



Review

Multiple Sclerosis: Immune Cells, Histopathology, and Therapeutics

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Abstract: Multiple sclerosis (MS) is an inflammatory demyelinating disease affecting the central nervous system (CNS). In MS, oligodendrocytes and myelin that surround axons to facilitate transmission of neuronal signals are destroyed by adaptive and innate immune cells, resulting in the formation of demyelinating plaques. For many years, research into MS pathophysiology has identified immune cell populations in lesions such as T cells, B cells, and myeloid and innate lymphoid cells. In this review, we discuss the involvement of these immune cells in MS pathophysiology and demonstrate how findings from histopathology studies and single-cell analyses in animal and human models have identified which immune cell subsets contribute to disease. This knowledge has facilitated the introduction of numerous immune-targeted therapeutics towards CD20, CD52, interferon-beta, sphingosine-1-phosphate receptor, Bruton's tyrosine kinase, and many more. These treatments have shown effective reduction in new lesion formation and management of symptoms in MS patients. Furthermore, as MS is a chronic disease, these therapeutics slow disease progression, reduce cognitive disabilities, and prevent relapses. Further research is required to develop a cure for MS with limited side effects. The ongoing research that utilises innovative methods to identify and assess MS pathophysiology could transform the treatment landscape for patients in the future.

Keywords: multiple sclerosis; inflammation; histology; single-cell analysis; disease-modifying therapy



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1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) affecting approximately 2.8 million people worldwide [1]. MS is the most significant cause of neurological disability in young adults aged between 20 and 40 years, detrimentally altering their most productive years in life [2]. In MS, myelin and myelin-producing cells, the oligodendrocytes, are destroyed by the body's immune system, preventing neurons from transmitting information in the CNS [3]. As a result, patients experience cognitive deficits, fatigue, muscle weakness, sensory disturbances, impaired coordination, and permanent neurological disability, which significantly reduce quality of life [4]. This inflammatory demyelinating disease occurs when the blood–brain barrier (BBB) is compromised, enabling immune cells to bypass and infiltrate the CNS [5]. The BBB is a highly regulated interface that separates circulating blood from the CNS; however, in MS, this interface is disrupted by pro-inflammatory cytokines and chemokines [6]. Expression of adhesion markers on the endothelium and increased permeability of the BBB permit transmigration of activated leukocytes, T cells, B cells, and monocytes/macrophages into the perivascular space. These immune cells generate cytotoxic factors and react with autoantigens and microglia to perpetuate inflammation and damage myelin and axons [2,5]. Moreover, recent therapeutic advances in MS have targeted these immune cell populations, with strategies aimed at

modulating their activation, trafficking, and effector functions. Extensive research into MS pathogenesis has introduced many targets for treatment of MS. In this review, we discuss these novel targets and the medications currently used by MS patients. Furthermore, the valuable contribution of methods such as histopathology and single-cell analysis in human and animal models of MS is discussed, as well as how they contribute to our knowledge of MS pathogenesis.

1.1. Aetiology

Because MS involves a complex and multifaceted inflammatory response, the precise aetiology remains elusive. However, extensive research now shows genetic predisposition, environmental triggers, and intricate immunological dysregulation as pivotal factors influencing the onset and progression of MS [7,8]. Genetic factors influence an individual's susceptibility to MS, with over 233 gene variants identified [9]. Findings from family and epidemiological studies identified specific human leukocyte antigen (HLA) alleles on chromosome 6 linked to the risk of developing MS [10]. Among the HLA class alleles, DRB1*15:01 [11], *03:01 [12], and *13:03 [13] exhibit a robust association with MS diagnosis [14]. Several non-HLA genes associated with immune system function and myelin maintenance have also been identified. For example, the interleukin-2 receptor alpha (*IL2RA*) gene, which is involved in T-cell regulation, has a single-nucleotide polymorphism (SNP) that impacts CD4 T-cell differentiation and T regulatory cell (Treg) suppressive function [15]. Similarly, gene variants for immune cell development such as interleukin-7 receptor [16,17], tumour necrosis factor receptor superfamily member 1A, and interferon regulatory factor 8 [18] have been linked with high risk of MS. Indeed, genetic predispositions also influence an individual's response to medications. For example, interferon-beta-treated MS patients with the *IFNAR1* allele respond more positively to treatment [19]. Interestingly, MS patients with the *CCR5* deletion allele presented beneficial interferon-beta treatment efficacy, whereas interferon-beta non-responders expressed the *CCR5* gene [20]. Similarly, a significant proportion of relapsing–remitting MS patients responding to glatiramer acetate displayed the presence of the HLADRB1 1501 allele [21]. Furthermore, a study identified a positive association between the anti-VLA-4 drug natalizumab with *GSTP1* and *NQO1* wild-type genotypes where treatment efficacy was increased [22]. More work is required to investigate the allelic combinations in response to anti-CD20 and anti-CD52 treatment. These studies confirm pharmacogenetic associations of therapeutics in MS patients, highlighting the importance of tailoring treatments for individuals to ensure beneficial outcomes.

Environmental factors also play a significant role in MS and can synergise with genetic predisposition to influence disease susceptibility. Vitamin D deficiency as a result of reduced sun exposure, inadequate diet, or mutations in the vitamin D receptor (*VDR*) gene has been linked to an increased risk of developing MS [23–25]. Vitamin D supplementation has reduced the risk of developing MS by 40% [23,24]. Cigarette smoking is another environmental factor, increasing the risk of MS 1.5 times compared to non-smokers [26,27]. Epigenetic mechanisms also play a role in MS pathogenesis. Specifically, DNA methylation patterns, histone modification, and miRNA profiles have been identified in MS patient plasma and B and T cells, and these are distinctly different to healthy individuals [28,29]. These epigenetic changes can be a potential diagnostic tool since markers were detected in patient blood and cerebrospinal fluid [30]. In addition, viral infections have also been implicated as potential triggers for MS development, and this has been observed with Epstein–Barr virus (EBV). In a longitudinal study monitoring adults recruited in the US military over 20 years, the risk of MS after EBV infection increased 32-fold [31]. Indeed, these findings support more recent studies showing EBV-specific T cells in MS patient peripheral blood and post-mortem brain tissue [32–34]. Further investigations have suggested the possibility of EBV triggering CNS autoimmunity through EBV nuclear antigen 1 and glial cell adhesion molecule, which can lead to MS [35]. Further investigations are required to interpret this link between EBV and MS, which could promote novel treatment options and

strategies for prevention. There are numerous other pathogens that have been potentially linked to MS aetiology, including viruses, notably Semliki Forest virus, Japanese macaque encephalomyelitis rhadinovirus, Coxsackie viruses, herpes viruses (particularly HHV6), human endogenous retroviruses, cytomegalovirus (CMV), as well as measles, rubella, and varicella zoster viruses, and even SARS-CoV2, but also *Helicobacter pylori*, *Chlamydia pneumoniae*, or *Borrelia burgdorferi* [36–39].

1.2. Clinical Course of MS

Often, MS progression adheres to distinct patterns with initial relapses and remissions giving way to gradual accumulation of disability in later phases. Patients with MS are classified into one of four categories according to the 2017 revised McDonald criteria [40]: (1) clinically isolated syndrome (CIS) describes the first episode of neurological symptoms that lasts at least 24 h, often considered an initial manifestation of MS [41,42]; (2) relapsing–remitting MS (RRMS) is characterised by intermittent episodes of worsening neurologic function followed by periods of partial or complete recovery [43]; (3) secondary progressive MS (SPMS) prominently involves axonal loss and neurodegeneration, resulting in gradual worsening of the disease without distinct relapses and remissions [44,45]; (4) primary progressive MS (PPMS) is characterised by a continuous deterioration in neurological function, resulting in severe disability over time [46,47]. RRMS is the most prevalent form of MS, comprising 85% of total MS patients, while 10–15% of patients are classified under PPMS [46,48]. Approximately 90% of untreated individuals with RRMS will transition to SPMS, where the disease worsens more steadily [49,50].

2. Immune Cells Involved in MS Pathology

MS is characterised by inflammatory lesions and demyelinating plaques in the CNS composed of immune cells (Figure 1). These immune cells transmigrate through the BBB at post-capillary venules into the perivascular space [51] and accumulate around blood vessels to form perivascular cuffs. Perivascular cuffs observed in acute phases of inflammation differ from chronic lesions, which are characterised by B-cell aggregations known as follicles [52]. It is known that adaptive immune cells (T and B cells) are a key contributor to MS, but more studies have emphasised a pivotal role for innate lymphoid and myeloid cells. Discussed below are the immune cells that promote neuroinflammation in MS.

2.1. Adaptive Immune Cells: T and B Cells

Autoreactive T cells, particularly CD4⁺ T cells, play a pivotal role in initiating and perpetuating the autoimmune response against the CNS. They recognise myelin antigens, such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG), which are essential components of the myelin sheath [53]. Recognition of these self-antigens activates the autoreactive T cells, resulting in proliferation and differentiation into effector T cells [54]. Primary responses to these auto-antigens likely occurs outside the CNS [55]. The activated T cells transmigrate through the BBB via upregulation of adhesion molecules on endothelial cells such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and are further influenced by chemokines [56,57]. Once in the CNS, autoreactive T cells release pro-inflammatory cytokines, such as interferon- γ (IFN- γ) and tumour necrosis factor (TNF), triggering an inflammatory cascade [58]. This inflammatory milieu further contributes to the disruption of the BBB, allowing infiltration of other immune cells into the CNS [59]. CD8⁺ T cells can also detect CNS antigens, resulting in their activation and release of pro-inflammatory cytokines, which exacerbates the immune response [60] and destroys oligodendrocytes and axons through the release of perforin and granzyme [61]. The persistent inflammation and demyelination creates a self-sustaining loop that perpetuates the chronic nature of MS. Additionally, Tregs play a vital role in modulating the immune response and maintaining peripheral tolerance [54]. However, in MS, Tregs become dysfunctional and contribute

to the breakdown of immune tolerance by releasing greater amounts of IFN- γ and have reduced suppressive function [62], allowing for the sustained attack on myelin [63].

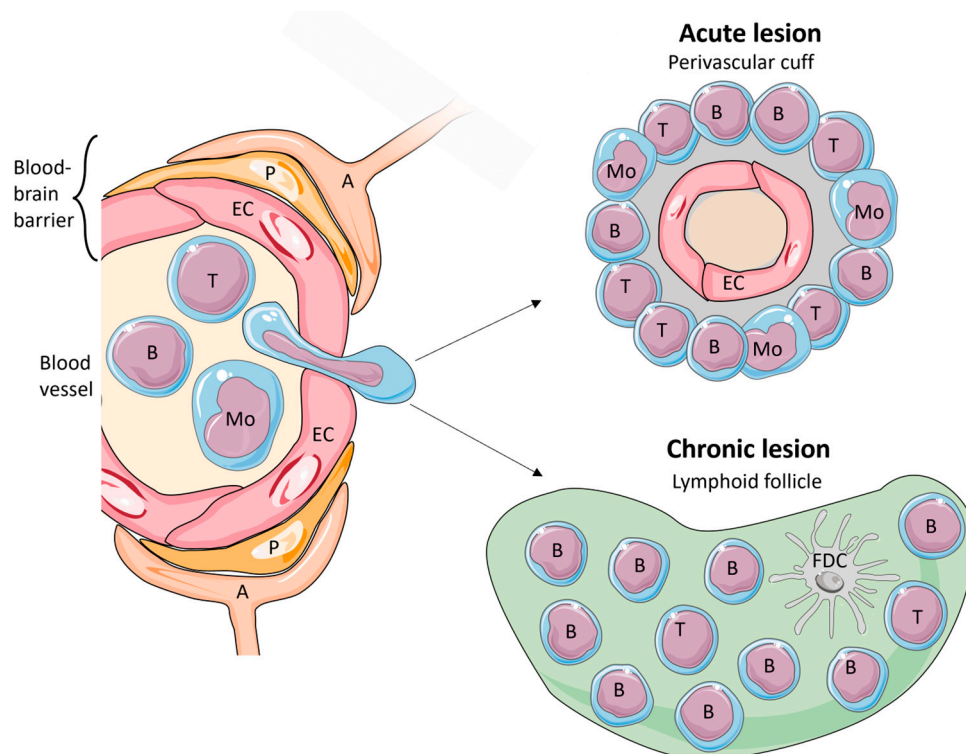


Figure 1. Transmigration of immune cells across the blood–brain barrier in multiple sclerosis. The blood vessel wall is lined with endothelial cells and supported by pericytes and astrocytes to form the blood–brain barrier. In multiple sclerosis, the blood–brain barrier is compromised and permits the migration of monocytes, T cells, and B cells across the wall into the central nervous system. These immune cells form perivascular cuffs and B-cell follicles in the brain observed in acute and chronic multiple sclerosis, respectively. Abbreviations: EC: endothelial cell; P: pericyte; A: astrocyte; Mo: monocyte; FDC: follicular dendritic cell.

B cells play multifaceted roles, by functioning as antigen-presenting cells (APCs) and producing antibodies against myelin components [64], such as MBP and MOG. These autoantibodies contribute to the formation of immune complexes that activate complement cascades and induce inflammation, resulting in demyelination [64,65]. The presence of these autoantibodies in the CNS is indicative of B-cell dysregulation. Additionally, B cells interact with T cells and facilitate the activation of autoreactive T cells specific to myelin antigens [66]. This process perpetuates the immune response and sustains chronic inflammation in the CNS, contributing to the progression of MS. Moreover, regulatory B cells (Bregs) can modulate the immune response, but in MS, Bregs are dysregulated, which compromises their ability to restrain pro-inflammatory responses, exacerbating the autoimmune attack on myelin [62,67]. The balance between pro-inflammatory and regulatory B-cell functions is crucial for maintaining immune homeostasis, and thus dysregulation contributes to the chronic inflammation observed in MS [62,68,69].

The interplay between T cells and B cells is central to the pathogenesis and progression of MS. T cells recognise myelin antigens and secrete pro-inflammatory cytokines to activate B cells and monocytes [70]. B cells, in turn, amplify the autoimmune response by antigen presentation to T cells via co-stimulatory molecules CD80 and CD86, resulting in T-cell activation and proliferation [71]. Furthermore, B cells induce pro-inflammatory T-cell function, notably via CD40–CD40L (CD154) interaction [72]. In addition, B cells release cytokines that promote inter-cell signalling; for example, IL-6 production by B cells induces Th17 differentiation [73]. B cells also influence monocyte activity by regulating TNF

production in pro-inflammatory monocytes [71]; how this contributes to MS pathogenesis requires further investigation. Collectively, these immune cells interact with each other to promote and exacerbate CNS inflammation in MS.

2.2. Myeloid Cells: Monocytes, Macrophages, and Dendritic Cells

In MS, monocytes contribute to the phagocytosis of myelin debris, leading to the presentation of myelin antigens to T cells and the amplification of the adaptive immune response. Within the monocyte population, there are three main subsets: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$), which exhibit diverse functions [74]. Classical monocytes can infiltrate the CNS and contribute to the clearance of myelin debris. However, they can also exacerbate inflammation by releasing pro-inflammatory cytokines and promoting the activation of other immune cells [75]. Intermediate monocytes have been implicated in the pathogenesis of MS through their ability to produce inflammatory cytokines and chemokines. These cells exhibit an enhanced capacity for antigen presentation, contributing to the activation of autoreactive T cells and perpetuating the inflammatory cascade in MS lesions [76]. Non-classical monocytes are involved in immune surveillance and patrolling the vasculature [77]. In MS, they have been associated with the resolution of inflammation through anti-inflammatory cytokine production and phagocytosis of apoptotic cells [78]. However, dysregulation of non-classical monocytes leads to an impaired resolution of inflammation.

Upon entering the CNS, classical monocytes undergo maturation into macrophages, adopting a pro-inflammatory phenotype [79]. Activated macrophages release cytokines, such as TNF and IL-1, which exacerbate the inflammatory milieu and contribute to the demyelination observed in MS lesions [80]. The dysregulation of the balance between M1 and M2 macrophages is a key factor in the pathogenesis of MS. An excess of M1 polarisation contributes to the initiation of inflammation and demyelination. As the disease progresses, there is a shift from M1 to M2 polarisation to resolve the inflammation and promote tissue repair [81]. However, this response is often insufficient, leading to chronic inflammation and neurodegeneration. M1 macrophages, often referred to as pro-inflammatory or classically activated macrophages, are implicated in the initiation and propagation of the inflammatory cascade in MS. These macrophages release pro-inflammatory cytokines such as IL-1 β , TNF, and IL-6 and recruit other immune cells, such as T cells, that further amplify the inflammatory response [82]. Additionally, M1 macrophages contribute to the oxidative stress and tissue damage by producing reactive oxygen species (ROS) and nitric oxide, exacerbating the neuroinflammatory environment in MS lesions. Conversely, M2 macrophages, known as anti-inflammatory macrophages, are associated with tissue repair and resolution of inflammation. In MS, M2 macrophages promote tissue regeneration and clearance of myelin debris [83]. They also secrete anti-inflammatory cytokines, such as IL-10 and TGF- β , to dampen the inflammatory response and support tissue repair. Additionally, M2 macrophages release factors to stimulate oligodendrocyte precursor cell differentiation and myelin formation [84].

Perivascular macrophages (PVMs) are located around blood vessels in the perivascular space and have been implicated in various CNS pathologies (Figure 2). PVMs function as APCs by extending their cellular processes across the endothelium into the vessel lumen to present antigens to T lymphocytes to encourage CD8 T-cell transmigration into the CNS [85–87]. Brain PVMs present antigens derived from MBP to induce an autoimmune response that damages myelin [88]. In MS, PVMs accumulate around blood vessels in the brain and have shown to associate with disease progression [89].

Dendritic cells (DCs) are involved in the initiation and modulation of the autoimmune response against CNS antigens. DCs and follicular DCs (FDCs) within the peripheral lymphoid organs and at the CNS in the perivascular space function as APCs to present myelin-derived antigens to activate autoreactive T cells [90]. Activated T cells migrate into the CNS, perpetuate inflammation, and contribute to the demyelination observed in MS lesions [91]. Furthermore, DCs are implicated in the maintenance of immune

tolerance, and alterations in DC function can lead to the presentation of self-antigens in an immunogenic manner [92], exacerbating autoimmune responses [93]. DCs also contribute to the modulation of other immune cells, such as B cells and Tregs, influencing the overall immune balance in MS [92]. Follicular DCs expressing CXCL13 act as APCs to B cells [94]. Additionally, DCs participate in the formation of organised lymphoid aggregates known as tertiary lymphoid structures. These structures can form within the CNS, further highlighting the role of DCs in sustaining local inflammation [95].

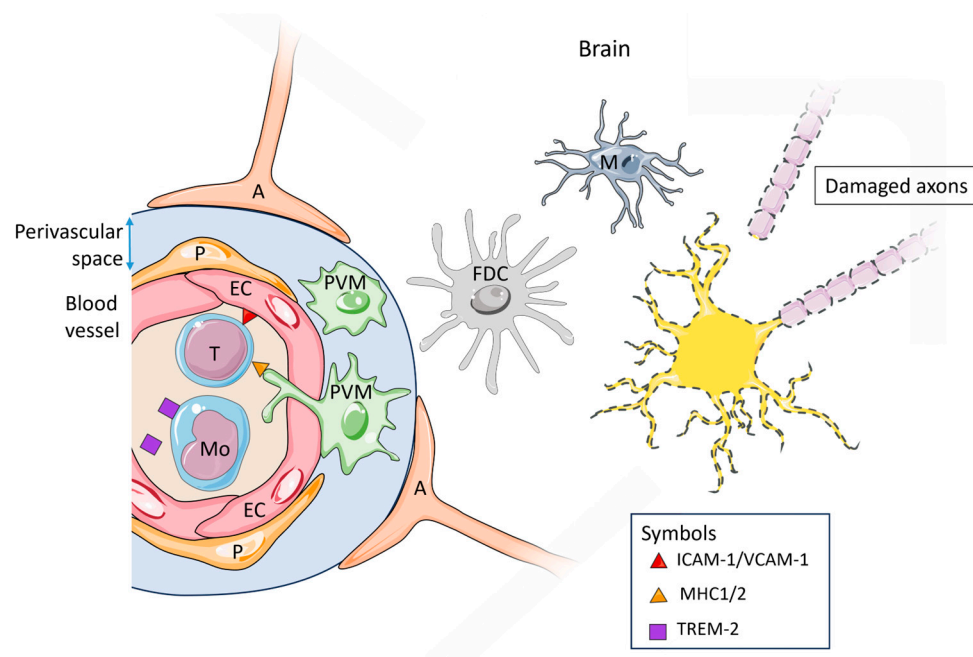


Figure 2. The involvement of perivascular macrophages and follicular dendritic cells in multiple sclerosis. Perivascular macrophages reside within the perivascular space around blood vessels, acting as antigen-presenting cells. These cells can extend their processes through the endothelium to interact with T cells in the blood vessel lumen and promote transmigration. Follicular dendritic cells also present similar functions by acting as antigen-presenting cells to leukocytes in the brain. These immune cells in the brain promote autoimmune responses, leading to axonal damage. Abbreviations: EC: endothelial cell; P: pericyte; A: astrocyte; PVM: perivascular macrophage; Mo: monocyte; FDC: follicular dendritic cell; M: microglia; VCAM-1: vascular cell adhesion molecule-1; MHC: major histocompatibility complex; TREM-2: triggering receptor expressed on myeloid cells 2.

2.3. Microglia

Microglia, the resident macrophages of the CNS, play a pivotal role in the initiation and propagation of neuroinflammation in MS through a myriad of interactions with other immune cells, neurons, and glial cells [96]. Microglia act as key regulators of the immune response in the CNS; however, in MS, these cells become activated in response to the presence of myelin debris and inflammatory signals [97]. Activated microglia release pro-inflammatory cytokines, such as TNF and IL-1 β , and contribute to inflammation and recruitment of peripheral immune cells into the CNS, resulting in demyelination [98]. Additionally, microglia play a crucial role in phagocytosing myelin debris and apoptotic cells [99]. However, the process is dysregulated in MS, leading to the degradation of healthy myelin and axons. This dysregulated phagocytosis further exacerbates the neuroinflammatory response. Moreover, chronic microglial activation leads to the release of ROS and nitrogen species, creating an oxidative stress environment that contributes to neurodegeneration [100,101]. Continuous activation of microglia in the absence of effective resolution mechanisms further contributes to the disease process.

2.4. Innate Lymphoid and Natural Killer Cells

Innate lymphoid cells (ILCs) represent a subset of lymphocytes that lack antigen-specific receptors but play crucial roles in the innate immune response. While their involvement in MS is not well understood, evidence suggests that ILCs contribute to the initiation and progression of the disease [102]. There are five different ILC subsets: group 1 (ILC1), group 2 (ILC2), group 3 (ILC3), lymphoid tissue inducers, and cytotoxic natural killer (NK) cells. ILCs are non-cytotoxic tissue resident cells that resemble CD4 T helper (Th) cells in function by producing cytokines in response to different stimuli [103]. For example, ILC1 (which is similar to Th1 cells) protects against intracellular bacteria, parasites, viruses, and tumour cells, while ILC2 (similar to Th2 cells) defends against extracellular parasites, asthma, and allergens, and ILC3 (similar to Th17 cells) combats bacteria and fungi and is involved in chronic inflammation [104]. In MS pathogenesis, ILC1 and ILC3 subsets promote inflammation within the CNS [104]. These cells produce pro-inflammatory cytokines such as IFN- γ and IL-17, which can exacerbate the inflammatory response and contribute to tissue damage [105]. ILCs may also modulate the activity of other immune cells, such as T cells and microglia [106]. Interactions between ILCs and resident immune cells can further amplify the inflammatory cascade, creating a microenvironment conducive to autoimmune responses [107]. In fact, excessive pro-inflammatory cytokine production from ILCs has been suggested to influence blood–brain barrier function, further facilitating immune cell migration and neuroinflammation in the CNS [108].

NK cells are cytotoxic cells that play a crucial role in immune surveillance by detecting and eliminating infected or transformed cells without prior sensitisation [109,110]. In MS, studies have reported differences in the numbers and functions of NK cells in the peripheral blood and CNS, suggesting dysregulation of NK-cell activity [111–113]. NK cells can modulate the adaptive immune response by influencing the activation and differentiation of T cells that are involved in the development of MS lesions [112,114]. Dysfunctional NK cells create an imbalance between regulatory and effector T-cell populations, leading to an exaggerated autoimmune response against myelin components [115–117]. NK cells can contribute to the breakdown of the BBB through the release of cathepsin D, cytokines, and chemokines, which can enhance the migration of immune cells into the CNS [118]. This process further exacerbates inflammation and formation of demyelinating lesions [119]. In addition, NK cells are also involved in the clearance of stressed or damaged cells, including oligodendrocytes in the CNS [120]. Dysregulated NK-cell activity may contribute to the elimination of oligodendrocytes, thereby exacerbating demyelination and axonal damage [121].

3. MS Pathogenesis with Histopathology and Single-Cell Analysis

Understanding how immune cells contribute to neuroinflammation in MS is critical for developing new treatments. Research is ongoing to characterise the pathogenesis of MS, and here, we discuss the significant discoveries identified by histopathology and single-cell analysis.

3.1. Histopathology

Histopathology involves the study of diseased tissues, and this can be performed *in vivo* using magnetic resonance imaging (MRI) or by preparation of tissue *ex vivo* for immunohistochemistry. MRI is a key tool used clinically to visualise active MS lesions and measure atrophy in patients that allows monitoring of disease progression [122]. It is sensitive at detecting white and grey matter T1 and T2 weighted ratio to determine lesion size and volume; however, the level of neurodegeneration at different phases of MS is difficult to determine [123]. Greater insight into the extent of neuronal damage can be determined with magnetic resonance spectroscopy and optical coherence tomography [124,125]. Indeed, combining techniques provides greater pathological details such as PET and MRI imaging, which differentiates MS subtypes by representing greater homogeneous and rim lesions in patients with PPMS and SPMS compared to RRMS [126]. Furthermore, a combinatorial

assessment of MRI with single-nucleus RNA sequencing identified the interplay of different immune cells in the inflammatory edge of demyelinated white-matter lesions; however, this only assessed mRNA transcripts [127]. While in vivo imaging is a practical diagnostic modality, knowledge about the immune components that form the lesion is still lacking.

Histology has been the most valuable and well-established technique to identify demyelinated focal plaques and can additionally determine localisation of immune cell sub-populations in lesions. Acute active plaques observed in RRMS and CIS are identified by hypercellular regions entirely filled with macrophages, myelin debris, lymphocytes, and microglia [128]. Chronic active lesions in SPMS and PPMS are advanced and distinctively demarcated with macrophages, myelin-filled macrophages, microglia, and lymphocytes, while inactive chronic MS lesions are identified by a demyelinated, hypocellular core, surrounded by activated microglia and macrophages at the lesion rim [129]. Immunohistochemical staining of active demyelinating MS lesions shows that these microglia and astrocyte-like cells express CD86 and PD-L1, respectively, which were absent in normal white matter from healthy donors [130]. These active demyelinating MS lesions also present expression of M1 markers such as CD40, CD32, CD64, and CD86 in activated macrophages and microglia [131]. Further, macrophages containing myelin express M2 markers, mannose receptor, and CD163, and they are localised to the perivascular space in active lesions. PVMs expressing CD163 and MHC class II molecule HLA-DR are found localised around blood vessels in demyelinating lesions and are associated with myelin degradation [132]. Further analysis of these PVMs in acute active lesions showed double positive staining for MBP and CD163, indicating that PVMs can ingest MBP and act as APCs to promote immune attack on myelin [88].

MS patients with acute lesions also display perivascular cuffs made of T and B cells [7,133]. CD3⁺, CD4⁺, and CD8⁺ T cells populate white-matter MS lesions, and these CD8⁺ T cells have a tissue resident effector memory cell phenotype and express CD69, CD44, CXCR6, GPR56, granzyme, and CD103 [34,130,134,135]. Furthermore, perivascular T-cell cuffs express large populations of CD3⁺ and CD103⁺ tissue resident memory T cells, which are co-localised with CD20⁺ B cells and HLA- and CD163-expressing macrophages, indicating the presence of antigen presentation and reactivation of T cells in lesions [134]. Interestingly, lymphocytes stained with the proliferation marker PCNA revealed greater T-cell proliferation in lesions of RRMS patients compared to PPMS, whereas B-cell proliferation was observed in some patients [135]. B cells are commonly localised to the perivascular space, with very few found in the centre of plaques, and indeed, B-cell infiltration in MS patients is uniquely higher compared to other inflammatory diseases except autoimmune human encephalitis [135]. CD20⁺ B cells, localised to perivascular regions, were identified in PPMS and SPMS patients and associated with mitochondrial damage, an indication for severe pathology [136]. Furthermore, CD20⁺ B cells can also accumulate in meningeal lymphoid-like structures and are associated with cortical grey matter in SPMS patients [94]. These ectopic B-cell follicles can be extensive in SPMS and also contain a population of CD35⁺ and CXCL13⁺ stromal cells and FDC, suggesting their involvement in promoting B-cell recruitment and activation since B cells were positive for Ki67 [137]. PPMS patients can present with diffuse follicles that contain significant B- and T-cell infiltration but do not have FDC, positive staining for Ki67, or follicle-like organisation as observed in SPMS patients; however, both clinical forms are associated with severe CNS damage [138,139]. The study of MS pathology using histology is a valuable source for providing information about the location of immune cells involved in MS neurodegeneration and disease severity; however, this requires post-mortem tissue. Human tissues remain a valuable source for studying MS pathology; however, animal models of MS have been pivotal in unravelling the complex pathophysiology of the disease. Experimental autoimmune encephalomyelitis (EAE) remains the most used animal model, induced by immunisation with myelin-derived antigens or peptides to induce autoimmune activation that causes axonal damage and mimics MS pathology [140]. These models have been vital in understanding the role of

various immune cells, cytokines, and inflammatory pathways in disease development and progression.

3.2. Single-Cell Analysis

Single-cell analysis of each immune cell involved in MS pathogenesis can be investigated with single-cell RNA sequencing (scRNA-seq), flow cytometry, and mass cytometry. RNA sequencing is a genomic assessment of the expression of RNA molecules, and in scRNA-seq, each individual cell is profiled. Transcriptomics analysis of cells in RRMS and CIS patient cerebrospinal fluid (CSF) identified an increased population of myeloid DCs, monocytes, B cells, CD4⁺ T cells, Tregs, and helper T cells compared to healthy patients [141]. Furthermore, scRNA-seq of isolated peripheral blood mononuclear cells (PBMCs) from MS patients demonstrated increased inflammatory pathways activated in RRMS, while SPMS patients showed reduced activity for neuronal repair [142]. Whether these RNA readouts reflect the protein level is unclear, but combining scRNA-seq with immunohistochemistry could confirm this. For example, scRNA-seq analysis of normal-appearing white matter (NAWM) brain tissue samples from PPMS patients identified an enrichment in expression of inflammatory and cellular stress genes in brain macrophages [143]. Further immunohistochemistry found these to be PVMs localised near blood vessels and parenchyma with positive expression of FCGR2B and HLA-DR, markers associated with inflammation and antigen presentation. Transcriptomic studies provide massive datasets about single-cell activity, biological processes, and pathways altered with disease, which can be complex to interpret and require expertise in bioinformatics.

Flow cytometry uses fluorophore conjugated antibodies and lasers to rapidly analyse single cells in solution, generating a scattered and fluorescent light signal that provides biological and physical properties of cells. Because lymphocyte subpopulations can be characterised by a variety of molecules expressed on the cell surface, antibodies are utilised to distinguish sub-populations of lymphocytes that are otherwise morphologically similar. This has been demonstrated where flow cytometry analysis of isolated PBMCs showed increased CD14⁺HLA-DR- monocytic myeloid-derived suppressor cells, CD14⁺CD16⁺ inflammatory monocytes, and CD4⁺T-helper in RRMS patients compared to healthy controls [144]. Furthermore, targeted flow cytometry presented a loss in circulating CD8⁺CD161^{high} T cells in PPMS, defining a shift in expression of markers with disease progression [145]. More recent studies use flow cytometry to assess extracellular vesicles (EVs) in MS patients, an emerging area that suggests EVs as a biomarker for disease [146]. Interestingly, we combined flow cytometry with trans-endothelial migration (TEM) to identify immune cell migration across a BBB. In this in vitro BBB model, fresh PBMCs from patients were assessed on their migratory ability across an inflamed endothelial monolayer and examined for their phenotypic expression of immune cell markers using flow cytometry [56]. This study showed that leukocytes treated with the sphingosine-1-phosphate modulator drug, fingolimod, had markedly reduced migration. Specifically, these cells were CD3⁺ T cells, effector memory CD4⁺ T cells, naïve CD19⁺ B cells, and NK cells. In another study investigating the effect of cladribine treatment on patient PBMCs, cell migration of intermediate monocytes and both CD4⁺ effector memory and CD8⁺ central memory T-cell migration were reduced [147,148]. These methods provide critical knowledge about the immune cell subtypes that are involved in MS pathogenesis.

Mass cytometry enables high-dimensional assessment of complex phenotypes by replacing fluorochromes with heavy metal isotopes conjugated to antibodies [149]. Samples may be analysed in solution, or tissue sections may be prepared for analysis by imaging mass cytometry, both of which provide valuable information [150]. By using heavy metals, mass cytometry eliminates the issue of spectral overlap observed in flow cytometry. Compared to scRNA-seq, cytometry allows the assessment of post-translational modification of proteins including protein phosphorylation. Mass cytometry has provided new insights in the field of MS. For example, CyTOF differentiated myeloid cell populations in active lesions and NAWM between PPMS and non-MS donors, by measuring 74 proteins, which

indicated an enrichment of phagocytic and activated microglia in active PMS lesions [151]. Similarly, our study designed a panel of B-cell markers to assess differences between CIS vs. MS and active vs. inactive MS, whereby nine IgG3⁺ B-cell subsets were identified. We further observed significant changes in memory B-cell subsets between CIS and MS patients that corresponded with serum IgG3 levels [152]. Another study demonstrated an increased abundance of both a T-bet-expressing B-cell subset and a CD206⁺ classical monocyte subset in early MS [153]. Compared to immunohistochemistry, imaging mass cytometry provided better discrimination of the location of immune cell subsets in patient tissues [154]. Single-cell analysis has the potential to provide a comprehensive analysis of cell phenotypes, functional states, and cell–cell interactions in relation to lesion morphometry and demyelinating activity. These techniques have the potential to lead to new discoveries, improved diagnosis, and novel treatments for MS patients.

4. Disease-Modifying Therapies (DMTs) to Treat MS

Significant findings from histopathology in animal MS models and human samples and single-cell analysis have identified immune cell subsets involved in MS pathogenesis. As a result, this has contributed to the development of many targets for MS treatments, including CD20, CD52, $\alpha_4\beta_1$ -integrin, sphingosine 1-phosphate receptor, interferon-beta, and Bruton's tyrosine kinase (Figure 3). These targets have generated DMTs that improved MS prognosis by modulating the immune system to suppress inflammation, inhibit migration of lymphocytes across the BBB, and decrease the formation of new lesions [155]. DMTs alleviate symptoms, slow disease progression, and reduce disabilities and relapses while improving overall quality of life, thereby ameliorating the impact of MS [156]. Most DMTs are effective at treating RRMS but have been far less effective in influencing the course of SPMS and PPMS [157,158]. It is also important to note that the impact of DMTs for newly diagnosed RRMS patients is more effective when introduced at early stages of the disease [156,159]. There are many Food and Drug Administration (FDA)-approved DMTs commercially available to MS patients, with several others being assessed in clinical studies as discussed below. Research is still ongoing to develop new DMTs that treat MS patients with limited side effects.

4.1. Immunomodulators

Immunomodulatory therapies aim to stimulate or suppress the immune system to help induce an effective response to disease. The first FDA-approved DMT for MS was interferon-beta (IFN- β) therapy in 1993 [160]. IFN- β is a signalling protein of the immune system, though its mechanism of action is not completely understood. Studies suggest IFN- β is involved in antiviral, anti-proliferative, and immunomodulatory properties [161]. Recombinant IFN- β 1-a and 1-b are FDA-approved and used to treat MS patients. Clinical trials demonstrated significant reductions in disease severity supported by reduced lesions in MRI scans [160,162–165]. However, side effects were frequently observed where patients develop flu-like symptoms and reactions to intramuscular injections. Another immunomodulatory drug is glatiramer acetate (GA), which is a random polymer of the four amino acids found in MBP that suppresses T-cell activity and promotes APCs to switch to an anti-inflammatory phenotype [166]. GA was approved in 1996 as a daily subcutaneous injection with no severe side effects, with the exception of transient post-injection reactions [167]. GA treatment vs. placebo showed reduced relapse rates in RRMS patients over 2 years, with improved disability outcomes [168]. Dimethyl fumarate (DF) is an oral immunomodulator involved in nuclear factor erythroid 2-related factor 2 activation, which promotes an anti-inflammatory and anti-oxidative effect on immune cells [169]. In a phase 3 clinical trial, relapse rates reduced by 44% for MS patients treated with DF compared to placebo over 2 years [170]. However, some patients experienced flushing and gastrointestinal side effects.

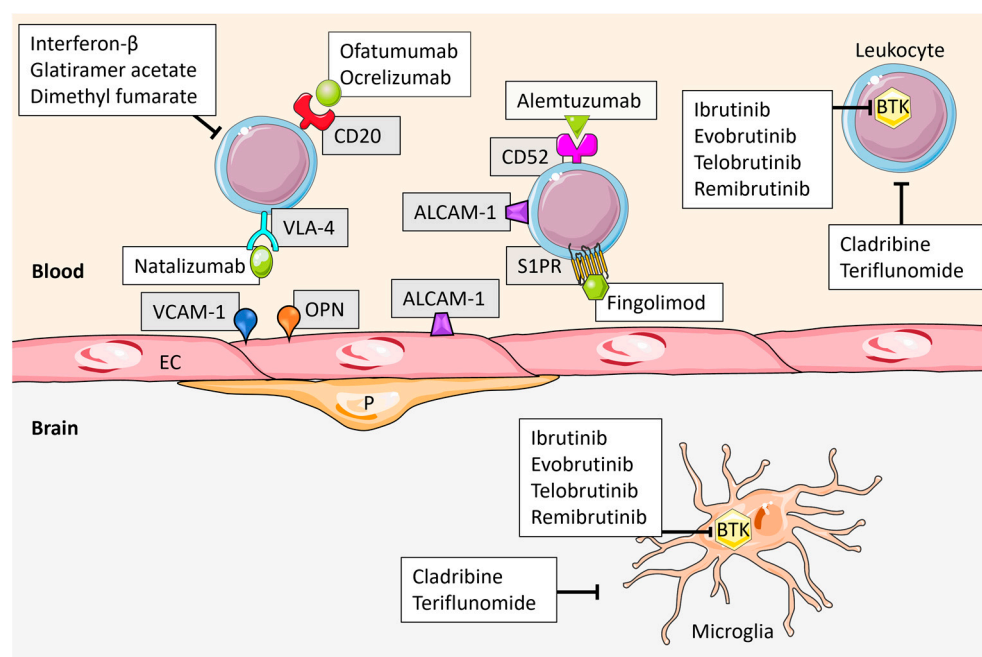


Figure 3. Targets for disease-modifying therapies in multiple sclerosis. Immunomodulatory therapies like interferon- β , glatiramer acetate, and dimethyl fumarate target immune cells to reduce inflammatory responses in disease. Leukocyte depletion and cytotoxicity are induced by anti-CD20 therapies such as ofatumumab and ocrelizumab, anti-CD52 by alemtuzumab, and small molecule drugs such as cladribine and teriflunomide. Leukocyte transmigration into the CNS is inhibited by the anti-VLA-4 antibody natalizumab, and cell emigration from lymph nodes is prevented by sphingosine-1-phosphate receptor modulator fingolimod. An emerging target for multiple sclerosis treatment is Bruton's tyrosine kinase inhibitors, which include ibrutinib, evobrutinib, telobrutinib, and remibrutinib. These BTK inhibitors block BTK activity in leukocytes and microglia, preventing inflammation. Additional markers such as ALCAM-1 and osteopontin are possible targets for treatment. Abbreviations: EC: endothelial cell; P: pericyte; S1PR: sphingosine-1-phosphate receptor; VCAM-1: vascular cell adhesion molecule-1; BTK: Bruton's tyrosine kinase; OPN: osteopontin.

4.2. Leukocyte Depletion and Cytotoxicity

Many of the DMTs used to treat patients display potent effects on leukocyte numbers and activity in MS. For example, ofatumumab and ocrelizumab are human anti-CD20 monoclonal antibody therapies that induce cell cytotoxicity in CD20⁺ cells [171–173]. Patients treated with ofatumumab showed depleted B-cell numbers, reduced lesion size, and improvements in symptoms with limited infusion-related reactions [174–179]. Because low doses of ofatumumab are self-administered monthly, this treatment has become convenient, cost-effective, and burden-free [180]. Alemtuzumab is another human monoclonal antibody that binds to CD52⁺ cells to induce complement-dependent cytotoxicity [181,182]. MS patients treated with alemtuzumab had reduced relapses and disability compared to IFN- β treatment [183]. After a year of treatment, MRI scans showed reduced disease activity [184]. Our lab studied the effect of alemtuzumab on circulating immune cells isolated from RRMS patients before and after treatment and assessed immune markers by mass cytometry. We identified that alemtuzumab treatment restored expression of B-cell linker protein, CD40, and CD210 on IgA⁺ and IgG1⁺ B cells that were altered in RRMS patients [185]. In a prospective study, circulating ILC1 levels increased with alemtuzumab treatment with no changes in NK levels except during relapse [186]. These findings suggest alemtuzumab promotes a more tolerant immune phenotype in MS. We also found that alemtuzumab decreased CD4 effector memory T cells and CD8 central memory T-cell migration in our TEM study [187]. As an immunosuppressive drug, side effects experienced by patients treated

with alemtuzumab include autoimmune hyperthyroidism and immune thrombocytopenic purpura [188–190].

Small-molecule drugs are also available as oral medications such as cladribine and teriflunomide. Cladribine is a synthetic adenosine deaminase taken up by cells and phosphorylated to its active metabolite cladribine triphosphate to inhibit DNA synthesis and repair, resulting in consecutive cell apoptosis [191,192]. Clinical trials showed a reduction in relapse rates and disability progression in cladribine-treated MS patients compared to placebo [193–195]. Remarkably, lesion numbers were reduced as early as six months after starting cladribine treatment in RRMS patients [196]. In a TEM assay, we showed that cladribine reduced the migratory ability of CD4 effector memory T cells, CD8 central memory T cells, and intermediate monocytes during MS [147,148]. Furthermore, mass cytometry found cladribine-treated RRMS patients had a reduction in all ILC subsets except for ILC2 [197]. These findings suggest cladribine tablets are semi-selective at immune cell depletion, resulting in the emergence of a new, expectedly more tolerant, immune system [198]. Teriflunomide is an active metabolite derived from leflunomide that selectively inhibits dihydroorotate dehydrogenase, a mitochondrial enzyme involved in lymphocyte production [199]. Clinical trials studying the effect of teriflunomide treatment reported reduced relapse rates and disability outcomes for MS patients [200]. Side effects were observed with teriflunomide such as alopecia, nausea, and diarrhoea.

4.3. Leukocyte Transmigration Inhibitors

Leukocyte TEM across the BBB is promoted by the expression of adhesion molecules such as VCAM-1, ICAM-1, E-, and P-selectin on endothelial cells [201]. A selective adhesion molecule inhibitor such as natalizumab blocks the ability of leukocytes to attach to the endothelium. Natalizumab is a humanised IgG4 monoclonal antibody that binds to $\alpha_4\beta_1$ -integrin or VLA-4 on leukocytes to prevent interaction with VCAM-1 on endothelial cells [202,203]. Clinical trials showed effective results in 90% of RRMS patients with reduced relapses, lesions size, and disability outcomes [204,205]. Compared to fingolimod, natalizumab-treated patients have reduced rates of treatment discontinuation and display no sign of relapse after two years [206]. However, a 10-year follow-up study showed 52% of patients discontinued natalizumab treatment, with 27% displayed worsening disabilities and 1 in 1000 treated patients presenting with progressive multifocal leukoencephalopathy, a rare immunosuppressive disease [207,208]. These observations suggest that its long-term effects may not be apparent.

Another inhibitor of leukocyte migration is the sphingosine-1-phosphate (S1P) receptor modulator, which includes fingolimod, ozanimod, siponimod, and ponesimod [167]. Fingolimod is a small-molecule oral medication that binds to S1P receptors on immune cells causing internalisation and degradation of the receptor, thus preventing lymphocyte egress from lymphoid tissue and into the CNS [209]. Clinical trials comparing fingolimod to placebo treatment in MS patients showed reduced lesions on MRI scans, and these effects were still observed in the long term with low disability progression, improved quality of life, and no side effects [210–212]. Indeed, our studies demonstrated the immunomodulatory effect of fingolimod on T-cell, B-cell, and NK-cell migration across a BBB model. Specifically, CD4 effector memory T-cell migration was reduced following fingolimod treatment [56]. Clinical trials reported patients experiencing side effects with fingolimod treatment such as fingolimod-associated macular oedema and cardiac complications [211,213]. Hence, the more recently FDA-approved second-generation S1P receptor modulators, ozanimod, siponimod, and ponesimod, have better specificity and activity for S1P receptors to reduce side effects and improve clinical benefits [214].

Additional studies have been conducted demonstrating potential targets for regulating leukocyte transmigration in MS. For example, activated leukocyte cell adhesion molecule (ALCAM-1) is involved in B-cell migration across the CNS, whereby ALCAM+ B cells have been identified in peripheral blood and MS brain lesions [215]. Further, blocking ALCAM in a mouse model of EAE reduced B-cell transmigration and disease progression.

Osteopontin (OPN) is another potential target, with recent studies detecting elevated levels of serum anti-OPN autoantibodies in MS patients [216]. These novel targets represent a promising treatment option for inhibiting leukocyte transmigration in MS.

4.4. Bruton's Tyrosine Kinase (BTK) Inhibitors

BTK inhibitors are an emerging field for MS treatment. BTK is an intracellular signalling protein that belongs to the tyrosine kinase family and is downstream of the B-cell receptor (BCR) [217]. Signalling pathways modulated by BTK activity include nuclear factor of activated T cells (NFAT), nuclear factor- κ B (NF- κ B), mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK). These signalling pathways promote B-cell development, maturation, activation, proliferation, survival, and differentiation into memory B cells and antigen-presenting plasma cells [218]. BTK is also expressed by other immune cells such as T cells for activation and proliferation [219,220], microglia for neuroinflammation, and macrophages to promote phagocytosis and cytokine production [221]. Increased BTK activity contributes to CNS inflammation in MS [222]. RRMS and SPMS patients displayed higher levels of phosphorylated BTK protein expression in blood-derived class-switched memory B cells compared to CIS and healthy controls [223]. Histopathological studies assessing MS brain tissue have also presented increased expression of BTK protein in acute and chronic active lesions [224]. Furthermore, these active lesions showed positive BTK staining in B cells, microglia, and macrophages while in chronic active lesions, only the rim showed positive BTK staining in microglia. These findings demonstrate the importance of BTK activity in MS pathogenesis and its potential to be a promising therapeutic target.

BTK inhibitors modulate immune cell function by reducing inflammation and neurodegeneration, and include ibrutinib, evobrutinib, orelabrutinib, tolebrutinib, remibrutinib, and fenebrutinib [225,226]. All are currently being evaluated in clinical trials except for ibrutinib, which is FDA-approved [227]. Each BTK inhibitor differs in its selectivity, binding ability, and strength of inhibition; for example, higher concentrations of evobrutinib are required to induce the same effects as other inhibitors [228]. Phase 2 clinical trials assessing evobrutinib demonstrated reduced lesion size in one year and number of lesions over two years [229,230]. Likewise, tolebrutinib treatment demonstrated a reduced number of new lesions in RRMS patient over 12 weeks in a phase 2b study [231]. Remarkably, these studies observed limited side effects to treatments. Furthermore, these BTK inhibitors are small molecules that can pass the BBB to target activated immune cells within the CNS and promote effective treatment [232].

5. Conclusions

MS is a chronic disease that initially presents as relapses and then progressively leads to severe disability if not managed and treated early. MS pathogenesis is complex and influenced by various immune cells. Research is ongoing to determine the cause and define the pathology that affects many young individuals. Our understanding of immune cell subsets involved in MS pathogenesis has expanded with the contribution of histopathology and single-cell analysis. Using histopathology and immuno-staining, the localisation of CD3⁺ and CD103⁺ T cells and CD20⁺ B cells in perivascular cuffs has been established, and the contribution of stromal cells and FDC to ectopic B-cell follicles has been specified. Furthermore, single-cell analysis by techniques such as flow cytometry, scRNA-seq, and mass cytometry developed the ability to identify immune cell phenotypes in MS patient tissue, blood, and CSF samples. These studies have led to the introduction of targeted therapeutics to CD52, CD20, S1P1, and BTK for the treatment of MS. With ongoing research and novel treatments being tested in clinical trials and investigations to further understand MS pathogenesis, more effective therapeutics to treat MS are a promising goal for the near future.

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