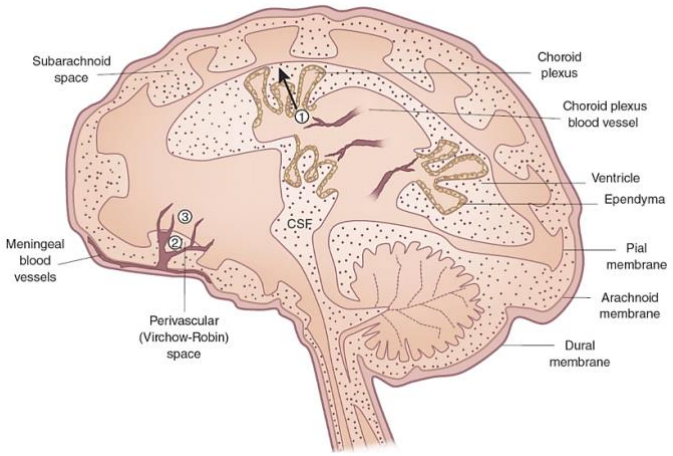

Figure 2. Possible routes of entry into the CNS for immune cells *(Ousman and Kubes, 2012). Permission conveyed through Copyright Clearance center, Inc.*

Autoimmune responses towards CNS antigens can be triggered by autoreactive T cells. Immature T lymphocytes have specific receptors that were generated by random gene rearrangement. Therefore, our T cell repertoire includes specificities for foreign and self-antigens. T cells expressing receptors towards self-antigens are called 'autoreactive T cells' and they have to be eliminated in the thymus. Nevertheless, their elimination sometimes is not completed, as a result autoreactive T cells remain in our system. Autoreactive T cells may never meet their autoantigen because it can be located inside the CNS and these T cells are in the periphery. Yet they can be activated in the periphery by foreign antigens that have similar structure to autoantigens (cross reactivity) (Hemmer et al., 2015).

1.3 MULTIPLE SCLEROSIS (MS)

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the CNS. The pathological feature of MS is the presence of focal lesions (Figure 3A), which are demarcated areas of tissue damage containing large infiltrates of immune cells (Figure 3B). In contrast to lesions seen in other brain diseases, MS lesions are primary demyelinating, meaning that the myelin is lost while axons can remain intact (Lassmann, 2018; Ryan et al., 2013). Demyelinated lesions are also called 'plaques' because of its appearance when the myelin is completely lost (Lassmann, 2018). Within the inflammatory infiltrates there are T and B lymphocytes, plasma cells, activated macrophages and microglia, which can be found in the meninges, perivascular space and parenchyma (Machado-Santos et al., 2018). It has been suggested that MS is an autoimmune disease in which T cells specific for myelin antigens start the inflammatory response in the CNS. However, the specificity of the adaptive immune response remains unknown (Gold et al., 2006).

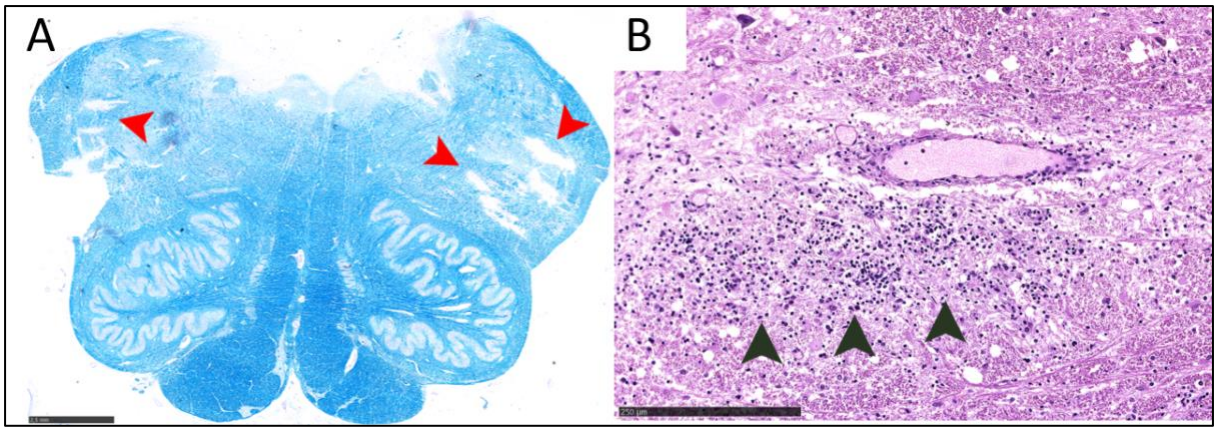

***Figure 3. Focal lesions in multiple sclerosis (MS)**. A) Klüver-Barrera staining on a medulla oblongata section from a patient with MS. Red arrows indicate demyelinated lesions in the white matter. Scale bar: 2.5 mm. B) Hematoxylin staining on the same section. Dark purple round structures are the cell nuclei. Black arrows indicate inflammatory infiltrates in one of the lesions. Scale bar: 250 µm. Pictures were taken with NDP.view 2.*

Despite the vast number of studies done with MS, the etiology remains unknown. Genomic studies revealed more than 100 common genetic variants have been identified and associated with MS. These common genetic variants are called single nucleotide polymorphism (SNP), which is a change at a single position in a DNA sequence among individuals. Most of the SNPs associated with MS are linked with genes implicated in differentiation of T cells and effector functions. Regarding the environmental risk factors contributing to the development of the disease, Epstein-Barr virus (EBV) and cigarette smoking are the most implicated ones (Hemmer et al., 2015; Hoppenbrouwers et al., 2008; Pakpoor et al., 2013). The implication of EBV in MS has been extensively studied. Indeed, it has been suggested that EBV triggers MS (Bar-Or et al., 2020; Bjornevik et al., 2022). High titers of serum antibodies towards EBV have been found in many MS

patients in comparison with healthy individuals. Moreover, EBV-infected cells have been found in the CNS from MS patients. However, the mechanism by which EBV triggers MS remains unknown. The following mechanisms have been hypothesized: (1) Molecular mimicry, EBV viral proteins mimic human myelin proteins and other CNS proteins leading to autoimmune response towards self-CNS proteins. (2) The virus may induce B cell transformation because the viral latent membrane protein 2A (LMP2A) mimics the B cell receptor signaling pathway and the LMP1 viral protein mimics CD40 receptor signaling, which is important for interaction between B and T cells. (3) Persistent EBV infection in the CNS may stimulate $CD8^+$ T cell responses that eventually cause CNS injury (Robinson & Steinman, 2022). The hypothesis that MS is triggered by EBV was recently tested in 10 million adults from the US military cohort. During their period of service, 955 men were diagnosed with MS. The study have shown that there is a 32-fold increased risk to develop MS after being infected with EBV, but not after other viral infections (Bjornevik et al., 2022).

1.3.1 Clinical course

MS can be classified into different subtypes according to the clinical course of the disease. Usually, MS has its onset in early adult life. Generally, MS patients can be classified into 'acute MS' (AMS) or 'chronic MS'. The term 'acute' refers as a clinically fulminant disease course that leads to death within weeks or months. On the other hand, the term 'chronic MS' has been used to describe a progressive disease course. Within the chronic MS group, we can distinguish between three types of progressive MS: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). In most of the individuals, the disease starts with RRMS, which is characterized by periodic relapses followed by remissions. RRMS can transform into SPMS, in which there is a gradual neurologic deterioration and worsening of symptoms. Less frequently, patients do not show a relapsing-remitting course but a continuous progressive clinical deterioration from the disease onset, called primary progressive MS (PPMS). In contrast with the other MS types, PPMS has its disease onset within the 5th decade of life (Kuhlmann et al., 2017; Lassmann, 2018a; Steinman, 2014).

1.3.2 MS lesions

MS lesions can be classified histologically into active and inactive lesions. Myelin degradation occurs inside macrophages and microglia. Therefore, the amount of these cells as well as their absence provide information about the age of a given lesion. Active lesions are the initial phenotype and are more frequently found in patients with AMS or RRMS. These lesions are characterized by loss of myelin and many infiltrates of CD68 positive cells (i.e., macrophages or microglia), as well as high numbers of T and B cells. In active lesions, demyelination process is

still going on, thus myelin fragments can be observed inside macrophages and microglial cells. Furthermore, astrogliosis with increased GFAP expression is also a feature of active lesions. On the other hand, inactive lesions dominate in patients with a longer disease duration. Here the myelin is completely degraded and only a few microglia/macrophages are present. However, the numbers of T and B cells can be similar as those observed within active lesions. Axonal swelling, which indicates ongoing axonal damage is a feature of inactive lesions (Kuhlmann et al., 2017)

Plaques can be in part remyelinated. Remyelination is the process by which new myelin sheaths are generated by oligodendrocytes around axons in the adult CNS; Remyelinated axons are difficult to identify, there is no specific magnetic resonance imaging (MRI) marker to identify them; The current method used for it is via post-mortem histopathological analysis, performing immunohistochemistry (IHC) stainings. Plaques which are remyelinated are called 'shadow plaques' because the new myelin sheaths are thinner and as a result they look like a shadow when analyzing the stained tissue. The extent of remyelination varies from patient to patient. Moreover, it has been hypothesized that remyelination is transient because shadow plaques can be demyelinated again (Patrikios et al., 2006). The first step for the remyelination process is the recruitment and proliferation of oligodendrocyte progenitor cells (OPC). Next, OPCs find demyelinated axons and start wrapping around them generating new myelin sheaths. The signaling events that drive the remyelination process are related with the inflammatory response. It is thought that a failure on these signaling events results in a chronic demyelinated lesion (Franklin, 2002).

1.3.3 Inflammation in MS

Inflammation in MS is characterized by the presence of T and B cells found in the perivascular space, meninges and parenchyma. As in other brain inflammatory diseases, $CD8^+$ T cells are the dominant population. A study revealed that $CD8^+$ T cells from CNS parenchyma of MS patients were clonally expanded, thus indicating that these T cells responded towards an antigen. Nevertheless, it is unknown if the $CD8^+$ T cells were expanded by CNS self-antigens or by viral antigens not related to the disease (Korn & Kallies, 2017). In contrast, in rodent experimental models $CD4^+$ T cells are the major drivers of inflammation (Rangachari & Kuchroo, 2013), while they are scarce or even not present in the CNS from MS patients. A possible explanation could be that $CD4^+$ T cells are involved at disease onset but not later on. In addition, it has been shown that many of the $CD8^+$ T cells found in the MS brain have a phenotype of tissue memory cells, meaning that these cells survive and stay in the CNS. Nonetheless, their function has not been defined yet (Lassmann, 2018b) On the other hand, therapies targeting B cells have been prof to reduce

disease severity in RRMS and progressive MS, highlighting a major role for B cells in the disease (Kappos et al., 2011; Montalban et al., 2017).

1.3.3.1 The role of B cells in MS

Increasing evidence suggests that B cells are important contributors to MS pathogenesis. High levels of clonal immunoglobulin G (IgG) (also known as oligoclonal bands) have been found in the CSF from MS patients, which implies that antibodies are being produced towards a specific antigen. The possible pathogenic role of these antibodies in MS is supported by the benefits observed by plasma exchange in patients (Keegan et al., 2005), although it may be due to removal of pro-inflammatory plasma components. In addition, It has been hypothesized that proteins of the myelin sheaths such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) are the targets of the humoral response (Weber et al., 2011). Many studies have been focused on the immune response towards myelin proteins and the results have been extensively disputed. Antibodies against MOG have been found in MS lesions, as well as in CSF from patients. Nevertheless, myelin-specific antibodies have been also found in other neurological diseases and in healthy controls (Karni et al., 1999; Lampasona et al., 2004).

Histopathological studies have shown high numbers of $CD20^+$ B cells in brain from MS patients at early stages of the disease (Machado-Santos et al., 2018). In contrast, plasma cells have been found in the meninges and perivascular space in patients with progressive MS indicating a gradual differentiation of infiltrating B cells into plasma cells. Their functions may include direct pro-inflammatory effects through cytokine production or indirect effects on T cells via antigen presentation. Nevertheless, the target antigen recognized by T and B lymphocytes remains unknown (Machado-Santos et al., 2018). Moreover, little is known regarding the antibody-independent role of B cells. Their antigen presenting role was tested using the MS model experimental autoimmune encephalomyelitis (EAE). Mice expressing the MOG-specific B cell receptor (IgH^{MOG}), named rhMOG mice, were compared to a group of transgenic mice in which the antibody secretion was invalidated; Both groups of mice were susceptible to EAE which demonstrates that the invalidation of antibody secretion did not modify the pathogenicity of B cells in rhMOG EAE (Molnarfi et al., 2013). In addition, ocrelizumab, which is a monoclonal antibody specific for the B cell marker CD20, has been shown to reduce disease severity in RRMS and progressive MS. In patients treated with ocrelizumab, oligoclonal bands persisted and plasma cells were spared because they do not express CD20. These results suggest that B cells in MS act as mediators of inflammation through mechanisms independent of antibody production (Bankoti et al., 2014; Kappos et al., 2011; Montalban et al., 2017).

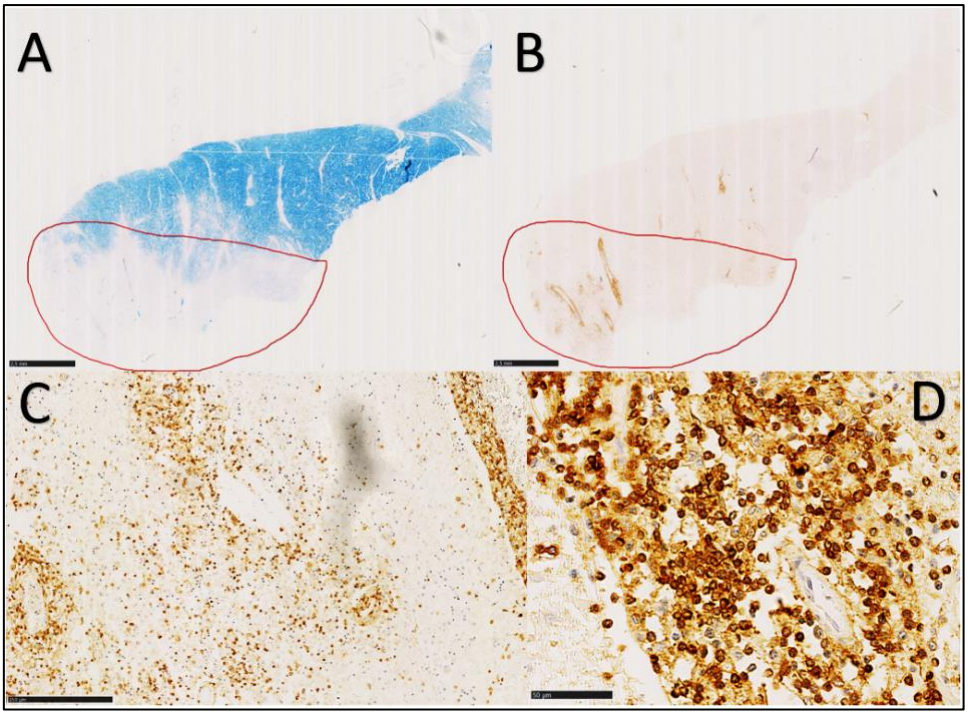

Figure 4. B cell infiltrates in acute MS brain. *A) Klüver-Barrera staining showing demyelination. Red circle demarcates the lesion. Scale bar: 2.5 mm. B) Immunohistochemistry for CD20⁺ B cells. Scale bar: 2.5 mm. Red circle delimitates the lesion. C) and D) CD20⁺ B cells at higher magnification. Scale bars: 250 and 50 µm. Pictures were taken with NDP.view 2.*

Recently, a novel B cell subset, 4-1BBL⁺ B cells (also called 4BL cells), has been found increased with age in humans, macaques and mice. In addition, the accumulation of 4BL cells induced Granzyme B⁺ CD8⁺ T cells in elderly (Lee-Chang et al., 2014, 2016). 4-1BB (CDw137) is a member of the tumor necrosis factor (TNF) family. The TNF molecules have a key role in the activation, proliferation, differentiation, and migration of immune cells into the CNS. 4-1BB is expressed on activated CD8⁺ T cells, CD4⁺ T cells, B cells, dendritic cells (DC), natural killer cells (NK) and mast cells. The 4-1BB ligand (4-1BBL), has been found expressed on activated B cells, macrophages and DCs. The interaction between 4-1BB and the ligand provides a co-stimulatory signal to CD8 T cells, triggering proliferation and cytokine (IFN-γ) production *in vitro* (Shuford et al., 1997). Another study done with transgenic mice revealed that 4-1BB is also expressed on DCs, and that the interaction with the ligand directly activates DCs (Futagawa et al., 2002).

Moreover, 4BL cells have been recently found to make up about the 23% of the B cells in peripheral blood and about 70% in CSF from MS patients (*Dr Lennart Mars*, n.d.).

1.4 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

Several attempts have been made to reproduce MS disease in animal models. Animal models are non-human species used for research which allow us to study biological processes as well as disease pathogenesis. Experimental autoimmune encephalomyelitis (EAE) is the most common animal model used for MS studies. A wide spectrum of EAE models is available. This variety of models reflect different clinical, immunological and histological features of human MS. The pathophysiology of EAE is based on the immune response towards brain-specific antigens that induces inflammation and the elimination of the antigen carrying structures. The neurological and pathological consequences are, to a certain point, similar to those observed in human MS (Gold et al., 2006).

Two main approaches can be distinguished to induce EAE on animals: Actively-induced EAE and passively transferred EAE (Figure 5). In the actively-induced EAE model, rodents are immunized with peptides of myelin proteins such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP). In addition, pertussis toxin (PT) is added to induce EAE in rodents because it promotes cytokine production by T cells. Additionally, it has been suggested that PT induces BBB aperture facilitating the entry of T cells into the CNS (Hofstetter et al., 2002). In order to induce sensitization, strong adjuvants are also needed, which are substances that stimulate and enhance the immune response towards an antigen. One of the most used for animal models is Freund's adjuvant, which greatly stimulates phagocytic uptake and presentation of antigens, and as a result, activation and expansion of $CD4^+$ T cells. Therefore, in EAE the immune response is mediated by MHC class II restricted $CD4^+$ T cells (Bittner et al., 2014; Gold et al., 2006). On the other hand, in passively transferred EAE, T cells towards specific self-antigens are directly transferred into mice (Mannara et al., 2012). The target autoantigen in part determines the pattern of lesion distribution in the CNS; Immune responses towards MBP or PLP induce lesions located in the spinal cord while MOG induces optic nerve and spinal cord lesions (Bettelli et al., 2003).

Although EAE is one of the most widely used in vivo models to study brain inflammation and MS, the following limitations must be taken into account. First of all, EAE is induced by active sensitization with brain tissue antigens, while MS is a spontaneous disease. Secondly, EAE protocols require strong immune adjuvants which induce a strong reaction in the organism that would not occur under physiological conditions. Moreover, for reproducibility and practical reasons, EAE is induced in genetically homogenous populations, while MS is a genetically heterogeneous disease (Gold et al., 2006). Finally, some of the key aspects of MS pathology, including the role of $CD8^+$ T cells and B cells, is covered by experimental models only to a very

limited degree (Lassmann & Bradl, 2017). Nevertheless, EAE has been very useful to get a better understanding regarding brain inflammation and MS pathology.

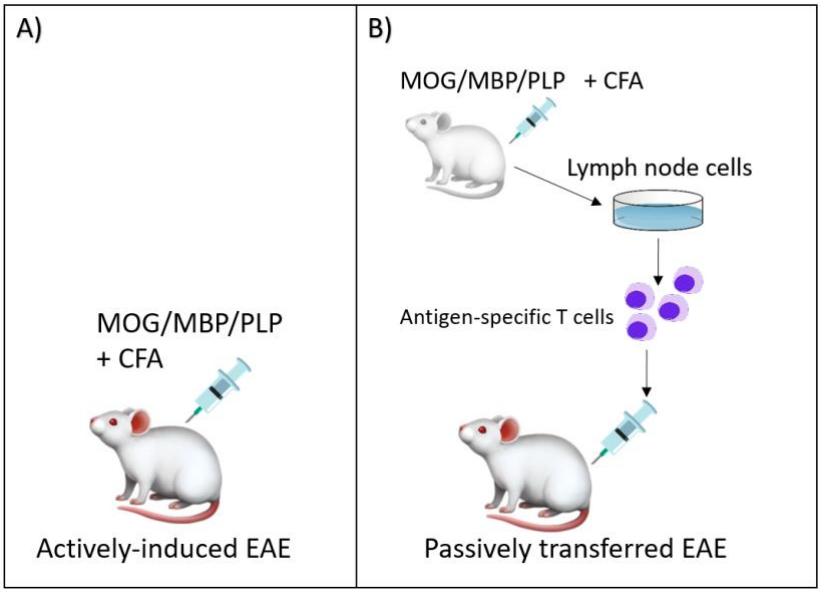

Figure 5. EAE induction in mice. *A) Actively-induced EAE: mice are injected with myelin proteins such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP) and Complete Freund's adjuvant (CFA). B) Passively transferred EAE: myelin proteins and CFA are injected into mice. One/two weeks later lymphocytes are isolated from the lymph node. Next, antigen-specific T cells purified are transferred into other mice. (Adapted from Denic et al., 2016).*

1.4.1 Transgenic mice 2D2 for EAE experiments

In 2D2 mice most of the $CD4^+$ T cells express a transgenic T cell receptor (TCR) that recognize MOG 35-55 peptide. These mice thus are predisposed to develop spontaneous EAE. However, this depends on the immunization protocol. A study reported that when 2D2 mice are immunized with MOG 35-55 peptide, adjuvants, and PT, they develop EAE (based on the clinical and histological signs). In contrast, 2D2 mice immunized only with MOG peptide alone develop isolated optic neuritis without showing clinical nor histological evidence of EAE. Therefore, 2D2 mice can be used to study isolated optic neuritis as well as the autoimmune response towards MOG (Bettelli et al., 2003).

Optic neuritis (ON) is a disorder in which there is inflammation and demyelination of the optic nerve. It has been reported that in many patients with multiple sclerosis the disease starts with a clinically isolated syndrome of optic neuritis, which is a clinical demyelinating event isolated in time (Kale, 2016). However, the relationship between optic neuritis and MS is not really understood.

1.4.2 B cell infiltration in 2D2 mice

B cell infiltration in EAE has been studied using transgenic GF23-2D2 mice. These mice show astrocyte-specific expression of interleukin 23 (IL-23). IL-23 has been found to be increased in serum and CSF from MS patients, however its role is not understood. After EAE induction, high amounts of B cells have been found in GF23-2D2 mice, as well as pronounced demyelination and upregulation of pro-inflammatory cytokines. These results indicate that there is an interaction between encephalitogenic T cells and B cells via cytokines that exacerbates the EAE disease (Nitsch et al., 2021).

1.4.3 IgH^{MOG} transgenic mice

IgH^{MOG} mice are knock-in mouse expressing the immunoglobulin heavy chain of a MOG-specific monoclonal antibody. This model has been used to study the development and control of conditionally pathogenic B lymphocytes *in vivo*. The transgenic B cells are conditionally pathogenic because the mutant mice do not spontaneously develop neurologic deficits. However, when confronted with encephalitogenic antigens or T cells, the autoimmune potential of the B cells is revealed in the transgenic mice, leading to an increased incidence, severity, and accelerated EAE disease onset (Litzenburger et al., 1998).

2 AIMS OF THE THESIS

Despite the vast knowledge in immunology, molecular biology, genetics and cell biology, MS etiology remains unclear. Intensive studies with experimental animal models have helped to understand mechanisms of T cell-mediated damage of the CNS. However, the information regarding the involvement of B lymphocytes in MS pathology is limited. The success of clinical trials of selective B cell depletions in patients with relapsing MS and primary progressive MS indicates that B cells are key players in the immune response. In this study, we focused on a novel B cell subset, 4-1BBL$^+$ B cells, which might be a potential novel mediator of inflammatory damage in MS.

Therefore, the following aims were addressed in this thesis

- Analyze the presence of 4-1BBL$^+$ B cells in MS brain.
- Study the pathogenicity of induced 4-1BBL$^+$ B cells in animal models.

3 MATERIALS AND METHODS

In order to achieve the goals of this project, we performed histopathological analysis of several brain samples from patients who had MS. Furthermore, we were provided with different groups of transgenic mice in which 4BL cells were transferred. In summary, we stained MS brain specimens as well as brain, spinal cord and optic nerve from mice for several immunological markers After that, the samples were evaluated.

3.1 MS SAMPLES

For this study we used in total 24 MS cases (Table 1). Each case corresponds to one patient who had MS. The autopsies were performed in Austria, Hungary, Denmark, Belgium, and Spain.

Table 1. MS cases used in this study.

MS case (ID)	MS type	Sex	Age at death
MS1	AMS	F	46
MS2	AMS	M	59
MS3	AMS	M	45
MS4	AMS	M	52
MS5	AMS	F	45
MS6	AMS	M	78
MS7	SPMS	M	65
MS8	SPMS	M	28
MS9	SPMS	F	45
MS10	SPMS	M	76
MS11	SPMS	F	48
MS12	SPMS	F	53
MS13	SPMS	F	62
MS14	SPMS	M	41
MS15	SPMS	F	46
MS16	PPMS	F	55
MS17	PPMS	M	54
MS18	PPMS	M	34
MS19	PPMS	M	67
MS20	PPMS	F	83
MS21	PPMS	F	75
MS22	RRMS	F	20
MS24	RRMS	F	44
MS23	RRMS	F	40

MS: multiple sclerosis. AMS: Acute MS. SPMS: Secondary progressive MS; PPMS: Primary progressive MS; RRMS: relapsing remitting MS.

3.2 TRANSGENIC MICE

Transgenic 2D2 mice were used in order to study the pathogenicity of induced 4BL cells (i4BL). All mice used for this experiment were provided by Dr. Lennart Mars and colleagues. The experimental design that they carried out is pictured in Figure 6. First of all, B cells were purified from two different mice: wild type (WT) mice and IgH-MOG transgenic mice. The last ones are MOG-specific Ig heavy-chain knock-in mice, thus their B cells are highly efficient in presenting MOG to transgenic T cells. Next, these purified B cells were in vitro differentiated into 4BL cells and then transferred into 4 different groups of 2D2 mice:

1. **PBS group**: no B cell/4BL B cell injection.
2. **WT group**: injected with B cells.
3. **WT i4BL group**: injected with 4BL cells.
4. **IgH i4BL group**: injected with 4BL cells

2D2 mice are expressing MOG-specific T cell receptors. These transgenic mice are predisposed to develop EAE (Bettelli et al., 2003) (Krishnamoorthy et al., 2009).

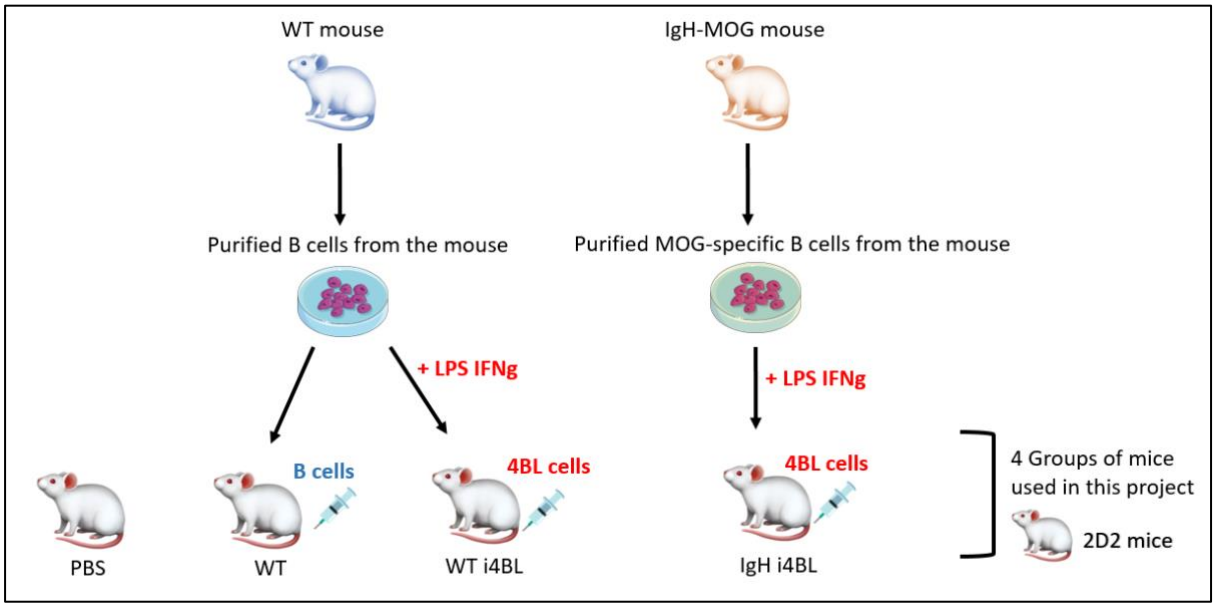

Figure 6. Schematic representation of mice used in the experiments. *PBS: Phosphate-buffered saline. 4BL: 4-1BBL+ B cells. LPS: lipopolysaccharide. IFNg: Interferon gamma. IgH: Immunoglobulin H. 2D2 mice: Transgenic mice expressing a myelin oligodendrocyte glycoprotein (MOG)-specific T cell receptor.*

In addition, the mice used in this study have been separated into two experiments: experiment 1 (mice sacrificed at day 13) and experiment 2 (mice sacrificed at day 40). The groups and the conditions used for both experiments were the same.

After transcardiac perfusion, the animals were preserved with 4% paraformaldehyde (PFA) in a phosphate-buffered saline solution (PBS) with 30% sucrose in 50 ml falcon tubes. Finally, Dr Lennart Mars and colleagues sent us the falcon tubes containing the mice. Once received, we proceeded with the sample preparation. From each mouse we sampled the optic nerve, spinal cord, brain and spleen.

3.3 TISSUE PREPARATION

The human autopsy specimens and mice specimens were fixed in formalin and embedded in paraffin (FFPE). FFPE tissues are resilient and can be used for microscopic anatomy studies for almost indefinitely. After embedding, cross-sections of 2-5 µm were made with a slide microtome.

3.4 IMMUNOHISTOCHEMISTRY FOR SINGLE AND DOUBLE-LABELLING

Brain specimens from MS patients as well as brain, spinal cord and optic nerve from mice were stained for several inflammatory markers according to previously described protocols (Bauer & Lassmann, 2016). As a control for human immune cell staining, we used tonsil sections, and for mice immune cell staining we used the spleen. First of all, FFPE samples were deparaffinized incubating them two times for 15 minutes in xylene, and were rinsed with 96% ethanol. In order to block the endogenous peroxidase activity, the samples were incubated 30 minutes in H_2O_2 - methanol. Then rehydration was performed gradually with 96% ethanol 70% ethanol, 50% ethanol and finally, deionized water. Digestion to unmask epitopes with proteinase type XXVI was used in some cases. Alternatively, heat induced epitope retrieval (HIER) was done by heating the slides in Ethylenediamine tetraacetic acid (EDTA) (pH 9 or pH 8.5) or citrate-buffer (pH 6) in a household food steamer device (Braun) for one hour. After one hour, the slides were rinsed with Tris-buffered saline solution (TBS) 3 to 5 times. The following step was blocking unspecific background reactions by incubating the samples with 10% Fetal calf serum (FCS)-DAKO buffer (commercial buffer solution from Dako corporation) for 15 minutes. Then the primary antibody (Table S1) was applied to the slides and these were incubated at 4°C overnight. The next day, the slides were rinsed with TBS buffer 3 to 5 times. Then, the secondary antibody (Table S2) was added and the slides were incubated for one hour. After one hour, the slides were rinsed with TBS-buffer 3-5 times and incubated with the peroxidase-conjugated streptavidin (1:500) in 10% FCS-DAKO buffer for another hour. Depending on the antibody, catalyzed signal amplification (CSA) system was performed with tyramide. For that, biotinylated tyramide was diluted 1:1000 in phosphate-buffered saline solution (PBS) containing 30% H_2O_2. After that, incubation was done again using peroxidase conjugated streptavidin (1:500) in 10% FCS-DAKO buffer for half hour. Finally, the slides were rinsed again with TBS-buffer and were developed under microscopic control in 3,3'-Diaminobenzidine (DAB). The enzymatic reaction was stopped with deionized

water. Counterstaining was done by incubating the slides for 15-20 seconds in Mayer's hematoxylin, washed two times with water, rinsed with HCl-ethanol, incubated 4 minutes in Scott's solution and rinsed with water. Next, dehydration of the samples was performed gradually with ethanol (50%, 70%, 95%, 95%, 95%) ending with n-butyl acetate. Finally, the samples were mounted with cover slips using Eukitt®. When the development was done with Fast Blue, counterstaining was not performed, and the mounting medium used was Geltol.

3.5 MULTIPLEX IMMUNOFLUORESCENT LABELLING

We performed combinations of immunofluorescent labelling for several antibodies using Akoya Fluorescent Multiplex kit according to manufacturer's protocol. The samples were deparaffinized dipping them two times for 15 minutes in xylene. Next, rehydration was performed gradually with 96% ethanol 70% ethanol, 50% ethanol and finally, deionized water. Next, fixation was performed with 4% paraformaldehyde (PFA) during 20 minutes. Sections were rinsed with deionized water and steamed for 1 hour in antigen retrieval buffer pH 9.0 (AR9) in a household food steamer device (Braun). After that, sections were rinsed with Tris-buffered saline with Tween 2.0 (TBST) pH 7.5, followed by a blocking step with Opal antibody diluent/block solution for 10 minutes. The incubation time for primary antibodies was either 2 hours at room temperature or overnight at 4°C. Next, slides were rinsed with TBST and the secondary antibody (Opal Polymer horseradish peroxidase (HRP) Ms + Rb) was applied for 10 minutes at room temperature. The sections were rinsed with TBST and then, incubated with one fluorophore (Opal dye 480, 520, 570, 620, 690 or 780) diluted 1:100 for 10 min. Next, the slides were rinsed with TBST and again, fixed with 4% PFA only for ten minutes, and steamed with antigen retrieval buffer pH 6.0 for 30 minutes. From here, the steps mentioned before were performed again for each primary antibody. Finally, counterstaining was done with 4',6-diamidino-2-phenylindole (DAPI) and slides were mounted with a specific medium for fluorescent stainings. The stainings were scanned with Vectra Polaris scanner at 20x magnification.

3.6 CELL QUANTIFICATION

The IHC sections were scanned with a slide scanner (NanoZoomer Digital Pathology, Hamamatsu Photonics) at 20x magnification. Positive cells for the following markers were quantified: CD3, CD4, CD8, CD19 and immunoglobulin (Ig). The quantification was done semi-automatically with Qupath, which has incorporated a tool for object classification. First, the areas of interest were selected manually and then the object classifier detected all positive cells within the area selected. Finally, the absolute number of quantified cells was divided by the analyzed area in order to obtain the number of cells per mm^2. For double-labelled samples, cells were counted manually.

3.7 ANALYSIS OF DEMYELINATION

First of all, the spinal cord sectios were stained following the Klüver-Barrera (KB) method (Appendix 1.2). The KB method is used to stain the myelin sheaths in brain and spinal cord, this allow us to see which areas of the CNS are demyelinated. Demyelination was measured in spinal cord cross sections. For this, a morphometric grid with 10x10 squares was placed on the spinal cord section. The center of the grid was aligned with the central canal of the spinal cord. Next, we counted the myelinated (green) and demyelinated (yellow) cross points in the white matter at 5x magnification (Figure 7). The proportion of demyelinated white matter was calculated as a percentage of total white matter. For each animal, we analyzed 5-10 spinal cord sections.

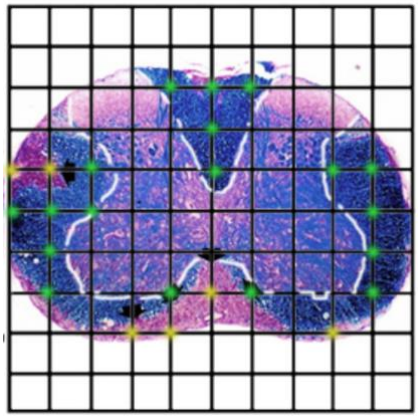

Figure 7. Demyelination scoring system in mice spinal cord. *Demyelination was counted with a morphometric grid. Yellow point: demyelinated area. Green point: normal myelin.*

3.8 CLINICAL SCORE

The clinical score was evaluated everyday on each mouse after the immunization. The data was provided by Dr Lennart Mars team. The following scoring system was used based on the clinical signs that the mice exhibit:

Table 2. Clinical scoring.

Clinical signs	**Scoring value**
No clinical signs	0
Tail weakness	1
Absence of straightening with tail weakness	2
Severe paralysis of the lower limbs	3
Quadriplegia	4
Moribund state or death	5

Clinical signs were evaluated everyday on after the day of B cells or 4BL cells injection on all mice.

3.9 STATISTICS

Statistics were performed using the program GraphPad Prism 9. The analysis selected was ordinary one-way ANOVA + T-test nonparametric for comparison between the different groups of mice. **Important note**: The PBS group from both mice experiments were not included in the statistical analysis since there was only one PBS individual for each experiment.

4 RESULTS

4.1 ANALYSIS OF 4BL CELL PRESENCE IN MS BRAIN

In this study we attempted to find out if 4BL cells are present in the MS brain. First of all, we analyzed the amounts of $CD20^+$ B cells, $CD19^+$ cells (B cell and plasma cell marker) and $CD38^+$ cells (plasma cell marker) in different MS types. Next, we selected the cases in which there were moderate or high amounts of B cells or plasma cells within the brain. The analysis performed was semi-quantitative: 0= no B/plasma cells present, 1= small number of B/plasma cell present, 2= moderate amount of B/plasma cells, 3= Many B/plasma cells. In addition, the localization of B and plasma cells was divided into meninges, perivascular space, and parenchyma (Table 3, Figure 7).

Table 3. B cell and plasma cell semi-quantitative analysis.

		Marker		
ID	Area	CD20	CD19	CD38
MS 1 (Acute MS)	Meninges	2	2	2
	Perivascular	3	1	2
	Parenchyma	3	1	2

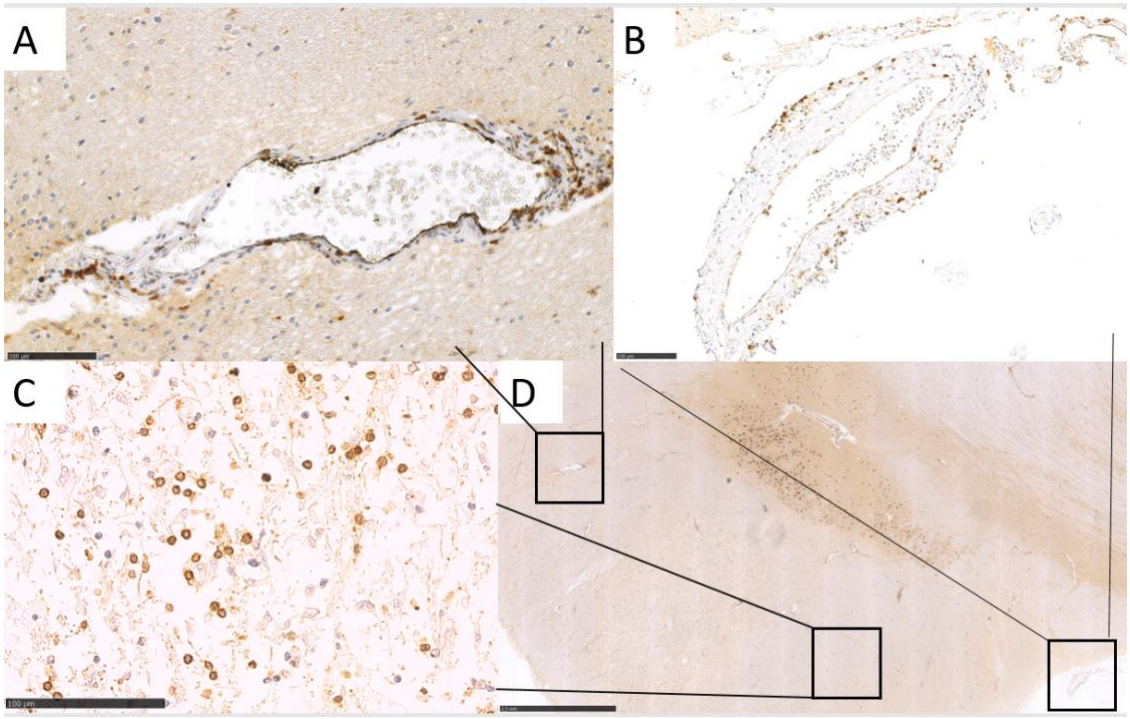

Figure 7. B cell localization in the MS brain. *IHC staining for $CD20^+$ cells. A) Perivascular space; B) Meninges; C) Parenchyma. Scale bars: 100 µm. D) Squares indicate the areas (meninges, perivascular, parenchyma). Scale bar: 2.5 µm.*

In total, 24 MS cases in which there were considerable amounts of B cells or plasma cells were selected. Several attempts were made to study the presence of 4BL cells; Single-labelling immunostainings, double-labelling and fluorescent multiplex labelling stainings were performed with different antibody dilutions and pre-treatments. The first 4-1BBL antibody that we purchased did not work at all since we did not find positive cells in our control tissue (human tonsil). The second that we ordered worked properly, we found many 4-1BBL$^+$ B cells in the B cell areas of the tonsil (Figure 8). In addition, we tried the 4BL antibody on a brain lymphoma in which there were lots of B cells; Nevertheless, we did not find any 4BL positive cell there (data not shown).

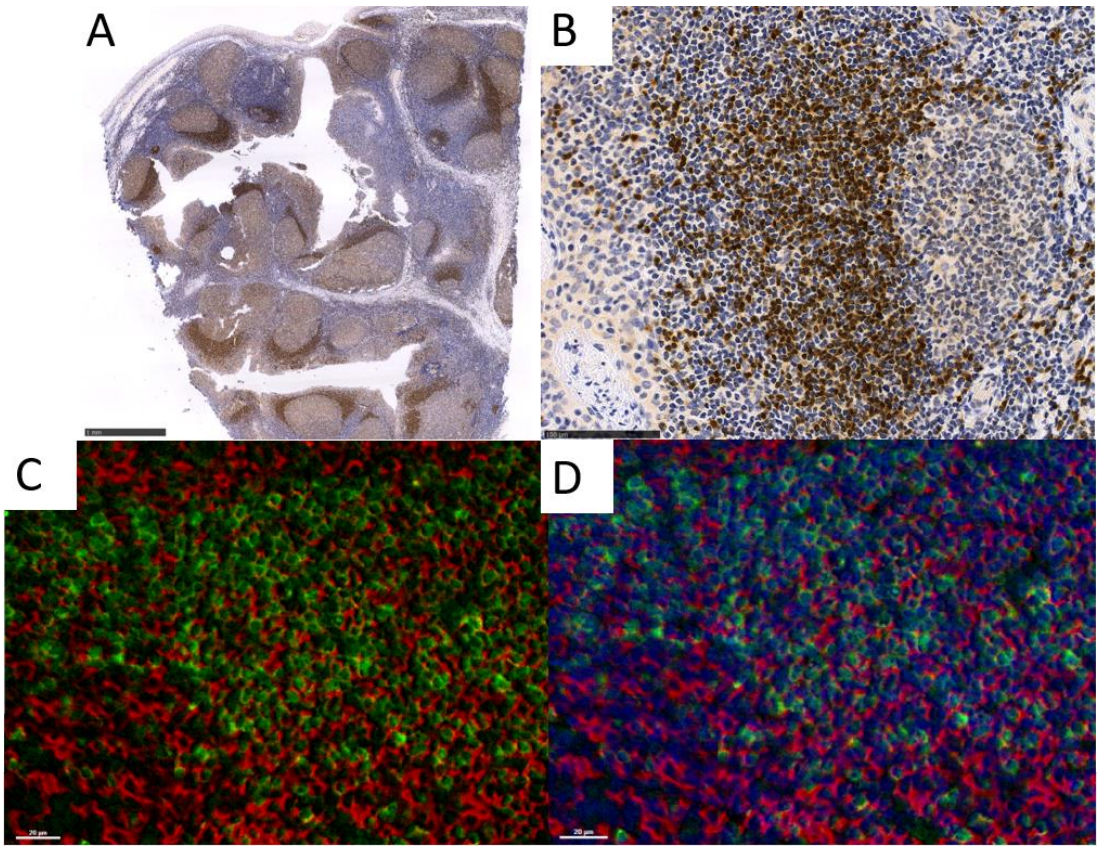

Figure 8. 4BL and B cells on human tonsil*. A) IHC staining for 4-1BBL. Cell nuclei stained in blue, 4-1BBL+ cells in brown. Scale bar: 1 mm. B) Picture A at higher magnification. Scale bar: 100 µm. C) Fluorescent staining for CD20+ cells (red) and 4-1BBL+ cells (green) Scale bar: 20 µm. D) DAPI for cell nuclei (blue) with CD20 and 4BL. Scale bar: 20 µm.*

We tried to optimize our stainings by trying several antibody dilutions (1:8000 + CSA, 1:1000, 1:500, 1:300, 1:250). Moreover, we tested if a protease enzymatic pre-treatment would help unmasking epitopes. Overall, we found 4-1BBL$^+$ B cells in two MS cases out of 24. One of them was an acute MS case. The positive cells were found in the perivascular space (Figure 9 A). In order to proof that these positive cells were actually 4BL B cells, we performed double-labelling using two antibodies: anti-4-1BBL and anti-CD20 (Figure 9 B). Unfortunately, the performed double staining did not clearly reveal which cells were double positive. The second case where

we found positive 4BL cells was a chronic MS case. 4-1BBL+ cells were found in the meninges (Figure 10 A, B). Based on the morphology of some of these cells, they were probably monocytes or macrophages (Figure 10 C). The fluorescent multiple-labelling staining performed did not provide further insight (not shown).

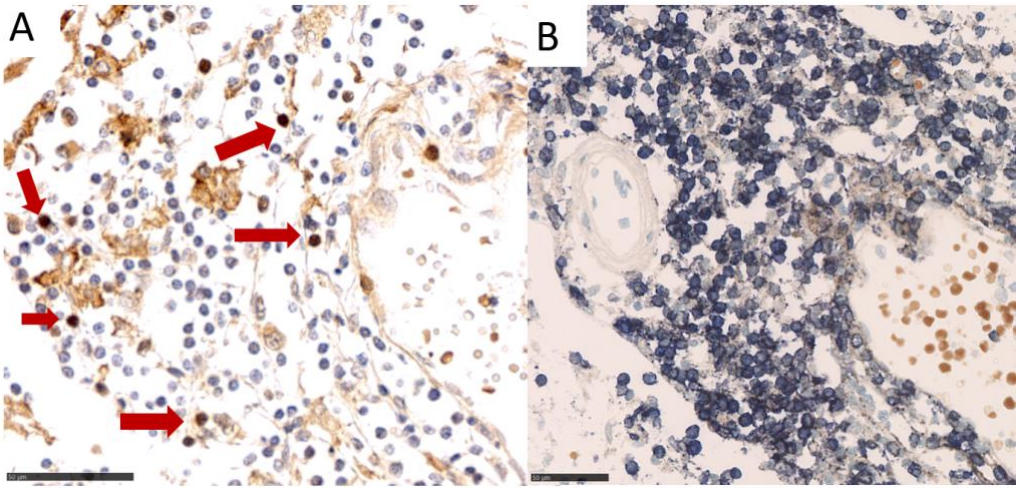

Figure 9. Analysis of 4BL cells presence in acute multiple sclerosis (AMS) brain. *A) Immunohistochemistry (IHC) for 4-1BBL+ cells. Red arrows indicate positive cells. The antibody was developed with DAB (brown) and counterstaining was done with hematoxylin for cell nuclei (blue). Scale bar: 50 µm. B) Double-labelling IHC for CD20+ cells (blue) and 4-1BBL+ cells (brown). Scale bar: 50 µm. Pictures were taken with NDP.view 2.*

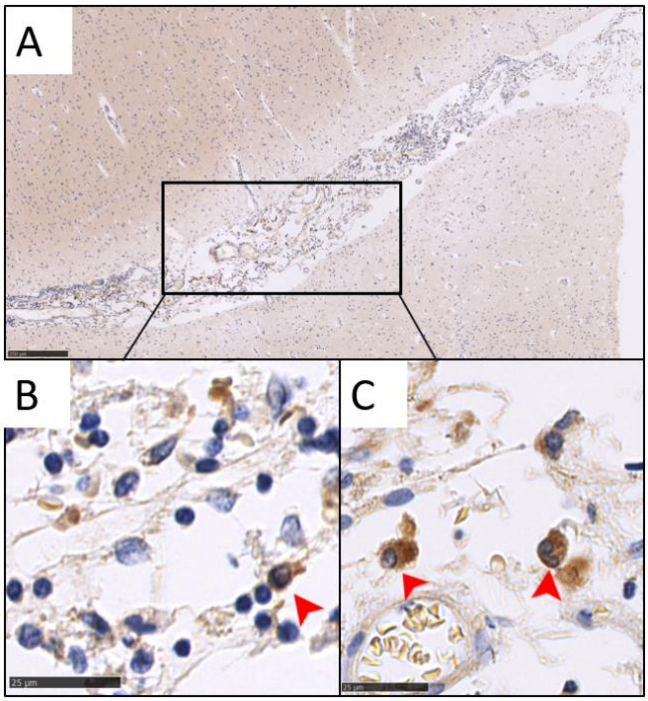

Figure 10. Analysis of 4BL cells presence in chronic MS. *Immunohistochemistry (IHC) for 4-1BBL+ cells. Red arrows indicate positive cells. The antibody was developed with DAB (brown) and counterstaining was done with hematoxylin for cell nuclei (blue). A) Meningeal area. Scale bar: 250 µm. B) Possible 4-1BBL+ B cell Scale bar: 25 µm. C) Monocytes positive for 4-1BBL⁺ marker. Scale bar: 25 µm. Pictures were taken with NDP.view 2.*

4.2 ANALYSIS OF INDUCED 4BL CELLS PATHOGENICITY

As mentioned in the materials and methods, in this study we used 4 different groups of mice which were non-manipulated or transferred with B cells or 4BL cells. In total there were 24 mice, but they were divided into two experiments: In the 1st experiment mice were sacrificed at day 13 after injection (here on referred as **D13 mice**) while in the 2nd experiment mice were sacrificed at day 40 after injection (here on referred as **D40 mice**) (Figure 11). The difference in the sacrifice day is reflected in the results because at day 40 there are almost no inflammatory cells remaining in the CNS and the lesions can be remyelinated. On the contrary, in mice that were sacrificed at day 13 we still can find many immune cells in the CNS and demyelination.

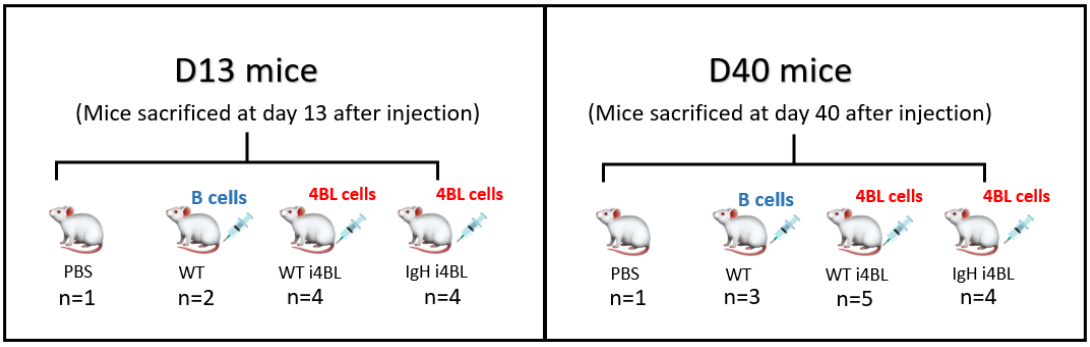

Figure 11. Schematic representation from 1st and 2nd experiments.

4.2.1 Clinical score

The clinical score values were provided by Dr Lennart Mars and colleagues. The clinical score was 0 every day for both WT groups and the PBS group (Figure 12). The only group that showed clinical scores >0 was the IgH i4BL group. These results suggest that the IgH-MOG specific 4BL cells exacerbated the EAE disease.

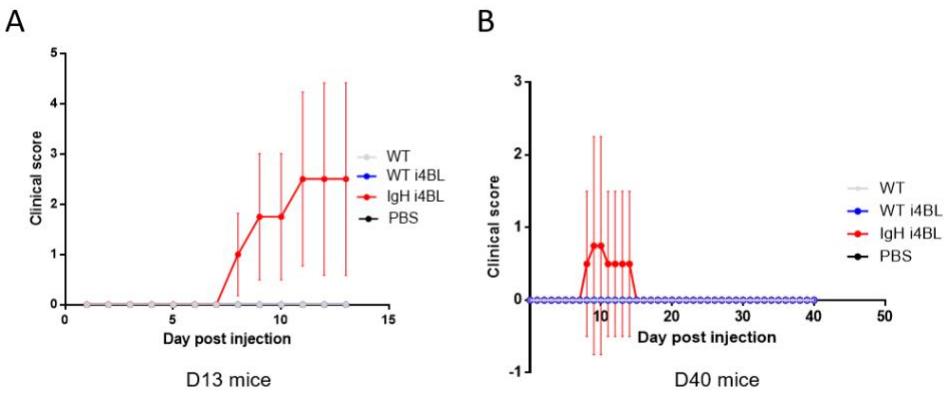

Figure 12. Clinical score values for each day post injection till sacrifice. *A) Clinical score from D13 mice. B) Clinical score from D40 mice. The graphs show mean and error. Clinical scores were provided by Dr Lennart Mars team.*

4.2.2 Demyelination

In order to measure the extent of demyelination, KB stainings in spinal cord sections were analyzed as indicated in the methods. Demyelination was scored as a percentage. In D13 mice, the % of demyelination was much higher in the IgH i4BL group than in the other groups (Figure 13 A). In D40 mice overall there was almost no demyelination, most likely because all lesions were remyelinated (Figure 13 B).

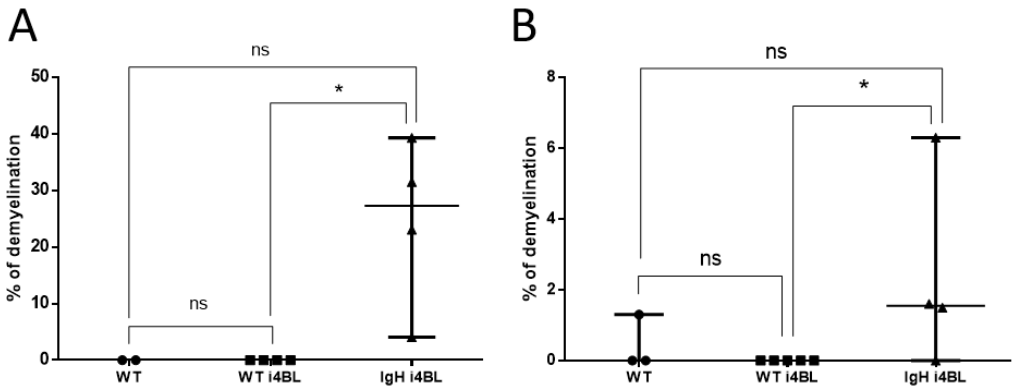

Figure 13. Demyelination in the spinal cord. *Demyelination scored as a percentage in all groups. A) Demyelination scored in D13 mice. B) Demyelination scored in D40 mice. *= p-value < 0.05. The graphs show median with range.*

4.2.3 T cell infiltrates

The number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells was analyzed in the spinal cord sections from D13 mice. Overall, the presence of T cells was higher in the IgH i4BL mice (Figure 14). Elevated numbers of $CD3^+$ T cells and $CD4^+$ T cells were found in the spinal cord sections from IgH i4BL D13 mouse. (Figure 14 A, C). The amount of $CD8^+$ T cell infiltrates was much lower but still significant between the WT i4BL and the IgH i4BL group (Figure 14 B).

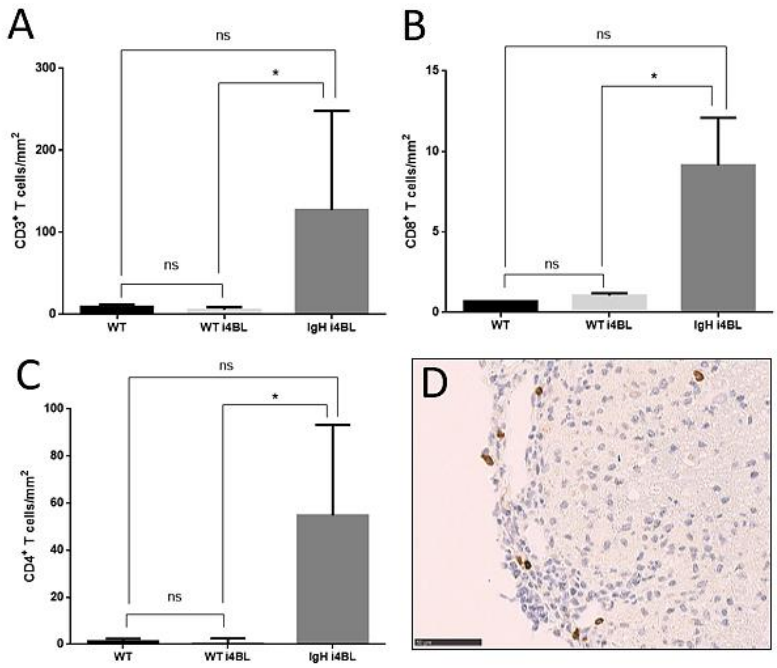

Figure 14. T cells in the spinal cord from D13 mice. *A) CD3⁺ T cells/mm². B) CD8⁺ T cells/mm². C) CD4⁺ T cells/mm². ns= not significant. *= p-value <0.05. D) CD8⁺ T cells in a mouse spinal cord. Positive cells are stained brown and cell nuclei blue. Scale bar: 50µm. The graphs show median with range.*

In D40 mice, only CD3⁺ and CD8⁺ T cells were analyzed (Figure 15). Due to technical reasons, we could not perform the staining for CD4⁺ T cells thus it will be done in our future work. The amount of T cells was low in all groups. One mouse from the WT group had higher amounts CD3⁺ T cell and CD8⁺ T cell infiltrates in the spinal cord in comparison with the others (Figure 15 A, B).

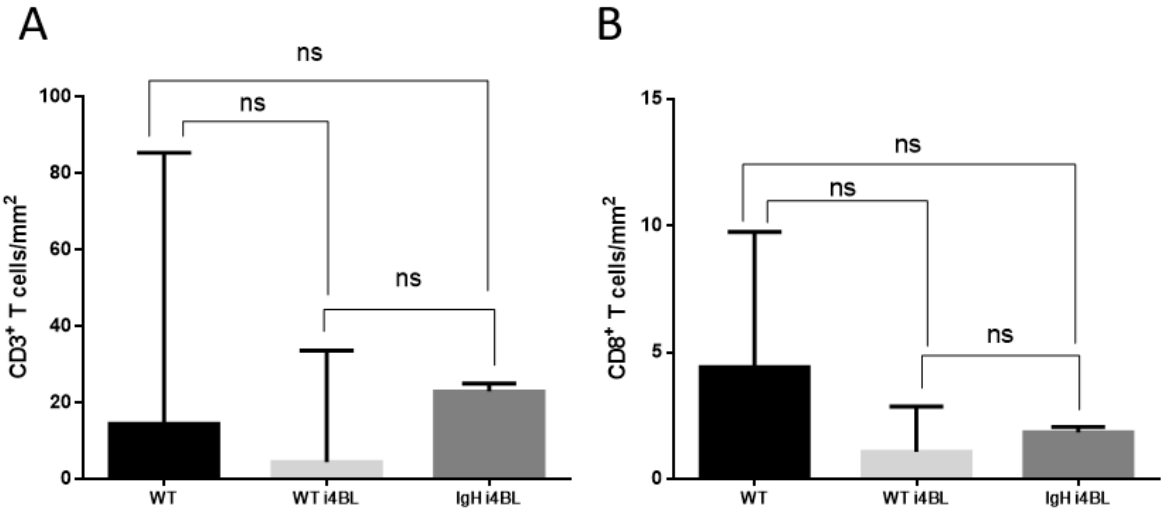

Figure 15. T cell numbers in the spinal cord from D40 mice. *A) CD3⁺ T cells/mm². B) CD8⁺ T cells/mm². ns: not significant. The graphs show median with range.*

4.2.4 B cell infiltrates

B cell infiltration was analyzed in D13 and D40 mice. For that, we quantified $CD19^+$ B cells in the spinal cord. Overall, the number of $CD19^+$ B cells was small in all groups. However, we had significant results between WT i4BL and IgH i4BL mice sacrificed at day 13 (Figure 16 A). All B cells were mostly found in the meninges. Almost no B cells were present in D40 mice (Figure 16 B).

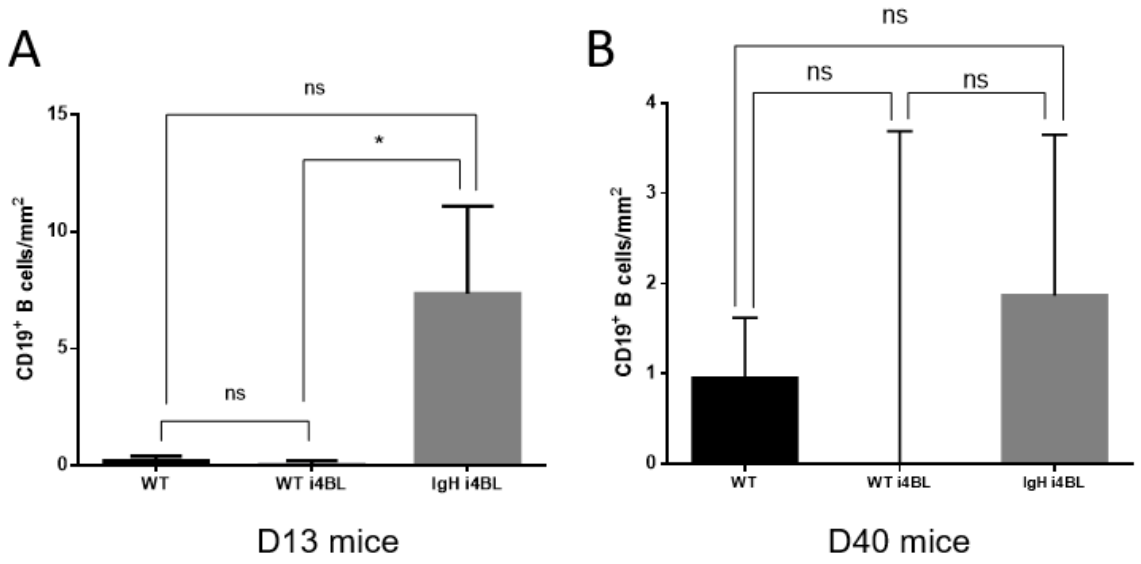

Figure 16. $CD19^+$ B cells in the spinal cord. *A) $CD19^+$ B cells/mm^2 in D13 mice. B) $CD19^+$ B cells/mm^2 in D40 mice. *= p-value <0.05; ns= not significant. The graphs show median with range.*

4.2.5 Plasma cell infiltrates

To analyze the amount of plasma cells in the CNS, Ig stainings were performed in brain and spinal cord from D40 mice. Almost no plasma cells were present in the spinal cord (Figure 17) and brain (data not shown). The results are not significant between the groups. Plasma cell infiltrates were not analyzed in D13 mice since it takes more time for the B cells to differentiate into plasma cells. Therefore, we don't expect to find plasma cells in the CNS from D13 mice.

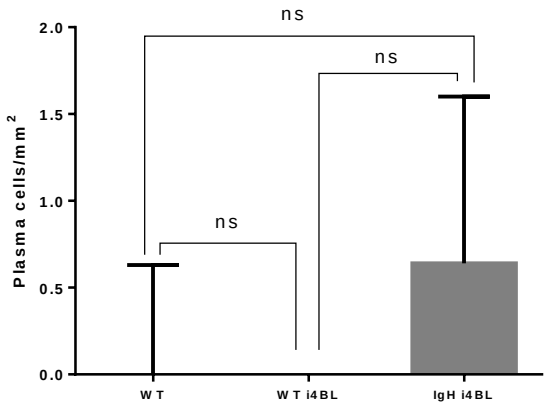

Figure 17. Plasma cells/mm² in the spinal cord from D40 mice*. ns= not significant. The graph shows median with range.*

4.2.6 Co-localization of CD19⁺ B cells and CD4⁺ T cells

Spinal cord sections in which there were higher amounts of CD19⁺ B cells were selected to perform double-labelling staining using CD19⁺ and CD4⁺ markers (Figure 18). In total we had 24 spinal cord sections from 4 mice from the first experiment. We found co-localization of CD19⁺ B cells with CD4⁺ T cells in 19 spinal cord sections.

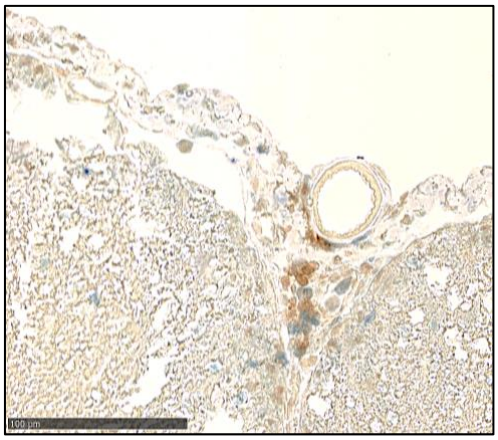

Figure 18. T cells and B cells in the spinal cord from a D13 mouse. *CD19⁺ B cell marker was developed with DAB (brown) and CD4⁺ T cell marker was developed with Fast Blue (blue). Scale bar: 100 µm. Picture was taken with NDP.view 2.*

4.2.7 4BL cell infiltrates

Finally, we analyzed if the 4BL cells infiltrate into the brain and spinal cord. First, we performed single IHC staining using the anti-4-1BBL$^+$ antibody. We observed many positive cells, however, most of them were oligodendrocytes and not B cells. Therefore, we performed stainings using the OPAL system for multiple labelling (Figure 19). We used three markers: DAPI for cell nuclei, B220 for B cells and 4-1BBL. We found a few double positive cells (B220$^+$ 4-1BBL$^+$) in the perivascular space (Figure 19 A, B, C). As shown in figure 19 D, many oligodendrocytes were 4-1BBL$^+$ (red).

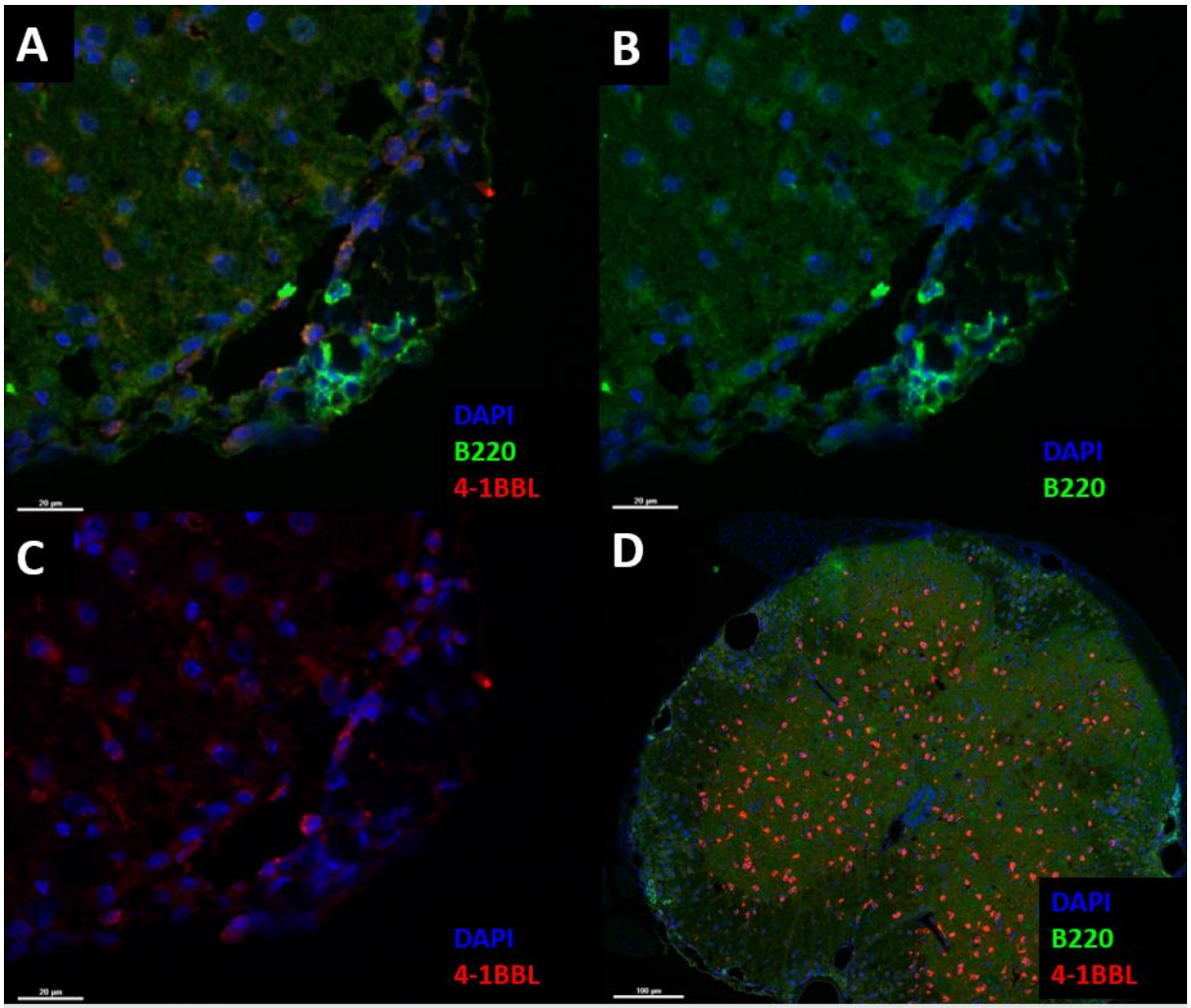

Figure 19. B cells and 4BL cells in mouse spinal cord. *Multiple labelling staining with OPAL system. DAPI (cell nuclei) in blue, B220 (B cells) in green, and 4-1BBL in red. A) DAPI, B220 and 4-1BBL; B) DAPI and B220; C) DAPI and 4-1BBL. All belong to the same area. Scale bar: 20 µm. D) DAPI, B220 and 4-1BBL. Scale bar: 100 µm.*

5 DISCUSSION

In recent years, several studies have focused on the role of B cells in MS pathogenesis due to the benefits of therapies targeting B cells in MS patients (Kappos et al., 2011; Montalban et al., 2017). Both antibody-dependent and independent mechanisms mediated by B cells are thought to be contributing to the pathology. The presence of oligoclonal bands in the CSF of MS patients is one of the hallmarks of MS, indicating that there is an antibody-mediated response going on. Moreover, antigen presentation to B cells and production of pro-inflammatory cytokines are B cell functions which are likely to mediate CNS injury in MS (Comi et al., 2021). In acute MS, CD20+ B cells dominate in the meninges, perivascular space and brain parenchyma. In contrast, plasma cells numbers are higher in chronic MS, indicating that infiltrating B cells transform into plasma cells with disease progression (Machado-Santos et al., 2018). Recently, a novel B cell subset called '4BL cells' has been found in the peripheral blood and CSF from MS patients (Dr Lennart Mars, n.d.). In this study, we analyzed if 4BL cells infiltrate into the brain from MS patients.

Our methods revealed 4BL cells in the brain from only two MS patients, one who had acute MS and one who had SPMS. It is important to point out that in our AMS cases there were more B cell infiltrates than in the chronic MS sections. Thus, the probabilities of finding 4BL cells are higher in the AMS brain. However, we were expecting to find more 4BL cells in chronic cases because these cells have been reported to increase with age (Lee-Chang et al., 2014). Two main limitations regarding our methods must be taken into account; First of all, MS tissue is strongly formalin-fixed because of the post-mortem delay after an autopsy. It has been documented that formalin can cause cross-linking in antigens and epitope masking. Therefore, resulting in a reduced antigen detection (Webster et al., 2009). Moreover, most of our MS samples were embedded about 20 years ago; Several studies have reported a loss of antigenicity in old paraffin sections (Bertheau et al., 1998; Blind et al., 2008; Jacobs et al., 1996; van den Broek & van de Vijver, 2000). Tissue dehydration seems to have an impact in protein degradation and immunoreactivity decrease in FFPE tissue (Blind et al., 2008; Xie et al., 2011). Hence, three possibilities can be contemplated regarding our results: (1) The antibody used for 4BL detection cannot bind its ligand due to cross-linking, epitope masking or protein degradation, (2) 4BL cells do not infiltrate into the MS brain, (3) 4BL cells infiltrate into the CNS from MS patients but lose the 4BL phenotype after entrance into the CNS. The inflammatory profile from 4BL B cells (Futagawa et al., 2002), their presence in the CSF from MS patients (data not published) and their stimulatory capacity for GrB+CD8+ T cells (Lee-Chang et al., 2014) open up the possibility of being potential mediators of inflammatory damage in MS. Further work will be needed in order to determine their role in MS disease and to determine a possible therapeutic target.

In addition, we studied the pathogenicity of induced 4BL cells in transgenic mice prone to develop EAE. Overall, our results suggest that induced 4BL cells exacerbated both CD4+ T cell response and EAE disease. In the first experiment (D13 mice), only mice who had the MOG-specific BCR and were injected with 4BL (IgH i4BL group) showed clinical scores >0. Moreover, white matter demyelination in the spinal cord was only observed in the IgH i4BL group. The number of CD3+ T cells and CD4+ T cells was very high in the IgH i4BL in comparison with the WT groups. However, in our histopathological analyses there was almost no difference between the WT group transferred with B cells and the WT group transferred with 4BL cells. Thus, the exacerbated CD4+ T cell response and EAE disease severity could be a consequence of the MOG-specific BCR from the IgH i4BL group. Bettelli and colleagues reported that transgenic mice who have MOG-specific T cells and MOG-specific B cells develop spontaneous and severe EAE (Bettelli et al., 2006). Therefore, a control group with MOG-specific B and T cells without transferred 4BL cells is needed to confirm the pathogenic effects of i4BL cells.

On the other hand, we analyzed the co-localization of CD4+ T cells with CD19+ B cells in D13 mice. We found CD4+ T cells positioned very close to CD19+ B cells in the meningeal areas. In addition, we performed a fluorescent multiple-labelling staining using markers for CD3+, CD4+ and CD8+ T cells, as well as CD19+ B cells (data not shown). Unfortunately, it did not work properly. Working with IHC double-labelling as well as fluorescent multiple-labelling is sometimes a challenge. For this, is essential to find the most optimal antibody concentration. Otherwise, if there is too much signal from one marker it will overlap with the other markers and it will be difficult to distinguish single positive cells. The antibody concentration used for single-labelling IHC is often not the same as used for double- and multiple-labelling, thus control stainings need to be done for each system. Nonetheless, double-labelling and multiple-labelling IHC staining are a valuable technique when performed properly. In our future work, we will perform more multiple-labelling stainings to analyze if there are 4BL cells positioned close to CD8+ and CD4+ T cells.

Giving the fact that none of the D40 mice had clinical scores >0, we suspected that in the second experiment none of the mice developed EAE. However, our analysis showed that inflammatory T cells (although lower than at day 13) could be detected in the spinal cord. These findings thus suggest that inflammation occurred but the inflammatory cells were not present anymore in the CNS by the time when the mice were sacrificed (day 40). Furthermore, we thought that plasma cells could be present in the spinal cord from D40 mice since B cells had time to differentiate. However, almost no plasma cells were found in the spinal cord and brain from D40 mice. Finally,

we did not observe demyelination in the white matter from the spinal cord sections of D40 mice. It is unclear if demyelination did not take place or if demyelination was not found because remyelination took place.

Further analyses will be needed to study the presence of 4BL cells in the mice CNS. As shown in the results, the antibody used for 4-1BBL+ B cells did not seem specific since we found positive T cells as well as many positive oligodendrocytes. Thus, for future analyses we will try to find another 4BL antibody for murine tissue.

6 OUTLOOK

The presence of 4BL cells in the CNS from MS patients needs to be further investigated. First of all, more MS cases will need to be added to our study. Fortunately, we have many brain autopsies from patients with acute MS as well as from patients with chronic MS. Therefore, we will first evaluate the amount of B cell and plasma cell infiltrates and then we will select the cases with higher numbers. Secondly, we need to optimize the 4BL as well as other antibody concentrations for multiple-labelling system. We will perform IHC staining with new markers such as T-bet, which is a nuclear factor expressed on 4-1BBL+ cells. Besides this, another possibility will be to analyze MS brain autopsies with spatial transcriptomics. This is a genomic method which allows to study gene expression in a given area within a sample. In this way, we will be able to select a meningeal area or perivascular space of blood vessels to analyze if the expression of 4-1BBL is upregulated. Furthermore, if we find more 4BL cells in other MS cases we can investigate their role in the disease by analyzing their immunological phenotype.

Regarding the i4BL EAE experiments, new groups of mice can be added to this project; The pathogenicity of i4BL cells can be studied in active EAE models. Moreover, we should include a MOG-IgH control group with no i4BL cells transferred since this control group presently is missing. With these experiments, we will be able to determine if the exacerbated EAE disease as well as the CD4+ T cell response is due to the transferred 4BL cells or due to the MOG-specific BCR.

The present experiments were performed with one anti-4BL antibody. It would be good if we could confirm our findings with another antibody. For this, we should purchase another (mouse-specific) anti-4-1BBL antibody to analyze if 4BL cells infiltrate into the mice CNS. In addition, we could try our 4BL antibody in fresh frozen tissue instead of in FFPE tissue; Fresh frozen tissue has been reported to be a better option when evaluating new markers (Shi et al., 2008).

We can distinguish donor 4BL cells (which express CD45.2) from recipient B cells (expressing CD45.1) by using anti-CD45.2 specific antibody. Thus, we will evaluate if the mice produced 4BL cells. Finally, the co-localization of 4BL cells with CD4+T cells and CD8+ T cells still needs to be assessed to provide further insight about a possible interaction between them.

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8 LIST OF ABBREVIATIONS

AMS: Acute multiple sclerosis

APC: Antigen-presenting cell

AR: Antigen retrieval

BCR: B cell receptor

BBB: Blood-brain barrier

BCB: Blood-cerebrospinal fluid barrier

CD: Cluster of differentiation

CFA: Complete Freund's adjuvant

CMI: Cell-mediated immunity

CNS: Central nervous system

CSA: Catalyzed signal amplification

CSF: Cerebrospinal fluid

CTL: Cytotoxic T cell

DAB: 3,3'-Diaminobenzidine

DAPI: 4',6-diamidino-2-phenylindole

DC: Dendritic cell

EAE: Experimental autoimmune encephalomyelitis

EBV: Epstein-Barr Virus

FCS: Fetal calf serum

EDTA: Ethylenediamine tetraacetic acid

FFPE: Formalin-Fixed Paraffin-Embedded

GFAP: Glial fibrillary acidic protein

HIER: heat induced epitope retrieval

IFN: Interferon

IHC: Immunohistochemistry

Ig: Immunoglobulin

IL: Interleukin

i4BL: induced 4BL

KB: Klüver-Barrera

LMP2A: Latent membrane protein 2A

MALT: Mucosa associated lymphoid tissue

MBP: Myelin basic protein

MHC: Major histocompatibility complex

MOG: Myelin oligodendrocyte glycoprotein

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

NK: Natural killer

ON: Optic neuritis

OPC: Oligodendrocyte progenitor cell

PBS: Phosphate-buffered saline solution

PFA: paraformaldehyde

PLP: Proteolipid protein

PNS: Peripheral nervous system

PPMS: Primary progressive multiple sclerosis

RRMS: Relapsing-remitting multiple sclerosis

SPMS: Secondary progressive multiple sclerosis

TBS: Tris-buffered saline solution

TBST: Tris-buffered saline with Tween 2.0

TCR: T cell receptor

TNF: Tumor necrosis factor

WT: Wild type

9 APPENDIX

9.1 SUPPLEMENTARY TABLES

Table S1. Primary antibodies used in the study.

Antibody name and manufacturer	Species	Target	Pretreatment	Dilution
B220 (Pharmingen)	Rat	B cells	Citrate	1:250
CD3 (DAKO #A0452)	Rabbit	T cells	EDTA	1:1000
CD4 (eBiosciences)	Rat	T helper cells	EDTA	1:250
CD8a (abcam #ab209775)	Rabbit	Cytotoxic T cells	EDTA	1:500
CD19 (Cell signaling #90176)	Rabbit	B cells, plasma cells	EDTA	1:300
CD20 (Thermoscientific #MS-340-50)	Mouse	B cells	EDTA	1:500
CD137L (Cell signalling #90204)	Rabbit	4-1BBL+ cells	EDTA	1:250
4-1BBL (Abcam #ab64912)	Rabbit	4-1BBL+ cells	EDTA	1:250
4-1BBL (Invitrogen #MA5-29838)	Rabbit	4-1BBL+ cells	EDTA	1:250
CD138 (Serotec #MCA681H)	Mouse	Plasma cells	EDTA	1:500
B220 (Pharmingen)	Rat	B cells	Citrate	1:250
CD3 (DAKO #A0452)	Rabbit	T cells	EDTA	1:1000
CD4 (eBiosciences)	Rat	T helper cells	EDTA	1:250
CD8a (abcam #ab209775)	Rabbit	Cytotoxic T cells	EDTA	1:500
CD19 (Cell signaling #90176)	Rabbit.	B cells, plasma cells	EDTA	1:300
CD20 (Thermoscientific #MS-340-50)	Mouse	B cells	EDTA	1:500
CD137L (Cell signalling #90204)	Rabbit	4-1BBL+ cells	EDTA	1:250
4-1BBL (Abcam #ab64912)	Rabbit	4-1BBL+ cells	EDTA	1:250
4-1BBL (Invitrogen #MA5-29838)	Rabbit	4-1BBL+ cells	EDTA	1:250
CD138 (Serotec #MCA681H)	Mouse	Plasma cells	EDTA	1:500

EDTA: Ethylenediamine tetraacetic acid.

Table S2. Secondary antibodies used in this study.

Antibody name and manufacturer	**Target**	**Dilution**
Biotin anti-rabbit (Jackson #711-065-152)	Rabbit	1:1000
Biotin anti-mouse (Jackson #715-065-150)	Mouse	1:500
Biotin anti-rat (Jackson #712-065-153)	Rat	1:500
AP anti-mouse (Jackson #715-055-151)	Mouse	1:100
AP anti-rabbit (Jackson #711-035-152)	Rabbit	1:100
AP anti-rat (Jackson #112-055-062)	Rat	1:200
Cy2 Streptavidin (Jackson #016-220-084)	Biotin	1:100
Cy3 anti-rabbit (Jackson 111-165-144)	Rabbit	1:100

9.2 KLÜVER-BARRERA METHOD

KB staining protocol for FFPE tissue:

1. Deparaffinization in xylene 2x15 min
2. Rinse with 96% ethanol
3. Dip slides into 0.1% luxol fast blue solution and incubate at 57°C over night
4. Cool down at room temperature
5. Rinse with 96% ethanol
6. Rinse with deionized water
7. Dip slides into 0.1% aqueous lithium carbonate for 5 min
8. Differentiate slides with 70% ethanol until grey and white matter are distinguishable
9. Rinse with deionized water
10. Dip slides into 0.8% periodic acid for 10 min
11. Rinse with deionized water
12. Dip slides into Schiff's reagent for 20 min
13. Dip slides into sulfite washing solution for 2 min 3 times
14. Rinse with tap water for 5 min
15. Dehydrate slides through graded ethanol
16. Dip slides into n-butyl acetate
17. Mount slides using Eukitt medium