Chapter 1

Introduction

1 Introduction

Multiple sclerosis (MS), meaning scars in legion, is a chronic inflammatory autoimmune neurological disease with the characteristic of axonal demyelination due to immune hyper-reactivity in the central nervous system (CNS) and peripheral nervous system (PNS) [1]. It involves the active involvement of various immune cells like antigen-presenting cells, dendritic cells, B and T lymphocytes, NK cells, and glial cells like microglia, astrocytes, and oligodendrocytes [2]. MS starts merely as an inflammation mediated by the immune cells like auto-reactive CD4+ T cells and T helper cells (Th)17 secreting interferon-gamma (IFN-γ) and (interleukins) IL-6, respectively [3]. Still, eventually, it generates autoimmunity against the components of the myelin sheath, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) whose exact cause is unknown [4, 5]. The hyperactivated immune cells start targeting the myelin sheath [6] and oligodendrocytes, each of which provides support to many neurons as compared to the Schwann cells, which are abundant in the PNS, therefore, yielding fewer chances of recovery or remyelination in the CNS [7]. The clinical symptoms obtained depend on the damage of the region of the brain attacked by immune cells, like cortical lesions can be seen in later stages, which also includes damage to the grey matter as well. Common clinical symptoms like double vision, motor dysfunction, and sexual dysfunction are seen in MS patients owing to demyelination in the optic nerve, cortical and hypothalamic regions, respectively [8].

Based on the course of the disease and extent of demyelination [9], MS can be categorized into four types, the first type is relapsing-remitting multiple sclerosis (RRMS) which affects 85% of MS patients with clinical manifestations of relapses or exacerbating attacks that

continues from days to weeks followed by a recovery period in which there are no flaring symptoms [10]. The second type is secondary progressive multiple sclerosis (SPMS) which has a more pronounced cortical lesion than RRMS. Also, the disease severity and attacks are more frequent [11]. The third type is primary progressive multiple sclerosis (PPMS affects approximately 10% of MS patients having a very progressive development of the disease from the onset of the disease with no relapses, but along with demyelination, there is grey matter atrophy localized explicitly to the spinal cord [12]. Progressive-relapsing multiple sclerosis (PRMS) is the fourth type, affecting approximately 5% of the patients, which is progressive in nature since the onset of the disease, with intermittent flare-ups and worsening symptoms without any remission period [10]. Subpial demyelination and abundance of microglial activation are the mark features of PRMS [13].

1.1 Epidemiology

3.1 million people live with MS worldwide in 2020 (35.9 per 100,000 population), a 30% increase since 2013, and MS prevalence has increased in every world region since 2013. There has been an increase in juvenile-onset MS, with more than 30,000 cases of MS diagnosed as reported by 47 countries in comparison to 7,000 cases reported in 2013 by 34 countries [14]. Most individuals are diagnosed with MS at the age of 20–50 years. There is the rarity of MS in Samis, Turkmen, Uzbeks, Kazakhs, Kirgizis, native Siberians, North and South Amerindians, Canadian Hutterites, Chinese, Japanese, African blacks and New Zealand and it is high risk in Sardinians, Parsis, and Palestinians [14]. There were 18932 deaths due to multiple sclerosis and 1151478 disability-adjusted life years (DALYs) due to multiple sclerosis in 2016 [15]. The prevalence of MS in India was estimated to be nearly 1/100,000 in the early 1980s, but the occurrence rate has increased tremendously to 5-20 per

100,000 [16]. Moreover, MS is dominantly found in the Parsi population with a prevalence ratio of 26–58/100,000 has been reported [17].

1.2 Causes of multiple sclerosis

The exact cause implicated for multiple sclerosis is still unclear but there are many factors that are responsible for the occurrence of the disease, as shown in **Table 1.1**. There is the involvement of complex genetics and environmental factors that determine susceptibility to disease development. The most prominent environmental factors are early-life Epstein-Barr virus infection, ultraviolet light exposure, and vitamin D status [18]. The human leukocyte antigen (HLA) has been known to have the largest genetic contribution to MS susceptibility. In the early stages of life, there are protective factors such as having protective HLA haplotypes during *in-utero* or childhood. Human leukocyte antigens are a cluster of genes on the short arm of chromosome 6 and certain haplotypes such as HLA-DR1 and HLA-DR53 are considered to be protective against MS [19].

Autoimmunity is characterised by the presence of autoreactive factors as there is the presence of autoreactive CD8+ T cells in the cause of MS patients, it is undoubtedly regarded as an autoimmune disease. Moreover, these CD8+ T cells secrete lysosomal apoptotic enzymes like perforin and granzyme, which directly damage the oligodendrocyte leading to demyelination [20].

Table 1: Protective and risk factors associated with MS, adapted from the reference [21].

Stage of life	Protective factors	Risk Factors
In-utero and early childhood	Protective HLA haplotypes	Family history or genetic factors Female sex Being born in May Born in high-latitude regions
Adolescence	Amount of sun exposure Vitamin D supplements Diets high in fish oils	Exposure to Epstein Barr virus Smoking Vitamin D deficiency Living in high-altitude regions
Adulthood	Not identified	Exposure to Epstein Barr virus Smoking

1.3 Clinical symptoms and diagnosis of MS

The Clinical features and symptoms are heterogeneous among MS patients. The common clinical features are mobility impairments, bladder and bowel problems, fatigue, memory and other cognitive problems, balance impairments, weakness, stiffness and spasms, pain and unpleasant sensations, visual changes, and dizziness. Moreover, the same symptoms may vary tremendously for example, mobility impairments may range from slight leg weakness to being fully wheelchair dependent [22]. The diagnosis of MS is done according to McDonald's criteria which depend on the clinical symptoms. According to the criteria, the number of attacks and number of lesions that are present are used to indicate whether an

individual has MS or not. The number of lesions in the brain can be visualised by Magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) is investigated for the presence of IgG and any other autoreactive cells for the diagnosis [23].

1.4 Animal models of multiple sclerosis

Multiple Sclerosis is an autoimmune disease and the most common neurodegenerative disease affecting the young population. MS is a complex disease with an opaque etiology and thus no effective cure despite decades of extensive research that led to the development of several partially effective treatments [24].

Due to limited access to early and immunologically active MS tissue samples, the modification of experimental circumstances is much more restricted in human studies compared to studies in animal models. For these reasons, animal models are needed to clarify the underlying immune-pathological mechanisms and test novel therapeutic and reparative approaches. It is not possible for a single mouse model to capture and adequately incorporate all clinical, radiological, pathological, and genetic features of MS [25].

The three most commonly studied significant categories of animal models of MS include:

(1) the purely autoimmune experimental autoimmune/allergic encephalomyelitis (EAE)

(2) the virally induced chronic demyelinating disease models, with the main model of Theiler's Murine Encephalomyelitis Virus (TMEV) infection and;

(3) toxin-induced models of demyelination, including the cuprizone model and focal demyelination induced by lyso-phosphatidyl choline (lyso-lecithine).

Experimental autoimmune encephalomyelitis, originally designated experimental allergic encephalitis (EAE), has been proposed as animal model to investigate pathogenetic hypotheses and test new treatments in the field of central nervous system inflammation and demyelination. EAE is known to be motivated by convalescence from some viral diseases by Thomas M. Rivers, D. H. Sprunt and G. P. Berry back in 1933. They have found acute monophasic disease symptoms in primates after the transfer of inflamed patient tissue to them [26, 27]. EAE in the mouse was first induced over 60 years ago by active immunization with spinal cord homogenate [28].

EAE is induced by subcutaneous injecting an emulsion that contains an adjuvant and synthetic peptides derived from myelin proteins: myelin proteolipid protein (PLP) or oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP). This leads to the immunization of animals and causes activation and further expansion of peripheral antigen-specific immune cells specifically the T-cells [29]. These cells enter the CNS, encounter the specific myelin antigen and subsequently induce disease. Myelin protein-specific CD4+ T cells are generally considered necessary for EAE induction, as the adoptive transfer of these cells from immunized into normal animals elicits EAE in 100% of animals [30]. EAE disease course is significantly influenced by several factors, including species, strain and the autoantigen used [31].

1.4.1 Mouse models of EAE

Mice are the most common species used in EAE research. C57BL/6 mice are one of the most popular strains used in biomedical research. C57BL/6 mice are generally considered to be EAE resistant; however, the disease can be induced with $MOG_{35–55}$. Rat MOG proteins were

associated with distinct CNS infiltrates, with a largely mononuclear infiltrate in mice immunized with rat MOG protein [32].

1.4.2 Rat models of EAE

Although the mouse model of EAE is the most utilized animal model for MS, rat EAE has provided significant insight into the pathology of MS as well. In the rat model (usually the Lewis rat or Dark Agouti (DA) strains) of EAE, induced with either MBP or one of its encephalitogenic epitopes, the disease consists of inflammatory MNC infiltration into the spinal cord cerebellum and brainstem, but not the cortex [33]. Although Wistar and Sprague-Dawley rats are capable of developing EAE, but they were used less commonly than Lewis and DA rats in the past [34]. Now they are used quite frequently to study autoimmune diseases as well as other immunologically mediated diseases [35, 36]. EAE induced in these rats by guinea pig spinal cord homogenates (GPSCH). GPSCH-induced EAE is characterized by extensive tissue inflammation with a typical chronic disease course. The mechanisms by which GPSCH mediate tissue destruction and influences the course of the disease, which is an acute monophasic or chronic disease and activation of immune cells and they release cytokines, namely, IL-2, TNF-α , ILs and IFN-ɤ are expressed at the onset of disease [36, 37].

1.4.3 Clinical comparison with EAE

The age of the animal model used imparts a significant role in EAE development and severity. As in the case of EAE induction in young Wistar rats (7 weeks) results in the commencement of neurological deficits 12–16 days post-induction, from which they spontaneously recover. However, induction in middle-aged rats (15 weeks) results in a later onset of disease with reduced clinical signs [38]. This can be correlated with clinical

implications as in MS. If disease onset begins in younger people under 30 years, the disease usually begins with a relapsing-remitting phase and eventually into a secondary progressive course. However, disease manifestation typically follows a primary progressive phenotype if disease onset begins in people over 40 years [39].

1.4.4 Choice of autoantigen

Myelin basic protein (MBP) is one of the most studied myelin proteins involved in MS second most abundant after PLP. It possesses an easier way to isolate them owing to its physicochemical characteristics. It was the first to be extensively used in EAE [40]. MBP is a key candidate autoantigen involved in MS on the basis of abundant MBP-specific T cells that can be isolated from MS patients [41] and unlikely to MOG and PLP, MBP is found in significant quantities in central and peripheral myelin [42]. The disease course induced by MBP and its peptides is characterized by an acute paralytic episode from which the mice recover either partially or completely [43].

Although MOG is present in minor proportion but is expressed on the outer surface of CNS myelin [44], which makes it a better autoantigen than other autoantigens, MOG has emerged as an important target in MS because MOG-reactive T cells seem to be more readily detected in MS patients than T cells reactive to PLP and MBP [45]. C57BL/6 mice develop a chronic disease [46]. $MOG_{35–55}$ could induce a potent and chronic form of EAE in C57BL/6, and this model became useful in the evaluation of candidate drug molecules in the development and regulation of EAE [46].

PLP is a major transmembrane protein of the CNS and is an essential component for the compaction of the CNS myelin [47]. The PLP-induced disease in SJL/J mice offers a unique and exciting relapsing-remitting course [48]. Symptoms can be observed 12–18 days post-

induction. Generally, the first acute relapse is more severe than the subsequent relapse [49]. PLP is also encephalitogenic in SJL/J mice with an indistinguishable disease phenotype from PLP-induced mice, in terms of incidence, severity and histologic features [50].

1.4.5 Role of adjuvants

Adjuvants are used in an animal model to enhance autoantigens' immunogenicity which plays a major role in the induction of EAE. Adjuvanticity can be defined as the efficacy of an immunological adjuvant substance to increase the immunogenicity of an antigen [51]. Complete Freund's adjuvant (CFA) is considered the key adjuvant for inducing cell-mediated immunity and due to this, it has been an adjuvant of choice for EAE. CFA is an adjuvant preparation of killed *mycobacteria*, most commonly *M. tuberculosis* in a water and oil emulsion. CFA has been shown to induce preferentially a cell-mediated immune reaction, although it is also often used as an adjuvant for the generation of antibodies and humoral immune reactions. Through *Mycobacterium*, a strong inducer of 1L-12, preferentially inducing the Thl responses [52] and additionally activating the macrophages, ensuring antigen persistence and facilitating recruitment of memory T cells, *Mycobacterium* in CFA preparation may also have an important role in increasing the blood-brain barrier (BBB) permeability due to antibodies to a mannan component of the bacteria [53]. The CFA-induction increased permeability to various proteins of the blood-brain barrier by itself does not result in reactive gliosis or microglia activation as shown in the experiment [54]. Induction of active EAE in mice is greatly facilitated by Pertussis toxin (Bordetella pertussis). The mechanisms of facilitation of EAE induction by pertussis toxin are complex [55]. A histamine-sensitizing factor has been extracted from B. pertussis. The susceptibility to EAE in mice was shown to be in large part dependent on the MHC and the histamine

sensitization genes responsible for vasoactive amines leading to an increased vascular permeability of the brain and spinal cord [56]. The adjuvant effect of pertussis, however, appears to involve both Thl and Th2 immune responses which emancipate the expansion and stimulation of CD4+ T cells and other antigen-presenting cells and T cells [57].

1.5 Treatment and prevention of multiple sclerosis

There is no cure for MS now but many treatment approaches are available for minimizing the symptoms of the disease which include medication, stem cell therapy, venous angioplasty, urinary catheterization, psychotherapy and rehabilitation; however, medication is the most widely available and used for the treatment. The medication includes drugs like dimethyl fumarate, beta interferons, glatiramer acetate, mitoxantrone, natalizumab, fingolimod etc [58]. There are complementary and alternative treatment (CAM) options like exercise, food and diet, stress management and acupuncture. Exercise is one type of complementary treatment which has been shown to improve balance and locomotion among MS patients. In addition, exercise reduces stress and promotes recovery in patients with MS.

1.6 Limitations of current treatments for multiple sclerosis

The limitation of current treatments for multiple sclerosis has been summarised in **Table 2.**

Table 2 Limitations of current treatments of multiple sclerosis

Medication	Route	Mechanism of action	Limitation
Dimethyl fumarate	oral	DMF/MMF acts on nuclear factor-E2-related factors, with immunomodulatory properties and neuroprotective effects.	Flushing, gastrointestinal upset, PML, headache, Safety partly established.

Fingolimod	oral	Sphingosine 1-phosphate modulator and sequesters T lymphocytes in secondary lymphoid organs	Bradycardia due to slowing of AV conduction, macular edema, hypertension, disseminated herpes infections, lymphopenia, and skin malignancies.
Teriflunomide	oral	Metabolite of leflunomide, reversible noncompetitive inhibitor of the mitochondrial enzyme dihydroorotate dehydrogenase. It inhibits pyrimidine synthesis and has cytostatic effects on T and B cells. Inhibition of tyrosine kinases results in reduced T-cell activation and cytokine production).	Diarrhea, elevation of liver enzymes, nausea, hair loss, paresthesias. upper respiratory tract infections, Leflunomide is pregnancy category X.
Lequinimod	oral	Modulates cytokine expression with an effect on antigen presentation, T cells, B cells, and microglia.	Elevates liver function enzymes. pro-inflammatory increased C-reactive protein and erythrocyte sedimentation rate.
Natalizumab	*IV*	Monoclonal antibody directed at α4-integrin inhibits leukocyte migration across the BBB.	Allergic reactions, PML, Monitoring of JCV serology yearly; natalizumab antibodies at 6 months; liver function tests every 6 months
Alemtuzumab	*IV*	Humanized monoclonal antibody against CD52.	Autoimmune reactions include immune thrombocytopenic purpura, Goodpasture's syndrome and respiratory infections.

1.7 Dimethyl fumarate

Dimethyl fumarate (DMF) is an FDA-approved oral drug for the management of RRMS. DMF is not a very new drug as it has been used to treat psoriasis since 1959 [59]. The human dose of DMF is 120 mg and 240 mg twice/thrice a day and the maximum dosage of dimethyl fumarate is 240 mg three times daily, which is a well-tolerated dose and results in improved clinical outcomes when compared to the placebo [60]. DMF is a prodrug and its active metabolite monomethyl fumarate (MMF), both are available in the blood after absorption [61]. They act as electrophiles or alkylating agents readily attacking the cysteine thionyl groups, which owe to their desirable and undesirable effects [62]. It has immunomodulatory effects on both innate and adaptive immunity, which are implicitly and explicitly related to its antioxidant effects [3]. DMF being an electrophile, has an enormous affinity for electron-rich compounds like proteins, specifically some functional groups like thionyl in cysteine amino acid, is responsible for attacking the conjugation in the DMF [63]. DMF covalently interacts with the cysteine residues, the phenomenon called as “succination” of the protein, which makes the protein inactive [64]. This is the basic principle behind the activity and interaction of DMF with its target molecule as shown in the **Figure. 1-1.**

The core mechanism by which DMF exerts its effects is immunomodulatory, as it brings a shift in the population of Th1 and Th17 into a Th2 phenotype which reduces the Inflammatory cytokine production and reduced expression of adhesion molecules followed by a diminished inflammatory infiltrate within the effector tissues like CNS and psoriatic plaques [65]. The cellular targets of DMF include monocytes, macrophages, microglial cells, astrocytes, dendritic cells, natural killer cells, naive and activated T cells (Th, effector memory B cells, T cells, regulatory T cells) [66].

1.7.1 Limitations of Dimethyl fumarate

The precise mechanism of DMF remains uncertain however, DMF is known to influence intracellular glutathione (GSH) levels. Another reported mechanism includes activation of Nrf2 by DMF thus regulating cellular antioxidant responses and stimulation of cytoprotective and anti-inflammatory factors like NFκB [3]. The reported mechanism of action is mainly due to its antioxidant effect which is a general mechanism and many drugs have antioxidant effects but they all are not immunomodulatory like DMF. CD4+/CD8+ T lymphocytes play a crucial role in the pathophysiology of MS, but the mechanism of DMF on these T lymphocytes has not been explored thoroughly. Moreover, DMF has a crucial impact on the important effector cells including microglia, dendritic cells, macrophages, astrocytes and neurons. Therefore, there is a need to find an immunological mechanism of DMF that can help explore the exact mechanism of action of DMF in the immune cells.

Moreover, DMF owes some serious gastrointestinal (GI) adverse effects anal incontinence, diarrhoea, dyspepsia, irritable bowel syndrome, gastritis, erosive gastritis, gastric ulcer and gastroduodenitis [67, 68]. These gastrointestinal side effects are much more common in the patients undergoing DMF treatment and the gastrointestinal tract irritations are not completely understood. These symptoms may worsen with the increase in the dose [69].

DMF has a physiochemical disadvantage as it sublimates at a relatively low temperature as when processed conventionally, about 15-20% of DMF is lost from the final formulation and upon storage. This is most likely because of sublimation during production. Sublimation also leads to the loss of DMF during long-term storage from bulk and its formulations as well [70].

1.7.2 Cathepsin C as a novel target in multiple sclerosis

Cathepsin C is an abundant lysosomal cysteine protease from the papain superfamily with a mol. wt of 200 kDa and is widely expressed in many tissues of mammals and other animals. DPP1 is the only member of this family which is functional in its tetramer form, which consists of four identical subunits. Each subunit is composed of an N-terminal fragment, a heavy chain, and a light chain DPPI is the physiological activator of groups of serine proteases from immune and inflammatory which is vital for the defence mechanism of an organism [71].

1.8 Cocrystals

Cocrystals are crystalline materials formed by the combination of two or more components, typically an active pharmaceutical ingredient (API) and a coformer, in a specific stoichiometric ratio within the same crystal lattice. Unlike salts, cocrystals are not held together by ionic bonds. Instead, they are stabilized by various intermolecular interactions such as hydrogen bonding, π-π stacking, and van der Waals forces [72].

Cocrystals offer certain advantages over amorphous API forms or solid dispersions due to their combination of high solubility associated with high-energy solids and a thermodynamically stable crystalline structure [73]. They are characterized by the European Medicines Agency (EMA) as crystalline structures consisting of two or more components in a specific stoichiometric ratio, with the arrangement in the crystal lattice not based on ionic bonds like salts. The components of a cocrystal can be neutral or ionized. According to the United States Food and Drug Administration (USFDA), cocrystals are defined as crystalline materials composed of two or more different molecules, typically an API and cocrystal formers (coformers), in a specific stoichiometric ratio, all within the same crystal lattice.

Cocrystals are distinct from salts, polymorphs, solvates, and hydrates. The hydrogen-bonding interactions between the API and the coformer result in changes to the physicochemical properties of the API and contribute to improved pharmaceutical characteristics [74].

In the field of pharmaceuticals, cocrystals, salts, and polymorphs play important roles in drug development and formulation. Here's a brief explanation of each term in the context of pharmaceuticals:

1. Pharmaceutical Cocrystals: Pharmaceutical cocrystals are crystalline structures formed by the combination of an active pharmaceutical ingredient (API) with one or more coformers through non-covalent interactions. Coformers are typically small molecules that can form hydrogen bonds or other interactions with the API. Cocrystals offer a way to modify the physicochemical properties of APIs, such as solubility, dissolution rate, stability, and bioavailability. By forming cocrystals, it is possible to enhance the properties of drugs, improve their formulation, and address challenges related to poor solubility or stability.

2. Pharmaceutical Salts: Pharmaceutical salts are formed when an API reacts with an acid or a base, resulting in the formation of charged species (ions). Salt formation is often employed to improve the solubility and stability of APIs. The choice of an appropriate counterion can significantly impact the properties of the salt, such as its crystal structure, solubility, and pharmacokinetics. Salt formation can also help overcome challenges related to API's intrinsic properties and aid in the development of more effective and stable drug formulations.

3. Pharmaceutical Polymorphs: Pharmaceutical polymorphs are different crystalline forms of the same API. These polymorphic forms have identical chemical compositions but differ in their crystal structure, which can lead to variations in physical properties. Polymorphism can significantly impact the bioavailability, stability, and processing of drugs. Different polymorphs may exhibit distinct dissolution rates, melting points, or solid-state properties, potentially affecting drug efficacy, safety, and formulation development.

Understanding and characterizing cocrystals, salts, and polymorphs are crucial in the pharmaceutical industry. These forms can have implications on drug performance, formulation strategies, intellectual property, and regulatory aspects. Extensive studies are conducted to identify, characterize, and evaluate these crystal forms to optimize drug development and ensure consistent quality in pharmaceutical products.

1.9 Rationale

S-(2-succinyl) cysteine (2SC) is a chemical modification of proteins formed by a Michael addition reaction (**Figure 1-1**) between the Krebs cycle intermediate, fumarate, and thiol groups in protein-a process known as succination of protein. Chemical modification of either of these thiol groups is known to cause the inactivation of the enzyme [75]. Interestingly, DPP1 also contains cysteine (Cys-234) in its active site [76].

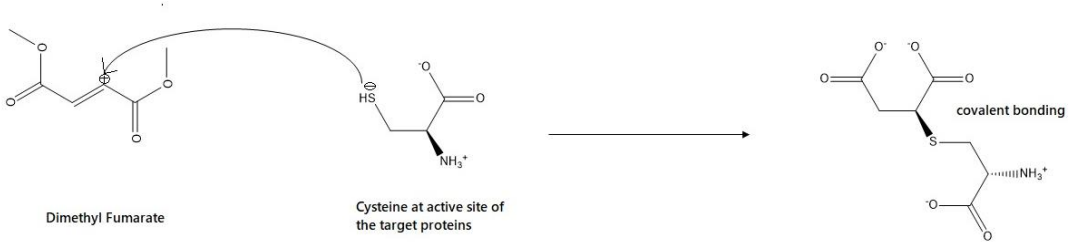

Figure 1-1 Succination of cysteine amino acid by DMF.

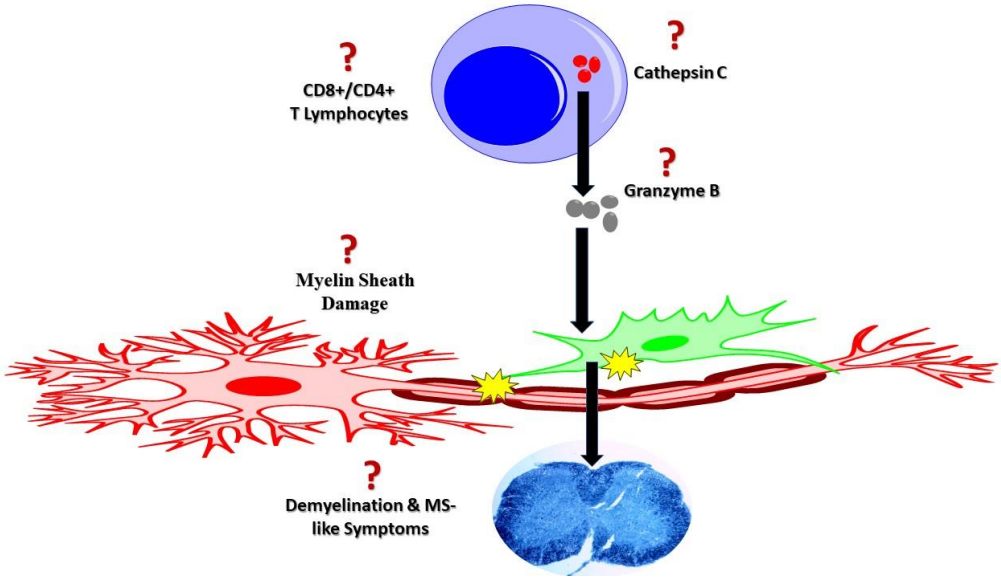

Figure 1-2 Proposed hypothesis

1.10 Objectives

Objective I: Evaluation of the immunological mechanism of dimethyl fumarate in the EAE model of multiple sclerosis

Objective II: Preparation, characterization, *in-vitro* and *in-vivo* pharmacokinetic evaluation of dimethyl fumarate cocrystal with basic coformer (Nicotinamide)

Objective III: Preparation, characterization, *in-vitro* and *in-vivo* pharmacokinetic evaluation of dimethyl fumarate cocrystals with acid-based coformers