Université de Montréal

Role of MCAM^{+} Regulatory T cells in Multiple Sclerosis

par

Jennifer Sebali

Département de Neurosciences

Faculté de Médecine

Mémoire présenté en vue de l'obtention du grade de Maîtrise ès sciences (M. Sc.) en Neurosciences

Juillet, 2020

Université de Montréal

Département de Neurosciences, Faculté de Médecine

Ce mémoire intitulé

Role of MCAM^{+} Regulatory T cells in Multiple Sclerosis

Présenté par

Jennifer Sebali

A été évalué par un jury composé des personnes suivantes

Nicolas Chomont
Président-rapporteur

Alexandre Prat
Directeur de recherche

Samuel David
Membre du jury

Résumé

Chez les patients atteints de la sclérose en plaques (SEP), les lymphocytes T autoréactifs utilisent des molécules d'adhérence (CAM) pour traverser la barrière hémo-encéphalique (BHE), pénétrer dans le système nerveux central (SNC) et médier la détérioration de la myéline. Les lymphocytes T régulateurs (Treg) constituent l'un des éléments clés de la tolérance immunitaire, protégeant contre les réactions auto-immunes. Cependant, l'entrée et la fonction des Treg dans le SNC restent largement inconnues. Notre laboratoire a démontré la contribution de plusieurs CAM, dont la molécule *melanoma cell adhesion molecule* (MCAM), dans la migration des lymphocytes pathogéniques à travers la BHE. L'objectif de cette étude est de déterminer si les Treg migrent dans le SNC en utilisant MCAM et s'ils exercent des fonctions anti-inflammatoires qui pourraient atténuer l'inflammation du SNC.

L'expression de MCAM, des marqueurs fonctionnels de Treg (CTLA-4, CCR6, CCR5), ainsi que leur sécrétion de cytokines (IL-10, GrzmB, TGF-ß, IFN-γ, TNF α, GM-CSF, IL-17a), ont été étudiées sur des Treg du sang périphérique, du liquide céphalo-rachidien (LCR) et de la culture *in vitro*, provenant de patients atteints de SEP et d'individus sains (HC), par cytométrie de flux, en corroboration avec qPCR et ELISA. De plus, la présence de $MCAM^+$ Treg dans le SNC a été évaluée par immunohistofluorescence (CD4, CD25, Foxp3, MCAM, noyaux) sur des souris atteintes d'encéphalomyélite auto-immune expérimentale (EAE).

Nos données ont montré une augmentation de l'expression de MCAM sur les Treg de patients atteints de la forme cyclique de SEP (RRMS) par rapport aux HC. Nous avons observé une tendance vers une fréquence plus élevée de $MCAM^+$ Treg dans le LCR par rapport au sang périphérique des patients atteints de SEP, ce qui suggère que MCAM pourrait jouer un rôle important dans la migration des Treg. Ces cellules $MCAM^+$ Treg semblent avoir un phénotype plus fonctionnel et anti-inflammatoire que leurs contreparties $MCAM^-$. De plus, nous avons trouvé des niveaux plus élevés de $MCAM^+$ Treg dans les périodes de rémission de l'EAE, ce qui souligne leur implication durant cette phase de la maladie. Dans l'ensemble, nos données montrent que MCAM est une CAM essentielle pour la migration des Treg vers le SNC.

Mots-clés : Sclérose en Plaques, Système Nerveux Central, Inflammation, Cellules T Régulatrices, MCAM, Barrière Hémo-encéphalique, Migration, Cytométrie en Flux, Imagerie, Encéphalomyélite Auto-immune Expérimentale.

Abstract

In multiple sclerosis (MS), autoreactive T cells upregulate cellular adhesion molecules (CAMs) to cross the blood brain barrier (BBB), enter the central nervous system (CNS) and mediate damage to myelin. Regulatory T cells (Treg) are one of the key components of immune tolerance, protecting against autoimmune reactions. However, Treg's entry and function in the CNS remains largely unknown. Our lab has demonstrated the contribution of several CAMs, including melanoma cell adhesion molecule (MCAM), in the migration of pathogenic lymphocytes across the BBB. The goal of this study is to determine whether Treg migrate into the inflamed CNS using MCAM and exert anti-inflammatory functions, possibly dampening CNS inflammation.

The expression of MCAM and Treg functional markers and chemokine receptors (CTLA-4, CCR6, CCR5,), as well as cytokine secretion (IL-10, GrzmB, TGF-ß, IFN-γ, TNF α, GM-CSF, IL-17a), were studied on MS patients and healthy individuals (HC) Treg from the peripheral blood, cerebrospinal fluid (CSF), and *in vitro* culture, by flow cytometry, in corroboration with qPCR and ELISA. Moreover, the presence of $MCAM^+$ Treg in the CNS was assessed by immunohistofluorescence (CD4, CD25, Foxp3, MCAM, nuclei) on experimental autoimmune encephalomyelitis (EAE) affected mice.

Our data showed an increase in the expression of MCAM on Treg during relapse-remitting MS patients (RRMS) compared to HC. We observed a trend for a higher frequency of $MCAM^+$ Treg cells in the CSF versus the peripheral blood of MS patients, suggesting that MCAM might play an important role in the migration of Treg. These $MCAM^+$ Treg seem to have a more functional and anti-inflammatory phenotype than their $MCAM^-$ counterparts. Moreover, we found higher levels of $MCAM^+$ Treg in periods of EAE remission, underlining their involvement during this disease phase. Overall, our data depicts MCAM as an essential CAM for Treg homing to the CNS.

Keywords: Multiple Sclerosis, Central Nervous System, Inflammation, Regulatory T cells, MCAM, Blood Brain Barrier, Migration, Flow cytometry, Imaging, Experimental Autoimmune Encephalomyelitis .

Table des matières

Liste des tableaux

Liste des figures

Liste des sigles et abréviations

1a,25$(OH)_2$D3: 1,25-dihydroxyvitamin D3

ac: Activation

AF: Alexafluor

AJ: Adherens Junctions

ALCAM: Activated Leukocyte Cell Adhesion Molecule

ANOVA: One-Way Analysis of Variance

APCs: Antigen Presenting Cells

ATP: Adenosine Triphosphate

ATRA: All Trans Retinoic Acid

BBB: Blood Brain Barrier

BBB-EC: Blood Brain Barrier-Endothelial Cells

bFGF: Basic Fibroblast Growth Factor

BMs: Basement Membranes

BMB: Blood Meningeal Barrier

Breg: B Regulatory Cells

cAMP: Cyclic Adenosine Monophosphate

CAMs: Cell Adhesion Molecules

CIS: Clinically Isolated Syndrome

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

CTLA-4: Cytotoxic T Lymphocyte Associated Molecule-4

d3Tx: Day 3 Of Mice Life

DAPI: Diamidino-2-Phenylindole

DCs: Dendritic Cells

DMTs: Disease Modifying Therapies

EAE: Experimental Autoimmune Encephalomyelitis

EBi3: Epstein-Barr Virus-Induced Gene 3

EBV: Epstein-Barr Virus

EC: Endothelial Cells

ECM: Endothelial Cell Culture Media

EM: Extracellular Matrix

EDSS: Expanded Disability Status Scale

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

FACS: Fluorescence-Activated Cell Sorting

FasL/Fas: Fas Cell Surface Death Receptor

FBS: Fetal Bovine Serum

FLAIR: Fluid-Attenuated Inversion Recovery

Foxp3: Forkhead Box Protein 3

GDNF: Glial-Derived Neurotrophic Factor

GFAP: Glial Fibrillary Acidic Protein

GM-CSF: Granulocyte–Macrophage Colony-Stimulating Factor

GrzmB: Granzyme B

Genome-wide association studies (GWAS)

HBEC: Human Brain Endothelial Cells

HC: Healthy Controls

HLA: Human Leukocyte Antigen

HRP: Horseradish Peroxidase

IBD: Inflammatory Bowel Disease

ICAM-1: Intercellular Adhesion Molecule

IDO: Indoleamine 2,3-Dioxygenase

IF: Immunofluorescence

IFN-γ: interferon γ

Ig: Immunoglobulin

IL: Interleukin

IPEX: Immunodysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome

iTreg: *In Vitro*–Induced Treg

JAMs: Junctional Adhesion Molecules

LAG-3: Lymphocyte-Activation Gene 3

LFA-1: Leucocyte Function-Associated Antigen

LHE: Luxol Fast Blue and Haematoxylin and Eosin

MAG: Myelin Associated Glycoprotein

MAGNIUMS: Magnetic Resonance Imaging in Multiple Sclerosis

MBP: Myelin Basic Protein

MCAM: Melanoma Cell Adhesion Molecule

MCAM-l: MCAM-Long Isoform

MCAM-s: MCAM-Short Isoform

MHC: Major Histocompatibility Complex

MMPs: Matrix Metalloproteinases

MOG: Myelin Oligodendrocyte Glycoprotein

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

MAdCAM-1: Mucosal Addressin Cell Adhesion Molecule-1

NVU: Neurovascular Unit

OCBs: Oligoclonal Bands

OCT: Optimal Cutting Temperature Compound

OPCs: Oligodendrocyte Progenitor Cells

PB: Pacific Blue

PBS: Phosphate Buffer Solution

PBMCs: Peripheral Blood Mononuclear Cells

PC: Pericytes

PECAM: Platelet EC Adhesion Molecules

PLP: Proteolipid Protein

PMA: Phorbol 12-Myristate 13-Acetate

PNS: Peripheral Nervous System

pTreg: Peripherally *In Vivo*–Induced Treg

RA: Retinoic Acid

RD: Reagent Diluent

rpm: Rotation Per Minute

RRMS: Relapsing-Remitting MS

SA: Streptavidin

SEM: Standard Error of the Mean

SPMS: Secondary Progressive MS

Tc: T Cells

TCR: T Cell Receptor

TCR^{1640}: T Cell Receptor Transgenic Mice

Teff: T effector Cells

TGF-ß: Transforming Growth Factor beta

Th: T Helper cells

TJ: Tight Junctions

TNF: Tumor Necrosis Factor

Tr1: T Regulatory Type 1 Cells

TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Treg: Regulatory T Cells

tTreg: Thymus-Derived Treg

UV: Ultraviolet

VCAM-1: Vascular Cell Adhesion Molecule

VE: Vascular-Endothelial

VLA-4: Very Late Activation Antigen 4

ZO: Zona Occludens

To my family, thank you for your love and support every step of the way.

Remerciements

I would like to extend my deepest, most sincere gratitude for my supervisor, Dr. Alexandre Prat, for giving me the opportunity to work on this research project in the highly enriching environment that is the CRCHUM. Dr. Prat is unmatched in his phenomenal knowledge, passion, dedication, and generosity. He is a tremendous source of inspiration to me. I would like to thank Dr. Stephanie Zandee for guiding me throughout my project. I would also like to thank members of my advisory committee, Dr. Catherine Larochelle and Dr. Nicolas Chomont, for their help and advice along the way. I am thankful for all the technical assistance and friendship of former and current Prat lab members: Lyne, Sandra, Camille, Roxaneh, Elizabeth, Karine, Evelyn, Paula, Rose-Marie, Chloe, Olivier, Marc, and Antoine.

My work could not have been possible without the support and care of my loving family members, Nabil, Wafa, Jason, Jeremy, and Caren. To my dear friends, Sarah, Sara and Roxaneh, thank you for all your encouragement. Family and friends near and far, your constant support has meant the world to me!

Chapter 1 – Introduction

1. Multiple Sclerosis

The understanding of multiple sclerosis (MS) has rapidly evolved over the last 100 years. Following discoveries made in the early 1800's, MS was recognized as a distinct disease called *sclérose en plaques disséminée* by French neurologist Dr. Charcot around 1868. Since then, our knowledge in terms of epidemiological features, clinical presentations, neuropathological characteristics, and treatment approaches have greatly deepened (1). However, to date the cause of MS remains unknown and the cure unattained.

1.1 Clinical Aspects

MS is a chronic idiopathic neuro-inflammatory and neurodegenerative disorder of the central nervous system (CNS) characterized by multifocal lesions disseminated in time and space. These lesions are formed through the infiltration of immune cells to the CNS, leading to demyelination, inflammation, axonal degeneration, and gliosis in the white and grey matter (2). The loss of myelin sheath along axonal projections, alters the neuronal signal conduction, causing heterogeneity in the presentation and progression of the disease.

Prevalence

MS has an estimated worldwide prevalence of 2.5 million cases, with an increasing prevalence gradient in regions more distant from the equator (3). With around 100,000 patients affected, Canada holds one of the highest rates of MS in the world (4). The average age of disease onset is around 30 years old, with a globally increased female to male ratio (3:1). However, it is worth noting that there are also cases of pediatric MS onset (2-10% of cases) and late MS onset, (over the age of 50, 4-10% of cases) (5, 6). Being the most common chronic non-traumatic neurologic disease in young adults, MS poses a heavy burden on the day to day lives of affected individuals, as well as their support system, and the health care system (7).

MS subtypes

The disparity in the disease course is revealed by the different MS subtypes (Figure 1). Some people can have symptoms suggestive of a first episode of neurologic symptoms often referred to as clinically isolated syndrome (CIS); while others may be asymptomatic individuals and yet demonstrate typical MS lesions on their CNS-magnetic resonance imaging (MRI) (8). More than 85% of CIS patients progress to having the relapsing-remitting (RRMS) form of the disease (9). RRMS is the most common form of MS (80-90%) and is characterized by the appearance of neurological symptoms that can last from 24 hours to several days or months (relapse), followed by a period of partial or complete recovery (remission). An incomplete recovery is associated with a poorer prognosis on the long term (10, 11) . The relapsing-remitting cycle may repeat itself numerous times, at an unpredictable relapse frequency and severity. With time, 80% of RRMS patients will experience a gradual increase in clinical deterioration that coincides with a reduction of cerebral volume, known as secondary progressive MS (SPMS). In contrast, 10-20% of patients face a steady decline from disease onset and are diagnosed with primary progressive MS (PPMS) (7).

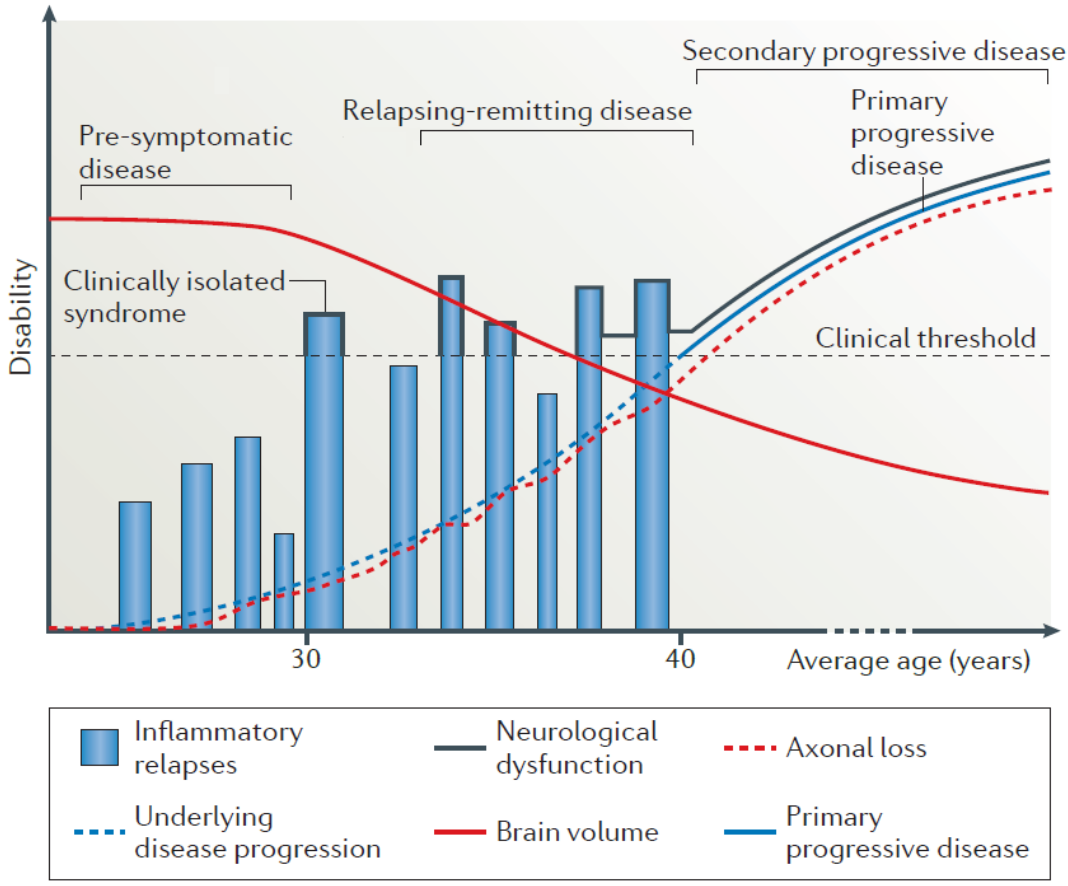


Figure 1. – Graphical representation of the disability progression of the different MS subtypes over time (clinically isolated syndrome, relapse-remitting, secondary progressive, primary progressive).

Neurological dysfunction is determined by a clinical threshold and is correlated with inflammation, axonal loss, and brain volume. Image extracted and adapted from Dendrou, Fugger, and Friese, 2015 (7).

Clinical manifestations

An MS attack can affect any part of the CNS, but sites that have a preference for demyelination include the optic nerves, the spinal cord, the brainstem, the cerebellum, and the white matter of the cerebral hemispheres (12). Thus, clinical manifestations vary from sensory and visual disturbances, to motor and cognitive impairments. Still, some presentations are more common and typical than others. Unilateral optic neuritis (loss of monocular vision; reduction in visual acuity; red desaturation) is the first presenting symptom in 20% of patients, and features in up to 50% of MS patients (13). However, the most common CIS symptom is asymmetric transverse myelitis, a spinal cord syndrome responsible for the loss of sensation and movement

below the cord lesion, including urinary disorders. When the lesion involves the cervical cord, Lhermitte's symptoms may occur (neck flexion causes electrical shooting sensation). Moreover, the distribution of sensory and motor impairments can help pinpoint the location of the lesion (14, 15). In more progressive forms of MS, spinal injuries with gait disorders become dominant such as chronic progressive myelopathy, and asymmetric paraparesis (16, 17). Another site that can be affected is the brainstem. Brainstem syndromes represent 25% of CIS cases and include diplopia (specifically, internuclear ophthalmoplegia - horizontal eye movement impairment), isolated cranial nerve deficits (trigeminal neuralgia - pain from nerve V; Bell's palsy - lower motor neuron lesion to nerve VII), and vertigo (2, 14). Impairments in the cerebellum such as lack of coordination, cerebellar ataxia, gaze-evoked nystagmus, dysarthria, and tremors, may occur at acute (10% of cases) and progressive (80% cases) phases of the disease. However, the more cerebellar symptoms are apparent at disease onset, the worse the prognosis (18, 19). Finally, patients experience extreme fatigue, depression, emotional changes, and cognitive impairments which are considered to be the most debilitating symptoms of MS (20, 21).

Diagnosis

Up until 2001, the diagnosis of MS was made based on the recognition of clinical signs, and the exclusion of other resembling diseases. With the development of the MRI and the desire to intervene and treat patients at early stages, the diagnosis of MS has known continuous progress leading to the establishment of specific diagnostic criteria. The criteria of McDonald in 2001, and of the Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIUMS) in 2007, have always shared the common principle of lesion dissemination in time and space (at least 2 lesions occurring at different times, and affecting different parts of the CNS)(22-24). MRI sequences revealed the presence of demyelinating lesions that are typical of MS in terms of their morphology (oval hyperintense appearance), distribution (periventricular, juxtacortical, brainstem, spinal cord, corpus callosum), and signal abnormalities on T2-weighted, T2-weighted fluid-attenuated inversion recovery (FLAIR) scans, T1-weighted, and gadolinium-enhanced scans. Moreover, the use of contrast agents such as gadolinium allows the detection of BBB leakage, which is indicative of an active lesion (22, 25, 26). Thus, attesting to the valuable role of the MRI in bridging the gap between visible symptoms and silent lesions. It is important to note that the

MRI does not make the diagnosis alone but is an aid to the clinical diagnosis. Other paraclinical tests including cerebrospinal fluid (CSF) examination, ocular coherence tomography, evoked potentials recordings, and urodynamic studies may be used to support a diagnosis of MS (27). Although CSF examination is not mandatory, it remains a valuable test used to determine the presence of oligoclonal bands (OCBs). OCBs are visible as an increased concentration of restricted bands of specific immunoglobulins (mainly IgG) after CSF electrophoresis, which are not present in peripheral blood. While OCBs are detected in nearly 90% of MS patients, their presence is not specific to MS as they can be found in a variety of CNS disorders like paraneoplastic disorders, CNS lupus, neurosarcoidosis (24, 28, 29). Once the MS diagnosis has been made through a combination of clinical and paraclinical tests, other tools such as MRI and Expanded Disability Status Scale (EDSS) are used to assess disability and monitor disease progression (30, 31).

Risk factors

To date, the etiology of MS remains unknown. However, different genetic, environmental, and infectious triggers have been proposed. Having a first-degree relative affected with the disease increases the risk of MS by 10-50 times compared to the general population (0.1% risk)(32, 33). In fact, the recurrence risk of developing MS is higher for monozygotic twins (18.2%), than for dizygotic twins (5%), and siblings (2.7%), thereby alluding to the contribution of a genetic component (34). Genome-wide association studies (GWAS) have identified more than 200 gene variants associated with MS susceptibility. Most of these MS candidate genes are found to be immune system related. One polymorphism with a high prevalence for MS is the haplotype Human Leukocyte Antigen (HLA)-DRB1-15:01 allele (odds ratio of 3.08), associated with the Major Histocompatibility Complex (MHC) class II (35, 36). The main function of MHC class II molecules is to present antigens to $CD4^+$ T lymphocytes, which is critical in initiating the antigen-specific immune response. MS patients carriers of DRB1-15:01 are more likely to be female and have an earlier disease onset (37). Other genes implicated in multiple immunological pathways have been identified as influencing MS susceptibility. Such genes are involved in chemokine and cytokine signaling (e.g. CXCR5, IL2RA, IL7R), immune stimulation (CD80, CD40), migration signaling (e.g. STAT3, CD6, ALCAM)(7, 38, 39). Genes implicated in neurodegenerative pathways seem to be

absent (39). Thus, genetic factors further support the role of immunological mechanisms in the development of MS.

Although genetic predisposition explains the increased risk in monozygotic twins, their relatively low risk (18.2%) suggests the involvement of other non-genetic MS risk factors. Studies of migration and geographic gradients point to the environment as a significant influence on MS susceptibility. As previously mentioned, MS has an uneven distribution, with higher latitudes correlating with increased prevalence. When people migrate before adolescence, their risk of MS is equivalent to that of the migrated region, while those who migrate after adolescence, carry with them the incidence of MS from their original region (40, 41). As such, many environmental factors have their effect early in life and have been identified as MS risk factors.

The increase in MS prevalence with greater distance from the equator is correlated with exposure to sunlight, specifically ultraviolet (UV) radiation. UVB exposure is one of the pathways by which vitamin D is synthesized. Active vitamin D, 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3), has been shown to have immunoregulatory properties. In fact, 1,25$(OH)_2$D3 can activate the innate immune response and suppress the adaptive immune response. Specifically, 1,25$(OH)_2$D3 promotes the differentiation of monocytes into macrophages and enhances their phagocytic capacity, but inhibits the differentiation of monocytes into dendritic cells (DCs) (42). Moreover, 1,25$(OH)_2$D3-modulated myeloid DCs increase the number and suppressive abilities of regulatory T cells (Treg) (43, 44). Independently of DCs, 1,25$(OH)_2$D3 promotes the development of Treg, but not of T helper 1 (Th1) or T helper 17 (Th17) cells. Serum levels of vitamin D (25(OH)D) regulate the suppressive capacities of circulating Treg. Also, 1,25(OH)2D3 reduces the differentiation of B cells and their capacity to produce antibodies (45). In addition to its anti-inflammatory role, vitamin D may also have a neuroprotective role in a variety of neurological disorders (e.g. vitamin D helped prevent Amyloid- ß derived neuronal cytotoxicity in Alzheimer's disease) (46, 47). In the context of MS, lower serum levels of 25(OH)D are associated with higher rates of disease susceptibility. In fact, vitamin D supplementation may reduce the risk of MS in the general population, as well as in children of mothers supplemented before and during pregnancy (48). Studies have found MS patients to have low 25(OH)D levels compared to healthy individuals (49). In MS patients, increased levels of vitamin D are associated with attenuated clinical activity,

including a reduced risk of relapse, and a decreased lesion activity on brain MRI (50-52). Moreover, there's a positive correlation between serum levels of 25(OH)D and Treg suppressive ability in RRMS patients (53). Furthermore, UV radiation, independent of vitamin D, can have an effect on disease susceptibility and progression through other mediators (e.g. Vitamin A, melatonin)(54, 55). It is therefore questionable whether UV exposure, vitamin D supplementation, or both, are necessary for a decrease in MS susceptibility.

There is an increasing gender prevalence bias in MS, primarily in RRMS, in which women are 3 times more affected than men. Moreover, developmental periods including prenatal environment, puberty, pregnancy and reproductive ageing seem to influence the risk and progression of MS. Earlier puberty in girls is associated with an increased risk of MS and an earlier onset of symptoms (56, 57). As such, hormonal factors are involved in MS. Women generally have earlier disease onset, lower prevalence of PPMS, and less progression of disability than men (58). Pregnancy has a protective effect on MS relapses, but is followed by a disease rebound during the postpartum period (59, 60). The use of oral contraceptives could delay the onset of MS, but would not affect the risk of developing the disease (61, 62) .

Various infectious agents have been found to be associated with MS. To date, the most prominent virus candidate identified is the Epstein-Barr Virus (EBV), a herpesvirus causing infectious mononucleosis (63). According to epidemiological and meta-analysis studies, individuals that have previously experienced clinically overt infectious mononucleosis have a greater risk of developing MS (64). In fact, people who are EBV-negative are 15 times less likely to develop MS than those who are EBV-positive (65). One apparent association with MS is that EBV mediates immune-modulating changes through the reduced activity of EBV-specific cytotoxic $CD8^+$ T cells and the prolonged lifespan of B cells leading to aberrant T cell responses in MS patients (66). Some hypothesis supporting EBV molecular mechanisms in MS include molecular mimicry (activation of autoreactive cells by cross reactivity between self-antigens and foreign agents), and bystander activation (autoreactive cells are activated because of nonspecific inflammatory events that occur during infections)(67-69). Moreover, the heterogeneity of the disease suggests the involvement of more than one infectious agent, like Human Herpesvirus 6, Varicella-Zoster virus in increasing MS predisposition (69).

Another lifestyle risk factor is obesity. Men and women who experienced adolescence obesity have a two-fold increase risk of developing MS compared to normal weight people (70, 71). However, obesity at later ages is not associated with an increased MS risk (72) . Adolescent obesity can lead to chronic fat-related inflammation, while promoting Th1 responses and decreasing Treg number. In this way, obesity may increase the risk of recruitment of autoimmune cells to the CNS (73). In addition, obesity reduces the bioavailability of vitamin D (74). Interestingly, the odd ratio of obesity and EBV/infectious mononucleosis is around 2 each, but approaches 14 when combined (75). Many other environmental factors have been proposed such as smoking, concussions in adolescence, antibiotic exposure, alcohol, caffeine, sodium intake, diet, shift work, air pollutants exposure (e.g. particulate matters, heavy metals, lipopolysaccharide) (71, 76, 77). It is important to note that many risk factors that have been associated with MS also affect the gut microbiota composition (78-80). In fact, studies have shown that people suffering from inflammatory bowel disease have an increased risk of developing MS (81, 82). Distinctive bacteria of the MS flora can promote Th1 differentiation, and reduce Treg differentiation (83, 84). Therefore, dysbiosis of gut microbiota is implicated in the pathogenesis of MS.

1.2 MS Pathology

Pathological features

MS is one of the inflammatory demyelinating diseases of the CNS, whereby myelin is damaged. Myelin sheaths consist of glial plasma membranes that wrap in a compact multilamellar spiral around axons (85, 86). These membranes form an insulating layer that increases the resistance and decreases the capacitance across axonal membranes. As such, the main role of myelin is to propagate electrical signals rapidly along neurons for long distances, by allowing the voltage gated sodium channels to cluster at nodes of Ranvier, thus promoting rapid salutatory conduction (86). Moreover, myelin can provide metabolic support to the axons by ensuring the transport of lactate (87, 88). Myelin is synthesized by oligodendrocytes in the CNS, and by Schwann cells in the peripheral nervous system (PNS). In fact, an oligodendrocyte can myelinate from 2 to 50 portions of adjacent axons segments. Although CNS myelin differs slightly from PNS

myelin, the dry mass of both is comprised of a high proportion of lipid (70 to 85%) and a low proportion of protein (15 to 30%). Some of the CNS myelin proteins include myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin associated glycoprotein (MAG)(89, 90). Thickness, length and compaction of myelin can have an impact on the conduction velocity of action potentials (91-93). Thus, damage to myelin and myelin producing cells may impair the complex connections of the nervous system, leading to a myriad of adverse neurological symptoms.

A defining pathological hallmark of MS is the formation of focal inflammatory plaques in the CNS. The plaques carry a number of immune cells, which are responsible for an autoimmune-mediated damage of the myelin sheath. Thus, MS lesions are defined as focal areas of demyelination, inflammation, and glial reaction. As previously mentioned, plaque structures can be detected by MRI, however they can be further studied and classified by immunohistochemistry in post-mortem tissue (Figure 2). Immuno-staining with Luxol fast blue and Haematoxylin and Eosin (LHE) reveals the localization and identification of lesions in CNS tissue. Using this histology technique, we can see areas of demyelination (blue staining represents myelinated fibers), as well as inflammatory infiltrates in the CNS (purple staining represents nuclei; pink staining represents the cytoplasm).

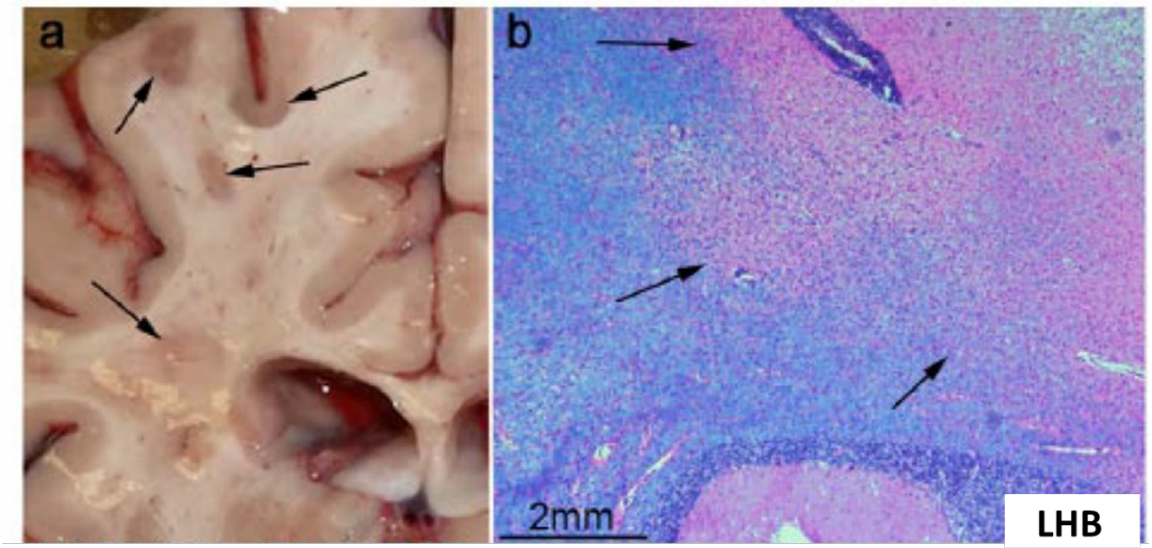


Figure 2. – Characterization of MS inflammatory plaques in the CNS.

(a) Example of a post-mortem MS brain section with apparent demyelinating lesions (black arrows). (b) Representative immune-staining with Luxol fast blue and hematoxylin and eosin (LHE) identifying areas of demyelination and immune infiltration. Image extracted from Larochelle et al., 2016 (94).

There are several different methods of histological classification systems for CNS lesions. One can retain the approach of Kuhlmann et al. as a guideline (95). This method mainly characterizes MS lesions according to two criteria; their inflammatory activity, which is based on macrophages and microglia distribution, and their demyelinating activity, which is based on the continuous level of myelin damage (Table 1). These activities are dynamic and heterogeneous processes that evolve with time. Typically, in a lesion, macrophages and microglia first take up damaged myelin, break it down in their lysosomes, and then gradually withdraw from the lesion site. Thus, the composition of myelin degraded products and the numerical density of macrophages and microglia are studied to provide information on the time of active demyelination and the age of a lesion. Other immune cells, such as T cells and B cells are present in a lesion, however, their numbers and distribution dynamics are lower than macrophages/microglia. Accordingly, MS lesions are classified as follows: active lesions, mixed active/inactive lesions, and inactive lesions. Both active and mixed active/inactive lesions may be further subdivided into demyelinating lesions (ongoing myelin damage), and post-demyelinating lesions (when myelin damage has arrested, but macrophages remain present)(95).

Lesion activity / Lesion Type	Inflammatory activity: macrophages/microglia	Demyelinating activity
Active lesion	Present throughout the lesion	Yes for demyelinating lesions; No for post-demyelinating lesions
Mixed active/inactive lesion	Limited to the lesion border	Yes for demyelinating lesions; No for post-demyelinating lesions
Inactive lesion	Absent	No, completely demyelinated

Table 1. - MS lesion classification criteria.

MS lesions can be characterized according to their inflammatory activity, and their demyelinating activity. These criteria classify MS lesions into 3 main types: active lesions, mixed active/inactive

lesions, and inactive lesions. Table adapted with permission from Paula Lepine's mémoire, 2018, Université de Montréal.

Pathological processes in MS are not limited to the white matter, as they can also affect the grey matter. Although less appreciated historically, cortical demyelinating lesions are common in MS. There are different types of cortical lesions depending on their location (subpial, intracortical, leukocortical, and pancortical lesions). In early stages of MS, these lesions are highly inflammatory, whereas in more chronic stages, these lesions have lower T cell infiltrates and a more prominent microglia population (96). In general, cortical lesions are less inflammatory than white matter lesions and have less BBB permeability (97).

In addition to activated macrophages and microglia, and immune cell infiltrates, acute MS plaques show antibody deposition, complement activation, astrocyte activation and oligodendrocyte apoptosis (95). In fact, the infiltrated cells, with the help of CNS resident cells (e.g. astrocytes, microglia), cause the selective death of oligodendrocytes either through direct contact or through inflammatory and soluble neurotoxic factors (98). As such, demyelination is only secondary to the damage of oligodendrocytes. Although neurons are mostly preserved in early MS, axonal transections are frequent (99). However, ongoing disease leads to degeneration of the vulnerable denuded axons and deposition of a dense astrocytic scar, resulting in brain atrophy and ventricular enlargement (7, 100). Accordingly, neuroaxonal degeneration correlates with the progressive disability that characterizes later stages of MS.

The long-term fate of a lesion is unpredictable, as MS lesions may undergo a spontaneous process of partial or complete regeneration. Limiting the axonal degeneration that follows demyelination, remyelination proves to be a neuroprotective mechanism (101). Remyelination requires the activation, proliferation, migration and differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes capable of forming new myelin sheaths (102). These sheaths are thinner and therefore represent distinct areas of remyelination called shadow plaques. However, only 20% of lesions exhibit significant remyelination and the process ultimately fails (95). Factors such as ongoing inflammation, myelin debris, axonal loss, lack of trophic factors and mitochondrial damage can hinder the recruitment and function of OPCs and thus limit remyelination (103). Moreover, as adults age, the efficiency of remyelination gradually decreases

(104). This makes the restoration of remyelination an important therapeutic goal to further prevent neurodegeneration in MS (101).

Pathogenic involvement of T cells in MS

It is the complex pathological interactions between the immune system, glial cells, and neurons that ultimately lead to MS. Whether the disease starts in the CNS (inside-out hypothesis) or in the periphery (outside-in hypothesis) remains a subject of great debate. However, studies in animal models of MS (e.g. murine experimental autoimmune encephalitis, EAE), together with human blood and CSF samples, support the undoubted role of the adaptive immunity in the development and sustention of MS. In sum, activated autoreactive T cells directed to CNS myelin specific antigens upregulate cell adhesion molecules (CAMs), to migrate over the BBB and penetrate the CNS. In humans, the specific antigens at the origin of this reaction have not yet been conclusively defined, whereas in EAE studies, immunization with myelin proteins such as MOG and MBP induced disease (105, 106). Upon local contact with antigen presenting cells (APCs) in the CNS, T cells are reactivated and mediate damage to myelin by secreting pro-inflammatory cytokines and by recruiting other immune cells like B cells (autoantibodies), monocytes, macrophages (phagocytosis, cytokine production, antigen presentation), natural killer cells (cytotoxicity) and mast cells (degranulation)(107). The accumulation of pro-inflammatory mediators, and oxygen and nitric oxide radicals ultimately leads to demyelination and axonal loss. Although several immune actors are implicated in MS, the following section will focus on the different populations of T lymphocytes, as these are the cell types of interest studied in this thesis.

$CD8^+$ T lymphocytes

MHC class I-restricted $CD8^+$ T cells dominate in MS lesions compared to $CD4^+$ T cells, which are present in lower levels (108, 109). Within a lesion, enhanced levels of MHC class I antigens are found not only on inflammatory cells, but also on neurons and glial cells (110, 111). These $CD8^+$ T cells can infiltrate the CNS through adhesion molecules (e.g. α4-integrin binding) (112). In the CNS, $CD8^+$ T cells retain their cytotoxic activity which is correlated with acute axonal damage (113). Indeed, these cytotoxic T cells (Tc) can release cytotoxins such as granzymes A and B, and

perforin, which in turn infiltrate the target cell and induce apoptosis (114, 115). Tc expressing granzyme B (GrzmB) are found close to or attached to oligodendrocytes and demyelinated axons (116). Another way these cells exhibit their effector function in MS is through the secretion of pro-inflammatory cytokines interferon γ (IFN-γ), tumor necrosis factor (TNF), interleukin (IL)-2, and IL-17 (117-119). Specifically, increased levels of pathogenic IL-17^{+}CD8^{+} T cells are present in lesions and in CSF of MS patients (120, 121). Conversely, a population of regulatory CD8^{+} T cells have been reported in EAE and in MS patients (see chapter 2, tolerance section, CD8^{+} regulatory T cells) (114, 122) . Thus, different subsets of CD8 T cells may play a detrimental or beneficial role in MS.

CD4^{+} T lymphocytes

CD4^{+} T cells are also known to initiate MS lesion formation. CD4^{+}:CD8^{+} ratio in MS patients is 2:1 in the peripheral blood and ranges from 3:1 to 6:1 in the CSF (107, 123, 124). There are different subtypes of CD4^{+} T cells; only the following 4 T helper (Th) subsets and their implication in MS will be discussed in this section: Th1, T helper 2 (Th2), Th17, and Treg. In order for a particular phenotype to be defined, a combination of cytokine signaling pathways, together with lineage-specific transcription factor activation are necessary. Each differentiated cell will then secrete a specific set of cytokines, thereby determining their effector functions. The key players involved in differentiating different subtypes are explicitly presented by Tato et al. in figure 3 (125). Additional cytokines are also involved. However, in the case of a deregulatory immune disease, the activation of these cells and their production of pro-inflammatory cytokine proves to be deleterious. In MS, the roles of CD4^{+} T cells have been supported by patient observations and animal models, such as EAE. These revealed that the pro-inflammatory activity of Th1 and Th17 is associated with disease exacerbation, while the anti-inflammatory activity of Th2 and Treg seem to play a protective role.

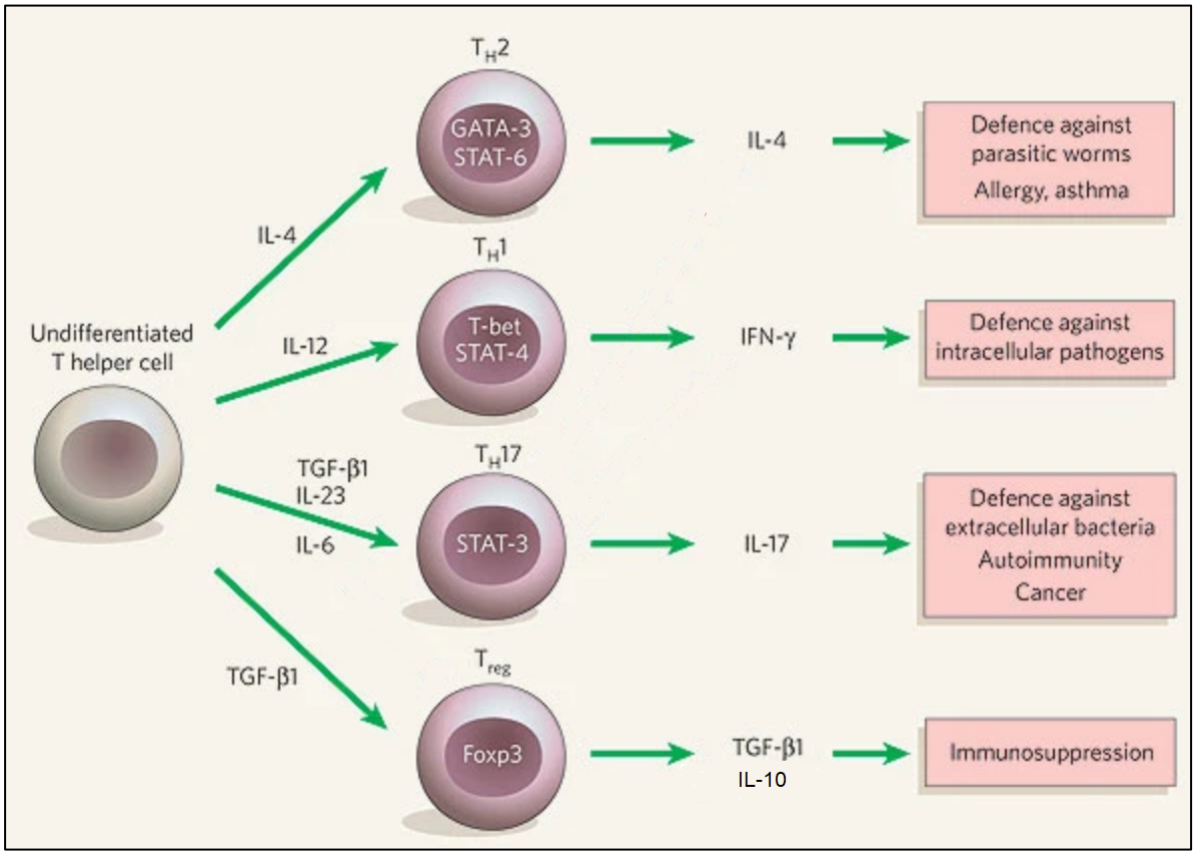


Figure 3. – Key players in Th polarization.

Depending on the cytokine environment laid down by APCs as well as the activation of specific transcription factors, previously antigen-activated naïve $CD4^+$ T cells will differentiate into distinct pathways. Each phenotype will have a specific effector function. Image extracted and adapted from Tato and O'Shea, 2006 (125).

Th1 cells

In the presence of IL-12 and IFN-γ, signaling through the STAT1 pathway induces the expression of transcriptional regulator T-box transcription factor TBX21 (T-bet), which drives the differentiation of naïve $CD4^+$ T cells into Th1 cells. Th1 cells are characterized by the production IFN-γ, as well as lymphotoxin, IL-2, TNF, and granulocyte–macrophage colony-stimulating factor (GM-CSF)(126) . They are known to preferentially express the receptors CCR5 and CXCR3(127). Their role is critical for immunity against intracellular pathogens (128). Specifically, Th1 production of IFN-γ can activate and recruit macrophages and dendritic cells to the site of inflammation (129).

In MS patients, Th1 responses have been positively correlated with disease activity (130). In fact, increased clinical activity was associated with IFN-γ and IL-12 levels in the CNS and CSF (131). CSF derived $CD4^+$T cells of MS patients produced larger amounts of IFN-γ and IL-2 (132). Many Th expressing IFN-γ cells were found in CNS lesions of patients and EAE (133, 134). Furthermore, the pathogenic role of IFN-γ was highlighted by a trial in 1987 in which administration of IFN-γ seemed to exacerbate MS (135). High levels of GM-CSF were detected in lesions and CSF of patients (136, 137).

Th17 cells

Naïve $CD4^+$T cells are driven to differentiate into Th17 cells by early exposure to TGF-β, IL-6, and IL-23. Their master transcriptional regulator is retinoic-acid-receptor-related orphan receptor ROR-γt. Th17 cells are pro-inflammatory cells that secrete IL-17A, IL-17F, IL-21, IL-22, and GM-CSF, and provide defense against extracellular pathogens (138). The number of Th17 cells in the CSF of RRMS patients was found higher during relapse than during remission, which implicates Th17 responses with disease activity (139). It has been shown that Th17 cells are present in MS lesions and can transmigrate across the BBB more efficiently than Th1 cells (140). Moreover, higher levels of IL-17 mRNA and proteins were found in the blood, CNS and CSF of patients (118, 141).

Th2 cells

IL-4 signaling to naïve $CD4^+$ T cells results in induction of the master transcription factor GATA-3. This induces the production of Th2 defining cytokines IL-4, IL-5, and IL-13 (126). In health, Th2 immune responses play an important role in eradicating extracellular parasites and bacterial infections. These cells mediate allergic responses by directly or indirectly activating inflammatory and residential effector pathways (142). Although the exact mechanisms remain unclear, Th2 cells are thought to have a protective role in MS. Higher levels of Th2 cytokines were detected in the blood of MS patients during remission (143). Decreased MS disease activity during pregnancy has been found to be associated with Th2 responses (144).

Regulatory T cells (Treg)

Treg are a subset of $CD4^+$ T cells that express the transcription factor Forkhead box protein 3 (Foxp3) and the IL-2 receptor α chain (CD25). IL-2 and Transforming Growth Factor beta (TGF-ß) appear to favor the differentiation and development of Treg. These cells are best known for suppressing the functions of effector T cells (Teff) and APCs by secreting anti-inflammatory cytokines such as TGF-ß1, IL-10, and IL-35. The function of Treg has proven to be essential in preventing autoimmune diseases (145). Their role in suppressing inflammation within the CNS is controversial and will be elaborated in chapter 2.

1.3 Therapeutic Approaches

At present, treatments for MS consist of immunomodulating and immunosuppressive drugs whose efficacy is positively correlated with the risk of toxicity. These disease modifying therapies (DMTs) can help reduce the number and damage caused by relapses in RRMS patients but have not proved beneficial in managing the progressive phases of the disease (146). Briefly, the following text will discuss the mechanism of action of some common treatments currently used.

Interferon beta is a polypeptide naturally found in the body and is used in its injectable form as a first line approach to treat MS. Known for its anti-inflammatory nature, interferon beta decreases the expression of MHC class II, favors the production of anti-inflammatory cytokines, reduces the proliferative activity of Th1 and Th17 cells, and enhances the BBB stability (147). Glatiramer acetate, polymer of four amino acids, is another injectable form of MS treatment that has anti-inflammatory and neuroprotective effects, by promoting a Th2 deviation and Treg induction (148). Dimethyl fumerate is an anti-inflammatory agent that targets the redox master regulator Nrf2 and is implicated in monocyte and T cells oxidative processes (149). An anti-metabolite drug, Teriflunomide, although not fully understood, can inhibit the *de novo* pyrimidine synthesis needed for proliferating immune cells as well as reduce their migratory ability to the CNS (150, 151).

From studies done with Teriflunomide, it was evident that B cells were involved in activating pro-inflammatory T cells in MS, thus other monoclonal antibodies were developed,

notably, rituximab and ocrelizumab, that target CD20 and limit the proliferation of B cells (152). Another immune selective intervention worth mentioning is Fingolimod, an oral agonist of sphingosine 1-phosphate receptor that causes the internalization and degradation of the receptor in multiple cell types, including lymphocytes. It is known to reduce the amount of circulating lymphocytes in MS by preventing T cells from leaving secondary lymph organs (153). Clinical trials revealed the beneficial effects of Fingolimod, including a 50% reduction in relapse lesions and a reduced level of brain atrophy.

Another type of DMT, is an inhibitor of immune migration. Natalizumab is a monoclonal antibody that prevents the migration of leukocytes by blocking the α4β1 integrin of very late activation antigen 4 (VLA-4) involved in the adhesion of cells and their diapedesis across the BBB into the CNS. Favorable outcomes have been reported from RRMS patients as their relapses and lesions are reduced. However, when treatment is stopped, disease activity recurs with excessive reactivation. Moreover, Natalizumab comes with the risk of developing progressive multifocal leukoencephalopathy (a demyelinating disease caused by reactivation of JC virus infection in immunocompromised patients)(154, 155).

Other agents such as mitoxantrone, alemtuzumab and cyclophosphamide are known for providing general immunosuppression. For instance, alemtuzumab targets the depletion of T cells and B cells expressing the CD52 marker and preserves the Treg population due to their low CD52 expression (156). Although the depletion of activated immune cells decreases the number of effector cells capable of entering the CNS, they are associated with higher risks of severe complications (157).

Over the past decade, FDA-approved therapies for MS have greatly expanded, which have mainly benefited newly diagnosed patients with the RRMS disease course. Since most patients advance towards a neurodegenerative phenotype, additional research towards preventive and neuroprotective treatments are needed. Thus, determining biomarkers to aid in the diagnosis, prognosis, and treatment option is a valuable new research direction in MS.

1.4 Animal models of MS

MS has been extensively investigated by using the animal model of experimental autoimmune encephalomyelitis (EAE) in which CNS autoimmunity is induced in susceptible animals. In fact, mice and rats were found the best models for assessing acute, relapse-remitting, and chronic progressive stages of EAE disease course (158). Currently, there are three main types of EAE: active, passive, and spontaneous.

Active EAE is carried out by immunizing a susceptible mouse with a myelin peptide selected according to the mouse strain (e.g. peptide $PLP_{139-151}$ in SJL/J mice, or peptide MOG_{35-55} in C57BL/6 mice). To potentiate the humoral immune response, a complete Freund's adjuvant emulsion containing inactivated amounts of Mycobacterium tuberculosis is added (159). Following the first few days of immunization, injections of pertussis toxin are administered to promote BBB destabilization and immune infiltration to the CNS, leading to symptoms of relapsing-remitting disease like those of MS patients (160, 161). In fact, EAE mice symptoms are characterized by an ascending paralysis starting at the tail, followed by limb and forelimb paralysis, the severity of which is evaluated on a five points scale. Th1 and Th17 cells are considered as the important mediators of EAE pathology. Passive EAE is induced by the passive transfer of encephalogenic T cells from an immunized donor mouse into a naïve recipient. In general, EAE developed by passive induction is more aggressive than that induced actively and has additional symptoms to ascending paralysis, such as ataxia and proprioceptive disorder (162). Spontaneous EAE occurs in transgenic mice models in which mutant T cell receptors (TCR), expressed by most T cells, can recognize a myelin peptide. Consequently, these animals spontaneously develop EAE symptoms. A particular mouse model used in our lab is the TCR^{1640} transgenic mouse, which express a TCR specific for MOG_{92-106}. This model was developed from the Vα8,3 and Vβ4 gene segments of TCR from the Th1 encephalitogenic clone of immunized SJL/J mice with recombinant rat MOG (163). In addition to allowing the study of acute disease phase, this model reflects more accurately the disease presentation of humans. For instance, in TCR^{1640}, disease is more prevalent in females (80%) that develop a relapse-remitting course, than in males (60%) that develop a more progressive course (164).

Overall, studies involving the EAE model have greatly contributed to the identification and understanding of MS mechanisms. The use of these experimental models makes it possible to study different aspects of CNS inflammation. In addition, the EAE model has contributed to the development of MS therapies such as glatiramer acetate, and mitoxantrone (165).

2. Regulatory T Cells

To maintain immune homeostasis, there must be a balance between immune activation (processing of foreign antigens) and immune suppression (tolerance). Immune checkpoints exist to control the activation of immune cells and eliminate the function of pathogenic cells. However, the loss of self-tolerance leads to the activation of self-reactive T cells and the development of autoimmune diseases, like MS. Over the years, growing interest in tolerance mechanisms has led to the discovery of immune-regulatory cells. One of the main "suppressive" subsets identified are regulatory T cells (Treg). In health, Treg are key players in maintaining immune tolerance to self- and non-self-antigens, thereby preventing autoimmune reactions.

2.1 Tolerance

To achieve tolerance, the immune system, depended on antigen-specific reactions, must be educated. In this way, the non-reactivity of the immune response to self-molecules establishes tolerance. Hematopoietic precursor cells migrate from the bone marrow to the thymus where they undergo positive and negative selection based on the nature of their T cell receptor (TCR) interactions with peptide-MHC complex and auto-antigens. Highly self-reactive lymphocytes are deleted during this clonal selection: process known as central tolerance. However, some self-reactive cells escape the clonal selection and enter the periphery. Mechanisms of peripheral tolerance ensure the control of self-reactive T cells by directly (T cell intrinsic mechanisms) or indirectly (T cell extrinsic mechanisms) acting on them. T cell intrinsic mechanisms work in concert and in redundancy to prevent autoimmunity by deleting or inactivating cells; these include clonal anergy (no immune response to cognate antigenic stimuli), clonal deletion (engagement with self-peptide-MHC complex induces apoptosis pathway), clonal ignorance (circulating T cells have low immunogenicity to self-antigens located in immune privileged sites), and inhibitory receptors (e.g. Cytotoxic T Lymphocyte associated molecule-4 (CTLA-4) upregulated upon TCR stimulation leading to T-cell-cycle arrest) (166, 167). T cell extrinsic mechanisms control immune responses via a subset of regulatory cells that differ in their phenotypical and functional characteristics.

Similar to Treg cells, these regulatory cells may inhibit the activation or promote the suppression of other immune cells. Some of these regulatory cells will be briefly discussed in this section.

Tolerogenic dendritic cells

Tolerogenic DCs are known to exert multiple immunosuppressive effects to induce tolerance. Tolerogenic DCs often have an immature phenotype characterized by a low expression of co-stimulatory ligands and MHC molecules. Thus, presenting antigens to self-reactive T cells in the absence of co-stimulatory signals can induce T cell mediated anergy (168). Moreover, they can trigger T cell apoptosis by expressing indoleamine 2,3-dioxygenase (IDO), CTLA-4 and death receptors like tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas cell surface death receptor (FasL/Fas). They can also secrete IL-10, TGF-ß and retinoic acid (RA) leading to the induction of tolerogenic DCs, the inhibition of Teff cells and the promotion of Treg differentiation and function (169, 170).

B regulatory cells (Breg)

IL-10 producing B cells have been recently characterized as B cells with regulatory properties known as Breg (171, 172). Their primary mechanism of action is the secretion of IL-10, IL-35 and TGF-ß, thereby favoring Treg development and inhibiting Teff cell function. Breg have been reported to play a role in the development of malignancies, infections, organ transplantation, and autoimmune diseases (173).

$CD8^+$ regulatory T cells

As previously mentioned, regulatory $CD8^+$ T cells help maintain immune homeostasis. These cells are identified by the expression of CD122, CD28, CD103, and HLA-G (174-177). They are able to secrete IL-10, and are thought to contribute to the suppression of the immune response and MS disease activity (177, 178).

T helper 3 cells (Th3)

Initially Th3 cells were described in the context of oral tolerance and were later found to be important in the gut immune regulation (179). It has been demonstrated that EAE mice fed with several low doses of MBP developed T cells that primarily secreted TGF-ß, as well as IL-4 and

IL-10. These T cells were characterized as Th3 cells that have suppressive abilities that could prevent the induction of EAE (180, 181).

T regulatory type 1 cells (Tr1)

A regulatory subset of $CD4^+$ T cells, Tr1 cells are induced in the periphery in the presence of IL-10 (182). Tr1 cells are characterized by the co-expression of CD49b and lymphocyte-activation gene 3 (LAG-3) and have low levels of CD25 and CTLA-4 (183, 184). They control immunological responses in several immune-mediated diseases by secreting IL-10, TGF-ß and GrzmB (182).

2.2 Origin of CD4 Regulatory T Cells

Over the past 20 years, Treg's contribution to the immune response has become more evident as their understanding and identification have progressed. Treg constitute approximately 1% of developing CD4 thymocytes, about 10 to 15% of CD4 T cells in secondary lymphoid organs, and about 5 to 10% of all circulating CD4 T cells (185). Although the Treg population is relatively small, their impact on the immune responses is substantial. Like other regulatory cells, Treg are responsible for maintaining immune homeostasis and are crucial for survival. While Treg can be characterized as T cells with immunosuppressive activity, several studies have reported distinct populations of Treg in terms of development, phenotype, and function. Throughout the years, the terminology used to classify Treg has been inconsistent. Currently $Foxp3^+$ Treg are classified as follows: thymus-derived Treg (tTreg), peripherally *in vivo*–induced Treg (pTreg), and *in vitro*–induced Treg (iTreg). In the past, tTreg were referred to as natural Treg (nTreg), whereas pTreg and iTreg were collectively referred to as induced or adaptive Treg (186).

Thymus-derived Treg (tTreg)

Early work in the 1960s involving thymectomies on day 3 of mice life (d3Tx), was found to induce autoimmune diseases, highlighting the importance of the thymus and the role of thymus-derived T cells in immune responses and tolerance (187). A seminal study in 1969 by Nishizuka and Sakakura revealed that thymectomies at day 1, day 7 or later, did not result in the development of autoimmune diseases as d3Tx mice did. They also showed that autoimmunity of

d3Tx mice can be rescued by a thymus transplant. These discoveries were later attributed to Teff cells exiting the thymus by day 3 post-partum, followed by a delayed exit of "suppressor T cells"(188, 189). It was in 1995 that Sakaguchi et al. identified the IL-2 receptor, CD25, as a marker of suppressive T cells, further characterizing this population (190). In a subsequent study, they showed that d3Tx inhibited the accumulation of $CD25^+$ T cells in the periphery, and that $CD25^+$ T cells transferred into d3Tx mice rescued their autoimmunity, thereby revealing the importance of thymus-derived $CD25^+$ T cells in preventing autoimmunity (191).

To date, our knowledge of tTreg development has expanded but is not yet fully elucidated. Treg cell progenitors originate in the bone marrow and mature into tTreg in the thymus, where they undergo a number of immune checkpoints before migrating to the periphery. These checkpoints include gene rearrangement for TCR expression, positive selection (capacity to interact with MHC), and negative selection (capacity of self-reaction), in which most of the immature T cells are eliminated while a minority differentiate into tTreg (192). The interaction between TCR and tTreg is crucial for tTreg generation whereby tTreg have a high self-antigen reactivity compared to conventional T cells. In parallel with TCR stimulation, CD28-B7 or CD40-CD40L co-stimulation are essential for the generation of tTreg. In fact, CD28 or CD40 deficiency in mice resulted in an 80% decrease in tTreg number (193). In addition, it has been shown that TCR and CD28 signaling induce the expression of TNF receptor superfamily protein, most notably OX40, GITR and TNFR2, further promoting tTreg generation. Additional molecules of stimulation and cell adhesion are involved to ensure the appropriate reaction of Treg with APCs (CTLA-4, CD18/CD11a). Following TCR and CD28 signaling, transcription factors NF-κB, AP-1 and NFAT become activated and induce the expression of Foxp3, thereby determining the tTreg cell lineage (167). Studies revealed that CD28 co-stimulation can induce Foxp3 expression in thymocytes independently of IL-2, but that IL-2 is crucial for the maintenance and survival of the tTreg population in the thymus and the periphery (193). In fact, IL-2 and CD25 signaling prevent tTreg from Foxp3-induced death, thus promoting their survival (194). Combined deficiency in IL-2 and TGF- ß nearly completely depleted tTreg number (195, 196). Once developed in the thymus, tTreg migrate to the periphery, circulating through secondary lymphoid organs and non-lymphoid tissues. In the periphery, tTreg are subject to phenotypic and functional changes depending on

the target cells and the local environmental signals. Thus circulating T cells may enter and exhibit anti-inflammatory properties in several tissues such as lungs, liver, pancreas, gastrointestinal tract, adipose tissue and other (195, 197) .

Peripherally in vivo–induced Treg (pTreg)

In the periphery, naïve $CD4^+$ T cells may also upregulate Foxp3 expression, thereby acquiring Treg cell properties. This population, called pTreg, is induced under specific conditions involving *in vivo* chronic suboptimal antigenic stimulation, and *in vivo* targeting of antigen to immature DCs (198, 199). Some suggest that differentiation into pTreg is a way to redirect potentially pathogenic T cells into protective T cells (192). Although the specifics of pTreg differentiation are not fully understood, it is now recognized that TCR interaction, CD28, IL-2, TGF-ß and retinoic acid are required (200). In fact, IL-2 and TGF-ß play an important role in peripheral induction by activating the Foxp3 locus through transcription factors STAT5 and those of the Smad family (201). Moreover, the tissue specific environment can provide additional factors required for pTreg generation. For instance, pTreg are mostly present in the gastrointestinal tract, where T cells are continually activated by commensal organisms (e.g. Clostridium spp). These microbes can lead to DC-derived TGF-ß induction of pTreg, as well as the secretion of short-chain fatty acids (e.g. butyrate), which in turn contribute to pTreg development (202, 203). While the extent to which these pTreg contribute to self-tolerance is not yet known, they are thought to play a major role in maintaining gut-immune tolerance and in controlling dynamic responses in pathological context.

In vitro–induced Treg (iTreg)

Following *in vitro* treatment with α-CD3, α-CD28, IL-2, TGF-ß and retinoic acid, naïve T cells upregulate Foxp3 expression and acquire Treg phenotypes. Such induced cells are referred to as iTreg. Although the use of *in vitro* iTreg to investigate *in vivo* Treg mechanisms is considered a valid approach, iTreg lack the epigenetic patterns and transcriptional characteristics of *in vivo* Treg, thus rendering them unstable (204). Because they do not have the complete demethylation that drives Treg signature genes, the expression of some Treg markers may differ. For example, human and murine tTreg express Helios and Neuroplilin-1, while most iTreg fail to do so (205).

Despite differences in mRNA transcripts, protein expression, epigenetics, and stability, iTreg exhibit phenotypic and functional characteristics similar to other Treg subsets (206).

2.3 Characterization of Treg

In humans, both thymic and peripheral Treg are essential for the maintenance of immune tolerance. Currently, one of the main challenges in advancing Treg research is the lack of markers that differentiate between Treg and conventional T cells, and between different Treg subsets. This section will cover some of the general markers used for Treg characterization.

Initially identified as $CD4^+$ $CD25^+$ T cells in mice, Treg were later characterized in humans as $CD4^+$ $CD25^{high}$ T cells in the thymus and in the peripheral blood (207). CD25 proves to be important in the development, survival, and function of Treg, and is a useful marker for Treg given its stable and high expression. However, CD25 is expressed by 5 to 10% of mouse $CD4^+$ T cells, and by 1 to 2% of human $CD4^+$ T cells, whereby all conventional $CD4^+$ T cells upregulate CD25 upon activation (167, 208). Moreover, many $CD4^+Foxp3^+$ Treg cells have mid or low expression of CD25 (209). Thus, $CD4^+CD25^+$ T cells do not represent a distinct Treg population. *In vitro* studies showed that $CD4^+CD25^+$ T cells that lack the α-chain of the IL-7 receptor, CD127, are suppressive cells (209, 210). However, later studies demonstrated that CD127 is downregulated by conventional $CD4^+$ T cells upon activation, rendering the differentiation between Treg and activated T conventional cells challenging (211).

As previously alluded to, the expression of Foxp3 is crucial for Treg development and suppressive function (212). The importance of Foxp3 was revealed by mutation studies of *Foxp3* gene in mice, which resulted in massive proliferation of $CD4^+$ T cells, extensive multiorgan infiltration, overproduction of pro-inflammatory cytokines, and spontaneous autoimmunity, otherwise known as Scurfy mutant mice (213). These mice suffered an X-linked mutation of the *Foxp3* gene, leading to fatal lymphoproliferative disease. Similarly, mutations in the *Foxp3* gene in humans have led to an immunodeficient disease, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), and are known to be associated with immune dysregulation, autoimmune polyendocrinopathy syndrome, autoimmune enteropathy, inflammatory bowel disease (IBD), allergic dermatitis, food allergy, hematological disorders,

immunodeficiency hyperimmunoglobulinemia E (199, 214), but not MS, for which GWAS studies did not provided evidence of a significant genetic role for *Foxp3* in MS (215). In both mice and humans, *Foxp3* mutations resulted in deficient numbers of tTreg and pTreg. In addition, deletion of the *Foxp3* gene in mature Treg cells resulted in the loss of their suppressive function *in vivo* and their secretion of pro-inflammatory cytokines (216). Foxp3 is a member of the forkhead family of transcriptional regulators that can act as a transcriptional enhancer or repressor of about 1500 genes in humans. These genes include several cytokine genes, T cell proliferation and activation genes (e.g. NF-κB), which can inhibit the effector functions of T cells (217). To date, Foxp3 is known to be the master regulator of Treg cells and a valuable marker for Treg in mice and humans. $CD4^+CD25^+$ T cells are shown to specifically express Foxp3 protein. However, in humans, other T helper cells transiently express high levels of Foxp3 upon activation, making it difficult to distinguish between Treg cells and conventional T cells under inflammatory conditions (218). In addition, due to its intracellular localization, its use as a defining marker is restricted in the purification and sorting of Treg for functional studies.

Currently, the combination of $CD4^+CD25^{high}CD127^{low}Foxp3^+$ T cells allows to discriminate Treg from non-Treg subsets. It is also largely assumed that the majority of isolated $CD4^+Foxp3^+$ T cells are in fact tTreg, although pTreg contribution is recognized, there are is presently no way to separate theses subtypes. The identification of cell surface markers that uniquely characterize Treg remains an area of intensive research. Many other markers have been associated with, but are not limited to, human Treg (e.g. CTLA-4, CCR6, GITR, CD62L, PD-1, Helios)(167). Having specific Treg markers would allow to better understand Treg physiology, function, and therapeutic application.

2.4 Treg Mechanisms of Suppression

Treg can limit and inhibit the proliferation and cytokine production of a wide range of immune cells (T cells, B cells, natural killer cells, monocytes, macrophages, and DC). Treg exert their immunosuppressive function either directly (by cell-cell contact), or indirectly (soluble mediated factors). Here, an overview of the four main Treg mediated suppression mechanisms will be presented (Figure 4).

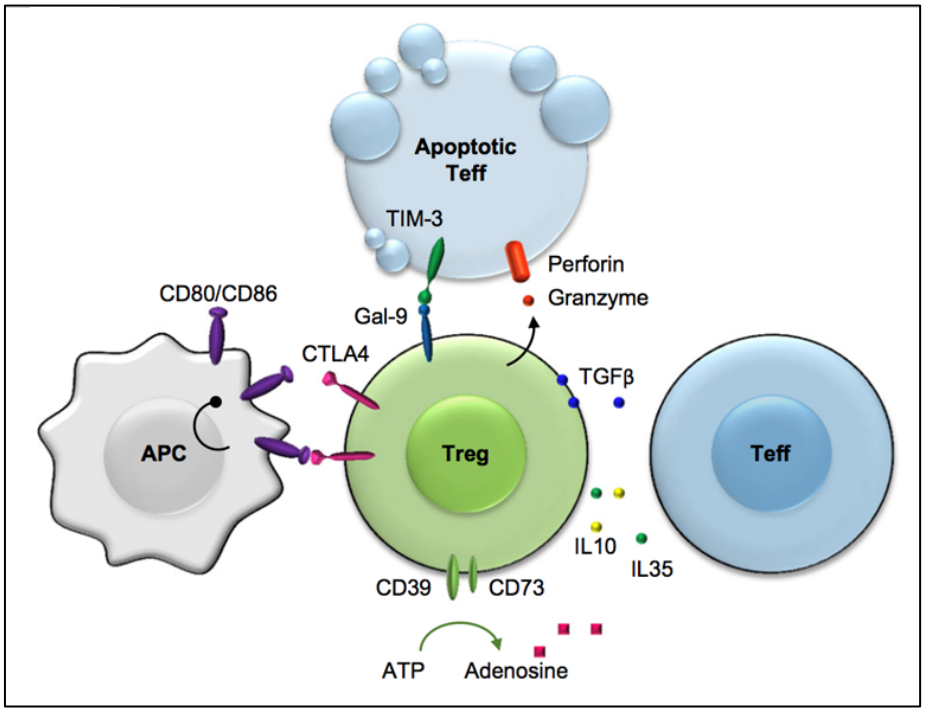


Figure 4. – Schematic representation of Treg's suppression mechanisms of other immune cells.

To mediate their immunosuppressive functions, Treg may limit APCs role via CTLA-4 effects, disrupt metabolic pathways (e.g. adenosine), induce apoptosis via cytotoxic molecules (e.g. Gal-9, granzyme B) and secrete anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35. Image extracted from Grant et al., 2014 (219).

One form of Treg contact dependent suppression is the modulation of APC activity. During T cell activation, CD80/CD86 expressed by APCs bind to CD28 expressed by T cells to stimulate their proliferation. However, Treg express CTLA-4, which competes with CD28 and in turn binds with higher affinity to CD80/CD86 (220). This interaction causes the internalisation and downregulation of CD80/CD86 on APCs, thereby limiting the initiation of an adaptive immune response. In addition, these signals can activate the enzyme IDO, generating the immunosuppressive metabolite kynurenin, as well as activate the transcription factor Foxo3, inhibiting DC-cytokine production (221, 222). Moreover, Treg can express LAG-3, a surface receptor that binds MHC class II molecules on APCs, which negatively regulates the expansion of activated T conventional cells (223).

Treg may disrupt the metabolic pathway of effector cells, thereby limiting their survival and function. IL-2 is produced by T cells during an immune response and is known for its

immunostimulatory and immunoregulatory functions. In fact, IL-2 is a necessary growth factor promoting naïve T cells proliferation and differentiation into Teff cells, in addition to expanding the Treg population. Since Treg express high levels of CD25 on their surfaces, it has been suggested that they limit T effector activity by depleting the local environment of IL-2 (224, 225). Moreover, Treg are found to have elevated levels of intracellular cyclic adenosine monophosphate (cAMP), a long-known suppressor of T cell activation and function (226). While Teff have minimal levels of cAMP, Treg are able to directly transfer their intracellular cAMP to Teff via cell contact-depended gap junctions. This cAMP acts as a second messenger to impair IL-2 synthesis and inhibit Teff proliferation (227). Furthermore, Treg express the ectoenzymes CD39 and CD73 on their surfaces which enables the hydrolysis and conversion of the pro-inflammatory extracellular adenosine triphosphate (ATP) into anti-inflammatory adenosine.

Similar to NK cells and $CD8^+$ T cells, in highly inflammatory environments, Treg may induce T cell, B cell, granulocyte, and APC apoptosis by releasing cytotoxic molecules such as granzymes A and B, that enter target cells via perforin pores, leading to the activation of the caspase pathway (228-230). Galectin-1, Galectin-9 and Galectin-10 have also been reported to trigger Treg-mediated apoptosis (231-233).

Treg can mediate their suppressive ability by secreting anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35. Although the role of these immunosuppressive cytokines is not yet completely understood, they are of great importance for Treg suppressive function *in vivo,* particularly at interfaces of the gut, skin and lungs (234, 235). TGF-β is widely expressed by various cells and has pleiotropic functions such as limiting T cell proliferation and NK responses, and inhibiting cytolytic and effector functions of T cells (e.g. by downregulating cytokines such as TNF and IFN, and inhibiting IL-2 transcription). Treg can produce large amounts of membrane-bound and soluble TGF-β, and highly express the Glycoprotein A Repetitions Predominant, GARP, a transmembrane protein that binds to the latent form of TGF-β, which is correlated with their suppressive capacity (236, 237). Defects in TGF-β signaling have led to the development of autoimmune diseases in mice (238). IL-10 is another cytokine that is produced by $Foxp3^+$ and $Foxp3^-$ T cells, B cells, macrophages and DCs, and has immunosuppressive effects on various immune cells (239-241). In fact, IL-10 production by these immune cells were shown to indirectly

influence suppression by helping Treg maintain Foxp3 expression (242). IL-10 is known for its key role in Treg-mediated suppression of intestinal inflammation (243). However, depending on the tissue and the disease, IL-10 may have different roles. For example, in the inflamed skin, IL-10$^+$ Treg were necessary to control IFN-γ $^+$ T cells, whereas in the lymph nodes, IL-10 was dispensable for IFN-γ regulation and T cell proliferation (244). Moreover, Treg derived from the spleen seem to barely secrete any IL-10 *in vitro*, suggesting that their suppressive ability is not dependent on IL-10 (245). Recently, IL-35, composed of IL-12α and the Epstein-Barr virus-induced gene 3 (EBi3), has been reported as an immune suppressive cytokine implicated in Treg-mediated suppression (246). Although human Treg do not constitutively produce IL-35, IL-35 is preferentially expressed by Treg since EBi3 is a downstream target of Foxp3 (247). Deficiency in one of the IL-35 chains reduced the suppressive ability of Treg *in vitro* and *in vivo* in an IBD model (246). In addition to their suppressive function, TGF-β, IL-10, and IL-35 are involved in the maintenance of tolerance, by which they enhance Treg suppression and stimulate the conversion of activated conventional T cells into T cells with a regulatory phenotype (eg. Foxp3$^+$ T cells, Tr1 cells) (248-250).

2.5 Treg in MS

In healthy individuals, maintaining central and peripheral immune tolerance is crucial to prevent the activation of autoreactive T cells. In fact, dysfunctional self-tolerance mechanisms leading to autoimmunity distinguish MS from other neuro-inflammatory diseases, such as Parkinson's disease, Alzheimer's disease, and stroke. Throughout the years, controversial evidence regarding Treg activity in MS have emerged and their involvement has been a subject of great debate.

A study by Putheti et al. in 2004 first showed that circulating Treg in the peripheral blood of MS patients have no altered frequency or function compared to those of healthy controls (251). However, an opposing study in the same year reported a significant reduction in the suppressive capacity of circulating CD4$^+$CD25^{high} T cells from RRMS patients versus healthy donors (252). At that time, most studies only analyzed peripheral blood samples to study Treg dysfunction in MS. While an initial study by Tzartos et al. confirmed the absence of Treg in MS brain lesions, later studies, although in low levels, have highlighted the presence of Treg in the CNS (120, 253). In

2017, Zandee et al. showed that $IL10^+$ Treg were present in the CNS of a subset of MS patients, suggesting that Treg might have anti-inflammatory properties in MS lesions (254). In addition, an increased frequency of $CD4^+CD25^+Foxp3^+$ was detected in the CSF compared to the peripheral blood of untreated MS patients (253).

The role of Treg in regulating neuro-inflammation is also well supported by EAE studies. Although some studies have reported an accumulation of Treg in the CNS of EAE, their failure in controlling CNS derived pro-inflammatory T cells suggested that Treg abnormalities involved their loss of suppressive abilities (255). However, when Treg were depleted using anti-CD25 antibodies in $PLP_{139\text{-}151}$ immunized EAE, an enhancement of disease severity and mortality was reported (256). In $MOG_{35–55}$ active and passive EAE, accumulation in the CNS of IL-10-producing $CD4^+CD25^+Foxp3^+$ T cells positively correlated with disease recovery. In fact, the importance of IL-10 in Treg mediated function was documented in EAE models. McGeashy et al. have demonstrated that a transfer of these Treg cells to mice before EAE induction resulted in a reduced disease severity (257).

Together, these data suggest that Treg might be needed for the resolution of disease. Such findings point to the possibility that Treg might play an important role in dampening CNS inflammation. Therefore, further in-depth research is needed to reconcile these discrepancies and advance MS research.

3. Barriers of the CNS

The proper functioning of the CNS is vital to our survival. To this end, constant exchanges between the CNS and the blood compartment are necessary. Importantly, blood vessels supply the CNS with oxygen and nutrients and remove carbon dioxide and metabolic waste. However, a varying number of toxins and cells are also present in the blood. Because of the limited regenerative capacity of neuroglial cells and their requirement of a stable extracellular environment to function, blood components may pose a threat to the brain. Thus the importance of heavy restricting barriers that tightly regulate the movement of molecules, ions, and cells between the blood and the CNS (258).

3.1 Neuro-anatomical composition

The Blood Brain Barrier (BBB)

The BBB outlines the unique properties of CNS microvasculature (259). CNS vessels are continuous, non-fenestrated vessels that allow for an extensive but selective area of surface exchange (260). Together, vascular cells (endothelial cells, smooth muscle cells, pericytes), neural cells, glial cells (astrocytes, microglia, oligodendrocytes) and the extracellular matrix (EM) form a functional and anatomical unit, known as the neurovascular unit (NVU) (Figure 5) (261). The contribution of each cell type will be discussed in this section.

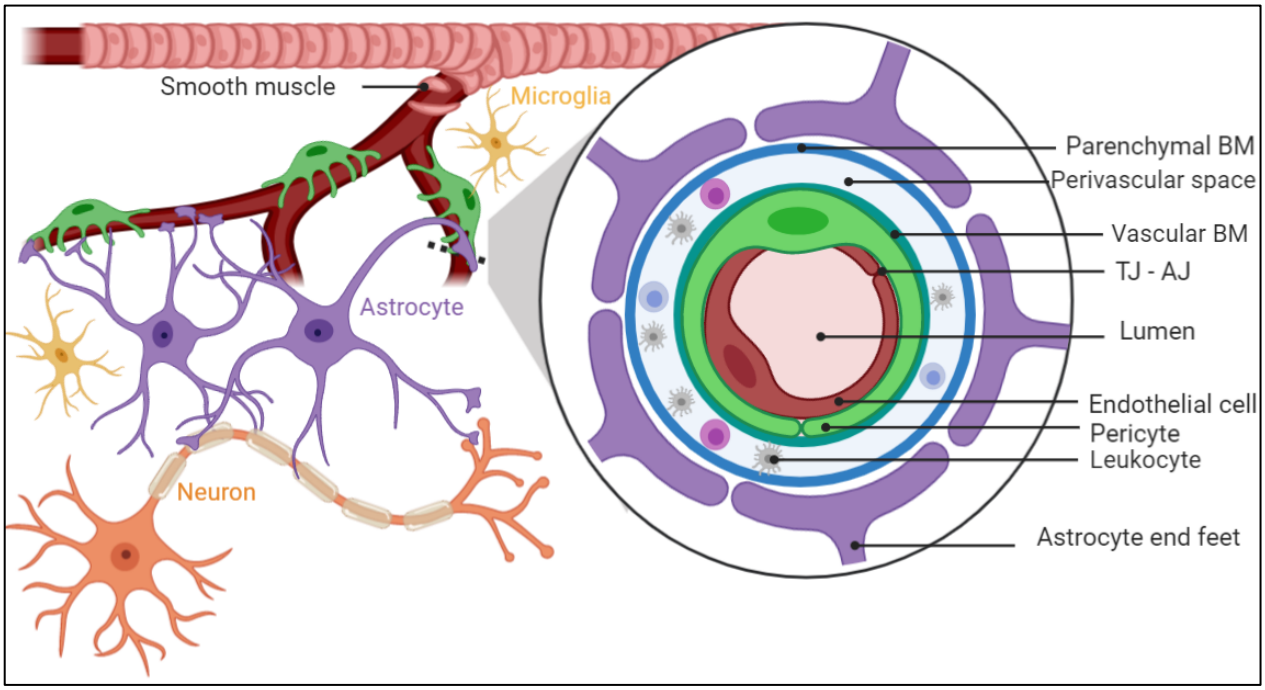


Figure 5. – Schematic representation of the neurovascular unit (NVU) and the blood brain barrier (BBB).

The NVU enables intercellular communication between cells of the vasculature, neurons, and glia. The BBB acts as a module within the NVU to tightly regulate blood-CNS exchanges. As vessels penetrate the CNS, they lose their smooth muscle coverage and pial layer, and become surrounded by pericytes, perivascular astrocytes and microglia. Astrocytes have fine processes near synapses and occupy separate non-overlapping spatial domains. The BBB composition is represented in the magnified window. Endothelial cells (EC) line CNS vessels and are connected by tight junctions (TJ) and adherens junctions (AJ). Between the EC and the astrocytic feet are two basement membranes (BM) (Vascular BM and Parenchymal BM) separated by the perivascular space where T cells are re-activated in the context of neuroinflammation. Illustration was created using Biorender.com.

Endothelial cells (EC)

The mesodermally derived EC of the BBB (BBB-EC) constitute an extremely thin layer of cells, held together by tight junctions (TJ) and adherens junctions (AJ) proteins, thereby restricting the movement of large and hydrophilic molecules between the cells (paracellular), and reducing BBB permeability. This paracellular barrier consists of the interactions between transmembrane proteins such as claudins, occludin and junctional adhesion molecules (JAMs) for TJ, and vascular-endothelial (VE)-cadherin and platelet EC adhesion molecules (PECAM)1 for AJ. In turn these intercellular zipper structures are anchored to the cytoskeleton by adaptor molecules including zona occludens (ZO)-1, ZO2, ZO3 for TJ, and catenins (α, β and p120) for AJ (262, 263). In addition,

EC lack fenestrations and exhibit low levels of pinocytic and transcytotic activity, thereby limiting diffusion of molecules through the cells (transcellular) (264, 265). BBB-EC are highly polarized cells characterized by high efflux transporters on their luminal surface that restrict the passive diffusion of lipophilic toxins into the CNS, and by nutrient transporters that allow the transport of specific nutrients and the removal of specific waste products across the BBB (266, 267). Moreover, they are known to express very low levels of leukocyte adhesion molecules (LAM), thereby restricting the entry of immune cells to the CNS (262, 268). Thus, the barrier properties of BBB-EC further maintain a rigorous control of CNS homeostasis.

Pericytes (PC)

A cellular layer of pericytes (PC) incompletely lines the abluminal surface of EC (269). Nevertheless, CNS-PC provide a high microvascular coverage with an endothelial:pericyte ratio between 1:1 and 3:1 (270). These neural crest derived PC are embedded within the vascular basement membrane and have anchor points with EC via N-cadherin (271). They are able to extend their lengthy processes over several EC which grants them the ability to control capillary diameter and regulate blood flow in response to neural activity (272). In addition to their contractile capacity, PC provide support, guidance and barrier properties during embryogenesis and maintain BBB function during aging (273).

Basement Membranes (BM)

Together, EC and PC are surrounded by a vascular basement membrane (BM) composed of EM molecules, including collagens, laminins (α4 and α5), and heparan sulfate proteoglycans. A second BBB BM, called the parenchymal BM or glia limitans perivascularis, is secreted by astrocytic processes and is characterized by proteins such as laminins (α1 and α2) and dystroglycan (259, 274). The area between the vascular BM and the parenchymal BM is called the perivascular space, and it is where the initial reactivation of CNS invading lymphocytes takes place (275).

Astrocytes

Astrocytes are CNS-specific cells derived from the neuroepithelium of the embryonic neural tube. Historically, they were viewed as structural support to neurons, but today their

function has proven to be essential for regulating the micro-environment of CNS cells. Using a staining for glial fibrillary acidic protein (GFAP) as a cellular marker, one can appreciate their extended polarized cellular processes that can ensheath neural cells and blood vessels (276). As part of the tripartite synapse, astrocytes maintain a bidirectional communication with neurons via gliotransmitters (277). Their strategic position between neurons and blood capillaries ensures a neurometabolic and neurovascular (regulating PC and smooth muscle cells) coupling in an activity dependent manner (278). Moreover, astrocytic endfeet are known to cover over 90–99% of the abluminal surface of CNS micro-vessels. Through the dysroglycan–dystrophin/agrin complex the endfeet are linked to the BM (279). Thus, their close proximity to CNS microvasculature allows them to provide metabolic and functional support to the BBB. They can release essential soluble factors including TGF-β, basic fibroblast growth factor (bFGF), glial-derived neurotrophic factor (GDNF), angiotensinogen, angiopoietin I, and members of the Hedgehog family (280, 281). They have been shown to be crucial for BBB formation and maturation, particularly in establishing barrier properties (282, 283). Astrocytes can also regulate immune responses in the CNS and secrete inflammatory cytokines (281, 284).

The blood-meningeal-barrier (BMB)

The CNS is surrounded by connective tissue coverings, known as meninges, which act as a protective shield to the brain and spinal cord. The meninges consist of 3 main layers; the dura mater (the outermost dense, inelastic sheet that contains a rich vascular network lacking TJ between EC, thus allowing the extravasation of solutes), followed by the leptomeninges: the arachnoid mater (middle barrier), and the pia mater (innermost layer in contact with the CNS)(285). The arachnoid is a translucent avascular membrane that lacks blood vessels. This loose connective tissue is composed of epithelial cells and collagen fibers, and is involved in CSF metabolism via the subarachnoid space (286). Clusters of arachnoid granulations (arachnoid villi) protrude into the dura mater, allowing CSF to exit the CNS and pass into the systemic venous system. Having TJ and efflux pumps, the arachnoid is as an important barrier between the fenestrated vasculature of the dura mater and the CSF (287). Beneath the arachnoid mater is the subarachnoid space, which is filled with CSF. Blood vessels entering or leaving the CNS travel in this subarachnoid space. The pia matter is closely adherent to the surface of the CNS as it follows

its numerous invaginations, surrounding the initial portions of each blood vessel infiltrating the brain. This sheathing creates an interstitial space, the perivascular or Virchow-Robin space, between the pia mater and the vessels. Thus, the pia further regulates exchanges of macromolecules between the CSF and perivascular space (288). The pia mater is composed of epithelial and fibroblast-like cells. Unlike the arachnoid, the pia does not have TJ and lack astrocytic end feet (289).

The blood–CSF barrier

The CNS contains five communicating cavities, called ventricles, which are lined with ependymal cells and filled with CSF. The choroid plexus is a circumventricular organ, localized in the ventricular system, forming an interface between the blood and the CSF. The blood–CSF barrier consists largely of the fenestrated endothelium of the choroid plexus. These EC differ from the BBB-EC in that they lack TJ. The epithelial cells of the choroid plexus, however, are linked together by TJ and specialize in CSF secretion, thereby contributing to brain homeostasis maintenance, intracranial volume adjustment, buffering of extracellular solutes, nutrient supply to CNS cells, as well as limiting access to circulating cells (290, 291).

3.2 Recruitment of immune cells to the CNS

The CNS compartment is regarded as an immunoprivileged site. Although the term "immune privilege" implies a lack of immune response, it is now well established that the immune privilege of the CNS is not absolute, but rather highly regulated. Moreover, studies showed that the CNS has its own lymphatic drainage system and is directly connected to secondary cervical lymph nodes (292, 293). In fact, immune cells constantly circulate through the blood-CNS in order to patrol the CNS environment, process of immune surveillance.

APCs that reside next to CNS barriers are responsible for initiating CNS immune responses during immune surveillance. These specialized immune sentinel cells include DCs, meningeal macrophages, perivascular macrophages, CP macrophages, and microglia (the resident macrophages in the CNS parenchyma) (294-296). Under physiological conditions, a very limited number of lymphocytes can access the CNS through the CP, CSF, meninges, perivascular spaces,

and eventually parenchymal tissue. Regardless of their antigen specificity, only activated T cells expressing the necessary CAMs, chemokine receptors and integrins, may cross CNS barriers and conduct immune surveillance (297-299). The expression of CAMs, chemokine, and chemokine receptors by the CNS barriers is an additional immunological checkpoint that prevents (during homeostatic conditions), or promotes (during inflammation) the penetration of leukocytes to the CNS parenchyma, ventricular and subarachnoid spaces (291). However, in the absence of inflammation, BBB-EC do not express much of the necessary CAMs for activated T cells to enter, thus, immune surveillance happens primarily at the BMB and the blood-CSF barrier (300). In pathological conditions such as MS, CNS barriers are disrupted, resulting in ion dysregulation, altered signaling homeostasis, and infiltration of leukocytes and molecules to the CNS, leading to neuro-axonal degeneration (259).

3.3 Leukocyte migration into the CNS

The structure of blood vessels entering the CNS progresses as follows: leptomeningeal arteries, parenchymal arterioles, capillaries, post-capillary venules, venules, and, veins. Leukocytes reach the CNS either through parenchymal capillaries and post-capillary venules, in order to cross the BBB and access the perivascular space; or through the leptomeningeal arteries and CP, in order to cross the BMB and blood-CSF barrier and access the CSF (280, 301). To cross the EC layer of the barriers, leukocytes use a multistep process of migration that involves their capture-rolling-tethering, adhesion, arrest-crawling, and diapedesis (280, 302, 303) (Figure 6).

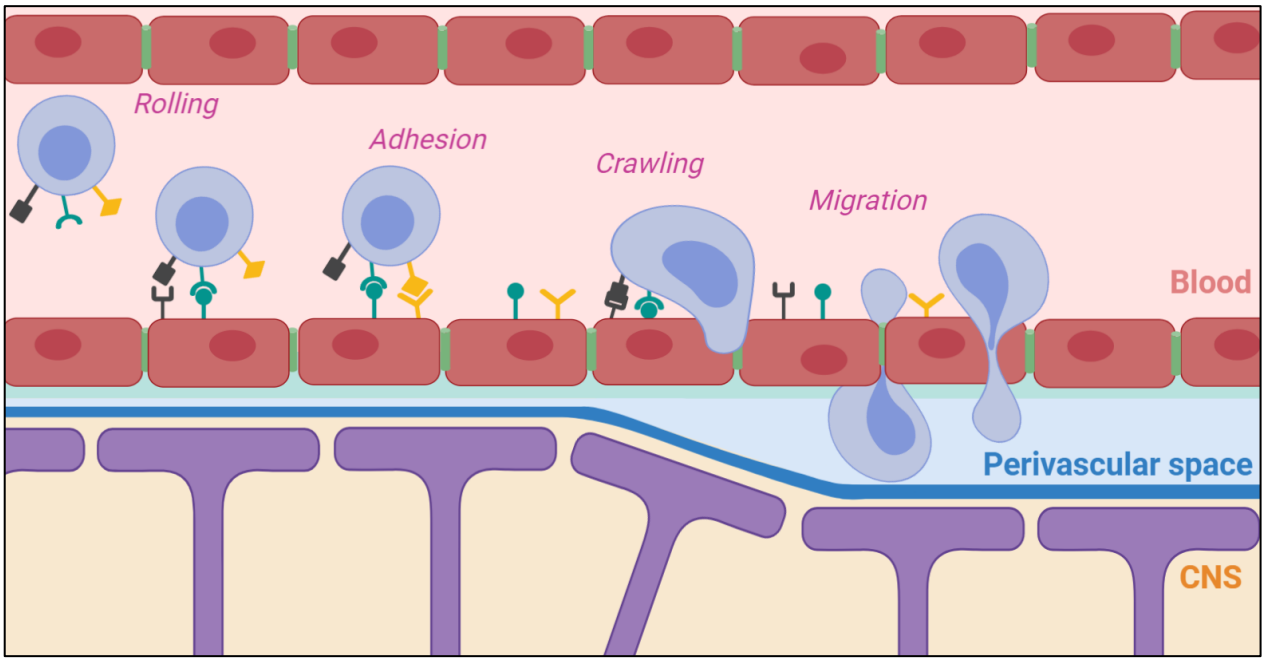


Figure 6. – Schematic representation of the multistep extravasation of leukocytes at the BBB.

Leukocytes tether to BBB-EC through selectin binding, allowing their rolling. Chemokine signaling lead to leukocyte firm adhesion and arrest. Leukocytes led by chemokine signaling extend their protrusions for migration. Following migration, extravasating leukocytes in the periventricular space await their reactivation. Entry into the CNS parenchyma requires the action of matrix metalloproteinases (MMPs) which degrade the BBB. Illustration was created using Biorender.com.

During an inflammatory process, interactions between leukocytes and EC involve selectins, chemokines, cytokines, and CAMs. Leukocytes undergo E- and P-selectin-mediated rolling along the surface of EC (304). EC secrete pro-inflammatory chemokines like CCL2, CCL5, and CXCL10, to recruit monocytes and lymphocytes into the CNS (140, 305, 306). Meanwhile, pro-inflammatory cytokines like IL-17, IL-22, IFN-γ, TNF and GM-CSF, promote the upregulation of pro-inflammatory mediators by EC, as well as the disruption of EC TJ and AJ, thereby weakening CNS barrier properties and facilitating immune cell infiltration (259, 307). The expression of CAMs by BBB-EC, such as intercellular adhesion molecule (ICAM)-1 and vascular CAM (VCAM)-1, and the expression of their cognate ligands on leukocytes, such as αLβ2 (leucocyte function-associated antigen (LFA)-1) and α4β1 (very late antigen (VLA)-4) respectively, are crucial for the firm adhesion of leukocytes on the surface of EC (308-311). This direct interaction EC-leukocytes, leads to the activation and conformational change of integrins, in turn inducing intracellular changes in

leukocytes, enabling them to perform a para- or trans-cellular migration across the BBB (312, 313). Leukocyte diapedesis increases BBB permeability, thus promoting subsequent immune infiltration (314, 315).

In MS, different CAMs were found upregulated on BBB-EC and were reported to play an important role in the transmigration of immune cells across CNS barriers. For example, activated leukocyte CAM (ALCAM) can interact with high affinity with its ligand CD6, and is involved in the recruitment and transmigration of monocytes, CD4 cells and B cells to the CNS (308, 316, 317). Melanoma CAM (MCAM), is also involved in the recruitment of a subset of pathogenic CD4 and CD8 cells to the CNS, whereas Ninjurin-1 promotes the migration of APCs and activated T cells to the CNS (318, 319). Moreover, controversial involvement of Mucosal addressin CAM (MAdCAM-1) in MS and EAE is reported (320-322). The identification of CAMs involved specifically in MS can have a great therapeutic potential in limiting the entry of immune cells to the CNS.

Following migration across EC, immune cells will first have to cross the vascular BM, followed by the parenchymal BM, in order to reach the CNS. In fact, the EM components of the BMs are important in regulating the extent of perivascular infiltration. Studies have demonstrated that vessels expressing high levels of laminin-α4, and low levels of laminin-α5 contain large immune infiltrates, whereas normally vessels express high levels of laminin-α5 and have very low immune infiltrates (323, 324). In MS, the secretion of matrix metalloproteinases (MMPs) highly contributes to BM degradation, thereby facilitating immune cell entry to the parenchyma (325). In the perivascular space, leukocytes need to be reactivated by perivascular APCs to secrete MMPs, destroy the parenchymal BM, overcome astrocytic feet, and finally, infiltrate the CNS (275, 326). In MS, the entry of pathogenic leukocytes to the CNS is an early phenomenon that leads to neuro-inflammation and MS plaque formation (327).

3.4 Role of Melanoma Cell Adhesion Molecule (MCAM)

MCAM Structure, Expression and Localization

The MCAM molecule, also reported as MUC18, S-endo-1, gicerin, HEMCAM and CD146, was initially described as a membrane antigen that increased in expression with melanoma

progression and metastatic potential (328). MCAM is a 113-118 kDa glycoprotein that belongs to the immunoglobulin (Ig) superfamily of proteins. The CD146 gene is found on the q23.3 arm of chromosome 11 in humans, and on chromosome 9 in mice. The molecule consists of 5 Ig domains (2 variable (V) and 3 constant type 2 (C2)) arranged as V-V-C2-C2-C2, a transmembrane domain and a cytoplasmic region (329). Murine MCAM has an amino acid sequence that is 76.2% identical to human MCAM (330). Alternative splicing generates 2 CD146 isoforms that differ in the length of their cytoplasmic domain: A long isoform discovered in human melanocytes (MCAM-l) and a short isoform first identified in chickens (MCAM-s) (331). A soluble form of CD146, that is about 10 kDa smaller than cell-associated CD146, was also identified in endothelial cell culture supernatant (332). This soluble isoform is generated by a metalloprotease-dependent shedding of CD146 extracellular domain, and can be detected in human plasma and serum (333).

In addition to being expressed in melanoma, MCAM was found present in various cancers such as pancreatic, breast, ovarian, and kidney cancers, osteosarcomas, angiosarcomas, Schwann cell tumors and leiomyosarcomas. Currently, the differential expressions and localizations of CD146 isoforms on these cancer cells is not known. However, MCAM overexpression was observed in biopsies of patients and was correlated with a poor prognosis (334). Thus, MCAM increased tumor growth, invasiveness, MMP activity and metastatic potential (335). In healthy adults, MCAM was found to be expressed on bone marrow fibroblasts, blood vessel components (smooth muscle cells, EC, PC), as well as on a subpopulation of activated T cells (336-338). Peripheral vascular EC express MCAM, notably when activated, proliferating, or reaching confluence. MCAM was found highly expressed at EC-intercellular junctions, but outside of TJ and AJ sites (339). Studies showed that MCAM-l has a preferred junctional localization, whereas MCAM-s has a preferred apical position, suggesting different roles for each isoform (340, 341). However, it was shown that MCAM is involved in the rolling, adhesion and migration of leukocytes across vascular EC and in VEGF-induced angiogenesis (341-343) . In fact, MCAM can interact with the actin cytoskeleton to increase leukocytes adhesion. Moreover, it was shown that MCAM is present on approximately 1% of mononuclear leukocytes in the peripheral blood (344). However, upon stimulation, the amount of $CD146^+$ T lymphocytes is increased (338, 345). Furthermore,

MCAM can mediate both homotypic and heterotypic interactions with numerous ligands on leukocytes and EC, such as laminin-α4, VEGFR-2, Wnt5a, Galectin-1, Netrin-1 (346-351).

MCAM involvement in MS

The Prat lab has previously used proteomic techniques in order to uncover new targets in MS. These studies revealed the potential expression and implication of MCAM in MS (308). This led to an in-depth study in 2012 by Larochelle et al. where they identified the expression of MCAM on human BBB-EC, as well as on a subset of human effector memory $CD4^+$ lymphocytes ($CD161^+CCR6^+CD4^+$ lymphocytes) that were highly inflammatory (strong correlation with ROR-γ, IL-23R, IL-17, IL-22, GM-CSF and GrzmB)(311). Specifically, they showed that $MCAM^+$ lymphocytes expressed significantly higher levels of GM-CSF and GrzmB than $MCAM^-$ lymphocytes under inflammatory conditions. Moreover, this population was more abundant in the peripheral blood, CSF and CNS of MS patients and EAE, compared to healthy individuals and controls. In fact, they reported an upregulation of MCAM at the BBB within inflammatory lesions. In EAE, the blockade or depletion of $CD4^+MCAM^+$ T cells restricted the adhesion and migration of Th17 cells across BBB-EC and decreased disease severity. In 2015, another study by Larochelle et al. identified an upregulation of MCAM on circulating $CD8^+$ lymphocytes during MS relapses and on infiltrating $CD8^+$ lymphocytes in MS CNS lesions (352). They showed that these $MCAM^+$ effector lymphocytes ($CD161^+CCR6^+CD8^+$ lymphocytes) express higher levels of IL-17, IFN-γ, GM-CSF and TNF and significantly lower levels of IL-10, IL-4 and FoxP3 than $MCAM^-$ lymphocytes. Moreover*, in vitro* work revealed the enhanced ability of $MCAM^+$ $CD8^+$ lymphocytes to kill human oligodendrocytes compared to $MCAM^-CD8^+$ lymphocytes. As well, *in vivo* blockade of $MCAM^+CD8^+$ lymphocytes in EAE and TCR1640 reduced disease severity. A recent study in 2018 showed that MCAM blockade in EAE and *in vitro* reduced the migration of encephalogenic cells particularly across the CP endothelium (353). Moreover, they detected laminin-α4 on CP endothelium, suggesting that interactions between MCAM-laminin-α4 facilitate the migration of $MCAM^+$ cells to the CNS. These studies conclude that MCAM is an adhesion molecule used by pathogenic T cells to cross CNS barriers, enter the brain, thereby allowing disease progression. Thus, MCAM could be a valuable therapeutic target for treating MS. However, whether MCAM is used by other cell types is yet to be determined.

Chapter 2 - Objectives and Hypothesis

Currently, little is known about how Treg enter the CNS or how they function within the CNS, particularly in the context of MS. Over the years, the Prat lab has demonstrated the contribution of several CAMs, including MCAM, in the migration of pathogenic lymphocytes across the BBB. However, the specific CAMs involved in Treg recruitment remain to be established.

Objective

The main goal of this study is to determine whether MCAM is necessary for the migration of Treg across the BBB, and whether these $MCAM^+$ Treg have higher suppressive capabilities than the non-migrating Treg.

Hypothesis

Treg upregulate MCAM in order to migrate over the BBB into the CNS, after which they could possibly contribute to reducing inflammation in MS. Therefore, Treg migration mechanisms are a potential target for future therapeutic treatments.

Aim 1. Assess the expression of MCAM on Treg in steady state compared to inflammatory conditions

Evaluated the expression of Treg functional markers, chemokine receptors, and cytokine secretion on $MCAM^+$ versus $MCAM^-$ Treg by flow cytometry, in corroboration with qPCR, ELISA and cytospins.

Aim 2. Investigate the migratory potential of MCAM+ Treg in resting and inflammatory conditions

Studied $MCAM^+$ Treg migratory potential across human BBB-EC compared to $MCAM^-$ Treg by using *in vitro* migration assays modeling BBB properties.

Aim 3. Evaluate the expression of MCAM+ Treg in the CNS

Immunofluorescence (IF) and confocal microscopy on brain and spinal cord sections allowed the detection of $MCAM^+$ Treg in the CNS of EAE and TCR^{1640}.

Chapter 3 - Materials and Methods

Donors Classification

Patients were diagnosed and classified into CIS, RRMS, SPMS, and PPMS by certified neurologists from the Centre Hospitalier de l'Université de Montréal – MS clinic, according to McDonald's revised criteria (24). Upon obtaining informed consent from each donor in accordance with institutional guidelines (Centre de Recherche du Centre Hospitalier de l'Université de Montréal research ethic committee approval number BH07.001), blood and CSF samples were collected. Patient's samples were further subgrouped for analysis depending on disease stage, sex, and age. Similarly, blood samples from consenting healthy individuals were obtained. Donors characteristics are described in table 2.

Type	Number	Female	Male	Average age
RRMS	22	18	4	49
SPMS	3	3	0	59
PPMS	2	2	0	49
DMTs	30	26	4	41
HC	31	19	12	32

Table 2. - Description of the characteristics of patients and donors included in the immune-staining experiments.

Human immune cell isolation

Peripheral blood samples were obtained by venous puncture and processed using 1X Phosphate Buffer Solution (PBS) – 2mM ethylenediaminetetraacetic acid (EDTA; Sigma) by density gradient centrifugation on Ficoll-Paque™ (GE Healthcare). A 1800 rotation per minute (rpm) centrifugation for 30 minutes (min) separated different blood cell types according to their density. As a result, a distinct layer of peripheral blood mononuclear cells (PBMCs) can be observed and recollected. These PBMCs were subject to multiple washes with 1X PBS-2mM EDTA buffer, and 2 rounds of centrifugation (1500rpm-10min, 800rpm-10min) for purification. 10µL of the isolated PBMCs diluted in 20mL of 1X PBS-2mM EDTA were then counted in a 1:10 dilution of 0.1X Trypan Blue using a cell count Hemacytometer. Cells ($1x10^6$ PBMCs) were immediately processed for flow

cytometry (referred to as FACS, fluorescence-activated cell sorting), while the rest were put into culture for direct Treg isolation or Th/iTreg polarization.

CSF isolation

CSF samples were collected from untreated MS patients undergoing lumbar puncture (n=4). Following a 1400rpm-10min centrifugation, cells were collected and immediately processed for flow cytometry.

Isolation of human Treg

After PBMC isolation of healthy donors, Treg isolation was performed using $CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell isolation kit II for human (Miltenyi Biotec, 130-094-775) following the manufacturer's protocol. Briefly, using a cocktail of biotinylated antibodies and anti-Biotin MicroBeads, non-$CD4^+$ and $CD127^{high}$ cells were depleted while the cell fraction passed through LD MACS isolation columns in a MACS magnetic field. The flow-through fraction of pre-enriched $CD4^+$ $CD127^{dim/-}$ T cells is labeled with CD25 MicroBeads for subsequent positive selection of $CD4^+$ $CD25^+$ $CD127^{dim/-}$ Treg through MS MACS isolation columns in a MACS magnetic field.

In vitro immune cell polarization (iTreg, Th1, Th2, Th17)

Following PBMC isolation of HC and MS patients, $CD4^+$ T cells were labelled by EasySep™ Human $CD4^+$ T cell Enrichment kit (STEMCELL Technologies, 19052) and isolated using an EasySep™ magnet. MACS buffer (1X PBS, 2mM EDTA, 0.5% Fetal Bovine Serum (FBS)) at 4°C was used. A second isolation using Naïve $CD4^+$ T cell isolation kit II (Miltenyi Biotec, 130-094-131) was performed to separate the naïve $CD4^+$ $CD45RA^+$ T cells from the memory $CD4^+CD45RO^+$ T cells using MACS isolation columns (negative selection) according to manufacturer's protocol. From each fraction $0.5x10^6$ cells were set aside for a FACS purity check staining. Purity was measured by flow cytometry and was greater than 90%.

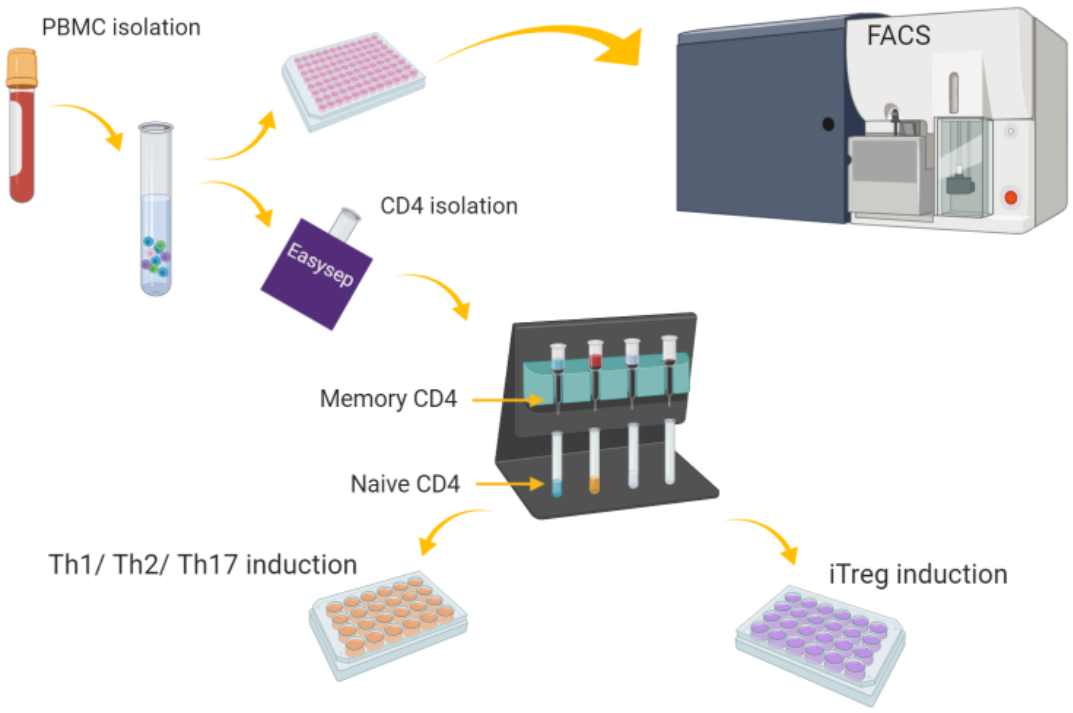


Figure 7. – Schematics of sample processing from PBMC isolation to Th polarization.

Naïve $CD4^+$ T cell fraction

In a 24-well plate (Corning) pre-coated with 5µg/mL α-CD3 (eBioscience, clone OKT3), 1mL of iTreg stimulation mix containing, 1 µg/mL of α-CD28 (BD Biosciences), cytokines, All Trans Retinoic Acid (ATRA) (see table 3) and X-VIVO™ 15 medium (Lonza™) supplemented with 2mM L-glutamine, 100U/mL penicillin, and 100µg/mL streptomycin (all from Sigma), was added per well. $1x10^6$ cells from the naïve CD4 fraction were added into each well and were cultured for 6 days at 37ºC for iTreg polarization. Wells with $1x10^6$ cells and 1mL of α-CD28 (2µg/mL)-X-VIVO™ 15 were also cultured as controls.

iTreg - Stimulation mix in X-VIVO	Concentration	Company
rhTGF-ß1	10 ng/mL	R&D Systems
ATRA (All Trans Retinoic Acid)	20nM	Sigma Aldrich
rhIL-2	200 U/mL	R&D Systems

Table 3. - Mix of antibodies used to polarize naïve $CD4^+$T cells into iTreg.

Memory $CD4^+$ T cell fraction

In a 6 well-plate (Corning) pre-coated with 2.5μg/mL α-CD3 (eBioscience), 1mL of Th stimulation mix (see table 4) containing 2 μg/mL of α-CD28 (BD Biosciences) was added per well. $1x10^6$ cells from this fraction were added into each well and were cultured for 5 days at 37ºC for Th1, Th2, Th17 polarization.

	Concentration	Company
Th1 - Stimulation mix in X-VIVO		
rhIL-12	10 ng/mL	R&D Systems
α-IL-4	5 μg/mL	Bio X Cell
Th2 - Stimulation mix in X-VIVO		
α-IL-12	10 ng/mL	R&D Systems
rhIL-4	200 ng/mL	R&D Systems
α-IFN-γ	5 μg/mL	Bio X Cell
Th17 - Stimulation mix in X-VIVO		
α-IL-4	5 μg/mL	Bio X Cell
α-IFN-γ	5 μg/mL	Bio X Cell
rhIL-23	25 μg/mL	R&D Systems

Table 4. - Mix of antibodies used to polarize memory $CD4^+$T cells into Th1, Th2, Th17.

Following *the in vitro* culture (5 days for the Th1, Th2, Th17 condition, and 6 days for the iTreg condition) cells were collected and harvested. The supernatant of different conditions was saved for future Enzyme-linked immunosorbent assay (ELISA). Cells were counted, divided for immediate FACS staining, and saved for future RNA isolation ($2x10^6$ cells in Trizol reagent (Life Technologies)), Western blot ($8x10^6$ cells in RIPA Buffer (Thermoscientific)), and cytospin slides.

Flow cytometry extracellular and intracellular staining

Samples were transferred to 96-well V-bottom plates for flow cytometry staining. Cells were stained with surface markers against antibodies presented in table 5, for 20 min at 4°C. Fluorochrome-matched isotypes and unstained cells were used as controls. Cell viability was measured with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Life Technologies). Spinning cycles of 2000rpm-3min and 1X PBS-washes were performed. Cells were then fixed and permeabilized for 30 min at 4°C with eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, 00-5523-00), and incubated overnight at 4°C in the dark. The next day, cells were

incubated for 15 min at room temperature in 5% normal mouse IgG serum to avoid nonspecific immunoglobulin binding, and stained for intracellular expression of Foxp3 for 20 min at 4°C. Two washes were performed before resuspending cells in FACS buffer (1% FBS, 0.1% NaN_3 in PBS). Cells were acquired on a LSR II flow cytometer (BD Biosciences), and analysed using BD FACSDiva Software.

For intracellular cytokine staining, cells were re-stimulated for 4 hours with 20ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma), 1μg/mL ionomycin (Sigma) and 2μg/mL Golgi Plug™ inhibitor (BD Biosciences). Cells were first stained for the following surface markers: CD4-PB, CD25-PerCP Cy5.5, MCAM-PE-vio (all from BD Biosciences). Cells were then fixed, permeabilized and incubated overnight as previously described. The next day, cells were incubated for 15 min at room temperature in 5% mix of normal mouse and rat IgG serum. Intracellular staining was performed by incubating cells with antibodies presented in table 6. Cells were treated as previously described before acquisition.

Antibody mix	Fluorochromes	Company
CD3	AF700	BD Biosciences
CD4	PerCP Cy5.5	BD Biosciences
CD8	APC-Cy7	BD Biosciences
CD25	PE-Cy7	BD Biosciences
CD127	PB	BioLegend
MCAM	APC	Miltenyi Biotec
CCR5	FITC	BD Biosciences
CTLA4	BV605	BD Biosciences
CCR6	BV785	BioLegend
CD45RA	FITC	BD Biosciences
CD45RO	APC-Cy7	BD Biosciences

Table 5. - Antibodies used for extracellular staining and purity check.

Antibody mix	Fluorochromes	Company
TGF-ß1	FITC	R&D Systems
IL-10	APC	BD Biosciences
GrzmB	AF700	BD Biosciences
Foxp3	PE	Thermo Fisher Scientific
TNF-α	BV785	BD Biosciences
IL-17a	AF488	Thermo Fisher Scientific

GM-CSF	biotin	BD Biosciences
Streptavidin	605	BD Biosciences
IFN-γ	AF700	BD Biosciences

Table 6. - Antibodies used for intracellular staining.

Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was performed to measure recombinant human IL-10 and TGF-ß1 secreted by iTreg and Th17 polarized cells. To this end, supernatants were collected from the *in vitro* culture and processed according to the manufacturer's instructions: Human IL-10 DuoSet ELISA (R&D Systems, DY217B) and Human TGF-ß1 DuoSet ELISA (R&D Systems, DY240). In a 96-well microplate, 100µL/well of capture antibody diluted in 1X PBS was incubated overnight at room temperature. The next day, plates were blocked by adding 300µL/well of Reagent Diluent (RD) (1% BSA in PBS) for IL-10 conditions, and 300µL/well of buffer (5% Tween 20 in PBS) for TGF-ß1 for 1 hour to ensure the binding of non-specific sites. Prior to this step, TGF-ß1 conditions were activated from latent TGF-ß1 to immunoreactive TGF-ß1 by 1N HCl and neutralized by 1.2N NaOH/0.5 M HEPES. 100µL/well of samples and standards diluted in RD were added and incubated for 2 hours. Next, 100µL/well of Detection Antibody – RD (2 hours), followed by Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase) (20 min) were added to each well to bind the antigen of interest. A Substrate Solution (H_2O_2, Tetromethylbenzidine) and Stop Solution (2N H_2SO_4) were added to allow for detection. Two series of washes were performed between each step using 400µL of Wash Buffer per well (1X PBS, 0.05%Tween 20). The optical density at wavelengths 450nm and 540nm was determined using a plate reader.

quantitative Polymerase Chain Reaction (qPCR)

Cell pellets of $1x10^6$ iTreg and Th17 dissolved in Trizol reagent (Life Technologies) were preserved at -80°C. RNA was isolated from these samples using RNease Mini kit according to manufacturer's protocol (Qiagen). The amount of RNA present in each sample was measured using a nanodrop (Implen). cDNA was then synthesised from 0.5µg RNA using QuantiTect Reverse Transcription Kit (Qiagen). A Veriti 96 Well Thermal Cycler (Applied Biosystems) allowed the cDNA transcription based on the following program: 2 min at 42°C in gDNA Wipeout Buffer (RNeasy Mini Kit - Qiagen) to remove genomic DNA, followed by reverse transcription for 30 min at 42°C

and 3 min at 95°C. Gene expression was determined using primers and TaqMan MGB polarization probes from Thermo Fisher Scientific, labelled with the FAM reporter for FOXP3 (Hs01085834), ROR-γt (Hs01076112_m1), TGF-ß1 (Hs00998133_m1), IL-10 (Hs00961622_m1), IL-12A (Hs01073447_m1) and EBI3 (Hs00194957). Human 18S ribosomal endogenous control (Thermo Fisher Scientific) labelled with the VIC reporter was used to normalize samples. CT values were quantified by qPCR using QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies).

Cytospins - Immunocytofluorescence

iTreg cells obtained from *in vitro* culture were resuspended in 1X PBS and transferred into cytospin wells (375 000 cells/slide). Slides were spun for 2 min. Supernatants were aspirated and slides were fixed in acetone for 20 seconds at room temperature before being stored at -80°C. Immunocytofluorescence was performed on iTreg slides to better assess the expression of IL-10 and TGF-ß1. iTreg cytospins were permeabilized and fixed with cold acetone for 10 min at –20°C and immunostained with goat-anti-IL-10 (1:13.3, R&D Systems) for 30 min at room temperature. Then, a donkey-anti-goat Cy3 antibody (1:400, Jackson ImmunoResearch) was added for 1 hour at room temperature. TGF-ß1 is still being optimized. 0.05% PBS-Tween was used for washes. Slides were incubated with the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma) for 10 min before being mounted (Mowiol reagent, homemade). Slides with only secondary antibody and DAPI were used as negative control. Images were acquired on a Leica TCS SP5 confocal microscope (Leica Microsystems)

BBB-EC isolation and culture

CNS tissue was obtained from patients undergoing epilepsy surgery. Prior to surgery, informed consent from every patient and ethical approval was obtained (Centre de Recherche du Centre Hospitalier de l'Université de Montréal research ethic committee approval number HD04.046). The non-epileptic part of the CNS tissue was processed to isolate Human Brain endothelial cells (HBEC) as previously published (354-356). Briefly, meninges were removed, and specimen was washed with 1X PBS. The specimen was minced, and homogenized before being filtered (Nalgene filtration apparatus, VWR) over 3 different Nitex mesh (Filmar) (first a 350µm mesh, followed by two 112µm mesh) to obtain different fraction sizes. Filters were removed and

fractions were spun at 2200rpm for 15min. Filtrates were treated with collagenase type IV (2mg/ml, Sigma) for 15 min and inactivated with Endothelial Cell Culture Media (ECM). This ECM contained: Medium 199 (Invitrogen) enriched with 20% murine melanoma cell conditioned medium (clone M3, ATCC), 10% FBS (Gibco), 5% human serum (Gibco), 0.2% endothelial cell growth supplement (BD Biosciences) and 0.13% insulin selenium-transferrin premix (Sigma). HBECs were grown to confluence in 0.5% gelatin (Sigma) coated T25 flasks (Corning) in ECM for 3 weeks before being transferred into T75 flasks. After 1 week, HBECs were confluent and passaged into new T75 flasks.

BBB transmigration assay

Transmigration assays were performed using modified Boyden chambers to model the BBB *in vitro*, as previously published (140, 308, 356). Briefly, 3×10^4 HBECs were grown to confluence on gelatin coated 3 µm pore size Boyden chambers (Corning) in ECM supplemented with 40% Astrocyte Conditioned Media (ACM) for 3 days. After 48h, HBECs were stimulated with 200 U/ml TNF and 100 U/ml IFN-γ (Invitrogen) (inflamed) or ECM-ACM alone (rested). On day 3, iTreg cells cultured *in vitro* were harvested and separated into $MCAM^+$ iTreg and $MCAM^-$ iTreg using anti-CD146-APC and anti-APC magnetic beads (Miltenyi Biotec, 130-090-855) on MACS isolation columns (positive selection) according to manufacturer's protocol (mean purity over 95% was verified by FACS). One million cells of each population were added to the upper chamber and allowed to migrate for 18 hours. Cells in the upper chambers and those that migrated to the lower chambers were collected, counted, and stained for FACS. Each condition was performed in triplicates for every donor.

Active EAE disease induction and scoring

Dr. Stephanie Zandee and Marc Charabati induced active EAE in 6 to 10-week old female wildtype C57BL/6 mice procured from Charles River Laboratories (Montreal, QC, Canada). All animal protocols were conducted in accordance with the Canadian Council on Animal Care and institutional guidelines (Centre de Recherche du Centre Hospitalier de l'Université de Montréal animal care committee approval N11023APs). Briefly, mice were immunized subcutaneously with 200µg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; Alpha Diagnostic International) in

100µl emulsion of complete Freund's adjuvant (CFA) with 4 mg/mL Mycobacterium tuberculosis (Fisher Scientific). On day 2, an intraperitoneal injection of Pertussis toxin (500 ng PTX, Sigma) was administered. A score reflecting disease severity was daily attributed on a scale of 0 to 5: 0 = no clinical symptoms; 1 = limp tail; 2 = slow righting-reflex; 2.5 = ataxia; 3 = hind limb monoparalysis; 3.5 = hind limb monoparalysis and severe weakness in the other hind limb; 4 = hind limbs paraparalysis; 4.5, hind limb paraparalysis and forelimb weakness; 5 = moribund. Based on disease course, mice were sacrificed on specific days after post-immunization (p-i): on day 9 p-i, onset mice were sacrificed (score 2); on day 14 p-i, peak mice were sacrificed (score 4), on day 15 p-i, remission mice were sacrificed (score 3); on day 31 p-i, chronic mice were sacrificed (score 2.25). CNS tissue was harvested for histology and immunofluorescence staining.

In addition, spleen and spinal cord sections from TCR^{1640} transgenic mice from our lab's mouse biobank were used. This mouse model expresses a T-cell receptor (TCR)-specific for $MOG_{92–106}$, which were kindly provided by the group of Dr Wekerle.

Cryo-sectioning

Brain and spinal cord EAE tissues were embedded in optimal cutting temperature compound (OCT) (Tissue-Tek) and frozen on dry ice before transferal to -80°C. Using a Leica CM3050S cryostat (Leica Microsystems), tissue blocks were cut into 10 µm sections, mounted on Superfrost slides (Thermo Fisher Scientific), and left drying overnight at room temperature. The next day, slides were prefixed for 20 seconds with acetone before being wrapped in aluminium foil and stored at -80°C.

Histology of frozen EAE sections

In order to identify EAE lesions and areas of demyelination, Luxol Fast Blue and Haematoxylin and Eosin (LHE) stainings were performed on EAE brain and spinal cord sections. Sections were fixed with 10% formaldehyde (Fisher Scientific) for 3 hours at room temperature, and washed in 1X PBS for 3 min. Then, sections were incubated in Luxol Fast Blue solution (1% Solvan Blue 38 (Sigma), 95% ethanol and 0.05% glacial acetic acid (Fisher Scientific)) for 2 hours at 60°C. After cooling down for 15 min, excess stain was removed by 95% ethanol for 1 minute. Sections were washed under running tap water and then differentiated with 1 dip in Luxol

differentiator (0.4% hydroquinone (Sigma), 5% sodium sulphite (Sigma)). Sections were placed in Harris Haematoxylin (Sigma) for 5 min for nuclear staining, and then differentiated with 1 dip in Haematoxylin differentiator (1% hydrochloric acid in a 70% ethanol solution). The sections were dipped in 0.05% NaOH and dehydrated in 95% ethanol for 1 min before using Eosin Y for 30 seconds to stain the cytoplasm. Lastly, slides were immersed in successive baths of 95% and 100% ethanol and were cleared in toluene (Chaptec) for 5 min before being mounted in Entallan medium (Millipore). Thus, nuclei were stained in purple, cytoplasm in pink, and myelin in blue, allowing identification of lesions. Images were acquired on a Leica DM4000B microscope (Leica Microsystems) and processed on the Leica Application suite V3 program (Leica Microsystems).

Immunofluorescence (IF) staining of CNS mouse

Frozen mouse CNS sections from active EAE and TCR^{1640} were processed similarly. Slides were fixed in cold acetone for 10 min at -20°C, then transferred to 70% ethanol for 5 min at -20°C, before being hydrated in 1X PBS for 3 min at room temperature. A PAP-pen (Dako) was used to draw a hydrophobic barrier around each section. First, sections were blocked with streptavidin/biotin endogenous blocking kit (Invitrogen, E21390), and then blocked for nonspecific immunoglobulin binding with 10% serum of the secondary antibody host species for 30 min at room temperature. Primary antibodies (table 7) diluted in 3% serum of the secondary antibody host species were incubated overnight at 4°C. The next day, sections were washed 3 times for 5 min with 0.05% PBS Tween and incubated with secondary antibodies (table 7) for 40 min at room temperature in a humidity chamber.

For the 4-color Treg staining in EAE, primary antibodies with their respective secondary antibodies were incubated sequentially as presented in table 8. The use of a Tyramide Superboost™ kit (Invitrogen, B40922) allowed the detection of weakly visible targets (MCAM) by providing signal amplification using the catalytic activity of horseradish peroxidase (HRP) enzymes conjugated with short polymers (poly)(providing an additional signal enhancement). The kit enables the combination of AlexaFluor (AF) dyes with poly-HRP-mediated tyramide labeling, in which the reaction generates a greater signal. On the first day of staining, MCAM rabbit anti mouse (1:200) antibody was incubated overnight. The next day, endogenous peroxidase was

blocked for 15 min using a 0.3% HRP solution from the Tyramide Superboost kit before adding a goat anti rabbit poly-HRP-conjugated secondary antibody for 1 hour (from the Tyramide Superboost kit). After washing 4 times for 5 min with 0.05% PBS Tween, 100uL per slide of tyramide working solution (containing tyramide-AF488) was added for 5 min and blocked using reaction stop reagent working solution to halt the HRP reaction, as per the kit's instructions. Since PB emits its fluorescence out of the range of the visible scope (maximum detection at 455 nm), CD4-Pacific Blue (PB) rat anti-mouse (1:70) primary antibody was used followed by the secondary antibody donkey anti rat AF647 in order to better detect the staining in a visible channel (594 nm - 633 nm). Sections were then blocked with 10% rat serum for 1 hour before being incubated with Foxp3-biotin rat anti mouse (1:20) followed by a 1-hour incubation with streptavidin (SA)-Cy3. The use of biotin-SA provides an additional amplification of the Foxp3 signal detection (table 7 – 8).

For the 5-color proof of concept staining in EAE, an additional 15 min blocking step with 10% goat serum followed by an incubation with anti-Laminin for 1 hour and its secondary antibody were done on the third day.

Before being mounted, slides were washed with 0.05% PBS Tween and 1% Triton-X, and were incubated with the appropriate nuclear counterstain TOPRO-3 (1:300, Invitrogen) or DAPI (1:1000, Sigma) diluted in 1X PBS for 10 min, then mounted with Mowiol mounting reagent. For negative control sections, primary antibodies were omitted, and for single stain sections, nuclear counterstains were omitted. Images were acquired on a Leica TCS SP5 confocal microscope (Leica Microsystems) with a 40X/1.4 oil immersion objective or a Leica TCS SP5 MP confocal microscope with a 63X/1.4 oil immersion objective. Images were analyzed by Leica Application Suite Advanced Fluorescence (Leica Microsystems) and ImageJ software.

Primary Antibodies	Dilution	Host	Company
CD4-FITC	1:20	rat	BD Biosciences
CD4-PB	1:70	rat	BD Biosciences
Foxp3-biotin	1:20	rat	e-bioscience
CD6	1:10	goat	R&D Systems
MCAM	1:200	rabbit	Abcam

CD25	1:50	goat	Invitrogen
Laminin	1:500	rat	Dako
GFAP-Cy3	1:750	mouse	Sigma
MHC II	1:30	rat	BD Biosciences

Secondary Antibodies	Dilution	Company
Strepavidin-Cy3	1:1500	Jackson ImmunoResearch
donkey anti goat AF488	1:350	Jackson ImmunoResearch
donkey anti goat	1:500	Invitrogen
goat anti rabbit AF594	1:200	Invitrogen
donkey anti rat AF647	1:200	Jackson ImmunoResearch

Table 7. - List of primary and secondary antibodies used for IF.

Time	Overnight	15 min	1 hour	5 min	1 hour	45 min	1 hour	1 hour	1 hour
Antibodies	MCAM	Block HRP	Goat poly-HRP	Tyramide AF488	CD4-PB	Donkey anti rat AF647	Block 10% rat serum	Foxp3-biotin	SA-Cy3

Table 8. - Optimized IF staining protocol of 4 markers (MCAM, CD4, Foxp3, DAPI) on EAE spinal cord sections.

Representation of the sequential order of antibody incubation with time.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. Paired or unpaired Students'*t*-test, as well as one-way analysis of variance (ANOVA) statistics with post-test Turkey were performed when appropriate. Results are presented as the mean ± standard error of the mean (SEM). Only p values < 0.05 were considered statistically significant.

Chapter 4 - Results

Expression of $CD4^+CD25^{high}CD127^{low}Foxp3^+$ Treg cells and increased frequency of $MCAM^+$ Treg in RRMS

Our first objective was to detect the presence of Treg and assess the expression of MCAM on Treg in healthy controls (HC) compared to MS patients. First, PBMCs were isolated from HC and MS patients at different disease stages by gradient density centrifugation using Ficoll™ , and then were immuno-stained for flow cytometry processing. Treg cells were identified by FACS using the gating strategy described in Figure 8. As such, in this thesis, $CD4^+CD25^{high}CD127^{low}Foxp3^+$ T cells detected by FACS from freshly isolated PBMCs and CSF cells will be referred to as Treg or *ex-vivo* Treg, in contrast with the naïve $CD4^+$ induced cells, referred to as iTreg.

A preliminary study in our lab revealed similar levels of $Foxp3^+$ T cells (about 5% $CD25^{high}$ $CD127^{low}$ $Foxp3^+$ cells of CD4 T cells) between HC, untreated MS patients (RRMS, PPMS, SPMS) and treated MS patients (DMT) in the peripheral blood (Figure 9A). Thus, Treg levels are maintained during different diseases phases of MS. We then looked whether those cells expressed MCAM by using an MCAM isotype control to position the positive and negative MCAM selection gate on the Treg population (Figure 8). We detected significantly higher levels of $MCAM^+$ Treg in untreated RRMS patients (mean $MCAM^+$ Treg from CD4 = 0.5%; mean $MCAM^+$ cells from Treg = 9.4%) compared to HC (mean $MCAM^+$ Treg from CD4 = 0.3% ; mean $MCAM^+$ cells from Treg= 7.0%) (Figure 9B-C). There were no significant $MCAM^+$ Treg expression in PPMS, SPMS or DMT groups compared to the RRMS group. Consequently, we decided to focus our study of MCAM expression on Treg in HC compared to untreated RRMS patients.

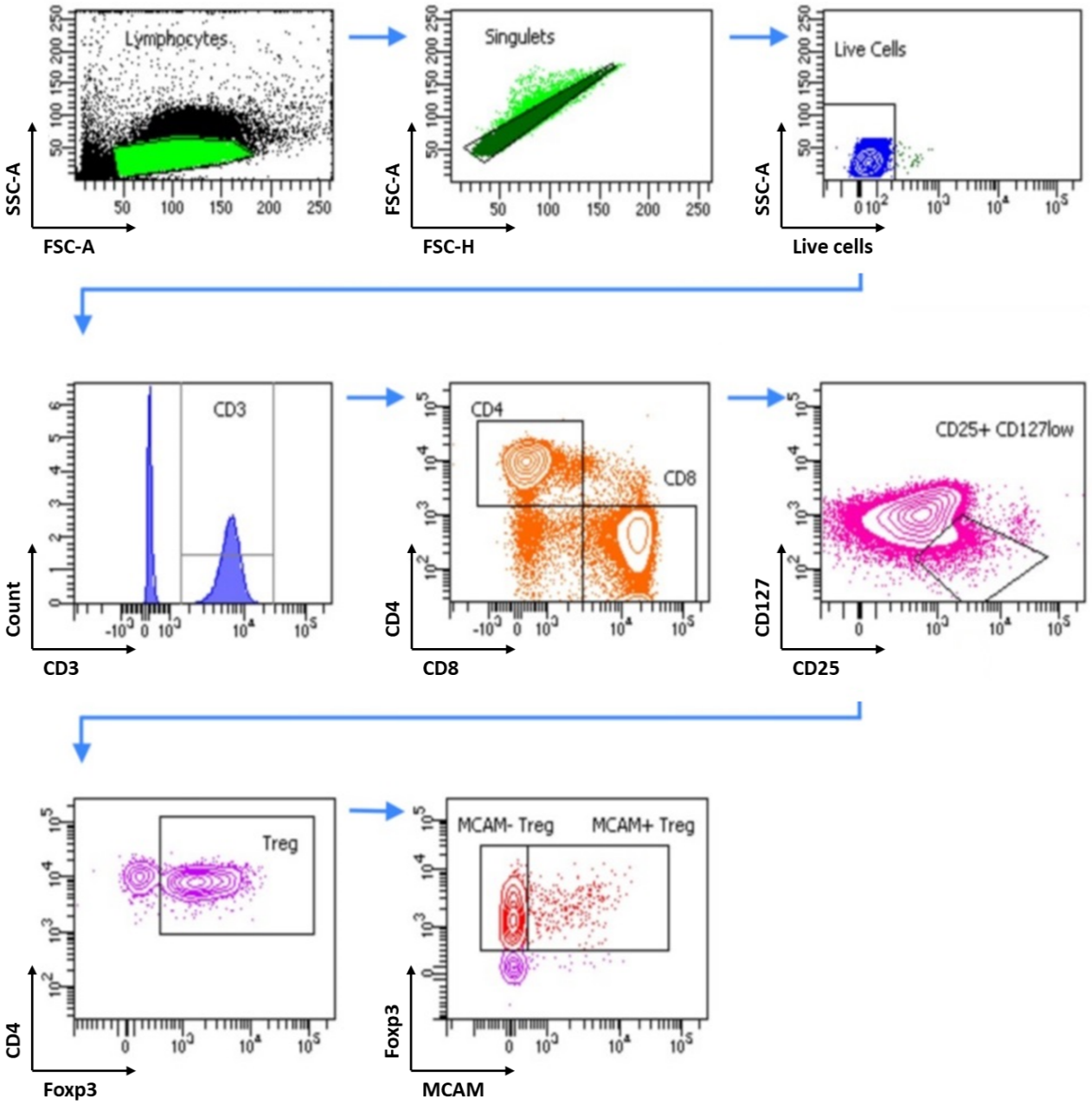
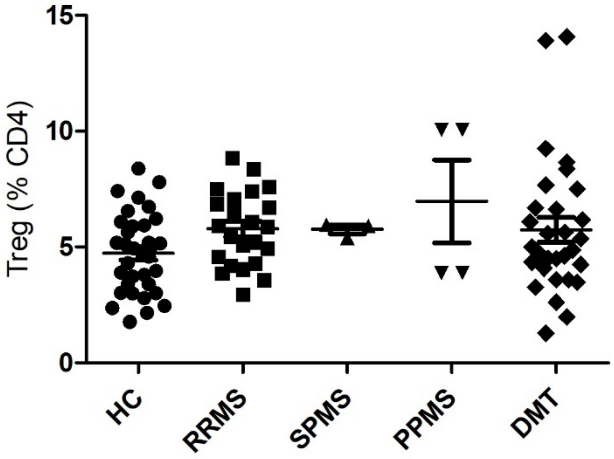
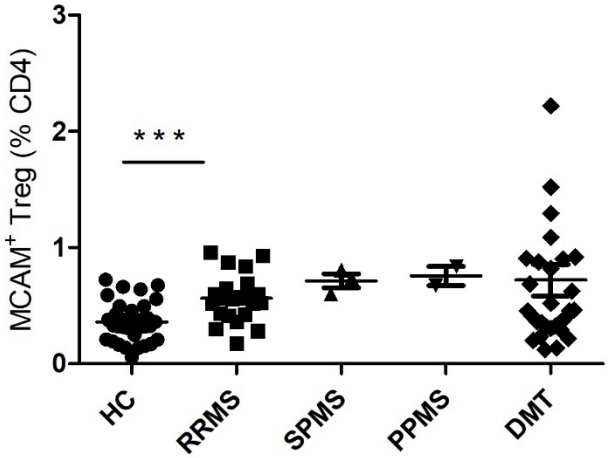


Figure 8. – Gating strategy for identifying the $CD3^+$ $CD4^+$ $CD25^{high}$ $CD127^{low}$ $Foxp3^+$ T cell population representing the population of interest, Treg, and further determining $MCAM^+$ Treg and $MCAM^-$ Treg by flow cytometry analysis (FACS).

First, lymphocytes are selected according to their characteristics of FSC and SSC. Then, single cells (singlets), and living cells are conserved using a scatter gate procedure. From these, we selected the $CD3^+$ lymphocytes cells and identified the CD4 population. From the CD4 population, we gated on the $CD25^{high}$ and $CD127^{low}$ cells. From the $CD3^+$ $CD4^+$ $CD25^{high}$ $CD127^{low}$ T cells, those that are $Foxp3^+$ were retained and

considered as Treg cells in this project. In order to determine the MCAM^{+} versus MCAM^{-} Treg populations, an MCAM isotype control was used to position the positive and negative selection gate.





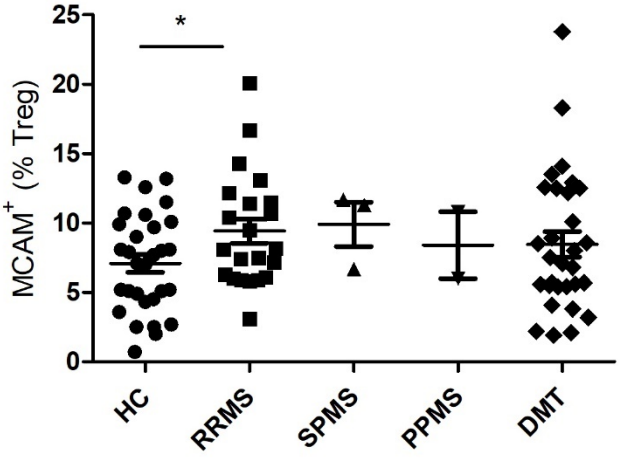


Figure 9. – Expression of $CD25^{high}$ $Foxp3^{+}$ CD4 T cells and $MCAM^{+}$ Treg from peripheral blood (PBMCs) of healthy individuals (n=31) and MS patients at different disease phases (n=2-30 per group).

(A) Percentage of CD4 cells positive for CD25 and Foxp3 population from PBMCs of HC and MS patients. (B) Percentage of Treg positive for MCAM from PBMCs of HC versus different phases of MS. Combined data in collaboration with Dr. Stephanie Zandee. HC = healthy control, RRMS = untreated relapsing-remitting MS, PPMS= primary progressive MS, SPMS = secondary progressive MS, DMT = RRMS patients receiving disease modifying therapy. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

Since studying Treg expression in PBMCs is not completely representative of what is happening in the CNS, CSF samples were used as an indication of the circulating CNS environment. When available, CSF samples from untreated RRMS patients were collected, processed, and analyzed by FACS according to the same gating strategy described in Figure 8. PBMC and CSF samples from the same untreated RRMS patients were matched and compared. Treg functional markers, CTLA-4, CCR6, and CCR5, were reported in the literature and were chosen to assess Treg functionality in PBMC versus CSF samples of untreated RRMS patients (Figure 10A). We observed a general increase of CTLA-4, CCR6 and CCR5 levels in CSF-Treg versus PBMC-Treg, suggesting that functional Treg might be present in the CNS of MS patients and could potentially exert their suppressive function in the CNS. To determine whether these Treg prefer the use of MCAM for their migration across CNS barriers, we investigated the expression of $MCAM^{+}$ versus $MCAM^{-}$ Treg in PBMCs compared to CSF (Figure 10B). We observed a trend for a higher frequency of $MCAM^{+}$ Treg in the CSF compared to the peripheral blood of untreated RRMS patients, suggesting that MCAM might be important for Treg homing to the CNS. However, data from some patient samples revealed a decrease in the expression of MCAM on Treg which could be explained by interpatient variability and the early stage of disease at which the CSF samples were processed. Moreover, increased expression of CTLA-4, CCR6 and CCR5 was also detected in $MCAM^{+}$ Treg versus $MCAM^{-}$ Treg which was significant in Treg from PBMC, and showed a trend but did not reach significance in Treg from CSF (Figure 10B).

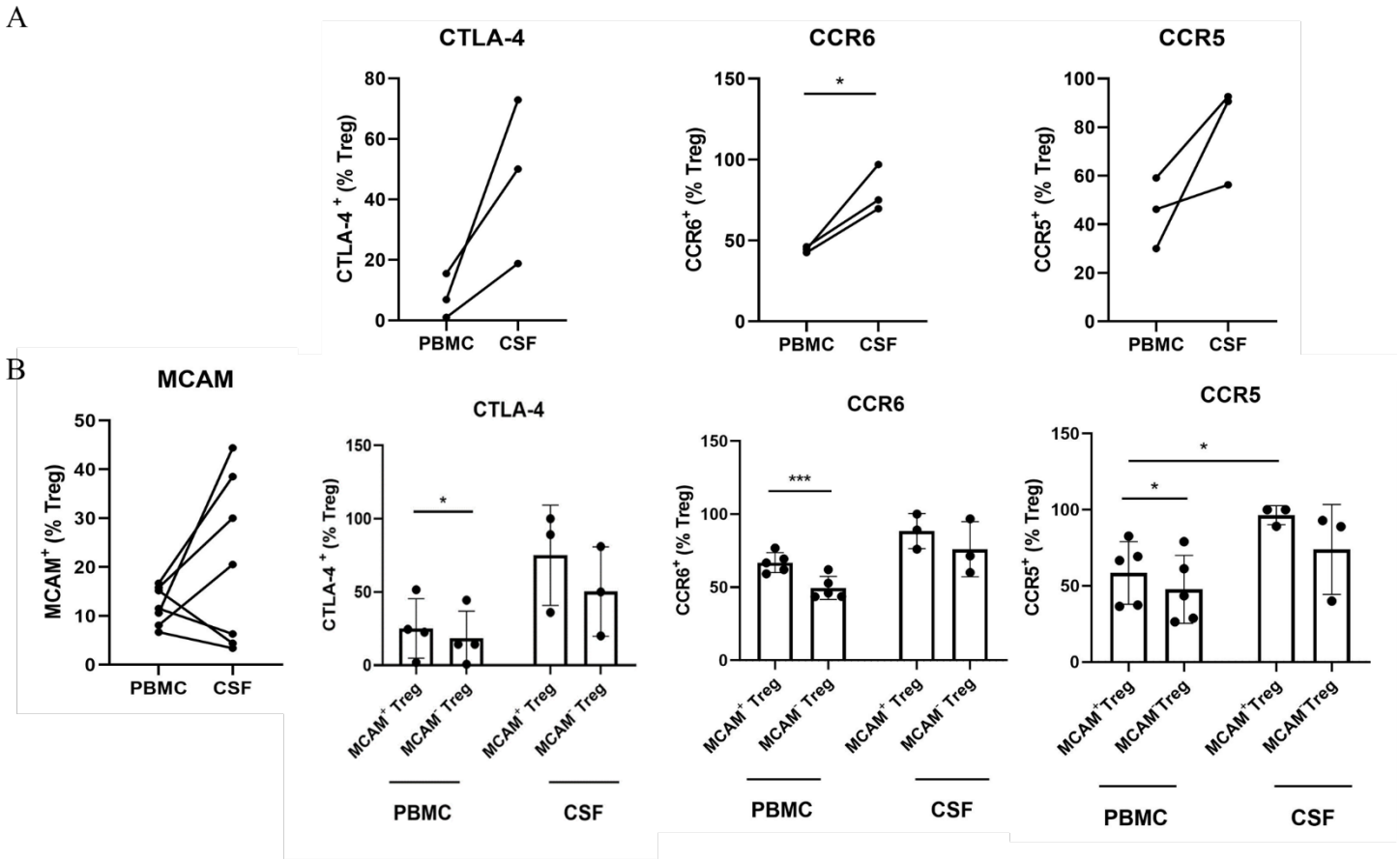


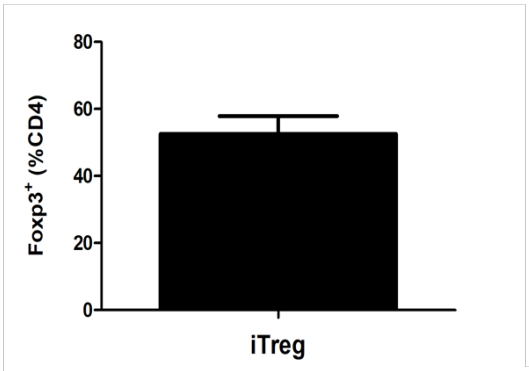
Figure 10. – Expression of Treg functional markers CTLA-4, CCR6, and CCR5 in peripheral blood (PBMCs) versus cerebrospinal fluid (CSF) of untreated RRMS patients.

(A) Percentage of Treg expressing CTLA-4, CCR6, CCR5 in PBMC compared to CSF (n=3). (B) Percentage of $MCAM^+$ versus $MCAM^-$ Treg expressing CTLA-4, CCR6, CCR5 in PBMCs versus CSF (n=3-7). All plots are presented as mean standard error of the mean. "*" $p < 0.05$.

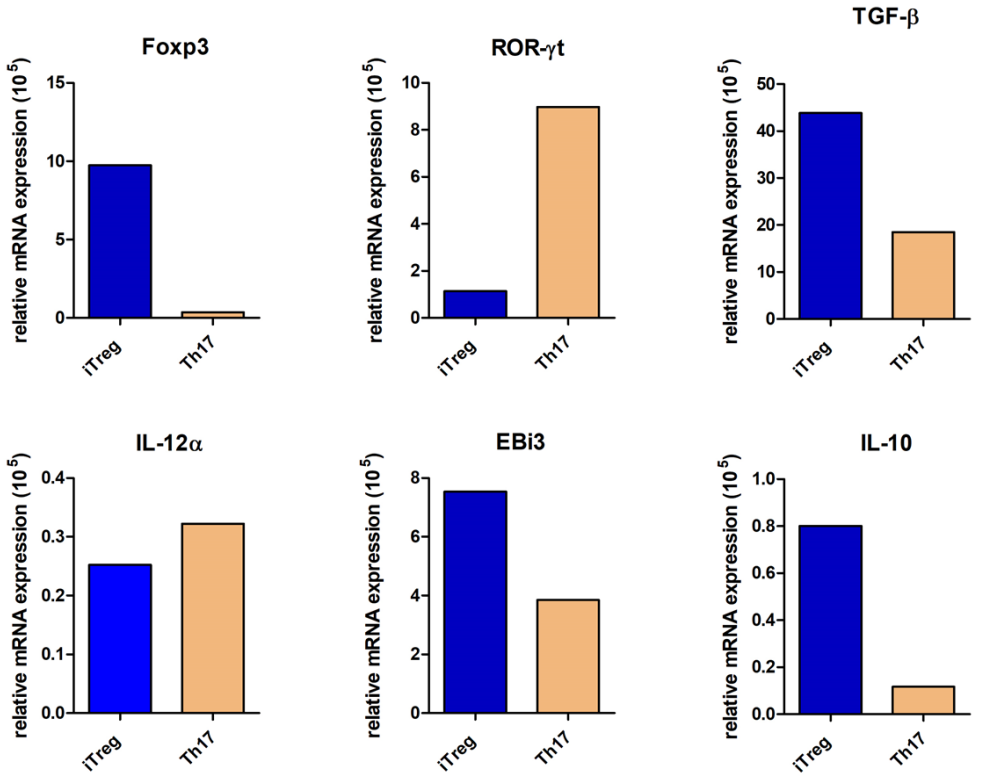
Modified expression of Treg functional markers in iTreg population

One of the main challenges in working with Treg is the low number of cells retrieved from the peripheral blood (357). In order to investigate Treg migratory potential via functional assays (e.g. migration assay, flow adhesion assay), additional number of cells were needed (> $1x10^6$/condition). In fact, when we tried sorting Treg from the peripheral blood using magnetic beads from the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell isolation kit (Miltenyi Biotec, 130-094-775), we obtained 0.215 x 10^6 isolated Treg cells from a total of $215x10^6$ PBMCs isolated from 12 tubes of blood of HC. The low cell count was not enough to proceed with functional assays. Thus, it was first necessary to optimize the culture of Treg *in vitro* to obtain higher numbers of $CD4^+$ $CD25^{high}$ $CD127^{low}$ $Foxp3^+$ T cells. From freshly isolated PBMCs, naïve $CD4^+$ $CD45RA^+$ T cells and memory $CD4^+CD45RO^+$ T cells were sorted using magnetic beads. By putting in culture the naïve $CD4^+$ $CD45RA^+$ T cells in a plate coated with α-CD3, in the presence of α-CD28, TGF-ß1, IL-2, ATRA, and X-VIVO medium for 6 days, we generated an iTreg cell population. We obtained around a 60% purity of $Foxp3^+CD4^+$ iTreg cells (Figure 11A), in line with the iTreg published literature (358). In the same way, we cultured the memory $CD4^+CD45RO^+$ T cells and used different cytokines and antibody mixes to induce the polarization of Th1, Th2, and Th17 cells as per previous published protocols in the Prat lab. These were used as controls to compare the phenotypes of different cell populations. With qPCR we detected mRNA expression of transcription factors (Foxp3 and ROR-γt) and cytokines (IL-10, TGF-ß, and IL-35 compromised of EBi3 and IL-12α) in HC iTreg and Th17 cells. The mRNA expression of Foxp3, IL-10, TGF-ß, and EBi3 by iTreg cells confirmed their Treg-like phenotype in contrast to Th17 cells that had higher expression of ROR-γt and IL-12α (Figure 11B). In addition, we detected IL-10 expression on iTreg cells by immunocytofluorescence analysis (Figure 11C), further characterizing iTreg phenotype.

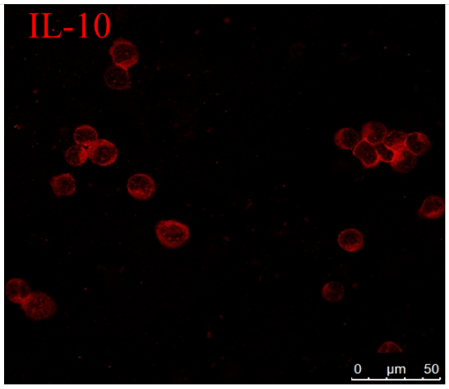
A


Foxp3+ (%CD4)
80
60
40
20
0
iTreg

B


Foxp3
ROR-γt
TGF-β
IL-12α
EBi3
IL-10
relative mRNA expression (10 5)
iTreg
Th17

C


IL-10
0 µm 50

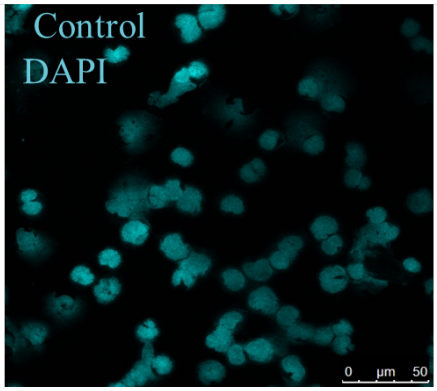

Control
DAPI
0 µm 50

Figure 11. – Confirmation of iTreg purity and phenotype.

(A) Treg were isolated and cultured *in vitro* for 1 week to generate iTreg population (*in vitro* induced Treg). After culture, around 60% of iTreg were Foxp3^{+} cells. (B) Relative mRNA expression of Foxp3 and ROR-γt in iTreg versus Th17 (n=1) by qPCR. (C) iTreg expression of IL-10 optimized by immunocytofluorescence (n=1).

In order to determine whether iTreg cells are capable of producing and releasing IL-10 and TGF-ß1, we measured the concentration of these cytokines in the supernatant of HC and RRMS-iTreg collected after 6 days of culture by ELISA. We were able to detect IL-10 in the iTreg supernatant of HC with an increased concentration in the iTreg supernatant of RRMS (IL-10 DuoSet ELISA, R&D Systems, DY217B) (Figure 12). Unlike other cytokines that are produced in an active form, TGF-ß is a precursor protein that is synthesized in a latent form and must be converted to its active form to bind to its receptor. We performed TGF-β1 ELISA on samples with latent TGF-β1 activation (ac) and without latent TGF-β1 activation (not ac). To activate latent TGF-β1 to immunoreactive TGF-β1 we used acid activation and neutralization solutions as described by the TGF-β1 DuoSet ELISA kit (R&D Systems, DY240). Since we add TGF-ß1 in the culture mix to induce iTreg polarization, we measured control samples that only contained iTreg stimulation mix (α-CD3, α-CD28, TGF-ß1, IL-2, ATRA, and X-VIVO) with the TGF-β1 ELISA kit and subtracted the mean concentration of TGF-ß1 detected in ac and not ac control samples to the mean TGF-ß1 concentration detected in iTreg supernatant samples. We detected higher concentrations of not ac TGF-ß1 than ac TGF-ß1 samples in iTreg HC and RRMS supernatants. Moreover, higher levels of TGF-ß1 were measured in RRMS samples than HC samples. Altogether, the data suggest that iTreg cells from HC and RRMS can produce and secrete anti-inflammatory cytokines.

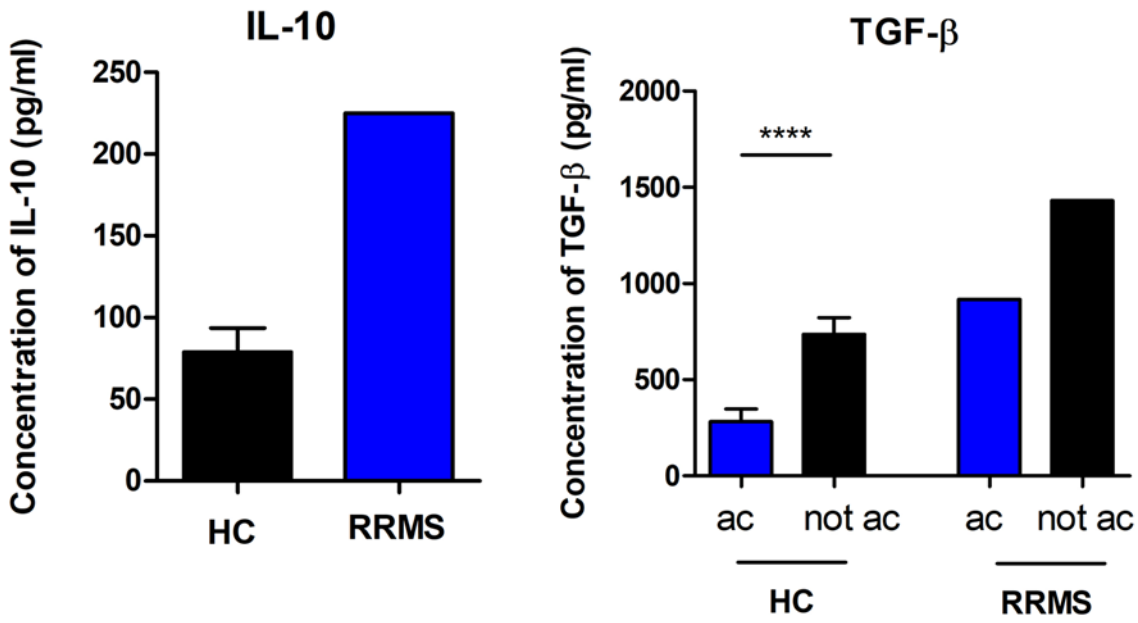


Figure 12. – Concentration of IL-10 and TGF-ß1 measured by ELISA in iTreg supernatant of HC (n=4) and RRMS (n=1).

TGF-ß1 ac = activated form, TGF-ß1 not ac = not activated form. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

Treg have higher expression of CTLA-4, CCR6, and CCR5

In order to investigate iTreg functionality, FACS immuno-staining for CTLA-4, CCR6, and CCR5 was performed to further assess differences in Treg phenotypic expression between HC and RRMS iTreg, as well as between *ex-vivo* Treg and iTreg (Figure 13). Unpaired t test was used to evaluate statistical differences between HC and RRMS groups. We observed similar expression of CTLA-4, CCR6, and CCR5 in PBMC derived *ex-vivo* Treg between HC and RRMS groups, hinting to the maintained functionality of Treg in RRMS (Figure 13A). In the iTreg population, there were no significant changes in CTLA-4 and CCR5 expression between HC iTreg and RRMS iTreg. However, CCR6 seems to be significantly more expressed by HC iTreg than by RRMS iTreg (Figure 13B), suggesting that some differences in iTreg derived from $CD4^+$ $CD45RA^+$ T cells from RRMS patients and HC exist. To compare the expression of functional makers on *ex-vivo* Treg (Figure 13A) versus iTreg (Figure 13B), ANOVA statistics with post-test Turkey were performed (not represented on graphs), with a significance level measured at 95% confidence intervals. A significant increase in the levels of CTLA-4 and CCR6 of HC iTreg was detected compared to HC *ex-vivo* Treg. There were no significant changes in CCR5 levels between HC *ex-vivo* Treg and iTreg. Furthermore, RRMS *ex-vivo* Treg and RRMS iTreg revealed similar expression of functional markers. Taken together these results demonstrate that iTreg is a reliable alternative to expand and study the Treg population *in vitro*.

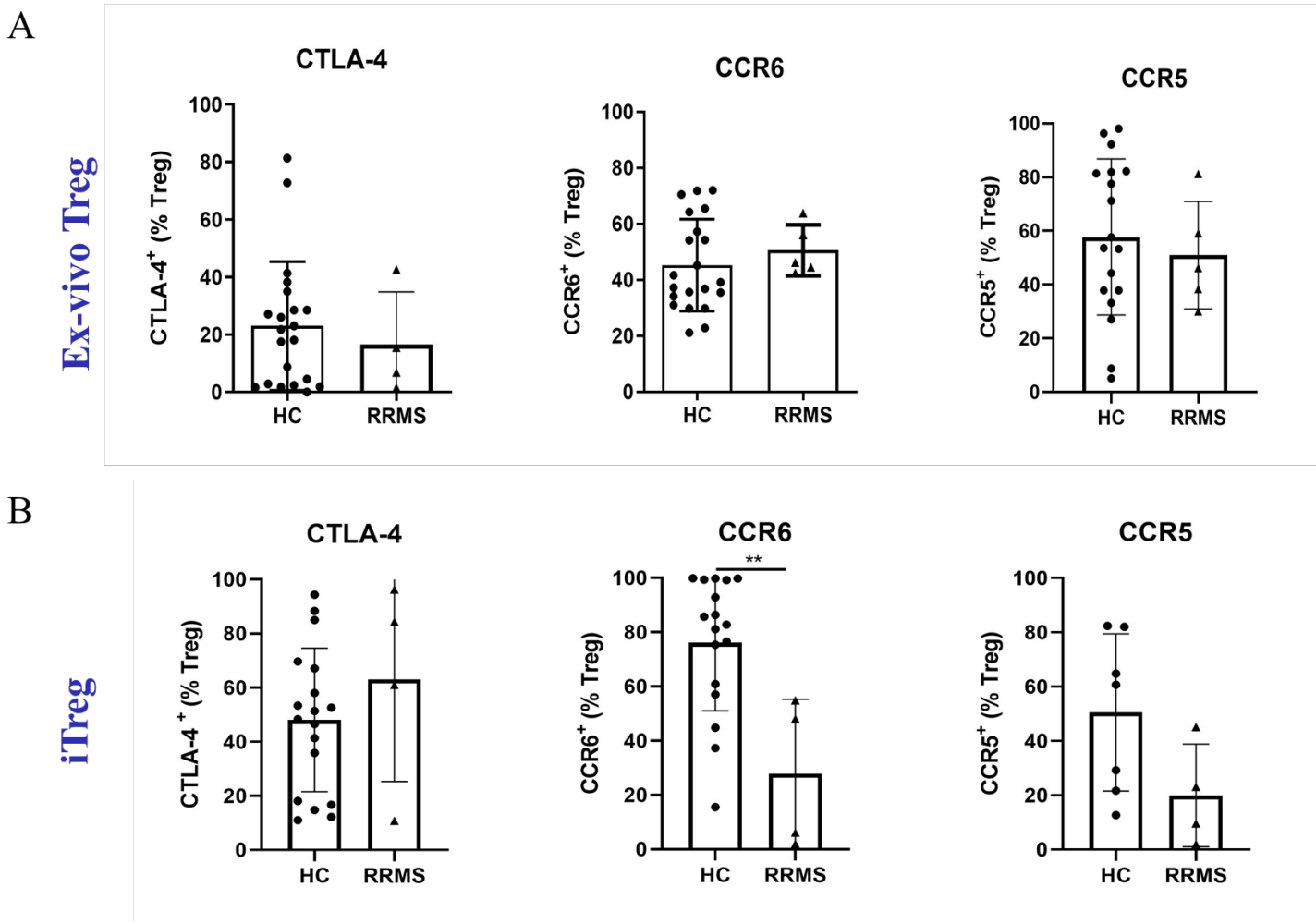


Figure 13. – Expression of Treg functional markers CTLA-4, CCR6, and CCR5 in *ex-vivo* Treg and iTreg in HC (n=21) and untreated RRMS (n=4) by FACS.

(A) Similar expression of CTLA-4, CCR6, and CCR5 in PBMC derived *ex-vivo* Treg between HC and RRMS. (B) Modified expression of CTLA-4, CCR6, and CCR5 in CD4+ CD45RA+ T cells derived iTreg between HC and RRMS. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

MCAM$^+$ Treg have increased expression of CTLA-4, CCR6, and CCR5

We further characterized the expression of MCAM on *ex-vivo* Treg versus iTreg from HC and RRMS. We found that MCAM levels were significantly increased in iTreg cells compared to *ex-vivo* Treg in both HC and RRMS groups, suggesting that the activation of cells in culture conditions for a week without a specific antigen could enhance the expression of cellular adhesion molecules on the surface of cells (Figure 14A).

Our goal is to determine whether these MCAM$^+$ Treg, which may have a preferred migratory potential, express higher functional markers than MCAM$^-$ Treg. We determined that MCAM$^+$ HC *ex-vivo* Treg express significantly higher levels of CTLA-4, CCR6 and CCR5 than their MCAM$^-$ counterparts (Figure 14B). Likewise, MCAM$^+$ RRMS *ex-vivo* Treg express significantly higher levels of CTLA-4 and CCR5 than their MCAM$^-$ counterparts, as well as an increased trend of higher CCR6 levels than MCAM$^-$ RRMS *ex-vivo* Treg (Figure 14B). In addition, similar trends are observed in the iTreg population, where MCAM$^+$ HC and RRMS iTreg significantly upregulate CTLA-4 and CCR5, with a slight increase in CCR6 expression compared to their respective MCAM$^-$ counterparts (Figure 14C). These results imply that MCAM$^+$ Treg, which may be better equipped to migrate, may also be better at exerting their suppressive functions.

It is worth noting that MCAM$^-$ Treg, although in lower levels than MCAM$^+$ Treg, still expressed Treg functional markers. We further investigated the possibility of these Treg being involved in MS by looking at the expression of other CAMs (CD6, ALCAM, CD49d) on Treg from PBMCs (Annex 1). In addition to MCAM, we found that RRMS Treg can express significantly higher levels of CD6, ALCAM, and CD49d, suggesting that Treg in RRMS are functional and upregulate CAMs for their potential to migrate.

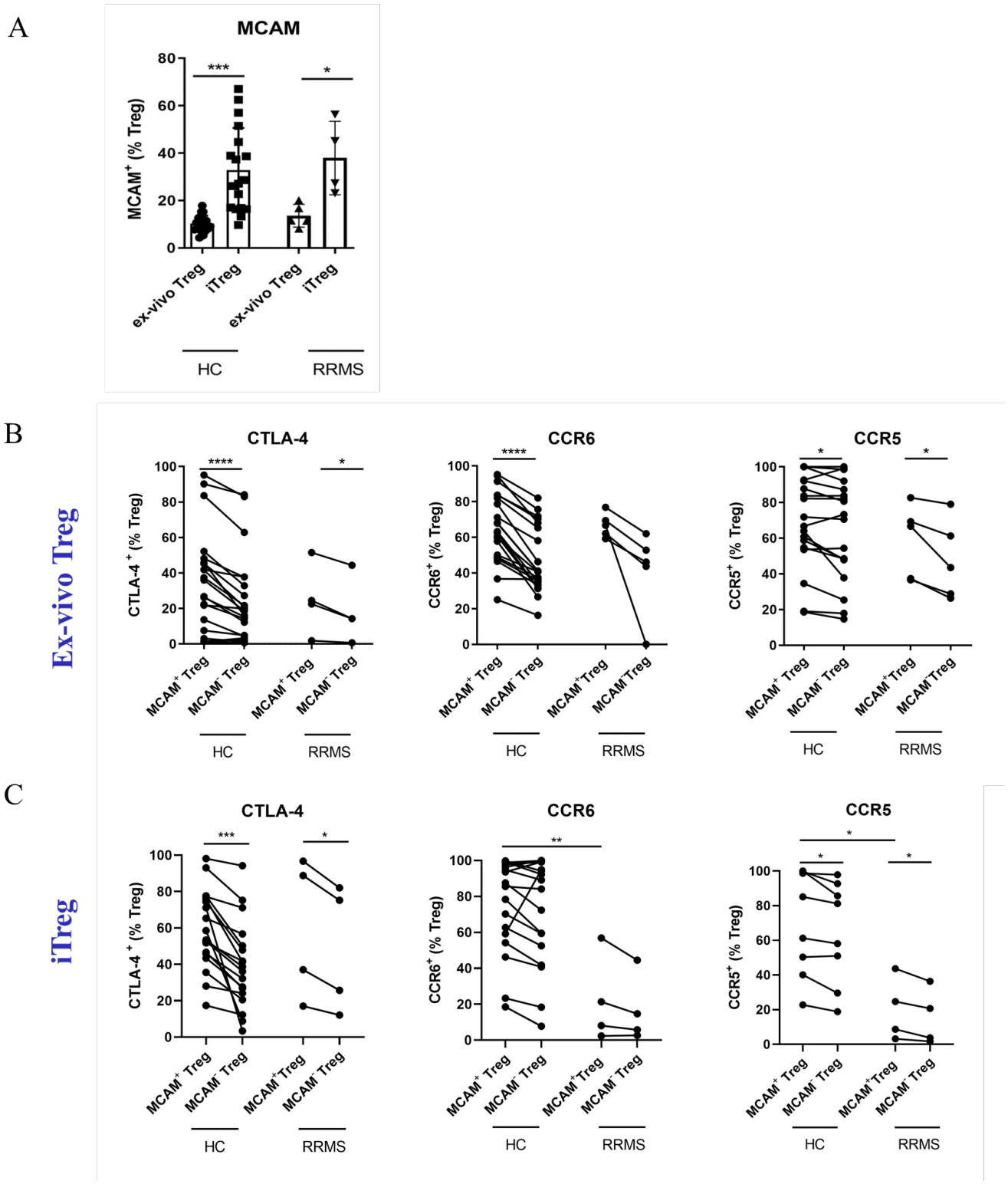


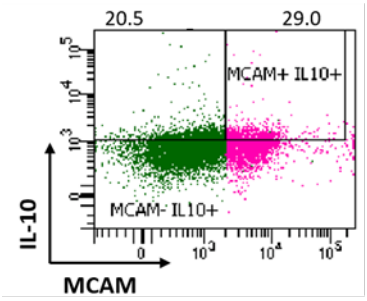
Figure 14. – Expression of Treg functional markers CTLA-4, CCR6, and CCR5 in MCAM$^+$ and MCAM$^-$ *ex-vivo* Treg and iTreg in HC (n=21) and untreated RRMS (n=4) by FACS.

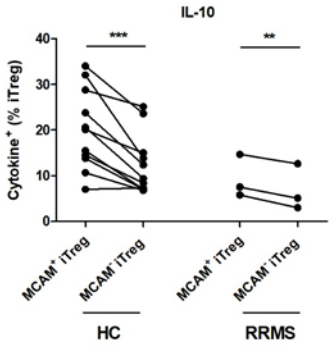
(A) Increased MCAM expression in iTreg versus *ex-vivo* Treg. (B) MCAM+ HC and RRMS *ex-vivo* Treg express higher levels of CTLA-4, CCR6 and CCR5 than their MCAM- counterparts. (C) MCAM+ HC and RRMS iTreg express higher levels of CTLA-4, CCR6 and CCR5 than their MCAM- counterparts. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

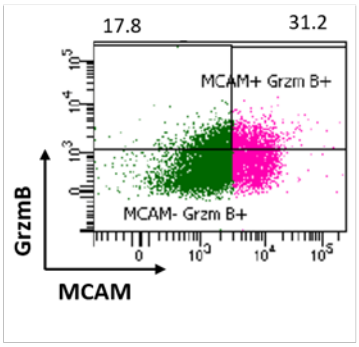
MCAM$^+$ versus MCAM$^-$ iTreg cytokine secretion

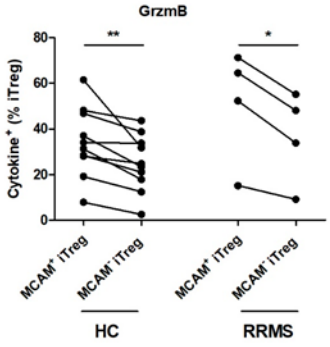
Our next goal was to determine whether MCAM$^+$ Treg could be further characterized as anti-inflammatory or pro-inflammatory in MS. We first evaluated the expression of anti-inflammatory cytokines (IL-10, TGF-ß), pro-inflammatory cytokines (IFN-γ, TNF α, GM-CSF, IL-17a), and cytotoxic mediator (GrzmB) on MCAM$^+$ and MCAM$^-$ iTreg by FACS. After 1 week of culture, iTreg cells were harvested and re-stimulated for 4 hours with PMA, ionomycin, and Golgi Plug inhibitor to detect the maximum potential of iTreg cytokine secretion, prior to immuno-staining. The percentage of MCAM$^+$ iTreg cells expressing IL-10 (20% HC; 10% RRMS) and GrzmB (34% HC; 50% RRMS) is significantly higher than in MCAM$^-$ iTreg in both HC and RRMS groups (Figure 15). These results suggest that MCAM$^+$ Treg have a more favorable anti-inflammatory phenotype than MCAM$^-$ Treg. Since we detect very few expressions of TGF-ß1 by FACS compared to qPCR and ELISA we think that TGF-ß1 FACS antibody is not properly working and need further optimization. Thus, results with iTreg expression of TGF-ß1 could not be interpreted. We also detected high levels of pro-inflammatory cytokine TNF α in MCAM$^+$ iTreg (69% HC; 76% RRMS) and MCAM$^-$ iTreg (65% HC; 76% RRMS) in both HC and RRMS, as well as low levels of pro-inflammatory cytokines IFN-γ and GM-CSF, and almost no IL-17a. Moreover, there were no significant differences in cytokine secretion between HC iTreg and RRMS iTreg, further supporting Treg unchanged functional abilities in MS.

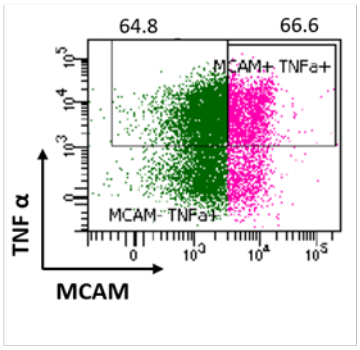
We further investigated whether IL-10$^+$ MCAM$^+$ iTreg cells were positive for other cytokines by FACS gating on this population (Figure 16). ANOVA statistics with post-test Turkey were performed (not represented on graphs) with a significance level measured at 95% confidence intervals. HC MCAM$^+$ iTreg producing IL-10 expressed significantly increased levels of GrzmB and TNF α compared the other cytokines, while RRMS MCAM$^+$ iTreg producing IL-10 only expressed significantly increased levels of GrzmB and TNF α compared to IL-17. Overall, this shows that MCAM$^+$ iTreg producing anti-inflammatory IL-10 is also capable of producing GrzmB and TNF α.

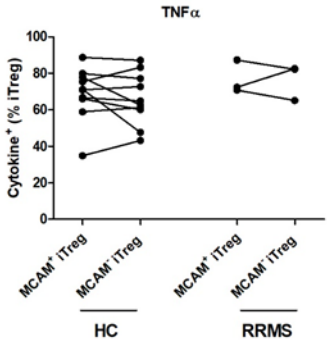

20.5
29.0
MCAM+ IL10+
MCAM- IL10+
IL-10
MCAM

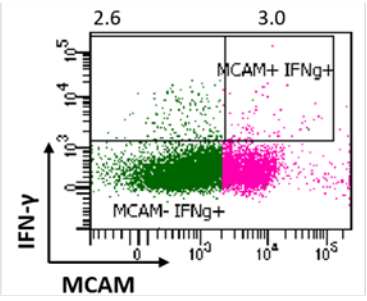

IL-10
***
**
Cytokine+ (% iTreg)
MCAM+ iTreg
MCAM- iTreg
HC
RRMS

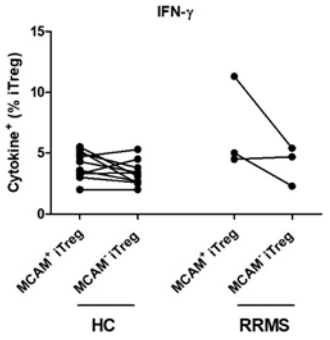

17.8
31.2
MCAM+ Grzm B+
MCAM- Grzm B+
GrzmB
MCAM


GrzmB
**
*
Cytokine+ (% iTreg)
MCAM+ iTreg
MCAM- iTreg
HC
RRMS


64.8
66.6
MCAM+ TNFa+
MCAM- TNFa+
TNF α
MCAM


TNFα
Cytokine+ (% iTreg)
MCAM+ iTreg
MCAM- iTreg
HC
RRMS


2.6
3.0
MCAM+ IFNg+
MCAM- IFNg+
IFN-γ
MCAM


IFN-γ
Cytokine+ (% iTreg)
MCAM+ iTreg
MCAM- iTreg
HC
RRMS

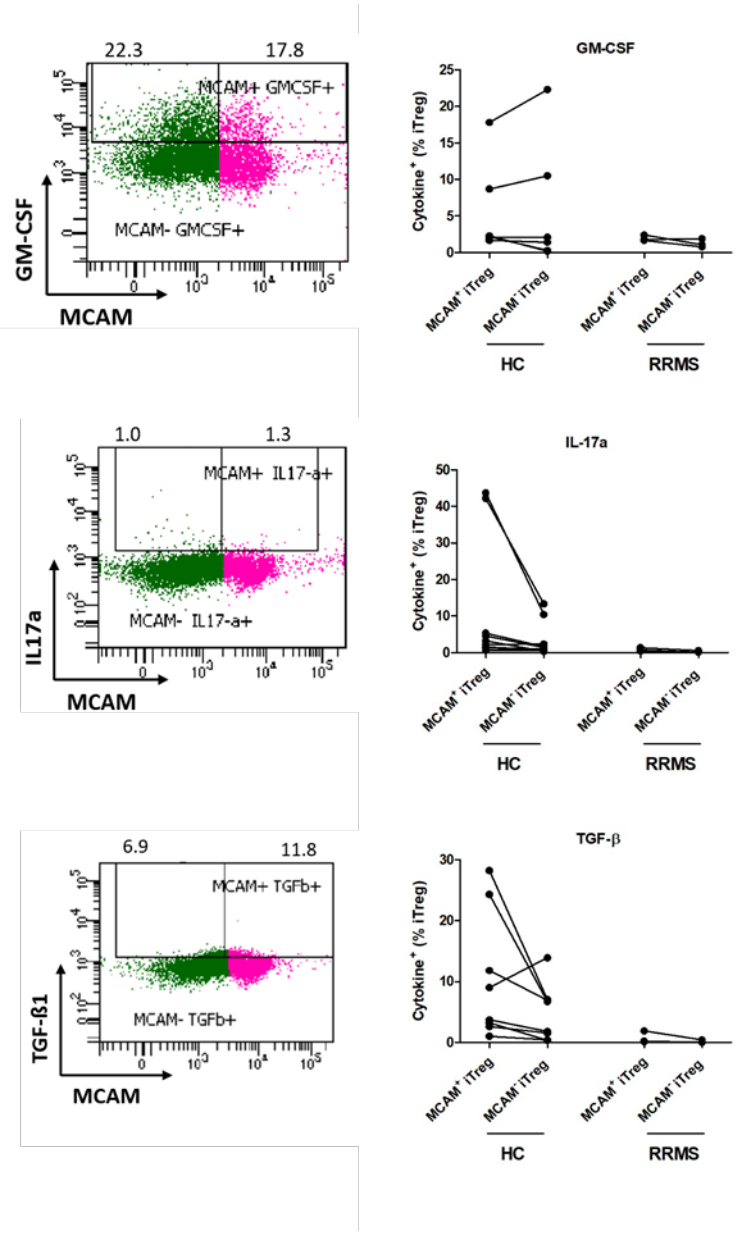


Figure 15. – MCAM$^+$ and MCAM$^-$ iTreg cytokine expression from HC (n=11) and RRMS (n=4).

First column FACS dot plot example of cytokine gating strategy on MCAM+ and MCAM- iTreg of 1 HC. MCAM$^+$ iTreg seem to have a more anti-inflammatory phenotype than their MCAM$^-$ counterparts. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

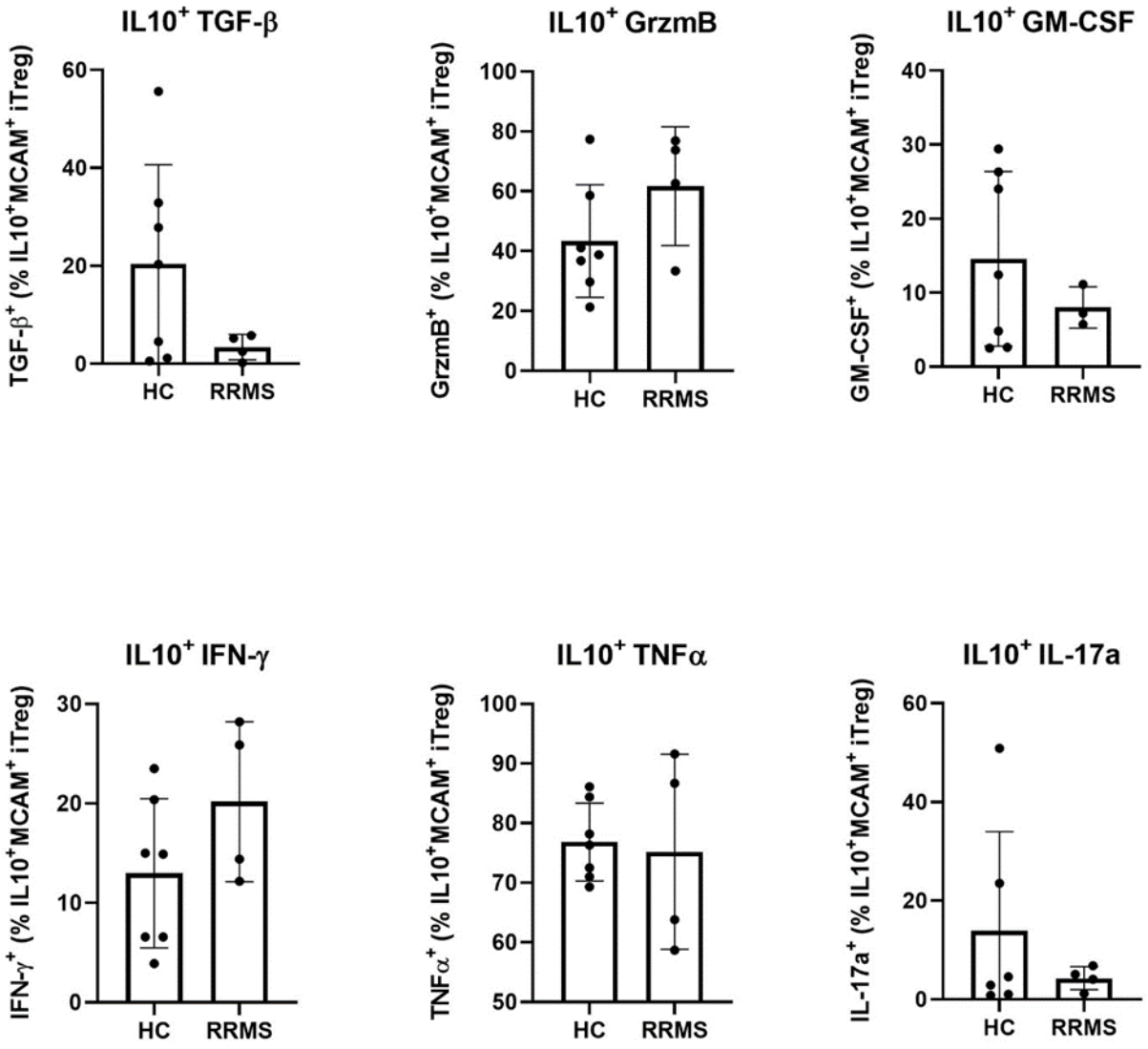


Figure 16. – Cytokine (IFN-γ, TNF α, GM-CSF, GrzmB, IL-17a) expression of IL-10^{+} MCAM^{+} iTreg from HC and RRMS by FACS.

All plots are presented as mean ± standard error of the mean.

iTreg migratory potential *in vitro*

Previous studies by the Prat lab have shown that human BBB-ECs express MCAM, particularly under inflammatory conditions. Given that MCAM interacts with itself and with other heterophilic ligands expressed at the BBB and that MCAM is expressed on Treg, we investigated whether MCAM may be involved in the transmigration of Treg across the BBB. To that end, an *in vitro* human BBB model was used to perform iTreg transmigration studies. Modified Boyden chambers consisting of inserts (upper chamber) with 3µm pores were placed in 24-well plates (lower chamber) (Figure 17A). In the upper chamber, HBECs were grown in primary culture on a gelatin layer until forming a confluent monolayer of ECs. HBECs were either stimulated with TNF α + IFN-γ (inflamed EC) to mimic the inflamed BBB, or only had added ECM-ACM (resting EC) to mimic resting conditions. iTreg cells were added to the upper chamber and left to migrate for 18 hours across the monolayer of HBECs, after which iTreg cells in the lower chamber were collected and counted. We detected a significantly increased iTreg transmigration in inflammatory conditions (mean number of cells counted= 20 390) compared to resting conditions (mean number of cells counted= 5 897) (Figure 17B).

Our next goal was to study the migratory potential of $MCAM^+$ iTreg compared to $MCAM^-$ iTreg across the BBB. To this extent, we sorted iTreg cells into subsets of $MCAM^+$ iTreg and $MCAM^-$ iTreg by using anti-CD146-APC followed by anti-APC magnetic beads (positive selection of MCAM) on magnetic columns. One million cells of each population were then added to the upper chambers and allowed to migrate for 18 hours. When cells were counted, it seemed that $MCAM^-$ iTreg transmigrated in higher numbers than $MCAM^+$ iTreg in both resting and inflamed conditions (Figure 17C). We suspect that the beads selection of MCAM interfered with the potential of $MCAM^+$ iTreg cells to migrate across HBECs laid on a porous membrane. We therefore need to repeat this study with a different design approach in order to validate $MCAM^+$ iTreg migratory potential.

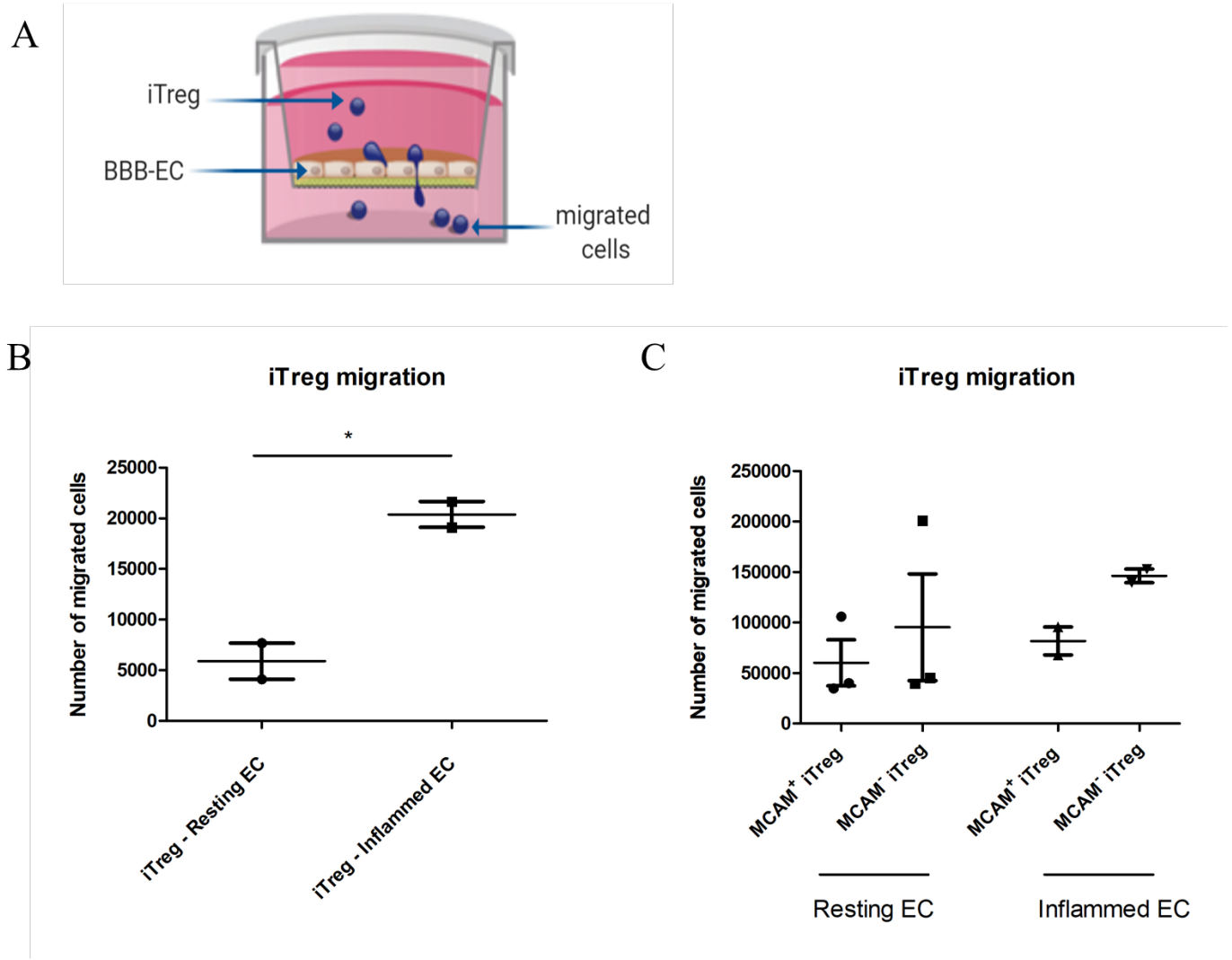


Figure 17. – Migration of iTreg across resting and inflamed BBB-ECs in a 2D *in vitro* 2D model of the human BBB.

(A) Schematic representation of modified Boyden chamber assay. A total of 0.5 x10^6 iTreg cells were added to the upper chamber and allowed to migrate for 18 hours across cultured BBB-EC. Cells recovered from the lower chamber were counted. Migration is expressed as number of cells in the lower chamber. (B) Graph representing the number of iTreg migrated under resting and inflammatory conditions (TNF α + IFN-γ) (n=2). (C) Graph representing the number of $MCAM^+$ versus $MCAM^-$ migrated iTreg under resting and inflammatory conditions (n=1-3). All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

MCAM^{+} Treg expression in the CNS

We aim to confirm the migration of MCAM^{+} Treg across CNS barriers by detecting their presence in CNS tissue. We used immunofluorescence and confocal microscopy to determine the expression of MCAM and Treg and their localization in the CNS. We first set out to optimize and validate antibodies and staining protocols for Treg markers. Since the spleen is a secondary lymphoid organ that stores immune cells, tissue sections of C57BL/6 mouse spleen were used as a positive control to evaluate the expression of CD4 and Foxp3 on T cells (Figure 18A). In addition, we optimized laminin staining as a blood vessel marker for future assessment of MCAM co-localization with vessels and visualization of CNS meninges, in order to detect MCAM^{+} Treg distinctive entry points within the CNS. We were able to successfully detect CD4^{+}Foxp3^{+} T cells in the spleen by verifying the nuclear localization of Foxp3 transcription factor (Figure 18B). Thus, we confirmed that the 4 fluorochromes used (CD4, Foxp3, Laminin, nuclei) work well together in the mouse spleen.

Since a more precise characterization of Treg would be CD4^{+}CD25^{high}CD127^{low}Foxp3^{+} T cells, we wanted to verify that these CD4^{+}Foxp3^{+} T cells were indeed Treg cells by their positive expression of the CD25 marker using IF. We used brain sections from the TCR^{1640} mouse model, known for the presence of Treg in the CNS, to evaluate the expression of Treg markers CD4, Foxp3 and CD25. We found that almost all CD4^{+}Foxp3^{+} cells were also positively stained for CD25 (Figure 19). Due to limitations in the use of multiple markers by IF, we therefore define Treg with IF as the overlay of CD4 and Foxp3 markers.

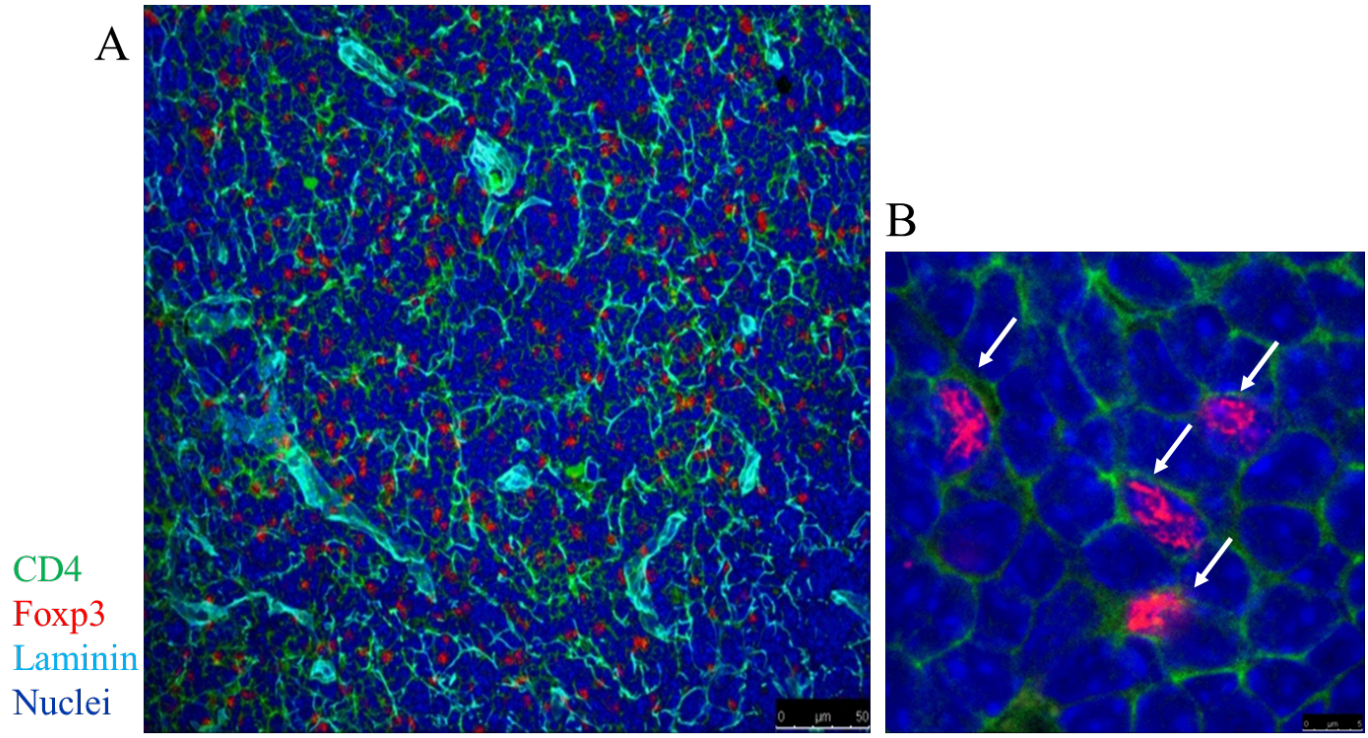


Figure 18. – Immunofluorescence staining of $CD4^+$ $Foxp3^+$ T cell in mouse spleen.

(A) Expression of CD4 (green), Foxp3 (red), Laminin (cyan), and nuclei (blue) in spleen sections from wildtype C57BL/6 mice. Scale bar = 50µm. (B) Zoom in spleen section demonstrating double staining of CD4 (green) and Foxp3 (red). White arrow = $CD4^+$ $Foxp3^+$ T cells defining Treg. Scale bar = 50µm.

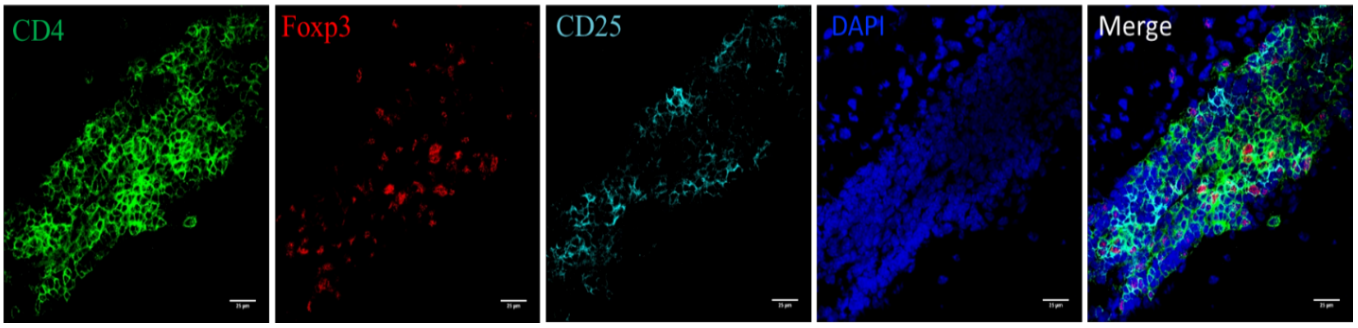


Figure 19. – Immunofluorescence images of Treg in brain section of MS mouse model, T cell receptor transgenic mice (TCR^{1640}).

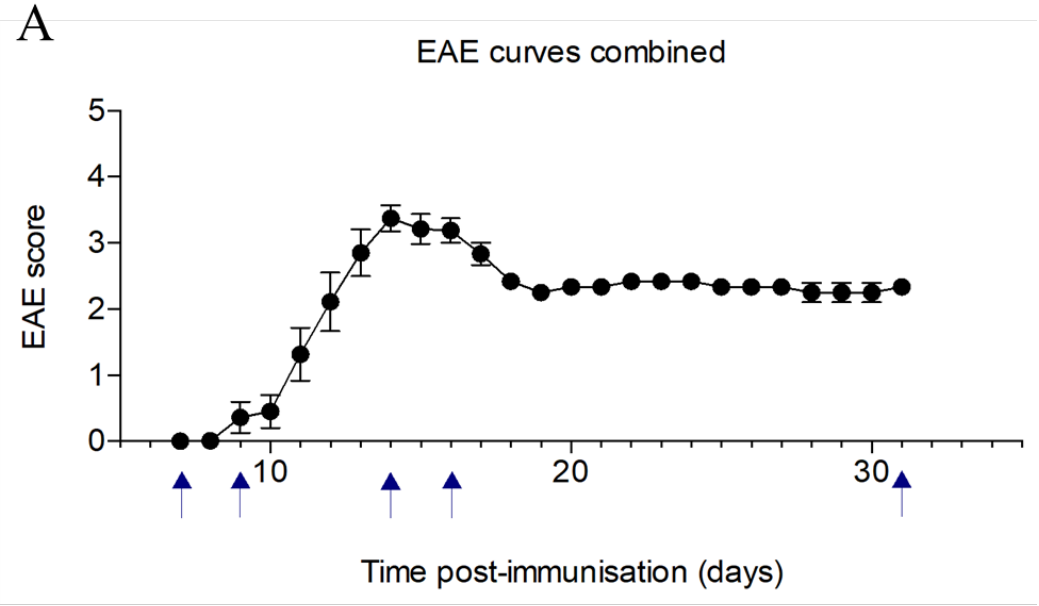
Treg defined by immunofluorescence as $CD4^{+}$ $Foxp3^{+}$ $CD25^{+}$ cell, 25 µm.

MCAM$^+$ Treg expression at different EAE phases

To confirm that MCAM$^+$ Treg play a role in CNS inflammatory lesions, we evaluated the expression of MCAM and Treg in MOG_{35-55} induced active EAE mice at different time points. EAE mice were harvested based on their disease score, at different time points, each representing a distinct disease phase as revealed by the EAE curve (Figure 20A). Days 0 to 7 post-immunization (p-i), mice did not show any symptoms related to disease, which represented the pre-symptomatic phase. Symptoms began to appear on day 9 p-i, representing the onset phase, reaching the highest score 4 on day 14 p-i, representing peak of disease. On days 15-16 p-i, a partial clinical recovery phase, herein referred to as a remission phase is observed with a score drop to 2.25, which stabilizes with time, representing the chronic phase (days 17 to 31 p-i). For each time point, CNS tissue was harvested for histology and immunofluorescence staining. LHE staining was performed on spinal cord sections in order to identify demyelinating and inflammatory lesions (Figure 20B, black arrows). Immune infiltrates were primarily localized on the ventral side of the spinal cord rather than the dorsal side, with most immune infiltrates at onset and peak phases. Moreover, it seems that the distribution of infiltrates was centred in the meninges. The LHE staining revealed the presence and localization of immune infiltrates in the CNS which can now be studied in more detail using immunofluorescence and confocal microscopy.

Next, we investigated the presence of Treg and MCAM *in situ* within immune infiltrates of EAE spinal cord sections at different disease time points (onset, peak, remission, chronic) (Figure 21). Looking at the overlap of CD4 and Foxp3 markers, we detected the presence of Treg cells at different disease phases. We observed a fewer proportion of Treg at early time points (onset and peak), while an enrichment of Treg within the CNS was more persistent at later disease timepoints (remission and chronic). In addition, we found strong MCAM expression particularly on blood vessels at all disease time points, while some expression was also detected on single cells. Furthermore, we detected the presence of MCAM$^+$ Treg within EAE perivascular infiltrates at all disease time points with a growing proportion over time. Thus, we have confirmed the presence of Treg in the CNS of MS mouse models as well as the accumulation of MCAM$^+$ Treg in the inflamed CNS specifically at remission and chronic disease phases.

A


EAE curves combined
EAE score
5
4
3
2
1
0
10
20
30
Time post-immunisation (days)

B

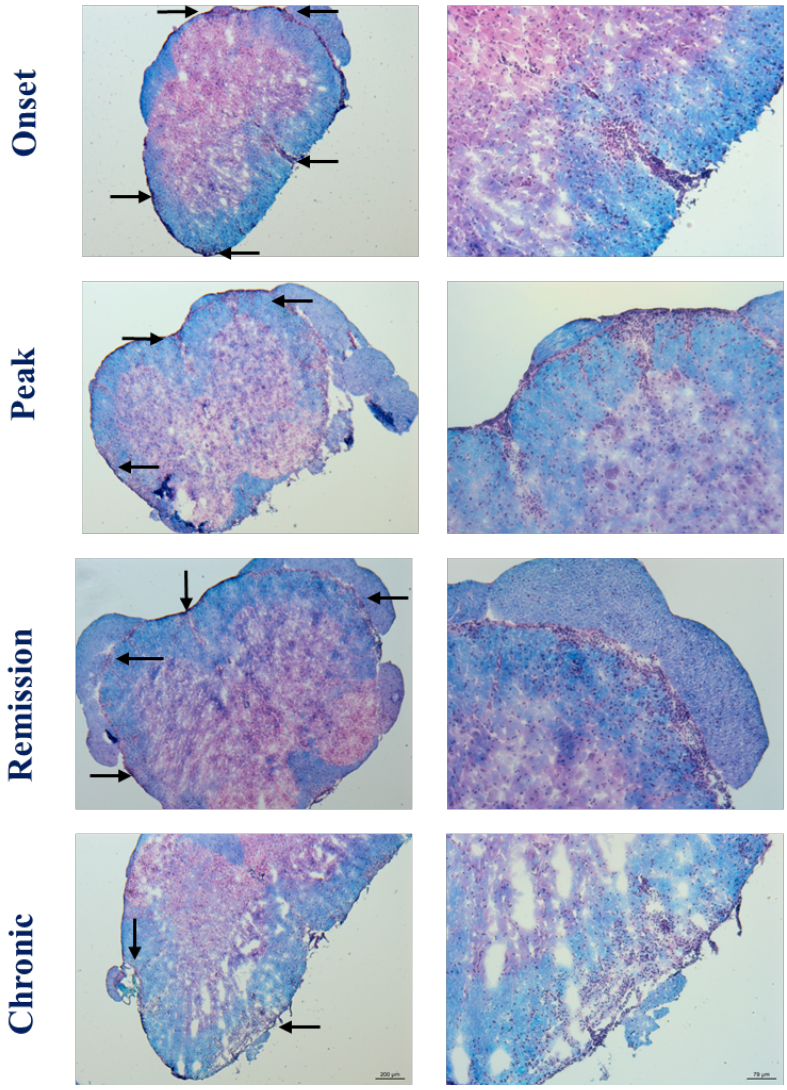

Onset
Peak
Remission
Chronic

Figure 20. – Active EAE disease course and CNS immune infiltrates.

(A) EAE curve representing disease development (days post-immunization, p-i) of MOG35–55 induced active EAE in C57BL/6 mice, measured according to 5-point scale reflecting disease severity. Data representative of n=15 animals (n=3 per group, per time point). Blue arrows indicate day of harvest of chosen mice at different disease time points (onset, peak, remission – defined as the partial recovery phase, chronic). (B) Frozen EAE spinal cord sections stained with Luxol Fast Blue and Haematoxylin and Eosin (LHE) at specific disease phases: onset (EAE score= 2), peak (EAE score=4), remission (EAE score=3), and chronic (EAE score =2.5). Images in the first column display x4 magnification, scale bar= 200µm. Images in the second column display x10 magnification, scale bar=79µm. Black arrows indicate areas of immune infiltrates in the CNS.

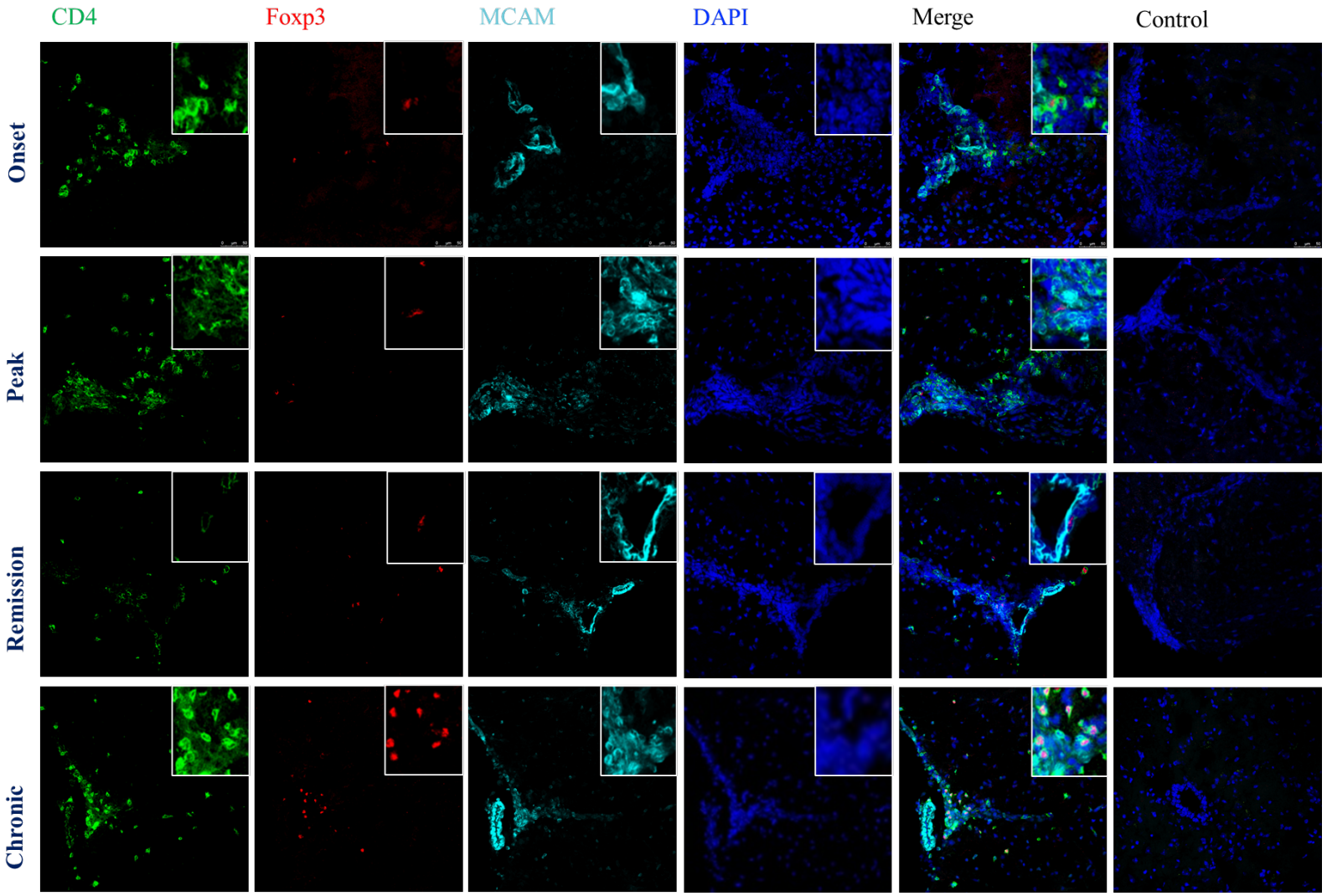


Figure 21. – *In situ* immunofluorescence images of MCAM^{+} Treg in spinal cord sections of active EAE mice at different disease time points (onset, peak, remission, chronic).

Overlap of CD4 (green) and Foxp3 (red) define a Treg cell. Evidence of MCAM (cyan) expression on Treg and blood vessels. DAPI was used to define nuclei (blue). Scale bar =50 µm.

Chapter 5 - Discussion

The main objective of this project is to identify the implication of Treg in MS by investigating their migratory potential to cross the BBB and enter the CNS. Previous work by the Prat lab have demonstrated the importance of the adhesion molecule MCAM in the infiltration of pathogenic immune cells to the CNS. We determined that MCAM is also an essential adhesion molecule for Treg homing to the CNS.

The study of human peripheral blood has revealed a major role for Treg in MS pathology. Although some studies have reported lower numbers of circulating $CD4^+CD25^{high}$ $Foxp3^+$ T cells , our data, along with others, demonstrate a normal frequency of $CD4^+CD25^{high}$ $Foxp3^+$ T cells in the blood of MS patients at different disease stages compared to HC or RRMS (251, 252). Furthermore, many studies have documented Treg enrichment in the CSF in relation to the peripheral blood by flow cytometry (359, 360). A recent study, using unbiased comparative single-cell RNA-seq approach confirmed Treg abundance in the CSF of MS patients compared to the peripheral blood (361). We propose a possible migration mechanism for Treg involving the upregulation of MCAM, thereby enabling them to cross CNS barriers. Flow cytometry study of immune cells in the peripheral blood and CSF samples can provide essential data to understand the transfer of cells across CNS barriers in health and disease (362). We found significantly higher levels of $MCAM^+$ Treg in the peripheral blood of untreated RRMS patients compared to HC, as well as an increased trend of $MCAM^+$ Treg in the CSF of untreated RRMS patients compared to peripheral blood levels. It is worth noting that CSF samples are collected from patients who are suspected of RRMS to confirm their clinical diagnosis. Thus, this time point might be representative of a potential MS diagnosis or an early disease stage. Moreover, interpatient variability might be responsible for the decreased trend observed in some patients. Previous studies have shown an increased frequency of $MCAM^+$ $CD4^+$ T cells and $MCAM^+$ $CD8^+$ T cells in the blood and CSF during MS relapses, thus contributing to the pro-inflammatory environment of disease activity (311, 352). We suggest that $MCAM^+$ Treg could potentially be intervening at this time point to help control disease activity which could be translated into a remission state.

We verified that MCAM$^+$ Treg, which might have an enhanced migratory potential, are functional cells by assessing the expression of functional markers CTLA-4, CCR6, and CCR5 on their surface. Human Treg cells are known to constitutively express elevated intracellular levels of CTLA-4, while surface expression of CTLA-4 is upregulated on all CD4$^+$ T cells upon activation (363, 364). Although surface expression of CTLA-4 is not limited to Treg, it is used as a functional marker of activated Treg cells. From the homing signals known to drive Treg accumulation over Teff in disease are the chemokine receptors CCR6 and CCR5 (365). Studies have shown the importance of CCR6 in the recruitment of Th17 as well as Treg to inflammatory tissues (366). In addition, CCR6 was found to define a suppressive-memory subset of Treg cells, as CCR6$^+$ Treg exhibit markers of activation, memory, and expansion (367). Moreover, CCR6 expression on Treg was necessary for efficient suppression in the T cell-transfer model of colitis (368). CCR5 was demonstrated to be expressed on a subset of Treg that preferentially infiltrates extra-lymphoid sites and sites of inflammation (369). CCR5 expression was also shown important for Treg-mediated suppression mechanisms in models of transplantation (370, 371). We found a general increase in CTLA-4, CCR6 and CCR5 expression in MCAM$^+$ Treg compared to MCAM$^-$ Treg in the peripheral blood and CSF of untreated RRMS patients. Thus, we conclude that activated and functional Treg from MS patients might have an enhanced propensity to migrate to the CNS via MCAM.

To further confirm the migratory potential of MCAM$^+$ Treg, we needed to perform an *in vitro* migration assay that mimics human BBB properties. However, when isolating Treg from PBMCs using magnetic beads we obtained a low number of cells (less than 0.5 x10^6 cells). In fact, the low number of Treg cells in the peripheral blood, which constitute around 1% of PBMCs, is one of the main hurdles of studying Treg (372). Thus, we proceeded with the *in vitro* polarization of Treg from naïve CD4 cells and generated an iTreg population. Although Treg differentiation efficiency might seem low (around a 60% purity of Foxp3$^+$CD4$^+$ iTreg cells), it is in line with current iTreg protocols and is generally accepted for studying certain Treg features (206, 358). Like others, our protocol resulted in reproducible expression of Foxp3 and other Treg markers, which enable the study of Treg migration and suppressive mechanisms (373). While iTreg is accepted and used for Treg studies, differences between Treg and iTreg have been reported. One limitation of using iTreg is that they might be functionally unstable since they do not have the complete

demethylation that underlies Treg signature genes (374). With qPCR we verified gene expression of Foxp3, IL-10, TGF-ß, and IL-35 in iTreg cells thereby confirming their Treg-like phenotype. We also detected IL-10 producing iTreg cells by immunocytofluorescence, as well as IL-10 and TGF-ß levels in the culture supernatant of iTreg cells by ELISA, further confirming the anti-inflammatory properties of iTreg. Moreover, future use of an ELISPOT assay in addition to the ELISA data would give a more complete understanding of the cytokine secreted and a more reliable interpretation of the TGF-ß1 concentration *in vitro*.

When we compared the expression of markers CTLA-4, CCR6, CCR5 and MCAM between *ex-vivo* Treg and iTreg, we observed an increase in CTLA-4, CCR6 and MCAM expression in HC iTreg compared to HC *ex-vivo* Treg, while CCR5 levels remained similar. We detected a significantly increased expression of MCAM in RRMS iTreg compared to RRMS *ex-vivo* Treg, while no significant changes in CTLA-4, CCR6 and CCR5 expression was detected between RRMS *ex-vivo* Treg and iTreg. The increased expression of certain markers in the iTreg population could be attributed to the activation of cells in culture conditions for a week without a specific antigen. This polarization could enhance the expression of markers on the surface of cells rendering the iTreg population in a more activated state than *ex-vivo* Treg. Using a non-specific antigen activation could resemble the polyclonal activation associated with inflammation (7). Moreover, other studies have reported changes in the suppressive capacity of iTreg compared to PBMC derived HC Treg isolated by magnetic beads, where iTreg displayed a superior suppressive activity (373). Although minor changes were noted between *ex-vivo* Treg and iTreg populations, we demonstrate that iTreg phenotype is a reliable alternative to further investigate Treg functional mechanisms in MS.

Furthermore, in comparing CTLA-4, CCR6 and CCR5 levels between HC and RRMS *ex-vivo* Treg, we observed similar expression, hinting to the maintained functionality of Treg in RRMS. When comparing these same functional markers between HC and RRMS iTreg, we did not detect significant changes in CTLA-4 and CCR5 expression, however significantly higher levels of CCR6 were detected in HC iTreg, thereby further confirming the increased activated state of HC iTreg due to induction. Since iTreg cells are derived from naïve $CD4^+$ $CD45RA^+$ cells, we suspect that differences in naïve CD4 T cell biology between HC and RRMS patients also could account for the

results observed. In fact, differences in TCR and TLR signaling pathways in naive CD4 T cells were able to characterize a subsets of RRMS patients with a more rapid conversion to SPMS (375). Moreover, our ELISA data revealed an increased trend of IL-10 and TGF-ß1 in RRMS samples compared to HC. Therefore, although iTreg from RRMS may have biological differences in their naïve CD4 T cells, they still generated a functional population of Treg cells. We confirm that iTreg cells maintain Treg-like phenotype by being functionally activated and producing anti-inflammatory cytokines, which allowed us to investigate the role of $MCAM^+$ Treg in MS.

We evaluated the expression of Treg functional markers, chemokine receptors, and cytokine secretion on $MCAM^+$ versus $MCAM^-$ iTreg by flow cytometry. We found that $MCAM^+$ HC and RRMS iTreg significantly upregulate CTLA-4 and CCR5, and slightly increase CCR6 expression compared to their respective $MCAM^-$ counterparts. These results imply that $MCAM^+$ Treg may be more apt at exerting their suppressive functions. To better characterize the role of $MCAM^+$ iTreg we looked at their cytokine signature by FACS. We did not detect significant differences in cytokine secretion between HC iTreg and RRMS iTreg, further supporting Treg unchanged functional abilities in MS. Contrary to initial studies that reported defects in the suppressive capacity of $CD4^+$ $CD25^+$ Treg in MS, more recent studies that characterize Treg as $CD4^+$ $CD25^+$ $CD127^{low}$ T lymphocytes, reported Treg unchanged functional abilities in MS, in line with our data(376, 377). In fact, many controversies about the contribution of Treg in MS are found because Treg were not well characterized.

We detected a significantly increased expression of IL-10 and GrzmB in $MCAM^+$ iTreg compared to $MCAM^-$ iTreg in both HC and RRMS. It is well documented that IL-10 and GrzmB expression are associated with Treg, conferring their role in immune suppression. At first sight these results suggest that $MCAM^+$ Treg might have a more favorable anti-inflammatory phenotype than $MCAM^-$ Treg. However, the role of immunosuppressive cytokines in Treg mediated suppression is not well understood. Anti-inflammatory cytokine IL-10 was found crucial for mediating Treg suppression in EAE, whereas it appeared dispensable in other autoimmune models such as gastritis (378, 379). GrzmB is known as a cytotoxic mediator normally associated with $CD8^+$ T cells and NK cells that induces programmed cell death in targeted cells. Similarly, human Treg are known to express GrzmB that aim to kill Teff cells. However, recent studies in

transplant recipients suggest that Treg expressing GrzmB have an activated phenotype that is more apoptotic than Treg that do not express GrzmB (380). Thus, although GrzmB has been shown to be important in controlling self-reactive cells, it may be responsible for the self-induced apoptosis of Treg. Therefore, conclusions about Treg suppressive function in our study requires deeper investigation based on suppression and proliferation assays.

Moreover, we also detected different levels of pro-inflammatory cytokines TNF α, IFN-γ, GM-CSF and IL-17a in $MCAM^+$ iTreg and $MCAM^-$ iTreg. These results could point to Treg's plasticity in acquiring Th-like phenotypes to more effectively regulate Teff cells. Treg are known to adept their phenotype and function to the type of immune response they control. As a result, Treg can co-express Th lineage transcription factors which influence Treg expression patterns in various infectious and inflammatory conditions (e.g. Treg acquire expression of T-bet to restrain inflammation)(381). However, aberrant Treg plasticity was suggested in autoimmune diseases, whereby Treg acquired Th-like phenotype, expressed pro-inflammatory cytokines, and displayed diminished suppressive function (382). For instance, in patients with autoimmune diseases, such as type 1 diabetes, autoimmune hepatitis, and MS, increased frequency of IFN-γ^+ $Foxp3^+$ T cells were reported and correlated with their decreased suppressive ability (383-385). Additionally, considering the well-established developmental relationship between Treg and Th17, whether Th17-like Treg are a transient step in the de-differentiation of Treg into Th17 is yet to be established (386). Th17-like Treg identified in the gastrointestinal tract appear to have a beneficial role as their depletion has been shown to exacerbate disease in models of mucosal autoimmunity (387). Thus, future research focusing on Treg plasticity would provide insight into the function of Treg in MS.

To study iTreg transmigratory potential, we used the *in vitro* 2D model of the human BBB well-established in the Prat lab (311). We detected a significantly increased HC iTreg transmigration in inflammatory conditions versus resting conditions, suggesting that in an inflammatory context iTreg can migrate across the BBB. A similar increased motility was observed in Th17 cells under inflammatory conditions (311). Schneider-Hohendorf et al. have demonstrated that $CD4^+Foxp3^+$ Treg from HC had enhanced migratory abilities compared to non-Treg cells, confirming Treg innate migratory advantage to maintain immune homeostasis and CNS

surveillance. In contrast with our data, their *in vitro* simulation with IFN-γ and TNF-α did not significantly alter the migratory superiority of Treg compared to resting conditions (388). Differences between BBB models and Treg characterization could explain the observed discrepancies. In addition, Schneider-Hohendorf et al. found that RRMS derived $CD4^+$ $Foxp3^+$ T cells had an impaired migratory capacity compared to HC under resting conditions, but had an increased migratory capacity reaching similar levels to HC under inflammatory conditions. They speculated that Treg in MS patients had a migratory dysfunction in early phases of MS (388). Our FACS data revealed an increased expression of CAMs on RRMS *ex-vivo* Treg (MCAM, CD6, ALCAM, CD49d) suggesting an enhanced Treg migratory potential in RRMS. An early event in the development of CNS inflammation is the upregulation of CAMs by BBB-EC and leukocytes leading to leukocyte transmigration into the CNS parenchyma (303, 389). Future work with iTreg RRMS transmigration assay should attest to Treg's migratory potential.

As demonstrated by the clinical efficacy of Natalizumab, interactions between BBB-EC and leukocytes are important (154). However, the lack of specificity in restricting immune cell entry to the CNS could inhibit immune patrolling and possibly anti-inflammatory Treg migration to the site of inflammation, resulting in detrimental effects. Hence, the need to characterize CAMs expression on leukocytes. The expression of MCAM on human BBB-EC, effector memory $CD4^+$ T cells, and $CD8^+$ T cells, has been previously well established (311, 352). Our goal is to investigate the role of MCAM in Treg transmigration by comparing the migratory potential of $MCAM^+$ iTreg and $MCAM^-$ iTreg across the *in vitro* 2D model of the human BBB. After 2 individual experiments, we observed a higher cell number of $MCAM^-$ iTreg transmigration compared to $MCAM^+$ iTreg in both resting and inflamed conditions, contradicting our previous suggested FACS data. We suspected a flawed experimental approach as the use of magnetic beads in selecting the $MCAM^+$ iTreg fraction, in contrast with a bead free $MCAM^-$ iTreg fraction, could have obstructed the porous membrane of our *in vitro* BBB model. Thus, we need to first optimize our MCAM selection approach. To circumvent this problem, future work should focus on redefining $MCAM^+$ and $MCAM^-$ fractions as follows: iTreg put through a magnetic column in the absence of magnetic beads would constitute the $MCAM^+$ fraction, while iTreg depleted from MCAM using anti-CD146 magnetic beads would constitute the $MCAM^-$ fraction. Moreover, further studies using dynamic

migration assays (Ibidi flow system) could be performed to better evaluate the role of MCAM in the various stages of Treg vascular extravasation (112, 352).

To attest to the migration of Treg across CNS barriers, our next goal was to determine the presence of $MCAM^+$ Treg within the CNS parenchyma. EAE studies have long supported the role and possible protective implication of Treg in MS (257, 378). Importantly, depletion of Treg before disease onset exacerbated EAE severity and inhibited the remission phase of the disease (257). We sought to determine the presence of $MCAM^+$ Treg by immunofluorescence and confocal microscopy. We first optimized and validated antibodies for immunofluorescence staining of $CD4^+$ $Foxp3^+$ Treg in the spleen of C57BL/6 mice. Since Foxp3 and CD25 Treg markers are also known be transiently expressed upon activation of T lymphocytes, we verified the proper detection of Treg by assessing the colocalization of CD4, Foxp3, and CD25 in the CNS of TCR^{1640} mouse model known for the frequent presence of Treg within CNS infiltrates (164). Almost all $CD4^+$ $Foxp3^+$ cells were positively stained for CD25; thus, we confirm that colocalization of CD4 and Foxp3 markers suffice to characterize *in situ* Treg cells by IF.

Treg have been found to accumulate in the CNS of EAE during the course of disease (257). To determine whether $MCAM^+$ Treg accumulate within CNS inflammatory lesions, we evaluated the expression of MCAM and Treg in MOG_{35-55} induced active EAE mice at different disease time points. MOG_{35-55} induced active EAE in C57BL/6 mice are known to display immune infiltrates in the spinal cord and cerebellum rather than in the brain (390, 391). We therefore studied the distribution of immune infiltrates by LHE in EAE spinal cord sections at different disease phases. We found increased number of CNS infiltrates with increased disease severity, in line with previous reports (391). LHE staining revealed the localization of immune infiltrates in the CNS whereby their composition was studied by IF. We found high MCAM expression on CNS blood vessels highlighting the important role of MCAM in the diapedesis of leukocytes to the CNS, as previously demonstrated (311). Additionally, we detected various levels of $MCAM^+$ Treg in all disease phases. At early disease phases (onset and peak), LHE revealed more immune infiltrates than any other time point, however we only detected very few Treg, as well as $MCAM^+$ Treg, suggesting a deficiency of Treg involvement in the CNS at these time points. Although later disease phases are associated with long term axonal damage and reduced CNS inflammation, we

detected the persistence presence of immune cells (392). We found increasing numbers of MCAM$^+$ Treg in later disease phases (remission and chronic), which are associated with disease amelioration and declined inflammation. Others have consistently reported that Treg isolated from EAE CNS are a major source of IL-10 (257, 378, 393). The direct correlation between Treg numbers and disease amelioration is yet to be established. We speculate that MCAM$^+$ Treg enter the CNS at later disease time points, in contrast with pathogenic immune cells, thereby contributing to dampening inflammation and disease resolution.

We were interested in locating the different entry points of MCAM$^+$ Treg into the CNS. Since laminin is a blood vessel marker, we optimized the laminin antibody in order to assess whether Treg enter the CNS through vessels in the meninges or through vessels in the parenchyma, and whether they upregulate MCAM specifically at distinctive locations. Our goal was to stain for a combination of 5 markers CD4, Foxp3, MCAM, laminin, and nuclei in EAE CNS. We successfully optimized a staining protocol using spectral unmixing to separate fluorochromes and reduce crosstalk (Annex 2). We used markers widely expressed in the CNS perivascular infiltrates and confirmed their interchangeable use with other primary antibodies. Future work using this image acquisition technique will allow a better evaluation of the expression and location of MCAM$^+$ Treg in the CNS.

Though the results from this study are promising, there are a few identifiable limitations. Since a small CSF sample size was obtained from RRMS patients, future work including a larger sample size would better determine whether a general trend of increasing MCAM$^+$ Treg is detected in the CSF of MS patients. Another limitation is the lack of defining Treg markers specifically under inflammatory conditions. Since upon activation CD4 cells may transiently upregulate CD25 and downregulate CD127, additional markers should also be used in combination with CD25, CD127 and Foxp3 to better discriminate between Treg and non-Treg populations (208, 211). To this end, new Treg panels have been optimized for future FACS experiments to include a wide array of Treg-related markers recently reported in the literature (e.g. Helios, GARP) and study their expression in MS (Annex 3) (394, 395). Moreover, in order to better understand the balance between autoimmunity and tolerance, a Th17/Treg ratio needs to be calculated and account for disease progression. Thus, future work should consider both Th17

and Treg frequencies from PBMCs of the same HC and RRMS individuals. Although the low number of Treg was worked around with the iTreg culture, a limiting population was still obtained and only $1x10^6$ iTreg cells were set aside for qPCR analysis. This number has not always been sufficient to isolate an adequate concentration of RNA, and a larger sample size should be allocated to the qPCR study. Another limiting factor was the detection of Foxp3 in human CNS by immunofluorescence and confocal microscopy. Due to the complex and highly lipidic environment of the human CNS, detection of a faint signal such as Foxp3 proves to be challenging. Many optimization protocols including varying antibody concentrations, antibody incubation time, permeabilization and fixing solutions were attempted but require further development.

Investigations concerning the role of Treg in a variety of diseases has been recently resurfacing. With the growing interest in utilizing their suppressor function to restore immune balance, the translation of Treg biology to therapeutic applications proves to be exciting yet challenging with many unanswered questions involving Treg number, function, dosage, *in vivo* stability, etc. One approach relies on the development of a highly personalized therapy, adoptive Treg cell-based immunotherapy, in which patients' own cells are subject to *ex-vivo* expansion followed by their re-infusion. One important advantage account for the capacity of these cells for self-regulation based on therapeutic demand. Such an approach was recently used in an ALS clinical trial in which intravenous administration of *in vitro* expanded Treg and IL-2, contributed to slowing disease progression while being well-tolerated by patients (396). These results show promise for developing similar strategies for treating MS. Though it is important to consider that the use of polyclonal Treg of undefined antigenic specificity may lead to a systemic immunosuppression. A way to prevent this is to use antigen specific Treg engineered, for example, through chimeric antigen receptor T cell (CAR-T) (397). Cellular modifications to Treg before their transfer may enable and allow for a more localized suppression. In a study performed by Kim et al. in 2018, MBP-specific transgenic T cell receptors expressed by programmed human CAR-Treg were able to improve the pathogenesis of the disease in EAE, which is promising for human Treg manipulations in the context of MS (398). By investigating and defining the specific CAM signature that promote Treg migration to the CNS, one can imagine the use of similar technological advances to expand a specific sub-population of Treg or induce the upregulation of

CAMs *in vitro* to boost the migratory potential of the cells, before administration to patients. Being a multi-faceted disease, combining Treg cell-based therapy with traditional treatments could be envisioned to treat MS.

Chapter 6 - Conclusion

Our data depicts MCAM as an essential CAM for Treg homing to the CNS. In untreated RRMS patients, we found an increased expression of $MCAM^+$ Treg in the peripheral blood and a prospective increased trend of $MCAM^+$ Treg in the CSF. These $MCAM^+$ Treg seem to seem to have a more functional and anti-inflammatory phenotype than their $MCAM^-$ counterparts. Moreover, we found higher levels of Treg in periods of remission in EAE, underlining their involvement during this disease phase. Although further investigation is needed, $MCAM^+$ Treg seem to maintain anti-inflammatory properties important for dampening inflammation in MS and resolution of symptoms. By exploring the migration mechanisms of Treg, we would not only be advancing knowledge on the pathophysiology of MS but would also be painting a target on a potential therapeutic novelty.

Références bibliographiques

1. Charcot J-M. Histologie de la sclérose en plaques. Paris: [s.n.]; 1868.
2. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000;343(13):938-52.
3. Wade BJ. Spatial analysis of global prevalence of multiple sclerosis suggests need for an updated prevalence scale. Mult Scler Int. 2014;2014:124578.
4. Amankwah N, Marrie RA, Bancej C, Garner R, Manuel DG, Wall R, et al. Multiple sclerosis in Canada 2011 to 2031: results of a microsimulation modelling study of epidemiological and economic impacts. Health Promot Chronic Dis Prev Can. 2017;37(2):37-48.
5. Narula S. Pediatric multiple sclerosis: updates in epidemiology, clinical features and management. Neurodegener Dis Manag. 2016;6(6s):3-7.
6. Martinelli V, Rodegher M, Moiola L, Comi G. Late onset multiple sclerosis: clinical characteristics, prognostic factors and differential diagnosis. Neurol Sci. 2004;25 Suppl 4:S350-5.
7. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nature reviews Immunology. 2015;15(9):545-58.
8. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. Neurology. 2014;83(3):278-86.
9. Hou Y, Jia Y, Hou J. Natural Course of Clinically Isolated Syndrome: A Longitudinal Analysis Using a Markov Model. Sci Rep. 2018;8(1):10857-.
10. Freedman MS, Selchen D, Arnold DL, Prat A, Banwell B, Yeung M, et al. Treatment optimization in MS: Canadian MS Working Group updated recommendations. Can J Neurol Sci. 2013;40(3):307-23.
11. Bsteh G, Ehling R, Lutterotti A, Hegen H, Di Pauli F, Auer M, et al. Long Term Clinical Prognostic Factors in Relapsing-Remitting Multiple Sclerosis: Insights from a 10-Year Observational Study. PLoS One. 2016;11(7):e0158978.
12. Miller DH, Weinshenker BG, Filippi M, Banwell BL, Cohen JA, Freedman MS, et al. Differential diagnosis of suspected multiple sclerosis: a consensus approach. Mult Scler. 2008;14(9):1157-74.
13. Balcer LJ. Clinical practice. Optic neuritis. N Engl J Med. 2006;354(12):1273-80.
14. Brownlee WJ, Miller DH. Clinically isolated syndromes and the relationship to multiple sclerosis. Journal of Clinical Neuroscience. 2014;21(12):2065-71.
15. Palace J. Making the diagnosis of multiple sclerosis. J Neurol Neurosurg Psychiatry. 2001;71 Suppl 2(Suppl 2):ii3-ii8.
16. Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. The Lancet. 2017;389(10076):1336-46.
17. Antel J, Antel S, Caramanos Z, Arnold DL, Kuhlmann T. Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity? Acta Neuropathologica. 2012;123(5):627-38.
18. Weinshenker BG, Rice GP, Noseworthy JH, Carriere W, Baskerville J, Ebers GC. The natural history of multiple sclerosis: a geographically based study. 3. Multivariate analysis of predictive factors and models of outcome. Brain. 1991;114 (Pt 2):1045-56.
19. Wilkins A. Cerebellar Dysfunction in Multiple Sclerosis. Front Neurol. 2017;8:312-.

20. Braley TJ, Chervin RD. Fatigue in multiple sclerosis: mechanisms, evaluation, and treatment. Sleep. 2010;33(8):1061-7.
21. Grzegorski T, Losy J. Cognitive impairment in multiple sclerosis - a review of current knowledge and recent research. Rev Neurosci. 2017;28(8):845-60.
22. Bove RM, Hauser SL. Diagnosing multiple sclerosis: art and science. The Lancet Neurology. 2018;17(2):109-11.
23. Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, et al. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. The Lancet Neurology. 2016;15(3):292-303.
24. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. The Lancet Neurology. 2018;17(2):162-73.
25. Bakshi R, Hutton GJ, Miller JR, Radue EW. The use of magnetic resonance imaging in the diagnosis and long-term management of multiple sclerosis. Neurology. 2004;63(11 Suppl 5):S3-11.
26. Traboulsee AL, Li DK. The role of MRI in the diagnosis of multiple sclerosis. Adv Neurol. 2006;98:125-46.
27. Gelfand JM. Chapter 12 - Multiple sclerosis: diagnosis, differential diagnosis, and clinical presentation. In: Goodin DS, editor. Handbook of Clinical Neurology. 122: Elsevier; 2014. p. 269-90.
28. Jeanne Billioux B, Alvarez Lafuente R, Jacobson S. Chapter 7 - HHV-6 and Multiple Sclerosis. In: Flamand L, Lautenschlager I, Krueger GRF, Ablashi DV, editors. Human Herpesviruses HHV-6A, HHV-6B & HHV-7 (Third Edition). Boston: Elsevier; 2014. p. 123-42.
29. Dobson R, Ramagopalan S, Davis A, Giovannoni G. Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. Journal of Neurology, Neurosurgery & Psychiatry. 2013;84(8):909.
30. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology. 1983;33(11):1444-52.
31. McDonald WI, Compston A, Edan G, Goodkin D, Hartung H-P, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on the diagnosis of multiple sclerosis. Annals of Neurology. 2001;50(1):121-7.
32. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. New England Journal of Medicine. 2018;378(2):169-80.
33. Garg N, Smith TW. An update on immunopathogenesis, diagnosis, and treatment of multiple sclerosis. Brain and Behavior. 2015;5(9):e00362.
34. O'Gorman C, O'Gorman C, Lin R, Stankovich J, Broadley SA. Modelling Genetic Susceptibility to Multiple Sclerosis with Family Data. Neuroepidemiology. 2013;40(1):1-12.
35. Misra MK, Damotte V, Hollenbach JA. Structure-based selection of human metabolite binding P4 pocket of DRB1*15:01 and DRB1*15:03, with implications for multiple sclerosis. Genes Immun. 2019;20(1):46-55.
36. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. J Autoimmun. 2015;64:13-25.

37. Hensiek AE, Sawcer SJ, Feakes R, Deans J, Mander A, Akesson E, et al. HLA-DR 15 is associated with female sex and younger age at diagnosis in multiple sclerosis. J Neurol Neurosurg Psychiatry. 2002;72(2):184-7.
38. Wagner M, Bilinska M, Pokryszko-Dragan A, Sobczynski M, Cyrul M, Kusnierczyk P, et al. ALCAM and CD6--multiple sclerosis risk factors. J Neuroimmunol. 2014;276(1-2):98-103.
39. Sawcer S, Hellenthal G, Pirinen M, Spencer CCA, Patsopoulos NA, Moutsianas L, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214-9.
40. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. Prog Neurobiol. 1995;47(4-5):425-48.
41. Berg-Hansen P, Moen SM, Sandvik L, Harbo HF, Bakken IJ, Stoltenberg C, et al. Prevalence of multiple sclerosis among immigrants in Norway. Mult Scler. 2015;21(6):695-702.
42. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. Current Opinion in Pharmacology. 2010;10(4):482-96.
43. Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S, et al. 1,25-Dihydroxyvitamin D₃ Selectively Modulates Tolerogenic Properties in Myeloid but Not Plasmacytoid Dendritic Cells. The Journal of Immunology. 2007;178(1):145.
44. van der Aar AMG, Sibiryak DS, Bakdash G, van Capel TMM, van der Kleij HPM, Opstelten D-JE, et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. Journal of Allergy and Clinical Immunology. 2011;127(6):1532-40.e7.
45. Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? Nature Reviews Immunology. 2011;11(9):584-96.
46. Nimitphong H, Holick MF. Vitamin D, neurocognitive functioning and immunocompetence. Curr Opin Clin Nutr Metab Care. 2011;14(1):7-14.
47. Dursun E, Gezen-Ak D, Yilmazer S. A novel perspective for Alzheimer's disease: vitamin D receptor suppression by amyloid-beta and preventing the amyloid-beta induced alterations by vitamin D in cortical neurons. J Alzheimers Dis. 2011;23(2):207-19.
48. Munger KL, Aivo J, Hongell K, Soilu-Hanninen M, Surcel HM, Ascherio A. Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort. JAMA Neurol. 2016;73(5):515-9.
49. van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Taylor BV, Kilpatrick T, et al. Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia. J Neurol. 2007;254(5):581-90.
50. Smolders J, Menheere P, Kessels A, Damoiseaux J, Hupperts R. Association of vitamin D metabolite levels with relapse rate and disability in multiple sclerosis. Mult Scler. 2008;14(9):1220-4.
51. Auer DP, Schumann EM, Kumpfel T, Gossl C, Trenkwalder C. Seasonal fluctuations of gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. Ann Neurol. 2000;47(2):276-7.
52. Sintzel MB, Rametta M, Reder AT. Vitamin D and Multiple Sclerosis: A Comprehensive Review. Neurol Ther. 2018;7(1):59-85.
53. Smolders J, Thewissen M, Peelen E, Menheere P, Tervaert JWC, Damoiseaux J, et al. Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. PloS one. 2009;4(8):e6635-e.

54. Becklund BR, Severson KS, Vang SV, DeLuca HF. UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production. Proceedings of the National Academy of Sciences. 2010;107(14):6418.
55. Mehta BK. New hypotheses on sunlight and the geographic variability of multiple sclerosis prevalence. Journal of the Neurological Sciences. 2010;292(1):5-10.
56. Ramagopalan SV, Valdar W, Criscuoli M, DeLuca GC, Dyment DA, Orton SM, et al. Age of puberty and the risk of multiple sclerosis: a population based study. Eur J Neurol. 2009;16(3):342-7.
57. Sloka JS, Pryse-Phillips WE, Stefanelli M. The relation between menarche and the age of first symptoms in a multiple sclerosis cohort. Mult Scler. 2006;12(3):333-9.
58. Bergamaschi R. Prognostic factors in multiple sclerosis. Int Rev Neurobiol. 2007;79:423-47.
59. Runmarker B, Andersen O. Pregnancy is associated with a lower risk of onset and a better prognosis in multiple sclerosis. Brain. 1995;118 (Pt 1):253-61.
60. Airas L, Saraste M, Rinta S, Elovaara I, Huang YH, Wiendl H, et al. Immunoregulatory factors in multiple sclerosis patients during and after pregnancy: relevance of natural killer cells. Clin Exp Immunol. 2008;151(2):235-43.
61. Hernan MA, Hohol MJ, Olek MJ, Spiegelman D, Ascherio A. Oral contraceptives and the incidence of multiple sclerosis. Neurology. 2000;55(6):848-54.
62. Alonso A, Jick SS, Olek MJ, Ascherio A, Jick H, Hernan MA. Recent use of oral contraceptives and the risk of multiple sclerosis. Arch Neurol. 2005;62(9):1362-5.
63. Sheik-Ali S. Infectious mononucleosis and multiple sclerosis - Updated review on associated risk. Mult Scler Relat Disord. 2017;14:56-9.
64. Handel AE, Williamson AJ, Disanto G, Handunnetthi L, Giovannoni G, Ramagopalan SV. An Updated Meta-Analysis of Risk of Multiple Sclerosis following Infectious Mononucleosis. PLOS ONE. 2010;5(9):e12496.
65. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol. 2007;61(4):288-99.
66. Ahmed SI, Aziz K, Gul A, Samar SS, Bareeqa SB. Risk of Multiple Sclerosis in Epstein-Barr Virus Infection. Cureus. 2019;11(9):e5699-e.
67. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science. 1985;230(4729):1043-5.
68. Duke RC. Self recognition by T cells. I. Bystander killing of target cells bearing syngeneic MHC antigens. J Exp Med. 1989;170(1):59-71.
69. Virtanen JO, Jacobson S. Viruses and multiple sclerosis. CNS Neurol Disord Drug Targets. 2012;11(5):528-44.
70. Hedström AK, Olsson T, Alfredsson L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. Multiple Sclerosis Journal. 2012;18(9):1334-6.
71. Hedstrom AK, Alfredsson L, Olsson T. Environmental factors and their interactions with risk genotypes in MS susceptibility. Curr Opin Neurol. 2016;29(3):293-8.
72. Wesnes K, Riise T, Casetta I, Drulovic J, Granieri E, Holmoy T, et al. Body size and the risk of multiple sclerosis in Norway and Italy: the EnvIMS study. Mult Scler. 2015;21(4):388-95.

73. Matarese G, Carrieri PB, La Cava A, Perna F, Sanna V, De Rosa V, et al. Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells. Proc Natl Acad Sci U S A. 2005;102(14):5150-5.
74. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr. 2000;72(3):690-3.
75. Hedstrom AK, Lima Bomfim I, Hillert J, Olsson T, Alfredsson L. Obesity interacts with infectious mononucleosis in risk of multiple sclerosis. Eur J Neurol. 2015;22(3):578-e38.
76. Montgomery S, Hiyoshi A, Burkill S, Alfredsson L, Bahmanyar S, Olsson T. Concussion in adolescence and risk of multiple sclerosis. Ann Neurol. 2017;82(4):554-61.
77. Gould AL, Black L, Smith J, Gonzales E, Lucas R, editors. Fresh fish consumption is associated with a lower risk of multiple sclerosis independent of serum 25OHD levels. MULTIPLE SCLEROSIS JOURNAL; 2017: SAGE PUBLICATIONS LTD 1 OLIVERS YARD, 55 CITY ROAD, LONDON EC1Y 1SP, ENGLAND.
78. Shamriz O, Mizrahi H, Werbner M, Shoenfeld Y, Avni O, Koren O. Microbiota at the crossroads of autoimmunity. Autoimmun Rev. 2016;15(9):859-69.
79. Ly NP, Litonjua A, Gold DR, Celedón JC. Gut microbiota, probiotics, and vitamin D: Interrelated exposures influencing allergy, asthma, and obesity? Journal of Allergy and Clinical Immunology. 2011;127(5):1087-94.
80. Biedermann L, Zeitz J, Mwinyi J, Sutter-Minder E, Rehman A, Ott SJ, et al. Smoking Cessation Induces Profound Changes in the Composition of the Intestinal Microbiota in Humans. PLOS ONE. 2013;8(3):e59260.
81. Kimura K, Hunter SF, Thollander MS, Loftus EV, Jr., Melton LJ, 3rd, O'Brien PC, et al. Concurrence of inflammatory bowel disease and multiple sclerosis. Mayo Clin Proc. 2000;75(8):802-6.
82. Gupta G, Gelfand JM, Lewis JD. Increased risk for demyelinating diseases in patients with inflammatory bowel disease. Gastroenterology. 2005;129(3):819-26.
83. Cekanaviciute E, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, et al. Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. Proceedings of the National Academy of Sciences. 2017;114(40):10713.
84. Kirby TO, Ochoa-Repáraz J. The Gut Microbiome in Multiple Sclerosis: A Potential Therapeutic Avenue. Med Sci (Basel). 2018;6(3):69.
85. Bunge MB, Bunge RP, Pappas GD. Electron microscopic demonstration of connections between glia and myelin sheaths in the developing mammalian central nervous system. J Cell Biol. 1962;12:448-53.
86. Chang K-J, Redmond SA, Chan JR. Remodeling myelination: implications for mechanisms of neural plasticity. Nat Neurosci. 2016;19(2):190-7.
87. Bercury KK, Macklin WB. Dynamics and mechanisms of CNS myelination. Dev Cell. 2015;32(4):447-58.
88. Funfschilling U, Supplie LM, Mahad D, Boretius S, Saab AS, Edgar J, et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. Nature. 2012;485(7399):517-21.
89. Bradl M, Lassmann H. Oligodendrocytes: biology and pathology. Acta Neuropathol. 2010;119(1):37-53.

90. Jahn O, Tenzer S, Werner HB. Myelin proteomics: molecular anatomy of an insulating sheath. Mol Neurobiol. 2009;40(1):55-72.
91. Waxman SG. Determinants of conduction velocity in myelinated nerve fibers. Muscle Nerve. 1980;3(2):141-50.
92. Babbs CF, Shi R. Subtle paranodal injury slows impulse conduction in a mathematical model of myelinated axons. PLoS One. 2013;8(7):e67767.
93. Waxman SG. Axon-glia interactions: building a smart nerve fiber. Curr Biol. 1997;7(7):R406-10.
94. Larochelle C, Metz I, Lécuyer M-A, Terouz S, Roger M, Arbour N, et al. Immunological and pathological characterization of fatal rebound MS activity following natalizumab withdrawal. Multiple Sclerosis Journal. 2016;23(1):72-81.
95. Kuhlmann T, Ludwin S, Prat A, Antel J, Brück W, Lassmann H. An updated histological classification system for multiple sclerosis lesions. Acta Neuropathologica. 2017;133(1):13-24.
96. Gh Popescu BF, Lucchinetti CF. Meningeal and cortical grey matter pathology in multiple sclerosis. BMC Neurology. 2012;12(1):11.
97. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Ann Neurol. 2001;50(3):389-400.
98. Grigoriadis N, van Pesch V. A basic overview of multiple sclerosis immunopathology. Eur J Neurol. 2015;22 Suppl 2:3-13.
99. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. N Engl J Med. 1998;338(5):278-85.
100. Buss A, Brook GA, Kakulas B, Martin D, Franzen R, Schoenen J, et al. Gradual loss of myelin and formation of an astrocytic scar during Wallerian degeneration in the human spinal cord. Brain. 2004;127(Pt 1):34-44.
101. Franklin RJM, ffrench-Constant C. Regenerating CNS myelin — from mechanisms to experimental medicines. Nature Reviews Neuroscience. 2017;18(12):753-69.
102. Podbielska M, Banik NL, Kurowska E, Hogan EL. Myelin recovery in multiple sclerosis: the challenge of remyelination. Brain Sci. 2013;3(3):1282-324.
103. Lassmann H. Mechanisms of white matter damage in multiple sclerosis. Glia. 2014;62(11):1816-30.
104. Hampton DW, Innes N, Merkler D, Zhao C, Franklin RJ, Chandran S. Focal immune-mediated white matter demyelination reveals an age-associated increase in axonal vulnerability and decreased remyelination efficiency. Am J Pathol. 2012;180(5):1897-905.
105. Miller SD, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol. 2010;Chapter 15:Unit 15.1.
106. Willis SN, Stathopoulos P, Chastre A, Compton SD, Hafler DA, O'Connor KC. Investigating the Antigen Specificity of Multiple Sclerosis Central Nervous System-Derived Immunoglobulins. Front Immunol. 2015;6:600-.
107. Baecher-Allan C, Kaskow BJ, Weiner HL. Multiple Sclerosis: Mechanisms and Immunotherapy. Neuron. 2018;97(4):742-68.
108. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med. 2000;192(3):393-404.

109. Traugott U, Reinherz EL, Raine CS. Multiple sclerosis: Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. Journal of Neuroimmunology. 1983;4(3):201-21.
110. Hoftberger R, Aboul-Enein F, Brueck W, Lucchinetti C, Rodriguez M, Schmidbauer M, et al. Expression of major histocompatibility complex class I molecules on the different cell types in multiple sclerosis lesions. Brain Pathol. 2004;14(1):43-50.
111. Gobin SJ, Montagne L, Van Zutphen M, Van Der Valk P, Van Den Elsen PJ, De Groot CJ. Upregulation of transcription factors controlling MHC expression in multiple sclerosis lesions. Glia. 2001;36(1):68-77.
112. Ifergan I, Kebir H, Alvarez JI, Marceau G, Bernard M, Bourbonnière L, et al. Central nervous system recruitment of effector memory CD8+ T lymphocytes during neuroinflammation is dependent on α4 integrin. Brain : a journal of neurology. 2011;134(Pt 12):3560-77.
113. Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Bruck W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain. 2000;123 (Pt 6):1174-83.
114. Goverman J, Perchellet A, Huseby ES. The role of CD8(+) T cells in multiple sclerosis and its animal models. Curr Drug Targets Inflamm Allergy. 2005;4(2):239-45.
115. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nature Reviews Immunology. 2002;2(6):401-9.
116. Neumann H, Medana IM, Bauer J, Lassmann H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. Trends Neurosci. 2002;25(6):313-9.
117. Zang YC, Li S, Rivera VM, Hong J, Robinson RR, Breitbach WT, et al. Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. J Immunol. 2004;172(8):5120-7.
118. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 Production in Central Nervous System-Infiltrating T Cells and Glial Cells Is Associated with Active Disease in Multiple Sclerosis. The American Journal of Pathology. 2008;172(1):146-55.
119. Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. J Clin Invest. 2013;123(1):247-60.
120. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol. 2008;172(1):146-55.
121. Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. The Journal of clinical investigation. 2013;123(1):247-60.
122. Zozulya AL, Wiendl H. The role of CD8 suppressors versus destructors in autoimmune central nervous system inflammation. Hum Immunol. 2008;69(11):797-804.
123. Traugott U, Reinherz EL, Raine CS. Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. J Neuroimmunol. 1983;4(3):201-21.
124. Friese MA, Fugger L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain. 2005;128(Pt 8):1747-63.
125. Tato CM, O'Shea JJ. What does it mean to be just 17? Nature. 2006;441(7090):166-7.

126. Brummelman J, Pilipow K, Lugli E. Chapter Two - The Single-Cell Phenotypic Identity of Human CD8+ and CD4+ T Cells. In: Galluzzi L, Rudqvist N-P, editors. International Review of Cell and Molecular Biology. 341: Academic Press; 2018. p. 63-124.
127. Rothhammer V, Heink S, Petermann F, Srivastava R, Claussen MC, Hemmer B, et al. Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. J Exp Med. 2011;208(12):2465-76.
128. Reiner SL. Development in motion: helper T cells at work. Cell. 2007;129(1):33-6.
129. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J Exp Med. 2008;205(7):1535-41.
130. Hofman FM, von Hanwehr RI, Dinarello CA, Mizel SB, Hinton D, Merrill JE. Immunoregulatory molecules and IL 2 receptors identified in multiple sclerosis brain. The Journal of Immunology. 1986;136(9):3239.
131. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. J Clin Invest. 2007;117(5):1119-27.
132. Merrill JE, Kagan JM, Schmid I, Strom SR, Quan SG, Chen ISY. T cell lines established from multiple sclerosis cerebrospinal fluid T cells using human retroviruses. Journal of Neuroimmunology. 1989;21(2):213-26.
133. Renno T, Krakowski M, Piccirillo C, Lin JY, Owens T. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. J Immunol. 1995;154(2):944-53.
134. Pierson E, Simmons SB, Castelli L, Goverman JM. Mechanisms regulating regional localization of inflammation during CNS autoimmunity. Immunol Rev. 2012;248(1):205-15.
135. Panitch HS, Hirsch RL, Haley AS, Johnson KP. Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet. 1987;1(8538):893-5.
136. Rasouli J, Ciric B, Imitola J, Gonnella P, Hwang D, Mahajan K, et al. Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-beta Therapy. J Immunol. 2015;194(11):5085-93.
137. Imitola J, Rasouli J, Watanabe F, Mahajan K, Sharan AD, Ciric B, et al. Elevated expression of granulocyte-macrophage colony-stimulating factor receptor in multiple sclerosis lesions. Journal of Neuroimmunology. 2018;317:45-54.
138. Yang J. Chapter 5 - Th17 Cells. In: Tan S-L, editor. Translational Immunology. Boston: Academic Press; 2016. p. 133-63.
139. Brucklacher-Waldert V, Stuerner K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. Brain. 2009;132(Pt 12):3329-41.
140. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Med. 2007;13(10):1173-5.
141. Matusevicius D, Kivisakk P, He B, Kostulas N, Ozenci V, Fredrikson S, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Mult Scler. 1999;5(2):101-4.

142. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med. 1998;187(1):129-34.
143. Clerici M, Saresella M, Trabattoni D, Speciale L, Fossati S, Ruzzante S, et al. Single-cell analysis of cytokine production shows different immune profiles in multiple sclerosis patients with active or quiescent disease. Journal of Neuroimmunology. 2001;121(1):88-101.
144. Al-Shammri S, Rawoot P, Azizieh F, AbuQoora A, Hanna M, Saminathan TR, et al. Th1/Th2 cytokine patterns and clinical profiles during and after pregnancy in women with multiple sclerosis. Journal of the Neurological Sciences. 2004;222(1):21-7.
145. Read S, Maloy KJ, Powrie F. CHAPTER 9 - Regulatory T Cells. In: Rose NR, Mackay IR, editors. The Autoimmune Diseases (Fourth Edition). St. Louis: Academic Press; 2006. p. 119-31.
146. Torkildsen Ø, Myhr K-M, Bø L. Disease-modifying treatments for multiple sclerosis – a review of approved medications. European Journal of Neurology. 2016;23(S1):18-27.
147. Kieseier BC. The mechanism of action of interferon-β in relapsing multiple sclerosis. CNS Drugs. 2011;25(6):491-502.
148. Racke MK, Lovett-Racke AE, Karandikar NJ. The mechanism of action of glatiramer acetate treatment in multiple sclerosis. Neurology. 2010;74 Suppl 1:S25-30.
149. Carlström KE, Ewing E, Granqvist M, Gyllenberg A, Aeinehband S, Enoksson SL, et al. Therapeutic efficacy of dimethyl fumarate in relapsing-remitting multiple sclerosis associates with ROS pathway in monocytes. Nature Communications. 2019;10(1):3081.
150. Allison AC, Eugui EM. Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs. Transplant Proc. 1993;25(3 Suppl 2):8-18.
151. Vermersch P, Czlonkowska A, Grimaldi LM, Confavreux C, Comi G, Kappos L, et al. Teriflunomide versus subcutaneous interferon beta-1a in patients with relapsing multiple sclerosis: a randomised, controlled phase 3 trial. Mult Scler. 2014;20(6):705-16.
152. Tintore M, Vidal-Jordana A, Sastre-Garriga J. Treatment of multiple sclerosis - success from bench to bedside. Nat Rev Neurol. 2019;15(1):53-8.
153. Kappos L, Radue E-W, O'Connor P, Polman C, Hohlfeld R, Calabresi P, et al. A Placebo-Controlled Trial of Oral Fingolimod in Relapsing Multiple Sclerosis. New England Journal of Medicine. 2010;362(5):387-401.
154. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med. 2006;354(9):899-910.
155. Clifford DB, De Luca A, Simpson DM, Arendt G, Giovannoni G, Nath A. Natalizumab-associated progressive multifocal leukoencephalopathy in patients with multiple sclerosis: lessons from 28 cases. Lancet Neurol. 2010;9(4):438-46.
156. Havari E, Turner MJ, Campos-Rivera J, Shankara S, Nguyen TH, Roberts B, et al. Impact of alemtuzumab treatment on the survival and function of human regulatory T cells in vitro. Immunology. 2014;141(1):123-31.
157. Castro-Borrero W, Graves D, Frohman TC, Flores AB, Hardeman P, Logan D, et al. Current and emerging therapies in multiple sclerosis: a systematic review. Ther Adv Neurol Disord. 2012;5(4):205-20.
158. Baxter AG. The origin and application of experimental autoimmune encephalomyelitis. Nature reviews Immunology. 2007;7(11):904-12.

159. Freund J, McDermott K. Sensitization to Horse Serum by Means of Adjuvants. Proceedings of the Society for Experimental Biology and Medicine. 1942;49(4):548-53.
160. Munoz JJ, Bernard CCA, Mackay IR. Elicitation of experimental allergic encephalomyelitis (EAE) in mice with the aid of pertussigen. Cellular Immunology. 1984;83(1):92-100.
161. Miller SD, Karpus WJ. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol. 2007;Chapter 15:Unit-15.1.
162. Stromnes IM, Goverman JM. Passive induction of experimental allergic encephalomyelitis. Nature Protocols. 2006;1(4):1952-60.
163. Pöllinger B, Krishnamoorthy G, Berer K, Lassmann H, Bösl MR, Dunn R, et al. Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. J Exp Med. 2009;206(6):1303-16.
164. Dhaeze T, Lachance C, Tremblay L, Grasmuck C, Bourbonnière L, Larouche S, et al. Sex-dependent factors encoded in the immune compartment dictate relapsing or progressive phenotype in demyelinating disease. JCI Insight. 2019;4(6):e124885.
165. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol. 2006;60(1):12-21.
166. Mueller DL. Mechanisms maintaining peripheral tolerance. Nat Immunol. 2010;11(1):21-7.
167. Immune Regulation in Human Health and Disease. eLS. p. 1-17.
168. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. Blood. 2002;99(7):2468-76.
169. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity. 2008;29(5):758-70.
170. Domogalla MP, Rostan PV, Raker VK, Steinbrink K. Tolerance through Education: How Tolerogenic Dendritic Cells Shape Immunity. Frontiers in Immunology. 2017;8(1764).
171. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol. 2012;30:221-41.
172. Mizoguchi A, Bhan AK. A case for regulatory B cells. J Immunol. 2006;176(2):705-10.
173. Ray A, Dittel BN. Mechanisms of Regulatory B cell Function in Autoimmune and Inflammatory Diseases beyond IL-10. J Clin Med. 2017;6(1):12.
174. Vuddamalay Y, van Meerwijk JPM. CD28(-) and CD28(low)CD8(+) Regulatory T Cells: Of Mice and Men. Frontiers in immunology. 2017;8:31-.
175. Uss E, Rowshani AT, Hooibrink B, Lardy NM, van Lier RA, ten Berge IJ. CD103 is a marker for alloantigen-induced regulatory CD8+ T cells. J Immunol. 2006;177(5):2775-83.
176. Feger U, Tolosa E, Huang YH, Waschbisch A, Biedermann T, Melms A, et al. HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation. Blood. 2007;110(2):568-77.
177. Rifa'i M, Shi Z, Zhang SY, Lee YH, Shiku H, Isobe K, et al. CD8+CD122+ regulatory T cells recognize activated T cells via conventional MHC class I-alphabetaTCR interaction and become IL-10-producing active regulatory cells. Int Immunol. 2008;20(7):937-47.
178. Zhang J, Medaer R, Stinissen P, Hafler D, Raus J. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. Science. 1993;261(5127):1451-4.

179. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science. 1994;265(5176):1237-40.
180. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev. 2001;182:207-14.
181. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. J Immunol. 2007;178(1):179-85.
182. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. Immunity. 2018;49(6):1004-19.
183. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med. 2013;19(6):739-46.
184. Ronchetti S, Ricci E, Petrillo MG, Cari L, Migliorati G, Nocentini G, et al. Glucocorticoid-induced tumour necrosis factor receptor-related protein: a key marker of functional regulatory T cells. J Immunol Res. 2015;2015:171520-.
185. Owen DL, Sjaastad LE, Farrar MA. Regulatory T Cell Development in the Thymus. The Journal of Immunology. 2019;203(8):2031.
186. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, et al. Regulatory T cells: recommendations to simplify the nomenclature. Nature Immunology. 2013;14(4):307-8.
187. Miller JFAP, Haddow A. Effect of neonatal thymectomy on the immunological responsiveness of the mouse. Proceedings of the Royal Society of London Series B Biological Sciences. 1962;156(964):415-28.
188. Sakaguchi S. Regulatory T Cells: History and Perspective. In: Kassiotis G, Liston A, editors. Regulatory T Cells: Methods and Protocols. Totowa, NJ: Humana Press; 2011. p. 3-17.
189. Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T Cells. The Journal of Immunology. 1972;108(3):586.
190. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. The Journal of Immunology. 1995;155(3):1151.
191. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med. 1996;184(2):387-96.
192. Li MO, Rudensky AY. T cell receptor signalling in the control of regulatory T cell differentiation and function. Nature reviews Immunology. 2016;16(4):220-33.
193. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol. 2005;6(2):152-62.
194. Tai X, Erman B, Alag A, Mu J, Kimura M, Katz G, et al. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. Immunity. 2013;38(6):1116-28.
195. Wing JB, Sakaguchi S. 18 - Regulatory Immune Cells. In: Rich RR, Fleisher TA, Shearer WT, Schroeder HW, Frew AJ, Weyand CM, editors. Clinical Immunology (Fifth Edition). London: Content Repository Only!; 2019. p. 261-71.e1.

196. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat Immunol. 2008;9(6):632-40.
197. Li MO, Rudensky AY. T cell receptor signalling in the control of regulatory T cell differentiation and function. Nature reviews Immunology. 2016;16(4):220-33.
198. Wing K, Fehérvári Z, Sakaguchi S. Emerging possibilities in the development and function of regulatory T cells. Int Immunol. 2006;18(7):991-1000.
199. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nature Immunology. 2010;11(1):7-13.
200. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol. 2009;27:313-38.
201. Horwitz DA, Zheng SG, Wang J, Gray JD. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. Eur J Immunol. 2008;38(4):912-5.
202. Xu L, Ma C, Huang X, Yang W, Chen L, Bilotta AJ, et al. Microbiota metabolites short-chain fatty acid butyrate conditions intestinal epithelial cells to promote development of Treg cells and T cell IL-10 production. The Journal of Immunology. 2018;200(1 Supplement):53.16.
203. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science. 2011;331(6015):337-41.
204. Schmidt A, Eriksson M, Shang MM, Weyd H, Tegnér J. Comparative Analysis of Protocols to Induce Human CD4+Foxp3+ Regulatory T Cells by Combinations of IL-2, TGF-beta, Retinoic Acid, Rapamycin and Butyrate. PLoS One. 2016;11(2):e0148474.
205. Lin X, Chen M, Liu Y, Guo Z, He X, Brand D, et al. Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. Int J Clin Exp Pathol. 2013;6(2):116-23.
206. Shevach EM, Thornton AM. tTregs, pTregs, and iTregs: similarities and differences. Immunol Rev. 2014;259(1):88-102.
207. Taams LS, Vukmanovic-Stejic M, Smith J, Dunne PJ, Fletcher JM, Plunkett FJ, et al. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. European Journal of Immunology. 2002;32(6):1621-30.
208. Kmieciak M, Gowda M, Graham L, Godder K, Bear HD, Marincola FM, et al. Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function. J Transl Med. 2009;7:89-.
209. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006;203(7):1693-700.
210. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203(7):1701-11.
211. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. Nat Rev Immunol. 2007;7(2):144-54.
212. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. Nature Immunology. 2007;8(5):457-62.
213. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001;27(1):68-73.

214. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27(1):20-1.
215. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med. 2007;357(9):851-62.
216. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. 2007;8(3):277-84.
217. Ziegler SF. FOXP3: Of Mice and Men. Annual Review of Immunology. 2006;24(1):209-26.
218. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol. 2007;37(1):129-38.
219. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions. Autoimmun Rev. 2015;14(2):105-16.
220. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, et al. The interaction properties of costimulatory molecules revisited. Immunity. 2002;17(2):201-10.
221. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol. 2002;3(11):1097-101.
222. Dejean AS, Beisner DR, Ch'en IL, Kerdiles YM, Babour A, Arden KC, et al. Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. Nat Immunol. 2009;10(5):504-13.
223. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). J Immunol. 2005;174(2):688-95.
224. Barthlott T, Moncrieffe H, Veldhoen M, Atkins CJ, Christensen J, O'Garra A, et al. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. Int Immunol. 2005;17(3):279-88.
225. Scheffold A, Hühn J, Höfer T. Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango. European Journal of Immunology. 2005;35(5):1336-41.
226. Klein M, Bopp T. Cyclic AMP Represents a Crucial Component of Treg Cell-Mediated Immune Regulation. Frontiers in immunology. 2016;7:315-.
227. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. The Journal of experimental medicine. 2007;204(6):1303-10.
228. Zhao D-M, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood. 2006;107(10):3925-32.
229. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, et al. Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. Immunity. 2007;27(4):635-46.
230. Askenasy N. Enhanced killing activity of regulatory T cells ameliorates inflammation and autoimmunity. Autoimmunity Reviews. 2013;12(10):972-5.
231. Garín MI, Chu C-C, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. Blood. 2006;109(5):2058-65.
232. Leitner J, Rieger A, Pickl WF, Zlabinger G, Grabmeier-Pfistershammer K, Steinberger P. TIM-3 Does Not Act as a Receptor for Galectin-9. PLOS Pathogens. 2013;9(3):e1003253.

233. Kubach J, Lutter P, Bopp T, Stoll S, Becker C, Huter E, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. Blood. 2007;110(5):1550-8.
234. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. Immunity. 2008;28(4):546-58.
235. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17–producing human T helper cells. Nature Immunology. 2007;8(9):942-9.
236. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. GARP (LRRC32) is essential for the surface expression of latent TGF-β on platelets and activated FOXP3⁺ regulatory T cells. Proceedings of the National Academy of Sciences. 2009;106(32):13445-50.
237. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. Proceedings of the National Academy of Sciences. 2009;106(32):13439-44.
238. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359(6397):693-9.
239. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. Journal of Experimental Medicine. 1999;190(7):995-1004.
240. O'Garra A, Vieira P. TH1 cells control themselves by producing interleukin-10. Nature Reviews Immunology. 2007;7(6):425-8.
241. Grazia Roncarolo M, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunological Reviews. 2006;212(1):28-50.
242. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nature Immunology. 2009;10(11):1178-84.
243. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. The Journal of experimental medicine. 1999;190(7):995-1004.
244. Sojka DK, Fowell DJ. Regulatory T cells inhibit acute IFN-γ synthesis without blocking T-helper cell type 1 (Th1) differentiation via a compartmentalized requirement for IL-10. Proceedings of the National Academy of Sciences. 2011;108(45):18336-41.
245. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol. 1998;10(12):1969-80.
246. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007;450(7169):566-9.
247. Bardel E, Larousserie F, Charlot-Rabiega P, Coulomb L, Herminé A, Devergne O. Human CD4⁺CD25⁺Foxp3⁺

Regulatory T Cells Do Not Constitutively Express IL-35. The Journal of Immunology. 2008;181(10):6898.
248. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature. 1997;389(6652):737-42.
249. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, et al. IL-35-mediated induction of a potent regulatory T cell population. Nature Immunology. 2010;11(12):1093-101.
250. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3+ regulatory T cells under inflammatory or non-inflammatory conditions. Seminars in Immunology. 2011;23(6):424-30.
251. Putheti P, Pettersson A, Soderstrom M, Link H, Huang YM. Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs. J Clin Immunol. 2004;24(2):155-61.
252. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med. 2004;199(7):971-9.
253. Fritzsching B, Haas J, König F, Kunz P, Fritzsching E, Pöschl J, et al. Intracerebral human regulatory T cells: analysis of CD4+ CD25+ FOXP3+ T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients. PloS one. 2011;6(3):e17988-e.
254. Zandee SEJ, O'Connor RA, Mair I, Leech MD, Williams A, Anderton SM. IL-10-producing, ST2-expressing Foxp3(+) T cells in multiple sclerosis brain lesions. Immunol Cell Biol. 2017;95(5):484-90.
255. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med. 2007;13(4):423-31.
256. Zhang X, Koldzic DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S, et al. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. International Immunology. 2004;16(2):249-56.
257. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol. 2005;175(5):3025-32.
258. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178-201.
259. Daneman R, Prat A. The blood-brain barrier. Cold Spring Harb Perspect Biol. 2015;7(1):a020412-a.
260. Daneman R. The blood-brain barrier in health and disease. Ann Neurol. 2012;72(5):648-72.
261. Netto JP, Iliff J, Stanimirovic D, Krohn KA, Hamilton B, Varallyay C, et al. Neurovascular Unit: Basic and Clinical Imaging with Emphasis on Advantages of Ferumoxytol. Neurosurgery. 2017;82(6):770-80.
262. Daneman R, Zhou L, Agalliu D, Cahoy JD, Kaushal A, Barres BA. The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. PLoS One. 2010;5(10):e13741.

263. Furuse M. Molecular basis of the core structure of tight junctions. Cold Spring Harb Perspect Biol. 2010;2(1):a002907.
264. Brightman MW, Reese TS. Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol. 1969;40(3):648-77.
265. Westergaard E, Brightman MW. Transport of proteins across normal cerebral arterioles. J Comp Neurol. 1973;152(1):17-44.
266. Mittapalli RK, Manda VK, Adkins CE, Geldenhuys WJ, Lockman PR. Exploiting nutrient transporters at the blood-brain barrier to improve brain distribution of small molecules. Ther Deliv. 2010;1(6):775-84.
267. Loscher W, Potschka H. Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx. 2005;2(1):86-98.
268. Henninger DD, Panes J, Eppihimer M, Russell J, Gerritsen M, Anderson DC, et al. Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. J Immunol. 1997;158(4):1825-32.
269. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res. 2005;97(6):512-23.
270. Shepro D, Morel NM. Pericyte physiology. Faseb j. 1993;7(11):1031-8.
271. Gerhardt H, Wolburg H, Redies C. N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. Dev Dyn. 2000;218(3):472-9.
272. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21(2):193-215.
273. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010;468(7323):562-6.
274. Sorokin L. The impact of the extracellular matrix on inflammation. Nature reviews Immunology. 2010;10(10):712-23.
275. Owens T, Bechmann I, Engelhardt B. Perivascular spaces and the two steps to neuroinflammation. J Neuropathol Exp Neurol. 2008;67(12):1113-21.
276. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. Nature Reviews Neuroscience. 2006;7(1):41-53.
277. Perea G, Navarrete M, Araque A. Tripartite synapses: astrocytes process and control synaptic information. Trends Neurosci. 2009;32(8):421-31.
278. Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010;468(7321):232-43.
279. Noell S, Wolburg-Buchholz K, Mack AF, Beedle AM, Satz JS, Campbell KP, et al. Evidence for a role of dystroglycan regulating the membrane architecture of astroglial endfeet. Eur J Neurosci. 2011;33(12):2179-86.
280. Larochelle C, Alvarez JI, Prat A. How do immune cells overcome the blood–brain barrier in multiple sclerosis? FEBS Letters. 2011;585(23):3770-80.
281. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41-53.
282. Janzer RC, Raff MC. Astrocytes induce blood-brain barrier properties in endothelial cells. Nature. 1987;325(6101):253-7.

283. Al Ahmad A, Taboada CB, Gassmann M, Ogunshola OO. Astrocytes and pericytes differentially modulate blood-brain barrier characteristics during development and hypoxic insult. J Cereb Blood Flow Metab. 2011;31(2):693-705.
284. Miljkovic D, Timotijevic G, Mostarica Stojkovic M. Astrocytes in the tempest of multiple sclerosis. FEBS Lett. 2011;585(23):3781-8.
285. Snyder JM, Hagan CE, Bolon B, Keene CD. 20 - Nervous System. In: Treuting PM, Dintzis SM, Montine KS, editors. Comparative Anatomy and Histology (Second Edition). San Diego: Academic Press; 2018. p. 403-44.
286. Hartman AL. CHAPTER 2 - Normal Anatomy of the Cerebrospinal Fluid Compartment. In: Irani DN, editor. Cerebrospinal Fluid in Clinical Practice. Philadelphia: W.B. Saunders; 2009. p. 5-10.
287. Yasuda K, Cline C, Vogel P, Onciu M, Fatima S, Sorrentino BP, et al. Drug transporters on arachnoid barrier cells contribute to the blood-cerebrospinal fluid barrier. Drug Metab Dispos. 2013;41(4):923-31.
288. Pizzo ME, Wolak DJ, Kumar NN, Brunette E, Brunnquell CL, Hannocks MJ, et al. Intrathecal antibody distribution in the rat brain: surface diffusion, perivascular transport and osmotic enhancement of delivery. J Physiol. 2018;596(3):445-75.
289. Mastorakos P, McGavern D. The anatomy and immunology of vasculature in the central nervous system. Sci Immunol. 2019;4(37).
290. Strazielle N, Ghersi-Egea J-F. Choroid Plexus in the Central Nervous System: Biology and Physiopathology. Journal of Neuropathology & Experimental Neurology. 2000;59(7):561-74.
291. Williams JL, Holman DW, Klein RS. Chemokines in the balance: maintenance of homeostasis and protection at CNS barriers. Front Cell Neurosci. 2014;8:154-.
292. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337-41.
293. Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. Journal of Experimental Medicine. 2015;212(7):991-9.
294. Mundt S, Mrdjen D, Utz SG, Greter M, Schreiner B, Becher B. Conventional DCs sample and present myelin antigens in the healthy CNS and allow parenchymal T cell entry to initiate neuroinflammation. Science immunology. 2019;4(31).
295. Bartholomaus I, Kawakami N, Odoardi F, Schlager C, Miljkovic D, Ellwart JW, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. Nature. 2009;462(7269):94-8.
296. Nayak D, Zinselmeyer BH, Corps KN, McGavern DB. In vivo dynamics of innate immune sentinels in the CNS. Intravital. 2012;1(2):95-106.
297. Kivisakk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, et al. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. Proc Natl Acad Sci U S A. 2003;100(14):8389-94.
298. Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. J Neurosci Res. 1991;28(2):254-60.
299. Ludowyk PA, Willenborg DO, Parish CR. Selective localisation of neuro-specific T lymphocytes in the central nervous system. J Neuroimmunol. 1992;37(3):237-50.

300. Piccio L, Rossi B, Scarpini E, Laudanna C, Giagulli C, Issekutz AC, et al. Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors. J Immunol. 2002;168(4):1940-9.
301. Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature reviews Immunology. 2003;3(7):569-81.
302. Mrass P, Weninger W. Immune cell migration as a means to control immune privilege: lessons from the CNS and tumors. Immunol Rev. 2006;213:195-212.
303. Engelhardt B. Molecular mechanisms involved in T cell migration across the blood-brain barrier. J Neural Transm (Vienna). 2006;113(4):477-85.
304. Kubes P, Ward PA. Leukocyte Recruitment and the Acute Inflammatory Response. Brain Pathology. 2000;10(1):127-35.
305. Biernacki K, Prat A, Blain M, Antel JP. Regulation of Th1 and Th2 lymphocyte migration by human adult brain endothelial cells. J Neuropathol Exp Neurol. 2001;60(12):1127-36.
306. Prat A, Biernacki K, Wosik K, Antel JP. Glial cell influence on the human blood-brain barrier. Glia. 2001;36(2):145-55.
307. Alvarez JI, Cayrol R, Prat A. Disruption of central nervous system barriers in multiple sclerosis. Biochim Biophys Acta. 2011;1812(2):252-64.
308. Cayrol R, Wosik K, Berard JL, Dodelet-Devillers A, Ifergan I, Kebir H, et al. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. Nat Immunol. 2008;9(2):137-45.
309. Dodelet-Devillers A, Cayrol R, van Horssen J, Haqqani AS, de Vries HE, Engelhardt B, et al. Functions of lipid raft membrane microdomains at the blood-brain barrier. J Mol Med (Berl). 2009;87(8):765-74.
310. Greenwood J, Heasman SJ, Alvarez JI, Prat A, Lyck R, Engelhardt B. Review: leucocyte-endothelial cell crosstalk at the blood-brain barrier: a prerequisite for successful immune cell entry to the brain. Neuropathol Appl Neurobiol. 2011;37(1):24-39.
311. Larochelle C, Cayrol R, Kebir H, Alvarez JI, Lecuyer MA, Ifergan I, et al. Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system. Brain. 2012;135(Pt 10):2906-24.
312. Grabovsky V, Feigelson S, Chen C, Bleijs DA, Peled A, Cinamon G, et al. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. J Exp Med. 2000;192(4):495-506.
313. Shulman Z, Shinder V, Klein E, Grabovsky V, Yeger O, Geron E, et al. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. Immunity. 2009;30(3):384-96.
314. Biernacki K, Prat A, Blain M, Antel JP. Regulation of cellular and molecular trafficking across human brain endothelial cells by Th1- and Th2-polarized lymphocytes. J Neuropathol Exp Neurol. 2004;63(3):223-32.
315. Seguin R, Biernacki K, Rotondo RL, Prat A, Antel JP. Regulation and functional effects of monocyte migration across human brain-derived endothelial cells. J Neuropathol Exp Neurol. 2003;62(4):412-9.

316. Michel L, Grasmuck C, Charabati M, Lécuyer M-A, Zandee S, Dhaeze T, et al. Activated leukocyte cell adhesion molecule regulates B lymphocyte migration across central nervous system barriers. Science Translational Medicine. 2019;11(518):eaaw0475.
317. Lécuyer M-A, Saint-Laurent O, Bourbonnière L, Larouche S, Larochelle C, Michel L, et al. Dual role of ALCAM in neuroinflammation and blood–brain barrier homeostasis. Proceedings of the National Academy of Sciences. 2017;114(4):E524.
318. Odoardi F, Sie C, Streyl K, Ulaganathan VK, Schläger C, Lodygin D, et al. T cells become licensed in the lung to enter the central nervous system. Nature. 2012;488(7413):675-9.
319. Ifergan I, Kebir H, Terouz S, Alvarez JI, Lécuyer MA, Gendron S, et al. Role of Ninjurin-1 in the migration of myeloid cells to central nervous system inflammatory lesions. Ann Neurol. 2011;70(5):751-63.
320. Kanwar JR, Kanwar RK, Wang D, Krissansen GW. Prevention of a chronic progressive form of experimental autoimmune encephalomyelitis by an antibody against mucosal addressin cell adhesion molecule-1, given early in the course of disease progression. Immunol Cell Biol. 2000;78(6):641-5.
321. Allavena R, Noy S, Andrews M, Pullen N. CNS elevation of vascular and not mucosal addressin cell adhesion molecules in patients with multiple sclerosis. Am J Pathol. 2010;176(2):556-62.
322. Döring A, Pfeiffer F, Meier M, Dehouck B, Tauber S, Deutsch U, et al. TET inducible expression of the α4β7-integrin ligand MAdCAM-1 on the blood-brain barrier does not influence the immunopathogenesis of experimental autoimmune encephalomyelitis. Eur J Immunol. 2011;41(3):813-21.
323. Sixt M, Engelhardt B, Pausch F, Hallmann R, Wendler O, Sorokin LM. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J Cell Biol. 2001;153(5):933-46.
324. Wu C, Ivars F, Anderson P, Hallmann R, Vestweber D, Nilsson P, et al. Endothelial basement membrane laminin alpha5 selectively inhibits T lymphocyte extravasation into the brain. Nat Med. 2009;15(5):519-27.
325. Graesser D, Mahooti S, Madri JA. Distinct roles for matrix metalloproteinase-2 and alpha4 integrin in autoimmune T cell extravasation and residency in brain parenchyma during experimental autoimmune encephalomyelitis. J Neuroimmunol. 2000;109(2):121-31.
326. Agrawal S, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J Exp Med. 2006;203(4):1007-19.
327. Raine CS, Cannella B, Duijvestijn AM, Cross AH. Homing to central nervous system vasculature by antigen-specific lymphocytes. II. Lymphocyte/endothelial cell adhesion during the initial stages of autoimmune demyelination. Lab Invest. 1990;63(4):476-89.
328. Lehmann JM, Holzmann B, Breitbart EW, Schmiegelow P, Riethmuller G, Johnson JP. Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. Cancer Res. 1987;47(3):841-5.
329. Sers C, Kirsch K, Rothbacher U, Riethmuller G, Johnson JP. Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. Proc Natl Acad Sci U S A. 1993;90(18):8514-8.

330. Yang H, Wang S, Liu Z, Wu MH, McAlpine B, Ansel J, et al. Isolation and characterization of mouse MUC18 cDNA gene, and correlation of MUC18 expression in mouse melanoma cell lines with metastatic ability. Gene. 2001;265(1-2):133-45.
331. Taira E, Nagino T, Taniura H, Takaha N, Kim CH, Kuo CH, et al. Expression and functional analysis of a novel isoform of gicerin, an immunoglobulin superfamily cell adhesion molecule. J Biol Chem. 1995;270(48):28681-7.
332. Bardin N, Frances V, Combes V, Sampol J, Dignat-George F. CD146: biosynthesis and production of a soluble form in human cultured endothelial cells. FEBS Lett. 1998;421(1):12-4.
333. Bardin N, Moal V, Anfosso F, Daniel L, Brunet P, Sampol J, et al. Soluble CD146, a novel endothelial marker, is increased in physiopathological settings linked to endothelial junctional alteration. Thromb Haemost. 2003;90(5):915-20.
334. Wu Z, Wu Z, Li J, Yang X, Wang Y, Yu Y, et al. MCAM is a novel metastasis marker and regulates spreading, apoptosis and invasion of ovarian cancer cells. Tumour Biol. 2012;33(5):1619-28.
335. Wu GJ, Peng Q, Fu P, Wang SW, Chiang CF, Dillehay DL, et al. Ectopical expression of human MUC18 increases metastasis of human prostate cancer cells. Gene. 2004;327(2):201-13.
336. Shih IM, Elder DE, Speicher D, Johnson JP, Herlyn M. Isolation and functional characterization of the A32 melanoma-associated antigen. Cancer Res. 1994;54(9):2514-20.
337. Filshie RJ, Zannettino AC, Makrynikola V, Gronthos S, Henniker AJ, Bendall LJ, et al. MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies. Leukemia. 1998;12(3):414-21.
338. Pickl WF, Majdic O, Fischer GF, Petzelbauer P, Fae I, Waclavicek M, et al. MUC18/MCAM (CD146), an activation antigen of human T lymphocytes. J Immunol. 1997;158(5):2107-15.
339. Bardin N, Anfosso F, Masse JM, Cramer E, Sabatier F, Le Bivic A, et al. Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. Blood. 2001;98(13):3677-84.
340. Kebir A, Harhouri K, Guillet B, Liu JW, Foucault-Bertaud A, Lamy E, et al. CD146 short isoform increases the proangiogenic potential of endothelial progenitor cells in vitro and in vivo. Circ Res. 2010;107(1):66-75.
341. Guezguez B, Vigneron P, Alais S, Jaffredo T, Gavard J, Mege RM, et al. A dileucine motif targets MCAM-l cell adhesion molecule to the basolateral membrane in MDCK cells. FEBS Lett. 2006;580(15):3649-56.
342. Zhuang J, Jiang T, Lu D, Luo Y, Zheng C, Feng J, et al. NADPH oxidase 4 mediates reactive oxygen species induction of CD146 dimerization in VEGF signal transduction. Free Radic Biol Med. 2010;49(2):227-36.
343. Bardin N, Blot-Chabaud M, Despoix N, Kebir A, Harhouri K, Arsanto JP, et al. CD146 and its soluble form regulate monocyte transendothelial migration. Arterioscler Thromb Vasc Biol. 2009;29(5):746-53.
344. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106(8):2923-4.
345. Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 2005;106(8):2923-4.

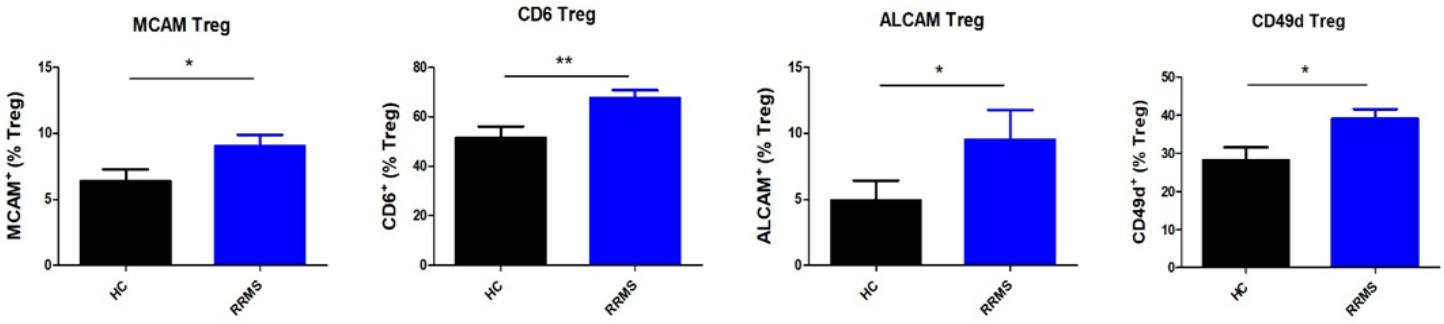
346. Staquicini FI, Tandle A, Libutti SK, Sun J, Zigler M, Bar-Eli M, et al. A subset of host B lymphocytes controls melanoma metastasis through a melanoma cell adhesion molecule/MUC18-dependent interaction: evidence from mice and humans. Cancer Res. 2008;68(20):8419-28.
347. Flanagan K, Fitzgerald K, Baker J, Regnstrom K, Gardai S, Bard F, et al. Laminin-411 is a vascular ligand for MCAM and facilitates TH17 cell entry into the CNS. PLoS One. 2012;7(7):e40443.
348. Jiang T, Zhuang J, Duan H, Luo Y, Zeng Q, Fan K, et al. CD146 is a coreceptor for VEGFR-2 in tumor angiogenesis. Blood. 2012;120(11):2330-9.
349. Ye Z, Zhang C, Tu T, Sun M, Liu D, Lu D, et al. Wnt5a uses CD146 as a receptor to regulate cell motility and convergent extension. Nature Communications. 2013;4(1):2803.
350. Yazawa EM, Geddes-Sweeney JE, Cedeno-Laurent F, Walley KC, Barthel SR, Opperman MJ, et al. Melanoma Cell Galectin-1 Ligands Functionally Correlate with Malignant Potential. J Invest Dermatol. 2015;135(7):1849-62.
351. Tu T, Zhang C, Yan H, Luo Y, Kong R, Wen P, et al. CD146 acts as a novel receptor for netrin-1 in promoting angiogenesis and vascular development. Cell Res. 2015;25(3):275-87.
352. Larochelle C, Lecuyer MA, Alvarez JI, Charabati M, Saint-Laurent O, Ghannam S, et al. Melanoma cell adhesion molecule-positive CD8 T lymphocytes mediate central nervous system inflammation. Ann Neurol. 2015;78(1):39-53.
353. Breuer J, Korpos E, Hannocks M-J, Schneider-Hohendorf T, Song J, Zondler L, et al. Blockade of MCAM/CD146 impedes CNS infiltration of T cells over the choroid plexus. Journal of Neuroinflammation. 2018;15(1):236.
354. Prat A, Biernacki K, Pouly S, Nalbantoglu J, Couture R, Antel JP. Kinin B1 receptor expression and function on human brain endothelial cells. J Neuropathol Exp Neurol. 2000;59(10):896-906.
355. Prat A, Biernacki K, Lavoie JF, Poirier J, Duquette P, Antel JP. Migration of multiple sclerosis lymphocytes through brain endothelium. Arch Neurol. 2002;59(3):391-7.
356. Ifergan I, Wosik K, Cayrol R, Kebir H, Auger C, Bernard M, et al. Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: relevance to multiple sclerosis. Ann Neurol. 2006;60(1):45-55.
357. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol. 2001;167(3):1245-53.
358. Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, et al. Generation and Large-Scale Expansion of Human Inducible Regulatory T Cells That Suppress Graft-Versus-Host Disease. American Journal of Transplantation. 2011;11(6):1148-57.
359. Feger U, Luther C, Poeschel S, Melms A, Tolosa E, Wiendl H. Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. Clin Exp Immunol. 2007;147(3):412-8.
360. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens J-L, et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology. 2008;123(1):79-89.

361. Schafflick D, Xu CA, Hartlehnert M, Cole M, Schulte-Mecklenbeck A, Lautwein T, et al. Integrated single cell analysis of blood and cerebrospinal fluid leukocytes in multiple sclerosis. Nature Communications. 2020;11(1):247.
362. Kleine TO, Benes L. Immune surveillance of the human central nervous system (CNS): Different migration pathways of immune cells through theblood–brain barrier and blood–cerebrospinal fluid barrier in healthy persons. Cytometry Part A. 2006;69A(3):147-51.
363. Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, et al. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. J Exp Med. 2002;196(3):379-87.
364. Jago CB, Yates J, Câmara NO, Lechler RI, Lombardi G. Differential expression of CTLA-4 among T cell subsets. Clin Exp Immunol. 2004;136(3):463-71.
365. Huehn J, Hamann A. Homing to suppress: address codes for Treg migration. Trends in Immunology. 2005;26(12):632-6.
366. Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, et al. CCR6 regulates the migration of inflammatory and regulatory T cells. J Immunol. 2008;181(12):8391-401.
367. Kleinewietfeld M, Puentes F, Borsellino G, Battistini L, Rötzschke O, Falk K. CCR6 expression defines regulatory effector/memory-like cells within the CD25+CD4+ T-cell subset. Blood. 2005;105(7):2877-86.
368. Kitamura K, Farber JM, Kelsall BL. CCR6 marks regulatory T cells as a colon-tropic, IL-10-producing phenotype. J Immunol. 2010;185(6):3295-304.
369. Herman AE, Freeman GJ, Mathis D, Benoist C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med. 2004;199(11):1479-89.
370. Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity. 2009;30(3):458-69.
371. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, Su L, et al. Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. Blood. 2005;106(9):3300-7.
372. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. The Journal of experimental medicine. 2001;193(11):1285-94.
373. Schmidt A, Éliás S, Joshi RN, Tegnér J. In Vitro Differentiation of Human CD4+FOXP3+ Induced Regulatory T Cells (iTregs) from Naïve CD4+ T Cells Using a TGF-β-containing Protocol. J Vis Exp. 2016(118).
374. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? Nature reviews Immunology. 2009;9(2):83-9.
375. Zastepa E, Fitz-Gerald L, Hallett M, Antel J, Bar-Or A, Baranzini S, et al. Naive CD4 T-cell activation identifies MS patients having rapid transition to progressive MS. Neurology. 2014;82(8):681-90.
376. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology. 2008;123(1):79-89.

377. Michel L, Berthelot L, Pettré S, Wiertlewski S, Lefrère F, Braudeau C, et al. Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor alpha-chain are excluded from the analysis. J Clin Invest. 2008;118(10):3411-9.
378. Zhang X, Koldzic DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S, et al. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. Int Immunol. 2004;16(2):249-56.
379. Suri-Payer E, Cantor H. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. J Autoimmun. 2001;16(2):115-23.
380. Sula Karreci E, Eskandari SK, Dotiwala F, Routray SK, Kurdi AT, Assaker JP, et al. Human regulatory T cells undergo self-inflicted damage via granzyme pathways upon activation. JCI Insight. 2017;2(21).
381. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol. 2009;10(6):595-602.
382. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune disease. Nature Immunology. 2018;19(7):665-73.
383. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. Nat Med. 2011;17(6):673-5.
384. McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. J Immunol. 2011;186(7):3918-26.
385. Arterbery AS, Osafo-Addo A, Avitzur Y, Ciarleglio M, Deng Y, Lobritto SJ, et al. Production of Proinflammatory Cytokines by Monocytes in Liver-Transplanted Recipients with De Novo Autoimmune Hepatitis Is Enhanced and Induces TH1-like Regulatory T Cells. Journal of immunology (Baltimore, Md : 1950). 2016;196(10):4040-51.
386. Radhakrishnan S, Cabrera R, Schenk EL, Nava-Parada P, Bell MP, Van Keulen VP, et al. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. J Immunol. 2008;181(5):3137-47.
387. Yang BH, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, et al. Foxp3(+) T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. Mucosal Immunol. 2016;9(2):444-57.
388. Schneider-Hohendorf T, Stenner M-P, Weidenfeller C, Zozulya AL, Simon OJ, Schwab N, et al. Regulatory T cells exhibit enhanced migratory characteristics, a feature impaired in patients with multiple sclerosis. European Journal of Immunology. 2010;40(12):3581-90.
389. Man S, Ubogu EE, Ransohoff RM. Inflammatory Cell Migration into the Central Nervous System: A Few New Twists on an Old Tale. Brain Pathology. 2007;17(2):243-50.
390. Kuerten S, Kostova-Bales DA, Frenzel LP, Tigno JT, Tary-Lehmann M, Angelov DN, et al. MP4- and MOG:35-55-induced EAE in C57BL/6 mice differentially targets brain, spinal cord and cerebellum. J Neuroimmunol. 2007;189(1-2):31-40.
391. Kuerten S, Gruppe TL, Laurentius L-M, Kirch C, Tary-Lehmann M, Lehmann PV, et al. Differential patterns of spinal cord pathology induced by MP4, MOG peptide 35–55, and PLP peptide 178–191 in C57BL/6 mice. APMIS. 2011;119(6):336-46.

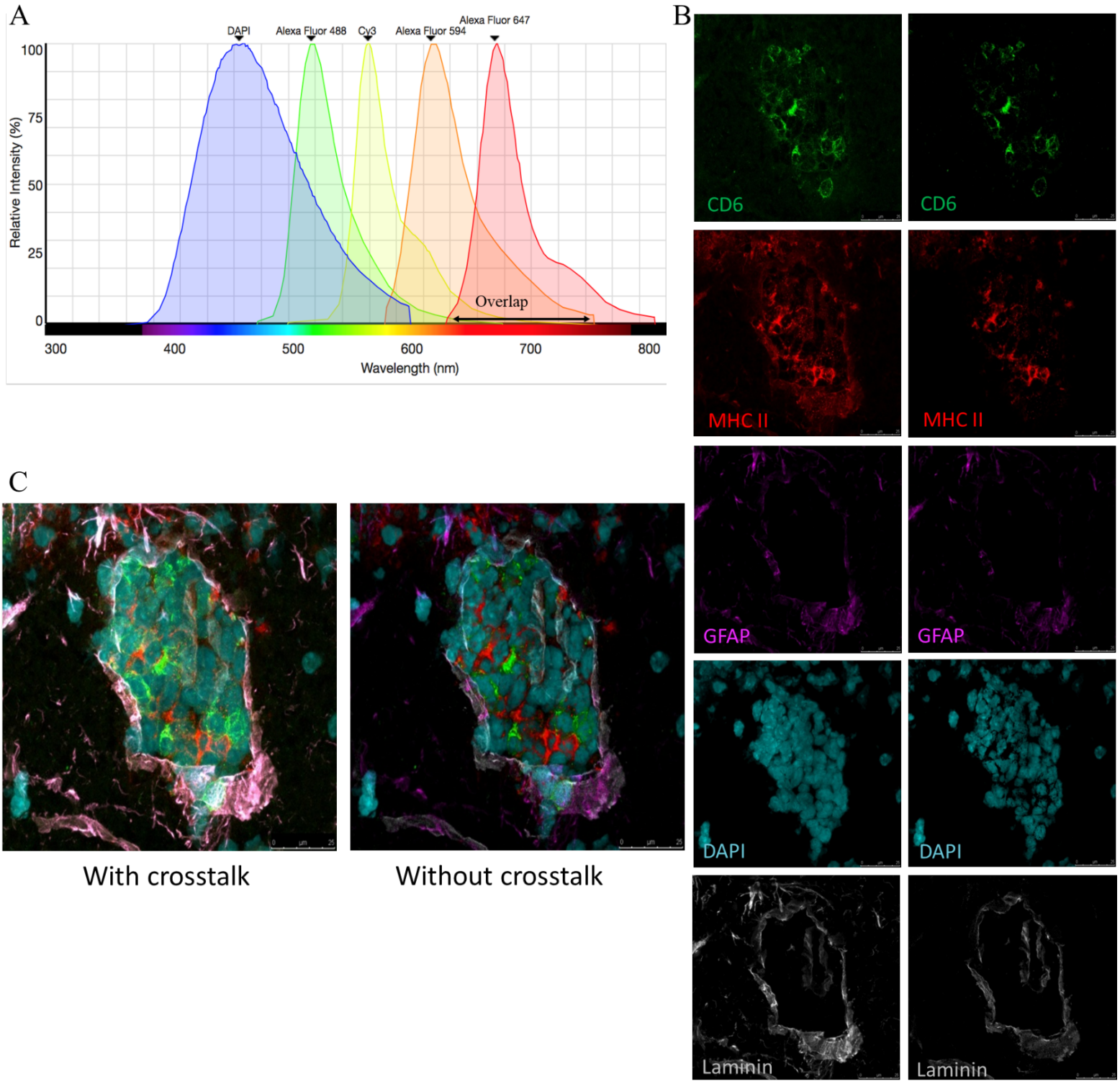
392. Wujek JR, Bjartmar C, Richer E, Ransohoff RM, Yu M, Tuohy VK, et al. Axon Loss in the Spinal Cord Determines Permanent Neurological Disability in an Animal Model of Multiple Sclerosis. Journal of Neuropathology & Experimental Neurology. 2002;61(1):23-32.
393. Yu P, Gregg RK, Bell JJ, Ellis JS, Divekar R, Lee HH, et al. Specific T regulatory cells display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. J Immunol. 2005;174(11):6772-80.
394. Chougnet C, Hildeman D. Helios-controller of Treg stability and function. Transl Cancer Res. 2016;5(Suppl 2):S338-S41.
395. Sun L, Jin H, Li H. GARP: a surface molecule of regulatory T cells that is involved in the regulatory function and TGF-β releasing. Oncotarget. 2016;7(27):42826-36.
396. Thonhoff JR, Beers DR, Zhao W, Pleitez M, Simpson EP, Berry JD, et al. Expanded autologous regulatory T-lymphocyte infusions in ALS. Neurology - Neuroimmunology Neuroinflammation. 2018;5(4):e465.
397. Zhang Q, Lu W, Liang CL, Chen Y, Liu H, Qiu F, et al. Chimeric Antigen Receptor (CAR) Treg: A Promising Approach to Inducing Immunological Tolerance. Front Immunol. 2018;9:2359.
398. Kim YC, Zhang AH, Yoon J, Culp WE, Lees JR, Wucherpfennig KW, et al. Engineered MBP-specific human Tregs ameliorate MOG-induced EAE through IL-2-triggered inhibition of effector T cells. J Autoimmun. 2018;92:77-86.

Annex



Annex 1- CAMs expression on Treg from the peripheral blood of HC and RRMS patients. Graphs show the percentage of Treg positive for MCAM, CD6, ALCAM, CD49d in HC and RRMS. Combined data in collaboration with Dr. Stephanie Zandee. All plots are presented as mean ± standard error of the mean. * $p < 0.05$.

To better assess the expression of MCAM on Treg and their localization within the CNS, we aimed to stain for a combination of CD4, Foxp3, MCAM, laminin, and nuclei. Since detecting 5 markers simultaneously is a rather challenging task, we first sought to optimize the staining settings using markers widely expressed in the CNS. We selected the following primary antibodies pre-validated by our lab for IF: MHC II, GFAP, CD6, Laminin and DAPI. The 5 fluorochromes associated with these markers have significant overlap in their spectral emission (Annex 2). Thus, to allow efficient discrimination between these fluorochromes, we optimized image acquisition technique to attain maximum signal with minimum fluorescence crosstalk. We performed spectral unmixing, which consist of subtracting a fluorochrome's spill over from another one's channel by first recording single stain fluorochromes as reference controls while maintaining similar parameters between dyes. The system then computed the distribution coefficient of all the recorded dyes in the different channels and generated a successful image without crosstalk (Annex 2 B-C). After spectral unmixing, we observed a reduced background and specific signal detection in processed images (without crosstalk) compared to initial acquired images (with crosstalk). Thus, we have proof of principle that it is possible to image up to five different fluorochromes in the CNS simultaneously.



Annex 2- Five-color proof of concept immunofluorescence staining and confocal microscopy acquisition.

(A) Emission spectra of 5 chosen fluorochromes (DAPI, AF488, cy3, AF594 and AF647) revealing spectral spill over. Excitation of different fluorochromes by confocal lasers: 405 laser – DAPI; 488 laser-AF488; 561

laser- Cy3 and AF594; 633 laser - AF647. (B) EAE spinal cord sections stained with CD6 – donkey anti goat AF488 (green), MHCII – donkey anti rat AF647 (red), GFAP directly conjugated to Cy3 (magenta), nuclei – DAPI (cyan), and Laminin – goat anti rabbit AF594 (grey). First column images were acquired before spectral unmixing. Second column images reveal reduced crosstalk after spectral unmixing processing. (C) Merged 5 staining markers revealing perivascular infiltrate in EAE CNS. Left image acquired before spectral unmixing, with presence of emission crosstalk. Right image acquired after spectral unmixing, reduced/without presence of emission crosstalk. Technique initially developed by Dr. Stephanie Zandee and further optimized with her collaboration. Scale bar = 25µm.

Antibodies	Fluorochromes
CD39	PerCP Cy5.5
Tigit	BV605
KLRG1	BV605
GiTR	PerCP Cy5.5
TNFRII	APC
ST2	FITC
OX40	BV605
Nrp1	APC
Helios	FITC
GARP	PerCP Cy5.5
CD28	APC
LAG3	PerCP Cy5.5
PD1	BV786
ICOS	BV650

Annex 3- Additional markers optimized for Treg phenotyping by FACS. All antibodies are from BD Biosciences.