ABSTRACT

DERIVATION OF TOLEROGENIC THERAPEUTIC VACCINES FOR MOUSE MODELS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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Multiple sclerosis (MS) is a chronic inflammatory disease that results in autoimmune demyelination of the human central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a widely-studied animal model of multiple sclerosis which reflects many of the histopathological and immunological features of MS. Because currently used therapies for multiple sclerosis result in broad-spectrum immunosuppression, antigen specific vaccines may be an important alternative. Whereas the concept of traditional anti-microbial vaccination is to introduce the immune system with the foreign microbes to develop memory T and B cells, self-reactive T cell suppression is the fundamental principle of tolerogenic vaccines. For EAE, contemporary research is focused on the development of potent tolerogens. Fusion proteins of cytokine and neuroantigen (NAg) are assumed to be such potent tolerogens. Antigen presenting cells (APC) are targeted by the fusion proteins in a cytokine dependent manner. These APC have been shown to play a fundamental role in inducing sustainable self-tolerance. That is, autoimmune effector T cells are eliminated by regulatory T (Treg) cells that are stimulated and

expanded in a negative thymic selection process where dendritic cells (DC) play an inevitable role. The principle is that the cytokine domain of the fusion protein will interact with cytokine receptors on the APC and facilitate the presentation of covalently linked NAg to self reactive T cells and thereby, induce a co-inhibitory signal leading to cytotoxicity and killing of NAg-specific autoreactive T cells. In an approach to induce tolerogenic response in EAE, I have studied two fusion proteins consisting of interferon-beta (IFN-β) fused to myelin oligodendrocyte glycoprotein (MOG) and IFN-β fused to proteolipid peptide (PLP). Both fusion proteins were potent tolerogens. The fusion genes were cloned in pIRES2 expression plasmids and then expressed stably in human embryonic kidney cells, and the fusion proteins were purified by affinity chromatography. Functional integrity of the IFN-β domain within the fusion protein was confirmed by a T cell anti-proliferative assay and a class-I MHC induction assay. Biological activity of NAg domains were tested by NAg specific T cell proliferative assays. The tolerogenic effect of the fusion protein was assessed in two ways. First, the preventive role of IFNβ-PLP was examined by administering the protein before EAE induction which inhibited the subsequent incidence of EAE. Second, IFNβ-MOG was administered after disease onset which halted disease progression. In both cases, covalent linkage between the IFN-β and NAg domain was required to restore the tolerance.

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This dissertation is dedicated to my beloved parents, S.M. Abdul Momen and Sultana Razia Begum, for their love, affection, support and endless encouragement to succeed in life.

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APC	Antigen presenting cell(s)
CFA	Complete Freund's adjuvant
CNS	Central nervous system
DC	Dendritic cell(s)
EAE	Experimental autoimmune encephalomyelitis
EK	Enterokinase
GFP	Green fluorescent protein
GMCSF	Granulocyte macrophage colony stimulating factor
GPMBP	Guinea pig myelin basic protein
HLA	Human leukocyte antigen
IFA	Incomplete Freund's adjuvant
IFN-β	Interferon beta
i.p.	Intraperitoneal
mAb	Monoclonal antibody
MBP	Myelin basic protein
MCSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAg	Neuroantigen
OVA	Ovalbumin
PLP	Proteolipid protein

s.c.	Subcutaneous
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TNF-γ	Tumor necrosis factor-γ
Treg	Regulatory T cell
TTV	Tolerogenic therapeutic vaccine

CHAPTER 1: INTRODUCTION

1.1 Multiple sclerosis and its etiology

Multiple sclerosis (MS) is a progressive demyelinating inflammatory neurodegenerative disorder involving the loss of fatty myelin sheath around the axons of brain and spinal cord. Neurodegeneration occurs as a consequence of demyelination which is characterized by the formation of plaques and glial scars in the white matter due to the stripping of myelin from axons accompanied by activation of microglial cells. Demyelination in MS is mediated by an inflammatory response characterized by the recruitment of self-reactive $CD4^+$ and $CD8^+$ T-cells as well as macrophages. Axonal damage as a consequence of demyelination results in calcium influx and mitochondrial collapse. Immune cell populations are activated in the peripheral immune system by antigen presentation by mature dendritic cells (DC). $CD4^+$ T cells (Th-1 and possibly Th-17) play the role of initial inducer of disease, whereas $CD8^+$ cells are probably more relevant for central nervous system (CNS) tissue damage. T cells proliferate, cross the blood brain barrier, and enter the CNS under the influence of cellular adhesion molecules (CAMs) and proinflammatory cytokines. Autoreactive T-cells and B-cells are particularly directed against myelin self antigens which contribute to this deleterious immune response (1-3).

The CNS has long been considered as an immunoprivileged organ. This immune privilege was classically attributed to several mechanisms. First, the blood-brain barrier was assumed to prevent the trafficking of resting lymphocytes. Second, the CNS was assumed to be devoid of antigen presenting cells and was thought to contain few resident cells that express major histocompatibility complex (MHC) gene products. Third, the CNS has local tolerogenic mechanisms, e.g. expression of Fas-ligand, prostaglandins E2, TGF-β, and galectin-9 which have

been associated with functional silencing or killing of incoming T-cells. This immune privilege of the CNS has been challenged by recent studies. First, recent studies shows that the CNS is not prohibited from immune cells, because naïve T-cells have been shown to traffic into the inflamed brain. In steady state, both naïve $CD4^+$ and $CD8^+$ T-cells are able to patrol nonlymphoid tissues, including the CNS. Second, the CNS is not devoid of antigen presentation. In a pro-inflammatory environment, oligodendrocytes and neurons express MHC class I molecules, while astrocytes and microglial cells can express both MHC I and II molecules. In addition, CNS dendritic cells can initiate epitope spreading to induce self immune reactions during chronic tissue inflammation. Third, TGF-β which was known to show regulatory properties, has recently been shown to induce the differentiation of $CD4^+$ T-cells into pathogenic IL-17 secreting Th-17 cells (1).

Self tolerance is maintained through the elimination of nascent auto-reactive T-cells expressing T-cell receptors (TCR) that form high-affinity interactions with self-antigens to induce apoptosis. A dedicated subset of antigen presenting cells, called the cortical and medullary thymic epithelial cells, mostly carries out the task of expressing a large array of self-antigens. Tissue restricted antigens are expressed under the control of several transcription factors e.g. autoimmune regulator (AIRE). Mutation in AIRE may result in the incomplete elimination of auto-reactive T-cells in the thymus and in the development of autoimmunity (1). Alternatively, weak HLA (human leukocyte antigen) binding in the thymus can translate into inefficient negative selection and a larger pool of peripheral auto-reactive T-cells. In this case, T cells with high avidity TCR for epitopes can be spared from negative thymic selection because of poor HLA binding (2).

1.2 EAE and its relevance to multiple sclerosis

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model of MS that has contributed to the current understanding of MS. Although EAE is considered as an ideal model of multiple sclerosis, there are some major differences between EAE and MS. First, MS is a spontaneous disease, whereas EAE is induced by active sensitization with CNS tissue antigens. Recently, spontaneous models of EAE have been developed using transgenic approaches to override the intrinsic regulatory mechanisms that normally suppress tissue-specific auto-immune reaction. Second, for practical reasons and for the sake of reproducibility, EAE is studied mainly in inbred animals and in genetically homogeneous populations. On the other hand, genetic heterogeneity is so critical in multiple sclerosis that it can only be reflected by using multiple different animal models of EAE studied in parallel. In spite of these limitations, most of our current knowledge regarding the principle mechanisms of CNS inflammation has been distilled from experimentation on EAE. Without this knowledge, the understanding of the pathogenesis of MS and development of new therapies would not be feasible (4).

1.3 Currently available therapies of multiple sclerosis

Therapeutic drugs, currently available for the treatment of multiple sclerosis are mostly dependent on non-antigen specific approaches that may entail a significant impairment of the entire immune system. One of the most currently used treatments for individuals with MS is IFN-β, which is efficacious due to a combination of actions. First, expression of HLA class II (which is required to activate pathogenic $CD4^+$ T cells) is elevated in the brain of individuals with MS, and IFN-β downregulates the expression of HLA class II. Second, after a few weeks of treatment, IFN-β downregulates the expression of IFN-γ which has a role in exacerbating the

disease. Third, IFN-β downregulates the expression of matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9, both of which play a role in increasing the permeability of the blood-brain barrier (BBB) (3). Some monoclonal antibodies have been developed which also have been shown to have promising effect against MS. Natalizumab is a humanized mAb that binds to α-4 subunit of α4β1 and α4β7 integrins, which are expressed on the surface of activated T-cells. This interaction blocks the binding of these activated lymphocytes to their endothelial receptors (VCAM-1 and CAM-1) which is an important step in T cell transmigration through the BBB into the CNS (5). Alemtuzumab is a monoclonal antibody (mAb) directed against the CD52 antigen, which is present on the cell surface of T and B lymphocytes, monocytes, macrophages and eosinophils but not stem cells. It depletes these cells through complement mediated lysis, antibody-dependent cell toxicity, and induction of apoptosis. Daclizumab is a mAb directed against the α-chain of the interleukin (IL)-2 receptor (CD25), which is crucial for T cell proliferation and activation. Rituximab is another mAb directed against the CD20 antigen that depletes the CD20-expressing pre-B cells and B cells, but not the antibody producing plasma cells or stem cells. Some anti-metabolic or anti-proliferative drugs have also been used to treat MS patients. Fingolimod is an orally available derivative of the ascomycete metabolite myriocin, which was identified as a new class of immune modulators targeting sphingosine-1-phosphate (S1P) receptors. After *in vivo* phosphorylation, Fingolimod leads to internalization of the S1P receptor, resulting in a rapid and reversible inhibition of lymphocyte egress from peripheral lymph nodes, which prevents auto-reactive T cells from reaching sites of inflammation. Teriflunomide is the active metabolite of leflunomide. Triflunomide is an inhibitor of the mitochondrial dihydroorotate dehydrogenase, an enzyme crucially involved in pyrimidine synthesis. Because of the dependence of activated lymphocytes largely on *de novo* synthesis of

pyrimidine, pyrimidine depletion might result in inhibition of immune cell proliferation (6, 7). Cladribine is a deaminase-resistant deoxyadenosine analogue. As an analogue of ATP, Claridine triphosphate gets incorporated into the DNA in dividing cells and halts replication and thereby leads to DNA damage and finally cell death. All of these currently used therapies are broad-spectrum inhibitors of the immune system. The efficacy of these drugs may be compromised by the severity of the side-effects. Therefore, the success of a drug for multiple sclerosis is directly related to the ability of the drug to inhibit the disease without compromising the immune system.

1.4 Prospect of antigen specific therapy for EAE

Antigen-specific therapy is considered to be a potential alternative therapy for multiple sclerosis. The concept of antigen-specific approaches is taken from the ability of immunological tolerance to inhibit the autoimmune response. EAE has been used as an animal model of multiple sclerosis to develop antigen-specific approaches. In mucosal tolerance, tolerance is induced through the oral and nasal administration of self antigens. Inducible T cell co-stimulator (ICOS), whose ligands are known as B7h (also called B7RP-1, GL-50, B7-H2, and LICOS) is assumed to play the key role in mucosal tolerance. ICOS is up-regulated on $CD4^+$ and $CD8^+$ T cells after TCR stimulation. In addition, cross linking of CD28 on T cells with B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules on antigen presenting cells (APC) can stimulate ICOS expression. While B7-1 and B7-2 are expressed predominantly on APC, ICOS ligand (ICOSL) is expressed not only on cells of hematopoietic origin, but also on fibroblasts, endothelial cells and some epithelial cells. Expression of ICOSL on these nonhematopoietic cells contributed to the concept of mucosal tolerance. ICOS has positive co-stimulatory activities, including enhancing cytokine production, up-regulating CD40L expression and providing help for Ig isotype class switching by B cells. The most important role of ICOS is the production of IL-10, a key

immunoregulatory cytokine involved in the induction of regulatory T type 1 (Tr1) cells and suppression of autoimmunity (8).

In high dose tolerance, intravenous administration of antigen is used to induce tolerance. The exact mechanism of high dose tolerance is still unknown. Recent findings show that high dose antigen therapy induces the increased level of TNF-α accompanied by considerable induction of IFN-γ and inducible nitric oxide (NO) synthase (iNOS) expression. Increased level of IFN-γ is also partly attributed the increased expression of iNOS. NO inhibits the production of IL-2 and IFN-γ, and thereby has an anti-proliferative effect on T cells, and inhibits antigen presentation by down-regulating MHC class II molecules in macrophages. Moreover, NO may induce apoptosis of myelin reactive T cells (9).

Elimination of self-reactive T cell in the thymus is the principle of deletional tolerance. In an experiment, transplantation of genetically manipulated bone marrow (BM) cells that express myelin oligodendrocyte glycoprotein (MOG) through retroviral transduction was shown to prevent the induction and progression of EAE induced by MOG. Thymus progenitors arising from the BM continuously seed the thymus to develop into T cells DC. Exposure of T cells to self antigens expressed by these bone marrow derived DC cells promotes deletion of self-aggressive T cells with high avidity for self antigens through negative selection (10).

In immune deviation, adoptive transfer of T cell lines generated with the altered peptide ligand (APL) of a self antigen can confer protection from EAE. In one experiment, EAE was induced with proteolipid protein 139-151 (PLP139-151) in which a majority of the T cell clones recognized the original peptide with tryptophan at position 144 as the primary TCR contact residue and histidine (147) and leucine (141) as the secondary contact residues. EAE was

prevented by the adoptive transfer of T cell specific for an altered peptide of PLP139-151(Tryptophan → Glutamine). A possible explanation is that naive T cells specific for Tryptophan 144 had the potential to become Th1 cells if activated with naive peptide, but instead differentiated into Th2-like cells when activated with the Glutamine 144 peptide. These Th2-cells inhibited Th1 cells and protected mice from EAE upon adoptive transfer (11, 12).

These therapies are based on different strategies. An altered peptide ligand of a self antigen was used to develop tolerance against EAE by immune deviation (12). Another antigen-specific approach for EAE suppression is via administration of antigen-antibody fusion proteins. In an experiment, a fusion protein consisting of myelin basic protein (MBP) linked to mouse anti-rat immunoglobulin (Ig) D was used in a pretreatment protocol to protect Lewis rats from EAE induced by MBP. This resistance was ascribed to the fact that the antibody-peptide conjugate targeted the antigen to B cells and that the presentation of the peptide by B cells would induce Th2 like cytokine response which is assumed to antagonize Th1 cell responses responsible for mediating EAE (13). However, targeting a single auto-reactivity by tolerization with individual peptide had no significant effect on complex EAE induced with multiple auto-antigens. A multitude of diverse antigenic epitopes may be important auto-antigens driving pathogenesis in MS. To test a multivalent tolerogen, a synthetic gene was constructed to encode 'multi-epitopic antigen' containing selected disease -relevant epitopes of myelin basic protein (MBP), proteolipid lipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). The protein product was processed for antigen presentation of the relevant integral epitopes *in vitro* and *in vivo*. Systemic administration of the protein not only suppressed and treated EAE induced by PLP epitope, but also abrogated complex EAE transferred by multi-specific T-cell lines reactive against epitopes of MBP, PLP and MOG. This result indicated the promising use of a

synthetic gene encoding a 'multi-epitopic antigen' for the therapy of complex EAE (14). In a DNA vaccine method, DNA encoding the self myelin antigen was injected into the tissue. In one experiment, a DNA vaccine encoding either proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) in combination with IL-4 DNA proved to be a powerful method of modulating an immune response by causing encephalitogenic T cells to shift to a protective Th2 phenotype. In another experiment, a DNA cocktail composed of full-length cDNAs encoding the four major components of myelin, myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) with or without the addition of a plasmid encoding IL-4, reduced the spread of autoantibody responses to epitopes on various myelin molecules, concomitant with a reduction in relapse rate, when given after the initial disease onset (15).

1.5 Limitations of traditional antigen specific therapies

An important disadvantage of all of these currently used strategies of antigen specific therapies of EAE is the uncertainty of which self-antigens should be used to inhibit the initiation and progression of the disease, because use of inappropriate antigens may lead to anaphylaxis, inadvertent sensitization, and exacerbation of the disease (16, 17). Despite all of these potential risks, antigen-specific therapies offer the most promising inhibitory approach against the pathogenic autoimmune responses of multiple sclerosis without compromising the immune system and may be able to restore the immune tolerance to self-antigens in a safe and effective way.

1.6 Promise of cytokine-NAg fusion proteins as therapeutic tolerogenic vaccines

In search of a suitable antigen to induce tolerance in autoimmune disease, it has been found that cytokine-NAg fusion proteins are potential candidates for antigen-specific induction of tolerance. Fusion protein consisting of IL-2 and encephalitogenic determinant of guinea pig myelin basic protein (GPMBP) was found to be effective for treatment and prevention of EAE. Similarly, a fusion protein consisting of IL-16 and an encephalitogenic peptide of GPMBP was also reported to have prophylactic and curative potential against EAE (18, 19).

1.7 Rationale of therapeutic tolerogenic vaccine

The principal goal of antigen-specific therapy is to eliminate or suppress the self reactive T cells by four possible mechanisms. The first one is to achieve specific T cell ablation (by activation induced cell death) through negative selection which eliminates T cells by strong avidity with MHC-peptide complex during early T cell development. A second strategy is to achieve specific T cell anergy where T cells bind to MHC-peptide complex but lack appropriate co-stimulation due to a lack of an interaction with CD28 on T cells with B7 on antigen presenting cells (APC). The third mechanism is to achieve the induction of regulatory T cells (primarily $CD4^{+}CD25^{+}$ regulatory T cells) which suppress the proliferation of self reactive antigen specific T cells. A fourth approach is to cause a shift in the predominant phenotype of the T cell anti-self response from a T helper cell 1 (Th1) to a T helper cell 2 (Th2) phenotype. Th1 cells are responsible for classic cell-mediated functions, such as delayed type hypersensitivity and the activation of cytotoxic T lymphocytes, which are assumed to be suppressed by Th2 cells (16). In EAE, a neurodegenerative self reaction is mediated by autoreactive T cells which are induced by administration of a self NAg. Therefore, the rationale for using therapeutic fusion proteins consisting of the cytokine and NAg was to target the covalently linked NAg to a particular subset of APC through the interaction with cell-surface cytokine receptors to achieve

substantially enhanced presentation of NAg by that subset of APC. The cytokine domain of the fusion protein was predicted to modulate the function of antigen presenting cell to stimulate the inhibitory or tolerogenic activities and simultaneously present the NAg through the MHC class II antigen processing pathways. The NAg-specific Th cells that recognize the NAg presented by the APC may have two fates, one is the desensitization of those self-reactive T cells and another is the differentiation into regulatory T (Treg) cells (19). Treg cells, also known as suppressor T cells, are specialized T cells that suppress the immune system in an antigen-specific manner.

1.8 Suppressive mechanism of Treg cells

Treg cells are assumed to suppress the effector T cells by four mechanisms: by inhibitory cytokines, cytolysis, metabolic disruption, and modulation of DC maturation. Inhibitory cytokines, such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β), are the focus of cytokine-mediated suppression by Treg cells. In allergy and asthma models, adoptive transfer of allergen-specific Treg cells induced significant IL-10 production by $CD4^+$ effector T cells in the lung following allergen challenge, and this Treg cell mediated-suppression was inhibited by the administration of a neutralizing IL-10 receptor specific antibody. Several studies support the direct involvement of TGF-β from Treg cells as a mechanism to suppress the effector T cells. For instance, effector T cells resistant to TGF-β suppression cannot be controlled by Treg cells in an IBD (inflammatory bowel disease) mouse model. In addition TGF-β produced by Tregs has been found to have an important role in the control of the host immune response to *Mycobacterium tuberculosis*, suppression of allergic responses, and prevention of colitis in an IBD model. Recently, a new inhibitory cytokine, IL-35 has been found to be preferentially expressed by Treg cells required for their maximal suppressive activity. Suppression by cytolysis mediated by Treg cells was found from their ability to suppress the clearing of tumors by NK

cells and CTLs by killing these cells in a granzyme B and perforin dependent manner. A recent study suggested that Treg cells can induce apoptosis of effector T cells through a TRAIL-DR5 (tumor necrosis factor related apoptosis inducing ligand-death receptor 5) pathway. Metabolic disruption of effector T cells is also an important suppressive mechanism of Treg cells. In this case one important mechanism is the high expression of CD25 which empowers Treg cells to consume local IL-2 and thereby, make it unavailable to effector T cells. Treg cells have been proposed to induce the intracellular and extracellular release of adenosine nucleosides. Concordant expression of ectoenzymes CD39 and CD73 was shown to generate pericellular adenosine, which suppressed effector T cell function through activating the adenosine A2A receptor. Binding of adenosine to A2A receptor appears to not only inhibit effector T cell function but also to enhance the generation of adaptive Treg cells by inhibiting IL-6 expression while promoting TGFβ function. Treg cells have also been shown to suppress effector T cell function by transferring the potent inhibitory messenger cAMP into the effector T cells via membrane gap function. Targeting dendritic cells is another suppressive mechanism of Treg cells. Treg cells can condition DC upon the interaction between CTLA4 and CD80 and/or CD86 and express indoleamine 2,3-dioxygenase (IDO) that induces the catabolism of tryptophan into pro-apoptotic metabolites that result in suppression of effector T cells. Recent studies have shown that lymphocyte activation gene 3 (Lag 3) expressed by Treg cells binds MHC class II expressed on immature DC with high affinity and induces an immunoreceptor tyrosine-based activation motif (ITAM)- mediated inhibitory signaling pathway (20). By these mechanisms, Treg cells maintain the immune system homeostasis and tolerance to self-antigens by eliminating the autoimmune effector T cells. One major cause of autoimmune disease is assumed to be an inappropriate or inadequate activity of self-antigen-specific Treg cells. Therefore, it became the

focus of research interests to derive potential fusion proteins of cytokines and NAg to engender antigen-specific tolerance in EAE and ultimately in MS.

1.9 Recent successes in development of tolerogenic fusion proteins

In an approach to induce tolerance in EAE, the two fusion proteins, IL2-NAg and IL16-NAg, were analyzed in rats for their efficacy to inhibit the disease state. For both fusion proteins, IL2-NAg and NAg-IL16, covalent linking was crucially important for effective modulation of EAE (18,19).

In another study, GM-CSF and M-CSF were chosen as cytokine domains of the fusion proteins because the two cytokines perform the key role in driving the differentiation, survival, and growth of myeloid-derived APC. Although GM-CSF and M-CSF have many common biological features, they also have unique properties. One of the key features of GM-CSF is that the cytokine modulates the immunogenic pathways in an activation dependent manner. On the other hand, M-CSF is constitutively produced by some cell types and plays an important role in the homeostatic regulation of inactive and dormant macrophages. GMCSF-NAg and MCSF-NAg fusion proteins were found to stimulate DC and macrophages and were very effective as tolerogens to prevent the initiation and progression of EAE (21). All of these studies validate the concept of tolerogenic vaccines formed by the fusion of cytokines and NAg and identify some cytokine partners for the generation of such vaccines to prevent the initiation and inhibit the progression of EAE.

1.10 Objective of this study

IFN-β is one of the most widely used therapies for multiple sclerosis. Although IFN-β can reduce the attack rate up to ~30%, it has some generalized effects on the immune system. On

the other hand, the cost efficiency and substantial inter-patient variability in tolerability and performance are disadvantages. IFN-β most likely inhibits MS through the modulation of general regulatory pathways instead of eliminating a particular cell type. Because IFN-β suppresses the overall adaptive immune response and doesn't stimulate long lasting immunological tolerance, lifetime administration of IFN-β is required. On the other hand, antigen-specific therapies may provide sustainable interruption of self-reactive immunological attacks after a limited series of administrations (22). EAE is also inhibited by IFN-β. An inhibitory role of endogenous IFN-β in EAE has been shown by the exacerbated condition of disease in mice deficient with IFN-β (20). In an attempt to utilize the anti-encephalitogenic response of IFN-β in an antigen-specific manner, a fusion protein consisting of IFN-β and an encephalitogenic peptide from (GPMBP) was used to provide effective inhibition of the initiation and progression of EAE. Pretreatment with IFN-β-NAg attenuated the bout of disease and post-treatment after the onset of disease improved the condition (22).

In this project, a fusion protein consisting of IFN-*β* and two different NAg, an encephalitogenic peptide of myelin oligodendrocyte glycoprotein (MOG35-55) and proteolipid peptide (PLP139-151) have been examined individually to determine their efficacy as tolerogenic vaccines to prevent the initiation and progression of disease in the C57BL/6 and SJL mouse models of EAE respectively.

1.11 Hypothesis developed for this study

A current goal of contemporary immunological research is to induce antigen-specific tolerance as an effective way to re-establish self-tolerance to auto-antigens in the case of autoimmune disease. This project was based on two novel fusion proteins IFN-β-MOG and IFN-

β-PLP and was designed to analyze their effectiveness as tolerogenic vaccines in models of EAE elicited by the respective antigens MOG35-55 or PLP139-151. The project was based on the hypothesis that the IFN-β moiety of the fusion protein will condition the APC and enhance the presentation of covalently linked antigen by those conditioned APC and thereby, will attenuate the subsequent bout of EAE in the case of pretreatment and inhibit the progression of the disease in the case of administration after the onset of disease through the elimination of MOG and PLP specific T cell and expansion of antigen specific Treg cells.

1.12 Adopted strategy

The strategy for this study was to transfect human embryonic kidney (HEK) cells with a pIRES2 plasmid encoding IFN-β and either MOG or PLP linked by an enterokinase (EK) linker. After purification of fusion proteins from expression supernatants, the protein was analyzed for bioactivity of the IFN-β and antigen domains and then examined for inhibitory activity in regard to the initiation and progression of EAE in mice.

1.13 Significance of this study

Successful development of tolerogens for EAE will be a milestone for the prevention and treatment of MS which is the ultimate objective of these studies. MOG35-55 and PLP139-151 are novel fusion partners of IFN-β, which is one of the important innovative aspects of this project. The success of these fusion proteins will be instrumental to develop a human tolerogenic vaccine as a potential breakthrough for the development of therapy for autoimmune disease.

1.14 Net Findings from this study

From *in vitro* bioassays, we found that the IFN-β moiety of both of the fusion proteins was biologically active and retained anti-proliferative activity on T cells. *In vitro* antigen stimulation assays for the antigen domain confirmed myelin-specific T cell stimulatory activity. For *in vivo* experiments, in the case of IFNβ-PLP, SJL mice were significantly protected from EAE compared to the control animals when treated before PLP139-151 sensitization. On the other hand, IFNβ-MOG treatment halted the progression of EAE in MOG sensitized C57BL/6 mice.

CHAPTER 2: METHODS AND MATERIALS

2.1 Recombinant construct development

The IFN-β-NAg fusion protein consisted of the following order from the N terminus to C terminus: IFN-β, EK linker and neuroantigen. Mouse IFN-β was cloned in two plasmids as pVAX1murineIFN-β and pCEP4murineIFN-β containing the IFN-β gene followed by seven-histidine tag (22). In pVAX1murineIFN-β-MOG-M ('M' stands for Met from the first amino acid residue of MOG peptide) and pCEP4murineIFN-β-MOG-M , mouse IFN-β cytokine (NM_019127) was fused by a GDDDDKG enterokinase linker to a dominant 35-55 encephalitogenic epitope (MEVGWYRSPFSRVVHLYRNGK) of MOG (NM_022668, www.ncbi.nlm.nih.gov/protein) followed by consecutive H residues to comprise a C-terminal 7-histidine (7his) tag (Mannie, unpublished data, 2010). In pVAX1murineIFN-β-PLP-139-151 and pCEP4murineIFN-β-PLP-139-151, mouse IFN-β cytokine (NM_019127) was fused by a GDDDDKG enterokinase linker to a dominant 139-151 encephalitogenic epitope of PLP followed by consecutive H residues to comprise a C-terminal 7-histidine (7his) tag (Mannie, unpublished data, 2010). The genes of these fusion proteins were assembled by overlap extension PCR, and the resulting fusion genes were inserted into an expression plasmid by directional, restriction endonuclease free, whole plasmid PCR. To make pVAX1murineIFN-β-MOG-M, overlapping extension PCR ((96^{o}C-2', 68^{o}C-5')-39x; 68^{o}C-10'; 4^{o}C-α) was performed using 50 ng template DNA (pVAX1murineIFN-β) and 10 µl of 10 times diluted product of an overlapping PCR (94^{o}C-3'; (94^{o}C-1', 59^{o}C-30", 68^{o}C-1')-30x; 68^{o}C-10'; 4^{o}C-α. In the case of IFN-β-MOG, nine other mutant fusion proteins were developed where the first amino acid residue M (Met) in the MOG (35-55) was replaced by Ser-Ala (SA), Arg-Pro-Ser-Pro-Pro-Gly (RPSPPG), Gly-Gly-Gly-Gly-Gly-Ser (GGGGGS), Gly (G), Gly-Ser-Gly-Ser-Gly-Ser

(GSGSGS), Gly-Glu-Ser-Gly (GESG), Gly-Gly-Gly-Gly-Gly (GGGGG), Asp-Glu-Arg-Glu-Lys (DEREK) and Thr-His-Asp (THD) sequence through site directed mutagenesis. The mutagenic PCR profile for these constructs was 95^{o}C-2'; (94^{o}C-1',60^{o}C-30", 68^{o}C-5')-5x; (95^{o}C-2', 68^{o}C-5')-30x; 68^{o}C-10'; 4^{o}C-α. The primers that were used for making the IFNβ-MOG constructs are listed in Table I. To make pVAX1murineIFN-β-PLP, overlapping extension PCR (95^{o}C-2', (94^{o}C-2', 60^{o}C-30", 68^{o}C-5')-5x; (94^{o}C-2', 68^{o}C-5')-30x; 68^{o}C-10'; 4^{o}C-α) was done using 50 ng of template DNA (pVAX1murineIFN-β) and two overlapping primers. To make stable cell line, pIRES2AcGFP1IFNβ-MOG and pIRES2AcGFP1IFNβ-PLP139-151 constructs were made. To make pIRES2AcGFP1IFNβ-MOG, a PCR was done using pCEP4 IFNβ-MOG as template (94^{o}C-3'; (94^{o}C-1', 59^{o}C-30", 68^{o}C-1')-30x; 68^{o}C-10'; 4^{o}C-α. The product of this PCR was used as template for another PCR (94^{o}C-3'; (94^{o}C-1', 59^{o}C-30", 68^{o}C-1')-30x; 68^{o}C-10'; 4^{o}C-α. Finally an overlapping extension PCR was done using the product of the second one and pIRES2AcGFP1 template ((96^{o}C-2', 68^{o}C-5')-39x; 68^{o}C-10'; 4^{o}C-α). To make pIRES2AcGFP1IFNβ-PLP139-151, a PCR was done using pCEP4 IFNβ-PLP139-151 as template (94^{o}C-3'; (94^{o}C-1', 59^{o}C-30", 68^{o}C-1')-30x; 68^{o}C-10'; 4^{o}C-α. The product of this PCR was used as template for another PCR (94^{o}C-3'; (94^{o}C-1', 59^{o}C-30", 68^{o}C-1')-30x; 68^{o}C-10'; 4^{o}C-α. Finally an overlapping extension PCR was done using the product of the second one and pIRES2AcGFP1 template ((96^{o}C-2', 68^{o}C-5')-39x; 68^{o}C-10'; 4^{o}C-α). Primers used for making pIRES2AcGFP1IFNβ-NAg constructs are listed in Table II. All the inserts were subjected to forward and reverse sequencing to verify the predicted DNA sequence.

Table I: List of primers used to make pVAX1-IFN-β-NAg constructs

Construct name	Primers
IFNβ-MOG(M)	Forward: 5'-TTCCAAACCGGTGACGATGACAAAGGAATGGAGGTGGGTTGGTACCGTTC TCCCTTCTCAAGAGTGGTTCACCTC-3'
	Reverse: 5'-GGCCGCTCAATGGTGATGGTGATGGTGATGCTTGCCATTTCGGTAGAGGT G AACCACTCTTGAGAAGGGAGAACG-3'
IFNβ-MOG(SA)	Forward: 5'-AACGGTGATGACGATGACAAAGGAAGCGCTGAGGTGGGTTGGTACCGTT CTCCCTTCTCA-3'
	Reverse: 5'-TCCTTTGTCATCGTCATCACCGTTTTGGAAGTTTCTGGTAAGTCTTCGAATG ATGAG-3'
IFNβ-MOG(RPSPPG)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACAGGCCTAGCCCACCCGGGATGGAGG TGGGTTGGTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(GGGGGS)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACGGAGGCGGAGGGGGTTCTATGGAGG TGGGTTGGTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(G)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACGGCATGGAGGTGGGTTGGTACCGTTC TCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(GSGSGS)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACGGATCTGGCTCCGGATCAGGAGTATG GAGGTGGGTTGGTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(GESG)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACAGGCCTAGCCCACCCGGGATGGAGT GGGTTGGTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(GGGGG)	Forward: 5'-AGACTTACCAGAAACTTTTAAAACGGAGGCGGTGGGGGAGGGGGAATGG AGGTGGGTTGGTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(DEREK)	Forward: 5'-AGACTTACCAGAAACTTCAAAACGGAGACGAGAGAGAGAGAAGATGGAG GTGGGTTGGTTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-MOG(THD)	Forward: 5'-AGACTTACCAGAAACTTCCAAAACGGAACCCACGACATGGAGGTGGGTG GTACCGTTCTCCCTTCTCA-3'
	Reverse: 5'-GTTTTGGAAGTTTCTGGTAAGTCTTCGAATGATGAGAAAGTTCCTGAAGAT CTCTGC-3'
IFNβ-PLP	Forward: 5'-GCTGAGACGATGACAAAGGACATTCTTTGGGAAAATGGCTAGGACATCCC GACAAGTTTCATCACCATCACCATCACCATTGAGCG-3'
	Reverse: 5'-TCCTTTGTCATCGTCATCACCGTTTTGGAAGTTTCTGGTAAGTCTTCG-3'

Table II: Primers used to make pIRES2AcGFP1IFNβ-MOG and pIRES2AcGFP1IFNβ-PLP139-151

constructs

Construct name	Primers
pIRES2AcGFP1IFNβ-MOG-PCR-1	Forward: 5'-GAGCTGGTTTAGTGAACCGTCAGATCCGCTGCCGCCACCATGGCCAACAA CAGG TGGATC-3'
	Reverse-5'-GAGGGGCGTTTAAACGATATCTCAGTGATGGTGATGGTGATGGTGATGCT TGCCATTTCGGTAGAGGTGAAC-3'
pIRES2AcGFP1IFNβ-MOG-PCR-2	Forward: 5'-GAGCTGGTTTAGTGAACCGTCAGATCCGCTGCCGCCACCATGGCCAACAACAGG TG GATC-3'
	Reverse-5'-AGTAACGTTAGGGGGGGGGGAGGGAGAGGGGCGTTTAAACGAT ATCTCA -3'
pIRES2AcGFP1IFNβ-PLP139-151-PCR-1	Forward: 5'-GAGCTGGTTTAGTGAACCGTCAGATCCGCTGCCGCCACCATGGCCAACAACAGGTGG ATC-3'
	Reverse-5'-GAGGGGCGTTTAAACGATATCTCAGTGATGGTGATGGTGATGGTGATGCTTGCCATT TCGGTAGAGGTGAAC-3'
pIRES2AcGFP1IFNβ-PLP139-151-PCR-2	Forward: 5'-GAGCTGGTTTAGTGAACCGTCAGATCCGCTGCCGCCACCATGGCCAACAACAGGTGG ATC-3'
	Reverse-5'-AGTAACGTTAGGGGGGGGGGAGGGAGAGGGGCGTTTAAACGATATCTCA-3'

2.2 Transfection and gene expression

Plasmid DNA was isolated using Marligen high purity Midi Prep kit or a similar kit. Fusion gene constructs were expressed by transfection of human embryonic kidney cells (HEK293) cells. Transfection was done in two scales: minitransfection and megatransfection. In minitransfection, 75000 HEK293 cells contained in 1 ml Gibco® Freestyle™ 293 expression medium (Invitrogen™) were transfected with 1.25 µg DNA diluted in 20 µL Optipro SFM 20 and 1.25 µL Turbofect transfection reagent and the protein supernatant was used directly for in vitro anti-proliferative assay without purification. In megatransfection, 30 x 10^6 cells contained in 30 ml Gibco® Freestyle™ 293 expression medium were transfected with 37.5 µg DNA diluted in 200 µL Optipro SFM 20 and 37.5 µL Turbofect transfection reagent in a spinner flask. After 2 days of incubation at 37^{o}C, cell number was counted, centrifuged at 660 rpm for 6 minutes, the expression medium was collected, and 10x10^6 cells were moved from SFM medium in spinner flask to 30 ml complete RPMI1640 medium (10% heat inactivated FBS, 2mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin (Whittaker Bioproducts), 50 µM 2-ME (Sigma Aldrich)) in a T75 cell culture flask in the presence of 400 µg/ml of geneticin G418 drug (Gemini Bioproducts) and incubated at 37^{o}C. The complete RPMI medium was changed with fresh media once a week until the bottom of the flask was a confluent HEK293 cell layer. The cell layer was released using 10 ml release buffer (0.5% BSA, 0.5M EDTA (pH8.0), 1xPBS) and sorted with a flow cytometer (BD Biosciences) in 1 ml complete RPMI. The sorted cells were put back and grown in 30 ml complete RPMI1640 medium in a T75 flask in the presence of 400 µg/ml of G418 drug until they formed a layer and then the cells were released using release buffer and cultured again in SFM medium in the presence of 400 µg/ml of G418 drug. The cell

number was maintained between $60x10^6$ and $90x10^6$. The supernatant was then collected every 2 or 3 days and used for protein purification.

2.3 Purification of fusion proteins

Expression supernatants were concentrated on YM10 ultrafiltration membranes and were subjected to consecutive affinity chromatography steps (23). The first affinity chromatography step was based on the use of a single-chain Fv anti-6his Ab fused to two tandem chitin-binding domains (scFv-CBD2) (24). This recombinant protein was immobilized on a chitin resin column by stable binding of the tandem chitin-binding domains to the chitin bead resin. Immobilization of the scFv anti-6his single-chain Ab onto chitin columns enabled purification of recombinant proteins bearing C-terminal 6-histidine tags from HEK293 cell supernatants. These columns were maintained in TBST buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 0.01% Na azide (pH 8.0)). Before each use, columns were equilibrated in MBS buffer (20 mM MES, 500 mM NaCl, 0.1 mM EDTA (pH 6.5)), and concentrated expression supernatants were passed through the column to trap the 6his-tagged protein. The fusion proteins were eluted in CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffer (50 mM CAPS, 500 mM NaCl, 0.1 mM EDTA (pH 10.0)), concentrated, and directly applied to Ni-NTA agarose columns (Qiagen) followed by extensive washing of the resin (50 mM NaH2PO4, 500 mM NaCl, 10 mM imidazole (pH 8.0)). IFNβ-NAg was eluted by acid elution (pH 4.5) and was concentrated and diafiltrated in Millipore Ultra-15 centrifugal filter devices. Protein quantity was assessed by absorbance at 280 nm and purity was assessed by SDS-PAGE.

2.4 *In vitro* anti-proliferative assay

The SJL.PLP2.$CD4^+TCR^+$ mouse T hybridoma cell is a stable PLP specific, IL-2 dependent line derived from spleen of a SJL mouse sensitized with PLP in complete Freund's

adjuvant (CFA) plus Ptx in Dr. Mannie's lab. To assess the IFN-β mediated anti-proliferative activity of expression supernatants, supernatants from IFN-β-NAg minitransfection expression system were incubated for 2 days in designated titrations (0, 10^{-4}, $10^{-3.5}$, 10^{-3}, $10^{-2.5}$, 10^{-2}, $10^{-1.5}$, 10^{-1}) with 10,000 SJL.PLP2.CD4^{+}TCR^{+} mouse T cells in 200 µl complete RPMI supplemented with recombinant rat IL-2 (0.4% (v/v) Sf9 supernatant) in a 96 well plate (25). Expression supernatant collected in the same way from the minitransfection of IFN-β was used as control. To assess the IFN-β mediated anti-proliferative activity of purified IFN-β-NAg, the protein was added to IL-2 supplemented complete RPMI with SJL.PLP2.CD4^{+}TCR^{+} (10,000 cells per well) mouse T cells at designated concentrations of purified proteins (0, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM) and incubated for 2 days. Purified IFN-β and antigens were used as control. Cultures were pulsed with 1 µCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) on the second day of culture.

2.5 *In vitro* antigen stimulated proliferation assay

The "2D2 TCR" mouse strain is transgenic for mouse T-cell receptor alpha and beta chain (*Tcra* and *Tcrb*) transgenes. Hemizygous animals of this strain are viable and fertile. The majority of CD4^{+} splenocytes express the *Tcra* and *Tcrb* transgenes (as defined by Valpha 3.2 and Vbeta 11 expression). MOG specific T cells were stimulated by culturing the splenocytes from MOG specific TCR alpha and beta chain transgenic 2D2 mouse in complete RPMI at the presence of 1 µM concentration of MOG peptide for 3 days. MOG specific T cells (10,000 cells/well) were then cultured with irradiated mouse C57BL/6 splenocytes (500,000 cells/well) in 200 µl complete RPMI with designated concentrations of purified IFNβ-MOG (0, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM) in a 96 well plate at 37^{o}C for 2 days. MOG35-55 antigen and purified IFN-β were also used as controls. For the antigen stimulation assay of IFNβ-PLP, SJL

mice were sensitized with PLP 139-151 antigen by subcutaneous injection of an emulsion containing 200 µg of PLP-139-151 peptide in 50 µl 1xPBS and 50 µl CFA (200 µg of *Mycobacterium tuberculosis* H37 RA (Difco Bacto) in 50 µl incomplete Freund's adjuvant (IFA) (Difco Bacto) on either side of the base of the tail. When initial symptoms (paralysis of limp tail) of disease were seen, the animals were euthanized, the spleen was removed and splenocytes were processed to a single cell suspension. Most of T cells of these splenocytes are PLP-139-151 specific. These splenocytes were then cultured (500,000 cells/well) in 200 µl complete RPMI with designated concentrations of purified IFNβ-PLP (0, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM) in a 96 well plate at 37^{o}C for 2 days. PLP-139-151 antigen and purified IFN-β were also used as controls. 5% anti-MHC-II mAb was also added as control to see its suppressive effects on ell proliferation. Cultures were pulsed with 1 µCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) on the second day of culture. Cultures were pulsed on the second day of culture and were harvested onto filters on the third day by use of a Tomtec Mach III harvester. [^{3}H]thymidine incorporation into DNA was measured by use of a Wallac 1450 MicroBeta Plus liquid scintillation counter. Error bars represent standard deviation of triplicate or quadruplet sets of wells.

2.6 Flow cytometric analysis

Thymocytes ($2x10^{6}$) from 3 week-old C57BL/6 mouse were individually incubated with 0.25 µM concentration of IFN-β, IFNβ-MOG and IFNβ-PLP in 2 ml complete RPMI for 2 days at 37^{o}C. After 2 days cells were washed once and incubated individually with designated concentration of anti-PE-H-2 (Biolegend) and anti-PE-H-2K^{b}/D^{b} anti-MHC-I Ab (Biolegend). Then the cells were washed three times in 4^{o}C and run in a FACScan flow cytometer (BD

Bioscience) and were analyzed with the CellQuest software program. Dead cells were excluded from analysis by forward versus side scatter profiles.

2.7 Animals

C57BL/6 and SJL mice were maintained at East Carolina University School of Medicine. Animal care and use was performed according to the protocols and guidelines approved by East Carolina University Institutional Animal Care and Use Committee (IACUC).

2.8 *In vivo* preventive experiment for IFNβ-PLP

To determine whether IFNβ-PLP could prevent active induction of EAE, SJL mice were given injections of 2 nmol of IFNβ-PLP in saline on days -21, -14, and -7. In the same way, PLP alone, a mixture of 2 nmol of (IFN-β + PLP), or saline were also injected in control animals. Then 7 days after the last injection, mice were challenged on day 0 to induce EAE with the emulsion containing 200 µg of PLP peptide in CFA (200 µg of heat-killed *Mycobacterium tuberculosis* in 50 µl IFA) in a total volume of 1 ml. The emulsion was injected subcutaneously in two 0.05-ml volumes on either side of the base of the tail. The following scale was used to score EAE: no disease, 0; partial paralysis of tail without ataxia, 0.5; flaccid paralysis of tail or ataxia but not both, 1.0; flaccid paralysis of tail and ataxia or impaired righting reflex, 2.0; partial hind limb paralysis marked by inability to walk upright but with ambulatory rhythm in both legs, 3.0; same as above but with full paralysis of one hind leg, 3.5; full hind limb paralysis, 4.0.

2.9 *In vivo* curative experiment for IFNβ-MOG

To test the curative role of the IFNβ-MOG, C57BL/6 mice were injected subcutaneously to induce EAE on day 0 with the emulsion containing 200 µg of MOG peptide in CFA (200 µg of heat-killed *Mycobacterium tuberculosis* in 50 µl IFA) in a total volume of 1 ml. Disease was enhanced by intraperitoneal injection of 0.2 µg of pertussis toxin (Calbiochem) in 500 µl 1xPBS

on day 0 and day 2. Then the mice were injected subcutaneously with IFNβ-MOG or control reagents (only MOG, mixture of 2 nmol of IFN-β + MOG and saline) on day 10 (2 nmol), day 12 (2 nmol), day 14 (2 nmol) and day 16 (2 nmol). Same scale of scoring used for preventive experiment was used for scoring the animals of curative experiment.

2.10 Statistical analysis

Mean cumulative score, mean maximal score, mean percent of maximal weight loss and the mean number of days with severe EAE were analyzed by parametric ANOVA. The mean cumulative score was calculated by summing the daily scores for each mouse and then averaging the cumulative scores to obtain the mean cumulative score for the group. The mean maximal score was calculated by averaging the most severe score of EAE for all mice in each group. The mean percent of maximal weight loss was calculated as a percent of the value obtained by the subtraction of the daily weight from the maximal weight and dividing with the same maximal weight of each mouse and then averaging the maximal values of all mice in each group. Means were reported together with the SD. Median cumulative score and median maximal score were listed as the median values for all mice in each group and were analyzed by nonparametric ANOVA based on ranked data. ANOVA was interpreted with the Bonferroni post hoc test. One-way ANOVA was used to assess data from a single experiment. "Incidence of severe EAE" was analyzed pair-wise with the Fisher's exact test.

2.11 Histological analysis of EAE

On the day of termination of animal pretreatment experiment, IFNβ-PLP treated and control animals were humanely euthanized. Then the whole spine was put in 4% paraformaldehyde to fix the spinal cord and after one month the spinal cord was carefully removed from the spinal cage. Then the cervical part was sent to Harris Histology Relief Service,

Inc. for block formation in 100% paraffin. 10 micron thick parasagittal sections were mounted onto glass slides and stained with hematoxylin and eosin. Section images were taken with a spot insight digital camera connected to an Olympus Bx51 microscope at 10× magnification.

CHAPTER 3: RESULTS

3.1 Development of construct and purification of protein

All the constructs were made in an orientation where the N-terminal IFN-β was connected by an intervening enterokinase linker to the C-terminal NAg followed by a seven His tag (Fig. 1A). The linker between the NAg and IFN-β domain facilitated their free movement and retention of their own structure without any interference. The His tag was used to facilitate the purification of the protein. After stable transfection using a recombinant pIRES2AcGFP1 plasmid, GFP expressing HEK293 cells were sorted using a flow cytometer (BD Biosciences). Fig. 1B shows the gene order in the mRNA transcript in which the internal ribosomal entry site (IRES) lies in between the IFNβ-NAg fusion gene and green fluorescent protein (GFP) gene and facilitates the separate translation and expression of GFP. Fig. 2Aa and Fig. 2Ba show the percent of total cells which were viable before sorting (for IFNβ-MOG 46.7% and for IFNβ-PLP 36.6%). Fig. 2Ab and Fig. 2Bb show the percent of viable cells that expressed the fluorescence (for IFNβ-MOG 63.0 % and for IFNβ-PLP 21.1%). The purity of sorted cells for IFNβ-MOG and IFNβ-PLP was 92.6% and 86.9% (Fig. 2.A.c and Fig. 2.B.c). This purity was good enough to produce sufficient amounts of protein (around 1 mg protein from the purification of 1L of HEK293F expression media). After purification, purity of the protein was checked by SDS-PAGE run (Fig. 3). Absence of any conspicuous smear or unexpected band confirmed about 95% purity of the protein. This protein preparation was used for all bioassays and animal experiments. The actual molecular weight of IFNβ-MOG and IFNβ-PLP is 24 Kd and 23 Kd respectively, but gel picture shows their ladder position at 35 Kd which is due to their extensive post-translational glycosylation.

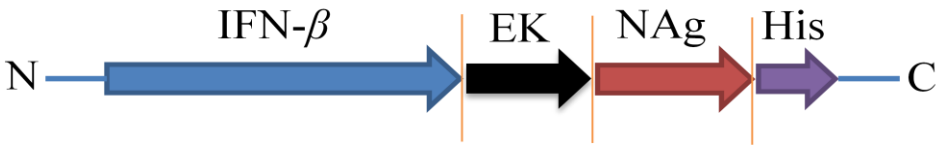


Figure 1A. General organization of IFNβ-NAg. The N-terminal IFN-β was linked to the C-terminal NAg by enterokinase (EK) linker followed by a seven His tag to facilitate the purification.

Figure 1B. The gene order in the mRNA transcript. There was an internal ribosomal entry site (IRES) between the IFNβ-NAg fusion gene and green fluorescent protein (GFP) gene.

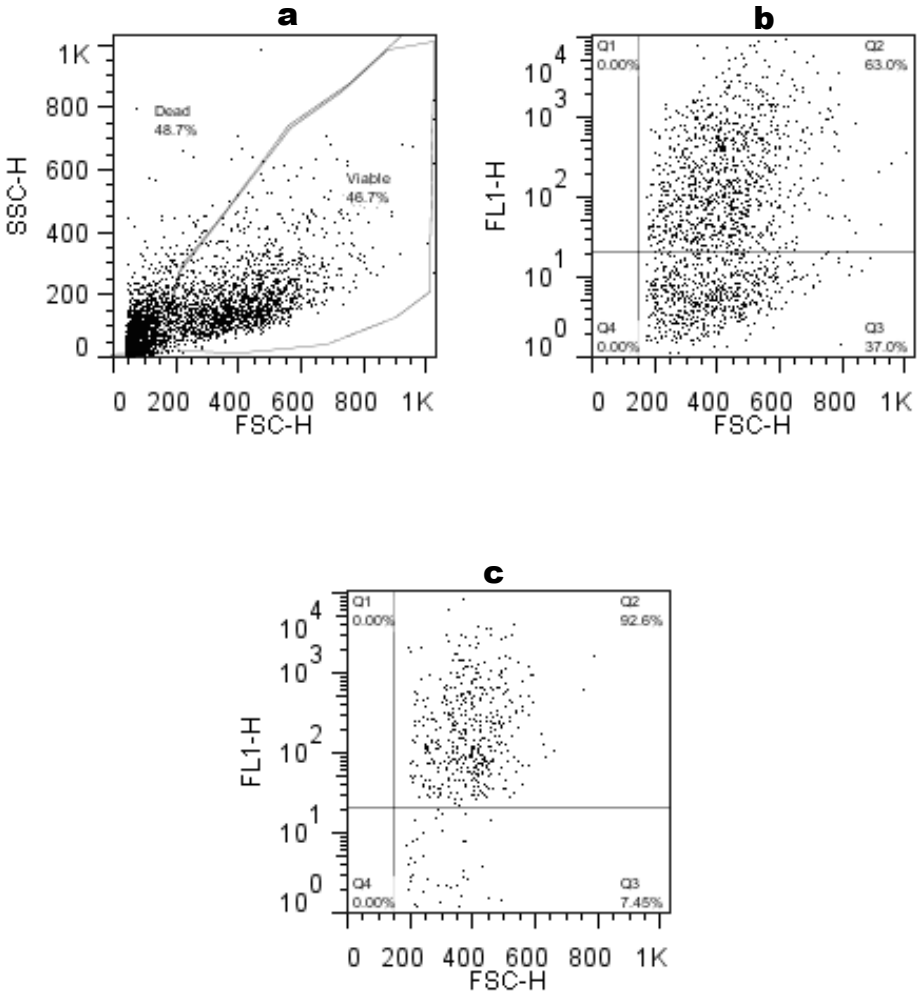


Figure 2A. Flow sort of HEK293 cell lines stably transfected with pIRES2AcGFP1IFNβ-MOG where (a) shows the forward and side scatter of total unsorted cells, (b) shows percentage of viable cells expressing GFP before the sort and (c) shows the purity of sorted cells.

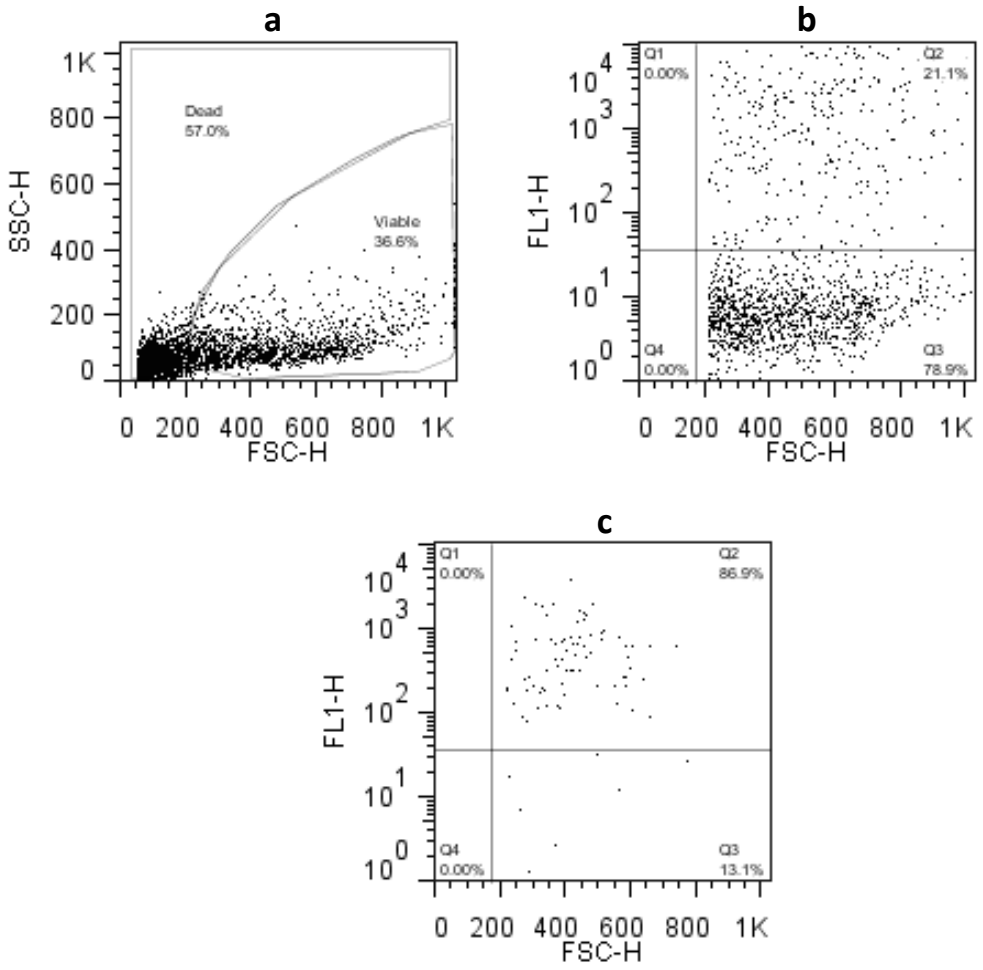


Figure 2B. Flow sort of HEK293 cell lines stably transfected with pIRES2AcGFP1IFNβ-PLP where (a) shows the forward and side scatter of total unsorted cells, (b) shows percentage of viable cells expressing GFP before the sort, and (c) shows the purity of sorted cells.

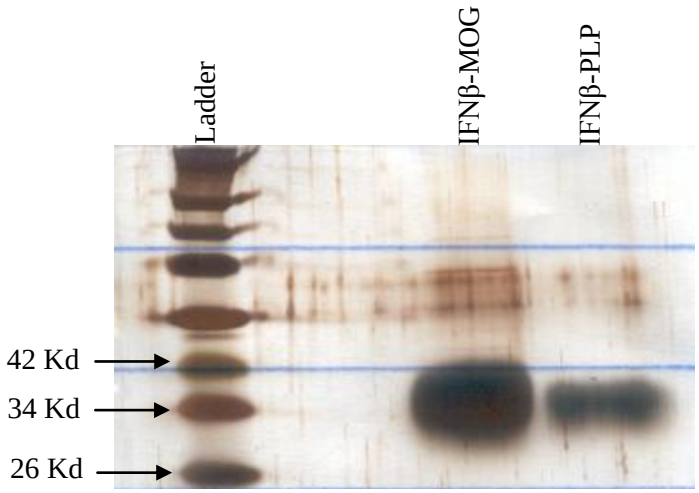


Figure 3. 12% SDS-PAGE gel of purified IFNβ-MOG and IFNβ-PLP

3.2 Cytokine domain of the fusion protein was biologically active

The main objective of this study was to test IFN-β as a fusion partner for generation of tolerogenic cytokine-NAg vaccines. The initial question was whether the cytokine and NAg domains of an IFNβ-NAg fusion protein were biologically active. The activity of IFN-β domain might be anticipated to be affected by its fusion partners. The activity of IFN-β domain of the fusion proteins was assessed by specific bioassays. IFNβ-NAg fusion proteins and relevant controls were expressed by transient transfection of HEK293 cells. The biological activity of the IFN-β domain of the fusion protein was assessed by assays measuring IFN-β-mediated induction of T-cell anti-proliferative and MHC-I expression activity. The anti-proliferative assays were done in two steps. First, the sups from minitransfection were used for IFN-β mediated IL-2 dependent T-cell anti-proliferative bioassay. Expression supernatants containing IFNβ-NAg inhibited IL-2 dependent proliferation of T cells by induction of cell stasis followed by extensive cell death (22). Out of ten candidate constructs for IFNβ-MOG only IFNβ-MOG (EK-M) and IFNβ-MOG (EK-SA) showed anti-proliferative activity (Fig. 4A). Therefore, only these two were used for the subsequent assays and experiments. Anti-proliferative activity of IFNβ-MOG (EK-M) and IFNβ-MOG (EK-SA) expression supernatants was less than that of IFN-β (Fig. 4A). For instance, the titrations of IFNβ-MOG and IFN-β that elicited half maximal anti-proliferative activity (50% of the maximal response was ~ 200,000 cpm) were 10^{-2} and $10^{-2.5}$ respectively. This indicates that the bioactivity of IFN-β in IFNβ-MOG fusion protein was 3.2 times less than IFNβ if equitable expression levels were achieved in these expression supernatants. For IFNβ-PLP, the minitransfection sup for IFNβ-PLP showed a clear anti-proliferative activity (Fig. 4B),

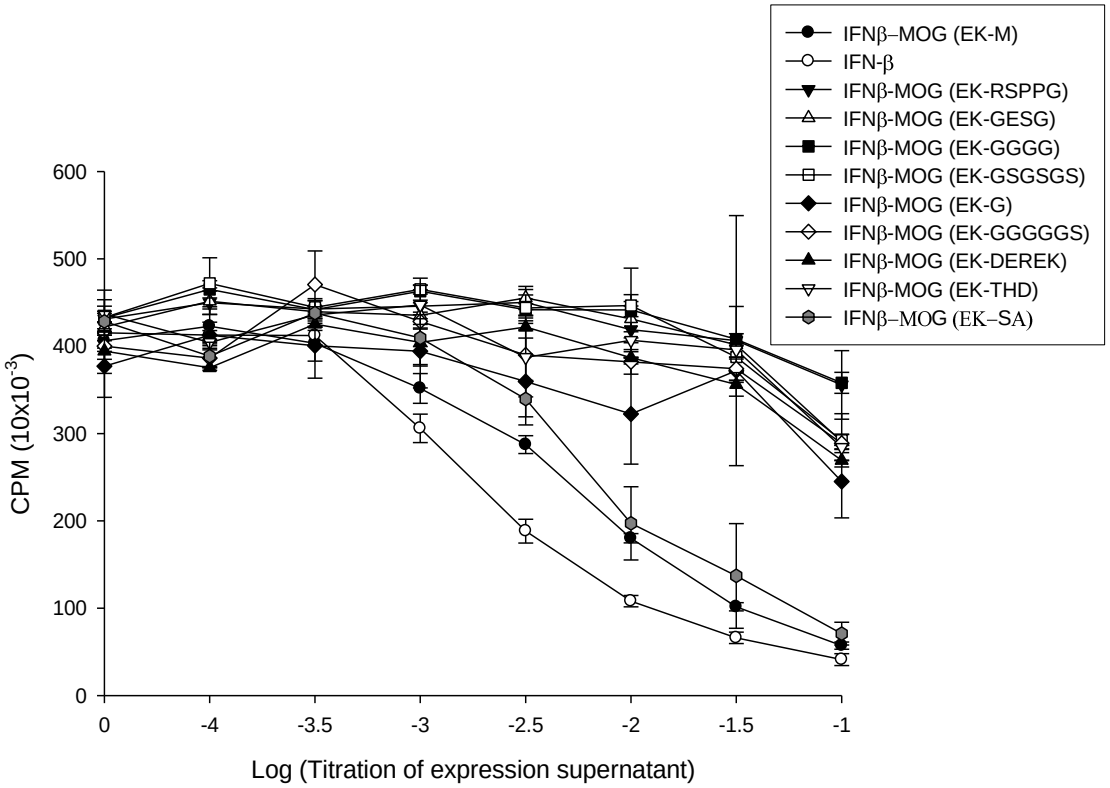


Figure 4A. T cell anti-proliferative assay for IFNβ-MOG minitransfection sup. SJL.PLP2.CD4^{+}.TCR^{+} T cells (10,000/well) were cultured in IL-2 supplemented complete RPMI for 2 days with transfection supernatants in designated titrations representing different constructs for IFNβ-MOG. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day. Out of ten different constructs, only IFNβ-MOG (EK-M) and IFNβ-MOG (EK-SA) showed clear anti-proliferative activity.

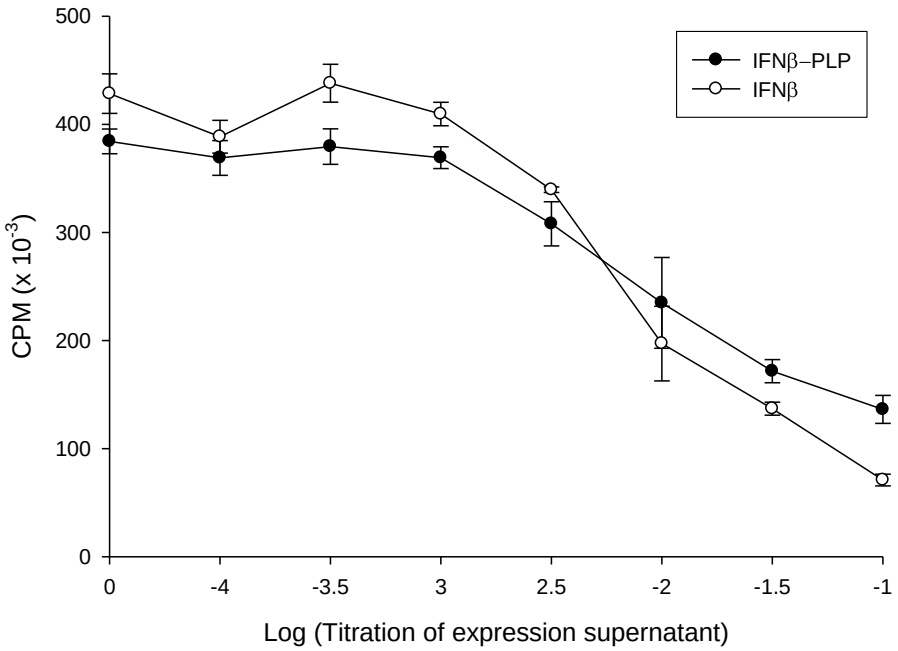


Figure 4B. T cell anti-proliferative assay for IFNβ-PLP minitransfection sup. SJL.PLP2.CD4^{+}TCR^{+} T cells (10,000/well) were cultured in IL-2 supplemented complete RPMI for 2 days with transfection sups in designated titrations for IFNβ-PLP. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day. IFNβ-PLP showed anti-proliferative activity to T cell.

but the activity of IFNβ-PLP expression supernatant was approximately 2 times less than that of IFN-β. Second, the constructs chosen after minitransfection sup bioassay were used for making stable HEK293 cell lines for protein purification. Purified protein for both IFNβ-MOG and IFNβ-PLP showed T cell killing activity as did IFN-β (Fig. 5A and Fig. 5B respectively). Fig. 5A shows that the T cell killing activity of IFNβ-MOG is 40 fold less than IFN-β, and Fig. 5.B shows that the T cell killing activity of IFNβ-PLP is 3.2 fold less than IFN-β. The bioassay using purified protein of IFNβ-PLP gave the similar picture of bioassay done by minitransfection supernatant. At the case of IFNβ-MOG, bioassay using purified protein gave much less activity (40 fold less than IFN-β) compared to the bioassay done by using minitransfection supernatant (3.2 fold less than IFN-β). The other assay done for testing the IFN-β activity of the fusion protein was the stimulation of class I MHC expression on naive thymocytes. Naive C57BL/6 thymocytes were cultured in complete RPMI in the presence and absence of IFN-β and IFNβ-NAg fusion proteins for 2 days. After 2 days, the cells were stained for 1 hour with PE-anti-MHC-I antibody (PE-anti-H-2 and PE-anti-H-2K^b/H-2D^b) and run for flow cytometry. Flow analyses showed a very conspicuous stimulation of class I MHC molecule for IFN-β and IFNβ-NAg fusion proteins compared to the cells cultured without any protein (Fig. 6). In both cases of anti-PE-H-2 (Fig. 6A) and anti-PE-H-2K^b/H-2D^b (Fig. 6B) antibody, a similar stimulation of MHC-I expression was found for IFN-β and IFNβ-NAg fusion proteins. Cultures that lacked IFN-β did not exhibit enhanced expression of class I MHC. Cells that were incubated without antibody showed only a background level of expression (Fig. 6C). These data confirmed the biological activity of IFN-β inside the fusion protein which is consistent with the results obtained from the T cell anti-proliferative assay.

3.3 NAg was biologically active

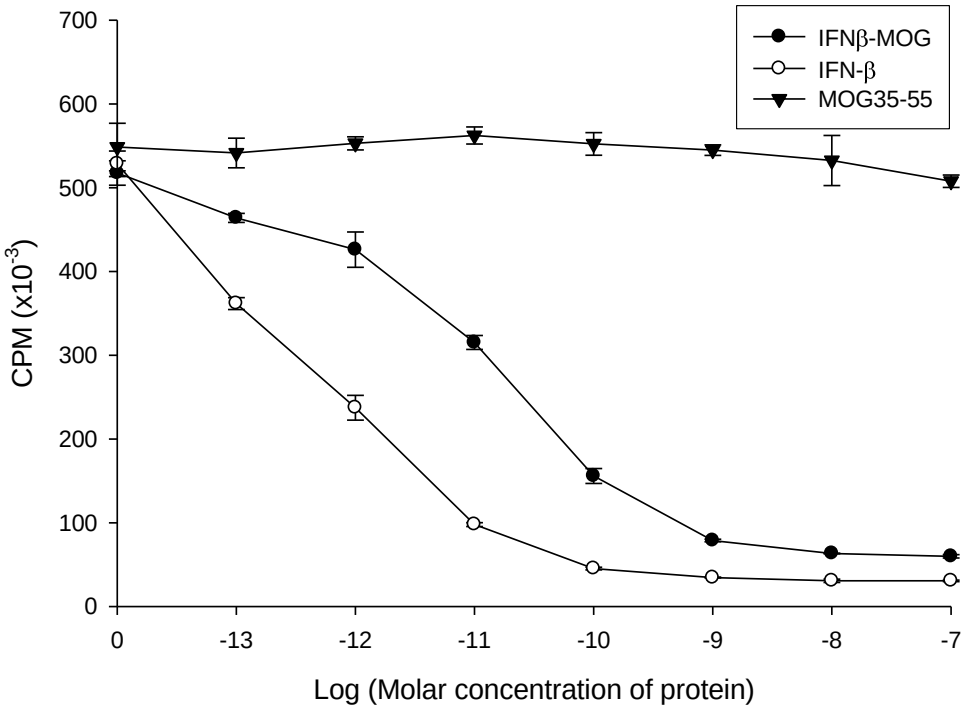


Figure 5A. T cell anti-proliferative assay for purified IFNβ-MOG fusion protein. SJL.PLP2.CD4^{+}TCR^{+} T cells (10,000/well) were cultured in IL-2 supplemented complete RPMI for 2 days with purified IFNβ-MOG in designated concentrations. The x-axis was based on the exponent of the molar concentration. For example, a concentration of 100 nM was represented as $\log_{10}[10^{-7}M]$= -7 and was plotted by the exponent of -7. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day. IFNβ-MOG showed anti-proliferative activity.

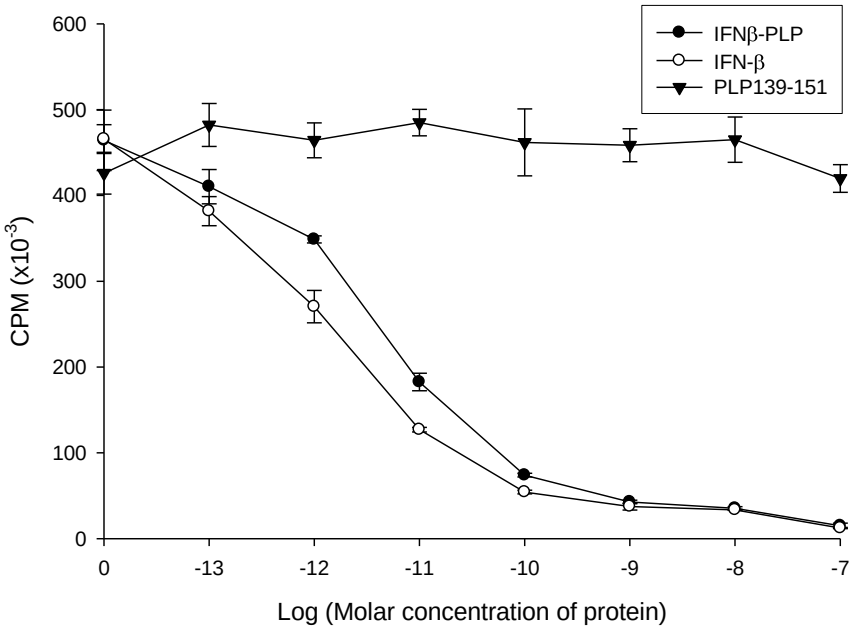


Figure 5B. T cell anti-proliferative assay for purified IFNβ-PLP fusion protein. SJL-CD4^{+}PLP2-T cells (10,000/well) were cultured in IL-2 supplemented complete RPMI for 2 days with purified IFNβ-PLP in designated concentrations. The x-axis was based on the exponent of the molar concentration. For example, a concentration of 100 nM was represented as $\log_{10}[10^{-7}M]$= -7 and was plotted by the exponent of -7. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day. IFNβ-PLP showed anti-proliferative activity.

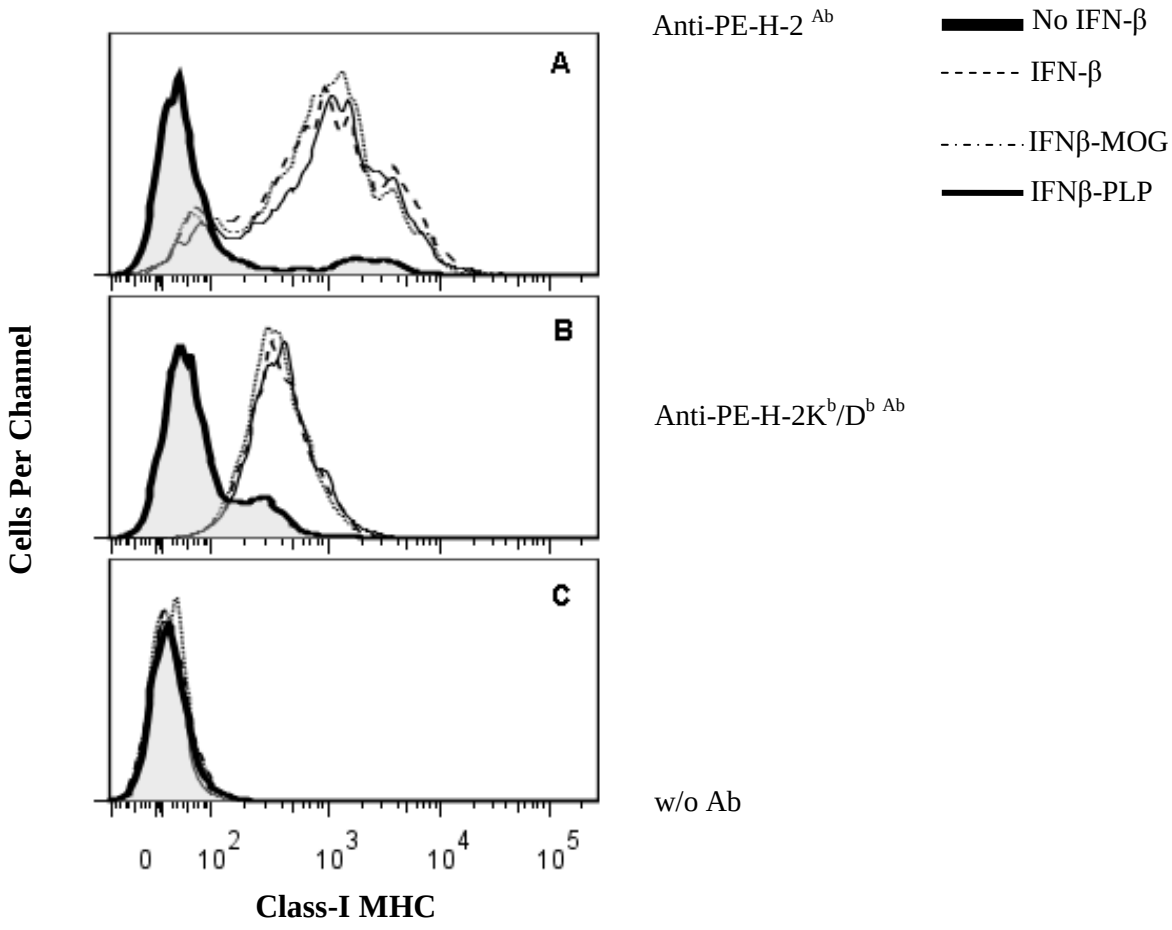


Figure 6. IFN-β domain of IFNβ-NAg was biologically active and able to stimulate the expression of class I MHC glycoproteins in thymocytes. To assess whether IFNβ-NAg could induce class I MHC expression, thymocytes (2 x 10^6) were cultured with and without IFNβ-NAg for 2 days and then stained with anti-PE-H-2 ^{Ab} and anti-PE-H-2K^b/$D^{b\ Ab}$ and analyzed by flow cytometry. Flow data shows class I MHC induction on thymocytes cultured with IFN-β, IFNβ-MOG and IFNβ-PLP when stained with anti-PE-H-2 ^{Ab} (A) and anti-PE-H-2K^b/$D^{b\ Ab}$ (B). A background level of fluorescence was always found for mock treatment (C).

Antigen specific T cell proliferation assays were done to test the antigenic activity of the encephalitogenic peptide domain of the fusion protein. Because of residing with a comparatively larger, folded protein domain IFN-β and being positioned between the enterokinase linker and seven His tag, the NAg domain might be buried by other protein and peptide structures and might be inaccessible to the immune system, but the antigen stimulated T cell proliferation assay revealed the presence of biological activity. The activity of the NAg domain of IFNβ-MOG was confirmed by assaying NAg-specific proliferation of MOG specific 2D2-T cell from '2D2 TCR' mouse in the present of APC (Fig. 7A). IFNβ-MOG and MOG35-55 showed clear stimulation whereas only IFN-β did not show any proliferation. In the case of IFNβ-MOG, clear stimulation of MOG specific T cells were found at 1 µM and 100 nM concentrations. At other concentrations (from 0 to 10 nM) no stimulation was found. One reason for the lack of reactivity at low concentrations may because the response was masked by the inhibitory activity of IFN-β. Splenocytes were irradiated before adding to the T cell culture so that they cannot proliferate and contribute to the final cell count by adding their own number, but they can still internalize the protein, process it and present the antigen through the MHC molecule. Antigen stimulated proliferation of T cells was lower in the case of IFNβ-MOG compared to the MOG35-55 which may be due to the simultaneous anti-proliferative effect of IFN-β linked to the fusion protein. This confirmed the proper T cell stimulatory activity of NAg domain of the fusion protein. In the presence of Y3P anti-class II MHC mAb, T cell proliferation was completely inhibited which verified the processing and presentation of IFNβ-MOG by MHC-II to NAg-specific T cell. In the same way, IFNβ-PLP showed a clear stimulation of PLP-specific T cells (Fig. 7B). In this case, splenocytes from PLP sensitized mice were used for PLP specific T cell stimulation. There was a

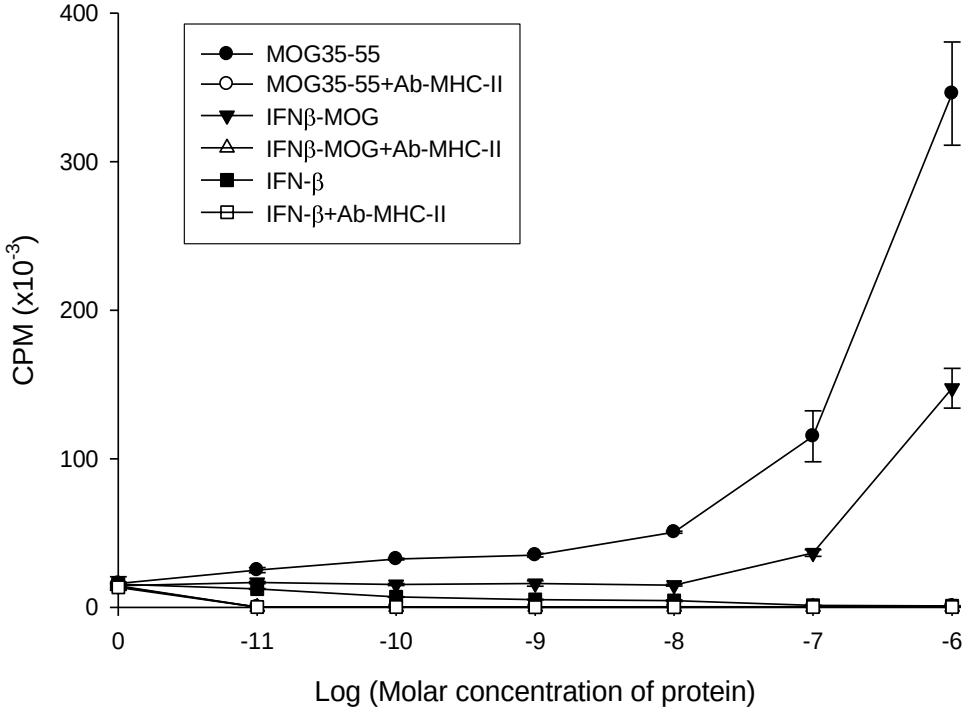


Figure 7A. MOG-specific T cell proliferative assay for purified IFNβ-MOG fusion protein. MOG specific 2D2 T cells (10,000/well) were cultured in complete RPMI for 2 days with purified IFNβ-MOG in designated concentrations in the presence of irradiated splenocytes (500,000/well). The x-axis was based on the exponent of the molar concentration. For example, a concentration of 1 μM was represented as $\log_{10}[10^{-6}M]$= -6 and was plotted by the exponent of -6. 5% anti-MHC-II mAb was used to suppress the cell proliferation. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day.

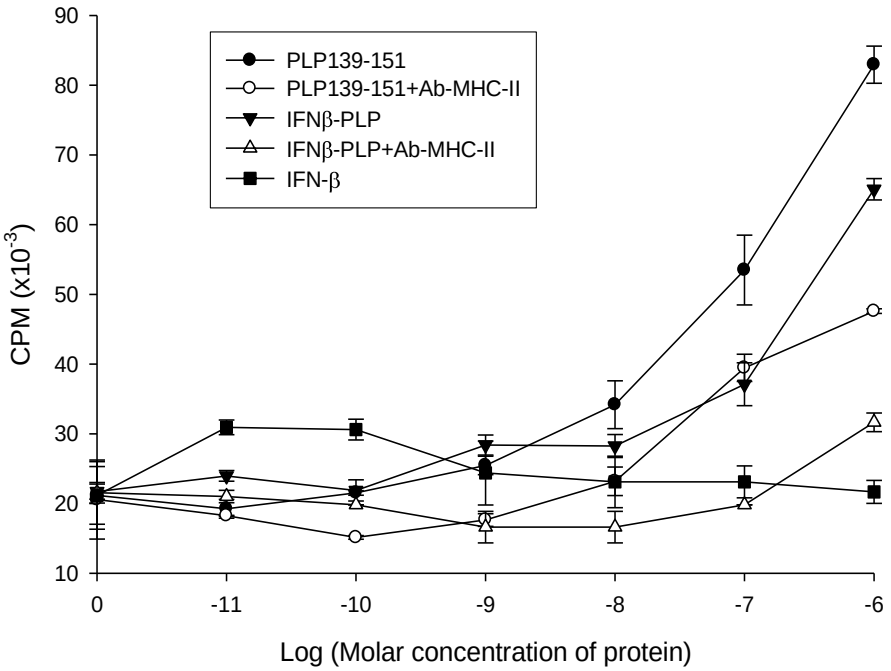


Figure 7B. PLP-specific T cell proliferative assay for purified IFNβ-PLP fusion protein. Splenocytes (500,000/well) from PLP sensitized SJL mouse containing PLP specific T cells (10,000/well) were cultured in complete RPMI for 2 days with purified IFNβ-PLP in designated concentrations. The x-axis was based on the exponent of the molar concentration. For example, a concentration of 1 μM was represented as $\log[10^{-6}]=\log_{10}[10^{-6}M]= -6$ and was plotted by the exponent of -6. 5% anti-MHC-II mAb was also used to suppress the cell proliferation. The culture was pulsed with [^{3}H]-thymidine on the 2nd day and harvested on the 3rd day.

clear stimulation at the concentration of 1 μM and 100 nM of the fusion protein. In this case, higher stimulation was found for only PLP139-151 compared to the fusion protein IFNβ-PLP. In the presence of Y3P anti-class-II MHC mAb, PLP-specific T cell proliferation was partially inhibited indicating that suboptimal preparations of anti-MHCII antibody were used in this assay This partial suppression of stimulation may be due to the lack optimization of the antibody preparation.

3.4 IFNβ-PLP could prevent EAE

After assessing the bioactivity of fusion domains of IFNβ-NAg, the most important question was whether the fusion proteins can efficiently prevent and cure the disease. IFNβ-PLP was used for assessing the preventive capacity. SJL mice were subcutaneously injected with IFNβ-PLP, a mixture of IFN-β and PLP, PLP139-151 or saline on days -21, -14 and -7 followed by an encephalitogenic challenge with PLP in CFA on day 0. Pretreatment of SJL mice with IFNβ-PLP inhibited EAE induced by a subsequent encephalitogenic challenge of PLP-139-151 in CFA (Table III). IFNβ-PLP significantly inhibited the cumulative and maximal score and maximal weight loss. It also significantly decreased the incidence of EAE and mean days with severe EAE. Pretreatment with the mixture of IFNβ + PLP-139-151 or PLP-139-151 did not inhibit EAE. Time course of disease showed that IFNβ-PLP pretreatment significantly improved the clinical EAE score (Fig. 8) and percent of initial body weight (Fig. 9). Whereas IFNβ-PLP pretreatment maintained a healthy score, control pretreatments (mixture of IFNβ + PLP, the PLP139-151 peptide or saline) did not prevent the relapsing remitting disease. In the same way, only IFNβ-PLP pretreatment inhibited the weight loss. This experiment provided evidence that the capability of IFNβ-PLP fusion protein to prevent the EAE in mice.

3.5 Covalent linkage between IFN-β and NAg was required for tolerance

Table III: Vaccination with IFNβ-PLP fusion protein prevented the subsequent induction of EAE

Treatment	Incidence of EAE[a]	Mean cumulative score[b]	Median cumulative score[b]	Mean maximal score[b]	Median maximal score[b]	Mean mximal wt. loss (%)[b]	Incidence of severe EAE[bc]	Mean no. of days with severe EAE[bc]
IFNβ-PLP	2 of 8	1.2 ± 2.8	0.0	0.3 ± 0.5	0.0	13.7 ± 4.2	0 of 8	0.0 ± 0.0
IFNβ+PLP	8 of 8*	38.6 ± 17.1*	41.0	3.5 ± 1.1*	4.0	24.2 ± 6.1*	7 of 7*	8.4 ± 4.6*
PLP	7 of 7*	25.4 ± 14.9*	19.5	3.3 ± 0.8*	3.5	24.4 ± 4.9*	8 of 8*	5.3 ± 5.1
Saline	8 of 8*	40.2 ± 22.3*	34.8	3.9 ± 0.2*	4.0	25.4 ± 7.0*	8 of 8*	9.4 ± 7.5*

[a] SJL mice were injected subcutaneously with saline, 2 nmoles of IFNβ-PLP fusion protein, 2 nmoles of the synthetic peptide PLP139-151 in saline and 2 nmoles of mixture of (IFN-β + PLP) in saline on days -21, -14 and -7. Mice were then challenged with on day 0 with 200 µg PLP-139-151 in CFA. Mice were weighed and scored daily for clinical signs of EAE through day 42.

[b] Cumulative scores were calculated by summing daily scores for each mouse. Maximal scores were calculated as the most severe EAE score for each mouse. The mean cumulative and mean maximal scores included all mice within a group including both afflicted and non-afflicted mice. Differences in median values for cumulative and maximal scores were analyzed non-parametric ANOVA based on ranked scores. Differences in mean values for percent of maximal weight loss and number of days with severe EAE were assessed by parametric ANOVA. ANOVA was interpreted with the Bonferroni post hoc test. Incidence of EAE was analyzed by Fisher's Exact Test.

[c] Severe EAE was defined as hindlimb paresis or paralysis (clinical score of 2.0 or greater).

* Mean difference from IFNβ-MOG is significant at 0.05 level.

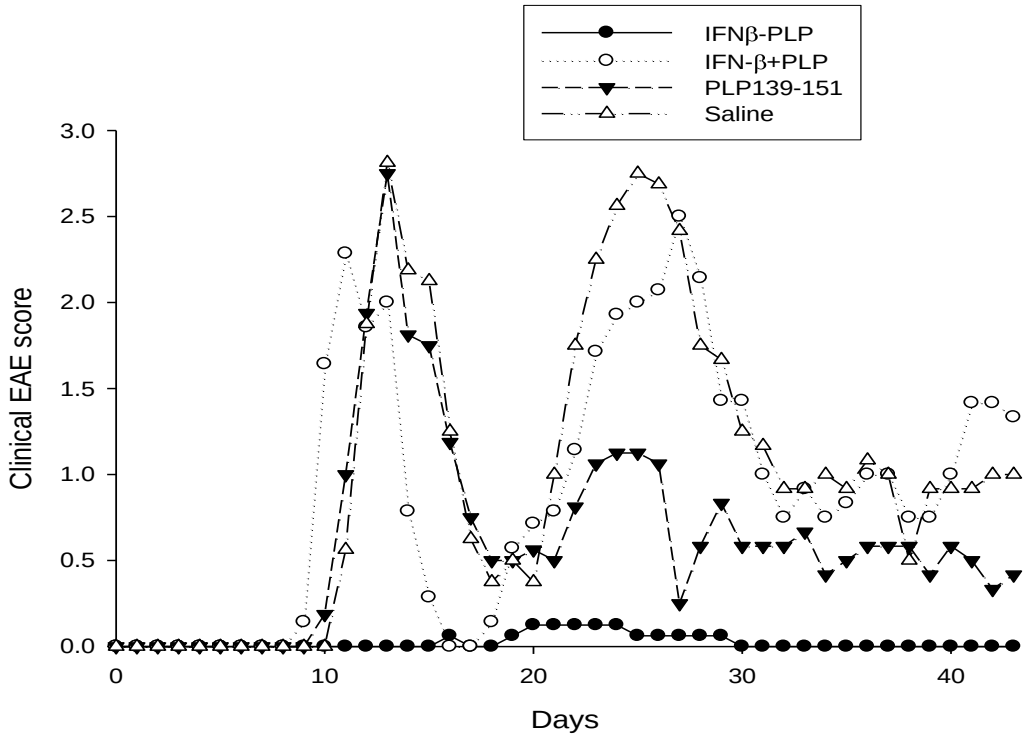


Figure 8. Time course of EAE inhibitory activity of IFNβ-PLP. SJL mice were pretreated with 2 nmoles of IFNβ-PLP, saline, PLP139-151 or equimolar mixture of IFN-β and PLP (2 nmoles of each) on days -21, -14 and -7 and challenged with 200 µg of PLP139-151 in CFA on day 0 and scored every day for clinical signs of disease. Pretreatment with saline, PLP139-151 or an equimolar mixture of IFN-β and PLP did not prevent the relapsing remitting course of disease, but pretreatment with IFNβ-PLP prevented it very efficiently.

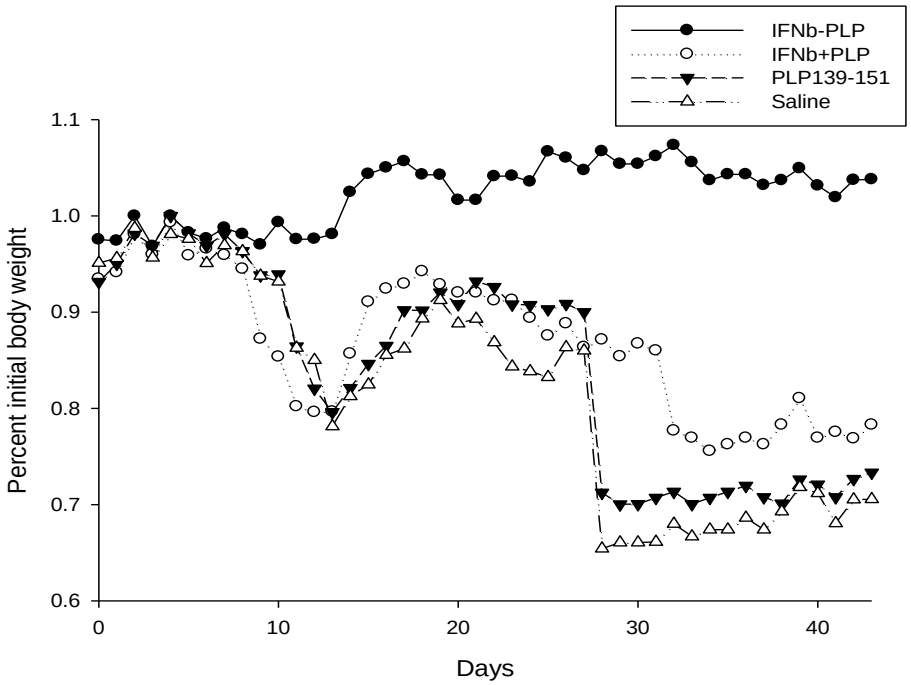


Figure 9. IFNβ-PLP pretreatment maintained a uniform initial body weight. SJL mice were pretreated with 2 nmoles of IFNβ-PLP, saline, PLP139-151 or equimolar mixture of IFN-β and PLP (2 nmoles of each) on days -21, -14 and -7 and challenged with 200 µg of PLP139-151 in CFA on day 0 and weighed every day. Only IFNβ-PLP pretreatment prevented weight loss and maintained a uniform weight even after encephalitogenic challenge.

The tolerogenic activity of IFNβ-PLP was contingent upon the covalent linkage of IFN-β and PLP-139-151 (Table III).SJL mice pretreated with IFNβ-PLP exhibited significant reductions in all measures of EAE severity including the incidence of EAE. In contrast, mice pretreated with the mixture of IFN-β + PLP-139-151as separate molecules were fully susceptible to EAE. Therefore, physical linkage between IFN-β and PLP-139-151 was required for tolerance induction.

3.6 IFNβ-MOG halted the disease when administered during the onset of EAE

As a tolerogenic vaccine, IFNβ-NAg is assumed to not only prevent the EAE but also to halt the ongoing disease. IFNβ-MOG fusion protein was used test its efficacy to inhibit EAE in C57BL/6 mice. To assess whether IFNβ-MOG could inhibit the ongoing EAE, treatment was initiated after the onset of disease. To do that, mice were matched for clinical severity of EAE on day 10 after encephalitogenic challenge. Matched groups were treated with IFNβ-MOG and control reagents (mixture of IFN-β + MOG, only MOG peptide and saline) on days 10, 12, 14 and 16. IFNβ-MOG treatment significantly reduced incidence of EAE, mean maximal score, incidence of severe EAE and mean number of days with severe EAE (Table IV). Although not significant, IFNβ-MOG also reduced the cumulative score and maximal weight loss. Mice treated with the mixture of IFN-β and MOG also showed an apparent reduction in mean cumulative scores, maximal scores and mean number of days with EAE. The later finding probably reflected the inhibitory activity of IFN-β by itself, but this curative effect of IFN-β was not statistically significant. The time course of clinical score (Fig. 10) and initial percent of weight (Fig. 11) also gave a very consistent picture of IFNβ-MOG to inhibit the EAE by maintaining a healthy EAE score and consistent body weight. Treatment with the mixture of

Table IV: Treatment with IFNβ-MOG fusion protein inhibited the progression of EAE

Treatment	Incidence of EAE[a]	Mean cumulative score[b]	Median cumulative score[b]	Mean maximal score[b]	Median maximal score[b]	Mean mximal wt. loss (%)[b]	Incidence of severe EAE[bc]	Mean no. of days with severe EAE[bc]
IFNβ-MOG	2 of 4	1.6 ± 2.6	0.5	0.4 ± 0.5	0.25	4.7 ± 6.4	0 of 4	0.0 ± 0.0
IFNβ+MOG	2 of 4	13.4 ± 16.5	9.3	1.5 ± 1.6	1.25	7.9 ± 5.6	2 of 4	4.5 ± 5.4
MOG	4 of 7	27.8 ± 37.0	17	1.5 ± 1.6	1.0	8.7 ± 10.6	2 of 7	7.0 ± 11.4
Saline	6 of 7*	58.4 ± 28.7	68	3.4 ± 1.5*	4.0	21.2 ± 8.5	6 of 7*	19.9 ± 9.7*

[a] C57BL/6 mice were challenged with on day 0 with 200 µg MOG35-55 in CFA and were given intra-peritoneal injections of 0.2 µg pertussis toxin in PBS on day 0 and day 2. Mice were then injected subcutaneously with saline, 2 nmoles of IFNβ-MOG fusion protein, 2 nmoles of the synthetic peptide MOG-35-55 in saline and mixture of 2 nmoles of IFN-β and 2 nmoles of synthetic peptide on days 10, 12, 14 and 16. Mice were weighed and scored daily for clinical signs of EAE through day 39.

[b] Cumulative scores were calculated by summing daily scores for each mouse. Maximal scores were calculated as the most severe EAE score for each mouse. The mean cumulative and mean maximal scores included all mice within a group including both afflicted and non-afflicted mice. Differences in median values for cumulative and maximal scores were analyzed non-parametric ANOVA based on ranked scores. Differences in mean values for percent of maximal weight loss and number of days with severe EAE were assessed by parametric ANOVA. ANOVA was interpreted with the Bonferroni post hoc test. Incidence of EAE was analyzed by Fisher's Exact Test.

[c] Severe EAE was defined as hindlimb paresis or paralysis (clinical score of 2.0 or greater).

* Mean difference from IFNβ-MOG is significant at 0.05 level.

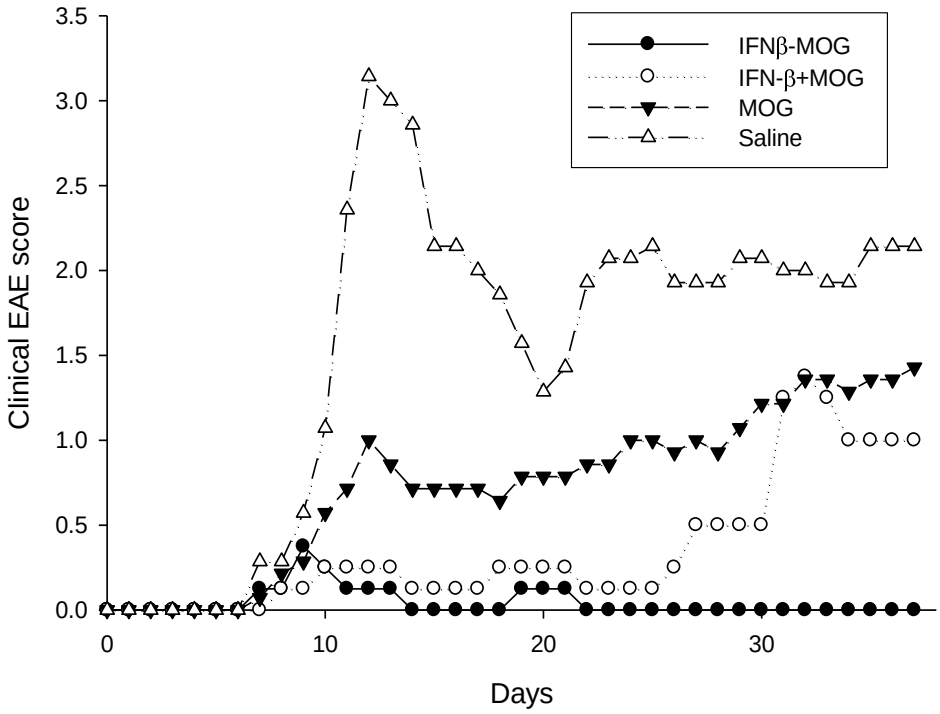


Figure 10. IFNβ-MOG strongly inhibited the progression of EAE when administered after encephalitogenic challenge. C57BL/6 mice were immunized with 200 µg MOG35-55 in CFA on day 0 and treated with 2 nmoles of IFNβ-MOG, saline, MOG35-55 or an equimolar mixture of IFN-β and MOG35-55 (2 nmoles of each) on days 10, 12, 14 and 16 after the onset of disease and scored every day for clinical signs. While treatment with saline, MOG35-55 or a mixture of IFN-β and MOG35-55 did not inhibit the disease, IFNβ-MOG inhibited EAE.

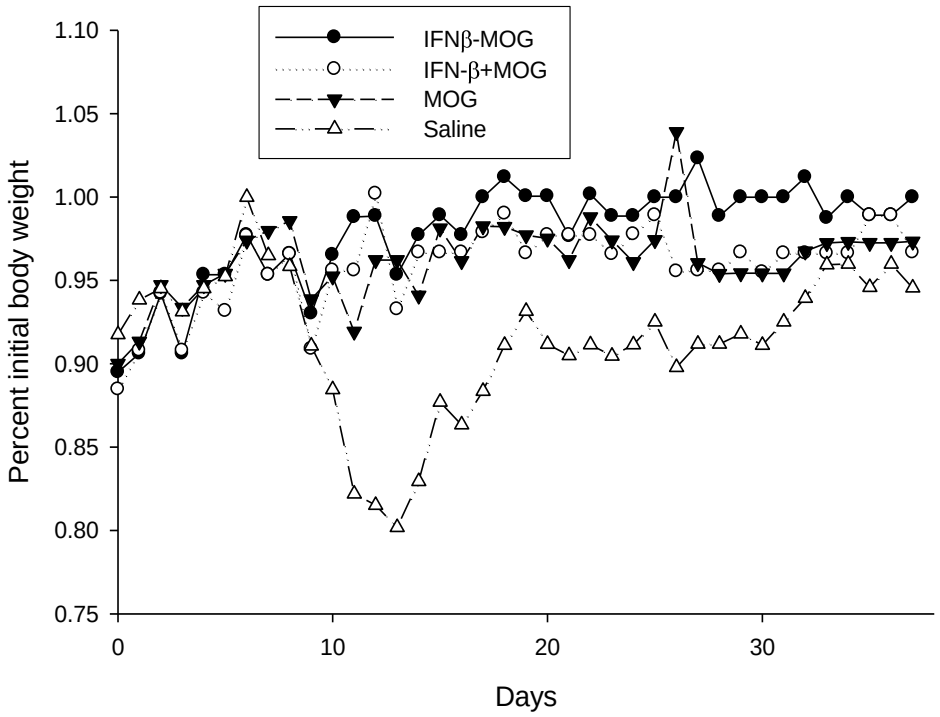
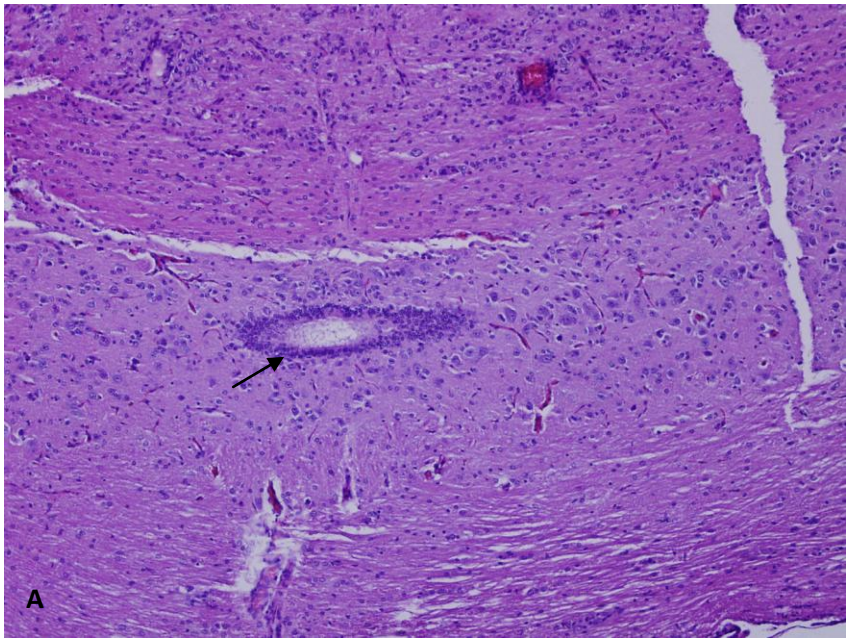


Figure 11. Time course of initial percent of body weight of mice treated with IFNβ-MOG. C57BL/6 mice were immunized with 200 µg MOG35-55 in CFA on day 0 and treated with 2 nmoles of IFNβ-MOG, saline, MOG35-55 or an equimolar mixture of IFN-β and MOG35-55 (2 nmoles of each) on days 10, 12, 14 and 16 after the onset of disease and weighed every day. Mice treated with IFNβ-MOG, MOG35-55 or mixture of IFN-β and MOG35-55 maintained a uniform body weight, but the mice treated with saline did not maintain a uniform body weight.

IFN-β and MOG or MOG35-55 peptide maintained a consistent body weight which gave a close scenario to the case of IFNβ-MOG (Fig. 11). Based on these data, it is worthy to conclude that IFNβ-MOG is an effective inhibitor EAE when administered after the onset of EAE.

3.7 Histological evidence of EAE

Mice pretreated with IFNβ-PLP did not exhibit any histological evidence of EAE (Fig. 12B). On the other hand, mice, pretreated with no IFNβ-PLP, were afflicted with EAE and exhibited conspicuous focal lesions in CNS (Fig. 12A). These lesions were identified as a perivascular infiltration of mononuclear cells in the white matter of the spinal cord. These lesions are manifestation of classical EAE affliction in the animals which were not vaccinated with the IFNβ-PLP.



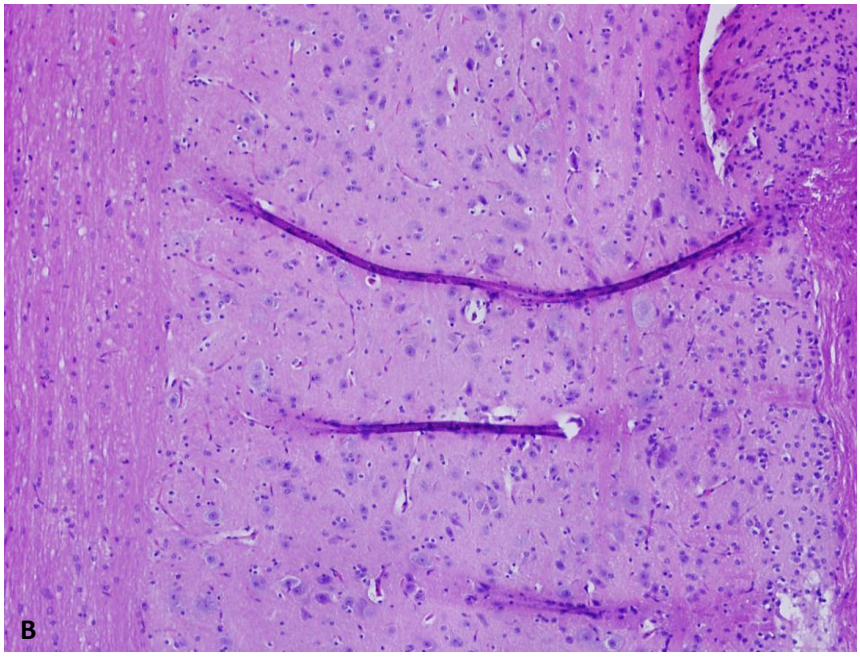


Figure12. IFNβ-PLP prevented histological signs of EAE. Shown are representative histological sections of mice. Control mice that were not pretreated with IFNβ-PLP were afflicted with severe EAE and had perivascular infiltrations of mononuclear cells (arrow shows the lesion) which is typical of EAE in cervical region of the spinal cord (A). In contrast, mice pretreated with IFNβ-PLP were not afflicted with severe EAE and did not show any inflammatory lesions in cervical regions of the spinal cord (B).

CHAPTER 4: DISCUSSION

4.1 IFN-β was covalently linked to NAg as a tolerogenic cytokine partner

IFN-β is currently used as one of the most universally accepted therapies for the treatment of MS. Although the chronic administration of IFN-β can reduce the frequency of clinical signs and overall disease burden, its beneficial effects are limited due to its patient-to-patient variability and difficulty in assessing efficacy in a particular patient (22). In this study, IFN-β was used as a fusion partner for MOG35-55 and PLP139-151 to derive novel tolerogenic therapeutic fusion proteins for EAE. To validate the domains of these fusion proteins, we tested the activity of the fusion components. Specific bioassays were performed to confirm whether the fusion components of the proteins were biologically active. For the IFN-β domain, a T cell anti-proliferative assay was done which showed inhibitory activity of IFNβ-MOG. These results indicated that the IFN-β retained biological function as a fusion partner (Fig. 5A). Previous studies showed that IFN-β stimulated the expression of class I MHC molecule on naive thymocytes (22). We designed a parallel experiment to confirm the biological function of the IFN-β domain of the fusion protein by assessing its ability to stimulate the expression of class I MHC on naïve thymocytes. Flow data clearly showed that IFNβ-NAg induced the expression of class I MHC identical to that of the control IFN-β (Fig. 6). The result obtained from this flow cytometry experiment was consistent with the flow data reported for another IFNβ-NAg fusion protein which showed enhanced expression of class I MHC in rat T cells (22). According to that report, IFN-β domain augmented the expression of class I MHC molecule and did not affect the expression of TCR-β or other markers such as CD4, Thy1.1, LFA-1, Class-II MHC, CD2, CD5, CD28, CD45 or CD48. Exactly same strategy was undertaken to test the biological activity of IFN-β domanin as a fusion partner in IFNβ-PLP (Fig. 5B and Fig. 6).

An antigen-specific T cell proliferation assay was designed to test the antigenic activity of the encephalitogenic peptide domain of the fusion protein. The NAg domain is adjacent to the comparatively larger, folded protein IFN-β and in between the enterokinase linker and the 7-his tag. Therefore, the NAg domain was potentially buried in the other protein and peptide structures and hidden from the immune system. However, the antigen-stimulated T cell proliferation assay confirmed the biological activity of the NAg domain (Fig. 7.A). In an antigen-stimulated proliferation assay, the presence of an anti-MHC-II antibody (Y3P) suppressed the T cell stimulation. These data indicated that the APC-mediated presentation of IFNβ-NAg through MHC-II molecule to MOG specific TCR transmitted a T cell activation signal to stimulate proliferation. Similarly, the antigen-specific assay for IFNβ-PLP confirmed the biological activity of the PLP domain (Fig.7.B). All of these different bioassays for IFN-β and NAg confirmed the respective biological activities within the fusion protein structure.

4.2 Novel IFNβ-NAg proteins have tolerogenic therapeutic promise

To test the efficacy of IFNβ-NAg as TTV, murine IFNβ-MOG (35-55) and IFNβ-PLP (139-151) TTVs were derived and tested in the C57BL/6 chronic progressive and the SJL relapsing-remitting model of EAE, respectively. IFNβ-PLP prevented the relapsing-remitting EAE in SJL mice when administered as three injections before challenging with antigen (Table III and Fig. 8 & 9) and IFNβ-MOG suppressed the progression of disease in C57BL/6 mice when administered as four injections after the onset of disease (Table IV Fig. 10 & 11). Previous studies have shown that the IFN-β has strong suppressive effects (27). Therefore, some inhibition of disease was observed with treatment of equimolar mixture of IFN-β and MOG compared to treatments with either saline or MOG35-55 (Table IV). On the other hand, IFN-β prevented the disease only when used as a cytokine partner in the fusion protein. Pretreatment with an

equimolar mixture of IFN-β and PLP was also ineffective for prevention of disease (Table III). These data indicate, in mouse model of EAE, the physical linkage between IFN-β and NAg had a crucial role in effectively targeting, processing and presenting the NAg through the APC to the T cell clones specific for the respective NAg.

IFNβ-MOG halted the progression of disease when administered at the onset of the disease (Table IV and Fig. 10 & 11). The ability of IFNβ-MOG to suppress the progression of the disease could not be strictly attributed to the IFN-β domain, because the mixture of IFN-β and MOG treatment did not inhibit the chronic form of EAE (Fig. 10). These data imply that IFNβ-MOG eliminates the self reactive T cells depending on the antigen specificity rather than by the general cytotoxic effects of IFN-β on the T cells. The NAg domain may attract antigen specific T cells, and the IFN-β domain may activate the cytotoxic pathways to kill already-existing self reactive T cells. Another central finding was that equimolar mixture of IFN-β and MOG did not suppress ongoing disease (Fig. 10). This indicates that the linkage between the cytokine and NAg domains was required for long term protection. Treatment was started at the early onset of disease, because this is the first transitioning stage of effector T cells to CNS. Administration of IFNβ-NAg at this stage halted the progression of disease. In contrast, treatment with MOG35-55 or saline could not stop the progression to paralytic disease. These data indicate that IFN-β covalently linked to NAg had tolerogenic reactivity in both preventing and curing EAE.

4.3 Certain cytokine-NAg fusion proteins enhance the availability of NAg to the APC

In one study, antigen specific tolerance was achieved by targeting covalently linked foreign antigen to DC for enhanced presentation by use of a recombinant antibody-antigen fusion

protein specific for DEC-205 (CD205) (28, 29). Targeting of antigen to DC led to an initial proliferative burst by antigen-specific T cells followed by a later collapse and emergence of tolerance (28, 29). In another study, a recombinant anti-DEC205 antibody containing the MOG35-55 peptide as the C-terminus was administered 7 days before encephalitogenic challenge that suppressed the subsequent development of EAE in C57BL/6. Similarly, a recombinant anti-DEC205 antibody containing PLP139-151 peptide suppressed the subsequent induction of EAE in SJL mice. Tolerance was induced not only by elimination of self-reactive pathogenic $CD4^+$ T cells but also by the expansion of regulatory T cells (30, 31). In another study, immunization of mice with recombinant anti-DEC205 antibody fused with the extracellular domain of human epidermal growth factor receptor (HER2) gave protection from a neu-expressing mammary tumor challenge mediated by anti-tumor T cell induction (32).

In our study, pretreatment of SJL mice with IFNβ-PLP prevented the subsequent bout of disease, but pretreatment with the unlinked mixture of IFN-β and PLP139-151 failed to give any protection (Table III). Similarly, treatment of C57BL/6 mice with IFNβ-MOG fusion protein right after the onset of disease suppressed the progression of disease more effectively than treatment with the unlinked mixture of IFN-β and MOG35-55 (Table IV). These studies gave strong evidences for the strategy of targeting encephalitogenic NAg to APC by using fusion proteins to diminish the susceptibility to EAE. These studies also strengthen the hypothesis that enhanced and efficient presentation of self antigens by APC may contribute to the restoration of self tolerance. Administration of fusion protein consisting of IFN-β and encephalitogenic antigen can be a very efficient mode of enhanced presentation of antigen by APC. IFN-β is a very promising cytokine for carrying the encephalitogenic antigen as a fusion partner to the APC for enhanced and efficient presentation to the pathogenic T cells.

4.4 Rationale of IFN-β as a tolerogenic fusion partner

Like other cytokines, interferons have a multitude of functions in the mammalian immune system. They play a pivotal role in inducing many signaling pathways of the immune system. There are two major types of interferons; i.e. Type I (IFN-α and IFN-β) and Type II (IFN-γ). Interferons transmit their signal through interactions with specific cell surface receptors mediated by JAK-STAT and other signal transduction pathways (33). Type I interferons interact with the IFNAR receptor complex. IFNAR consists of two chains, IFNAR1 and IFNAR2 (major ligand binding chain). The receptor chains for IFN-γ are known as IFN-γR1 and IFN-γR2. IFN-α and IFN-β have an important role in antiviral and anti-proliferative activity of different cell types (e.g. T cells, natural killer cells, macrophages, dendritic cells). Their anti-proliferative effects are mediated by the stimulation of different cytotoxic activities. Expression of molecules such as tumor antigens and Class I MHC is the typical end-result of interferon activity. Over-expression of these molecules sensitizes these cells as targets for killing by cytotoxic T cells and natural killer cells. Another important mechanism is to induce the expression of pro-apoptotic genes (e.g., caspases, bak, bax), and repression of anti-apoptptic genes (e.g., Bcl-2) (33). IFN-β has both pro-inflammatory and anti-inflammatory roles in immune modulation depending on the cytokine milieu. In the case of viral infection, IFN-β is produced by APC-like macrophages and DC. IFN-β then binds to its receptor on APC and stimulates the production of other Type I and Type II IFNs and expression of high levels of MHC class I molecules that promote activation of T cells (34). On the other hand, IFN-β is believed to mediate autoimmune regulation in MS by induction of cytotoxic activity and by increasing the frequency and suppressive activity of Treg cells (34). In regard to inflammation of the CNS, it is believed that Th1 and Th17 cells are causative and Th2 cells are protective (35). The notion that IFN-β induces a Th2 shift in MS was

supported by a reduced activity of MBP-specific T cell clones and Th2 shift upon IFN-β treatment (35).

Inhibitory effect of IFN-β in EAE has also been shown in previous studies (22, 27, 35). In these cases, the administration of IFN-β was concurrent with or subsequent to the encephalitogenic challenge. Studies in mice deficient in IFN-β or type I IFN receptor gene showed that the beneficial effect of IFN-β is exerted during the effector phase of EAE (22). The administration of a fusion protein consisting of IFN-β and NAg not only prevented the disease caused by subsequent encephalitogenic challenge but also halted the ongoing disease (22). IFN-β can provide protection against self-reactive antigen-specific pathologic T cells by two probable mechanisms. First, IFN-β may eliminate the self-reactive T cells by activating cytotoxic pathways. Second, IFN-β may convert a subset of pathologic T cells to Treg cells that can provide long term protection against EAE (22). In these cases, the role of the fusion protein would be to facilitate the efficient presentation of self antigen to self reactive T cells by APC for enhanced elimination of self-reactive T cells by cytotoxic activity or development of Treg cells.

4.5 Cytokine-NAg fusion proteins can control the balance of tolerance and immunity

Some cytokine-NAg fusion proteins have been reported to efficiently act as antigen specific tolerogens in EAE. GMCSF-NAg, IL2-NAg and NAg-IL16 fusion proteins inhibited EAE when administered before encephalitogenic challenge and suppressed the ongoing disease when administered after the onset of disease (18, 19, 21). The common requirement for all of these prevention and treatment cases was the physical linkage between the cytokine and NAg. Linkage in all of these cases increased the antigenic targeting to APC in vitro. GMCSF-NAg facilitated the presentation of antigen by DC and macrophages by more than 1000-fold (21). IL2-

NAg facilitated the presentation of NAg by blastogenic T cells by ~1000-fold (18). NAg-IL16 increased the presentation by Con A-activated splenocytes by ~10-fold (19).

Cytokine-NAg fusion proteins have also been reported to elicit immunity rather than tolerance. In determining whether the immunity or the tolerance would be induced, not only the cytokine domain but also the antigen domain may play an important role. Self antigens are known to stimulate the differentiation of Treg cells. In contrast, foreign antigens are exposed to the immune system too transiently to drive the efficient differentiation of Treg cells. Antigen specific effector T cells and Treg cells are maintained in balance. In the case of self antigen, Treg cells dominate to cause self tolerance by suppressing the self reacting pathologic T cells. In the case of foreign antigen exposure, effector cells predominate to eliminate the foreign elements. In the cytokine-NAg fusion protein, if multiple foreign epitopes are used as antigenic domain, then the antigen may be recognized by higher frequencies of conventional majority T cells and limited number of minority Treg cells leading to the clonal proliferation of effector T cells to give immunity against that specific antigen. If self antigen is used as the antigenic domain of the fusion protein, then the self antigen will tip the Treg cell:effector cell balance toward the Treg cells (36). In both cases, role of cytokine is to facilitate the increased presentation of the antigen by APC through the interaction with corresponding receptor. The relative balance of conventional and Treg cells that interact with a particular APC may in turn control the activation of APC and thereby, contribute to maintain the balance of tolerogenic versus immunogenic signals during antigen presentation.

4.6 Stimulation of Treg cells is the key strategy to restore tolerance

In both pre-challenge and post-challenge treatment regimens, the strategy was to stimulate the proliferation of the Treg cell clone specific for a self antigen. In one study, depletion of Treg cells before self-immunization intensified the resulting disease (36). Depletion of Treg cells was also reported to render a genetically resistant strain susceptible to EAE (36). In another study, naïve B cells were incubated with OVA323-339 conjugated with cholera toxin B (CTB) subunit. This conjugation allowed the enhanced presentation of Ag. When naïve T cells were co-cultured with B cells pretreated with OVA-CTB, there was an increase in antigen specific Treg cells by more than 50 fold followed by an effective suppression of effector T cells in secondary co-culture. Adoptive transfer of OVA-CTB treated B cells to mice followed by immunization with OVA in CFA resulted in an increase of Treg cells and suppression of effector T cells (37). Similarly, adoptive transfer of MOG-CTB treated B cells to mice followed by immunization with MOG in CFA increased the number of Treg cells, suppressed the MOG specific effector T cells and prevented EAE (37). These studies showed that Treg cells were involved in the tolerance.

In the case of pre-challenge treatment of IFNβ-NAg, antigen-specific Treg cells may be attracted to the antigen which was tethered to the IFN-β and thereby, targeted to corresponding cytokine specific receptor on APC and then processed and presented by those APC. The attracted Treg cells may then undergo a clonal proliferation upon the reception of a co-stimulatory signal and inhibit the self-reactive pathogenic T cells. In the case of the post-challenge treatment regimen, Treg cells may not only suppress the already existing pathologic T cells but may also work as a safeguard for future protection. Both effector T cells and Treg cells are known to exist for a self antigen. In autoimmune disease, effector T cells outnumber the Treg cells, and in normal people, self-reactive Treg cells dominate the self-reactive effector cells. The role of the innovative fusion protein is to facilitate the clonal proliferation of Treg cells so that they can

suppress the effector T cells. Both conventional effector T cells and Treg cells may be attracted to the antigen targeted to and presented by the APC, but probably different signals are sent to them due to the IFN-β domain in the fusion protein. Possibly Tregs cells receive co-stimulatory signals, and pathologic effector T cells receive co-inhibitory signals. Therefore, Treg cells may undergo clonal proliferation, and effector T cells go through cell death and thereby, the balance between them moves toward the Treg cells which facilitates the suppression of effector T cells by Treg cells. This strategy can also be applied for other autoimmune diseases. This also implies that stimulation of Treg cells against tumor antigens which are also normal constituents may activate both Treg cells and effector T cells. These activated Treg cells can aggravate the disease by eliminating the effector T cells which are supposed to kill the antigen bearing tumor cells (20). The whole purpose of this study is to find a novel fusion protein that can stimulate the antigen-specific Treg cell to restore tolerance in mice with EAE caused by that specific antigen.

4.7 Future directions

The ultimate goal of this project was to examine the efficacy of IFNβ-MOG and IFNβ-PLP to prevent EAE when administered prior to encephalitogenic challenge and to halt the ongoing disease when administered at the onset of disease. Post-challenge treatment regimen was done to test the curative role of IFNβ-MOG and pre-challenge treatment regimen was done to test the preventive efficacy of IFNβ-PLP. To draw decisive conclusions about the tolerogenic efficacy of these fusion proteins, a pre-challenge treatment experiment with IFNβ-MOG and a post-challenge treatment experiment with IFNβ-PLP will need to be done.

In the case of a post-challenge treatment regimen, fusion proteins were administered at the onset of disease with EAE score ≤2. Therefore, future experiments will address whether the

fusion protein can cure animals with a more severe diseased condition. To make these data more reliable, another post-challenge treatment experiment will need to be performed by administration of protein after the first remission of severe paralytic disease in SJL mice. These data would extend our knowledge of the vaccine's therapeutic role.

Another experimental hurdle is the variable susceptibility of animals (particularly SJL mice) to EAE. Therefore, it is difficult to match the animals with the same EAE score prior to the administration of IFNβ-NAg. To solve this problem, splenocytes from NAg sensitized mice will be cultured with the NAg to get antigen-specific stimulated T cell lymphoblasts, and those lymphoblasts will be injected i.p into the animals. The adoptive transfer of pathogenic T cell will make the animals sick at the same level and have very similar day of onset.

In our previous report, IFNβ-MBP and IFN-β were shown to have an equipotent activity in the anti-proliferative assay, but the anti-proliferative activity of IFNβ-MOG and IFNβ-PLP was less than IFN-β by 40 and 2 folds respectively. In one study, antiviral activity of a fusion protein consisting of human serum albumin and interferon-alpha2b was increased by 68% and 115%, respectively, by engineering a rigid linker (PAPAP) and a helix-forming linker (AEAAAKEAAAKA) between the two protein domains (38). The same linkers can be used between IFN-β and NAg to increase the activity of the fusion proteins.

Another important experiment is planned where the antigenic domain would contain multiple encephalitogenic epitopes to constitute a multivalent vaccine. In this case, the same fusion protein would be able to restore tolerance in EAE mice challenged with different self antigens. For example, if the antigenic domain of the IFNβ-NAg fusion protein contains epitopes

for MBP, MOG and PLP, then the same fusion protein could be used as TTV for animals immunized with those different antigens.

Last of all, the ultimate success of this project lays in the applicability of the devised fusion proteins to MS patients. To test the efficiency of these fusion proteins to prevent and inhibit MS, humanized forms of these fusion proteins will be developed and tested in human patients.

CHAPTER 5: CONCLUSION

The most commonly used treatment for MS is IFN-β. Although IFN-β can decrease the clinical signs of MS, its success is limited by individual variability and overall suppressive effects on the immune system because IFN-β cannot discriminate between normal T cells and the auto-reactive T cells. Therapeutic tolerogenic vaccines can open a new dimension for the treatment of MS. This study has examined the efficacy of IFN-β-MOG and IFN-β-PLP fusion proteins to ameliorate the altered tolerance in a murine model of EAE. This study provides suggestive evidence that IFNβ -NAg fusion proteins have therapeutic action at two levels. First, the fusion protein interacts with the cytokine receptors on APC and may induce the cytokine-mediated differentiation of Ag-specific T cells to Treg cells that will eventually suppress self-reactive NAg-specific T cells. By this mechanism, the fusion protein will exert its tolerogenic activity. Second, the cytokine may enhance the presentation of NAg by APC through MHC class II. Self-reactive T cells that interact with this antigen will ultimately be desensitized. By this mechanism, the fusion protein will eliminate the already existing self-reactive T cells and thereby mediate therapeutic activity. The role of the antigenic domain of the fusion protein is to mediate the targeted killing of autoimmune T cells rather than a general suppression as may be the case with unlinked IFN-β.

In summary, this project was designed to show the potency of the IFNβ-MOG and IFNβ-PLP fusion protein as a tolerogen. Administration of IFNβ-PLP before encephalitogenic challenge ameliorated the subsequent episode of EAE and administration of IFNβ-MOG after disease onset inhibited the progression of disease.

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APPENDIX: IACUC APPROVAL LETTER

East Carolina University AUPK147

1907-2007 CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

March 31, 2010

Mark Mannie, Ph.D.
Department of Micro/Immuno
Brody 5E-106
ECU Brody School of Medicine

Dear Dr. Mannie:

Your Animal Use Protocol entitled, "Antigen-Specific Tolerogenic Vaccines for Murine EAE," (AUP #K147a) was reviewed by this institution's Animal Care and Use Committee on 3/31/10. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure