1.1 General introduction
Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that affects roughly 2.3 million people globally. Clinical symptoms are diverse in nature and are a reflection of the pathology developing within the CNS. Classical symptoms of MS include, but are not limited to: fatigue, numbness or tingling, pain, muscle spasms, bladder dysfunction, visual problems, a loss of motor capabilities and cognitive issues. For patients in late stage disease, disability accumulates and impairment of pulmonary function as a result from weakened ventricular muscles can be fatal. It was estimated in 2013 that nearly 20,000 people lost their lives due to complications associated with the disease.

MS has a predilection for disease occurrence in young adults. Hence, unlike other degenerative diseases of the CNS, such as Parkinson’s or Alzheimer’s disease, MS adversely affects the quality of life during prime activity years. The chronic nature of this disease, coupled with the predilection for early onset, makes MS a tremendous financial burden for western societies as treatment options are expensive and may be required for long periods of time. Despite recent advances in both knowledge and development of therapeutics, there is still much to be learned about the mechanisms underlying disease initiation and development.

Clinical progression of MS

The clinical course of MS is heterogeneous and varies per patient. There are several patterns of disease phenotypes, characterized based upon progression of disability; or more specifically the refractory period of activity and intensity of disease symptoms.

Accurate diagnosis of the clinical course, or phenotype of disease, is vital for the treatment decision-making process and for enrollment into clinical trials. Recently, guidelines on categorizing clinical phenotypes have been changed by the US National Multiple Sclerosis Society Advisory Committee on Clinical Trials in MS; an effort to clarify terminology and to lead to more accurate clinical diagnosis. Patients frequently visit the clinician following a first bout of neurological deficit. During this time, patients are categorized with clinically isolated syndrome (CIS), compatible with inflammatory/demyelination and will be diagnosed with clinically definite MS following another attack and confirmation of CNS lesions via MRI. For CIS patients with abnormal MRI presentation, there is an 80% chance of going on to develop MS. Moreover, patients with white matter (WM) abnormalities consistent with demyelination, but which are lacking neurological deficits, have been noted. These patients are categorized with radiologically isolated syndrome (RIS). The risk of future development of clinically definite MS is lower in RIS cases than in patients presenting with both CIS and abnormal MRI.

Disease progression can be broadly categorized as either a relapsing-remitting (RR) or progressive course. RRMS describes a disease course where periods of disability alternate with recovery (Figure 1). Although 85% of patients with MS have the RRMS form, the ability of patients to recover from neurological deficit is frequently diminished as time progresses and roughly 80% of patients will go on to develop secondary progressive MS (SPMS). In a minority of cases (±15%) the disease is progressive from onset; this is primary progressive (PP) MS.
forms of the disease, either PPMS or SPMS, are characterized by the gradual worsening of neurologic functions without intermittent recovery. Other variants are benign MS, a mild form, and progressive relapsing MS, which is progressive from onset with super-imposed attacks.

Figure 1. Clinical course of MS. A majority (85%) of clinically diagnosed MS patient’s start with a relapsing-remitting (RR)MS form of the disease characterized by intermittent neurological symptoms followed by recovery. In > 80 % of patients with RRMS, the ability to recover is diminished as time progresses, leading to secondary progressive (SP)MS.

Pathological characterization of MS

General background

Pathological characterization of post mortem CNS tissue has yielded an abundance of knowledge about underlying disease mechanisms and potential therapeutic targets. One of the most prominent pathological hallmarks of MS is the presence of multiple focal demyelinated areas (i.e. lesions or “plaques”), which are variably associated with a humoral and/or cellular autoimmune attack on myelin. Oligodendrocytes produce the myelin sheaths, which wrap around axons to ensure rapid salutatory pulse conduction and adequate trophic support to axons. With respect to composition of myelin, a high proportion of myelin is composed of various lipids (70-85% w/w). A lesser proportion of myelin is protein (15-30%), such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). Myelin loss is readily detected by immunohistochemistry using monoclonal antibodies (mAb) to myelin specific proteins or general histology stains such as Luxol Fast Blue. Demyelination is a feature of many neurological disorders, but in early MS, myelin loss is associated with relative axonal preservation.

Lesions in the MS brain are disseminated through WM and grey matter (GM) and both spinal cord and brain tissue. Yet, there is a clear topographic predilection for certain areas of the CNS, including optic nerves, brainstem, and cerebellum and periventricular WM regions. Oligodendrocyte injury, inflammation and astrogliosis are variable and depend on the activity of the lesion. Inflammation of the active lesion predominantly consists of infiltrated macrophages,
activated microglia, and T cells, with CD8+ T cells outnumbering CD4+ T helper cells. B cells and plasma cells can be observed, to a much lesser degree, in lesions, and these are often associated near perivascular spaces.

**Lesion classification**

A hallmark of the active MS lesions is the presence of myelin-laden macrophages. This feature along with cellular composition and myelin content of the lesion has led several prominent groups to propose lesion staging systems. Lesion staging systems (Table 1) include the Bö/Trapp system, later modified by De Groot/van der Valk and the commonly referenced Lassman/Brück method. In November of 1997, a workshop was held for prominent neuropathologist and MS researchers to discuss staging of MS lesions (reviewed in ). Despite an agreement to combine features of both Bö/Trapp and the Lassman/Brück systems into a single staging system termed the Vienna consensus, this classification system is infrequently used. Regardless of the classification system used, the research question and aim dictate the system used, and clarification of such key words has provided guidelines for neuropathologists to compare lesions of different studies.

**Table 1. Lesion classification.**

<table>
<thead>
<tr>
<th>Bö/Trapp</th>
<th>De Groot/van der Valk</th>
<th>Lassman/Brück</th>
<th>Vienna consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active (demyelination, hypercellular)</td>
<td>Preactive (microglia cluster, no demyelination)</td>
<td>Early active (MOG + Mφ)</td>
<td>Infl.-, demyel.-</td>
</tr>
<tr>
<td>Active demyelinating (myelin + Mφ)</td>
<td></td>
<td>Infl.+ , demyel.+</td>
<td></td>
</tr>
<tr>
<td>Chronic active (hypocellular center, hypercellular rim)</td>
<td>Active non demyelinating (no myelin+ Mφ)</td>
<td>Late active (PLP + Mφ)</td>
<td>Infl.+ , demyel.-</td>
</tr>
<tr>
<td>Chronic inactive</td>
<td>Inactive</td>
<td>Early remyelinating (thinly myelinated axon)</td>
<td></td>
</tr>
<tr>
<td>Chronic inactive (hypocellular)</td>
<td>Chronic inactive</td>
<td>Late remyelinating (shadow plaque)</td>
<td>Infl.-, demyel.-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Infl.-, demyel.+</td>
</tr>
</tbody>
</table>

Mφ, macrophage. Infl.= inflammation, demyel.= demyelination. Adapted from .

**WM lesion heterogeneity**

Inter-individual lesion heterogeneity in WM demyelination has been described. Lucchinetti and colleagues characterized biopsies and autopsies for immunological and neurobiological markers. These authors proposed four different patterns of WM demyelination defined by myelin loss, oligodendrocyte destruction, and antibody deposition (Table 2). It was suggested that these patterns of demyelination were homogenous within a patient, but heterogeneous between patients. These findings however must be interpreted with caution as other groups failed to replicate lesion heterogeneity between patients, and inherent difficulties identifying apoptotic oligodendrocytes to apoptotic lymphocytes may confound results derived from such a system. Others have argued that these different types of WM lesions more likely represent different stages of demyelination, or a spectrum of activity, than distinct pathological disease.
Table 2. Patterns of demyelination in white matter lesions.

<table>
<thead>
<tr>
<th>Clinical phenotypes</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Type IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute, RR, SP, PP</td>
<td>Acute, RR, SP, PP</td>
<td>Acute, RR, SP</td>
<td>PP</td>
</tr>
<tr>
<td>Demyelination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perivenous pattern</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>Lesion edge</td>
<td>Sharp</td>
<td>Sharp</td>
<td>Ill-defined</td>
<td>Sharp</td>
</tr>
<tr>
<td>Concentric pattern</td>
<td>–</td>
<td>–</td>
<td>~30% of cases</td>
<td>–</td>
</tr>
<tr>
<td>Myelin protein loss</td>
<td>Even</td>
<td>Even</td>
<td>MAG &gt;&gt; others</td>
<td>Even</td>
</tr>
<tr>
<td>Shadow plaques</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>B cells/plasma cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Complement activation</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Oligodendrocytes in plaque</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density in plaque</td>
<td>+++</td>
<td>+++</td>
<td>+ (↓)</td>
<td>+ (↓)</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>±</td>
<td>±</td>
<td>++ (apoptosis)</td>
<td>+++ (periplaque white matter)</td>
</tr>
</tbody>
</table>

RR, relapsing-remitting; SP, secondary progressive; PP, primary progressive. Adapted from 27,31.

Grey matter pathology

Historically, MS was viewed as a mainly WM disease. However, it is increasingly clear that the extent of focal WM damage alone cannot account for the symptoms observed in the MS patients; issues such as cognitive deficits are more strongly associated with GM injury 32-35. GM lesions can be observed in the hippocampus, cerebellum and in all layers of the cerebral cortex 15. Several distinct lesion patterns have emerged based upon the location in the cortex where they develop. Leukocortical lesions extend from the WM into the GM; yet spare superficial cortical layers 36,37. Leukocortical lesions, which can be seen in the earliest disease stages, frequently appear to start from subcortical WM lesions and exhibit a relatively reduced number of immune cells compared to the WM portion of the lesion 38. Intracortical lesions lie completely inside the GM and are generally associated with a blood vessel. Subpial lesions are the most abundant type of cortical lesion and are unique to MS as they are typically not observed in other human brain diseases 39. The predilection for subpial demyelination to occur in deep sulci regions of the cortex has led some to suggest that these lesion types may be mediated by factors present in the cerebrospinal fluid (CSF); yet no single factor has been isolated to explain this phenomenon 40. Cortical GM lesions are characterized by microglia activation, myelin loss and axonal injury, but are paucicellular with respect to immune cell infiltration and only limited BBB disruption is observed 32,41. In early stages of MS, cortical lesions have been observed to be highly inflamed and suggested to precede WM demyelination 42. The degree of subpial cortical demyelination and neurodegeneration has been related to the presence of meningeal follicle like structures that may play a significant role in disease progression 43. Others, however, observed no correlation between meningeal inflammation and subpial lesions 44. Furthermore, it is unknown whether GM injury observed in the MS brain results from a primary (i.e. neuronal susceptibility) or secondary
pathogenic process (i.e., virtual hypoxia); it is also unclear if WM and GM tissue destruction are induced by the same mechanism.

**Oxidative injury and mitochondrial defects in the MS brain**

**Oxidative Injury**

The human brain is vulnerable to oxidative stress due to the high polyunsaturated fatty acid content of the neuronal membranes and the high oxygen consumption relative to the rest of the body. Recent attention has focused on oxidative damage and mitochondrial dysfunction as causative factors in axon-related energy deficits and degeneration in the MS brain (reviewed in [46,47]). The active MS lesion is characterized by the expression and activation of an oxiradical generating nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; NOX2) in macrophages and microglia. Once the membrane-bound (p22phox, gp91phox) and cytosolic subunits (p40phox, p47phox & p67phox) are assembled (Figure 2), NOX2 produces superoxide anion (O$_2^-$); a key first step in the oxidative injury cascade (Figure 3). Superoxide anion can be dismutated to hydrogen peroxide (H$_2$O$_2$) by antioxidant enzymes, particularly superoxide dismutase (SOD) 1 and 2, which are markedly expressed in hypertrophic astrocytes and myelin-laden macrophages of the MS brain. The active MS lesion is also characterized by abundant expression of inducible nitric oxide synthase (iNOS) in astrocytes and macrophages. The nitric oxide synthase family mediates the conversion of L-arginine to nitric oxide (NO*) and L-citrulline; the coupling of nitric oxide with superoxide anions yields highly toxic peroxynitrite. Iron accumulates as ferric ion (Fe$^{3+}$) in myelin and oligodendrocytes of the human brain upon aging and is liberated during demyelination or oligodendrocyte death. The short-living superoxide anion can transfer its free electron to ferric iron yielding ferrous iron (Fe$^{2+}$), which is thought to amplify oxidative injury via the production of highly reactive hydroxyl radicals (OH$^*$). Iron metabolic markers, such as lactoferrin and transferrin, are altered upon demyelination and iron rims are present in chronically expanding lesions.

**Figure 2. The NADPH oxidase complex.** Generation of superoxide radicals by the NADPH oxidase involves translocation of cytosolic subunits (p40phox, p47phox, & p67phox) to membrane-bound subunits (p22phox & gp91phox).
Collectively, reactive oxygen species (ROS) and reactive nitrogen species (RNS) metabolites cause metabolic stress, deoxyribonucleic acid (DNA) alkylation and peroxidation of phospholipids and proteins. Oxidative injury is extensively observed in the MS brain and the presence of markers associated with oxidative insult coincides with a marked upregulation of anti-oxidant enzymes.

**Mitochondrial impairment**

A major consequence of oxidative stress and oxidative tissue injury is impairment of mitochondria, and a growing body of evidence implicates mitochondrial dysfunction as a key contributing factor in MS pathogenesis. The mitochondrial respiratory chain is the major site of energy production, which involves the (oxidative) phosphorylation of adenosine diphosphate to adenosine triphosphate (ATP). The respiratory chain is composed of four complexes (Complexes I-IV) that are made of protein subunits encoded by both nuclear and mitochondrial DNA (mtDNA).

Due to the lack of protective histones mtDNA is particularly susceptible to oxidative stress leading to deletions and point-mutations that may be propagated during clonal expansion. As cells contain many copies of mtDNA, a high ratio of deleted versus healthy mtDNA (heteroplasmy) copy numbers must exceed a certain threshold for biochemical defects to be manifested at the cellular level. The MS brain is characterized by the presence of neurons functionally deficient in respiratory chain complex IV and by mtDNA deletions throughout the GM. Respiratory deficient neurons have been shown to contain clonally expanded mtDNA deletions or high levels of oxidative stress.

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**Figure 3. The oxidative injury cascade.** The NADPH oxidase, xanthine oxidase and mitochondrial defects generate oxygen radical species. The anti-oxidant superoxide dismutase (SOD1/2) catalyzes the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. Hydrogen peroxide is reduced to water via the energetically demanding glutathione redox cycle. If ferrous iron is present, hydrogen peroxide can form highly toxic hydroxyl radicals. Concomitantly, the coupling of superoxide radicals with nitric oxide forms toxic peroxynitrite. Collectively these molecules contribute to oxidative tissue injury. L.Arg= L-arginine.
of heteroplasmy. The functional consequences of the oxidative damage induced by mitochondria impairment are numerous. As ATP production is diminished, the ability of the sodium/potassium pump (Na+/K+ ATPase) to function and effectively remove intra-axonal sodium becomes compromised, resulting in the reversal of the axolemmal Na+/Ca2+ exchanger and increased intracellular calcium content.

The energy failure and ensuing altered calcium homeostasis not only contribute to axonal degeneration; intra-axonal ROS production would likely increase as electrons would be liberated from the dysfunctional respiratory chain, resulting in further amplification of oxidative injury. Mitochondrial injury may also induce histotoxic hypoxia, a state of energy failure and reduced oxygen consumption. Such mechanisms have been described in the MS brain and would contribute to degeneration.

Figure 4. Mitochondrial dysfunction contributes to axonal degeneration.

NO= Nitric oxide. Adapted from 47.
Risk factors

Genetic

Although there is no single determinant risk factor for the development of MS, the use of patient databases for epidemiological studies and the advancement of genetic technologies enabling genome-wide association screening has identified several environmental and genetic factors that predispose an individual to MS. With respect to genetic risk factors, the risk to develop MS is genetically linked as first degree relatives of MS patients are at a 10-25 times greater risk for developing the disease than individuals of the general population. Moreover, the MS concordance in identical twins (30%), is substantially higher than in non-identical twins (3%). While no single gene dictates the susceptibility of MS, the strongest genetic associations are immune-oriented. Polymorphisms in the genomic region encoding human leukocyte antigen (HLA) complex class II have been identified as conferring the strongest influence on MS risk or resistance depending on the polymorphism. For instance, whereas alleles HLA-DRB1*1501, -DRB5*0101, -DQA1*0102, -DQB2*0602, and -DRB*0101 may confer risk, alleles such as HLA-DRB1*14 and -DRB1*11 confer some degree of resistance to MS development. The main susceptibility risk allele in MS has been shown to be HLA-DRB1*1501, yet this risk can be completely abrogated by the co-inheritance of HLA-DRB1*14. Despite the strongest genetic associations being within HLA class II region on chromosome 6p21, more than 110 non-HLA associated genetic risk factors have been noted thus far.

The vast majority of non-HLA genetic risk factors encodes a function in the immune response and ± 70% overlaps with other chronic autoimmune diseases (e.g. rheumatoid arthritis, colitis and diabetes). One such a risk factor is a single nucleotide polymorphism (SNP) in the interleukin-7 receptor-α chain (IL-7Rα /CD127). IL-7 is a type 1 cytokine family member that plays a crucial role in both lymphopoiesis and homeostasis of B cells and T cells. Signaling is mediated through binding of IL-7 to a heterodimeric receptor composed of IL-7Rα paired with the common γ chain (CD132). Under normal circumstances, engagement of the IL-7R by IL-7 on naïve and memory T cells exerts anti-apoptotic effects, resulting in increased cell survival. Whereas IL-7R is typically down-regulated on T cells upon activation, it is selectively up-regulated on populations destined to develop into memory T cells. A functional association of IL-7 and IL-7R polymorphism to MS has also been established. MS patients have a lower threshold for IL-7-induced CD8+ cytotoxicity and most CD8+ T cell subsets exhibit increased expression of IL-7Rα. The ratio of membrane-bound to soluble IL7Rα is altered in individuals with MS and they also have lower systemic levels of IL-7 and soluble IL-7Rα; potentially eliciting aberrant activation of T cells. Furthermore, IL-7 has been shown to drive the expansion of high avidity, myelin-specific CD4+ T cells isolated from peripheral blood mononuclear cells (PBMC) from MS patients. These findings, along with observations that blockade or genetic knock out of IL-7R in rodent models is sufficient to ameliorate disease, has led some to suggest this may be an attractive therapeutic target for MS.
**Latitude and low vitamin D**

As previously noted there are several environmental risk factors for MS, of which geographic location, serum vitamin D, and a history of Epstein-Barr virus (EBV) infection are the most prominent. Just like genetic factors, no single environmental association can explain the development of MS. Variation in MS prevalence with respect to geographic latitude has been noted in Europe, North America, Australia and Asiatic countries. A meta-analysis of 650 prevalence estimates from 59 countries in 321 peer-reviewed studies confirmed a latitude gradient effect, and this evidence supported previous findings of a reduction in risk of MS when migrating from high to low risk areas before the age of 15. The correlation of latitude may, in part, be explained by the observation that low serum vitamin D levels are linked to MS. For instance, a 41% decrease in risk per every 50 nmol/l increase of serum 25-hydroxyvitamin D (25(OH)D) levels has been observed. As the primary source of biologically active 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) is predominantly derived by sunlight mediated conversion of cutaneous 7-dehydrocholesterol to pre-vitamin D3; living in latitudes of low sunlight exposure may predispose an individual to low serum levels of active vitamin D. The effects of vitamin D levels on the human immune system is supported by a plethora of literature data, and converge with evidence derived from animal models of autoimmune disease. Vitamin D-deficient mice experience accelerated onset of experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Direct injection of 1,25(OH)2D3 completely prevented disease development and 1,25(OH)2D3 supplementation attenuated disease severity. In rodent EAE, disease severity attenuation was a result from reduction in IL-17A and IL-17F expression. Moreover, the Th1/Th2 T cell ratio in MS was found to be correlated to serum 25(OH)D levels, and thus low serum vitamin D could contribute to MS progression by promoting a more pro-inflammatory T-cell compartment.

**Epstein-Barr virus**

Another environmental risk factor for MS is infection with EBV; a member of the γ-herpes virus family, known to preferentially infect B cells via CD21, the receptor of complement factor C3d. Although well known as the main etiological agent of classical infectious mononucleosis (IM) and Burkitt’s lymphoma, it has also been associated with neoplastic diseases such as Hodgkin’s, T cell and natural killer (NK) cell lymphomas and autoimmune diseases including Systemic Lupus Erythematosus, Sjögren’s syndrome and MS (for review).

While EBV’s relationships to neoplastic malignancies are well characterized, the exact role of EBV in autoimmune diseases, particularly MS, is unclear. Although EBV infects >90% of the general population, individuals with a history of IM have a two- to three-fold increased risk of developing MS. A temporal relationship between immunoglobulin (Ig)G1 titers against the EBV nuclear antigen-1 (EBNA-1) and gadolinium-enhancing (inflammatory active) lesions has been established. Elevated IgG1 titers against EBNA-1 are also predictive of disease onset. Despite epidemiological and humoral immune responses implicating EBV as a causative agent in MS, a direct role has yet to be elucidated. This relationship has been complicated by conflicting reports of EBV in the MS brain and a lack of temporal association between viral reactivation and gadolinium contrast-enhancing lesion identification. Recently, Pender and colleagues demonstrated the MS T cell is defective in controlling EBV infection.
Immunopathogenic mechanisms in MS

The recent failures and successes of therapeutics in Phase II and Phase III clinical trials has yielded a greater appreciation for the complexity of the immunopathogenic mechanisms governing MS. Although it has yet to be resolved if MS is primary a neurodegenerative disease with secondary autoimmune attack, or a primary autoimmune disease, substantial evidence supports a clear role of the immune system in disease progression. T cells are observed in the active lesion, and T-cell related pro-inflammatory cytokines are enriched in the CSF and plasma of MS patients. Despite conflicting reports regarding the relative importance of Th1 versus Th17 subsets, administration of interferon (IFN)-γ exacerbates MS and IL-17 expression is enhanced in the active lesion. Interestingly, IL-17 is detected at similar rates in the CD8+ and CD4+ T cell in the MS brain. While early focus has been placed on the CD4+ Th subset in disease progression, due to the CD4+ T cell bias of rodent EAE models, CD8+ T cells are more frequent in the MS lesion and axonal damage correlates with the number of CD8+ T cells.

The B cell is now recognized to play a prominent role in disease pathogenesis. IgG and complement are characteristic features of both type II and active MS lesions and oligoclonal IgG bands from the CSF are a long-standing hallmark of disease. Furthermore, B cell laden germinal center (GC)-like structures are frequently observed in RRMS and SPMS patient brains; a link between meningeal inflammation B cell follicles and the onset SPMS has been observed. As will be discussed in the next section, therapeutic depletion of the B cell with monoclonal antibodies against the CD20 surface antigen (e.g. rituximab, ofatumumab or ocrelizumab) provided the strongest evidence of a role of B cells in MS.

Although the role for the adaptive immunity has been the focus of much preclinical MS research, the innate system also plays a clear role in disease progression and has significant influences on adaptive immune cell effector function. Resident microglia cells and activated macrophages up-regulate key molecules involved in antigen presentation and interaction with effector T cells, secrete cytokines and regulatory molecules, and generate ROS as previously detailed. Macrophages and microglia may also play a beneficial role by secretion of anti-inflammatory cytokines (i.e. IL-10) or neurogenic factors such as brain derived neutrophic factor (BDNF). Furthermore, dendritic cells (DC) function as key antigen presenting cells (APC) in the immune system, and exhibit an altered phenotype in the MS patient. Peripheral derived DC exhibit elevated expression of activation markers such as CD40 and CD80, reduced expression of the checkpoint inhibitor programmed death ligand-1 (PDL1), and secrete pro-inflammatory cytokines, suggesting that these cells play a role in promoting a pro-inflammatory phenotype.

Therapeutics

Pharmacological intervention

Despite the discovery of MS occurring in 1868, and documented MS-like cases being chronicled as early as the 15th century, disease-modifying therapies were lacking until recently. Prior to the 1990s the only treatment option offered to patients were corticosteroids, which are neither effective in terms of reduction of relapses or slowing disease progression. Fortunately,
the past few decades have brought tremendous advances in the development of disease modifying therapies (shown in Table 3). There are currently 13 approved therapies for RRMS that have demonstrated an ability to modify the disease severity and/or course; half of these treatment options have become available in the last 5 years 127.

Table 3. Therapeutics approved for RRMS.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trade name</th>
<th>Method</th>
<th>Therapeutic target</th>
<th>Anticipated mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glatiramer acetate</td>
<td>Copaxone</td>
<td>Injectable</td>
<td>MHC class II molecules</td>
<td>Not fully elucidated, but mimicks MBP for MHC class II molecules and is Th2 skewing</td>
</tr>
<tr>
<td>β Interferons</td>
<td>Rebif, Betaseron, Extavia, Plegridy, Avonex</td>
<td>Injectable</td>
<td>Leukocytes</td>
<td>Modulation of T cell responses and cytokine profile, down-modulation of MHC class II expression</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Novantrone, Ralenova</td>
<td>Infused</td>
<td>Topoisomerase</td>
<td>Type II topoisomerase inhibitor</td>
</tr>
<tr>
<td>Natalizumab</td>
<td>Tysabri</td>
<td>Infused</td>
<td>Cell adhesion molecule VLA-4 on lymphocytes and monocytes</td>
<td>Inhibits cell migration into CNS</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>Lemtrada</td>
<td>Infused</td>
<td>Cell surface marker CD52 on lymphocytes and monocytes</td>
<td>Depletes CD52 expressing cells</td>
</tr>
<tr>
<td>Dimethyl Fumarate</td>
<td>Tecfidera</td>
<td>Oral</td>
<td>Nrfl pathway</td>
<td>Stimulates Nrfl pathway and is immunomodulatory</td>
</tr>
<tr>
<td>FTY720 (Fingolimod)</td>
<td>Gilenya</td>
<td>Oral</td>
<td>Cell surface marker S1P receptor on immune cell or CNS cells</td>
<td>Prevents lymphocyte lymph node egression</td>
</tr>
<tr>
<td>Daclizumab</td>
<td>Zenapax</td>
<td>Infused</td>
<td>Cell surface marker CD25/IL-2R on T cells</td>
<td>Inhibits proliferation of T cells</td>
</tr>
<tr>
<td>Ocrelizumab*</td>
<td>Ocrevus</td>
<td>Infused</td>
<td>Cell surface marker CD20 on B cells</td>
<td>Depletes CD20+ B cells</td>
</tr>
<tr>
<td>Teriflunomide</td>
<td>Aubagio</td>
<td>Oral</td>
<td>Pyrimidine synthesis</td>
<td>Inhibits proliferation of lymphocytes</td>
</tr>
</tbody>
</table>

* Also approved for PPMS.

The 1990s heralded a new era for both patient and researcher alike. The observation that treatment with IFN-γ exacerbated disease and that the enhanced disease activity was associated with activation of the immune system was a very seminal finding, demonstrating that immune modulation could indeed alter the disease course 114. Interest shifted back to IFNβ-1b and IFNβ-1α, with particular interest in IFNβ1-b as it was known to inhibit the IFN-γ pathway and was more tolerable than interferon alphas 126. IFNβ-1b was approved by the Food and Drug Administration (FDA) for treatment of RRMS, being the first biological agent to demonstrate an ability to modify disease course and heralding a new era of therapy research for patients 128. Several different formulations of both IFNβ-1a or IFNβ-1b have been approved for therapeutic use in MS, despite common side effects including injection site reactions, headaches and flu-like symptoms (reviewed in 127). Mechanistically it is thought that IFNβ’s induce anti-inflammatory cytokines
such as interleukin-10 (IL-10) while inhibiting pro-inflammatory cytokines such as IL-12/-23 and IFN-γ, and alter B-cell trafficking through the blood brain barrier (BBB) 129,130.

Glatiramer acetate (GA), commonly marketed as Copaxone and Glatopa, became the second disease modifying therapy approved for RRMS 131. GA is not a cytokine, but a random polymer based on amino acids prevalent in MBP, namely alanine, glutamic acid, lysine and tyrosine. The original aim of the inventors was to antagonize the activation of anti-MBP T cells. The therapeutic benefit of GA lies in a sound safety profile, with lower injection site reactions than IFNβ, the ability to use it during pregnancy and minimal long term monitoring requirements 132. The first oral therapeutic available for patients was Fingolimod (Gilenya), a drug that alters lymphocyte migration by binding to the sphingosine-1-phosphate (S1P) receptor thereby blocking S1P-driven lymphocyte egression from lymphoid tissue 133-135.

The development of reagents such as monoclonal antibodies (mAb) that selectively target individual molecules expressed by cells participating in the pathogenic process, thus targeting individual cells, has resulted in tremendous progress in the treatment of relapsing forms of MS 136. The most noticeable clinically tested mAbs targeting specific receptors include natalizumab (anti-α4β1 integrin/VLA-4), alemtuzumab (anti-CD52), daclizumab (anti-IL-2Rα) and mAb directed against CD20 such as rituximab, ofatumumab and ocrelizumab 137-141. Natalizumab binds to a cell adhesion molecule expressed on lymphocytes and monocytes thus restricting interaction with the counterstructure vascular cell adhesion molecule-1 (VCAM-1) on BBB endothelial cells and impeding CNS immigration of inflammatory cells. Alemtuzumab depletes CD52+ cells such as T and B cells 140-143. Although these therapies exhibit better efficacy with respect to modulation of disease activity and progression in comparison to the interferon class of drugs, significant side effects exist 127,141. For instance, treatment with natalizumab is associated with increased risk of progressive multifocal leukoencephalopathy (PML), due to reactivation of John Cunningham virus inside the CNS. Alemtuzumab is associated with autoimmune thyroid disease, idiopathic thrombocytopenic purpura, and can cause reactivation of herpes viruses and common infections 144-147. For these reasons, efficacy must be carefully balanced against safety risk, and treatment with natalizumab and alemtuzumab often is used as second or third line therapies in aggressive forms of disease.

The remarkable clinical success of CD20 depletion with rituximab has not only greatly benefited the patient, but also has led to a major paradigm shift for the immunopathogenesis of MS by emphasizing the importance of the B cell in disease progression 148. With respect to RRMS, in two identical phase 3 clinical trials, treatment with the fully human mAb ocrelizumab reduced gadolinium-enhancing lesions by 94% and 95% compared to IFNβ-1α, and was significantly favored with respect to the ability to improve the multiple sclerosis functional composite score; a composite measurement of walking speed, limb mobility and cognition 138. Recently, promising results from the ORATORIO trial were presented demonstrating a beneficial therapeutic effect of the anti-CD20 antibody, ocrelizumab, to reduce disability progression in PPMS patients 149. Although the clinical beneficial outcome of this trial were modest in that disability progression was temporarily delayed and not halted, this is the first FDA approved therapeutic to modify disease activity in PPMS 150.
Non-pharmacological intervention

Alternative pharmacological treatment options, such as cell-based strategies, are also currently being developed. Ablation of the adaptive immune system and installment of a new immune repertoire via immune system ablation and hematopoietic stem cell transplantation (HSCT) has recently been demonstrated as a very successful treatment option for individuals with aggressive forms of MS. In a multi-center phase II clinical trial, HSCT therapy eliminated disease activity in 70% of the patients for a period of 3 years or more. However, one patient died of transplantation-related complications and therefore this treatment option may be riskier than mAb approaches. Cell-based approaches to stimulate autologous remyelination or neuron and oligodendrocyte replenishment are potentially attractive strategies in late stages of disease. Recent studies have led to the advancement of protocols isolating induced pluripotent stem (iPS) cells from PPMS patients and the ability to differentiate these cells into oligodendrocyte progenitor cells (OPCs) and functioning mature oligodendrocytes for basic research. Clinical trials are now underway to assess the safety of implanting human iPS-derived OPCs into the WM of PMS patients. Another stem cell type under investigation are bone marrow-derived autologous mesenchymal stem cell (MSC). Transplantation of MSC has demonstrated a suitable safety profile and there are currently ongoing phase II clinical trials (MESEMS; NCT01854957) that will provide evidence on the efficacy of this type of therapy for disease modulation.

Although mitigating the CNS directed autoimmune attack is beneficial for many RRMS patients, there is a true therapeutic need for PPMS and SPMS as during the transition of RR to SP disease immune modulation becomes ineffective. The apparently diverse mechanisms driving RRMS and PMS led some to suggest that any effective treatment regimen must include a combination of neuro-protective, regenerative and anti-inflammatory strategies. The failure of immunosuppressive medications in trials once patients are in the progressive phase of the disease indicate that supplementation with neuroprotective therapies will be needed. Neuroprotection broadly includes preservation of axons, neurons, glia and myelin integrity and function and several pharmacological agents have been shown to be effective in EAE, yet few have made it to clinical trial.

Animal models for MS

The elected animal model for MS, experimental autoimmune encephalomyelitis (EAE), was first developed in primates by Thomas Rivers in 1933 and is now widely used to study human CNS demyelinating disease. Although EAE can be induced in rabbits, guinea pigs, primates and rats, the mouse is currently the most commonly used species. Currently, the C57BL/6 mouse is the most frequently used and best characterized strain, which is also favored due to the availability of well-characterized genetic strains. Less frequently used strains include the Biozi antibody high (AB/H) and the Swiss Jim Lampert (SJL) mouse.

Mouse models

Induction of EAE in the mouse is most commonly done by either active immunization with myelin antigen, or adoptive transfer of myelin specific T cells into naïve mice (reviewed in...
To break peripheral immune tolerance, myelin or myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG), is emulsified in complete Freund’s adjuvant (CFA). In addition, Bordetella pertussis toxin is injected with the aim to elicit synchronous development of robust disease. Moreover, there are currently several transgenic models that develop disease spontaneously and those that are induced by virus and toxins. A selection of frequently utilized models is listed in Table 4.

Table 4. Rodent models for MS.

<table>
<thead>
<tr>
<th>Model type</th>
<th>Species/strain</th>
<th>Induction</th>
<th>Features/notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active immunization</td>
<td>Mouse: C57BL/6</td>
<td>Immunization with MOG35-55 in CFA*</td>
<td>Chronic progressive disease course</td>
</tr>
<tr>
<td>Mouse: Biozzi ABH</td>
<td>Immunization with SCH, or MOG, MOG8-21 &amp; MOG35-55 in CFA*</td>
<td>Chronic relapsing to progressive disease course</td>
<td></td>
</tr>
<tr>
<td>Mouse: NOD</td>
<td>Immunization with PLP48-70 or MOG35-55</td>
<td>Chronic relapsing to progressive disease course</td>
<td></td>
</tr>
<tr>
<td>Mouse: SJL</td>
<td>Immunization with MOG92-106, MBP &amp; PLP epitopes in CFA*</td>
<td>Acute monophasic disease course</td>
<td></td>
</tr>
<tr>
<td>Rat: Lewis</td>
<td>Immunization with MBP69-88 in CFA</td>
<td>Monophasic disease course</td>
<td></td>
</tr>
<tr>
<td>Rat: Dark Agouti</td>
<td>Immunization with SCH or MBP63-81 in IFA</td>
<td>Protected relapsing disease course</td>
<td></td>
</tr>
<tr>
<td>Adoptive transfer (passive)</td>
<td>Mouse and rat</td>
<td>Transfer of myelin specific T cells from donor mice</td>
<td>Disease varies according to specificity of T cell transferred</td>
</tr>
<tr>
<td>Mouse: C57BL/6</td>
<td>Oral administration of cuprizone</td>
<td>Non inflammatory toxicity to oligodendrocytes</td>
<td></td>
</tr>
<tr>
<td>Mouse and rat</td>
<td>Focal injection of LPC</td>
<td>Focal demyelination</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Ethidium Bromide</td>
<td>Death of oligodendrocytes and axonal loss</td>
<td></td>
</tr>
<tr>
<td>Viral</td>
<td>Mouse: Biozzi ABH</td>
<td>Semliki Forest virus</td>
<td>IP injection of virus resulting in infection and demyelination of CNS</td>
</tr>
<tr>
<td>Mouse: SJL</td>
<td>mouse Hepatitis virus</td>
<td>Acute encephalitis to chronic neuroinflammation and demyelination depending on strain</td>
<td></td>
</tr>
<tr>
<td>Mouse: SJL</td>
<td>Theiler’s virus (TMEV)</td>
<td>Early acute phase followed by chronic demyelinating phase</td>
<td></td>
</tr>
<tr>
<td>Transgenic</td>
<td>Mouse: SJL</td>
<td>TCR for PLP 139-151</td>
<td>Moderate spontaneous incidence (40-60%) of disease</td>
</tr>
<tr>
<td>Mouse: 2D2 C57BL/6</td>
<td>TCR for MOG35-55</td>
<td>Low incidence of spontaneous (30%) optic neuritis</td>
<td></td>
</tr>
<tr>
<td>Mouse: B10.PL</td>
<td>TCR for MBP1-11</td>
<td>High incidence (100%) of spontaneous EAE on RAG-1 background</td>
<td></td>
</tr>
<tr>
<td>Mouse: C57BL/6</td>
<td>TCR MOG × IgM MOG</td>
<td>Severe spontaneous EAE</td>
<td></td>
</tr>
</tbody>
</table>

MOG, myelin oligodendrocyte glycoprotein; CFA, complete Freund’s adjuvant; MBP, myelin basic protein; PLP, proteolipid protein; SCH, spinal cord homogenate; IFA, incomplete Freund’s adjuvant; LPC, lysophosphatidyl choline; TCR, T-cell receptor; BCR, B-cell receptor. * Addition of bordetella pertussis toxin required. Reviewed in 159
Rodent models have substantially contributed to our understanding of the MS relevant autoimmune and neurodegenerative mechanisms, and the successful development of therapeutics such as natalizumab, fingolimod, and glatiramer acetate. Nevertheless, the high number of failures in the translation of data from pre-clinic research to useful medication in the clinic has shed doubts about the relevance of these models for therapy development. Some failures may be attributed to poor design of clinical trials or pre-clinical testing that does not adequately mimic clinical application. However, many other failures can be attributed to limitations in the predictive value of the models themselves.

One considerable critique of the classical rodent EAE model is that the pathogenic process is dominated by CD4+ T cells, in particular Th1 and Th17 cells, and poorly stimulates CD8 responses which is reflected by dominance of the CD4+ T cell in the active EAE lesion. This contrasts with the MS patient where CD8+ T cells outnumber CD4+ T cells in brain lesions, regardless of the stage or activity of disease. Frequently used standard models such as the MOG EAE model in C57BL/6 mice also have a strong predilection for spinal cord WM pathology in contrast to MS where also the brain is affected with prominent cortical involvement. That said, pathogenic mechanisms of cortical demyelination can be studied using Lewis rat models, ABH mice immunized with neurofilament proteins or with stereotactically cortical cytokine injection models.

While oxidative damage, iron aberration and mitochondrial dysfunction are critical aspects of MS disease progression, these features are incompletely (if at all) reflected in current rodent EAE models. In the C57BL/6 mouse EAE model, ROS and RNS do indeed contribute to the initiation of focal axonal degeneration. Disease severity is also ameliorated in EAE of the Lewis rat when treated with the H_2O_2 scavenger, catalase. Macrophages isolated from the EAE rat brain also exhibit ex vivo elevated ROS production. Impairment of NOX subunits gp91 and p47 has also been shown to decrease EAE severity in rodent models. Despite the ability to develop some degree of oxidative stress, and a proven contribution of ROS to EAE development in Lewis rat and C57BL/6 mice, MS-like oxidative tissue injury is absent in most EAE models, with only the mouse hepatitis virus strain (JHM-MHV) model of demyelination displaying the ability to elicit MS-like oxidative injury in the CNS. In addition, iron accumulation as observed in the MS brain is not reflected in the rodent brain. Iron accumulation in C57BL/6 mice at the age used in EAE experiments (range 9-12 weeks) is restricted to spinal cord and brain stem nuclei and does not occur in myelin and oligodendrocytes of brain WM. While iron-deficient mice fail to develop autoimmune disease, this is mechanistically different from the situation in MS as disease modification was associated with alteration of CD4+ T cell development and not modification of free radical formation in the brain; unsurprising given the limited accumulation of iron in the brain WM. Thus, lack of brain myelin iron accumulation and oxidative tissue injury represents a major fundamental difference between mouse and man and represents a significant challenge for the selection and development of therapeutics.

**Marmoset EAE**

The common marmoset (Callithrix jacchus) is a neotropical primate that is increasingly being utilized in biomedical research as an animal model in fields such as neurodegeneration, neuroscience, autoimmunity, regenerative medicine and toxicology. Inherent biological
features of these monkeys such as small body size, bone marrow chimeric twin birth, and high fertility rates have made this monkey a popular choice for modeling human diseases (see also Table 5). Moreover, unlike mice that are used in EAE studies, these animals are genetically outbred and have a pathogen-educated mature immune system, reflecting the human immune system. Whereas the evolutionary distance between mouse and man is approximately 90 million years, the distance between marmoset and man is only 35-40 million years. As a result of phylogenetic relationship to humans, the marmoset exhibits a more complex neuroanatomical development, and this is reflected in a human comparable WM to GM ratio. Whereas the rat brain only weighs 0.5% of total body weight, the marmoset brain has an average weight of 7.9 gram, or 2.7% of total body weight. This is much more comparable to the human situation in which the brain is 2% of total body weight.

Table 5. Advantages and disadvantages of using marmosets for biomedical research.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow chimeric twins</td>
<td>Ethical restrictions and experimental limitations not observed in research with lower species. Low animal numbers hinders statistical significance for heterogenous responses.</td>
</tr>
<tr>
<td>Reproductive efficiency</td>
<td>Costs</td>
</tr>
<tr>
<td>Pathogen educated immune system</td>
<td>Experiments are much more expensive than lower species due to housing and animal husbandry staff requirements.</td>
</tr>
<tr>
<td>CalHV3 infection</td>
<td>Size</td>
</tr>
<tr>
<td>Neuroanatomical</td>
<td>Housing requirements are greater than rodents, but tissue volume available for experimentation (i.e. blood) is much less than other NHPs.</td>
</tr>
<tr>
<td>Size</td>
<td>Cross-reactivity</td>
</tr>
<tr>
<td>Age associated β-amyloid deposition in cerebral cortex, reduced neurogenesis in hippocampus, and development of presbycusis.</td>
<td>Fewer cross-reactive diagnostic agents, such as flow cytometry and IHC antibodies, available.</td>
</tr>
</tbody>
</table>

The marmoset EAE model has emerged as an ideal animal model of MS that mirrors many key pathological and clinical features of MS disease progression. Induction of EAE does not require artificial innate stimulation with bacterial components in the immunization cocktail. Whereas many murine models require the inclusion of myelin antigen emulsified in CFA, EAE can be induced in these monkeys with a peptide from human myelin oligodendrocyte glycoprotein (MOG; residues 34-56) or a recombinant MOG protein (residues 1-125) emulsified in IFA.
Induction of EAE results in multi-focal demyelination of WM that mirrors many characteristics of the MS lesion\(^\text{183}\). Like MS, these monkeys are capable of developing cortical GM pathology\(^\text{183-185}\). Development of cortical GM pathology in the marmoset model occurs in all layers of the GM, and reflects similar patterns of intracortical, leukocortical and subpial demyelination as found in the MS brain\(^\text{186}\). Particularly striking in this model are the MS-like intracortical plaques that project radially from microvessels, and the band-like subpial demyelination that frequently spans gyri and seems to extend intracortically from the pia matter. Thinning of the GM has been observed in marmoset EAE suggesting that these monkeys also exhibit neurodegenerative aspects of MS\(^\text{185}\).

Marmoset EAE lesions exhibit a pattern of vesicular demyelination, or concentric areas of macrophage mediated demyelination, with relative axon sparing and focal remyelination, which is in contrast to inflammatory panencephalitis with sparse demyelination that is observed in CD4+ T-cell mediated rodent EAE models\(^\text{148}\). Like MS, the disease is also juxtaposed over a pathogen-educated immune system as housing design exposes these monkeys to environmental pathogens\(^\text{187}\). Marmosets are naturally infected with a lymphocryptovirus called CalHV3, an EBV homolog, and thus these monkeys are valuable in delineating a role of the lymphocryptovirus in disease progression\(^\text{188}\). As marmoset EAE is a central theme of this thesis, this model will be further discussed in detail in chapter 1.2.

**Rhesus monkey EAE**

Models of EAE have also been established in the rhesus macaque (\textit{macaca mulatta}). Despite a closer biological and phylogenetic relationship of rhesus monkeys to humans than both marmoset and mouse, the EAE model in rhesus macaques is missing many key pathological and clinical features of adult forms of RRMS, SPMS or PMS\(^\text{183}\). In comparison to marmoset EAE, the rhesus monkey model much more closely resembles acute disseminated encephalomyelitis (ADEM)\(^\text{189,190}\). Clinically, EAE evolution in these primates mirrors the aggressive nature observed in its human counterpart ADEM. Like ADEM, disease in these monkeys is short-lasting and aggressive, with clinical symptoms including vomiting, vision impairment, and neurological deficits such as hemiparesis\(^\text{183}\). Pathologically this model also closely resembles ADEM with respect to development of peri-vascular confluent lesions and a role of neutrophilic granulocytes in disease induction. It is unclear why these two primate species diverge from each other so drastically in terms of pathological and clinical disease course.
References


General introduction


General introduction


Chapter 1  |  Introduction


