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The expression of IL-22 during the development of experimental autoimmune encephalomyelitis (EAE)

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Contents

Abstract

Interleukin (IL)-22 (IL-22) is a member of the IL-10 family, which is an important regulator of the inflammatory response. IL-22 is produced by Th17 cells, which play a key immunopathogenic role in many immune disorders. However, the exact function of IL-22 in the development of central nervous system (CNS) inflammatory diseases such as multiple sclerosis (MS) remains unclear. This study aims to understand better the role of IL-22 in the development of MS disease by examining its expression levels in the peripheral immune organs and CNS spinal cord (SC) tissues of experimental autoimmune encephalomyelitis (EAE) mice and investigates whether the expression levels of IL-22 correlate with the CNS inflammation.

EAE was induced by immunizing C57BL/6 female mice with MOG_{35-55} peptide emulsified in Complete Freunds Adjuvant (CFA) subcutaneously, together with intraperitoneal injection of pertussis toxin (PTX). EAE clinical score was recorded daily and spleen and SC tissues were harvested at days 9, 17 and 28 post-immunization to assess cytokine secretion profile and tissue pathology. Our data demonstrate that MOG_{35-55} immunized mice start to develop EAE around day 9 and reached peak at day 15 while PBS-CFA immunized mice remained unaffected. ELISA data of the spleen cell cultures show that cells from MOG-CFA immunized mice produced higher levels of antigen-specific IL-22, IL-17A and IFN-γ at day 9 and day 17 when compared with PBS immunized mice. Furthermore, immunohistological staining data show that whilst naïve/PBS-CFA SC tissues expressed IL-22, the expression level was significantly increased in the SC of MOG-CFA mice at day 9 and 17. Furthermore, IL-22 was generally detected in both white and grey matter within the SC and was highly expressed

by astrocytes (and not axons or neurons) in EAE at the peak of inflammation. Our data therefore suggest that IL-22 may play a pathogenic role in the neurological autoimmune diseases, possibly through divergent roles in both the peripheral immune and CNS systems.

List of abbreviations

Ags: antigens

APC: antigen presenting cell

BBB: blood brain barrier

BBB-ECs: blood brain barrier- endothelial cells

BECs: brain endothelial cells

CCR: chemokine receptor

CFA: complete freunds adjuvant

CNS: central nervous system

CSF: cerebrospinal fluid

DA: EAE susceptible

DC: dendritic cells

DC1P: DA.PVG-*Eae29*

EAE: experimental autoimmune encephalomyelitis

FCS: foetal calf serum

FOXP3^{+}: forkhead box P3

GA: glatiramer acetate

GWAS: genome-wide association studies

HLA: human leukocyte antigen

HRP: horseradish peroxidase

IFN-γ: interferon gamma

IHC: immunohistochemical staining

IF: immunofluorescence staining

IL: interleukin

IL-22: interleukin-22

IL-22BP: IL-22 binding protein

IMT: immunomodulatory therapies

Jak: janus kinase

LN: lymph node

LTi: leukocyte lymphoid tissue inducer cells

MBP: myelin basic protein

MOG_{33-35}: myelin oligodendrocyte glycoprotein (MOG) 33-35 peptide

MHC: major histocompatibility (MHC)-II

MS: multiple sclerosis

MRI: magnetic resonance imaging

NK: natural killer cells

NKT: natural killer T-cells

ON: overnight

PBS: phosphate buffered saline

PLP: proteolipid protein

PPMS: primary progressive multiple sclerosis

PRMS: progressive relapsing multiple sclerosis

PTX: pertussis toxin

PVG: EAE resistant

RBC: red blood cell

ROR-γt: retinoid-related orphan receptors

ROS: reactive oxygen species

RRMS: relapsing remitting multiple sclerosis

RT: room temperature

SAS: subarachnoid space

SC: spinal cord

SP: spleen

SPMS: secondary progressive multiple sclerosis

STAT: signal transducer and activator of transcription

STEWS: scott’s tap water substitute

T-bet: transcription factor

TBS: tris-buffered saline

TBST: TBS-tween

TGF-β: transforming growth factor-β

Th1: T-helper 1

Th17: T-helper 17

T_{reg}: regulatory T-cells

Tyk2: tyrosine kinase 2

Chapter 1: Introduction

The immune system has evolved and developed in many different ways to protect the body against many threats such as evolving pathogens including parasites and bacteria (1). The immune cells express many receptors to ensure the evolving pathogens are constantly recognized as the pathogen-antigens by lymphocytes specific receptors leading to clonal expansion of pathogen-specific lymphocytes and the clearance of these pathogens. However it is crucial for immune cells to have the ability to distinguish a self-antigen from a foreign antigen so that they will not mount an immune response towards self-tissues (2). The immune system overcomes this problem by mechanisms of self-tolerance. During the lymphocyte development in the central lymphoid organs, once the receptors have been generated randomly, those self-reactive immune cells will become eliminated. Some self-reactive immune cells that escape to the periphery will become potential threat to health by reacting towards self-tissues and inducing autoimmunity (3, 4). The associated pathogenesis in autoimmunity most often involves self-reactive T helper (Th) cells that cause secretion of pro-inflammatory cytokines leading to inflammation. These self-reactive T-cells have the ability to help autoreactive B-cells in expansion and maturation leading to production of not only autoantibodies but also secretion of inflammatory cytokines, which will contribute to more tissue inflammation and damage. Therefore, these autoreactive T-cells are the prime cause of autoimmunity induction and tissue inflammation as they are not only able to bind specifically to self-antigens but more importantly can induce inflammation through their effector functions (5). When self or cross-reactive antigens interact with receptors, T helper cells are activated followed by expansion and differentiation into different T-cell subsets. The T-cell subsets each have different properties depending on the cytokine they produce. The

currently recognized effector subsets include Th1, Th2, Th17, Th22 and the regulatory T-cells (T_{Regs}). The contribution of these T-cell subsets in induction and immune regulation is discussed in more depth later.

1.1 Multiple sclerosis (MS)

MS is prolonged and progressive inflammatory autoimmune disease of CNS resulting in demyelination and neurodegeneration (6). The cause of MS is not clear, but different factors such as vitamin D deficiency, genetic background and many environmental and immune factors can contribute to the disease development (7). The main hallmark of MS is the presence of inflammatory plaques in CNS that can be determined either histopathologically or by using magnetic resonance imagining (MRI) (8). Different studies looking at the brain lesions of MS samples have demonstrated the presence of inflammatory cells mediated by pathogenic T cells against antigens of myelin. Thus these pathogenic T cells are regarded as the main effector cells in MS development (9). The main T lymphocytes involved are the Th1 and Th17 cells, which produce pro-inflammatory cytokines such as IFN-γ and IL-17 (10-12). For decades MS has been accepted as the CD4+ T-helper1 (Th1) mediated disease. This hypothesis was based on the early results obtained from studies using animal models of MS: experimental autoimmune encephalomyelitis (EAE), where IFN-γ producing $CD4^+$ T cells were able to induce EAE in naïve mice after adoptive transfer (13-15). However the Th1 pathogenic effector dichotomy was questioned when Kroenke *et al* reported that either the Th1 or Th17 cells can induce EAE with clinical paralysis after being adoptively transferred into naïve mice however with different levels of pathology (16), suggesting Th1 cells are unlikely to be the sole player in causing tissue destruction in EAE and that the intensity of EAE may vary depending on cytokine production by the disease inducing T cells (17). The data suggest Th1 cells are unlikely to be the sole player in causing tissue destruction in EAE and that the intensity of EAE may vary depending on cytokine production profile

by the disease inducing T cells (12). Thus it becomes important to understand the precise role of these pro-inflammatory cytokines in MS and EAE pathogenesis.

1.1.1 Etiology and epidemiology of MS

It is estimated that there are about 127,000 and 2.5 million MS patients in UK and world wide respectively. MS is a disease of all ages, the symptoms can start initially any time between the ages of 10 to 80 but they commonly begin between the age of 20 and 40 with an average age of 32 (18). Interestingly, the incidence of MS varies depending on the residence location with general increase the further away from the equator in either hemisphere, suggesting an important environmental factor that impacts the prevalence of MS. This however remains ambiguous whether the increase in MS incident represents a genuine environmental influence, or genetic difference or simply a result of variable surveillance. The Genome-Wide Association Studies (GWAS) have been conducted with an aim of identifying common genetic variants important in disease susceptibility of MS. The effect of an environmentally dependent genetic risk locus was modeled and showed that despite a relatively low overall odds ratio, environmental exposure was able to conceal a large true effect size on disease susceptibility in exposed individuals. This model was applied to vitamin-D deficiency in MS and raised the possibility of large magnitudes of concealed effect in loci responsive to vitamin D in this disease. However, in the post-GWAS era, efforts should be made to elucidate potential gene environment interactions involved in complex traits in order for true effects on disease risk to be realised (19). Other genetic studies have been carried out to show associations between MS and polymorphic alleles of candidate genes, which regulate either the immune response or the myelin production and found that major histocompatibility complex

(MHC)-II is the most consistent gene in case-control studies (20-22). MHC-II molecules play a crucial role in controlling adaptive immune responses through selection of CD4+ T-cell repertoire in the thymus and antigen presentation in the periphery. Patients with autoimmune disorders have shown inherited susceptibility associated with particular MHC-II alleles (23). The gene of MHC-II, human leukocyte antigen (HLA), is responsible for functioning of many immune responses as well as influencing susceptibility to over 40 diseases.

1.1.2 MS clinical subtypes and current therapies

The key neurological symptoms of MS is determined by the damage against myelin sheaths (demyelination) and neurons, which leads to neuronal signal conduction blockage or conduction slowing (24). During clinical recovery, inflammation and oedema in the CNS resolves and it is thought that CNS conduction is repaired due to glial ensheathment and re-myelination however axonal loss is irreversible.

The diagnosis of MS is difficult as it is based on patient clinical signs and symptoms, which are very broad and non-specific. The diagnosis often relies on MRI examination together with the clinical evidence. There are a few criteria clinically to identify MS from other neurological diseases. The Schumacher criteria were established in 1965 capturing the essence of the MS diagnosis that required dissemination of CNS lesions in space and over time. In addition the patients were required to be between the age of 10 and 50 and possess objective abnormalities on examination, both of which are now outdated. The new criteria from the Revised International Panel on MS diagnosis are the latest attempt to clearly define diagnostic criterion for MS (18).

Clinically MS has been divided into four different subtypes based on their clinical manifestations, however the different categories do not reflect specific biological pathophysiology (25). Relapsing-remitting MS (RRMS) is one of the most common forms of MS where symptoms usually occur for a short period of time, lasting several days to several weeks, and the experienced symptoms usually resolve spontaneously after they appear. However sometimes the inflammatory accumulation and tissue damage persists after many years, this typically enters the patients to secondary progressive stage of MS (SPMS) where the previous neurological impairments gradually worsens. Relapses associated with SPMS are usually detected at the early stages but it becomes uncommon as the disease progresses over time. 15% of MS patients show gradual worsening symptoms from the onset of the disease without any clinical relapses, this is defined as the primary progressive MS (PPMS). The PPMS patients are generally older and present less abnormalities on brain MRI (26). Another form of MS is the progressive relapsing MS (PRMS), which is defined by the gradual worsening of the neurological symptoms from disease onset followed by superimposed relapses (27). PRMS is suspected to represent SPMS in which initial relapses were un-recognized, or clinically silent. The typical symptoms associated with relapses involve: episodes of numbness, weakness and dyscoordination of an arm, leg or both. MS affects any area of the brain, optic nerve or SC and cause changes to sensory and/or motor neuron effecting one side of the body. Also brainstem damage can manifest as diplopia and resulting in altered sensation in face and/or ataxia (28).

Currently there is no cure for MS. The initial treatment used for MS starts during the acute relapse phase where corticosteroids are given to shorten the length of relapse with possible long-term improvement outcome. After the acute relapse is treated, disease-modifying therapy becomes the focus. Current disease-modifying therapies attempt to target the neural tissue damage by either preventing or reducing the long-term risk of clinically significant disabilities (28), and immune-suppression immunomodulatory therapies (IMTs) are the most effective in active relapsing stages of the disease (29). IFN-1β clinical treatments such as Avonex, Betaseron and Rebif work by altering the inflammatory response through specific receptors that help to modulate T and B cells cytokine expression. IFN-β is considered as one of the most effective first line therapies (30). However, one third of RRMS patients using IMTs tend to develop recurrent relapses and/or show an increase in sustained disability and some patients develop neutralizing antibodies to IFN-β regardless (31). Another first line therapy for RRMS is the Glatiramer acetate (GA), a random polypeptide based on the amino-acid structure of myelin protein (Alanine, Glutamine, Lysine, Tyrosine) that works as a potential immunochemical mimic of myelin and suppresses antigen-specific immune responses through altering autoreactive regulatory T-cells (T_{Regs}) more than it decreases inflammation (32-34). For patients with worsening RRMS and early SPMS with on-going inflammation, Mitoxantrone chemotherapy is used to reduce clinical relapses and progression of disability by arresting the cell cycle and inhibiting DNA replication as well as interfering with DNA repair and RNA synthesis that reduces immune proliferation. Another significantly more effective therapy than IFN-β and GA is the Natalizumab therapy (35). Natalizumab is a monoclonal antibody that inhibits cell trafficking from circulating into CNS and is proven useful in

reducing inflammation and improving clinical symptoms of patients (18, 36). However this therapy imposes risks and potential fatal complications as there are many cases of multifocal leukoencephalopathy (PML) developed after receiving the treatment (37). Additionally cyclophosphamide has been used in MS treatment for over 40 years, it has selective effects on the immune response and works by suppressing the Th1 and Th17 responses as well as enhancing the secretion of IL-4, IL-10 and TGF-β anti-inflammatory cytokines. Like most IMTs, cyclophosphamide has limited efficacy in PPMS or SPMS, as the clinical symptoms are often latent. This latency leads to slow clinical deterioration that can go unnoticed in the absence of relapses or inflammatory changes on MRI. Cyclophosphamide can however be used as a second-line therapy in patients unresponsive to IFN-β or GA for treating relapsing or actively progressive MS (38, 39). All the mentioned therapies, however efficient in reducing clinical relapses or progression of disability in patients, have numerous side effects, some of which are expected whilst others unanticipated and rare. This highlights the potential risks for serious and rare conditions that may arise during the process of these therapies, thus it is important that we understand better of the immunopathogenic mechanisms of MS disease in the aim to develop novel therapeutic strategies for patients.

1.2 Experimental Autoimmune Encephalomyelitis (EAE)

To understand better the immunobiology of MS disease and the other heterogeneous disorders in CNS, EAE animal model is often used to a heterogeneous group of disorders that affect the CNS tissues (40-43). The histopathological observations obtained during the disease course of EAE as well as the immune mechanisms and risk genes resemble those found in MS patients, thus EAE has generally been shown to be a useful model to understand the progressive clinical course of MS and study the CNS pathology mediated by the immune system. EAE can be induced in different species and strains of animals. The mono-phase EAE in C57BL/6 mouse is one of the most common MS models with immunization of myelin oligodendrocyte glycoprotein (MOG_{35-55}) emulsified in Complete Freud's Adjuvant (CFA) together with injection of pertussis toxin (PTX). However EAE in other background murine strains immunized with different CNS antigens can develop acute/chronic progressive or relapsing-remitting course (44). Despite EAE being a valuable tool for developing a better understanding of the immune inflammatory processes in MS disease and providing an invaluable tool for drug development and treatment against MS, the differences between EAE and MS must be accredited (45, 46), and noted that the findings in EAE models may not necessarily be reflected in MS disease clinically. For example different dendritic cell (DC) subsets have been found in mouse and human. The lymphoid tissues in mouse contain particular DCs with a CD8α subset that distinguishes them from other DC subsets. These non-migrating resident $CD8^+$ DC cells can cross present exogenous cell-bound molecules and soluble antigens on MHC-I and once activated lead to excessive production of IL-12 and stimulate inflammatory responses. On the contrary, humans have similar DC subsets to mice but lack the CD8

expression. The exposure of DC cells to inflammatory cytokines can quickly activate other innate cells including NK/T cells, which may mediate the balance between Th1 and Th2 responses. This becomes crucial as various DCs have been identified in both mouse and human (47), where human DC1 cells and mouse $CD8^+$ DC subset can promote Th1 proliferation (48, 49), whilst human DC2 and mouse $CD8^-$ DC subsets promote Th2 response (50-52). In addition to different DC subsets driving proliferation of T-helper cells in mouse and human, evidences suggest a difference between Th17 differentiation in mouse and man. Th17 is another T-cell subset capable of inducing severe autoimmunity and secrete pro-inflammatory interleukins such as IL-17 and IL-22 (53-55). The divergence began with defining the role of transforming growth factor (TGF)-β in differentiation of Th17 cells. The role of TGF-β is highly pleiotropic and contrary evidences have been reported regarding its role in mice and man. In mice, IL-6 and TGF-β are essential for the differentiation of Th17 cells, whereas in human studies, IL-1β and IL-6 were shown to be essential for priming Th17 response and not TGF-β (56, 57). However despite many human studies showing the presence of TGF-β could suppress IL-17 production (56), more recent data revealed that TGF-β, IL-1β, IL-6, IL-21 and IL-23 could induce IL-17 production from naïve human CD4 T-cells (58). The controversy remains as TGF-β and IL-6 were shown in a different human study to be critical for Th17 differentiation (59), yet another study showed TGF-β was only capable of enhancing the expression of IL-17 in peripheral T-cells (60). These evidences emphasize on the importance of acknowledging the existed differences between mice models and man, thus observations from EAE studies will have limitations in their application to MS patients.

1.3 Immunopathogenesis of MS and EAE

The current understanding of the pathophysiological process in MS disease through studies in MS and EAE suggest that T-cells recognizing myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP) or MOG, become activated in the periphery and migrate to the CNS via blood brain barrier (BBB) and cause autoimmune inflammation leading to CNS tissue damage and clinical paralysis (61). The CNS has been regarded as an immune-privileged site that is protected against immune response by preventing the accessibility of immune cells and molecules to the site in three major ways; the presence of tight endothelial junctions of BBB, the absence of lymphatic vessels, and the lack of parenchymal DCs (62-64). These characteristics are able to maintain the absence of immune cells in CNS during normal conditions; however, the disruption in balance can trigger several CNS pathologies by allowing the T-cells to accumulate and develop inflammation. The ambiguity remains as to how CNS specific T-cells are initially activated in periphery and what mechanisms enable them to increase in number and trigger cascade of events that leads to accumulation of immune cells. The recent data attempt to understand the mediating T-cells in EAE and elucidate the distinct T-cell phenotypes and their effector mechanisms to broaden our knowledge of T-cell infiltrations and BBB disruption. Current understanding of the immunopathogenesis of MS/EAE is that: MS/EAE is mediated by myelin-specific T-cells that are activated in the periphery and transmigrate into the CNS through BBB (65, 66). After entering CNS, T-cells are then re-reactivated by local and infiltrating activated APCs that present MHC class-II associated peptides, resulting in subsequent inflammatory processes and eventually in demyelination and axonal damage.

For CNS inflammation to be initiated, myelin-specific T-cells are first required to activate in the periphery, gain access to the CNS and reactivate by APCs presenting self-antigens. The reactivation of T-cells causes the production of soluble mediators that could recruit other inflammatory cells to the site. However the recruitment of soluble mediators to the CNS becomes more challenging as the CNS has a unique anatomy and is protected from cellular infiltrations via the BBB that surrounds parenchymal venules and also the blood-cerebrospinal fluid (CSF) barrier that surrounds the choroid plexus and meningeal venules. The activated T-cells are however able to cross the tight junction between the endothelial of BBB and the epithelial cells of blood-CSF barrier by carrying out immune surveillance as they increase the expression of specific chemokines, integrins and adhesion molecule that allows them to pass through the tight junction (62). It is worth mentioning that the activated T-cells may not necessarily take the same route of entry to CNS. They can either cross the blood-CSF barrier and enter the region between arachnoid and pial membrane, known as subarachnoid space (SAS) where CSF circulates or cross the BBB and enter the perivascular region that separates basement membrane attached to the endothelial cells of the vessel from a second membrane known as glial limitans which comprises astrocyte feet and microglial cells (Figure 1). Moving on, many studies have focused on determining the T-cells preferential route of entry to CNS and the primary site of inflammation in CNS. The endothelial cells of the brain were discovered to express selectins and adhesion molecules that allow activated T-cells to adhere to the vessel wall in the presence of inflammation. This suggests that the immune surveillance of T-cells that cross blood-CSF barrier first occurs in SAS where adhesion molecules are expressed abundantly (67). Another study has also confirmed the presence of chemokine receptor

(CCR) CCR6 by a subset of pathogenic T-cells, which facilitates the entry of T-cells into the CNS in EAE. Interestingly the ligand for CCR6, CCL20, is expressed by the choroid plexus epithelial cells present in both man and mice, supporting the idea of T-cells crossing blood-CSF barrier first to induce EAE (68). Lassmann *et al* have also detected the SAS as the primary inflammation site in EAE, where the previously activated $CD4^+$ T-cells in the periphery are reactivated by MHC-II APCs (69). The reactivation of T-cells and recognition of cognate ligand leads to T-cell proliferation and formation of large T-cell aggregates in SAS (70). This process of reactivation itself, leads to activation of perivascular endothelial cells that allow the recruitment of T-cells into the perivascular space. Although the details of this process are poorly understood, one study suggested that the activation of T-cells in the SAS could trigger the activation of both microglial cells and axonal damage, leading to Wallerian degeneration of axons (71). This may lead to distal activation of microglial cells and up-regulation of adhesion molecules on parenchymal vasculature that are distant from initial T-cell site infiltration in SAS region (Figure 2).

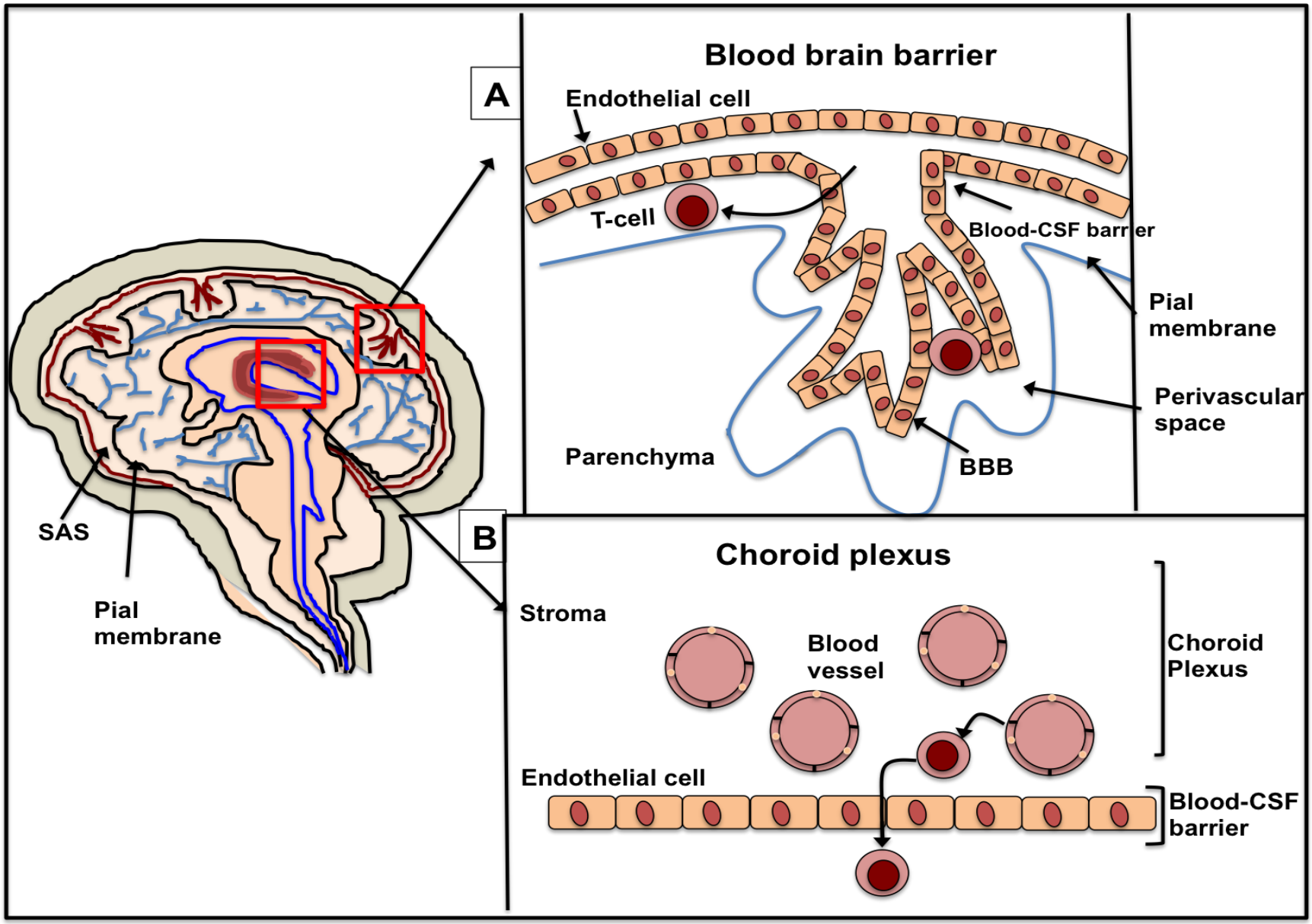
A
Blood brain barrier
Endothelial cell
T-cell
Blood-CSF barrier
Pial membrane
Perivascular space
Parenchyma
BBB
SAS
Pial membrane
B
Choroid plexus
Stroma
Blood vessel
Choroid Plexus
Endothelial cell
Blood-CSF barrier

Figure 1. Schematic drawing of possible routes of activated T-cell entry into CNS. Redrawn from "Autoimmune T-cell responses in autoimmunity", by J. Goverman, 2010, *Nature Reviews Immunology*, 9, p. 393-407. Reprinted with permission.

The activated T-cells can enter the CNS via different routes, (A) by entering the subarachnoid space (SAS) region through meningeal cell walls; (B) by entering the SAS region via blood vessels into stroma of choroid plexus and then crossing blood-CSF barrier surrounding the choroid plexus stroma. The BBB is made of endothelial cells that are connected by tight junctions making entry to CNS more difficult. T-cells crossing BBB enter perivascular space where T-cells are proliferated.

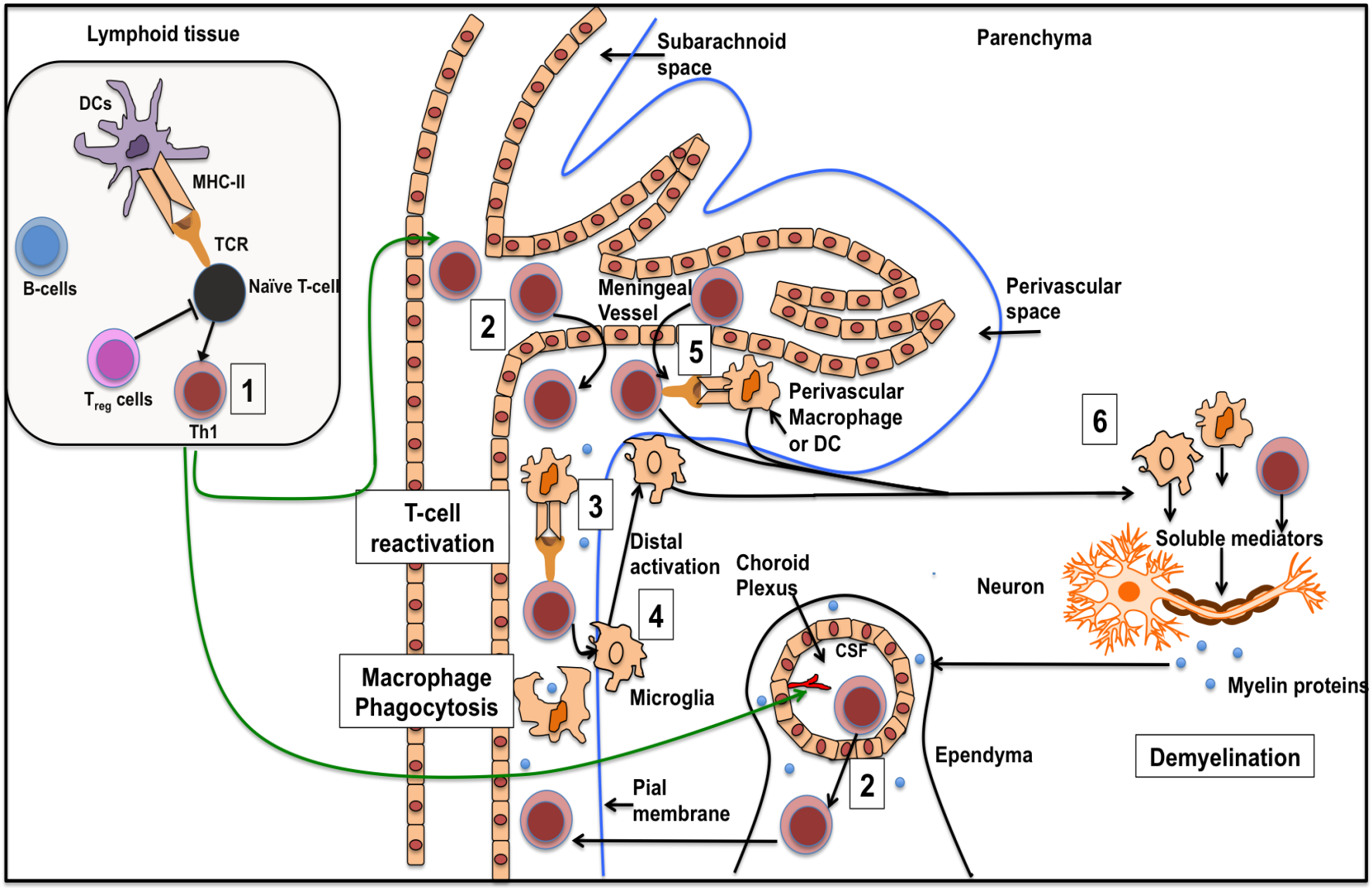
Lymphoid tissue
DCs
MHC-II
TCR
B-cells
Naïve T-cell
T_{reg} cells
Th1
1
Subarachnoid space
Parenchyma
2
Meningeal Vessel
5
Perivascular space
Perivascular Macrophage or DC
6
3
T-cell reactivation
Distal activation
Choroid Plexus
Soluble mediators
Neuron
4
CSF
Macrophage Phagocytosis
Microglia
Myelin proteins
Ependyma
Demyelination
Pial membrane
2

Figure 2. Schematic drawing of the myelin-specific $CD4^-$ T-cell activation in periphery and CNS. Redrawn from "Autoimmune T-cell responses in autoimmunity", by J. Goverman, 2010, *Nature Reviews Immunology*, 9, p. 393-407. Reprinted with permission.

Autoantigens (soluble myelin antigens) are phagocytosed by APCs and presented to naïve $CD4^+$ T-cells in lymphoid tissues. The activated $CD4^+$ T-cells then enter SAS by crossing blood-CSF barrier in either choroid plexus or meningeal vessels via chemo-attraction (2). These T-cells are then re-activated in SAS by local or infiltrating APCs (3), resulting in microglial activation and distal activation of microglial cells (4). The activated T-cells adhere to the vessel walls with the aid of adhesion molecules and cross the BBB barrier to enter the perivascular space where they become reactivated by perivascular macrophages or DCs (5). The release of T-cells, macrophages/DCs and microglial cells into parenchyma triggers secretion of soluble mediators that causes demyelination. The protective myelin sheath is damaged as a result production of protease, glutamate, reactive oxygen species and other cytotoxic agents that promote myelin breakdown. Damage to the myelin sheath surrounding axons is followed by axonal damage and neurological impairment.

1.3.1 $CD4^+$ T-cell function in CNS autoimmunity

Emerging data suggests a prime role for Th1 cells and possible contribution of Th17 cells to CNS autoimmunity through different mechanisms (13-15). For decades MS has been accepted as the $CD4^+$ T-helper1 (Th1) mediated disease. Th1 cells play a crucial role in adaptive immune response by defending body against intracellular pathogens. However over response of Th1 cells can lead to excessive inflammation and consequently tissue damage. To avoid this, the immune regulatory mechanisms normally keep the Th1 responses in a delicate balance of effective host-protection without deleterious damage. Self-reactive Th1 cells were initially regarded as the major effector cells in MS pathology (72, 73). This hypothesis was based on the early results obtained from EAE models, where IFN-γ producing $CD4^+$ T-cells were able to induce EAE with clinical symptoms and CNS inflammation in naïve mice after adoptive transfer (13). The main cytokine to drive the differentiation of IFN-γ secreting Th1 cells is IL-12 (74-76). The generation of Th1 cells begins from a naïve T-helper cell by TCR engagement and STAT1 signaling that are induced by activation of the IFN-γR and IFNs. Once STAT1 is phosphorylated, it then induces the expression of transcription factor T-bet which predominately drives the Th1 differentiation by trans-activating IFN-γ and subunit of IL-12Rβ2. As a result the cells are becoming responsive to IL-12 that are produced by activated APCs and subsequent IL-12 signaling through STAT4 that further stabilises the Th1 phenotype (77). The presence of Th1 cytokines in the inflammatory lesion in the CNS during the peak stage of EAE and the loss of Th1 cytokines along the recovery marks the importance of Th1 in pathogenicity of EAE (78, 79). In support with previous findings, EAE mice lacking the Th1 transcription factor, $T\text{-}bet^{-/-}$ and $STAT4^{-/-}$ were shown to be highly

resistant to EAE (80-82) and when treated with IFN-γ, the disease was exacerbated in MS patients (83-85). However the Th1 pathogenic effector dichotomy was questioned when surprisingly the mice models deficient in IL-12α (IL-12p35), IL-12Rβ2, IFN-γ, and transcription factor STAT1 were all found to be highly susceptible to EAE, despite inability to produce IFN-γ (86-90). The mice deficient in p35 chain of IL-12 were shown to be highly susceptible to EAE however on the contrary the p40 deficient and p19 deficient mice were highly resistant to EAE. The p19 deficient mice however showed normal generation of Th1 response, but failed to produce substantial numbers of IL-17 producing T-cells. These paradoxical evidences suggested the potential involvement of other T-helper subsets that may induce autoimmunity and has therefore shed light on the identification of Th17 lineage. The development of Th17 lineage is driven by two transcription factors, retinoid-related orphan receptors (ROR)γt and RORα (91, 92). The development of Th17 is antagonized by the expression of IFN-γ secreting-Th1 cells and IL-4 secreting Th2 cells and hence result in suppression of Th17 differentiation if present (93, 94). IL-23 is known as an essential component for later stage of Th17 differentiation into mature effector cells (95). Interestingly the IL-23 knockout mice were unable to generate Th17 cells and showed full susceptibility to EAE (95-97). The main drivers of Th17 differentiation are the cytokine TGF-β and IL-6 (98, 99). The differentiation of Th17 cells can be induced either with signaling of IL-6 to phosphorylate STAT3 or in absence of IL-6, IL-21 and TGF-β will endure this effect. Despite IL-6 being a stronger driver of Th17 response, IL-21 can act in an autocrine fashion to amplify Th17 differentiation (100-102). Moreover, Th17 cells can produce series of cytokines including IL-17A/F, IL-21, IL-22 and TNF-α that can promote inflammation and contribute to

pathogenicity of autoimmune disorders. Th17 and other leukocytes such as natural killer cell (NK), natural killer T-cells (NKT), $CD117^+$ $CD127^+$ leukocyte lymphoid tissue inducer (LTi) cells and mucosal $NKp46^+$ cells can also release IL-17 cytokine. IL-17 is a key pathogenic molecule in many disease processes including EAE, showing that the IL-17 deficient model can develop attenuated EAE and that the neutralizing IL-17 antibodies can ameliorate EAE (53, 103, 104). However these findings were questioned when another study showed IL-$17A^{-/-}$ and IL-$17F^{-/-}$ mice models were still susceptible to EAE with mild disease severity after neutralizing IL-17 in vivo (105). Also despite 50% homology between IL-17A and IL-17F and similar pattern of expression, IL-17F showed no decrease in EAE incidence or strong differences in EAE severity when IL-17A was neutralized in IL-$17F^{-/-}$ mice compared with wild-type, nor caused disease exacerbation when IL-17A was overexpressed in $CD4^+$ T cells (106). Despite the debates many studies have moved on to clinically investigate the sole pathogenic role of Th17 in MS patients by looking at the expression of IL-17 in CNS lesions. As a result high levels of IL-17 in chronic MS lesions were detected compared to other acute lesions and naïve controls of patients without CNS pathology (107). A full induction of EAE by adoptive transfer of highly purified Th1 cells were observed where on the contrary Th17 cells lacking IFN-γ producing cells were unable to induce the disease, highlighting the importance of IFN-γ in both Th1 and Th17 cells. Other studies have focused on the importance of Th17 in disease incidence and contribution to pathology. Lees *et al* discovered lower incidence of EAE in IL-$17^{-/-}$ upon transfer of Th1 cells compared to the wild type (108). Therefore it was suggested that Th1 cells are primarily responsible for crossing the tight BBB junction and accessing CNS leading to facilitated subsequent recruitment of Th17 cells. T-cells

responsible for production of both IL-17 and IFN-γ that express T-bet and RORγt are recruited to CNS during EAE. Expression of T-bet have been described as the main encephalogenicity of T-cells rather than their cytokine expression, and inhibition of T-bet has been shown to ameliorate EAE by inhibiting both Th1 and Th17 cells (109). Interestingly the transferred Th17 cells can switch between production of IL-17 and IFN-γ, indicating the existent level of plasticity between these populations (110, 111). In an attempt to understand the cellular behavior of Th17 and Th1 cells in pathogenicity of EAE/MS, Korn *et al* demonstrated that after active immunization, Th17 cells frequency peaks earlier during induction of disease whereas IFN-γ producing Th1 cells reach the highest levels at the peak of clinical disease where symptoms are worsened and disability ensues. This may suggest that Th17 could potentially be the first cells to infiltrate CNS and propagate tissue inflammation before Th1 cells (112). Jager *et al* have observed different nature of disease development and symptomology induction by Th1 and Th17. The Th17 cells seem to promote lesion formation localised in brainstem and cerebellum leading often to atypical signs of EAE, whereas Th1 cells induce lesions in SC causing classical signs of EAE (113). On the basis of the Th1 and Th17 studies, it is difficult to attribute a sole pathogenic role to either T-cell subsets. Given all the controversies over the functional differences between Th1 and Th17 cells and the lack of consensus on their relative pathogenicity in EAE, it becomes crucial to expand our understanding of $CD4^+$ mediated T-cells in CNS autoimmunity.

1.3.2 T-regulatory cells in MS/EAE

While the effector T-cells are promoting and perpetuating inflammation in MS, the regulatory T-cells serve to sustain balance by controlling expansion and activation of autoreactive $CD4^+$ T-cells as well as maintaining self-tolerance. The effector Th17 cells are hypothesized to have reciprocal relationship with the protective $FOXP3^+$ T-regulatory cells ($CD4^+CD25^+$ T_{Regs}). If the encounter of high avidity MBP-specific T-cells with lymphoid endogenous MBP cells is suppressed, an anti-inflammatory response is acquired which is characterized by secretion of IL-10 and TGF-β (114). In myelin-specific $CD4^+$ TCR transgenic mouse models, the transcription factor for T_{Regs}, forkhead box P3 $(FOXP3)^+$ suppresses the activation of myelin-specific $CD4^+$ T-cells within the periphery and thereby preventing the development of spontaneous EAE (115, 116). However in the presence of immunogenic stimuli and absent of T_{Regs} the reactions between MBP-specific T-cells and APCs will overcome active tolerance and autoimmunity will be endured. The generation of the pro-inflammatory Th17 cells requires the presence of TGF-β together with IL-6 to drive the process. However the presence of TGF-β alone induces $FOXP3^+$, as the essential component for developing Tregs within the periphery. On the other hand the presence of IL-6 pro-inflammatory cytokine alone, inhibits the induction of $FOXP3^+$ T_{Regs} and instead promotes the differentiation of Th-17 cells simultaneously (117). To further support the hypothesis of Th17 and T_{Regs} reciprocal relationship, Zhou and Du J *et al* have shown the transcription factors RORγτ/RORα and $FOXP3^+$ for Th17 and T_{Regs} to inhibit each other's functions (118, 119). Additionally cytokines such as IL-2 favors T_{Regs} production and inhibits Th-17 cell differentiation, whilst IL-21 promotes Th-17 differentiation and instead inhibits the T_{Regs} expansion (120). Despite the similar numbers

of T_{Regs} in peripheral blood and CSF in both MS and healthy patients, some studies have pointed to the defects in T_{Regs} capacity from MS patients to suppress myelin-specific T-cells in periphery (121, 122). This can imply that either there is a lack of T_{Regs}-mediated suppression in autoimmune responses towards CNS or the defects lie in migration of T_{Regs} to the CNS (112). Other studies have shown the impaired functions of $CD4^+$ T_{Regs} cells in MS patients as well as decreased ability or absence of $CD4^+$ T_{Regs} cells to prevent activation of myelin-specific T-cells in the periphery (123), suggesting the lack of $CD4^+$ T_{Regs} cells in CNS could be a result of defective migration or inability to survive in CNS microenvironment. Indeed the mechanism of T_{Regs} in active EAE has generally been proven to be protective and to reduce inflammation. Zhang *et al* report the application of anti-CD25 antibody, which reduces T_{Regs} production in vivo, increased the onset and severity of EAE with associated decrease of an important anti-inflammatory cytokine IL-10. Furthermore it was reported that transfer of T_{Regs} from naïve SJL mice decreases EAE severity but surprisingly has no effect on IL-10 deficient mice (124). This may indicate that T_{Regs} may play a role in down-regulation of effector T-cells via mechanisms that involves IL-10. IL-10 is considered to be a multi-functioning cytokine that initially was described to be a product of Th2 cells that can negatively regulate the activation and secretion of Th1 cell cytokines (125). IL-10 is now known to inhibit the Th1 response by reducing antigen presentation and cytokine production from APC (126). Furthermore IL-10 can also act directly on T-cells to reduce proliferation and cytokine production (127). Many studies have particularly focused on the effects of IL-10 in both MS and EAE and not surprisingly IL-10 was observed to elicit beneficial effects. Moreover MS patients have shown an increased level of IL-10 in their serum during disease remission (128)

indicating the potential protective role of IL-10 during remission. The two widely used treatments of MS, IFN-β and GA, both have also demonstrated efficacy in part by inducing IL-10 production from immune cells (129). More evidence also suggests the genetic study where IL-$10^{-/-}$ model showed development of severe EAE where symptoms were exacerbated, while the over-expression protected mice against EAE (130). Although some studies have suggested correlation between the presence of FOXP3 and IL-10 cells in CNS with disease recovery (131), others have shown T_{Regs} to be ineffective in suppressing T-peripheral $CD4^{+}$ T-cells whilst the levels of IL-6 and TNF are still elevated (112). Further research in understanding the exact function of IL-10 and T_{Regs} will help to develop new therapeutic strategies for patients.

1.4 IL-22 and its role in inflammation

IL-22 was first discovered in 2000 as an IL-9 induced gene and termed IL-10-related T cell-derived inducible factor (IL-TIF) (132, 133). IL-22 is induced by IL-9 in mouse T lymphoma cells and shows 22% amino acid similarity with IL-10 and 80.8% identity with human IL-22 (134, 135). IL-22 receptor (IL-22R) contains two subunits of IL-22R1 and IL-10R2 (136). The IL-22R1 is expressed by a variety of non-immune tissues (skins, lung and kidney) while IL-10R2 is expressed on immune cells (T, B and NK cells). Another form of IL-22R encoded by different gene is the soluble IL-22R, IL-22 binding protein (IL-22BP), which counteracts to IL-22 with affinity four times higher than those observed in IL-22R1 in vitro (137). In general, the biological role of IL-22 involves the increase of innate immunity and providing protection from damage as well as enhancing regeneration of damaged tissues (136, 138-145). However, IL-22 can also play a pathogenic role in some chronic inflammatory disease depending on the nature of effected tissue and local cytokine milieu (146-149). These paradoxical effects of IL-22 are dependent on the context of IL-22 production as IL-22 can synergistically act with other inflammatory cytokines, including IL-17 and TNF-α (54). Therefore a tight regulation of IL-22 is critical in maintaining he beneficial effects of IL-22 and avoiding deleterious inflammatory effects. Recent studies have shown the activated T and NK cells to express IL-22 upon immune cell isolation whereas other immune cells such as B-cells, monocytes and DCs were unable to produce IL-22 (133, 150). Among all these immune cells, IL-22 expression was mainly restricted to $CD4^+$ memory cells but human Th1 cells were shown to be important IL-22 regulators (151). In the murine system Th17 cells are thought to be the main IL-22 producers whereas in humans Th22 and Th1 cells are likely to play a more

important role for IL-22 production (59, 152). The IL-10 family members mainly signal via activation of the Jak/STAT pathway (153). The signaling of IL-22 begins with IL-22 binding to IL-22R causing the activation of JAK1 and Tyk2 kinases; primarily leading to STAT3 and to a lesser extends STAT1 and STAT5 phosphorylation (136). STAT3 has recently been discovered to be constitutively linked to the C-terminus of the IL-22 that leads to mediating the expression of variety of genes involved in cellular processes such as cell growth and apoptosis (154). As IL-22 signaling pathway affects multiple molecular processes in autoimmune diseases, therefore manipulating these pathways may have potentials to treat autoimmune disorders (155-159). Interestingly IL-22 also exhibits similar pattern of expression as IL-17, a key molecule in MS and EAE development (160). A recent genomic study by Beyeen *et al*, in an attempt to identify EAE-regulating quantitative trait locus that harbors the EAE risk genes, has demonstrated the presence of *Eae29* on rat chromosome 1. The *Eae29* allele from the resistant strain both conferred milder EAE and lower production of pro-inflammatory molecules in macrophages as demonstrated by the congenic line, (DA, susceptible to EAE and PVG, resistant to EAE) DA.PVG-*Eae29* (Dc1P). Interestingly soluble IL-22R α2 gene was discovered within the *Eae29* locus and its expression was reduced in Dc1P, both in activated macrophages and splenocytes from immunized rats (40) supporting the potential pathogenic role of IL-22 in EAE/MS development. Indeed, there are a few recent reports suggest an emerging role of IL-22 in MS disease (161). Durelli *et al* reported that the IL-22-producing $CD4^+$ T-cells were dramatically increased in the serum samples of MS patients together with Th17 cells but not Th1 cells. Furthermore, they observed two distinct CD4 subsets producing either IL-17/IL-22 or only IL-22 in the peripheral blood samples of acute MS patients (161).

Almolda and colleagues have also investigated the potential role of IL-22 and other cytokines in MS disease using EAE model (162). Their results revealed a specific cytokine expression profile along the EAE course characterized by no changes of IL-10 and IL-17 levels throughout the disease course, but a decrease of IL-21 on the peak, and an increase of IL-22 levels in SC tissues during the induction and peak phases that was markedly decreased during recovery. An elevated increase in $CD3^+$ and $CD4^+$ cells was reported during clinical symptomatology of the disease, showing a Th1 phenotype. Unexpectedly during the recovery phase, although the clinical signs progressively decreased, the number and proportion of $CD3^+$ and $CD4^+$ populations remained unaltered. More interestingly there was a marked decrease in Th1 and an important increase in Th17 and T_{Regs} cells, which was also coupled with dramatic decrease of IL-22 during the recovery phase of acute EAE in rats (162). This could illustrate a potential pathogenic role for IL-22 in induction and pathogenicity of the disease. In contrast to these findings Kreymborg *et al* have exclusively looked at the impact of IL-22 in pathogenicity of EAE and found IL-$22^{-/-}$ deficient mice were fully susceptible to EAE development (163). As controversies remain, to determine the exact role of IL-22 in EAE development, I studied the expression of IL-22 in CNS and peripheral immune tissues during the induction, peak and recovery stages of EAE and examined the correlation of the expression level with clinical severity of EAE.

1.5 Research aims

Current data suggest that IL-22 may have an important role in MS/EAE development, but the exact function of this molecule is less clear. This project aims to understand the mechanisms of IL-22 function in MS/EAE by studying the expression levels of IL-22 in immune and CNS systems and examining the correlation of the expression levels with disease severity. The data obtained from this study will indicate the potential role of IL-22 in the peripheral immune system and in the CNS system during the development of CNS inflammation, thus provide better insights of the immunopathogenesis of MS/EAE.

Chapter 2: Material and methods

2.1 Buffers and solutions

Avidin/Biotin: TBS containing 5%HOS/2%MOS and PBS plus 4 drops of Avidin per ml, pH 7.4

Blocking buffer reagent (BB): 10% Normal, 2.5% Normal Mouse Serum, 1% BSA, pH 7.2

BB + Avidin: BB, 4 drops of Avidin/ml, pH 4.5

BB + Biotin: BB + 4 drops of Biotin/ml, pH 3.5

Cresyl Echt Violet: Cresyl Echt Violet Acetate 0.5 gm. Distilled water 100 ml, pH 3.5

Formalin: 25% ethanol + 75% acetone, pH 6.8

PBST: PBS + 0.05% Tween 20, pH 7.4

Complete RPMI: RPMI medium +L-glu+ 10% FCS+ S/P (Streptomycin 10000 μg/ml, Penicillin10000 U/ml), pH 7.2

TBS: 116.8 g NaCl, 40ml 2M Tris, 4 ml of Tween® 20, 4 L with H_2O, pH 7.6

TBST: TBS + 0.05% Tween® 20, pH 7.5

2.2 EAE induction and clinical evaluation

C57BL/6 female mice were purchased from Harlan (UK) and maintained in the Biological Procedure Unit at University of Strathclyde under UK Home Office guidelines. Female mice at the age of 7-8 weeks were used in all experiments. For EAE induction, mice were immunized subcutaneously at the back region with MOG_{35-55} peptide (100 μg/50μl of (Sigma Genosys, USA) emulsified with an equal volume of CFA (5 mg/ml of *Mycobacterium tuberculosis*, strain H37RA, Difco, Detroit MI)) on day 0. Each mouse also received 100 ng/100μl of PTX (Sigma, USA) in PBS intraperitoneally on day 0 and 2. Mice immunized with an equal volume of PBS in CFA together with PTX were used as controls. Following the immunization the mice were monitored closely of their body weight and clinical signs of EAE disease. Mice were weighed on day 0 and from day 7 to day 28 post-immunization on daily basis. Percentage of body weight change was calculated as (the weight of the day-the weight of day 0)/the weight on day 0 x 100%. EAE severity was scored according to a 0-5 scale as follows: 0, no clinical sign; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb involvement; 5, moribund.

2.3 Tissue harvesting and cryosectioning

Naïve mice and mice from MOG-CFA or PBS-CFA immunized groups were sacrificed on day 9, day 17 and day 28 post immunization for histology and immune response examination. Mice were sacrificed by asphyxiation in CO_2, then blood, spleen, inguinal LNs, and SC tissues were harvested. The blood from each mouse was harvested using 1ml syringe from the open chest cavity and transferred to eppendorf tubes. Samples were left at room temperature (RT) for 4 hour and then centrifuged at 200 g for 5 minutes, serum samples were collected and stored at -20°C until used. Once the blood was collected, mice were perfused with 20 ml of cold PBS buffer, spleen and LNs were then harvested and suspended in PBS with 2% FCS and Penicillin (100 units/ml)/Streptomycin (100 µg/ml) for tissue culturing and stimulation assay.

Mouse SC was then flushed out with PBS by hydrostatic pressure using a syringe attached to an 18-gauge needle. In brief, the spinal column just below the skull and at the hill level was cut and removed. The 2 ml syringe filled with PBS was inserted to the lumbar region of the spinal column and the plunger pushed forcefully to inject PBS into the column to allow the SC to slip out. SCs were collected and snap frozen on dry ice in mounting medium O.C.T (VWR International BVBA) for cryotomy or individually suspended in RNA*later*® (Life technologies) for PCR.

SC molds embedded in OCT were put onto metal grids that fit into the cryostat. The sections were cut 7 µm thick at −20°C and placed on poly-l-lysine coated slides (SuperFrost® Plus, VWR Int.) for staining purposes. The slides were then air dried at RT for 3-4 hours before storing at -20°C.

2.4 Haematoxylin and Eosin (H&E) staining

The slides were taken out from the -20°C freezer and left to defrost at RT for 40 minutes, followed by fixation in formalin solution for 10 minutes. Slides were then left for 20 minutes at RT before rehydrating in TBS buffer for 10 minutes. The slides were then placed in haematoxylin for 7 minutes, followed by washing under running tap water until water runs clear. Slides were then placed in 1% Acid/Alcohol for 12 dips followed by 2-3 minutes wash under running water. After washing, the slides were placed in Scott's Tap Water Substitute (STEWS) for 2 minutes and were checked under microscope for staining morphology before washing under tap water again. Slides were then put in Eosin for 30 seconds. Eosin is a strong dye and it requires longer washing step but this can be checked regularly under the microscope until a suitable pink colour is obtained. Slides were then put in 70% Ethanol for 10 dips followed by 20 dips in absolute alcohol and 3 minutes in Xylene, followed by mounting in DPX medium (Sigma-Aldrich®).

2.5 Luxol Fast Blue staining

Frozen sections were taken out from -20°C freezer and left to defrost at RT for 40 minutes. Sections were then fixed in formalin for 10 minutes. The slides were then placed in Luxol® Fast blue solution (Sigma-Aldrich®) containing 10% acetic acid and were left overnight (ON) at 58°C oven. The staining cylinder was tightly capped to prevent the alcoholic solution evaporating easily. After the ON incubation, the sections were then rinsed in 95% alcohol to remove excess stain followed by rinsing in distilled water for 1-2 minutes. The differentiation step is the most crucial and sensitive step of the staining and it begins by immersing the slides in 0.05% lithium carbonate solution for 10-20 seconds. The differentiation step was then continued in 70% alcohol solution until the grey and white matter could be distinguished. The slides were microscopically checked to observe a clear grey matter and sharply defined white matter. The differentiation step can be repeated a couple of times if necessary. Once the differentiation step is completed, the slides were rinsed in distilled water thoroughly and were placed in pre-heated Cresyl-Echt-Violet solution for 6 minutes at 60°C. The slides were then differentiated in several changes of 95% alcohol and further dehydrated in absolute alcohol and cleared in Xylene. The slides were then mounted with DPX mounting medium.

2.6 Antigen specific immune responses of immune cells

LNs and spleens were collected from naïve, MOG-CFA and PBS-CFA immunized mice and pooled within each group. Tissues were placed on Nitrex nylon mesh and mashed gently using syringe plunger. The single cells from LNs and spleens were collected and washed with sterile PBS and centrifuged at 200 g for 5 and 7 minutes respectively. This step was repeated several times. The spleen pellets were then lysed in 5 ml of red blood cell (RBC) lysis buffer (BD Pharmingen) to lyse the RBCs for 5 minutes and re-filtered through Nitrex nylon again. LNs do not require the lysis buffer and therefore pellets are directly suspended in complete RPMI media (Invitrogen) and cell number were counted using Trypan blue with haemocytometer. $4x10^6$ cells in 2ml were then cultured with or without MOG peptide (50 µg/ml) in 24 well plates (Test plates, Zellkultur, Switzerland) as shown in Figure 3. After incubation of cells for 72 hours, cell supernatants were harvested and then centrifuged for 5 minutes before transferring the supernatant to new eppendorf tubes and storing at -20°C for ELISA test.

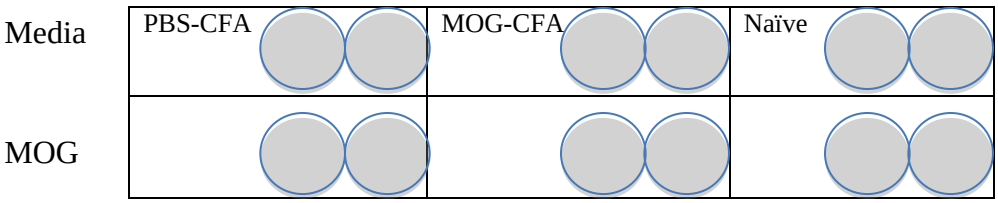

Figure.3 Antigen specific stimulation of immune cells in culture

The MOG-CFA, PBS-CFA and naïve spleen cells were harvested and pooled within each group, single cell suspensions were then counted and $4x10^6$ cells in 2ml complete RPMI medium were cultured with or without MOG_{35-55} (50 µg/ml) for 72 hours.

2.7 ELISA

To analyse the secretion of cytokines IL-17A, IL-22 and IFN-γ (reagents details see Table 1, all from eBioscience, UK) by the LN and spleen cells, ELISA was performed. The appropriate capture antibody (purified antibody) was diluted in the coating buffer (eBioscience, UK) according to the manufacturer's instruction and 100 µl was added to each well of the high-binding 96 ELISA well plates (Microlon, Greinerbio-one) and left ON at 4°C. The plates were then washed using a washing buffer PBST five times before treating with 50 µl/well of Assay Diluent (Phosphate buffered solution containing fetal bovine serum) to prevent non-specific binding of ELISA plates for 30 minutes at 37°C. After five washes, 100 µl/well of cell supernatant samples or double diluted standards were added into appropriate wells and sealed for further 2 hours of incubation at RT. Plates were then washed for five more times before adding 100 µl/well of prepared detection antibody (Biotin-conjugated antibody) and left for an hour incubation at RT. Following that, plates were washed thoroughly again and added 100 µl/well of Avidin-HRP (eBioscience) for 30 minutes. After further washing, plates were added with 100 µl/well of tetramethylbenzidine peroxidise substrate TMB (BD OptEIA^{TM,} BD Bioscience) and incubation for 15 minutes at RT and stopped by adding 50 µl of stop solution (Sulphuric Acid, 1 M H_2SO_4) to each well. The plates were then read at 450 nm within 30 minutes of adding the stop solution, using an Epoch (BioTek, Highland park, U.S.A) automatic microplate reader and Gen5^{TM} software.

Table 1. ELISA reagents from eBioscience

Cytokine	Capture Ab	Standard	Detection Ab
IL-22	Purified α-mouse IL-22	Recombinant mouse IL-22	Biotin conjugate α-mouse IL-22
IL-17A	Purified α-mouse IL-17A	Recombinant mouse IL-17A	Biotin conjugate α-mouse IL-17A
IFN-γ	Purified α-mouse IFN-γ	Recombinant mouse IFN-γ	Biotin-conjugate α-mouse IFN-γ

2.8 RNA extraction and reverse transcription (RT-PCR)

The SC samples suspended in RNA*later*® were taken out from 4°C fridge and were added with 1.5 ml of Trizol® (Invitrogen™ Carlsbad CA, USA). The samples were then homogenized using homogenizer T25 basic (IKA LABORTECHNIK). To each ml of Trizol, 0.5 ml Chloroform (Sigma-Aldrich, Germany) was added in each eppendorf tube vigorously mixed by hand and kept at RT for 2 minutes. Samples were then centrifuged at 10,000 g for 15 minutes at 4°C and the top aqueous layer was transferred to a new micro-centrifuge tube with 0.6 ml ice cold Isopropanol. Further centrifuging was required at 10000 g for 10 minutes at 4°C where the pellet was formed and washed in 1mL 70% Ethanol, before centrifuging it again at 7000 g for 15 minutes at 4°C. At the final step, ethanol was carefully removed and pellet air dried before adding 50 µl RNase free water to each sample and heat tube at 65°C for 10 minutes to solubilise RNA. The RNA was then quantified using Nano-drop 2000 c spectrophotometer (Thermo Scientific).

After quantification of RNA sample by Nano-drop, 2µg of total RNA per 20µl reaction is taken and thawed on ice. Then the aliquot RT reaction mix (Table 2) is loaded into plate and sealed for brief centrifugation. The thermal cycle began with plate incubation at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and paused at 4°C when the cycle is completed. Once the cycle stops, the cDNA is ready for long-term storage in freezer (-20°C).

Table 2. RT-PCR reaction mix

Component	**Component Volume/ Reaction (µl) (Without RNase Inhibitor)**
10x RT Buffer	2.0
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per reaction	10.0

2.9 Real-time PCR

Once tissue cDNA is ready, it can be used in real-time PCR application. Fast SYBR® Green real-time PCR was performed according to the manufacturer's instructions (AB Applied Biosystems) using the primers described below. Each reaction volume contained 20 µl, which was loaded on MicroAmp™ Fast Optical 96-well reaction plate. Each sample contained 10 µl of Fast SYBR® Green Master Mix, forward and reverse primers‡ (Table 3, minimum of 200 nM of each primer), RNase-free water and 1 µl of cDNA template which should give a total volume of 20 µl. The plate is then placed in Applied Biosystems real-time quantitative PCR instrument (StepOne Plus) and appropriate programming for thermal cycling conditions is then applied. The first step begins with AmpliTaq® Fast DNA Polymerase, UP Activation for 20 seconds at 95°C, denaturing step for 3 seconds at 95°C for 40 cycles, following anneal/extension step for 30 seconds at 60°C for further 40 cycles. Once the cycles are complete, the plate is ready for data analysis. The used primers with both forward and reverse sequences are tabulated below.

Table 3. Cytokine PCR primers

Gene	**Company**	**Forward primer 5′⟹ 3′**
IL-22	Invitrogen™	TTGAGGTGTCCAACTTCCAGCA
IL-22R	Invitrogen™	CTGCCAACCTGACTATGGAGA
IL-17A	Sigma®	CTCAACCGTTCCACGTCAC

2.10 Immunohistochemical colour staining

The slides were fixed in formalin solution for 10 minutes before being air-dried. Tissues on the slides were then circled with wax pen and left at RT for 20 minutes to air-dry again. The slides were then rehydrated in staining cylinder with TBST for 25 minutes while placed on the shaker. 100 µl of blocking buffer (BB)+Avidin was then added to each tissue and incubated for 30 minutes. This step helps to reduce non-specific binding in the tissues. Following the blocking step, a fluorescence-conjugated antibody can be added to react directly with the antigen in tissue sections. However this method is insensitive due to low signal amplification. In comparison, indirect staining method has proven to be more sensitive due to signal amplification through biotinylated antibody and the following enzyme horseradish peroxidase (HRP). We used purified primary antibody, which reacts with the tissue antigen followed by a biotinylated antibody, which specifically bound to the primary antibody. Thus tissues were incubated with blocking buffer first, which is followed by 100 µl of pre-diluted primary antibody (Rat α-mouse IL-22, or other antibodies, details see Table 2) for either 1 hour at RT or incubation ON at 4° C in dark stain tray with wet paper towels to allow humidity. Following the incubation ON, the slides were taken out and left at RT for 30 minutes before washing in TBST for further 30 minutes. Appropriate biotinylated antibody in BB was applied to the tissues for 30 minutes at RT. Sections were then thoroughly washed in TBST before being incubated with HRP diluted in BB for 30 minutes. After further washing, substrate DAB/AEC (Vector Laboratories, Inc. Burlingame, CA.) (1 µl in 100 µl) was added to the samples for about 10 minutes. Slides were then washed in TBST and water before being

counterstained with haematoxylin, and mounted in hydromount solution. Matched isotypes (Table 5) were used as controls for the immunohistochemical staining.

2.11 Double fluorescence immunohistochemical staining

The slides were fixed and treated as before. Upon incubation with first primary antibody the slides were treated with 100 μl of pre-diluted primary antibody (Rat α-mouse IL-22) in BB + biotin (4 drops per 1 ml) for 1 hour at RT placed in dark tray with cooled paper towels to allow humidity and prevent the tissue drying quickly. Rehydration step was then applied using TBS+0.05% Tween® 20, for 30 minutes as before. Appropriate biotinylated antibody in BB were then applied to the tissues for 30 minutes and washed as before. For indirect immunofluorescence method the tissues require to be incubated with Strepavidin or species specific secondary antibodies conjugated with FITC or TRITC in BB for 45 minutes to allow colour development. The slides were then washed in TBST before drying and mounting with Vectashield containing DAPI (Vector Laboratories, Inc. Burlingame, CA.).

Table 4. Primary antibodies used in immunohistochemical staining

Primary Antibody	Company	Species	Catalogue No
IL-22	R & D system	Rat α mouse	IC582P
GFAP	DAKO	Rabbit α mouse	Z0334
NeuN	Millipore	Mouse α mouse	MAB377
Iba1	Abcam	Goat α mouse	ab5076
SMI-31	Abcam	Mouse α mouse	ab24570

Table 5. Isotype antibodies used in immunohistochemical staining

Isotype control Antibody	Company	Catalogue No
Rat IgG	Vector	I-4000
Mouse IgG	Vector	AI-9200
Rabbit IgG	Vector	I-1000
Goat IgG	Invitrogen	026202

Table 6. Secondary antibodies used in immunohistochemical staining

Biotinlyated Antibody	Company	Species	Catalogue No
α Rabbit Biotin	BD Pharmingen™	Goat α rabbit	555616
α Rat Biotin	eBioscience	Mouse α rat	13-7341-85
α Goat biotin	Vector	Rabbit α goat	BA-5000
Fluorescent Antibody			
Strep FITC	Vector	-	SA-5001
Strep Texas Red	Vector	-	SA-5006
TRITC	Invitrogen	Mouse α mouse	A11036
TRITC	Invitrogen	Goat α mouse	A21124
TRITC	Invitrogen	Rabbit α goat	A10532

2.12 Statistical analysis

All the data are presented as mean ± S.E.M in all experiments or as readings from individual mouse as appropriate. The cytokine production by immune cells were tested using ELISA and the experiments were repeated three times fully and each time in duplicate unless stated otherwise. All statistics along the study were performed using the Graph Pad Prism® software. Unpaired T-tests were also performed for calculating the significant difference of the cytokine levels in the splenocyte culture supernatants between EAE immunize mice and control group. T-test was also used to analyze the difference of EAE severity and percentage of body weight change between the two groups.

Chapter 3: Results

Introduction

During the development of MS/EAE, various immune molecules such as IL-22 are released by tissues and immune cells through different pathways and contribute to the disease pathology or recovery. A few recent papers reported that increased percentage of IL-22$^+$ CD4 T cells in the peripheral blood of MS patients, suggesting an emerging role of IL-22 in MS development (161, 164, 165). Almolda *et al* have reported increased CD3$^+$ and CD4$^+$ cells particularly Th1 cells in SC tissues of Lewis rat models during the induction and peak phases of EAE, however during the recovery phase despite the reduced clinical scores, the proportion of CD3$^+$ and CD4$^+$ remained unchanged (162). They further observed reduced levels of Th1 cells paralleled with increased Th17 and T_{Reg} cells during the recovery phase. Furthermore, they also observed while there was no change of IL-17 and IL-10 levels in SC during the course of EAE, a high up-regulation of IL-22 during the induction and peak onset, followed by marked decrease during recovery. Interestingly, despite the elevated expression of IL-22 during induction and peak in CNS tissues, Kreymborg *et al* have reported the IL-22 deficient mice were fully susceptible to EAE development (163). To understand the role of IL-22 in EAE development, we studied the expression of IL-22 in CNS and peripheral immune tissues during the induction, peak and recovery stages of EAE and examined the correlation of the expression level with clinical severity of EAE. ELISA assay was used to test the levels of IL-22; IL-17A and IFN-γ in the spleen cell supernatant and serum. Following on the ELISA experiment, the mRNA expression levels of these cytokines in CNS were examined by real-time PCR. Furthermore, we investigated the expression and cellular

localization of IL-22 in control and EAE CNS tissues using colour and double immunofluorescence staining.

3.1 EAE development

As described in the Chapter 2, C57BL/6 mice were immunized with MOG_{35-55} peptide emulsified with an equal volume of CFA to induce EAE. Furthermore each mouse received PTX in PBS intraperitoneally on day 0 and 2. The control group received PBS in CFA instead of MOG_{35-55} peptide together with PTX. Each mouse was monitored on daily basis to record any weight loss and the development of clinical symptoms (166-168). The percentage body weight change of MOG-CFA and PBS-CFA immunized mice is presented in Figure 4. Both group mice gained some weight from day 0 to day 9 before any clinical symptoms were observed. However this was followed by a rapid decrease in the percentage body weight of the MOG_{35-55} immunized mice starting around day 10 while the control PBS-CFA immunized group mice continued to gain weight. There is a significant difference in the percentage of the body weight change between the two groups from day 11, throughout the EAE course even after the EAE mice started to regain weight around day 17.

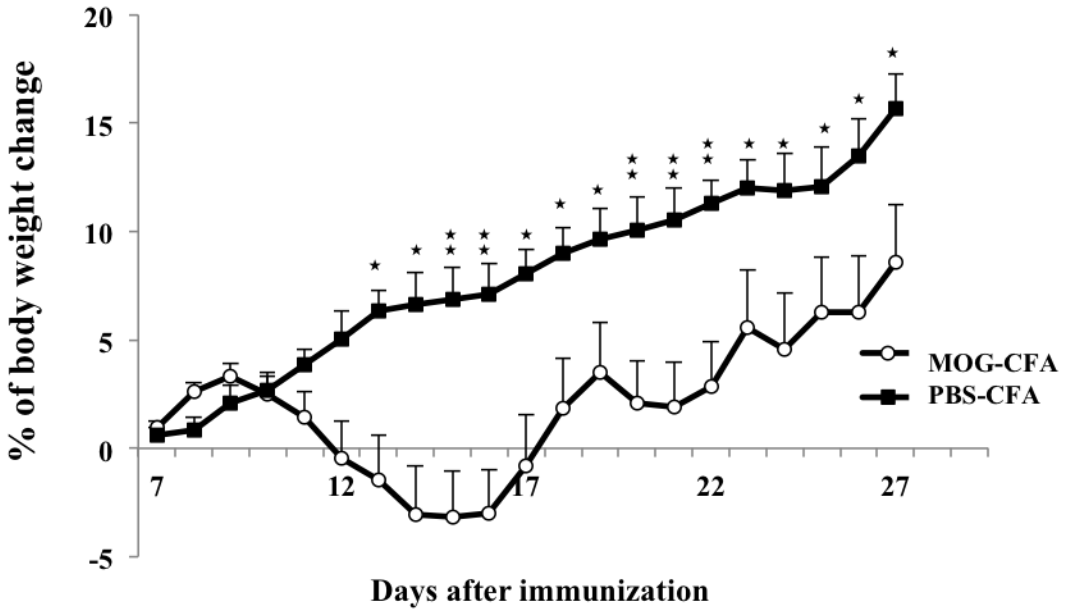

Figure 4. Percentage body weight change of MOG and PBS immunized mice

C57/BL/6 mice were immunized s.c at day 0 with 100 µg/50 µl of MOG_{35-55} peptide emulsified in CFA together with 100 ng/100 µl of PTX i.p on day of immunization and 2 days later. Control animals were immunized with PBS-CFA and PTX. Individual mice were monitored for their body weight change daily. Both group mice showed increase in weight from day 0 to 9, followed by rapid decrease of body weight for MOG immunized mice starting at day 10. The weight change continued to fluctuate throughout the EAE course even after the EAE mice started to gain weight around day 17. Data show the mean ± SEM, n= 20 (days 0-9), n=14 (days 10-17) and n=7 (days 18-28) in EAE group and n=15 (days 0-9), 9 (days 10-17) and 7 (days 18-28) in PBS groups. * $p < 0.05$, ** $p < 0.01$.

The clinical scores of each individual mouse were also recorded according to a 0-5 scale as described in Materials and Methods. Some photos were taken to show the score system (Figure 5). The clinical signs began mainly from day 12 starting with tail paralysis, and became more severe each day until day 17 where the disease peaked. The MOG-CFA groups presented clinical signs varying from tail paralysis to hind limb paralysis and one with forelimb paralysis. Each individual mouse developed EAE at different rate and one failed to show any clinical signs at all, however the majority of mice developed EAE with evident clinical phenotype. The recovery period for each mouse also differed as some showed complete disease reversibility while others showed slow but steady improvements after the peak on-set.

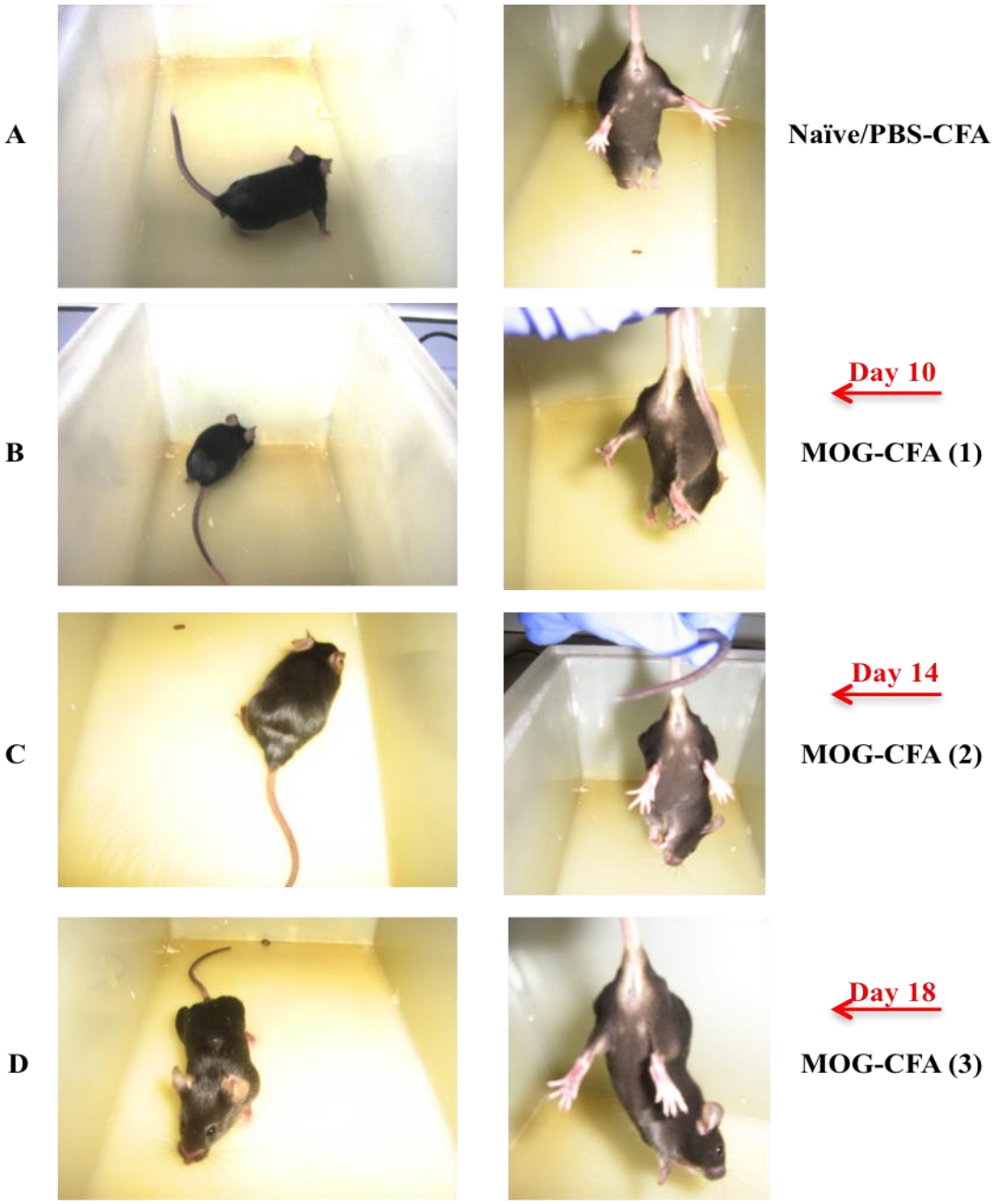

Figure 5. EAE clinical evaluation system

C57BL/6 female immunized mice were evaluated with the standard EAE score system over the 28 days. The naïve/PBS-CFA groups of mice showed no signs of EAE (A). The MOG immunized mice developed tail signs of paralysis with a score of 1 at day 10 (B). The symptoms increased in severity gradually resulting in hind limb weakness with a score of 2 at day 14 (C), followed by hind limb paralysis with a score of 3 at around day 18 (D).

The EAE clinical scores of both immunized mice were recorded and the daily average EAE scores are presented in Figure 6. The MOG-CFA group mice had severe EAE and the symptoms begin with tail paralysis at day 10, then developed to hind limb weakness and full paralysis at day 17. The PBS-CFA group did not show any clinical signs of EAE and continue to behave normally.

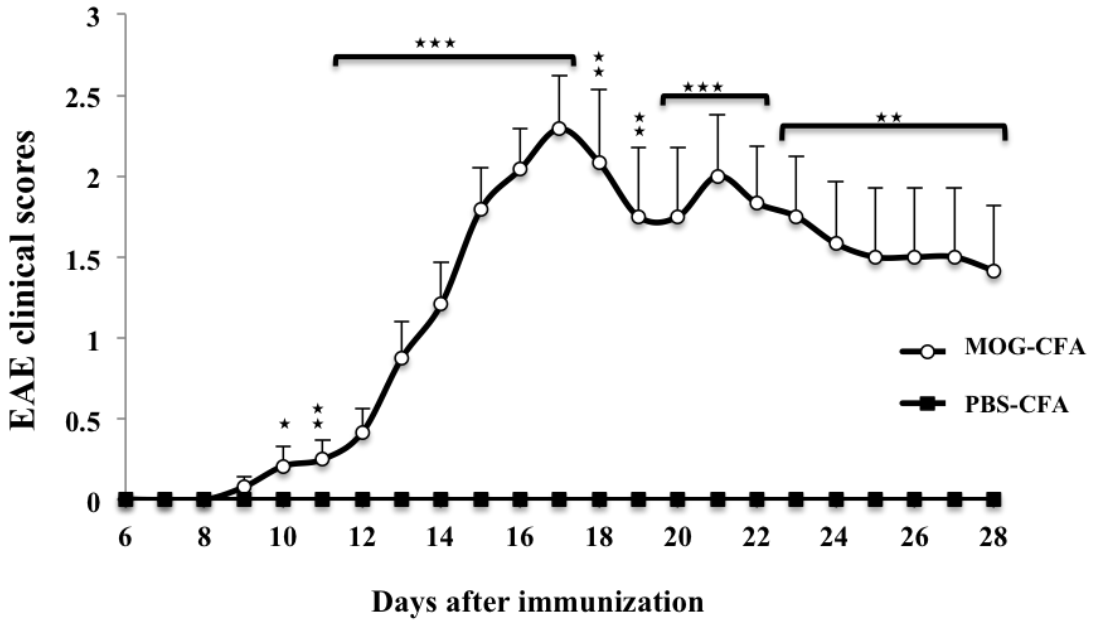

Figure 6. Clinical score of MOG_{35-55} and PBS immunized mice

C57/BL/6 mice were immunized s.c with 100 µg/50 µl of MOG_{35-55} peptide emulsified in CFA together with 100 ng/100 µl of PTX i.p. Control animals were immunized with PBS-CFA and PTX. The MOG-immunized mice showed EAE clinical symptoms starting at day 10 with tail paralysis and continuing in severity until reached peak at day 17 where severe disability ensued. Following on, the clinical symptoms began to improve from day 18 resulting in mice recovery. PBS-immunized mice showed no signs of EAE. The clinical scores of individual mice were monitored daily using the clinical scoring shown in Figure 5. Data show the mean ± SEM, animal numbers were the same as in Figure 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.2 Histology of SC tissues in naïve and EAE mice

To confirm the clinical observation, I carried out H&E staining to examine the histopathology of the SC tissues of MOG-CFA, PBS-CFA and compared with that of the naïve mice. The MOG-CFA tissues were from mice with no clinical symptoms for day 9, score 3 for day 17 and score 1.5 for day 28. The H&E staining presents visible look of the cell nucleus and their state of activity. The cell nucleus was stained in both the white and grey matter of naïve and PBS-CFA samples with no abnormal morphology (data not shown). However abnormal morphology with increased infiltrating cells was observed particularly in the white matter region of the SC starting at day 9 and became more obvious during peak of the disease sited. The MOG-CFA immunized EAE SC at day 28 the recovery phase of the disease showed reduced levels of inflammation (Figure 7). These changes in the SC morphology, suggests the change of the expression levels of many inflammatory cytokines, chemokines and cytolytic enzymes in periphery and CNS systems, which enabled the access of immune cells and molecules to the CNS through the disruption of BBB. To identify these cytokines responsible, we examined the expression of some specific immune cytokines particularly IL-22 in serum, spleen and in CNS using cell culture, ELISA, PCR and immunohistochemical staining methods.

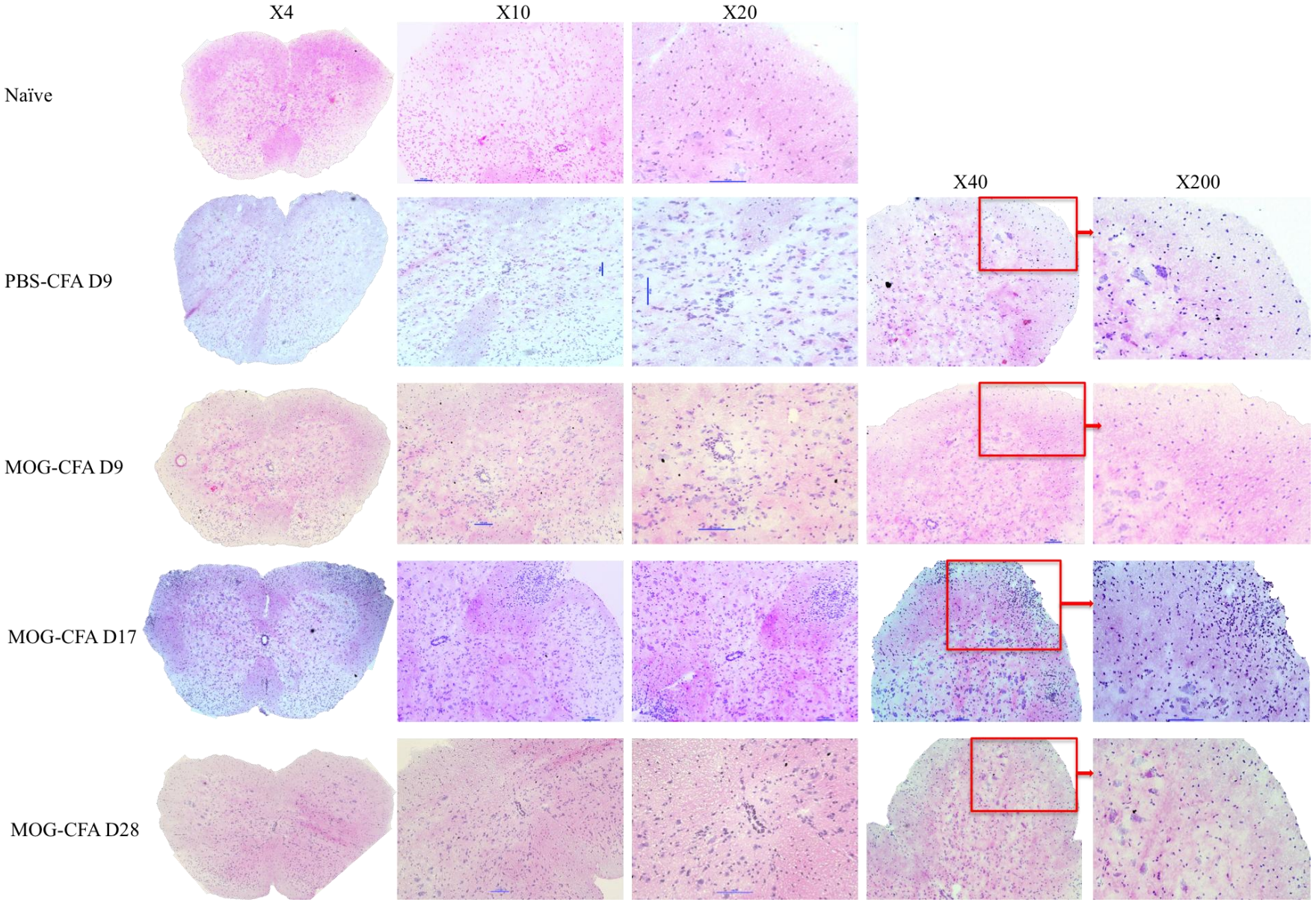
X4
X10
X20
X40
X200
Naïve
PBS-CFA D9
MOG-CFA D9
MOG-CFA D17
MOG-CFA D28

Figure 7. H&E staining of SC tissues of naïve, MOG-CFA and PBS-CFA immunized mice

The SC tissues were harvested at day 9, 17 and 28 during the disease course. Frozen sections were stained with H&E staining to evaluate the inflammation in the SC tissues. The MOG-immunized mice showed abnormal morphology with increased infiltrating cells in the white matter at day 9 where this site activity became more enhanced during peak phase at day 17. The MOG-immunized mice showed reduced levels of inflammation at day 28 during recovery phase of the EAE. The control groups of PBS-CFA and naïve mice both showed no abnormal morphology. Haematoxylin stains the nuclei blue/purple, and Eosin-Y stains other eosinophilic structures in various shades of red, pink and orange. The images are representative of mice SC tissues in each group.

3.3 Cytokines expression in serum and spleen cell supernatant

To understand how systemic immune responses correlate with the development of EAE, I also harvested the spleens from MOG-CFA and PBS-CFA mice at day 9, 17 and 28 post-immunization and pooled within each group. Spleen cells from naïve mice were also collected as controls. After lysing the RBCs, single cell suspension was then counted with haemocytometer and cultured in 24 well plates with MOG_{35-55} or medium. After 72 hours incubation, cell supernatants were collected for cytokine measurement using ELISA. In addition to spleen cells, inguinal LNs were also harvested and processed for measuring different cytokine expression. However, the cell numbers for LNs were very low, and I was unable to detect any cytokine secretion, therefore the LNs data are not presented. The cytokine levels of IL-22, IL-17A and IFN-γ produced by spleen cells collected from mice at day 9, 17 and 28 post immunization is presented in Figure 8. In both the naïve and PBS-CFA immunized mice groups, very low levels of cytokines were observed in the supernatant treated with medium alone or with MOG. Only in the supernatants collected from MOG-CFA group mice spleen cells, high levels of IL-17, IL-22 and IFN-γ were detected in the supernatant of the spleen cells from day 9 and 17 mice and stimulated with MOG_{35-55} peptide in the culture but not with medium alone. The data suggested an antigen specific production of these cytokines at the initiation and peak of the disease. At day 28 after immunization, the spleen cells were not able to produce any of the three cytokines with or without MOG in the culture. The similar production pattern of IL-22 with IFN-γ and IL-17A by the spleen immune cells may suggest that IL-22 is perhaps also involved in pathogenicity of EAE similar to IL-17A and IFN-γ, the key pathogenic molecules in MS/EAE development. We further measured the expression levels of IL-22, IL-17A and

IFN-γ systemically in the serum samples collected from PBS-CFA and MOG-CFA groups. We were unable to detect any of these cytokines.

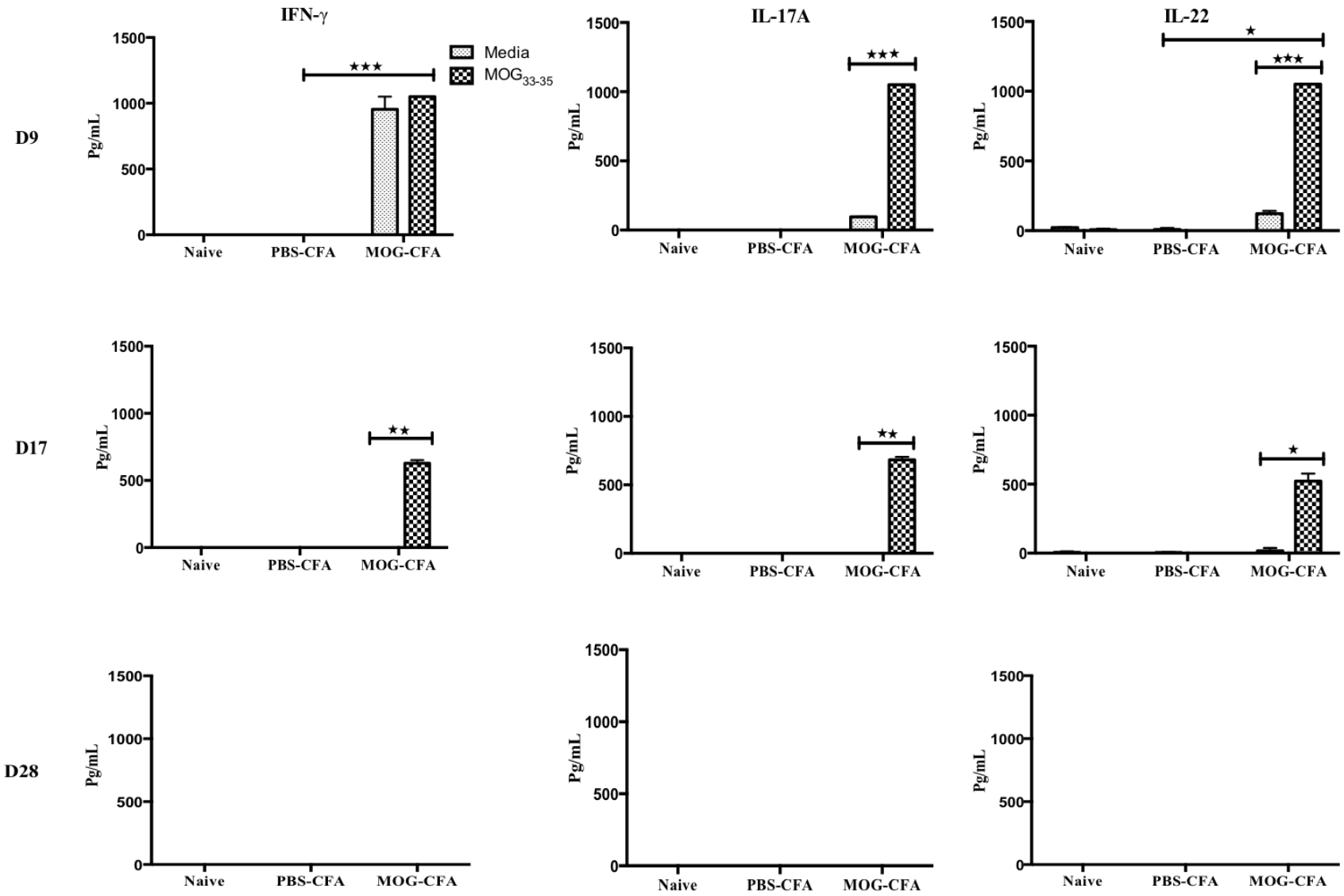
IFN-γ
IL-17A
IL-22
Media
MOG33-35
D9
D17
D28
Pg/mL
1500
1000
500
0
★★★
★★
★
Naive
PBS-CFA
MOG-CFA

Figure 8. Cytokine production of spleen cells in EAE and control mice

The spleens from naïve, or MOG-CFA and PBS-CFA immunized mice were collected at day 9, 17 and 28 post-immunization and then pooled within each group. The cells were then counted and stimulated with MOG_{35-55} (Black pattern) or with medium (Grey filled) for 72 hours. The supernatants were collected to examine cytokine production of IL-22, IL-17A and IFN-γ. MOG-immunized samples of spleen cells showed high levels of IL-17A, IL-22 and IFN-γ at day 9 and 17, stimulated with MOG_{35-55} but not with medium. At day 28 the spleen cells were unable to produce any specific cytokines with or without MOG in the culture. Very low levels of cytokines were observed in PBS-CFA and naïve control groups treated with MOG or medium. Data show the mean ± SEM of duplicate culture samples of same animal, representative of 3 separate repeats of ELISA experiments. Each group's MOG and media were compared with each other and with the MOG and media of the two other groups. (E.g. naïve MOG compared with naïve media, PBS-CFA MOG and MOG-CFA MOG) and (naïve media compared with PBS-CFA media and MOG-CFA media). This was carried out for PBS-CFA and MOG-CFA groups also. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.4 mRNA expression of IL-22, IL-22R and IL-17A in SC of naïve and EAE mice

During the induction and peak stages of EAE, spleen cells produced high levels of antigen specific IL-22 and IL-17. To investigate whether IL-22 is also produced by the CNS cells during EAE development, I examined the expression of IL-22, IL-22R and IL-17A in CNS using real time PCR as described before, SCs were harvested from naïve, or PBS-CFA and MOG-CFA immunized groups at day 9, 17 and 28 post-immunization. Using the Trizol method, RNA was extracted and then quantified via Nano-drop before cDNA was synthesized using RT-PCR. We then examined the mRNA expression using real-time PCR with Fast SYBR® Green according to the manufacturer's instructions. The PCR results were analyzed using $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ method and data are shown in Figure 9. The S.E.M were calculated using the final $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ values, however for statistics it is more accurate to use average ${}^{\Delta C}{}_{\mathrm{T}}$ values, therefore the average ${}^{\Delta C}{}_{\mathrm{T}}$ values were used to calculate T-test between each groups. Similar to spleen cell data, very low levels of IL-22 mRNA was detected in naïve and PBS-CFA CNS samples throughout the course of EAE (Figure 9). Interestingly, the PCR results showed that mRNA expression of IL-22 in CNS was also very low during disease onset and peak of EAE with small increase in expression during the disease recovery in MOG-CFA samples. In contrast to IL-22 expression, IL-22R was up regulated in MOG-CFA CNS samples throughout the disease course, with lower levels of expression during day 9 and 17 followed by marked increase at day 28 (Figure 9 (ii)). The data may support the previous findings of IL-22R presence in BBB endothelial cells with a potential pathology for IL-22 cytokine in MS/EAE. Interestingly IL-17A also showed very little expression at day 9 and 17 followed by an elevated

increase in MOG-CFA during the recovery phase at day 28 (Figure 9 (iii)). In contrast to previous findings where IL-17A was shown present in BBB during pathology, my data showed an up regulated expression of IL-17A mRNA during the recovery phase of the disease. It would be important to verify our findings with further PCR experimentations, but I was unable to repeat these experiments due the time limitation of my study.

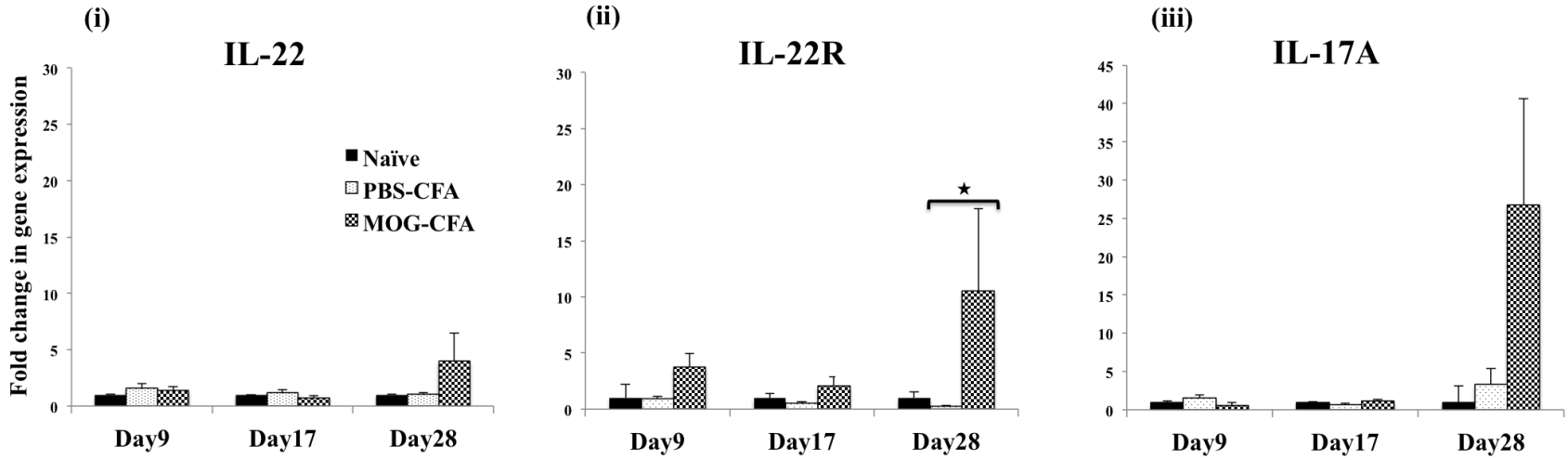

Figure.9 mRNA level of IL-22, IL-22R and IL-17A in CNS of EAE mice

The cDNA was synthesized from naïve, PBS-CFA and MOG-CFA CNS samples at day 9, 17 and 28 during EAE. n=4 in naïve group, n=8 in PBS-CFA group and n=8 in MOG-CFA group. Duplicate samples from each CNS samples were tested for each sample from each time point. The observed mRNA expression levels of IL-22 appeared to be very low at day 9 and 17 with small increase at day 28. In contrast IL-22R and IL-17A levels were only up regulated at day 28 with low levels observed at day 9 and 17 during EAE. Error bars are representative of duplicate samples representing as S.E.M using $2^{-\Delta\Delta C}{}_{T}$ values. The unpaired T-tests were run between each group using ${}^{\Delta C}{}_{T}$ values. Each three groups (naïve, PBS-CFA and MOG-CFA) were compared with each other at each day and compared with other two time points. (E.g. naïve group was compared with PBS-CFA and MOG-CFA of day 9 and with naïve samples of day 17 and 28). The data are representative of two experiments. * $p < 0.05$.

3.5 IL-22 expression in naïve and EAE SC tissues

Although there is very little expression of IL-22 mRNA in the SC tissues, our preliminary data suggested the protein expression of IL-22 in SC tissues, I next investigated the specific protein expression of IL-22 in SC during EAE development. Tissues were harvested from MOG-CFA and PBS-CFA immunized mice during three different phases of EAE at day 9, 17 and 28 post-immunization. Naïve tissues were also used as controls to determine whether IL-22 expression was observed under normal conditions. The embedded SC tissues in O.C.T were sectioned and stained according to the IHC staining protocol described in Materials & Methods. Looking at the control groups of naïve and PBS-CFA mice, brown staining of IL-22 was observed in both white and grey matter of SC (Figure 10) tissues of both groups. This demonstrated the IL-22 is expressed in SC under normal conditions and the similar level of IL-22 expression was observed in SC of PBS immunized mice. In comparison to the control groups, the MOG-CFA SC tissues had small increase of IL-22 expression at day 9. This increased expression was apparent in the white matter of SC tissues where inflammation is likely to occur at later stage during the disease (162, 163). At day 17, the expression of IL-22 in SC was dramatically increased, mostly in areas where inflammation was evident with foci of infiltrating leukocytes. The cluster appearance of IL-22 expression in the white matter elucidated a potential participation of IL-22 in pathology of EAE. Furthermore, the expression of IL-22 was significantly reduced during the recovery phase of the disease at day 28 when mice had reduced clinical symptoms. Thus despite the low levels of mRNA levels detected, the protein expression of IL-22 in SC correlates with EAE severity, suggesting that IL-22 may be important in the development of MS and EAE.

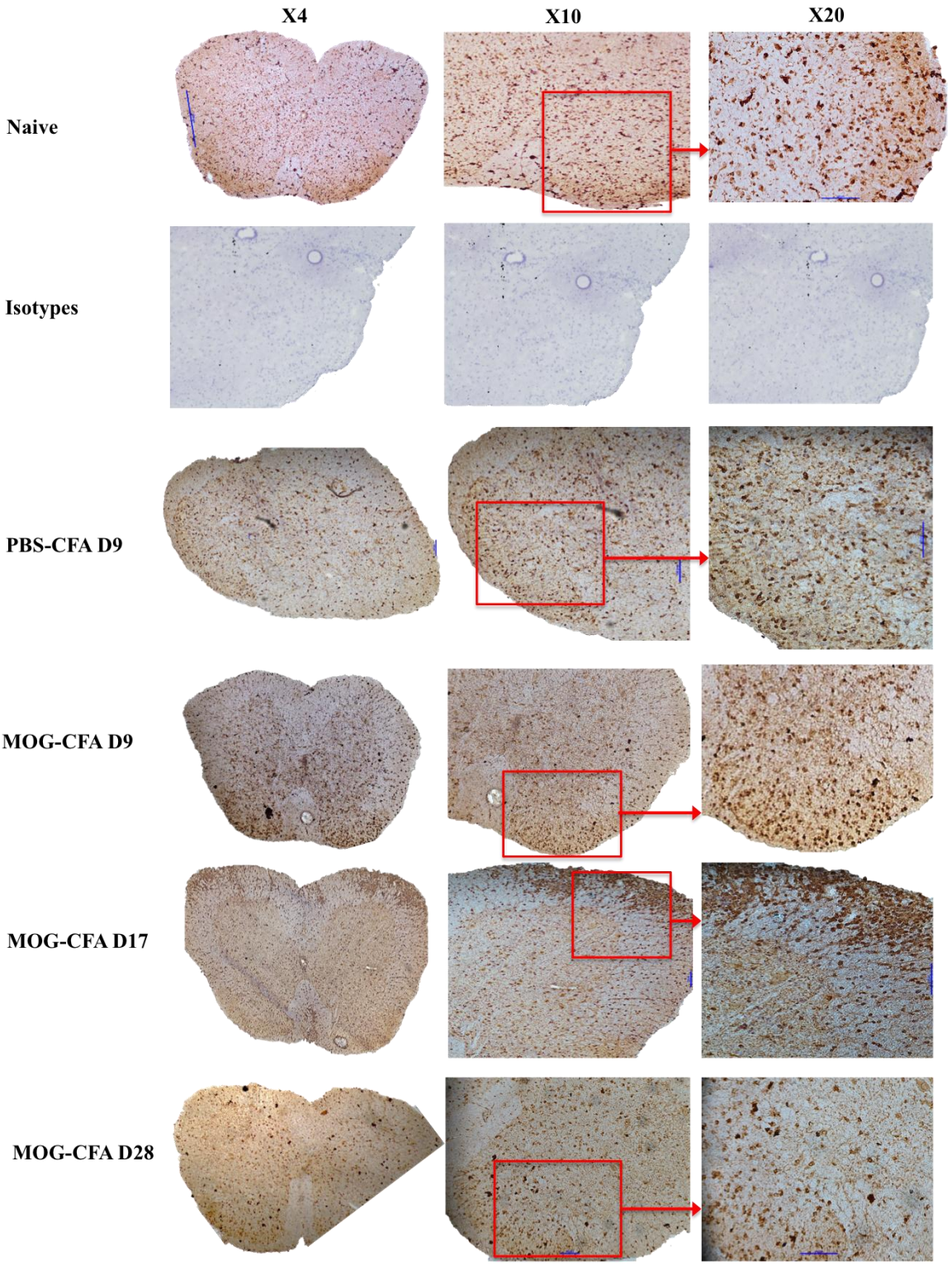
X4
X10
X20
Naive
Isotypes
PBS-CFA D9
MOG-CFA D9
MOG-CFA D17
MOG-CFA D28

Figure.10 IL-22 expression in SC tissues of EAE and control mice

The SC tissues of MOG-CFA and PBS-CFA immunized mice were harvested at day 9, 17 and 28 post-immunization. Naïve mice SC tissues were also harvested as controls. Tissues were sectioned and stained with IL-22 antibody (DAB, brown) while isotype antibody staining showed no staining in tissues. MOG-immunized tissues showed small increase of IL-22 expression in both white and grey matter at day 9 with increased expression of IL-22 in white matter at day 17 followed by marked decrease during recovery at day 28. PBS-CFA and naïve control groups showed IL-22 expression under normal conditions in both white and grey matter. The images are representative of tissues from the same group of mice.

3.6 Co-localization of IL-22 with CNS resident cells

We have showed in Figure 10 that CNS-resident cells in both white and grey matter can express IL-22. To understand the role of IL-22 during EAE development it is crucial to identify the CNS cells, which express IL-22. Double immunofluorescence staining (IF) was performed to investigate the expression of IL-22 (FITC-conjugated antibody, green) in CNS astrocytes (GFAP antibody), or neurofilament (SMI-31 antibody) or neuron cells (NeuN antibody), which were visualized with appropriate TRITC-conjugated secondary antibody (red).

First, we examined the co-localization of IL-22 with GFAP. Our data show that GFAP positive astrocytes were mainly in the white matter region with elongated "star" like morphology, and IL-22 is expressed by GFAP positive astrocytes in tissues of both PBS-CFA and MOG-CFA mice (Figure 11). In naïve isotype staining, isotype for IL-22 was used together with the second primary antibody GFAP, no green staining was observed suggesting IL-22 staining in following groups was IL-22 specific. The PBS-CFA tissue showed the expression of IL-22 in both white and grey matter with GFAP positive cells mainly in the white matter. This is consistent with our data of IHC of IL-22. In comparison to PBS-CFA control group, MOG-CFA tissue showed a generally increased expression of IL-22 in the SC during disease onset at day 9 and the expression was further enhanced in tissues of day 17 EAE mice. Again the data are in good agreement with our IHC staining results that the expression levels of IL-22 in MOG-CFA tissues were increased in the clusters of cells at the site of inflammation in the white matter during early and peak phases of EAE. However in some regions of the white matter of SC in EAE mice, there is a loss of IL-22 expression together with reduced expression of GFAP,

suggesting a more complicated role of IL-22 possible involving of both immune and CNS cells. The accumulation of nuclei (Dapi/blue), IL-22 (FITC/green) and astrocytes (TRITC/red) caused the pink colour change and due to high intensity of colour change some areas to appeared white. Expectedly the IL-22 expression of MOG-CFA tissue for day 28 the recovery phase of the disease showed much lower expression of IL-22 in white matter. Overall these data demonstrated the expression of IL-22 in CNS correlates with EAE severity and more importantly IL-22 is likely to be expressed by CNS astrocytes. The data suggest IL-22 may exert its important role in EAE development through astrocytes.

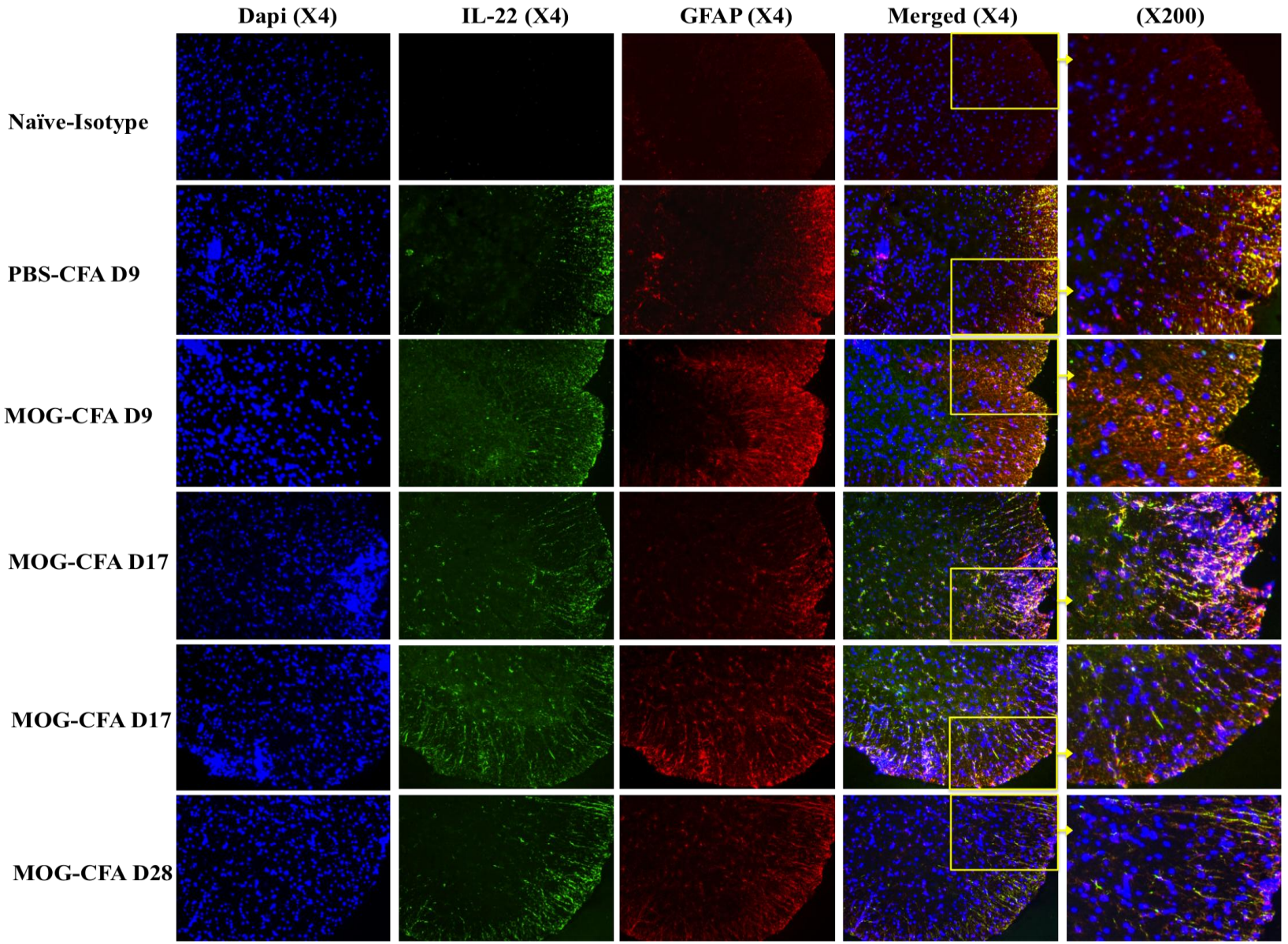
Dapi (X4)
IL-22 (X4)
GFAP (X4)
Merged (X4)
(X200)
Naïve-Isotype
PBS-CFA D9
MOG-CFA D9
MOG-CFA D17
MOG-CFA D17
MOG-CFA D28

Figure.11 Expression of IL-22 by CNS resident cells in SC of Naïve, PBS-CFA and MOG-CFA of immunized mice

The Naïve, PBS-CFA and MOG-CFA mice SC tissues were harvested at day 9, 17 and 28 post-immunization. Frozen sections were then stained with IL-22 (FITC/green), GFAP (TRITC/red) and DAPI (blue). IL-22 was co-localized with GFAP marker of astrocytes. The images are representative of tissues from the same group mice.

Next we examined whether IL-22 is expressed by axons using SMI-31 (TRITC/Red) antibody. In naïve isotype staining, again isotype for IL-22 was used together with the second primary antibody SMI-31, no green staining was observed suggesting IL-22 staining in other tissues was IL-22 specific. Our data show that SMI-31 positive axons red at the white matter and (Figure 12). The figures represented the nuclei staining of DAPI (blue), IL-22 (green) and SMI-31 (red) in single channels. . The PBS-CFA tissue showed the expression of IL-22 in both white and grey matter with SMI-31 markers staining axons in the white matter. This correlated with the previous histology images of IL-22 where the PBS-CFA tissues showed uniformed expression of IL-22 across the SC in both white and grey matter. However no co-localization had been observed between IL-22 and SMI-31 marker in merged channel, indicating that axons did not express IL-22 in the CNS. In comparison to control groups, MOG-CFA tissue showed increased expression of IL-22 in the SC during disease onset at day 9. This was apparent in the white matter where more significant fluorescent staining was observed. However no clinical signs were observed at this stage of EAE. The slight increase in the expression of IL-22 during disease onset agrees with our previous findings where IL-22 expression was marginally higher in the white matter of SC from day 9 EAE mice. This may suggest that IL-22 plays a role in induction phase of EAE. Surprisingly the expression levels of IL-22 were not significantly increased at this tissue, although higher than those observed in PBS-CFA, during the peak phase of the disease as anticipated. The DAPI image of MOG-CFA tissues of day 17 showed the presence of inflammatory cells in the white matter. Similar to our observation of the regional reduction of IL-22 in SC when stained with IL-22 and GFAP, the levels of IL-22 expression in day 17 EAE tissues were not dramatically

increased which reflects the different inflammation levels at different sites of SC, and it could also be due to the fact that the mouse sacrificed for this staining had already entered the recovery phase. Expectedly the IL-22 expression of MOG-CFA tissue for day 28 was reduced again in the SC, similar to our IHC staining data. Overall these data confirmed the high level of IL-22 expression in SC, and it was not expressed by SMI-31 positive axons.

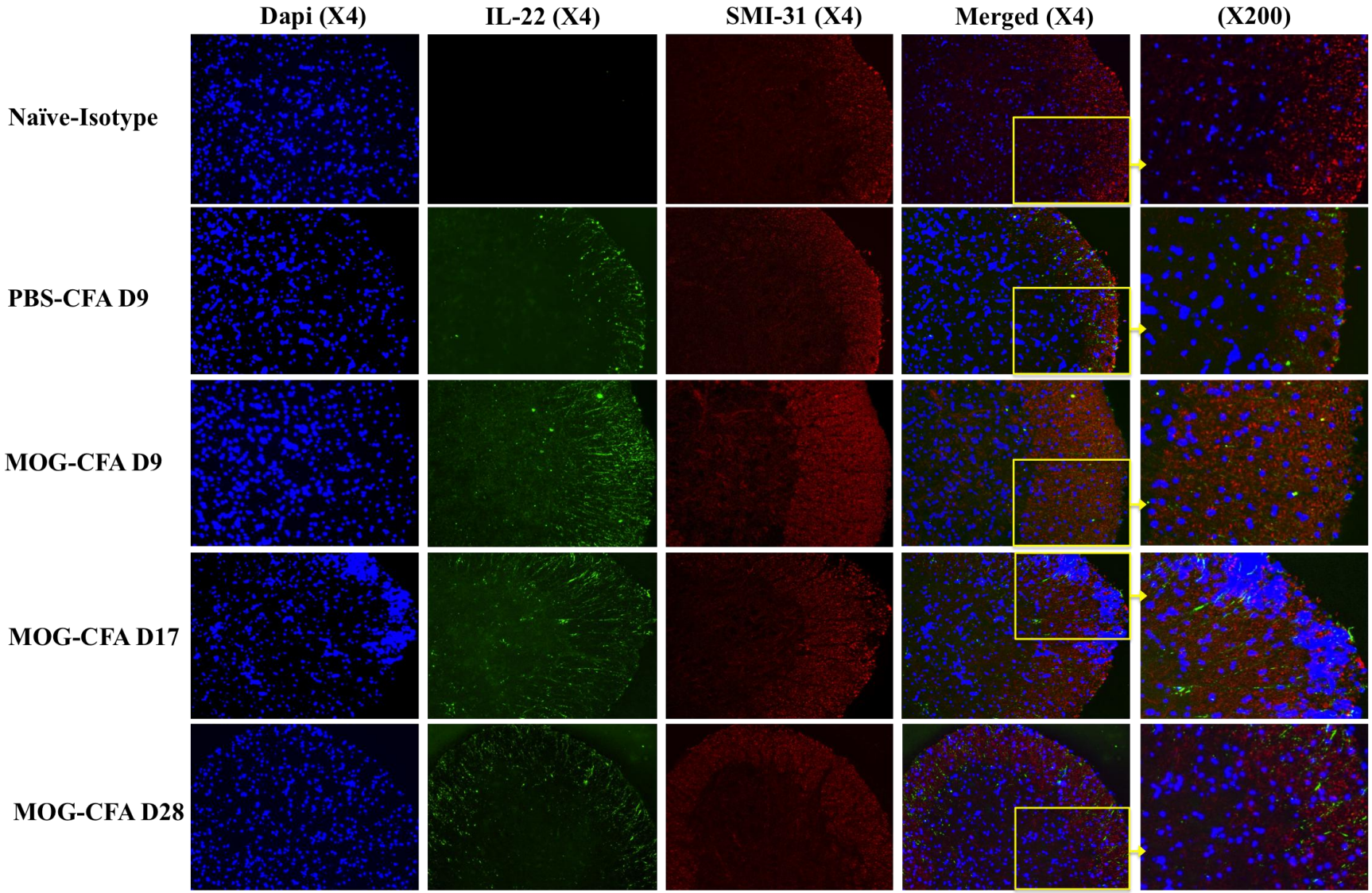
Dapi (X4)
IL-22 (X4)
SMI-31 (X4)
Merged (X4)
(X200)
Naïve-Isotype
PBS-CFA D9
MOG-CFA D9
MOG-CFA D17
MOG-CFA D28

Figure.12 Expression of IL-22 by CNS resident cells in SC of Naïve, PBS-CFA and MOG-CFA of immunized mice

The Naïve, and PBS-CFA and MOG-CFA SC tissues at day 9, 17 and 28 post-immunization were harvested. Frozen sections were then stained with IL-22 (FITC/green), SMI-31 (TRITC/red) and DAPI (blue). IL-22 did not co-localize with SMI-31 positive axons. The images are representative of tissues from the same group mice.

We further compared the localization of IL-22 in astrocytes and axons in the whole SC tissue by putting together the individual images in PowerPoint. The white matter is represented as the periphery area of SC, characterized by glial cells, astrocytes (GFAP/red) and myelinated axons (SMI-31/red) (Figure 13). The grey matter is presented in the middle, butterfly section, composing of neurons mainly. The SMI-31 marker of axons stained the white matter of MOG-CFA and showed no co-localization with IL-22. On the contrary the co-localization of astrocytes gave a colour change of yellow/orange in the white matter (B).

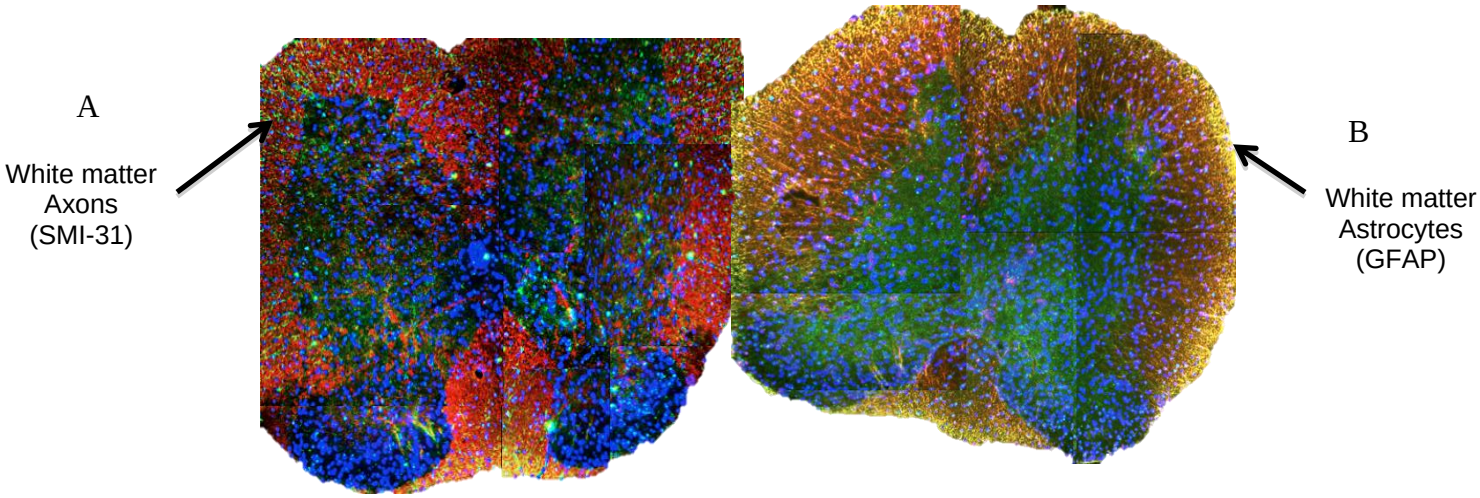

Figure.13 Expression of IL-22 by CNS resident cells in SC

The MOG-CFA SC tissues of day 9 were harvested. Frozen sections were then stained with IL-22 (FITC/green) together with SMI-31 (TRITC/red) (A) or GFAP (TRITC/red) (B) and mounted with Mounting medium containing DAPI (blue). IL-22 co-localization with GFAP marker of astrocytes appears in yellow/orange colour.

I further examined whether neuron cells express IL-22 by performing double staining of IL-22 (FITC/green) with NeuN (TRITC/red), which stains neuron-nuclei cells. The naïve staining showed expression of neuron cells bodies to be mainly concentrated in the grey matter. The double staining showed that IL-22 expression in the grey matter did not co-localize with the NeuN positive neuron cells, suggesting neuron cells did not express IL-22 (Figure 14). We also performed experiments comparing the expression of IL-22 in neuron cells in SC tissues collected from PBS-CFA and MOG-CFA group mice, however limited data were obtained due to contaminated antibodies and with time limitation in the laboratory, I was unable to repeat the experiment again.

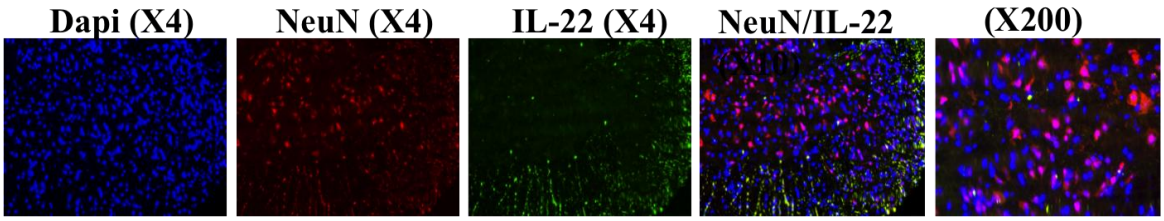

Figure.14 Expression of IL-22 by CNS resident cells in naive mouse SC

The Naïve tissue was harvested and the frozen sections were stained with IL-22 (FITC/green), NeuN (TRITC/red) marking the expression of neurons and DAPI (blue) staining cell nuclei. IL-22 is not co-localized with NeuN marker of neurons but stains the nuclei in the grey matter and therefore appears in pink.

Chapter 4: Discussion

MS is an autoimmune CNS disorder in both men and women (169). The cause of MS is still unknown however research evidence from MS patients and animal models suggest multifactorial etiology including genetic predisposition and environmental factors. Evidences suggest that MS can be developed in genetically susceptible individuals with exposure to critical environmental conditions, resulting in myelin damage and accumulation of pathological lesions in CNS. Despite the familial study lacking any abnormal genes associated in patients, the risk of MS developing in close relatives- sister- of MS female patient is shown to be increased by 25-fold (170). Environmental factors such as vitamin-D deficiency can also contribute to development of MS as well as increasing the risk of relapses, advanced disability, lesion injury and brain atrophy (171, 172). The current understanding of the immunopathogenesis of MS/EAE states that myelin-specific T-cells are activated in the periphery, cross BBB to transmigrate into the CNS and then are reactivated by local and infiltrating APCs that present MHC class-II associated peptides, resulting in demyelination and axonal damage (173, 174). However the secreted pro-inflammatory cytokines by these mediated T-cells along the course of EAE and their contribution to development and pathology of EAE is still poorly understood. An overwhelming attention has been focused on understanding the mediating T-cells and their pro-inflammatory cytokines that initiate and perpetuate CNS inflammation along EAE phases, making my research significant in discovering the roles of these potentially pathogenic cytokines. Th1 cells were originally thought to be the main pathogenic T-cells in EAE/MS, however this hypothesis was soon challenged with discovery of Th17 cells. The main Th1 produced pro-inflammatory cytokine is IFN-γ with the ability to exacerbate EAE/MS upon treatment (175). Th1 cells activate macrophages that secrete IL-23, a critical cytokine for ex-

pansion of Th17 cells and synthesis of inflammatory cytokine IL-17A (176). IL-17 was initially identified as the key pathogenic population in autoimmune disorders, however recent evidences indicate that the cytokine may play a less important role towards development and pathogenicity of EAE as originally suggested. In contrast, IL-22 may be an important contributor to the main pathogenic function of Th17 cells (163). IL-22 secreting Th17 cells have received an overwhelming attention as they have been postulated to mark the pathogenic population of self-reactive T-cells in EAE (17, 177). Thus understanding the specific expression pattern of this cytokine at different EAE phases will be helpful in determining its role in CNS inflammation.

My study aimed to determine the role of IL-22 in MS/EAE development by examining its expression in the periphery and CNS tissues of EAE mice at early, peak and recovery stages, and whether the expression levels correlate with disease severity. C57BL/6 mice immunized with MOG_{35-55} peptide were used as the inflammatory demyelinating model of MS with the histopathological observations and immune mechanisms resembling those found in MS patients and is well-established in our laboratory. Female C57BL/6 mice were s.c. immunized on day 0 with MOG_{35-55} emulsified in CFA together with i.p. injection of PTX on day 0 and 2. MOG_{35-55} is the immuno-dominant epitope for $CD4^+$ T-cells that induces progressive form of EAE by initiating expansion and differentiation of MOG specific autoimmune T-cells. C57BL/6 mice immunized with MOG peptide were used as the inflammatory demyelinating model of MS with the histopathological observations and immune mechanisms resembling those found in MS patients. In comparison to other EAE immunization techniques, MOG_{35-55} is proven the most effective peptide for studying the pathogenic role of T-cell responses and conducting neurological

impairment than those induced by MBP or PLP (178). Also the PTX injection works as immunization booster and enhances EAE development by providing additional adjuvant and facilitating entrance of autoimmune T-cells into CNS (179). Our data show MOG immunized mice developed EAE around day 9 then reached peak at day 17, while control mice received PBS+CFA+PTX did not show any clinical signs of disease. Tip tail paralysis was the first apparent signs among the MOG immunized mice followed by hind limb weakness and hind paralysis with some involving the forelimbs towards the peak phase of the disease. It is possible for some mice to develop disease with maximum severity until the end of study whilst others would stay at the peak severity for one day before recovering. The recovery period for each mouse is different as some showed complete disease reversibility whilst others failed to show any improvements after the peak. The clinical score data is consistent with many other research studies where MOG induced mice have developed similar pattern of clinical pathology starting with the disease onset at around day 8-9 with the peak disease appearing 3-4 days after onset following recovery (53, 178, 180-183). We also monitored mice body weight on a daily basis. The body weight change of MOG-CFA immunized mice showed no profound changes until disease onset at day 9 with a sharp weight loss until day 17 where disease peaked and animals started to gain weight again. Overall the immunized mice consistently lost about 5-10% of their body weight with the loss reaching around 20% in severe cases during the progression of EAE possibly due to severe inflammation caused paralysis and inability to intake food (soft wet food were supplied to mice after disease onset). The data in Figures 4 and 5 suggest that the percentage of body weight loss negatively correlates with EAE severity. As this EAE in C57BL/6 is a mono-phase self-remitting disease

model, once the peak is reached the mice slowly regained weight although the paralysis was not completely recovered. The PBS-CFA control group continued to gain weight with no clinical symptoms throughout the study.

To confirm the clinical observation, I first carried out H&E staining to examine the histopathology of the SC tissues of both MOG-CFA and PBS-CFA immunized mice and compared with naïve SC tissues with EAE mice showing the obvious clinical signs as described the control mice did not show any clinical signs. The histology results of immunized MOG-CFA mouse for day 9 showed no obvious inflammation in comparison to the control group. SC tissues from MOG-CFA immunized mice at day 17 however showed massive cellular infiltration mainly in the white matter of SC. During the recovery phase at day 28, MOG-CFA immunized showed much reduced inflammation and less inflammatory cells in SC. Our findings are similar to other reports that MOG induced EAE in C57BL/6 had optimal inflammation at peak of the disease (184). Following on to examine whether the inflammation in SC leads to myelin damage and/or loss, I then performed Luxol fast blue (LFB) staining to look at demyelination of the CNS in MOG-CFA immunized mice. LFB is a method requiring over-staining and differentiation of the fiber tracts (white matter) from cell bodies (grey matter) to reveal myelin. This technique marks the difference between white and grey matter where the myelin cells sited in white matter would appear in light blue whilst the grey matter is stained purple with the nucleus. Unfortunately the demyelination staining was unsuccessful after many attempts because the samples were not fixed correctly.

To understand the importance of inflammatory cytokines in CNS inflammation and whether the systemic immune responses correlate with the development and pathogenesis of EAE, I harvested the spleens from MOG-CFA and PBS-CFA immunized mice at day 9, 17 and 28 post-immunization and analysed the production of IL-17, IL-22 and IFN-γ by these splenocytes. Single suspended spleen cells were stimulated with Media or MOG_{35-55} for 72 hours before supernatants were harvested for ELISA test. The main goal of this approach was to compare the levels of IL-22, IL-17A and IFN-γ with the inflammation observed during the course of EAE disease. These cytokines chosen were relevant for MS and EAE, as shown by other researches (53, 185-190). My study shows that both naïve and PBS immunized mice spleen cells were unable to produce any detectable levels of IL-17, IL-22 and IFN-γ with or without the stimulation of MOG peptide in the culture. Only spleen cells collected from day 9 and day 17 EAE mice were able to produce high levels of all three cytokines upon MOG peptide stimulation in the culture, suggesting antigen specific responses of these cytokines. The expression of IL-22 from MOG-CFA samples stimulated with MOG treatment revealed similar pattern of expression as IL-17A and IFN-γ, with high levels of expression at day 9 and 17 followed by no expression at day 28. This similarity may suggest the possible involvement of IL-22 in pathogenicity of EAE. The high expression of IL-22 at day 9 may propose the significance of this cytokine for initiating and driving the inflammatory events during EAE onset. Interestingly the expression levels of IFN-γ in MOG-CFA stimulated media during disease onset also appeared to be considerably high.

The antigen specific production of all three cytokines at day 9 and 17 suggests that these cytokines play important roles in EAE development particularly at the initial and peak

stages of the disease, but unlikely at the recovery stage of the disease. The expression levels of IL-22, IL-17A and IFN-γ were also measured systemically using the serum samples taken from PBS-CFA and MOG-CFA immunized mice and the levels of expression for these cytokines were undetectable. This was perhaps due to insufficient amount of serum and result of dilution. The exact cell populations that are responsible for producing these cytokines are not known in our study. However, generally the high levels of $CD4^+$ lymphocytes are observed during induction and peak of the disease in different EAE models (191-193), as many studies have shown improvements of EAE symptomatology after receiving treatments that decreases the lymphatic infiltrations (194). Similarly activated macrophages/microglia were reported to remain elevated during the recovery and post-recovery of EAE, potentially pointing towards cross talk between the activated macrophages/microglia and lymphocyte population along the course of the disease (195-197). Also it is important to consider that $CD4^+$ T-cells encompass wide range of sub-populations including both pathogenic and protective functions (198-200). The suppressive role of $CD4^+$ T-cells functions as $CD4^+CD25^+$ regulatory cells (T_{regs}), which could explain the high levels of $CD4^+$ T-cells throughout the disease course.

The exact roles of IFN-γ and IL-17 in the pathogenesis of many autoimmune diseases including MS are controversial and not fully understood. Th expression of IFN-γ was reported high at the EAE onset, reaching the maximum at peak and decreasing during recovery of disease (162) but IFN-γ deficient mice had exacerbated EAE. Although IL-17 was reported to have an important role in MS/EAE development surprisingly Kreymborg *et al.* observed that low levels of IL-17A during the peak of the disease (163). The authors suggested IL-17A could only moderately impact EAE alone. Another study using IL-17A deficient (IL-17A$^{-/-}$) mice further confirmed that IL-17A is not essential in EAE development as the deficient mice only showed moderate resistance to EAE development (53) emphasizing on either synergistic function of these cytokines or perhaps the only minor role it plays during EAE development. Interestingly the IL-17A$^{-/-}$ mice lymphocytes stimulated with MOG peptides showed marked increase in expression levels of IFN-γ, despite the lack of evidence suggesting any direct effect of IL-17 on production of IFN-γ by CD4^{+} T-cells. These findings perhaps suggest the ability of IL-17 to negatively regulate the development of IFN-γ. However, despite the current controversy in the roles of IFN-γ and IL-17 in MS/EAE, The current view is that both cytokines are important effector cells in the development of the CNS inflammation, possibly with different mediating mechanisms and they work in synergy in MS/EAE pathogenesis. The significant increase of antigen specific production of both IFN-γ and IL-17 cytokines by the spleen cells of EAE mice in my study also confirmed the importance of both cytokines in EAE disease.

Interestingly, similar to the IFN-γ and IL-17 production by the spleen cells in early and peak stage of EAE mice, antigen specific production of IL-22 was also observed. Our data may suggest the importance of IL-22 in MS/EAE development. A report by Kreymborg *et al* investigating the expression of IFN-γ and IL-22 in both isolated inguinal LN and spleen cells at the peak disease (day 21), thus confirmed our findings. They further discovered that the induction of IL-22 in pathogenic $CD4^+$ T-cells to be time and dose dependent on IL-23 cytokine (163). IL-22 was reported the most prominent gene of Th cells after treatment with IL-23 and that IL-23 was essential for self-reactive Th cells to produce IL-22 by MOG stimulation (54). To further investigate the sole impact of IL-23 in EAE, IL-$23^{-/-}$ mice were immunized for EAE induction and surprisingly the results showed that these mice were completely resistant to EAE development (90, 201) suggesting a pivotal role of IL-23 in MS/EAE disease. Therefore identifying the cytokine signature expression of IL-23 derived genes, such as IL-22 becomes paramount in understanding MS/EAE pathogenicity. Further to confirm the sole pathogenic role of IL-22 in development of EAE, Kebir *et al* immunized IL-$22^{-/-}$ mice with MOG peptide and unexpectedly these mice developed severe EAE similar to those found in wild type (202). This finding rejects the conclusion that IL-22 is a sole key pathogenic player of EAE development in CNS inflammation, however the contribution of this cytokine to EAE pathology cannot be dismissed completely based on this study. Similarly, IL-23 is also a key cytokine for the proliferation of Th17 cells but IL-17 deficient mice were able to develop EAE. To better understand the role of IL-22 in MS/EAE development, the expression of IL-22 in CNS tissues during the course of the disease was investigated via IHC. The constitutive expression of IL-22 in CNS tissues of both naïve and PBS-CFA control groups suggest a

likely role of IL-22 in the CNS apartment. However the increased expression of IL-22 in the SC of EAE mice, particularly at the white matter area where inflammation resided at both the induction and peak phase of EAE, could indicate the potential pathogenic impact of this cytokine in EAE, this is consistent to the findings reported by Almoda *et al.* where the systemic production of IL-22 in SC was increased during disease onset and peak followed by marked decrease during recovery in a rat EAE model (162). Although IL-22 is abundantly expressed by CNS tissues, it is still not clear which CNS cells can express IL-22. The answers to the question may help to understand the role of IL-22 in the development of CNS inflammation. I studied the co-localization of IL-22 with CNS resident cells such as astrocytes and neuron cells using appropriate markers for performing double Immunofluorescence staining (IF). The strong co-localization of IL-22 with GFAP marker in both MOG-CFA and PBS-CFA tissues confirmed the ability of astrocytes markers to express IL-22. Surprisingly we observed some sporadic reduction of IL-22 expression mainly suited in white matter of the MOG immunized SC. Although high expression levels of IL-22 are anticipated during the peak phase of EAE, the intermittent reduction in IL-22 during peak is not known. However it is possible that the MOG-immunized SC used in the double IF staining had come from a mouse undergoing early recovery just before sacrificed. Further investigation with more EAE tissues around the peak days of EAE may help to answer the question.

The works of Keyser *et al* have shown that astrocytes are becoming more important for their key roles in CNS function. They comprise various functions such as providing energy metabolism for neuron and oligodendrocytes, managing the extracellular water and electrolyte homeostasis, regulating neurotransmitter release, producing trophic factors

and modulating the immune response (203). It becomes evident that alteration in astrocyte functionality can be catastrophic to CNS homeostasis, making them crucial key player in pathogenesis of CNS disorders. An overwhelming attention has been focused on astrocytes role CNS disorders and many hypotheses have imposed the dysfunction of astrocytes to be derivative cause of MS. In agreement with these findings Kang *et al* has further investigated the impact of astrocytes in EAE pathogenicity and interestingly found the targeted ablation of Act1 in astrocytes, a component essential for IL-17 signaling, resulted in much reduced EAE severity and impaired IL-17-mediated inflammatory gene induction (204). Normal Th17 infiltration into CNS was reported, however Th17 cells in mice with Act1 deficient astrocyte were unable to recruit other lymphocytes and leukocytes to the site of inflammation in CNS. This strongly suggests IL-17 Act1 mediated signaling to plays a key role in effector stage of EAE via astrocytes. These findings clearly suggest astrocytes to be vital component of CNS. The co-localization results of IL-22 with astrocyte markers in SC and the similar pattern between IL-17 and IL-22 protein expression by spleen cells and CNS tissues in EAE mice may indicate that IL-22 could perhaps play a role during EAE development. IL-17 and IL-22 producing astrocytes could therefore become potential therapeutic target cells for MS disease. The double IF staining of IL-22 with SMI-31 marker showed no co-localization in any of the tissues tested, indicating that axons markers are unable to express IL-22 cells. I also examined whether CNS neuron cells express IL-22. The naïve tissues used for this staining clearly showed no co-localization of IL-22 with NeuN positive neurons. Unfortunately, the double staining of NeuN with MOG-CFA and PBS-CFA groups was

unsuccessful due to non-specific binding and also due to lack of time I was unable to repeat the experiment again.

Overall these data from IHC staining confirmed the expression of IL-22 in both white and grey matter of SC tissues and the expression is up regulated in some areas of SC tissues in EAE mice. Furthermore, IL-22 is shown to be likely expressed by CNS astrocytes but not the neuron cells or axons. Despite the dismissal of either IL-17A and IL-22 cytokines as sole pathogenic players of EAE, both cytokines were shown to infiltrate the CNS and disturb BBB integrity in MS patients and EAE models (14, 205). Also the expression levels of IL-17R and IL-22R were reported on endothelial cells and CNS-resident astrocytes (146). To further investigate the expression and correlation of IL-22 and IL-17A levels in CNS, along with IL-22R I carried out Real-Time (RT)-PCR using RNA samples collected from MOG-CFA, PBS-CFA and naïve tissues at day 9, 17 and 28. The naïve control showed very low mRNA expression of IL-22, IL-22R or IL-17A. Different to the cytokine production by spleen cells in the periphery and IL-22 protein expression in CNS for the reasons not known, the data here showed IL-22 and IL-17A mRNA levels in CNS were unchanged at day 9 and 17 while the expression was slightly increased on day 28. However the levels of IL-22R were up regulated in CNS of EAE mice on day 9, 17 and most obvious on day 28-post immunization when compared with naïve and PBS control mice. Kebir *et al* have detected both IL-22R and IL-17R on BBB-ECs primary culture and reported their strong expression in MS lesions. They further investigated the capacity of IL-17 and IL-22 in controlling the entrance of lymphocytes across the barrier, and showed that both IL-17 and IL-22 were able to disrupt the integrity of BBB in both *in vitro* and *in vivo* (202), thus responsible for promoting the transmigration of $CD4^+$ T-cells

across the BBB. They suggested that these cytokines might play a unique role in altering the BBB and promoting the recruitment of additional $CD4^+$ lymphocytes across the barrier. It is also well documented that IL-17A increases the production of reactive oxygen species (ROS) in brain endothelial cells (BECs), resulting in oxidative stress and mediating the activation of endothelial contractile machinery leading to loss and disorganization of tight junction proteins and eventually BBB breakdown, therefore allow the opening of BBB and entrance of lymphocytes to the CNS (187, 206-210). Surprisingly only high dose of IL-17A but neither IL-22 induced barrier disruption in bEnd.3 monolayer culture nor IL-22R detected by RT-PCR despite the expression of IL-17A receptor in the monolayer (187). Whether IL-17A alone or indeed a combined effect of both IL-17A and IL-22 are able to alter the permeability of BBB thus facilitating the immune cell infiltration in CNS is not fully understood. However the data from my study and from current publications suggest IL-22 is likely to play an important role in MS/EAE development. Further research to elucidate the underlying mechanisms of IL-22 function in MS/EAE is essential to gain a better understanding of its role in EAE pathogenicity. The concept of a single cytokine responsible for pathogenicity of EAE merely based on its presence is proven unviable. The previous interpretation that a cytokine could solely be liable for development of autoimmune disease had major impact on misinterpreting the function of Th1 and Th17 cells in EAE/MS (211-214). MS is caused by multiple factors, including cytokines that function in many ways to either mediate inflammation or exert immunosuppressive functions in MS (215-218). These inflammatory cytokines may provide the necessary tools for developing potential anti-inflammatory drugs targeting the pro-inflammatory cytokines responsible for the pathogenesis of MS. This demonstrates

the importance of studying the function and the mechanisms of these immune cytokines in the development of autoimmune MS disease. Thus despite several genes deficient studies suggesting that none of IL-22, IL-17A and IFN-γ are solely responsible for pathogenicity of EAE, many other reports strongly suggest that IL-22, IL-17A and IFN-γ are important players in MS/EAE development (205, 219, 220). My study examined the expression profile of IL-22, IL-17A and IFN-γ in spleen cells and in CNS during EAE development, similar to the expression pattern of IL-17A and IFN-γ, IL-22 protein production by splenocytes and its expression in CNS was elevated at early and peak phases of EAE disease compared with naïve and PBS control tissues. The data indicate IL-22 has an important function in the development of MS/EAE together with IL-17A and/or IFN-γ, possibly through peripheral immune cells or CNS astrocytes.

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