

**IL-10-differentiated dendritic cells treatment for  
Experimental Autoimmune Encephalomyelitis (EAE),  
a model of human Multiple Sclerosis.**

A Thesis Submitted to the College of  
Graduate Studies and Research  
In Partial Fulfillment of the Requirements  
For the Degree of Master of Science  
In the Department of Veterinary Microbiology  
University of Saskatchewan  
Saskatoon

By

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## **ABSTRACT**

Multiple sclerosis is a chronic autoimmune neurological disease characterized by inflammatory cell infiltration and demyelination in the central nervous system (CNS). It is considered to be mediated by Th1 and Th17 immune responses. Experimental autoimmune encephalomyelitis (EAE) is widely used as a mouse model to study MS as it has features and histopathology similar to that of MS. Tolerogenic dendritic cells (DC) are reported to efficiently prevent sensitization for EAE. In this research, we induced tolerogenic DC (DC10) by differentiating them with IL-10. Compared to immature DC, DC10 did not show increased expression of MHC II or the co-stimulatory molecules CD40, CD80 and CD86, and produced low levels of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and IL-12 but higher levels of IL-10. This is consistent with their possessing a tolerogenic phenotype. We found that three intraperitoneal (i.p.) injections of DC10 successfully inhibited the signs of established, ongoing EAE: DC10 significantly reduced the clinical scores, demyelination and cell infiltration in the spinal cord, as well as the production of IL-4, IL-6, IL-10, IL-17 and IFN- $\gamma$  by spleen and lymph node (LN) lymphocytes. DC10 treatments did not significantly affect inflammatory cytokine mRNA levels in the CNS. We found that there was higher FoxP3 expression in the CNS in response to DC10 treatments relative to PBS-treated

animals. We also found that DC10 treatments significantly enhanced IgG1, IgG2a and IgG2b production and total spleen and LN lymphocyte proliferation following challenge with myelin oligodendrocyte glycoprotein (MOG) antigen. As far as we know, this is the first report showing the successful therapeutic treatment with tolerogenic DC10 of established EAE in mice.

## ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my supervisor, Dr. John Gordon, for his guidance and support throughout the whole project. His suggestions and critical thinking on science not only successfully bring me to the completion of this project, but also strongly help me develop my professional skills and experience on scientific research. I also want to express my sincere thanks to my committee members, Dr. Volker Gerdts, Dr. Henry Tabel and Dr. Vikram Misra for their valuable suggestions and discussions in my projects.

I would like to thank my lab members, Ms. Jennifer Town, Mrs. Xiaobei Zhang and Dr. Hui Huang for their help and support throughout the whole project. They provide me excellent technical support and suggestions, which are essential for the success of my experiments and thesis. I also want to thank Dr. Wojcieck Dawicki, Dr. Xixing Zhao and his wife Dr. Aimei Yang, Ms. Nicole Paur, Mrs. Chunyan Li, Dr. Xiuling Li and Dr. Meiping Lu for their help and suggestions on my experiments and thesis.

In addition, I would like to thank Dr. Kelly M. McNagny and Dr. Jami Bennett from University of British Columbia for their help and suggestions in setting up our EAE model. I also want to express my gratitude to all the staff in animal care unit, as they provided excellent service and support in taking care of our mice.

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**Figure 6 General mechanisms by DC10 reduce EAE clinical signs and CNS**

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## LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
APC	antigen-presenting cell
ABTS	2-2'-azino di-[3-ethylbenzthiazoline sulphonic acid]
ANOVA	one-way analysis-of-variance
BBB	blood brain barrier
BM-DC	bone marrow-derived dendritic cell
CD	cluster of differentiation
CNS	central nervous system
CSF	cerebrospinal fluid
CTLA-4	cytotoxic T lymphocyte antigen-4
CPM	counts per minute
DC	dendritic cell
DTH	delayed-type hypersensitivity
DNA	deoxyribonucleic acid

EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3 transcription factor
GM-CSF	granulocyte/monocyte-colony stimulating factor
GA	Glatiramer acetate
HE	Hematoxylin and eosin (histology stain)
HLA	human leukocyte antigen
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule-1
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.v.	intravenous
LAG-3	lymphocyte activation gene-3
LFB	luxol fast blue

LN	lymph node
LPS	lipopolysaccharide
MBP	myelin basic protein
MFI	mean fluorescence intensity
MOG	myelin oligodendrocyte glycoprotein
MHC	major histocompatibility complex
MS	multiple sclerosis
NGF	nerve growth factor
mRNA	messenger RNA
ml	millilitre
ng	nanogram
OD	optical density
OVA	ovalbumin
PAF	platelet activating factor
PAR	proteinase activating receptor
PBMC	peripheral blood leukocyte
Pg	picogram
PLP	proteolipid protein
PP	primary-progressive

PR	progressive-relapsing
qRT-PCR	quantitative real-time polymerase chain reaction
RAG	recombination activating gene
RBC	red blood cell
RNA	ribonucleic acid
RR	relapsing-remitting
s.c.	subcutaneous
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SP	secondary-progressive
TCR	T cell receptor
Teff	T effector cell
TGF	transforming growth factor
Th	T helper cell
CTL	cytotoxic T cell
TNF	tumor necrosis factor
Treg	T regulatory cell(s)
$\mu$ g	microgram
$\mu$ l	microlitre

## CHAPTER 1

### Introduction

Multiple Sclerosis (MS) was first discovered in 1868. It is a chronic autoimmune neurological disease characterized by inflammatory cell infiltration and inflammation of the central nervous system (CNS) [1, 2]. MS is considered to be driven by Th1 and Th17 immune responses [2, 3]. Though the mechanisms mediating MS are still not clear today, it is suggested that both genetic and environmental factors affect MS development [2]. Currently, several drugs like Glatiramer acetate and IFN- $\beta$  are used to reduce the symptoms of MS in patients [4, 5]. Experimental autoimmune encephalomyelitis (EAE) is widely used as a model to study MS, as it demonstrates similar symptoms and histopathology with MS, such as demyelination and cell infiltration in the CNS, production of inflammatory cytokines like IL-17, IL-6 and IFN- $\gamma$ , and myelin-specific antibody [6]. Immunizing mice or rats with myelin-specific antigens or peptides can induce different types of EAE, like chronic EAE in C57BL/6 mice induced by myelin oligodendrocyte glycoprotein (MOG)<sub>(35-55)</sub>, relapsing-remitting EAE induced by proteolipid protein (PLP)<sub>(139-151)</sub> in SJL mice, and acute monophasic EAE induced by CNS homogenates in SJL mice [7]. But the

mechanisms of EAE induction and development, as well as the roles of Th1/Th2/Th17 responses in EAE are still not well understood [6].

Tolerance is defined as an immunological state wherein the immune system does not react against self-antigens and tissues, as well as exogenous antigens (e.g., allergen and gut bacteria) [8]. Scientists today are trying to induce tolerance in autoimmune diseases like MS/EAE, for example, by inducing the deletion and anergization of autoreactive T cells, or by induction of suppressive or regulatory cells like Tregs [8, 9]. As professional antigen presenting cells (APC) dendritic cells (DC) play important roles in mediating immune responses. Our lab has already reported that IL-10 can induce tolerogenic DC *in vitro* [10]. These DC10 express lower levels of APC co-stimulatory molecules and pro-inflammatory cytokines. Allergen-presenting DC10 treatment can reverse airway hyperresponsiveness and induce tolerance to allergic asthma in mice [10] by inducing CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) [11], which suppressed the Th2 response. Studies using peripheral blood Th2 cells from asthmatic donors, performed by Dr. Xiuling Li in our lab, also showed that DC10 suppress the activity of effector T cells *in vitro* [12]. Though several reports showed that tolerogenic DC could prevent induction of EAE when administrated before sensitization [13-15], we wished to determine whether DC10 could be used as an therapy for established EAE. In this study, we generated DC10 *in vitro*, pulsed them with specific antigen (MOG<sub>(35-55)</sub>), and injected them into EAE mice intraperitoneally (i.p.) after the mice had



developed advanced clinical symptoms. We found that EAE mice that had been treated with DC10 showed decreases in clinical scores as well as demyelination and inflammatory cell infiltration in the CNS, and decreased expression of inflammatory cytokines by spleen and LN cells. However, this reduction in EAE scores was accompanied by an increased production of MOG<sub>(35-55)</sub>-specific IgG1, IgG2a and IgG2b, as well as enhanced proliferation of total spleen and LN cells, suggesting that these could be associated with the reductions in EAE scores. We did not observe significant differences in the cytokine mRNA expression in CNS between DC10-treated and PBS-treated EAE mice, but did find a higher expression of FoxP3, which suggested a potential induction of Tregs in response to DC10 treatment.

To our knowledge, it is the first study showing that IL-10-differentiated dendritic cells reduce established, chronic EAE in C57BL/6 mice. Our studies provide a novel insight into the therapeutical treatment with MS, in which patients could be treated with their own DC, which have been rendered tolerogenic *ex vivo*.

## CHAPTER 2

### Literature review

#### 2.1. Autoimmune disease

Autoimmunity is defined as a disease in which the immune system responds against self-antigens and thereby induces pathologic manifestations. Autoantigens are characterized as self-antigens, usually proteins, carbohydrates, lipids or DNA. About 5% of the world's population is affected by autoimmune diseases, the incidence of which is higher today than over the past 50 years, especially in developed countries such as the United States and Canada [2, 8].

Autoimmune diseases have several immunological features [8]: 1) different kinds of autoantibodies can be detected in the serum of the patients with autoimmune disease; 2) antibodies can bind to their autoantigens, leading to the deposition of immune complexes in the affected tissues; 3) cellular infiltration, generally lymphocytes and monocytes, are widely detected in the affected organ.

The aetiology and development of autoimmune disease are still not clear, but previous studies indicated that both genetic and environmental factors are associated with their progression. The concordance rate in identical twins for development of autoimmune disease is about 25%, while

the concordance rate in non-identical twins is only about 5% [2, 8]. Major histocompatibility complex (MHC) class II genes contribute by about 50% to the total genetic risk of developing autoimmunity, because most autoimmune diseases are CD4<sup>+</sup> T cell-dependent, and APC (e.g., DC) present autoantigens that are associated with MHC II molecules to CD4<sup>+</sup> T cells, resulting in autoimmune responses [8, 16]. Other non-MHC genes, for example, the sex-related genes, also affect the susceptibility of autoimmune disease, as many autoimmune diseases such as MS and systemic lupus erythematosus (SLE) are more frequent in females. This could be in part due to the influence of sex hormones [8].

People with specific combinations of susceptibility genes are considered to be at high risk of getting autoimmune disease, and environment factors may well play important roles in triggering disease. Two main mechanisms, known as molecular mimicry and tissue damage, have been proposed. In molecular mimicry, if a pathogen has an epitope that is similar to a self-peptide, T cells specific for the foreign epitope can cross-react with the self-peptide and thus be activated as autoreactive T cells [8]. Tissue damage caused by the invasion of pathogens and the ongoing inflammatory response can lead to the release of self-antigens which can be presented by DC to T cells, resulting in the activation of autoreactive T cells [8].

Recent studies have found that viral infections can break peripheral self-tolerance and trigger autoimmune disease. The generation of

self-epitopes is up-regulated during infection-induced inflammation, leading to the subsequent release and the processing of self-antigens. Then viral proteins which were expressed during chronic infections can cross-react with self-antigens by activating DCs and priming the new autoreactive T cells [8, 17]. Infectious agents, drugs, food, toxins and pollutants are other types of environmental factors that may trigger autoimmune diseases, as some may similarly cross-react with host self-antigens [8].

## **2.2. Multiple Sclerosis (MS)**

### **2.2.1. Introduction to MS**

Multiple Sclerosis (MS), first discovered in 1868 by Charcot, is a chronic autoimmune neurological disease characterized by inflammatory cell infiltration and inflammation of the central nervous system [1, 2]. MS can be divided into several phenotypes: 1) relapsing-remitting (RR) –MS, which affects about 85%-90% of patients, and is especially prevalent in women; 2) primary-progressive (PP)-MS, 10%-15% frequency; 3) secondary-progressive (SP)-MS, a sequel to RR-MS; 4) progressive-relapsing (PR)-MS, which is relatively rare; 5) benign MS, with long periods of remission and little disability after 15 years (20%-25% of patients diagnosed with RR-MS also have benign MS); and 6) malignant MS, a rare form with rapidly progressive disability within the first 5 years of diagnosis [5].

Loss of balance, impaired speech, extreme fatigue, double vision and paralysis can be seen in MS patient [5]. Histopathologically, the brain and the spinal cord lesions are present as plaques within the white matter in which myelin and oligodendrocytes are absent. In active disease these plaques show infiltration by lymphocytes, plasma cells and macrophages, which cause destruction of the myelin surrounding the nerve cell axons. There are also tissue oedema, apoptotic oligodendrocytes and infiltrating cells within the plaques at the early stages. Lymphocytes and macrophages are also seen around the venules in the area [18].

MS is considered to be a T cell-mediated autoimmune disease [19, 20], but the pathogenic mechanisms of MS are still not clear. Previous studies indicated that MS was associated with the up-regulation of proinflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and IL-12 [21]. Myelin basic protein (MBP) is considered to be one of the major autoantigens involved in the immunopathogenesis of multiple sclerosis, and MBP-specific T cells were found at high frequency in the cerebrospinal fluid and blood of MS patients [22].

Molecular mimicry and bystander activation have been proposed to explain how microbial infections may induce MS, though again the mechanisms are not clear [2]. If a pathogen has an epitope that crossreacts with a myelin self-antigen, any of the T cells that are specific for the self-antigen will be activated. These T cells can migrate across the blood-brain barrier and cause tissue damage if they recognize the

autoantigens expressed in the brain and/or spinal cord, leading to autoimmune demyelinating disease [2]. On the other hand, autoreactive cells could be activated through nonspecific inflammatory events during the process of infection. For example, the destruction of self-tissue caused by pathogens could release autoantigens that could be presented by APCs to T cells, resulting in the activation of autoreactive T cells [2]. Proinflammatory cytokines such as TNF- $\alpha$  or IFN- $\gamma$  produced by T cells may directly damage the myelin [21].

According to Multiple Sclerosis International Federation, about 2,000,000 people over the world have MS, and most of them are between 20 to 50 years of age [23]. We must remember that Canada has one of the highest rates of multiple sclerosis in the world. According to the Multiple Sclerosis Society of Canada, three more people are diagnosed with MS every day in Canada and women are three times more likely than men to develop MS [5].

### **2.2.2. Current treatment of MS**

Current drugs used in the treatment of MS are mostly based on IFN- $\beta$  and Glatiramer acetate [5]. IFN- $\beta$  has been approved for the treatment of relapsing-remitting MS patients, wherein it reduces the relapse rate and CNS lesions [4, 5]. The mechanism of action for IFN- $\beta$  in the treatment of MS is still not well understood, but it was suggested that IFN- $\beta$  could suppress effector T cell proliferation and thereby reduce CNS lesions. Unfortunately,

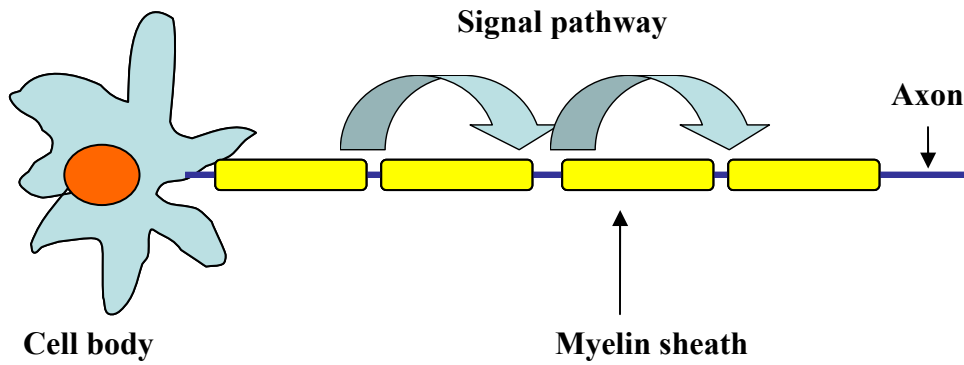
IFN- $\beta$  was found to have several side effects, such as flu-like symptoms, fatigue, depression, headache and abnormal liver function, etc [4]. Glatiramer acetate (GA) is a synthetic peptide (composed of tyrosine, glutamic acid, alanine and lysine) resembling myelin basic proteins [24], which can also reduce the relapse rate of MS patients [25]. How it works is also not well understood, but it could down-regulate Th1 responses and reduce CNS inflammation [26]. A study in an EAE model also showed that GA treatment inhibited IL-12 and IFN- $\gamma$  production while promoting Treg induction [24]. But GA may also have severe side effect on MS patients, like chest tightness, shortness of breath and anxiety, etc [25].

### **2.2.3. MS models: Experimental Autoimmune Encephalomyelitis (EAE)**

EAE has been used as a model to study MS for a long time, as it bears a resemblance to the feature of MS in humans. It was first used as a model of human demyelinating disease in 1933 by Rivers [27]. Rats and mice have widely served as the species of choice for EAE for the past 30 years [28]. Previous studies had reported that EAE can be induced by myelin basic protein (MBP), proteolipid protein (PLP) [28], myelin oligodendrocyte glycoprotein (MOG) [29], or brain spinal cord homogenates (BSCH) [30]. The brain contains a large amount of specialized cells called neurons. Neurons are the basic processing unit of the brain and convey signals by passing electrical impulses in the form of action potentials from one end of the cell to another (See Fig.2.1. below). MBP, PLP, MOG are the most

abundant and the best-studied CNS myelin proteins; these myelin proteins cover the axons [2]. Once autoreactive anti-nerve T cells are generated, they will produce inflammatory mediators and specifically attack the myelin sheath and destroy them. The destruction of myelin short-circuits electrical impulse transduction, leading to the clinical symptoms as described above. EAE can be induced in several strains of mice and rats by subcutaneous injection of myelin peptides or by the adaptive transfer of effector T cells from EAE mice. Interestingly, induction of EAE in different strains of mice or by different immunogens is associated with different phenotypes of EAE. For example, in female SJL/J mice induced with PLP<sub>(139-151)</sub> or PLP<sub>(178-191)</sub>, EAE is characterized as the RR-MS type. EAE induced by MOG<sub>(35-55)</sub> shows a chronic clinical phenotype in C57BL/6 mice [31].





**Figure 2.1. Diagram of a neuron.** Myelin proteins are covered around the axons. The function of myelin is to conduct the electrical signals from node to node, as indicated in the diagram. If the myelin sheath is destroyed, axons lose their action potentials to conduct signals. (Adapted from Neil A. Campbell. Lawrence G. Mitchell. Jane B. Reece. Biology concepts and connections. Third Edition, 1999. Pg: 565.).

The progression of active EAE can be divided into the induction and effector phases [32]. The priming of the myelin-specific T cells following immunization with myelin protein or peptide occurs in the induction phase. In the effector phase, myelin-specific T cells extravasate across the tight endothelial junctions comprising the blood brain barrier and migrate into the CNS. Chemokines and cytokines released from the T cells attract peripheral inflammatory cells into the CNS, and then these cells are activated by pro-inflammatory cytokines, resulting in inflammatory and cytotoxic effects and, hence, enhanced demyelination. In addition, within the CNS the local and infiltrating APC present myelin peptides in the context of co-stimulatory molecules to the infiltrating myelin-specific T cells, resulting in more inflammatory cytokine production and development of a cascade of CNS inflammation and myelin damage [29]. In the studies of histopathology, CNS-infiltrating cells in EAE include T cells, B cells, macrophages and neutrophils, while demyelination in the white matter of the CNS is similar to the pathology seen in MS patients [29]. In addition, it was reported that CNS local glial cells (e.g., astrocytes and microglia) are also present in the CNS lesions in EAE [33, 34].

Inflammatory cytokines such as IFN- $\gamma$ , IL-6 and IL-17 produced by T cells or other cells (e.g., macrophages) are considered to be one of the major factors causing EAE/MS [2]. Increased levels of IFN- $\gamma$  and myelin-specific IFN- $\gamma$ -producing T cells are found in the blood of MS patients [35, 36]. Injection of IFN- $\gamma$  induced a significantly higher

exacerbation rate, increasing the numbers of attacks from 1.42 to 4.67 per year, as compared with pretreatment and follow-up periods [37]. Intraventricular injection of IFN- $\gamma$  induces a severe EAE with more relapses in a chronic-relapsing EAE model in rats [38]. Other inflammatory cytokines like IL-6 also contribute to the pathology of EAE/MS; IL-6-deficient mice were shown to be resistant to the induction of EAE in mice [39], and spleen cells derived from IL-6-deficient mice showed a significantly lower ability to produce IL-2 and IFN- $\gamma$  in response to myelin-specific antigens [39]. High levels of IL-6 were detected in the plasma and cerebrospinal fluid (CSF) in MS patients [36, 40]. IL-6 was also shown to promote Th17 responses by suppressing Treg development, which will be discussed later in this chapter.

Recently, another process called epitope spreading was considered to be involved in the pathogenesis of MS and EAE. Epitope spreading is defined as reactivity to neuroepitopes or neuropeptides other than that used to induce the initial phase of disease [41]. T cells which are specific for a particular epitope of MBP or PLP could be activated to respond against other MBP or PLP epitopes during the later stage of disease development [9, 42, 43], and it was suggested that these T cells specific for other epitopes or peptides also contribute to the pathogenesis of EAE. Adoptive transfer of splenocytes from MBP<sub>(84-104)</sub>-induced EAE mice can transfer EAE to naive mice following activation by PLP<sub>(139-151)</sub> *in vitro* [43]. The release of such alternate epitopes or peptides may be due to the destruction of CNS tissue

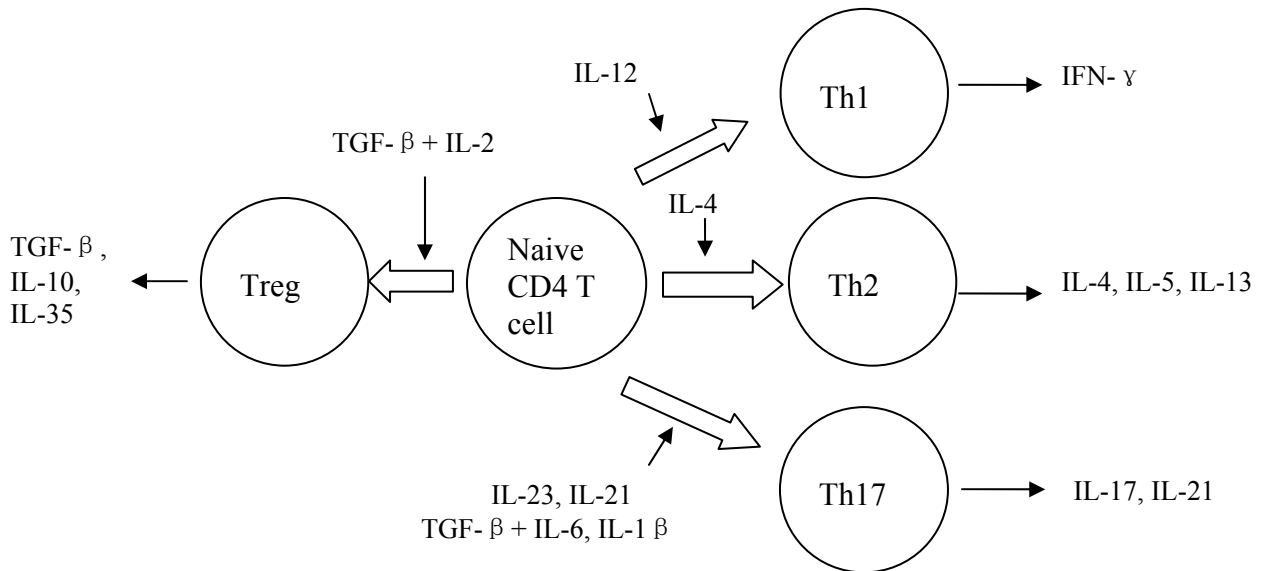
caused by the specific T cells during the initial acute phase of disease development. It was also suggested that APC in the periphery could potentially capture these various peptides and present them to T cells, leading to the re-activation of other neuroantigen-specific T cells.

#### **2.2.4. T cells in EAE/MS**

T cells are originally generated from bone marrow but develop into mature naive T cells in the thymus. T cells recognize antigens that are associated with MHC molecules expressed on APC via the T cells' receptors (TCR) [8]. Two special mechanisms, positive selection and negative selection, are integral to T cells development in the thymus. During positive selection, T cells whose TCR can engage self-MHC receive survival signals and proceed into the next stages of development. Those T cells whose TCR don't bind to MHC die by apoptosis. After positive selection, T cells which survive positive selection then undergo another process called negative selection. During negative selection, those T cells which react with self-peptides/self-MHC are eliminated via apoptosis, and only those T cells that do not react with self-peptides/self-MHC survive and leave the thymus as naive T cells [8].

Effector CD4<sup>+</sup> T cells can be divided into three subsets: Th1, Th2 and Th17 cells (See Fig. 2.2.below). Th1 cells are characterized as producing large amounts of IFN- $\gamma$  and are predominantly involved in the clearance of intracellular pathogens, cell-mediated immunity and delayed-type

hypersensitivity (DTH) responses; Th2 cells mainly produce IL-4, IL-5 and IL-13 and are important for the elimination of extracellular pathogens and parasites, as well as allergic responses [44, 45]. Naive CD4 T cells may also differentiate into Th17 cells in response to several cytokines, including TGF- $\beta$  plus IL-6, IL-23 and IL-21 [3]. Th17 cells mainly produce IL-17 and IL-21, and they are involved in clearance of extracellular parasites as well as the induction of autoimmunity [46].



**Figure 2.2. Summary of the differentiation of Th cell subsets.** Naive CD4 T cells can differentiate into Th1, Th2, Th17 or Tregs subsets in response to different cytokines. For example, IL-12 promotes Th1 cells differentiation and Th1 cells produce IFN- $\gamma$ . IL-4 promotes Th2 differentiation and Th2 cells mainly produce IL-4, IL-5 and IL-13. IL-23, IL-21, TGF- $\beta$ +IL-6 and IL-1 $\beta$  preferentially differentiate naive Th cells into Th17 subsets and Th17 cells predominantly produce IL-17 and IL-21, TGF- $\beta$  plus IL-2 promote Tregs development, and Treg mainly produce IL-10, IL-35 and TGF- $\beta$  (Reference: [3, 6, 21, 46-48]).

EAE is considered to be a T cell-mediated disease [2, 49, 50]. It was indicated that Th1 cells, which are characterized by specific cytokine production like IFN- $\gamma$ , are involved in the development and pathology of EAE. The strongest supportive evidence is that adoptive transfer of myelin-specific Th1 cells could induce EAE in mice [51, 52]. IFN- $\gamma$ -producing CD4<sup>+</sup>T cells and IFN- $\gamma$  were found to be present in the CNS lesions during the peak of EAE development [44, 45]. Th1 cells were also essential for inducing EAE by promoting the further entry of Th17 cells into CNS, as it was reported that the i.v. injection of Th1/Th17 cells together induce severe EAE, while the injection of Th17 cells alone did not induce severe symptoms. When transferred together, significantly higher levels of Th1 cell infiltration than Th17 infiltration were found in the CNS. Th17 cells are found in the CNS only when co-injected with Th1 cells [53]. CD8<sup>+</sup> T cells are also involved in EAE pathogenesis as the transfer of myelin-specific CD8<sup>+</sup> T cells into mice induces rapid and severe EAE [54], suggesting that CD8<sup>+</sup> T cells could potentially attack neurons and destroy the myelin, leading to axonal destruction and demyelination [2].

Though Th1 cells are considered to be important in EAE pathogenesis, some evidence also challenges a role for Th1 responses in EAE induction and development [46]. IL-12 is considered to be a Th1-type cytokine as well as IFN- $\gamma$  [46], but IL-12-deficient mice were also shown to be susceptible to EAE induction [55], suggesting IL-12 was not critical in EAE induction. A controversial role of IFN- $\gamma$  in EAE has also reported by others. IFN- $\gamma$  or

IFN- $\gamma$  receptor-knock-out mice were showed to be susceptible to EAE induction as compared with WT mice [56, 57]. CNS-delivery of IFN- $\gamma$  before EAE induction or at the early stage, or after onset, of disease decreased EAE clinical scores and demyelination by inducing apoptosis of lymphocytes in the CNS [58]. Another interesting study showed that neutralization of IFN- $\gamma$  by anti-IFN- $\gamma$  mAb in WT EAE mice 1 week post-immunization worsened EAE clinical scores, but the same treatment decreased the clinical symptoms in IFN- $\gamma$  R<sup>-/-</sup> mice [59]. The mechanisms are still not clear but it was suggested that a likely alternative mechanism or an unidentified second IFN- $\gamma$  receptor mediated the different function of IFN- $\gamma$  in EAE development [59]. The role of IFN- $\gamma$  in EAE may be complex, but taken together the data suggest that IFN- $\gamma$  is not critical in the induction of EAE, or that it might also function in a beneficial role, potentially during EAE development.

Recent studies demonstrated that CD4<sup>+</sup> Th17 cells, strongly contribute to the pathogenesis of EAE/MS [60, 61]. The development of Th17 cells is considered to be a key event in the pathogenesis of EAE [62, 63], as the neutralization of IL-17 responses or IL-17-deficiency is associated with reduced EAE symptoms [3, 64]. The incidence and development of EAE in wild-type mice given IL-17-deficient CD4<sup>+</sup>T cells from EAE mice was significantly reduced as compared with those receiving wild-type CD4<sup>+</sup> T cells [64], suggesting that CD4<sup>+</sup> T cells' IL-17 fosters EAE development. In MS patients, Th17 lymphocytes are found to efficiently



migrate across the blood brain barrier (BBB) [65]. Human BBB-endothelial cells (BBB-ECs) in MS lesions had a higher expression of IL-17 receptor (IL-17R), while IL-17R is undetectable in normal CNS tissues, suggesting that the activation of IL-17R expression in MS patients is involved in BBB permeability [65]. IL-6 was demonstrated to have a key role in the differentiation of Th17 cells in EAE; the blockade of IL-6 by anti-IL-6 receptor monoclonal antibody (anti-IL-6R mAb) significantly suppressed EAE and the production of IL-17 and IFN- $\gamma$  [62, 66]. Higher numbers of Treg and lower numbers of Th17 cells are also found in anti-IL-6R-mAb-treated EAE mice [62]. TGF- $\beta$  signaling is also required for the differentiation of Th17 cells, as it was reported that the blockade of TGF- $\beta$  by anti-TGF- $\beta$  prevented IL-17 production and EAE onset [66, 67]. It was also reported that TGF- $\beta$  plus IL-6 strongly induced Th17 cells in WT mice, but T cells from IL-6R-deficient mice did not differentiate into Th17 cells in response to TGF- $\beta$  plus IL-6, suggesting IL-6 signaling was important in the induction of Th17 cells in the presence of TGF- $\beta$  [68].

IL-23 can be produced by DC and macrophages [69, 70], and it is a potent activator of Th17 cell development, whereby it can activate pathogenic Th17 T cells. Monocyte-derived DCs from MS patients show production of IL-23, suggesting that IL-23 plays a role in the pathogenesis of MS [60]. IL-23 is composed of two subunits, p40 and p19, while IL-12 is composed of p40 and p35 [46]. IL-23-deficient mice are EAE-resistant, and this was confirmed by their significantly decreased clinical scores and low

production of IL-17 and IFN- $\gamma$  [71, 72]. It was reported that p19-deficient mice (i.e., lacking IL-23) and wild-type (WT) mice developed EAE symptoms with similar severity after adoptive transfer of purified CD4<sup>+</sup>T cells from WT EAE donor mice, but that WT mice developed EAE with delayed disease onset and lower clinical scores when cells from p19-deficient EAE mice were injected. Neutralization of IL-23 during the effector phase of disease did not protect mice from EAE, suggesting that EAE could develop in the absence of IL-23 once the encephalitogenic T cells had developed [72], and that IL-23 plays an important role in the induction but not the effector phase of EAE [72]. It had also been reported that the p40-deficient (i.e., lacking IL-23 and IL-12) or p19-deficient mice (i.e., lacking IL-23) had less or no Th17 response and were resistant to EAE, while p35-deficient mice (i.e., lacking IL-12) showed normal Th17 response and were susceptible to EAE [46], suggesting IL-23 is required for the generation of the Th17 response, while IL-12 alone was not required in the induction of EAE.

IL-21 is mostly produced by Th17 cells themselves, and it can further promote the activation of Th17 cells in an autocrine manner [73]. IL-21<sup>-/-</sup> mice show a marked decrease in the production of IL-17, such that the frequency of Th17 cells induced in IL-21-deficient CD4<sup>+</sup> T cells is less than that in WT CD4<sup>+</sup> T cells in the presence of TGF- $\beta$  and IL-6, but significantly higher than that without TGF- $\beta$  and IL-6 in culture [73]. TGF- $\beta$  plus IL-21 significantly increased the frequency of Th17 T cells in IL-6<sup>-/-</sup> mice, but

TGF- $\beta$  plus IL-6 did not show any effect in inducing Th17 cells from IL-6R<sup>-/-</sup> T cells [68]. This suggested that TGF- $\beta$  plus IL-21 represents an additional pathway which contributes to the generation of Th17 responses, and it was likely that IL-6 and IL-21 work independently in the presence of TGF- $\beta$ , but that their cooperation can amplify the Th17 response [73]. The mechanisms are still not clear but it was considered that IL-21 and IL-6 can induce the activation of STAT3 and cooperate with TGF- $\beta$  signaling to induce and amplify Th17 differentiation [73].

A recent study with human Th17 cells reported that TGF- $\beta$  plus IL-1 $\beta$ , IL-6, IL-21 and IL-23 strongly increased IL-17 production *in vitro*, while the highest production of IL-17 was found in cells cultured with TGF- $\beta$ , IL-1 $\beta$ , IL-6, IL-21 and IL-23 together. Higher level IL-17 production is also found in the cells cultured with IL-1 $\beta$ , IL-6, IL-21 and IL-23 in the absence of TGF- $\beta$  as compared with control cultures (i.e, without IL-1 $\beta$ , IL-6, IL-21 and IL-23), though IL-17 production is less than that with IL-1 $\beta$ , IL-6, IL-21 and IL-23 in the presence of TGF- $\beta$  [74]. Taken together, these data suggest that TGF- $\beta$  is important but not critical in promoting IL-17 production when cooperating with other cytokines. It is likely that different combinations of cytokines could play different roles in the induction or inhibition of Th17 responses. Interestingly, TGF- $\beta$  might inhibit IL-17 production at later stages of inflammatory response, while it was required for the induction of IL-17 production at the beginning, as it was reported that

the neutralization of TGF- $\beta$  decreases IL-17 production in naive T cells but it does not affect IL-17 production by memory T cells [74]. It is likely that TGF- $\beta$  is not critical for the maintenance of Th17 memory cells, and that other cytokines instead of TGF- $\beta$  contribute to the development of Th17 cells at this stage. Or it could be possible that high concentrations of TGF- $\beta$  deliver negative signals to Th17 cells and suppress IL-17 production [74].

IL-27 limits EAE pathogenesis by suppressing the development of Th17-producing cells [75, 76]. IL-27 is produced by DC and macrophages [46]. IL-27R-deficient mice are highly susceptible to EAE as they have a higher disease incidence, but there is no change in the day of disease onset, which probably indicates that IL-27 is important in disease induction but not its development. Increase of IL-17 production in LN and the CNS was found in IL-27R-deficient mice [75]. IL-27 inhibits the development of Th17 cells directly, and not through effects on Tregs. This was confirmed by experiments showing that CD4<sup>+</sup> effector T cells from IL-27R-deficient and wild-type mice both had similar capacity to proliferate when co-cultured with wild-type Tregs. Tregs from IL-27R-deficient mice similarly suppressed the proliferation of wild-type T cells in the absence or presence of IL-27. IL-27 could also suppress the proliferation of effector cells from wild-type in the absence of Tregs [75]. Therefore, IL-27 was thought to be unnecessary for normal Tregs to suppress the development of Th17 cells. The suppressive effects of IL-27 on Th17 cells were dependent on the transcription factor STAT 1 [75]. It had been shown that IL-27 did not

inhibit the IL-17 production in STAT 1<sup>-/-</sup> mice, but it significantly inhibited the IL-17 production in STAT 1 WT mice [75] and reduced the differentiation of Th17 cells induced by IL-6 and TGF- $\beta$  [75].

EAE is considered to be attributable to Th1 and Th17 responses, but it was found that Th2 responses are also involved in EAE [51, 77, 78]. It was reported that the transfer of MBP-specific Th2 cells (e.g., CD4<sup>+</sup> T cells stimulated with MBP in the presence of IL-4) to RAG-1<sup>-/-</sup> mice also caused >90% EAE incidence, as does transfer of the same numbers of Th1 cells to these mice, although there was a few days delay in the disease onset. There was no evidence showing that these Th2 cells were converted into Th1 cells, as the spleens and CNS of Th2 cell recipients expressed IL-4, but not IFN- $\gamma$  [51, 52]. IL-4-deficient C57BL/6 mice are also susceptible to EAE induction as compared with WT mice [79]. Mast cells, a major effector cells which is involved in the Th2 response, were shown to contribute to the EAE development, as mast cell-deficient mice exhibited a significantly lower disease incidence, delayed disease onset and lower clinical scores than wide-type mice [80]. These findings suggest the possibility that Th2 response may be involved in EAE. But we must note that the roles of Th1, Th2 and Th17 response in the regulation of EAE are still not well understood.

### **2.2.5. B cells and antibodies in EAE/MS**

B cells are generated in the bone marrow, circulate through the bloodstream, spleen and LN. They express IgM and IgD on their surface as antigen receptors and make antibodies [8]. B cells can bind antigen via the B cell receptor (i.e, immunoglobulin), process the antigen and present peptides in association with cell surface MHC II to T cells. These T cells recognize peptide/MHC II complexes via their TCR, and produce cytokines essential for the differentiation of B cells, as well as to antibody class switching [8]. Several cytokines are involved in the switching and production of antibody. For example, IL-4 and IL-13 are important in the production of IgG1, IFN- $\gamma$  promotes IgG2a production, while TGF- $\beta$  is involved in IgG2b production. IL-4 and IL-6 can promote the differentiation of B cells into plasma cells and increased the antibody production, while the interaction of CD154 on Th cells and CD40 on B cells can promote memory B cells generation [8].

B cells and the antibodies they secret also have a specific role in EAE [81]. Previous studies indicated that B cells and antibody contribute to the recovery from EAE, inasmuch as myelin-specific antibodies can promote CNS remyelination and prevent further demyelination in EAE [82]. Increased production of MOG-specific IgG was found in IL-17-deficient mice during EAE as compared with wild-type EAE mice, suggesting that IL-17 influences MOG-specific IgG production by B cells [64]. The depletion of B cells before EAE sensitization significantly exacerbated disease symptoms and CNS demyelination [83]. Transfer of serum from rats

that had recovered from EAE to naive recipients suppresses the induction of EAE [84, 85]. In addition, the co-transfer of B cells and CD4<sup>+</sup> suppressor T cells from rats that have recovered from EAE protects the recipients from EAE induction, suggesting that the suppressor T cells may stimulate the B cells to produce the antibodies that inhibited EAE development [86]. In the study of humoral immune responses that enhanced remyelination, some specific autoantibodies like IgG were found to be beneficial [82, 87]. To assess the importance of B cells in the regulation of EAE, Fillatreau, et al [88] found that B cells played an important role in the regulation of EAE by provision of IL-10. The transfer of B cells from WT mice that had recovered from EAE could protect against EAE in the recipients, but the transfer of IL-10-deficient B cells failed to do that.

Conversely, B cells and CNS-specific autoantibodies are also involved in the pathogenesis of EAE and MS [89]. Increased levels of IgG are found in the CSF in MS patients [89, 90], and myelin-specific antibody-secreting B cells are present in the CNS lesions of MS patients [89, 91-93]. EAE mice induced by MOG<sub>(35-55)</sub> is associated with increased production of MOG<sub>(35-55)</sub>-specific IgG [94]. Myelin-specific B cells are found to accumulate in the CNS in EAE [95]. The depletion of B cells in mice with EAE significantly reduces clinical symptoms and the numbers of IFN- $\gamma$ -and IL-17-producing CD4<sup>+</sup>T cells, as well as CNS demyelination [83]. The suggestion is that B cells could be related to an enhancement of antigen-presentation to antigen-specific T cells [96]. Thus, the roles of B

cells, antibodies and even complement in MS and EAE are complicated, and may include both pathogenic and reparative roles. One explanation is that different B cells subsets play different roles in the regulation of EAE. One kind of regulatory B cell, the IL-10-producing CD1d<sup>hi</sup> CD5<sup>+</sup> regulatory cells, has been found to suppress EAE severity. As discussed above, depletion of B cells by anti-CD20 monoclonal antibody (mAb) before EAE sensitization significantly exacerbates EAE, and anti-CD20 mAb also depletes CD1d<sup>hi</sup> CD5<sup>+</sup> regulatory B cells [83]. But the depletion of B cells shortly after disease onset inhibits EAE, probably because it inhibits the cooperation of antigen-specific T cells and B cells in EAE [83, 96].

#### **2.2.6. Microglia and astrocytes in EAE**

Microglia and astrocytes are types of glial cells in the CNS. Microglia are CNS-resident macrophages, the function of which includes phagocytosis, antigen presentation, and cytokine and nitric oxide production [33]. Astrocytes are characterized as star-shaped glial cell which are involved in the maintenance of the BBB structure and barrier, neuronal replacement and signal conduction [97]. They were involved in the regulation of inflammatory responses in EAE, though the mechanisms are still not well understood [33, 98]. Increased numbers of microglia and astrocytes are observed in EAE lesions, suggesting they might potentially play a role in EAE pathogenesis [34]. Microglia and astrocytes could function as APCs during EAE development, as they express MHC II and co-stimulatory



molecules (e.g. CD80 and CD86), and have an ability to present antigen and co-stimulatory signals to autoreactive T cells [6, 33, 99]. *In vitro* experiments have shown that microglia can produce IL-12 and IFN- $\gamma$ , while astrocytes produce IL-12 and IL-23 under inflammatory condition, suggesting that they could potentially activate Th1 and Th17 cells and lead to further CNS inflammation [33, 100-102].

But controversial results indicate that microglia and astrocytes may play a beneficial role in inhibiting EAE development. Microglia are capable of producing IL-10 in the normal CNS, which indicates that they might participate in immune surveillance and inflammation prevention in the normal CNS [33, 103]. Apoptosis of infiltrating cells in the CNS has been observed in EAE [104, 105], and these apoptotic cells are closely associated with astrocytes and microglia, such that it was thought that astrocytes and microglia could eliminate these inflammatory cells by inducing their apoptosis [105]. Astrocytes are also thought to participate in the remyelination in the CNS because of their ability to produce nerve growth factor (NGF) [98]. Taken together, it has been suggested that microglia and astrocytes may contribute to a balance in regulating the induction and prevention of inflammatory responses in the CNS during EAE development, and that their different roles may also depend on the local environment of CNS [98].

## **2.3. Immunologic Tolerance**

### **2.3.1. Introduction to Tolerance**

Tolerance is an immunological response in which the immune system does not mount aggressive responses against self-antigen and tissues. The loss of immunologic tolerance may result in several kinds of autoimmune diseases, like MS [8, 16]. The immune system has several mechanisms, including central and peripheral tolerance, to maintain self-tolerance and prevent autoimmune responses. T cells which are specific for foreign peptides are selected by the process of negative selection in the thymus, whereas T cells that react with self-antigen are eliminated, leading to central tolerance, although these processes are not 100% efficient [8]. Thus, a peripheral mechanism for induction of T cell tolerance (i.e. outside the thymus) is necessary to prevent the autoantigen-specific T cells from damaging the host tissues. T cells are important in the maintenance of self-tolerance. The related mechanisms include [106]: 1) deletion by apoptosis, wherein autoreactive T cells could be induced to undergo apoptosis, leading to the elimination; 2) clonal anergy, which stops self-reactive T cells from responding to the signals of activation (for example, recognition of self-antigens by TCR leads to inactivation in the absence of co-stimulatory molecules) [8, 106, 107]; 3) the activation of immunosuppressive regulatory /suppressor T cells [16] and/or tolerogenic dendritic cells (DCs) which suppress autoreactive T cells. Similar mechanisms also prevent B cells from reacting against self-antigens.

Developing B cells recognizing self-antigens are induced to die by apoptosis, which is known as clonal deletion. In the process of clonal anergy B cells are rendered anergic if their receptors recognize self-antigens – these B cells can no longer respond to specific antigen [8].

### **2.3.2. Characteristics and function of CD4<sup>+</sup>CD25<sup>+</sup> Tregs**

CD4<sup>+</sup>CD25<sup>+</sup> Tregs account for 5-10% of peripheral CD4<sup>+</sup>T cells [108]. Adoptive transfer of CD4<sup>+</sup>CD25<sup>-</sup> T cells to thymus-deficient mice leads to a higher risk of developing autoimmune disease, but the co-transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells to the same mice does not lead to autoimmune disease [109]. This indicates that CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress the development of autoimmune disease [109]. CD4<sup>+</sup>CD25<sup>+</sup> T cells express high levels of IL-10, TGF- $\beta$  and IL-35, which suppress autoreactive T cells via cytokine-dependent mechanisms [47, 48, 110].

Autoreactive T cells are eliminated or inactivated via negative selection in the thymus, leading to central tolerance [107]. CD4<sup>+</sup>CD25<sup>+</sup> Tregs are selected in the thymus by positive selection when interacting with intrathymic cortical epithelium-expressing MHC II molecules [107]. The TCR expressed on CD4<sup>+</sup>CD25<sup>+</sup> Tregs with higher avidity than those required for positive selection for effector T cells lead to the generation of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (naturally-occurring Tregs) [106]. Selection of CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the thymus is also controlled by FoxP3, a transcription factor that is necessary for the development of CD4<sup>+</sup>CD25<sup>+</sup> Tregs [111].

Tregs could also be induced in peripheral organs (induced Tregs), as suggested by the observation that Tregs reach normal numbers in thymectomized mice [106]. The main difference between naturally-occurring Tregs and induced Tregs is that nTregs are fully functional at the time of thymic export, whereas the development of iTregs in the periphery requires exposure to antigens under specific conditions [106, 112]. Current research has suggested that TGF- $\beta$  and IL-2 are necessary for the induction of FoxP3 and generation of FoxP3<sup>+</sup> iTregs [113]. When CD4<sup>+</sup>FoxP3<sup>-</sup> T cells are cultured with TGF- $\beta$  in the presence of IL-2, there was significant conversion of the CD4<sup>+</sup>FoxP3<sup>-</sup> T cells into CD4<sup>+</sup>FoxP3<sup>+</sup> T cells as compared to cultures with TGF- $\beta$  alone [113]. Similarly, CD4<sup>+</sup>T cells cultured with IL-2 alone did not induce CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs [113]. In IL-2<sup>-/-</sup> mice the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells is significantly reduced even when cultured with TGF- $\beta$ , suggesting that TGF- $\beta$  and IL-2 are essential for the generation of CD4<sup>+</sup>FoxP3<sup>+</sup> iTregs from CD4<sup>+</sup>FoxP3<sup>-</sup> T cells in the periphery [113]. It has also been shown that the addition of exogenous IL-4 or IFN- $\gamma$  reduces the ability of TGF- $\beta$  to induce FoxP3 expression in CD4<sup>+</sup>T cells [114]. The neutralization of IL-4 and IFN- $\gamma$  by anti-IL-4 and anti-IFN- $\gamma$  antibodies not only significantly increased the TGF- $\beta$ -induced FoxP3 expression, but also enhanced the suppressive ability of these cells [114]. It has also been reported that IL-6 can prevent the conversion of CD4<sup>+</sup>FoxP3<sup>-</sup> T cells to CD4<sup>+</sup>FoxP3<sup>+</sup>T cells in the presence of TGF- $\beta$  and, in the same vane, that IL-6R-deficient EAE mice have an enhanced frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>

T cells [68, 73]. Similarly, TGF- $\beta$  plus IL-21 or IL-27 also reduces the frequency of FoxP3<sup>+</sup> T cells, although the stimulation with TGF- $\beta$  alone strongly induces CD4<sup>+</sup>FoxP3<sup>+</sup> T cells [73]. Taken together, this suggests that different combinations of cytokines may play different roles in promoting or inhibiting Treg development and function.

Previous studies revealed that CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs are one of the major regulators of autoimmune tolerance [107]. They are potent suppressor cells, capable of suppressing the activity or the functions of effector T cells [47, 115]. This suppressive function is antigen non-specific, which is called bystander suppression [107, 116]. Several cytokines, such as IL-10 and TGF- $\beta$  are important in the function of CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs [107, 117]. The mechanisms by which CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs regulate the immune response can be divided in two ways: 1) cell-cell contact dependent; and 2) cytokine secretion-dependent. In the cell contact-dependent pathway, it was found that the suppressive function of Tregs was not mediated by cytokines, as the physical separation of Tregs from effector cells by a semi-permeable membrane resulted in no inhibition of proliferation of the effector cells [118]. Previous studies also confirmed that Treg derived from TGF- $\beta$ -deficient or IL-10-deficient mice could still retain some of their suppressor function [106, 119], which indicated that the function of Treg were only partially mediated by cytokine production. In addition, our lab also reported that IL-10-differentiated DC could induce Treg and these Treg suppressed the proliferation of effector T cells in a cell-cell contact-dependant mechanism,

as confirmed in transwell assays [12]. In the cytokine secretion-dependent mechanisms, IL-10 and TGF- $\beta$  are involved in the development and function of CD4<sup>+</sup>CD25<sup>+</sup> Treg [48]. Treg produce high levels of IL-10 and TGF- $\beta$  [120]. Tregs from IL-10<sup>-/-</sup> mice fail to suppress airway hyperresponsiveness (AHR), and neutralizing antibodies against TGF- $\beta$  also reduce the suppression mediated by Treg and restored AHR [120], suggesting the suppressive function of Treg require the presence of IL-10 and TGF- $\beta$  [48].

There are several characteristic markers of CD4<sup>+</sup>CD25<sup>+</sup> Treg, including CD25, FoxP3, cytotoxic T lymphocyte-associated antigen (CTLA-4) and lymphocyte-activation gene (LAG-3) [107, 117]. CD25, known as IL-2R  $\alpha$  chain, is a classical marker for Treg and it is expressed stably by these cells [121], but it is also expressed by other T cells with non-regulatory function. Most activated T cells, like CD8<sup>+</sup>T cells, express low/intermediate levels of CD25 and can differentiate into functional effector cells with high type-specific cytokine (e.g, IFN- $\gamma$ ) production [47, 122]. The transcription factor FoxP3 is necessary for the development and the function of CD4<sup>+</sup>CD25<sup>+</sup> Treg, IL-2 and TGF- $\beta$  contribute to the induction of FoxP3 [111, 117]. Several genes, such as *Gpr83*, *Ecm1*, *Cmtm7*, *Nkg7*, *Socs2* and *glutaredoxin*, are found to be under the transcriptional control of FoxP3 [123]. Many studies have shown that mice lacking FoxP3 are deficient in CD4<sup>+</sup>CD25<sup>+</sup> Treg, and thereby develop multiple autoimmune diseases [117, 124, 125]. FoxP3 is also considered to be a specific marker CD4<sup>+</sup>CD25<sup>+</sup> Tregs [111, 117]. It confers suppressive function on CD4<sup>+</sup>CD25<sup>+</sup> Treg [126].

CTLA-4 is constitutively expressed by Treg and is essential for the activation of suppression and the maintenance of self-tolerance [48, 127, 128]. Anti-CTLA-4 mAb does not alter the numbers of Tregs, but mice treated with anti-CTLA-4 mAb spontaneously developed autoimmune gastritis [129]. Treg from CTLA-4-knockout mice fail to suppress T effector cell activity [127]. LAG-3 is expressed by Treg upon activation and is necessary for their regulatory function. Thus, LAG-3-deficient Treg show a reduced regulatory function, while blockade of LAG-3 by anti-LAG-3 antibodies also significantly reduces the suppressive function of Treg [12, 130]. CTLA-4 and LAG-3 can also bind to CD80/86 and MHC II on DC, leading to reduced expression of these molecules [48].

### **2.3.3. CD4<sup>+</sup>CD25<sup>+</sup>Treg in EAE/MS**

Recent studies revealed that CD4<sup>+</sup>CD25<sup>+</sup> Treg are the major players in the maintenance of immunologic self-tolerance (i.e., they can protect against EAE; [131]). Transfer of CD4<sup>+</sup>CD25<sup>+</sup> Treg can induce tolerance to EAE induced by MOG<sub>(35-55)</sub> by suppressing IFN- $\gamma$  production by MOG<sub>(35-55)</sub>-specific T cells and preventing CNS inflammation [132]. A study in a MBP-induced EAE model found that adoptive transfer of MBP-specific Treg induce bystander suppression in EAE, inasmuch as MBP-specific Treg provide significant protection against EAE induced by MBP, PLP and a mixture of MBP and PLP [133], indicating that the suppressive effects of these Treg was antigen-non-specific. In addition to these prophylactic effect,

Treg also showed a therapeutic effect in EAE, as i.v. administration of Treg on day 18 after EAE induction significantly reduces the severity of disease [133].

As described above, FoxP3 is a specific marker of CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Reductions in FoxP3 expression are observed in MS patients, although there are no differences in the frequency of CD4<sup>+</sup>CD25<sup>+</sup> Tregs when compared with healthy controls. This confirms that FoxP3 expression is required for the function of CD4<sup>+</sup>CD25<sup>+</sup> Tregs [9, 126]. Tregs isolated from the circulation of MS patients poorly suppress the proliferation of, and IFN- $\gamma$  production by T effector cells, but Tregs from healthy controls efficiently suppress both. There was no difference in the frequency of the Tregs from these 2 sources, suggesting that the suppressive function of Tregs in MS patients may be impaired [1, 9]. It is still not clear whether the loss of FoxP3 expression was due to a reduction in the numbers of FoxP3<sup>+</sup> Tregs, or to decreased FoxP3 expression at the cellular level [1, 9]. IL-10 expression is similar with cells from both populations, but blockade of IL-10 does not alter the suppressive function of Tregs [9], indicating that the operative mechanisms for these Tregs are cell contact-dependent.

It had also been reported that IL-6 controls the induction of Th17 cells by suppressing the generation of Tregs in the presence of TGF- $\beta$ . IL-6R-deficient mice show a significantly lower production of IL-17 as compared with wild-type mice and they are resistant to EAE induction, but a significantly higher frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs are found in their



spleens [68]. CD4<sup>+</sup>FoxP3<sup>-</sup> T cells from WT and IL-6R-deficient mice were transferred into RAG<sup>-/-</sup> mice after which the mice were immunized with MOG<sub>(35-55)</sub>/CFA. Twenty days later a significant conversion of FoxP3<sup>-</sup> T cells into FoxP3<sup>+</sup> T cells as observed in the spleens of recipients receiving IL-6R-deficient CD4<sup>+</sup>FoxP3<sup>-</sup> T cells [68], suggesting that IL-6 signaling was capable of preventing the conversion of FoxP3<sup>-</sup> T cells into FoxP3<sup>+</sup> T cells. Similarly, IL-6<sup>-/-</sup> EAE mice induced by MOG<sub>(35-55)</sub>/CFA also demonstrate a defect in IL-17 production, but have a significantly higher frequency of LN FoxP3<sup>+</sup> Tregs. The depletion of Tregs by anti-CD25 in IL-6<sup>-/-</sup> mice rendered the mice more susceptible to EAE induction, suggesting that Tregs are important in preventing the development of EAE [73]. Interestingly, it had been reported that the deletion of Tregs could lead to a reappearance of Th17 cells in IL-6<sup>-/-</sup> mice, and that co-culture with combination of TGF- $\beta$  and IL-21 reconstitutes the generation of Th17 cells and IL-17 production in these mice and suppress FoxP3<sup>+</sup> T cells generation [68, 73]. Taken together, these recent findings suggest that there is a new paradigm in the balance of Th17 and Tregs, and this may contribute to the pathogenesis and regulation of EAE. Different combinations of Th1, Th2 and Th17 cytokines may regulate the balance of Th17 cells and Tregs during the immunological response, leading to the exacerbation or prevention of disease development.

#### **2.3.4. The role of DCs in the immunologic stimulation and tolerance**

Dendritic cells (DCs), described early in the late 19th century, are considered to be professional APC and to play a significant role in the induction and regulation of immunological responses [16]. DCs, specialized bone marrow-derived cells that are found in most tissues of the body, are important to the induction of immunity and peripheral tolerance [134]. DCs have several characteristics in common. They are derived from hemopoietic bone marrow cells and their precursors travel through the blood to the tissues where they transform into immature DCs. These immature DCs constantly take up antigens from their surrounding environment, such as bacterial and viral antigens, but also normally processed self-tissues antigens, such as from apoptotic cells [8, 135]. Immature DCs become activated to mature upon interacting with pathogens. The captured antigens are degraded into small peptides capable of binding to MHC molecules within the phagolysosomes, and then the peptide-MHC complexes are transported to and expressed on the cell surface. Upon stimulation, DC traffic to secondary lymphoid organs like LN, where they can stimulate naive T cells. The high density of MHC I- or MHC II-peptides complexes can be recognized by the T cell receptor (TCR) on the T cells (signal 1), resulting in the activation of effector CD8 or CD4 T cells, respectively. The expression of costimulatory molecules on the surface of DCs (signal 2) such as CD80/86 are increased following interaction with T cells, which deliver the second signal to T cells, leading to their further activation [8]. The secretion of cytokines signals by

DCs is important in the T cell differentiation. DCs that produce IL-12 promote development of CD4 T cells into Th1 cells, whereas DC secretion of IL-4 drives development of Th2 cells [21]. DC are also important in Th17 cells development. IL-6 and IL-23 produced by DC promote Th17 differentiation, whereas IL-27 was involved in the suppression of Th17 response [75].

Dendritic cells play a significant role in immunologic tolerance in many diseases. Mature DCs induce immune responses, while tolerogenic DCs induce tolerance such as T cell anergy or Tregs which act as suppressor cells in the prevention of autoimmune disease [106]. Our lab had reported that tolerogenic DCs induced by IL-10 can inhibit Th2 response and reverse airway hyperresponsiveness in a mouse model of asthma [10]. IL-10 is produced by several kinds of cells, such as T cells, DC, B cells and macrophages [136, 137]. IL-10-treated DCs express lower levels of co-stimulatory molecules like CD40, CD54, CD80 and CD86 and surface MHC II, are actively endocytotic and secrete reduced levels of proinflammatory cytokines such as IL-1 $\beta$ , IL-6 and IL-12. Activation of Treg markers such as LAG-3, CTLA-4 was found in response to tolerogenic DC treatment [11, 12]. As discussed above, the activation of effector T cells need 2 signals delivered by APCs, if T cells received only signal 1 without signal 2, they are rendered anergic, leading to the peripheral tolerance [8]. The down-regulation of co-stimulatory molecules induced by IL-10 is thought to be one of the mechanisms that mediates tolerance, as blocking this

signal delivery results in the inactivation of naive T cells. It is suggested that this suppressive function was partially due to a cell contact-dependent process [138, 139]. It was reported that DCs matured with TNF also induce tolerance to EAE by i.p. injection, but s.c. injection of these DCs did not, suggesting the protective effect of tolerogenic DC was partially depended on the delivery route [15].

In humans, IL-10 treated-dendritic cells generated from peripheral blood mononuclear cells (MNC) induce T cells tolerance *in vitro*. These DC10 expressed low levels of HLA-DR, CD86, CD80, CD83 and proinflammatory cytokines IL-12 and IL-6 [12, 139, 140]. The suppressive functions of DC10 appear to be dependent on a cell-cell contact mechanisms partially, which was confirmed in transwell assays [12, 139]. The soluble factors produced by DC 10 in the upper chambers of the transwell apparatus did not have any influence on effector T cells proliferation, suggesting that the suppressive function was not directly mediated by soluble factors such as cytokines and that Treg-T effector cell contact was required [139]. Our lab has also reported that DC derived from human peripheral blood could be differentiated into tolerogenic phenotype by treatment with IL-10. These tolerogenic DC10 also express low levels of several surface markers like CD40, CD54, CD80, CD83, CD86 and HLA-DR as well as low production of proinflammatory cytokines like IL-6 and IL-12, but express high levels of IL-10 and inhibitory receptor immunoglobulin-like transcripts 2 (ILT2). These DC10 could efficiently induce Th2 cell allergen tolerance and

suppress effector T cell activity by activating Tregs *in vitro* [12]. Taken together, this suggests a potential therapeutic strategy for MS and other autoimmune diseases by use of IL-10-induced-tolerogenic DC in human.

### **2.3.5. DC in the prevention of EAE/MS**

Several labs have reported that DC can prevent sensitization for EAE as described above [15, 141]. Huang, et al. [14] reported that bone marrow-derived DCs that have been pulsed with encephalitogenic MBP<sub>(68-86)</sub> *in vitro* could prevent sensitization for EAE in Lewis rats. These DC enhance apoptosis among spleen and LN cells. In a MOG<sub>(35-55)</sub>-induced EAE model in C57BL/6 mice, it has also been reported that the administration of IL-10-treated bone marrow derived-DC before sensitization prevents and reduces the severity of disease induction [13]. Injection of TNF- $\alpha$  treated-DC that have been pulsed with MOG<sub>(35-55)</sub> can prevent EAE induction in C57BL/6 mice given MOG<sub>(35-55)</sub>, but unpulsed DC or DC pulsed with OVA *in vitro* failed to induce tolerance to EAE subsequently immunized with MOG<sub>(35-55)</sub>. This indicates that the tolerance to EAE induced by such DC is antigen-specific[142].

Several reports had indicated that tolerogenic DC induced by vasoactive intestinal peptide (VIP) [143] and Galectin-1 [144] could suppress established EAE. Based on the discovery of EAE prevention and treatment by tolerogenic DCs, we hypothesized that tolerogenic DC10 could be used to treat MS patients. IL-10-treated monocyte-derived DCs from MS patients

showed a significantly lower expression of surface molecules like CD80, CD83, CD86 and HLA-DR, and produce lower level of proinflammatory cytokines like IL-6 and IL-12 as compared with LPS-matured DCs [140]. This supports the suggestion that IL-10-differentiated DCs from MS patients could potentially be used therapeutically.

## CHAPTER 3

### Research hypothesis and objectives

#### 3.1. Research hypothesis

The purpose of this research is to determine whether that IL-10-differentiated dendritic cells can be used to therapeutically induce immunologic and disease tolerance in experimental allergic encephalomyelitis, a mouse model of multiple sclerosis.

#### 3.2. Objectives

1. To generate bone marrow derived DC10 by differentiating DC in the presence of IL-10.
2. To establish a chronic EAE model by immunizing C57BL/6 mice with MOG<sub>(35-55)</sub>.
3. To test the therapeutical effect of DC10 in mice with established EAE.

## CHAPTER 4

### Materials and Methods

#### 4.1. Generation of IL-10- differentiated dendritic cells

Bone marrow cells were flushed from the femora and tibiae of normal mice. We lysed the red blood cells by hypotonic lysis with sterile distilled water. We then resuspended the nucleated cells in RPMI-1640 supplemented with 1% antibiotics/antimycotics, 50  $\mu$ M 2-mercaptoethanol and 10% fetal bovine serum (FBS), and containing 20 ng/ml recombinant mouse granulocyte/monocyte colony-stimulating factor (rmGM-CSF), then we seeded these cells into 6-well plates (VWR, Mississauga, ON). On days 3, 6 and 8, we removed half of the medium from each dish, centrifuged it to recover any cells, and resuspended any sedimented cells in 4 ml RPMI-10%FBS supplemented with 20 ng/ml rmGM-CSF, then returned the cells to the plates. To generate immature DC, on day 10 the cells were collected by gentle pipetting and sedimented by centrifugation for 10 min at 1300 rpm (room temperature), then resuspended in 4 ml fresh RPMI-10% FBS containing 7.5 ng/ml rmGM-CSF. These cells were cultured for another 3 days before use as immature DC. To generate tolerogenic DC, the day 10 cells were instead



cultured for 3 days in 7.5 ng/ml rmGM-CSF and 50 ng/ml IL-10 [145]. On day 13, the cells were incubated for 4 hours at 37°C with antigen MOG<sub>(35-55)</sub> (MEVGWYRSPFSRVVHLYRNGK) (Genway, San Diego, CA; 50 µg/ml), or 50 µg/ml ovalbumin (OVA; Sigma, Mississauga, ON.) before use.

#### **4.2. Characterization of IL-10-differentiated dendritic cells**

On day 13,  $1-2 \times 10^5$  DC were collected and analyzed by FACS for the expression of selected APC-relevant surface markers. FITC-anti-mouse CD40 (BD, Mississauga, ON), CD80 (BD, Mississauga, ON), CD86 (BD, Mississauga, ON) and MHC II (eBioscience, San Diego, CA) were used to stain the DC (Isotype controls: CD40, hamster IgM; CD80, hamster IgG2; CD86, rat IgG2a; and MHC II, rat IgG2b) (BD, Mississauga, ON). The cells were then washed with PBS two times and fixed with equal volumes of 1% paraformaldehyde, then analyzed by FACS (Counter EPICS XL Flow cytometer, Beckman coulter, Mississauga, ON).

To assess their cytokine secretion, at the end of the 13 days of culture, the DC were washed twice with PBS and cultured in RPMI-10% FBS supplemented with 7.5 ng/ml rmGM-CSF for two more days. Supernatants were then collected and their cytokines (IL-1 $\beta$ , IL-6, IL-10 and IL-12) analyzed by ELISA.

### **4.3. Induction of EAE**

Animals: female C57BL/6 (B6) mice (6-8 week old), were purchased from our institutional breeding colony. The following reagents were purchased: MOG<sub>(35-55)</sub> (MEVGWYRSPFSRVVHLYRNGK) (Genway, San Diego, CA ; >95% purity), *Mycobacterium tuberculosis* H37Ra (Difco, Michigan), pertussis toxin (Calbiochem, CA), and Incomplete Freund's adjuvant (IFA) (Difco, Michigan).

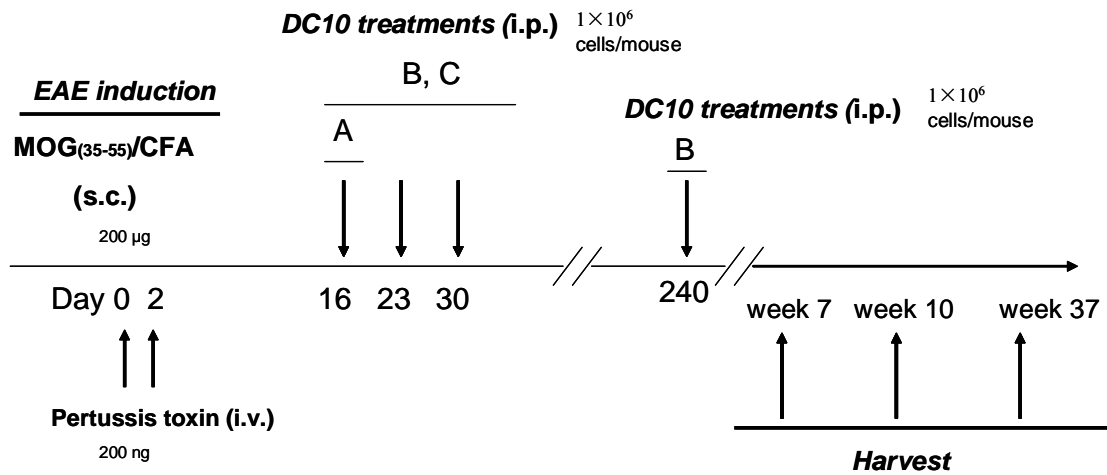
B6 mice were injected s.c. into three sites on the shaved back on day 0 with 100  $\mu$ l of 200  $\mu$ g MOG<sub>(35-55)</sub> in CFA emulsion containing 400  $\mu$ g *M tuberculosis* H37Ra. We injected 200 ng of pertussis toxin i.v (100  $\mu$  L/mouse) in the tail vein of each mouse on days 0 and 2. Pertussis toxin can promote the activation of T cells and contribute to the destruction of blood-brain barrier [146, 147]. The mice were monitored and scored in a blinded manner (experiments 2 and 3 only) for the development of clinical symptoms using the following scale: 0, normal; 1, limp tail; 2, hind limb weakness; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; 5, hind-limb and forelimb paralysis, or the animal is moribund.

### **4.4. DC10 treatment**

DC10 were pulsed with 50  $\mu$ g/mL MOG<sub>(35-55)</sub> in RPMI-10%FBS medium for 4 hours at 37°C, then the cells were washed in PBS twice to remove excess antigen and re-suspended at  $1 \times 10^6$ /ml PBS before injection ( $1 \times 10^6$

cells/mouse, i.p.). For therapeutic treatments, we injected the mice either once, or three times at weekly intervals, beginning when their clinical score had reached  $\approx 2$ . The mice were killed at 7, 10 or 37 weeks post-treatment under isoflurane (Abbott Laboratories, QC) inhalant anesthesia. In our pilot experiment, we gave EAE mice a single treatment (n=7) while in the following experiments we gave them three injections of DC10 to improve the efficiency of DC10 treatment. We also increased our animal numbers to 14-15 per group in order to reduce the standard error.

Experiments design is showed in Figure 4 below.



**Figure 4.1. Experiments design.** EAE is induced by s.c. injection of MOG<sub>(35-55)</sub>/CFA on day 0. Pertussis toxin is i.v. injected on day 0 and 2. For therapeutic treatments, we injected the mice either once, or three times at weekly intervals, beginning when their clinical score had reached  $\approx 2$ . Mice are killed on week 7, 10 or 37 after EAE induction. A, pilot exp (n=7), DC10 was injected on day 16; B, Exp 1 (n=5), DC10 was injected on day 16, 23, 30 and 240; and C, Exp 2 & 3 (n=14-15), DC10 was injected on day 16, 23 and 30.

#### **4.5. Histology staining and analysis**

Brains and spinal cords were harvested and fixed in 80% ethanol, 15% formaldehyde and 5% acetic acid for 3 hours on ice, then transferred into 70% ethanol and stored at -70°C before use. Sections (5  $\mu$  m) were prepared and stained with Hematoxylin-eosin (H&E) for evaluation of inflammatory cell infiltrates and Luxol fast blue (LFB) for demyelination. We estimate the area percentages of white matter by visual observation under light microscopy in a blinded manner using the following scoring scale:

For Demyelination (spinal cord): 0, normal; 1, mild demyelination (<10% of the total area of the dorsal or ventral white matter); 2, moderate demyelination (10%-50% of the total area of the dorsal or ventral white matter); 3, severe demyelination (>50% of the total area of the dorsal or ventral white matter).

For inflammatory cell infiltration (spinal cord): 0, normal; 1, mild infiltration (a single vessel affected, 1 layer of cells); 2, moderate infiltration (>2 vessels affected, 1-2 layers of cells); 3, severe infiltration (>2 vessels affected, >2 layers of cells).

For inflammatory cell infiltration (brain): 0, normal; 1, mild infiltration (single vessel affected, 1 layer of cells); 2, moderate infiltration (single vessel affected, >2 layers of cells or >2 vessels affected, 1-2 layers of cells); 3, severe infiltration (>2 vessels affected, >2 layers of cells).

#### **4.6. Spleen and lymph node cell culture**

Mice were killed on week 7, 10 or 37 after EAE induction. Splenocytes and LN (cervical, mesenteric and mediastinal nodes) cells were counted and cultured in RPMI-10% FBS medium at  $5 \times 10^6$  cells/ml for 72 hours in the presence ( $100 \mu\text{g/mL}$ ) or absence of MOG<sub>(35-55)</sub>. Supernatants were collected at the end of culture and cytokine (IL-4, IL-6, IL-10, IL-12, IL-17, IFN- $\gamma$  and TGF- $\beta$ ) levels were measured by ELISA.

#### **4.7. Enzyme-linked Immunosorbant Assay (ELISA)**

ELISA was used for the detection of cytokines (IL-1 $\beta$ , IL-4, IL-6, IL-10, IL-12, IL-17, IFN- $\gamma$ , TGF- $\beta$ ) in the supernatants of DC, spleen or LN cell cultures. Immulon-4 ELISA plates were incubated with capture antibodies ( $2 \mu\text{g/ml}$ ) (R & D Systems, Minneapolis, MN) in  $50 \mu\text{L/well}$  coating buffer ( $1\text{M NaHCO}_3$ ,  $1\text{M Na}_2\text{CO}_3$ ; pH 9.6) overnight at  $4^\circ\text{C}$ , and then were washed three times with PBS-0.5% Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma Chemical Co.) (PBST). We then blocked non-specific binding by adding blocking buffer (PBS-10%FBS) ( $200 \mu\text{L/well}$ ) for 2 hours at room temperature and then washed 4 times with PBST. Next, we added the recombinant cytokine standards (R & D Systems, Minneapolis, MN) diluted in PBST ( $100 \mu\text{L/well}$ ) or samples ( $100 \mu\text{L/well}$ ) to the wells, and incubated them overnight at  $4^\circ\text{C}$ , then washed the wells 4 times with PBST. Next, we added the detection antibodies (IL-1 $\beta$ , IL-6, IL-10, IL-12 and IL-17,  $200\text{ng/mL}$ ; IL-4 and IFN- $\gamma$ ,  $1 \mu\text{g/mL}$ ; TGF- $\beta$ ,  $100\text{ng/mL}$ ) (R & D Systems,

Minneapolis, MN) (100  $\mu$  L/well) and incubated for 1 hour at room temperature, and then washed 6 times with PBST. Then, 100 $\mu$ L/well of streptavidin-conjugated horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA), diluted 1:1000 in PBST, was added to each well and the plates were incubated at 37°C for 1 hour. Following 8 washes with PBST, we added 100  $\mu$  L of 12, 2'-azinobis (3-ethylbenthiiazoline-sulfonic acid) (ABTS) peroxidase substrate solution (Gaithersburg, MD) to the plates and incubated them for 5-15 min at room temperature for color development, and finally read the plates at OD 405 nm using a NOVOSTAR plate reader (BMG LABTECH, Ontario). We did not perform ELISA for CNS cytokine expression because of the limiting amounts of material available from CNS homogenates.

For antibody (IgG1, IgG2a, IgG2b) detection, 5  $\mu$  g/mL MOG<sub>(35-55)</sub> was diluted in coating buffer and 50  $\mu$  L/well was added as the capture reagent. Sera were diluted in 1:10 in PBS and added (100  $\mu$  L/well). Anti-mouse IgG1, IgG2a and IgG2b (BD, Mississauga, ON) was diluted in PBST (1  $\mu$  g/mL) and added (100  $\mu$  L/well) as the detection antibody. Streptavidin-conjugated horseradish peroxidase and ABTS Substrate Solution were subsequently added as above, and the plates were read at OD 405nm as described above.

#### **4.8. Spleen/ lymph node cell proliferation**

Proliferation was assessed by measuring the levels of  $^3\text{H}$ -methylthymidine incorporation into cellular DNA using liquid scintillation counting. Splenocytes and LN cells were harvested from mice and cultured in RPMI-10% FBS medium (200  $\mu\text{L}$ ,  $5 \times 10^5$  cells/well) in the presence (100  $\mu\text{g/ml}$ ) or absence of MOG<sub>(35-55)</sub>. The cells were cultured in triplicate for 72 hours, then pulsed with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine (GE HealthCare, Mississauga, ON) for the last 16 to 18 hours of the culture and the levels of  $^3\text{H}$ -thymidine incorporation determined by a liquid scintillation counting (Beckman coulter LS6000IC, Fullerton, CA).

#### **4.9. RNA purification and qRT-PCR analysis:**

Total RNA was purified from the CNS using RNeasy Mini Kits (Qiagen, Mississauga, ON). Briefly, brain and spinal cord tissues were harvested from mice and 600  $\mu\text{L}$  Buffer RLT (i.e., guanidinium isothiocyanate) was added. The tissues were disrupted and homogenized using a homogenizer (Kinematca, NY). The lysates were transferred into QIAshredder spin columns and centrifuged for 2 min ( $>10,000$  rpm), and the supernatants collected and mixed with 1 volume of 70% ethanol. About 700  $\mu\text{L}$  of sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at  $>10,000$  rpm. The flow-through was discarded and 700  $\mu\text{L}$  Buffer RW1 (contains guanidine salt) was added to the RNeasy column and centrifuged for 15 s at  $>10,000$  rpm to wash the spin column.



The flow-through was again discarded and 500  $\mu$  L of Buffer RPE was added to the RNeasy spin column to wash the column 2 times. We then placed the RNeasy spin column in a new 1.5 ml collection tube and add 50  $\mu$  L RNase-free water directly to the column membrane and spun the column for 1 min at >10,000 rpm to elute the RNA, which was stored at -80°C for later use.

The RNA was reverse-transcribed to DNA using quantitative real time (qRT)-PCR. qRT-PCR analysis was carried out using Brilliant II SYBR Green qRT-PCR Master Mix Kits (Stratagene, La Jolla, CA). The reactions were run using a Mx3005P Instrument (Stratagene, La Jolla, CA) as follows: segment 1 (1 cycle), 50°C for 30 min and 95°C for 10 min; segment 2 (40 cycles), 95°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec; segment 3, the reading was taken at 72°C during the 30-sec plateau. All mRNA levels were normalized to mouse  $\beta$ -actin levels. The results were calculated as quantities relative to the calibrators, where the calibrator samples were assigned a value of 1 and all the samples are expressed as the fold-changes relative to the calibrators. The formula used for the analysis was [148]:

$$\text{Relative quantity to calibrator} = 2^{-(\Delta\Delta Ct)};$$

$$\text{Where } \Delta\Delta Ct = (Ct_{GOI} - Ct_{Normalizer})_{Calibrator} - (Ct_{GOI} - Ct_{Normalizer})_{unknown}$$

**Table 4.1. The sequences of primers for qRT-PCR**

Host	Primer Name	Length (bp)	Primer Sequence
Mouse	$\beta$ -actin F	20	AGAGGGAAATCGTGCGTGAC
Mouse	$\beta$ -actin R	21	CAATAGTGATGACCTGGCCGT
Mouse	IFN- $\gamma$ F	23	TCAAGTGGCATAGATGTGGAAGA
Mouse	IFN- $\gamma$ R	22	TCAGGTGTGATTCAATGACGCT
Mouse	IL-4 F	19	ACAGGAGAAGGGACGCCAT
Mouse	IL-4 R	21	GAAGCCCTACAGACGAGCTCA
Mouse	IL-10 F	22	AAGCCTTATCGGAAATGATCCA
Mouse	IL-10 R	22	GCTCCACTGCCTTGCTCTTATT
Mouse	IL-12 p40 F	24	GAAGTTCAACATCAAGAGCAGTAG
Mouse	IL-12 p40 R	20	AGGGAGAAGTAGGAATGGGG
Mouse	IL-17 F	23	ATCTGTGTCTCTGATGCTGTTGC
Mouse	IL-17 R	20	CACGCTGAGCTTTGAGGGA
Mouse	IL-23 p19 F	23	CACCTGCTTGACTCTGACATCTT
Mouse	IL-23 p19 R	22	GCTGCCACTGCTGACTAGAACT
Mouse	TGF- $\beta$ F	20	GACTCTCCACCTGCAAGACCAT
Mouse	TGF- $\beta$ R	22	GGGACTGGCGAGCCTTAGTT
Mouse	FOXP3 F	23	CTTTCACCTATGCCACCCTTATC
Mouse	FOXP3 R	23	TCAAATTCATCTACGGTCCACAC

(F=Forward, R=Reverse; GOI: gene of interest; Ct: the numbers of cycles required for the fluorescent signal to cross the threshold (background level))

## **5.0. Statistics**

All data were expressed as the mean  $\pm$  SEM. Clinical scores were analyzed by repeated measure analysis-of-variance (ANOVA). Other group differences were analyzed by one-way ANOVA with Fisher's LSD post-hoc testing. Differences were considered significant when P-values were less than 0.05.

## CHAPTER 5

### Results

#### 5.1. *In vitro* characterization of IL-10-differentiated DC

##### 5.1.1. DC treated with IL-10 do not show increased expression of CD40, CD80, CD86 or MHC II relative to immature DC

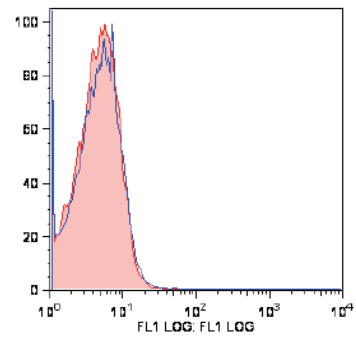
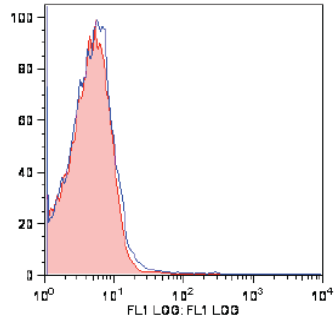
IL-10-differentiated DC were generated *in vitro* by differentiation in the presence of IL-10 and then their expression of CD40, CD80, CD86 and MHC II was assessed by FACS using FITC-labeled Abs. DCs were harvested on day 13 of culture. The results were shown in Fig. 5.1.1. FACS analysis confirmed that IL-10-differentiated DC (DC10) did not show increased expression of the DC markers CD40 (Mean fluorescence intensity [MFI]: 6.56 vs 12.7), CD80 (MFI: 61 vs 88.7), CD86 (MFI: 19.1 vs 17.8) or MHC II (MFI: 86 vs 107) relative to immature DC (DC(GM-CSF)), indicating that IL-10 does not induce high expression of these molecules as compared with DC generated without IL-10 (DC(GM-CSF)).

**Figure. 5.1.1. Expression of CD40, CD80, CD86 and MHC II by the DC populations assessed by FACS.** Bone marrow cells were cultured in RPMI-10% FBS containing 20 ng/ml GM-CSF for 10 days. On day 10, cells were cultured in RPMI-10%FBS containing 7.5 ng/ml GM-CSF and IL-10 (50 ng/ml) to generate tolerogenic DC (DC10). For immature DC generation (DC(GM-CSF)), cells were cultured in RPMI-10%FBS containing 7.5 ng/ml GM-CSF alone. On day 13, the cells were stained for CD40, CD80, CD86 and MHC II and analyzed by FACS using FITC-labeled specific (solid line) and isotype controls (shaded) antibodies.

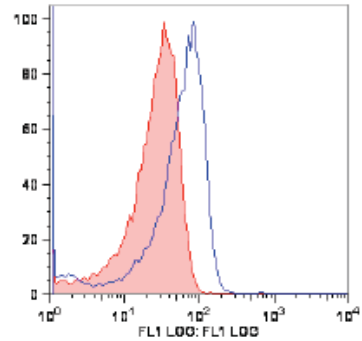
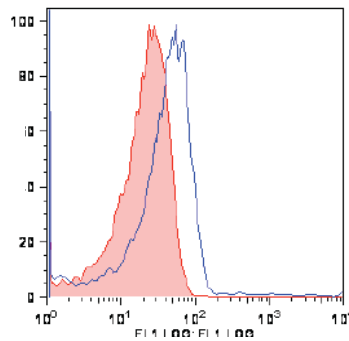
DC(GM-CSF)

DC10

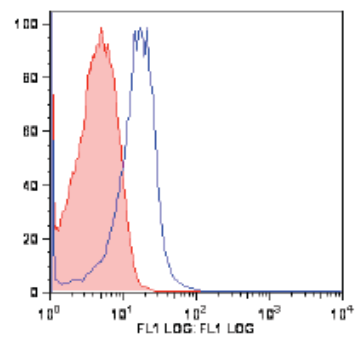
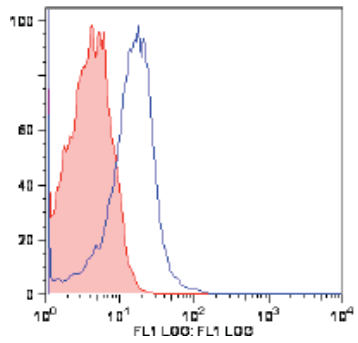
CD40



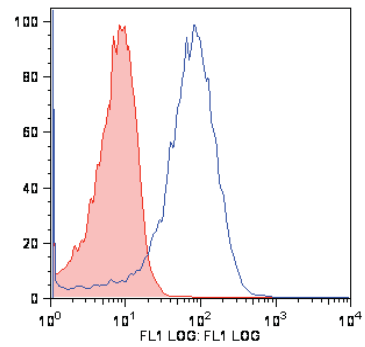
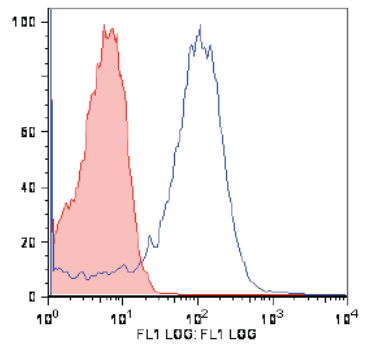
CD80



CD86

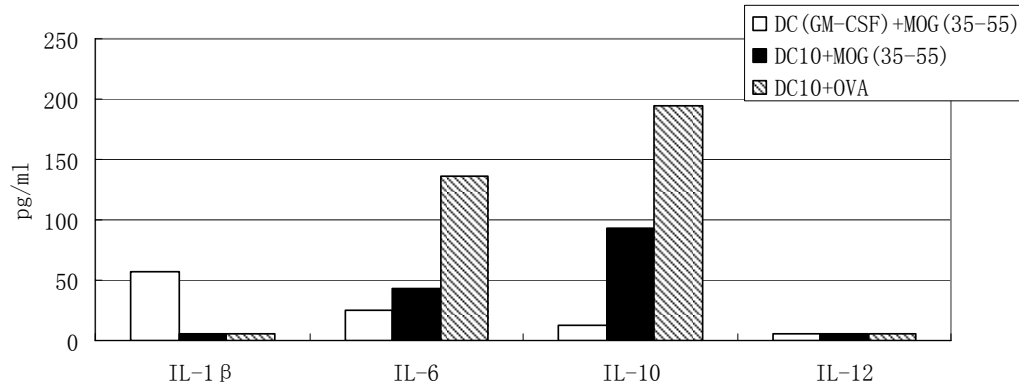


MHC II



### **5.1.2. IL-10-differentiated DC produce similar levels of IL-1 $\beta$ , IL-6 and IL-12, but higher levels of IL-10 relative to immature DC**

At the end of the DC culture (day 13) from Fig 5.1.1, DC were washed twice with PBS and culture for two more days. Supernatants were collected and their IL-1  $\beta$  , IL-6, IL-10 and IL-12 levels were assessed by ELISA. Fig. 5.1.2 shows that DC10 secreted low levels of IL-1  $\beta$  , IL-6 and IL-12, like DC(GM-CSF), but higher levels of IL-10. It was interesting that OVA-pulsed DC10 (DC10+OVA) showed a higher secretion of IL-6 and IL-10 as compared with MOG<sub>(35-55)</sub>-pulsed DC10 (DC10+MOG<sub>(35-55)</sub>), suggesting that OVA may provide stimulatory signals for production of these cytokines by DC10. Taken together, the secretion of low levels of proinflammatory cytokines like IL-1  $\beta$  , IL-6 and IL-12 but higher levels of IL-10 further suggested that these DC10 express a tolerogenic phenotype. We don't think DC10+OVA here could be used as effectively tolerogenic treatment to EAE because of its antigen non-specificity. The relative tolerogenic potentials of MOG-DC10 vs OVA-DC10 is discussed further in the 'Discussion' section.



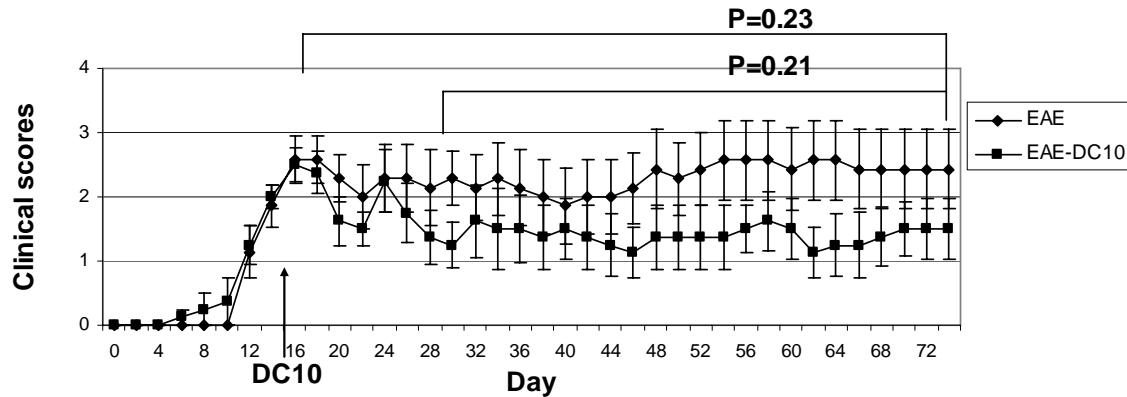
**Figure 5.1.2. Cytokine production by DC10 and DC(GM-CSF).** DC 10 and DC (GM-CSF) were generated as described in Fig.5.1.1. At the end of culture (day 13), DC were pulsed with MOG<sub>(35-55)</sub> or OVA (50 μg/ml) for 4 hours and then washed twice with PBS and cultured for two more days. Supernatants were collected and IL-1β, IL-6, IL-10 and IL-12 levels were assessed by ELISA. Statistic was not done because cells from all mice were pooled. Data are representative of two experiments.



## **5.2. Treatment of established EAE mice with DC10**

### **5.2.1. Impact of DC10 treatment in EAE**

The aim of our research was to test whether DC10 could be used to effectively treat on-going EAE. For the treatment, we tried both a single and repetitive injections of DC10. For the single treatment, DC10 were injected i.p. ( $1 \times 10^6$  cells/mouse) when the average clinical score had reached  $\approx 2$  (i.e., when the mice had developed readily discernible symptoms of EAE [specifically, hind limb weakness]). As shown in Fig. 5.2.1., we found that the EAE mice treated with DC10 showed a lower mean value for their clinical score (1.5 vs 2.0-2.5), although there was no significant difference between the DC treatment group and non-treatment group (From day 16 to 74,  $p=0.23$ ; from day 30 to 74,  $p=0.21$ ).

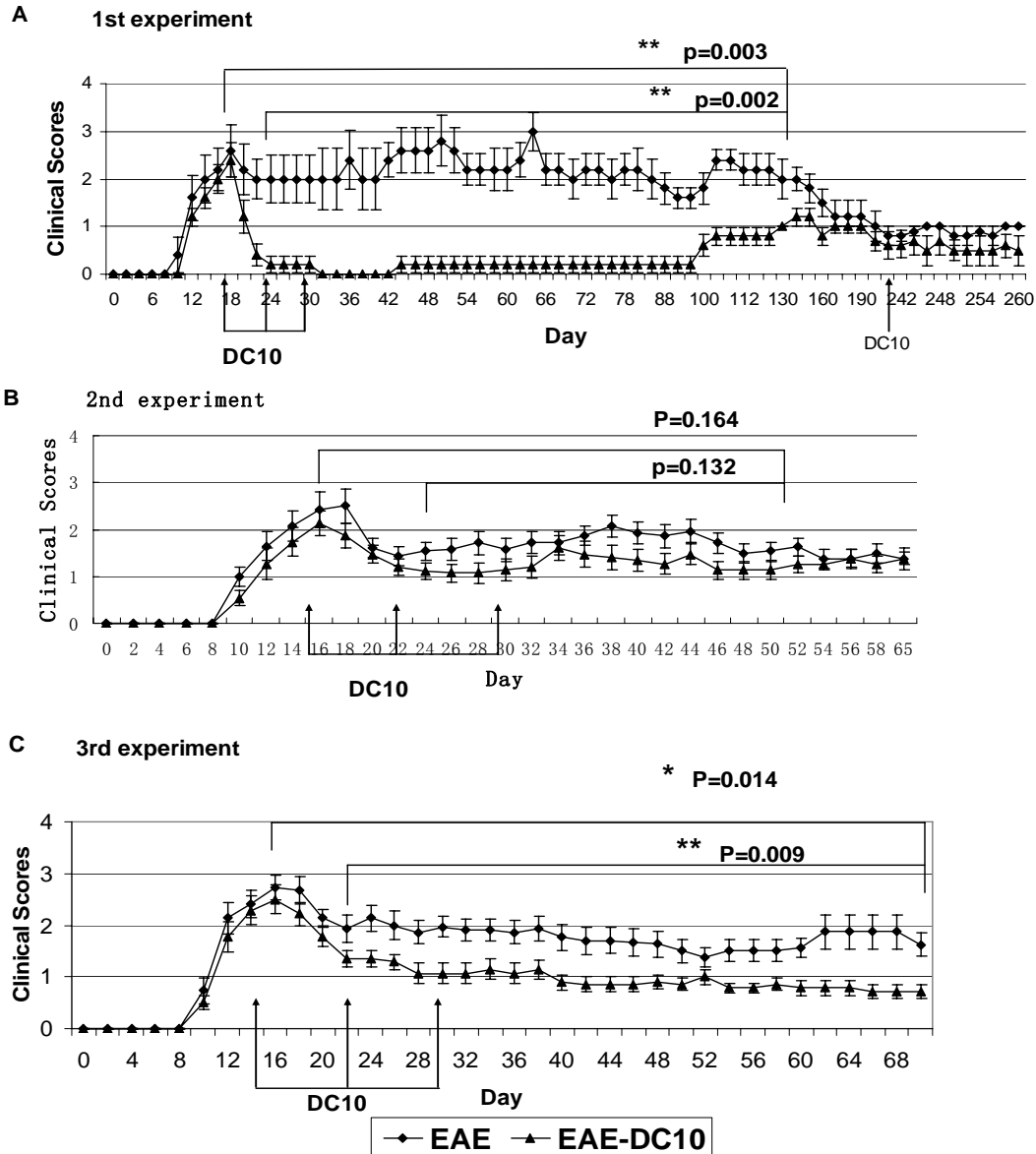


**Figure 5.2.1. Clinical scores of EAE mice in response to a single DC10 treatment.** C57BL/6 mice were immunized with 200 ng MOG<sub>(35-55)</sub>/CFA containing 400  $\mu$ g *M. tuberculosis* H37Ra into three sites on the shaved back on day 0. Pertussis toxin was given i.v. (200 ng/mouse) in the tail vein of each mouse on days 0 and 2. (Disease incidence: 71%). For the single DC10 treatment, DC10 were injected i.p. ( $1 \times 10^6$  cells/mouse) when the clinical score had reached  $\approx 2$  (day 16, black arrow). The mice were monitored and scored (not blinded in this experiment) for the development of clinical symptoms (n=7) using the following scale: 0, normal; 1, limp tail; 2, hind limb weakness; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; 5, hind-limb and forelimb paralysis or the animal is moribund. This experiment was done once. All P values were determined by repeated measure analysis-of-variance (ANOVA).

### **5.2.2. Assessment of DC10 as a therapeutic approach for EAE**

To improve the efficiency of DC10 treatment, we performed repetitive injections, giving DC10 i.p every week for 3 weeks beginning when their clinical scores were  $\approx 2$ . As shown in Fig. 5.2.2., we repeated this experiment 3 times. The clinical scores in the 1<sup>st</sup> experiment were not determined in a blinded fashion while the 2<sup>nd</sup> and 3<sup>rd</sup> experiments were. In 2 of our experiments, we found that DC10 significantly inhibited EAE development ( $p < 0.05$ ), as determined using repeated measure ANOVA assays. As shown in Fig. 5.2.2.A (the 1<sup>st</sup> experiment), DC significantly decreased the clinical scores in EAE mice (From day 16 to 130,  $p = 0.003$ ; from day 24 to 130,  $p = 0.002$ ). The therapeutic effect started from about day 24 (i.e, 8 days after the treatment started) and lasted for about 120-130 days after EAE induction (mean clinical score: 0-1.0). Compared to the DC10 treatment group, the PBS-treated group maintained their EAE clinical scores at around 1.5-2.5. The DC10 treatment group mice reverted to the EAE phenotype at about day 100-112. We then gave these reversed DC10 treated-mice one more DC10 treatment on day 240 to see whether this could reestablish tolerance, but we did not observe any significant difference subsequent to this 4<sup>th</sup> DC10 treatment.

In our second experiment, we did not observe any statistical difference in clinical scores. As shown in Fig. 5.2.2.B, although there was no



**Figure 5.2.2. Clinical scores of EAE mice in response to repeated treatment with DC10.** C57BL/6 mice were immunized and scored as described. For the repetitive DC10 treatment, DC10 were injected i.p weekly for 3 wk ( $1 \times 10^6$  cells/mouse) when the clinical score had reached  $\approx 2$  (Day 16, 23, 30 or 240; black arrows)). The mice were monitored and scored for the development of clinical symptoms (A,  $n=5$ ; B and C,  $n=14-15$ ). Only experiment 2 and 3 were scored in blinded manner. Disease incidence in 1<sup>st</sup> exp., 69%. 2<sup>nd</sup> exp., 97%. 3<sup>rd</sup> exp. 100%. All P values were determined by repeated measure ANOVA.

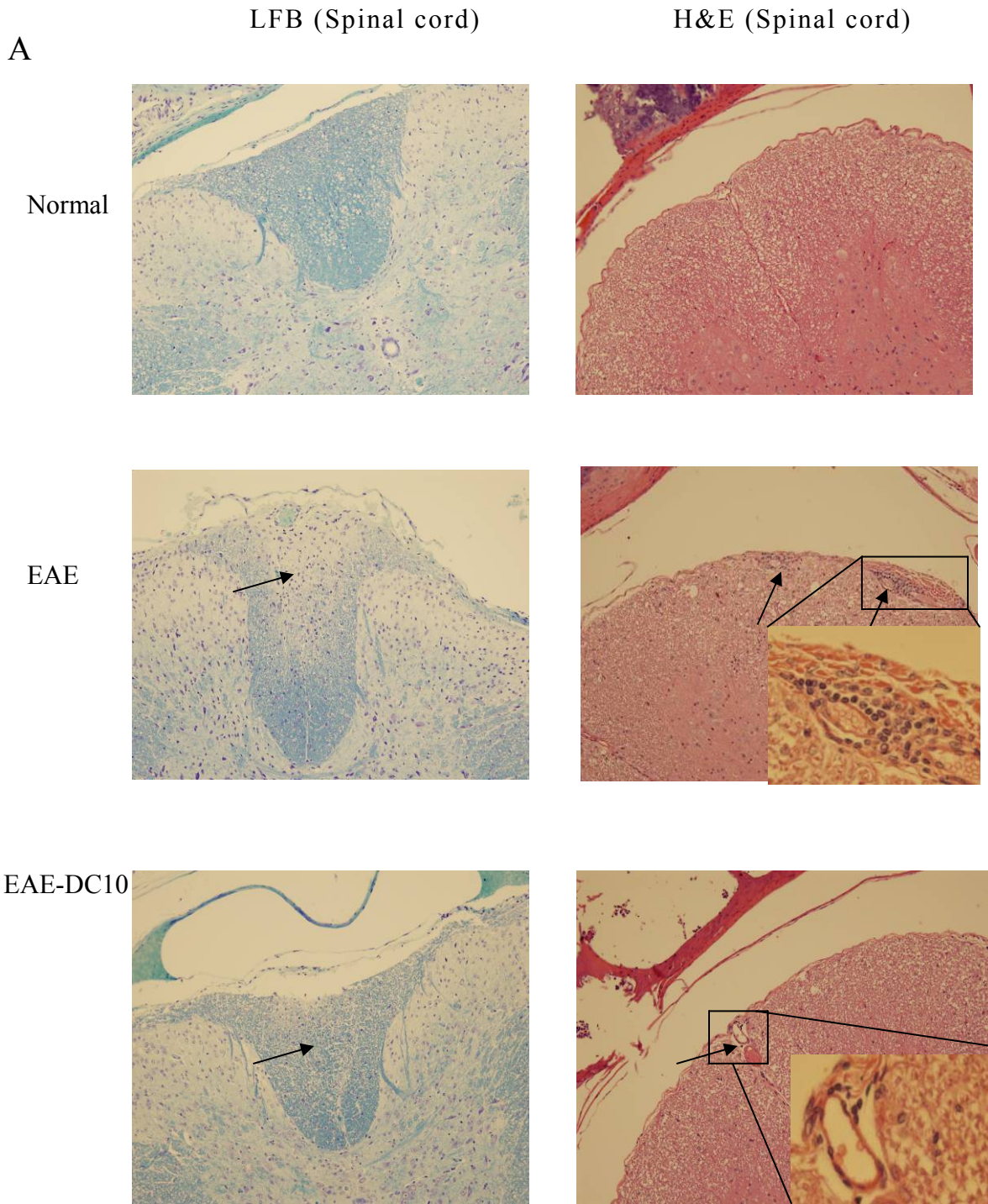
significant difference (From day 16 to 52,  $p=0.164$ ; from day 24 to 52,  $p=0.132$ ) in the clinical scores between the PBS-treated and DC10-treated EAE groups, nevertheless the DC10-treatment group had lower clinical score values than the PBS-treatment group. The reasons why there was no statistical difference in clinical scores in this experiment are unclear, but could probably due to the variance of DC10 treatment effects (i.e, the activity and the function of DC10 generated for these experiments). Such effects could also in part explain the reason why the DC10 treatments work so well in the first experiment (i.e, DC10 treatments reduced clinical scores to almost 0.). Other factors such as stress could also affect the clinical scores, as it has been suggested that stress can influence EAE susceptibility [149].

Similarly, in the 3<sup>rd</sup> experiment, as shown in Fig. 5.2.2.C, DC10 were also injected i.p on days 16, 23 and 30. In the DC10 treatment group, the clinical score decreased significantly after the DC10 treatments. After 3 injections, we did not see a relapse of the EAE clinical scores as compared with PBS-treatment group (From day 16 to 70,  $p=0.014$ ; from day 24 to 70,  $p=0.009$ ).

### **5.3. DC10 treatment reduces demyelination and cell inflammation in the spinal cord**

EAE is characterized by demyelination and inflammatory cell infiltration within the CNS, both of which contribute to the paralysis observed clinically. Herein, we wanted to determine whether our DC10 treatments could reduce demyelination and cell infiltration in the CNS in our EAE model. In our histological studies, MOG-induced EAE mice developed cellular infiltration and demyelination within the white matter of the spinal cord (Fig. 5.3A). We also found foci of cell infiltration in the brains of the EAE mice, but there was no significant difference as compared with normal mice ( $p>0.05$ ), and there was also no demyelination observed in the brains of the EAE mice (Fig. 5.3.B, C and D). Fig. 5.3.A depicts the spinal cord histology in normal, EAE and EAE-DC10 mice in our experiments. In the 1<sup>st</sup> experiment (Fig. 5.3 B), the mice were harvested at 37 wk. At this time we did not see any significant difference in demyelination or cellular infiltration into the CNS between the DC10- and PBS-treated groups, although there is a lower level of demyelination in the DC10 treatment group ( $p=0.063$ ) within the spinal cords. One potential reason why we did not see significant differences in demyelination and infiltration could be that these effects had declined to background at this a late stage of disease, although we have no evidence to support this. In the 2<sup>nd</sup> experiment (Fig. 5.3C), the DC10 treatments significantly reduced demyelination in the spinal cord at 7 wk, but there was

**Figure 5.3. Histological evaluation of the impact of DC10 treatment on brain and spinal cord inflammation and demyelination in EAE mice.** C57BL/6 mice were immunized and treated with DC10 as described. Mice were killed on week 7, week 10 or week 37. Spinal cords and brains were fixed and stained with hematoxylin-eosin (H&E) to determine cellular infiltration or luxol fast blue (LFB) to evaluate demyelination (original magnification, X200; or under oil immersion lens, X600). (A), Photomicrographs of the spinal cord lesions in normal, EAE and EAE-DC10 mice (7 wk, 3<sup>rd</sup> experiment). (B), Histology scores of spinal cords (LFB and H&E) and brains (H&E) in the 1<sup>st</sup> experiment (37 wk, n=5). (C), Histology scores of spinal cords (LFB and H&E) and brains (H&E) in the 2<sup>nd</sup> experiment (7 wk, started with 14 animals, 7 from them were killed at 7 wk). (D), Histology scores of spinal cords (LFB and H&E) and brains (H&E) in the 3<sup>rd</sup> experiment (7 and 10 wk, started with 15 animals, 7 from them were killed at 7 wk, then with 7 from them until wk 10). ND: Not determined. No demyelination was observed in the brain. Photomicrographs were representative of 3 independent experiments. Histology scores were examined by light microscopy in a blinded manner using the following scoring scale: For Demyelination (spinal cord): 0, normal; 1, mild demyelination (<10% of the total area of the dorsal or ventral white matter); 2, moderate demyelination (10%-50% of the total area of the dorsal or ventral white matter); 3, severe demyelination (>50% of the total area of the dorsal or ventral white matter). For inflammatory cell infiltration (spinal cord): 0, normal; 1, mild infiltration (a single vessel affected, 1 layer of cells); 2, moderate infiltration (>2 vessels affected, 1-2 layers of cells); 3, severe infiltration (>2 vessels affected, >2 layers of cells). For inflammatory cell infiltration (brain): 0, normal; 1, mild infiltration (single vessel affected, 1 layer of cells); 2, moderate infiltration (single vessel affected, >2 layers of cells or >2 vessels affected, 1-2 layers of cells); 3, severe infiltration (>2 vessels affected, >2 layers of cells). All P values were determined by ANOVA. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001.





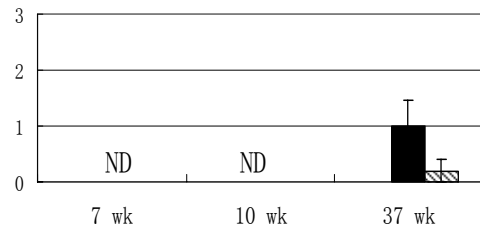
## Spinal cord-Demyelination

## Spinal cord-Infiltration

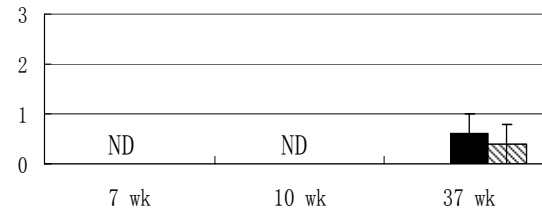
## Brain-Infiltration

B

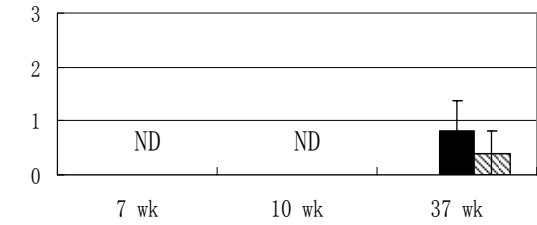
1st experiment



1st experiment

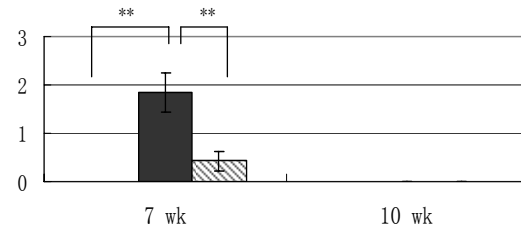


1st experiment

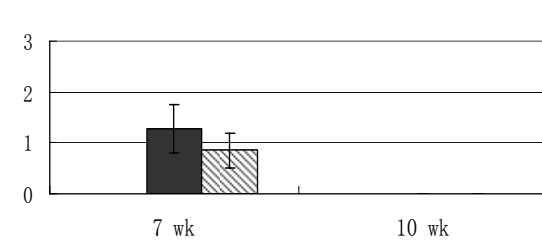


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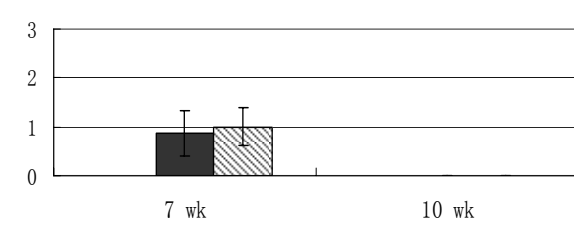
2nd experiment



2nd experiment

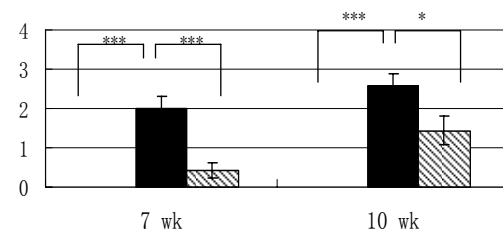


2nd experiment

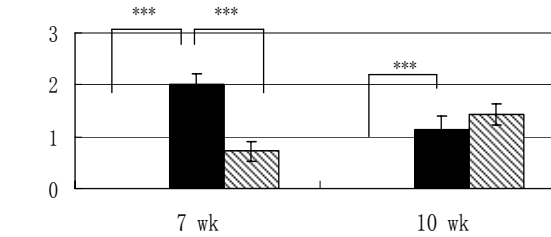


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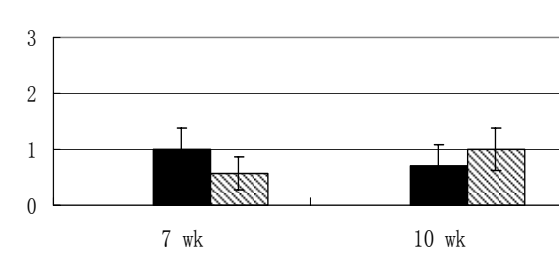
3rd experiment



3rd experiment



3rd experiment



Normal



EAE



EAE-DC10

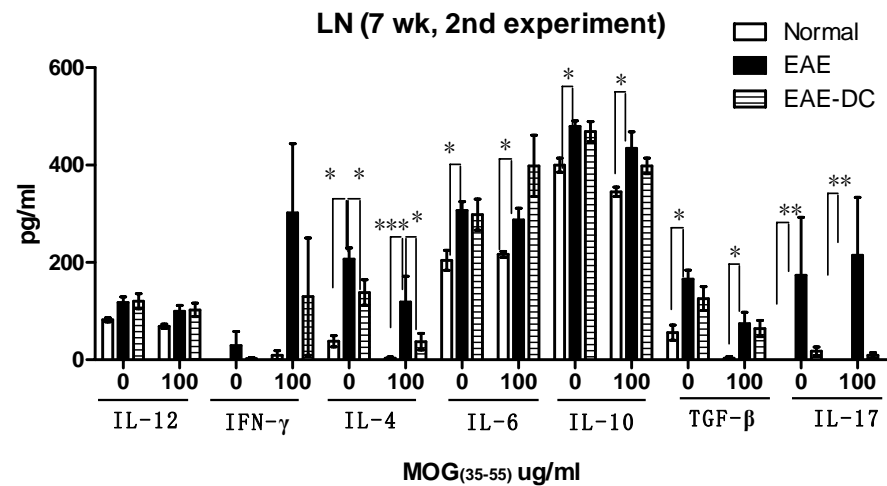
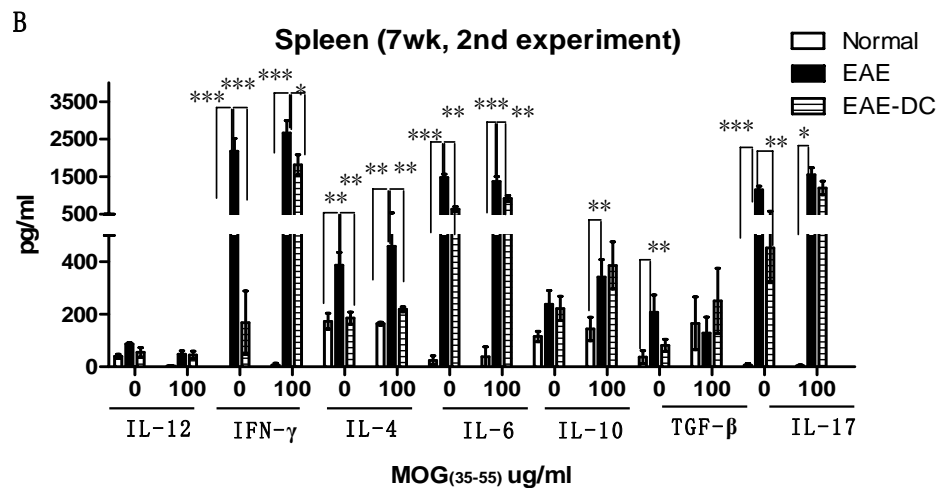
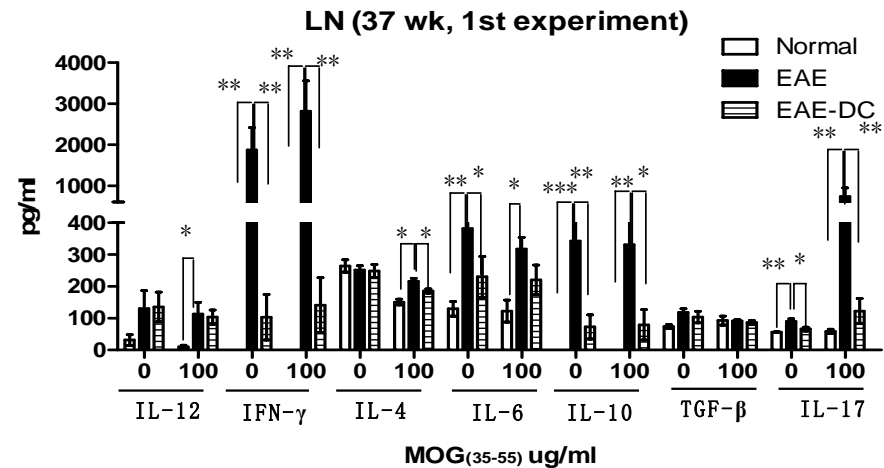
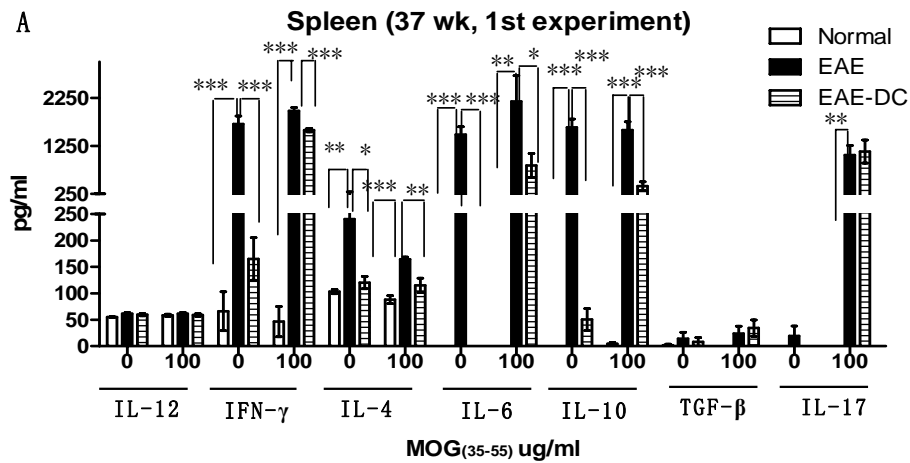
no significant difference ( $p>0.05$ ) in inflammatory cell infiltrates in spinal cord or brain. In the 3<sup>rd</sup> experiment (Fig. 5.3D), the DC treatment significantly reduced demyelination in the spinal cord at 7 wk and 10 wk, as well as cell infiltration at 7 wk, but there was no significant difference in inflammatory cell infiltrates in the spinal cord at 10 wk. We did not observe any significant differences in cell infiltration of the brain at 7 wk or 10 wk.

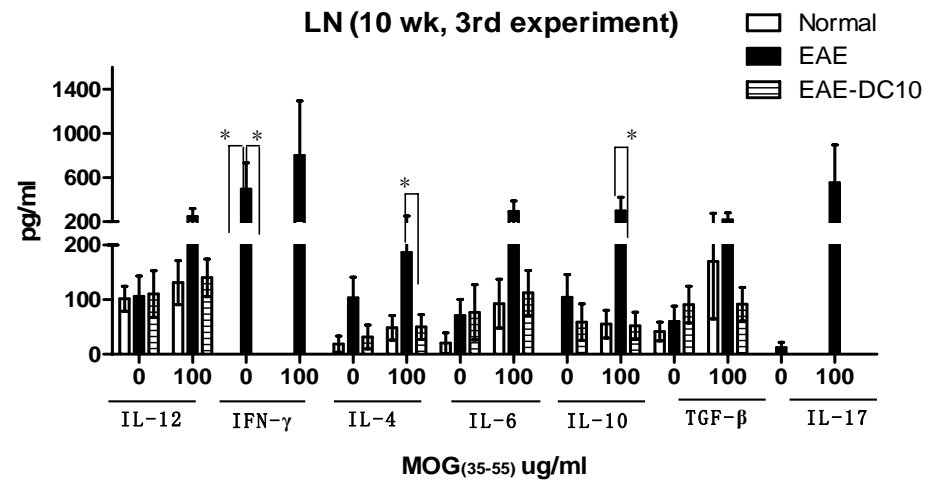
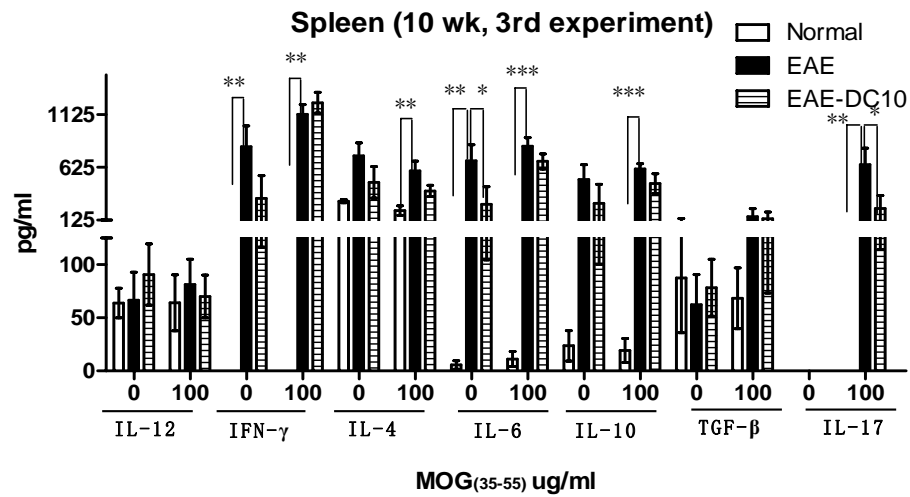
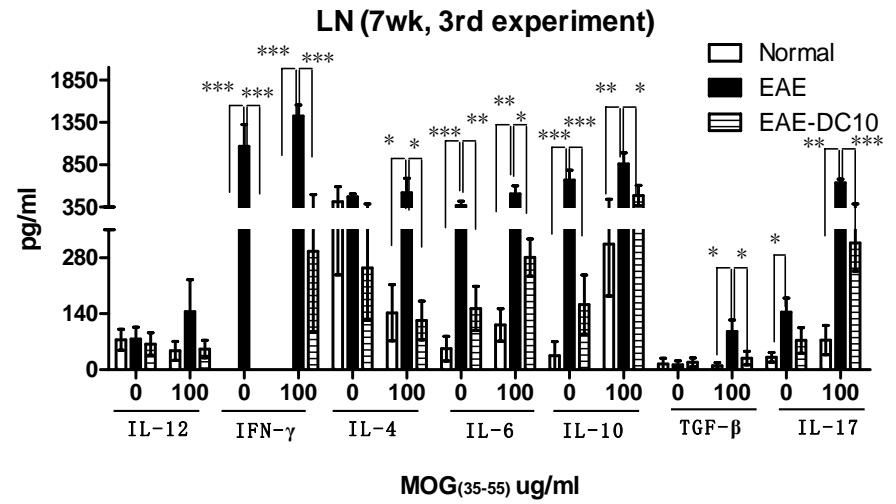
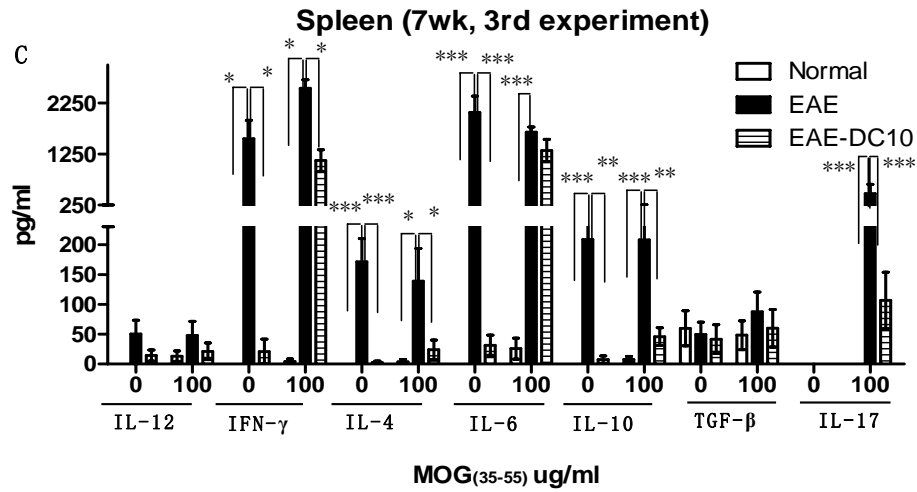
#### **5.4. DC10 treatment reduces inflammatory cytokine production in the spleens and lymph nodes**

To investigate how DC10 treatments affected the production of Th1/Th2/Th17 cytokines, we performed ELISA on the 3 day culture supernatants generating from total spleen and LN cells that were stimulated with or without MOG<sub>(35-55)</sub>. In the 1<sup>st</sup> experiment (Fig. 5.4 A), we showed that the DC10 treatments had significantly reduced the production of IFN- $\gamma$ , IL-4, IL-6 and IL-10 in the spleen and IFN- $\gamma$ , IL-4, IL-6, IL-10 and IL-17 in the LN. We did not see much difference in IL-12 and TGF- $\beta$  expression in between the PBS- and DC10-treated EAE groups. In the 2<sup>nd</sup> experiment (Fig. 5.4B), we found that the DC10 treatments had significantly reduced IFN- $\gamma$ , IL-4, IL-6, and IL-17 expression in the spleen (7 wk). In the LN, the DC10 treatment significantly decreased only IL-4 production (7 wk), but there was no significant difference in the IL-17 expression ( $p=0.056$ ) in the LN. In the 3<sup>rd</sup> experiment (Fig. 5.4C), we found that DC10 treatments significantly reduced IFN- $\gamma$ , IL-4, IL-6, IL-10 and IL-17 in the spleen and IFN- $\gamma$ , IL-4, IL-6, IL-10, TGF- $\beta$  and IL-17 in the LN (7 wk). We also found that the DC10 treatment had significantly reduced IL-6 and IL-17 expression in the spleen and IFN- $\gamma$ , IL-4 and IL-10 in the LN at 10 wk, but there were no statistically significant differences in any other cytokines. Nevertheless there was a lower level of IFN- $\gamma$  ( $p=0.062$ ) and IL-4 ( $p=0.06$ ) in the spleen

and IL-6 ( $p=0.065$ ) and IL-17 ( $p=0.073$ ) in the LN in response to the DC10 treatment.

**Figure 5.4. The impact of DC10 treatment on spleen and lymph node cell cytokine production in EAE.** C57BL/6 mice were immunized as described. Mice were killed on week 7, 10 or 37 wk after EAE induction. Total cells from the spleens and lymph nodes were counted and cultured in RPMI-10% FBS medium at  $5 \times 10^6$  cells/mL. These cells were cultured for 72 hours in the presence or absence of MOG<sub>(35-55)</sub> (0 or 100  $\mu$ g/mL) (n=5-7). Supernatants were collected at the end of culture and IL-4, IL-6, IL-10, IL-12, IL-17, IFN- $\gamma$  and TGF- $\beta$  levels were measured by ELISA. A, 1<sup>st</sup> experiment; B, 2<sup>nd</sup> experiment; C, 3<sup>rd</sup> experiment. All P values were determined by ANOVA. \*P<0.05, \*\* P<0.01, \*\*\* P<0.001.



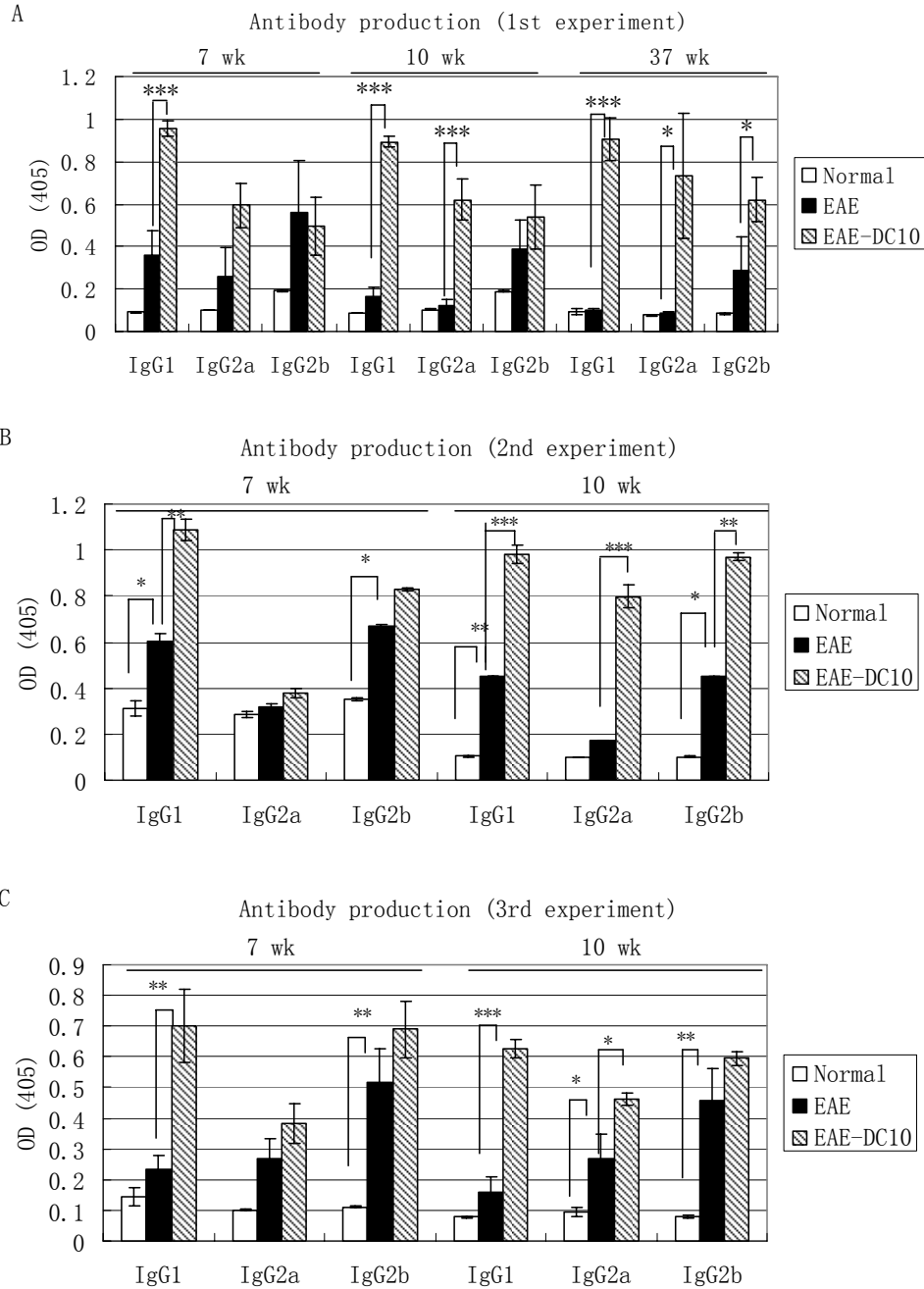


In these experiments, IL-4, IL-6, IL-10, IL-12, IFN- $\gamma$  and TGF- $\beta$  levels did not markedly increase in response to antigen, suggesting that the production of these cytokines might not be antigen-specific, or the cells were already activated to produce cytokines *in vivo*, perhaps due to the leakage of myelin antigens from the CNS, or to *in situ* antigen presentation by CNS-derived DC. Taken together, despite some occasional exceptions, our data shows that DC10 treatments suppressed the production of inflammatory cytokines in secondary lymphoid organs, suggesting that DC10 treatments suppress the activation of inflammatory cells in the spleens and LNs of mice with EAE.



### **5.5. Effect of DC10 treatment on the production of IgG1, IgG2a and IgG2b:**

The roles of autoantibodies in EAE are still not clear, although increase in their levels have been reported by others [94]. To investigate the influence of DC10 treatments on the production of MOG<sub>(35-55)</sub>-specific IgG1, IgG2a and IgG2b in EAE, we collected the serum on week 7, 10 or 37 after EAE induction and measured the levels of these antibodies by ELISA. In the 1<sup>st</sup> experiment (Fig. 5.5A), we found that DC10 treatments significantly increased IgG1 production on week 7, 10 and 37; IgG1 and IgG2a production on week 10 and 37; and IgG1, IgG2a and IgG2b production on week 37; In the 2<sup>nd</sup> experiment (Fig. 5.5B), we also found that the DC10 treatments increased IgG1 on week 7, although no differences in IgG2a and IgG2b levels in the PBS- and DC10-treated groups were observed at this time. We also found that DC10 treatments significantly increased IgG1, IgG2a and IgG2b levels on week 10. Similarly, in the 3<sup>rd</sup> experiment (Fig. 5.5 C), we showed that DC10 treatments significantly increased IgG1 on week 7, as well as IgG1 and IgG2a in week 10. Though it is not clear why DC10 increased these antibody responses, we query whether the DC10 treatments may have in part inhibited EAE through their effects on the MOG<sub>(35-55)</sub>-specific IgG1, IgG2a and IgG2b responses [82].

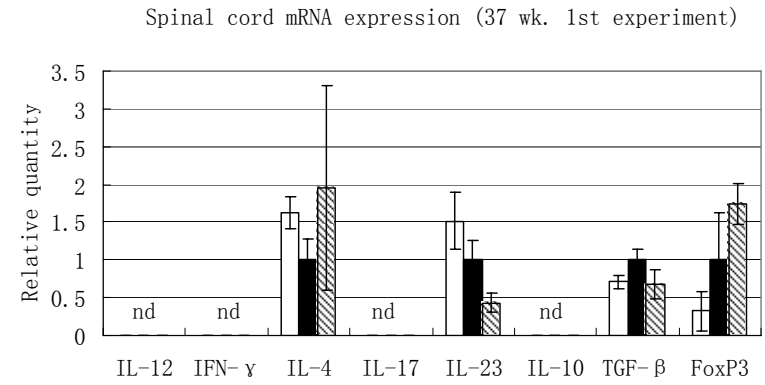
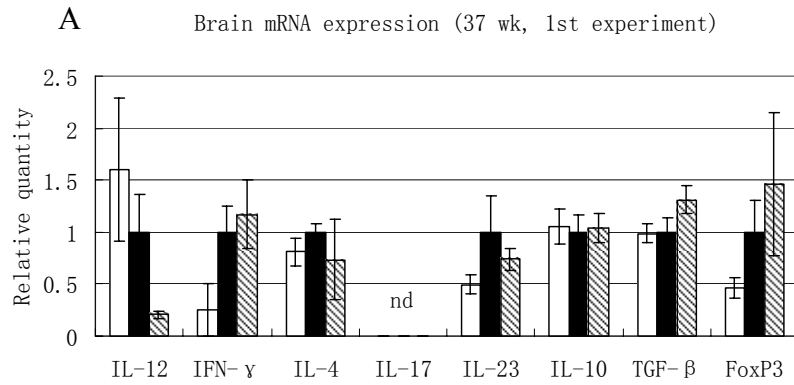


**Figure 5.5. Impact of DC10 treatments on MOG-specific antibody production in EAE mice.** C57BL/6 mouse were immunized as described. Serum was collected on week 7, 10 or 37 after EAE induction, and MOG<sub>(35-55)</sub>-specific IgG1, IgG2a and IgG2b levels were measured by ELISA as described (n=5-7). Fig. 5.5 shows the data from the 1<sup>st</sup> (A), 2<sup>nd</sup> (B) and 3<sup>rd</sup> (C) experiment. All P values were determined by ANOVA. \*P<0.05, \*\* P<0.01, \*\*\* P<0.001.

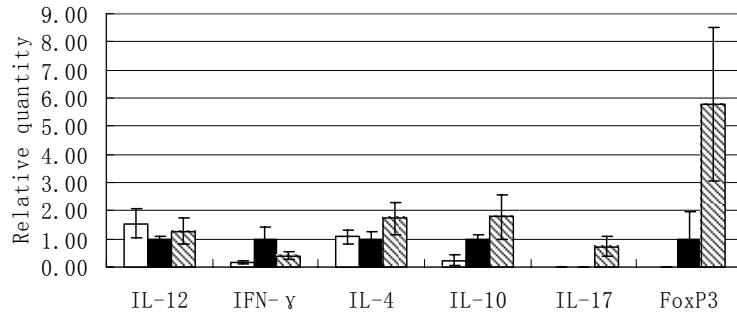
## **5.6. DC10 treatments did not have significant effects on inflammatory or immunoregulatory cytokine mRNA expression in the CNS of EAE mice**

Inflammatory cytokines produced by infiltrating cells contribute to the inflammatory response in the CNS and thereby can significantly affect EAE [2, 46]. Herein we used qRT-PCR to compare the expression of a number of cytokines in the CNS of DC10- and PBS-treated EAE mice, using total RNA purified from the CNS. In none of the three experiments (Fig. 5.6) we observed significant differences in the expression of IL-12, IFN- $\gamma$ , IL-4, IL-17, IL-23 or TGF- $\beta$  in the CNS of EAE mice as compared with normal controls. In the 1<sup>st</sup> experiment (Fig. 5.6A) in which the mice were harvested at a late stage (37 wk after EAE induction), we did not detect any expression of IL-12, IFN- $\gamma$ , IL-10 or IL-17 in spinal cords or IL-17 in the brains in this experiment. In response to the DC10 treatments, we did not observe significant differences in expression of most of these cytokines. In the 2<sup>nd</sup> experiment (Fig. 5.6B), we observed a similar pattern, but we also found a significant higher expression of IL-10 in the spinal cord in response to DC10 treatments (7 wk), and a higher expression of FoxP3 ( $p=0.07$ ) in the brain (7 wk), but there was no significant difference. In the 3<sup>rd</sup> experiment (Fig. 5.6C), on wk 7 we found a significant increase in FoxP3 mRNA in the brains and IL-10 mRNA expression in the spinal cords. It suggests that Tregs were induced in the CNS or migrated from periphery into the CNS. But it still needs to be investigated further, as in two of our experiments there was no discernible induction of Foxp3, while in the third there was.

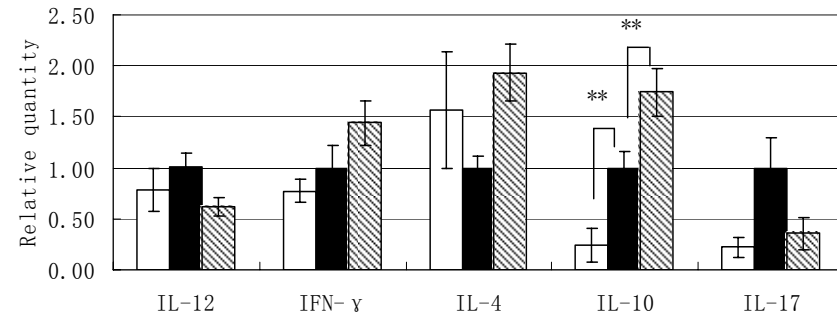
**Figure 5.6. The impact of DC10 treatments on inflammatory cytokine expression in the CNS of EAE mice.** C57BL/6 mice were immunized as described. Mice were killed on week 7, 10 or 37 and total RNA was purified from the brains and spinal cords (n=5-7). IL-4, IL-10, IL-12, IL-17, IL-23, IFN- $\gamma$ , FoxP3 and TGF- $\beta$  expression in the brains and spinal cords were measured by qRT-PCR. We did not analyze FoxP3 for spinal cord, 7 weeks in the 2<sup>nd</sup> experiment because of the limitation of low mRNA extraction. The reaction conditions used were: segment 1 (1 cycle), 50°C for 30 min and 95°C for 10 min; segment 2 (40 cycles), 50°C for 30 min, 95°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec; segment 3, the reading was taken at 72°C during the 30-sec plateau. All mRNA levels were normalized to mouse  $\beta$ -actin levels. Results were calculated as relative to the calibrators, where the calibrator samples were assigned a quantity of 1 and all the samples are expressed as fold-changes relative to calibrators. All samples were processed individually. Fig. 5.6 shows the data from the 1<sup>st</sup> (A), 2<sup>nd</sup> (B) and 3<sup>rd</sup> (C) experiment (nd, not detectable). All P values were determined by ANOVA. \*P<0.05, \*\* P<0.01.



**B** Brain mRNA expression (7 wk, 2nd experiment)



Spinal cord mRNA expression (7 wk, 2nd experiment)



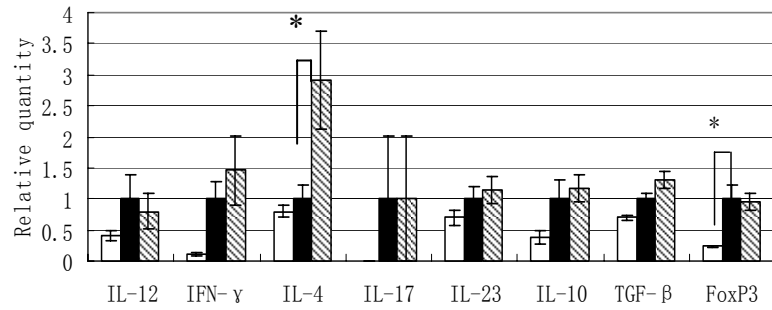
□ Normal

■ EAE

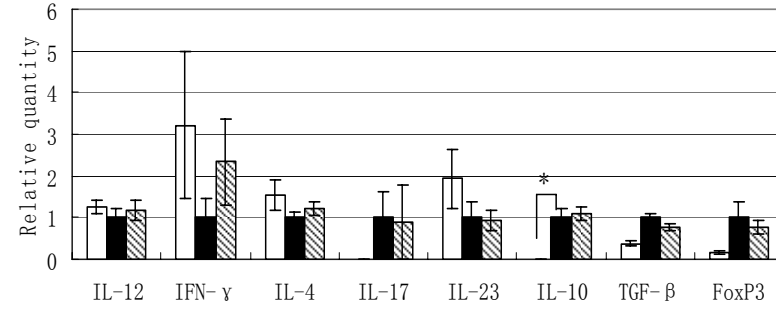
▨ EAE-DC10

C

Brain mRNA expression (7 wk, 3rd experiment)

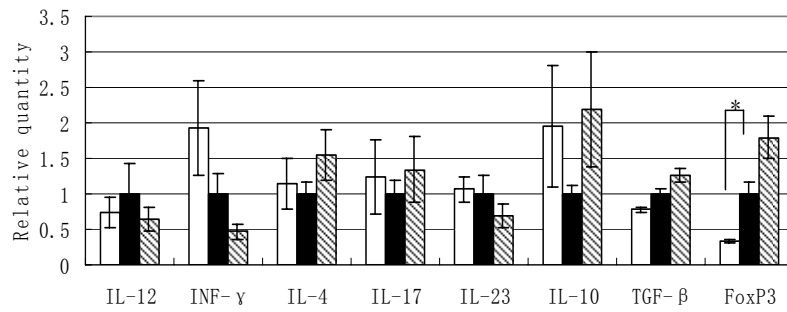


Spinal cord mRNA expression (7 wk, 3rd experiment)

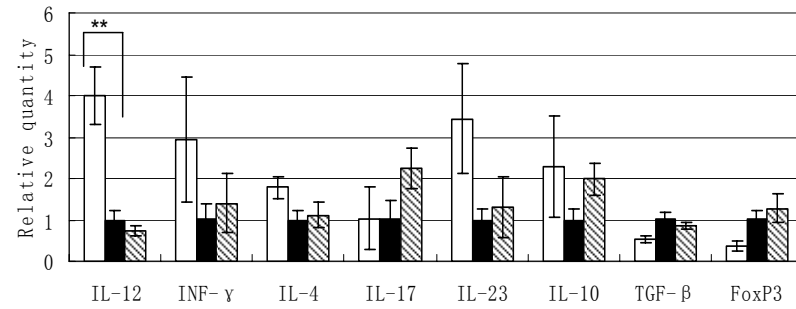


08

Brain mRNA expression (10 wk, 3rd experiment)



Spinal cord mRNA expression (10 wk, 3rd experiment)



□ Normal

■ EAE

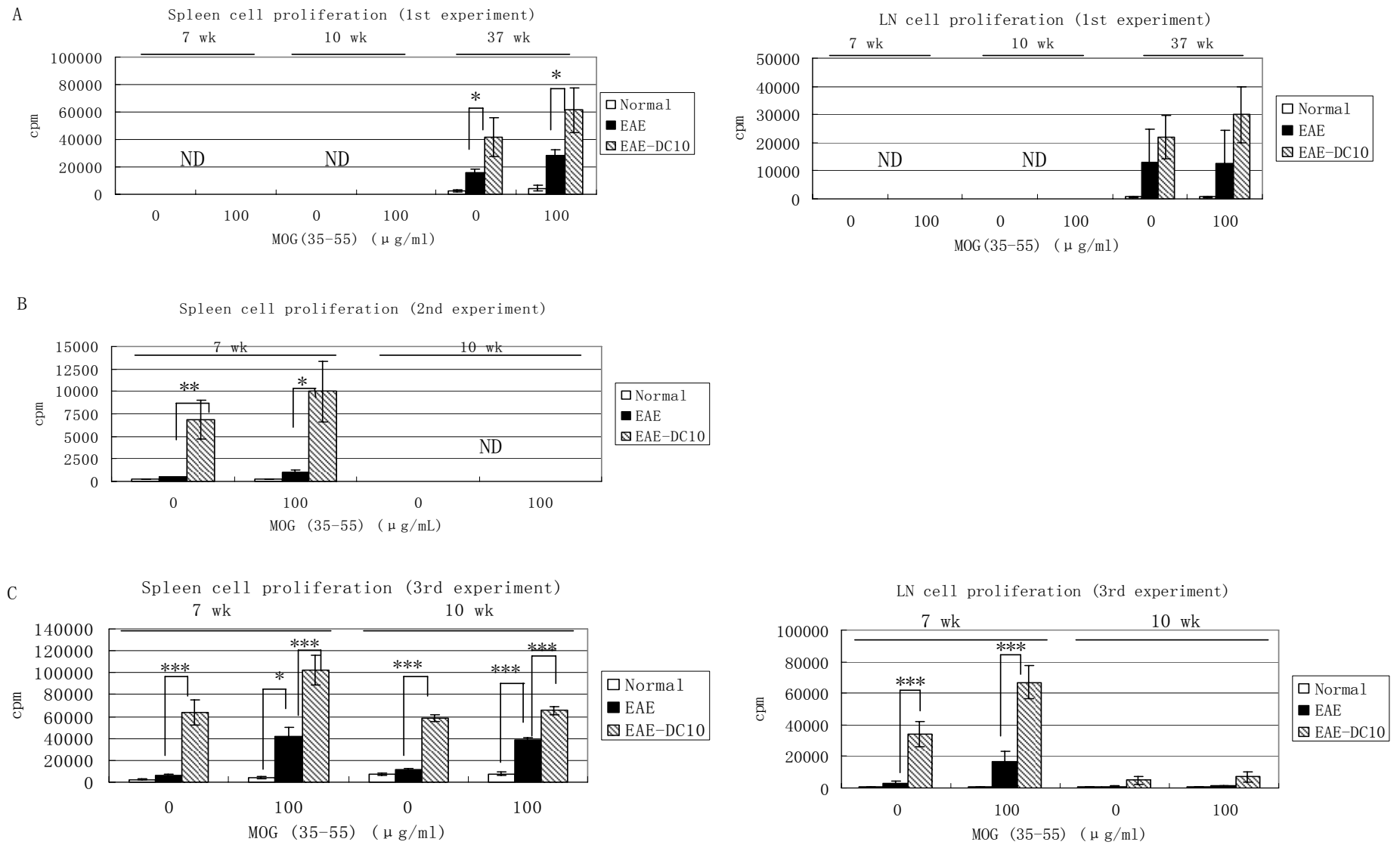
▨ EAE-DC10

### **5.7. DC10 treatments increased the proliferation of spleen and lymph node cells**

EAE is considered to be mainly mediated by T cells [2, 49, 50]. To investigate whether DC10 treatments had any effect on T cell responses while they were decreasing clinical scores, we measured the proliferation of total spleen and LN cells in response to MOG<sub>(35-55)</sub> antigen challenge. Total spleen and LN cells were cultured with 0 or 100 µg/mL MOG<sub>(35-55)</sub> and proliferation was measured using a <sup>3</sup>H-thymidine incorporation assay. In the 1<sup>st</sup> experiment (Fig. 5.7 A), we found that DC10 treatments significantly increased spleen cell proliferation in the cultures on week 37, irrespective of whether we added exogenous MOG<sub>(35-55)</sub>. In the 2<sup>nd</sup> and 3<sup>rd</sup> experiment (Fig. 5.7 B and C), we also found that the proliferations of spleen and LN cells in the culture were enhanced on week 7 or 10 in response to the DC10 treatments. Our results are similar to those of previous reports in which higher proliferation of spleen and LN cells was observed for immature MBP-pulsed prophylactically DC-tolerized EAE rats [14].

**Figure 5.7. Impact of DC10 treatments on spleen and lymph node cell proliferation in EAE mice.** C57BL/6 mice were immunized as described. Mice were killed on week 7, 10 or 37 after EAE induction. Total nucleated cells from the spleens and lymph nodes were counted and cultured in RPMI-10% FBS medium in a total volume of 200  $\mu$  L ( $5 \times 10^5$  cells/well). These cells were cultured in triplicate for 72 hours in the presence or absence of MOG<sub>(35-55)</sub> (0, 100  $\mu$  g/mL), then pulsed with 1  $\mu$  Ci of  $^3$ H thymidine for the last 16 to 18 hours. The levels of  $^3$ H-thymidine incorporation were determined by liquid scintillation counting (n=5-7). (ND, Not determined). Fig. 5.7 shows the data from the 1<sup>st</sup> (A), 2<sup>nd</sup> (B) and 3<sup>rd</sup> (C) experiments. All P values were determined by ANOVA. \*P<0.05, \*\* P<0.01, \*\*\* P<0.001.





## CHAPTER 6

### Discussion and Conclusions

#### 6.1. Discussion

Our lab has already reported that OVA-specific DC10 treatments can reverse airway hyperresponsiveness and induce tolerance in allergic asthma in mice [10, 11, 150]. Herein, we wanted to test the therapeutic effects of MOG<sub>(35-55)</sub>-presenting IL-10-differentiated DC (DC10) in ongoing EAE. DC10 were characterized as expressing low levels of CD40, CD80, CD86 and MHC II, which is consistent with an immature phenotype. It suggests that DC10 have lower antigen-presenting and stimulatory abilities for T cell activation. In addition, DC10 produced lower levels of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and IL-12. Similar results have been reported by others, who showed that DC10 do not efficiently produce IL-6 or IL-12, when compared to mature DC [13, 140], suggesting that DC10 are not capable of strongly inducing inflammatory responses [8]. IL-1 $\beta$  and IL-6 play important roles in the development of Th17 cells and IL-17 production, and IL-17 is considered to be important in the inflammatory responses in EAE [46, 68]. Thus we query whether the low production of IL-1 $\beta$  and IL-6 by DC10 might be associated with the reduced Th17 response.

Our lab has already reported that DC10 express high levels of IL-10 [10-12, 150]. The induction of Th2 tolerance in asthmatic mice by DC10 is dependent on IL-10 production, as Dr. Huang in our lab found that IL-10-specific siRNA-treated DC10 have no tolerogenic effects in asthmatic mice. This data suggests that IL-10 expression by DC10 is important for their tolerogenic functions, as reported by others [151]. In this study, we found higher level expression of IL-10 in our MOG<sub>(35-55)</sub>-specific DC10 cultures as compared to immature DC, but the levels of IL-10 produced by MOG<sub>(35-55)</sub>-pulsed DC10 is lower than that by OVA-pulsed DC10. The reasons are not clear but it is thought that the OVA-specific DC10 may be stimulated by LPS that is known to contaminate commercial preparations of OVA [152]. It has been reported that LPS can activate DC via TLR-mediated signaling pathways, and thereby augment IL-10 expression *in vitro* [153, 154]. We have also transfected IL-10-mRNA into DC and found significantly increased IL-10 production by these IL-10-transfected DC when LPS is added into the cultures (J.R. Town, J.R. Gordon, unpublished observations). These data further suggest that DC10 may receive stimulatory signals from LPS which triggers and amplifies their IL-10 secretion, though we do not have direct evidence to support this. Variable coincidental LPS contamination could potentially have contributed to the variable efficacy of our DC10 treatments in our three experiments. This could potentially occur. For example, because LPS can adhere strongly to glass and plasticware and because it is difficult to remove completely during cleaning [155], we query

whether such putative and adventitious LPS contamination of our cell culture reagents could have delivered stimulatory signals to our differentiating DC10 to induce greater IL-10 expression.

One of the most important and difficult parts of this research was to set up the chronic EAE model in mice. EAE is widely used as an animal model to study MS because it presents symptoms similar to those of human MS. Our research showed that MOG<sub>(35-55)</sub> could induce severe EAE symptoms in C57BL/6 mice. However, we found that the day of onset and severity of disease could vary from mouse to mouse, and that the disease incidence was not always 100%, as also indicated by others [29]. The reasons are still unknown, but decreases in incidence or severity of EAE might be attributed to the stress of the mice [156]. According to our experience, several steps should be taken in order to decrease such stress: the mice should be acclimatized in the animal room for at least one week before immunization, inasmuch as new environments are stressful to animals. They should also be acclimatized to human handling before immunization. They must be treated gently when immunizing and anesthesia should be avoided, as we found that using anesthesia greatly decreases the incidence of EAE.

It has already been shown that antigen-pulsed DC, which were induced by IL-10 or other mediators (e.g, TNF- $\alpha$ ), can protect mice or rats from EAE induction [13, 142]. Most of these researches treated the mice or rats with their tolerogenic cells before EAE sensitization or after induction but before disease onset. We found that the clinical scores decreased from  $\approx 2.5$  to 1.5

after one injection of DC10 (Fig. 5.2.1), although there was no significant difference between the PBS-treated and DC10-treated EAE groups. The lack of significance could be explained by the small sample size and therefore high standard error in each group. After treating the EAE mice a second and third time with DC10, their clinical scores decreased significantly (Fig. 5.2.2). Menges, et al. [142] found that single injection of MOG<sub>(35-55)</sub>-specific DC matured by TNF- $\alpha$  did not prevent EAE induction, but three such treatments did. As far as we know, this is the first report showing the successful therapeutic treatment with IL-10-differentiated DC10 to the established EAE mice.

In addition to following the clinical scores, we performed histological studies to visualize how the DC10 treatment affected the CNS tissues in EAE mice. In the 1<sup>st</sup> experiment there was a lower level of demyelination in the DC10 treatment group ( $p=0.063$ ). In the 2<sup>nd</sup> experiment, we found that DC10 treatments significantly reduced demyelination in the spinal cord at wk 7, but there was no difference in the cellular infiltration. In the 3<sup>rd</sup> experiment, we also found that DC10 treatments significantly reduced demyelination in the spinal cord at wk 7 and 10. DC10 treatments also reduced cellular infiltration at wk 7, but not wk 10 in the 3<sup>rd</sup> experiments. Interestingly, in our 2<sup>nd</sup> experiment, there was no significant reduction in clinical scores. The reasons for this are unknown to us, but other factors such as stress or further damage of axons may affect the clinical scores [156, 157]. We did not observe any reductions in cell infiltration in the brains in response to the

DC10 treatments. This may be due to the structural differences between the brain and spinal cord, or perhaps to our DC10 treatments preferentially targeting the inflammatory cells which migrate into the spinal cord rather than into the brain.

In our experiments, significantly higher levels of inflammatory cytokines (e.g., IL-6, IL-17 and IFN- $\gamma$ ) were expressed by lymphocytes in the EAE mice, suggesting that these cytokines could play an important role in EAE development, and thus they serve as specific makers of EAE development. This is consistent with previous reports that IL-6, IL-17 and IFN- $\gamma$  play important roles in the pathogenesis of EAE [39, 62, 158, 159]. We also found that there was a significant increase of IL-10 expression in the spleens and LNs in EAE mice in our model, which was in part consistent with previous research. It had been shown that polarized Th1 cells derived from MOG-induced EAE mice were capable of producing IL-10 [53]. IL-6 can increase the production of IL-10 by CD4<sup>+</sup>T cells in the presence of TGF- $\beta$  [160]. In MS patients, elevated numbers of IL-10 mRNA-expressing cells are found in the blood [136]. The role of IL-10 in EAE and MS is complex and still needs to be confirmed, but IL-10 is an anti-inflammatory cytokine produced by Th2 cells, B cells, macrophages [161], and it is known for its ability to down-regulate Th1 responses [162]. Previous studies found that IL-10 levels were correlated with recovery from EAE [163], but Cannella et al. [164] showed that administration of IL-10 had no effect in the protection from EAE. In our studies, we found higher levels of IL-10 in the

spleen and LN in EAE mice, suggesting that IL-10 was involved in the development of EAE. Another study also reported that neutralization of IL-10 with anti-IL-10 monoclonal antibodies did not protect mice from EAE, suggesting that IL-10 might not play a critical role in the pathogenesis of EAE, even though it is expressed during EAE development [165]. In our experiments, we also found higher levels of IL-4 expression in the spleen and LN as compared to normal mice, which was similar to previous results, describing high levels of IL-4-producing cells and IL-4 mRNA expression in the peripheral blood of MS patients [36, 166]. This opened the possibility that Th2 responses might be mixed with Th17 and Th1 responses. Our DC10 treatments significantly reduced IL-4 expression in the spleens and LNs, indicating that DC10 treatments could affect Th2 responses in EAE mice. However, we must note that the role of Th1/Th2 cytokines in the induction and development of EAE is not well understood. Chitnis and Khoury showed that knock-out of Th1 or Th2 cytokines like IFN- $\gamma$  or IL-4 renders mice more susceptible to EAE induction, while partial reduction of IFN- $\gamma$  or administration of IL-4 could reduce severity of disease [167]. This suggests that the presence of these cytokines might be involved in the protection from EAE, but that their over-expression might potentially worsen EAE [167, 168].

It was also interesting that we observed high levels of IL-4, IL-6, IL-10 and IFN- $\gamma$  in the supernatants of spleen and LN cells that had not been challenged with antigen. One explanation is that, during EAE, CNS antigens

are consistently presented in the spleen and LN. This could be due to the destruction of the blood brain barrier (BBB), one of the characteristics of EAE, leading to the leakage of myelin antigens to these organs, with subsequent stimulation of antigen-specific cells *in situ*. Such *in vivo* activation of T cells could continue to proliferate and / or produce cytokines *ex vivo*. Epitope spreading contributes to the activation of autoreactive T cells at later stages of EAE development, when T cells specific to other CNS Ag are observed in the spleen and LN [41, 42, 169]. Release of endogenous myelin antigens or peptides following the CNS tissue damage caused by specific T cells might also potentially activate inflammatory T cells with diverse specificity, leading to further activation of T cells and cytokine production [170]. For example, Tuohy, et al. found that autoreactive T cells from PLP<sub>(139-151)</sub>-induced EAE mice were capable of producing IL-4, IL-10 and IFN- $\gamma$  in response to both PLP<sub>(139-151)</sub> and MBP<sub>(87-99)</sub> [170, 171]. In addition, local dendritic cells in the CNS could carry myelin peptides across the blood brain barrier and present these to peripheral T cells in the spleen and LN [172], it may lead to inflammatory cytokine production and further inflammatory cell infiltration and myelin destruction in the CNS. Another potential explanation is that there is activation of antigen-non-specific T cells. In MS patients, the combination of inflammatory cytokines such IL-6 and IFN- $\gamma$  produced by antigen-specific inflammatory cells could activate peripheral resting T cells by increasing intracellular calcium concentration. This activation was independent of specific antigens [173, 174]. The



production of IL-4 and IL-6 in EAE mice was also contributed by cytokine-mediated bystander reactions [175], wherein MOG<sub>(35-55)</sub>-specific CD4<sup>+</sup>T cells from IL-4<sup>-/-</sup> and IL-6<sup>-/-</sup> mice could induce bystander IL-4 and IL-6 production in spleen cells from naive WT mice. The supernatants of MOG<sub>(35-55)</sub>-specific CD4<sup>+</sup>T cells cultured from WT EAE mice could also induce IL-4 and IL-6 production in spleens from naive WT mice [175]. Taken together, these antigen-non-specific responses are considered to be involved due to the additional production of inflammatory cytokines like IL-6 and IFN- $\gamma$ , and may potentially promote further activation of T cells, thereby amplifying the inflammatory response [174]. We did not see a significant increase of spleen and LN cell proliferation in the absence of specific antigen as compared with normal mice, while proliferation increased in response to antigen. This means that there was antigen-specific proliferation, and suggests that the high production of these cytokines without antigen in culture was from other antigen non-specific cells, which partially support the hypothesis above. We also observed that IL-4, IL-10, IL-12, TGF- $\beta$  and IL-6 were not markedly increased in response to antigen, suggesting that the production of these cytokines might be non-antigen-specific. The relatively low production of IL-12 and TGF- $\beta$  by spleen and LN cells in culture with or without antigen may suggest that these cytokines were not important to pathogenesis at this stage of the disease. Also, our restimulation culture conditions (3 days culture *in vitro*) might

have failed to optimally induce their maximum production, or they were already consumed in the early days of culture by other cells.

Our DC10 treatments significantly reduced clinical symptoms and inflammatory cytokine production in the spleen and LN, suggesting that DC10 treatments are capable of suppressing the activation of inflammatory lymphoid cells and CNS inflammation in EAE. During the development of EAE, myelin-specific T cells are activated in secondary lymphoid organs and escape immune tolerance such that these autoreactive T cells infiltrate the CNS and induce neuroinflammatory responses [29]. Our data clearly shows that the DC10 treatments suppressed spinal cord demyelination [32, 99]. Though we did not further look at the mechanisms of DC10-mediated tolerance in EAE in this study, our data is consistent with the DC10 treatments having induced Tregs in our EAE mice. Our lab has reported that Tregs are induced and activated by DC10 treatments of asthmatic mice, and these cells suppressed the effector T cell response [11, 12, 150]. Min et al. [176] also reported that there was a self-maintaining regulatory loop in which tolerogenic DC could promote the generation of Tregs while Tregs induced generation of tolerogenic DC from bone marrow progenitors, suggesting that a self-renewal mechanisms might exist *in vivo* for a long time after treatment, thus it is possible that DC10 could potentially educate other DC to become tolerogenic via the induction of Tregs. This is fully consistent with other data from our lab, wherein DC10 induce both regulatory T cell populations and regulatory DC populations [11, 12, 150]. Herein we found

that DC10 treatments maintained tolerance up to 70 -130 days, and it is unlikely that these DC10 could have survived for such a long time. Thus we speculate that the down-regulatory effects induced by DC10 treatments would be maintained by other cells, such as Tregs or other induced-tolerogenic DC, which could keep interacting with each other to maintain a tolerogenic environment [176]. We also speculate that other possible mechanisms, like the induction of regulatory B cells and / or CD8<sup>+</sup> regulatory T cells, could contribute to the tolerance via alternate pathways. It had been reported that CD1d<sup>hi</sup>CD5<sup>+</sup> regulatory B cells effectively inhibit EAE development during the initiation stage [83], and that CD8<sup>+</sup> regulatory T cells are capable of suppressing ongoing EAE [177-179].

We also assessed the levels of inflammatory cytokine expression in the CNS by qRT-PCR, but we didn't see significant differences in expression of most of these cytokine in the PBS- and DC10-treated EAE mice. We found that there was a higher expression of FoxP3 in the brains and spinal cords of the both groups of mice, even though there were no significant differences between these groups. We have reported previously that there are no difference in the numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in saline- and DC10-treated (i.e., tolerant) asthmatic mice, but that the cells from the latter mice are highly activated and express a regulatory phenotype [11, 12, 150]. Our data suggested that Tregs were stimulated in the CNS or migrated from the spleen or LN into the CNS, and that these Tregs could have inhibited cytokines production in the CNS locally. This is supported by the evidences

that Tregs accumulate in the CNS during the recovery from EAE and that these CNS-derived Tregs can suppress EAE induction [180]. Similarly, Korn, et al. reported that the highest numbers of MOG-specific T effector cells were found at the peak of disease but that they decrease markedly in the recovery stage, while the numbers of Treg do not decline. They also compared the ratio of Treg to T effector cells during EAE and found that this ratio increased from 1:13 at the peak of disease to 1:4 during remission [181], suggesting that Treg may contribute to the infiltrating cell population. In addition, T cell apoptosis is reported to occur in the CNS after the acute phase of EAE development, leaving few T cells present in the lesions of CNS [104]; such apoptotic T cells would have lost their ability to produce cytokines, despite the fact that infiltrating cells would still be observed histologically in the CNS. Tregs are thought to be involved in inducing apoptosis of CD4<sup>+</sup>T cells [25]. In our experiments, we harvested the mice either 6-9 week or 35 week after the peak of the disease, such that very likely inflammatory cytokine expression would have declined in the CNS by this time.

Another interesting finding in our report was the induced increase in antibody levels associated with the DC10 treatments. Our data showed that DC10 treatments significantly increased MOG-specific IgG1, IgG2a and IgG2b levels. The reasons for this are unknown, as is the role of antibody in EAE. It has been reported that increased production of IgG occurs in MS patients [89] but, conversely, transfer of serum from rats that had recovered

from EAE to naive recipients suppresses the induction of EAE [84]. Increases in IgG, IgG1, IgG2a and IgG2b production might also be related to the decreases in IL-17, as enhanced MOG-specific IgG, IgG1, IgG2a and IgG2b production are found in IL-17-deficient mice during EAE. It was suggested that IL-17 could prevent IgG production by B cells [64]. Our DC10 treatments significantly decreased IL-17 production in the EAE mice, and this could similarly have facilitated the production of MOG-specific-IgG by B cells during EAE. Myelin-specific antibodies are potentially beneficial to CNS remyelination in EAE [82], as EAE rats that received i.p. injections of myelin-specific serum or myelin-specific IgG develop significantly increased CNS remyelination [82, 87]. Wolf et al. [182] also showed that B cell-deficient EAE mice failed to completely recover after the peak of disease as compared to WT mice, though they had similar disease incidence, suggesting B cell might play a role in the regulation of EAE during the recovery stage. The mechanisms were not clear, but it was suggested that these myelin-specific IgG could facilitate the growth and activation of oligodendrocytes, the cells which promote myelin production and thereby remyelination [82]. We can not exclude the possibility that the increased antigen-specific antibodies levels induced by the DC10 treatment in our experiments were involved in the remyelination and inhibition of further demyelination, although we have no direct evidence to suggest that remyelination had occurred in our DC10 treated EAE mice.

We also found something interesting related to the proliferation of spleen and LN cells. There was a significantly higher proliferation of these cells in cultures of cells (with or without antigen) from the DC10-treated groups. Previous research also reported similar results as antigen-pulsed immature DC significantly induced proliferation of spleen and LN cells in DC-tolerized EAE rats [14], but the reason why our DC10 treatments increased spleen and LN cell proliferation is unknown. There are many different kinds of cells in the spleen and LN, and the interactions between these cells are complicated. Bone marrow derived-DC can not only induce differentiation of FoxP3<sup>+</sup> Tregs from FoxP3<sup>-</sup> precursors, but also directly stimulate Treg proliferation *in vitro* [128, 183], and these Treg could potentially have contributed to the increases in the total cell proliferation in our experiments. This observation also suggests a mechanism whereby DC treatment could actively expand Treg to suppress autoreactive response in EAE. DC10 treatments significant increased spleen and LN cell proliferation even without antigen in the cultures, suggesting that antigen-specific cells were activated *in situ*. It might be possible that this proliferation was contributed not only by T cells but also other cell types such as B cells, as it has been reported that DC can directly stimulate B cells to proliferate [184-187]. In addition, it had been reported that higher proliferation of spleen and LN cells was observed for immature MBP-pulsed prophylactically DC-tolerized EAE rats [14]. Zhang, et al. also suggested that this suppression of EAE was due to the apoptosis of T cells that had proliferated

[188] (i.e., T cells that had proliferated would be deleted by apoptosis [189, 190]). Thus this provides another potential mechanism whereby DC10 treatments could suppress inflammatory cell activation (i.e., via induction of apoptosis).

To our knowledge, this is the first study showing that IL-10-differentiated dendritic cells can reverse established, chronic EAE. This DC10-induced tolerance in EAE provides us with a novel approach that could potentially be applicable to MS treatment in the clinic. But we must keep in mind that there are still many challenges which need to be further resolved. For example, in only two of our three experiments did we observe significant reductions in EAE clinical scores following DC10 treatments. Further experiments should be needed to confirm the effect of DC10 treatment in this model. Specific computer program and image software can be used to analyze the exact area of demyelination in spinal cord to provide us a better understanding of the pathological changes.

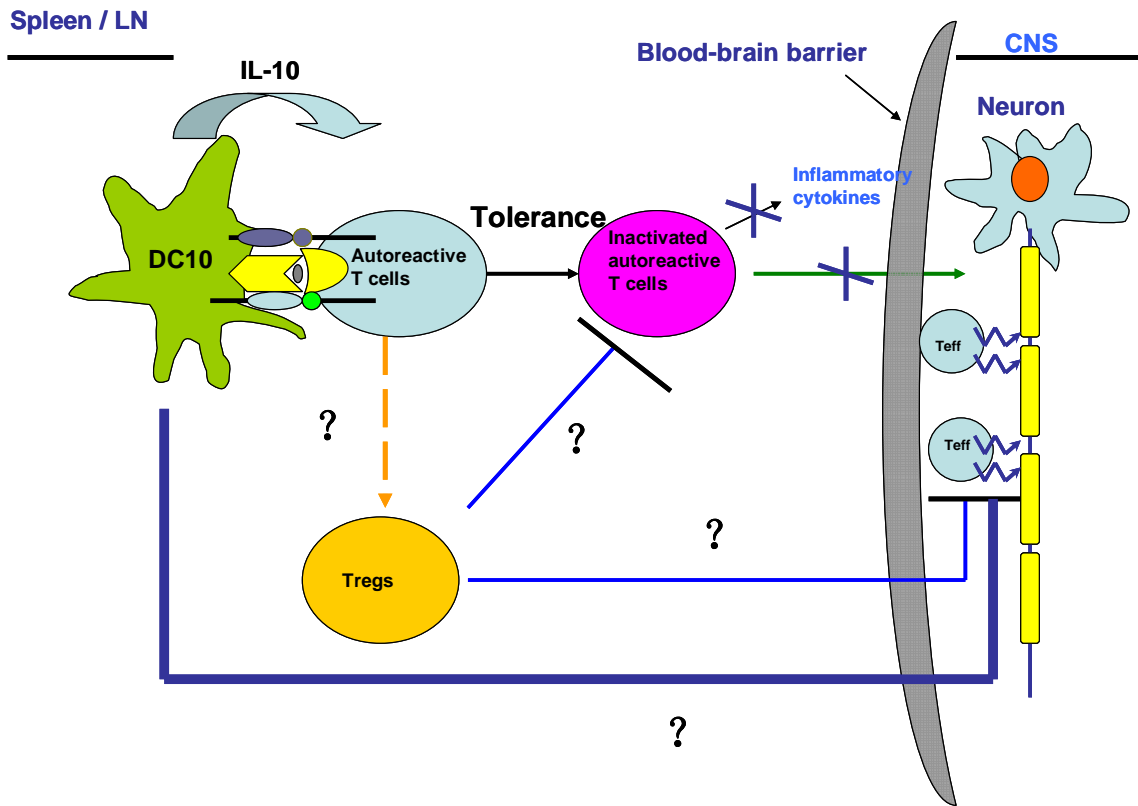
## 6.2. Conclusions and future directions

In conclusion, it is clear that:

1. IL-10-differentiated dendritic cells (DC10) did not express high levels of CD40, CD80, CD86 and MHC II;
2. DC10 produce low levels of IL-1 $\beta$ , IL-6, and IL-12 similar to DC(GM-CSF) but high levels of IL-10;
3. A single injection of DC10 did not significantly inhibit EAE development, but three DC10 injections reversed the course of established EAE;
4. DC10 treatments significantly reduced demyelination in the spinal cord;
5. DC10 treatments significantly reduced the production of inflammatory cytokines in spleen and LN;
6. DC10 treatments did not show significant effects on inflammatory cytokine expression in the CNS of EAE mice;
7. DC10 treatments significantly increased MOG<sub>(35-55)</sub>-specific IgG1, IgG2a or IgG2b levels in EAE mice; and
8. DC10 treatments significantly enhanced proliferation of spleen and LN cells in EAE mice.



Figure 6 hypothesizes general mechanisms by which DC10 mediate EAE tolerance. In general, IL-10-differentiated DC show low expression of MHC II and costimulatory molecules and high production of IL-10. These DC10 induce autoreactive T cell anergy possibly via their reduced costimulatory signals and the IL-10 production, which would lead to autoreactive T cells tolerance. DC10 suppress inflammatory cytokines production by these T cells, as well as their migration into the spinal cord, resulting in the suppression of CNS local inflammation and demyelination. For our future directions, we would investigate the suggestion that DC10 induce Tregs activation in the CNS. We are also interested in investigating whether DC10 and these putative Tregs migrate into the CNS and mediate immune tolerance locally. Due to the multiple phenotypes of MS, we are similarly interested in whether DC10 could be used to treat clinical signs in other EAE models of multiple sclerosis (e.g., acute)[7]. Furthermore, in the human *in vitro* studies, Dr. Xiuling Li in our lab has already reported that DC10 could induce Th2 tolerance *ex vivo* in human asthmatic subjects [12], and this provides us with insights into potential treatments with DC10 in MS patients. Thus we are interested to test whether DC10 could suppress effector T cells response in MS patients *ex vivo*



**Figure 6 General mechanisms by which DC10 may reduce EAE clinical signs and CNS pathology.** IL-10-differentiated DC show low expression of MHC II and costimulatory molecules and high production of IL-10. These DC10 induce autoreactive T cell tolerance and suppress production of inflammatory cytokines by these T cells, as well as their migration into the spinal cord, resulting in the suppression of CNS local inflammation and demyelination. We also suggest a possibility that DC10 induce Tregs which could directly suppress autoreactive T cells activation and CNS inflammation.

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