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# Multiple sclerosis patient's lymphocytes induce smoldering lesions in mouse spinal cord

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# Sorbonne Université

Ecole doctorale Cerveau-Cognition-Comportement

*Paris Brain Institute - ICM / Myelin plasticity and regeneration team*

## **Multiple sclerosis patient's lymphocytes induce smoldering lesions in mouse spinal cord**

Par Océane PERROT

Thèse de doctorat de Neurosciences

Dirigée par Violetta ZUJOVIC

Présentée et soutenue publiquement le 26/09/2024

Devant un jury composé de :

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## Résumé

La sclérose en plaques (SEP) est une maladie inflammatoire et démyélinisante du système nerveux central (SNC). La maladie est déclenchée par l'invasion du SNC par des macrophages et des lymphocytes qui, de concert avec les cellules microgliales du SNC, détruisent les gaines de myéline et les oligodendrocytes (OL). Dans la SEP, l'accumulation des handicaps est due à une progression indépendante de l'activité des rechutes (PIRA). Malheureusement, les PIRA ne sont pas ciblés par les traitements actuels et aucun modèle de la SEP disponible ne recrée ce type de progression. L'une des caractéristiques des PIRA est la présence de lésions chroniquement activées, marquées par un centre inactif, une faible remyélinisation et une bordure de microglies activées. Ces lésions sont des marqueurs d'une maladie grave et représentent un risque important d'aggravation du handicap chez les patients.

Nous avons développé un modèle de souris humanisé pour combler cette lacune. En combinant une démyélinisation focale induite par la lysophosphatidylcholine (LPC) et la greffe de lymphocytes de patients atteints de SEP, une lésion partageant les caractéristiques des lésions chroniques humaines est formée.

De plus, nous avons découvert une sous-population pathogène de cellules T régulatrices exprimant Notch3 dans le sang et le liquide céphalorachidien de patients atteints de SEP grâce à l'analyse FACS.

En conclusion, cette thèse présente un nouveau modèle murin humanisé de démyélinisation chronique focale et d'inflammation, qui sera utile pour comprendre la transition d'une démyélinisation focale à une démyélinisation et une inflammation généralisée.

## Summary

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS). The disease is triggered by macrophages and lymphocytes invasion of the CNS that in concert with CNS microglial cells, destroy myelin sheaths and oligodendrocytes (OL). In MS disability accumulation is driven by progression independent of relapse activity (PIRA). Unfortunately, PIRA is not targeted by current treatment, and no available models of MS recreate this type of progression. One characteristic of PIRA is the presence of smoldering lesions, marked by an inactive center, poor remyelination, and a border rim of activated microglia. These lesions are a hallmark of severe disease and pose a significant risk for greater disability in patients.

We developed a humanized mouse model to fill the gap. By combining focal lysophosphatidylcholine (LPC) -induced demyelination and the graft of MS patients' lymphocytes, a lesion which shares features of human smoldering lesions is formed.

Additionally, we identified a pathogenic subpopulation of T regulatory cells expressing Notch 3 in the blood and cerebrospinal fluid of MS patients.

In conclusion, this thesis presents a novel humanized mouse model of focal chronic demyelination and inflammation, which will be useful for understanding the transition from focal demyelination to widespread demyelination and inflammation.

*In loving memory of Manon.  
Thank you for the memories,  
I miss you.*



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# Abbreviations

<b>AIMS:</b>	Astrocytes inflamed in multiple sclerosis	<b>MIMS:</b>	Microglia inflamed in multiple sclerosis
<b>APOE:</b>	apolipoprotein E	<b>MOG:</b>	Myelin oligodendrocytes protein
<b>BBB:</b>	Blood-brain barrier	<b>MRI:</b>	Magnetic resonance imaging
<b>Breg:</b>	B regulator	<b>mRNA:</b>	Messenger RNA
<b>CAL:</b>	Chronic active lesion	<b>MS:</b>	Multiple sclerosis
<b>CC1:</b>	Adenomatous polyposis coli clone	<b>MYRF:</b>	Myelin regulatory factor
<b>CD:</b>	Cluster of differentiation	<b>OL:</b>	Oligodendrocytes
<b>CFA:</b>	Complete Freund's adjuvant	<b>OLIG2:</b>	Oligodendrocyte transcription factor 2
<b>CIS:</b>	Clinically isolated syndrome	<b>OPC:</b>	Oligodendrocytes precursor cell
<b>CLEC7A:</b>	C-type lectin domain containing 7A	<b>P0:</b>	Protein zero
<b>CNPase:</b>	2',3'-cyclic-nucleotide 3'-phosphodiesterase	<b>PET:</b>	Positron emission tomography
<b>CNS:</b>	Central nervous system	<b>PIRA:</b>	Progression independent of relapse activity
<b>CSF:</b>	Cerebrospinal	<b>PLP:</b>	Proteolipid protein
<b>DAM:</b>	Disease-associated microglia	<b>PNS:</b>	Peripheral nervous system
<b>DMT:</b>	Disease-modifying treatment	<b>PPMS:</b>	Primary progressive MS
<b>EAE:</b>	Experimental autoimmune encephalomyelitis	<b>PRL:</b>	Paramagnetic rim lesion
<b>EBV:</b>	Epstein-Barr virus	<b>PwMS:</b>	People with MS
<b>EDSS:</b>	Expanded disability status scale	<b>RAW:</b>	Relapse associated worsening
<b>GM-CSF:</b>	Granulocyte-macrophage colony-stimulating factor	<b>RRMS:</b>	Relapsing remitting MS
<b>GML:</b>	Grey matter lesions	<b>SCP:</b>	Schwann cell precursors
<b>GWAS:</b>	Genome-wide association studies	<b>ScRNA-seq:</b>	Single cell RNA sequencing
<b>HLA:</b>	Human leukocytes antigen	<b>SEL:</b>	Slowly expanding lesions
<b>hNSC:</b>	Human neural stem cells	<b>SL:</b>	Shadow lesion
<b>IFN:</b>	Interferon	<b>SNPs:</b>	Single nucleotide polymorphisms
<b>Ig:</b>	Immunoglobulin	<b>SnRNA-seq:</b>	Single nuclei RNA sequencing
<b>IL:</b>	Interleukin	<b>SOX10:</b>	SRY-box 10
<b>KO:</b>	Kock-out	<b>SPMS:</b>	Secondary progressive MS
<b>LPC:</b>	Lysophosphatidylcholine	<b>Th:</b>	T helper
<b>LPS:</b>	Lipopolysaccharide	<b>TMEV:</b>	Theiler's murine encephalomyelitis virus
<b>MBP:</b>	Myelin basic protein	<b>TNF:</b>	Tumor necrosis factor
<b>MHC:</b>	Major histocompatibility complex	<b>TO:</b>	Theiler's original
		<b>Treg:</b>	T regulator
		<b>TREM2:</b>	Triggering receptor expressed on myeloid cells 2
		<b>TSPO:</b>	translocator protein



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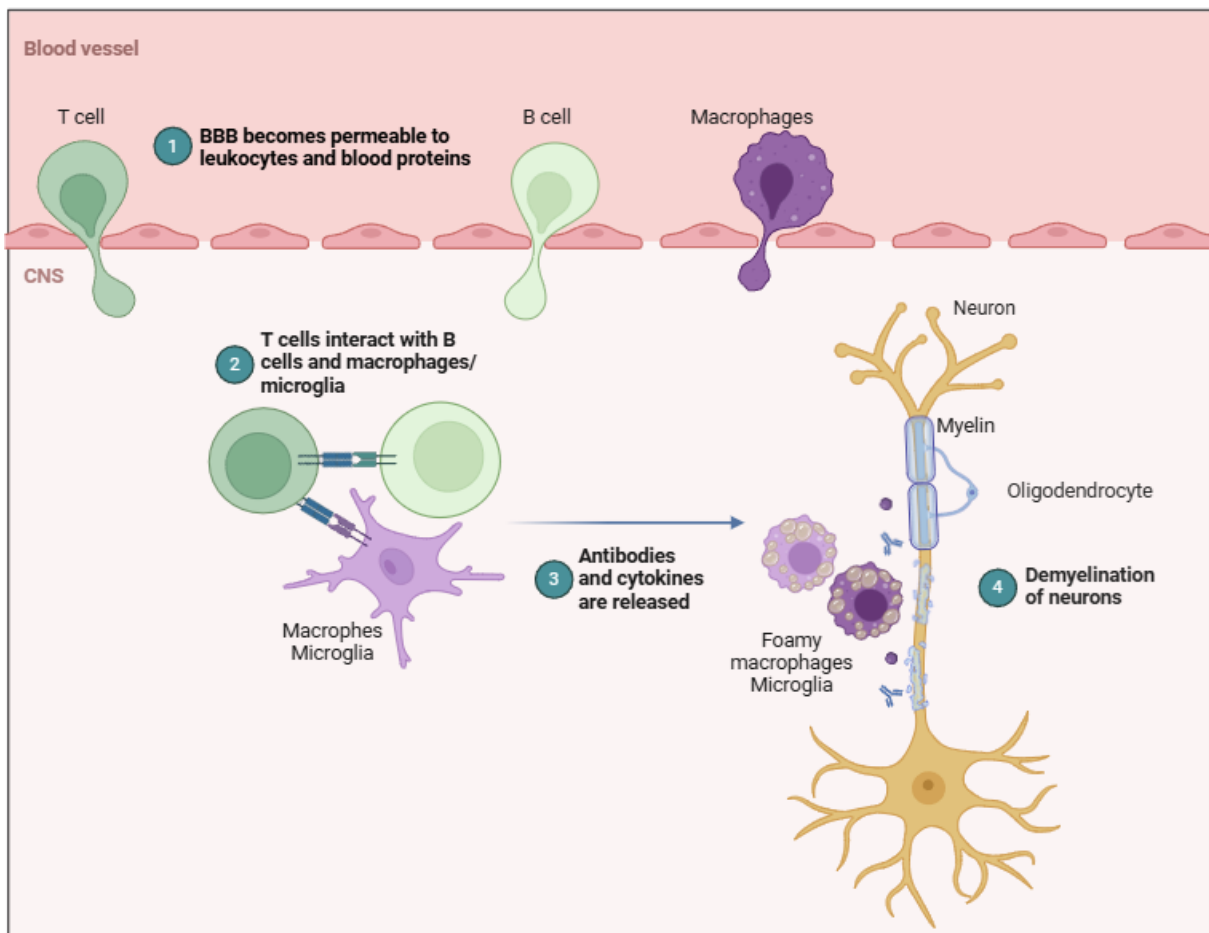
# **INTRODUCTION**





## A. Multiple Sclerosis

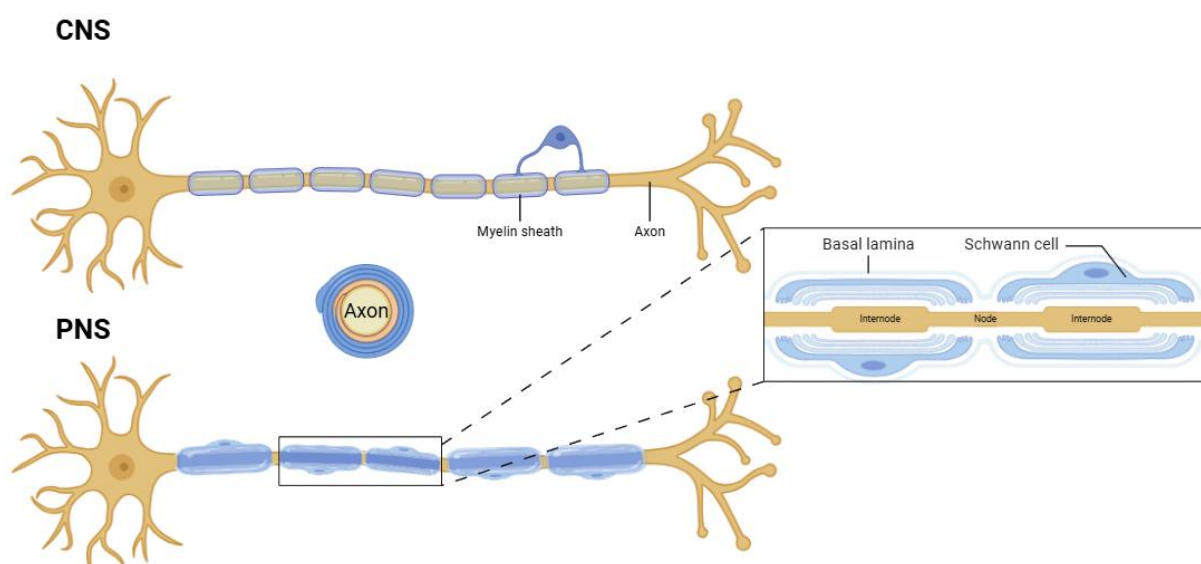
Multiple sclerosis (MS) is an inflammatory, autoimmune, and demyelinating disease of the central nervous system (CNS). In this pathology, peripheral immune cells (i.e., macrophages and lymphocytes) invade the brain and the spinal cord. The combined efforts of the invading cells and resident microglial cells lead to the destruction of myelin, a membrane ensheathing the axons and formed by the oligodendrocytes (OL). Demyelinated axons are thereafter exposed and can degenerate (Figure 1). The degeneration of axons in MS patients leads to a wide range of symptoms that vary depending on the affected part of the CNS. The etiology of MS is still unknown, but genetic predisposition and environmental factors increase disease risks. There are disease modifying treatments targeting neuroinflammation that ameliorate MS patients' life but there is no cure for MS.



**Figure 1: Physiopathology of MS.** Peripheral immune cells (T cells, B cells, and macrophages) cross the blood brain barrier (BBB), they are reactivated in the CNS and with resident activated microglial cells, they destroy myelin. Created with Biorender

## 1. Myelin

Myelin is a complex, lipid-rich structure formed by myelinating cells, the OL in the CNS, and by the Schwann cells in the peripheral nervous system (PNS). Myelin is formed by the wrapping of multiple layers of OL or Schwann cells membrane around the axons. While OL can myelinate multiple axons, Schwann cells wrap only one axon with a myelin surrounded by a basal lamina (Figure 2). Myelin forms segments along the axons, it is called internodes, and these internodes are separated by nodes of Ranvier. CNS and PNS myelin are quite similar but have distinct features specific to each system that allow us to differentiate with specific immunological markers. For example, OL express the myelin oligodendrocytes protein (MOG) while Schwann cells the protein zero (P0).



**Figure 2: Myelin in the CNS and in the PNS.** In the CNS OL form multiple myelin segments and in the PNS Schwann cells form only one myelin segment and are surrounded by a basal lamina. CNS: central nervous system; PNS: peripheral nervous system; OL: oligodendrocyte; SC: Schwann cell. Created with Biorender

### a) Oligodendrocytes and Schwann cells generation

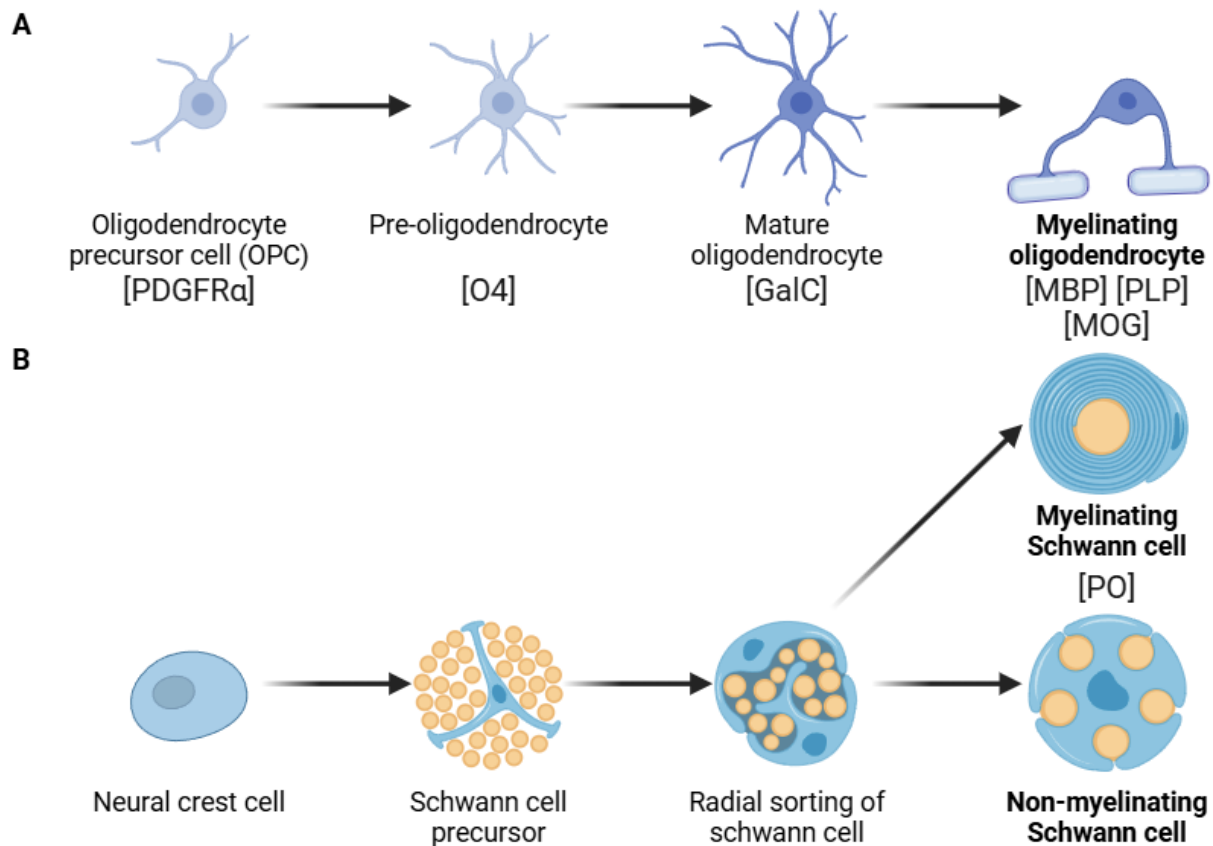
In the CNS, the oligodendrocyte precursor cells (OPC) are derived from neural stem cells (NSC). During embryonic life, OPCs migrate from ventral and dorsal domain, 3 waves of OPCs in the brain and 2 waves in the spinal cord are sufficient to populate the entire CNS (van Tilborg et al., 2018). Then OPCs will be able to differentiate into OL with different intrinsic and extrinsic factors (such as neuronal activity). During the differentiation into myelinating OLs, OPCs undergo a multistep process. Each stage has specific markers that enable us to

identify the step of OPC differentiation. First, OPCs become pre-oligodendrocytes that are characterized by the expression of O4 protein, then they differentiate into mature OL that express adenomatous polyposis coli clone (CC1). Myelinating OL express myelin proteins such as MOG, proteolipid protein (PLP) and myelin basic protein (MBP). Interestingly, all the cells from the OL lineage also express the oligodendrocyte transcription factor 2 (Olig2) (Figure 3A). After the waves of CNS colonization, some of the OPCs will remain undifferentiated and form an adult OPC pool representing 5% of total brain cells (Polito & Reynolds, 2005). However, when needed (such as CNS injury, or demyelination) with extrinsic and intrinsic signals this pool can differentiate into myelinating OL. However, when needed (such as during CNS injury or demyelination) with extrinsic and intrinsic signals this pool can differentiate into myelinating OL. For example, electrical activity of neurons is positive stimuli for OPCs differentiation and myelination (Gibson et al., 2014; Mitew et al., 2018), immune cells activation can also play a role in OPCs proliferation and differentiation (Butovsky et al., 2006; Miron et al., 2013). New roles of OPCs have been discovered, OPCs can also present antigen to T-cells during demyelination context (Kirby et al., 2019).

In the PNS, myelin is formed by Schwann cells. Schwann cells derived from cells located in the dorsal part of the neural crests. When they encounter axons, these cells will differentiate into Schwann cell precursors. Schwann cell precursors will then become immature Schwann cells and surround several axons without forming myelin. Finally, immature Schwann cells can either form myelin around one axon and differentiate into a myelinating Schwann cell, or become a non-myelinating Schwann cell, this process depends on the axon diameter, Schwann cells will myelinate larger axons, and this process is called radial sorting (Feltri et al., 2016) (Figure 3B).

## **b) Functions**

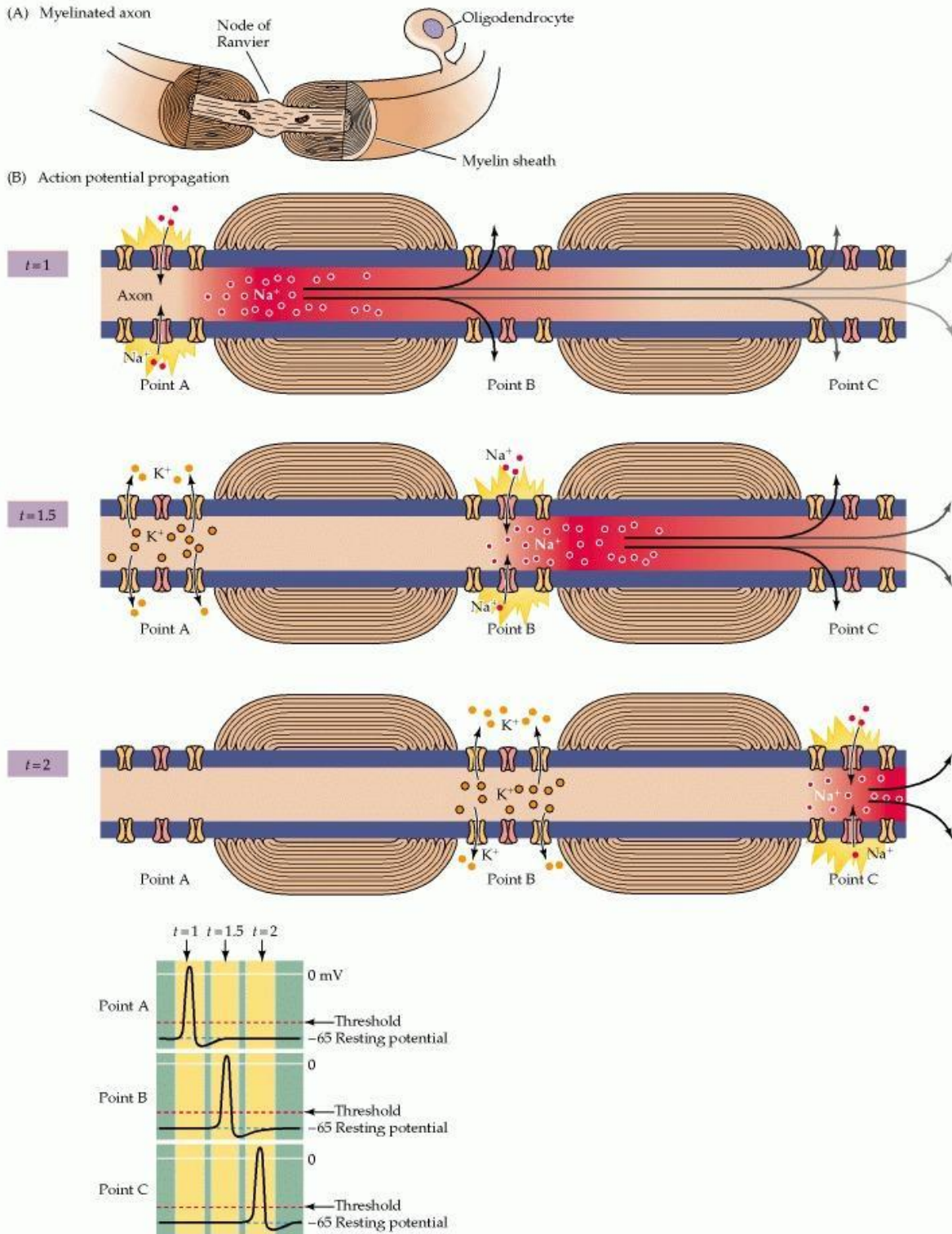
The first function of myelin is to accelerate the action potential's conduction speed along the axons up to 100 times faster. The first mechanism allowing this increase in speed of conduction is the membrane composition of myelin. Membranes are a lipidic bilayer, and while classically composed of 50% lipids and 50% proteins, myelin composition differs with 70% lipids and 30% proteins. This composition is enriched in fat, which makes myelin less permeable and a natural insulator for axons.



**Figure 3: Oligodendrocytes and Schwann cells differentiation.** The OL lineage express *Olig2+*, and during differentiation each stage have specific makers. Pre-OL express *O4*; Mature OL express *CC1* and myelinating OL express *MBP*, *PLP* and *MOG*. Myelinating Schwann cells express *P0*. Created with Biorender

In addition, myelin is not continuous along the axon; myelin sheaths form internodes separated by nodes of Ranvier (Figure 4A). There is a high density of voltage-dependent channels in nodes of Ranvier allowing the saltatory conduction of the action potential from nodes of Ranvier to nodes of Ranvier (Figure 4B) (Purves et al., 2001).

Myelin also has other functions. As a natural insulator for axons, it also serves as a physical barrier, shielding the axons from the environment and protecting them during neuroinflammatory events, for example. Furthermore, oligodendrocytes metabolically support the axons through the myelin sheath as shown with knock-out (KO) mouse model of a myelin protein, the 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase). Indeed, in the CNPase KO mouse model, myelination occurs but the mice develop axonal damage (Lappe-Siefke et al., 2003). The metabolite exchange between OL and neurons is impaired in these mice and this leads to axonal transport defects in neurons and an early death of animals.



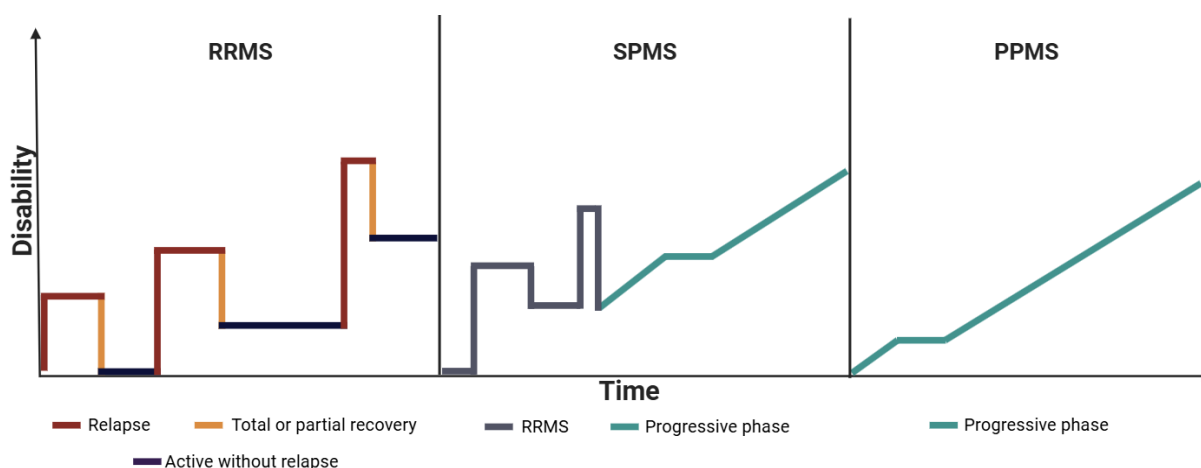
**Figure 4: Saltatory conduction of the action potential along a myelinated axon.** (A) Diagram of a myelinated axon. (B) Local current in response to action potential initiation at a particular site “jump” locally. However, the presence of myelin prevents the local current from leaking across the internodal membrane; it therefore “jump” farther along the axon than it would in the absence of myelin. Moreover, voltage-dependent  $\text{Na}^+$  channels are present only at the nodes of Ranvier. This arrangement means that the generation of active, voltage-gated currents need only occur at these unmyelinated regions. The result is a greatly enhanced velocity of action potential conduction. Panel to the left of the figure legend shows the changing membrane potential as a function of time at the points indicated. From: (Purves et al., 2001).

## 2. Epidemiology, diagnosis and symptoms

### a) Epidemiology

MS prevalence in France is 155 cases per 100,000 people and affects approximately 2.9 million people worldwide (Multiple Sclerosis International Federation, 2024). This pathology is more prevalent in young women (the first symptoms appear around 30 years old) compared to men (3 women affected for 1 man (Multiple Sclerosis International Federation, 2024; Wallin et al., 2019)).

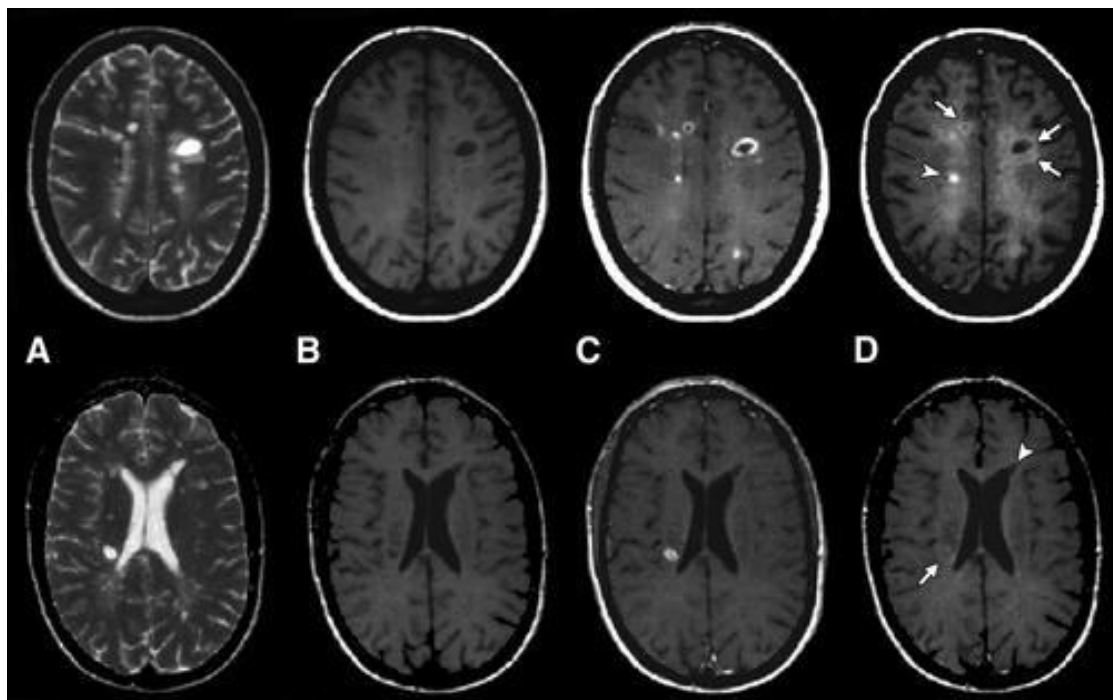
Three forms of MS can be distinguished depending on the evolution of the symptom. A relapsing-remitting form, named RRMS, affects 85% of people with MS (PwMS) and is characterized by the manifestation of symptoms (called relapses) (Figure 5; RRMS: red line) followed by complete or partial recovery (called remission) (Figure 5; RRMS: yellow line). Eventually, 80% of these patients will develop secondary progressive MS (SPMS), where the symptoms accumulate without any more remissions (Figure 5; SPMS: blue line). Fifteen percent of PwMS develop a primary progressive form with a progressive course at the onset of disease (PPMS) (Figure 5) (Reich et al., 2018). People experiencing one RRMS-like event are considered to have clinically isolated syndrome (CIS), until or unless they have a second event.



**Figure 5: Graphical representation of the different forms of MS.** A: Relapsing remitting MS (RRMS) is characterized by manifestation of symptoms followed by partial or complete recovery., B: 80% of PwRRMS will convert to secondary progressive MS (SPMS) characterized by an accumulation of symptoms without remission and C: Primary progressive MS (PPMS) is a progressive course from the onset of the disease. Created with Biorender

## b) Diagnosis

MS is diagnosed following the McDonald criteria revised in 2017 (Thompson et al., 2018). These criteria are a combination of magnetic resonance imaging (MRI), symptoms history and cerebrospinal fluid (CSF) analysis. To diagnose MS, clinician must show dissemination in space and in time (Schmidt-Mengin et al., 2022; Thompson et al., 2018). To do so, MRI is widely used to assess the presence of lesions or plaques (demyelinated areas). In T2-weighted images, lesions appear white (Figure 6A); in T1-weighted, lesions appear as “black holes” (Figure 6B) and in gadolinium-enhanced T1-weighted images, lesions will appear in white (Figure 6C) (Vellinga et al., 2008). MRI is also used to assess the extent of brain atrophy, which is a marker of MS. Furthermore, clinicians can use additional clinical signs to diagnose MS. One method involves testing evoked potentials which are slowed in MS patients due to demyelination. The latest McDonald criteria include also detecting B cells activation by searching for oligoclonal immunoglobulin bands in the CSF of patients (Compston & Coles, 2008; Correale & De los Milagros Bassani Molinas, 2002; Thompson et al., 2018). The detection of a unique oligoclonal band in the CSF suggests CNS inflammation (Winger & Zamvil, 2016). Interestingly, people having a CIS with the presence of an oligoclonal band in their CSF are at higher risk of converting to MS (Dobson et al., 2013).

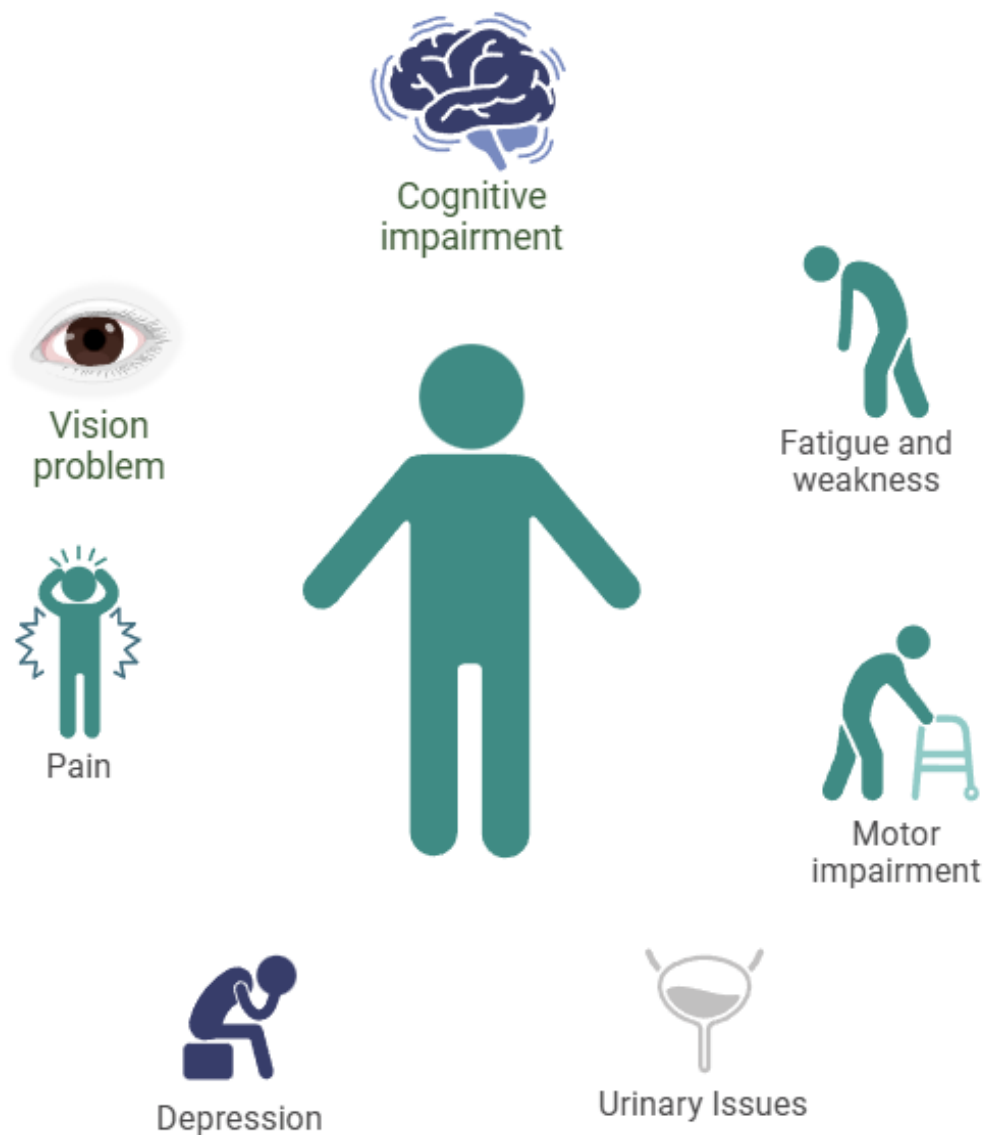


**Figure 6: MRI in a patient with MS.** A, T2-weighted images. B, T1-weighted images show hypointensity of some of the MS lesions. C, Gadolinium-enhanced T1-weighted images show that several lesions enhance in focal and ringlike patterns. Modify from: (Vellinga et al., 2008)



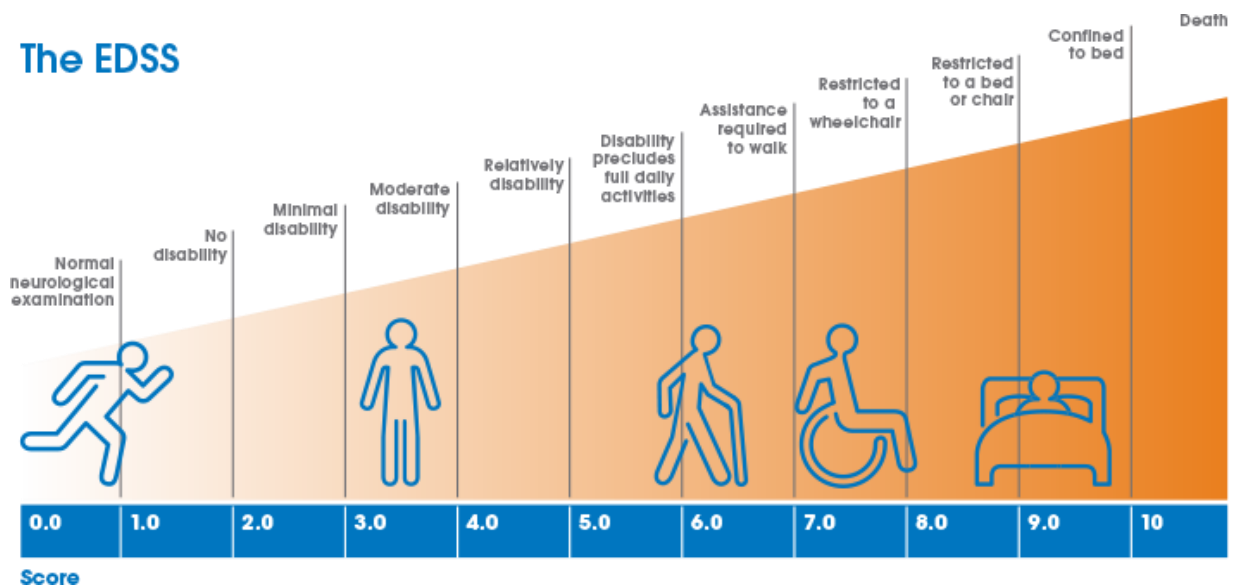
### c) Symptoms

MS symptoms are heterogeneous from one individual to another. Depending on where the demyelinating lesions are formed in the CNS, a multitude of symptoms can appear. However, some symptoms are more recurrent than others. The most common are: motor deficits, fatigue, pain, vision problems, depression, and cognitive decline (this list is not exhaustive) (Figure 7) (Meca-Lallana et al., 2021; National Multiple sclerosis society, 2024; Sumowski et al., 2018).



**Figure 7: Representation of the most common MS symptoms.** The most common symptoms are motor impairment, fatigue and weakness, vision problems, urinary issues, pain but also depression and cognitive impairment. Created with Biorender

Different scales to measure the clinical disability of MS patients have been developed. The most used is the expanded disability status scale (EDSS). This scale goes from 0 to 10 (Figure 8) and has been developed by Kurtzke in 1983 (Kurtzke, 1983). The multiple sclerosis severity score (MSSS) evaluates the clinical disabilities (EDSS) considering the disease duration (Roxburgh et al., 2005). However, the EDSS and therefore by extension the MSSS are mostly based on walking difficulties. However, it is known that 50 to 60% of MS patients experience cognitive decline (Meca-Lallana et al., 2021; Sumowski et al., 2018). In most PwMS, the cognitive impairment usually seen is a reduced information processing speed and memory impairment (Grzegorski & Losy, 2017; Sumowski et al., 2018). To assess cognitive decline, and particularly the information processing speed, the symbol digit modalities test is widely used and is an efficient test to differentiate healthy individuals from PwMS (Sumowski et al., 2018).



**Figure 8: Graphic representation of the extended disability status scale (EDSS). The EDSS score, goes up to 10 and evaluate walking disability in MS patients. Source: MSonetone**

### 3. Etiology

MS is a complex pathology, and its etiology is still unclear. There are no factors or associations of factors that are sufficient to induce MS. However, genetic predisposition and environmental factors increase the risk of developing MS.

#### a) MS genetic predisposition

MS is not a genetic disease, but several studies support a genetic contribution. Indeed, some studies have found evidence toward familial clustering, and an association between ancestry and MS (Beecham et al., 2022; Chi et al., 2019; Compston & Coles, 2008; O’Gorman et al., 2012). Another evidence of genetic contribution is the study of twins, a meta-analysis of eight twin studies has shown that the heritability proportion of variance of MS is 0.5 (Fagnani et al., 2015).

Alleles of the human leukocyte antigen (HLA) were the first genetic risks factors identified. HLA is the major histocompatibility complex (MHC) of humans, it plays a role in the defense of the organism. This gene is located on the chromosome 6 and is highly polymorphic with multiple alleles. The *HLA-DRB1\*15:01* is the first haplotype described and the one with the strongest risk effect still today. The odds ratios (OR) vary around 3 (2.9 to 3.17) (Brynedal et al., 2007; Hollenbach & Oksenberg, 2015; Osoegawa et al., 2021; Stürner et al., 2019). Protective alleles have also been discovered such as *HLA-A* (Brynedal et al., 2007; Sawcer et al., 2011), And when the lack of *HLA-A* is associated with the presence of *HLA-DRB1* the combined OR is over 20 (Brynedal et al., 2007).

Single nucleotide polymorphisms (SNPs) are variations at a specific location of one base pair in the genome. Genome-wide association studies (GWAS) identify the association between these SNPs and diseases. Three GWAS studies have been conducted for MS (Beecham et al., 2013; Patsopoulos et al., 2019; Sawcer et al., 2011). In 2019, the last MS-GWAS identified over 200 SNPs that significantly increase the risks of developing MS, 32 of these variants are located within the MHC, and the others are unrelated to HLA (Patsopoulos et al., 2019). Interestingly, the SNPs variants identified by GWAS are enriched in microglia and peripheral immune cells (Ma et al., 2023), and in 20 X-linked SNPs increased in MS, 14 of them are in genes with immune and myelination functions (Borziak & Finkelstein, 2022).

These data suggest that genetics play a significant role in the development of MS. However, while genetic predisposition is important, it alone does not cause MS. Other non-genetic risk factors, such as epigenetic modifications and environmental factors, also contribute to the risk of developing the disease (Alfredsson & Olsson, 2019; Küçükali et al., 2015; Zarghami et al., 2021).

## **b) Environmental factors**

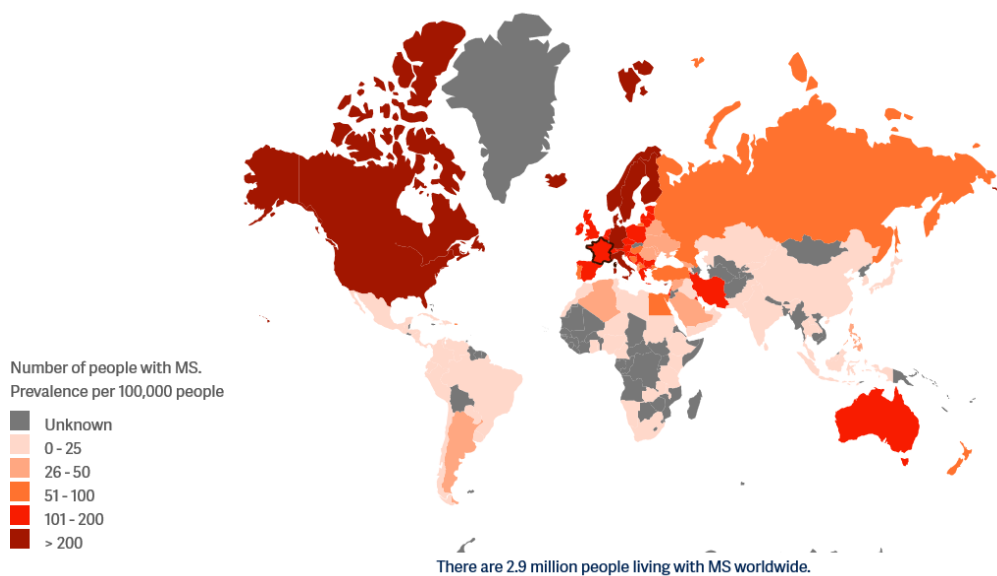
Some environmental factors can also participate to increase MS risk. In the next paragraphs, I will present some of the environmental factors shown to contribute to MS risk (Alfredsson & Olsson, 2019; Zarghami et al., 2021).

### **(1) Viral infection**

It has been suggested that viral infection in early life, with a specific genetic predisposition, could induce an immune-mediated CNS attack (Sundaresan et al., 2023). In MS cases, the Epstein-Barr virus (EBV), a gamma-herpes virus that affects 90% of the population, has been considered a prerequisite to developing the disease (Hedström, 2023). In a longitudinal study in the US military of more than 10 million young adults, they showed that after EBV infection the risk of developing MS is increased 32-fold and that only after EBV infection there is an increase in serum of a neuroaxonal degeneration's marker: the neurofilament light chain (Bjornevik et al., 2022). These data suggest that EBV may play a causal role in the development of MS pathology (Bjornevik et al., 2022). One proposed mechanism is the molecular mimicry between EBV and myelin proteins (Soldan & Lieberman, 2023; Sundaresan et al., 2023), where the immune system mistakes myelin proteins for EBV antigens due to their structural similarity. As a result, adaptive immune cells initially targeting EBV might attack myelin, leading to MS pathology (Sundaresan et al., 2023).

## (2) Latitude, sun exposure and vitamin D

Latitude of the living country in early life plays a role in MS risk, indeed, people living in a country with higher latitude have a higher risk of developing MS (Multiple Sclerosis International Federation, 2024; Sabel et al., 2021; Simpson et al., 2011, 2019) (Figure 9). There is a link between ultraviolet radiation exposure (and therefore sun exposure) and the risk of MS. This could be linked to lower sun exposure in higher latitudes. It has been demonstrated that low sun exposure is associated with MS risk and disease progression, while high sun exposure decreased MS risk (Tremlett et al., 2018; Wu et al., 2024). Sun exposure leads to vitamin D synthesis in the skin (Raymond-Lezman & Riskin, 2023) and is the main source of vitamin D. Low sun exposure increases MS risk directly and indirectly by leading to low vitamin D levels (Hedström et al., 2020). Low levels of vitamin D in the blood has also been associated with a higher risk of MS (Munger et al., 2006), and a higher risk of relapses in patients with RRMS (Simpson et al., 2010). The immunomodulatory role of vitamin D could explain its protective effect in MS (Athanassiou et al., 2022; Hamza et al., 2023; Rizwan et al., 2024).



**Figure 9: World map of the number of people with MS.** We can see that there is a gradient from the latitude to the Equator. From: (Multiple Sclerosis International Federation, 2024)

### (3) Other environmental factors

Smoking is a modifiable environmental risk factor for MS (Hedström et al., 2016). On average smoking increases the risk of MS by around 50% in people who smoke compared to non-smokers (Manouchehrinia et al., 2022), while being passively in contact with smoke (being close to someone that smoke) increases the risk by 30% in adults (Hedström et al., 2011) and by more than two times in children, with a dose-response association (Mikaeloff et al., 2007). Smoking also plays a role in disease progression, for instance, PwMS that are smokers have more severe motor symptoms compared to non-smokers PwMS (Emre & Decker, 1992), they also, are at risk to reach 4.0 and 6.0 EDSS score (Hernán et al., 2005), and are also more at risk of converting into SPMS (Hernán et al., 2005; Ramanujam et al., 2015). This effect could be the result of the action of smoking on immunity, indeed smoking induces a pro-inflammatory reaction and activation of the immune system (Alrouji et al., 2019).

Other environmental factors are implicated, such as the gut microbiota, young and adolescent obesity, alcohol and coffee consumption (Alfredsson & Olsson, 2019; Zarghami et al., 2021). Interestingly, association and interaction between different risk factors can potentiate the effect of one risk factor. For example, an interaction between *HLADRB1\*15:01*; sun exposure and Vitamin D (Hedström et al., 2020); or the interaction between the presence of *HLADRB1\*15:01*; the absence of *HLA-A* and smoking (Olsson et al., 2016) increase MS risk more than just the presence of one of the risk factors.

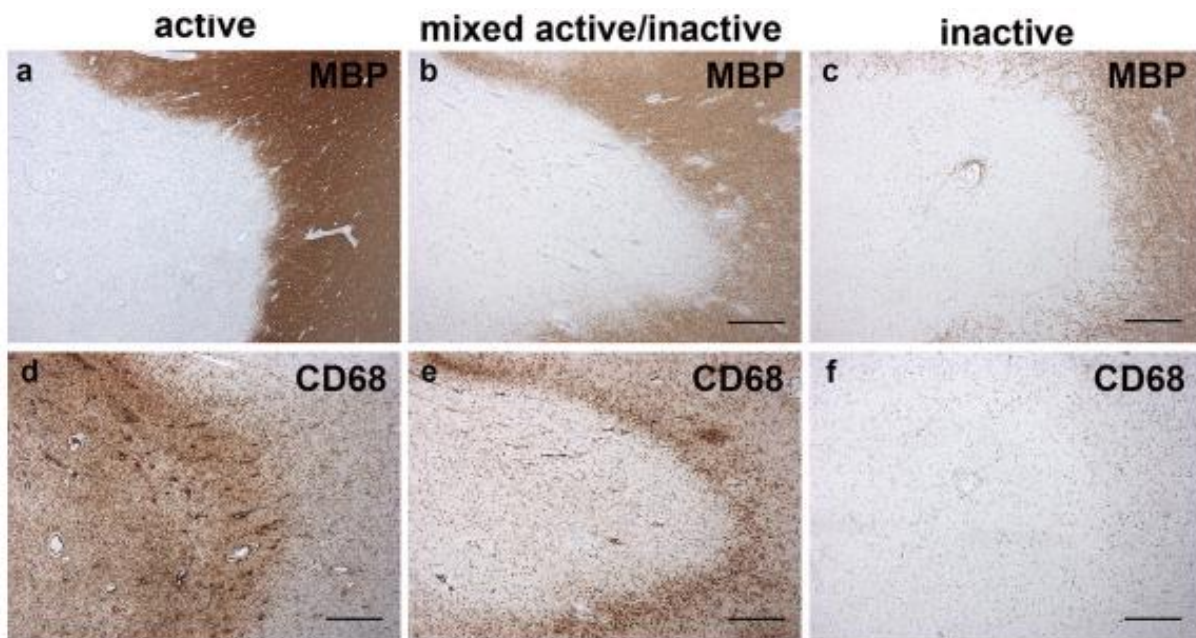
In conclusion, MS is a multifactorial disease, and there is no specific factor or combination of factors that could induce the development of MS. Rather it is the interaction between genetic predisposition, epigenetics modification, and environmental factors that will induce dysregulation of the immune system homeostasis (Baranzini & Oksenberg, 2017) that increase the risk of developing MS.

## B. Histopathology of MS

The analysis of post-mortem tissue from MS patients enabled the characterization of the different subtypes of lesions. In MS post-mortem tissue analysis, lesions are heterogeneous. The primary characteristics used to describe lesion patterns include demyelination, immune infiltration, and glial activation (Bö et al., 1994; Frischer et al., 2015; Kuhlmann et al., 2017; C. Lucchinetti et al., 2000).

### 1. Lesion types

To identify lesion types in human post-mortem tissue, immunohistochemistry staining is used, typically staining myelin with antibodies against myelin protein such as MOG, PLP, or MBP (Figure 10A-C) and immune cells with the clusters of differentiation (CD)-68 (Figure 10D-F) (Kuhlmann et al., 2017). It is now admitted that white matter lesions can be: 1) active 2) inactive and 3) mixed active/inactive (Kuhlmann et al., 2017). Luxol fast blue, which stains myelin, is also commonly used to assess the extent of demyelination and remyelination.



**Figure 10: Immunohistochemistry of MS lesions.** (a–c) immunohistochemistry for myelin stained with MBP; (d–f) immunohistochemistry for immune cells stained with CD68. All lesions are characterized by a loss of myelin and a relatively sharp border. The anti-CD68 immunohistochemistry reveals the variable inflammatory activity. Active lesions with loss of myelin (a) and numerous CD68 positive foamy macrophages within the lesion (d). (b) and (e) display a mixed active/inactive lesion. The lesion is demyelinated (b), CD68 positive phagocytes form a rim at the lesion border (e). Inactive lesion in c; only a few ramified phagocytic cells are found within the lesion (f). Scale bars 500  $\mu$ m. From: (Kuhlmann et al., 2017)

### **a) Active lesions**

Active lesions in MS, show demyelinated areas (Figure 10A) and are hypercellular due to dense infiltration of activated macrophages and lymphocytes (Figure 10D). Macrophages and microglia are evenly distributed throughout the lesion, while lymphocytes (T and B cells) typically cluster around blood vessels in perivascular spaces, with some also present in the parenchyma (Absinta et al., 2019; Bö et al., 1994; Fransen et al., 2020; Frischer et al., 2009; Hsiao et al., 2021; Machado-Santos et al., 2018; Smolders et al., 2018). Increased astrogliosis, a marker of active lesions, is also observed. In these lesions, demyelination is driven by T-cell and macrophage-mediated inflammation, accompanied by myelin-specific antibodies and complement activation, which are key indicators of demyelination (Breij et al., 2008). Active lesions can occur throughout the disease course (Kuhlmann et al., 2017; C. Lucchinetti et al., 2000).

### **b) Inactive lesions**

Inactive lesions, as observed in histopathological studies, have well-defined borders (Figure 10C). These lesions are hypocellular, featuring sparse infiltrates of T cells and macrophages (Figure 10F). The density of microglia and macrophages in these areas is lower than in normal-appearing white matter (Hametner et al., 2013), and the macrophages or microglia present do not contain myelin debris (Bö et al., 1994; Kuhlmann et al., 2017). Additionally, these lesions lack mature oligodendrocytes, with a glial scar formed by astrocytes at the lesion center (Ludwin, 2000). Axonal loss can also be detected in these lesions (Mews et al., 1998). Such lesions are typically found in PwMS for more than 15 years or in those with progressive MS (Frischer et al., 2015).

### **c) Mixed active and inactive lesions**

Mixed active/inactive lesions are also called “smoldering-like” or chronic active lesions (CALs). For simplicity, for the following part, I will call them smoldering-like lesions.

These lesions are characterized by an inactive hypocellular center (Figure 10B) with the presence of a glial scar and an active border of the lesion. The border or the rim of these lesions



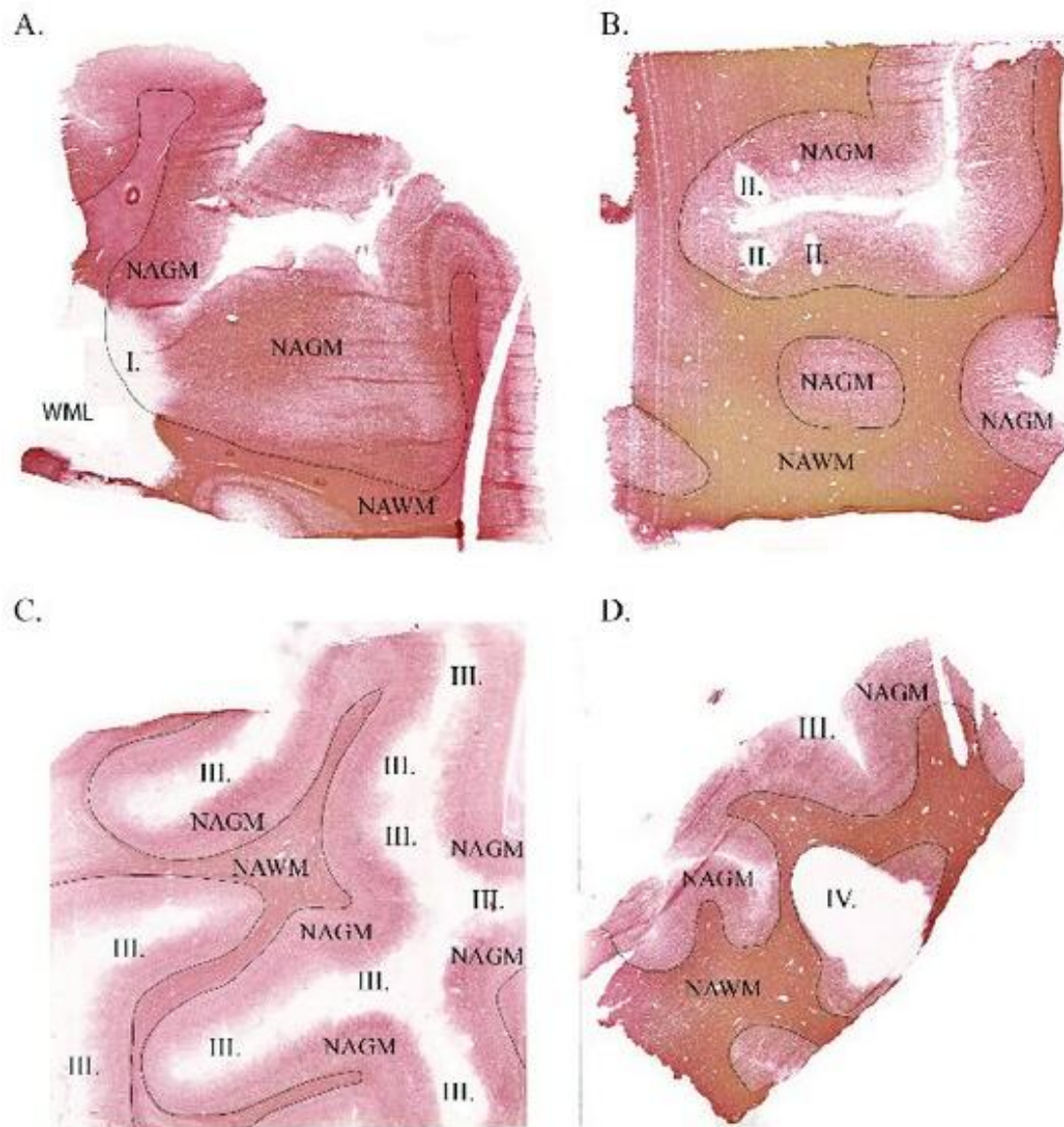
is hypercellular with the presence of immune cells (Figure 10E), such as foamy macrophages/microglia full of myelin, or macrophages/microglia full of iron named microglia inflamed in MS (MIMS) (Absinta et al., 2021; Maggi et al., 2020). The presence of foamy macrophages/microglia in the rim of the lesion suggests an ongoing demyelination, another hint toward ongoing demyelination is the upregulation in MIMS of complement genes (Absinta et al., 2021), which are markers of demyelination (Breij et al., 2008). In addition to macrophages and microglia, there is also the presence of T and B lymphocytes in smaller numbers, with T lymphocytes being more prevalent compared to B lymphocytes (Absinta et al., 2021; Machado-Santos et al., 2018). CD8+ T cells are the dominant component of the infiltrates, primarily located in perivascular cuffs, and they exhibit tissue-resident memory (TRM) features (Absinta et al., 2021; Fransen et al., 2020; Frieser et al., 2022; Hsiao et al., 2021; Machado-Santos et al., 2018; Smolders et al., 2018).

Interestingly, this type of lesion is associated with disability *in vivo* (Absinta et al., 2019), more relapses and worse cognitive recovery after relapse (Reeves et al., 2023). Therefore, smoldering lesions are a hallmark of patients with severe disease and at risk of developing more disabilities.

#### **d) Cortical lesions**

MS is a primary white matter disease, however, in PwMS, we can also detect cortical lesions, also called gray matter lesions (GML) in post-mortem analysis (Figure 11) (Enz et al., 2021). There is different types of GML, the type I or also called leukocortical is characterized by cortical demyelination and adjacent subcortical white matter demyelination (Figure 11A; I.); the type II or intracortical, is characterized by cortical demyelination and do not contact the meninges and the white matter (Figure 11B; II.); the type III or subpial lesions are in contact with the meninges, they are the more abundant GML, and are only seen in PwMS (Junker et al., 2020), these lesion do not contact the white matter (Figure 11C; III.); and the type IV or pancortical, are characterized by demyelination of all the cortical layers, and are in contact with the meninges and the white matter (Figure 11D; IV.) (Enz et al., 2021; Lucchinetti et al., 2011). The GML are associated with disability in PwMS (Chase, 2015).

Even if these lesions are more present in people with progressive MS (Kutzelnigg et al., 2005), they are also seen early in the disease (Kuhlmann et al., 2017; Lucchinetti et al., 2011). In addition to the different types of GML, they can also be classified as active, inactive and smoldering lesions such as white matter lesions (Kuhlmann et al., 2017).

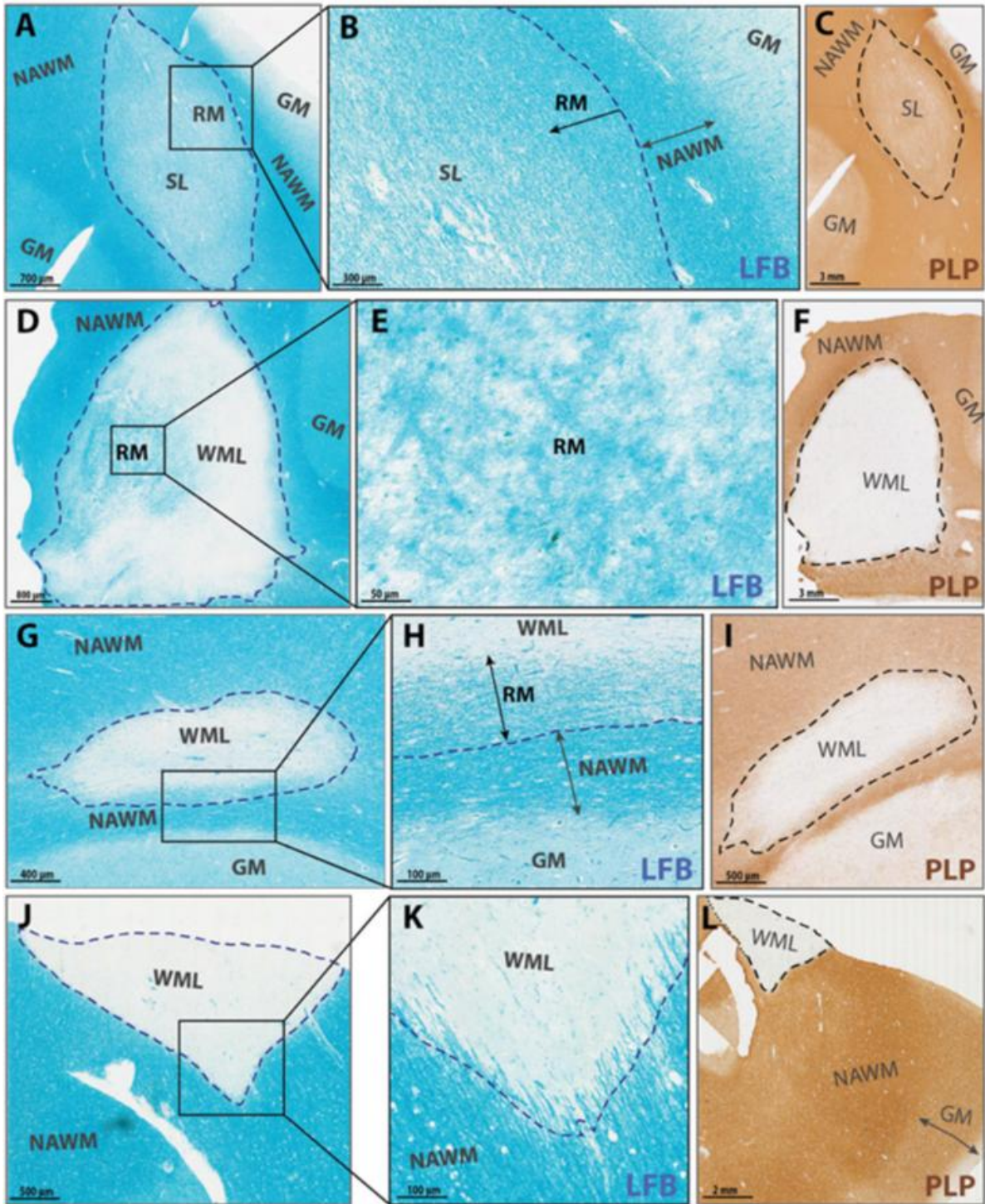


**Figure 11: Types of cortical grey matter lesions.** Representative images of anti-MOG staining of human cortical grey matter tissue and subcortical white matter. (A.) Type I leukocortical lesion (I.) with adjacent subcortical White Matter Lesion (WML) (B.) Type II intracortical lesions (II.). (C.) Type III subpial lesion (III.) spanning multiple gyri. (D.) Type IV pancortical lesion (IV.). Abbreviations: NAGM: Normal-Appearing Grey Matter; NAWM: Normal-Appearing White Matter. The black line delineates the border between grey and white matter. From: (Enz et al., 2021)

### e) **Shadow plaques**

Shadow plaques (also called shadow lesions (SL)) are the histopathological representation of remyelination. For a long time, the CNS was considered to have poor intrinsic capacity to repair (Cajal, 1928). However, remyelination, that is the formation of new myelin sheath after demyelination (or injury), is a spontaneous repair process in the adult CNS (Chari, 2007). Remyelination can occur in all lesion types and both cortical and white matter areas (Dulamea, 2017; Franklin, 2002). Interestingly, remyelination can be extensive (Albert et al., 2007; Patani et al., 2007), and decrease with disease chronicity (Goldschmidt et al., 2009).

A scoring of the remyelination can be assessed based on the extent of remyelination (Huitema et al., 2021) observed using luxol fast blue staining. Different areas can be detected, white matter lesions (WML); the normal-appearing white matter (NAWM) that surrounds the WML; the grey matter (GM), and finally the remyelinated area (RM) (Figure 12). A score 3 correspond to completely remyelinated or remyelination covering >60% of the entire lesion area (Figure 12A-C); score 2: substantial remyelination, between 21–60% of lesion area remyelinated (Figure 12D-F); score 1: little remyelination that is restricted to the lesion rim and characterized by a paler gradient (Figure 12G-I) and score 0: demyelinated (Figure 12 J-L) (Huitema et al., 2021).

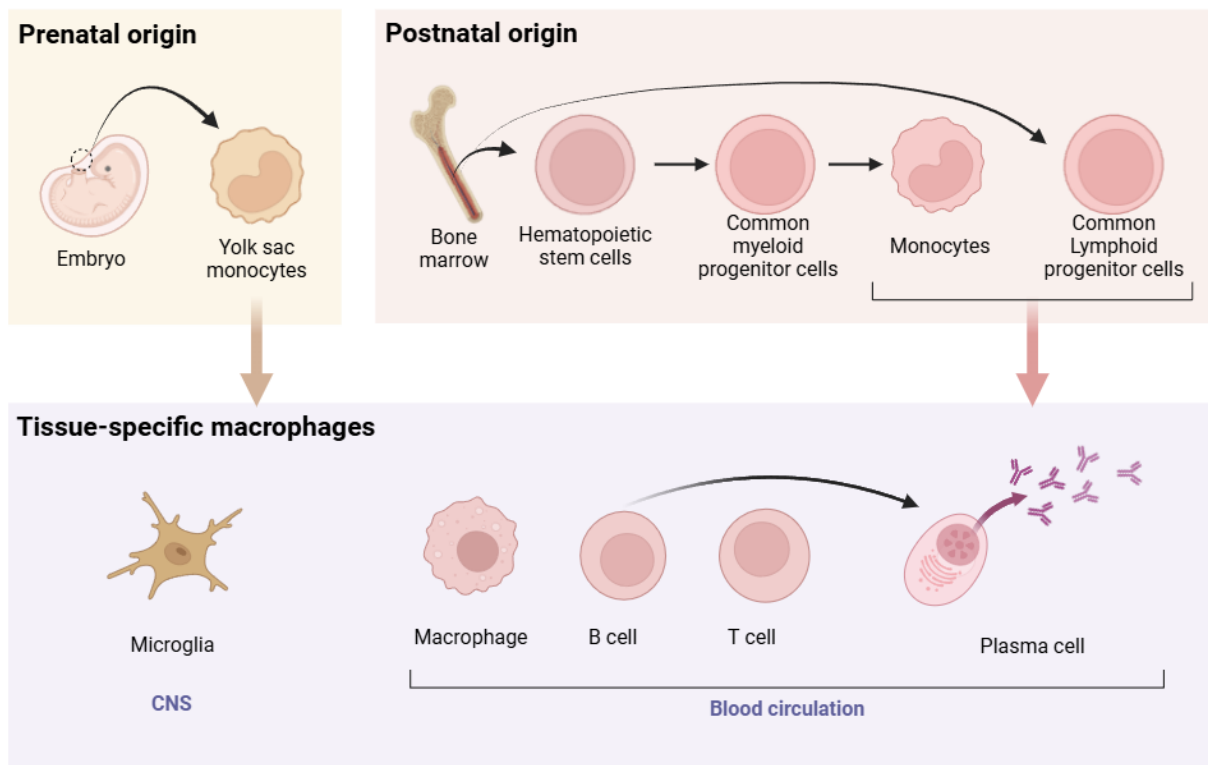


**Figure 12: Luxol fast blue (blue) stained WM Lesions with remyelination score 3, 2, 1.** (A–C) represent a shadow lesion (SL, remyelination score 3). (A) showing an almost completely remyelinated SL, covering almost the entire surface, but still with lower myelin density in the center. Fibers are running almost similar to the fibers of adjacent NAWM. (B) The lesion border is still visible, with a visible gradient in myelin density (indicated by arrows). (D–F) Lesion with substantial remyelination (remyelination score 2), with thin, patchy myelin fibers spread throughout the lesion area (E). (G–I) Lesion with partial remyelination (remyelination score 1), showing a thinner myelin gradient restricted to the lesion rim (indicated by the arrow in (H)). (J–L) Demyelinated lesion (remyelination score 0) with at the border normal myelin thickness and cut in the same fiber direction as the surrounding NAWM. (C,F,I,L) Corresponding immunohistochemical myelin staining (PLP; brown) showing NAWM, WML, GM and SL. GM: grey matter; NAWM: normal appearing white matter; RM: remyelination; SL: shadow lesion; WML: white matter lesion From: (Huitema et al., 2021)

## 2. Myelin destruction and repair in MS

### a) Immunopathology of MS

Most MS polymorphisms (SNP) are linked with immune system related genes confirming the autoimmune component of the disease where immune cells attack myelin (Beecham et al., 2013; Patsopoulos et al., 2019; Sawcer et al., 2011). Cells from both the innate and adaptive immune system participate in this inflammatory attack. The innate immune system includes macrophages and CNS resident microglia, while the adaptive immune system comprises lymphocytes, specifically T and B cells. Macrophages, T cells, and B cells are generated in the bone marrow, differentiating from hematopoietic stem cells. Hematopoietic stem cells give rise to either myeloid stem cells (which become monocytes) or lymphoid stem cells (which become lymphocytes). Both lymphocytes and macrophages circulate through the bloodstream to monitor the body. In contrast, microglia are the resident innate immune cells of the CNS. They originate from early progenitors in the embryonic yolk sac and migrate to colonize the CNS. Each cell type contributes uniquely to the pathophysiology of MS (Figure 13).



**Figure 13: Origins and Differentiation Pathways of Immune Cells.** Microglia has a prenatal origin and comes from the yolk sac, while macrophages and lymphocytes have a postnatal origin and are differentiated from hematopoietic stem cells. Created with Biorender

## **(1) T cells in MS**

We can distinguish two groups of T cells depending on the expression of specific CD at their cell surface; they can express CD4 or CD8. Both groups of T cells have specific functions. CD4<sup>+</sup> T cells coordinate the adaptive immune response and are called T helper (Th), whereas the CD8<sup>+</sup> T cells are the effectors and cytotoxic cells, their role is to kill infected cells by producing perforin and granzyme molecules. The activation of CD4<sup>+</sup> T cells involved the MHC class II whereas CD8<sup>+</sup> T cells activation involved the MHC class I.

### **(a) CD4<sup>+</sup>T cells in MS**

Depending on the type of stimulation CD4<sup>+</sup> T cells receive during antigen presentation, naïve CD4<sup>+</sup> T cells can differentiate into various subsets with distinct functions. They can be polarized into pro-inflammatory Th1 or Th17 cells, which drive inflammation. Th1 cells are involved in cellular immunity and are known for their role in fighting intracellular pathogens, while Th17 cells are associated with combating extracellular bacteria and fungi and play a significant role in autoimmune conditions.

After the initial inflammatory response, Th2 and regulatory T cells (Tregs) work to resolve the inflammation. Th2 cells inhibit Th1 activation, thereby dampening the inflammatory response. On the other hand, Tregs suppress the proliferation of other T cells, helping to maintain immune tolerance and prevent excessive immune responses. Together, Th2 cells and Tregs ensure that the immune response is controlled, and that tissue damage is minimized.

Th1 cells secrete interferon (IFN)- $\gamma$ , and the levels of IFN- $\gamma$  in PwMS was correlated with the frequency of active lesions (Olsson, 1992). An upregulation of IFN- $\gamma$  secretion can be detected just before disease activity (Dettke et al., 1997). Importantly, IFN- $\gamma$  can activate microglia, and also directly kill OL (Aloisi et al., 2000).

Th17 cells express Interleukin (IL) 17, in PwMS brain lesions, IL-17 messenger RNA (mRNA) can be detected (Lock et al., 2002; Montes et al., 2009). Interestingly, in active lesions of PwMS there is an upregulation of cells expressing IL-17 (glial cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells) compared to inactive lesions (Tzartos et al., 2008). In PwMS blood there is also an upregulation of IL-17 producing cells and levels of IL-17 mRNA is associated with disease activity (Durelli

et al., 2009; Matusevicius et al., 1999), and a higher IL-17 mRNA and protein levels, in both blood and CSF of PwMS is detected during relapses (Brucklacher-Waldert et al., 2009; Durelli et al., 2009; Matusevicius et al., 1999; Vaknin-Dembinsky et al., 2006). IL-17A levels in CSF of PwMS is associated with blood brain barrier (BBB) disruption (Kostic et al., 2015), and correlates with glutamate levels in CSF of PwMS (marker of glutamate excitotoxicity for neuron) (Kostic et al., 2014). *In vitro* study on a model of BBB crossing showed that human Th17 cells are more efficient to cross the BBB and have neurotoxic effects (Kebir et al., 2007). There is also a subset of CD4<sup>+</sup> T cells that can express both IL-17 and IFN- $\gamma$  that have been detected as enriched in MS brain tissue suggesting that they could play a role in disease (Kebir et al., 2009).

In MS patients Tregs do not proliferate efficiently to stop the inflammation (Danikowski et al., 2017; Viglietta et al., 2004). In addition, they also are no longer able to suppress the proliferation of myelin-specific T cells (Dombrowski et al., 2017). Therefore, loss of functions and low number of Tregs could explain the attack of oligodendrocytes and myelin by inflammatory CD4<sup>+</sup> T cells.

## **(b) CD8<sup>+</sup>T cells in MS**

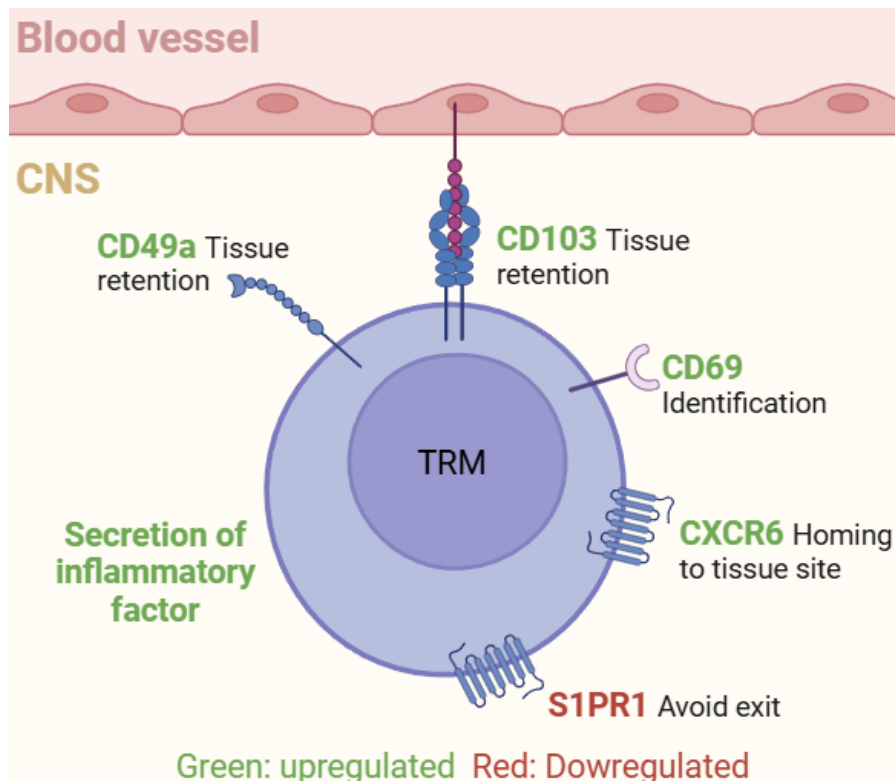
The primary model for studying MS is the experimental autoimmune encephalomyelitis (EAE), which is driven by CD4<sup>+</sup> T cells. Consequently, it was initially believed that MS was also driven by CD4<sup>+</sup> T cells. However, increasing evidence suggests that MS is actually driven by CD8<sup>+</sup> T cells. In fact, as mentioned above, MS lesions contain more CD8<sup>+</sup> T cells infiltrate compared to CD4<sup>+</sup> T cells (Absinta et al., 2021; Bitsch et al., 2000; Kuhlmann et al., 2002; Machado-Santos et al., 2018).

Studies have shown that EAE induction with CD8<sup>+</sup> T cells is more severe and results in greater axonal degeneration compared to EAE induction with CD4<sup>+</sup> T cells (Sun et al., 2001). In MS post-mortem tissue, CD8<sup>+</sup> T cells are located close to damaged axons (Fletcher et al., 2010), and their number is correlated to axonal damage (Bitsch et al., 2000). CD8<sup>+</sup> T cells are also located in cortical lesions, and these cortical lesions are associated with clinical progression in MS (Lucchinetti et al., 2011). This data suggests that CD8<sup>+</sup> T cells could be the main cells contributing to axonal damage in MS.

Several clues show the implication of CD8<sup>+</sup> T cells in MS pathogenesis. The early upregulation of MHC class I molecules observed in PwMS, occurring before demyelination, suggests an intensified immune response involving CD8<sup>+</sup> T cells that likely contributes to the autoimmune attack on myelin (Gobin et al., 2001; Hayashi et al., 1988; Ransohoff & Estes, 1991). It has also been demonstrated that in PwMS, CD8<sup>+</sup> T cells undergo oligoclonal expansion within the CNS, in peripheral blood, and in the CSF (Babbe et al., 2000; Friese & Fugger, 2009; Junker et al., 2007). This oligoclonal expansion indicates a targeted immune response, suggesting that these CD8<sup>+</sup> T cells are actively involved in the pathology of MS by recognizing and attacking specific antigens within these regions. Studies have also shown that a subpopulation of CD8<sup>+</sup>T also expresses CD161. CD161<sup>+</sup> CD8<sup>+</sup>T cells producing IFN are found in MS lesions and are enriched in MS patient blood (Annibaldi et al., 2011).

In MS lesions, CD8<sup>+</sup> T cells aggregate to form perivascular cuffs, and many of these cells are classified as tissue-resident memory T cells (TRM). Unlike circulating T cells that move through the bloodstream, TRM will remain within the CNS and continuously interact with the surrounding environment by sending signals to nearby cells (Figure 14) (Kumar et al., 2017). Cellular and molecular mechanisms allow TRM cells to persist in the CNS (Figure 14), contributing to the chronic inflammation seen in MS lesions.





**Figure 14: Signature of human TRM.** TRM loses the ability to return in the blood circulation by downregulation of *S1PR1*, and upregulation of factors that induce tissue retention such as *CD103*, *CD49a* and *CXCR6*. *CD69* is a marker that allows their identification. When in the tissue of residency such as brain in MS, they will secrete inflammatory factors. Modify from: (Kumar et al., 2017)

## (2) B cells in MS

Immunoglobulins, which are produced by B lymphocytes, are found in the CSF of 90% of people with MS (PwMS) (Thompson et al., 2018). The presence of immunoglobulins in the CSF of PwMS is a significant disease marker (also used to diagnosis MS pathology), indicating active B cell involvement in the pathogenesis of MS. Moreover, specific types of immunoglobulins, such as IgM, have been associated with a more severe disease course (Villar et al., 2002, 2003).

*Ex vivo* studies have shown that activated B cells from PwMS produce excessive amounts of pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bar-Or et al., 2010; Duddy et al., 2007; R. Li et al., 2015). For instance, elevated levels of TNF are found in both the serum and CSF of PwMS, and the receptors for these cytokines are upregulated in MS lesions. Additionally, B cells from PwMS secrete factors that are toxic to OL (Lisak et al., 2012) and

neurons (Lisak et al., 2017). *In vivo* and *in vitro* studies have demonstrated that B cells from PwMS can induce demyelination by secreting IgG antibodies that target myelin components such as MBP and MOG (Claes et al., 2015; Elliott et al., 2012). Furthermore, B cells secrete cytokines that activate macrophage (R. Li et al., 2015) but they can also stimulate self-reactive CD4+T cells proliferation (Jelcic et al., 2018). Within MS lesions, B cells are also found in perivascular cuffs and are less frequently observed in the parenchyma (Esiri, 1977; ESIRI, 1980; Prineas & Wright, 1978). These cells tend to accumulate in the meningeal and perivascular spaces, contributing to the formation of lymphoid-like structures (Choi et al., 2012; Howell et al., 2011; Magliozzi et al., 2007, 2010; Serafini et al., 2004; H. Y. F. Yong & Yong, 2022). In PwMS with oligoclonal band in the CSF, and B cells accumulation in perivascular and meningeal space there is a high level of inflammation, active subpial grey matter demyelination and a rapid progression of clinical course (Androdias et al., 2010; Bevan et al., 2018; Choi et al., 2012; Haider et al., 2016; Howell et al., 2011; Magliozzi et al., 2007, 2010; Serafini et al., 2004)

Another subset of B cells, known as Bregs, exhibits immunomodulatory properties by expressing IL-10. In people with MS, Bregs have been shown to be dysfunctional, expressing less IL-10 (Okada et al., 2018), which results in a diminished ability to suppress Th1 functions (Cencioni et al., 2021). Additionally, the number of Bregs is reduced during relapse phases compared to remission phases (Y. Kim et al., 2018; Knippenberg et al., 2011).

### **(3) Macrophages and microglia in MS**

As mentioned above, microglia are the innate immune cells of the CNS, and macrophages are the peripheral innate immune cells. In the healthy CNS, microglia are the only innate immune cells present in the parenchyma, but under pathology (such as MS or other neurodegenerative diseases) macrophages infiltrates the CNS. However, it is difficult to differentiate activated microglia and macrophages as they express the same molecules. Therefore, for the following part, I will refer to them as myeloid cells, unless it is specified.

Myeloid cells can be differentially activated in response to different stimuli. For example, in response to pro-inflammatory stimuli (such as: IFN- $\gamma$  or lipopolysaccharide (LPS)) myeloid cells will be “classically activated” also called “M1” phenotype and will have pro-

inflammatory properties, while in response to anti-inflammatory stimuli (such as: IL-4) myeloid cells will be “alternatively activated” also called “M2” phenotype and have pro-regenerative properties (Martinez & Gordon, 2014). However, this is a simple vision, nine different activation phenotypes were found in a transcriptomic study of human macrophages (Xue et al., 2014). In addition, the M2 activation can be further subdivided in M2a, M2b and M2c (Martinez & Gordon, 2014). Nonetheless, this dichotomic view of M1/M2 is challenged, I will refer to pro-inflammatory and pro-regenerative in the next paragraphs for convenience.

Myeloid cells are thought to participate in demyelination since they are present in active demyelinating lesions (Kuhlmann et al., 2017) and detected containing myelin products (foamy cells) (Absinta et al., 2021). They are also thought to contribute to neurodegeneration as their number correlates with the extent of axonal damage in MS lesions (Bitsch et al., 2000). In addition, pro-inflammatory markers of myeloid cells are higher in areas of active demyelination (Jäckle et al., 2020; Zrzavy et al., 2017), suggesting that pro-inflammatory activation induces demyelination. In blood and CSF of MS patients, there is an upregulation of monocytes CD16<sup>+</sup> (Gjelstrup et al., 2018; Waschbisch et al., 2016), these monocytes are pro-inflammatory, and they are also present in MS lesions (Waschbisch et al., 2016). Interestingly, MS patients' monocytes-derived macrophages show a higher pro-inflammatory activation independently of the stimuli they received compared to healthy control, this is associated with a dysregulation of their metabolic profile (Fransson et al., 2021). These data suggest that myeloid cells from MS patients are more prone to pro-inflammatory polarization, and therefore to induce demyelination.

In addition to contributing to myelin destruction, myeloid cells function as antigen-presenting cells (APCs). For CD4<sup>+</sup> T cell activation, antigen recognition is necessary, involving interaction between the T cell receptor (TCR) on T cells and HLA class II molecules on APCs. Subsequently, APCs upregulate co-stimulatory ligands like CD80 and CD86. In MS patients, myeloid cells show increased expression of HLA class II (Hendrickx et al., 2017; Jack et al., 2005), and CD80 is upregulated in post-mortem myeloid cells from MS patients. These findings suggest that myeloid cells act as APCs and reactivate T cells within the CNS. Additionally, myeloid cells can secrete chemoattractant molecules which are highly expressed in MS lesions, to recruit T cells to the CNS.

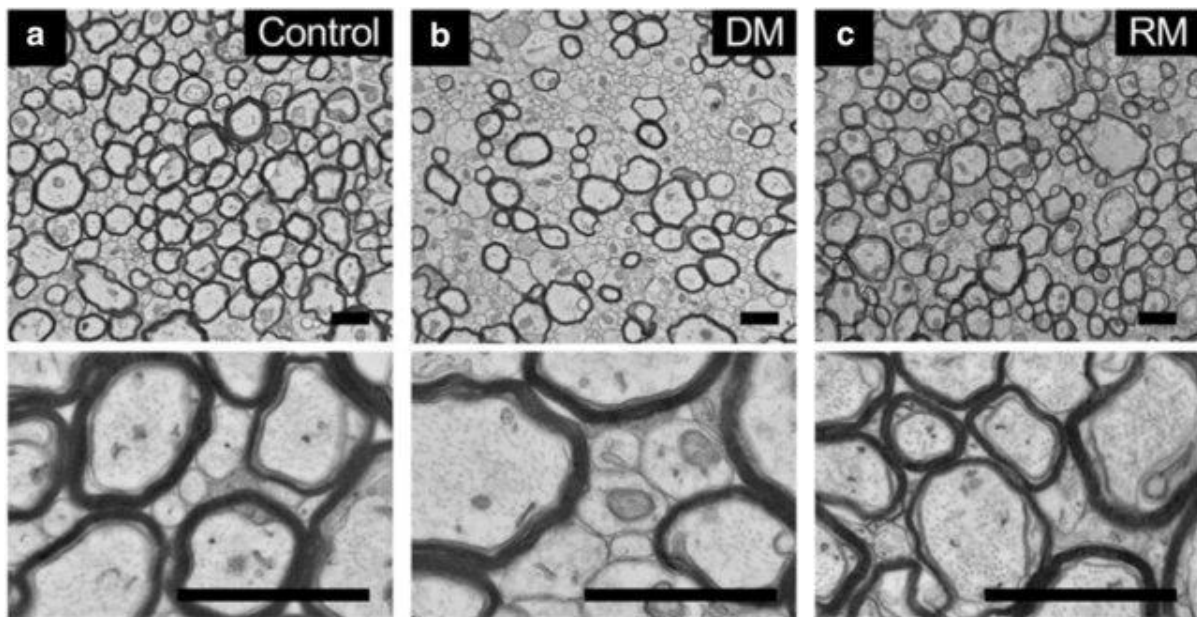
Even if microglia and infiltrating macrophages have been classically considered a homogeneous population due to similar roles and expression, there is more and more evidence

that they could have distinct roles. The purinergic receptor P2Y<sub>12</sub> is a homeostatic marker of microglial cells. P2Y<sub>12</sub> is decreased in lesions and normal appearing white matter in MS patients (Zrzavy et al., 2017) suggesting that there is an activation of innate resident cells of the CNS in MS patients, in areas with no infiltration of circulating immune cells. *In vitro* assessment of phagocytic properties of human macrophages and microglia from healthy individuals, showed a higher phagocytic capacity of human primary microglia when compared to macrophages (Healy et al., 2016). Moreover, with the use of new technology such as single-cell RNA sequencing (scRNA-seq), single-nucleus RNA sequencing (snRNA-seq) and also single-cell mass cytometry (CyTOF) on MS tissues provide new insight on microglia and macrophages contribution (Absinta et al., 2021; Böttcher et al., 2019; Healy et al., 2022; Masuda et al., 2019). In PwMS post-mortem tissue snRNA-seq analysis revealed that microglia downregulate homeostatic genes (Masuda et al., 2019). In parallel, they also appear to be highly activated and phagocytic (Böttcher et al., 2019). snRNA-seq also allowed the identification of specific microglia subtypes in MS, such as: microglia inflamed in MS (MIMS), either full of myelin debris named MIMS-foamy or loaded with iron named MIMS-iron and disease associated microglia (DAM) (Absinta et al., 2021). Microglia differentiate into DAM through a two-step process. The first step involves a reduction in homeostatic gene expression, followed by the second step, which includes the activation of phagocytosis and lipid metabolism through triggering receptor expressed on myeloid cells 2 (TREM2) activation. DAM express genes associated with both pro-inflammatory and pro-regenerative phenotypes (Song & Colonna, 2018). While homeostatic microglia can suppress T-cell proliferation *ex vivo*, DAM lack this ability (Krasemann et al., 2017). DAM are characterized by the upregulation of apolipoprotein E (APOE) and C-type lectin domain containing 7A (Clec7a), making APOE and Clec7a markers of DAM cells (Krasemann et al., 2017).

## **b) Remyelination**

In 1961, a study demonstrated for the first time that spontaneous remyelination occurs after demyelination (BUNGE et al., 1961). The newly formed myelin is thinner (Figure 15C) compared to developmental myelin (Figure 15A) (Teo et al., 2019); however, it has been demonstrated to support functional recovery. Indeed, after demyelination in the cat, remyelination restores saltatory conduction (Smith et al., 1979), and locomotor function loss

induced by demyelination in the rat (Jeffery & Blakemore, 1997). Remyelination also protects against neurodegeneration (Duncan et al., 2023).

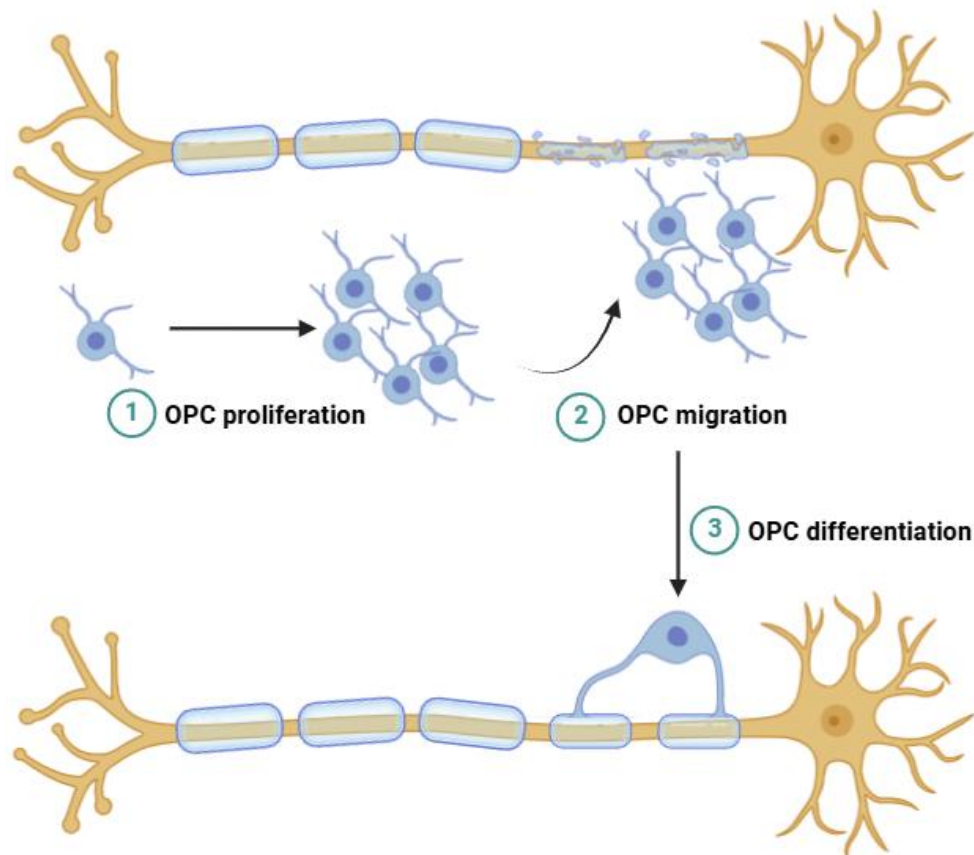


**Figure 15: Ultrastructural analysis of myelinated axons following demyelination in the corpus callosum of wild-type (WT) mice.** (a–c) Representative electron microscopy images of the corpus callosum of WT mice belonging to the (a) developmental myelin, (b) demyelination, and (c) remyelination. Scale bar = 1  $\mu$ m. From: (Teo et al., 2019)

In the CNS, the cells of origin of new myelin sheaths are mainly the adult pool of OPCs (Bacmeister et al., 2020), but new evidence showed that surviving OL can participate in remyelination (Bacmeister et al., 2020; Duncan et al., 2018; Franklin et al., 2021; Mezydło et al., 2023). However, surviving OL remyelination is inefficient, because internode formation by surviving OL is a rare phenomenon and outweighed by internodes loss happening at the same time (Mezydło et al., 2023). Interestingly, in the spinal cord, Schwann cells can participate in the remyelination process (El Behi\*, Sanson\* et al., 2017). Some evidence shows that remyelinating Schwann cells are derived from CNS glial precursors (Zawadzka et al., 2010), and other results have shown that a large amount of Schwann cells participating in remyelination are derived from the periphery (Oudega & Xu, 2006).

## (1) Remyelination steps

After an event of demyelination (or CNS injury), remyelination can occur. The remyelination process can be divided into distinct phases. First, OPCs need to populate the lesioned area, to do so they proliferate and migrate to colonize the demyelinated area, this phase is called the recruitment phase (Figure 16; 1-2). Then in order to make new myelin, OPCs need to differentiate into myelinating OL. This is called the differentiation phase (Figure 16; 3).



**Figure 16: Schematic representation of remyelination process.** For remyelination, OPCs need to migrate to the site of lesion, then they proliferate and finally they differentiate into myelinating OL. Created with Biorender

After demyelination, OPCs are activated, this activation induces morphological (Levine and Reynolds, 1999) and gene expression changes (Franklin & Ffrench-Constant, 2008). OPCs start to express OLIG2 and NKX2.2 in demyelinating lesions, these genes that are known in OL development (Fancy et al., 2004; Franklin & Ffrench-Constant, 2008). In demyelinating area, the expression of PDGF and FGF-2 is increased (Messersmith et al., 2000; Woodruff et al., 2004), and associated with an increase of both receptors on OPCs in response to demyelination (Redwine & Armstrong, 1998), this will induce proliferation and migration

towards the demyelinated area (Zhao et al., 2005). Interestingly, the OPCs recruited to the lesion are located at a small distance from the injury site (Franklin et al., 1997).

When the OPCs have proliferated and migrated to populate the demyelinated area they form new myelin around axons. To do so, OPCs are believed to go through the same stage of differentiation as for developmental myelination, OPCs extend their processes to contact axons and wrap them to form new myelin. The expression of Olig2 in OPCs induces the expression of the transcription factor SRY-box 10 (Sox10) that is critical for OL maturation (Küspert et al., 2011). Sox10 is required for the differentiation of OPCs into myelinating OL, its actions are direct and indirect. Indeed, Sox10 directly activates several genes of the myelination process such as MBP and the myelin regulatory factor (MYRF) (Hornig et al., 2013; H. Li et al., 2007). MYRF is essential to OPCs differentiation, as in KO model there is a loss of OL (Emery et al., 2009), and is also important for the remyelination process (Duncan et al., 2017).

In animal models of demyelination/remyelination, this process (recruitment and differentiation) takes up to three weeks to be totally complete (Blakemore & Franklin, 2008; Jeffery & Blakemore, 1995).

## **(2) Immune system orchestrates de and re-myelination**

Inflammation undoubtedly contributes to the pathogenesis of MS. However, the remyelination process heavily depends on the surrounding environment, particularly the inflammatory environment. Inflammation plays a significant role in remyelination. First, myelin debris inhibits the remyelination process, so its clearance by myeloid cells is crucial. Additionally, the differential activation or polarization of immune cells has been shown to orchestrate the various steps of remyelination.

### **(a) Innate immune system**

For a long time, macrophages were viewed as detrimental, particularly because their infiltration into the CNS was known to exacerbate disease progression in EAE (Ajami et al.,

2011). However, research using a rat model of demyelination induced by lysophosphatidylcholine (LPC) has shown that depleting macrophages from day 1 to day 21 (the early stages of remyelination) impairs the remyelination process. In contrast, depleting macrophages from day 8 to day 21 (the later stages of remyelination) does not impact remyelination. Nevertheless, depleting macrophages throughout the entire remyelination process hinders oligodendrocyte remyelination. These findings indicate that, despite their role in driving demyelination, macrophages are essential for the early stages of remyelination (Kotter et al., 2001).

But in a model of secondary progressive MS (SPMS), obtained by the induction of EAE in nonobese diabetic mice, the depletion of microglia cells using the PLX3397 CSF1R inhibitor increases the secondary progression of EAE and the mortality. In addition, increased inflammation, demyelination, axonal degeneration and number of proliferating CD4<sup>+</sup> T cell is observed in treated mice (Tanabe et al., 2019). It suggests that microglia inhibit CD4<sup>+</sup> T cells proliferation (Tanabe et al., 2019). In another model of CNS demyelination with the infection of mice with mouse hepatitis virus (MHV) strain JHMV the depletion of microglia using another inhibitor of CSF1R the PLX5622 during the time of clearance of the MHV impaired remyelination (Sariol et al., 2020).

These results demonstrate that myeloid cells drive demyelination as their inhibition in specific times reduces demyelination, but they also are essential for the remyelination process as their depletion decreases the repair process.

#### (i) **Debris clearance**

*In vitro* studies have demonstrated that myelin substrates impair OPC differentiation (S. Robinson & Miller, 1999). *In vivo* studies further revealed that injecting myelin three days after focal demyelination decreased remyelination due to the inhibition of OPC differentiation (Kotter et al., 2006). Therefore, myelin debris act as inhibitors of remyelination by preventing OPC differentiation, underscoring the importance of clearing this debris for the remyelination process (Neumann et al., 2009).

The clearance of myelin debris is done by myeloid cells and is mediated by the fatty acid translocase CD36. In the EAE mouse model, uptake of myelin by CD36 reduces



neuroinflammation, and its inhibition induces pro-inflammatory properties *in vivo* (Grajchen et al., 2020). It is known that phagocytosis of myelin by phagocytosing cells promotes a switch in their phenotype toward one that is associated with repair and reduces the expression of pro-inflammatory mediators (Bogie et al., 2011; Boven et al., 2006; Hikawa & Takenaka, 1996), this shift is mediated via liver X receptor and peroxisome proliferator-activated signaling pathways (Bogie et al., 2012, 2014). The pro-inflammatory activation through MyD88 is essential for myelin degradation once it is uptaken by myeloid cells, without this pro-inflammatory activation, myeloid cells will not be able to degrade myelin (Cunha et al., 2020). Long-term exposure to myelin increases intracellular cholesterol and induces inflammatory phenotype in human macrophages-derived monocytes, mouse bone marrow-derived macrophages and microglia (Bogie et al., 2020).

Therefore, myelin uptake and clearance should be quick and effective to allow the remyelination process to take place.

#### **(ii) Recruitment of OPCs**

Pro-inflammatory activation of myeloid cells is known to induce the recruitment (proliferation and migration) of OPCs. As mentioned above PDGF and FGF-2 induce OPC proliferation, both can be secreted by astrocytes and myeloid cells. TNF is a molecule secreted by activated myeloid cells and is a pro-inflammatory cytokine. In TNF receptor 1 KO mice, there is a decrease in OPCs proliferation after demyelination (Arnett et al., 2001). Moreover, in an *in vitro* study, the conditioned media of pro-inflammatory macrophages induces the proliferation and migration of OPCs, and pro-inflammatory macrophages depletion *in vivo* decreases the proliferation of OPCs (Miron et al., 2013). These data indicate a crucial role of pro-inflammatory activated myeloid cells in OPC recruitment.

#### **(iii) Differentiation of OPCs**

After myelin debris clearances and the recruitment phase, which are possible because of pro-inflammatory activation, there is a switch in the activation. Myeloid cells will become pro-regenerative and secrete immunomodulatory and pro-regenerative factors. Pro-regenerative

myeloid cells are known to induce OPC differentiation via molecule secretion (Miron et al., 2013; Patel & Klein, 2011; V. W. Yong & Rivest, 2009). For example, Insulin growth factor (IGF-1) increases OPCs differentiation and myelin production *in vitro*, and in EAE model injection of IGF-1 increases the number of remyelinated axons (Yao et al., 1995). Activin A, another factor secreted by myeloid cells increases OPCs differentiation *in vitro* and remyelination *in vivo* (Miron et al., 2013).

These data indicate a crucial role of pro-regenerative activated myeloid cells in OPC differentiation.

### (b) Adaptive immune system

Lymphocytes were not originally thought to participate in the myelin repair process. However, being present during remyelination in acute plaques of PwMS (Popescu et al., 2013), lymphocytes could participate in the remyelination process. Using KO mice or antibody depletion, it was demonstrated that lymphocytes are necessary for remyelination (Bieber et al., 2003). In B6-Rag<sup>tm1Mom</sup> mice, lacking both T and B cells, 35 days after focal demyelination (when the remyelination should be complete) the density of remyelinated axons was reduced. In mice lacking CD4<sup>+</sup> (CD4<sup>tm1Mak</sup> KO model or depletion induced by anti-CD4 antibodies) or CD8<sup>+</sup> cells (CD8<sup>tm1Mak</sup> or depletion induced by an anti-CD8 antibody), remyelination was also impeded (Bieber et al., 2003). This pioneer study demonstrated for the first time the essential role of adaptive immunity in remyelination (Bieber et al., 2003).

However, now new data have unraveled the contribution of lymphocytes to remyelination. There are studies showing a direct regenerative role of Tregs in remyelination: First, there is a Tregs induction at the initiation of remyelination (Plaisted et al., 2016). In addition, in a KO mouse for Foxp3, in which Tregs are absent, remyelination was impeded and rescued by the graft of exogenous wild type Tregs (Dombrowski et al., 2017). More interestingly, in the EAE, mice grafted with human neural stem cells (hNSCs) showed more remyelination, this is associated with an increase of Tregs cells in the spinal cord. Depletion of Tregs before hNSCs graft suppresses the regenerative effect (McIntyre et al., 2020). Tregs were shown to have a direct pro-differentiation effect on OPCs *in vitro* mediated by the secretion of CCN3. CCN3 is also up-regulated in spinal cord during remyelination process after LPC demyelinating injection, but the same study using CCN3 KO mice showed that

remyelination isn't impaired without CCN3, those results suggest that CCN3 isn't essential for remyelination (de la Vega Gallardo et al., 2020). More interestingly, when comparing CCN3 expression between patient and controls, there is no CCN3 deficiency in MS but show a dynamic expression profile regarding disease type and treatment (Naughton et al., 2020). Even if the well-known immunomodulatory role of Tregs cannot be excluded to have played a role in the rescue of remyelination, Tregs seem to have a direct regenerative role on promoting OPC maturation. Interestingly, it was also shown that myelin-specific T cells can induce a proliferation of OPC after axonal damage through an unknown mechanism (Hvilsted Nielsen et al., 2011).

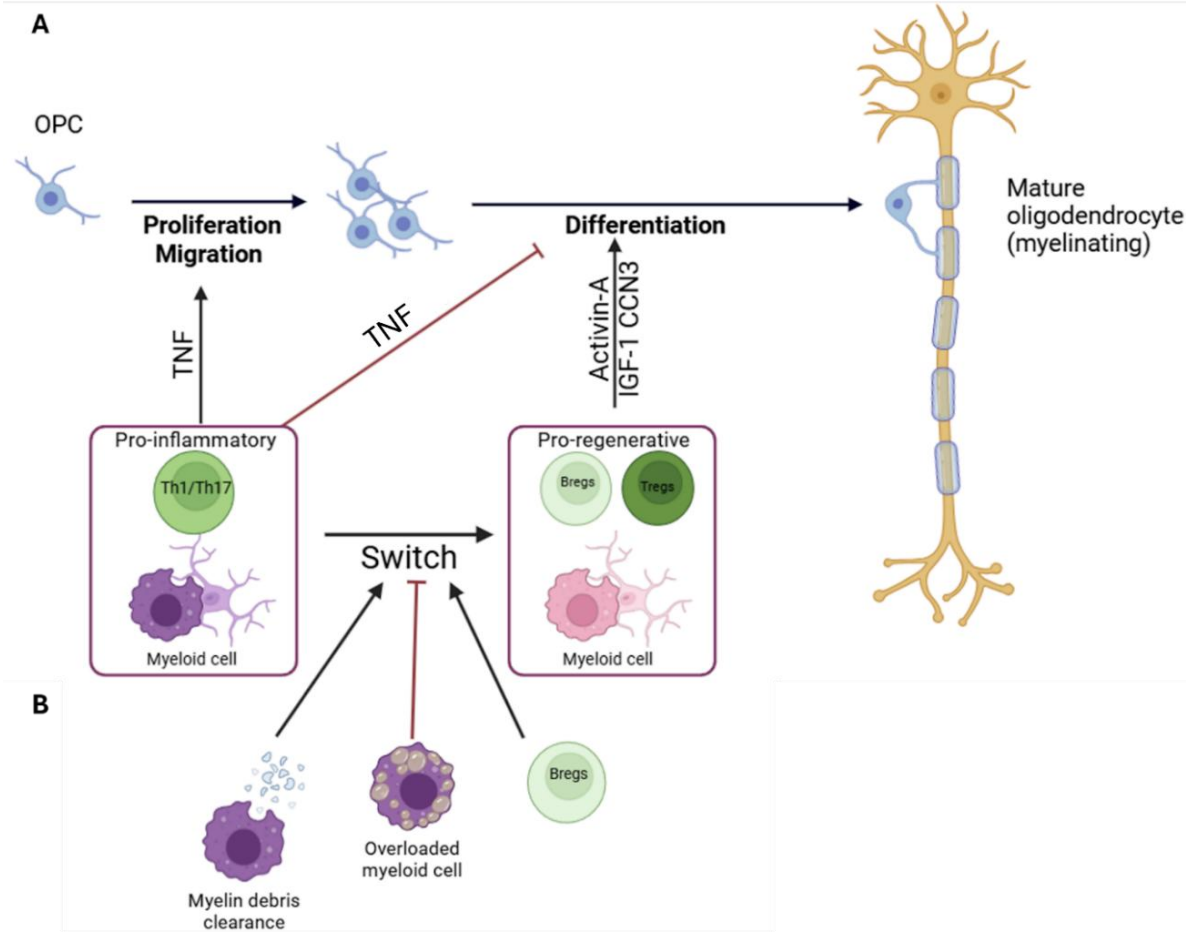
In a new humanized mouse model of demyelination/remyelination, that combined LPC injection and grafting of human lymphocytes, healthy donor lymphocytes did not disrupt the remyelination process. In contrast, lymphocytes from MS patients impeded the remyelination process (El Behi\*, Sanson\* et al., 2017)

Recently a new study showed the importance of B regulators (Bregs) in the remyelination process. In a murine model of EAE, at the onset of the first sign of clinical disability (tail paralysis) the adoptive transfer of Bregs isolated from naïve mice improves remyelination and increases the size of newly formed myelin sheath. Those results are linked to a diminution of immune cell infiltration in the CNS and a switch in myeloid cells phenotype toward pro-regenerative (Pennati et al., 2020). Furthermore, the same teams previously demonstrated that Bregs induced IL10 production by CD4+ activated T cells *in vitro*. *In vivo* experiment showed that adoptive transfer of Bregs in EAE mice increased the number of Tregs (Pennati et al., 2016).

These shows that both innate and adaptive immune cells are important for the repair process (Figure 17A). To resume, while pro-inflammatory cells (Th1/Th17; pro-inflammatory activated myeloid cells) will induce the first steps of remyelination, the proliferation and migration of OPCs, the pro-regenerative cells (T- and B-regs; pro-regenerative activated myeloid cells) will induce the differentiation of OPCs into myelination OL (Figure 17A). It is therefore important to also switch from pro-inflammatory to pro-regenerative phenotypes (Figure 17B).

Taken together, all these data prove that, even if the immune system drives demyelination in MS, the same immune system is needed for efficient remyelination. Instead

of trying to stop inflammation, it is therefore extremely important for successful remyelination that the inflammatory response is well controlled in time and intensity.



**Figure 17: The immune system plays a role in remyelination.** (A) Pro-inflammatory cells (Th1, Th17, pro-inflammatory myeloid cells) induce proliferation and migration of OPCs and inhibits OPCs differentiation by secreting pro-inflammatory cytokines, whereas pro-regenerative cells (Tregs, Bregs and pro-regenerative myeloid cells) secrete pro-regenerative cytokines that induce OPCs differentiation. (B) The switch from pro-inflammatory to pro-regenerative is necessary. Created with Biorender

## C. Animal models of MS

To study MS pathology, the formation of demyelinated lesions, or the repair process, animal models have been developed. By definition, there is no perfect animal model. In the study of MS, different models are used to study different parts of the disease. There are three types of animal models mostly used: (1) experimental autoimmune encephalomyelitis (EAE); (2) virally induced chronic demyelination; and (3) toxin-induced demyelination. All those models will recreate some features of MS, but with some differences.

### 1. Experimental autoimmune encephalomyelitis (EAE)

EAE is widely used as a model for MS because this model shares pathological and clinical features (Fletcher et al., 2010). Interestingly, the first EAE has been developed to study the pathogenesis of rabies post-vaccinal encephalomyelitis (Rivers et al., 1933). They try to reproduce the acute disseminated encephalomyelitis (ADEM) in monkeys (*Macacus Rhesus*) by repeated intramuscular injection of normal rabbit brain. In this first publication, only 2 out of 8 monkeys developed symptoms and analysis of their brain showed perivascular infiltrates and demyelination (Rivers et al., 1933). In a second experiment 6 out of 8 monkeys had symptoms development and demyelination (Rivers & Schwentke, 1935). However, to develop symptoms and induce demyelination they need to do a high number of injections. In 1934, using homologous injections of emulsions or extracts from rabbits' brains, they showed that the more antigenic the emulsions were, the higher the proportion of rabbits affected, and they also developed brain-specific antibodies. The association between the pathology and the presence of brain-specific antibodies suggest that demyelination could be caused by a specific immune response (Schwentker & Rivers, 1934). To test this hypothesis, several groups tried to boost the antibodies production with complete Freund's adjuvant (CFA) that was just newly described (Freund & Mcdermott, 1942). This resulted in the induction of the now named EAE with only a few numbers of injections (Freund et al., 1947; Kabat et al., 1947; Morgan, 1947)

Now two method of EAE induction are used, 1) active immunization (active EAE) of the animal with myelin peptide such as MBP, MOG and PLP in CFA (Freund & Mcdermott, 1942) with the injection of pertussis toxin to open the blood-brain barrier (BBB) (Munoz et al., 1984), or 2) passive immunization (passive EAE), firstly describe with the transfer of cells

extract from lymph node of rats immunized with guinea pig spinal cord (PATERSON, 1960). Now passive EAE is obtained by the adoptive transfer of activated myelin-specific T cells (Robinson et al., 2014). One of the biggest differences between passive and active immunization is the percentage of mice that will be developing EAE, while it is known that active EAE will not induce clinical sign in all mice, 100% of mice immunized by adoptive transfer of activated myelin-specific T cells will develop clinical sign. The first symptoms appear around 7 to 12 days post-immunization. To evaluate the evolution of the disease, clinical disability is quantified using a standard EAE scoring system, on a 0 to 5 disease severity scale: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb paralysis or weakness; and finally, 5, moribund/death (Shahi et al., 2019).

Overall, EAE can be induced in different animal, such as monkeys (rhesus or marmoset are the most used) (Jagessar et al., 2012; Kabat et al., 1947), guinea pigs (Freund et al., 1947), rats (M. M. Lipton & Freund, 1952) and mice (Olitzky & Yager, 1949), but the rodents' models are the most used and described. The immunization process with different myelin peptides in different strains of mice will give different EAE disease types. For example, the immunization of C57BL/6J mice with high dose of MOG<sub>35-55</sub> peptide induce a chronic EAE (Tompkins et al., 2002) a relative low dose of MOG<sub>35-55</sub> peptide can induce a relapsing-remitting EAE (RR-EAE) such as immunization of SJL/J mice with PLP<sub>139-151</sub> peptide (Tuohy et al., 1989).

As mentioned above; to enhance the immune responses to the immunization, CFA is used in the emulsion. CFA has been shown to stimulate the antigen presentation, activation, and expansion of CD4<sup>+</sup>T cells (Billiau & Matthys, 2005). Therefore, EAE models are CD4<sup>+</sup>T cells mediated diseases. In this pathology, the immunization of the mice with antigen of myelin protein leads to the development of myelin-specific T cells that will enter the CNS and destroy myelin and OL. However, both CD4<sup>+</sup>T and CD8<sup>+</sup>T cells can induce passive EAE (Fletcher et al., 2010; Sun et al., 2001).

This model has been widely used and helpful to decipher the different types of cells involved in the pathogenesis of the disease. EAE is used to study neuroinflammation, immune activation, CNS invasion by immune cells and autoimmune mediated neurodegeneration. It provides a better understanding of the MS pathogenesis and allows the development of treatment (Constantinescu et al., 2011).

While EAE is a CD4<sup>+</sup>T cells mediated pathology, MS has been demonstrated to be CD8<sup>+</sup>T cells mediated (Absinta et al., 2021; Machado-Santos et al., 2018). In rodents' EAE model, lesions are formed essentially in the spinal cord, leading to paralysis of the animal, but in MS lesions are essentially formed in the brain parenchyma (Lassmann & Bradl, 2017), in addition, EAE lesions are formed randomly (Table 1).

## **2. Virus-induced demyelination**

There is more and more evidence that viral infection could possibly result in the development of MS, such as EBV infection (Bjornevik et al., 2022; Hedström, 2023; Houen et al., 2020; W. H. Robinson & Steinman, 2022). Interestingly in mice, the Theiler's murine encephalomyelitis virus (TMEV) was reported to cause paralysis (Theiler, 1934).

TMEV is an enteric mouse pathogen described by Max Theiler in 1934 (Theiler, 1934). He reported that some mice were paralyzed and exhibited some histological features, he discovered it was caused by a virus and maintained the virus. When the virus is injected in the mouse brain it induces encephalitis. However, the virus doesn't affect there are some differences between the strain in disease induction and can also induce heart pathology (For difference in strain and disease induced by TMEV (look for review: (Omura et al., 2018)). For example, susceptible SJL mice are more likely to develop CNS pathology compared to C57BL/6 mice (Omura et al., 2018).

There are two subgroups of strains based on the ability to induce CNS pathology, the GDVII and the Theiler's original (TO). While GDVII strains are fatal to mice 1 to 2 weeks after the injection (Dal Canto & Lipton, 1977; Oleszak et al., 2004; Sato et al., 2014). The strains from the TO are the most used to study MS. TMEV will induce a biphasic disease; the first phase consists of an acute polioencephalomyelitis, followed by a chronic progressive demyelinating phase that develops within a month (Lipton, 1975). During the first phase, TMEV will induce axonal damage that will be demyelinated during the chronic phase, therefore TMEV model is a secondary demyelinating model (Tsunoda et al., 2003).

TMEV model is used to study viral infection and CNS autoimmune disease development, and axonal damage. However, TMEV induces CNS pathology only in mice and no other animals, and is a secondary demyelinating disease, whereas MS is primary

demyelinating disease. These aspects of TMEV-induced demyelination raise the question of its use to characterize MS (Table 1).

### **3. Toxin-induce demyelination**

Toxin-induced demyelination models do not attempt to imitate MS, they are used to study the demyelination/remyelination process (Blakemore & Franklin, 2008). In those models, the location of demyelination is known, and the demyelination process is separated from the remyelination process allowing us to study them separately. In these models the demyelination process is independent of immune activation. We are going to focus on the cuprizone and lysophosphatidylcholine models, but other gliotoxic agent can be used (such as ethidium bromide (Woodruff & Franklin, 1999)).

#### **a) Cuprizone**

Cuprizone is a copper chelator agent. When mice are fed with cuprizone the mature OL located in the corpus callosum die leading to demyelination and glial cells activation (microglia and astrocytes) (Matsushima & Morell, 2001).

Cuprizone can have different effects on different strains of mice (Yu et al., 2017). Yu and collaborator, showed that 3 weeks of cuprizone feeding on CD1 mice have less effect compared to C57BL/6, suggesting that cuprizone will not affect the mice the same way because of genetic differences. Short-term feeding (5 weeks) induces acute demyelination whereas long-term feeding (>10 weeks) induces chronic demyelination. In both cases, remyelination occurs after the removal of cuprizone from the food even if it is decreased during chronic cuprizone feeding (Matsushima & Morell, 2001).

However, in this model, there is not the involvement of an autoimmune process, making it difficult to assess the demyelination/remyelination process in an inflammatory/immune context close to MS (Table 1).



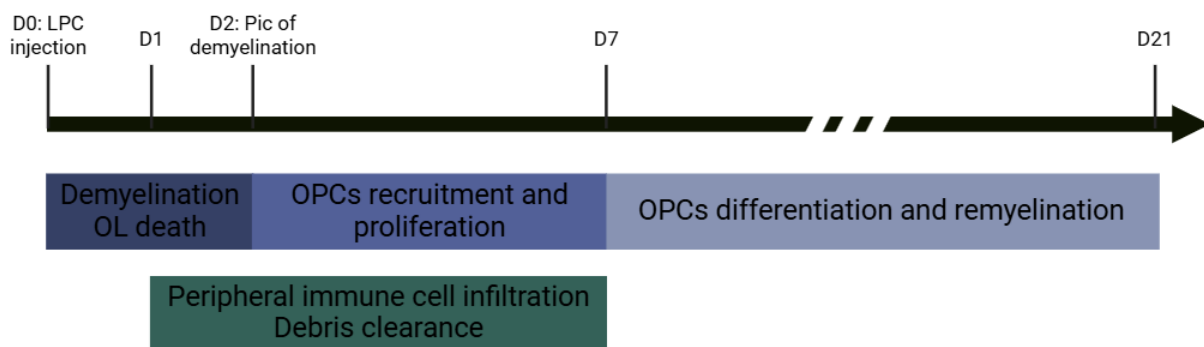
## **b) Lysophosphatidylcholine (LPC)**

Lysophosphatidylcholine (or lysolecithin (LPC)) is an endogenous phospholipid, and also a toxic compound used to induce demyelination. Firstly, the demyelination effect of LPC was described in CNS tissue *in vitro* (Birgbauer et al., 2004; PERIER, 1965). The first *in vivo* experiment was done in the sciatic nerve of adult mice (Hall & Gregson, 1971), and next in the spinal cord of mouse, cat, and cerebellar peduncle (Blakemore et al., 1977; Hall, 1972; Woodruff & Franklin, 1999).

For a long time, the mechanism of LPC induced demyelination was unknown. LPC integrates cellular membranes, this led to an increase in membranes permeability and lipid disruption. So, when LPC is injected in white matter abundant area it will integrate myelin membranes and disrupt myelin lipid leading to demyelination (Plemel et al., 2018). This demyelination led to the accumulation of myelin debris that will recruit peripheral immune cells and activate microglia for debris clearance (Procaccini et al., 2015). After the demyelination, the remyelination is observed, this process is spontaneous and extremely efficient (BLAKEMORE, 1978; Blakemore et al., 1977; Blakemore & Franklin, 2008; Jeffery & Blakemore, 1995).

LPC-induced demyelination is a well-documented model. At one day post-injection, peripheral macrophages, along with T and B cells, infiltrate the CNS parenchyma. By two days post-injection, demyelination is complete. OPCs, which are not affected by the LPC injection, are recruited to the lesion site and begin to proliferate. Remyelination starts after myelin debris is cleared, around 7 days post-injection, and the process is completed by 21 days post-injection (Figure 18). In the spinal cord, SC can also contribute to the remyelination process (El Behi\*, Sanson\* et al., 2017; Blakemore & Franklin, 2008).

One significant drawback of LPC-induced demyelination is the lack of involvement of an autoimmune process. However, the immune system plays a crucial role in the demyelination process in MS and in determining the success or failure of remyelination (Butovsky et al., 2006; Miron et al., 2013) (Table 1).



**Figure 18: Time course of LPC-induced demyelination/remyelination.** *LPC: lysophosphatidylcholine; OL: oligodendrocytes; OPCs: oligodendrocytes precursor cells.*

### **c) A model mimicking patient remyelination capacity heterogeneity**

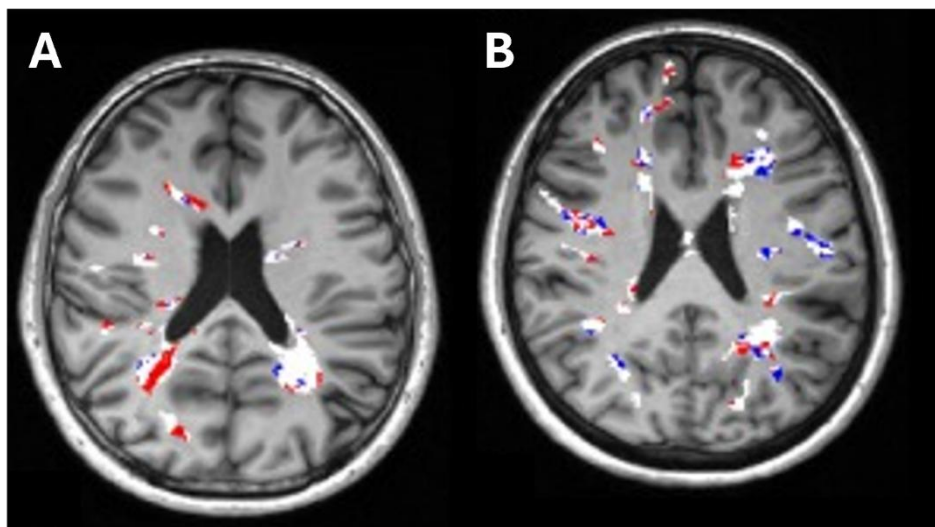
MS is characterized by significant heterogeneity in many of its aspects such as: its etiology, form of the disease, symptoms, lesions development, and repair capacity.

A variety of environmental factors, associated with epigenetic and genetic predisposition will increase the risks of developing MS, but there are no factors or association of factors that have been proven to induce MS. When MS is diagnosed the patients can follow different clinical courses. PwMS can either develop an RRMS course that will eventually convert into SPMS, or they will develop a PPMS course (Reich et al., 2018).

In addition, the heterogeneity in the etiology and clinical course, lesions formation, and evolution are highly heterogeneous among patients (Kuhlmann et al., 2017; Lucchinetti et al., 1999, 2000). PwMS will also experience a variety of symptoms, depending on lesion location. Among lesions heterogeneity can also be detected, four different patterns of active lesions have been identified on post-mortem analysis based on OL density, lesion edge, apoptosis, antibodies deposition, and myelin loss (Lucchinetti et al., 2000). The quantification of OL in MS post-mortem lesions also led to the categorization of five patterns (within one lesion). In 3 of the patterns OL recovering and remyelination were seen (those patterns are more present in RRMS and SPMS), in the last two patterns there is a lack of OL differentiation and remyelination was not seen, it was mostly seen in the center of the lesions analyzed (those patterns are more present in PPMS) (Lucchinetti et al., 1999)

There are more than ten therapies for PwMS, however there is heterogeneity in the response to those treatments. These responses depend on the activity of the diseases (Hauser & Cree, 2020), but also on patients' individuality (Buck & Hemmer, 2011). This heterogeneity in treatment responses could be caused by the inter-individual differences in the immune system (Pradeu, 2019).

There is a high variability in the remyelination capacity of MS patients (Bodini et al., 2016). Some PwMS have a high capacity to regenerate myelin (Figure 19B) whereas others have a low capacity of remyelination (Figure 19A). Interestingly, this capacity to regenerate myelin is correlated with disability, people who have a better capacity to remyelinate have less disabilities (Bodini et al., 2016). Enhancing remyelination in MS patients would have beneficial outcomes. However, it is not that simple, different mechanisms can lead to remyelination failure, such as diminution of OPCs pool, default in the recruitment of OPCs (Boyd et al., 2013), default of OPCs differentiation (Kuhlmann et al., 2008; Sim et al., 2002), and the inflammatory environment (El Behi\*, Sanson\* et al., 2017; Miron et al., 2013).

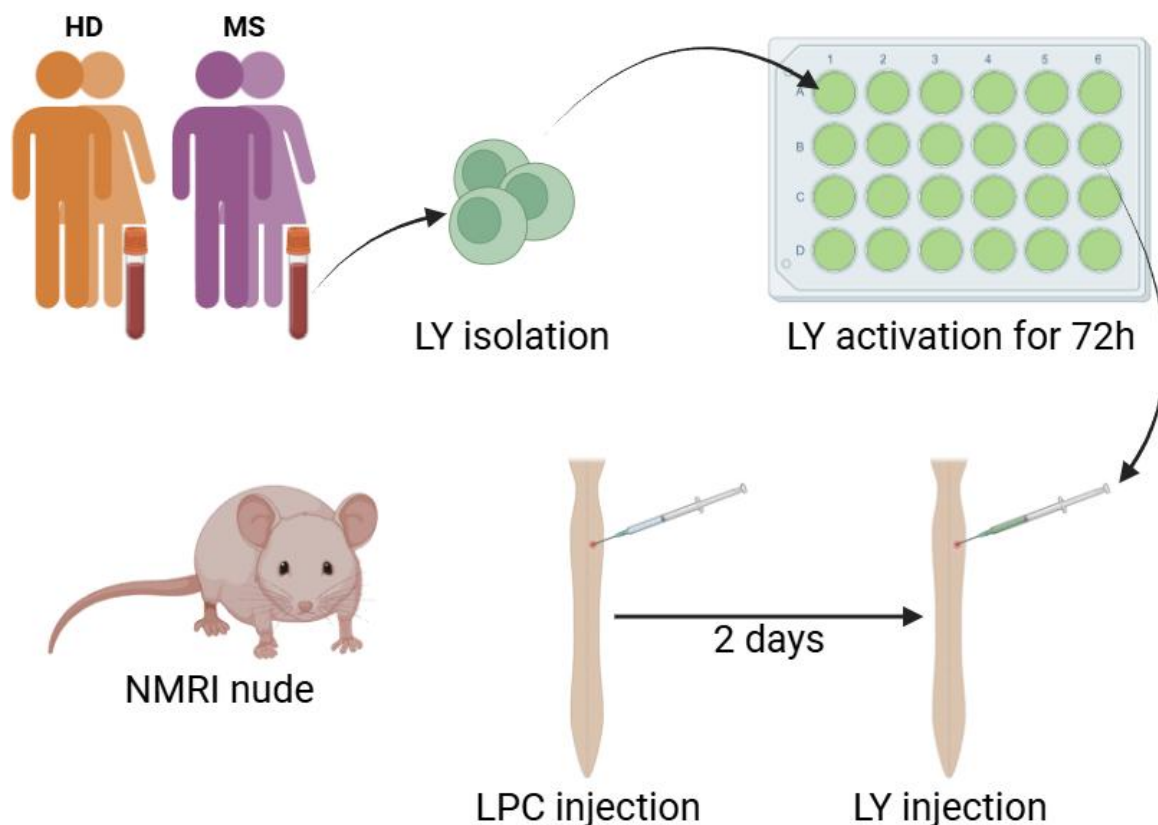


**Figure 19: Remyelination is heterogeneous.** Visualization of remyelination (in blue) and demyelination (in red) in two patients with MS. (A) Patients presenting low capacity of remyelination and (B) Patients with high capacity of remyelination. (Bodini et al., 2016)

In research, we strive for highly replicable models, often leading to homogenized results. However, as mentioned above, MS pathology is highly heterogeneous, which challenges the validity of using homogeneous models. To address the variability among MS

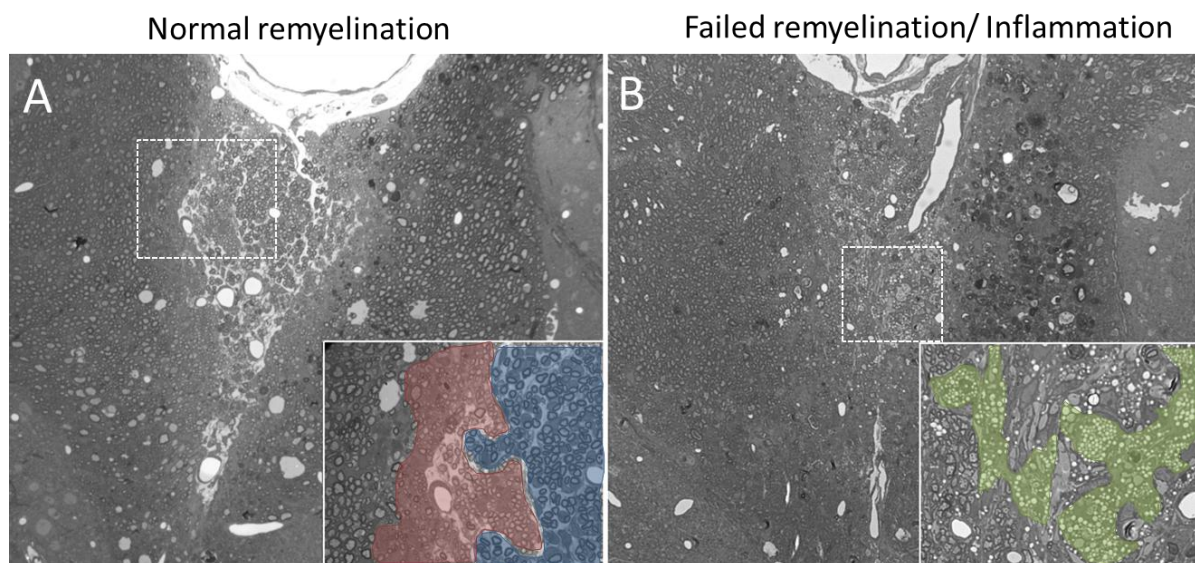
patients, new models must be developed. Consequently, a new model of demyelination and remyelination has been introduced (El Behi\*, Sanson\* et al., 2017).

To be able to study the demyelination and remyelination processes in a context closer to PwMS, our team developed a new humanized model (El Behi\*, Sanson\* et al., 2017). To obtain this new model, LPC-induced demyelination is associated with MS lymphocytes' graft in the site of the demyelinating lesion. Lymphocytes from MS patients and matched healthy donors (HD) are isolated from fresh blood and activated *in vitro* for 72 hours using anti-CD2/anti-CD3/anti-CD28 bead-conjugated antibodies. At the same time, Nude NMRI (RjOrl: NMRI-Foxn1<sup>nu</sup>/Foxn1<sup>nu</sup>) mice undergo injection of 1 $\mu$ L of 1% LPC in the dorsal horn of the spinal cord to induce demyelination. Two days after the LPC injection, activated lymphocytes are grafted into the lesion site (Figure 20) (El Behi\*, Sanson\* et al., 2017). Nude NMRI mice are used because they are athymic and lack an adaptive immune system, preventing the rejection of the grafted human lymphocytes.



**Figure 20: Experimental design of the humanized mice model of demyelination and remyelination.** Lymphocytes from HD and MS patients are isolated and activated *in vitro* using anti-CD2/anti-CD3/anti-CD28 bead-conjugated antibodies, while LPC induced demyelination is performed in Nude NMRI mice and two days after the demyelination lymphocytes are graft into the demyelinating lesion. Methods from (El Behi\*, Sanson\* et al., 2017). Created with Biorender.

Using this new model, they demonstrated that three weeks after the lymphocyte graft, lymphocytes from healthy donors (HD) and some MS patients (referred to as MS High) did not disrupt the remyelination process. Electron microscopy revealed remyelinated axons by oligodendrocytes (Figure 21A, in red) or by Schwann cells (Figure 21A, in blue). In contrast, lymphocytes from other MS patients (referred to as MS Low) impeded the remyelination process (Figure 21B), as electron microscopy showed macrophages with vacuoles (Figure 21B, in green). This decrease in remyelination in the MS Low group was attributed to a lack of OPC differentiation compared to the HD and MS High groups.



**Figure 21: Some MS patients lymphocytes impede the remyelination process.** Electron microscopy images of (A) normal remyelination as seen in HD and MS high grafted mice, we can observe remyelinated axons by OL (in red) or Schwann cells (in blue) or (B) impaired remyelination and inflammation as seen in MS low grafted mice, We can see the presence of macrophages (in green).

Next, they conducted in vitro experiments using the supernatant of activated human lymphocytes. They found that human lymphocytes did not directly affect OPCs; instead, the effect was mediated by the activation of microglial cells. Lymphocytes from MS patients induced a pro-inflammatory phenotype in mouse microglial cells. The supernatant from MS patients' lymphocytes activated the microglial cells, which in turn decreased OPC differentiation. This effect was mediated by the chemokine (C-C motif) ligand 19. (CCL19) (El Behi\*, Sanson\* et al., 2017).

To resume, this humanized model combines the demyelination/remyelination LPC model with the graft of human lymphocytes to assess these processes in a context similar to

MS. First this model recreates the heterogeneity of remyelination capacity seen in PwMS (Bodini et al., 2016). In addition, the discovery of CCL19 chemokine being involved in the blockage of OL differentiation proves that this model can also be used to assess potential therapeutic targets.

*Table 1: MS animal models, application and negative aspects.*

<b>Model</b>	<b>Application</b>	<b>Negative aspects</b>
<b>EAE</b>	Neuroinflammation and immune cell activation	Lesion appearance is random demyelination and remyelination happens at the same time
<b>TMEV</b>	Axonal damage, secondary induced demyelination	Secondary demyelination pathology, it is primary demyelination in MS
<b>Cuprizone</b>	Demyelination and remyelination	Do not involve auto-immunity remyelination is highly effective
<b>LPC</b>	Demyelination and remyelination	Do not involve auto-immunity remyelination is highly effective
<b>Humanized LPC</b>	Demyelination and remyelination	Human TCR, BCR and mouse MHC are not compatible

## **D. Silent progression**

Currently, there is no cure for MS. However, effective therapies for the relapsing phase of MS have been developed. Globally, the treatment for MS improved the quality of life of PwMS. Treatments are able to prevent relapses, new MRI lesions, but do not affect the progression of the disease. These treatments are called disease-modifying treatment (DMT) and target the peripheral inflammatory part of the disease (Hauser & Cree, 2020) (Figure 21). In this section, I will discuss DMTs and disease progression.

### **1. Disease modifying treatment (DMT)**

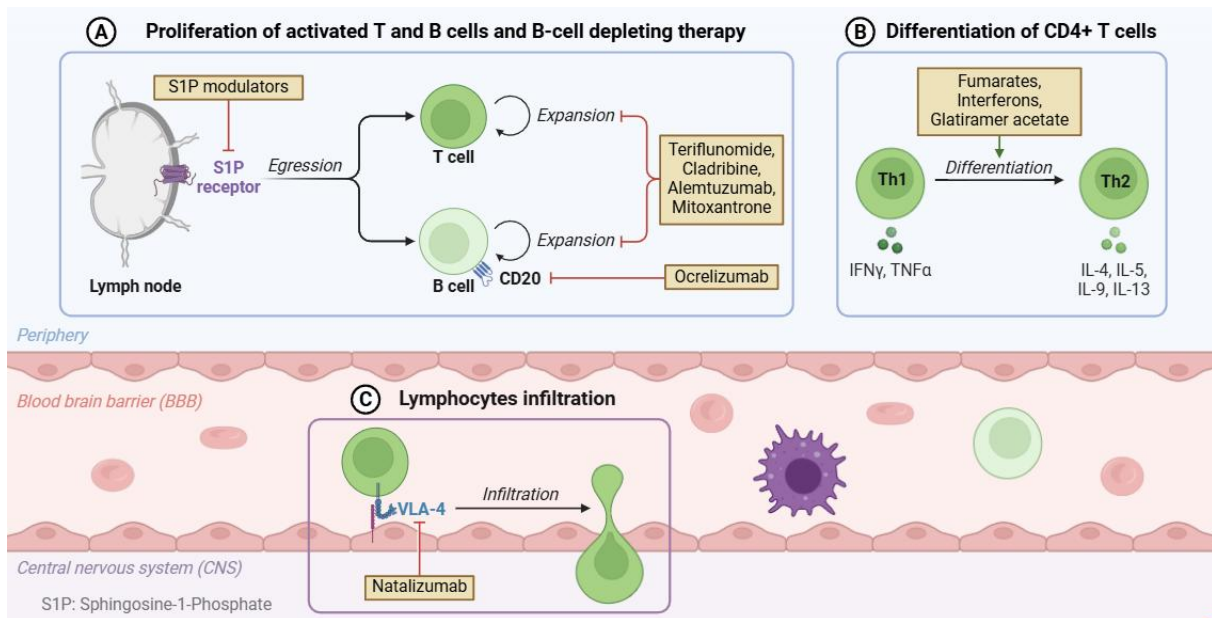
For RRMS, the first treatments developed in the early 1990s were injections of IFN- $\beta$  and the use of Glatiramer Acetate (GA). Both therapies have immunomodulatory and anti-inflammatory effects, offering similar efficacy, such as reducing the annual relapse rate, and they rarely cause serious side effects. Dimethyl fumarate and Dimethyl fumarate are other immunomodulatory drugs used in RRMS treatment; both are metabolized into mono-methyl fumarate, which activates nuclear factors with anti-inflammatory properties (Linker & Haghikia, 2016) (Figure 22B). Another treatment, Teriflunomide, works by inhibiting the proliferation of activated lymphocytes.

In the past years, the development of antibody-based therapy for the treatment of MS led to more efficient drugs. For example, Natalizumab is directed against the adhesion molecule  $\alpha$ 4-integrin that is used by T cells to cross the blood brain barrier (BBB) (Figure 22C). Therefore, activated T cells will not be able to enter the CNS, this led to efficient reduction of relapses rate and disability progression. Ocrelizumab is another antibody, directed against B cells, this treatment is also highly effective (Figure 22A).

However, with this highly effective treatment, more severe side effects are seen. For example, PwMS under Natalizumab treatment have the risk to develop progressive multifocal leukoencephalopathy (PML), PML is a rare condition and fatal in 30% of people.

For progressive MS, fewer treatments are available. In secondary progressive MS (SPMS), therapies include a sphingosine-1-phosphate (S1P) modulator for active disease (Figure 22A), as well as Ocrelizumab. For primary progressive MS (PPMS), Ocrelizumab is

the only approved treatment. This medication has been shown to improve both clinical and MRI markers of inflammation and degeneration and also to slow the progression of clinical disability in patients with PPMS (Montalban et al., 2017; Riederer, 2017)



**Figure 22: Disease modifying treatment of MS.** DMTs target the peripheral cells of the immune system. S1P modulators inhibit the egression of LY from lymph nodes. Teriflunomide, Cladribine, Alemtuzumab and Mitoxantrone stop the expansion of LY. Fumarates, Interferon and glatiramer acetate induce anti-inflammatory effects. Natalizumab inhibits the entry of LY in the brain parenchyma. Created with Biorender

DMTs in MS primarily target the peripheral immune system, specifically the immune cells involved in the disease's inflammatory processes. By modulating the activity of these immune cells, DMTs can reduce the occurrence of new inflammatory lesions in the CNS, which are visible on MRI scans. This reduction in new lesions correlates with a decrease in the frequency of clinical relapses, or flare-ups, which are periods when symptoms worsen, or new symptoms appear.

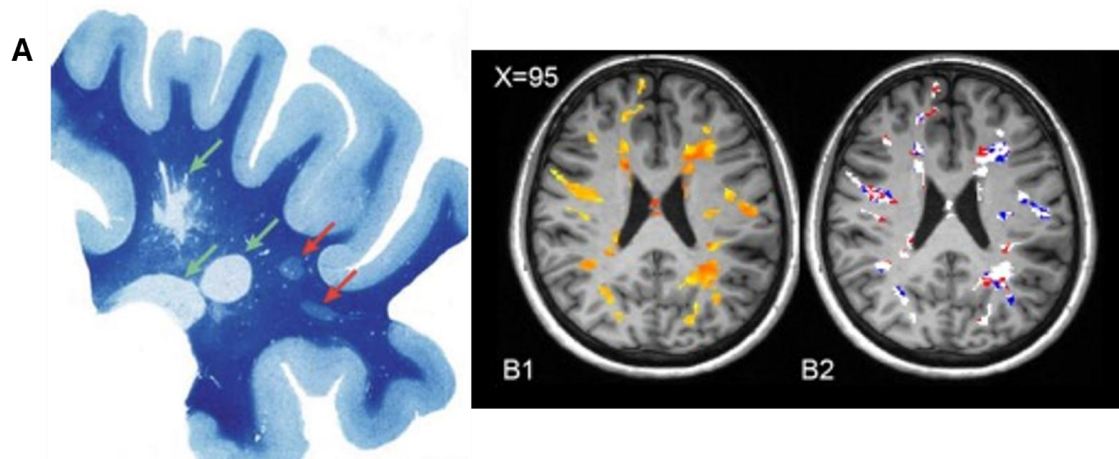
The prevention of new lesions and relapses through DMTs helps to manage the disease's activity and is referred to as relapse-associated worsening (RAW). RAW represents the part of MS progression that is directly linked to inflammatory events, such as relapses. By controlling RAW, DMTs not only improve immediate clinical outcomes but also contribute to a slower accumulation of neurological disability over time. However, it is important to note that while DMTs are effective in managing the inflammatory component of MS, they may not fully address the neurodegenerative aspects of the disease, which can continue to progress independently of relapse activity.



## 2. *In vivo* imaging of lesions and activity

Post-mortem analysis have been greatly useful to characterize the different types of MS lesions, however, these histological analyses are a snapshot at one moment. MRI imaging is a crucial tool in diagnosing and monitoring MS. It allows for the visualization of MS lesions in the brain and spinal cord, which appear as areas of inflammation, demyelination, and neurodegeneration. T2-weighted MRI scans typically reveal these lesions as hyperintense areas, while T1-weighted scans can show hypointense "black holes," indicating more severe tissue damage. Gadolinium enhancement on MRI can help differentiate between active and inactive lesions, with active lesions showing contrast enhancement due to blood-brain barrier disruption. More recently, to assess the evolution of lesions, new MRI technologies are now able to detect smoldering lesions *in vivo* in PwMS, and therefore follow their evolution as well as inflammation *in vivo*.

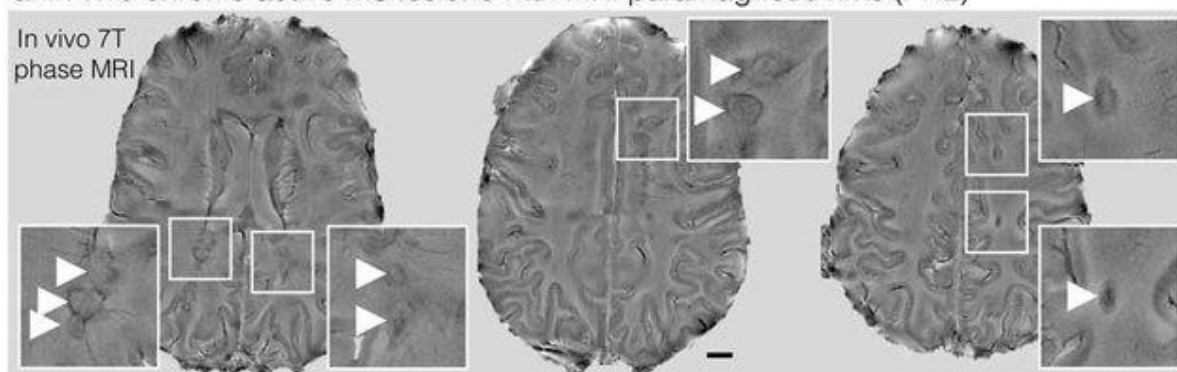
By combining MRI and positron emission tomography (PET) imaging, using a myelin marker, the Pittsburgh compound B ([<sup>11</sup>C] PiB), remyelination that was previously assess through luxol fast blue satining (Figure 23A), can now be visualized *in vivo* in humans (Figure 23B). Two PET-MRI scans are taken at two different times and the images are compared. The first image (Figure 23B1) is the baseline showing the extent of demyelination, and the second images (taken three months after the baseline image) show the area of demyelination (Figure 23B2; red) or remyelination (Figure 23B2; blue). This technique allows the study of the dynamic of demyelination and remyelination. Interestingly, there is a negative correlation between remyelination capacity and motor disability progression (established with the EDSS) (Bodini et al., 2016), these prove in humans that remyelination is also beneficial (Bodini et al., 2016).



**Figure 23: Remyelination in human.** (A) Post-mortem myelin observation. Developmental myelin appears dark blue. Green arrows: White areas, corresponding to demyelinated areas; red arrows: light blue areas corresponding to newly-formed myelin, and therefore less dense. from: (Adams 1989). (B) In vivo imaging of myelin. B1: Baseline myelin; B2: Longitudinal follow-up of the same patient 3 months after acquisition of B1. Demyelinated areas (in red) and remyelinated areas (in blue) are shown. From (Bodini et al., 2016).

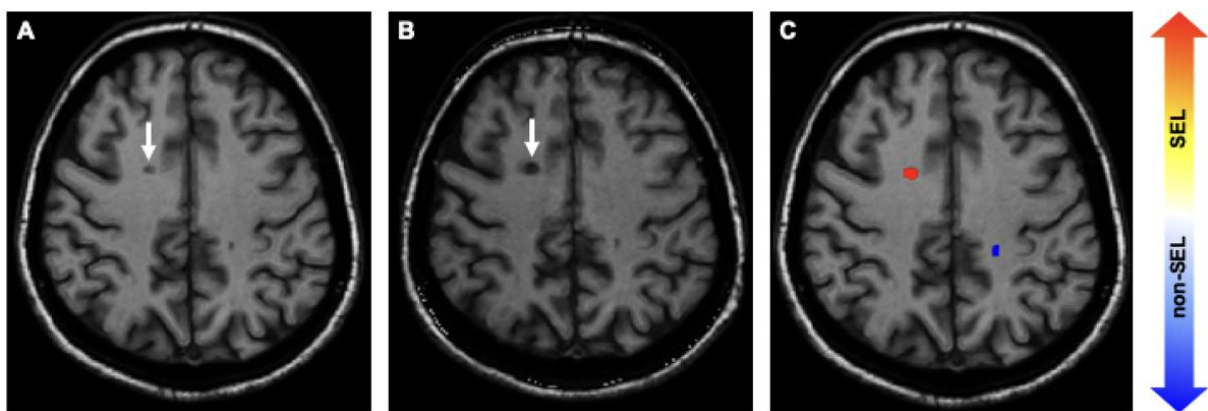
Two markers of smoldering lesions have been described, the first is the paramagnetic rim lesion (PRL) (Figure 24) (Absinta et al., 2019; Lou et al., 2021; Maggi et al., 2020; Martire et al., 2022). Using susceptibility-based MRI, some lesions have paramagnetic rims (Figure 24; lesions magnified view in insets arrows). PRL has been demonstrated to be specific to smoldering lesions in PwMS (Absinta et al., 2021; Maggi et al., 2020). This paramagnetic rim is due to an accumulation of iron-loaded microglia in the border of smoldering lesions (Absinta et al., 2021).

a. In vivo chronic active MS lesions with MRI paramagnetic rims (PRL)



**Figure 24: PRL detection using 7T MRI.** Susceptibility-based axial MRI at three levels showing the chronic active MS lesions with paramagnetic rims (magnified view in the insets, arrows) Scale bar = 10 mm. From: (Absinta et al., 2021).

The second MRI marker for identifying smoldering lesions is the slowly expanding lesion (SEL), which is detected through longitudinal deformation analysis of routinely acquired volumetric MRI scans in people with multiple sclerosis (PwMS) (Figure 25) (Calvi, Carrasco, et al., 2022; Calvi, Tur, et al., 2022; Preziosa et al., 2022). A baseline image is done (Figure 25A) and compared to an image done after (Figure 25B; 9 years between the two images), Deformation analysis is done to identify SEL (Figure 25C; SEL are shown in red, and non-SEL in blue). SELs have been associated with smoldering lesions (Dal-Bianco et al., 2017; Elliott et al., 2019). Notably, the presence of SELs can predict the transition from relapsing-remitting MS (RRMS) to secondary progressive MS (SPMS) (Preziosa et al., 2022).

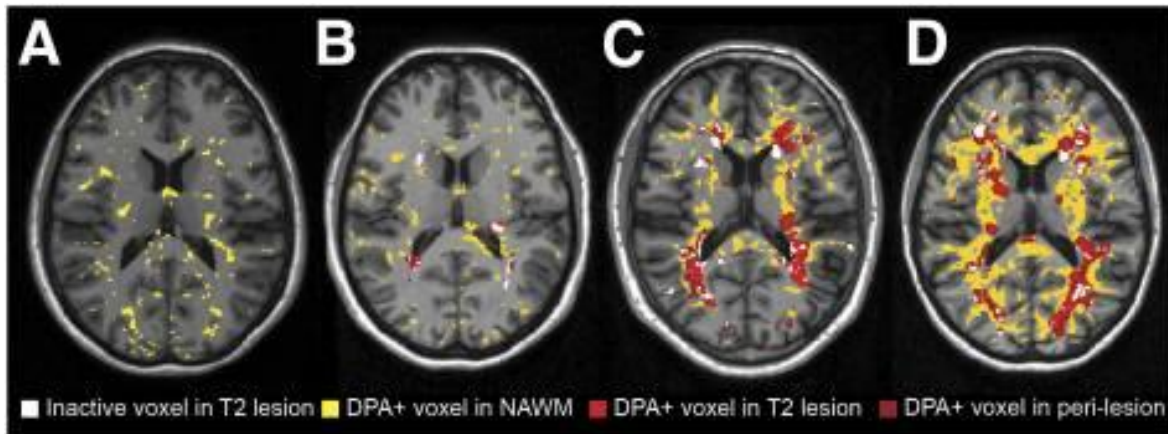


**Figure 25: SEL detection in PwMS (9 years follow-up).** (A) Baseline T1-weighted MRI, arrow represent black-hole (B) Follow-up MRI, arrow represent black-hole (C) SEL identification of the black-hole (Calvi, Tur, et al., 2022).

Both markers of smoldering lesions, PRL and SEL, are associated with disability in PwMS (Absinta et al., 2019; Blindenbacher et al., 2020; Calvi, Carrasco, et al., 2022).

The translocator protein (TSPO) is used as a PET marker to assess the extent of inflammation within the CNS (Figure 26). TSPO protein level is upregulated in activated glial cells, especially in microglia during CNS injury or disorders (E. J. Kim & Yu, 2015). PET-MRI scans using TSPO marker, show innate immune cell activation in healthy people (Figure 26A) and different activation in PwMS (Figure 26B-D). They can have a sparse (Figure 26B), intermediate (Figure 26C) or extensive (Figure 26D) immune cells activation (Bodini et al., 2015). They can also identify immune cells activation location within the lesion (Figure 26; in light red), in perilesional areas (Figure 26; in dark red), and in normal appearing white matter (Figure 26; yellow) (Bodini et al., 2020). When they looked at black hole lesions, a high

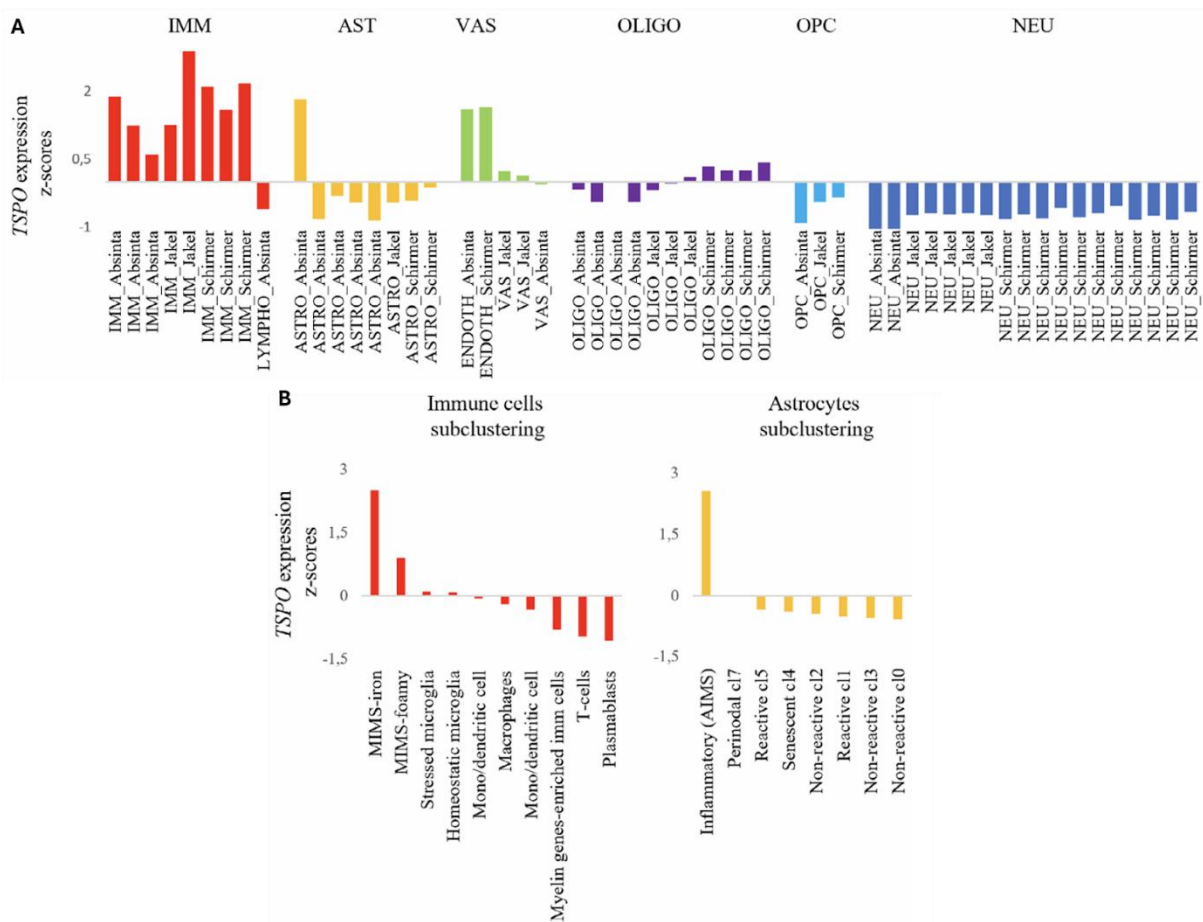
proportion of these lesions had an immune activation component, either homogeneously through the lesion or at the rim of the lesion (smoldering lesions). These results suggest that most lesions will develop a chronic inflammatory component, promoting neurodegeneration and clinical progression (Hamzaoui et al., 2023).



**Figure 26: PET-MRI scans of immune cell activation.** (A) Innate immune cells activation in healthy people (B) Sparse innate immune cell activation in MS patients (C) Intermediate innate immune cell activation in MS patients (D) Extensive innate immune cell activation in MS patients. Lesional inactive voxels in patients are represented in white, whereas voxels characterized by significant activation of innate immune cells are displayed in yellow in normal appearing white matter, dark red in perilesional areas and in light red inside T2 lesions (Bodini et al., 2015). From (Bodini et al., 2020)

In the study mentioned above, published in 2023, they also looked for TSPO expression within different CNS cell populations. To do so, they used different SnRNA-seq datasets of human CNS (from: Absinta et al., 2021; Jäkel et al., 2019; Schirmer et al., 2019) (Figure 27A). Interestingly they saw that TSPO is mainly expressed by immune cells and astrocytes (Figure 26A-B), principally expressed by MIMS-iron and astrocytes inflamed in MS (AIMS) (Figure 27B) (Hamzaoui et al., 2023). Another study also identifies microglia and astrocytes as the main cells expressing TSPO (Nutma et al., 2019).

There is more and more evidence suggesting that macrophages and microglia have different roles and act at different times in the disease progression (Faissner et al., 2019; Healy et al., 2022; Woo et al., 2024). For example, a study has shown that infiltrating macrophages communicate with microglia to suppress their inflammation, in the context of spinal cord injury when this crosstalk is suppressed there is a worsening of symptoms (Greenhalgh et al., 2018). In addition, in another study they showed that microglia prevent the spreading of infiltrating macrophages (Plemel et al., 2020).



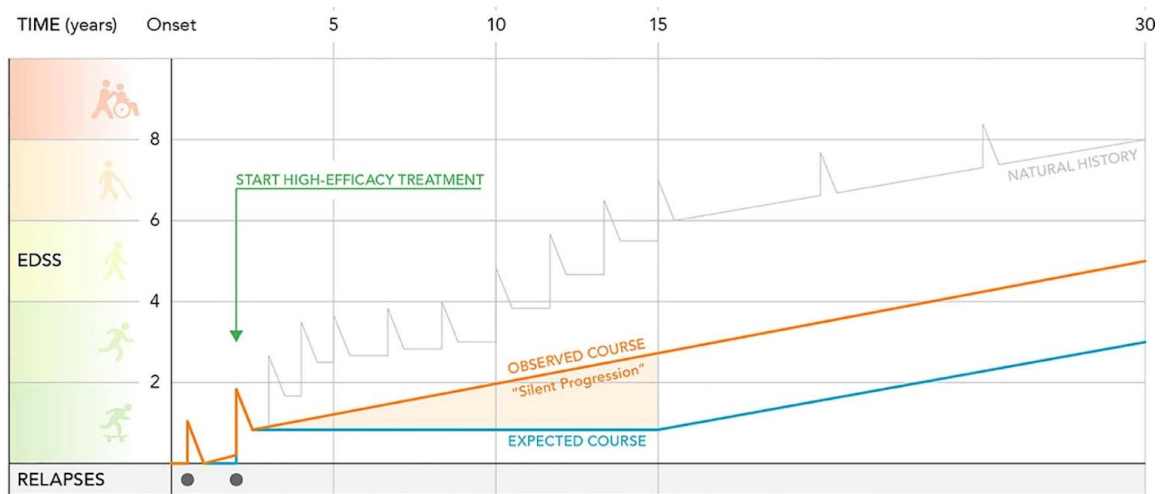
**Figure 27: TSPO expression in different ScRNA-seq.** showed that microglia is the main cell type expressing TSPO. (A) TSPO expression in the dataset of Absinta et al., 2021; Jäkel et al., 2019 and Schirmer et al., 2019. (B) Focus on the immune cells of Absinta et al., 2021. From: (Hamzaoui et al., 2023)

Now, with the new imaging techniques we are able to image *in vivo* in humans the smoldering lesions, and CNS inflammation and to follow their evolution. These new technologies allowed a better understanding of disease progression.

### 3. Disability accumulation

With the introduction of high-efficacy therapies, it was anticipated that PwMS would not experience disability progression (Figure 28; blue line: expected course). However, managing relapse-associated worsening (RAW) alone has proven insufficient to halt disease progression and the accumulation of disability. Even with treatment, patients have continued to experience silent progression (Figure 28; orange line: observed course) (Cree et al., 2019; Hauser & Cree, 2020; Kappos et al., 2020). This silent progression is known as progression independent of relapse activity (PIRA). PIRA is a significant factor in disease progression for

PwMS, even during the early stages of MS pathology (Figure 28) (Cree et al., 2019; Hauser & Cree, 2020; Kappos et al., 2020).



**Figure 28: PIRA in MS.** Evolution of the EDSS through time. In blue: the expected evolution of disability, in orange: the observed evolution of disability, in green: the beginning of high-efficacy treatment. From: (Hauser & Cree, 2020).

PIRA are associated with non-enhancing lesions and black-hole detected on MRI scans (Woo et al., 2024). As mentioned above, the majority of black hole lesions have an inflammatory component (either homogeneous inflammatory or in the rim) (Hamzaoui et al., 2023). Even if there is no more infiltration by peripheral immune cells, there is inflammation in CNS. This inflammation is due to the activation of microglial cells. Activated microglial cells, within the CNS even in normal appearing white matter (Hamzaoui et al., 2023), is called widespread inflammation.

Taken together these data suggest that a first event will trigger infiltration of peripheral immune cells, inducing demyelination, appearance of a contrast-enhancing lesion, and activation of microglial cells. Once the acute inflammatory phase is over, the lesion can either remyelinate, or the lesions can turn into black hole (not remyelinated), but microglia are still activated (Hamzaoui et al., 2023) and some lymphocytes will stay in the CNS form perivascular and meningeal cuffs leading to compartmentalized inflammation (Machado-Santos et al., 2018; Magliozzi et al., 2023). This compartmentalized inflammation and widespread activated microglia are the cause of neurodegeneration (Woo et al., 2024). However, no current therapies target this part of the disease, and there are no models to study this phase. Therefore, developing new models mimicking PIRA, or part of PIRA are of high importance.

## **E. A much-needed model**

The classical model used to study demyelination/remyelination, such as the injection of various gliotoxic agents such as lysolecithin, ethidium bromide and bacterial endotoxins in central nervous system white matter tracts, improved our understanding of the different steps leading to remyelination ((Blakemore & Franklin, 2008). Nonetheless, because of the high regenerative potential observed in these models, there is a lack of chronic lesion model mimicking important features of MS also, none of the animal models recreates the heterogeneity of MS disease (Procaccini et al., 2015). With growing evidence that these chronic lesions are associated with PIRA, and a more severe disease, it is important to better understand how they are formed and developed. However, in the current model that allows us to study MS, none allow us the study of this type of lesion specifically. Therefore, it is important to develop a new model that will allow this kind of study.

## Aim of the project

We undertook an original approach to examine the neuro-oligo-immune interactions leading to remyelination failure or success in conditions closer to human pathological conditions. We established a new *in vivo* experimental paradigm to investigate how human MS lymphocytes influence remyelination (El Behi\*, Sanson \*et al., 2017). We show that 3 weeks after their grafting MS patient lymphocytes impede remyelination *in vivo* when grafted in demyelinated lesions of Nude mouse spinal cord. By deciphering the mechanism of remyelination failure *in vitro*, we found that MS lymphocytes induce a higher pro-inflammatory activation of mouse microglial cells compared to HD lymphocytes, this process leading to an early blocking of OPC differentiation. Moreover, we found that patients lymphocytes influenced differentially the remyelination process, some showing a beneficial (high patients) and some a deleterious effect (low patients) on the repair process, a pattern mimicking what is observed in MS patients (El Behi\*, Sanson\* et al., 2017).

The aim of my thesis project is to investigate the long-term effects of MS lymphocytes graft in the demyelinating lesion of nude mice spinal cord. To evaluate the relevance of our new humanized animal model as a good model of chronic active lesion, we have 3 aims:

1. Firstly, evaluate how the evolution of the lesion influences different sensory motor skills and somatosensory conduction, by performing different behavioral tests and evoked somatosensory potential.
2. Secondly, evaluate the lesion volume and the extent of inflammation 3 months after the lymphocytes grafting by electron microscopy and immunohistochemistry.
3. Thirdly, establish the multicellular interaction driving remyelination or lesion development by single cell RNA sequencing.

This will help us decipher how do we transition from a focal demyelination to a widespread chronic demyelinated lesion.





## **RESULTS PART I**

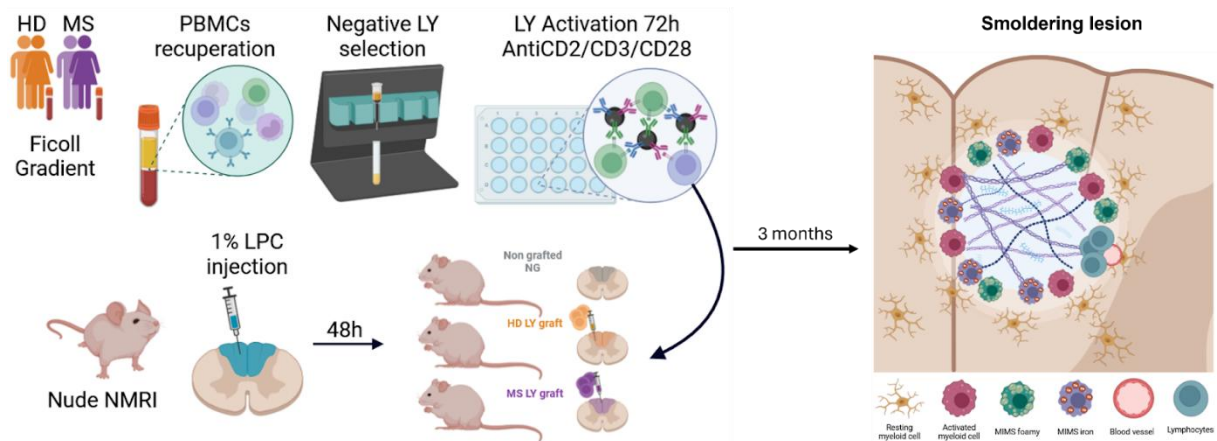


# Multiple sclerosis patient's lymphocytes induce smoldering lesions in the mouse spinal cord.

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## Graphical abstract



## Abbreviation:

CNS: Central Nervous System; EAE: Experimental Autoimmune Encephalomyelitis; HD: Healthy donor; LPC: Lysophosphatidylcholine; LY: Lymphocytes; MS: Multiple Sclerosis; NG: Non-grafted; OL: Oligodendrocytes; OPC: Oligodendrocytes Precursors Cell; PBMC: Peripheral Blood Mononuclear cell; PIRA: Progression Independent of Relapse Activity; PPMS: Primary Progressive Multiple Sclerosis; RAW: Relapse Associated Worsening; RRMS: Relapsing Remitting Multiple Sclerosis; SPMS: Secondary Progressive Multiple Sclerosis.

## **Abstract**

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS). The disease is triggered by macrophages and lymphocytes (LY) invasion of the CNS that in concert with CNS microglial cells, destroy myelin sheaths and oligodendrocytes (OL). In the pathophysiology of MS, smoldering lesions—marked by an inactive center, poor remyelination, and a border rim of activated microglia—are a hallmark of severe disease and pose a significant risk for greater disability in patients.

To understand the generation and the development of these lesions, we establish a new humanized mouse model of MS lesion by grafting MS patients or healthy donors LY in a demyelinated lesion of the nude mouse spinal cord. Previously, we showed that MS patients LY impeded remyelination 3 weeks after the lesion/graft. Here, we evaluated the long-term effect three months after LY grafting. We observed, only in mice grafted with MS patient LY that human LY form perivascular cuffs and contact both the vascular wall and mouse immune cells. Interestingly, LY forming perivascular cuff is a feature of MS smoldering lesions. MS LY grafts impaired OL differentiation and remyelination and induced a persistent microglial activation causing a glial scar in the lesion center and persistent demyelination at the lesion rim corresponding to the inactive center and active border of smoldering lesions. Behavioral analysis showed ongoing deficits in mice grafted with MS LY, whereas the control groups (both non grafted mice and mice grafted with healthy donor LY) exhibited improvements. We also measured the somatosensory evoked potential (SSEP), since the ascending sensory fibers are located in the lesion area, to assess the physiological changes due to the persistence (or not) of the lesion. SSEP indicated slower speeds in MS LY grafted mice, correlating with fewer mature OL. These results suggest that we developed a unique model mimicking several aspects of smoldering like lesions.

This novel humanized mouse model of focal chronic demyelination and inflammation helps us comprehend how the shift from a focal demyelination to widespread demyelination and inflammation occurs.

## Introduction

Multiple sclerosis (MS) is an autoimmune, neuro-inflammatory, and demyelinating disease of the central nervous system (CNS) (Compston & Coles, 2002) with different disease types. The most common form (85%), Relapsing/Remitting MS (RRMS) is characterized by relapse-associated worsening (RAW) with periods of neurologic symptoms followed by partial or complete recovery, and possible progression independent of relapse activity (PIRA) (Woo et al., 2024). The Secondary Progressive MS (SPMS), which can occur in RRMS patients and involves accumulating disability without remission phases, including both RAW and PIRA; and Primary Progressive MS (PPMS), which accounts for 15% of cases and features steady disability accumulation from onset, primarily driven by PIRA.

Disease progression and symptomatology has been associated with different cellular and molecular events including waves of inflammatory demyelinating episodes followed by remyelination attempts that would lead to the formation of lesions. The study of MS postmortem tissue led to the classification of different lesion type: (1) active lesions that present ongoing inflammation and demyelination, (2) inactive lesions that are demyelinated, with a glial scar and without active inflammation, and (3) “smoldering lesion” or mixed active/inactive lesion (Lucchinetti et al., 2000; Kuhlmann et al., 2017). These lesions are characterized by an inactive center (demyelinated, with glial scar and without active inflammation) and an active rim (ongoing inflammation and demyelination), with the presence of either iron-loaded or myelin-loaded microglia (Absinta et al., 2021). Active lesions are more detected in the early phases of RRMS and SPMS while smoldering lesions are more frequent in SPMS and PPMS (Frischer et al., 2015; Kuhlmann et al., 2017; Luchetti et al., 2018). The number of smoldering lesions is positively correlated with severity scores and lesion load, while being negatively correlated with the proportion of remyelinated lesions (Luchetti et al., 2018; Absinta et al., 2019). Furthermore, smoldering lesions are a hallmark of patients with severe disease and at risks of having higher disability (Absinta et al., 2019).

Classically, experimental autoimmune encephalomyelitis (EAE) is used as an animal model of MS (Procaccini et al., 2015). It helps decipher the autoimmune component of MS and it mimics the cyclical nature of RAW, characterized by episodes of neurological decline followed by periods of partial or complete recovery. This is crucial for understanding the triggers and recovery processes. Other animal models such as the injection of various gliotoxic

agents such as lysolecithin, ethidium bromide and bacterial endotoxins in CNS white matter tracts, as well as cuprizone ingestion, improved our understanding of the different steps leading to remyelination (Blakemore and Franklin, 2008). However few models recapitulate continuous and irreversible progression of PIRA, which is essential for understanding how and why the disease shifts from a relapsing-remitting phase to a progressive one. Indeed, the initial infiltration of peripheral immune cells into the CNS sets off a chain reaction involving microglial cells that impedes remyelination and perpetuates inflammation and demyelination. This central activation of glial cells is crucial to the gradual expansion of smoldering-like lesions, making it essential to understand their development.

To address this challenging question, we established a new *in vivo* experimental paradigm to investigate how human MS lymphocytes (LY) influence remyelination and demyelination. We demonstrate that three weeks after grafting, LY from MS patients hinders remyelination *in vivo* when grafted into demyelinated lesions of the Nude mouse spinal cord. By deciphering the mechanism of remyelination failure *in vitro*, we found that MS LY induce a higher pro-inflammatory activation of mouse microglial cells compared to healthy donor (HD) LY, this process leading to an early blocking of OPC differentiation. Moreover, we found that patients LY influenced differentially the remyelination process, some showing a beneficial (High Patients) and some a deleterious effect (Low patients) on the repair process, a pattern mimicking the heterogeneity of remyelination capacity observed in MS patients (El Behi\*, Sanson\* et al., 2017).

In this study, we analyze the long-term effects of LY graft in demyelinating lesions of nude mice spinal cord. Our results show that mice grafted with MS patients LY present mixed active-inactive lesions that share several aspects of smoldering like lesions including: perivascular cuffs of human LY, an inactive scarred tissue at its center, failed remyelination and presence at its border of myelin- or iron loaded microglial cells. The presence of these lesions in mice grafted with MS patients LY are correlated to behavioral deficits and slower axonal conduction reflecting the chronic nature of the lesion. Interestingly, we also replicate a range of effects depending on the MS patient from whom the LY were isolated, mirroring the heterogeneity observed in patients.

## **Material and methods**

### **Standard Protocol Approvals, Registrations, and Patient Consents**

Approval for human blood sampling was obtained from the French Ministry of research (NCT03369106) and written informed consent was obtained from each study participant. All animal protocols are performed in accordance with the guidelines published in the National Institute of Health Guide for the Care and Use of Laboratory Animals, EU regulations (agreement n°A75-1319) and the local “Charles Darwin” ethics committee.

### **Patients and healthy donors**

A total of 11 healthy donor (HD) and 21 MS patients paired by age and sex are included in the study (see table 1 and suppl table 1 for details). All patients fulfill MS diagnostic criteria and individuals with any other inflammatory or neurological disorders are excluded from the study.

### **Collection and activation of patients and HD PBMCs and LY**

Blood (80 ml) is collected in acid citrate dextrose tubes. Peripheral blood mononuclear cells (PBMC) are extracted on a Ficoll gradient plus (GE Healthcare Life Sciences). Cells were washed in PBS (2x10 min at 1500 rpm) and RPMI 1640 + 10% fetal bovine serum (FBS) (5 min at 1500 rpm) (all products from ThermoFisher). CD14<sup>-</sup> lymphocytes are isolated by magnetic beads sorting and activated using the T cell Activation/Expansion kit, human (Miltenyi; 130-091-441), consisting in anti-CD2/anti-CD3/anti-CD28 bead-conjugated antibodies (at a density of 1 bead for 2 cells) in RPMI/10%SVF medium. After 72h of activation, cells are collected for the graft in RPMI medium.

### **LPC injection and LY graft**

Eight weeks old female Nude (RjOrl: NMRI-Foxn1<sup>nu</sup>/Foxn1<sup>nu</sup>) mice are purchased from Janvier (France). Focal demyelination is performed as previously described (El Behi\*, Sanson\* et al., 2017). Briefly, Nude mice were anesthetized with a mix of ketamine (100mg/ml; Imalgene 1000) and Xylazine (10mg/ml; Paxman). A stereotaxic injection of 1μL of lysophosphatidylcholine (LPC) 1% (Sigma) is performed in the dorsal horn of the spinal cord (between T9-T10). After 48h, 1μL of 100 000 activated LY are grafted in the lesion site. Ten mice are grafted per individual, and 10 mice per batch for the non grafted group (NG) undergo LPC injection only.



## **Behavioral testing**

In order to assess the effect of LY grafting, we implemented three behavioral tests. Prior to testing, the animals undergo a familiarization phase of three trials/day for five days. This pre-surgery test established a baseline performance for comparison with post-surgery results. The mice are then retested with three trials for each test 1 week, 1 month, 2 months and 3 months after surgery.

The notched beam test allows us to assess mice motor coordination. The apparatus corresponds to a wooden beam one meter long and 8 mm wide, with a starting and ending plate of 80 mm and square slots: 17 mm deep, 17 mm wide and 17 mm long, spaced every 17 mm and 30cm high. For each test, the number of errors and the time of crossing are recorded.

The Grip test is a force measurement instrument for studying neuromuscular functions. The device allows the determination of the gram force of the front legs (using a bar) and the four legs (using a grid) of the mice. For each trial, the force in grams of the front legs and the four legs are recorded.

The rotarod allows us to evaluate sensorimotor coordination. Mice are placed on a rotating bar (2.5 cm Ø) in the direction of rotation. The speed of rotation is initially set at 4 rpm and then gradually accelerated over 5 minutes until 40 rpm is reached. For each trial, the fall latency, and the speed at the time of the fall are recorded.

## **Somatosensory evoked potential (SSEP)**

Since the lesion affects the dorsal part of the spinal cord, where the ascending somatosensory fibers are located, somatosensory evoked potential (SSEP) testing is performed three months post-grafting, prior to sacrificing the mice. Once the animal is secured on the pre-disinfected frame, electrodes will be attached. Stimuli (pulse 200 µs, frequency 3.5 Hz) will be applied to the middle of the tibial nerve using single-use electrodes positioned on the hind paws. The SSEPs will be recorded from electrodes placed on the somatosensory cortex of the mouse. Additionally, a ground electrode will be placed subcutaneously above the cervical spinal cord (monopolar needle electrode Spesmedica, Ref: MN3512P150).

## **Perfusion and tissue processing**

Mice are anesthetized with a mix of ketamine (100mg/ml; Imalgene 1000) and Xylazine (10mg/ml; Paxman). Euthanasia is performed after the assessment of SSEP with an overdose of pentobarbital (euthasol (150mg/kg). For immunohistochemistry (IHC), mice are perfused intracardially with a solution of PBS 4% paraformaldehyde (PFA), for electron microscopy (EM) mice are perfused with 4% PFA, 5% glutaraldehyde. Spinal cords were removed and post-fixed for 1h for IHC and EM and 2-3h for clearing. For IHC, tissues were cryoprotected overnight in a 20% sucrose solution, frozen in cooled isopentane and cryosected longitudinally (12- $\mu$ m slices).

For clearing spinal cord are kept in PBS with sodium azide to avoid bacterial contamination until the clearing process.

For EM, spinal cords were cut into 1 mm sections and contrasted first with 2% osmium tetroxide and with 5% uranyl acetate before several steps of dehydration. Spinal cord sections were embedded in Epon<sup>TM</sup> before being cut into semithin and ultrathin sections for analysis.

For single cell analysis, mice are euthanized by CO<sub>2</sub>. Then the lesioned area of the spinal cord (1cm) is extracted, and mechanical dissociation and enzymatic dissociation (Papain at 37°C) are performed. Cells are collected after a Percoll gradient and processed with the 10x genomics technology.

### **Immunohistochemistry and clearing**

Tissues are permeabilized, saturated and stained with antibodies described in supplementary table 2. All primary antibodies are incubated overnight at 4°C, secondary antibodies 1h at room temperature. Nuclei are counterstained with Hoechst (Sigma, 33342).

For clarification, tissues are dehydrated, bleached and rehydrated before staining. Tissues are incubated in primary antibody for 5 days at 37°C, wash multiple time a day and incubate with secondary antibody for 5 days at 37°C. Tissues are cleared in Rapiclear<sup>®</sup> (solution overnight at 4°C, nuclei counterstained with Dapi dilactate (Sigma, D9564) and spinal cord are imaged with a confocal microscope ( ).

### **Single cell RNA sequencing analysis**

XenoCell tool (v1.0) was used to classify reads barcodes between graft-specific (hg38) and host-specific (mm39) cells. Less than 30K barcodes was identified as graft for samples MS

and CTR (21K and 29K, respectively). Nearly 1M barcodes was assigned to host for samples MS and CTR (948K and 1.3M, respectively). The Cell Ranger Single-cell Software suite (6.1.2) was used to process the data. Count function was used on each GEM well that was demultiplexed by mkfastq to generate gene-cell matrices. FASTQ files are aligned on the mm39 reference genome. Then, filtered\_feature\_bc\_matrix output was loaded into Seurat bioconductor package v4.3.0 to filter the datasets and identify cell types using R v4.2.2. Genes expressed in at least 2 cells and cells with at least 50 features were retained for further analysis. To remove likely dead or multiplet cells from downstream analyses, cells were discarded when they had less than 100 UMIs (Unique Molecular Identifiers), greater than 40 000 UMIs, or expressed over 8% mitochondrial genes. All samples were merged together for downstream analysis. As no batch effects were observed among the four samples, no integration step was performed. Gene expression matrix was normalized using the negative binomial regression method implemented in the Seurat NormalizeData() function, via selection of the top 2000 variable genes and regressed out the mitochondrion expression percentage. The final dataset was composed of 17515 genes and 2113 cells. To cluster cells, we computed a Principal Components Analysis (PCA) on scaled variable genes, as determined above, using Seurat's RunPCA function, and visualized it by computing a Uniform Manifold Approximation and Projection (UMAP) using Seurat's RunUMAP function on the top 31 PCs. We also computed the k-nearest neighbor graph on the top 31 PCs, using Seurat's FindNeighbors function with default parameters, and in turn used Seurat's FindClusters function with varying resolution values with the Louvain algorithm. We chose a final value of 0.8 for the resolution parameter at this stage of clustering. Clusters were assigned preliminary identities based on expression of combinations of known marker genes for major cell types. The Seurat's Clustifyr function was used to refine the annotation with CellMarker database (Zhang et al., 2019) and the mouse brain dataset from Ximerakis et., al, 2019. The FindAllMarkers function with the default parameters (min.LogFC = 0.25, min.pct = 0.25, test.use = Wilcox) was used to identify differentially expressed genes for one cluster compare to the others. Functional enrichment analysis were done with clusterProfiler (v4.0.5) bioconductor package on the differentially deregulated genes with over-representation analysis (enricher function) on all the genes. The FindMarkers function with parameters min.LogFC = 0.25, min.pct = 0.1, test.use = Wilcox was used to identify differentially expressed genes for comparing the two samples in each cluster.

## **Biostatistical analysis**

Statistical tests were run by Prism GraphPad software (GraphPad Prism version 8.00 for Windows). For behavioral data analysis, two-way ANOVA test was performed followed by a Tukey post-hoc test. For the analysis of histological data (EM and IHC) and SSEP, Kruskal-wallis test was performed. The significance level was set at 0.05.

To evaluate the inter-individual variability among donors, we calculated Z-scores. A Z-score, or standard score, represents the distance of a value from the population mean. We computed the Z-scores for each donor (both HD and MS) for conduction speed, the percentage of mature oligodendrocytes, and the number of myeloid cells containing myelin or iron.

## Results

### **MS patient's LY form perivascular cuff.**

We clarified the spinal cord of grafted mice to detect the presence or absence of human LY (HLA, red) and their localization in the vicinity of blood vessels (CollagenIV, white) or mouse myeloid cells (mix F4/80; CD11b; CD68, green) (Figure 1A-D, Supplementary video 1 and 2). We do not detect human LY in HD grafted mice (Figure 1A;C), but we detect human MS patient LY (Figure 1B;D;G, HLA red) in the perivascular area (Figure 1B;D;G, Collagen IV in white, arrows). Interestingly, the 3D reconstruction enables us to observe these LY perivascular cuffs and the accumulation of murine myeloid cells in close contact with these human cells (Figure 1D;G; mix F4/80; CD11b; CD68, green, arrows). This location of MS patients LY around blood vessels was further confirmed by EM (Figure 1E-F). These observations confirm that after 3 months MS patients LY interact with mouse immune cells and might be implicated in the persistence of inflammation and a deleterious environment impeding the remyelination process.

### **MS patient's LY induce scar formation and decrease remyelination.**

Since our previous results after 3 weeks showed already a perturbed remyelination in mice grafted with MS patient LY, we assessed the evolution of remyelinated and scarred areas by electron microscopy between 3 weeks and 3 months post-graft in NG, HD, and MS grafted mice (Sup Fig 1A-I). While no differences were observed in NG and HD mice, MS grafted mice showed a decrease in remyelinated area and an increase in glial scar area, along with a shift from macrophages to microglia over time, indicating a progression from peripheral attack to CNS dysregulation (Sup Fig 1G;H;I, macrophages in blue). These observations, while not statistically significant, are informative because they were made in different mice, all grafted with LY from the same individual.

Next, we focus on 3 months time point following NG mice (Figure 2A, D, G, J; 7 mice), mice grafted with HD LY (figure 2B, E, H, K; 6 HD, 3 to 5 mice per individual) or mice grafted with MS LY (Figure 2C, F, I, L; 8 MS patients, 3 to 4 mice per individual). We observe that remyelination was complete in NG mice (Figure 2A, D) and HD LY grafted mice (Figure 2B, E) as illustrated by the presence of axons remyelinated either by oligodendrocytes (OL) (Fig 2J,K, green) or schwann cells (Fig 2 J, K, blue). However, in MS patient LY-grafted mice, we observe sustained demyelination with the formation of a glial scar at the center of the lesion

(Figure 2C, F, I). At the lesion's border, there were some remyelinated axons (Figure 2I, L, blue, by Schwann cells), but predominantly unmyelinated axons (Figure 2I, L, yellow). Quantification of the remyelinated and scar areas confirmed a lower percentage of remyelinated area in MS LY-grafted mice compared to NG (Figure 2M;  $p < 0.01$ ) and HD (Figure 2M;  $p < 0.05$ ) LY-grafted mice, and a higher glial scar area in MS LY-grafted mice compared to NG (Figure 2N;  $p < 0.01$ ) and HD (Figure 2N;  $p < 0.05$ ) LY-grafted mice. These data demonstrate that MS patient LY induces the formation of a glial scar and impedes the remyelination process.

Next to assess if this decrease in remyelination was due to a lack of OPCs recruitment or differentiation, we evaluated the density of OPCs (Olig2<sup>+</sup> cells in red) and the percentage of mature OL (Olig2<sup>+</sup>CC1<sup>+</sup> in red and in green) at the border of the lesion (0 $\mu$ m to 200 $\mu$ m) in NG (Sup Fig 2 A, D; 14 mice), HD LY (Sup Fig 2 B, E, 5 HD, 3 to 5 mice per individual) or MS LY grafted mice (Sup Fig 2 C, F, 11 MS patients, 3 to 4 mice per individual). While we see no difference in the number of Olig2<sup>+</sup> cells/mm<sup>2</sup> between the three groups (Sup Fig 2G;  $p > 0,05$ ) we see a diminution of the percentage of Olig2<sup>+</sup>CC1<sup>+</sup> cells/Olig2<sup>+</sup> cells in MS grafted mice compared to NG and HD grafted mice (Sup Fig 2H;  $p < 0.001$  and  $p < 0.0001$  respectively). These results demonstrate that while MS LY did not perturb the recruitment of OPCs, they impede the differentiation of OL.

### **Enhanced Myeloid Cell Activity and Demyelination at the Lesion Rim in in Mice Grafted with MS patient LY**

To evaluate the impact of LY grafting beyond the lesion graft area, we studied the myeloid cells in the normal appearing white matter at the rim of the lesion. To do so, we performed immunohistochemistry on the spinal cord using a mix of antibody F4/80-CD11b-CD68 antibodies to label myeloid cells (Figure 3, red); MOG antibody to label myelin (Figure 3, green) and assess the iron load with ferritin light chain antibody (FTL) (Figure 3, white). In MS grafted mice (Figure 3C, D, I) there are more myeloid cells in the border of the lesion compared to NG (Figure 3K;  $p < 0,0001$ ) and HD grafted mice (Figure 3K;  $p < 0,0001$ ). While we see that myeloid cells in HD are mostly empty (Figure 3B; I) in MS grafted mice they are either full of myelin (Figure 3D) or iron (Figure 3I) and are higher in proportion in MS grafted mice compare to NG (Figure 3E; J,  $p < 0.0001$ ) and HD grafted mice (Figure 3E; J,  $p < 0.01$ ;  $p < 0.0001$ ). Interestingly there is also more myeloid cells loaded with myelin (Figure 3L) and iron (Figure 3M) per mm<sup>2</sup> in MS grafted compared to NG (Figure 3L; M;  $p < 0,0001$ ) and HD

(Figure 3L; M;  $p < 0,0001$ ) grafted mice. These data demonstrate that in the border of MS patient LY grafted mice there is sign of ongoing demyelination with an active border of the lesion.

### **Longitudinal Behavioral Analysis Reveals Impaired Recovery in Mice Grafted with MS patient LY**

Next, we monitored lesion evolution through a longitudinal follow-up of mice sensory motor skills with behavioral tests at different time points in the 3 group of mice (Figure 4, NG mice grey, HD LY grafted mice orange, MS patient LY grafted mice purple). We set up a baseline the week before the surgery and then tested the mice 1 week; 1 month; 2 months and 3 months after the surgery. For the notched beam test (Figure 4A), there was no significant difference in the time to cross the notched beam at all time points (Supplementary Figure 3C;  $p > 0,05$ ) but we show that 1 week after the graft all the groups make significantly more errors compared to before surgery (Figure 4A). While NG, and HD grafted mice recover and even perform better than before the surgery, MS grafted mice continue to make more errors compared to NG (Figure 4A;  $p < 0,0001$ ) and HD grafted mice (Figure 4A;  $p < 0,0001$ ), and compared to before surgery and 3 months after (Figure 4A). At the grip test (Figure 4B), we do not see difference in the strength of all limb (Supplementary Figure 3D;  $p > 0,05$ ) but MS LY grafted mice have less front limb strength at 1; 2 and 3 months after the graft compared to NG (Figure 4B,  $p < 0,0001$ ) and HD grafted mice (Figure 4B,  $p < 0,01$ ;  $p < 0,0001$ ). NG and HD grafted mice recover and gain in strength, while MS grafted mice keep having a lower front limb strength. These results show that MS grafted mice do not recover after the induction of the lesion.

Since the demyelinating lesion is induced in the dorsal horn of the spinal cord where all the sensory fibers are, we measured the somatosensory evoked potential by recording the response delay of the somatosensory cortex to a hindpaw stimulation (Figure 4C). An example of the typical response in NG, HD, and MS grafted mice illustrates the delayed response seen in MS grafted mice (Figure 4E left panel). This delay was confirmed across all mice, as shown in the graph summarizing the mean conduction speeds for the three groups with a significant difference when compared to NG mice (Figure 4E right panel,  $p < 0,05$ ) and HD grafted mice (Figure 4E right panel,  $p < 0,001$ ).

## **Correlations Among Physiological, Behavioral, and Histological Findings**

Next, we examined correlations between physiological, behavioral, and histological findings. We found that conduction speed positively correlated with front limb strength (Figure 4E;  $r=0.33$ ;  $p<0.01$ ) and negatively correlated with the number of errors (Figure 4E;  $r=-0.35$ ;  $p<0.01$ ). Additionally, the number of errors and front limb strength were negatively correlated (Figure 4E;  $r=-0.57$ ;  $p<0.0001$ ). Conduction speed was positively correlated with the percentage of mature OL (Figure 4F;  $r=0.74$ ;  $p<0.01$ ) and negatively correlated with myeloid cells loaded with myelin (Figure 6B;  $r=-0.45$ ;  $p<0.01$ ) or iron (Figure 4F;  $r=-0.46$ ;  $p<0.01$ ). The percentage of myelin loaded myeloid cells was positively correlated with those iron loaded cells (Figure 4F;  $r=0.57$ ;  $p<0.0001$ ). These results indicate that mice with lower conduction speed have poorer behavioral performance, fewer mature OL, and more loaded myeloid cells, suggesting a correlation between behavioral, physiological, and histological assessments, with poor performance linked to mixed active/inactive lesion features.

## **Assessing the Impact of Individual Donor LY on Graft Outcomes Reveals Patient-Specific Variability**

So far, all the data have been presented regardless of the blood donors. We wanted to evaluate how LY of the individual donor might differently impact the outcomes of the graft. To do this, we calculated a Z-score for each individual for conduction speed, percentage of mature OL, and percentage of myeloid cells filled with either myelin or iron to track outcomes (Figure 5). High conduction speed and a high percentage of OL, along with a low percentage of myeloid cells filled with myelin or iron, were considered a good outcome, shown in blue on the heatmap (Figure 5). Conversely, the opposite was considered a bad outcome, shown in red (Figure 5). We observed that all HD belong consistently to the good outcome group while MS patients exhibit heterogeneous effects. Some MS patient LY induced poor outcomes, while others did not disrupt the regeneration process and had good outcomes. At 3 months after the graft, the model recreates the heterogeneity seen in patients with MS.

## **Presence of Microglia inflamed in MS (MIMS) signature with a dysregulation of complement activation**

To better understand the molecular changes occurring in the different cell population, we performed single cell RNA sequencing (scRNA-seq) on mice grafted with MS LY (MS), and age matched control nude mice (WT) (Figure 6). UMAP analysis was used to separate cell populations, each cell population being defined by a specific gene signature (ximerakis



database) (Figure 6A) with different CNS cell populations including cells of the OL lineage and microglial cells. Interestingly, microglial cells and OL are the two main cell populations impacted by MS LY graft since most of the differentially expressed genes between MS and control mice were identified in these two subpopulations of cells (data not shown). Focusing on the myeloid cell compartment, 4 subset of cells were identified: homeostatic like microglia (hom-MG) , microglia with pro regenerative features (M2-MG) and monocyte macrophages (MONO-MAC) as well as a subset Microglia inflamed in MS (MIMS) only detected in MS patient LY grafted mice (Figure 6B, MIMS); each subset with specific marker genes (Figure 8C). We have an upregulation of genes involved in the major histocompatibility complex (MHC) (such as H2-DA; H2-K1 and B2m) and genes involved in the complement (such as C1qa; C1qb and C1qc) in the myeloid cells of MS LY grafted mice compared to WT mice (Figure 6D). Interestingly, the complement gene upregulated in our dataset C1q (Figure 6E) is increased in active MS lesion edges and is a critical activator and marker of MIMS. Next we performed immunohistochemistry of C1q in HD and MS LY grafted mice (Figure 6F). C1q is detectable in MS LY grafted mice in the area of demyelination and not in HD grafted mice. These results show that in MS grafted mice microglia are activated and express more C1q genes, suggesting on going demyelination.

## Discussion

Animal models of MS present unique opportunities for studying various aspects of the disease. The EAE model, for instance, allows for the examination of inflammatory processes related to lesion formation, despite the random dispersion and timing of lesions which can add complexity to understanding myelin regeneration and lesion development (Procaccini et al., 2015). Meanwhile, the LPC model has been instrumental in reconstructing the steps leading to remyelination within a minimally inflammatory environment. Recently, we were able to combine a focal demyelination and the inflammatory component of MS by grafting human LY in LPC induced demyelinated lesions (El Behi\*, Sanson\* et al., 2017). Three weeks after the graft, LY from some MS patients prevent remyelination, whereas LY from HD or other MS patients do not interfere with this self repair process (El Behi\*, Sanson\* et al., 2017). This model helps us establish the key factors determining the success or failure of remyelination and, more importantly, raises questions about the future outcomes of these demyelinated lesions. Here we observe, three months after the graft, the formation of a lesion with shared features of MS smoldering lesions. The common features between our model and the human mixed active/inactive lesion include an inactive center with a glial scar and an active border (Kuhlmann et al., 2017). The active border consists of iron- or myelin-loaded myeloid cells, similar to those observed in MS (Kuhlmann et al., 2017; Absinta et al., 2021). We also observe that MS patients LY localize in the perivascular space, which is characteristic of smoldering lesions (Absinta et al., 2021; Machado-Santos et al., 2018; Fransen et al., 2020; Frieser et al., 2022). We also recreates some of the heterogeneity seen in MS patients (Bodini et al., 2016).

This model provides a unique insight into how human lymphocytes, even when injected directly into the parenchyma, remain confined to perivascular spaces for a period ranging from 3 weeks (El Behi et al.) to 3 months post-graft. Healthy donor lymphocytes were initially present 3 weeks after grafting but were no longer detectable at 3 months. In contrast, human lymphocytes persist only in mice grafted with lymphocytes from MS patients, indicating that specific signals are necessary to retain pathological lymphocytes within the tissue. Notably, MS lymphocytes grafted directly without a prior lesion do not persist in the parenchyma (data not shown), suggesting that both the appropriate pathological cells and a lesioned environment are required for the formation of these cuffs.

The formation of cuffs in the perivascular or meningeal spaces in MS patients has been linked to poorer clinical outcomes (Magliozzi et al., 2023). These cuffs are believed to consist

of invading T and B cells, which differentiate into tissue-resident memory cells TRM (Machado-Santos et al., 2018; Smolder et al., 2018; Hsiao et al., 2021; Absinta et al., 2021). These memory cells secrete various factors that activate the surrounding glial cells, including microglia and astrocytes. The activation of glial cells contributes to a chronic inflammatory environment, exacerbating tissue damage and inhibiting the repair processes, thus leading to a progressive worsening of the disease (Woo et al., 2024). In our model, the presence of these lymphocyte perivascular cuffs results in not only an impediment to remyelination but also ongoing demyelination, as evidenced by the increasing lesion load from 3 weeks to 3 months. This unique characteristic of active demyelination even 3 months after the initial chemical induction indicates a shift from focal demyelination to a continuous, slowly expanding demyelination. Even more compelling is the observation that, while macrophages appear to be the major players in the acute phase of demyelination at 3 weeks, CNS resident microglial cells seem to take over by 3 months. In our mouse model, we do detect a unique subset of microglial cells that were identified in the rim of smoldering lesions in human post mortem tissue (Absinta et al. 2021), the MIMS. MIMS upregulate complement genes (C1q). C1q expression is associated with demyelinated areas in MS postmortem tissue (Lucchinetti et al., 2000; Prineas et al., 2001; Breij et al., 2008) and also in our model. In addition, smoldering lesions, active lesions and normal appearing white matter are positive for complement proteins, regulators and activation products (Ingram et al., 2014). However, following our single cell analysis, the pro-inflammatory profile of MIMS is not that clear since we observe a downregulation of some genes involved in the induction of pro-inflammatory phenotype (such as: Akirin2; Ppp2r5a; S100a4; S100a6 and Ifitm6). MIMS have lower expression of homeostasis genes and also have upregulation of lipid metabolism genes (such as Lpl). Interestingly this association between a downregulation of homeostatic genes, upregulation of pro-inflammatory and lipid metabolism genes have been described in disease associated microglia (DAM) cells in Alzheimer disease (Keren-Shaul et al., 2017). Our data suggest that our MIMS could be DAM. Interestingly, DAM cells have been described in the EAE and cuprizone models (Barclay et al., 2024).

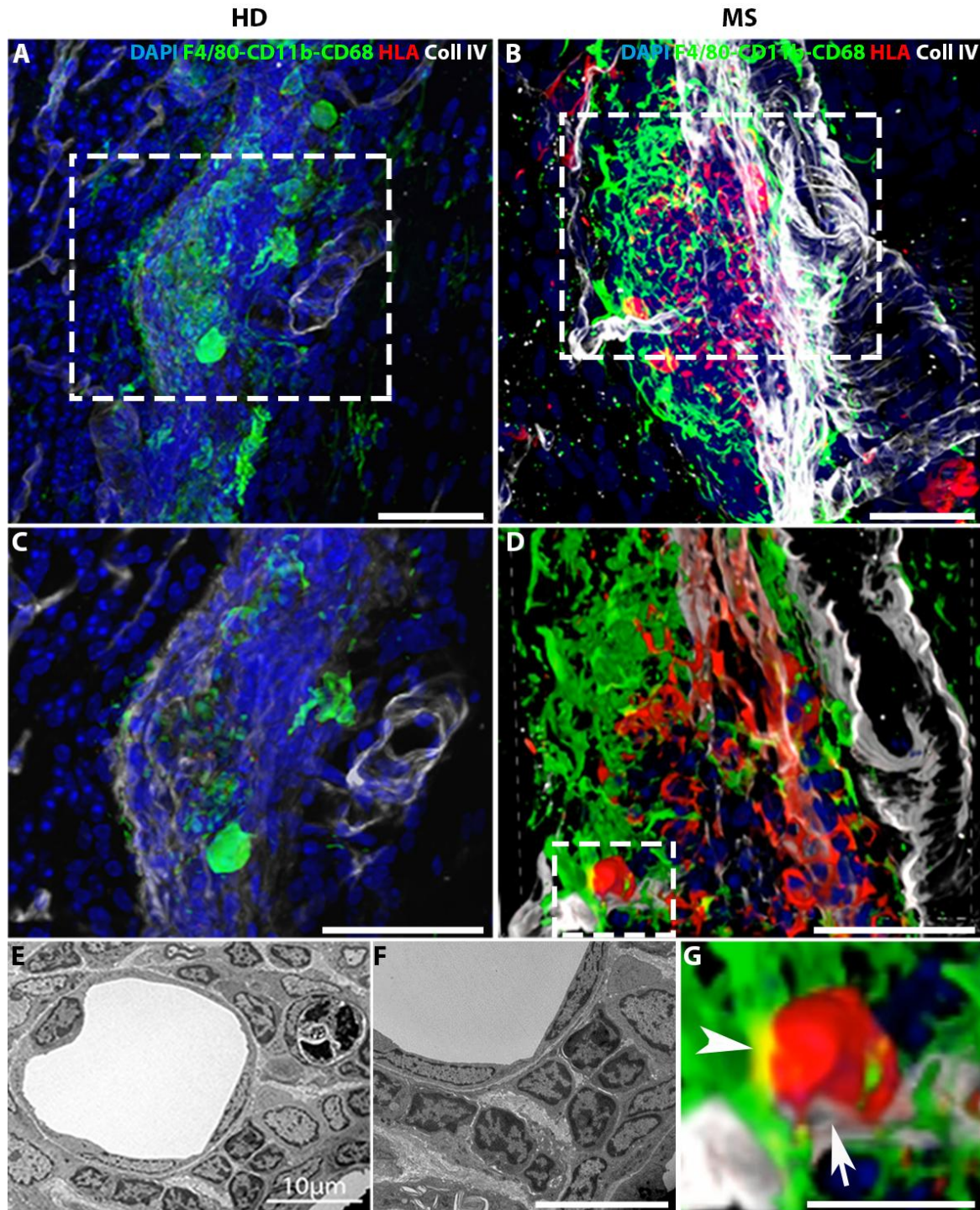
With this unique model, we will be able to decipher the steps and mechanisms leading to a transition from a peripheral inflammation to a chronic compartmentalized inflammation of the CNS. This compartmentalized inflammation is part of the PIRA mechanism, therefore we could identify and test potential therapeutic molecules on these inflammation driving progression in MS patients that is a critical unmet need. Additionally, we have behavioral and physiological readouts that enable us to study the evolution of the lesions non-invasively.

Notably, the physiological readouts correlate with histopathological findings, making them valuable for assessing potential drug effects.

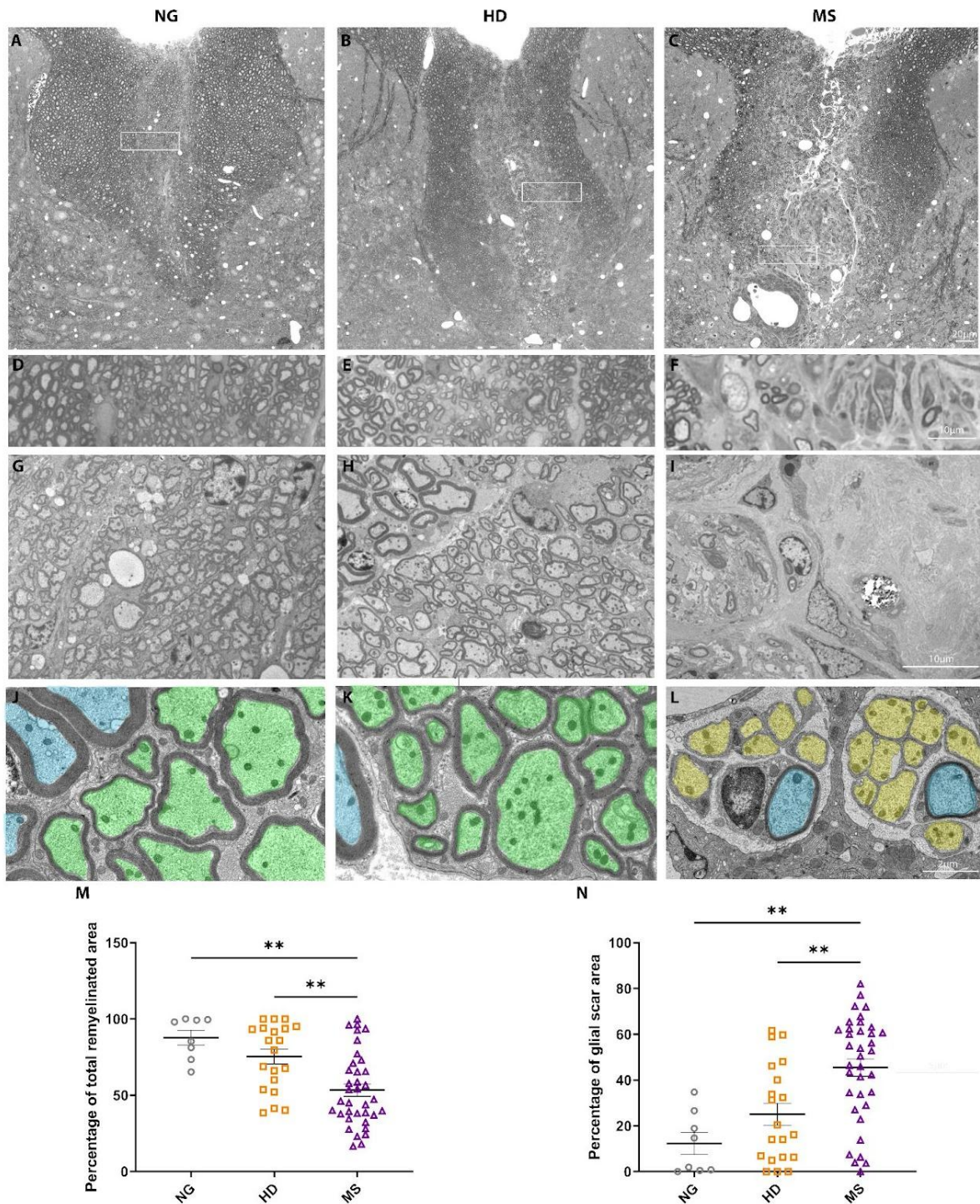
### **Limitation of the study:**

The use of the clearing method and three-dimensional imaging enable us to detect human LY three months after the graft in MS LY grafted mice. However, the clearing method does not allow the use of all antibodies due to high tissue processing (such as high permeabilization), in addition, with the few numbers of LY still present, we were not able to detect them on classical immunohistochemistry experiments. Due to this limitation, we cannot conclude on which subtypes of LY are the drivers of the development of smoldering lesions. To assess which subtypes of LY are necessary for the development of these lesions we could graft different subtypes of LY from one MS patient that we know to induce smoldering lesions. However, we hypothesize that these LY still present are T<sub>RM</sub> cells since they are still present in the tissue even three months after the graft. Then, in our model, LY are found in perivascular space, T<sub>RM</sub> are found in perivascular spaces of activated and smoldering lesions and have been implicated in the compartmentalized inflammation.

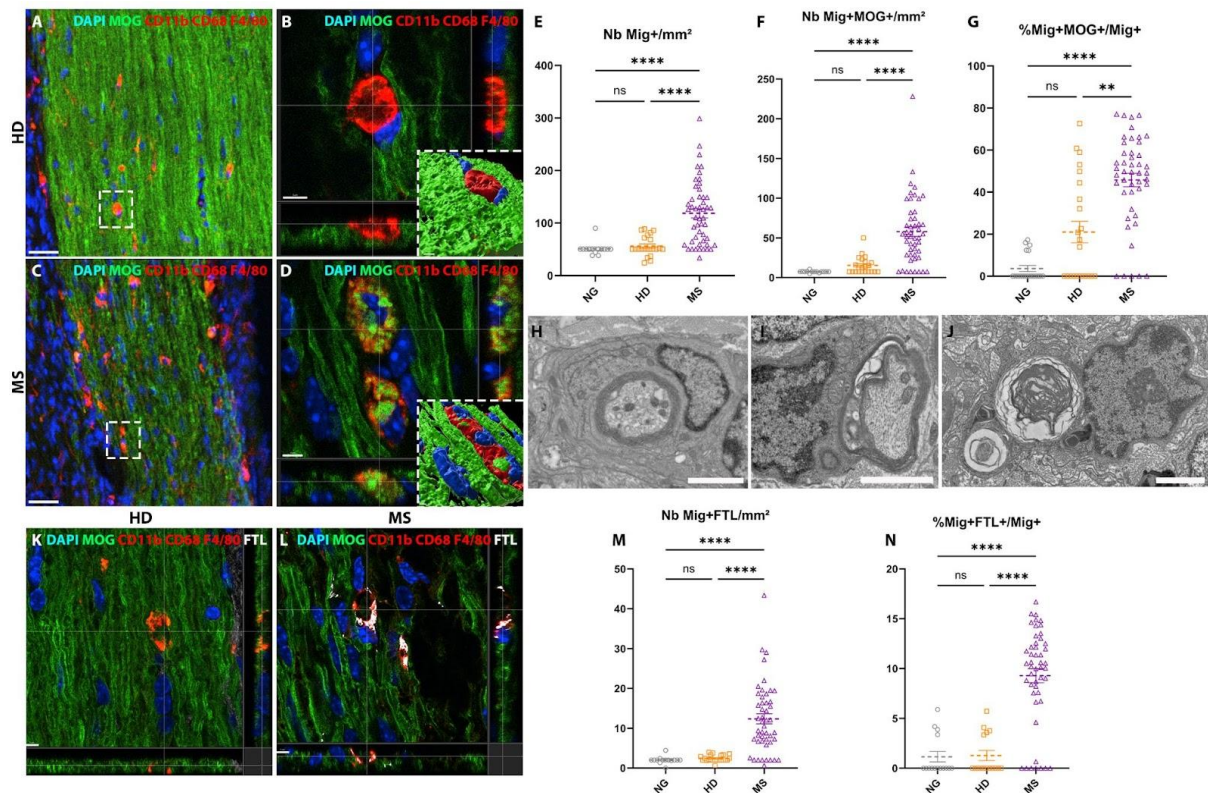
Figures:



**Figure 1: Human LY from MS patients, still present 3 months after the graft, form perivascular cuff and are in close contact with mouse myeloid cells. (A-D) Three-dimensional confocal image of mice grafted with (A;C) HD LY or mice grafted with (B;D) MS LY. Spinal cords are cleared using a modified iDISCO/RapiClear protocol. Scale bar: 50 $\mu$ m. (A) 3D image of HD grafted mice, (C) Zoom in the 3D confocal images. We do not observe LY in HD grafted mice, and we observe few mice myeloid cells (in green). (B) 3D image of MS grafted mice, (D) Zoom in the 3D confocal images. We observe MS LY (in red) close to mouse myeloid cells (in green) and around blood vessels (in white), interaction with mouse myeloid cells (arrowhead) and LY are found in perivascular space close to blood vessels (arrow). Scale bar: 20 $\mu$ m (E-F) Semi thin section of a mice grafted with MS LY. We observed LY around blood vessels. Scale bar: 10 $\mu$ m**

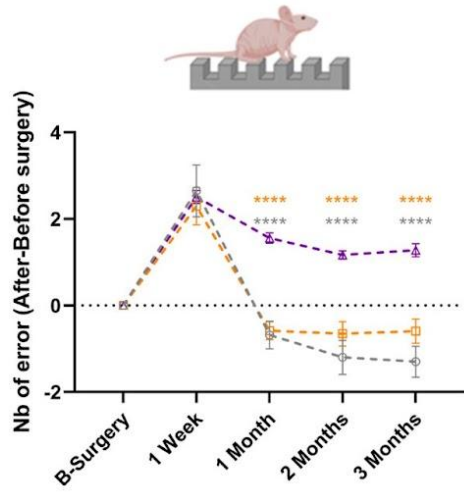


**Figure 2: MS patients LY graft induce the installation of a chronic demyelinated lesion with scaring tissue.** (A-C) Semi thin section of the dorsal horn of spinal cord of Nude mice after the lesion of (A) NG mice, (n= 6 mice); (B) and the grafting of HD LY (n= 6 HD with 2-3 mice per individual); (C) and MS patient LY (n=14 MS patients with 2-4 mice per individual) 3 months after the surgery. (D-F) Zoom inside the lesion of (D) NG mice, and mice grafted with (E) HD LY or (F) MS patient LY. (G-I) Lesion center of (G) NG mice and (H) HD LY grafted mice with remyelinated axons, and (I) MS mice exhibiting a glial scar. (J-L) Ultrathin sections visualization of lesion border of (J) NG mice and (I) HD LY grafted mice where we observe remyelinated (blue by OL or green by Schwann cells) axons or (L) MS patient LY grafted mice with some remyelinated axons but mostly unmyelinated axons (pink). (M) Mean percentage of remyelinated area in the lesion of each group and (N) mean percentage of glial scar area in the lesion for each group. (NG (n=6 mice, grey), HD (n=15 mice, orange) and MS (n=35 mice, purple)). One way ANOVA followed by Tukey's post-Hoc. \*:  $p < 0,05$ ; \*\*:  $p < 0,01$ .

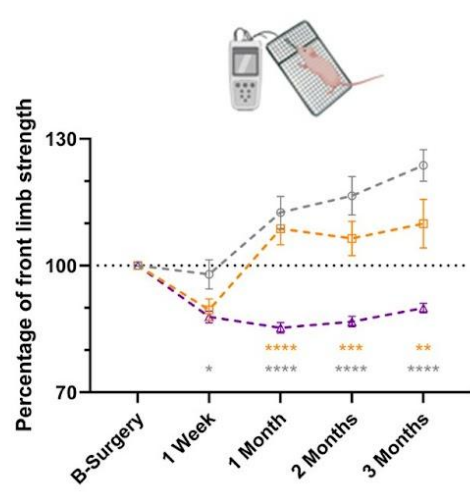


**Figure 3: MS LY graft increase phagocytic cells and the presence of iron-loaded myeloid cells in the white matter surrounding the lesion. (A;C) Representative images of cryosectioned spinal cord of mice grafted with (A) HD LY or (C) MS LY. Myeloid cells (MAC in red) are stained with a Mix (F4/80; CD11b; CD68, in red), myelin is stained with MOG (in green). Scale bar: 30 $\mu$ m. (B;D) Zoom and orthogonal projection of a myeloid cell in the white matter of mice grafted with LY from HD (B) or MS patients (D) and the associated 3D-reconstruction scale bar: 5 $\mu$ m. We observe that myeloid cells in MS are full of myelin. (E) Graph representing the mean density of myeloid cells for each group (NG (n=14 mice, grey), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200 $\mu$ m from myelin negative area (F) Graph representing the mean density of myelin-loaded cells for each group (MAC cells positive for MOG) (NG (n=14 mice, grey), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200 $\mu$ m from myelin negative area (G) Graph representing the mean percentage of phagocytic cells for each group (MAC cells positive for myelin) (NG (n=14 mice, grey), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200 $\mu$ m from myelin negative area. (H-J) EM images of myeloid cells in the border of MS LY grafted mice, (H) they are in close contact with myelinated axons (I) start to demyelinate the axons, (J) and have phagocytosed myelin. Scale bar = 20 $\mu$ m (K-L) Zoom and orthogonal projection of a myeloid cell in the white matter in HD (K) or MS (L) grafted mice. We observe that myeloid cells loaded with iron labeled with ferritin light chain (FTL) antibody are mainly observed in MS LY grafted mice. (M) Graph representing the mean density of iron-loaded cells for each group (MAC cells positive for FTL) (NG (n=14 mice, grey), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200 $\mu$ m from myelin negative area (N) Graph representing the mean percentage of iron-loaded cells for each group (MAC cells positive for FTL) (NG (n=14 mice, grey), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200 $\mu$ m from myelin negative area. Kruskal-Wallis test followed by Dunn's post-Hoc. \*\*:  $p < 0,01$ ; \*\*\*\*:  $p < 0,0001$ .**

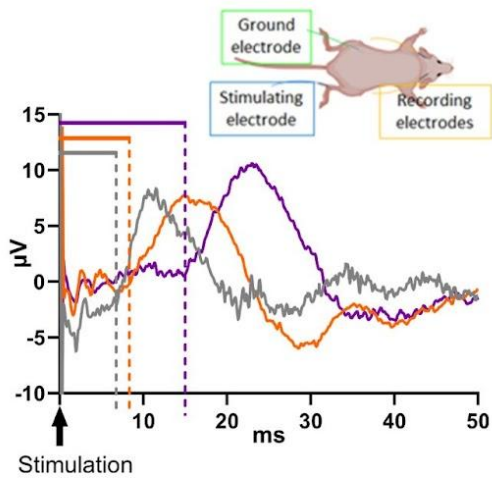
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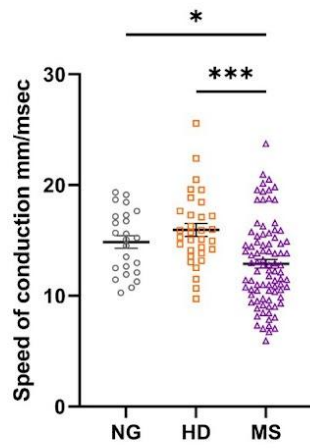
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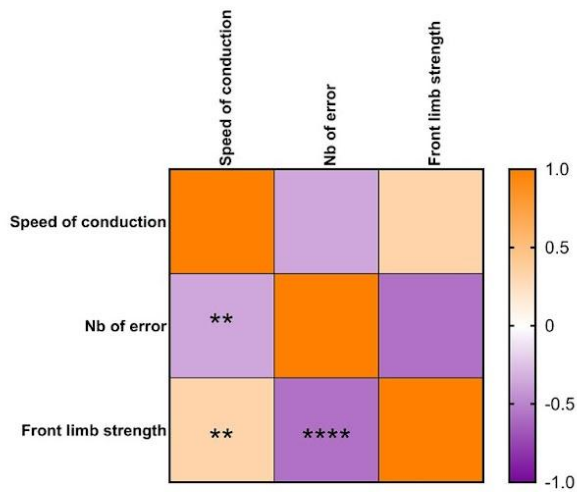
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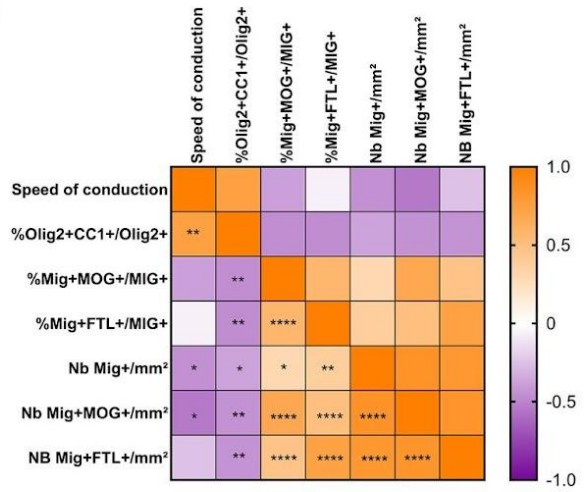
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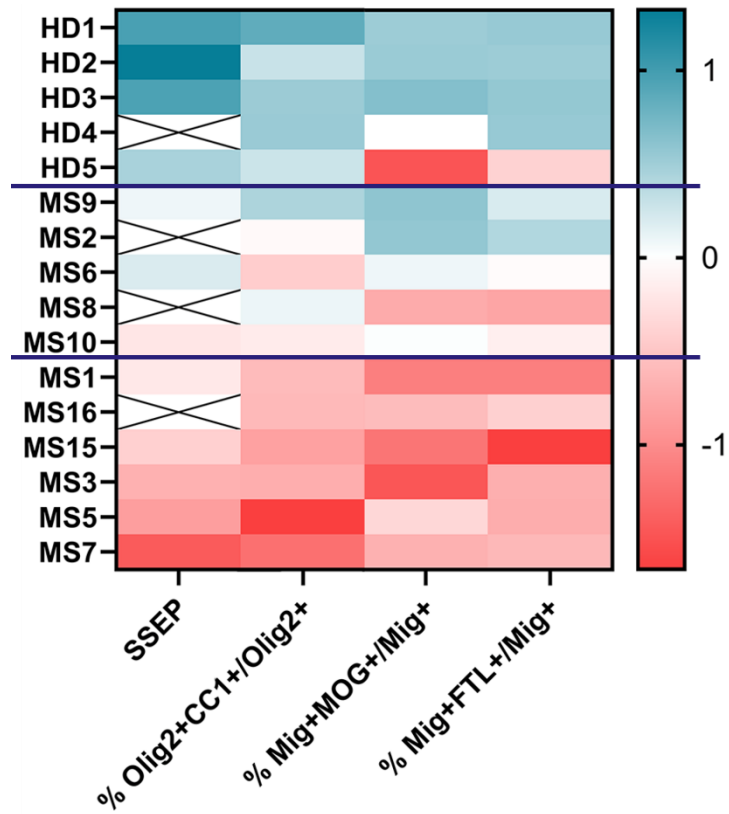


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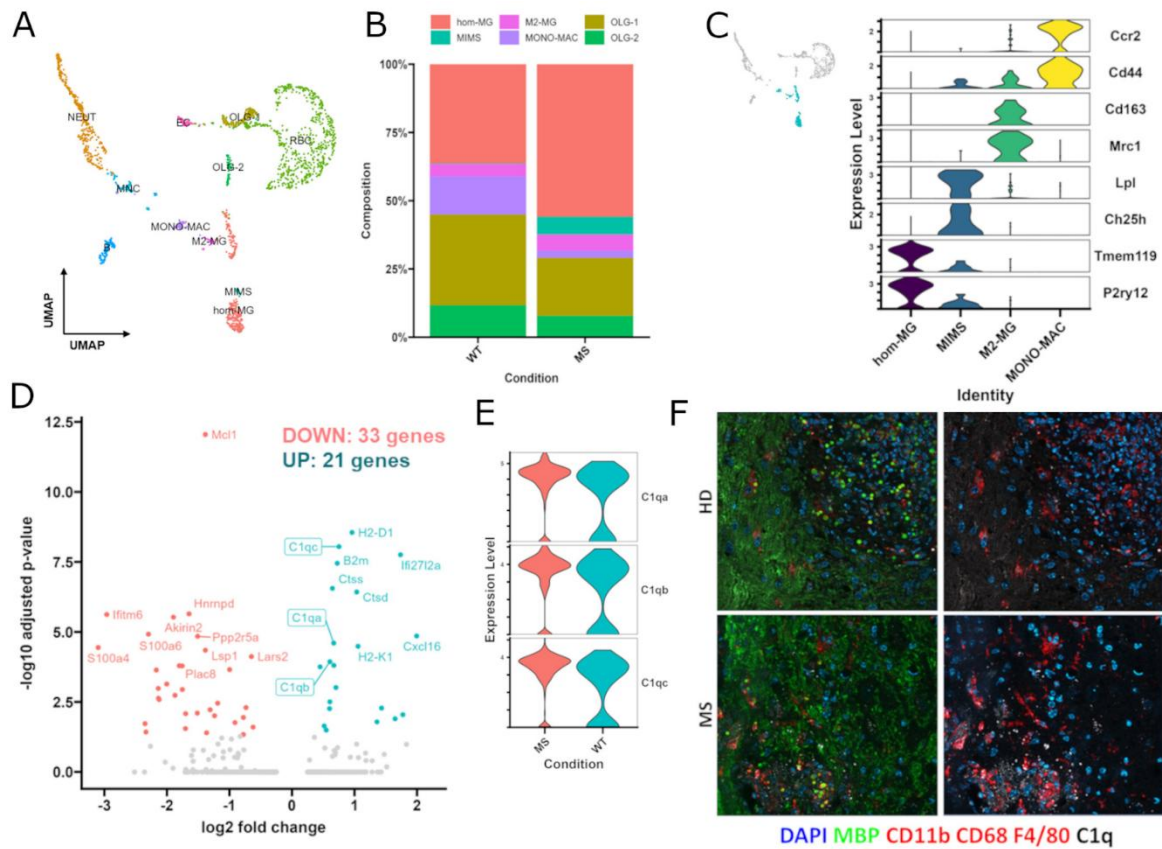




**Figure 4: Mice grafted with MS LY have impaired behavioral and physiological performances.** (A) Notched beam test and graph representing the mean number of errors after minus number of errors before the surgery on the notched beam test for each group (NG (n=20 mice, blue), HD (n=18 mice, green) and MS (n=39 mice, pink (4 MS patients))) at each time point comparing MS group versus NG (blue stars) or HD (green stars) group. (B) Grip test and graphs representing the percentage of the front limb strength for each group (NG (n=20 mice, gray), HD (n=27 mice, orange) and MS (n=77 mice, purple)) at each time point comparing MS group versus NG (blue stars) or HD (green stars) group. 2 way ANOVA followed by Tukey's post-Hoc. (C) Experimental set up. Briefly an electrical stimulation is performed on the tibial nerve while the response to the stimuli is measured in the somatosensory cortex area. Representative somatosensory evoked potential response of each group NG, HD and MS. Arrows represent the artifact of stimulation. Orange dotted line represent the beginning of the cortical responses. (D) Mean conduction speed in mm/ms for each group (NG (n=20 mice, gray), HD (n=23 mice, orange) and MS (n=87 mice, purple)). One way ANOVA followed by Tukey's post-Hoc. \*:  $p < 0,05$ ; \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0,001$ ; \*\*\*\*:  $p < 0,0001$  (E) The speed of conduction is positively correlated with the front limb strength ( $r=0,33$ ) and negatively correlated with the number of errors ( $r=-0,35$ ). The number of errors and the front limb strength are negatively correlated ( $r=-0,57$ ) \*\*:  $p < 0,01$ ; \*\*\*\*:  $p < 0,0001$  (F) The speed of conduction is positively correlated with the percentage of mature oligodendrocytes ( $r=0,74$ ), and negatively correlated to the number of myeloid cells/mm<sup>2</sup> ( $r=-0,45$ ) and myeloid cells containing myelin/mm<sup>2</sup> ( $r=-0,54$ ). The percentage of mature oligodendrocytes is negatively correlated with the percentage of myeloid cells either full of myelin ( $r=-0,45$ ) or iron ( $r=-0,46$ ) and the number of myeloid cells/mm<sup>2</sup> ( $r=-0,35$ ) myeloid cells full of myelin/mm<sup>2</sup> ( $r=-0,44$ ) or iron/mm<sup>2</sup> ( $r=-0,45$ ). The percentage of myeloid cells loaded with myelin is positively correlated with the percentage of myeloid cells loaded with iron ( $r=0,57$ ), and with the number of myeloid cells/mm<sup>2</sup> ( $r=0,30$ ) myeloid cells full of myelin/mm<sup>2</sup> ( $r=0,68$ ) or iron/mm<sup>2</sup> ( $r=0,47$ ). The percentage of myeloid cells loaded with iron is positively correlated with the number of myeloid cells/mm<sup>2</sup> ( $r=0,38$ ) myeloid cells full of myelin/mm<sup>2</sup> ( $r=0,50$ ) or iron/mm<sup>2</sup> ( $r=0,73$ ). The number of myeloid cells/mm<sup>2</sup> is positively correlated with the number of myeloid cells full of myelin/mm<sup>2</sup> ( $r=0,84$ ) myeloid cells full of iron/mm<sup>2</sup> ( $r=0,81$ ). The number of myeloid cells containing myelin/mm<sup>2</sup> is positively correlated with myeloid cells full of iron/mm<sup>2</sup> ( $r=0,83$ ). Pearson test of correlation, \*:  $p < 0,05$ ; \*\*:  $p < 0,01$ ; \*\*\*\*:  $p < 0,0001$



*Figure 5: The graft of MS patients LY has heterogenous effect. We calculated a Z-score for each patient for the different assessment. We can distinguish some MS patients LY that will induce a bad outcome in the assessment (in red) and some that will induce a good or mild outcome in the assessment (in blue).*



**Figure 6: scRNAseq analysis of Wild type (WT) and MS patient LY grafted mice 3 months after the lesion/graft.** (A) UMAP of cells coloured by populations after identification of the different subset of cells. (B) Bar plot showing the relative distribution of each cell population in WT (left) and MS LY grafted mice (right). (C) Identification of the specific markers of MIMS cells among the myeloid cells cluster (D) Volcano plot of gene expression changes in MIMS (downregulated in pink and upregulated in blue) (E) Violin plot showing expression of 3 complement genes C1qa, C1qb and C1qc in MS (pink) and WT (blue) condition. (F) Immunocytochemistry of myeloid cells (CD11b/CD68.F4/80, red) present at the border at the lesion (MBP, green) expressing C1q (White dots).

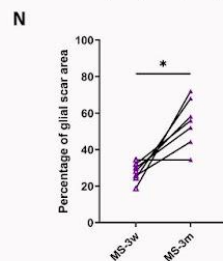
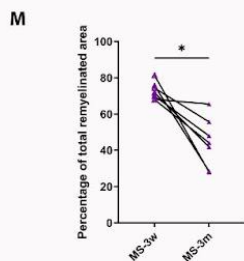
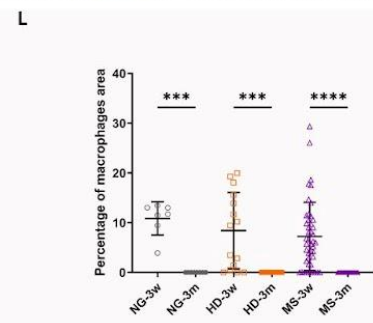
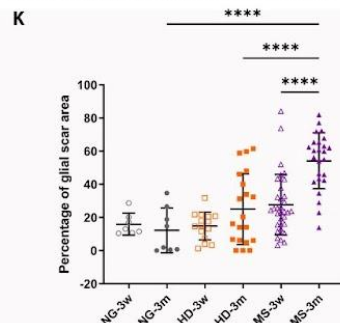
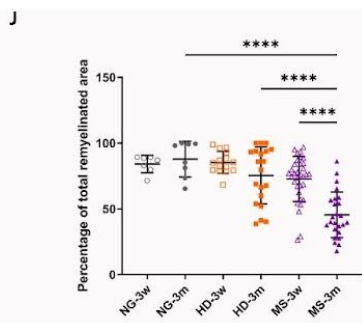
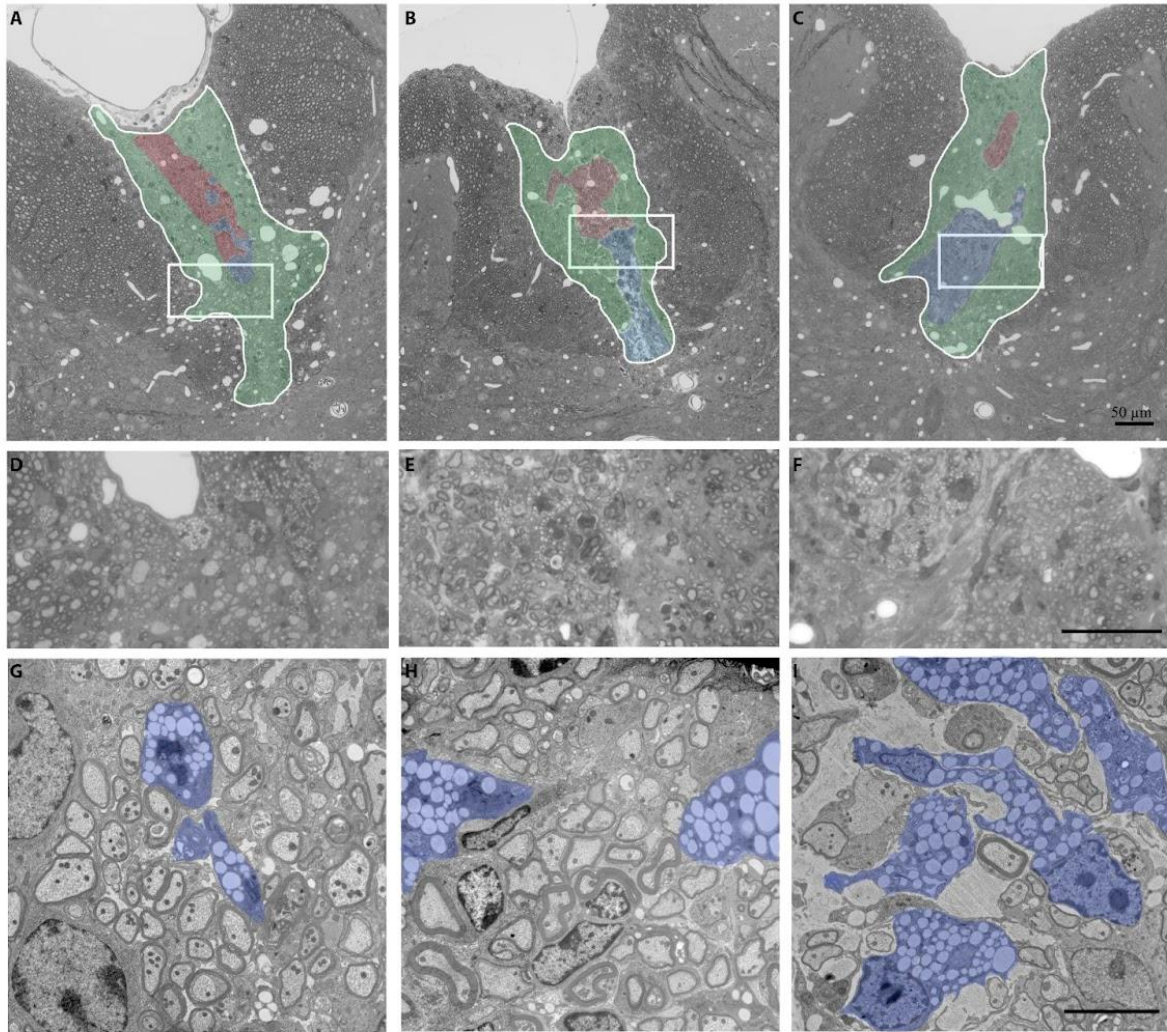
## Table:

**Table 1: Multiple sclerosis patients and healthy donor cohort description**

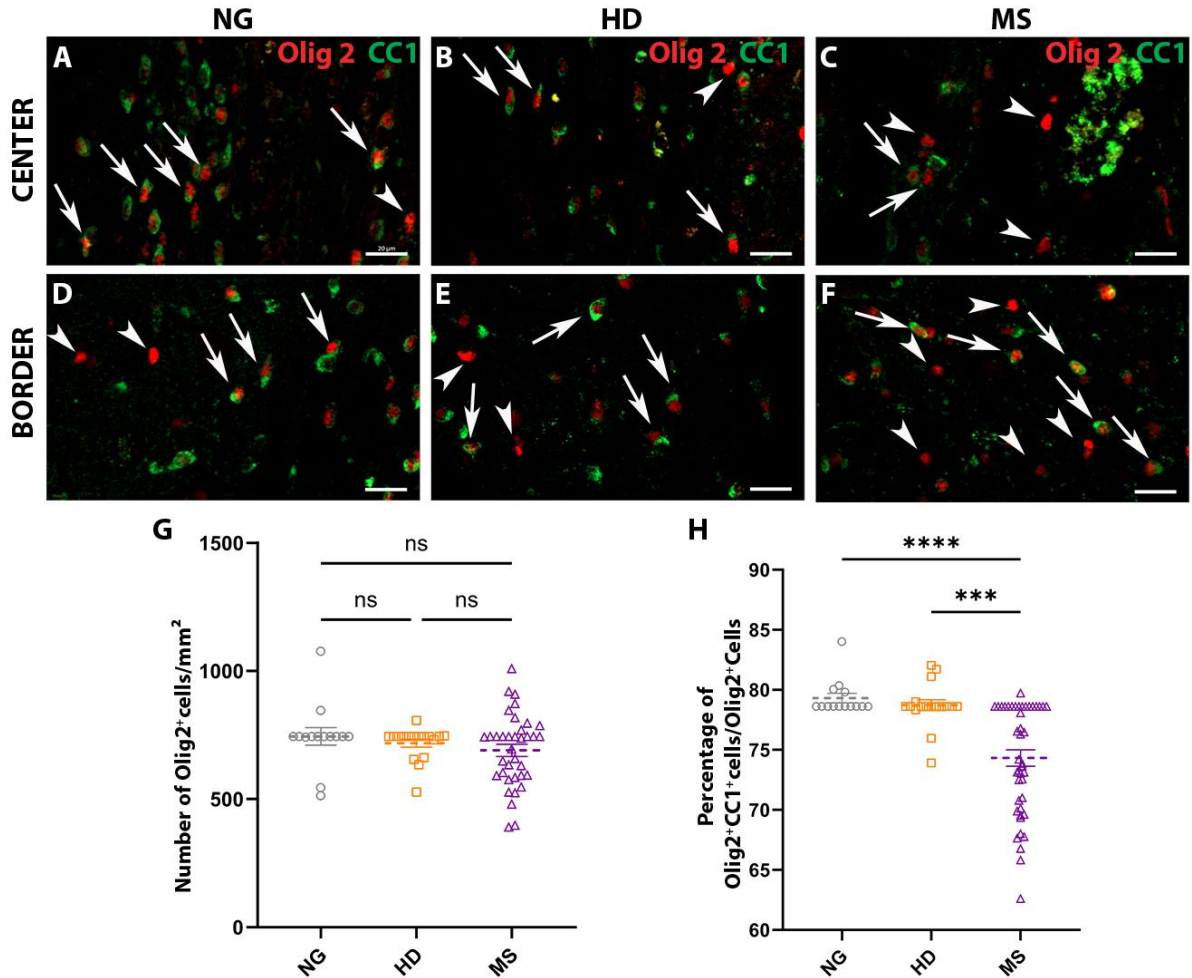
Characteristic	MS patients	RRMS	PPMS	HD
n	21	19	2	11
Female/male	11/10	10/9	1/1	8/11
Mean age (range) [standard deviation]	43.8 (23-70) [12.2]	44.3 (23-70) [12.3]	39.5 (29-50) [14.8]	38.4 (22-54) [10.6]
Under treatment/treatment-free	10/11	10/9	0/2	

MS: Multiple sclerosis; RRMS: Relapsing/remitting multiple sclerosis; PPMS: Primary progressive multiple sclerosis; HD: Healthy donors

# Supplementary Figure



**Supplementary Figure 1: Evolution of the lesion between 3 weeks and 3 months.** (A-C) Semi thin section of the dorsal horn of spinal cord of Nude mice after the lesion of (A) NG mice, (n= 7 mice); (B) and the grafting of HD LY (n= 5 HD at 3 weeks and 3 months with 2-3 mice per individual); (C) and MS patient LY (n=7 MS patients at 3 weeks and 6 at 3 months with 2-4 mice per individual) 3 weeks after the surgery. Remyelinated area either by OL (in green) or SC (in red), and macrophages (in blue). (D-F) Zoom inside the lesion of (D) NG mice, and mice grafted with (E) HD LY or (F) MS patient LY. (G-I) Macrophages with vacuoles (in blue) in (G) NG mice and (H) HD LY grafted mice and (I) MS mice. (J) Mean percentage of remyelinated area in the lesion at 3 weeks and 3 months of each group and (K) mean percentage of glial scar area in the lesion at 3 weeks and 3 months for each group. (L) Mean percentages of macrophages area in the lesion at 3 weeks and 3 months of each group NG (n=7 mice at 3 weeks and 3 months, grey), HD (n=15 mice at 3 weeks and 20 at 3 months, orange) and MS (n=33 mice at 3 weeks and 25 at 3 months, purple). One way ANOVA followed by Tukey's post-Hoc. \*\*\*:  $p < 0,001$ ; \*\*\*\*:  $p < 0,0001$ . (M) Mean percentage of remyelinated area in MS patients (6 patients) comparison between 3 weeks and 3 months (N) mean percentage of glial scar area in MS patients (6 patients) comparison between 3 weeks and 3 months. Wilcoxon, \*:  $p < 0,5$



**Supplementary Figure 2: MS patients LY decrease OL differentiation.** (A-C) Representative images of cryosectioned spinal cord in the center of the lesion of (A) NG mice or mice grafted with (B) HD LY or (C) MS LY. Dotted line represents the area of dense nuclei in the lesion. Cells from the oligodendroglial lineage are stained with Olig2 (in red), Differentiated oligodendrocytes are stained with Olig2 and CC1 (nuclei in red and cytoplasm in green). Scale bar: 100 µm. (D-F) Representative images of cryosectioned spinal cord in the border of the lesion of NG mice (D) mice grafted with LY from HD (E) or MS patients (F) 20 µm. We observe that myeloid cells in MS are full of myelin. (G) Graph representing the mean density of oligodendrocytes cells for each group (NG (n=14 mice, gray), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200µm from hypercellular area. (H) Graph representing the mean percentage of mature oligodendrocytes (NG (n=14 mice, gray), HD (n=22 mice, orange) and MS (n=58 mice, purple)) 0-200µm from the hypercellular area. Kruskal-Wallis test followed by Dunn's post-Hoc. \*\*\*:  $p < 0,001$  \*\*\*\*:  $p < 0,0001$ .

## Supplementary table

*Supplementary table 1: Individual description of multiple sclerosis patients cohort.*

Number	Age	Sex	Treatment	Disease
MS1	42	F	None	RRMS
MS2	32	H	None	RRMS
MS3	51	F	None	RRMS
MS4	29	F	None	RRMS
MS5	70	H	None	RRMS
MS6	69	F	None	RRMS
MS7	46	H	Copaxone	RRMS
MS8	36	F	Demethylfumarate	RRMS
MS9	50	F	None	PPMS
MS10	23	F	Demethylfumarate	RRMS
MS11	51	H	Teriflunomide	RRMS
MS12	37	H	Copaxone	RRMS
MS13	54	H	None	RRMS
MS14	42	F	Copaxone	RRMS
MS15	33	F	Tecfidera	RRMS
MS16	39	F	Copaxone	RRMS
MS17	50	H	None	RRMS
MS18	53	H	Teriflunomide	RRMS
MS19	29	H	None	PPMS
MS20	41	F	None	RRMS
MS21	43	H	Copaxone	RRMS

*Supplementary table 2: Antibody used in the study.*

Antibody	Dilution	Target	Isotype	Blocking/Permeabilisation solution	Source (Reference)
C1q	1/50	Complement	Mouse IgG2b-Biotin	PBS/BSA 4% Titron 0,2%	Thermofischer (MA1-40312)
CC1	1/100	Mature OL	Mouse IgG2b	PBS/BSA 4% Titron 0,2%	Millipore (OP80)
CD11b	1/400	Macrophages/ Microglia	Rat IgG	PBS/BSA 4% Titron 0,2%	Bio-rad (MCA74G)
CD69	1/400	Macrophages/ Microglia	Rat IgG	PBS/BSA 4% Titron 0,2%	Bio-rad (MCA1957)
Coll IV	1/400 IHC 1/200 Clearing	Blood vessels	Rabbit IgG	PBS/BSA 4% Titron 0,2%	Abcam (ab19808)
F4/80	1/100	Macrophages/ Microglia	Rat IgG	PBS/BSA 4% Titron 0,2%	Bio-rad (MCA497R)
FTL	1/100	Iron load	Rabbit IgG	PBS/BSA 4% Titron 0,2%	Abcam (ab69090)
HLA-DP,DQ, DR (Clone CR3/43)	1/100	Human LY	Mouse IgG1	PBS/BSA 4% Titron 0,2%	Dako (M0775)
MOG	1/100	Myelin	Mouse IgG1	PBS/BSA 4% Titron 0,2%	Home made
Olig2	1/200	OL lineage	Rabbit IgG	PBS/BSA 4% Titron 0,2% + Antigen retrieval	Millipore (AB9610)



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## **RESULTS PART II**



# Notch3 Directs a Regulatory T Cells-Microglia axis to Promote Autoimmune Neuroinflammation

Mehdi Benamar, Paola Contini, Klaus Schmitz-Abe, Corinne Bachelin, Olga Lanzetta, Manuel Leyva Castillo, Muyun Wang, Fatma Betul Oktelik, **Océane Perrot**, Sena Nur Arbag, Emmanuel Stephen-Victor, Hani Harb, Céline Louapre, Federico Ivaldi, Antonio Uccelli, Matilde Inglese, Claudia Angelini, Violetta Zujovic, Raffaele De Palma<sup>†</sup>, Talal A. Chatila<sup>†</sup>

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During my thesis project, we started a collection of PBMCs from PwMS that we had grafted in our humanized mouse model, creating a stock of MS PBMCs. We then collaborated with various teams to explore how lymphocytes from PwMS might promote CNS inflammation.

In this article, our collaborators used blood and CSF samples from various cohorts of PwMS, including ours, and discovered an increase in a subpopulation of Tregs expressing Notch 3 in both the blood and CSF of these patients. Additionally, this Notch3<sup>+</sup> Treg subpopulation was also identified in the CNS of EAE mice. Using this model, they found that Notch3<sup>+</sup> Tregs interact with microglia via the Notch Delta-like ligand 1 (DLL1), and this interaction induces the destabilization of Tregs into Th17 cells, which are known for their pathogenic role in MS and EAE. This study identifies the subversion of immune tolerance mediated by Notch3 as a fundamental mechanism enabling autoimmune neuroinflammation.

## Abbreviation:

CNS: Central Nervous System; CSF: CerebroSpinal Fluid; DLL1: Notch delta like ligand 1; EAE: Experimental Autoimmune Encephalomyelitis; MOG: Myelin Oligodendrocytes Protein ; MS: Multiple Sclerosis; ScRNA-seq: Single Cell RNA-Sequencing; TCR: T Cell Receptor; Tregs: Regulatory T cells.

# Notch3 Directs a Regulatory T Cells-Microglia axis to Promote Autoimmune Neuroinflammation

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## **Abstract**

Deciphering the mechanisms that promote central nervous system inflammation in multiple sclerosis is critical for developing precision therapies. We show that a specific regulatory T (Treg) cell subpopulation expressing Notch3 was increased in the peripheral blood and cerebrospinal fluid of individuals with multiple sclerosis (MS) and in the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE). Notch3 expression was upregulated by mechanisms involving the gut microbiota and Treg cell-specific toll-like receptor (TLR) signaling to promote EAE severity. Notch3 interaction with delta-like ligand 1 (DLL1) on microglia subverted Treg cells into T helper 17 (Th17) cells by Hippo pathway-dependent mechanisms while also inducing an inflammatory microglial response. Its deletion inhibited EAE by preventing Treg cell destabilization into Th17 cells while simultaneously promoting the expansion of a novel brain-protective neuropeptide receptor 1 (NPY1R)<sup>+</sup> Treg cell population that suppressed pathogenic IFN $\gamma$ <sup>+</sup> and GM-CSF<sup>+</sup> effector T cells. Our studies thus identify Notch3-mediated immune tolerance subversion as a fundamental mechanism that licenses autoimmune neuroinflammation.



## **Introduction**

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease driven by pathogenic autoreactive T cells <sup>1</sup>. Of particular interest is the role in disease pathogenesis of immune regulatory mechanisms normally operative to restrain autoreactivity, key among which are regulatory T (Treg) cells <sup>2</sup>. Studies have shown that the number and function of Treg cells are impaired in patients with MS, which may contribute to the development and progression of the disease <sup>3-5</sup>. In experimental autoimmune encephalomyelitis (EAE), a mouse model of MS <sup>6</sup>, Treg cells accumulate in the central nervous system (CNS) tissues but fail to control the autoimmune inflammation<sup>7</sup> and eventually lose their suppressive function and Foxp3 expression<sup>8,9</sup>. Subverted Treg cells may further convert into pathogenic Th cells under the influence of pro-inflammatory cytokines or antigenic stimulation <sup>5,8</sup>. Whether this Treg cell plasticity plays a role in the pathogenesis of EAE and MS remains unclear.

## **Methods**

### **Human Cohorts:**

*Multiple sclerosis and control patients:* Peripheral blood (PB) samples were obtained from MS patients (n=10) and healthy control (n=6) from Institut du cerveau, Paris, France. Moreover, PB and cerebrospinal Fluid (CSF) cells were obtained from MS patients (n=25) and non-MS patients (n=14) from Italy. The clinical characteristics of MS patients and Controls groups are reported in Extended Table Data 1.

*Sample Processing:* Peripheral blood samples were obtained in sodium heparin or ethylenediaminetetraacetic acid (EDTA) tubes at study enrollment. At each site, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen.

### **Human Subjects:**

Written informed consent (and assent when appropriate) was provided by the participants. The Institutional Review Boards (IRBs) approved the single-center research protocols.

### **Isolation of Human peripheral blood mononuclear cells (PBMCs)**

Human PBMCs were isolated from whole blood from either healthy control or MS patients via density gradient using Ficoll (GE Healthcare). PBMCs were then stored frozen in

Fetal Calf Serum (FCS) (Sigma Aldrich) and 15% Dimethyl sulfoxide (DMSO) (Sigma Aldrich). The cells were later thawed for analysis of their Notch or different markers and cytokine expression by flow cytometry.

### **Flow cytometric analysis of human cells.**

Antibodies against the following human antigens were used: CD3 (HIT3a, catalogue no: 300318, 1:200, Biolegend), CD4 (RPA-T4, catalogue no: 300530, 1:200, Biolegend), Foxp3 (PCH-101, catalogue no: 48-4776-42, 56-4716-41, 1:100 Thermofisher), Notch1 (HMN1-519, catalogue no: 566023, 1:300, BD Pharmingen), Notch2 (HMN2-25, catalogue no: 742291, 1:300, BD Pharmingen), Notch3 (HMN3-21, catalogue no: 744828, 1:300, BD Pharmingen), Notch4 (HMN4-2, Catalogue no: 563269, 1:300, BD Pharmingen), CD25 (BC96, Catalogue no: 12-0259-42 1:300, Thermofisher), CD127 (A019D5, Catalogue no: 351320 1:300, Biolegend). The specificity and optimal dilution of each antibody was validated by testing on appropriate negative and positive controls or otherwise provided on the manufacturer's website. Intracellular cytokine staining was performed as previously described<sup>9,10</sup>. Dead cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable Viability Dye (1:1000 dilution) (Thermofisher).

### **Statistical analysis.**

Student's two-tailed t-test, one- and two-way ANOVA and repeat measures two-way ANOVA with post-test analysis.

### **Data Availability.**

The data presented in the manuscript, including de-identified patient results, will be made available to investigators following a request to the corresponding author. Any data and materials to be shared will be released via a material transfer agreement. RNA sequencing datasets have been deposited in the Gene Expression Omnibus.

## **Results**

Here I present only the results with our PBMCs collection.

Notch signaling in T cells has been implicated in the pathogenesis of MS/EAE<sup>10-13</sup>. Our previous studies have shown that individual Notch receptor signaling in Treg cells licenses

inflammation in different tissues by promoting Treg cell destabilization and plasticity. Thus, Notch1 signaling drives Treg cell Th1 skewing and CD22-dependent Treg cell gut tissue homing to promote autoimmunity and inflammation<sup>14,15</sup>. In contrast, Notch4 is upregulated on lung Treg cells to promote their Th2 and Th17 cell skewing in allergic airway inflammation and Th1 skewing in lung viral infections, respectively<sup>16,17</sup>. Analysis of peripheral blood T cells revealed an increase in Notch3 expression, but not of other Notch receptors, specifically in Treg cells in patients with active MS (**Fig. 1a-c; Extended data Table 1**). Treg cell Notch3 expression was decreased in patients with inactive MS compared to those with active disease (**Fig. 1d,e**). Analysis of cerebrospinal fluid (CSF) T cells of patients with MS revealed a further increase in Notch3 expression in Treg cells compared to their matched counterparts in the peripheral blood (**Fig. 1f,g**). Notch3 expression was also increased but to a lesser extent in CD4<sup>+</sup> T conventional (Tconv) cells in the CSF of MS patients, while Notch3 expression was unchanged in the peripheral blood and CSF T cell subsets of control subjects (**Fig. 1g**). Notch3<sup>+</sup> Treg cells in the CSF expressed T helper (Th) cell cytokines implicated MS pathogenesis, including IL-17, GM-CSF and IFN $\gamma$ , indicative of their subversion into a Th cell-like phenotype (**Fig. 1h-i**). These results suggested a putative role for Notch3 in the pathogenesis of MS.

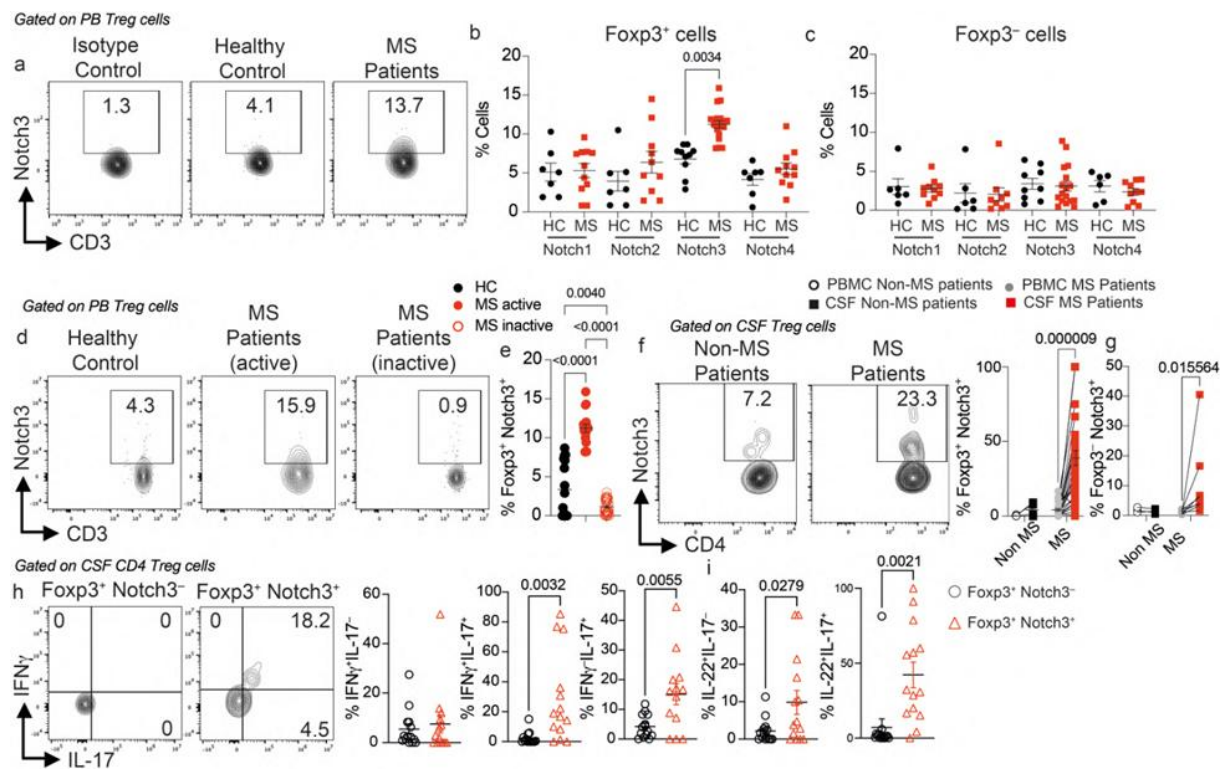
Overall, the identification of a cardinal role for Notch3<sup>+</sup> Treg cells in MS/EAE reconciles and integrates several previous findings including the respective roles of the microbiota, Treg cell destabilization and T cell-microglia interactions in disease pathogenesis. Importantly, our results indicate that the outcome of EAE is shaped by the balance of two opposing Treg cell populations: a pathogenic Notch3<sup>+</sup> Treg cell population enriched in MOG<sup>33-55</sup> reactivity that drives brain IL-17<sup>+</sup> T cell responses and simultaneously compromises a second, tissue-resident protective NPY1R<sup>+</sup> Treg cell population that normally restrains IFN $\gamma$ <sup>+</sup> and IFN $\gamma$ <sup>+</sup>GM-CSF<sup>+</sup> Tconv cells. Furthermore, our results show that the dichotomous IL-17<sup>+</sup> and IFN $\gamma$ <sup>+</sup>GM-CSF<sup>+</sup> CD4<sup>+</sup> T cell responses noted in MS/EAE<sup>38,40-44</sup> are linked to an underlying dynamic interaction between Notch3<sup>+</sup> and NPY1R<sup>+</sup> Treg cells. The distinct ontogeny of the respective Treg cell populations, the mechanisms by which they interact in disease pathogenesis including different subtypes of MS and means of manipulating their responses to promote long-term immune tolerance requires further investigations.

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## Figures



**Fig. 1. Increased Notch3 expression on peripheral and CSF Treg cells of MS patients.** a-c. Flow cytometric analysis of Notch3 expression in peripheral blood (PB) Treg cells and frequencies of the respective Notch receptor-expressing  $CD4^+$  Treg ( $Foxp3^+$ ) (a-b) and Tconv ( $Foxp3^-$ ) cells (c) in PBMC of healthy controls (HC,  $n=6$ ) and active relapsing-remitting multiple sclerosis (MS,  $n=10-17$ ) subjects. d,e. Flow cytometric analysis and cell frequencies of Notch3 expression in PB  $CD4^+$  Treg cells in HC ( $n=17$ ) and in MS subjects with active relapsing disease ( $n=17$ ) or in remission ( $n=30$ ). f,g. Flow cytometric analysis and frequencies of Notch3+ Treg and Tconv cells in the CSF of active MS subjects ( $n=27$ ) vs. non-MS control subjects ( $n=9$ ). h,i. Flow cytometric analysis and frequencies of IFN $\gamma$ , IL17 and IL22 expression by  $Foxp3^+$ Notch3+ or  $Foxp3^+$ Notch3-  $CD4^+$  T cells from CSF of MS patients ( $n=14$ ). Numbers in flow plots are percentages. Error bars are SEM. Each point denotes an individual. Statistical analysis: one-way ANOVA with Dunnett's post-hoc analysis (b,d) or two-way ANOVA (g) with Sidak posthoc analysis (f,g) or student two-tailed t-test (h,i).

## Extended Data

*Extended table 1: Multiple sclerosis patients cohort*

	MS patient (France) N=10	MS patient active (Italy) N=27	MS patient inactive (Italy) N=30	Non-MS patient (Italy) N=9
<b>Patient Demographics</b>				
Age-yrs (median, +/- SEM)	47+/-4.7	41.4+/- 2.6	37.9 +/- 2	70.5+/- 6.5
Sex (#, % female)	50%	62.96%	73.30%	66.60%
<b>Case Definitions/Criteria (#, %)</b>				
RRMS	70%	100%	100%	-
PP	10%	-	-	-
SP	10%	-	-	-
CIS	10%	-	-	-
dementia				18.18%
Narcolepsy				9.09%
Alzheimer				18.18%
hydrocephalus				27.27%
Heteroplastic Lesion				9.09%
Sepsis				9.09%
Backache				9.09%

## **DISCUSSION**





## **A. Resume of findings**

Animal models of MS have significantly enhanced our understanding of MS pathology. For instance, EAE has provided insights into the inflammatory processes leading to new lesion formation, while toxin-induced demyelination models have elucidated the fundamental steps of demyelination and remyelination in a non-inflammatory context. However, certain aspects of MS pathology, such as brain lesion formation, B-cell contribution, disease heterogeneity, and progression, remain inadequately modeled (Klotz et al., 2023; Procaccini et al., 2015).

Evidence suggests that MS inflammation is more widespread than previously believed (Woo et al., 2024). Beyond peripheral inflammation, there is compartmentalized inflammation within the CNS that current therapies do not target. Developing new models of this compartmentalized inflammation could lead to therapies addressing the chronic activation of microglia.

In Part I of the results, I demonstrate that lymphocytes from some MS patients induce smoldering lesions in the mouse spinal cord, which share characteristics with smoldering lesions in MS patients. This model also replicates some of the heterogeneity observed in MS patients and includes non-invasive tests to monitor lesion progression.

In Part II of the results, our collection of PBMS of PwMS helped reveal that a subpopulation of Treg cells from MS patients expresses Notch3. These Notch3+ Tregs are pathogenic in the chronic EAE model, promoting CNS inflammation through interactions with microglial cells.

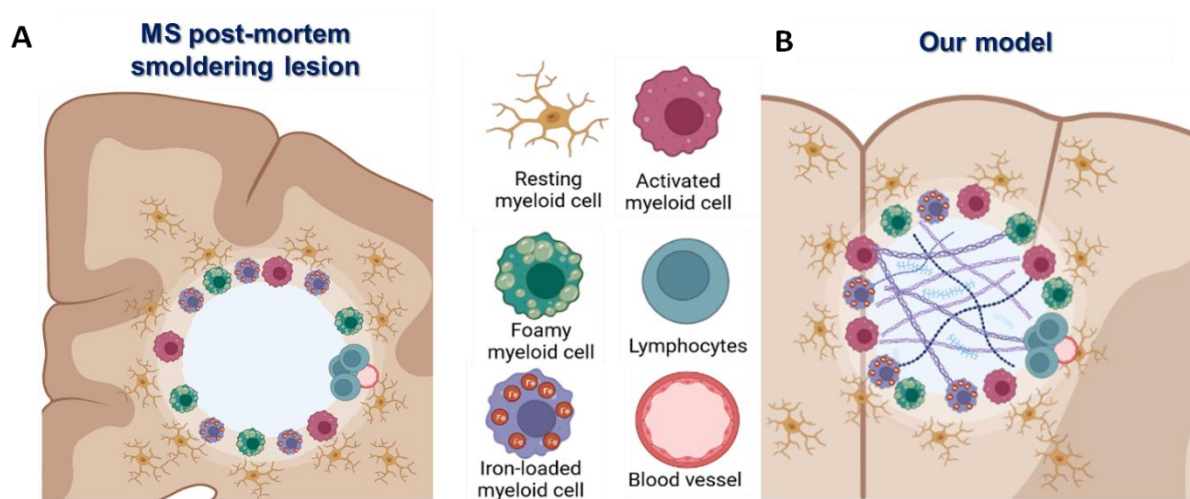
### **1. Advantages**

#### **a) A model of smoldering lesions**

With the combination of focal demyelination and MS inflammatory component, we have a new model that captures one of the components of PIRA progression: the smoldering lesion. The use of LPC-induced demyelination allows the study of demyelination and remyelination processes independently as they are separated in time. Combined with the graft

of MS patients' lymphocytes, it allows the study of remyelination failure in a patient's specific inflammatory context.

This model already provided evidence of dysregulation of CCL19 in patients' lymphocytes that could explain the heterogeneity of remyelination seen three weeks after the graft (El Behi\*, Sanson\* et al., 2017). When looking three months after the graft, we observe the formation of a lesion sharing features of the smoldering lesion in humans. These common features include an inactive center with a glial scar and an active border (Kuhlmann et al., 2017). The active border is composed of iron- or myelin-loaded myeloid cells, similar to those observed in MS (Absinta et al., 2021; Kuhlmann et al., 2017). We observe as well that MS patients lymphocytes localize in the perivascular space, which is characteristic of smoldering and active lesions (Absinta et al., 2021; Fransen et al., 2020; Frieser et al., 2022; Hsiao et al., 2021; Machado-Santos et al., 2018) (Figure 29). We also set up a range of behavioral and physiological indicators to non-invasively evaluate the progression of the lesion.



*Figure 29: Shared features between MS post-mortem smoldering lesion and our model. Schematic representation of the shared features between MS smoldering lesion and our model.*

### **b) A more suited model to test pro-regenerative molecules**

Most demyelination/remyelination models exhibit highly effective repair processes (Jeffery & Blakemore, 1995), allowing to assess only the acceleration of remyelination when using pro-remyelinating compounds. Additionally, these models provide an environment conducive to remyelination. In contrast, our model features an environment that is not favorable to remyelination. Therefore, using our model instead of the classic ones would more accurately

evaluate the potential of pro-remyelinating compounds. This approach allows us to test whether the compounds truly induce remyelination within an MS inflammatory context. With a stock of PBMCs from PwMS that we know will impede the remyelination process and induce a smoldering lesion, we could use this model at a bigger scale to test potential therapeutic molecules. In addition, we have two non-invasive behavioral tests to assess the lesion evolution, and also a physiological readout (the speed of conduction) that is correlated to the histopathological analysis. As a result, this model is well-suited for testing potential therapeutic molecules on a large scale.

### **c) A model to characterize and study microglial activation**

We also identify specific cells linked to smoldering lesions, such as MIMS-foamy and MIMS-iron, by comparing our ScRNAseq data with SnRNA-seq data from post-mortem human MS tissue (Absinta et al., 2021). Interestingly, the MIMS identified in our scRNA-seq also have similar patterns of DAM cells observed in EAE and cuprizone models (Barclay et al., 2024; Peruzzotti-Jametti et al., 2024). DAM activation is known to be pathogenic in chronic CNS diseases (Deczkowska et al., 2018). Notably, in the cuprizone model, tracking DAM activation during remyelination showed that DAM cells decrease their expression of DAM markers and increase their expression of homeostatic markers, suggesting a possible return to a homeostatic state (Barclay et al., 2024). Selectively depleting DAM cells in this model led to less severe demyelination but also resulted in diminished remyelination compared to control mice (Barclay et al., 2024). This highlights that although immune cell activation contributes to demyelination, it also plays a crucial role in the repair process. Additionally, a study comparing cells in active and chronic EAE stages found DAM cells in both, but persistent DAM in chronic EAE resembling microglial cell activation as MIMS in MS post-mortem tissue (Absinta et al., 2021; Peruzzotti-Jametti et al., 2024). This study also detected a shift between macrophages and microglia, with microglia emerging as the primary contributors to EAE pathology at the chronic stage (Peruzzotti-Jametti et al., 2024).

It has been demonstrated how myeloid cells activation (both pro-inflammatory and pro-regenerative polarization) can induce the repair process and that a shift from pro-inflammatory to pro-regenerative is necessary for efficient remyelination (Butovsky et al., 2006; Miron et al., 2013). This shift is induced by necroptosis of pro-inflammatory cells and repopulation by pro-

regenerative cells (Lloyd et al., 2019). However, in the referenced studies, microglia are not categorized as strictly pro- or anti-inflammatory (Absinta et al., 2021; Barclay et al., 2024; Peruzzotti-Jametti et al., 2024). Instead, the activation pattern of microglia is more complex than a simple pro-inflammatory/pro-regenerative dichotomy (Butovsky & Weiner, 2018; Zrzavy et al., 2017), often described as "intermediate microglia" (Zrzavy et al., 2017) exhibiting markers of both pro-inflammatory and pro-regenerative polarization. MIMS and DAM are also considered intermediate microglia. This raises the question of what drives this intermediate chronic activation. Our dataset indicates that our MIMS are not purely pro-inflammatory but are likely intermediate microglia. Thus, our model could be instrumental in characterizing these cells and understanding their induction, potentially leading to therapeutic targeting.

#### **d) Deciphering the role of different T cell population in the formation of smoldering lesions**

In addition to the use of the new humanized mice model, the study of PBMCs from PwMS allow to discover how lymphocytes from PwMS can induce CNS inflammation. Indeed, in results part II we show that Tregs expressing Notch3 are more present in PwMS and validated in EAE model their pathogenic role in CNS inflammation.

To assess which lymphocytes, induce smoldering lesions we could graft subpopulation of lymphocytes. For example, we know that in MS patients smoldering lesions CD8<sup>+</sup> T cells become TRM cells and form perivascular cuffs (Absinta et al., 2021; Fransen et al., 2020; Hsiao et al., 2021; Machado-Santos et al., 2018; Smolders et al., 2018). We could also assess if the graft of only CD8<sup>+</sup> T cells will induce perivascular cuffs and smoldering lesions. In addition, by using FACS analysis, we could find potential differences in lymphocyte subpopulation between patients and HD or among patients, we could therefore decide to graft different subpopulation of lymphocytes.

## **2. Limitation of our model**

Our model paves the way to better understand MS progression and a shift toward individualized therapies for PwMS. However, there are some limitations to our model.

The graft of non-activated lymphocytes has no effects on the lesion development (data not shown). Indeed, human T cell receptor (TCR), B cell receptor (BCR) and mouse MHC are not compatible due to species differences. Therefore, we have to activate human lymphocytes *in vitro* before the graft. Our model does not allow us to study reactivation of lymphocytes within the CNS, or part of the cross talk between adaptive and innate immune cells. However, the majority of cytokines, cytokines receptors and cytokines pathways are conserved between species. Therefore, there is communication between human lymphocytes and mouse myeloids cells through cytokine signaling.

In addition, when human lymphocytes either from HD or MS are grafted within the healthy mouse spinal cord (there is no prior demyelination), those lymphocytes do not stay in the CNS parenchyma, and therefore do not induce smoldering lesions. It suggests that both the appropriate pathological cells and a lesioned environment are required for the formation of these cuffs. It therefore suggests the inside-out hypothesis, first the initiation of injury in the CNS involving the OL leads to CNS inflammation and recruitment of peripheral immune cells (Kamm et al., 2014; Stys et al., 2012).

We used Nude NMRI (RjOrl: NMRI-Foxn1nu/Foxn1nu) mice, which are athymic and lack T cells, so the effects observed are likely due to human lymphocytes. However, these mice still have normal levels of B cells and natural killer cells, which might also influence the results. While lymphocytes, including CD4+ and CD8+ T cells, Tregs, and Bregs, are known to be crucial for remyelination (El Behi\*, Sanson\* et al., 2017; Bieber et al., 2003; Dombrowski et al., 2017; Pennati et al., 2020), Nude NMRI mice can still exhibit normal remyelination after LPC injection. This suggests that different mouse strains might affect the role of lymphocytes in remyelination, and that B cells, natural killer cells, and macrophages might compensate for the absence of T cells.

In the previous study, human lymphocytes were detected in both HD and MS grafted mice at three weeks after the graft (El Behi\*, Sanson\* et al., 2017). After 3 months, human lymphocytes persist only in mice grafted with lymphocytes from PwMS, indicating that specific signals are required to keep pathological lymphocytes within the tissue. In addition, if there is no prior lesion, PwMS lymphocytes are not detected either, suggesting that a lesioned environment signals are essential for the perivascular cuffs to be maintained. We grafted a mix of CD14-negative lymphocytes but could not identify which specific type induces smoldering lesions. Although the clearing method helped locate them around blood vessels, we couldn't

detect them on IHC analysis due to the thin cryosectioned slices (10-12  $\mu\text{m}$ ). Since these lymphocytes persist for three months post-graft, we hypothesize they are TRM cells. FACS sorting and/or grafting specific lymphocyte subpopulations could clarify this.

Our scRNA-seq analysis was conducted on a total of 2,000 cells from both wild-type and MS-grafted mice, and we did not capture all the cell types from the CNS (for example, astrocytes are missing). Given the relatively small sample size, these results should be interpreted with caution. The presence of a glial scar complicated the efficient dissociation of the tissue. Dissociating a glial scar typically requires more aggressive techniques, which, unfortunately, led to the loss of live cells necessary for scRNA-seq. To address this, we plan to employ SnRNA-seq. This method facilitates the effective dissociation of glial scar tissue by isolating nuclei rather than whole cells, thus circumventing the need for live cells and enabling a more comprehensive analysis.

### **3. A model to study progression**

The silent progression, also called PIRA, is the cause of disability accumulation in PwMS. PIRA are associated with the presence of black holes (non-contrast enhancing lesions) in MRI scans (Woo et al., 2024). It was previously believed that these black holes represented inactive lesions (Hamzaoui et al., 2023). However, recent findings revealed significant microglial activation within these lesions (Hamzaoui et al., 2023). This indicates that, despite the absence of further infiltration into the CNS, inflammation persists. And this inflammation is mediated by microglia as well as astrocytes (Hamzaoui et al., 2023). In addition to the activation of microglia and astrocytes, studies have demonstrated that although peripheral immune cell infiltration into the CNS is reduced (Frischer et al., 2009), meningeal and perivascular cuffs of lymphocytes remain. This inflammation is not targeted by current immuno-modulatory treatment, as these treatments do not affect progression (Woo et al., 2024). These data demonstrate that there is an activation within the CNS that drives disease progression and neurodegeneration.

Interestingly, in our model between three weeks and three months there is a shift, from macrophages, being more present in the earlier time (corresponding to active lesion) to microglia in the later time (smoldering lesion). This shift between macrophages and microglia is believed to have a role in the disease progression (Faissner et al., 2019; Healy et al., 2022),

interestingly this shift is also seen in chronic-EAE (Peruzzotti-Jametti et al., 2024). Our model appears to replicate the progression of an active demyelinating lesion, characterized by a high presence of peripheral immune cells, into a smoldering lesion, where CNS glial cells become more predominant.

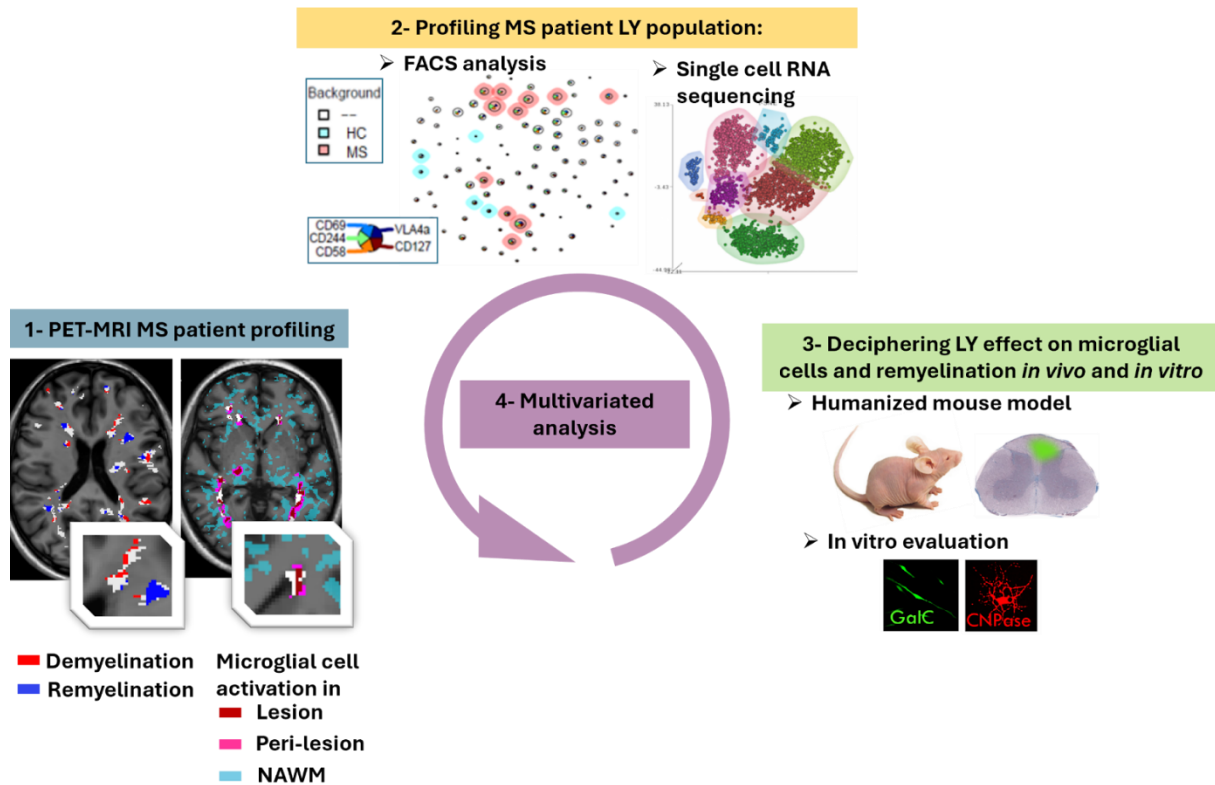
Increasing evidence suggests that astrocytes also play a significant role in MS. Firstly, they form a glial scar in demyelinated areas, which can hinder the remyelination process. Additionally, astrocytes express innate immune pathways, allowing them to participate in the inflammatory process (Ponath et al., 2018). Astrocytes also express TSPO (Hamzaoui et al., 2023; Nutma et al., 2019), meaning that when inflammation is assessed using TSPO PET-MRI scans, we are observing glial activation, including both microglia and astrocytes. This supports the notion that in progressive MS, the CNS, rather than the periphery, drives the inflammation. Moreover, there is a bidirectional crosstalk between microglia and astrocytes (Absinta et al., 2021; Healy et al., 2022). Using the SnRNA-seq data generated, we will analyze ligand–receptor pairs to infer intercellular communication. This will allow us to establish a cellular interactome between microglia, oligodendrocytes, astrocytes, and lymphocytes by examining the coordinated expression of specific gene subsets.

## **B. Perspective**

This project is part of a larger consortium study (Figure 30). In this project we will evaluate patients with either a relapsing-remitting or a progressive form of MS (secondary progressive or primary progressive), and determine their individual remyelination profiles using 18F-Florbetaben PET-MR (magnetic resonance), together with the extent of innate immune cells activation using 18F-DPA-714 PET. The relationship between individual remyelination capacities and neuro-inflammation will be investigated in vivo for the first time. In parallel, a high dimensional profiling of their lymphocytes will enable to define the lymphocytes subset associated with remyelination failure or success. Next, we will decipher experimentally how patients lymphocytes determine the remyelination success or failure, and trigger chronic activation of lesions, in an in vivo humanized animal model of MS lesion, elucidating more precisely the molecular and cellular actors in an in vitro culture system following both microglial activation and OPC differentiation steps. A powerful bio-informatic integrative analysis strategy will identify the lymphocytes population that inhibit repair. Novel biomarkers



potentially predicting bad remyelination capacities and disability worsening will be generated, and novel remyelination strategies will be opened.



**Figure 30: Overall project.** This project involves a consortium of neurologist, neurobiologist and biostatisticians with a complementary expertise in cerebral imaging, lymphocytes profiling and MS animal models.

Our team will assess the effect of MS patients' lymphocytes *in vitro* and *in vivo*. *In vitro* studies will focus on how lymphocytes can induce microglia activation or OPCs differentiation. Concerning the humanized model, the development of the smoldering lesion will be assessed through analysis at different timepoints between three weeks and three months, this will allow the determination the shift from the active lesion seen at three weeks (El Behi\*, Sanson\* et al., 2017) and the smoldering lesion we see at three months. In addition, deciphering the specific time, molecular and cellular mechanisms of lesion evolution will allow us to assess potential therapeutic molecules. To determine location of specific cells (such as MIMS, DAM and human lymphocytes), spatial transcriptomics will be done on the spinal cord of HD and MS lymphocytes grafted mice.

Another objective of the SMARTinMS project is to evaluate potential differences between Relapsing-Remitting Multiple Sclerosis (RRMS) and Primary Progressive Multiple

Sclerosis (PPMS) in the induction of smoldering lesions in a mouse model. To date, we have grafted lymphocytes from two PPMS patients who were not undergoing any treatment. Interestingly, we have not observed the development of smoldering lesions in mice that received lymphocytes from these patients (data not shown). This finding aligns with the "inside-out" model of progressive MS, which posits that disease progression in PPMS involves distinct mechanisms compared to RRMS.

### **C. Concluding remarks**

Disease progression in PwMS is driven by PIRA. The treatments of MS improved the quality of life of PwMS. However, PIRA being out of their range of action, ultimately the disease will continue to progress. PIRA are characterized by smoldering lesions and compartmentalized inflammation. We provide a unique *in vivo* model of smoldering lesions. This model will allow us to decipher the steps and mechanisms underlying the transition from a focal inflammation to a chronic compartmentalized inflammation of the CNS. Therefore, we could identify targets of PIRA, and test potential therapeutic molecules on this inflammation driving progression in PwMS that is a critical unmet need. Additionally, we have behavioral and physiological readouts that enable us to study the evolution of the lesions non-invasively. Notably, the physiological readouts correlate with histopathological findings, making them valuable for assessing potential therapeutic drugs effects. With the different scenario of outcomes in our model, we could screen potential therapeutic molecules, and provide an individualized therapy to PwMS, depending on their capacity to remyelinate or induce chronic inflammation within the CNS.



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