UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVI CYCLE

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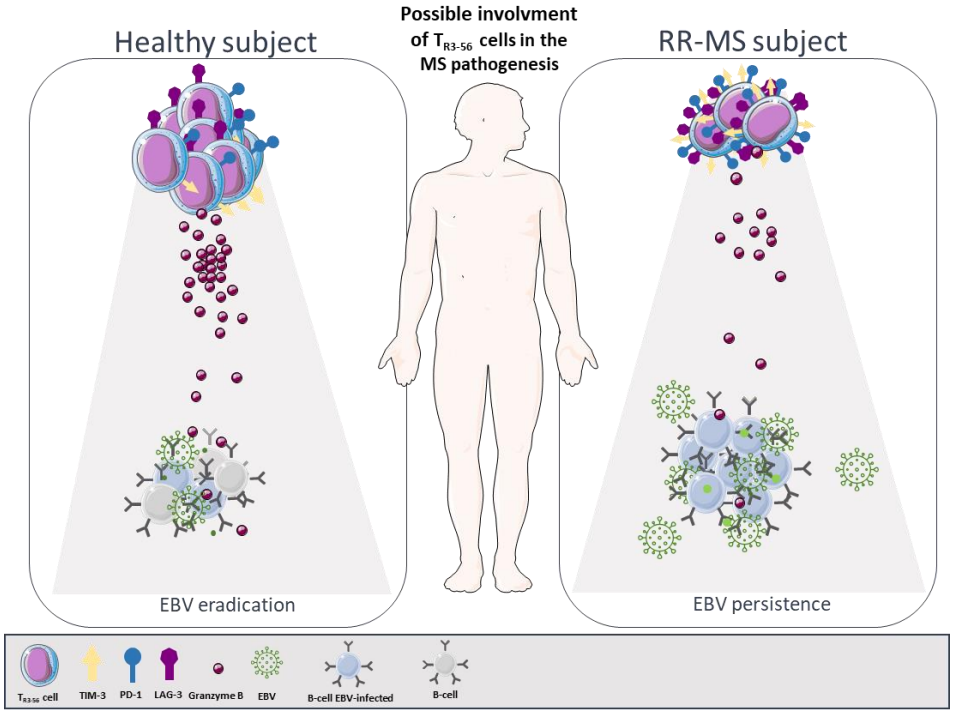

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"Human $T_{R3\text{-}56}$ cells: exploring a novel immune cell subset in Multiple Sclerosis: connection between EBV infection and disease pathogenesis."

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LIST OF ABBREVIATIONS

Abbreviation	Definition
MS	Multiple Sclerosis
CNS	Central nervous system
T1D	Type 1 diabetes
RR	Relapsing remitting
TCR	T Cell Receptor
EBV	Epstein-Barr virus
BBB	Blood-brain barrier
PP	Primary progressive
SP	Secondary progressive
RP	Relapsing progressive
CIS	Clinically isolated syndrome
RIS	Radiologically isolated syndrome
MRI	Magnetic Resonance Imaging
CSF	Cerebrospinal Fluid
EBNA 1	Epstein–Barr nuclear antigen 1
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
Th	T helper

LIST OF ABBREVIATIONS

Abbreviation	Definition
IFN-γ	Interferon γ
TNF- α	Tumor Necrosis Factor α
Interleukin	IL
Ig	Immunoglobulin
OCBs	Oligoclonal bands
EBV	Epstein-Barr virus
CTLs	Cytotoxic $CD8^+$ T lymphocytes
NK	Natural Killer
FoxP3	Forkhead Box P3
EDSS	Expanded Disability Status Scale
VOI	Volume of Interest
PBMCs	Peripheral Blood Mononuclear Cells

ABSTRACT

Multiple Sclerosis (MS) is an autoimmune demyelinating and neurodegenerative disorder, resulting from the infiltration of auto-reactive T lymphocytes into the central nervous system (CNS). The immune-pathogenetic mechanisms at the basis of loss of immunological self-tolerance in MS are not completely defined. It has become increasingly clear that several immune cell subsets have an undiscovered role in the initiation and progression of the disease. Recently published evidence from our research group has unveiled a poorly explored T cell subset known as $T_{R3\text{-}56}$ cells, which express CD56 surface molecules. We revealed that $T_{R3\text{-}56}$ cell frequency was reduced in type 1 diabetes (T1D) children and positively associated with β-cell function. Thus, we hypothesized that this T cell subset might be also involved in the occurrence of other autoimmune conditions, such as MS. To this purpose, the research activity in the PhD program was focused on the study of $T_{R3\text{-}56}$ cells in newly diagnosed treatment-naïve Relapsing-Remitting MS (RR-MS) subjects.

We found a reduction in the frequency of $T_{R3\text{-}56}$ cells in the blood of newly diagnosed, treatment-naïve RR-MS subjects, and this reduction was inversely correlated with disease severity. Additionally, $T_{R3\text{-}56}$ cells from these subjects exhibited decreased surface expression of activating/inhibitory receptors and cytotoxicity-related molecules compared to healthy controls. Next, $T_{R3\text{-}56}$ cells from RR-MS cohort displayed a significantly decreased expression of CD107a/LAMP-1 and Interferon-γ production, after T cell receptor (TCR) stimulation. Interestingly, $T_{R3\text{-}56}$ cells from RR-MS cohort showed an exhausted phenotype, evident not only from the increased expression levels of typical exhaustion surface markers but also from the reduced intracellular calcium flux following TCR stimulation. Then, we observed a reduced proliferative rate *in vitro* in $T_{R3\text{-}56}$ cells from RR-MS subjects, along with an impaired TCR signaling cascade. Finally, $T_{R3\text{-}56}$ cells from RR-MS cohort showed a significantly reduced response rate specifically to Epstein-Barr virus (EBV) peptides pool.

In conclusion, our results reveal a defect of $T_{R3\text{-}56}$ cell compartment in RR-MS individuals, characterized by an exhausted phenotype, probably due to their role in the elimination of EBV-infected cells. This study reveals an immunological cell population involved in the pathogenesis of MS, potentially representing a novel target for therapeutic interventions in the disease.

1. BACKGROUND

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune demyelinating and neurodegenerative disorder caused by auto-reactive T lymphocytes that cross the blood-brain barrier (BBB) and enter the central nervous system (CNS) where they cause local inflammation resulting in demyelination, gliotic scarring, and axonal loss (Reich 2018). Nowadays, MS is the most prevalent chronic inflammatory disease of the CNS, impacting >2 million people worldwide; it displays a higher occurrence in women, with a frequency double that of men and predominantly affects young individuals aged between 20 and 40 years (Reich 2018). Neuroinflammation and neurodegeneration are characteristic anatomopathological features of this disease, which contribute to the wide heterogeneity of symptoms and signs observed in MS subjects (Patejdl 2016). Postmortem examination of MS subject's brains reveals damage to myelin and associated axonal degeneration, as well as perivascular inflammatory infiltrates, specifically of T cells and macrophages (Morandi 2015). The diagnosis of MS primarily relies from clinical evaluation, necessitating the identification of neurological signs and symptoms after the emergence of white matter lesions. However, to discern MS from other neurological conditions, a set of criteria, including the well-known McDonald criteria, have been proposed (Milo 2014). These criteria are based on demonstrating the presence of CNS lesions that are disseminated both temporally and spatially, thereby excluding alternative disorders. The requirement for such widespread lesion dissemination is accomplished through complementary laboratory assessments and advanced imaging techniques, primarily encompassing magnetic resonance imaging (MRI) and cerebrospinal fluid analysis (Brownlee 2017).

1.2 Clinical forms of Multiple Sclerosis

Depending on the disease onset and the initial clinical course, MS can be classified into different categories (**Table 1**), including: relapsing-remitting MS (RR-MS), primary progressive MS (PP-MS), secondary progressive MS (SP-MS), relapsing progressive MS (RP-MS), clinically isolated syndrome (CIS), progressive relapsing MS (now considered under primary progressive MS with exacerbation), benign, and fulminant (Shah 2023).

Among them, RR-MS is the most common phenotype, affecting 85% of MS subjects. It is characterized by episodes of acute worsening of neurologic function, called relapses, lasting at least 24 hours, followed by remission periods with a variable degree of recovery (Lublin 1996, Brownlee 2017).
PP-MS affects 10-15% of MS subjects, and it occurs with a slowly progressive increase in neurological disability over time. Specifically, a characteristic of PP-MS is the gradual and continuous worsening of neurologic function with occasional plateaus and temporary minor improvements allowed, but without distinct relapse periods (Lublin 1996).
SP-MS represents the disease form characterized by an initial RR disease course followed by progression with or without relapses, minor remissions, and plateaus (Lublin 1996). About one-third of subjects with RR-MS onset will develop the progressive disability of SP-MS (Thompson 2018). Currently, there exist no established clinical or immunologic criteria to definitively determine the transitional point at which RR-MS transforms into SP-MS. However, when the baseline between relapses begins to progressively worsen, the disease has switched from the RR to the SP course (Lublin 1996).
Even though worsening proceeds at a similar rate in the two progressive phenotypes, PP-MS is considered a separate clinical course because of the absence of exacerbations before the clinical progression (Lublin 1996). RP-MS represents another important clinical form of MS defined by a combination of relapse and progression. Specifically, the RP phenotype presents a disease progression from onset with acute episodes of worsening (Lublin 1996).
The progressive-relapsing MS (PR-MS), instead, is a progressive disease from onset, with clear acute relapses and periods between relapses of continuous progression. It is considered a rare clinical course (Lublin 1996).

In 2013, the classical definition of MS clinical course was modified in order to take the disease progression into account (Lublin 2014). Indeed, two other disease phenotypes have been described and recognized: the CIS and the Radiologically isolated syndrome (RIS). CIS represents cases of a first clinical episode with features typical of MS. It usually affects optic nerves, the brainstem, or the spinal cord in young adults. While some individuals affected by MS recover from this episode, CIS is often the first clinical manifestation of MS (Miller 2012). For this reason, it is considered an early-stage MS, later becoming RR-MS if clinically active in the MS spectrum (Rival 2022). Indeed, CIS shows characteristics of inflammatory demyelination that could be MS but has not yet met the criteria of dissemination in time (Lublin 2014). It has been reported that CIS episodes in the presence of brain MRI lesions lead to a high risk of MS diagnosis. RIS is characterized by Magnetic Resonance Imaging (MRI)-typical brain lesions, detected in individuals

without clinical conditions suggestive of MS. It has been reported that about half of all RIS convert to MS phenotype in 10 years (Rival 2022).
In people affected by RIS form, MRI suggests inflammatory demyelination without clinical signs or symptoms, for this reason, RIS was not considered a real MS phenotype since clinical evidence of demyelinating disease is lacking and MRI results may be non-specific. RIS episodes represent the first suspicion of MS depending on the location and the morphology of the lesions. Imaging suggesting a high demyelinating course leads to a higher risk of future MS symptoms. It's reported that asymptomatic spinal cord lesions or positive cerebrospinal fluid (CSF) findings enhance the possibility of a future MS diagnosis (Rival 2022).

Table 1. MS clinical forms

Disease phenotype	Clinical manifestations	Frequency
Relapsing-remitting (RR-MS)	Acute relapses followed by partial or complete recovery (Joy 2001)	85%
Primary Progressive (PP-MS)	Disease progression starts right from disease onset without relapses (Joy 2001)	10%
Secondary Progressive (SP-MS)	Initial RR clinical course, then manifestation worsen gradually with or without acute relapses (Joy 2001)	30%
Progressive relapsing (PR)	A progressive clinical course from onset, with clear acute relapses (Joy 2001)	5%
Clinically Isolated Syndrome (CIS)	First clinical occurrence of neurological symptoms lasting at least 24 hours (Joy 2001)	

1.2.1 Disease severity evaluation

The scientific community has developed several clinical evaluation scales over time in response to the significant inter and intra-individual

variability characteristic of MS phenotypes. The "Expanded Disability Status Scale" (EDSS) is one of the most commonly used scales for assessing disability in MS subjects (Kurtzke 2008). This scale, which has evolved from the original "Disability Severity Scale" proposed by Kurtzke in 1955, evaluates eight functional systems (Piramidal, Cerebellar, Trunco-encephalic, Sensory, Sphincteric, Visual, Mental, and other neurological functions) by assigning a score of 0 (preservation of function) to 5/6 (total loss of function) (Kurtzke 1955). The final score of the scale, derived from the analysis of the scores obtained in each of the functional systems integrated with a judgment of the ability to walk independently, ranges from 0 (absence of symptomps) to 10 (death due to MS) (Kurtzke 1983). The EDSS is widely used in clinical practice due to its lack of special equipment requirements, however, this scale has several limitations (Cohen 2021). It is strongly dependent on the patient's mobility, uses subjective symptoms to evaluate certain functional systems, is insensitive to minor clinical variations, and does not provide a detailed picture of the patient's cognitive abilities or functional abilities in daily activities (Zanotto 2022). Since EDSS is unable to provide a precise picture of the patient's cognitive and functional abilities, in the last few years, neurologists have been using another diagnostic parameter, called Volume of Interest (VOI), which represents the total volume of active CNS lesions obtained through MRI. Compelling evidence from observational studies and clinical trials showed a strong association between brain volume loss and the accumulation of disability in MS (Matthews 2023).

1.2.2 Epstein-Barr viral infection and Multiple Sclerosis development

Among environmental factors related to MS occurrence, infectious diseases, particularly viral infections, seem to play a pivotal role in MS development (Christensen 2007). Scientific literature has always focused attention on viruses that lead to chronic and latent infections in humans, especially Epstein-Barr virus (EBV) infection, one of the most studied and discussed over the years (Tselis 2011). EBV is a ubiquitous human lymphotropic herpesvirus with a well-established causal role in several cancer types and compelling epidemiological evidence for a causal function also in MS (Ascherio 2007). EBV is recognized for its ability to survive within B cells throughout an individual's lifetime. During this persistent phase, the virus has the potential to intermittently reactivate, thereby enabling its transmission to new hosts. It is noteworthy that while primary infections in early childhood typically occur asymptomatically, a significant proportion — up to 75% — of individuals who contract the virus

for the first time during adolescence or adulthood may develop Infectious Mononucleosis (Balfour 2013). In the 1980s, pioneering studies unveiled a heightened prevalence and titers of antibodies directed against the EBV in individuals with MS in comparison to healthy controls (Sumaya 1980, Larsen 1985). In a 2022 investigation led by Bjornevik et al., the temporal relationship between EBV and MS was explored in 35 individuals initially negative for EBV but later diagnosed with MS. Through an analysis of repeated serum samples over the study's duration, the researchers established the EBV status of participants, revealing that all but one became infected with EBV before the onset of the initial neurological symptoms of MS (Bjornevik 2022). Additionally, a subset of participants underwent repeated measures of neurofilament light chain (NfL), a sensitive marker indicating neuroaxonal damage that can manifest years prior to the onset of MS symptoms (Khalil 2018, Bjornevik 2020, Jons 2022). The findings demonstrated that EBV infection preceded elevations in NfL, highlighting that EBV infection occurs before the manifestation of neurological symptoms and early signs of neuroaxonal damage during the preclinical phase of MS (Bjornevik 2022). Beyond its role in altering the immune system to induce CNS autoimmunity, EBV itself may contribute to the promotion of MS pathogenesis. Given its near-exclusive B cell tropism, EBV, during immune activation, generates a substantial pool of antigen-presenting B cells through EBV-mediated activation. Some of these cells may persist over time, continually stimulating autoimmune or cross-reactive T cell responses. Studies have shown that antigen-targeting B cells, particularly those transformed by EBV, including those targeting EBV-derived virus-like particles, efficiently stimulate $CD4^+$ T cells and support their expansion during primary immune responses (Leung 2013, Hong 2018, van Zyl 2018) (**Fig. 1**). Studies focused on $CD4^+$ T cells contribution to MS pathogenesis identified the HLA-DR15 allele as a key epitope source to autoreactive $CD4^+$ T cells (Chitnis 2007). Specifically, it is known that the autoreactive $CD4^+$ T clones able to cross-react with DR2a and DR2b-peptides represent a potential cause of MS (Chitnis 2007).

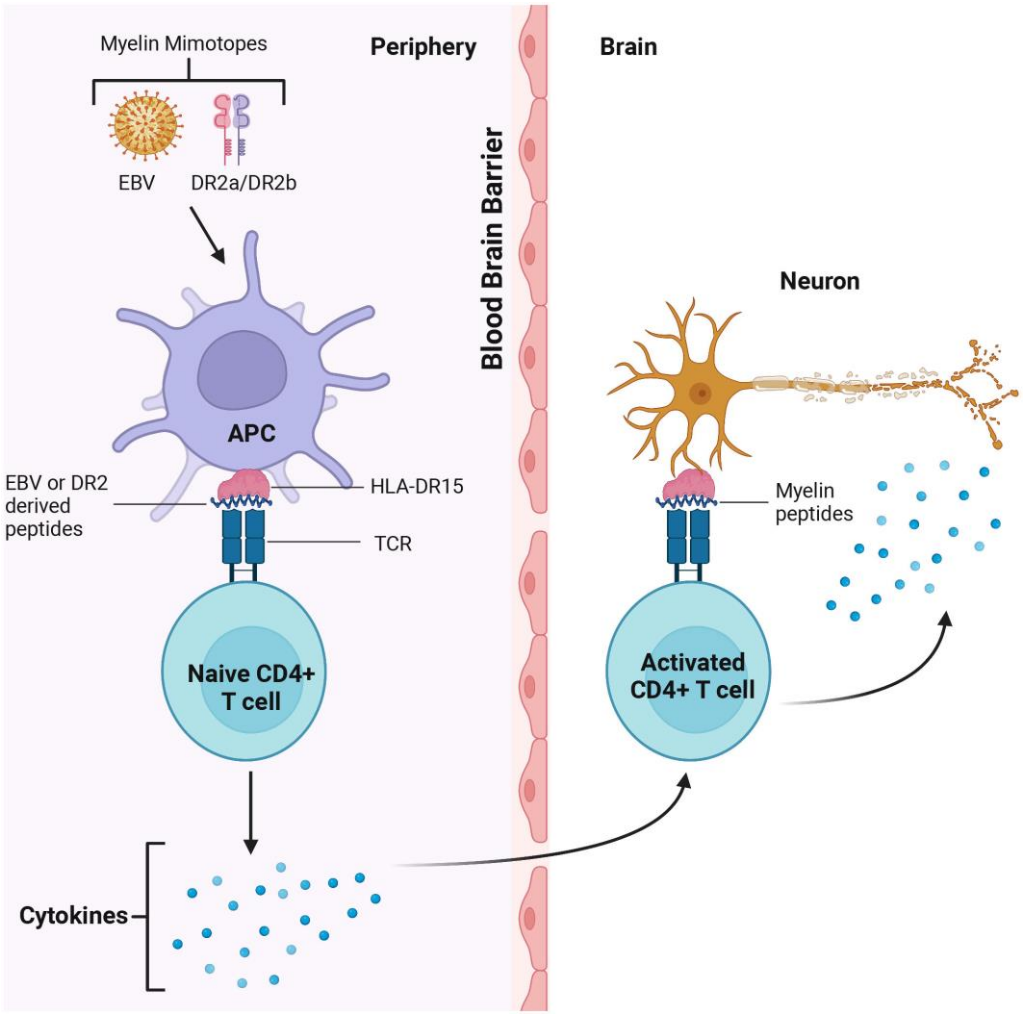

Figure 1. CD4⁺ T cells cross react with EBV in the periphery and myelin in the CNS. Myelin mimotopes can induce naïve CD4+ T cells in the periphery, by presenting EBV and DR2a/DR2b derived peptides similar to CNS self-antigens. Once in the CNS, these T cells could recognize the target antigens on microglia, leading to their activation. Activated T cells can release cytokines, inducing demyelination, axonal loss and tissue damage (Liu 2022).

One study reported a correlation between Epstein–Barr nuclear antigen 1 (EBNA1)-specific antibody titers at the onset of the initial clinical presentation of MS and measures of disease progression, such as CNS lesions and MS-associated disability (Lunemann 2010), although another study produced conflicting results (Munger 2015). Additional investigations revealed a correlation between MS disease activity, assessed through the detection of brain lesions by MRI, and levels of EBNA1-specific antibodies (Farrell 2009, Kvistad 2014). Moreover, these antibody levels were found to correlate with EBV viral loads in humanized mice (Zdimerova 2021). The elevated EBNA1-specific antibody responses observed in these studies may signify a poorly controlled

reservoir of EBV-specific B cells in individuals with MS. Evidence strongly suggests an intricate interaction between EBV and the major genetic risk factor for MS, the Major histocompatibility complex (MHC) class II molecule Human leukocyte antigen (HLA)-DRB1**1501. Carriers of HLA-DRB1**1501 were found to exhibit higher levels of EBNA1-specific antibodies compared to individuals lacking HLA-DRB1**1501* (Sundstrom 2008, Waubant 2011). Notably, on a genetic background positive for HLA-DRB1**1501*, even a slight increase in EBNA1 antibodies was associated with a significant elevation in MS risk (Sundstrom 2008). *When* HLA-DRB1**1501* and high EBNA1-specific antibody titers coexisted, the risk of MS was augmented more than 20-fold (Sundstrom 2009). These findings suggest that healthy individuals with an HLA-DRB1**1501* genetic background may exhibit heightened EBNA1-specific antibody responses, potentially indicating compromised immune control of EBV. In experiments involving EBV-infected humanized mice, immune compartments positive for HLA-DRB1**1501* demonstrated a diminished ability to control EBV viral load compared to those positive for HLA-DRB1**0401* or negative for HLA-DRB1**1501,* resulting in an increased expansion of $CD8^+$ T cells in the HLA-DRB1**1501*-positive immune compartments (Zdimerova 2021). Despite this compelling evidence, the precise mechanism through which HLA-DRB1*1501* compromises immune control over EBV remains elusive. Further research is needed to elucidate whether compromised immune control of EBV leads to the establishment of a reservoir of EBV-infected B cells possessing potent antigen-presenting cell function. The persistent stimulation of T cells by EBV may potentially trigger inflammation in the CNS, indeed EBV-infected B cells exhibit a marked preference for homing to submucosal secondary lymphoid organs, such as the tonsils within the Waldeyer ring, and to the CNS (Gasser 2007, Johnson 2019, Roschewski 2021, Zdimerova 2021). This preferential homing pattern could drive EBV-specific T cell stimulation and foster inflammation at these sites. Notably, in some studies, EBV-infected B cells were identified in meningeal tertiary lymphoid structures and brain lesions of patients with MS (Serafini 2007, Moreno 2018, Veroni 2018), although conflicting results were reported in other studies (Willis 2009, Peferoen 2010). The presence of EBV-infected B cells in these CNS-related structures raises the possibility that B cells may restimulate EBV-specific $CD8^+$ T cells at these sites, potentially contributing to inflammation in the CNS of patients with MS (Serafini 2019). Investigations have revealed an increased number of EBV-specific $CD8^+$ T cells in the CSF of MS patients, although the extent of this increase might not surpass that observed in other inflammatory neurological diseases (Erdur 2016, Lossius 2014). These findings suggest a complex interplay between EBV, B cells, and T cells within the CNS, shedding light on potential mechanisms driving inflammation in the context of MS.

1.3 Pathogenesis of Multiple Sclerosis

MS is an autoimmune, inflammatory, and neurodegenerative disorder of CNS with unclear etiology characterized by demyelination and variable degrees of axonal loss (Garg 2015).
It was described as a disseminated “plaque-like sclerosis” more than 150 years ago; indeed, MS diagnosis is based on the dissemination, in space and time, of lesions, recognized as focal areas of demyelination, inflammation, and glial reaction. It is now clear from MRI and biopsies, that the earliest stages of white matter demyelination, called “early active white matter lesions”, are heterogeneous (Reich 2018).
MS pathogenesis is caused by the auto-immune attack against CNS antigens, exerted mainly by $CD4^+$ and $CD8^+$ auto-reactive T lymphocyte subsets with the contribution of B cells and antigen presenting cells (APCs). Loss of tolerance toward myelin and other CNS antigens results in persistent peripheral activation of autoreactive T cells and consequent axonal damage (Garg 2015) (**Fig. 2**).

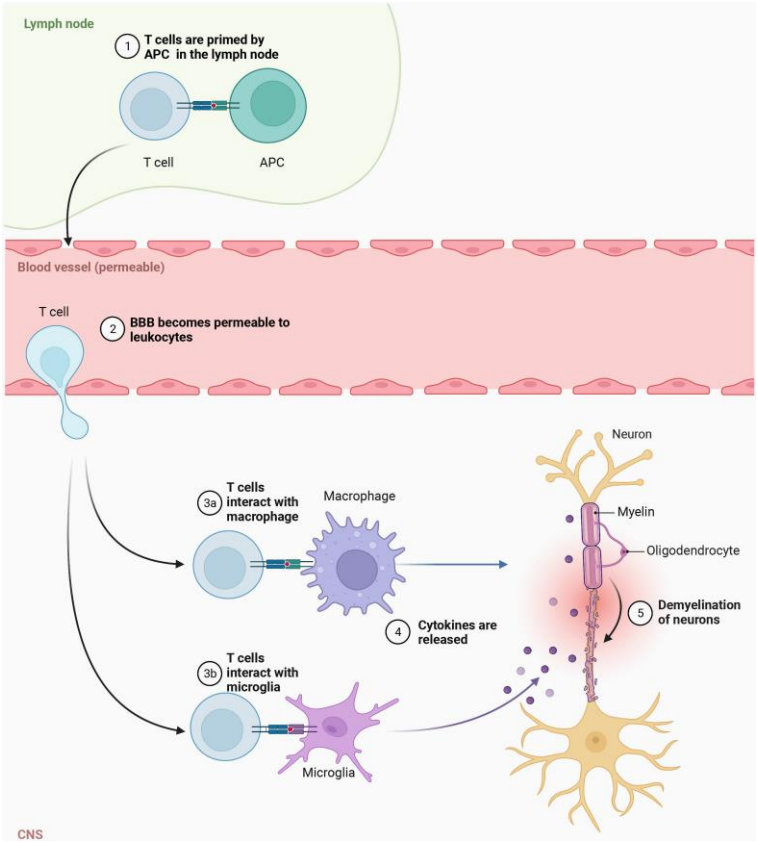

Figure 2. Autoreatcive T cells in the pathogenesis of MS. T cells are primed to CNS autoantigens by APCs in the lymph nodes, then they cross the BBB and enter the brain. Within the CNS, they activate microglia, which release pro-inflammatory cytokines leading to demyelination and tissue damage. (Liu 2022)

In recent years, there has been substantial progress in elucidating that the characteristic tissue damage in MS arises from intricate interactions involving the immune system, neurons, and various types of glial cells, including oligodendrocytes and their precursors, microglia, and astrocytes (Reich 2018). In this context, T helper type 1 (Th1) cells, Th17, $CD8^+$ T cells, and macrophages represent the main actors in generating the autoimmune attack and the pro-inflammatory environment. Usually, $CD4^+$ T cells are more present in the perivascular "cuff" of MS lesions, while $CD8^+$ T cells are broadly distributed in the parenchyma. These immune cells display the phenotype of resident memory cells and show focally restricted activation within active lesions (van Nierop 2017, Machado-Santos 2018). Insights gained from mouse model of MS have proposed that $CD4^+$ T cells play a pivotal role in driving the inflammatory processes into CNS. This notion aligns with the genetic correlation observed between MS and MHC class II haplotypes, as well as with the molecules that regulate the management of MHC Class II-restricted T-cell mediated inflammation (Lassmann 2018).

Even though myelin-reactive T lymphocytes have been found both in healthy and MS individuals, those found in MS subjects are in an activated state, displaying a Th1 and memory phenotype, while myelin-reactive T cells from healthy controls exhibit a naïve phenotype (Kaskow 2018). Thus, a fundamental difference in T lymphocytes between healthy and MS subjects seems to be an activation event. In this context, it has been widely demonstrated that the main immune cell population associated with autoimmune diseases, including MS, are the Th1 $CD4^+$ T cells, responsible for the secretion of Interferon γ (IFN- γ) and Tumor Necrosis Factor α (TNF- α), and the Th17 $CD4^+$ T cells, that secrete Interleukin (IL)-17, IL-21, and IL-22. Therefore, IFN- γ and IL-17 are believed to exacerbate immune activation by increasing both antigen presentation and proinflammatory cytokines release (Kaskow 2018).

1.3.1 B cell response

Diverging from the other chronic inflammatory diseases of CNS, B cells are one of the major components of the adaptive immune inflammation in the brain and spinal cord of MS-affected individuals (Machado-Santos 2018). B cells play a key role in acute and chronic inflammatory activity in MS (Michel 2015), through specific activated clones promoting antigen presentation, cytokine production, T cell activation, differentiation in plasma cells, and CNS invasion by immune cell subsets (Blauth 2015). Clonally expanded B and plasma cells in the CNS produce clonal Immunoglobulin G (IgGs), leading to the CSF-restricted oligoclonal bands (OCBs), one of the main hallmarks of MS, used at first as a biological marker for the MS diagnosis and to predict a

CIS conversion to clinically definite MS (Dobson 2013). OCBs derive from the intrathecal synthesis of clonal IgG and are reported in the CSF of over 90% of MS subjects (Freedman 2005). This intrathecal immunoglobulin synthesis is thought to be sustained by long-lived plasma cells, mustered or differentiating in the CNS (Prineas 1978). Several studies, over the years, showed various pathological implications of B cells in the MS context, such as the presence of anti-myelin antibodies inside phagocytic cells in MS active lesions (Genain 1999), the characteristic deposition of antibodies and complement proteins in most common demyelinating lesions (Lucchinetti 2000), and findings of antigen-experienced B-cell clones in meningeal aggregates (Lovato 2011, Magliozzi 2007, Lucchinetti 2011). Furthermore, molecular analyses focused on Ig variable region of B cells and plasma cells found in active parenchymal lesions, CSF or meninges of MS subjects revealed the presence of specific antigen-driven clonotypes in these different CNS regions (Lovato 2011, von Budingen 2012). Indeed, recent experimental evidence indicates that antibodies, generated from clonally expanded plasma cells found in CSF of MS subjects, are capable of binding human and mouse CNS tissue, and inducing complement-mediated demyelination in spinal cord explants (Blauth 2015). Even though plenty of studies are focused on clonally expanded B cells and aberrant antibodies in the CNS of MS subjects, the specific antigens recognized by these antibodies are still not clear. Recently, different targets have been suggested such as glycolipids, axoglial proteins and viruses (Brennan 2011, Ho 2012, Kanter 2006), yet it seems more clear that antibodies from CSF-expanded B cell clones of MS subjects are preferentially directed against neurons and astrocytes (Brennan 2011, Ho 2012, Kanter 2006, Ligocki 2015, Mathey 2007, Michel 2015). However, the efficacy of anti-CD20 monoclonal antibodies in limiting new relapsing MS activity has highlighted the pivotal role and involvement of B cells in the complex immune response underlying CNS inflammation in MS disease (Bar-Or 2008, Kappos 2011, Sorensen 2014). Among multiple functions recognized and addressed to B cells in MS pathogenesis, in addition to their known differentiation in antibody-secreting cells, they can also be highly efficient antigen-presenting cells to T lymphocytes (Pollinger 2009), thus presenting neuro-antigens recognized with their surface B cell receptor (Harp 2008, Harp 2010). Moreover, several studies demonstrated how activated B cells modulate the local inflammatory response of T lymphocytes and myeloid cells through secretion of both pro- and anti-inflammatory cytokines (Li 2015). Specifically, TNF-α, IL-6, Granulocyte-macrophage colony-stimulating factor and Lymphotoxin-α producing B lymphocytes support pro-inflammatory functions of other target cells (Duddy 2007, Rauch 2012, Dang 2014, Barr 2012, Li 2015), while IL-10 and IL-35 secreting B cells exert anti-inflammatory and regulatory roles (Shen 2014, Wolf 1996, Fillatreau 2002). However in MS context, B cells seem

to be polarized toward an abnormal pro-inflammatory signature (Li 2015), yet only some authors report a deficiency in their regulatory functions (Michel 2014).

1.3.2 Th1 cell response

IFN-γ production is the main signature cytokine of Th1 cells and it is responsible for the increased expression of MHC molecules by cells resident in the CNS (Ottum 2015) and also for the altered activation and viability of all the CNS occupant cells. IFN-γ receptors are ubiquitously expressed, thus all CNS cells are potentially responsive to this cytokine (de Weerd 2012). In this context, IFN-γ levels have been associated with the frequency of active CNS lesions in MS subjects (Olsson 1992) and specific genetic polymorphisms of IFN-γ have been associated with susceptibility to MS occurrence (Goris 2002, Kantarci 2005) . In 1997, Dettke et al. found that IFN- γ production by patient-derived T cells was increased before exacerbation of disease activity (Dettke 1997). While the overexpression of IFN-γ in the CNS leads to oligodendrocyte apoptosis and inhibition of myelination, low levels of this cytokine exert a protective effect against oxidative stress and enhance the proteasome activity of oligodendrocytes themselves (Balabanov 2007). Interestingly, IFN-γ is able not only to activate microglia to become phagocytic and present antigens, but it can also directly kill oligodendrocytes (Aloisi 2000), thus leading to a direct loss of neuronal myelination, typically observed in the CNS of MS subjects (Popko 1999). IFN-γ is also responsible of an enhanced expression of MHC molecules, and consequently of an increased antigen-presenting ability of myeloid cells to re-activate myelin-antigen-specific T cells, both $CD4^+$ and $CD8^+$ (Kaskow 2018). Indeed, APCs can activate myelin-reactive $CD8^+$ T cells using a process of antigen cross presentation to express exogenous myelin on MHC class I molecules (Kaskow 2018, Joffre 2012).

1.3.3 Th17 cell response

Th17 cell subset was discovered in 2005 and since then it has become quite evident that these cells do not only contribute to host defense against pathogens, but they also play a pivotal role in the pathogenesis of several autoimmune diseases (Stadhouders 2018). Th17 cells are typically considered the principal source of IL-17, a family of six structurally related cytokines, able to upregulate inflammatory gene expression either by inducing *de novo* gene transcription or by stabilizing mRNA transcripts. Specifically, in physiological contexts, IL-17 is a fundamental mediator for defense against bacteria and fungi (O'Quinn

2008) through a receptor ubiquitously expressed (Yao 1997). Elain G. et al in 2014 demonstrated that human astrocytes express 2 specific IL-17 receptor subtypes, IL17RA and IL17RC, at significant levels and that IL-17 is responsible for an increase in IL-6 levels in human astrocytes (Elain 2014). The release of cytokines from astrocytes is strictly related to the wave of immune cell infiltration into the CNS, which propagates and exacerbates the inflammatory milieu (Elain 2014). Furthermore, the direct communications with the endothelial cells allow astrocytes to control the BBB functions (Elain 2014).

Even though the response to IL-17 is dependent on target cell type, several previous studies showed that this cytokine promotes immune pathology, cooperating with other immune mediators and increasing the secretion of other pro-inflammatory cytokines (IL-6, granulocyte colony-stimulating factor and TNF-α), effector proteins (complement) and chemokines (CXCL8, CXCL2 and CCL20) (Onishi 2010). Through this complex interplay, IL17 is responsible not only for Th17 cell maintenance but also for microglia activation and the recruitment of neutrophils, macrophages and other T cell types . Interestingly, in 2008 Tzartos et al. found an increased number of IL-17-producing glial cells, $CD8^+$ and $CD4^+$ T cells in active MS brain lesions, compared to inactive brain regions (Tzartos 2008). Several studies showed not only elevated frequencies of IL-17-producing cells and IL-17 mRNA in patient-derived peripheral blood but also a positive correlation with disease activity (Durelli 2009). It has been widely demonstrated that human Th17 cells migrate across models of BBB more efficiently than Th1 cells, exerting, moreover, neurotoxic effects (Kebir 2007). Th17 cells, indeed, also secrete IL-21 and IL-22. The first influences lymphocytes infiltrating acute and chronic active white matter MS lesions, regulating both immune cell activation and survival (Tzartos 2008); the second one, in high levels, leads to BBB disruption, and has been detected in cerebrospinal fluid (CSF) and peripheral blood of MS subjects (Kebir 2007). Of note, IL-17A and IL-22 double expressing Th17 cells can also express granzyme B, a typical cytolytic enzyme (Kebir 2007), able to kill neurons through its extracellular release and consequent binding to the glutamate receptor, GluR3 (Ganor 2007).

1.3.4 $CD8^+$ T cell response

In physiological conditions, $CD8^+$ T cells are responsible for an important antiviral immune surveillance in the CNS (Salinas 2010), killing target cells by introducing cytolytic enzymes into the cytosol through a directional release of granules containing granzymes and perforin (Bevan 2004). In addition to their cytotoxic functions, different subsets of $CD8^+$ T

cells can also secrete specific cytokines like IFN-γ (Kish 2009). Studies on the pathogenic mechanisms at the basis of MS development have been focused mainly on $CD4^+$ T cells, due to the lack of CNS tissue samples from MS subjects with acute lesions (Johnson 2007). In one of the first studies focused on the role of $CD8^+$ T cells, the authors found that $CD8^+$ T cells outnumber the $CD4^+$ T cell subset in all MS subjects, regardless of the disease phenotype, duration and speed of disease progression (Booss 1983). Subsequently, analyzing brains from deceased progressive and acute MS subjects, Hauser et al. found that perivascular cuffs at the edges of active demyelinating plaques showed up to 50 times more $CD8^+$ than $CD4^+$ T cells (Hauser 1986). Even though nowadays is pretty clear the clonal expansion of $CD8^+$ T cells in MS subjects (Monteiro 1995) and their lesions (Babbe 2000), the specific epitopes recognized by these CD8+ T lymphocytes are still unknown. The abundance and preponderance of cytotoxic $CD8^+$ T lymphocytes (CTLs), together with their clonal expansion within inflammatory CNS lesions, clearly suggest their pivotal role in the pathogenesis of MS, indeed CTLs have been found close to demyelinating axons in MS brain tissue. Within parenchymal lesions, CTLs can be visualized with their cytolytic granules polarized toward injured axons, indicating a direct cytotoxic attack (Neumann 2002). Also in the CSF of MS subjects, there is an increase of activated effector or effector memory $CD8^+$ T cells ($CD45RA^{-/+}$, $CCR7^-$) (Jilek 2007), that lacking the C-C chemokine receptor type 7, are able to migrate into inflamed tissue and exert their effector functions (Debes 2005). Furthermore, it has been widely demonstrated that $CD8^+$ T cells show oligoclonal expansion in CSF, peripheral blood and CNS lesions of MS subjects (Babbe 2000, Jacobson 2002, Junker 2007, Friese 2009), indicating a clear antigen-driven activation of $CD8^+$ T cells in MS. The pro-inflammatory milieu found in the CNS of MS subjects increases $CD8^+$ T cell cytolytic function, supported also by MHC class I mediated antigen presentation. Indeed, under inflammatory conditions most CNS resident cells express MHC class I molecules, becoming, thus, potential targets for $CD8^+$ T cells, and it has been widely demonstrated how in MS early stages, before the demyelination phase, there is an enhanced up-regulation of MHC class I molecules (Ransohoff 1991, Gobin 2001). As concerns $CD8^+$ T cell subset in MS, several studies showed an association with cytokine-expressing $CD8^+$ T cells. Tzartos et al. found an increase in $CD8^+$ T cells expressing IL-17 specifically in active MS lesions compared to inactive CNS regions (Tzartos 2008).

Nevertheless, myelin-reactive T lymphocytes have been observed also in subjects not affected by MS, suggesting that these cells may be dysfunctional in MS patients or that other immune factors need to be involved (Reich 2018, Lassmann 2018).

1.4 $T_{R3\text{-}56}$ cells, a subset of CD56$^+$ T cells

CD56, also known as neural cell adhesion molecule (NCAM), is prominently present on the surface of most Natural Killer (NK) cells and on a heterogenous subset of CD3$^+$ T cells, constituting approximately 1 to 11% of peripheral blood lymphocytes (Van Acker 2017, Romero-Olmedo 2021). The nomenclature for CD56$^+$ T cells can be a subject of controversy; they are frequently designated as NK-like T cells or CD3$^+$CD56$^+$ NKT-like cells (Van Acker 2017). CD56$^+$ T cells may express additional NK cell markers and include TCRγδ$^+$ T cells, as well as CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ double-negative (DN) T cells expressing diverse or invariant (iNKT, CD1d-restricted) αβ chains of the TCR (Van Acker 2017). Also, within the TCRVα7.2$^+$CD161$^+$ mucosal-associated invariant T (MAIT) cell population, a CD56$^+$ subset has been identified, with heightened responsiveness to cytokine stimuli (Dias 2017).

CD56 expression on αβ T cells is associated with potent effector function in the human intestine, liver, and peripheral blood (Cohavy 2007, Correia 2011, Guia 2008). More specifically, CD56 surface expression on T cells correlates with the expression of CD16, NKG2A/D, NKp44/46, CD122, and DNAM-1, high intracytoplasmic perforin and granzyme B content, and cytotoxic functions (Van Acker 2017). Moreover, CD56$^+$ T cells are able to exert NK cell-like killing activity in a pro-inflammatory milieu (Correia 2011, Chan 2013). Historically, a CD3$^+$CD56$^+$ cell population exhibiting a CD3-dependent lytic activity and with T cell-like phenotype was first described by Schmidt-Wolf and co-workers in the peripheral blood of healthy subjects (Schmidt-Wolf 1993). Other observational studies found a positive correlation between circulating CD3$^+$CD56$^+$ cell number and immune tolerance towards the embryo in *in vitro* fertilization (IVF) techniques (Zhou 2013) and higher residual β-cell function in subjects with T1D (Galgani 2013), thus suggesting a potential involvement of this cell population in immune tolerance. In metastatic melanoma, a distinct population of CD56$^+$T cells increased in patients responding to anti-PD-1 therapy (Ribas 2016). In line with this, immune-radiotherapy increased the frequency of circulating CD8$^+$CD56$^+$ cells in advanced cancer patients (Hasumi 2013). However, not all CD56$^+$ T cell exhibits cytotoxic capacity. In the tumour tissue of hepatocellular carcinoma, subjects have been found a CD56$^+$ T cell subset expressing transcriptional factor Forkhead Box P3 (FoxP3) with immunosuppressive functions (Li 2015), which inversely correlated with patient survival (Li 2015). In human glioblastoma, a considerable proportion of tumour infiltrated lymphocytes were CD56$^+$ T cells, with a low frequency in the peripheral blood (Waziri 2008). Regarding autoimmune diseases, subjects affected with systemic sclerosis have decreased numbers of circulating CD56$^+$ T cells compared to healthy individuals (Almeida

2015). The low frequency of $CD56^+$ T cells was observed also in other autoimmune disease, such as psoriasis (Koreck 2002).
Recently published evidence from our research group revealed a $CD56^+$ T cell subset, defined as $T_{R3\text{-}56}$ cells, which was distinct from the iNKT cells and with a heterogeneous TCRβ repertoire. These cells were able to control $CD8^+$ T cell response in human healthy subjects, while are defective in autoimmune diabetes (Terrazzano et al. 2020). Specifically, we found reduced $T_{R3\text{-}56}$ cells in the peripheral blood of T1D children, compared with matched healthy controls, which positively correlated with pancreatic β-cell functions, in terms of fasting C-peptide levels (Terrazzano et al. 2020). Also, we found that $T_{R3\text{-}56}$ cells inhibited the proliferation of both $CD8^+$ and $CD4^+$ T cells, with the main suppressive effect on $CD8^+$ cell subset (Terrazzano et al. 2020). Phenotypical analysis showed that $T_{R3\text{-}56}$ cells from recent-onset T1D children showed that $T_{R3\text{-}56}$ cells from T1D children had reduced surface expression of the activating/inhibitor receptors NKG2A, NKG2D, DNAM-1, and also cytotoxicity-related intracellular enzymes, such as granzyme B, compared to healthy children. On the other hand, T1D $T_{R3\text{-}56}$ cells expressed increased surface expression levels of T cells typical chemokine receptors like CXCR3, CXCR4 and CCR7, responsible for T cell recruitment in the pancreas (Terrazzano et al. 2020).

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