ENCAPSULATED INTERFERON-TAU DURING THEILER'S VIRUS-INDUCED DEMYELINATING DISEASE: EFFICACY OF TREATMENT AND IMMUNE RESPONSE PROFILE

A Dissertation

by

DANA DEANNA DEAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Biomedical Sciences

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ABSTRACT

Encapsulated Interferon-Tau during Theiler's Virus-Induced Demyelinating Disease: Efficacy of Treatment and Immune Response Profile. (December 2008)

Dana Deanna Dean, B.S., Texas A&M University; M.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. C. Jane Welsh
Dr. Robert C. Burghardt

Multiple sclerosis (MS) is the most common primary demyelinating disease of the central nervous system in humans. Type I interferons are most frequently used to treat MS. However, their main mechanism of action remains elusive. Various biomarkers have been investigated for their ability to assess treatment efficacy, but results are often confounding due to differences in experimental design and variation in individual physiology. In fact, not all MS patients respond to IFN therapy and a significant number suffer severe negative side effects and must cease treatment. Thus, alternative therapeutics that offer less cytotoxicity and greater efficacy are a major objective of research.

This dissertation evaluated a novel type I interferon, interferon-tau (IFNT), and its ability to attenuate Theiler's virus-induced-demyelinating disease (TVID), a mouse model of MS. In this model, viral infection with the BeAn strain of Theiler's murine encephalomyelitis virus (TMEV) is the initiating factor leading to demyelination of the CNS. It was hypothesized that IFNT would: 1) provide therapeutic benefit as witnessed by a stabilization of clinical score, a decrease in CNS inflammation, and a decrease in CNS demyelination, and 2) shift the immune profile from a Th1 to a Th2 response.

Once mice developed chronic disability, IFNT treatment began. This novel IFN was delivered in an innovative way: encapsulation (eIFNT) in an alginate polymer, which allowed for slow and sustained release. eIFNT was delivered by a 100 μl intraperitoneal injection (i.p.)

containing 1.4M U of IFNT once every two weeks for 8 weeks. Mice were clinically scored weekly and BeAn-eIFNT mice demonstrated a decrease in clinical score. Bright field microscopy was used to evaluate CNS tissues where a decrease in demyelination and inflammation was noted in BeAn-eIFNT-treated mice. *Ex vivo* stimulation of virus-specific lymphocytes revealed an increase in both T helper 1 (Th1) and T helper 2 (Th2) cytokine production. Specifically, TNFA was produced at very high levels by splenocytes from BeAn-eIFNT mice in response to UV-inactivated BeAn alone and in the presence of IFNT when compared to BeAn-eMOPS mice under the same conditions. IFNG was produced at elevated levels from the splenocytes of BeAn-eIFNT mice versus BeAn-eMOPS mice when stimulated *in vitro* with UV-inactivated Bean and with BeAn in the presence of IFNT. IL-2 was produced at moderately elevated levels from the splenocytes of BeAn-eIFNT mice versus BeAn-eMOPS mice when stimulated *in vitro* with UV-inactivated Bean. Il-2 was elevated to a statistically significant level ($p<0.05$) from BeAn-eIFNT mouse splenocytes when stimulated with BeAn in the presence of IFNT when compared to BeAn-eMOPS mice and IL-10 was produced at elevated levels by splenocytes from BeAn-eIFNT mice versus that produced from BeAn-eMOPS mouse splenocytes in response to UV-inactivated BeAn alone and in the presence of IFNT. Quantification of T regulatory (Treg) cells in the spleen of eIFNT vs. eMOPS mice and blood of eIFNT vs. eMOPS mice revealed no difference between the two groups. There was no statistical difference in virus-specific serum antibodies at the pretreatment time point noted in the OD readings of eIFNT mice at a dilution of 1/200 compared to the eMOPS mice. A modest decrease in the OD values at the 1/200 dilution were noted in the eIFNT mice compared to the eMOPS mice, but this difference was not significant. Antibody secreting cells (ASCs) from eIFNT mice versus eMOPS mice were slightly lower in the spleen and brains whereas there was a slight

increase in ASCs from the spinal cord of eIFNT mice when compared to those from eMOPS mice.

Altogether, the results support efficacy of the eIFNT treatment in the mice with TVID. Actual mechanisms of disease attenuation remain elusive at this time as mice exhibited an increase in certain Th1 and Th2 cytokines rather than the hypothesized shift from a Th1 to a Th2 immune profile. Likewise, mice exhibited a modest decrease in virus specific antibodies as well as the number virus-specific ASCs which also refute the hypothesized increase in these values. A remarkable finding was the fact that immune cells derived from eIFNT treated mice appeared to be divided into two distinct types of biological responders although all of the mice responded to the in vivo treatment with a decrease in disease severity. It is hypothesized that this difference is a reflection of individual genetic variability in response to immune modulation which is surprising owing to the fact that the animals used for these studies are in-bred and considered to be as identical genetically as is feasible in a population of animals. Obviously, immune modulation can proceed through different mechanisms and still provide the desired result of a decrease in disease severity. However, this reality creates an added level of difficulty when one is trying to interpret biological data in order to determine whether a therapeutic regimen is efficacious within a patient population.

DEDICATION

I dedicate this manuscript to the members of the Brazos Valley Chapter of the Multiple Sclerosis Society. They have been and will remain my inspiration each moment I spend in the laboratory. I dedicate this manuscript to my family members: Denise and Terry Stanphill, Wanda Gail, Bubba, and Emorie Dornak, Morris Reed and especially my mother, Bobbie Reed, for enduring my absences at family gatherings, for listening to my scientific ramblings, and for supporting and believing in me every bit of the way. And finally, I dedicate this manuscript to my precious gem, James Martin, for being there for me in all ways at all times no matter my needs or request.

ACKNOWLEDGEMENTS

I would like to thank my committee co-chairs, Dr. C. Jane Welsh and Dr. Robert C. Burghardt. The degree path was long and full of pitfalls. The destination blurred at times making the challenges seem insurmountable, but I stayed the course with the support and guidance of Dr. Welsh and Dr. Burghardt and I have come through my degree process victoriously. Dr. Welsh, you have taught me to be more patient, more gracious, and more tolerant. You have been a friend, a mentor, and a confidante and there is not space enough to list all of the lessons I have gleaned under your tutelage. Most of all, thank you for helping me to find my "inner Jane". Dr. Burghardt, you have challenged me and pushed me and demanded that I be sure of my scientific process. Thank you for giving me my inner sensor for each data point I generate when I ask myself "What would Bob say/ask/require?" I would like to thank Dr. Bazer for his kind gift of as much IFNT as needed to accommodate my research objectives and for his interest in my project as well as solid advice and commentary throughout my degree process. I would like to thank Dr. Berghman for always being a positive force and a ray of sunshine when things got really tough. I extend extreme gratitude to Dr. Allison Ficht and to Dr. Angela Arenas for making the encapsulation of the IFNT possible. Dr. Arenas spent too many hours to mention slaving over the encapsulator to provide me with the capsules needed for my extensive research design. She and Dr. Ficht were invaluable to this project. Likewise, Dr. Ralph Storts has been a long-time participant in all of my studies and has provided uncountable hours of advice, mentoring, and training to allow me to evaluate my histopathology slides.

Special thanks must go out to all who have been involved in my scientific evolution. Dr. Mark Dahmer was my mentor while an undergraduate student worker. He was the first to encourage me to pursue a scientific path. He knew before I even knew myself that research and science would be my life-long passion. The next person in my evolvement was Dr. Larry

Johnson. By absolute serendipity my name ended up in his hands and I was hired as his histology technician. Histology blew me away and I have been awe-struck ever since. He taught me all of the basics of electron microscopy and was my foundation for all that has transpired since that time. He will always hold a very special place in my scientific recollections and I applaud his dedication to encouraging school-aged children to embrace science. Dr. Johnson, you are a true steward for scientific research and should be commended for your mentorship. Dr. Evelyn Tiffany-Castiglioni was my teaching mentor in histology. She inspired my teaching style and theory: A student will rise to the level of the bar that you set for them. Set it high or you do them a disservice. I will be forever grateful for the honor of witnessing her in a classroom setting. It is a pity that the BIMS students of today are not blessed by her presence within a classroom. It has been an honor to carry on in your stead, Dr. C., I only hope that I have made you proud. Once again, I must thank Dr. Burghardt. He provided me with my final scientific polish. He has a vast knowledge of immunohistochemical techniques, cell/tissue culture techniques, and imaging techniques. Thank you, Dr. Burghardt, for the opportunity to be surrounded by such a variety of techniques as are displayed on a daily basis in the Image Analysis Laboratory. I am certain that the knowledge gained therein will serve me well throughout my scientific career.

I am also grateful to Dr. Rola Mouneimne and Dr. Colin Young for their guidance and advice through the years as well as for their camaraderie. I thank JoAnn Flemming for her constant interest and support in my progress and for supplying me with aliquots of IFNT when needed. I would like to acknowledge Dr. Mwangi for the use of his ELISpot counter and Dr. Weston Porter for the use of his spectrophotometer during my research. I thank Dr. Melanie Landis and Dr. Marvin Cannon for welcoming me into their classroom and providing me with the opportunity to extend my teaching experience. It has been a pleasure teaching with both of you and I think the three of us made a dynamite team. I would like to thank Dr. Louise Abbott.

You have been a constant source of never-ending support, advice, and guidance during my time at the Vet School and I appreciate your open door policy. I thank Lin Bustamante for always going above and beyond the call of duty in order to provide me with the best slides possible; no matter what it took and for the warmth of her friendship. Thanks, Lin! There are no words to express the gratitude I have for Dr. Andrew "Drew-bie" Steelman, but I will attempt to enumerate the many ways I am grateful for his presence within the laboratory. Drew is a force to be reckoned with in the research lab and is constantly tweaking protocols and designing succinct and elegant experiments that cut to the heart of a research question. He saturates himself in current literature which makes him a fount of information as to the latest techniques and findings. He has been a constant source of support and an eager participant in many of my experiments and many protocols within this manuscript can be attributed to his expertise. My appreciation is boundless and I will forever cherish his "go time" work mantra. Thanks, Drew-bie! Gigantic thanks to my scientific gal pals: Dr. Tamy Frank-Cannon, Dr. Kate Dunlap, Dr. Stephanie McKay, Dr. DeeAnn Wallis Schultz, Kerry Thuett, Paulette Suchodolski, and Andrea Kroenig. Having go-to-gals is a must for every woman. When in science, it is especially important to have an inner sanctum of science friends that "get you" and can provide solace, solutions, and silliness as needed. Additional acknowledgement goes to Kerry Thuett, my "Wonder–Science-Twin", as we make our way through the last voyage of this degree together. I am grateful that I have you for commissary, Ker-Bear. PUSH! A special thanks to Dr. Kate Dunlap for being a dear friend as well as an exceptional scientist. Your opinion means everything to me because I can always count on you to shoot it to me straight. I trust your judgment and heed your advice. I am thankful to have you as a part of Team Dean. Thanks, Kate! To my non-science friends, Lynette "Netter" Lew, Anne-Marie "Aunt Bunny-Lady" Lindsey, and Andrea "ank" Kroenig (again) I REALLY appreciate all of you enduring my

constant scientific rantings and ravings and the neglect that you suffered at times due to my scientific schedule. The three of you have always supported my dreams and have never lost faith in my abilities and I would be lost without the three of you. Grateful Smewtches!

Enormous gratitude is extended to the Ladies of the Department of Veterinary Integrative Biosciences, past and present. I always feel at home when I go into our main office. It has been a great asset to me as a student to know I can always find solutions to problems, seek advice, and find comfort in the hallowed halls of Room 107 VMA. Thanks, Ladies, y'all have never let me down. Additionally, I must thank the personnel at LARR. Theirs is too often a thankless job. It would be impossible for me to maintain the number of animals housed during this project without their tireless efforts to sustain the animals while being gracious, warm and friendly to all. Your efforts have not gone unnoticed or unappreciated. I thank each of you.

It is with great respect and admiration that I thank my family: Denise and Terry Stanphill; Wanda Gail, Bubba, and Emorie Dornak; and my parents Morris and Bobbie Reed. I have missed many family functions: moves, birthdays, and hurricane preparations, to name a few, but your love and support have never faltered. All of you are my foundation no matter how far from home I may roam. To my mother, Bobbie Reed, you have been there for me physically, financially, and spiritually and I am forever grateful. You encouraged me when my resolve waned, cheered me on when times got rough, and applauded me after each success. Lucky for me, you forced me to take typing in high school because "one never knows", you said. I bet you never dreamed I would use my typing skills to type "Thanks, Liddle Mudder" in my dissertation. Lastly, I thank my precious gem, James Martin. You got here near the end of this degree, but you have endured some of its most anxious and harried times. The final push has been grueling and you have been there for me every step of the way. I love you and I am grateful for your presence in my life and the completion you bring to it.

NOMENCLATURE

ASC	Antibody Secreting Cell
BBB	Blood Brain Barrier
Br-ILs	Brain Infiltrating Lymphocytes
CD	Cluster of Differentiation
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
eIFNT	Encapsulated IFNT
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunosorbent Spot
eMOPS	Encapsulated MOPS buffer
H&E	Hematoxylin and Eosin
IFN	Interferon
IFNA	Interferon Alpha
IFNB	Interferon Beta
IFNG	Interferon Gamma
IFNT	Interferon Tau
IL-2	Interleukin 2
IL-10	Interleukin 10
IL-12	Interleukin 12
IRF	Interferon Regulatory Factor
ISG	Interferon Simulated Gene
LFB	Luxol Fast Blue
MBP	Myelin Basic Protein

MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MSRV	Multiple Sclerosis-Associated Retrovirus
PPMS	Primary Progressive Multiple Sclerosis
RRMS	Relapsing-Remitting Multiple Sclerosis
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
SPMS	Secondary Progressive Multiple Sclerosis
SC-ILs	Spinal Cord Infiltrating Lymphocytes
STAT	Signal Transducer and Activator of Transcription
TGFB	Transforming Growth Factor Beta
Th	T-Helper
TMEV	Theiler’s Murine Encephalomyelitis Virus
TNFA	Tumor Necrosis Factor Alpha
TVID	Theiler’s Virus-Induced Demyelinating Disease
VP	Viral Protein

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1. INTRODUCTION

1.1. Multiple Sclerosis

1.1.1. General Description of Multiple Sclerosis

Multiple sclerosis was originally described by Charcot in 1868. There have been few revisions to the original description of the pathology of the disease process since that time. Multiple sclerosis (MS) is a human primary demyelinating disease affecting the central nervous system (CNS). The etiology of MS is unknown, although viral infection during early adolescence is thought to initiate the disease (Acheson, 1977) and results in an autoimmune response that leads to demyelination over the next two decades of life in susceptible individuals. In support of this, many viruses have been isolated from MS brains including measles, mumps, parainfluenza type I **(**Allen and Brankin, 1993), human herpes simplex type 6 and Epstein-Barr virus (EBV) (Serafini et al., 2007). Currently, the most effective treatment for MS is interferon beta (IFNB), an antiviral agent, which has been linked to a decrease in the number of and severity of MS attacks (relapses).

1.1.2. Immune-Mediated Pathogenesis of Multiple Sclerosis

Prior to an episode of demyelination, immune cells, including T cells (CD4+ and CD8+), plasma cells, and macrophages, infiltrate the central nervous system (CNS) parenchyma and form perivascular cuffs (Prineas, 1985 Lucchinetti, et al., 2000). In fact, magnetic resonance

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This dissertation follows the style of Journal of Neuroimmunology.

imaging (MRI) has been used to detect the presence of gadolinium-enhanced lesions as a direct measure of brain-blood barrier (BBB) disruption and predict a relapse (Barkhof and van Walderveen, 1999). Gadolinium is an extracellular contrast agent that is restricted from entry into the CNS by the BBB. Thus, its presence in the CNS during MRI is an indicator of BBB breakdown. This technique has become a vital tool in tracing and predicting lesions within the CNS of patients with MS. As a result, both the number and volume of lesions that are gadolinium-enhanced correlate positively with the number and severity of new lesions upon relapse (Khoury et al., 1994; Miller et al., 1992). Likewise, the absence of gadolinium-enhanced lesions on serial MRI is an indicator of lesion resolution, such as found during treatment regimes (glucocorticoid and IFNB). Once immune cells gain entrance to the CNS, the parenchyma surrounding the perivascular cuffs contain immune cell infiltration, myelin degradation and activated macrophages engulfing debris. The number of macrophages and the amount of time they are present within a lesion are directly correlated with size and severity of the relapse (Prineas and Wright, 1978). Although myelin destruction is often severe during the early stages of MS, the axons are minimally damaged. However, as the disease progresses, axons are severed (Trapp et al., 1998) and this results in an increased incidence of irreversible disability. Neurologists now believe that patients with MS should commence treatment as early as possible to prevent permanent disability due to axonal loss.

The BBB is formed by a continuous layer of cerebrovascular endothelial cells (CVE) sealed by tight junctions that limit permeability functions (Joo and Klatzo, 1989). Also, there is a low level of major histocompatibility complex (MHC) expression on CVE, which limits their antigen presenting capacity under normal circumstances. However, lymphocytic surveillance of the CNS parenchyma does occur in healthy animals (Wekerle et al., 1986), but only previously activated immune cells access the cerebrovascular endothelial cells of the BBB under normal

circumstances. Thus, activation in peripheral lymphoid tissues (lymph nodes and spleen) is critical for T cell access to CNS parenchyma. When the permeability of the BBB is compromised, a higher number of immune cells are allowed access to the nervous system parenchyma (Mokhtarian and Griffin, 1984). Additionally, when CVE express adhesion molecules and MHC, activated lymphocytes are able to transmigrate across the BBB. In MS, EAE, and TVID, inflammatory cell trafficking into the CNS increases dramatically. In fact, induction of EAE requires the injection of pertussis toxin (Voskuhl and Palaszynski, 2001) 24 and 48 hours after initial antigen priming with myelin components and Freund's complete adjuvant. This 'booster' of pertussis toxin causes BBB disruption and enhances the ability of myelin specific T cells to gain access to the CNS. Therefore, during EAE induction, BBB disruption is necessary for disease progression, while the TVID animal model of MS provides a more natural (not enhanced by experimental manipulations) course of disease onset and progression. Overall, therapies for MS are being sought that decrease inflammation within the CNS. One possible source of reducing pro-inflammatory cytokines and the subsequent inflammatory reaction are interferons which may also suppress viral activity, as in the case of TVID, where autoimmunity results from a persistent viral infection within the CNS. Thus, decreasing viral replication via IFN treatment could allow the immune system to finally clear the CNS of the persistent virus or IFN treatment could decrease the intermittent production of virus that may trigger relapses. Either of these outcomes could produce disease resolution or reduce the number of future relapses.

1.2. Theiler's Virus

1.2.1. General Description of TMEV

Theiler's murine encephalomyelitis virus (TMEV) is a natural pathogen of mice that causes an enteric infection, which occasionally reaches the CNS resulting in an encephalomyelitis

(Theiler, 1937). There are several different strains of TMEV that produce different disease outcomes. GDVII is a neurovirulent strain that, when injected intracranially (i.c.), results in fatal encephalitis in all strains of mice (Theiler and Gard, 1940). BeAn and DA strains of TMEV are avirulent which, when injected i.c., result in a persistent infection that causes a demyelinating disease in certain susceptible strains of mice (SJL) that is similar to MS (Lipton, 1975). In fact, persistence of TMEV within CNS parenchyma is necessary for the development of TVID in susceptible strains of mice (Aubert et al., 1987).

1.2.2. Immunopathogenesis of TVID

There are two major animal models used to study MS: experimental allergic encephalomyelitis (EAE) and Theiler's virus-induced demyelination (TVID). EAE is induced by immunizing mice or rats with brain homogenate or myelin components or by transferring myelin specific T cells from immunized mice into naive mice (Rivers et al., 1933; Soos et al., 1997). TVID has a viral etiology that culminates in autoimmune reactions to myelin components (Lipton, 1975; Borrow et al., 1998). Thus, TVID is an animal model in which demyelination is initiated by viral infection.

During acute TMEV infection, T cells are the first to invade the CNS parenchyma (Dal Canto et al., 1995). During the early phase of the disease, both CD4+ and CD8+ T cells mediate viral clearance and CD4+ helper T cells (Th2 subset) assist B cell differentiation into plasma cells which are probably the most important mediators of TMEV clearance (Welsh et al., 1987; Borrow et al., 1993) during the acute phase of TVID. CD8+ T cells mediate viral clearance via their cytolytic capabilities or by producing IFNG. This evidence is supported by results from *in vivo* depletion experiments (Borrow et al., 1992) and studies with transgenic mice (Fiette et al., 1993; Pullen et al., 1993). CD8+ depleted mice developed more severe demyelinating disease than

immunocompetent controls (Borrow et al., 1992), most likely due to the elevated viral titers within the CNS which result in increased demyelinating disease.

During late disease, both T cell subsets are involved in the development of lesions via TMEV-specific DTH reactions (Clatch et al., 1990), cytotoxic T cell reactivity (Rodriguez and Sriram, 1988), and myelin autoimmunity (Welsh et al., 1989; Welsh et al., 1990; Borrow et al., 1998; Miller et al., 2001; Neville et al., 2002). Thus, it is evident that T cells play a dual role in TVID. In early phases of the disease, both CD4+ and CD8+ T cells mediate TMEV clearance, which is crucial to animal survival. In late phases of the disease, the immunologic reaction is driven more toward a Th1 (cell-mediated inflammatory) response and T cells take on a pathogenic role causing tissue destruction with lesions similar in location and composition to those seen in MS (Friedmann and Lorch, 1985). In order to cause myelin destruction, the T cells must first enter the CNS by transmigration across the BBB. Thus, understanding interactions between peripheral lymphoid organs, the BBB, and cells of the immune system should provide important insights into the mechanisms of pathogenesis during TVID.

1.3. Type I Interferons

1.3.1. General Description of Type I IFN

Type I interferons (IFN) include five families. These are referred to as α, β, ω, τ, and δ (Alexenko et al., 2000). IFN delta is only found in pigs. IFN tau (IFNT) is only found in 'true' ruminants (Roberts et al., 1999) and is discussed later. Type I interferons range in molecular weight from 15-25kD depending on family member (α, β, etc.), species of origin (human and mouse being most germane to this proposal), genetic isoform, and amount of glycosylation (Oritani et al., 2001). All type I IFNs have a predominantly α-helical structure (5 helices) that is heat stabile, acid stable, and induced by viral infection (except for IFNT; see below). All Type I

interferons exhibit antiviral and anti-proliferative activities transduced through a common Type I IFN receptor (IFNAR-1 and IFNAR-2). The subunits of these receptors signal via the Jak-Stat system. IFNAR-1 is associated with Tyk-2 and IFNAR-2 is associated with Jak-1 protein tyrosine kinases. In basic terms, upon IFN binding to its receptor, Jak tyrosine kinases phosphorylate Stat proteins which dimerize and form a tri-molecular complex (ISGF-3) that transduces the signal to the nucleus where it binds and interferon response element to exert negative or positive regulation of DNA transcription (Levy et al., 2003). The most common gene products of IFN stimulation within a cell are interferon regulatory factors (IRFs), myxovirus resistance protein A (Mx A), and 2'-5'-oligoadenylate synthetase (OAS) (Oritani et al., 2001). The IRFs primarily autoregulate subsequent IFN production (Paun and Pitha, 2007). MxA and OAS are key molecules in the induction of an antiviral state in cells exposed to Type I IFNs. Thus, due to their ability to inhibit both cellular proliferation (especially cells of the immune system) and viral replication, Type I IFNs have been assessed for their value for treatment of a variety of diseases (MS, rheumatoid arthritis, Hepatitis C infection, herpes simplex infection).

1.3.2. Use of IFNB in the Treatment of Multiple Sclerosis

Although MS was originally described 140 years ago, first line treatment options were only recently approved by the Federal Drug Administration (FDA). In 1993, the results of the first clinical trial of an IFN (IFN-β1b) for the treatment of MS (The IFNβ Multiple Sclerosis Study Group, 1993) demonstrated a decrease in the number and severity of MS attacks in the treatment group. Currently, there are three Type I interferons classified as first-line therapeutics for use in relapsing/remitting (80% of all MS patients) and secondary progressive (15% of all MS patients) forms of MS (reviewed in Bayas and Gold, 2003). The original clinical trials enlisted IFNB-1b, known commercially as Betaferon®/Betaseron®. More recently, there are two IFNB-1a forms,

known commercially as Avonex® and Rebif®. These three formulations differ in their routes of delivery (s.c. versus intramuscular), frequency of delivery (daily, 1 X/week, 3 X/week) and dosage (6 MIU, 8MIU, 12MIU). These three IFNs modify the number of relapses a patient has during an extended time period and slow progression of the overall disabilities that accumulate in MS patients over time. Currently, researchers agree that the sooner that IFN treatment commences, the better its treatment efficacy (Söderström 2003). The two most common side effects in MS patients are flu-like symptoms and injection site reactions (Lublin and Reingold, 1996; Mehta et al., 1998). These side effects can result in a patient having to withdraw from an IFN treatment regimen. Injection site reactions are caused by production of antibodies to the IFN molecule that begin to neutralize the IFN and cause the patient to become a 'biological non-responder'. The percentage of patients producing neutralizing antibodies in clinical trials (reviewed in Bertolotto et al., 2004) ranges from 2% (Avonex) to 50% (Betaferon/Betaseron). These differences in antibody production to commercial IFNs is based on the product itself (and how it is manufactured), as well as route of administration, dose, and frequency of dose. Therefore, a less antigenic and less toxic (flu-like symptoms and lymphopenia are indicative of toxicity) IFN would be ideal. Likewise, an IFN treatment with increased benefits (such as remyelination) to a broader range of MS patients (less biological non-responders) would further enhance the current situation of MS patients.

1.3.3. Type I Interferons in TVID

Type I IFNs are known to be vital in early viral clearance of Theiler's virus since Type I IFN receptor knock out mice died early after infection (Fiette et al., 1995). Only one study has used Type I IFNs in the TVID model. During the late demyelinating phase of TVID, the administration of Type I interferons, IFN-A/B, to SJL/J mice infected with the DA strain of

Theiler's virus, resulted in increased demyelination when given 4.5 months p.i for 16 weeks (Njenga et al., 2000). IFNA/B given for the short term (5 weeks of treatment) resulted in the promotion of remyelination. The authors noted that the mice lost weight during weeks 6-7 of treatment, developed reduced DTH responses to TMEV and decreased numbers of T and B cells infiltrating the CNS. No studies have been done on the treatment efficacy of IFNT with the TVID model.

1.4. Interferon-tau

1.4.1. General Description of IFNT

IFNT is produced by mononuclear trophectoderm of conceptuses of pecoran ruminants (sheep, bovine, giraffes, and goats). It was originally discovered as a product of sheep conceptus trophectoderm (Godkin et al., 1982) and is the maternal recognition signal for pregnancy in sheep (Bazer and Johnson, 1991). IFNT rescues the corpus luteum by inhibiting luteolysis, thereby maintaining progesterone levels and pregnancy. It also induces production of interleukins-4 (IL4) and -10 (IL10), Th2 cytokines, within the uterine environment during early pregnancy. These two cytokines suppress delayed type hypersensitivity (DTH) responses, which if uncontrolled would lead to immune rejection of the conceptus.

1.4.2. IFNT as a Treatment in EAE

Extensive research has been conducted using IFNT over the past 15 years. When compared with IFNA and IFNB, IFNT has many of the same beneficial properties of other Type I interferons (antiviral and antiproliferative) without the side effect of cytotoxicity *in vitro* (Pontzer et al., 1991; Assal-Meliani et al., 1993) or fever, weight loss, and flu-like symptoms *in vivo* (Soos and Johnson, 1999). Most importantly, IFNT is effective in the prevention and the

treatment of EAE disease progression even when challenged by superantigens (Soos et al., 1995). The ability of IFNT to be cross-species reactive (Khan et al., 1998; Martal et al., 1998; Assal-Meliani et al., 1993) is crucial to its use for treatment of human autoimmune diseases, such as MS. It has been successfully tested for cytotoxicity in bovine, porcine, ovine, murine, and human cell lines. Also, acute and chronic forms of EAE were prevented whether IFNT was administered orally or intraperitoneally (Soos et al., 1997). Oral administration is highly desired since many MS patients develop neutralizing antibodies to IFNB during the first year of administration which can result in loss of treatment efficacy (Bertolotto et al., 2004). With oral administration in mice, no antibodies were detected against IFNT. IFNT treatment of EAE also resulted in increased IL-10 levels and has been proposed to explain the mechanism of action of IFNT (Mujtaba et al., 1997). The importance of IL-10 in EAE has been shown in transgenic mice. IL-10 deficient mice develop more severe EAE and IL-10 over expressors were completely resistant to disease (Bettelli et al., 1998). Soos et al. (1997) also found increased levels of IL-10 in IFNT-treated mice and increases in transforming growth factor beta (TGFB) has also been reported (Khan, et al., 1998). This is very encouraging because a generally accepted goal of MS treatment is to attenuate a Th1 (proinflammatory) immune response by shifting to a Th2 suppressive cytokine profile (Baker et al., 1996).

Although the efficacy of IFNT has been tested in the context of the EAE model, an evaluation of its therapeutic value has not been investigated in a virus model of demyelinating disease. Thus, the current investigations are needed to fill this void within the Type I IFN literature.

1.5. Possible Mechanisms of Disease Modifying Drugs

1.5.1. Th1/Th2 Cytokine Profiles

The normal functioning immune system is thought to be in balance between a Th1 and Th2 type immune response. The cytokine environment encountered upon antigen activation polarizes a CD4+ T cell to become either a Th1 or Th2 type cell (Kaiko et al., 2007). CD4+ Th1 cells are the predominant cell type associated with disease pathology in MS and TVID. Polarization to a Th1 subtype is driven by IL-12 produced by antigen presenting cells (Kaiko et al., 2007 and Dittel 2007). Upon activation by MHC-II-antigen complex bound to an APC, the Th1 cell secretes IFNG. IFNG is the cytokine most commonly identified as the hallmark cytokine of the Th1 profile. Its major effect is the stimulation of macrophages which further enhance the Th1 cell's polarization. IL-2 is also produced by the Th1 cell as a growth factor for naïve cells and a survival factor for activated Th1 cells. Overall, the Th1 cytokine profile drives a cell-mediated immune response which is important in eradicating intracellular pathogens and tumor cells.

CD4+ Th2 cells produce a cytokine profile that drives humoral immunity through activation of B cells and the subsequent production of antibodies. IL-4 is the hallmark cytokine of the Th2 profile. Its major effect is to induce immunoglobulin class switching in activated B cells. IL-5, IL-10, and IL-13 are also associated with humoral immunity and act on various components of the innate immune system (eosinophils and mast cells) to assist in the eradication of extracellular organisms, such as parasites.

1.5.2. Th1 Suppression

Cytokines secreted by Th2 cells that suppress Th1 responses include IL-4, TGFB, and IL-10 (Arnason and Reder 1994). During remission of MS, patients' white blood cells produce increased levels of TFGB. Likewise, TVID is ameliorated by TGFB treatment (Drescher et al., 2000). PGE_2 is secreted by macrophages and has inhibitory effects on a Th1 response as well. It appears to act as a self-regulator for macrophages in that PGE_2 and IFNG show inverse production rates. IFNT, in sheep, favors PGE_2 production over PGF_{2a} (Hansen et al., 1999) within the uterine environment during pregnancy, which is vital to maintaining the corpus luteum in early pregnancy. If this mechanism of action occurs during TVID treatment with IFNT, then it could provide a positive effect of IFNT treatment via down-regulation of macrophage activity. Results from our laboratory have shown that IFNT increases MHC Class I expression and decreases IFN-γ induced MHC Class II expression on cerebrovascular endothelial cells (Tennakoon et al., 2001). This is additional evidence of a switch from a Th1 response to a Th2. The expression of MHC Class I is important for antiviral activity during infection. However, MHC Class II allows CD4+ Th1 cells to drive the cell-mediated response leading to more destruction in MS, EAE, and TVID. By suppressing MHC Class II, the immune response should favor a switch to a Th2 response and suppression of the destructive Th1 immune response. Overall, the suppression of a Th1 immune response, whether via a switch to a Th2 profile or through mechanisms provided by Tregs, is the common goal of MS therapies and the common criteria for studying mechanisms of disease amelioration during TVID.

1.5.3. T-regulatory Cells

Historically, there has been much debate about the existence of a CD4+ T-cell that provides a regulatory (suppressive) mechanism within the immune system. In the past four years, the T regulatory cell (Treg) has been accepted as a vital part of the normal functioning immune system. Sakaguchi and colleagues revitalized the issue of Tregs in the mid 90's when they described the CD4+CD25+ T cell phenotype as being important in maintaining self-tolerance (Sakaguchi, et al., 1995). Without the presence of this population of T cells, animals would succumb to a variety of spontaneous autoimmune diseases. Transfer of the CD4+ CD25+ T cell population into these immunocompromised mice could inhibit the formation of the autoimmune disorders. Since their phenotypical identification, research has focused on the ability of these cells to maintain self-tolerance and confer graft tolerance in new hosts, as well as their ability to suppress tumor immunogenicity (Sakaguchi, et al., 2001; McHugh and Shevach, 2002). A more recent addition to the Treg specific phenotype is the expression of forkhead box protein 3 (Foxp3) which is a transcription factor that is linked to the development and function of the Treg population (Fontenot et al., 2003 and Hori et al., 2003). Currently, the CD4+ CD25+ Foxp3+ T cell is the accepted phenotype for a Treg. There are two types of Tregs recognized: naturally occurring Tregs (nTregs) that are produced in the thymus and inducible Tregs (iTregs) that are primed during an inflammatory response (Shevach, 2002). A common link between these two cells is the need for IL-2 (Yarkoni et al., 2008). Naturally occurring Tregs are not able to produce IL-2, but it is necessary for maintenance of suppressor phenotype. Inducible Tregs need IL-2 and TGFB to achieve the suppressor phenotype (via Foxp3 expression), but unlike nTregs, they are able to produce their own IL-2 once differentiated. IL-2 is necessary for iTregs to maintain their suppressor functions as well. Both types of Tregs need antigen for stimulation (Horwitz et al., 2008). Self antigens stimulate nTregs whereas foreign antigens stimulate iTregs. Both types of

Tregs can induce suppressive activity in CD4+CD25- T cells in the presence of TGFB and IL-10 (Zheng et al., 2004). Therefore, IL-2, TGFB, and IL-10 are crucial for the generation and maintenance of all Tregs. By using IL-2, Tregs compete with Th1 cells for survival. Additionally, Tregs produce IL-10 and TGFB which is a mechanism of suppressing Th1 cell activities (Yarkoni et al., 2008). The effect of Treg activity can be beneficial or detrimental to an animal depending on the immune response being suppressed by these cells. During TVID and MS, inflammatory cell infiltrates are damaging self-antigens (myelin) within the CNS. CD4+ Treg function has been shown to be defective in MS patients during a relapse, but the function is restored upon remission. Tregs have been shown to decrease IFN-γ levels secreted by macrophages from MS patients (Arnason and Reder, 1994). Therefore, the TVID model is a valuable tool for testing the therapeutic potential of manipulating the Treg population in order to suppress a Th1 response as is seen in MS.

2. ATTENUATION OF TVID WITH ENCAPSULATED IFNT: DECREASES IN CLINICAL SCORE, CNS INFLAMMATION AND DEMYELINATION

2.1. Introduction

The need for efficacious treatments for MS patients is still an active area of research. Currently, three of the five most commonly used therapeutics in MS patients are Type I interferon therapies. These include Avonex® (2006, product insert) and Rebif® (2005, product insert) which are IFNB1a and Betaseron® (2003, product insert) which is IFNB1b. The route of administration, dosage, and frequency of treatment differ among the three IFN therapies (McDonagh, et. al., 2007). Avonex® is administered intramuscularly (i.m.) once a week at a dose of 30 micrograms (μg). Rebif® is administered subcutaneously (s.c.) at a dosage of 22 μg or 44 μg three times a week. Betaseron® is given s.c. every other day at a dosage of 250 μg. All three agents are recommended for RRMS. Additionally, Betaseron® is the approved treatment for SPMS.

Usage of IFNB results in adverse side effects in the majority of MS patients. The most common side effects are flu-like symptoms and include fever, myalgia, fatigue, and weight loss (Lublin and Reingold, 1996; Mehta et al., 1998). These side effects can be so pronounced in some individuals that IFNB treatment must cease. Another common side effect is injection site reaction. This is caused by the production of antibodies to the IFN treatment and can lead to neutralization of the IFNB. If the antibodies to the IFNB are neutralizing in nature, then the therapeutic benefit of the IFN treatment is lost and a patient becomes a non-responder. Neutralizing antibodies results in loss of therapeutic efficacy which is a common problem in protein-based therapies and is an important area of drug manufacturing.

The fact that IFNT exerts an effect across species and that the toxicity associated with IFNT is far less than that associated with other Type I interferons, has prompted investigations directed towards unraveling the value of IFNT as a highly desirable therapeutic agent. Therefore, the objectives of the current study are simplistic in nature: to demonstrate drug efficacy. It is hypothesized that IFNT treatment will result in: a) a stabilization in the clinical scores of mice upon treatment with IFNT; b) a reduction in inflammation and/or demyelination within the CNS of mice treated with IFNT; and c) evidence of tolerability (lack of toxicity) in mice treated with IFNT. For these studies, the novel IFNT was administered in an innovative way, i.e. by encapsulation in an alginate polymer. This innovation allows for slow, constant release of the IFNT while decreasing the number of injections needed for treatment purposes. The eIFNT was administered by i.p. injection of 100μl of capsules containing 1.4 MU/100 μl of IFNT. The estimated rate of release was 10^5 U/day. It is suggested that this mode of administration could provide greater benefits by reducing the stress caused by multiple injections within the mice and by providing a constant release of the IFNT.

2.2. Materials and Methods

2.2.1. Virus Stocks

The BeAn strain of TMEV was a gift from Dr. H. L. Lipton (Northwestern University, Evanston, IL.) The virus was propagated in BHK-21 cells, titered, aliquoted, and stored at -80^{o} C before use (Welsh et al., 1987).

2.2.2. Animal Infection

SJL/J mice were purchased from Harlan (Indianapolis, IL) and bred in-house. Twenty-eight day old female mice were anesthetized with Isoflurane (Vedco, Saint Joseph, MO) and inoculated intracranially (i.c.) into the right cerebral hemisphere with 20 μl of BeAn strain of TMEV (5 X 10^5 plaque forming units) suspended in media or 20 μl of PBS alone. The SJL/J mice were divided into two groups as follows: 21 received PBS only and 58 received 5x10^5 plaque forming units (pfu) of BeAn virus. The day of i.c. inoculation was considered day 0. All animal protocols were in accordance with NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee. See Figure 1 for experimental design.

2.2.3. IFNT

IFNT was a gift from Dr. Fuller W. Bazer (Texas A&M University, College Station, TX). IFNT was isolated as previously described in Ott et al., 1991. The protein concentration was determined to be 1mg/ml by the Lowry assay (Lowry et al., 1951) and 10^8U/ml by virus plaque assay.

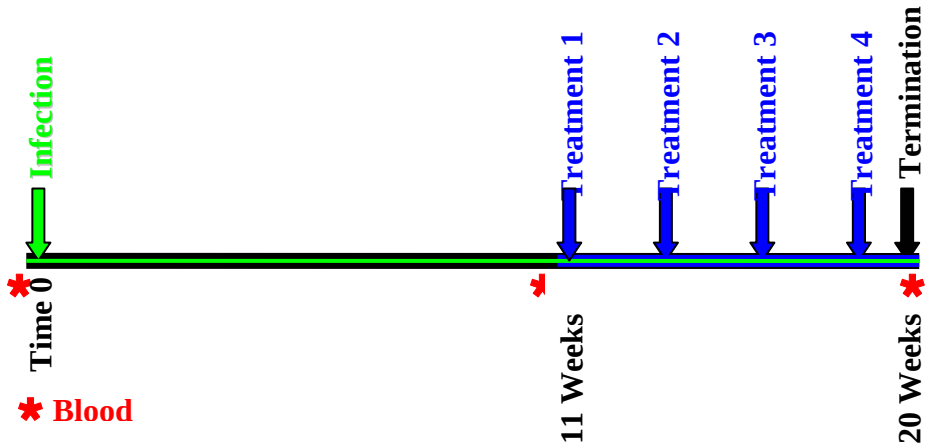


Figure 1. Experimental design: Section II. The following schematic denotes BeAn or PBS infection time point (green arrow), treatment (eIFNT or eMOPS) administration time points (blue arrows), and blood collection time points (red asterisks). Time points are in weeks post infection.

2.2.4. IFNT Encapsulation

Alginate capsules were prepared as previously described with some modifications (Abraham et al., 1996; Arenas-Gamboa et al., 2008). Briefly, 1 ml of 10^8 U/mg of IFNT was mixed with 5 ml of alginate solution (1.5% sodium alginate, 10 mM MOPS, 0.85% NaCl [pH 7.3]). Spheres were obtained (See Figure 2) by extruding the suspension through a 200 μm nozzle into a 100 mM calcium chloride solution and stirred for 15 min by using an Inotech Encapsulator I-50 (Inotech Biosystems International, Rockville, MD). After extrusion of the IFNT-alginate mixture into the $CaCl_2$, the capsules were washed twice with MOPS for 5 min and further cross-linked with 0.05% poly-L-lysine (molecular weight: 22,000; Sigma-Aldrich, St. Louis, MO) for 10 min. After two successive washes, the beads were stirred in a solution of 0.03% (wt/vol) alginate for 5 min to apply an outer shell and washed twice with MOPS. VpB was added to the shell of the capsule (*vjbR*::Tn*5*/VpB/shell) as a cross-linking agent by the addition of VpB in an equimolar ratio of poly-L-lysine/VpB. After two successive washes, beads were collected, aliquoted (600 μl capsules in 900 μl of MOPS buffer) and stored at 4°C.

Capsules (Figure 3) were kept at 4°C in the presence of excess IFNT until time of injection. Before injection, capsules were rinsed 3X in MOPS by resuspending capsules and allowing them to resettle. Finally, capsules were resuspended in the appropriate amount of MOPS buffer prior to injection (900μl).

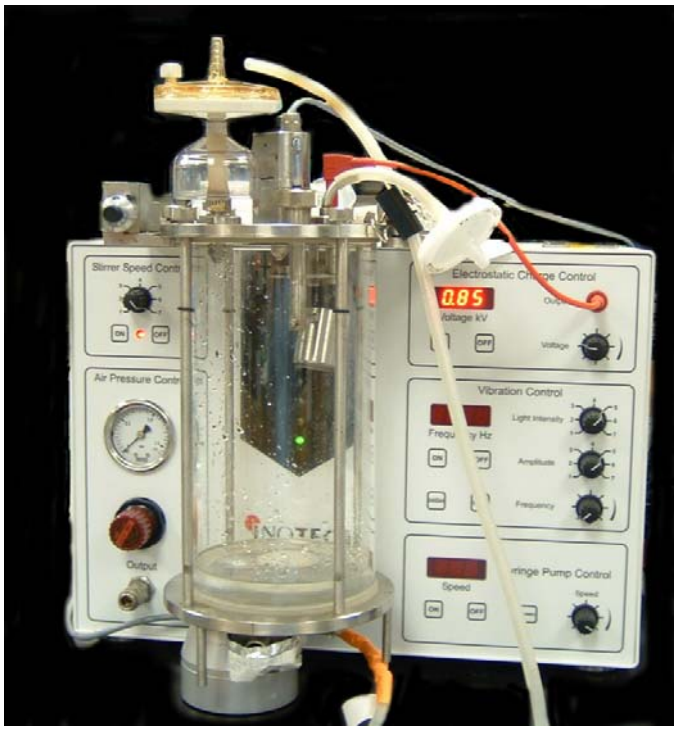


Figure 2. IFNT encapsulation apparatus. The IFNT solution enters the encapsulator via a syringe attached to the filtered port on top of the glass chamber. As the IFNT solution enters the apparatus it passes through a metal plate where it encounters an electrostatic charge as well as a vibrational frequency. This causes the stream of IFNT solution to form spheres that contain IFNT. The capsules are rinsed several times to remove any excess IFNT solution. The eIFNT is collected and stored at 4°C in excess IFNT to inhibit release from the capsules until needed.

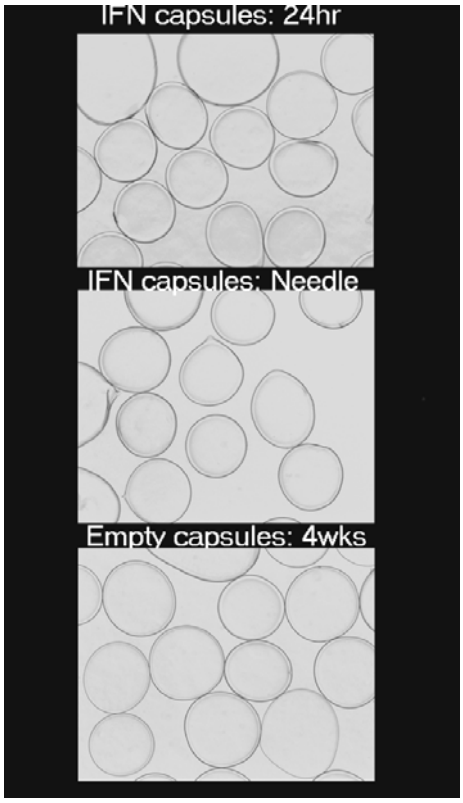


Figure 3. Alginate capsules containing IFNT or MOPS buffer. The capsules were stable for three months at 4°C. The images above show capsules after 24hrs at 4°C, after passage through a 23G needle, and after 4 weeks at 4°C

2.2.5. IFNT Treatment of Mice

Beginning at 11 weeks p.i., a 100 µl i.p. injection of encapsulated IFNT (eIFNT) was given every two weeks for a total of eight weeks. An estimated dose of 10^5 units of IFNT (Soos et al., 1995) was released daily from the capsules. Capsules containing MOPS buffer (eMOPS) were used as a vehicle control treatment. The treatment groups included: BeAn-infected/eIFNT-treated (n=18), BeAn-infected/ eMOPS-treated (n=18), and PBS mock infected/eIFN-treated (n=18).

2.2.6. Clinical Scores

Mice were weighed and evaluated weekly and assigned a clinical score based on disease severity. Mice were graded on overall body condition, posture, balance, front and hind limb spasticity, and activity level. Clinical scores were based on a scale from 0-6, where 0 indicates a healthy animal and 1-6 represent gradually increasing severity of signs as follows: 1:piloerection and/or hunched posture with slight gait abnormalities; 2: piloerection and lowered or hunched posture plus unsteady gait; 3: very unsteady gait, weak grasp response when placed on wire grid, decreased activity level, and occasional slight limb monoparesis; 4: severe hind limb weakness and/or spastic paralysis, weight loss, severe decrease in activity level, and loss of the righting response; 5: paraparesis of hind limbs and forelimbs, severe weight loss, incontinence, and complete loss of righting response; 6: highly moribund/dead (Borrow et al.,1998). For these observations, animals were examined while walking on a metal grid. The metal grid permitted inspection of an animal's movements from all sides (top, bottom, left and right side) allowing for close scrutiny of possible deficits. An inclined area was used to test an animal's balance and strength while ascending or descending.

2.2.7. Perfusion and Tissue Dissection for Paraffin Embedding

Mice were euthanized with 150 mg/kg of Beuthanasia-D special (Schering-Plough Animal Health, Boxmeer, The Netherlands) and perfused via the left ventricle with 4% paraformaldehyde in 1X PBS. The brain and spinal cord were dissected out and immersed in the same fixative. After an18-hour incubation in paraformaldehyde, tissues were rinsed once in PBS. Spinal cords were removed from the vertebral column and cut into 9 sections: 3 cervical, 3 thoracic and 3 lumbar. Brains were removed from the skull and cut into 3 sections in order to view the brain stem, pons, and cerebellar regions of the brain. Tissues were placed in cassettes and stored in PBS until ready for processing and embedding in paraffin. Once CNS tissues were removed, the remainder of the body was placed into a 50 ml conical tube and immersed in formalin and stored at 4°C until a full necropsy could be done.

2.2.8. Sectioning/Staining of Paraffin Embedded Tissues

For spinal cords, blocks from each animal (n=6 for each treatment group) were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) to observe inflammation and Luxol Fast Blue (LFB) to determine demyelination. Six slides were made from each spinal cord block with a minimum of 20 μm skipped between sections. The first slide and the last slide of each set were stained with LFB while the other four slides were stained with H&E.

2.2.9. Perfusion and Processing for Resin Embedding

Mice were euthanized with 150 mg/kg of Beuthanasia-D special (Schering-Plough Animal Health, Boxmeer, The Netherlands) and perfused via the left ventricle with 2% paraformaldehyde:3% glutaraldehyde in 0.1 M sodium cacodylate. The brain and spinal cord were dissected out and immersed in the same fixative. After an18 h incubation in the fixative, spinal cords were removed from the vertebral column and cut into 9 sections: 3 cervical, 3 thoracic and 3 lumbar. Dissected tissues were rinsed 3x in 0.1 M sodium cacodylate and post-fixed in osmium tetraoxide overnight. Tissues were rinsed 5x in 0.1 M sodium cacodylate and dehydrated through a graded series of ethanol, followed by propylene oxide. After dehydration, tissues were infiltrated overnight with resin. Tissues were embedded in beem capsules and resin was polymerized at 60ºC for 72 h.

2.2.10. Sectioning/Staining of Resin Embedded Tissues

For spinal cords, blocks from each animal (n=6 for each treatment group) were sectioned at .3 μm and stained with 1% toluidine blue. Two slides were made from each spinal cord block. Nine blocks were sectioned for each animal.

2.2.11. Microscopic Evaluation of Paraffin and Resin Embedded Tissues

Spinal cord tissues from the cervical, thoracic and lumbar levels were examined and scored based on the degree of demyelination within the cervical, thoracic, and lumbar regions and the amount of inflammation within the various dorsal, lateral, and ventral funiculi and/or meninges. A pathologic score of 0-4 was assigned to each section according to the following criteria: Stage 0= no lesions; Stage I=extremely mild, marginally identifiable inflammation, no demyelination (<5% of area affected); Stage II=mild, definitive inflammation with some demyelination, focal and limited in scope,(5-10% of area affected); Stage III=moderate inflammation and demyelination, multi-focal and moderately extensive in scope (25-50% of area affected); Stage IV=prominent inflammation and demyelination, moderate tissue destruction, multi-focal and prominently extensive in scope (50-75% of area affected); Stage V=very prominent demyelination with or without inflammation, severe tissue destruction, multi-focal and very prominently extensive in scope (75-100% of area affected). A mean score was derived for each animal. Individual animal means were pooled within treatment groups (n=12) to calculate means used in graphical representations.

2.2.12. Statistical Analysis

All data were reported as means ± standard error (SEM). Graphpad Prism 5.0 software generated all graphs and analyzed all data. Analysis of variance (ANOVA) was used for all parametric measurements, followed by Bonferroni post-hoc procedure when appropriate. Kruskal-Wallis Rank Sum was used for all nonparametric data. The confidence interval was set at 0.05 for all analyses.

2.3. Results

2.3.1. Mice Treated with IFNT Showed a Significant Decrease in Clinical Score, but No Difference in Body Weights.

The most obvious early indication of treatment efficacy was demonstrated when mice regained their normal posture while at rest as well as during ambulation. After two weeks of eIFNT treatment, mice began to show a decrease in clinical score (Figure 4). This decrease continued for the entire 8 week treatment period. Mice that received eMOPS had an increase in clinical score over the eight week treatment period ($p<0.001$). As shown in Figure 5, all of the eIFNT mice showed a decrease in clinical score after eIFNT treatment. The majority of Bean-eIFNT mice (28/30) showed a substantial decrease in clinical score of at least one point. Most BeAn-eIFNT mice (21/28) showed a decrease of 1.5-2 points. The majority of the BeAn-eMOPS mice (22/26) had an increase in clinical score of at least one point during this same time frame. PBS mock-infected mice showed no clinical signs and are not represented on Figures 3 and 4. There was no difference seen in the body weights of the BeAn-eIFNT-treated mice compared to the BeAn-eMOPS-treated mice (Figure 6). Once again, data for PBS mice are not shown.

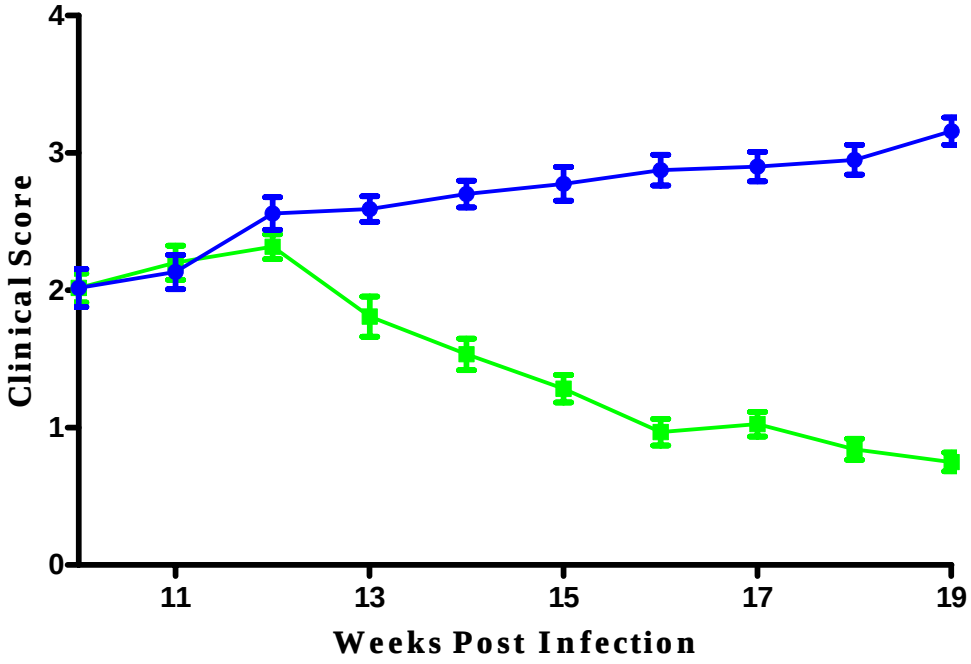


Figure 4. Clinical scores of mice decreased during eIFNT treatment. Mice were scored weekly starting at 9 weeks p.i. At 11 weeks p.i., BeAn-infected mice were treated with eIFNT (green squares) or eMOPS (blue circles) for eight weeks. A decrease in clinical score was evident the second week of treatment and continued for the duration of the treatment period. Results are combined means of five individual experiments with 6-7 mice in each group. Data from PBS controls are not shown. $P < 0.001$.

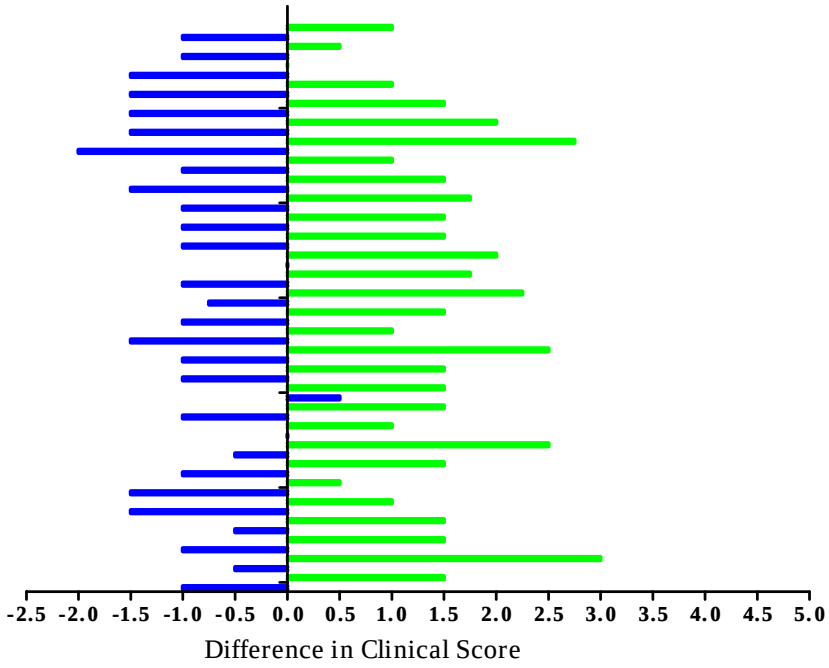


Figure 5. Difference in clinical scores of individual mice treated with eIFNT. Differences in pre-treatment and post-treatment scores demonstrate the efficacy of eIFNT treatment (green bars) versus eMOPS treatment (blue bars) in TVID mice. Post-treatment scores of individual mice at 20 weeks p.i. were subtracted from the pre-treatment clinical scores at 11 weeks p.i. A negative result indicates a clinical score that was higher at the post-treatment time point. All BeAn-eIFNT (green bars) mice had a reduction in clinical scores at the post-treatment time point, reflected graphically as a positive difference. All but one BeAn-eMOPS mouse (blue bars) had increased clinical scores, reflected graphically as a negative difference.

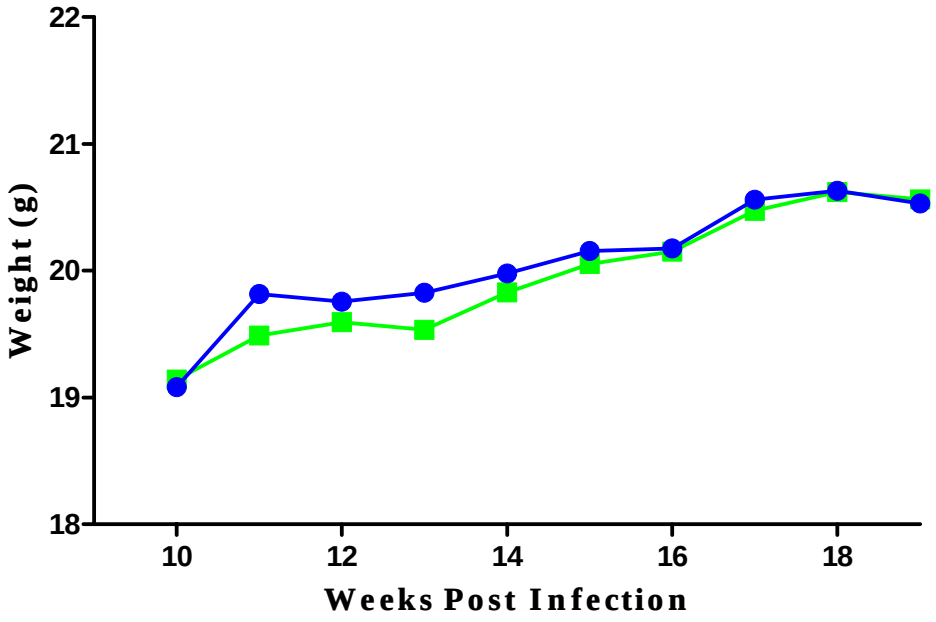


Figure 6. Body weights were not different between treatment groups. Mice were weighed weekly to monitor body condition. There were no differences in average body weight between the BeAn-eIFNT (green squares) treated mice and the BeAn-eMOPS (blue circles) treated mice. Results are shown as combined means of five individual experiments with 6-7 mice in each group. PBS controls are not shown.

2.3.2. Mice Treated with eIFNT Showed a Decrease in Inflammation and Demyelination.

Paraffin-embedded spinal cord sections were stained with H&E and LFB to determine extent of inflammation and demyelination (Figure 7 A-D). Resin-embedded spinal cord sections were stained with toluidine blue (Figures 8A and 8B) No demyelinating lesions were noted in the BeAn-eIFNT mice (Figures 7A and 7B; Figures 8A and 8B). Occasionally, minimal inflammation was noted in the meninges, but these inflammatory cells affected <2% of the meningeal area. The BeAn-eMOPS mice had definitive demyelinating lesions (Figures 7C and 7D; Figures 8C and 8D) and showed pronounced inflammation (Figure 7C) of the meninges as well as perivascular cuffing. Figure 9 shows the graphical representation of the histopathological scores of the BeAn-eIFNT, BeAn-eMOPS, and PBS-eIFNT mice. The amount of inflammation/demyelination of BeAn-eMOPS mice was significantly higher ($p< 0.05$) than BeAn-eIFNT mice. BeAn-eMOPS mice exhibited histopathology significantly higher ($p< 0.01$) than PBS-eIFNT mice. BeAn-eIFNT mice and PBS-eIFNT mice did not exhibit differences in histopathology scores. Brain tissues were examined and modest inflammation was seen in some BeAn-eIFNT mice. These were difficult to score due to variation in level of brain viewed on each slide making comparisons difficult. Thus, these data were not included. Overall, inflammation was rare in the brains of BeAn-eIFNT mice. The BeAn-eMOPS mice had obvious inflammation as well as some demyelination within the pons, brainstem, and cerebellar regions of the brain (data not shown).

2.3.3. Necropsies of Mice Revealed No Abnormalities.

At termination, the spinal cords and brains were removed and the mouse was placed in a 50 ml conical tube with fixative at 4°C and necropsies were performed at a later time. No animals treated with the capsules demonstrated pathology in any organ systems. Specifically, the

organs within the peritoneal cavity were examined for gross pathological changes. The thymus, within the chest cavity, was checked as well for signs of atrophy due to lymphopenia. No changes were seen in any organ systems. Occasionally, residual capsules were found in the peritoneal cavity, but histological examination of tissues revealed no pathological changes due to the presence of the capsules (data not shown).

2.4. Discussion

The first objective of this investigation was to evaluate treatment efficacy of eIFNT during TVID. The eIFNT treatment attenuated the clinical symptoms of TVID. In MS patients, IFNB treatment stabilizes the disease (The IFNB Multiple Sclerosis Study Group, 1993), but does not result in a decrease in clinical manifestation or a gain of function as was observed in the TVID mice during eIFNT treatment. Mice exhibited a decrease in clinical score within two weeks of eIFNT treatment and continued to improve over the eight week treatment period. The most obvious early indication of treatment efficacy was demonstrated when mice regained their normal posture while at rest as well as during ambulation. Mice had improved coordination as well as improved ambulation by the end of the treatment regimen. A substantial decrease in clinical score by 1-2 points within the BeAn-eIFNT-treated group was not expected. It was hypothesized that IFNT treatment would cause stabilization in disease. The fact that the animals demonstrated a marked clinical improvement is the best verification of treatment efficacy. As expected, the BeAn-eMOPS-treated mice showed progressive disability during the eight-week treatment period.

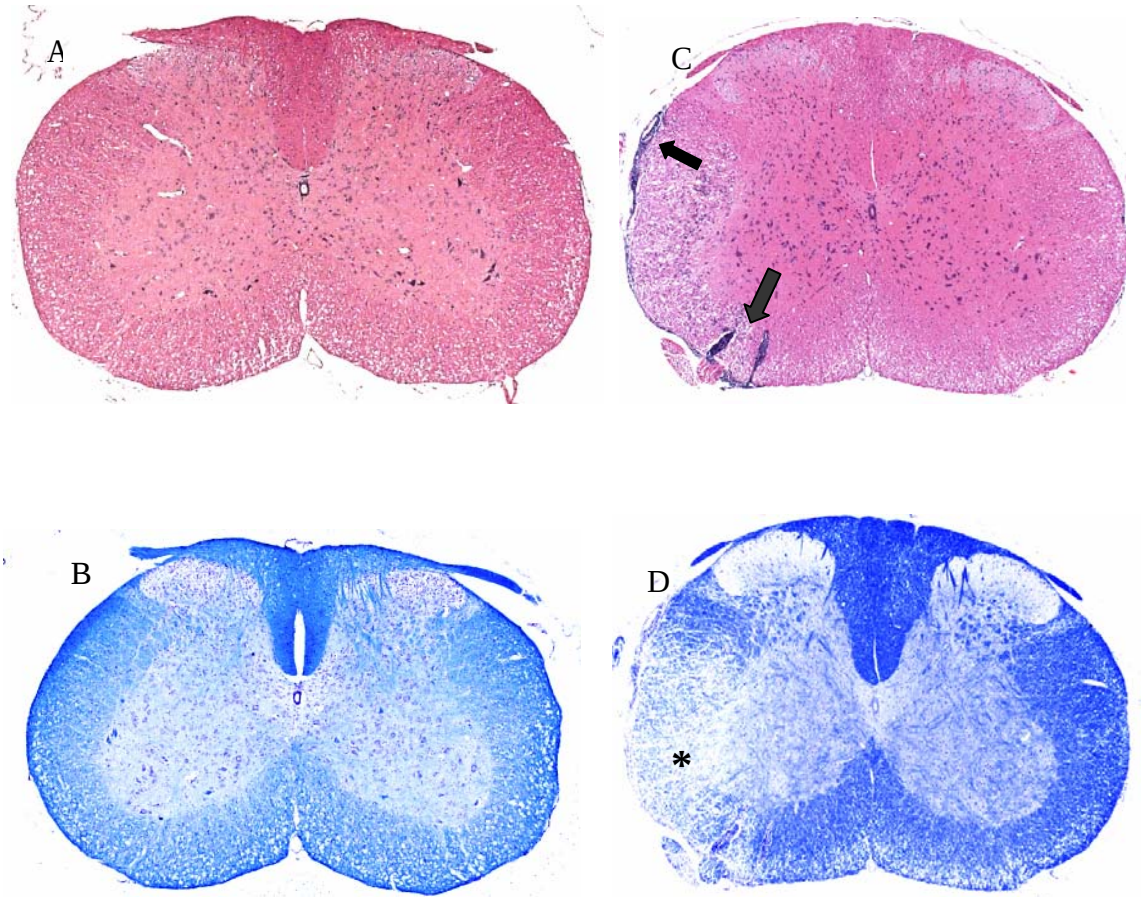


Figure 7. Spinal cord sections from BeAn-eIFNT-treated and BeAn-eMOPS-treated mice. Paraffin embedded spinal cords were inspected for inflammation and demyelination using H&E and LFB stains. A) H&E stained cervical spinal cord section from a BeAn-eIFNT-treated mouse. There were no indications of disease: no inflammation; no demyelination. B) Cervical spinal cord section from the mouse in A stained with LFB. No demyelination was present. C) H&E stained cervical spinal cord section from a BeAn-eMOPS-treated mouse. Note extensive dark bands of inflammation in the meninges (small arrow) as well as perivascular cuffing (large arrow) in parenchyma. The pallor in the lateral white matter bordered by regions of inflammation indicates demyelination. D) LFB stained cervical spinal section from the mouse in C. Note the pronounced area of pallor (asterisk) in the lateral white matter as above in C that is indicative of demyelination.

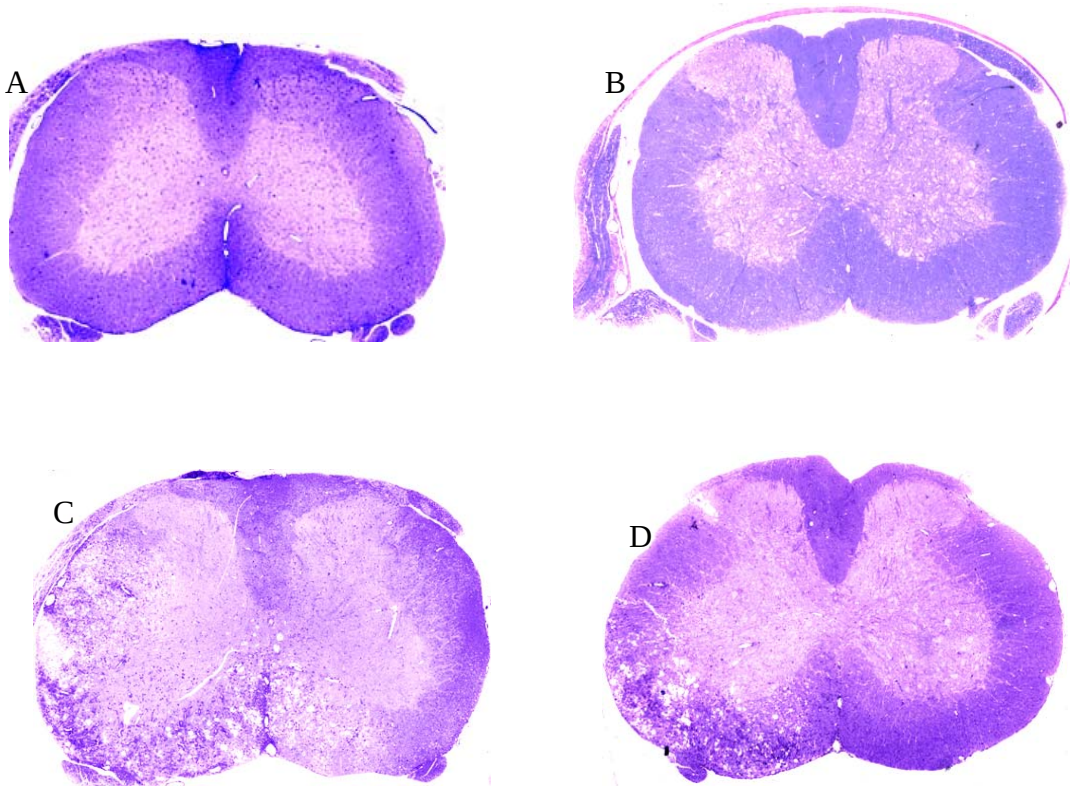


Figure 8. Resin-embedded sections of spinal cord from BeAn-eIFNT-treated and BeAn-eMOPS-treated mice. A and B are cervical spinal cord sections from BeAn-eIFNT-treated mice. Both of these sections represent a Stage 0=no inflammation or demyelination present. C and D are cervical spinal cord sections from BeAn-eMOPS mice. C represents a Stage IV=prominent inflammation and demyelination; moderate tissue destruction, multi-focal and prominently extensive in scope, ~ 70% of the area is affected. D represents a Stage III=moderate inflammation and demyelination, multi-focal and moderately extensive in scope ~30% of the area is affected.

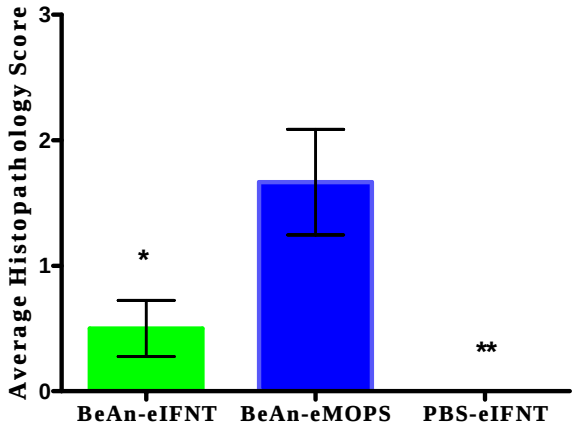


Figure 9. Graphical representation of histopathology scores of spinal cords from TVID mice. Spinal cord sections from BeAn-eIFNT, BeAn-eMOPS, and PBS-eIFNT mice were scored on amount of inflammation/demyelination based on the criteria listed in Section 2.2.11. BeAn-eMOPS mice had significantly higher histopathology scores ($p < 0.05$) than BeAn-eIFNT mice. BeAn-eMOPS mice had significantly higher scores ($p < 0.01$) than PBS-eIFNT mice. There was no significant difference seen in the histopathological scores between BeAn-eIFNT mice and PBS-eIFNT mice. n=12/group. *$p<0.05$; **$p<0.01$.

Many MS patients (~50%) endure side effects during IFNB treatment and many others must stop treatment due to the severity of these side effects (McDonagh et al., 2007). In this study, IFNT was well-tolerated and no obvious adverse effects (weight loss, lymphopenia, fever, lethargy) were noted during treatment. Likewise, the capsules did not present any negative side effects within the mice. None of the mice treated with IFNT-filled or MOPS-filled capsules ever suffered adverse effects from the treatment. Necropsies were performed on the mice to evaluate internal organs for the presence of pathology as well as the presence of peritonitis. Residual beads could be found from time to time, but they did not result in tissue damage, inflammation or organ pathology. Thus, the capsules were well-tolerated and the constant release of IFNT did not result in mice exhibiting flu-like symptoms of weight loss, fever, and lethargy nor did it result in detectable organ pathology.

It was surprising that an occasional inflammatory lesion was noted in the meninges of eIFNT treated mice without the presence of inflammation within the parenchyma or the presence of demyelination. It is suspected that the residual inflammation noted in the CNS of the eIFNT mice may be involved in the resolution of the demyelinating lesion and may even influence the process of remyelination. The necessity of the immune system during wound healing is well documented in the literature. Foote and Blakemore (2005) described the use of inflammation as a way to enhance remyelination by transplanted oligodendrocyte progenitor cells (OPCs) in a rat model of defective myelination. They found that the advanced meningitis seen in one inflammatory model of OPC transplantation resulted in the most widespread remyelination. Setzu et al (2006) found the same phenomena when injecting OPCs into the retina for the purpose of remyelination. If the site of injection was quiescent, then no remyelination occurred. If an inflammatory response was evoked by the injection of an inflammatory compound, then remyelination ensued. Thus, it is important that future investigations evaluate the types of cells

remaining in the CNS of BeAn-eIFNT-treated mice that have improved clinically in order to elucidate the possible role of these cells in CNS repair. The minimal amount of inflammatory cells noted in the brains and spinal cords of some of the eIFNT mice without the presence of any demyelinating pathology is supported by the aforementioned studies and it may be that the vestigial amounts of inflammation provided an environment conducive to remyelination within the eIFNT treated mice.

Overall, the treatment efficacy of eIFNT exceeds that seen by IFNB treatment in MS and IFNB and IFNT in EAE. In MS patients, disease progression is slowed by 30% during IFNB treatment (Tourbah and Lyon-Caen, 2007). In EAE mice, disease development is halted if IFNT is administered during the initiation of the disease. However, this effect is dose dependent and there is an incidence of disease in some animals at lower (5×10^4) levels of IFNT administration (Soos et al., 2002). More recently, investigators have delivered IFNB directly to the CNS with genetically engineered bone marrow stem cell (Makar et al., 2008). Even with this direct delivery system, the EAE mice had an 80% disease incidence, there was no difference in mean clinical score in the acute phase of the disease, and the mice still relapsed. Although the day of disease onset was significantly delayed, the duration of the first relapse was shortened, and the clinical scores during the relapse and the chronic phases of the disease were lowered, the intrathecal expression of IFNB did not improve clinical outcome as did eIFNT in the TVID mice in the current study. Like IFNB treatment in MS patients, the IFNB produced in the CNS of EAE mice was only able to slow disease progression. IFNB was not able to reverse symptoms nor did it result in a gain of function as was shown with eIFNT during TVID within the current study**.** This result is novel in Type I IFN treatment regimens and indicates that IFNT may be providing a mechanism that allows lesion resolution and/or encourages remyelination within the CNS. Future investigations will evaluate this mechanism using bromo-deoxyuridine to label dividing

cells and immunohistochemical markers for oligodendrocyte lineages, myelin, and immune cells in order to access the process of lesion resolution and the cells involved in this mechanism during eIFNT treatment of TVID.

3. CHARACTERIZATION OF CELL-MEDIATED IMMUNE PROFILE FOLLOWING IFNT TREATMENT

3.1. Introduction

It is well accepted that MS is an immune-mediated disease (Trapp and Nave, 2008). Although etiology is still unknown, viral infection is suspected as the initiating event that leads to autoimmune destruction of myelin (Acheson 1977). Therefore, in order to understand the pathogenesis of MS it is most appropriate to study an animal model of virus-induced demyelination such as Theiler's virus infection. Theiler's murine encephalomyelitis virus (TMEV) is a Picornavirus which causes an asymptomatic gastrointestinal infection and occasionally paralysis (Theiler, 1934). The BeAn and DA strains of TMEV are avirulent when injected i.c. However, these TMEV strains result in a persistent infection that causes a demyelinating disease in certain susceptible strains of mice (SJL) that is similar to MS (Lipton, 1975). In fact, persistence of TMEV within CNS parenchyma is necessary for the development of TVID in susceptible strains of mice (Aubert et al., 1987). Thus, Theiler's virus infection in mice represents not only an excellent model for the study of the pathogenesis of MS but also a model system for investigating the mechanisms of action of potential therapeutics in the treatment of MS.

A generally accepted goal of MS treatment is to attenuate a Th1 (pro-inflammatory) immune response by shifting to a Th2 (anti-inflammatory) cytokine profile (Baker et al., 1996). IFNT has been shown to inhibit Th1 cytokine production *in vivo* (Newton et al., 1989; Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993) and up regulate Th2 cytokines (Chaouat et al., 1995). It is believed that IFNB, a first line treatment for MS, provides disease modification by: a) decreasing cell trafficking into the CNS by down-regulating adhesion

molecules, such as α4 integrin on brain endothelium, b) decreasing levels of matrix metalloproteinases produced by lymphocytes, and c) by causing a shift in the Th1/Th2 immune profile (Billiau, et al., 2004). IFNT treatment of EAE resulted in increased IL-10 levels and has been proposed to explain the mechanism of action of IFNT **(**Mujtaba et al., 1997). The importance of IL-10 in EAE has been shown in transgenic mice. IL-10 deficient mice develop more severe EAE and IL-10 over-expressors were completely resistant to disease (Bettelli et al., 1998). Soos et al. (1997) also found increased levels of IL-10 in IFNT-treated mice and increased levels in transforming growth factor beta (TGFB) have also been reported (Khan, et al., 1998).

The objectives for this investigation were to elucidate the cell-mediated responses in TVID mice upon exposure to IFNT *in vivo*. After treatment, mice were terminated and their splenocytes were stimulated *in vitro* with virus in order to evaluate Th1 and Th2 cytokine production. Tregs were evaluated in the blood and spleens of animals to quantify any differences in the percentage of cells in these two biological compartments. It was hypothesized that Th1 cyokine production would be reduced for the eIFNT group and that Th2 cytokines would increase. Likewise, an increase in the number of Tregs in spleen and/or blood was hypothesized within the BeAn-eIFNT-treated mice compared to the BeAn-eMOPS placebo group.

During TVID and MS, inflammatory cell infiltrates cause damage to self-antigens (myelin) within the CNS. CD4+ Treg function is defective in MS patients during a relapse, but the function is restored upon remission. Tregs decrease IFN-γ levels secreted by macrophages from MS patients (Arnason and Reder, 1994). Therefore, the TVID model is a valuable tool for testing the therapeutic potential of manipulating the Treg population in order to suppress a Th1 response as occurs in MS.

3.2. Materials and Methods

3.2.1. Virus Stocks

The BeAn 8386 strain of TMEV was a gift from Dr. H. L. Lipton (University of Illinois at Chicago, IL.) The virus was propagated in BHK-21 cells, titered, aliquoted, and stored at -80^{o} C before use (Welsh et al., 1987).

3.2.2. Animal Infection

SJL/J mice purchased from Harlan (Indianapolis, IL) were bred in-house. Twenty-eight day old female mice were anesthetized with Isoflurane (Vedco, Saint Joseph, MO) and inoculated intracranially (i.c.) into the right cerebral hemisphere with 20 µl of BeAn strain of TMEV (5 X 10^5 plaque forming units) suspended in media or 20 µl of PBS alone. The SJL/J mice were divided into two groups as follows: 13 received PBS only and 26 received 5x10^5 plaque forming units (pfu) of BeAn virus. The day of i.c. inoculation was considered day 0. All animal protocols were in accordance with NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee. See Figure 10 for experimental design.

3.2.3. IFNT

IFNT was a gift from Dr. Fuller W. Bazer (Texas A&M Health Science Center, College Station, TX). IFNT was isolated as previously described in Ott et al., 1991. The protein concentration was determined by the Lowry assay (Lowry et al., 1951) to be 1mg/ml and was determined to be 10^8U/mg by virus plaque assay.

3.2.4. IFNT Encapsulation

Alginate capsules were prepared as previously described with some modifications (Abraham et al., 1996; Arenas-Gamboa et al., 2008). Briefly, 1 ml of 10^8 U/mg of IFNT was mixed with 5 ml of alginate solution (1.5% sodium alginate, 10 mM MOPS, 0.85% NaCl [pH 7.3]). Spheres were obtained by extruding the suspension through a 200 μm nozzle into a 100 mM calcium chloride solution and stirred for 15 min by using an Inotech Encapsulator I-50 (Inotech Biosystems International, Rockville, MD). After extrusion of the IFNT-alginate mixture into the $CaCl_2$, the capsules were washed twice with MOPS for 5 min and further cross-linked with 0.05% poly-L-lysine (molecular weight. 22,000; Sigma-Aldrich, St. Louis, MO) for 10 min. After two successive washes, the beads were stirred in a solution of 0.03% (wt/vol) alginate for 5 min to apply an outer shell and washed twice with MOPS. VpB was added to the shell of the capsule (*vjbR*::Tn*5*/VpB/shell) as a cross-linking agent by the addition of VpB in an equimolar ratio of poly-L-lysine/VpB. After two successive washes, beads were collected, aliquoted (600 μl capsules in 900 μl of MOPS buffer) and stored at 4°C.

Capsules were kept at 4°C in the presence of excess IFNT until time of injection. Before injection, capsules were rinsed 3X in MOPS by resuspending capsules and allowing them to resettle. Finally, capsules were resuspended in the appropriate amount of MOPS buffer prior to injection (900 μl).

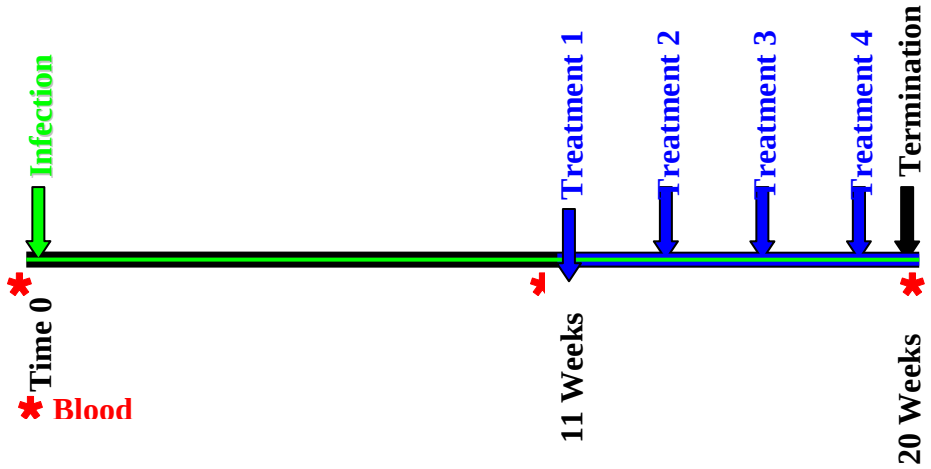


Figure 10. Experimental design: Section III. The following schematic denotes infection time point (green arrow), treatment administration time points (blue arrows), and blood collection time points (red asterisks). Time points are in weeks post infection.

3.2.5. IFNT Treatment of Mice

Beginning at 11 weeks p.i., a 100 µl i.p. injection of encapsulated IFNT (eIFNT) was given every two weeks for a total of eight weeks. An estimated dose of 10^5 units of IFNT (Soos et al., 1995) was released daily from the capsules. Capsules containing MOPS buffer (eMOPS) were used as a vehicle control treatment. The groups were as follows: BeAn-infected/eIFNT-treated (n=13), BeAn-infected/eMOPS-treated (n=13), and PBS mock-infected/eIFN-treated (n=13).

3.2.6. Clinical Scores

Mice were weighed and evaluated weekly and assigned a clinical score based on disease severity. Mice were graded on overall body condition, posture, balance, front and hind limb spasticity, and activity level. Clinical scores were based on a scale from 0-6, where 0 indicates a healthy animal and 1-6 represent gradually increasing severity of signs as follows: 1:piloerection and/or hunched posture with slight gait abnormalities; 2: piloerection and lowered or hunched posture plus unsteady gait; 3: very unsteady gait, weak grasp response when placed on wire grid, decreased activity level, and occasional slight limb monoparesis; 4: severe hind limb weakness and/or spastic paralysis, weight loss, severe decrease in activity level, and loss of the righting response; 5: paraparesis of hind limbs and forelimbs, severe weight loss, incontinence, and complete loss of righting response; 6: highly moribund/dead (Borrow et al.,1998). For these observations, animals were examined while walking on a metal grid. The metal grid allowed inspection of an animal's movements from all sides (top, bottom, left and right side) allowing for close scrutiny of possible deficits. There was an inclined area to test an animal's balance and strength while ascending or descending.

3.2.7. Single Cell Suspensions from Spleen

Mice were perfused through the left ventricle with heparinized (10 U/ml) Hank's balanced salt solution at pH 7.2. Spleens were removed and placed in RPMI-1640 media on ice. Single cell suspensions were prepared as described previously (Welsh et al., 2004). Briefly, the spleens were extruded through nylon mesh attached to a 50 ml beaker. Cells were pelleted by centrifugation and resuspended in 2 ml of red blood cell lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-Cl) and incubated for 2 minutes at room temperature. Following incubation, 10 mls of complete RPMI media was added. Any clot forming in the media was removed with a sterile Pasteur pipet prior to centrifugation to prevent cell loss. Cells were rinsed by centrifugation. Pellets were resuspended in 10 mls of complete RPMI media and counted with a hemacytometer using 0.1% Trypan blue dye. Once counted, cells were pelleted by centrifugation and resuspended to a concentration of $2x10^7$ cells/ml. Fifty microliters of cell suspension (per well) were used for ELISpot and *in vitro* stimulation procedures.

3.2.8. Virus: Enrichment, UV-Inactivation, and Peptides

The BeAn 8683 strain of TMEV (provided by Dr. H. L. Lipton, University of Illinois at Chicago, IL) were propagated in BHK-21 cells as previously described (Welsh et al., 1987).

Virus was enriched by ultracentrifugation pelleting through a 30% sucrose cushion at 80,000 x g for 3 hours in an Optima L-80 XP ultracentrifuge (Beckman Coulter) using an SW-28 rotor (Rueckert and Pallansch, 1981). Pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.4). Virus was UV-inactivated by exposing it to UV-light (1330 $\mu W/cm^3$ at 13 cm distance) for 1.5 hours. The immunodominant CD4 T-cell peptide QEAFSHIRIPLPH, corresponding to TMEV $VP2_{74-86,}$ was used to determine CD4 T-cell specific responses (Gerety et al., 1991 and 1994). The non-specific peptide sequence RLNRITKDSYPNS (NSP) was used

as a control peptide to determine non-specific immune responses. All peptides were purchased from Sigma-Aldrich (St. Louis, MO) at ≥ 95% purity.

3.2.9. *In Vitro* Stimulation

For cytokine profiling, 1.0 x 10^6 splenocytes were cultured with complete RPMI 1640, and stimulated with plate bound anti-CD3 (10 µg/ml) and anti-CD28 (2.0 µg/ml), or enriched UV-inactivated BeAn, or complete RPMI media in the presence (5X10^5U/ml) and absence of IFNT for 72 hours at 5.0% CO_2 and 37°C. The supernatants were then collected and stored at -20°C until analyzed to measure cytokine secretion with Bio-Plex kits (Bio-Rad Laboratories, Hercules, CA).

3.2.10. Th1/Th2 Cytokine Profiling

Th1/Th2 cytokine levels were determined in supernatants from in vitro BeAn-stimulated splenocytes using Bio-plex 5-plex luminescence kits according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The following cytokines were quantified: IL-2, IL-10, IL-12 (p70), IFNG, and TNFA

3.2.11. T-Regulatory Cell Quantification via Flow Cytometry

T-regulatory cells were quantified via flow cytometry in the spleens and blood of BeAn-infected mice treated with eIFNT or eMOPS for eight weeks. Splenocytes were isolated using the procedure described in Section 2.11. Upon isolation, splenocytes were diluted to a concentration of 1.0 x 10^6 splenocytes/50 µl and were incubated with 0.5 µg/10^6 cells of rat anti-mouse CD16/CD32 (#14-0161; clone 93; eBioscience, San Diego, CA) for 10 minutes on ice to block endogenous Fc receptors. Cells were rinsed with flow cytometry staining buffer (#00-

4222-26; eBioscience, San Diego, CA) and immunostained with the following antibodies from eBioscience: FITC conjugated rat anti-mouse CD4 (#11-0042; clone RM4-5), PE-Cy5 conjugated rat anti-CD45 (#15-0451; clone 30-F11), for 20 minutes at 4°C. Cells were washed 3X with flow cytometry staining buffer (eBioscience, San Diego, CA) and resuspended in 1 ml of fixation/permeabilization buffer (#72-5775 Foxp3 Kit; eBioscience, San Diego, CA) overnight at 4°C. The next day cells were rinsed 3X in permeabilization buffer (#72-5775 Foxp3 Kit; eBioscience, San Diego, CA) and stained with PE conjugated rat anti-Foxp3 (#72-5775 Foxp3 kit; clone FJK-16S) 30 minutes at 4°C. Cells were rinsed 3X in permeabilization buffer and then resuspended in 300µl of flow cytometry staining buffer and Treg cell percentages were determined using a FACScaliber (Becton Dickson, Franklin Lakes, NJ) and MoFlow software.

For blood cells, whole blood was collected into 7.5% EDTA. Blood was rinsed 2X with centrifugation in PBS + 7.5% EDTA to remove serum and wash blood cells. After the final rinse, 400 µl of whole blood (suspended in PBS) was blocked with Fc-block as listed above. After blocking endogenous Fc receptors, 50 µl of whole blood was stained according to the protocol described above for splenocytes. However, for blood samples, supernatants were removed with a Pasteur pipette after each washing step to decrease the number of cells lost by simply decanting the solution off of the cells after each wash step.

3.3. Results

3.3.1. IL-2 Secretion was Slightly Elevated from BeAn-eIFNT-Treated Mouse Splenocytes.

Cytokine secretion was quantified in the supernatants from splenocytes using Bio-Plex 5-plex luminescence systems from Bio-Rad. When stimulated *in vitro* with UV-inactivated BeAn (Figure 11A), splenocytes from BeAn-eIFNT mice (76.2±16.79) produced slightly higher mean levels of IL-2 than splenocytes from BeAn-eMOPS mice. (60.82±4.20), but the difference was not statistically significant. When splenocytes were stimulated with UV-inactivated BeAn in the presence of IFNT (Figure 11B), the splenocytes from BeAn-eIFNT-treated mice (96.49±13.56) produced more IL-2 than splenocytes from BeAn-eMOPS mice (56.3±3.05). This difference was statistically significant ($p < 0.05$). Control levels are shown in Figure 13A.

3.3.2. IFNG Secretion was Elevated from BeAn-eIFNT-Treated Mouse Splenocytes.

Cytokine secretion was quantified in the supernatants from splenocytes using Bio-Plex 5-plex luminescence systems from Bio-Rad. In response to BeAn stimulation (Figure 12A) *in vitro,* splenocytes from BeAn-eIFNT-treated mice (118±55.21) produced higher mean levels of IFNG than did the BeAn-eMOPS mice (15.84±7.83), but the difference was not significant. Mean IFNG levels increased further when splenocytes from BeAn-eIFNT-treated mice (129.5±70.33) versus those from BeAn-eMOPS mice (24.6±11.54) were stimulated with UV-inactivated BeAn in the presence of IFNT (Figure 12B) in the culture media, but this difference was not significant. Control levels are shown in Figure 13B.

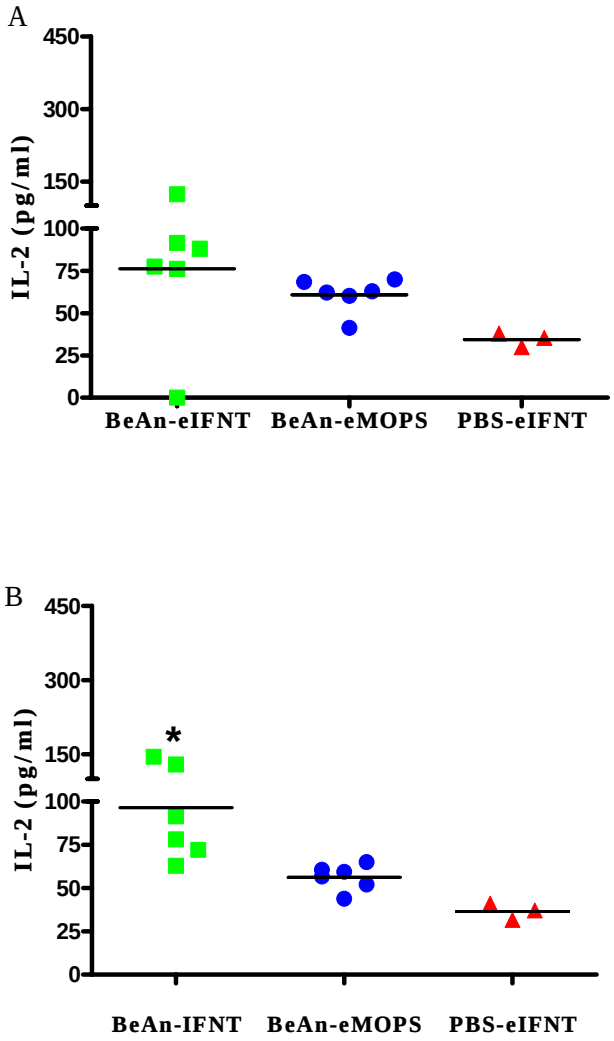
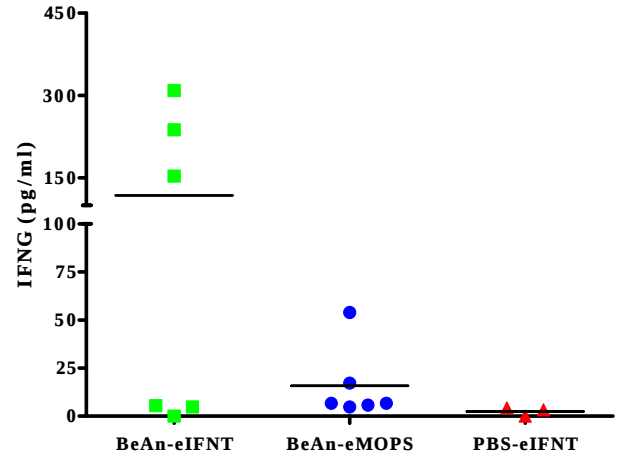


Figure 11. The effect of eIFNT on IL-2 secretion. Splenocytes from BeAn-eIFNT (green squares) mice and BeAn-eMOPS (blue circles) mice were stimulated with enriched BeAn for 72 h in the presence or absence of IFNT (10^5U/mg). Supernatants were assayed by Bio-Plex to determine IL-2 production levels. A) Splenocytes were stimulated with BeAn alone. The BeAn-eIFNT (green squares) treated mice had slightly higher mean levels of IL-2 when compared to BeAn-eMOPS (blue circles) mice, but this difference was not significant. B) Splenocytes were stimulated with BeAn in the presence of 10^5U/mg IFNT. The BeAn-eIFNT (green squares) treated mice produced more ($p<0.05$) IL-2 than the BeAn-eMOPS (blue circles) mice. The line designates the mean within a group. n=6 mice per group. *$p<0.05$

A



B

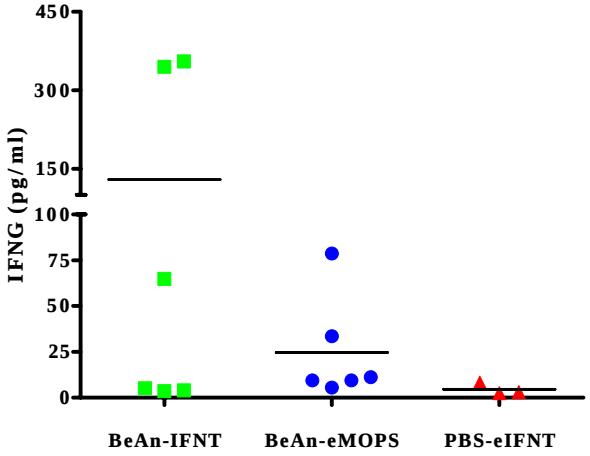


Figure 12. The effect of eIFNT on IFNG secretion. Splenocytes from BeAn-eIFNT (green squares) mice and BeAneMOPS (blue circles) mice were stimulated with enriched BeAn for 72 h in the presence or absence of IFNT (10^5U/mg). Supernatants were assayed by Bio-Plex to determine IFNG production levels. A) Splenocytes were stimulated with BeAn alone. The BeAn-eIFNT (green squares) treated mice had higher mean levels of IFNG when compared to BeAn-eMOPS (blue circles) mice, but this difference was not significant. B) Splenocytes stimulated with BeAn in the presence of 10^5U/mg IFNT. Splenocytes from the BeAn-eIFNT (green squares) treated mice produced more IFNG than the splenocytes from BeAn-eMOPS (blue circles) mice, but the difference was not significant. The line designates the mean within a group. n=6 mice per group.

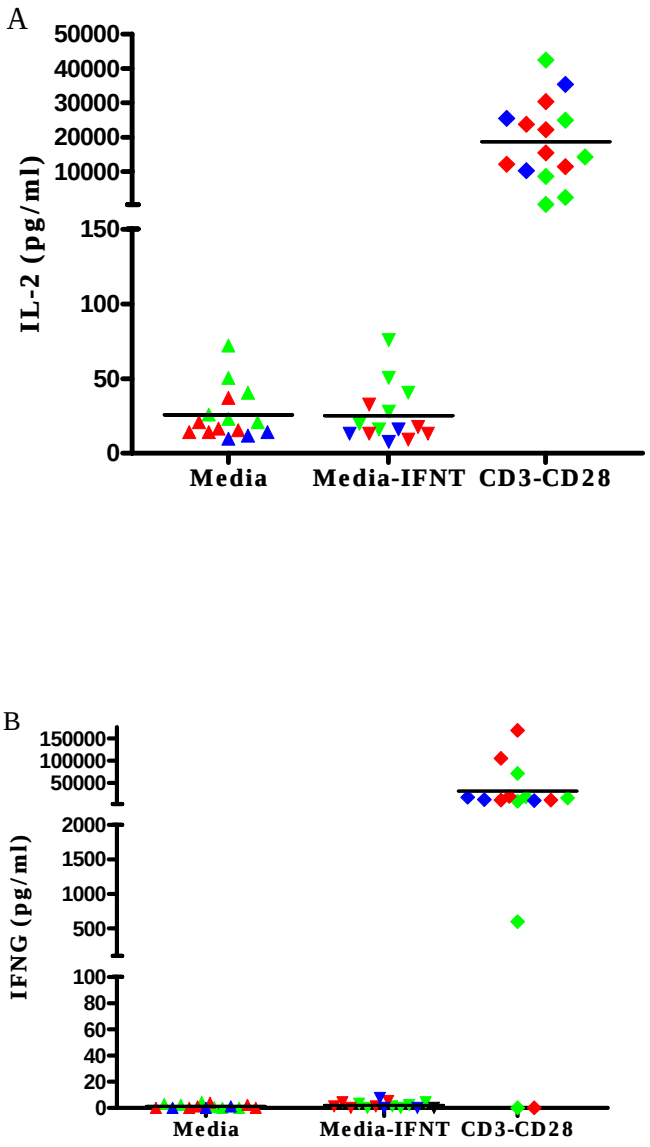


Figure 13. Media and anti-CD3/CD28 controls for IL-2 and IFNG cytokine data. A and B are the negative (media) and positive (anti-CD3/CD28) controls for the data in Figures 11 and 12. All data are represented by a diamond shape. Actual groups are designated by color as follows: eIFNT-green, eMOPS-blue, and PBS controls-red. A. Controls for IL-2 secretion. B. Controls for IFNG secretion. Lines represent the means for the designated condition.

3.3.3. IL-10 Secretion was Elevated from BeAn-eIFNT-Treated Mouse Splenocytes.

Cytokine secretion was quantified in the supernatants from splenocytes using Bio-Plex 5-plex luminescence systems from Bio-Rad. Splenocytes from BeAn-eIFNT mice (39.75±15.53) and BeAn-eMOPS mice (24.41±13.35) secreted low mean amounts of IL-10 when stimulated *in vitro* with UV-inactivated BeAn (Figure 14A). When splenocytes were stimulated with UV-inactivated BeAn in the presence of IFNT in the culture media (Figure 14B), BeAn-eIFNT mice (69.08±26.18) produced higher mean amounts of IL-10 as did the BeAn-eMOPS (33.66±9.26) mice, but differences in mean secretion of IL-10 from BeAn-eIFNT mice versus BeAn-eMOPS mice were not significant. Control levels are shown in Figure 16A.

3.3.4. TNFA Secretion was Elevated from BeAn-eIFNT-Treated Mouse Splenocytes.

Cytokine secretion was quantified in the supernatants from splenocytes using Bio-Plex 5-plex luminescence systems from Bio-Rad. The BeAn-eIFNT treated mice (783.10±336.5) produced higher mean levels of TNFA when compared to BeAn-eMOPS (318.40±79.64) mice when stimulated with UV-inactivated BeAn (Figure 15A), but this difference was not significant. When splenocytes were stimulated with UV-inactivated BeAn in the presence of IFNT (Figure 15B) in the culture media the BeAn-eIFNT mice (881.90±371.30) produced higher mean levels of TNFA when compared to BeAn-eMOPS mice (312.30±98.83), but the differences were not significant. Control levels are shown in Figure 16B.

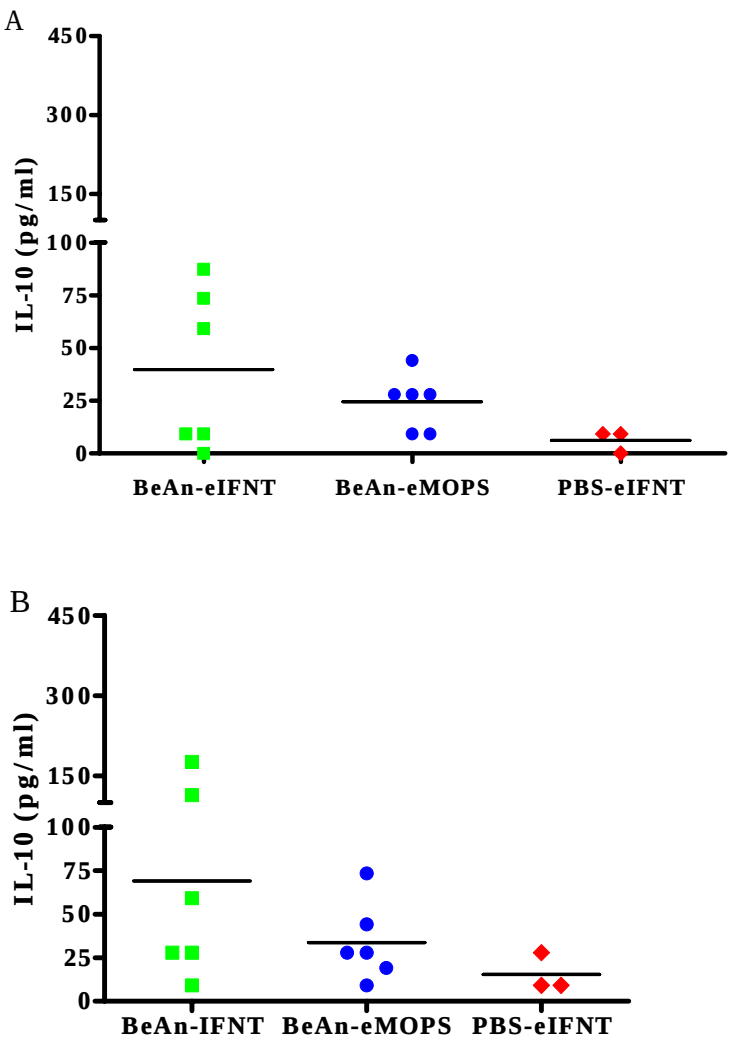


Figure 14. The effect of eIFNT on IL-10 secretion. Splenocytes from BeAn-eIFNT (green squares) mice and BeAn-eMOPS (blue circles) mice were stimulated with enriched BeAn for 72 h in the presence or absence of IFNT (10^5U/mg). Supernatants were assayed by Bio-Plex to determine IL-10 production levels. A) Splenocytes were stimulated with BeAn alone. The BeAn-eIFNT (green squares) treated mice had slightly higher mean levels of IL-10 when compared to BeAn-eMOPS (blue circles) mice, but this difference was not significant. B) Splenocytes were stimulated with BeAn in the presence of 10^5U/mg IFNT. The BeAn-eIFNT (green squares) treated mice produced more IL-10 than the BeAn-eMOPS (blue circles) mice, but the difference was not significant. The line designates the mean within a group. n=6 mice per group.

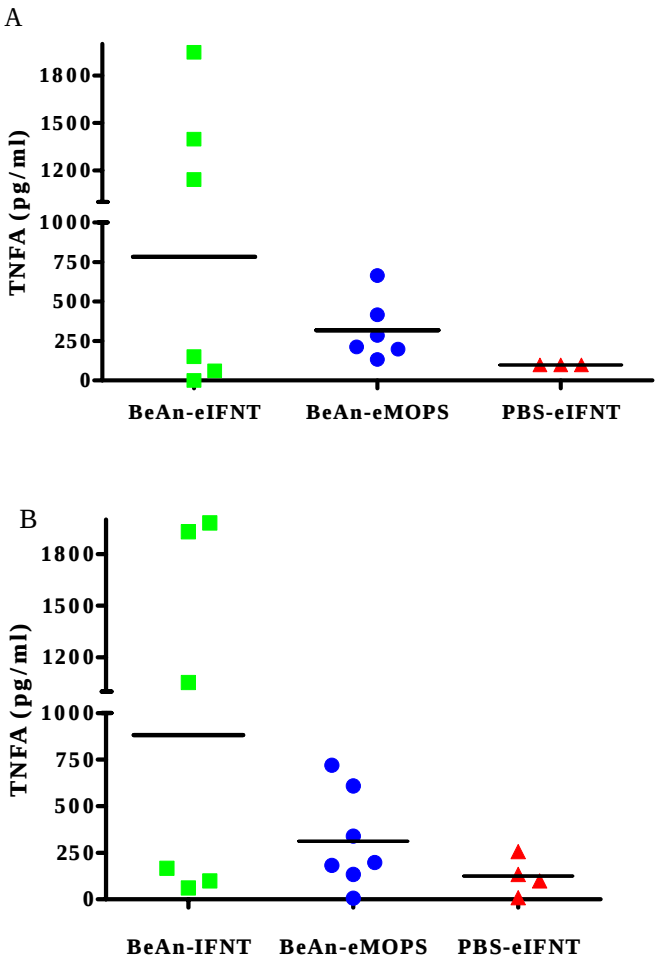


Figure 15. The effect of eIFNT on TNFA secretion. Splenocytes from BeAn-eIFNT (green squares) mice and BeAn-eMOPS (blue circles) mice were stimulated with enriched BeAn for 72 hours in the presence or absence of IFNT (10^5U/mg). Supernatants were assayed by Bio-Plex to determine TNFA production levels. A) Splenocytes were stimulated with BeAn alone. The BeAn-eIFNT (green squares) treated mice demonstrated higher mean levels of TNFA when compared to BeAn-eMOPS (blue circles) mice, but this difference failed to reach significance. B) Splenocytes were stimulated with BeAn in the presence of 10^5U/mg IFNT. The BeAn-eIFNT (green squares) treated mice produced higher mean levels of TNFA, but not significantly higher than the BeAn-eMOPS (blue circles) mice. The line designates the mean within a group. n=6 mice per group.

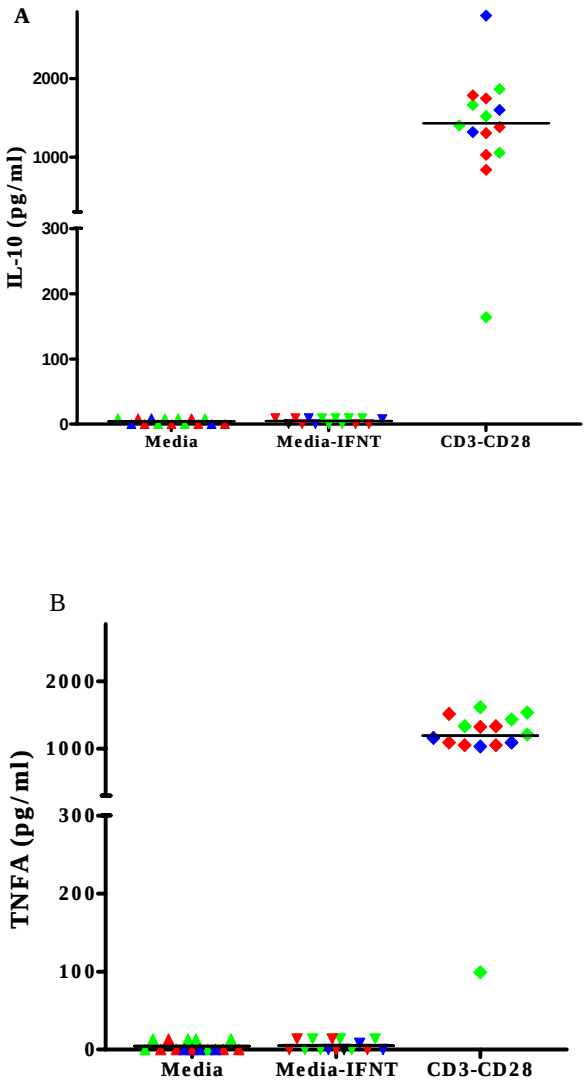


Figure 16. Media and anti-CD3/CD28 controls for IL-10 and TNFA cytokine data. A and B are the negative (media) and positive (anti-CD3/CD28) controls for the data in Figures 14 and 15. All data are represented by a diamond shape. Actual groups are designated by color as follows: eIFNT-green, eMOPS-blue, and PBS controls-red. A. Controls for IL-10 secretion. B. Controls for TNFA secretion. Lines represent the means for the designated condition.

3.3.5. IL-12 (p70) Secretion was Minimal from eIFNT-Treated Mouse Splenocytes.

Cytokine secretion was quantified in the supernatants from splenocytes using Bio-Plex 5-plex luminescence systems from Bio-Rad. The BeAn-eIFNT treated (9.84±3.10) and the BeAn-eMOPS (8.44±0.76) treated mice secreted minimal mean amounts of IL-12 (p70) when stimulated *in vitro* with UV-inactivated BeAn (Figure 17A). Likewise, there was minimal mean secretion of IL-12 from BeAn-eIFNT (8.98±3.11) and BeAn-eMOPS (6.73±1.24) treated mouse splenocytes when stimulated *in vitro* with UV-inactivated BeAn in the presence of IFNT (Figure 17B). No significant difference was seen between the mean levels of IL-12 from BeAn-eIFNT and BeAn-eMOPS groups with either culture condition. Control levels are shown in Figure 18.

3.3.6. Percentages of T-Regulatory Cells were Not Affected by IFNT Treatment.

Flow cytometry of the percentage of CD4+CD45+Foxp3+ T-cells in the spleen (Figure 19A) revealed high percentages in both BeAn-eIFNT (4.23±0.31) and BeAn-eMOPS (4.25±0.40) treated mice and these percentages were similar to those seen in PBS (4.17±0.22) control mice treated with eIFNT. The percentages of CD4+CD45+Foxp3+ T-cells in the blood (Figure 19B) were much lower than in the spleens in all three groups. No significance difference in percentages of Foxp3+ cells in the blood was seen between the BeAn-eIFNT (0.9±0.26), BeAn-eMOPS (1.07±0.19) or PBS-eIFNT (1.37±0.18) groups.

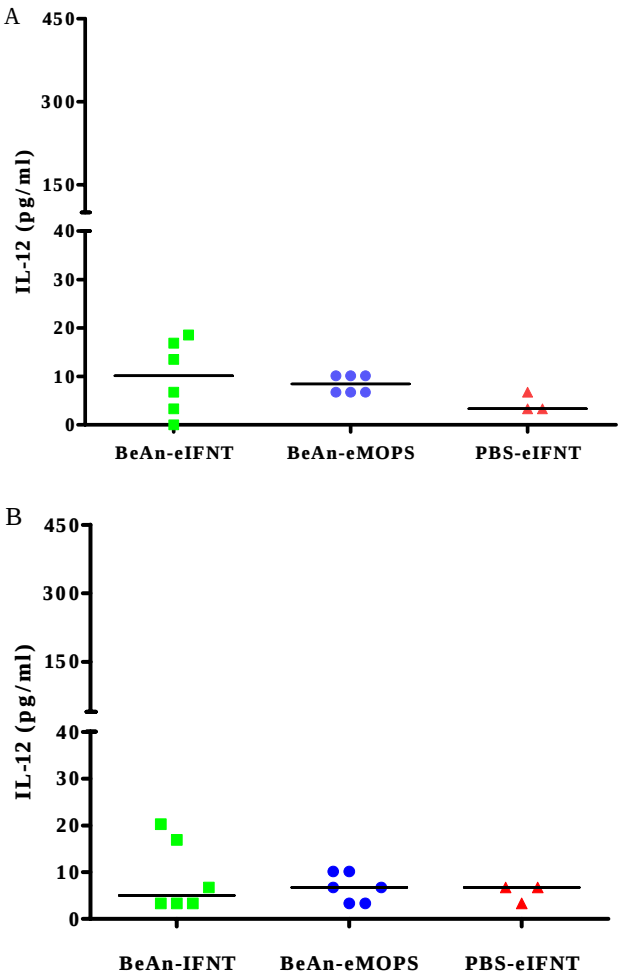


Figure 17. The effect of eIFNT on IL-12(p70) secretion. Splenocytes from BeAn-eIFNT (green squares) mice and BeAn-eMOPS (blue circles) mice were stimulated with UV-inactivated, enriched BeAn for 72 hours in the presence or absence of IFNT (10^5U/mg**).** Supernatants were assayed by Bio-Plex to determine IL-12(p70) production levels. A) Splenocytes were stimulated with BeAn alone. The BeAn-eIFNT (green squares) treated and the BeAn-eMOPS (blue circles) treated mice secreted minimal mean amounts of IL-12 (p70) B) Splenocytes were stimulated with BeAn in the presence of 10^5U/mg IFNT. The BeAn-eIFNT (green squares) treated mice and the BeAn-eMOPS (blue circles) treated mice produced minimal mean amounts of IL-12(p70). The presence of IFNT in the culture system caused no difference in the level of IL-12(p70) secretion within either group. The line designates the mean within a group. n=6 mice per group.

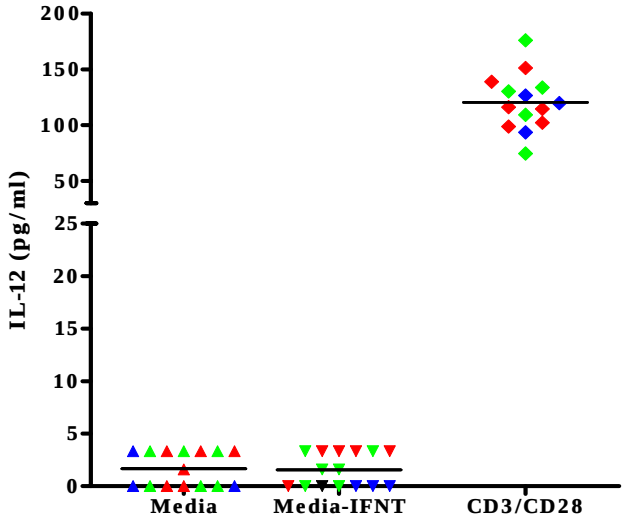
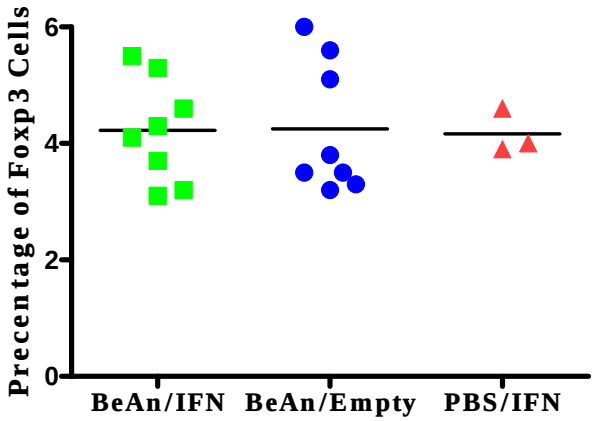


Figure 18. Media and anti-CD3/CD28 controls for IL-12 (p70) cytokine data. This graph shows the negative (media) and positive (anti-CD3/CD28) controls for the data in Figure 17. All data are represented by a diamond shape. Actual groups are designated by color as follows: eIFNT-green, eMOPS-blue, and PBS controls-red. Lines represent the means for the designated condition.

A



B

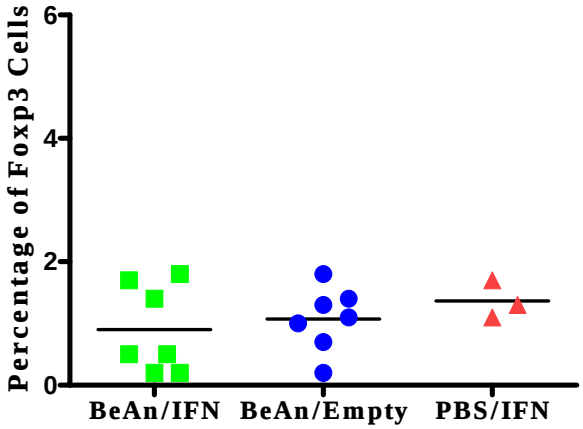


Figure 19. The effect of eIFNT treatment on percentage of Foxp3 cells in spleen and blood. Percentages of CD4+CD45+Foxp3+ T cells were enumerated in spleen and blood by flow cytometry. A) The spleen had a high percentage of Foxp3+ T cells. There were no differences seen between the eIFNT (green squares) and eMOPS (blue circles) groups. B) The percentages of Foxp3+ T cells in the blood were much lower than in the spleens in both groups. No significance difference was seen between the eIFNT (green squares) and eMOPS (blue squares) groups. Lines represent means for each group. n=7 -8 mice per group.

3.4. Discussion

The hypothesis tested in this investigation was that eIFNT mediated its therapeutic effect by shifting a Th1 immune profile to a Th2 immune profile. However, an obvious shift was not demonstrated. In fact, there were no elevated levels of Il-4, IL-5, or IL-6 noted during *in vitro* stimulation of BeAn-specific splenocytes.

The only cytokine to demonstrate statistical significance between BeAn-eIFNT-treated mice and BeAn-eMOPS mice was IL-2. When stimulated with UV-inactivated BeAn alone (Figure 11A), the splenocytes from Bean-eIFNT mice showed a very slight increase over the BeAn-eMOPS splenocytes (76.2 ± 16.79 pg/ml vs. 60.82 ± 4.20 pg/ml). When IFNT (Figure 11B) was added to the culture system during UV-inactivated BeAn stimulation, the mean amount of IL-2 produced by the splenocytes of BeAn-eIFNT mice was statistically increased from the mean amount secreted by BeAn-eMOPS splenocytes (96.49 ± 13.56 pg/ml versus 56.3 ± 3.05 pg/ml). IL-2 is required as a growth factor and a survival factor for Th1 T-cells. It is also a survival factor for Treg cells. Thus, its presence is to be expected during the stimulation with BeAn antigen in the culture condition used in this study. Although there was an increase of IL-2 production by splenocytes from the eIFNT mice when cultured in the presence of IFNT, the levels of production were not dramatic and it is not known if the IL-2 was from Th1 cells or Treg cells. It is also unclear which cell type may be using the cytokine for survival since mixed splenocyte cultures were used. The increase in IL-2 produced by IFNT mice may reflect increased populations of T-regulatory cells since iTregs are antigen-dependent like effector Th1 cells. This could provide a mechanism of disease modification if the increase in IL-2 reflects the presence and activity of Tregs rather than Th1 effector cells. An increased number of Tregs has been noted in MS patients after IFNB treatment (Venken et al., 2007). Future investigations will entail the use of a Miltenyi Magnetic Cell Sorting system to purify cell populations in order to

more clearly define cell source of cytokine. However, as demonstrated in Figures 19A and 19B, differences in Treg cell percentages do not appear to be a possible mechanism of disease modulation within the experimental conditions employed. However, their activation status may warrant further investigation.

Mean IFNG production by BeAn-eIFNT splenocytes was highly elevated compared to production by BeAn-eMOPs splenocytes when stimulated by UV-inactivated BeAn alone (118 ± 55.21 pg/ml versus 15.84 ± 7.83 pg/ml) or in the presence of IFNT (129.5 ± 70.33 pg/ml versus 24.6±11.54 pg/ml) in the culture media (Figures 12A and 12B). IFNG is produced by Th1 cells and is considered the hallmark cytokine of a Th1 type immune response. Thus, the increased production of IFNG by splenocytes from BeAn-eIFNT mice was unexpected as it suggests an up-regulation of the Th1 immune profile which refutes the stated hypothesis of this study. However, this increased production of IFNG from BeAn-eIFNT mice was not significant due to a broad range of secretion from the splenocytes of BeAn-IFNT mice. This broad range in secretion causes a large SEM and results in a non-significant difference between the BeAn-eIFNT and BeAn-eMOPS groups. However, the wide range of response does not appear to reflect outliers in the data set. The distribution pattern as seen in the scatter plot (Figures 12A and 12B) is more indicative of the presence of biological responders and non-responders within the eIFNT group. This situation is seen by other researchers and is a common obstacle when investigating Type I IFN disease modifying drugs and reporting biological measures that reflect treatment efficacy. However, all BeAn-eIFNT mice included in the present studies demonstrated improvement in clinical score indicating that all of the mice were biological responders to the eIFNT treatment. Thus, the explanation for the differences between mice that produce high levels of IFNG in response to IFNT stimulation and those that produce low levels of IFNG in response to IFNT stimulation remains elusive at this time. However, this difference suggests that

individuals may respond differently to a disease modifying drug, but still exhibit disease attenuation. It is possible that the increased production of IFNG is required for the induction of apoptosis in pathologic T-cells through the activation induced cell death pathway (AICD) suggested by other researchers (Refaeli et al., 2002). It is proposed that IFNG stimulates this effect through the activation of Stat1 and its stimulation of caspases. This alternative would decrease the number of pathologic Th1 cells in the periphery, thus, excluding them from the pool that is available to traffic to the CNS and contribute to demyelinating pathology. Another explanation is that the IFNG is being enlisted as an anti-viral agent and is lowering the viral load which would lower immune responses to the virus and should be reflected in a decrease in disease activity. SJL/J mice treated with antibodies to IFNG suffered an increase in demyelination (Rodriguez et al., 1995). In addition, IFNG knock-out mice on a TVID resistant background suffered increased demyelination and mortality when infected with Theiler's virus (Fiette et al., 1995). These studies suggest the importance of IFNG in the resistance to TVID. At this time, the discrepancy in amount of IFNG produced by BeAn-eIFNT mice does not reflect a lack of response to the IFNT as seen in the eMOPS group since all of the eIFNT mice showed clinical improvement. The splenocytes from BeAn-eMOPS mice did not exhibit a dramatic increase in IFNG production upon exposure to IFNT *in vitro*. This suggests that the splenocytes from the Bean-eIFNT group are biologically different from those found in the BeAn-eMOPS group. Future studies into the intracellular signaling pathways induced by IFNT stimulation by the high and low IFNG-secreting splenocytes should elucidate the cell machinery producing the variable responses and may provide information for future treatment targets.

Mean IL-10 production (Figure 14A) by BeAn-eIFNT splenocytes was slightly elevated over BeAn-eMOPS (39.75 ± 15.53 pg/ml versus 24.41 ± 13.35 pg/ml) splenocytes during *in vitro* stimulation with UV-inactivated BeAn virus. There was an even larger increase in mean

IL-10 production from the eIFNT splenocytes versus the eMOPS splenocytes when IFNT Figure 14B) was added to the culture system (69.08 ± 26.18 pg/ml versus 33.66 ± 9.26 pg/ml). Once again, this increase was not significant due to a wide range of responses from the eIFNT group. However, all mice responded to *in vivo* IFNT with a decrease in clinical score and are considered biological responders to the Type I IFN treatment regimen. IL-10 is one of the cytokines considered as anti-inflammatory. It is the counterpart of IL-12, the cytokine responsible for polarizing T-cells toward a Th1 profile. Thus, an increase in IL-10 was hypothesized for this study as a mechanism of IFNT immune modulation. In fact, an increase in IL-10 is considered one of the most reliable measures of biologic response to Type I IFN treatment in MS patients (Kumpfel et al., 2000; Kumpfel et al., 2007). However, the discrepancy in splenocyte response from the eIFNT group is a paradox to the decrease in disease severity witnessed in all of the Type I IFN- treated mice.

The most notable change in level of cytokine production was for TNFA. Mean TNFA production was increased dramatically from BeAn-eIFNT splenocytes (Figures 15A and 15B) and the levels were highly elevated compared to the levels produced by BeAn-eMOPS splenocytes in response to UV-inactivated BeAn stimulation alone (783.10 ± 336.5 pg/ml versus 318.40±79.64 pg/ml) and in the presence of IFNT (881.90 ± 371.30 pg/ml versus 312.30 ± 98.83 pg/ml). Similar to results from measurements of other cytokines, the levels produced by the eIFNT splenocytes demonstrated a wide range which resulted in a large SEM and a lack of statistical significance. However, all mice did respond to *in vivo* IFNT treatment with a decrease in disease activity. Such high levels of TNFA were unexpected as it is considered a pro-inflammatory cytokine and does not fit the clinical picture observed in the eIFNT mice. In fact, high levels of TNFA have been detected in the spinal cords of TMEV-infected SJL mice and disease was suppressed by treatment with monoclonal antibody to TNFA (Inoue et al., 1996). In

another study, administration of TNFA (Paya et al., 1990) to TVID susceptible mice resulted in reduced demyelination. Such high levels of *in vitro* TNFA production are similar to the results of our laboratory (Welsh et al, 1995) where high levels of TNFA have been shown to inhibit TVEV replication in cultured CVE cells. Likewise, its role in this study appears to be beneficial and result in disease modification, possibly through anti-viral effects.

The least notable mean level of cytokine production was seen for IL-12 (Figures 17A and 17B). The production of IL-12 from both the BeAn-eIFNT and BeAn-eMOPS splenocytes were at levels barely detectable with the Bio-Plex assay system whether stimulated with UV-inactivated BeAn alone (9.84 ± 3.10 pg/ml versus 8.44±0.76 pg/ml) or in the presence of IFNT (8.98 ± 3.11 pg/ml or 6.73 ± 1.24 pg/ml). These production levels are expected in the current culture conditions because T-cells do not produce IL-12 in response to antigen stimulation, as APCs, such as macrophages, produce IL-12. Since the BeAn was UV-inactivated, the APCs resident within the splenocyte population should not respond to the virus with activation and pro-inflammatory cytokine secretion. Thus, this measurement is more of an indication that the culture system utilized an antigen-specific condition and that other cytokines produced were antigen dependent in nature. However, it should be noted that even this cytokine revealed two populations of responders as noted by the scatter plot (Figures 17A and 17B) suggesting that even APCs from eIFNT treated mice exhibit a biological difference in response to Type I IFN treatment.

An additional test of the culture system's antigen-specific dependence is presented in Figures 13A, 13B, 16A, 16B and 18. The results presented in these figures are the medium controls (negative control) and the anti-CD3/CD28-stimulated positive controls. BeAn-eIFNT and BeAn-eMOPS splenocytes were assessed in negative and positive control conditions in order to evaluate response specificity. The anti-CD3/CD28 condition cross-links the T-cell receptor

and yields optimal antigen-independent activation. Thus, the anti-CD3/CD28 condition usually manifests the highest amount of cytokine production. The media (negative) control condition provides the amount of cytokines produced constitutively in the culture system. The levels of cytokines produced in all negative and positive controls supported the specificity of cytokines produced in the presence of UV-inactivated BeAn. An interesting observation is that the mice that responded with the highest production levels did so for all cytokines evaluated (IL-2, IFNG, TNFA and IL-10). However, these same mice did not produce the highest levels of cytokines during the anti-CD3/CD28 condition. This finding implies that the splenocytes that respond with high cytokine production are not just hyper-responsive cells. Their increased cytokine production is an actual manifestation of a biological difference in response within the splenocytes of mice primed *in vivo* with eIFNT. The “low responders” in the BeAn-eIFNT group easily produce high levels of cytokines in the anti-CD3/CD28 condition. Thus, their decreased cytokine production is not an indication of an inability to respond robustly to activation.

Overall, the cytokine findings warrant further investigations that: a) utilize purified cell cultures to better identify which cell type is producing each cytokine in response to antigen stimulation; b) examine intracellular signaling pathways to establish a difference between the high and low responses in cytokine production observed in these studies; and c) employ molecular techniques, such as microarray technology, to pinpoint genomic differences in relation to biological response.

4. CHARACTERIZATION OF HUMORAL IMMUNE PROFILE FOLLOWING IFNT TREATMENT

4.1. Introduction

MS is a chronic inflammatory demyelinating disease of the CNS. The etiology of MS remains elusive, yet viral infection is suspected. The presence of oligoclonal bands within the CNS of MS patients is considered one of the diagnostic criteria for this disease. However, the amount of research dedicated to this facet of the disease is sparse. Recently, this area has become more popular due to the observations of Serafini et al., 2007. These researchers noted the presence of Epstein-Barr Virus (EBV) positive B cells within the parenchyma of the majority of MS patients. These same researchers (Serafini et al., 2004) and others (Aloisi and Pujol-Borrell, 2006) have now described the presence of germinal centers like those seen in peripheral lymph nodes within the meninges of MS patients. Finally, the recent use of rituximab as a successful treatment in MS patients has shown that B-cell depletion is a potential disease modifying mechanism (Hauser, 2008). These results suggest that antibody production during MS is crucial to the disease mechanism and warrant further investigation.

The proposed mechanism of disease modifying drugs in MS is through a shift in a Th1/Th2 immune profile. If this is true, and MS pathology has a humoral component, then a shift to a more aggressive antibody response may result in disease exacerbation. If this simplistic view is correct, then a Th2 shift is not necessarily a desired goal of treatment regimens. In fact, the

most beneficial treatment would modulate then entire immune system and result in a quieting of the immune response and a switch to wound healing mechanisms. In this scenario, wound healing would entail the replacement of lost/damaged myelin. However, if MS has a viral etiology, then an increase in antibody production could be utilized to clear disease-initiating virus within the MS patient's CNS and decrease disease activity.

The purpose of the current study was to investigate the effect of eIFNT on the humoral response to TMEV. More specifically, sera from BeAn-infected mice treated with eIFNT and eMOPS were evaluated for virus-specific antibodies. It was hypothesized that: a) serum antibody levels specific for TMEV would increase; and b) ELISPot assays would detect an increase in virus-specific antibody secreting cells (ASCs) within the spleens and CNS of treated mice, supporting a switch in immune profile from a Th1 response to a Th2 response. It was predicted that increases in virus-specific antibodies and virus-specific ASCs would allow the SJL mice to finally clear the persistent TMEV infection within their CNS and that this outcome would decrease the severity of this demyelinating disease.

4.2. Materials and Methods

4.2.1. Virus Stocks

The BeAn strain of TMEV was a gift from Dr. H. L. Lipton (Northwestern University, Evanston, IL.) The virus was propagated in BHK-21 cells, titered, aliquoted, and stored at -80^{o} C before use (Welsh et al., 1987).

4.2.2. Animal Infection

SJL/J breeder mice were purchased from Harlan (Indianapolis, IL) and the mice used in this project were bred in-house. Twenty-eight day old female mice were anesthetized with Isoflurane (Vedco, Saint Joseph, MO) and inoculated intracranially (i.c.) into the right cerebral hemisphere with 20 μl of BeAn strain of TMEV (5 X 10^5 plaque forming units) suspended in media or 20 μl of PBS alone. The SJL/J mice were divided into two groups as follows: 18 received PBS only and 36 received 5x10^5 plaque forming units (pfu) of BeAn virus. The day of i.c. infection was considered day 0. See Figure 20 for experimental design.

4.2.3. IFNT

IFNT was a gift from Dr. Fuller W. Bazer (Texas A&M University, College Station, TX). IFNT was isolated as previously described in Ott et al., 1991. The protein concentration was determined to be 1mg/ml by Lowry assay (Lowry et al., 1951) and 10^8U/mg by virus plaque assay.

4.2.4. IFNT Encapsulation

Alginate capsules were prepared as previously described with some modifications (Abraham et al., 1996; Arenas-Gamboa et al., 2008). Briefly, 1 ml of 10^8 U/mg of IFNT was

mixed with 5 ml of alginate solution (1.5% sodium alginate, 10 mM MOPS, 0.85% NaCl [pH 7.3]). Capsules were obtained by extruding the suspension through a 200 μm nozzle into a 100 mM calcium chloride solution and stirred for 15 min by using an Inotech Encapsulator I-50 (Inotech Biosystems International, Rockville, MD). After extrusion of the IFNT-alginate mixture into the CaCl2, the capsules were washed twice with MOPS for 5 min and further cross-linked with 0.05% poly-L-lysine (molecular weight. 22,000; Sigma-Aldrich, St. Louis, MO) for 10 min. After two successive washes, the beads were stirred in a solution of 0.03% (wt/vol) alginate for 5 min to apply an outer shell and washed twice with MOPS. VpB was added to the shell of the capsule (*vjbR*::Tn*5*/VpB/shell) as a cross-linking agent by the addition of VpB in an equimolar ratio of poly-L-lysine/VpB. After two successive washes, beads were collected, aliquoted (600 μl capsules in 900 μl of MOPS buffer) and stored at 4°C.

Capsules were kept at 4°C in the presence of excess IFNT until time of injection. Before injection, capsules were rinsed 3X in MOPS by resuspending capsules and allowing them to resettle. Finally, capsules were resuspended in the appropriate amount of MOPS buffer prior to injection (900 μl).

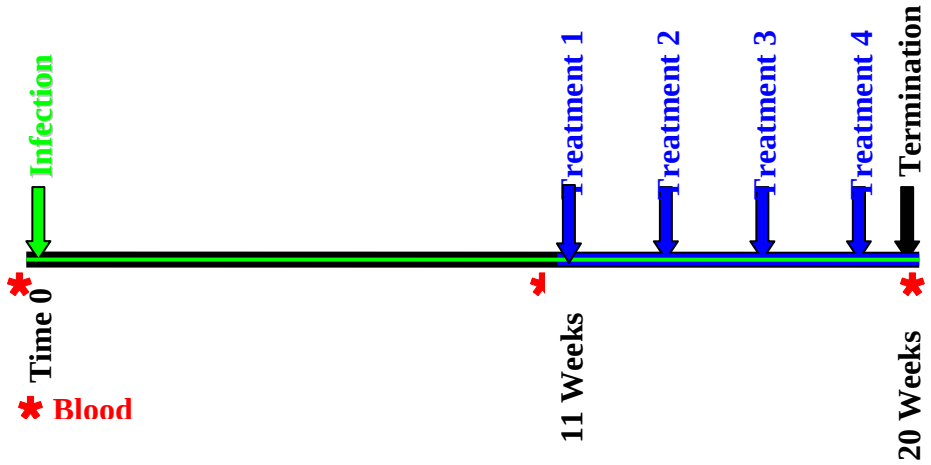


Figure 20. Experimental design: Section IV. The following schematic denotes infection time point (green arrow), treatment administration time points (blue arrows), and blood collection time points (red asterisks). Time points are in weeks post-infection

4.2.5. IFNT Treatment of Mice

Beginning at 11 weeks p.i., a 100μl i.p. injection of encapsulated IFNT (eIFNT) was given every two weeks for a total of eight weeks. A dose of 10^5 units of IFNT (Soos et al., 1995) was released daily from the capsules. Capsules containing MOPS buffer (eMOPS) were used as a vehicle control treatment. The groups were as follows: BeAn infected/eIFNT treated (n=13), BeAn infected/ eMOPS treated (n=13), and PBS mock infected/eIFN treated (n=13).

4.2.6. Clinical Scores

Mice were weighed and evaluated weekly and assigned a clinical score based on disease severity. Mice were graded on overall body condition, posture, balance, front and hind limb spasticity, and activity level. Clinical scores were based on a scale from 0-6, where 0 indicates a healthy animal and 1-6 represent gradually increasing severity of signs as follows: 1:piloerection and/or hunched posture with slight gait abnormalities; 2: piloerection and lowered or hunched posture plus unsteady gait; 3: very unsteady gait, weak grasp response when placed on wire grid, decreased activity level, and occasional slight limb monoparesis; 4: severe hind limb weakness and/or spastic paralysis, weight loss, severe decrease in activity level, and loss of the righting response; 5: paraparesis of hind limbs and forelimbs, severe weight loss, incontinence, and complete loss of righting response; 6: highly moribund/dead (Borrow, et al.,1998). For these observations, animals were examined while walking on a metal grid. The metal grid permitted inspection of an animal's movements from all sides (top, bottom, left and right side) allowing for close scrutiny of possible deficits. An inclined area was used to test an animal's balance and strength while ascending or descending.

4.2.7. Blood Collection

Blood samples were obtained from the tail vein of mice three times during the course of the experiment to determine TMEV-specific antibody titers. The mice were bled prior to infection (baseline), at 11 weeks post-infection, and at necropsy. The blood was collected in 1.5 ml microfuge tubes and placed on ice. Blood was allowed to clot for 24 h at 4°C. The clotted samples were centrifuged at 3000rpm for 10 min. Sera was collected and stored at -20°C until analyzed with ELISA.

4.2.8. Single Cell Suspensions from Spleen, Brain, and Spinal Cord Isolation

Mice were perfused through the left ventricle with heparinized (10 U/ml) Hank's balanced salts solution at pH 7.2. Brains, spinal cords, and spleens were removed and placed in complete RPMI-1640 (1.0% L-glutamine, 1.0% penicillin/streptomycin and 10% FBS) media on ice. Single cell suspensions were prepared from spleens as described previously (Welsh et al., 2004). CNS infiltrating lymphocytes (CNS-ILs) were prepared from brains (Br-ILs) and spinal cords (SC-ILs). CNS tissues were extruded through nylon mesh attached to a 50ml beaker using the plunger of a 5ml syringe. Tissue was pelleted by centrifugation and the pellet was resuspended in 2 mls of 250μg/ml of Collagenase IV (Worthington Inc., Lakewood, NJ) in RPMI-1640 with 2% FBS for 45 minutes at 37°C and 5% CO_2. Tissues were agitated every 15 min to enhance collagenase digestion. Following incubation, the digested tissues were rinsed in complete RPMI media by centrifugation. Lymphocytes were isolated by 35%/70% percoll gradient centrifugation (Irani and Griffin, 1991), resuspended in complete RPMI-1640 to the desired concentration, and placed on ice until needed.

4.2.9. Virus: Enrichment and UV- Inactivation

The GDVII and BeAn strains of TMEV (provided by Dr. H. L. Lipton, University of Illinois at Chicago, IL) were propagated in BHK-21 cells (Welsh et al., 1987).

Virus was enriched by ultracentrifugation pelleting through a 30% sucrose cushion at 80,000 x g for 3 h in an Optima L-80 XP ultracentrifuge (Beckman Coulter) using an SW-28 rotor (Rueckert and Pallansch, 1981). Virus pellets were resuspended in 0.1M sodium phosphate buffer (pH 7.4). Virus was assayed for total protein concentration with Bio-Rad Protein Assay reagent. Viral purity was confirmed by SDS-PAGE (Figure 21). Finally, virus was UV-inactivated by exposing it to UV-light (1330 μW/cm3 at 13 cm distance) for 1.5 h.

4.2.10. Serum Antibodies to TMEV

Serum antibody titers against TMEV were analyzed by ELISA. Briefly, enriched UV-inactivated GDVII virus was used to coat 96-well plates (Costar #3590) at a concentration of 0.25 μg/100 μl/well in carbonate buffer for 24 h at 4°C. Plates were rinsed 3 X with assay buffer and once with RO–H_2O. Plates were blocked by applying 100 μl SuperBlock buffer (#37515; Pierce) followed by immediate removal and replacement of blocking buffer for a total of three times according to manufacturer's instructions. Serum samples were diluted in assay buffer starting at 1/100 and serially diluted to 1/6400. Assay buffer served as the background control. Plates were incubated for 90 min at room temperature. Plates were washed 3X with assay buffer and once with RO–H_2O. HRP-conjugated goat anti-mouse polyvalent immunoglobulin (#A0412; Sigma-Aldrich, St. Louis, MO) was diluted 1/500 in assay buffer and plates incubated for 60 min at room temperature. Plates were rinsed 3X with assay buffer and once with RO-H_2O. The reaction was developed with 100 μl of OPD substrate (#P9187; Sigma-Aldrich, St. Louis, MO) for 10 min according to the manufacturer's instructions. The reaction was quenched with 50 μl

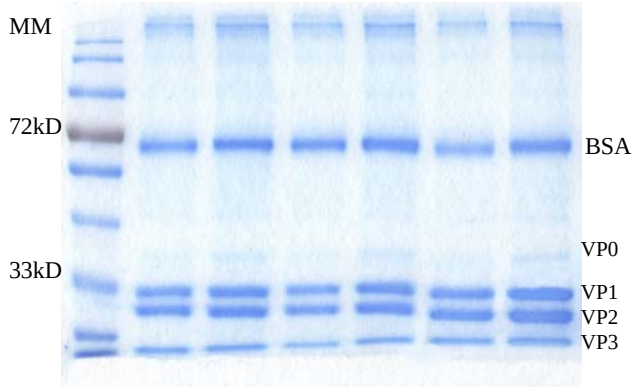


Figure 21. Enriched GDVII visualized by SDS-PAGE. The GDVII strain of TMEV was enriched by ultracentrifugation through a sucrose cushion. The gel image shown above represents three enriched virus samples diluted 1:6 in loading buffer. For each sample, 5 μl and 10 μl aliquots were loaded. The bands are identified as follows on the gel: BSA (69kD), VP0 (35kD), VP1 and VP2 (~32kD) and VP3 (20kD). The combined protein concentration of this virus enrichment was 382 μg/ml of pure virus (510 μg/ml of total protein). The BeAn strain of TMEV was enriched, assayed for protein concentration, and visualized by SDS-PAGE as seen above for the GDVII strain of TMEV. BSA=bovine serum albumin, VP=viral peptide, MM=molecular marker

of 2.0M sulfuric acid. Optical density was read at 490nm using a FLUOstar Optima spectrophotometer (BMG Inc., Offenburg, Germany). Background was subtracted from each column.

4.2.11. ELISpot Assay

Virus-specific antibody producing cells (APCs) were determined using an ELISpot assay. For this assay, 96-well PVDF membrane filtration plates (MAIPS4510; Millipore, Corp, Bedford, MA) were coated with 1.0μg/well of enriched GDVII strain of TMEV virus in 100 μl sterile PBS and incubated overnight at 4°C. The plates were blocked with 200μl RPMI-1640 + 10% FBS for 2 h at room temperature. After blocking, either 2.0 x 10^4 Br-ILs or SC-ILs or 1.0 x 10^6 splenocytes were added to each well. Samples were run in duplicate. The plate was incubated at 37° C and 5.0% CO_2 for 24 h. The plates were washed gently 6X with PBS containing 0.05% Tween-20 to preserve membrane integrity, but remove cells. Plates were rinsed once with water purified by reverse osmosis (RO- H_2O). Biotinylated goat anti-mouse IgG + IgM (#115-065-044, Jackson Laboratories, Bar Harbor, ME) was added at 1:500 as described (Pachner et al., 2007) and incubated for 2 h at room temperature. After incubation, plates were washed 6X with PBS containing 0.05% Tween-20. Then, 100 μl of avidin-HRP (horseradish peroxidase; #18-4100-51; eBioscience) diluted 1/1000 in assay diluent was added to each well and the plates incubated for 30 min at room temperature. After washing 6X with PBS + 0.05% Tween-20, spots were developed using 100 μl of 3-amino-9-ethyl-carbazole (AEC) substrate solution (1.0 mg AEC, 1.0 ml dimethylformamide, 14 ml 0.1M citrate-phosphate buffer pH 5.0, and 10.0 μl and H_2O_2). After development, the plates were rinsed 3X with 200 μl of RO-H_2O, and read with an ELISpot plate reader (AID ELISpot Reader System, Straberg,

Germany). Background was determined as the number of spots generated to RPMI-1640 media and this value was subtracted from each well of virus-specific antibody responses.

4.3. Results

4.3.1. No Differences were Seen in Number of Virus-Specific Antibody Secreting Cells in Spleens of Bean-eIFNT and Bean-eMOPS Mice.

Splenocytes from BeAn-eIFNT and BeAn-eMOPS treated mice were plated on UV-inactivated BeAn-coated membrane filtration plates for 24 h at which time cells were rinsed from the membranes and the plates were developed as ELISPots. The number of ASCs from the spleens (Figure 22) of BeAn-eIFNT mice were slightly lower (15.07±2.74) than the ASCs from the BeAn-eMOPS mice (19.50±2.82), but the difference was not significant. As expected, PBS control mice showed very low numbers of virus-specific ASCs (2.38±0.74).

4.3.2. Virus-Specific Antibody Secreting Cells in the Brains of BeAn-eIFNT Mice.

Br-ILs were isolated and plated on UV-inactivated BeAn-coated membrane filtration plates for 24 hours at which time cells were rinsed from the membranes and the plates were developed as ELISPots. The ELISpot assay quantifies the number of cells specific for the antigen utilized in the system. For these results (Figure 23A), the number of brain-isolated virus-specific ASCs were slightly lower in the BeAn-eIFNT mice (3.29±1.01) when compared to Bean-eMOPS mice (7.75±1.70), but this difference was not statistically significant. The PBS control mice (0.83±0.60) barely had detectable numbers of ASCs isolated from their brain parenchyma.

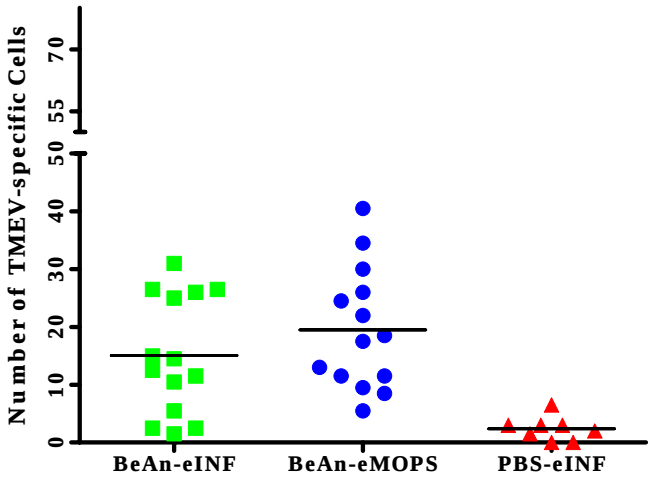


Figure 22. The effect of eIFNT on BeAn-specific antibody secreting cells in the spleen. Splenocytes were cultured for 24 hours in UV-inactivated GDVII coated membrane filtration plates. Cells were rinsed off and the plates were developed as ELISPots and read on an AID ELISpot Reader. The BeAn-eIFNT splenocytes (green squares) produced slightly lower mean numbers of virus-specific antibody producing cells (ASC) compared to BeAn-eMOPS splenocytes (blue circles). The difference was not statistically significant. PBS mice showed very few ASC. Lines represent means of each group. n=14 for BeAn-eIFNT and BeAn-eMOPS groups; n=8 for PBS group.

4.3.3. Virus-Specific Antibody Secreting Cells in the Spinal Cords of BeAn-eIFNT Mice.

SC-ILs were isolated and plated on UV-inactivated BeAn-coated membrane filtration plates for 24 hours at which time cells were rinsed from the membranes and the plates were developed as ELISPots. The ELISpot assay quantifies the number of cells specific for the antigen used in the system. For these results (Figure 23B), spinal cord-isolated virus-specific ASCs revealed slightly higher means in the BeAn-eIFNT mice (8.5±2.4) than from the BeAn-eMOPS mice (3.81±2.03). This difference was not significant. The PBS control mice (2.67±0.73) had numbers of virus-specific ASCs similar to those of BeAn-eMOPS mice.

4.3.4. Virus-Specific Serum Antibody Levels after eIFNT Treatment.

Mice were bled before treatment began (pre-treatment bleed) and at termination (post-treatment bleed). Sera from mice were used to quantify virus-specific antibody levels with a GDVII ELISA. Data are presented (Figure 24) as average OD readings from 12 mice per group whose sera was diluted from 1/100 to 1/6400. Mean pre-treatment OD values for virus-specific serum antibodies from Bean-eIFNT and Bean-eMOPS were similar at 1/200 dilutions (1.20±0.14 versus 1.47±0.13). At post-treatment, the mean OD values at the 1/200 dilution were lower in the Bean-eIFNT mice (0.99±0.22) compared to the BeAn-eMOPS mice (1.74±0.14). None of these differences were significant. As expected, the PBS control mice did not show OD readings above baseline.

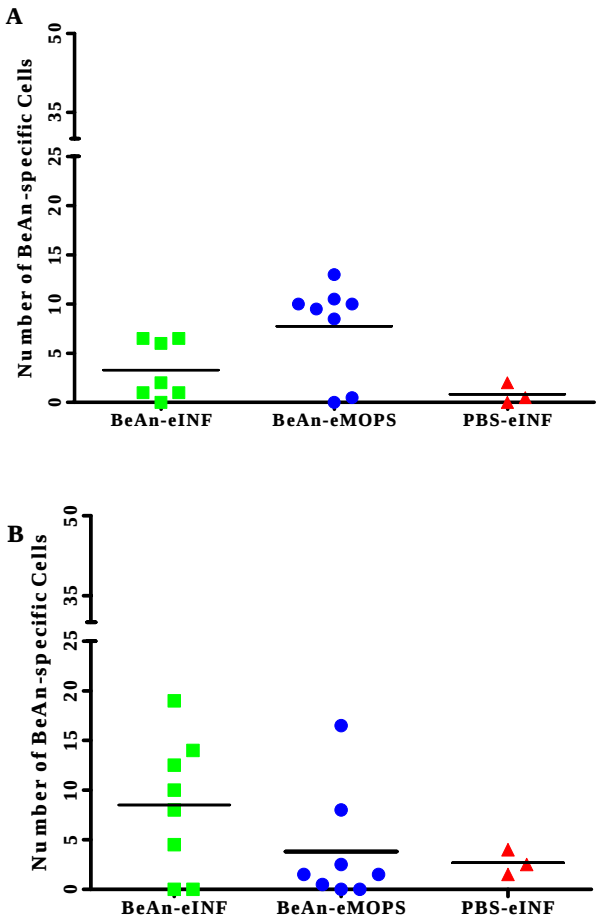


Figure 23. The effect of eIFNT on BeAn-specific ASCs in the CNS. Splenocytes were cultured for 24 h in UV-inactivated GDVII coated membrane filtration plates. Cells were rinsed off and the plates were developed as ELIPots and read on an AID ELISpot Reader. A) The number of virus-specific ASCs in the Br-ILs isolated from TVID mice treated with eIFNT (green squares) were lower than those from eMOPS (blue circles) treated mice. This difference is not statistically significant. The number of brain-derived ASCs in PBS control mice was very low. B) The number of virus-specific ASCs in the SC-ILs isolated from TVID mice treated with eIFNT (green squares) was higher than those from BeAn-eMOPS mice, but the difference was not significant. The number of virus-specific ASCs in the spinal cords of PBS control mice was very low. The lines represent the means of each group. n=8 for the eIFNT and eMOPS groups. n=3 for PBS control mice. Br-ILS=brain infiltrating lymphocytes, SC-ILs=spinal cord infiltrating lymphocytes

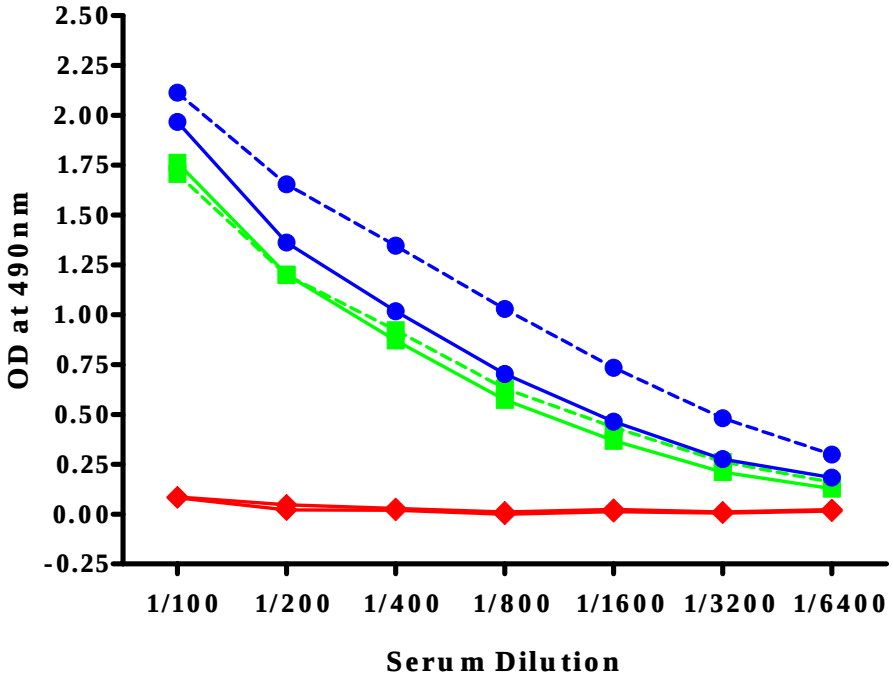


Figure 24. Effect of eIFNT on TMEV-specific serum antibodies. Mice were bled before treatment started (pre-treatment) and upon termination (post-treatment) of the experiment. Serum antibodies were tested using enriched GDVII in an ELISA system. OD readings reflect amount of antibody present to virus within the sera. The mean 1/200 readings are reported in the Results section. Mice treated with Bean-eIFNT (green squares) showed a decrease in mean antibody response to TMEV at the post-treatment time point (green broken line). The Bean-eMOPS mice (blue circles) had higher mean antibody titers at the post-treatment (broken blue line) time point. The differences in serum antibody levels to TMEV between the BeAn-eIFNT and Bean-eMOPS mice at the post-treatment time-point did not reach significance. PBS control mice did not have any titers for antibodies above baseline levels. Each point on the graph represents the mean OD reading for 12 mice within a group at the designated dilution and time-point. n=12 per group OD=optical density

4.4. Discussion

Mice infected with TMEV produce significant levels of virus-specific antibodies and numbers of virus-specific ASCs compared to non-infected mice. eIFNT treatment during TVID resulted in a slight decrease in humoral activity when compared to eMOPS-treated mice, as seen in a decrease in virus-specific ASC numbers within their spleens and a decrease in virus-specific antibodies in their sera. Although this decrease was modest, one must remember that the peripheral compartment is being tested rather than the actual site of disease pathology, which is within the CNS. The data generated from Br-IL and SC-IL isolations were inconclusive. Increased numbers of animals are needed to validate these findings since only six animals were used per group. This procedure is fraught with difficulty since it entails the removal of activated lymphocytes from the CNS parenchyma and a subsequent 24 h incubation period. The majority of the CNS-ILs recovered during the isolation were dead based on trypan blue dye exclusion. Thus, the actual numbers of cells that may have given a positive antibody response is unclear since it is possible that the most robust cells are those that are nearing the end of their life-cycle and these may be too delicate to survive the rigorous isolation techniques necessary for this assay system. Future immunostaining procedures should more clearly define the cell types inhabiting the CNS of the BeAn-eIFNT and BeAn-eMOPS mice and allow for the evaluation of the number of ASCs within the parenchyma, although the antigen-specificity of these cells will be unattainable with this protocol.

The slight decrease in post-treatment virus-specific serum antibody levels was not expected. One disease modifying mechanism of Type I IFN therapy is commonly held to be a shift from a Th1 to a Th2 type immune profile. The results of Section 3 refute this hypothesis as do the antibody data from this section (Figure 22). In fact, neither IL-4 nor IL-5 (data not shown) production was evident in any of the mice tested in Section 3. These results are in conflict with

previously published results (Pachner et al., 2007) indicating a high number of ASCs within the CNS during TVID. It is possible that the decrease in humoral response seen in the current studies reflect a direct down-regulation of B cells by the IFNT treatment rather than a bystander effect of a decrease in a Th1 response. This researcher theorizes that B-cells are being utilized within the current system as APCs and that down-regulation of B-cells results in down-regulation of pathological T-cells with the final outcome being a decrease in demyelinating pathology. To support this claim, researchers (Ghandi et al., 2008) have found B-cell activating factor (BAFF) to be a reliable biological marker for IFNB treatment in the serum of MS patients. The soluble form of BAFF is presumed to act as a down-regulator of B-cell activity. Treatment of MS patients with an antibody to delete B-cells, Rituximab, decreased disease progression by 50% over placebo controls, but total antibody concentration in patient serum did not decrease. (Hauser, 2008). Likewise, the removal of antibodies from MS patient sera by plasmapheresis has minimal benefit. Thus, it is plausible that the action of Type I IFN and Rituximab may be through the down-regulation of the B-cell as an APC. Since studies focusing on the B-cell are in their infancy, there is still much to learn about their place within MS disease pathology and exactly how one might exploit their position to ameliorate disease severity.

In summary, investigations to elucidate the humoral responses of the BeAn-eIFNT-treated mice within this dissertation have been inadequate and further investigation is needed. The quantification of Th1 and Th2 serum cytokine levels will bolster this data and provide more information about the actual cytokine environment that exists within the BeAn-eIFNT mice compared to the BeAn-eMOPS mice during TVID. It is hypothesized that the serum cytokine levels within the BeAn-eIFNT mice will reflect an increase in Th2 cytokines which strengthens the trend of lower viral antibody titers observed in the present study in these mice compared to the BeAn-eMOPS mice. Future investigations require that extra mice be added to the

experimental design for the quantification of viral load (by plaque assay) within the CNS of BeAn-eIFNT mice versus BeAn-eMOPS mice. A time-point series would be best, as mice could be terminated at different time-points post-treatment to quantify viral load within the CNS. It is hypothesized that the decrease in serum antibodies reported in this study reflects a decrease in or the total clearance of the BeAn virus within the CNS of eIFNT-treated mice. The aforementioned investigations should verify the humoral data gathered within this study and may provide correlational data between serum antibody levels and viral load within the CNS of TVID mice during eIFNT treatment.

5. CONCLUSIONS

Results of the current study demonstrate the effectiveness of eIFNT in the treatment of TVID. The efficacy of IFNT has been tested in the context of the EAE animal model of MS, in which animals are primed with myelin antigens to initiate demyelinating disease. However, an evaluation of the therapeutic value of IFNT has not been investigated in a virus-initiating model of demyelinating disease, such as TVID. The fact that IFNT exerts an effect across species and that the toxicity associated with IFNT is far less than that associated with the other type I interferons, prompted investigations directed towards unraveling the value of IFNT as a highly desirable therapeutic agent.

The major finding to emerge from this dissertation is that BeAn-eIFNT-treated mice demonstrated a marked clinical improvement (a decrease of 1-2 points). This is a novel finding amongst previous Type I IFN studies and the best verification of treatment efficacy. The decrease in clinical score (disability) was reflected in a decrease in inflammation and the absence of demyelination (pathology) within the CNS of BeAn-eIFNT-treated animals. The tolerability of the treatment was also informative since an innovative method of delivery (encapsulation) was enlisted for these studies (Arenas-Gamboa et al., 2008). The encapsulation did not hinder IFNT release or usage (as demonstrated by disease attenuation) nor did it cause pathology within any organ systems. Likewise, the constant release of IFNT from the capsules did not raise the level of toxicity within the treated animals as evidenced by the lack of weight loss (a common side effect of toxicity in animals). The treatment did not appear to result in the production of neutralizing antibodies to IFNT either as reflected in the animals improving throughout the 8 week treatment period. Thus, the disease modifying effects of IFNT did not appear to decrease as treatment progressed. This is an invaluable testament to the tolerability of IFNT since many MS patients develop neutralizing antibodies to the IFNB treatment which results in a decrease in

disease modification and the unwanted side effect of painful injection site reactions (Lublin and Reingold, 1996; Mehta et al., 1998) often leading to the cessation of therapy. Recent investigations of the pharmacogenomics of IFNB responses have elucidated which patients are biological responders to IFN therapy before precious time and money are wasted in an attempt to treat a patient that is an IFN non-responder (Van Baarsen et al., 2008). Additionally, *ex vivo* testing of peripheral blood lymphocytes is being evaluated (as was done in this dissertation) in order to predict treatment response to IFNB in MS patients (Wiesemann et al., 2008). Lastly, investigators have linked certain HLA alleles to the predisposition of a patient to produce neutralizing antibodies to IFNB during treatment (Barbosa et al., 2006; Hoffmann et al., 2008) before treatment begins.

Based on the findings of this investigation, caution may be needed when interpreting the data from studies designed to predict a patient's response to Type I IFN treatment. In the current study, animals responded with high and low cytokine secretory responses *in vitro* when stimulated with antigen, but both types of responders manifested clinical improvement when treated with eIFNT *in vivo*. It is not known from this study if the animals that were low secretory responders would have been low responders in a pre-treatment condition or if the low response was a manifestation of the eight week IFNT treatment regimen within this group of animals. Thus, until such a discrepancy in the data is resolved, caution should be used before decisions are made to exclude a patient from a Type I IFN treatment regimen. Since there are so few treatment options available to the MS patient, pre-screening that labels a patient as a non-responder could have huge repercussions for future coverage of such a medication by that patient's health insurance company.

The second objective of this dissertation was to elucidate the mechanism of disease modification during eIFNT treatment. It was hypothesized that a shift in immune profile from a

Th1 to a Th2 response would be evidenced by the production of cytokines in virus-stimulated cultures from eIFNT treated mice. However, an increase in both Th1 and Th2 cytokines was observed. In fact, the two most abundantly produced cytokines in the current studies were TNFA and IFNG. These two cytokines are the hallmarks of pro-inflammatory responses and were not expected. However, it should be noted that these cytokines were produced *in vitro* in response to antigen stimulation by UV-inactivated BeAn virus for 72 h and were not detected in sera or CSF of mice. Additional investigations are currently underway to test the sera from these same mice (via Bio-Plex luminescence kits) to determine if there is a correlation between *in vitro* and *in vivo* produced cytokines. There is evidence in the literature that these two cytokines may have pleiotropic effects and that considering them as only detrimental to demyelinating pathology is short-sighted. Within the EAE model of MS, mice lacking TNF have an increased susceptibility to EAE, whereas the reconstitution of TNF within these mice reduces disease severity (Liu et al., 1998). Additionally, TNF receptor 2 engagement was shown to promote remyelination through the proliferation of OPCs in a toxic model of demyelination (Arnett et al., 2001). Lastly, attempts to neutralize TNF in MS patients significantly enhanced disease severity (The Lenercept Multiple Sclerosis Study Group, 1999). Likewise, it has been shown in SJL/J mice that neutralizing IFNG with antibodies increases demyelination pathology (Rodriguez et al., 1995) and IFNG knock-out mice exhibited increased demyelination and mortality when infected with avirulent strains of TMEV (Fiette et al., 1995). Thus, the tendency to define a cytokine as detrimental or beneficial within a dynamic disease process, such as MS, is often too narrow to accommodate the pleiotropic behavior of many cytokines. Additionally, ex vivo data must be regarded as an indication of physiological conditions within the animal. Data gathered from sera, CSF, or CNS tissues should be enlisted whenever possible as data from these samples have more

merit since they provide information about the cytokine profile during the disease process at the time of data collection without additional experimental manipulation outside of the animal.

Results from the evaluation of humoral responses were surprising. It was hypothesized that there would be an increase in virus-specific antibodies and ASCs. The results refuted this hypothesis. There was a decrease in BeAn-specific antibody production and a very modest decrease in ASCs observed in the current studies. A search of the literature revealed no other studies that could support these conclusions. However, this is the first report of IFNT being used as a treatment of a virus-initiated demyelinating disease. Other studies have tested the disease modifying effects of Type I IFNs within the EAE animal model of MS. Thus, the current study may have elucidated a difference in disease modifying mechanism between these two animal models of MS. Within MS patients, it has been shown that IFNB treatment resulted in a decrease in serum levels of MS-associated retrovirus particles (MSRV). It is proposed that this could be a valuable biological marker for testing Type I IFN biological activity (Mameli et al., 2008). Likewise, MxA, a GTPase involved in anti-viral activity, is expressed after Type I IFN treatment and has been used as a biological marker in MS patients receiving IFNB therapy (Vallittu, 2007). Other anti-viral molecules, such as OAS and RNAse L, could be used to test the biological activity of IFNT within the current system which initiates demyelinating disease with a virus. These investigations could highlight how the response to a Type I IFN may be different within a demyelination model that includes viral infection as the initiator of disease. Such studies might also provide insight about how these anti-viral molecules are expressed during a condition of persistent infection, as is the case of TVID, and the way Type I IFN treatment may affect their patterns of expression.

Altogether the data support the efficacy of the eIFNT treatment in the mice with TVID. Actual mechanisms of disease attenuation remain elusive at this time as mice exhibited a mixed

increase of Th1 and Th2 cytokines rather than the hypothesized shift from a Th1 to a Th2 immune profile. Likewise, mice exhibited a modest decrease in virus-specific antibodies as well as the number virus-specific ASCs which also refute the hypothesized increase in these values due to an increase in Th2 immunity. A remarkable finding was the fact that immune cells derived from BeAn-eIFNT-treated mice appeared to be divided into two distinct types of biological responders although all of the mice responded to the *in vivo* treatment with eIFNT with a decrease in disease severity. It is hypothesized that this difference is a reflection of individual genetic variability in response to immune modulation which is surprising as mice used for these studies are in-bred and considered to be identical genetically. Obviously, immune modulation can proceed through different mechanisms and still provide the desired result of a decrease in disease progression and/or severity. However, this reality creates an added level of difficulty when one is trying to interpret biological data in order to determine whether a therapeutic regimen is efficacious within a patient population.

6. FUTURE DIRECTIONS

The bimodal secretion pattern of cytokines produced by splenocytes from BeAn-eIFNT treated mice warrants further investigation. Three possible hypotheses are proposed: a) the high/low secretion from the splenocytes may reflect an animal's phase of disease process when sampled for cytokine secretion, b) the high/low patterns of secretion may reflect variability upon chronic IFN receptor engagement, or c) the high/low patterns of secretion may be the result of different signaling pathways converging.

The first possibility for bimodal secretion may reflect phase of the disease process. During immune modulation in the BeAn-TVID mice, some mice may resolve their inflammatory lesions within the CNS quicker than others within the same group. This could be reflected as an overall decrease in cell responsiveness during the *ex vivo* experimental conditions used within this study to evaluate cytokine secretion patterns and levels upon exposure to BeAn antigen. Thus, the low secretors may reflect animals that have achieved disease resolution and whose splenocytes are no longer responsive to antigen stimulation, whereas the higher secretors reflect animals that are still clearing the virus and resolving inflammation within the CNS and are still responsive to antigen stimulation. At this time, this possibility seems unlikely since the clinical score data from 30 individual mice did not reflect a difference in recovery time as noted by the small SEMs among the data pool. However, future investigations will track the improvement of each individual mouse more closely to rule out this possibility.

The second possibility for bimodal secretion may reflect the genetic variation within the individual mice to respond to constant IFN receptor engagement. It is possible that the constant presence of IFNT within the present system could lead to differences in response from the individual mice. Low cytokine secretors may reflect habituation of the IFN receptor and a lack of

response reflects a down-regulation of the IFN receptor or its signaling mechanisms. The habituation may occur in down-stream components of the signaling pathway and may actually be induced by inhibitors of signaling such as SOCS (suppressors of cytokine signaling) molecules. Flow cytometric analysis of receptor numbers and signaling components of the splenocytes of low and high responders could answer such questions.

Lastly, the majority of cytokines up-regulated in this study use the JAK-STAT pathway. Thus, the constant presence of IFNT may affect the signaling of other cytokines in a positive or negative way depending on the individual mouse variation. This possibility could also be resolved with flow cytometric analysis of the various STATs being used in high and low cytokine secretors. Microarray analysis or high throughput gene sequencing may also prove beneficial in sorting out the broad range of genetic influence on cytokine signaling pathways during constant Type I IFN stimulation.

Overall, the cytokine findings warrant further investigations that: a) utilize purified cell cultures to better identify which cell type is producing each cytokine in response to antigen stimulation; b) examine intracellular signaling pathways to establish a difference between the high and low responses in cytokine production observed in these studies; and c) employ molecular techniques, such as microarray technology or high throughput gene sequencing, to pinpoint genomic differences in relation to biological response.

Future investigations should include the use of plaque assays to define levels of BeAn activity in mice treated with eIFNT during TVID. Additionally, mice should be evaluated at pre-treatment time-points for BeAn load as well as lesion load to assess these two parameters prior to treatment, setting a baseline for how far the disease has progressed before treatment begins. This data would demonstrate a direct effect of systemic IFNT treatment on CNS disease modification during TVID.

Lastly, future investigations will include studies designed to test the effect of eIFNT on CNS demyelinating pathology at earlier time-points in order to evaluate the mechanism of lesion resolution. The current study “missed” this aspect of eIFNT treatment because resolution of the lesions was not hypothesized. Future studies will evaluate the CNS of BeAn-eIFNT mice at 2-4 weeks post-treatment in order to catch a glimpse of the mechanism of lesion resolution enlisted during IFNT treatment. Tissues will be collected and evaluated with electron microscopy and immunohistochemistry to characterize cell type, myelin integrity, and oligodendrocyte maturation. Current therapies for MS slow disease progression and lengthen the amount of time it takes to reach the secondary progressive stage of the disease. A therapy that halts disease progression, restores function, and resolves demyelinating lesions is what is needed. In this study, IFNT provided those benefits to animals during TVID.

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