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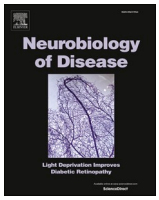

Review

An overall view of the most common experimental models for multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a complex chronic disease with an unknown etiology. It is considered an inflammatory demyelinating and neurodegenerative disorder of the central nervous system (CNS) characterized, in most cases, by an unpredictable onset of relapse and remission phases. The disease generally starts in subjects under 40; it has a higher incidence in women and is described as a multifactorial disorder due to the interaction between genetic and environmental risk factors. Unfortunately, there is currently no definitive cure for MS. Still, therapies can modify the disease's natural history, reducing the relapse rate and slowing the progression of the disease or managing symptoms.

The limited access to human CNS tissue slows down. It limits the progression of research on MS. This limit has been partially overcome over the years by developing various experimental models to study this disease. Animal models of autoimmune demyelination, such as experimental autoimmune encephalomyelitis (EAE) and viral and toxin or transgenic MS models, represent the most significant part of MS research approaches. These models have now been complemented by *ex vivo* studies, using organotypic brain slice cultures and in vitro, through induced Pluripotent Stem cells (iPSCs). We will discuss which clinical features of the disorders might be reproduced and investigated in vivo, ex vivo, and in vitro in models commonly used in MS research to understand the processes behind the neuropathological events occurring in the CNS of MS patients. The primary purpose of this review is to give the reader a global view of the main paradigms used in MS research, spacing from the classical animal models to transgenic mice and 2D and 3D cultures.

1. Introduction

Multiple sclerosis (MS) is the most frequent demyelinating disease of the central nervous system (CNS). It is a chronic, inflammatory, and degenerative disease with multifactorial etiology. The etymology of the term "multiple sclerosis" derives from the Greek word "skleros", which alludes to the formation of hardened areas in the lesions present in "multiple" portions of the CNS. In 1868, the French neurologist and

Abbreviations: MS, Multiple sclerosis; CNS, central nervous system; MRI, magnetic resonance imaging; EBV, Epstein-Barr virus; IgG, immunoglobulin G; RR, relapsing-remitting; SP, secondary progressive; PP, primary progressive; EAE, experimental autoimmune encephalomyelitis; TCR, T-Cell receptor; BCR, B-Cell receptor; iPSCs, induced Pluripotent Stem Cell; EB, ethidium bromide; OPCs, oligodendrocyte progenitor cells; BBB, blood brain barrier; LPC, lysolecithin; LPS, lipopolysaccharide; MBP, myelin basic protein; PLP, proteolipid proteins; MOG, oligodendrocytic myelin glycoprotein; CFA, complete Freund's adjuvant; Th, T-helper lymphocytes; TMEV, Theiler's murine encephalopathy virus; MHV, mouse hepatitis virus; SFV, Semliki forest virus; ABH, Biozzi antibody high; APCs, antigen-presenting cell; MHC, Major histocompatibility complex; IL, interleukin; PAMP, Pathogen associated molecular pattern; KO, knockout; GFAP, glial fibrillary acid protein; mAb, monoclonal antibody; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β1, cytokine transforming growth factor– β1; iNSCs, induced neural stem cells.

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pathologist Jean-Martin Charcot, combining the evidence of other scientists (Carswell, 1838; Cruveilhier, 1842; Rindfleisch, 1863), first described the clinical picture of MS as a "new disease of the nervous system" (Charcot, 1868). MS is characterized by lesions in different brain areas that show focal zones of demyelination and inflammation in the white matter that can be recognized by magnetic resonance imaging (MRI), while gray matter and cortical lesions can be visualized only with specific MRI sequences (Reich et al., 2018).

Almost 2.3 million people worldwide have been diagnosed with MS (Reich et al., 2018). The age of onset is typically between 20 and 40 years, but in about 20% of cases, the disease begins earlier (<20 years) or later (>40 years). The incidence of MS is higher in women, with a female: male ratio of about 2,5: 1. Pediatrics MS is rarer, but the prevalence is increasing worldwide: it is estimated that at least 5% of the total MS population are pediatric patients (Ghezzi et al., 1997). The prevalence of MS has steadily and dramatically increased in various regions over the past half-century, likely reflecting the impact of some environmental changes. As regards the geographical distribution, it has been observed that the prevalence of MS is correlated to latitude. The frequency of MS is high in countries with temperate climates, intermediate in the South of Europe and the USA, and low in Asia and Africa (Ghareghani et al., 2018, 2023).

MS is probably triggered by still unknown etiological factors in individuals genetically predisposed and exposed to environmental factors during adolescence. In particular, lifestyle, metabolism, reduction of vitamin D levels (less than 40-60 ng / mL), UV radiation, Epstein-Barr virus (EBV) infection, and tobacco smoke are all elements that individually or in combination may contribute to the development and outcomes of MS (Handel et al., 2011; Handel et al., 2015; Lucas et al., 2011; Munger et al., 2013; Munger et al., 2006; Patsopoulos et al., 2013). The existence of a predisposing environmental factor is suggested by the geographic distribution of the disease and by studies on populations that migrate between areas at different risk. Although MS is not hereditary, genetic predisposition plays an essential role in the onset of the disease. Among the genetic risk factors, the HLA-DR1*15:01 allele is the most frequently associated with MS in Caucasians (Patsopoulos et al., 2013).

Various data support the hypothesis that MS is linked to an autoimmune process, as suggested by the analogy between MS and chronic allergic encephalitis. Furthermore, the presence in the liquor of high levels of immunoglobulin G (IgG) and, even more frequently, of IgG oligoclonal bands suggests the production of antibodies within the CNS. All this is also supported by alterations in humoral and cellular immunity (Dobson and Giovannoni, 2019).

The onset of MS typically occurs in the vast majority (85-95%) of patients who experience acute neurological symptoms indicative of a mono or polysymptomatic alteration of the CNS. The most common initial presentations include optic neuritis, myelitis, and brainstem syndrome, and patients may have either a complete or partial recovery. This initial clinical manifestation is referred to as "clinically isolated syndrome". When a diagnosis of MS can be made after this type of onset, the clinical course is categorized as relapsing-remitting (RR). In the RR course, patients may experience multiple clinical relapses with varying frequencies. However, after an uncertain period of time from the onset, the clinical course may shift to secondary progressive (SP), characterized by a gradual worsening of disability. In 5-15% of patients, there is a progressive accumulation of disability from the onset of the disease, known as primary progressive (PP) MS. The most common symptom at the onset of PPMS is progressive spastic paraparesis. However, progressive visual loss, ataxia, and cognitive impairments are also possible. It's worth noting that both progressive clinical courses (PPMS and SPMS) can have superimposed relapses and/or gadolinium-enhancing lesions in MRI, indicating active disease and periods of neurological stability. Another distinct situation is when incidental MRI imaging suggests an inflammatory disease such as MS but without neurological signs or symptoms. This is called radiologically isolated syndrome (Dobson and Giovannoni, 2019; Lublin, 2014).

2. Neuropathology

The characteristic lesions of MS are represented by multifocal demyelinating lesions disseminated in the CNS, with a preference for the optic nerves, the periventricular areas, the corpus callosum, the brainstem, and the cerebellum. The symptomatology is various and heterogeneous and could follow the anatomical distribution of lesions. Typical manifestations include acute visual loss, weakness, paresis, and sensory alterations such as paresthesia. Some manifestations affect the brain stem, like dizziness, hearing loss, and facial sensory disorders (McGinley et al., 2021).

The typical lesions of MS were initially described in the pons, spinal cord, and periventricular region, but they can be observed in any area of the CNS. Other common localizations include the entire brainstem, the cerebellum, and the cortical and juxtacortical regions. Within these lesions, particularly in the relapsing course of the disease, an inflammatory infiltrate can be seen involving various immune cells, notably CD8+ T lymphocytes and B lymphocytes. Because of acute inflammation, damage to oligodendrocytes and demyelination occurs in the early stages of the disease. However, irreversible axonal loss is less evident initially and tends to increase as MS progresses.

During the progressive phase of the disease, acute inflammation becomes less prominent. An inactive core surrounded by activated microglia and macrophages inside the lesions becomes visible. Additionally, there is a higher proportion of B cells and plasma cells compared to the lesions observed in the relapsing course of the disease.

Furthermore, remyelination is a vital phenomenon present in all stages of MS but particularly prominent in the progressive course. However, this remyelination process is not efficient in fully restoring normal myelin.

Acute inflammation accompanied by demyelinating events corresponds to relapses, acute/subacute episodes characterized by the emergence of new neurological symptoms typical of an acute inflammatory demyelinating event that has not occurred previously, and/or the worsening of pre-existing symptoms. The damage to myelin alters the normal saltatory conduction, a mode of nerve impulse propagation where the electrical signal "jumps" from one node of Ranvier to the next rather than traveling continuously along the entire length of the axon. This type of conduction is facilitated by myelin, which insulates the axon and prevents ion leakage, thereby increasing the speed and efficiency of signal transmission.

When myelin is lost, the ability of the axon to conduct electrical signals is compromised, resulting in slower nervous conduction and neurological symptoms. These symptoms may exhibit complete or partial recovery and vary depending on the specific location of the active demyelinated lesion. The extent of recovery after a relapse is influenced by axonal damage and the loss of neuronal reserve, which leads to functional impairment in the affected area. This incomplete recovery contributes to long-term disability and disease progression (Dobson and Giovannoni, 2019; Raine and Scheinberg, 1988)

To understand the pathogenesis and develop new therapeutic strategies to treat MS, the use of in vivo, transgenic, and in vitro preclinical models is essential.

In vivo MS models are induced with several methods: the "experimental autoimmune encephalomyelitis" (EAE), the most widely used model, is usually induced by active immunization with myelin-derived proteins or peptides or by passive transfer of activated CD4+ T lymphocytes (Robinson et al., 2014).

In specific animal models, demyelination can be induced using toxins such as cuprizone and lysolecithin (Blakemore and Franklin, 2008). Additionally, transgenic models have been extensively investigated, particularly the T-cell receptor (TCR) (Olivares-Villagómez et al., 1998) and B-cell receptor (BCR) (Molnarfi et al., 2013) transgenic mouse lines that are specific to several myelin peptides.

Humanized models have also been generated expressing different HLAs and other human-derived molecules associated with the disease

(Madsen et al., 1999; Brynedal et al., 2007).

Ex-vivo and in vitro models are less used (Madill et al., 2016). Still, the new frontier of induced pluripotent stem cells (iPSCs) is opening new paths that will allow a greater understanding of this disease's molecular mechanisms (Mutukula et al., 2021).

This review will describe preclinical models, a powerful tool to investigate mechanisms underlying MS and fundamental to develop future effective therapeutic strategies. (Fig. 1)

3. The primary animal models utilized to investigate the etiology and pathogenesis of MS

The complexity of MS has made it very difficult to obtain a single animal model able to cover the broad spectrum of its manifestation; hence none of the models developed to date has been able to fully reproduce the entire spectrum of MS. Nevertheless, most of our knowledge regarding principal mechanisms of brain inflammation and progression of the disease has been gathered from preclinical studies on animal models. For MS, more than other diseases, it is crucial to be aware of the advantages and limitations of each animal model that can be used in preclinical studies. Models that use toxic agents to induce demyelination are crucial for studying the process of de- and remyelination as they ensure good reproducibility and well-defined anatomical localization of the demyelination area.

Ethidium bromide, lysolecithin, lipopolysaccharide, and cuprizone are among the agents mainly used to induce demyelination.

3.1. Ethidium bromide (EB)

Ethidium bromide preferentially interferes with DNA transcription (Kuypers et al., 2013) in glial cells rather than neurons or epithelial cells. Demyelination foci can be obtained by local administration of Ethidium bromide into specific white matter tracts. Optic neuritis can also be specifically induced by injecting the toxic agent directly into the subarachnoid space (Merril, 2009). Astrocyte and oligodendrocyte loss can be observed as early as 2-4 days after EB administration (Levine and Reynolds, 1999), while axons remain unaffected (Blakemore, 2005). Remyelination starts immediately after (Guazzo, 2005) with a significant increase in oligodendrocytes progenitor cells (OPCs) (about 10-fold ten days after EB injection) (Levine and Reynolds, 1999) and the migration of Schwann cells to the lesion location (Gilson and Blakemore, 2002). OPCs density in white matter is reduced 22 h after EB injection, increased after 48h, and remained significantly elevated up to 40 days after EB injection (Levine and Reynolds, 1999). The loss of astrocytes compromises the integrity of the blood-brain barrier (BBB), inducing the infiltration of inflammatory cells (Kuypers et al., 2013), causing an inflammatory response and possibly altering the permeability of the barrier to the invasion of Schwann cells, thus favoring their migration to the lesion site (Levine and Reynolds, 1999). The advantage of this model is that demyelination sites can be predicted, and the size of the lesion determined, as they depend on the concentration of Ethidium bromide used. Lesions of the hippocampus induced by local injection of EB can also be used as a model to study cognitive deficits (Goudarzvand et al., 2016).

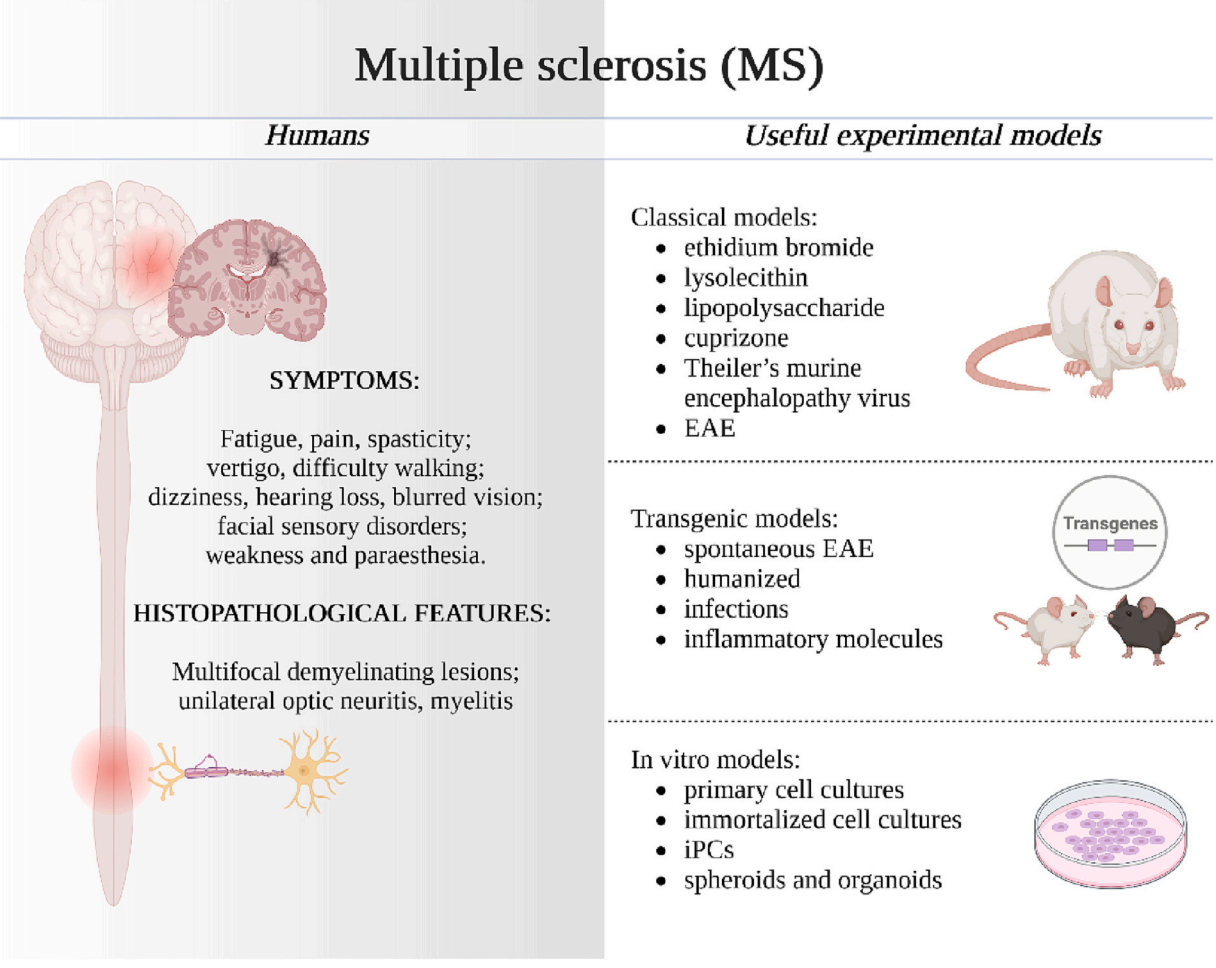

Fig. 1. Human MS and preclinical models.

3.2. Lysolecithin (LPC)

Lysolecithin is a toxic compound that induces demyelination by integrating into cellular membranes, where it increases their permeability and non-specific lipid disruption of myelin lipids (Plemel et al., 2018), and leads to complete loss of myelin markers in one-two day (Birgbauer et al., 2004). The accumulation of debris in the lesion site recruits T and B cells and activates microglia and macrophages (Procaccini et al., 2015). Remyelination begins after the elimination of debris about seven days after injection, and it is mainly due to the maturation of oligodendrocytes, whose progenitors are unaffected by the treatment and by Schwann cells (Blakemore and Franklin, 2008).

3.3. Lipopolysaccharide (LPS)

The administration of LPS can induce neuroinflammation, the key pathological event triggering the neurodegenerative process associated with many neurological diseases, including MS (Batista et al., 2019; Page et al., 2022). Mainly, LPS-induced demyelination resembles MS demyelinating lesions exhibiting the pattern III phenotype (Felts et al., 2005; Marik et al., 2007; Shen et al., 2009; Desai et al., 2016), reminiscent of virus- or toxin-induced demyelination rather than those consequent to autoimmunity (Lucchinetti et al., 2011).

Felts et al., 2005, found that injection of LPS resulted in an early invasion of inflammatory cells that was not limited to the injection site (dorsal funiculus) nor initially associated with apparent damage to the nerve fibers. Starting seven days after the LPS injection, however, a large, focal demyelinating lesion had formed within the dorsal funiculus. On day 14, mixed lesions containing demyelinated axons and debris-filled macrophages were even more evident, and remyelination appeared. After 28 days, many remyelinated axons were present, primarily due to Schwann cells.

LPS microinjection can be considered a suitable experimental model to reproduce the type III demyelinating lesions indicated by Lucchinetti et al., 2011.

3.4. Cuprizone

Cuprizone (oxalic acid bis [cyclohexylidene hydrazide]) is a copper-chelating compound whose oral administration induces oligodendrocyte apoptosis within few days, followed by activation of the innate immune cells in the brain and by demyelination of specific white and gray matter brain areas (Kipp et al., 2009; Praet et al., 2014). T- and B-cells do not play a crucial role in cuprizone-induced demyelination (Wolf et al., 2018; Hiremath et al., 2008). The cuprizone model is mainly used to recapitulate the key features of the RR form of MS as the intense demyelination observed after 5-6 weeks of oral administration of cuprizone, if normal chow is administered, is followed by partial or even complete remyelination (Zhan et al., 2020). On the contrary, if the treatment is prolonged for more than 12 weeks, the remyelination is significantly slower (Slowik et al., 2015; Lindner et al., 2009).

Although the exact mechanism by which cuprizone induces oligodendrocyte death is not fully understood, it has been suggested that, in these cells, cuprizone induces dysfunction of mitochondrial enzymes that, in turn, would cause oxidative stress and activate the apoptotic process (Miljković and Spasojević, 2013). Apoptosis of oligodendrocytes is observed as early as two days after the beginning of the treatment, while to detect demyelination, the treatment must be carried on for at least three weeks and correlates with significant microgliosis, astrogliosis, and axonal damage (Doan et al., 2013; Kipp et al., 2017). After six weeks of treatment, demyelination is diffused but is most prominent in the corpus callosum and posterior cerebellar peduncles (Bjelobaba et al., 2018).

The first week of treatment with cuprizone is characterized by the appearance of the first apoptotic oligodendrocytes - which can be observed as early as after two days of treatment - and by the alteration of the expression of several mRNAs that are preferentially expressed in these cells (Zhang et al., 2014). Other glial cells are also altered in the early phase of cuprizone intoxication, and in particular, activation of astrocytes and microglia has been observed (Zhan et al., 2020). Between weeks 1 and 3, the loss of oligodendrocytes and the activation of glial cells continues, and some sign of demyelination and acute axonal pathology becomes evident (Zhan et al., 2020). Between weeks 3 and 5, demyelination becomes visible, and OPCs are activated and recruited to replace the ones that have been lost (Brousse et al., 2015). Impaired motor coordination and behavioral deficits (mostly in spatial memory and social behavior) are now evident (Xu et al., 2009, 2010; Makinodan et al., 2009).

At this stage, if cuprizone-containing food is replaced with standard chow, the demyelination process stops, and remyelination occurs. If the treatment is prolonged for over 12 weeks, the demyelination becomes persistent and irreversible (Zhan et al., 2020).

While the cuprizone model represents a valid tool for studying the demyelination process and its consequences on MS, some of its characteristics must be considered. Sex, gender, and genetic background of the animals can significantly affect the extent of the cuprizone-induced changes (Valeiras et al., 2014; Taylor et al., 2009; Irvine and Blakemore, 2006), with the more reliable results observed in male mice. Wistar rats mostly show behavioral deficits without or with minor motor impairment (Valeiras et al., 2014).

3.5. Experimental autoimmune encephalomyelitis (EAE)

EAE belongs to the immuno-mediated animal models of MS. It represents the most used model of MS as it recapitulates the inflammation, demyelination, and neurodegeneration of the CNS typical of the pathology. It is also used as a model for autoimmune inflammatory diseases of the CNS (Gold et al., 2006; Steinman and Zamvil, 2005, 2006; Farooqi et al., 2010). Two distinct protocols can induce EAE: either by active immunization with myelin-derived proteins or peptides (active EAE), or by passive transfer of activated CD4+ T lymphocytes (passive EAE) (Robinson et al., 2014). Autoimmunity to CNS components in the active EAE model is induced through immunization with autoantigens derived from several peptides such as myelin basic proteins (MBP), proteolipid proteins (PLP), oligodendrocytic myelin glycoprotein (MOG), or other encephalitogenic epitopes (Delarasse et al., 2013). Active and passive EAE can be used to study different aspects of MS; in particular, passive EAE has been central to understanding the role of CD4+ T cells, although the loss of myelin is not always achieved with this model (Bjelobaba et al., 2018). An autoimmune reaction against myelin proteins in the CNS characterizes EAE. Experiments have been conducted in different animal species, such as guinea pigs (Freund et al., 1947) and monkeys (Kabat et al., 1947; Morgan, 1947); however, mice (Olitzky and Yager, 1949) and rats (Lipton and Freund, 1952) are the best models for mimicking acute monophasic, RR and chronic progressive EAE through immunogenetic, histopathological and therapeutic studies.

Immunization of mice with the immunodominant epitope of PLP ($PLP_{139–151}$) induces a RR disease course (Tuohy et al., 1989), while the immunodominant peptide $MOG_{35–55}$ induces a chronic form of the disease (Tompkins et al., 2002). Peptides are usually administered by subcutaneous injection. Potent adjuvants have been introduced in the solution containing the peptides to improve this protocol. One of them is the complete Freund's adjuvant (CFA). This antigen depot provides a slow, continuous, and prolonged release of active peptides from the inoculum and uses inactivated mycobacteria as a potent immune stimulant (Kabat et al., 1951). This preparation massively stimulates phagocytic absorption, antigen presentation, and subsequent activation and expansion of CD4+ T lymphocytes (Billiau and Matthys, 2001).

Three lymphocyte cell populations mediate the induction of EAE types: Type 1 and Type 17 t-helper CD4+ lymphocytes (Th1; Th17) and CD8+ T lymphocytes, with CD4+ lymphocytes as the main mediators.

After entering the CNS, these cells affect myelin proteins and mature oligodendrocytes, causing degradation of myelin, axonal damage, and apoptosis of oligodendrocytes (Sun et al., 2001; Bettelli et al., 2008; Huseby et al., 2001; Patel and Balabanov, 2012). The activation of macrophages and monocytes follows the migration of T cells (Yamasaki et al., 2014), which, in turn, release inflammatory substances, thus contributing to axonal damage and demyelination (Yamasaki et al., 2014; Ayers et al., 2004).

In the EAE model, the peak of demyelination – mainly confined to the spinal cord but also detected to a lesser extent in the optic nerve, cerebral cortex, and cerebellum – is reached 10-15 days after injection (Constantinescu et al., 2011; Plant, 2008). Also, axonal damage and generalized paralysis progressively develop following demyelination (Höflich et al., 2013). EAE is characterized by an ascending paralysis that starts from the tail (Batoulis et al., 2011), then affects the hind limbs, and finally compromises the forelimbs (McRae et al., 1992; Rangachari and Kuchroo, 2013; Berard et al., 2010). The chronic phase of the model, which can be observed about 30 days after infection, is characterized by inflammation-induced demyelination that primarily involves T cells, monocytes/macrophages, microglia proliferation in the brain stem, and perivascular inflammation in the spinal cord white matter (Procaccini et al., 2015).

Although this model is used to experimentally reproduce MS in animals, it presents some limitations and essential differences from human pathology. For instance, EAE is artificially induced through active or passive immunization, and the onset of symptoms occurs after a few weeks or even days after the immunization; in MS, on the other hand, sensitization to autoantigens is not artificially induced (Gran et al., 2007), can remain latent for years and be discovered only after the onset of evident symptoms. Furthermore, EAE is mainly characterized by spinal cord demyelination, while MS is a brain disease with marked cerebral and cerebellar cortex demyelination.

Another criticism of the EAE model is that it fails to reproduce some crucial characteristics of MS, especially those related to the activation of the immune system. At the same time, EAE is mainly mediated by CD4+ T cells, CD8+ T cells, and B cells play a predominant role in MS (t'Hart et al., 2011). CD8+ T cells, particularly, are emerging as critical players in MS (see for review Saxena et al., 2008). Evidence suggests that different subsets of CD8 T cells could be differently implicated in the mechanisms involved in tissue damage, and studies performed in animal models of MS have confirmed their crucial contribution to MS pathogenesis (Saxena et al., 2008). For all these reasons, EAE has sometimes been considered a misleading model of MS (Sriram and Steiner, 2005).

Considering the limitations, however, the animal model of EAE continues to play a key role as a front-line model in developing new therapeutic approaches for MS, representing the model that best reflects the autoimmune pathogenesis of MS.

Though essential for comprehending the mechanisms that underlie the pathology, the two described animal models of MS (EAE and cuprizone-induced demyelination) do not comprehensively reproduce all its characteristic features. While EAE reproduces the inflammation and the immune system's involvement, it is not helpful to study remyelination processes. On the other hand, the cuprizone model allows for better reproduce the RR form of MS, but its ability to induce chronic inflammation is weak. To overcome the flaws in the single models, it has been recently proposed (Gharagozloo et al., 2022) to combine cuprizone-induced demyelination with EAE, to recruit immune cells and induce the inflammatory response. The authors suggest that this combination may better recapitulate the main hallmarks of MS, thus obtaining a new model in which both inflammation and the ability to restore myelination can be incorporated.

3.6. Viral models of MS (TMEV)

Demyelination can be induced by viral infection, and this ability has been exploited to model MS. Three different viruses – Theiler's Murine Encephalopathy Virus (TMEV), Mouse Hepatitis Virus (MHV) and Semliki Forest Virus (SFV) – are mainly used to induce inflammatory demyelinating diseases that model different features of MS (Lassmann and Bradl, 2017). TMEV-induced encephalomyelitis is more persistent than those induced by MHV and SFV, making this model increasingly accepted and used (Pike et al., 2022). TMEV is an RNA virus of the Picornaviridae family able to induce neuropathology after intracerebral injection (Libbey and Fujinami, 2021). Among the three different strains of TMEV that have been categorized, only the TO subgroup, comprising BeAn and DA viruses, can be used to model MS, as the encephalomyelitis induced by the other two, GDVII and FA, is rapidly fatal in mice (Pike et al., 2022). After intracerebral injection, TMEV can infect CNS and persist in microglia, causing a chronic progressive demyelinating disease associated with adaptive inflammation in the CNS. One-two week after the injection of the DA strain of TMEV, mice develop acute encephalomyelitis, recovering in about two weeks. During the acute phase, transient limb flaccid paralysis can be observed. The second phase starts 1-2 months after infection and is characterized by chronic and progressive demyelinating disease of the white matter that mainly affects the spinal cord (Pike et al., 2022). During the acute phase, the DA strain of TMEV induces apoptosis in neurons, while in the chronic phase, apoptosis is induced in oligodendrocytes, thus causing demyelination (Tsunoda et al., 1997a, 1997b).

The TMEV model of MS also involves immune activation (CD4+ and CD8+ T cells), recapitulating B-cell specific disease phenotypes that are characteristic features of MS and has been revealed to be a good model of progressive MS (Pike et al., 2022). The model's main limitation is that TMEV can only induce encephalitis in susceptible strains of mice (Swiss, SJL) (Libbey and Fujinami, 2021).

3.7. Models of PPMS and SPMS

The animal models described in the previous paragraph are primarily used to model RRMS or SPMS. To date, very few models have been used to model PPMS, which has been proposed as a completely different form of the disease with differences in progression, clinical disabilities, and sensitivity to therapy (Correale et al., 2017). While TMEV has been proposed as a model for PPMS (Libbey and Fujinami, 2021), very recently, Wong and colleagues,2023, by administering the cerebrospinal fluid obtained by PPMS patients into the subarachnoid space of mice, were able to recapitulate in an animal model all the features of PPMS (forelimb motor deficits, demyelination, reactive astrogliosis, microglial activation, and axonal damage). They suggest that pathogenic antibodies transferred from PPMS patients to mice can play a unique role and might be crucial for understanding this form of MS.

A good model for reproducing PPMS and SPMS is represented by the Biozzi antibody high (ABH) mouse strain, which is of great importance for MS research thanks to its susceptibility to experimentally induced EAE and virus-induced demyelination (Amor et al., 2005). After subcutaneous injections with adjuvant spinal cord homogenate, these animals develop RR EAE, with weight loss, episodes of limb paralysis, demyelination axonal degeneration, and T $CD4^+$ lymphocytes infiltrates (Baker et al., 1990) during the acute phases, and secondary progressive EAE. Furthermore, when treated with the neurofilament light protein (NF-L), these animals develop inflammation and axonal degeneration at the spinal cord's level, leading to spastic paralysis (Huizinga et al., 2007). The clinical course of the symptoms in these animals after immunization mirrors what happens in human MS, in which the progressive form is observed principally in older patients (Scalfari et al., 2011). Indeed, after the immunization with the spinal cord homogenate, younger Biozzi ABH mice develop RR EAE that subsequently switches to the secondary progressive form. In contrast, older animals directly develop progressive EAE (Tutuncu et al., 2013). Interestingly, T-cell ablation in these animals fails to stop the course of the secondary progressive EAE, suggesting that the progression of the disease might be T-cells independent (Pryce et al., 2005); however, treatment with both

anti-CD20 and anti-CD8 and ablation of CD4+T cells were able to inhibit relapses (Sefia et al., 2017). Thus, the Biozzi ABH mouse strain is a valuable tool to investigate the molecular mechanisms underlying the progressive forms of MS and develop potential therapies to prevent the irreversible disability caused by progressive neurodegeneration in MS.

4. Transgenic mice models of MS

Considering the complex and, for a significant part, still uninvestigated etiology of MS, an increasing number of transgenic mice have been generated over the years to try to unravel the contribution of single molecules or cell type to the etiopathogenesis of the disease and provide new possible therapeutical targets.

4.1. Transgenic mice models of MS manifesting spontaneous experimental autoimmune encephalomyelitis (sEAE)

Some transgenic models spontaneously develop EAE; in others, proteins like PLP, MOG, and MBP are used to induce EAE. Furthermore, the injection of antigen-specific T cells and genetical manipulation of transgenic models that develop EAE are valuable methods for studying the mechanisms underlying MS. (Table 1)

- **Va2.3/Vb8.2**

CD4+ T cell responses to antigens of the white matter are thought to contribute to the pathogenesis of MS. Goverman et al., 1993, generated B10.PL transgenic mice Va2.3/Vb8.2 carrying the H-2^u haplotype that express a TCR specific for $MBP_{1\text{-}11}$. After immunization with MBP in CFA and pertussis toxin injection, these animals were highly susceptible to the disease. Some transgenic animals developed spontaneous EAE, with mononuclear infiltrates in spinal cord white matter. However, many animals recovered from the symptoms without relapses, suggesting that more factors other than a high number of MBP-specific T cells are needed for the onset of chronic EAE. Studying this transgenic model, Brabb et al., 1997, observed an increased incidence of spontaneous EAE in adolescent and young adult transgenic animals, and males are subjected to a higher risk of developing the disease spontaneously, suggesting that hormones can influence the onset of an autoimmune response. Furthermore, they demonstrated that the accumulation of transgenic T cells to the CNS is fundamental for the induction of the disease.

- **Va4/Vb8.2**

A year after Goverman, Lafaille et al., 1994, generated a transgenic mouse line Va4/Vb8.2 that expresses the H-2^u haplotype in which CD4+ T cells express a TCR specific for $MBP_{1\text{-}11}$ (T/R^+ H-2^u). By crossing these TCR transgenic mice with RAG-1-deficient mice, they obtained transgenic animals (T/R^- H-2^u) that present CD4+ T cells expressing the TCR transgene but no other lymphocytes to evaluate the pathological potential of the transgenic CD4+ T cells without the contribution of other immune cells. Starting from 2 months of age, with an average onset of 13 weeks, all the T/R^- H-2^u mice developed spontaneous EAE characterized by a rapidly progressing disease that starts with weakness and ends with paralysis of the hind and front legs. No remission was observed in any of the animals. In contrast with the high incidence of spontaneous EAE in T/R^- H-2^u mice, only 11% of T/R^+ H-2^u animals developed EAE by six months and 14% by 12 months. Although the absolute number of TCR transgenic CD4+ T cells was similar in both T/R^+ H-2^u and T/R^- H-2^u mice, T/R^+ H-2^u animals also expressed other immune cells without the transgene, including $CD4^-CD8^-$ B cells (30% of all lymphocytes), CD4+ T cells (5% of all T cells), and CD8+ T cells (3% of all T cells), suggesting a potential protective role of these cells towards the onset of EAE symptomatology, as already proposed by other authors (Kumar and Sercarz, 1993). A later study from the same authors (Olivares-Villagómez et al., 1998) showed that an early transfer of total splenocytes or purified CD4+ T cells from healthy donors could prevent the onset of EAE in T/R^- H-2^u mice, suggesting that CD4+ T cells that express endogenous TCR protect T/R^+ H-2^u from spontaneous EAE.

- **ND4**

Hemizygous ND4 transgenic mice, designed by Simons Johnson et al., 1995, carry 70 copies of a transgene encoding for DM-20, an alternative RNA splicing product of the PLP gene (Nave et al., 1987; Simons et al., 1987; Mastronardi et al., 1993). These animals develop a non-autoimmune PP demyelinating disease, often resulting in premature death. Studies carried out in these animals using non-invasive methods such as electroretinogram (PERG), MRI and optical coherence tomography (OCT), have revealed visual deficits and issues of the optic nerve (Enriquez-Algeciras et al., 2011).

- **2D2**

The pathogenic interaction between T and B lymphocytes is closely linked to developing pathological and/or autoimmune conditions, including MS, as seen by Bettelli et al., 2006. MOG-specific TCR transgenic mice, also known as TCR^{MOG} or 2D2 mice, express a TCR specific for $MOG_{33\text{-}55}$on CD4+ T cells. These animals exhibit spontaneous optic neuritis, and only a few of them develop encephalomyelitis (Bettelli et al., 2003); however, if cross-bred with MOG-specific Ig heavy-chain (IgH^{MOG}) mice show an increased incidence of spinal optical encephalomyelitis (Bettelli et al., 2006).

- **OSE**

IgH^{MOG} also known as Th mice, present B lymphocytes that produce antibodies with the heavy chain of a MOG-specific antibody (8.18C5) (Linington et al., 1988). IgH^{MOG} and TCR^{MOG} transgenic animals were crossed to obtain a TCR^{MOG}xIgH^{MOG} double-transgenic line, known as OSE, from opticospinal EAE. OSE mice developed spontaneous EAE, where lesions are almost only present in the optic nerve and spinal cord (Krishnamoorthy et al., 2006). Based on this evidence, Pöllinger et al., 2009, described a new transgenic SJL/J mouse model expressing a specific TCR for rat/mouse $MOG_{92\text{-}106}$ peptide in the context of the IA^s construct (TCR^{1640}), which were the first murine model to develop a RR spontaneous EAE. By crossing TCR^{1640} mice with IgH^{MOG}, the authors showed that female mice developed primarily a RR condition while males developed more often the PP form of EAE.

- **5B6**

The first description of the 5B6 transgenic mouse was made by Waldner et al., 2000. These transgenic animals with an SJL background express TCR α and β chains rearranged from encephalitogenic and non-encephalitogenic T-cell clones and specific to $PLP_{139\text{-}151}$. This study showed that transgenic mice expressing encephalitogenic and non-encephalitogenic TCR developed spontaneous EAE. The same authors tried to test the development of spontaneous EAE in the 5B6 transgenic mice model but on the B10.S background, which rarely develops spontaneous EAE, differently from the SJL background used in the previous study (Waldner et al., 2004). This study showed that antigen-presenting cells (APCs) cells in B10.S mice exhibit lower expression of major histocompatibility complex (MHC) II molecules and are less effective in stimulating transgenic 5B6 T cells in vitro. Another study showed that transgenic 5B6 TCR specific for $PLP_{139\text{-}151}$ develop EAE spontaneously and with great incidence; Zhang et al., 2008, observed that 80% of 5B6 mice develop EAE between 42 and 100 days of age. Histopathological investigations showed an intense infiltration of CD4+ T cells in the lumbar spinal cord, represented by an equal distribution of Th-17 and Th-1 cells (1:1). Other observations revealed that the lack of

Table 1
Overview of transgenic mouse models of MS.

Transgenic mice models of MS manifesting spontaneous experimental autoimmune encephalomyelitis (sEAE)		
Model	Clinical manifestations and histopathological features	Reference
Va2.3/Vb8.2	Spontaneous EAE with mononuclear infiltrates in spinal cord; increased incidence of spontaneous EAE in male adolescents and young adults; most animals recover, no relapses.	Goverman et al., 1993 Brabb et al., 1997
Va4/Vb8.2	Spontaneous EAE, progressive disease going from weakness to limb paralysis.	Lafaille et al., 1994
Va4/Vb8.2 Rag-1 deficient	Increased incidence of spontaneous EAE in Rag-1 deficient mice.	
ND4	Spontaneous EAE, non-autoimmune primary progressive demyelinating disease, often fatal; visual deficits and optic nerve degeneration.	Simons-Johnson et al., 1995 Enriquez-Algeciras et al., 2011
2D2	Spontaneous optic neuritis, some cases of spontaneous EAE.	Bettelli et al., 2006
OSE	Spontaneous EAE with lesions in optic nerve and spinal cord.	Krishnamoorthy et al., 2006
TCR^{1640}	Relapsing remitting EAE in females, primary progressive EAE in males.	Pöllinger et al., 2009
5B6	Spontaneous EAE.	Waldner et al., 2000
B7.2	Spontaneous demyelinating disease.	Fournier et al., 1997 Zehntner et al., 2003

Humanized mice as a transgenic model for multiple sclerosis		
Model	Clinical manifestations and histopathological features	Reference
HLA DR3.Aβo	Spontaneous EAE.	Mangalam et al., 2007
HLA DR3.AE^{o}	Severe EAE with inflammation and demyelination in spinal cord.	
Line 8	Impaired movement; chronic paralysis, CNS inflammation, perivascular infiltrates, with demyelination in the cerebellum after immunization. More severe phenotype in line 7.	Ellmerich et al., 2004; Ellmerich et al., 2005
Line7		
Hy^{+}-$DR2b^{+}$	Spontaneous severe EAE.	Gregersen et al., 2006
Hy^{+}-$DR2b^{+}$+$DR2a^{+}$	Reduction of the symptoms and clinical remission in the double transgenic line.	
HLA-DQB1*0602	Pathogenic autoimmunity against MOBP.	Kaushansky et al., 2009
HLA-DRB1*1501	Resistant to EAE development.	
CD20dbtg	Spinal cord injury in the ventrolateral tract (VLT), demyelination and axolysis.	Breakell et al., 2020
hu/m TNFR1-ki	Spontaneous EAE and demyelination.	Williams et al., 2018
DR2/TCR	Progressive disease going from weakness to limb paralysis, impaired movement and coordination after immunization with $MBP_{84\text{-}102}$ peptide.	Madsen et al., 1999
DR2/TCR/CD4		
DR2/TCR/$Rag2^{-/-}$	Spontaneous EAE.	

Exposure to environmental microbes such as viruses and bacteria in the occurrence of MS in transgenic mice models.			
Model	Virus	Clinical manifestations and histopathological features	Reference
huNSG	EBV	Increased CD4+ and CD8+ activation after EBV infection.	Zdimerova et al., 2021
β2M deficient animals	TMEV	Early onset, severe EAE. Lack in $CD8^{+}$ production, severe demyelination.	Koller et al., 1990; Begolka et al., 2001
MOG-HA	Influenza hemagglutinin (HA)	Inflammatory demyelinating lesions in the optic nerve, brain and spinal cord, after the transfer of the cytotoxic $CD8^{+}$ T cells.	Saxena et al., 2008; Cabarrocas et al., 2003

Inflammatory molecules involved in the etiopathogenesis of MS in transgenic models Cytokines and Chemokines		
Model	Clinical manifestations and histopathological features	Reference
GF-IL23	Spontaneous and progressive ataxia and cerebellar tissue damage, accumulation of inflammatory cells infiltrates.	Nitsch et al., 2019
GF23-2D2	Spontaneous EAE, myelitis, ataxia, microglial activation, B and T cells infiltrations.	Nitsch et al., 2022
GATA3-tg	Less severe EAE, reduced levels of anti-inflammatory cytokines.	Fernando et al., 2014
RORγtTg	No clinical symptoms; demyelinating lesions in spinal cord after TMEV infection.	Martinez et al., 2015
TNF-α tg	Spontaneous EAE with demyelinating course.	Taupin et al., 1997
IFN-γ tg	Hypomyelination, tremors.	Corbin et al., 1996
GFAP-IL-12	EAE after immunization with MOG peptide.	Pagenstecher et al., 2000
TGF-β1 mice	Increase susceptibility to immune-mediated EAE.	Wyss-Coray et al., 1997
TGF-β1 X Balb/c Byj's	Mononuclear cells infiltration in the spinal cord and the brain.	
GFAP-IL3	Low IL-3 expression causes a late-onset chronic motor disorder; High IL-3 expression causes a fatal early-onset acute neuroinflammatory disease.	Chiang et al., 1996
MBP- N51/KC	Strong microglial activation and activation of the blood brain barrier, hunched posture, rigidity, premature death.	Tani et al., 1996
MBP-CCL21	Tremors, ataxia, gliosis weight loss and premature death; Immune cells infiltrate in the spinal cord, cerebellum, and medulla.	Nagira et al., 1997
hGFAP-CCL2	Infiltration and accumulation of monocytes and lymphocytes in the CNS. Accelerated onset of the demyelinating disease after TMEV infection. Perivascular lesions and accumulation of mononuclear cells in the spinal cord.	Bennett et al., 2003
T/+ CCR2−/−	Reduced demyelination after immunization with MOG peptide.	Lagumersindez-Denis et al., 2017

functionality by natural regulatory T cells (nTregs) and the production of cytokines such as interleukin (IL)- 10 and IL-17could determine the development of age-dependent EAE in these mice.

- **B7.2**

The activation of APCs has been proposed to have a role in the induction of autoimmune diseases (Kissler et al., 2001). Pathogen-associated molecular pattern (PAMP) antigens activate PAMP receptors on APCs and lead to the expression of the costimulatory ligands B7.1 and B7.2 on these cells. These molecules interact with CD28 on T cells, triggering their activation and proliferation (Chambers, 2001). B7/CD28 interaction appears crucial for the induction of EAE (Khoury et al., 1995); furthermore, increased B7 expression has been detected in MS patients (Windhagen et al., 1995). Fournier et al., 1997, generated a transgenic mice strain that overexpressed B7.2 protein in microglia and lymphoid cells under the H-2K^{b} promoter. In another study, the same group reported the onset of spontaneous demyelinating disease in this transgenic line (Zehntner et al., 2003). Furthermore, by generating B7.2 transgenic mice lacking mature T cells (TCR$\beta^{-/-}$) or CD28 expression (CD28$^{-/-}$), they were able to demonstrate that an elevated expression of B7.2 on APCs in the nervous system together with T cells are needed for the induction of the disease. Brisebois et al., 2006, subsequently proved that the demyelination in this model is after an inflammatory response evoked by CD8+ T cells in the CNS.

4.2. Humanized mice as a transgenic model for multiple sclerosis

From the histopathological point of view, it has been proven that a lymphocytic infiltrate composed of T and B lymphocytes, plasma cells, and dendritic cells are the agents that support the active demyelinating lesions (Henderson et al., 2009). The involvement of CD4+ T lymphocytes as initiators of the inflammatory process in MS is known (Malpass and Multiple sclerosis, 2012). These cells then recruit other cells involved in adaptive immunity, particularly CD8+ T lymphocytes, that can be observed in the perivascular infiltrate and at the margins of the lesions (Traugott et al., 1983), resulting in myelin and axonal damage. The recurrence of MS in some families allowed the identification of HLA haplotypes (HLA-DR2) as predisposing genetic factors for developing the disease. The advent of transgenic mice expressing human class II HLA molecules helped to understand the mechanisms of susceptibility for the development of MS in more detail.

- **HLA DR3.Aβo** and **HLA DR3.AE^{o}**

By mating HLA-DR3 transgenic mice with MHC class II$^{\Delta/\Delta}$ (AE^{o}) knockout (KO) mice lacking all four conventional Class II MHC genes (Aα, Aβ, Eα, and Eβ), and mice Aβo, which possess a non-functional Aβ gene as well as Eα, the transgenic mice lines HLA DR3.Aβo and HLA DR3.AE^{o} were generated that lack complex endogenous molecules of class II.

These mice possess T cells that express HLA-DR molecules on their cell surface; therefore, these mouse CD4+ T cells behave like human T cells in the context of class II expression on their cell surface. Transgenic DR3.AE^{o} mice develop EAE more severely than DR3.Aβo mice, and they manifest increased inflammation and demyelination in the brain and spinal cord, concluding that T cells expressing MHC class II genes play an important role in EAE immunopathogenesis (Mangalam et al., 2007). What emerges from Friese et al., 2008, is that MHC Class I genes and CD8+ T cells contribute to the pathogenesis of MS. This has been deduced from the formulation of transgenic models expressing the MS-associated MHC class I alleles HLA-A(*)0301, HLA-A(*)0201, and a myelin-specific autoreactive TCR derived from a CD8+ T cell clone from an individual with MS (Friese et al., 2008). This study led to the assumption that HLA-A(*)0301 has a pathogenic role while HLA-A*0201 has a protective role. DRB1*1501, encoded by the DR15 haplotype (such as DRB5 and DQ6), is one of the earliest genetic risk factors for susceptibility to MS development. Humanized HLA-DR15 transgenic animals were proven to be helpful in studying specific phenomena possibly linked to the variety in the onset and clinical manifestations of MS. Epitope spreading is the phenomenon in which specific T cells modify their specificity and start to target different epitopes after chronic inflammation and tissue damage. This mechanism is thought to be involved in MS progression and relapse, and it could be partially responsible for the difficulty in translating to human immunotherapies for relevant epitopes studied in animal models (Tuohy et al., 1997).

- **Line 8 and line7**

To investigate this aspect, Ellmerich et al., 2004, generated a humanized transgenic line (line 8) with a null mutation for H2-A that expresses the human class II HLA-DR15 and a human HLA-DR15-restricted TCR specific for MBP$_{85\text{-}99}$ epitope. These mice maintain some endogenous TCR α chains that allow the investigation of potential epitope spreading for other HLA-DR15-restricted epitopes during EAE. Line 8 mice spontaneously show less movement ability compared to controls and after immunization with MBP$_{85\text{-}99}$/CFA followed by pertussis toxin, some of the animals develop chronic paralysis with CNS inflammation and perivascular infiltrates, with demyelination in the cerebellum. The authors identified different patterns of epitope spreading after 40 days from immunization with MBP$_{85\text{-}99}$, as TCR started to recognize also 80-99, 85-105, and 91-105 overlapping peptides. The epitope spreading was more evident in the animals who developed the most severe symptoms, suggesting that epitope spreading can influence the severity and progression of the disease. In line 8, the expression of the human TCR is restricted to CD4+ and results in a milder phenotype. However, Ellmerich et al., 2005, developed another transgenic line, called line 7, expressing the same HLA-DR15-restricted TRC in both CD4+ and CD8+ T cells, resulting in a more severe phenotype with 60% incidence of spontaneous paralysis, inflammation, frequent demyelination and axonal degeneration in cerebellum and spinal cord.

- **Hy.2E11 TCR (Hy), DR2a, DR2b**

As mentioned, the HLA-DR2 haplotype represents a genetic predisposition factor to MS. The HLA-DR2 haplotype presents two DR alleles, the DRB5*0101 (DR2a) and DRB1*1501 (DR2b), that are in strong linkage disequilibrium (Fernandez-Viña et al., 1991) and the region around DRB1 and DRB5 loci have been identified to predispose to MS (Lincoln et al., 2005). Gregersen et al., 2006, generated humanized transgenic mice that express Hy.2E11 TCR (Hy) and DR2a, DR2b, or both alleles to explore their contribution to provoking EAE (Wucherpfennig and Strominger, 1995). Hy TCR derives from patients affected with MS and recognizes MBP$_{85\text{-}99}$ presented by DR2b and the EBV$_{627\text{-}641}$ peptide from a DNA polymerase of the EBV presented by DR2a (Lang et al., 2002). The authors observed that Hy^{+}-DR2b^{+} spontaneously developed severe EAE, with a reduction in the symptoms if the DR2a transgene was also present. Furthermore, about 20% of the Hy^{+}-DR2b^{+}+DR2a^{+} transgenic mice underwent spontaneous clinical remission. These results suggest that the DR2b allele mediates the disease, while the DR2a allele acts as a genetic modifier of this disease.

- **DRB1*1501- DRB1*1502- DQB1*0601- DQB1*0602-Tg mice**

Kaushansky et al., 2009, tested DRB1*1501 and DQB1*0602 alleles or their combination in one HLA class-II transgenic mice (DRB1*1501-Tg and DRB1*1502-Tg, DQB1*0601-Tg and DQB1*0602-Tg, in addition, HLA(DRB1*1501×DQB1*0602) F $_{1}$ double Tg mice) to evaluate the sensitivity to EAE induced by the myelin-associated oligodendrocytic basic protein (MOBP). The results revealed that HLA-DQB1*0602 mice showed pathogenic autoimmunity against MOBP compared to HLA-DRB1*1501 mice, which are resistant to EAE

development. In contrast, previous studies identified DQB1*0601 as protective (Amirzargar et al., 1998; Marrosu et al., 1992).

- **hFcγR**

Mice immunized with a mouse monoclonal antibody (mAb), specific to MOG ($hMOG_{35-55}$) exacerbates encephalomyelitis in C57BL/6 mice (Bansal et al., 2013); based on this evidence, to assess the ability of MOG-specific antibodies derived from human patients affected by MS to exacerbate EAE, C57BL/6 mice were used to create the humanized model hFcγR (Khare et al., 2018). These animals express the Fc gamma receptor (FcγR), a receptor for the Fc portion of IgG; they lack the murine FcγR and express hFcγRI, $hFc\gamma RIIA^{R131}$, $hFc\gamma RIIB^{I232}$, $hFc\gamma RIIIA^{F158}$ and hFcγRIIIB under the control of their human endogenous regulatory elements. This research showed that immunization of mice with chimeric antibodies generated by fusing the VH and VL domain genes from MOG-specific hybridomas (1005 and 1011) with CH and CL genes (Cκ) derived from human IgG1 exacerbated EAE, demonstrating the pathogenicity of these chimeric antibodies in hFcγR mice.

- **CD20dbtg**

B cells are involved in the pathogenesis of human MS. Further to investigate the involvement of B cells in MS, the double transgenic mice huCD20xHIGR3 (CD20dbtg), which express the human CD20, a surface antigen of B lymphocyte (huCD20), have been used to assess the effect of the anti-human CD20 mAb obinutuzumab (OBZ) on CNS histopathology in EAE induced by MP4 (fusion protein consisting of MBP protein and the three hydrophilic domains of PLP) (Breakell et al., 2020). The treatment with OBZ resulted in a significant reduction in spinal cord injury in the ventrolateral tract (VLT), demyelination, and axonal lysis; in addition, it was observed a significant increase of remyelination. Confirming the importance of B cells in the clinical course of MS.

- **hu/m TNFR1-ki**

Humanized chimeric knock-in mice for Tumor necrosis factor receptor type 1 (TNFR1) hu/m TNFR1-ki, are useful for evaluating the effectiveness of human TNFR1 selective antibodies ATROSAB and H398. Subsequently to the immunization with the MOG protein and the development of EAE, mice treated with ATROSAB showed a reduction in the severity of the disease and a reduction in the demyelination typical of EAE (Williams et al., 2018), suggesting the importance of the TNF-α signaling pathway in the severity of the disease.

- **DR2/TCR, DR2/TCR/CD4 and $Rag2^{-/-}$ mice**

In the animal model of Madsen et al., 1999, the transgenic HLA-DR2 mice model expresses the DRA*0101 and DRB1*1501 genes under the control of a mouse MHC class II promoter, the TCR receptor derived from a patient with MS and specific for the MBP bound to HLA-DR2 (MBP_{84-102}) peptide 13, 14, and the human co-receptor CD4. After immunization with the peptide MBP_{84-102}, double transgenic mice DR2/TCR and triple-DR2/TCR/CD4 transgenic mice developed a disease characterized by tail weakness progressing to paralysis and hind leg incontinence. Other symptoms were observed, such as impaired coordination, balance, slow movement, paralysis of the front limbs, spasticity, or drowsiness. When they crossed their single and double transgenic models with $Rag2^{-/-}$ mice, they observed that DR2/TCR transgenic $Rag2^{-/-}$ mice developed the spontaneous disease, while no clinical signs of disease were shown by TCR or DR2 single-transgenic $Rag2^{-/-}$ mice.

4.3. Exposure to environmental microbes such as viruses and bacteria in the occurrence of MS in transgenic mice models

Exposure to environmental microbes is one of the possible etiological causes of autoimmune diseases. One of the factors to consider is molecular mimicry; Wucherpfennig and Strominger, 1995, showed that some microbial peptides are structurally like the MBP protein and able to stimulate MBP-specific T cell clones, generating autoimmunity effectively. In this study, among the 129 peptides synthesized, seven viral and one bacterial successfully mimicked the immuno-dominant MBP_{85-99} peptide. SFV induces encephalomyelitis and demyelination in the brain of C57Bl6/J (B6) mice, mimicking the MOG protein (Mokhtarian et al., 1999). In addition, Massilamany et al., 2010, showed that an epitope derived from *Acanthamoeba castellanii* (a protozoa) induces symptoms of EAE in SJL/J mice which develop chronic RR paralysis, mimicking the PLP protein. Considering this evidence, it is interesting to evaluate the role of some viruses on the development of EAE in transgenic models.

- **huNSG**

The NOD-scid IL2 receptorγ-chain-deficient mouse (NSG) lacks mature T and B lymphocytes, NK cells, macrophages, and dendritic cells. After human hematopoietic cells transplantation (huNSG), these animals develop a functional and efficient immune system capable of reproducing human immunity. In the field of MS research, these animals helped investigate the role of HLA-DR15, which, as mentioned above, represents the genetic risk factor most associated with MS (Patsopoulos et al., 2013). Zdimerova et al., 2021, reconstituted huNGS mice with HLA-DR15 from positive donors in blood and spleen. HLA-DR15-positive animals displayed higher activation of CD4+ and CD8+ T cells than controls at steady state and after immunization with EBV. These results suggest that HLA-DR15 positive hematopoietic cells progenitors have a role in the reconstitution of active T cells in huNSG mice. Furthermore, CD8+ T cells significantly increased after EBV sensitization, and the viral load was higher in HLA-DR15-positive animals, suggesting an enhanced susceptibility to the infection.

- **β2-microglobulin (β2M) deficient animals**

As mentioned above in the text, TMEV is often used to induce EAE in animal models, and SJL/J mice are the most susceptible strain to develop demyelinating disease after TMEV intracerebral inoculation (Miller et al., 1987). β2-microglobulin (β2M) deficient animals don't produce CD8+ T cells and cannot evoke a class I-restricted response (Koller et al., 1990). Begolka et al., 2001, used SJL/J mice deficient for β2M to investigate the role of CD8+ T cells in the progression of the demyelinating disease. These animals show a faster and more severe disease than control animals after TMEV inoculation, suggesting a possible protective and regulating role of CD8+ cells in the demyelination induced by TMEV.

- **MOG-HA and CL4 TRC**

The role of CD8+ T cells in MS pathogenesis is still debated, and contrasting results emerged from research in animal models. To better understand if CNS-infiltrating CD8+ T cells could contribute to the loss of oligodendrocytes and demyelination observed in MS, Saxena et al., 2008, using a transgenic mouse model that selectively expresses influenza hemagglutinin (HA) under the control of MOG promoter as a neo-self-antigen on oligodendrocytes (MOG-HA). Crossing MOG-HA animals with the CL4 TRC transgenic mice, which presents HA-specific TCR on most CD8 T cells, resulted in immune tolerance, with no activated CD8+ T cells, and none of the animals developed CNS-related symptomatology (Cabarrocas et al., 2003). When activated cytotoxic CD8+ T cells were transferred to the MOG-HA mice, the animals developed inflammatory

demyelinating lesions in the optic nerve, brain, and spinal cord. This result demonstrated that CD8+ T cell action specifically directed to oligodendrocytes could produce demyelinating lesions like those presented by MS patients.

4.4. Inflammatory molecules involved in the etiopathogenesis of MS in transgenic models

Some inflammatory cytokines and chemokines are crucial in the etiopathogenesis of autoimmune diseases such as MS.

- **GF-IL23**

Some evidence indicated that IL-23, (composed of IL-12Rβ1, which binds to the p40 subunit, and the IL-23R unit, which binds to p19) might be involved in the pathogenesis of MS (Hiltensperger and Korn, 2018)

To better understand the role of this inflammatory cytokine, Nitsch et al., 2019, generated a new transgenic mouse model "GF-IL23" with the astrocyte-targeted expression of the IL-23 subunits, IL-23p19 and IL-23p40, under the transcriptional control of the glial fibrillary acid protein (GFAP) promoter.

These mice develop spontaneous and progressive ataxia and cerebellar tissue damage by accumulating inflammatory infiltrates with a high percentage of B cells, clarifying the key role of IL-23 in neuroinflammation.

- **GF23-2D2**

A few years later, the same researchers studied IL-23 in the mouse model GF23-2D2, expressing a T cell receptor specific to the MOG protein (Nitsch et al., 2022) (the transgenic model 2D2 was also mentioned earlier).

GF23-2D2 mice develop spontaneous EAE with myelitis and ataxia, strong activation of microglia characterized by aggregates of B cells, and diffuse infiltration of T cells into the parenchyma, further confirming the role of IL-23 in the pathogenesis of MS.

- **GATA3-tg**

Type 2 t-helper (Th-2) cells secrete IL-4, IL-5, IL-6, IL-10 andIL-13). The Th-2s are often mentioned in studies that refer to MS and EAE (Tsunoda et al., 2005; McGeachy and Anderton, 2005). The Gata binding protein-3 (GATA3), a member of the Zinc-finger transcription factors, is the primary regulator of the protein Th-2 as it determines its differentiation. The GATA3-tg transgenic mouse model (mice that overexpress this transcription factor) is used to evaluate the progression of EAE induced by the MOG_{35-55} peptide (Fernando et al., 2014). This study showed that GATA3-tg mice exhibit a less severe EAE (in both sexes considered) compared to wild-type, and reduced levels of anti-inflammatory cytokines, confirming and defining the critical role of the Th-2 lymphocytes subpopulation.

- **RORγtTg**

Another fundamental cell type implicated in immunity and inflammation are the Th-17 lymphocytes which produce the IL-17. For the differentiation of these lymphocytes, the presence of the transcription factor of the orphan receptor related to retinoic acid (ROR) γt is essential. TMEV could determine demyelination, and IL-17 is involved in the susceptibility to the infection from this virus (Hou et al., 2009); Based on this assumption, Martinez et al., 2015, generated RORγtTg mice, and infected them with TMEV. During the acute phase of infection, the transgenic mice and their isotypic controls (C57BL/6) did not show signs of demyelination. In contrast, after two months from infection, histological signs of the disease are well detectable in transgenic animals, showing severe inflammatory demyelinating lesions in the spinal cord. However, these animals do not show clinical symptoms of the disease.

- **TNF-α tg**

As with other inflammatory mediators, tumor necrosis factor alpha (TNF-α) is implicated in different pathological conditions, including MS. Taupin et al., 1997, used transgenic mice in which this cytokine is expressed under the control of a 9.6-kb 5' MBP promoter showing that in comparison with non-transgenic controls, transgenic mice develop a spontaneous EAE with demyelinating course. Akassoglou et al., 1997, expressed a wild and mutant form of TNF-α in transgenic mice astrocytes or neurons; it emerged from this study that wild-type TNF in transgenic mice causes a neurological disorder with CNS inflammation and degeneration characteristics; moreover, the expression of this cytokine at the level of astrocytes determines similar symptomatology.

- **IFN-γ tg**

Transgenic mice expressing interferon gamma (IFN-γ) under transcriptional control of the MBP gene, present typical features of hypomyelinating disorders (such as tremors); compared to the control conspecifics, these mice have a much lower amount of myelin in the CNS (Corbin et al., 1996).

- **GFAP-IL-12**

IL-12 is a proinflammatory cytokine involved in the induction of Th-1 response and there is evidence that the expression levels of this cytokine are impaired in MS patients (Rohowsky-Kochan et al., 1999). To better understand the role of this molecule in the pathogenesis of MS, it was generated a transgenic mouse line that expressed both IL-12 subunits (p35 and p40) under the promoter of GFAP on astrocytes (Pagenstecher et al., 2000). These animals develop consistent perivascular and parenchymal inflammatory lesions characterized mainly through CD4+ and CD8+ T cells and NK cells infiltrates and strong inflammatory response, with increased TNF-α, IFN-γ, and IL-1 expression. In GFAP-IL-12 animals, EAE induced after immunization with MOG peptides occurred earlier than in the non-transgenic controls; however, the severity of the symptoms was not increased. These results suggest a potential involvement of IL-12 In the susceptibility to the disease but not in the clinical course and severity.

- **TGF-β1 mice**

Cytokine transforming growth factor-β1 (TGF-β1) increased in the blood of MS patients (Link et al., 1994). When transgenic mice overexpress TGF-β1 they are more likely to develop EAE (Wyss-Coray et al., 1997). Mouse spinal cord homogenate was emulsified in an equivalent proportion of CFA containing Mycobacterium tuberculosis H37 Ra and administered to controls and transgenic mice to trigger the EAE (Wyss-Coray et al., 1997). Ten days after the immunization, almost 70% of the immunized transgenic mice showed clinical manifestations of the disease. Moreover, GFAP-TGF-β1 mice, a strain susceptible to the development of EAE, and a resistant strain (Balb/c Byj) were back-crossed to evaluate if the TGF-β1 was able to determine an enhanced susceptibility to EAE in these mice. The findings revealed that Balb/c Byj's spinal cord and brain had a much higher level of mononuclear cell infiltration than the control group, demonstrating that TGF- β1 overexpression can encourage CNS inflammation.

- **GFAP-IL3**

MS have been linked to severe macrophage and microglia activation in the CNS (Airas and Yong, 2022). IL-3 promotes microglial activation and proliferation (Sugita et al., 1999). An innovative experimental paradigm to investigate the function of macrophages and microglia in

the pathogenesis of CNS demyelinating illness is represented by the GFAP-IL3 transgenic mice generated by Chiang et al., 1996. High IL-3 expression levels caused a fatal early-onset acute neuroinflammatory disease. In contrast, low IL-3 expression levels were linked to the emergence of a late-onset chronic progressive motor disorder in these transgenic animals. These results suggest that dose-related consequences were depending on the expression level of the transgene encoding IL-3, which can activate microglial cells and determine different grades of CNS inflammation.

- **GFAP-IL-6**

Campbell et al., 1993, generated a transgenic mouse in which the IL-6 expression was directed toward astrocytes using an expression vector derived from the mouse GFAP gene. In these mice, high levels of IL-6 (GFAP-IL6) cause some of the main characteristic traits of MS, like neurodegeneration and astrocytosis. Interestingly other researchers found that IL-6KO mice are resistant to the development of EAE (Eugster et al., 2001), clarifying the involvement of this cytokine in a multiple sclerosis-like disease. In the study of Giralt et al., 2013, EAE was induced by active immunization of mice with $MOG_{35–55}$ peptide. Crossing mice with overexpression of IL-6 and mice lacking IL-6 generated four different generations of littermates: wild-type mice showed leukocyte infiltration, demyelination, and gliosis.

IL-6 KO mice showed, as others, resistance in the development of EAE; GFAP-IL6 mice manifested severe ataxia; GFAP-IL6-IL-6 KO developed less severe ataxia. These data suggest that the production of IL-6 at the central level might predispose to an autoimmune response to an antigen of the CNS without peripheric IL-6 production.

To investigate the molecules responsible for the recruitment of leucocytes in the CNS, an event of fundamental importance in the pathogenesis and progression of MS, the role of different chemokines was investigated using specific transgenic mice lines.

- **MBP- N51/KC**

Tani et al., 1996, generated a mouse strain overexpressing the neutrophil-selective chemokine N51/KC, also known as CXCL1, under the MBP promoter on the oligodendrocytes. These animals displayed strong microglial activation and alteration of the blood-brain barrier, accompanied by neurological manifestations such as hunched posture and rigidity and premature death in some cases. Although demyelination was absent MBP-N51/KC mice, thus they do not represent a good model for MS; these animals were of crucial importance for identifying chemokines, such as CXCL1, as determinant molecules in the entry of leukocytes in the CNS.

- **MBP-CCL21**

C-C Motif Chemokine Ligand 21 (CCL21) is a chemoattractant protein specific for lymphocytes (Nagira et al., 1997). Although its involvement in MS has still to be fully elucidated, it might be of great importance considering the role of T cells in the pathophysiology of the disease. Mice that express CCL21 under the promoter of MBP developed tremors and ataxia starting from a very young age, accompanied by weight loss and premature death at only four weeks after birth. The most severe clinical symptoms were displayed when the transgene was expressed at higher levels. Infiltrates of inflammatory cells were evident at the level of the spinal cord, cerebellum, and medulla, and gliosis was present in several areas of the CNS. These results suggest a possible involvement of CCL21 in the pathological events that trigger the onset of MS.

- **hGFAP-CCL2**

The chemokine (C-C motif) ligand 2 (CCL2) was found to be expressed in active lesions in MS, with mononuclear cells and astrocytes being the primary source of this molecule (Sun et al., 1995). Mice that express the CCL2 under the control of the promoter of human GFAP (hGFAP on astrocytes present infiltration and accumulation of monocytes and lymphocytes in the CNS. After infection with TMEV, these animals showed an accelerated onset of the demyelinating disease, with perivascular lesions and accumulation of mononuclear cells in the spinal cord, compared to the controls (Bennett et al., 2003). These results suggest that the accumulation of monocytes/macrophages in the CNS due to the transgene is enough to elicit a more rapid disease development after infection.

- **T/+ CCR2−/−**

The receptor of CCL2, the C-C chemokine type 2 receptor (CCR2), has also been hypothesized to have a role in the recruitment and accumulation of mononuclear cells into CNS, contributing to the symptomatology of MS, since this molecule was found to be express in macrophages and microglia in chronic active lesions in the white matter during MS (Simpson et al., 1998). To further investigate this aspect, Lagumersindez-Denis et al., 2017, generated a transgenic line by crossing Th/+ mice (IgG^{MOG}) from Litzenburger et al., 1998, with the CCR2−/− KO mice from Kuziel et al., 1997. The IgG^{MOG} transgenic mouse line was obtained by substituting the original JH locus with the IgH variable gene of a pathogenic mAb specific for MOG, to obtain transgenic animals that present B lymphocytes capable to produce MOG-specific IgGs. Lagumersindez-Denis et al., 2017, crossed this transgenic line with mice deficient for CCR2 and generated the T/+ $CCR2^{-/-}$ transgenic mice. After immunization with $rMOG_{1–125}$, both T/+ $CCR2^{-/-}$ animals and controls developed EAE, but cortical demyelination was significantly reduced in T/+ $CCR2^{-/-}$ compared to T/+ $CCR2^{+/+}$. Furthermore, mRNA levels of monocyte-related genes were lower in T/+ $CCR2^{-/-}$ animals, further confirming the role of CCR2 in monocyte recruitment and progression of cortical lesions in EAE.

Despite the numerous models existing nowadays, none of them effectively reproduces every aspect of this complex disease. However, transgenic models contributed significantly to understanding some of the main pathological features of MS, such as immunity, genetics, inflammation, and remyelination, among others. In this context, using transgenic mice with defective remyelination, such as the Olig1-/-(Lu et al., 2002), might help determine the role of oligodendrocytes in the pathological processes behind MS. The most used models for MS research are mice that spontaneously develop the EAE. Starting from the ‘90s, these animals have been of great relevance for studying the pathophysiology of MS and therapeutical approaches. For example, T cell-mediated animal models of MS (Theien et al., 2001), and OSE mice (Häusler et al., 2015) were used to test a pharmacological compound against the α4β1, a fundamental protein implicated in the infiltration of the immune cells and therefore crucial for MS pathogenesis. Another transgenic mouse strain used to test pharmacological strategies is the ND4 mouse, which tested the efficacy of the synergic effect of paclitaxel and IFN-β plus vitamin B12CN against demyelination (Mastronardi et al., 2004, 2007).

FTY720, the first drug to be licensed by the food and drug administration (FDA) for the immunomodulatory therapy for the treatment of RRMS (Webb et al., 2004), was evaluated using SJL/J mice strains, the most popular model for the investigation of the Relapsing forms of EAE.

The transgenic model of MS also contributes to the investigation of the inflammatory and autoimmune aspects of the disease, which is helpful in evaluating therapies and treatments (Robinson et al., 2014).

As previously described, CD8+ T cells and the MHC Class I genes have fundamental roles in initiating the CNS autoimmune pathogenic process seen in MS.

To understand which populations of cells are involved in the inflammatory or neuroprotective mechanisms of MS, T-helper cells (secretors of a large variety of cytokines) have been investigated. Th-2 cells

have been proposed to cover a neuro-protective role in MS. Oreja-Guevara et al., 2012, carried out a study in which the balance between Th-1 and Th-2 in favors of Th-2 cells population led to the neuroprotection in patients affected by MS. The GATA3-tg transgenic mouse model (Fernando et al., 2014), which overexpresses the transcription factor GATA3 essential for Th-2 differentiation, has been demonstrated to develop a less severe EAE and a reduction in demyelination, corroborating the idea that Th-2 cells might have a protective role in MS, and this could be of translational importance for future prospectives and treatments.

Since inflammation is thought to have a principal role in the initiation and progression of MS, the involvement of single cytokines was investigated thanks to transgenic animals. For example, Probert et al., 1995, generated a transgenic mouse that constitutively expressed a murine TNF-α transgene that, starting from about 3 to 8 weeks of age, developed a persistent inflammatory demyelinating illness with a CD4+ and CD8+ T lymphocytes infiltration. These transgenic mice models are one of the most useful for the investigation of inflammation, as also demonstrated by Taupin et al., 1997 and Akassoglou et al., 1997, who used transgenic models that carry TNF-α modifications or over-express wild-type TNF-α. A further contribution in this field was given by the humanized knock-in model for the TNFR1 receptor (hum-TNFR1-ki), in which was observed the efficacy of a specific mAb ATROSAB in reducing the severity of the typical manifestations of EAE. In addition, the role of other cytokines has been evaluated in transgenic animals. For example, in transgenic mice lacking IL-23 subunits p19 or p40, EAE cannot be induced (Cua et al., 2003), like the IL-6KO mice (Eugster et al., 2001), indicating the fundamental role of these cytokines in the pathogenesis of the disease. Similar models have been developed for other cytokines such as IFN- γ (Corbin et al., 1996), IL-3 (Chiang et al., 1996) and IL-12 (Cua et al., 2003), and chemokines (Lagumersindez-Denis et al., 2017). For example, the chemokine CCL2 and its receptor CCR2 are potential therapeutic targets for treating MS (Mahad and Ransohoff, 2003). On the other hand, some of the findings regarding immunity and inflammatory cytokines are still of poor therapeutic translational relevance due to objective differences between human and murine immune systems (Mestas and Hughes, 2004). However, humanized mouse models for human immune system molecules are on the way to represent the best way to overcome these significant limitations (Allen et al., 2019). For instance, various transgenic humanized mice models have been developed to examine the function of human T cells and the MHC, which have been highly correlated with the genetic risk for MS. Moreover, the development of transgenic animals expressing the myelin-specific autoreactive TCR produced from a CD8+ T cell clone from an MS patient and the MS-associated MHC class I alleles HLA-A(*)0301 and HLA-A(*)0201, was crucial for better understanding the genetical basis behind MS, as already mentioned above (Friese et al., 2008). In addition, transgenic mice line HLA DR3.Aβo and HLA DR3.AE^{o,} which had a lack of complex endogenous molecules of class II confirmed that also CD4+ T cells are strongly implicated in the aetiology of MS.

Although the ideal transgenic model doesn't exist, transgenic animals nowadays represent a fundamental resource for studying different pathological aspects of the disease. The full potential of these models remains to be uncovered. Still, the value of these animals for research is clear, and the continuous development of this field gives hope for promising results in the future. Especially for what concerns humanized mice,

5. In vitro models in MS

Many in vitro methods have been created to comprehend the pathogenesis and etiology of a wide variety of neurodegenerative illnesses, including MS. The complexity of cellular models of MS varies from simple monolayers obtained from primary cultures or immortalized cell lines to intricate multicellular 3D tissue based on patient-derived iPSCs that can imitate MS (Fig. 1).

5.1. Ex vivo MD models

A widely used approach to study MS is the use of 3D organotypic brain slices from different animal sources such as mice (Sekizar and Williams, 2019; Mi et al., 2009), rats (Doussau et al., 2017; Gianinazzi et al., 2005), and humans (Nogueira et al., 2022). 2D monolayer cultures cannot reproduce the 3D cell architecture of neurons with their cellular microenvironment. In contrast, 3D slices are commonly used for this purpose, as they simultaneously represent multiple regions of the CNS. Additionally, organotypic brain slices enable experimental manipulations that are difficult to carry out in vivo. Brain slices culture protocols allow the preservation of different cell types with different structural features and synaptic organization. Brain slices between 100- and 400-μm are easy to obtain using a microtome and can be easily cultured on membranes (Madill et al., 2016). The choice of the most appropriate brain region to use relies upon the purpose of the study. For instance, the brain (Mi et al., 2009; Sekizar and Williams, 2019), cerebellum (Doussau et al., 2017) and spinal cord (Thomson et al., 2008; Sekizar and Williams, 2019) are all valuable sources for organotypic cultures. This cultural technique allows the study of axonal myelination in a system in which complex cell-cell connections are retained. Since the organotypic brain slices can be cultivated ex vivo for several months, it is possible to track the impact of therapies over time (Birgbauer et al., 2004). Because of this advantage, in vitro systems can also test potential remyelination-promoting medications (Schnädelbach et al., 2001). Research using 3D rodent slices has provided some intriguing findings but has also highlighted a significant drawback of using animals to study human diseases like MS (Ben-Nun et al., 2014). Therefore, new 3D in vitro methods must be developed to accurately simulate the human MS and uncover potential new targets for treatment.

5.2. Primary cell cultures

The use of in vitro models can create a link between the currently used preclinical animal models and humans. High data repeatability, the potential to cut expenses and experimental time, and the ease with which new mechanisms of action, new medications, and related hazards can be discovered are all benefits of in vitro testing. By cultivating single cells or mixed cell cultures that may simulate the interactions between CNS cells, in vitro models can now reflect a complex system like the CNS (De Vries and Boullerne, 2010).

Oligodendrocyte, astrocyte, and neuron primary CNS cultures are typically obtained from embryos to achieve a significant recovery and a safer cell culture. Among the several cellular elements in the brain, the oligodendroglia is the primary cell type of interest for the study of MS. Oligodendrocytes are the cells responsible for remyelination in MS, and the damage of these cells results in an altered function. In MS, myelin is destroyed by the immune system, and the oligodendrocytes are lost, while the endogenous OPCs are presumed to generate new oligodendrocytes. Oligodendrocytes work on elaborating myelin sheaths in vitro without axons (Bechler et al., 2015), and they can grow and be available for different weeks (Martinez and Peplow, 2020). It is crucial to monitor the cells' characteristics throughout time since they can be altered by prolonged subculturing (Spaas et al., 2021). One downside of oligodendrocyte primary cell cultures is that they are generally only accessible in limited quantities. However, mature oligodendrocytes can be created by cultivating glia progenitors in a serum-free medium (Tan et al., 2018).

Because astrocytes are crucial for cell homeostasis, cell repair, and the release of growth factors required for regeneration, many studies on MS also involve astrocytes. Astrocytes participate in the growth of MS lesions. The loss of oligodendrocytes and the resulting disruption of the astrocyte-oligodendrocyte networks in MS lesions might cause astrocytes to take on a hypertrophic appearance when tissue injury occurs. Moreover, this cell type sustains the damage by increasing the access of immune cells to the CNS, which determines retraction or loss of glia

(Brosnan and Raine, 2013).

They are often produced from rodents or extracted from human astroglioma. Furthermore, it is critical to guarantee the adequate clearance of microglia, which typically compromise primary astrocyte cultures and may alter culture responses. One benefit of using in vitro astrocytes is that these cells can be cryopreserved, which makes astrocytes easier to control and eliminates the need for an immortalized cell culture. Conversely, primary neuronal cultures are not easy to prepare; they require specific skills and are difficult to maintain. Furthermore, they are unsuitable for large-scale studies, and, of course, they cannot be cryopreserved as astrocytes (Lopes et al., 2017). It is known that it is challenging to obtain primary neurons or any of the other cell types cited above from adult animals. So far, few reports described the use of "adult" tissue for preparing primary cell cultures (Zuiderwijk-Sick et al., 2007; Mansilla et al., 2021).

5.3. Immortalized cell cultures

To circumvent some of these limitations in employing primary cultures, various neuronal-like cell lines have been introduced in MS research, such as HCN, NT2, and SHSY5Y cells. These cell lines undergo differentiation to generate cells that resemble neurons. To differentiate and obtain neuronal features, HCN and NT2 cells are treated with brain-derived neurotrophic factor (BDNF), and retinoic acid (RA) is used for SH-SY5Y cells. These cell lines grow slowly, are time-cost expensive, and often express only a few markers of mature neurons after differentiation. It is essential to realize that proper differentiation depends on cell passages, so cell cultures older than two months shouldn't be employed in research. Bromodeoxynucleotide treatment was used on four neuroblastoma cell lines, NCG, SK-N-DZ, GOTO, and NB-1, to investigate cell proliferation, morphological alterations, and S 100 cellular content. The GOTO cell line was the most sensitive and could be differentiated into S 100 protein-positive cells, indicating a glial cell type transition (Tsunamoto et al., 1998). The CG4 is another readily available non-neuronal cell line that resembles an oligodendrocyte-type 2 astrocyte (O-2A) precursor and is obtained from cultures of neonatal rat cerebral cortex. CG4 cells proliferate when grown in media containing basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) and differentiate into oligodendrocytes after the withdrawal of growth factors (Louis et al., 1992). This cell line must be preserved at a low level of confluency to allow a window for agents to stimulate differentiation. It is known that oligodendrocyte loss in MS lesions has been linked to excitotoxicity (Yoshioka et al., 1998). For instance, thanks to in vitro cell research, mature oligodendrocyte death was observed and replicated using differentiated CG4 cells cultivated in the presence of kainate (Yoshioka et al., 1995), as oligodendrocytes exhibit glutamate receptors, such as the AMPA-type and kainate one (Yoshioka et al., 1995).

Furthermore, Matute and co-workers found that most oligodendrocytes differentiated in vitro, expressed the AMPA receptor subunits GluR3 and GluR4 as well as the kainate receptor subunits GluR6, GluR7, KA1, and KA2 in cultured oligodendrocytes from perinatal rat optic nerves using reverse transcription-PCR and immunocytochemistry. Short and long-term kainate treatment resulted in widespread oligodendrocyte mortality in culture. This impact was somewhat reduced by the AMPA receptor antagonist GYKI52466 and entirely removed by the non-N-methyl-D-aspartate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), indicating that kainate toxicity is mediated by both AMPA and kainate receptors (Matute et al., 1997). Furthermore, persistent kainate administration to optic neurons in vivo resulted in extensive oligodendrocyte mortality, which, like in vitro, could be avoided by co-infusion of the toxin with CNQX. Then, Matute focused the investigation on the nature of the damage induced by kainate, when given directly to the optic nerve. Acute or chronic delivery of the agonist triggered massive nerve damage, which was wider in the second type. Both acute and chronic delivery also caused inflammation and demyelination in specific nerve areas. The CNQX receptor antagonist protected from both acute and chronic kainate lesions, whereas GYKI53655 failed to significantly diminish the extent of the lesion. The results revealed that changes in glutamate signaling could harm oligodendrocytes and play a role in the pathogenesis of MS and other demyelinating conditions (Matute, 1998).

The transplanted CG4 cells have shown the ability to travel several millimeters down the dorsal columns of the myelin-deficient rat's spinal cord, where they split and myelinated many axons over two weeks. Similar migration of transplanted CG4 cells down axonal pathways was seen in the brains of normal newborn rats (Tontsch et al., 1994). This oligodendrocyte cell line can also test compounds that produce or interfere with excitotoxicity and cell death (Casaccia-Bonnefil et al., 1996). The 6E12 line, generated from the spinal cord of MBP-SV40 large T-antigen transgenic mice, is another immortalized oligodendrocyte cell line (Jensen et al., 1993). However, this cell line was developed to evaluate drugs that promote differentiation and protect against cell death. Unlike typical oligodendrocyte precursors, 6E12 cells did not show a rise in MBP protein or mRNA, most likely due to an upregulation in the tSCIP/Tst-1, an MBP repressor.

The OLN-93, Oli-Neu, O-2A/myc, N1, N20.1 are oncogene-mediated immortalized OPCs from rodents, while the HOG, TC620, MO3.13, and KG-1C are tumoral-derived cell lines (Merrill and Matsushima, 1988; Kashima et al., 1993; Richter-Landsberg and Heinrich, 1996; Söhl et al., 2013; Barnett and Crouch, 1995; De Vries and Boullerne, 2010). For instance, OLN-93 cells are stimulated to develop and produce a strong arborization of processes, like immature oligodendrocytes, and were verified to show characteristics of immature oligodendrocytes (Buckinx et al., 2009). The murine cell line Oli-Neu, which is produced through oncogene transformation, grew in demyelinated lesions, and moved into adjacent tissues after being injected into experimentally demyelinated rat spinal cord and cells near demyelinated axons, exhibited a remarkable increase in the expression of the myelin-specific protein MAG, (Jung et al., 1995). HOG and TC620 oligodendrogliomas cells have been investigated for their potential use as replacements for more difficult-to-obtain human oligodendrocyte progenitors. In some experiments, such as the proliferation assay in the presence of IL-2, TC620 cells behaved like primordial oligodendrocytes. One disadvantage is that these cells are tumor-derived, and their usefulness as indications of regular cell activity and differentiation is limited. Within these cells, the N1 was the least developed and demonstrated to be the most vulnerable to NO-induced death by mitochondrial damage. The N20.1 cell line is the most developed in vitro phenotype; these cells produced membrane structures surrounding axons suggestive of myelin ensheathment when transplanted into the newborn brain of congenitally demyelinated shiverer mutant mice and are the least vulnerable to NO-induced cell death (Foster et al., 1995; MacKenzie-Graham et al., 1994). The effects of free radicals on normal main mouse oligodendrocytes and their progenitors were accurately recreated using these cell lines.

Pharmacological stimulation of endogenous OPC maturation and remyelination is currently acknowledged as a viable MS treatment strategy. Many data address the activation or inhibition of several cell signaling pathways to induce OPC development (Deshmukh et al., 2013; Zuchero and Barres, 2013). These signaling pathways comprise those that lead to cytoskeleton changes. For instance, Rho kinase is of particular interest since it is a pathway that provides therapeutic targets with the ability to impact the initial phases of OPC development (Pedraza et al., 2014).

Different astrocyte cell lines are available, such as C8-D1A and NHA (Kumar et al., 2004; Sato et al., 2012). One limitation to the use of these cells is represented by the variable response in comparison with primary cultures; for this reason, researchers often prefer to get them directly from rodents or isolate them from human astroglioma (Galland et al., 2019; Williams et al., 2007)

5.4. iPSCs in MS research

Recently, iPSC lines have come to light as a potential reproducible model more closely connected to human pathology.

The first iPSC line from an RRMS patient was generated by Song et al., 2012, and so far, more than 50 iPSC lines have been developed. Up to now, MS iPSCs have been created with the features of the most affected patients. For instance, iPSC cell lines have been generated starting from female subjects, from individuals aged between 30 and 50 years old, and from subjects suffering from RRMS (Mutukula et al., 2021). The cellular sources for MS iPSCs are mainly represented by fibroblasts, peripheral mononuclear blood cells, and mesenchymal and epithelial cells of the proximal renal tubule (Marangon et al., 2021; Mutukula et al., 2021).

In these last years, several research protocols have been developed to differentiate iPSCs into neural progenitor cells (NPCs) (Chambers et al., 2009; Xie et al., 2016), oligodendrocytes (Yamashita et al., 2017), neurons (Gunhanlar et al., 2018), microglia (Abud et al., 2017), astrocytes (Perriot et al., 2018; Shaltouki et al., 2013) and vascular cell types (Faal et al., 2019).

The requirement to continuously examine each line's DNA sequence as they undergo genetic modifications restricts the use of iPSCs (Rebuzzini et al., 2015). Moreover, recent data suggest that cell source origin may be critical to iPSCs creation because residual epigenetic memory may impact iPSC phenotype and transplantation results (Zhou et al., 2018). Additionally, because the genetic information collected using these cells can be directly linked to cellular phenotypes, using iPSCs enables researchers to concentrate on specific pathways connected to some genetic variants that are challenging to study using biopsy tissues (Madill et al., 2016). Moreover, improvement in remyelination was described after the transplantation of iPSC-derived neural cells into demyelinated models (Laterza et al., 2013; Zhang et al., 2014). Despite the progress achieved in recent years, iPSCs are currently little used in MS research. The main drawback of MS organotypic models, such as iPSCs-derived OPC cultures, is that they omit the peripheral immune system, which plays a vital role in MS development and regeneration. It should be emphasized that iPSCs constitute a fresh, legitimate potential for identifying specific phenotypes and evaluating remyelinating medications that may open new, more focused avenues in MS research.

Direct cell reprogramming, also called transdifferentiation, allows direct conversion or reprogramming of one somatic cell type to another (e.g., induced neural stem (iNSCs) and induced mesenchymal stem cells (iMSCs), which are often utilized to treat MS animal models due to their ability to differentiate into neurons and oligodendrocytes. These cells are beneficial because of a variety of factors, including paracrine action, an increase in trophic factor levels (BDNF and NGF), a decrease in CNS inflammation, and an improvement in the stability of the BBB (Pluchino et al., 2003; Pluchino and Martino, 2005; Xie et al., 2016; Uccelli, 2013; Einstein et al., 2006; Zappia et al., 2005; Payne et al., 2012; Peruzzotti-Jametti et al., 2018; Sullivan et al., 2020). iNSC and iOPC are autologous cells that are suitable for personalized therapies. It is now possible to generate iNSCs (Xie et al., 2016) that show high similarities with brain-derived ones. Future transplant-based brain or spinal cord therapies could use these cells' ability to regenerate myelin sheaths (Lujan et al., 2012).

5.5. Spheroids and organoids in MS research

Recent advancements in 3D in vitro models, known as organoids, provide new opportunities for studying human illnesses. iPSCs can be differentiated into simple monoculture or more complex 3D organoids, which can be utilized as a model to explore more intricate cellular interactions (Wray, 2021).

The induction of oligodendrogenesis and myelination in organoids was only recently reached. The oligodendrocyte growth factors PDGF, IGF-1, and T3 induced oligodendrocyte progenitors and myelinating oligodendrocytes in cortical 3D spheroids (Madhavan et al., 2018). By 20 weeks in culture, different analyses showed molecular features consistent with maturing oligodendrocytes, including the expression of MYRF, a nuclear marker of the oligodendrocyte lineage, of PLP1, the foremost representative oligodendrocyte membrane protein, and of MBP, a protein of early stages of myelin development, accompanied by the beginning of myelin wrapping of axons and compacted myelin by 30 weeks. The validated system showed two potential uses of the oligo-cortical spheroid for research in genetic disorder modeling and pre-clinical drug screening (Madhavan et al., 2018). In 2019 was proposed a protocol for reducing the time for the differentiation of cultures from 210 days (Madhavan et al., 2018) to 105 days, presenting evidence of oligodendrogenesis in forebrain organoids created with OLIG2-GFP knock-in human pluripotent stem cell (hPSC) reporter lines (Kim et al., 2019). Brain region-specific ventral forebrain organoids (VFOs) were generated by activating the SHH signaling pathway, and dorsal forebrain organoids (DFOs) were generated by blocking the same pathway. Fused forebrain organoids (FFOs) obtained by integrating DFOs and VFOs enhanced the maturation and differentiation of oligodendroglia.

Two years later, a human pluripotent stem cell line (hiPSC) was developed and reported on the SOX10 gene expression for creating human brain organoids, including OL in only 42 days. The SOX10-expression OPC in oligodendrocytes (OL) brain organoids mainly differentiate in oligodendrocytes, as evidenced by the presence of 2′,3′-Cyclic-nucleotide 3'-phosphodiesterase (CNPase), MBP and O4 oligodendrocyte markers. Furthermore, the deposition of myelin on neurons that co-localizes with MBP and the neuronal marker TUJ1 (class III beta-tubulin) was observed in this system.

It was demonstrated that this method could reduce the time required to create human cortical brain organoids containing neurons, astrocytes, and oligodendrocytes capable of myelinating endogenous neuronal axons (Shaker et al., 2021).

Ma and colleagues reveal that amplification of OLIG2 and SOX10 in hPSC-derived ventral forebrain neural progenitor cells (VF-NPCs) enables the efficient production of hPSC-derived forebrain OLs, particularly OL-spheroids able to myelinate axons in vivo and generate myelination in 60 days following in vitro differentiation.

These experimental methodologies, ranging from understanding demyelination to remyelination strategies for the treatment of MS, represents a first step towards a new research approach giving the potential to model and understand mechanisms underlying MS. Organoids appear to be a promising human model for studying MS and go beyond the limits of in vivo experiments, and utilizing patient-derived stem cells that enable the investigation of a patient's genetic history of MS, which will aid in the identification of prospective targets for treatment methods aimed at promoting myelin repair in MS.

Among 3D cultures, assembloids are the latest generation of brain organoids that may mix various brain areas and cell lineages to produce crosstalk between specific brain regions (Benito-Kwiecinski and Lancaster, 2020). They may be used to make connections within brain areas, catch cell-cell interactions, and research neural circuit formation. Assembloids containing microglia and neurons may help study immune-mediated mechanisms during synaptic loss, as it happens in MS (Marton and Pasca, 2019), and increase the possibilities of developing new therapeutic techniques.

6. Future developments

Current treatments for MS mainly target the immune system to reduce inflammation and ameliorate both severity of symptoms and the progression of the disease; a more recent approach is based on facilitating re-myelinization to increase neuroprotection (Lubetzki et al., 2020).

Consequently, new models have been developed to test compounds with potential re-myelinating abilities. Cortical organoids and

spheroids, three-dimensional cultures composed of oligodendrocytes and astrocytes (Lancaster et al., 2013), have been shown to recapitulate the main features of brain development (from cell proliferation to maturation) in which oligodendrocyte expression can be induced to obtain myelination (Madhavan et al., 2018). Brain organoids and spheroids represent a human-derived in vitro model in which the mechanism of de-myelination, myelination, and oligodendrocyte function can be studied, providing new insights and knowledge for MS and for other neurodegenerative diseases (see for review McCaughey-Chapman and Connor, 2023).

Daviaud and colleagues (Daviaud et al., 2023) obtained organoid cultures from iPSCs cells from healthy subjects and PPMS, SPMS, and RRMS patients. They demonstrated significant differences in proliferation and differentiation capacity suggesting that organoids can be used as a valid model of MS alternative to the use of animals.

While rodents are the most used animal models to study MS, the zebrafish (Danio rerio) is gaining interest as an animal model for neurodegenerative diseases, including MS (Bashirazade et al., 2022). Zebrafish present high physiological homology with mammals, their behavior can be easily tracked (Kalueff et al., 2014), and the characteristic optical clarity of the embryos and some adult strains facilitates visualization of individual genes that can be labeled with fluorescence (Araya et al., 2016; Tang et al., 2020). Moreover, their neuronal structural organization is like humans, and their genome has been fully sequenced, allowing manipulation of the genes involved in neurodegenerative disorders (Ebrahimie et al., 2017; Kumar et al., 2021; Razali et al., 2021). EAE has been validated in adult zebrafish that show motor deficits, reduced body weight, and microglia alteration (Burrows et al., 2019; Kulkarni et al., 2017). Zebrafish are also sensitive to the demyelinating action of lysolecithin (McGown et al., 2016). However, zebrafish rapidly compensate to the loss of oligodendrocytes and demyelination, thus complicating their use as a reliable MS model (Bashirazade et al., 2022). Recently (Häberlein et al., 2022), zebrafish have been proven a valuable model to evaluate the remyelinating ability of new compounds acting as antagonists at the GPR17 receptor, which physiologically inhibits the differentiation and maturation of oligodendrocyte (Chen et al., 2009; Simon et al., 2016; Merten et al., 2018). Zebrafish might therefore be a model with a significant predictive validity able to screen new drugs with remyelinating properties.

Among all the available in vitro and in vivo models for MS, the use of non-human primates offers the possibility to investigate more closely the mechanisms involved in the pathophysiology of MS (t'Hart and Bajramovic, 2008). Two non-human primates are mainly used in the EAE model, Macaca mulatta and marmoset (Callithrix jacchus). The marmoset model is particularly attractive because of their small size and even more because they are born as chimeric twins, making it possible to use one of the twins as a control for the test compound administered to the second one (t'Hart and Bajramovic, 2008). Moreover, non-human primates allow MRI to visualize pathological changes in the brain and test the therapeutic efficacy of new drugs.

7. Conclusions

MS is a complex disease, and its pathophysiology's mechanisms are not entirely understood.

The complexity of in vivo and cellular models of MS ranges from classical and transgenic models to simple monolayers derived from immortalized cell lines and more complex multicellular 3D tissue, which can replicate many MS hallmarks and physiological conditions in vivo. It is also important to note that traditional cell culture systems and animal models have helped us understand human neurodegenerative diseases. Translating results from animal experimental models to humans is the major challenge in investigating this disorder.

This review describes the main models currently used in MS research that allow scientists to study more variables and identify and test new therapeutic compounds. In vivo and ex vivo studies on MS are mainly directed towards investigating factors and drugs capable of influencing the immune system and the CNS cells to understand how to restore remyelination and neuronal functions. In contrast, in vitro, studies investigate the molecular mechanisms underlying MS. The future of MS research will be determined by how in vivo, ex vivo, and in vitro investigations will develop. These studies are crucial since they all clarify various facets of the intricate mechanisms behind MS.

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Data availability

No data was used for the research described in the article.

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