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To the Graduate Council:

I am submitting herewith a thesis written by Christopher Dean Pack entitled "Regulation of autoreactive T cell responses by neonatal exposure to a self-peptide Ig-chimera." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Habib Zaghouani, Major Professor

We have read this thesis and recommend its acceptance:

Barry T. Rouse, Robert N. Moore

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

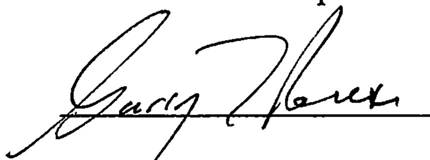
(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis by Christopher D. Pack entitled "Regulation of Autoreactive T Cell Responses By Neonatal Exposure to a Self-peptide Ig-chimera." I have examined the final copy of the thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Habib Zaghouani, Major Professor

We have read this thesis and recommend its acceptance:



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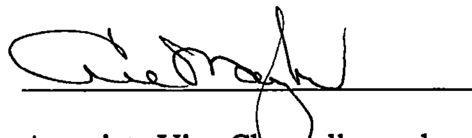

Associate Vice Chancellor and Dean of the Graduate School

REGULATION OF AUTOREACTIVE T CELL RESPONSES BY NEONATAL EXPOSURE TO A SELF-PEPTIDE Ig-CHIMERA

A Thesis Presented for the
Master of Science Degree

The University of Tennessee, Knoxville

Christopher Dean Pack
August 2000

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DEDICATION

This thesis is dedicated to the individuals who have supported me in every way imaginable throughout my life. They have given me the strength, courage, and confidence to achieve my dreams, whatever they might be. I will never be able to express enough gratitude and love to my father, Larry Pack, my mother, Susan Pack, and my sister, Kimberly Pack. Thanks for always believing in me.

ACKNOWLEDGMENTS

I would like to give special thanks to the members of my committee, Habib Zaghouani, Barry T. Rouse, and Robert N. Moore, for their invaluable time, advice, and patience over the years. I would also like to extend a sincere expression of gratitude to my fellow lab colleagues for their helpful assistance and effort: Booki Min, Kevin Legge, Aimee Cestra, Jacque Caprio, Randy Gregg, Jeremiah Bell, and Lequn Li. In addition, I will always be grateful for the undying support of those closest to me, my family and close friends. Lastly, I will never be able to express enough gratitude or love to one special person, Heather Johnson, who has patiently stood by me through these trying years.

ABSTRACT

Prior investigations from this laboratory reported that exposure of the neonatal immune system to an immunoglobulin (Ig) chimera carrying a proteolipid protein (PLP) peptide drives the adult response to a challenge with the PLP peptide towards T cell deviation in the lymph node and an IFN-γ-dependent T cell anergy in the spleen. In addition, such exposure protects against experimental allergic encephalomyelitis (EAE). Herein, we wished to determine whether such a biased response is intrinsic to the PLP peptide or emanates from the delivery by Ig, thus allowing a similar outcome to ensue with other myelin peptides. Accordingly, the amino acid sequence 87-99 of myelin basic protein (MBP) was expressed on the same Ig backbone, and the resulting Ig-MBP chimera was tested for induction of neonatal immunity and protection against EAE involving diverse T cell specificities. The results revealed Th2 deviated T cell responses in both lymphoid organs, and the animals resisted induction of EAE. More striking, the splenic T cells produced IL-10 in addition to IL-4, providing an environment that facilitated bystander deviation of responses to unrelated encephalitogenic determinants and promoting protection against autoimmunity involving diverse T cell specificities. Thus, exposure of the neonatal immune system to antigenic peptides in the context of a self-Ig molecule fosters the development of a broad-spectrum protective immunity emanating from organ specific regulatory functions.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
APC	antigen presenting cell
APL	altered peptide ligand
BSA	bovine serum albumin
C region	constant region of Ig
CD	cluster of differentiation
CDR3	complimentarity determining region 3
CFA	complete Freund's adjuvant
CNS	central nervous system
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CTLA-4	cytolytic T lymphocyte-associated antigen-4
D region	diversity region of Ig
DMEM	Dulbecco's modified Eagle's medium
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ER	endoplasmic reticulum
FCS	fetal calf serum
gpt	guanine phosphoribosyl transferase

HLA	human histocompatibility leukocyte antigens
ICAM	intracellular adhesion molecule
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
J region	joining region of Ig
L chain	light chain
LN	lymph node
LFA-1	leukocyte function-associated antigen-1
mAb	monoclonal antibody
MBP	myelin basic protein
MAG	myelin associated glycoprotein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte protein
MS	multiple sclerosis
OD	optical density
p	probability
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PLP	proteolipid protein
Sal	saline
s.c.	subcutaneous
SD	standard deviation
SFU	spot-forming units
SP	spleen
Sln	saline
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
Tol	tolerized
TNF	tumor necrosis factor
V region	variable region of Ig
W	wild-type

Part I: General Introduction and Overview

Chapter 1. T Cell Development, Activation, and the Concept of Tolerance

The immune system has acquired the remarkable ability to recognize and respond to a myriad of antigens, primarily through the receptors of specific cells in the blood known as T and B lymphocytes. Antibodies, the blood proteins that recognize and bind foreign antigens, were the first of the recognition structures of the immune system to be described. Antibodies, which are proteins designated as immunoglobulins (Ig), also act as receptors on the lymphocytes that produce them, the B cell. The B cell expresses a specialized antibody that contains a membrane-spanning domain, allowing it to function as a receptor. When this receptor interacts with the antigen it is specific for (each B or T cell expresses only one receptor at a given point in time, a concept known as the clonal selection theory) the B cell secretes a soluble form of its receptor, lacking the membrane-spanning domain. B cells arise from precursor cells that are found in the bone marrow. Once a mature B cell encounters its specific antigen it becomes activated, proliferates, and proceeds to develop into a plasma cell capable of secreting large amounts of soluble Ig (1,2).

T lymphocytes, the primary focus of this study, do not produce antibodies. They, however, are responsible for mediating the various effector functions of the immune system. T cells exist primarily as two distinctive subsets, the cytolytic CD8+ T cells and the helper CD4+ T cells. Cytolytic T cells, or CTL, are responsible for recognizing and killing cells of the body that have been infected by viruses or intracellular bacteria, as

well as tumor cells. Helper T cells secrete soluble factors, such as cytokines, that instruct the appropriate direction of an immune response. These cytokines may preferentially activate B cells or cytolytic T cells, depending on their nature. In contrast to B cells, the T cell receptor, or TCR, cannot bind to or recognize the antigen that they are specific for in a soluble form. Instead, they must see the antigen in a processed form of approximately 8-10 amino acids that is presented in the context of a major histocompatibility complex molecule (MHC) on the surface of an antigen presenting cell (APC) (3). Antigens may gain access to MHC molecules through one of two major pathways. In one pathway, the exogenous or endocytic pathway, antigens are acquired for presentation from outside of the APC. The APC may internalize such an antigen by phagocytosis, pinocytosis, or receptor-mediated internalization. The antigen is then digested in the endosome, generating peptides that are loaded onto MHC class II molecules, transported to the cell surface, and recognized by CD4 T cells (4). Alternatively, an antigen may be synthesized within the APC, as is the case in viral infections, intracellular bacterial infections, and tumor cells. In this instance the antigen is processed in the cytosol by the proteosome, transported into the endoplasmic reticulum (ER) by a peptide transporter in the ER membrane known as TAP (transporter associated with antigen processing), and loaded onto waiting MHC class I molecules The MHC/peptide complex is then transported to the cell membrane and recognized by CD8 cytolytic T cells. This pathway is known as the endogenous pathway (5). This study is primarily concerned with the exogenous pathway of antigen presentation.

As previously described, T cells recognize a peptide in the context of an MHC molecule bound to the surface of an APC. This interaction, known as signal one, generates a signal transduction cascade in the T cell that may ultimately lead to activation of the cell. However, this signal alone is not sufficient to activate a T cell. Another cell to cell interaction, referred to as signal 2, must also occur. This interaction is also known as costimulation. Several costimulatory pairs have been extensively characterized. Some examples on T cells include CD28/CTLA-4, LFA-1, LFA-2, and CD40L. The corresponding factors on the APC include B7-1/B7-2, ICAM-1, LFA-3, and CD40, respectively. If a T cell receives signal 1 in the absence of signal 2 the T cell may enter into a non-responsive state known as anergy (6,7). This regulation of the T cell activation requirement through costimulation is a major component of peripheral tolerance, thus ensuring that T cells are only activated at the appropriate time and place in the body (8).

The immune system, through eons of development, has developed ways to recognize virtually any foreign antigen through the interaction of the TCR and MHC/peptide complex. In addition, the immune system is also capable of discriminating between foreign, potentially harmful antigenic stimuli and harmless self-antigens, a concept referred to as self-tolerance. The ability of the T cell repertoire to achieve this feat is centered in the maturation process of the T cell. Lymphoid progenitors, which arise from stem cells in the bone marrow, migrate to the thymus in order to begin the maturation process. In the thymus the repertoire of the T cell population is expanded by a variety of mechanisms including rearrangement of the TCR receptor genes and N-region diversification. Such diversification of the population ensures that at least one TCR in the

repertoire should be able to recognize any given peptide (foreign or self) bound to any MHC allele. This process is random, and often nonfunctional or even self-reactive receptors are generated. Therefore, it is necessary for this population of immature T cells to be purged of cells that are either unable to recognize any antigen or are able to recognize self-antigens. First, T cells must demonstrate that they can recognize antigens on the particular MHC allele of that is expressed in the thymus and throughout the rest of the body, a process referred to as positive selection. T cells unable to recognize an antigen complexed to the specific MHC die by neglect. The remaining cells proceed to the next step of maturation, negative selection. It is during this process that T cells expressing a TCR that strongly binds self-antigen are deleted from the repertoire. Therefore, it is assumed that affinity of the TCR for self-peptide-MHC complexes determines the overall fate of a developing T cell. More specifically, a T cell whose TCR possesses a low, but sufficient, affinity for a self-peptide is allowed to pass positive selection. T cells that possess a TCR with a high affinity for a self-peptide also pass positive selection, but are eliminated during negative selection. Only the T cell with the low affinity for a self-peptide is allowed to completely mature, exit the thymus, and patrol the periphery (9-11). This process, although quite efficient, is not perfect. Some autoreactive T cells manage to exit the thymus, and autoreactive T cells have been detected in the periphery of healthy individuals. These cells, however, rarely pose a threat due to additional regulatory mechanisms present in the periphery.

Chapter 2. Neonatal Tolerance

The insightful and pioneering work of Medawar in the 1950s laid the foundation of what is now referred to as neonatal tolerance. He demonstrated that injection of allogeneic spleen cells into newborn mice resulted in the generation of tolerance to these cells, thus allowing these tolerized mice to accept skin grafts later in life from the same allogeneic donor (12). The neonatal period soon became recognized as a potential window during immune system development that could be exploited in efforts to generate tolerance to virtually any antigen. Neonatal tolerance was soon induced with a variety of soluble antigens, including peptides, which yielded similar results. This observed lack of an immune response to the antigen was initially believed to be mediated by deletion or inactivation of the targeted T cell (13,14). However, continued investigations revealed that a response was being induced to the antigen of interest (specific reactivity was detected against the allogeneic skin graft in mice that had received matching allogeneic spleen cells as a neonate). Further analysis unveiled that T cells responding to the antigen displayed a Th2 profile of cytokine production (14-19). Th2 cytokines (IL-4, IL-5, IL-10, IL-13) are mediators of non-inflammatory immune responses, such as is the case in the clearance of extracellular bactena, parasites, and worms. These cytokines do not provide the support necessary for the required Th1 response (IL-2, IFN-γ, TNF-α) necessary for a skin graft rejection. Such a response would require pro-inflammatory cytokines to activate the CTL necessary for a response involving tissue rejection, tumor killing, or lysis of a viral or bacterial infected cell (20). These findings suggested that neonatal

tolerance could prime an immune response, not just delete or inactivate the T cells of interest. Additional studies involving tolerance generated against peptides revealed that T cells in the spleen displayed this deviated phenotype (21,22). In addition, a host of other groups have demonstrated that the outcome as well as the mechanism of neonatal tolerance is dependent on the dose of antigen (23), the form of antigen (24), the adjuvant given with the antigen (22), the APC presenting the antigen (18), and the in vivo availability of the antigen (25). The most useful and intriguing aspect of this form of tolerance is the ability to generate a Th2 response as an adult against an antigen that would normally elicit a Th1 response. Since most autoreactive T cells display a Th1 profile, their specific deviation during the neonatal stage may prove beneficial in an effort to vaccinate against a host of autoimmune diseases mediated by tissue specific Th1 T cells.

Chapter 3. Autoimmune Disorders

Autoimmunity results when an individual's immune system reacts against autologous (self) antigens, ultimately leading to tissue injury or destruction. As previously described, these autoreactive T cell clones should have been deleted during negative selection, but somehow small numbers of such clones escape this process and circulate in the periphery. Such potentially dangerous autoreactive clones T cells generally pose no threat, provided that they do not become activated. A number of mechanisms have been proposed for the activation of these cells, all of which, in the end, lead to the breakdown of self-tolerance. Contributing factors include abnormalities of APCs or lymphocytes, genes that predispose to autoimmunity, microbial infections, and tissue injury revealing antigens previously sequestered from the immune system. A major challenge in delineating the mechanism of many human autoimmune diseases lies in the inability to define the distinct antigens responsible for the initiation of such responses.

A variety of autoimmune diseases have been described and extensively studied in humans. Some result from the B cell production of self-reactive antibodies, such as in systemic lupus erythematosus (SLE) and myasthenia gravis (MG). In SLE the cardinal sign of disease is the production of anti-nuclear antibodies (26), while in myasthenia gravis the antibody response is directed against the acetylcholine receptor (27). However, the majority of human autoimmune disorders stem from inflammatory T cell reactions to self-antigens. Examples include multiple sclerosis (MS) (28), insulin-dependent diabetes mellitus (IDDM) (29), and rheumatoid arthritis (RA) (30). These disorders involve T

cells specific for epitopes located in the central nervous system, the pancreas, and the joints, respectively. Animal models have been developed to mimic the pathology and symptoms of each of these maladies, allowing scientists to extensively investigate the multiple factors associated with the induction and progression of each.

Several factors, both genetic and environmental, have been associated with autoimmune disease development. It appears that one of the primary genetic predispositions for an autoimmune disease is the MHC haplotype of the individual (31-33). Since the MHC haplotype determines what set of self-peptides is presented to the T cell population, some haplotypes are associated with resistance, while others render susceptibility. Such varying degrees of susceptibility may also be linked to alterations in thymic selection. More specifically, susceptible MHC alleles may poorly bind the peptide required for negative selection of the autoreactive T cell, allowing it to escape the thymus. The most critical environmental factor to be examined is a prior microbial infection. If a particular virus or bacteria happen to express proteins that possess significant sequence homology to certain self-peptides, an autoreactive T cell might become activated. This sequence of events has been coined molecular mimicry, and several examples of viruses and bacteria with sequence homology to self-antigens have been proposed for MS, IDDM, and RA (34-36). In addition, these infections were able to override peripheral tolerance mechanisms and activate such autoreactive clones. For instance, viral derived peptides from human papilloma virus have been shown to activate myelin-specific T cells and induce experimental allergic encephalomyelitis (EAE) (37), a mouse model of human MS that will be discussed in greater detail later. Unfortunately,

the initiating microbial infection in humans is often completely cleared weeks or even months before any signs of an autoimmune disease become evident, making it rather difficult to link a specific pathogen to an ensuing autoreactive response.

Current medical treatments for most autoimmune diseases include the administration of anti-inflammatory drugs such as corticosteroids. Antagonists of proinflammatory cytokines (such as IL-1, IFN-β, IL-4, and IL-10) have also been employed in efforts to treat such diseases as MS (38). Other recently approved human therapies include the administration of antagonists against costimulators such as the B7 family (39) and CD40 (40). Also, activation of negative regulators, such as CTLA-4 (41) have been explored. Although effective, these non-specific approaches possess limitations, since the host may not be able to mount effective immune responses to a microbial infection. Efforts to induce tolerance include the oral administration of self-antigens (oral tolerance) and treatment involving altered forms of the self-peptide, known as altered peptide ligands (APLs). APLs possess a permutation in the TCR contact residues only, resulting in a partial signal for activation in the T cell. This incomplete signal may result in anergy or a deviated cytokine profile in the affected T cell (42).

In all, a variety of experimental approaches have been undertaken in efforts to unveil the pathogenic mechanisms, as well as to devise novel approaches to prevent or treat autoimmune disorders. Nevertheless, the initiators and the exact list of autoreactive epitopes involved in each disorder have yet to be unveiled. Once this information is verified, specific treatments will be able to be designed to either prevent the emergence

of the disease or effectively threat the condition in an antigen specific manner, allowing the rest of the immune system to function properly.

Chapter 4. Multiple Sclerosis and Experimental Allergic Encephalomyelitis

Multiple sclerosis is the most common neurological disorder affecting the central nervous system. Although the disease course is typically variable, the majority of affected individuals suffer from chronic or relapsing paralysis, impairments in vision and other sensory functions, as well as various other neurologic disfunctions. Pathologically, the disease acquired its name from the presence of multiple sclerotic lesions or plaques in the white matter. Such lesions result from the loss of myelin, which, in turn, affects the ability of neurons to transmit electrical impulses. Without the insulating layer of myelin covering the axon, saltatory signals are often too weak to be transmitted. Such a condition typically results in paralysis. It is now widely agreed that such demyelination is initiated by autoreactive CD4+ T cells of the Th1 phenotype that recognize certain epitopes present in the myelin sheath. The specific proteins that have been identified as targets include: myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), and myelin oligodendrocyte protein (MOG) (28,43).

Experimental allergic encephalomyelitis (EAE) evolved from studies of post vaccinal encephalomyelitis. It was noted at the turn of the century that some individuals that had received rabies vaccination with inactivated virus grown in rabbit spinal cord developed an acute neurological disease unrelated to rabies (43). Decades later it was demonstrated that if monkeys were injected with normal spinal cord homogenate they developed a disease manifest by acute demyelination. The pathological symptoms

(inflammatory lesions and demyelinating foci) resembled what had been previously observed in MS. This induced condition was then named experimental allergic encephalomyelitis (43). Extension of the disease to susceptible strains of rats and mice induced a relapsing/remitting disease with similar pathologic conditions to MS (44). Soon after, EAE was regarded as an appropriate animal model for the study of human MS. EAE can be induced by active immunization with myelin proteins or peptides in susceptible strains of mice (linked to MHC background), provided that *B. pertussis* bacteria or toxin are administered along with the immunization to break down the blood-brain barrier. In addition, adoptive transfer of myelin antigen-specific CD4+ T cells of the Th1 phenotype into naïve animals has been clearly demonstrated to induce EAE (44). As previously mentioned, EAE possesses a relapsing/remitting disease pattern similar to MS. This disease course is now believed to be the result of the sequential spreading of the immune response to different myelin epitopes, a process called epitope spreading (45). As the immune system regulates the response to the previous epitope (remission period), a new epitope from myelin has been exposed due to prior tissue damage in the CNS. The T cell response against the new epitope is then responsible for the subsequent relapse.

Modulation of the cytokine environment associated with EAE is the most widely studied and exploited avenue for possible treatment regimens. Since EAE appears to be induced by Th1 CD4+ T cells (secreting IL-2, IFN-γ, and TNF-α) most groups have focused on ways to either down-regulate this response or deviate the response to a non-inflammatory Th2 type response (secreting IL-4, IL-5, IL-10). Methods for down-regulation have included antibodies against IL-12 (a cytokine secreted by APCs critical

for Th1 T cell differentiation) (46) and TNF-α (47). Antibodies have also been used to specifically target TCRs known to use certain Vβ genes involved in responses to MBP (48). Other non-specific approaches include the systemic injection of recombinant anti-inflammatory cytokines (IL-4, IL-10, TGF-β, IFN-β) and the interference of certain costimulatory interactions between the APC and the T cell (such as B7 family/CD28 and CD40/CD40L) (48,49). Investigators have also devised a multitude of ways to deviate this Th1 response. A popular one is the use of APLs, altered forms of myelin derived peptides that cause Th1 T cells to become anergic after engagement or to become deviated in their cytokine profile (50-52). In addition, other groups have genetically modified T cells specific for myelin epitopes to secrete IL-4 or IL-10, thus delivering the cytokine to the specific location that it is needed (53,54). Oral administration of myelin antigens (oral tolerance), which leads to the generation of T cells producing TGF-β and IL-10, also appears promising (55). Moreover, neonatal tolerance has also been shown to induce prophylactic protection, generating a Th2 response to myelin antigens prior to disease induction (19). Therefore, one can assume that future treatment regimens for multiple sclerosis will most likely arise from experiments carried out in the EAE model system. It is also highly probable that new approaches will somehow involve modulation of the local cytokine environment in an antigen-specific manner.

Chapter 5. Objectives and Rational

Previous studies have demonstrated that immunoglobulins can be engineered as vehicles for the enhanced presentation of peptide antigens. Igs have been used for the presentation of both helper T cell and cytolytic T cell epitopes (56,57). Igs are autologous molecules, globular in nature (conferring resistance to degradation), and permissive for the insertion of foreign peptide sequences within certain hypervariable regions of the molecule (specifically the CDR3, or complementary determining region 3). Numerous studies have demonstrated that epitopes encoded within such Igs are presented to T cells 10-100 fold more efficiently than peptide alone. This increased efficiency has been attributed to the relative stability of half-life of the Ig in comparison to a peptide (weeks versus hours), as well as the ability of Igs to be internalized into APCs via the interaction of their Fc domain and the Fc receptor of the APC. This internalization greatly increases the efficiency of antigen presentation and grants the newly liberated peptide derived from the Ig access to newly formed class II molecules. In contrast, exogenous peptides must displace a self-peptide resting in the groove of an MHC class II molecule already on the surface of the APC. Peptide presentation, therefore, is also subject to physiologic turnover of MHC class II molecules, lowering efficiency even more. In addition, binding of Fc receptors has been shown to induce cytokine production of APCs, a process that may mimic the effects of adjuvant. The emulsification of the peptide in mineral oil either containing *Mycobacterium tuberculosis* (complete Freund's adjuvant or CFA) or not (incomplete Freund's adjuvant or IFA) is typically required to generate efficient

responses to peptides. Therefore, not having to utilize such a harsh, potentially dangerous substance would prove of great significance for human vaccine development (58).

Recent studies by Min *et al* (59) applied the use of such engineered Igs in an effort to induce neonatal tolerance against an immunodominant epitope of myelin, the proteolipid protein sequence encoding amino acids 139-151, or PLP1. This study revealed that when such an Ig-chimera (Ig-PLP1) was injected into neonates on the day of birth, the response as an adult to PLP1 peptide was deviated in the lymph node to IL-4 producing cells, while the spleen response was characterized by a novel form of anergy dependent on IFN-γ. The fundamental objectives of the study depicted in this thesis were threefold in perspective. First, we wished to determine whether this form of neonatal tolerance could be extended to other myelin epitopes in the context of an Ig (such as MBP 87-99, or MBP3), or whether this phenomenon was a characteristic solely dependent on the Ig-PLP1 chimera. Secondly, we wished to determine whether this form of "neonatal tolerance" might be capable of exerting a bystander suppressive effect on the response against a separate myelin antigen (such as the PLP 178-191 epitope, or PLP2) when introduced simultaneously with the MBP3 epitope for response and disease induction. Thirdly, we wished to further investigate this anergic state of the T cells in the spleen, possibly offering an explanation for their unresponsive condition. Analysis of these three objectives should shed light upon the mechanism responsible for this tolerance and its relative usefulness as a vaccine strategy to prevent the process of epitope spreading and subsequent relapses.

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Part II: Neonatal Exposure to an Ig-Chimera Carrying a Myelin Basic Protein Peptide Induces Differential T Cell Deviation in the Lymph Node Versus the Spleen and Confers Resistance to Experimental Allergic Encephalomyelitis Involving Diverse T Cell Specificities

Chapter 1. Introduction

Medawar and colleagues demonstrated almost half a century ago that rodents injected at birth with splenocytes from a genetically different donor were able to accept transplants from that donor as an adult (1). These landmark experiments suggested that neonatal T cells were somehow susceptible to the induction of tolerance. Ever since, the neonatal period has been viewed as a window during which the introduction of antigen leads to tolerance. Initially, inactivation and/or deletion of T cells were considered the leading mechanisms for antigen induced neonatal tolerance (2,3). However, recent investigations have demonstrated that neonatal exposure to antigen can prime rather than inactivate or delete T cells (4-7), and both Th1 and Th2 cells develop upon this initial antigen encounter. Surprisingly, challenge with the same antigen later in life elicits predominant Th2 response (8-12). Since this type of biased response does not support inflammatory reactions, neonatal tolerization with antigen has been applied to deviation of specific naïve T cells and has proven effective in the prevention of autoimmune diseases such as experimental allergic encephalomyelitis (EAE) (13) and type I diabetes (14). Although the exact mechanism for the apparent selective maintenance of the Th2 cells is unknown, it has been suggested that primary Th1 cells that arise upon neonatal exposure to antigen are more vulnerable to apoptosis (15). Recent investigations have even envisioned the involvement of regulatory T cells to maintain such biased neonatal immunity (16). Whatever the mechanism might be, factors such as the dose of antigen (17), the form of the antigen (18), the adjuvant administered in conjunction with the

antigen (12), the APC presenting the antigen (5), as well as the in vivo availability of the antigen (19) were shown to control the induction of neonatal tolerance.

Previous investigations using immunoglobulins (Igs) as a vehicle for peptide delivery revealed yet another bias in neonatal induced immunity (20-22). Indeed, Ig-PLP1 (23,24) a chimera encompassing the proteolipid protein (PLP) 139-151 sequence, or PLP1, given to mice on the day of birth induced an organ specific regulation of T cells involving a deviation in the lymph node and a novel form of anergy in the spleen (20-22). Specifically, mice given Ig-PLP1 on the day of birth and challenged with PLP1 peptide at 7 weeks of age developed PLP1-specific T cells in the lymph node that produced IL-4 instead of IL-2. In the spleen, the cells, although non-proliferative and unable to produce IFN-γ, secreted significant amounts of IL-2. Furthermore, when supplied with IL-12 or IFN-γ these cells regained proliferative and IFN-γ responsiveness. The study presented herein explores if this new form of tolerance is intrinsic to the PLP1 epitope or whether it is applicable to other epitopes, such as the myelin basic protein (MBP) 87-99 sequence, or MBP3 (25), incorporated into the same Ig vehicle. The results show that mice given Ig-MBP3 in saline on the day of birth and challenged with MBP3 in CFA at the age of 7 weeks developed deviated T cells producing IL-4 in both the spleen and the lymph node. This environment was also able to deviate unrelated T cells induced with a PLP derived peptide. Furthermore, the splenic, but not the lymph node T cells, produced elevated levels of IL-10 when subjected to prolonged (72 hours) peptide stimulation. IL-10, a well-characterized inhibitory paracrine cytokine, contributed to

bystander suppression of diverse T cell specificities and prevented the induction of EAE involving a mixture of epitopes.

Chapter 2. Materials and Methods

Mice

SJL/J (H-2^s) mice were purchased from Harlan-Sprague-Dawley (Frederick, MD) and maintained in our animal facility for the duration of experiments. For the generation of newborn mice, breeding sets of one adult male and three females were caged together. When pregnancy was visible the females were separated and caged individually. Offspring were weaned when they reached 3 weeks of age. All experimental procedures were carried out according to the guidelines of the institutional animal care committee.

Antigens

Peptides. All peptides used in this study were purchased from Research Genetics, Inc. (Huntsville, AL) and purified by HPLC to >90% purity. MBP3 peptide (VHFFKNIVTPRTP) encompasses an encephalitogenic epitope corresponding to amino acid residues 87-99 of MBP (25). PLP2 peptide (NTWTTCQSIAFPSK) encompasses an encephalitogenic sequence corresponding to amino acid residues 178-191 of PLP (26). PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to amino acid residues 139-151 of PLP (27). All three peptides are presented to T cells in association with I-A^s MHC class II molecules and induce EAE in SJL/J mice.

Ig-Chimeras. Ig-MBP3 is a chimera expressing MBP3 peptide, which corresponds to amino acid residues 87-99 of MBP. Construction of Ig-MBP3 used the genes coding for the light and heavy chains of the anti-arsonate antibody, 91A3, according to the procedures described for the construction of Ig-NP (28). In brief, the $91A_3V_H$ gene was subcloned into the EcoRI site of pUC19 plasmid and used as template DNA in PCR mutagenesis reactions to generate $91A_3V_H$ fragments carrying the MBP3 ($91A_3V_H$-MBP3) sequence in place of CDR3. The $91A3V_H$-MBP3 fragment was then subcloned into an expression vector (28) in front of the exons coding for the constant region of a BALB/c γ2b. This plasmid was then co-transfected into the non-Ig-producing SP2/0 myeloma B cell line with an expression vector carrying the parental 91A3 light chain. Transfectants producing Ig-MBP3 were selected in the presence of geneticin and mycophenolic acid. Ig-PLP2, which encompasses amino acid residues 178-191 of PLP, was previously described elsewhere (21). Ig-W, the parental Ig not encompassing any foreign peptide, has also been described elsewhere (28). Large-scale cultures of transfectoma cells were carried out in DMEM culture media containing 10% iron-enriched calf serum (BioWhittaker, Walkersville, MD). Purification of Ig-MBP3, Ig-PLP2, and Ig-W was carried out on separate columns of rat anti-mouse-κ mAb coupled to CnBr activated 4B sepharose (Amersham Pharmacia Biotech, Piscataway, NJ).

Radioimmunoassay

SP2/0 transfectants. Microtiter 96-well plates were coated with polyclonal rabbit anti-mouse-γ chain specific antibody (Zymed Laboratories, South San Francisco, CA) (2 μg/ml in PBS) overnight at 4°C and then blocked with 2% BSA in PBS for 1 hour at room temperature. The plates were then washed three times with PBS and 100 μl/well of supernatant from SP2/0 cells growing under selective pressure was incubated for 2 hours at room temperature. After three washes with PBS, captured Ig-chimeras were revealed by incubation with 1 x 10^5 cpm/well ^{125}I-labeled rat anti-mouse κ mAb (ATCC, Rockville, MD) for 2 hours at 37°C. The plates were then washed five times with PBS and counted using a Wallac LKB gamma counter.

Generation of T cell hybridoma

A T cell hybridoma specific for MBP3 was generated by immunizing SJL/J mice with 200 μg of MBP3 peptide in 200 μl PBS/CFA (vol/vol) s.c. in the foot pads and at the base of each limb. After 10 days the draining lymph nodes were removed, and T cells were stimulated in vitro for 2 rounds in the presence of irradiated, syngenic splenocytes, 5% T-Stim supplement (Collaborative Biomedical Products, Bedford, MA), and MBP3 peptide (15 μg/ml). The culture media used to carry out these stimulations and other T cells activation assays in this study was DMEM supplemented with 10% FCS (Hyclone, Logan, UT), 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μg/ml gentamycin sulfate. This MBP3-specific T cell line was then fused using polyethylene glycol 4000 (Sigma, St. Louis, MO) with the αβ TCR negative thymoma

BW1100 (ATCC). Hybrids were then selected by supplementing the culture media with hypoxanthine-azaserine (Sigma). Resulting hybridomas were then screened for reactivity to MBP3 peptide by testing for production of IL-2 and IFN-γ in the supernatant following stimulation with irradiated (3000 rads) spenocytes in the presence of 15 μg/ml MBP3 peptide. Positive hybridomas were then cloned by limiting dilution and used to assess presentation of MBP3 peptide from the Ig-MBP3 chimera.

Neonatal injections of tolerogen and adult immunizations with peptide

Neonatal injections of Ig-chimera were performed intraperitoneally (i.p.) in 100 μl saline within 24 hours after birth. When the mice reached 7 weeks of age they were subjected to immunization with peptide to analyze their proliferative and cytokine responses. The immunization of adult mice with either 200 μg MBP3 or a combination of 200 μg of MBP3 and 100 μg of PLP2 in 200μl PBS/CFA (vol/vol) was carried out subcutaneously (s.c.) in the food pads and at the base of the limbs. After 10 days the mice were sacrificed in order to examine the elicited immune response.

Induction of EAE

EAE was induced by s.c injection in the foot pads and at the base of the limbs with 200 μl IFA/PBS (vol/vol) solution containing 200 μg *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) and MBP3 peptide (200 μg); MBP3 (200 μg) + PLP2 (100 μg); or MBP3 (200 μg) + PLP1 (100 μg) peptides. Six hours later 200 ng of *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, CA) in 100 μl PBS

was given intravenously (i.v.). A second injection of *B pertussis* toxin was given to the mice after 48 hours. Mice were then scored daily for clinical signs of EAE as follows: 0, no clinical sign; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Proliferation assays

Lymph node (axillary, lateral axillary, and popliteal) and spleen cells were incubated in 96-well plates at 4×10^5 and 10×10^5 cells/100 μl per well, respectively, with 100 μl of stimulator for 3 days. MBP3, PLP2, and PLP1 peptides were used at 30 μg/ml. Subsequently, 1μCi [^{3}H]-thymidine (ICN Pharmaceuticals Inc., Costa Mesa, CA) was added per well, and the culture was continued for an additional 14.5 hours. The cells were then harvested and incorporated onto glass fiber filters. [^{3}H]-thymidine was measured using the Trace 96 program and an Inotech beta counter (Wohlen, Switzerland). A control of media without stimulator was included for each mouse and used as background. All the results presented in the figures represent cpms of test samples from which the background was deducted.

ELISA

Cytokine production by spleen cells was measured as previously described (20). Briefly, 10×10^5 cells/100 μl/well were incubated with 100 μl of stimulator for 24 hours, and cytokine production was measured by ELISA according to PharMingen's (San Diego, CA) instructions (except for TGF-β). TGF-β was measured according to

Genzyme's protocol. Some spleen cytokine measurements were conducted at 72 hours. The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Sunnyvale, CA) using SoftMAX PRO 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, IFN-γ, IL-10 (PharMingen), and TGF-β (Genzyme, Cambridge, MA) were included in all experiments to construct standard curves. The concentration of cytokines in culture supernatant was estimated by extrapolation from the linear portion of the standard curve. All anti-cytokine antibodies used in these studies were purchased from PharMingen except for the pair used for detection of TGF-β, which was obtained from Genzyme. Capture antibodies were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; rat anti-mouse IFN-γ, R4-6A2; and rat anti-mouse IL-10, JES5-2A5. Biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES5-5H4; rat anti-mouse IL-4, BVD6-24G2; rat anti-mouse IFN-γ, XMG1.2; and rat anti-mouse IL-10, JES5-16E3.

ELISPOT assay

ELISPOT assay was used to measure cytokines produced by lymph node T cells during antigen stimulation as previously described (20). Briefly, 5×10^5 cells/100 μl/well along with 100 μl of stimulator were added onto HA-multiscreen plates (Millipore, Bedford, MA) that had been previously coated with capture antibody. After 24 hours of incubation the plates were washed and subsequently incubated with biotinylated anti-cytokine antibody overnight at 4°C. Following incubation with avidin-peroxidase (Sigma, St. Louis, MO) for 1 hour at 37°C, spots were visualized by addition of substrate (3-amino-9-ethylcarbazole, Sigma), dried, and counted under a dissecting microscope. The

capture and biotinylated anti-cytokine antibodies used for ELISPOT were the same as those used for ELISA

Chapter 3. Results

Expression of MBP3 peptide on an Ig molecule drives efficient presentation to T cells

Recent studies revealed that neonatal exposure to an immunoglobulin (Ig) encoding the proteolipid protein (PLP) sequence 139-151 induced rather than suppressed an immune response (20-22). This response was organ specific and involved Th2 deviation in the lymph node and an IFN-γ mediated splenic T cell anergy in the spleen. This investigation addresses whether this organ specific response is intrinsic to the PLP139-151 sequence or whether delivery by an Ig is the key factor, thus allowing a similar response to develop with other peptides. In addition, we wished to explore whether any bystander effect of the Th2 response on the response to other myelin protein epitopes might occur. Therefore, an Ig-chimera was constructed to include the myelin basic protein (MBP) sequence 87-99, hereafter referred to as Ig-MBP3, within the CDR3 variable region of the γ2b heavy chain of the anti-arsonate BALB/c Ab 91A3 (28). The complete construction of the Ig-MBP3 chimera is detailed in figure 1*. DNA sequence analysis confirmed insertion of the nucleotide sequence for MBP3 in the correct reading frame (Fig. 1b). The resulting mutated V_H heavy chain gene was then subcloned into an expression vector upstream of the BALB/c γ2b constant region as described (28). To obtain complete Ig-chimera the mutated heavy chain gene was co-transfected with the gene encoding the parental 91A3 light chain gene into the non-Ig-secreting myeloma B

*all figures and tables can be found in the appendix

cell line SP2/0 (Fig 1c). Selection with the appropriate drugs allowed for the generation of transfectants producing complete Ig molecules, as depicted in figure 1d. Indeed, supernatant from an Ig-MBP3 transfectant incubated on a plates coated with anti-γ2b antibody bound a rat anti-mouse κ light chain mAb, indicating that the mutated heavy chain paired with the parental light chain and formed a complete Ig molecule. Ig-W, the parental 91A3 antibody with an intact CDR3 domain, yielded a similar result.

It has been previously reported that the CDR3 region of the 91A3 Ig is permissive for peptide expression, and both class I and II-restricted epitopes have been processed and presented to T cells when the peptide of interest was grafted in place of the D segment (23, 28-30). To test Ig-MBP3 for peptide delivery and presentation to T cells, the chimera was purified by affinity chromatography from the supernatant of large-scale cultures of transfectant and assayed for presentation using an MBP3-specific T cell hybridoma designated TCH-MBP3-A7. This hybridoma line was generated by fusing MBP3-specific short-term T cell lines with the $\alpha\beta$-T cell receptor ($\alpha\beta$-TCR)-negative thymoma BW1100. As is evident in figures 2a and b, the T cell hybridoma TCH-MBP3-A7 produced IL-2 and IFN-γ upon stimulation with irradiated APCs loaded with MBP3 peptide. PLP2 peptide, used as a negative control, did not stimulate the T cells. Similarly, Ig-MBP3 was able to induce both IL-2 and IFN-γ, while Ig-PLP2 (a chimera encompassing PLP2 peptide) did not. In addition, immunization of SJL/J mice with Ig-MBP3 in CFA induced MBP3 specific T cell responses in both the lymph node and spleen that were predominantly Th1 in nature, exhibiting MBP3 specific production of

both IL-2 and IFN-γ (data not shown). These results indicate that MBP3 peptide is cleaved from the Ig and presented to T cells.

Neonatal injection of Ig-MBP3 confers resistance to induction of MBP3 mediated EAE

Neonatal tolerance was examined by injecting Ig-MBP3 in saline into SJL/J pups within 24 hours after birth, challenging these mice as adults with a disease-inducing regimen of free MBP3 peptide, and then scoring daily for paralysis. As figure 3 illustrates, the group of mice that received no Ig at birth (Nil group) exhibited a disease course typical for MBP3, which generally manifests as a mild, monophasic, non-relapsing/remitting disease pattern (25). In contrast, mice that were injected with Ig-MBP3 as neonates showed virtually no clinical manifestations of EAE. The control mice that received Ig-W, the parental wild-type not containing the MBP3 epitope, developed a pattern of disease resembling the Nil group.

Neonatal injection of Ig-MBP3 leads to normal lymph node proliferation, reduced responsiveness in the spleen, and Th2 deviation in both organs

Investigation of the proliferation and cytokine profiles of the lymph node and spleen of mice injected at birth with Ig-MBP3 and challenged at seven weeks of age with MBP3 peptide in CFA yielded results similar to those obtained from neonatal studies carried out with Ig-PLP1 (20-22). Lymph node proliferation remained unaltered in Ig-MBP3 tolerized mice versus Ig-W tolerized mice (Fig. 4a). In addition, the lymph node

cytokine production of mice tolerized with Ig-MBP3 revealed a strong deviation from a Th1 response to a Th2 one. Indeed, Ig-MBP3 tolerized mice secreted high levels of IL-4 in response to stimulation with MBP3 peptide, while mice injected with Ig-W at birth secreted relatively none (Fig. 4b). In contrast, while the Ig-W tolerized mice produced IFN-γ in response to stimulation with MBP3 peptide, the Ig-MBP3 group did not show any significant IFN-γ production (Fig. 4c). These responses were specific for MBP3 peptide since the negative control, PLP2 peptide, generated no significant response in either group. In the spleen, mice that received Ig-MBP3 on the day of birth and challenged with MBP3 as adults demonstrated a significantly reduced proliferation (p=0.045) in response to stimulation with MBP3 when compared to mice that had been neonatally injected with Ig-W (Fig. 4d). Surprisingly, the cytokine profile of Ig-MBP3 tolerized mice revealed deviation to a Th2 phenotype as well. Indeed, Ig-MBP3 tolerized mice that had been primed as adults with MBP3 in CFA produced IL-4 upon stimulation with MBP3 rather than the usual production of IFN-γ (Fig. 4e, f). These responses were also specific for MBP3 peptide, as there was no detectable response to PLP2.

Neonatal exposure to Ig-MBP3 confers resistance to the induction of EAE with multiple epitopes

Since Ig-MBP3 neonatally tolerized mice exhibited Th2 deviation in both the lymph node and spleen, we hypothesized that such an environment might be able to modulate the response to a separate myelin epitope associated with EAE. In an attempt to address this issue, mice were injected with either Ig-MBP3 or Ig-W at birth and at the age

of seven weeks were assayed for resistance to EAE induction with multiple epitope regimens. As can be seen in figure 5, Ig-MBP3 tolerized mice showed a significant reduction in clinical paralytic severity when they were induced for disease with a mixture of MBP3 and PLP2 peptides. Mice that had received no Ig molecule (Nil group) during the neonatal period and those injected with the control Ig-W molecule had a normal disease pattern (Fig. 5a). In addition, Ig-MBP3 neonatal tolerization conferred resistance against a regimen including MBP3 and PLP1 peptides. Although Ig-MBP3 tolerized mice exhibited only a slightly reduced initial disease peak in comparison to both the Ig-W injected and nil control groups, they were completely protected from relapses (Fig. 5b). In contrast, both control groups suffered a severe relapse that resulted in a significant mortality rate (see Table I).

In order to show that MBP3 specific deviated T cells must be stimulated at the same time as the naïve PLP1 or PLP2 specific pathogenic T cells to achieve protection, EAE was induced in Ig-MBP3 or Ig-W tolerized mice with PLP2 peptide, and the signs of paralysis were assessed. As illustrated in figure 6, the two groups showed no significant difference in disease pattern. Therefore, it appears that MBP3 specific T cells must be activated to confer resistance to EAE induction involving multiple epitopes.

Production of IL-10 in the spleen of Ig-MBP3 neonatally tolerized mice

Since the above studies have suggested that deviated MBP3-specific T cells from mice that had been tolerized as neonates with Ig-MBP3 were possibly affecting the response to other epitopes, such as PLP2, we postulated that these cells might be

displaying bystander functions. To address this issue the MBP3-specific T cells were tested for production of cytokines defined as mediators of bystander suppression. The results indicated that, although after a 24 hour stimulation neither TGF-β or IL-10 was detectable, at 72 hours a high level of IL-10 was observed in the spleen (Fig. 7a). The lymph node T cells did not produce any detectable level of IL-10 in either group (Fig. 7b). Cells from the control mice tolerized with Ig-W did not secrete a detectable level of IL-10. TGF-β was not observed in either group (data not shown). This IL-10 production in Ig-MBP3 tolerized mice was specific for MBP3 peptide, since PLP2 stimulation yielded no significant IL-10 production.

Since recent studies have revealed that APCs could, under certain circumstances, produce IL-10 (31), we utilized purified T cells and mitomycin C treated APCs to ascertain whether the IL-10 seen in the culture was produced by MBP3-specific T cells. To this aim, purified T cells from Ig-MBP3 and Ig-W tolerized mice were stimulated with MBP3 peptide loaded, mitomycin C treated splenocytes from naive SJL/J mice. After 6 days of culture the cytokine production of both groups was analyzed. Adult splenic T cells derived from Ig-MBP3 tolerized mice demonstrated a polarized Th2 response characterized by elevated levels of both IL-4 and IL-10 (Fig. 8b, c) in response to free MBP3 peptide, as well as a significant decrease in the level of IFN-γ secretion (Fig. 8a). Mice that received Ig-W as neonates mounted only a characteristic Th1 response against MBP3 peptide, typified by IFN-γ release (Fig. 8a). These responses were specific to MBP3 in each case, as stimulation with the control peptide, PLP2, yielded negligible cytokine production.

IL-10 produced by neonatally induced MBP3-specific T cells displays bystander functions and suppresses responsiveness of diverse T cells

In an effort to delineate the mechanism underlying the resistance to EAE induction with multiple epitopes and to assess the bystander function of MBP3-specific T cells, mice recipient of either Ig-MBP3 or Ig-W at birth were immunized as adults with a combination of MBP3 and PLP2 peptides in CFA, and the T cell response to PLP2 was analyzed. The results indicate that the spleen proliferation of Ig-MBP3 tolerized mice was drastically reduced to both MBP3 (p=0.001) and PLP2 (p=0.023) peptide (Fig. 9a) when compared to Ig-W tolerized mice. Even more surprisingly, PLP2 specific T cells produced significant amounts of IL-4, and their IFN-γ levels were reduced (Fig. 9b, c). Further examination of IL-10 production indicated that substantial amounts were being produced by these cells (Fig. 9d). This provides an explanation for the low proliferation, as IL-10 has been known for its anti-proliferative function. The mice tolerized with Ig-W instead of Ig-MBP3 showed normal proliferation and IFN-γ production to both peptides, with neither IL-4 nor IL-10 being observed.

Restoration of multiple epitope induced disease severity in Ig-MBP3 tolerized mice by administration of anti-IL-10 antibody

In an effort to evaluate the contribution of IL-10 to the resistance against multiple epitope induced EAE, mice previously neonatally tolerized with Ig-MBP3 were subjected to induction of EAE with free MBP3 and PLP2 peptide and given anti-IL-10 mAb. As

can be seen in figure 10, administration of JES5-2A5 rat anti-mouse IL-10 mAb restored the severity of EAE to a level comparable to that obtained in the susceptible mice neonatally injected with Ig-W. The control group injected with rat IgG instead of JES5-2A5 mAb, like the Ig-MBP3 neonatally tolerized mice not given anti-IL-10 during disease induction, failed to restore the severity of paralysis. These results indicate that splenic IL-10 may contribute to bystander suppression and modulate T cells with diverse specificities.

Chapter 4. Discussion

In recent years it has become clear that neonatal exposure to antigen primes rather than ablates immunity. However, neonatal induced responses, in most cases, evolve to tolerate the antigen and do not mediate the inflammatory reactions that usually develop upon immunization of animals that have not been exposed to the antigen during the neonatal stage (8,9,13,14). Moreover, in addition to these qualitative differences, neonatal exposure to antigen seems to drive an organ specific regulation of T cells, supporting the development of responses in the spleen but leaving the lymph nodes unresponsive (12). However, if the antigenic peptide is given to newborns on an Ig, T cell responses develop in both the lymph node and spleen upon re-challenge with antigen later in life (20-22). Indeed, SJL/J mice given Ig-PLP1, an Ig-chimera carrying proteolipid protein (PLP) sequence 139-151 (PLP1), on the day of birth and challenged with PLP1 peptide as adults generated T cell responses in both lymphoid organs and resisted EAE induction (20). These responses, however, presented qualitative differences relative to control animals not given Ig-PLP1 at birth. Specifically, the lymph node T cells were deviated and produced IL-4 instead of IL-2, and the splenic cells, although non-proliferative and unable to produce IFN-γ, secreted significant levels of IL-2 (20). In the present studies using an Ig chimera incorporating the encephalitogenic MBP87-99 amino acid sequence (referred to as MBP3), we found that neonatal exposure to Ig-MBP3 also promoted immune responses in both the lymph node and spleen. However, the splenic T cells, unlike the Ig-PLP1 system, were more proliferative and produced IL-4, thereby extending

deviation to both lymphoid organs. As the deviation was broadened to both the lymph nodes and the spleen, we sought to test such deviated T cells for bystander modulation of neighboring cells and suppression of disease involving diverse T cell specificities. Indeed, mice tolerized with Ig-MBP3 at birth exhibited a significantly reduced disease severity when induced for EAE with a combination of MBP3 and PLP2 peptides or MBP3 and PLP1 peptides (Fig. 5a, b). Also, as resistance to disease was not present when Ig-MBP3 neonatally tolerized mice were induced for EAE with PLP2 peptide, we concluded that neonatally deviated MBP3-specific T cells had to be stimulated during disease induction in order for bystander T cell modulation to occur (Fig. 6). Furthermore, to determine how Ig-MBP3 induced Th2 cells were capable of regulating unrelated pathogenic cells and modulating the disease, two types of analyses were performed. On the one hand, we tested the neonatally induced MBP3-specific Th2 cells for production of IL-10, a cytokine that has previously been shown to mediate bystander suppression (32-34) and exert pleiotropic, down-regulatory effects upon ongoing immune responses, especially Th1-mediated ones (for review see 35). On the other hand, we analyzed the proliferative and cytokine responses to PLP2 peptide upon immunization of Ig-MBP3 neonatally tolerized mice with the MBP3 and PLP2 peptide mixture. Splenic MBP3-specific T cells that arise in Ig-MBP3 neonatally tolerized mice upon challenge with MBP3 peptide produced, along with IL-4, copious amounts of IL-10 when stimulated in vitro for 72 rather than 24 hours (Fig. 7a). IL-10 production was antigen specific and occurred only when the stimulator was MBP3 peptide. Neutralization of the splenic IL-10 during disease induction restored the severity of EAE, indicating that IL-10 plays a major

role in the resistance to disease induction (Fig. 10). IL-10 and IL-4 produced by MBP3-specific T cells provide an environment suitable for the development of Th2 cells, but unfavorable for the generation of Th1 cells. This statement is supported by the observation that immunization of Ig-MBP3 neonatally tolerized mice with a combination of MBP3 and PLP2 generates weakly proliferating, deviated T cells to both peptides (Fig. 9). More striking, the PLP2-specific T cells also produce significant amounts of IL-10 (Fig. 9d). One likely explanation for the deviation of PLP2 responses is that IL-4 and/or IL-10 from MBP3 cells guided the development of PLP2-specific cells into the Th2 pathway.

Concerning both MBP3 and PLP2 responses, whether IL-10 is produced by the cells secreting IL-4 or by a distinct population of cells is not clear at the present time. It has been previously shown that regulatory T cells can arise in an IL-10 rich milieu and secrete significant levels of IL-10 (36,37). Therefore, one could envision that the splenic cells include antigen specific regulatory cells that function as a source of IL-10 and regulate autoimmunity (38). However, these cells must be further characterized to support this assumption.

The other issues that arise from these observations relate to the factors driving neonatal T cells to deviate to Th2 and home either to the lymph node only in Ig-PLP1 induced neonatal immunity or to both the lymph node and the spleen in the Ig-MBP3 system. Neonatal T cells have been shown to express minimal levels of CD40L (39). Consequently, upon antigen recognition on APCs, CD40-CD40L interactions would be limited, resulting in little or no production of IL-12 (40) and a biased T cell

differentiation. In recent studies we have shown that the splenic T cells in Ig-PLP1 tolerized mice lack CD40L expression and could not progress in the differentiation pathway (Min *et al*, submitted). Since the same SJL mouse strain and Ig backbone are used in the Ig-MBP3 and Ig-PLP1 system, it may be that a discrepancy in peptide affinity among PLP1 and MBP3 influences T cell-APC interactions, contributing to a differential regulation of CD40L and leading to an unbalanced Th2 bias between the two systems.

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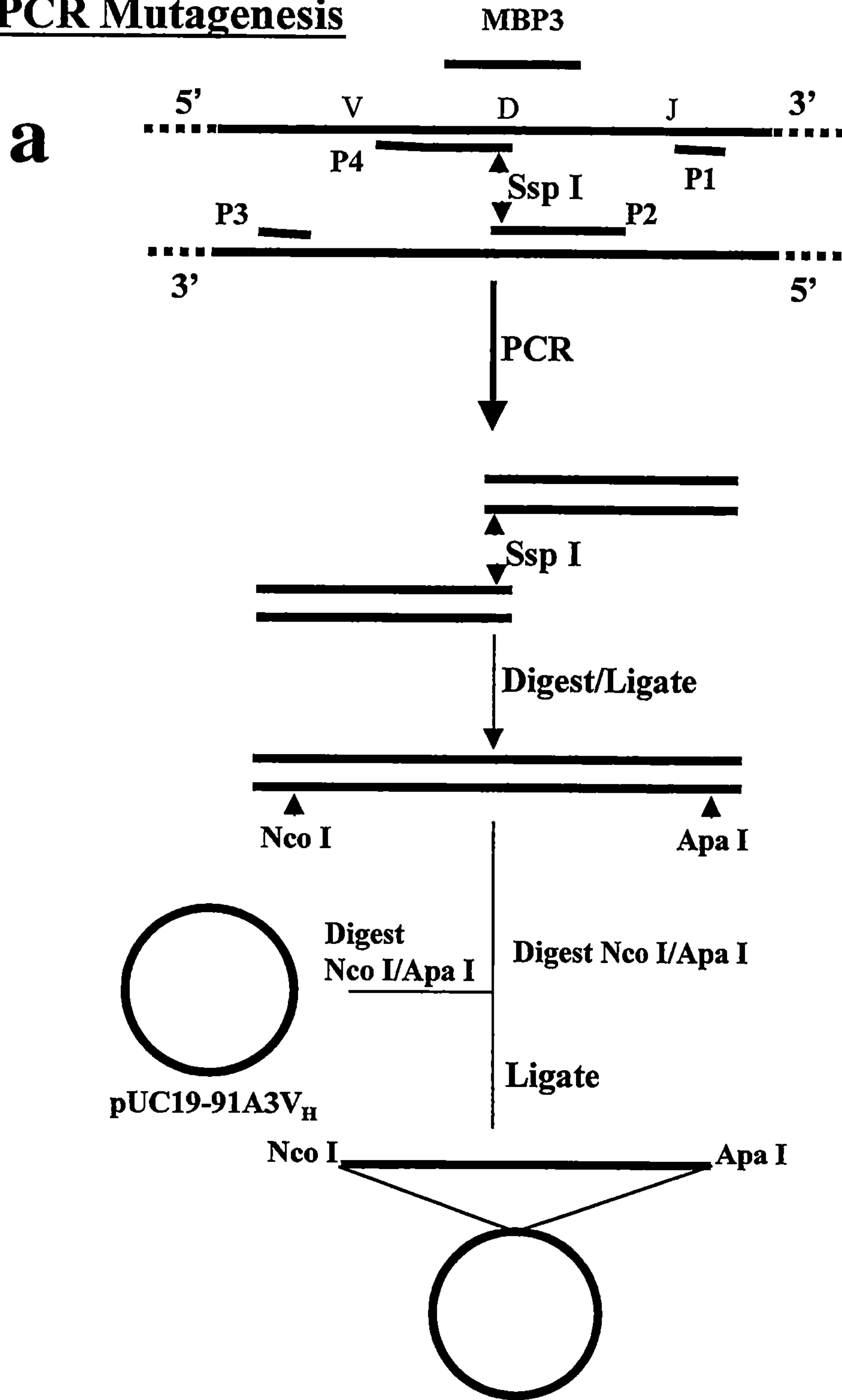
Appendix

Figure 1.

Complete depiction of the construction of the Ig-MBP3 chimera

Insertion of the MBP3 nucleotide sequence into the heavy chain variable region of the $91A_3V_H$ gene through PCR mutagenesis (a). The D segment was deleted and the sequences encoding MBP3 were inserted in its place. Two sets of primers were used to accomplish this. Primer 1 is complementary to sequences within the intron region downstream of the Apa I site, while primer 3 is complementary to noncoding sequences upstream of the Nco I site. Primer 2 contains 3'-sequences starting at the first base upsteam of the D region and 5'-sequences (unmatched) encoding a portion of the MBP3 sequence, while primer 4 contains 3'-sequences starting at the first base upstream of the D segment and 5'-unmatched sequences encoding part of the MBP3 sequence. Primers 2 and 4 encode overlapping sequences, so the generated fragments can be joined through an internally designed Ssp I site to create a complete MBP3 sequence. The complete fragment was then digested with Nco I/Apa I and ligated into a pUC19-$91A_3V_H$ vector from which the VDJ region had been excised with Nco I/Apa I. Comparison of the nucleotide sequences of the wild-type $91A_3V_H$ gene to those of the chimeric $91A_3V_H$-MBP3 indicates that the nucleotide sequence encoding full MBP3 was inserted in the correct frame in place of the D segment (b). The recombinant fragment was then excised from pUC19-$91A_3V_H$ and inserted into the mammalian expression vector pSV2-gpt-$91A_3V_H$ (c). The pSV2-gpt-$91A_3V_H$-MBP3 vector was then co-transfected with an expression vector encoding the parental light chain (pSV2-$91A_3$L) by electroporation into SP2/0, a non Ig-secreting myeloma B cell. Transfectants were selected in the presence of

G418-MA. Radioimmunoassay was then used to detect intact Ig-MBP3 chimera secreted from transfectants (d). Detection of complete Ig-MBP3 was carried out by incubation of supernatant of Ig-MBP3 or Ig-W transfectants on microtiter plates coated with rabbit anti-mouse γ–chain specific antibody and revelation of captured Ig-chimeras with [^{125}I]-labeled rat anti-mouse κ light chain mAb. Shown are the mean ± SD of triplicates. A schematic representation of Ig-MBP3 and Ig-W is illustrated in (e).



PCR Mutagenesis
MBP3
a
5'
V
D
J
3'
P4
Ssp I
P1
P3
P2
3'
5'
PCR
Ssp I
Digest/Ligate
Nco I
Apa I
Digest
Nco I/Apa I
Digest Nco I/Apa I
Ligate
pUC19-91A3V$_H$
Nco I
Apa I

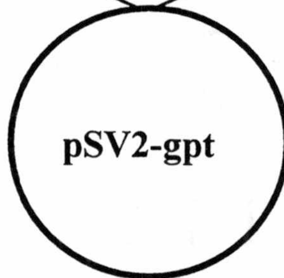
b **Figure 1 (continued)** **$91A_3V_H$**

```
            ------------D------------
TYR PHE CYS ALA ARG SER TYR TYR SER GLY ASP MET TYR CYS          PHE ASP TYR TRP

TAT TTC TGT GCA AGA TCG TAT TAC TCT GGT GAT ATG TAC TGC          TTT GAC TAC TGG
```

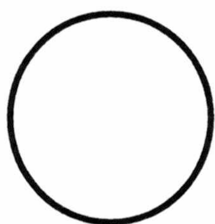
$91A_3V_H$-MBP3

```
            ----------------MBP3----------------
 -   -   -   -   -  VAL HIS PHE PHE LYS ASN ILE VAL THR PRO ARG THR PRO  -   -   -   -

--- --- --- --- --- GTA CAC TTC TTC AAG AAT ATT GTG ACC CCG AGG ACG CCG --- --- --- ---
```

c **Figure 1 (continued)**



pSV2-gpt-91A3H-MBP3



Cotransfect

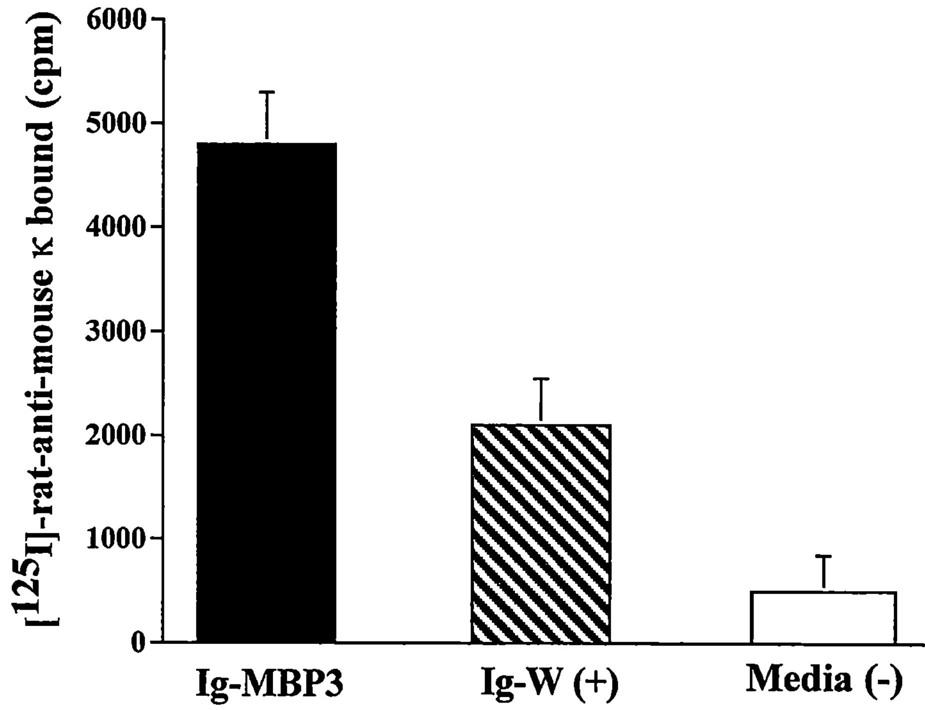
pSV2-neo-91A3L

SP2/0 (non Ig-secreting myeloma cells)

Selection in G418-MA

Radioimmunoassay
Cloning by Limiting Dilution
Large scale growing
Purification by rat-anti-kappa mAb coupled affinity column

d **Figure 1 (continued)**



e Figure 1 (continued)

Ig-MBP3

MBP3

V_H

V_L

C_{H1}

C_L

C_{H2}

C_{H3}

Ig-W

D Region

Figure 2.

Presentation of the Ig-MBP3 chimera to specific T cells

Irradiated (3000 rads) SJL/J splenocytes (5 x 10^5 cells/100 μl/well) were incubated with graded amounts of antigen and one hour later the culture was supplemented with 5 x 10^4 MBP3-specific T cell hybrıdoma, A7. After 24 hours the supernatant was harvested and 100 μl was used for detection of IL-2 (a) and IFN-γ (b). PLP2 and Ig-PLP2, antigens presented by I-A^s like MBP3 and Ig-MBP3, were used as negative controls. Each point represents the mean of triplicates.

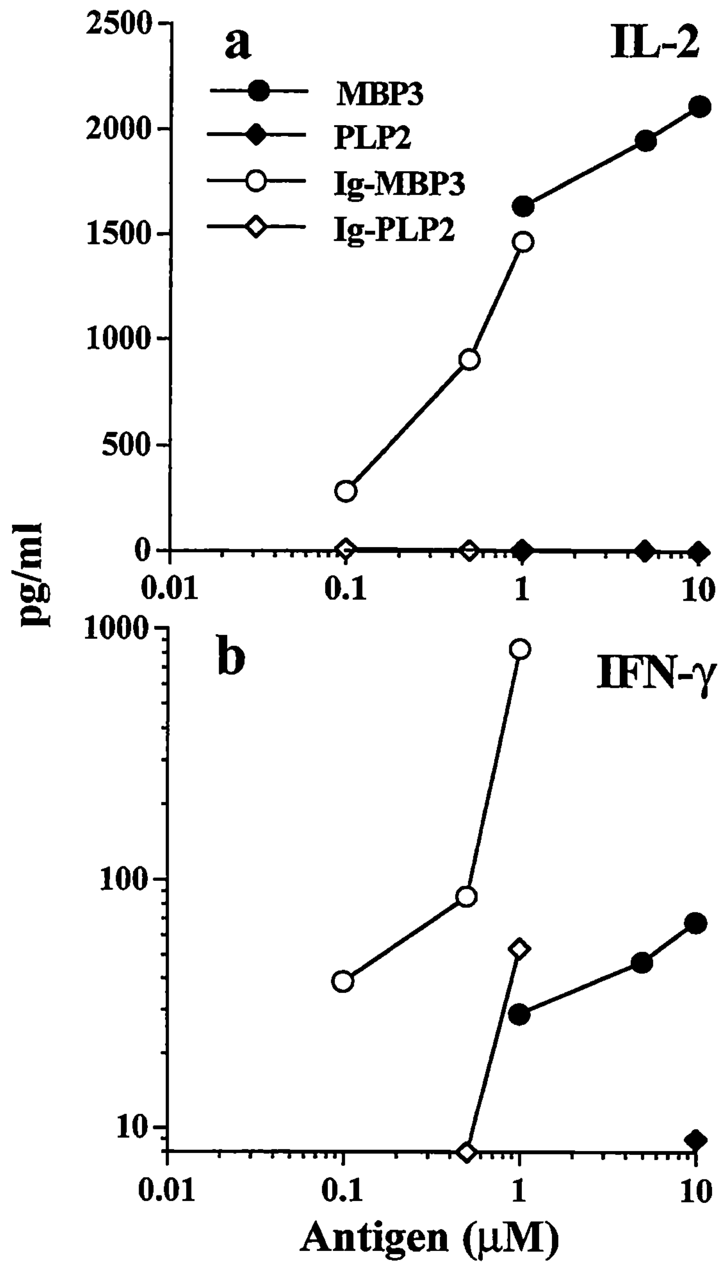

a
IL-2
MBP3
PLP2
Ig-MBP3
Ig-PLP2
2500
2000
1500
1000
500
0
0.01
0.1
1
10
pg/ml
b
IFN-γ
1000
100
10
0.01
0.1
1
10
Antigen (μM)

Figure 3.

Ig-MBP3 neonatally tolerized mice resist induction of EAE by MBP3 peptide Newborn mice (4-7 per group) were injected with 100 μg of affinity purified Ig-MBP3 (Ig-MBP3 Tol) or Ig-W (Ig-W Tol) in saline within 24 hours of birth and were induced for EAE at seven weeks of age with free MBP3 peptide as described in materials and methods. Mice were then scored daily for signs of paralysis. For comparison purposes a group of mice that did not receive any injection (Nil) was included.

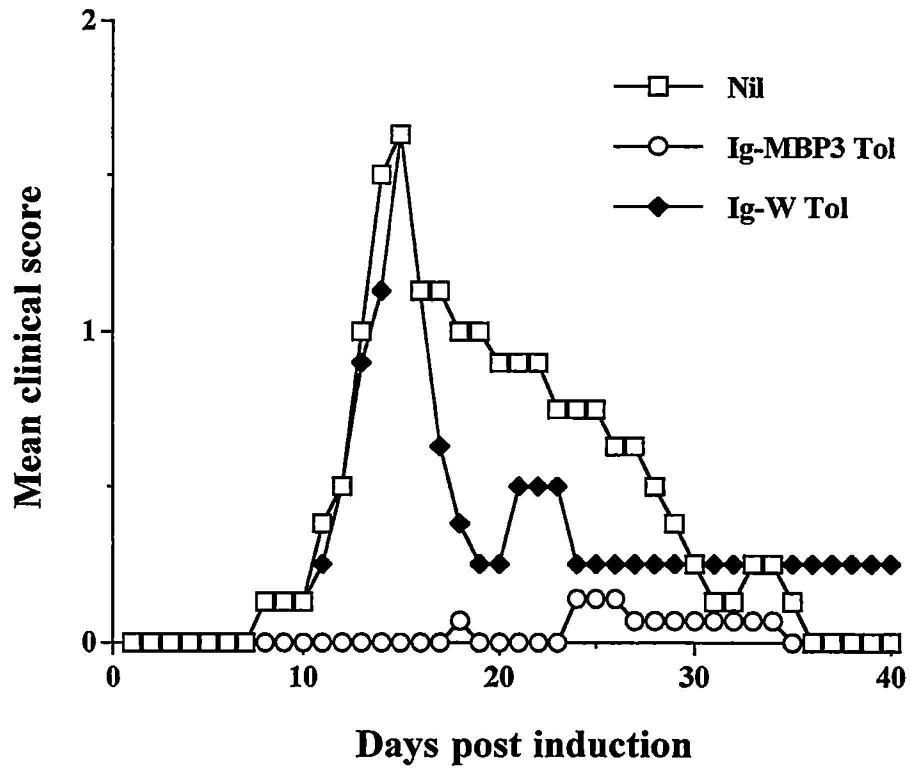
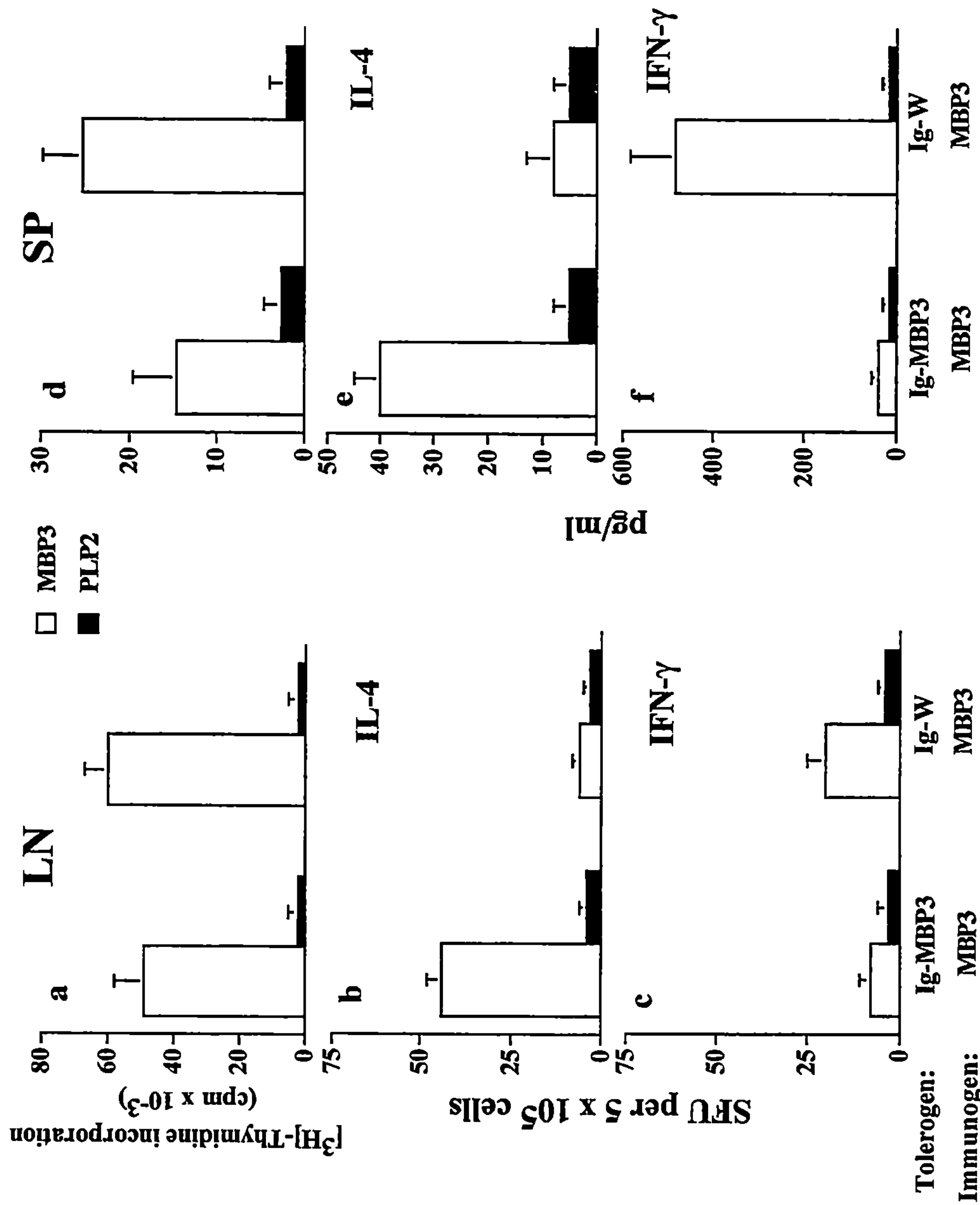

Nil
Ig-MBP3 Tol
Ig-W Tol
Mean clinical score
Days post induction
0
1
2
0
10
20
30
40

Figure 4.

Exposure to Ig-MBP3 on the day of birth drives T cell deviation in both the lymph node and spleen

Newborn mice (8 per group) were injected intraperitonally (i.p) within 24 hours after birth with either 100 μg of Ig-MBP3 or Ig-W in saline. When the mice reached seven weeks of age they were immunized with 200 μg of free MBP3 peptide in 200 μl CFA/PBS (vol/vol) subcutaneously (s.c.) in the foot pads and at the base of the limbs. Ten days later the mice were sacrificed, and the draining lymph node (0.4 x 10^6 cells/well) and spleen (1 x 10^6 cells/well) cells were stimulated with 30 μg/ml of free MBP3 or PLP2. The lymph node (LN) (a) and spleen (SP) (d) proliferation were measured by [^{3}H]-thymidine incorporation after 3 days of stimulation. Cytokine production was analyzed in the LN by ELISPOT (b and c) and in the SP by ELISA (e and f) after 24 hours of stimulation. The indicated values represent the mean ± SD of eight individually tested mice.



LN
SP
MBP3
PLP2
[3H]-Thymidine incorporation
(cpm x 10-3)
SFU per 5 x 105 cells
pg/ml
IL-4
IFN-γ
Tolerogen:
Immunogen:
Ig-MBP3
MBP3
Ig-W
MBP3

Figure 5

Ig-MBP3 neonatally tolerized mice show resistance to EAE induced with two epitopes

Newborn mice (5-10 per group) were injected with 100 μg of affinity purified Ig-MBP3 or Ig-W in saline within 24 hours of birth and were induced for EAE at seven weeks of age with either a mixture of MBP3 and PLP2 peptides (a) or MBP3 and PLP1 peptides (b) as described in the materials and methods. Mice were then scored daily for signs of paralysis. A group of mice that did not receive any injection on the day of birth (Nil) was included for control purposes.

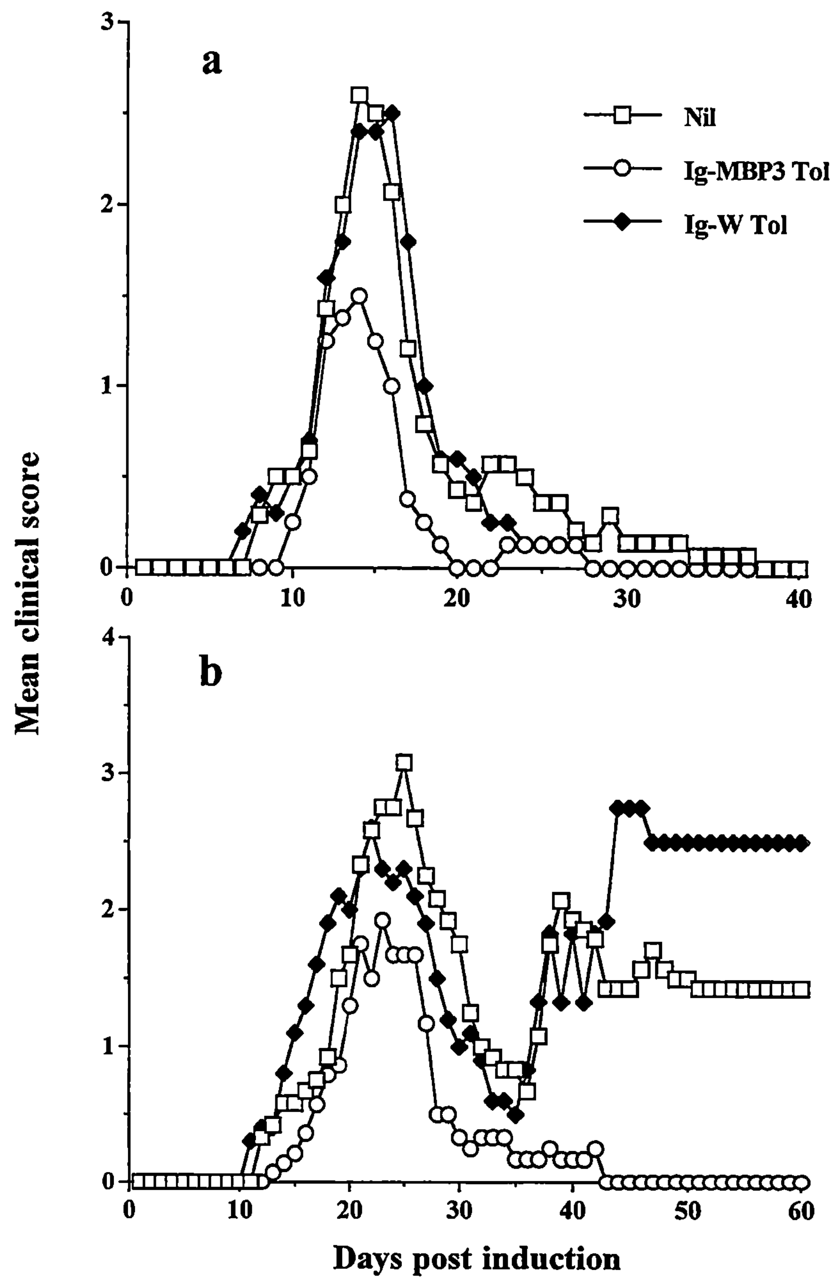

a
b
Nil
Ig-MBP3 Tol
Ig-W Tol
Mean clinical score
Days post induction
0
1
2
3
4
10
20
30
40
50
60

Figure 6.

Requirement for activation of MBP3-specific cells to ameliorate EAE

Newborn mice (4 per group) were injected with 100 μg of affinity purified Ig-MBP3 or Ig-W in saline within 24 hours of birth and were induced for EAE at seven weeks of age with free PLP2 peptide as described in materials and methods. Mice were then scored daily for signs of paralysis.

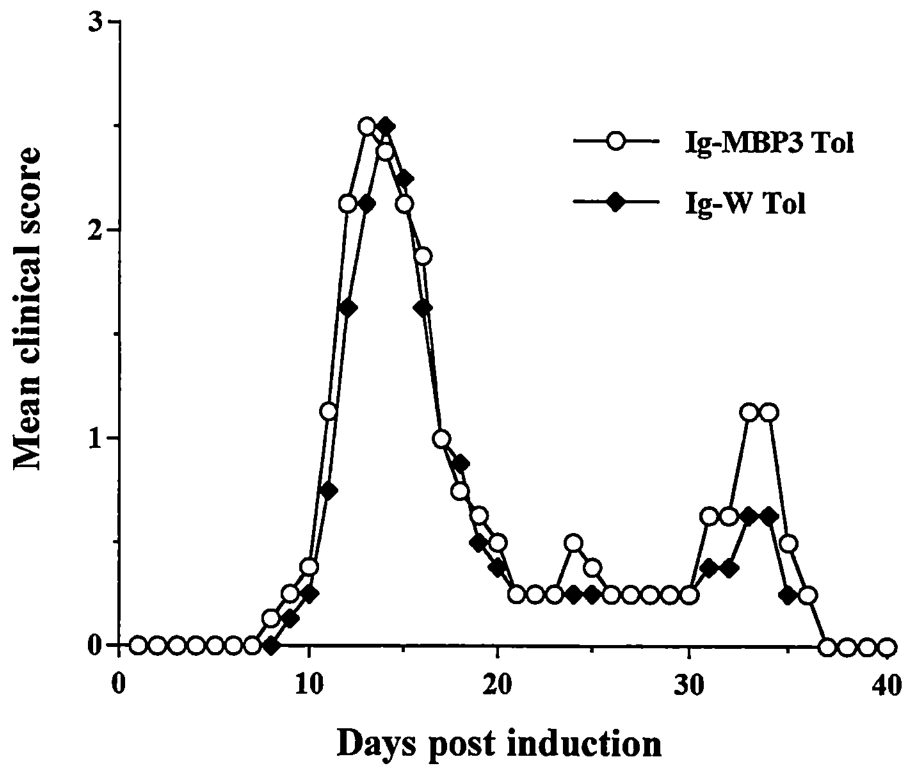
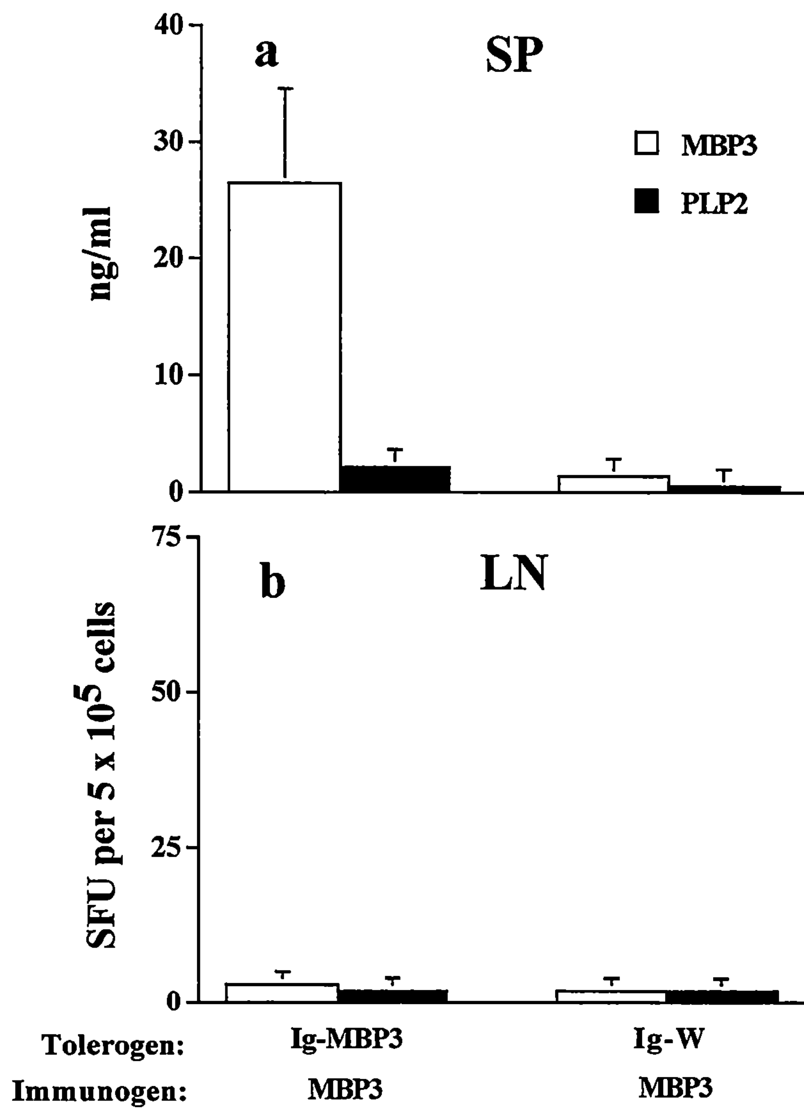

Mean clinical score
3
2
1
0
0
10
20
30
40
Days post induction
Ig-MBP3 Tol
Ig-W Tol

Figure 7.

Production of IL-10 in the spleen but not the lymph node of Ig-MBP3 tolerized and peptide immunized mice

Newborn mice (8 per group) were injected within 24 hours after birth with either 100 µg of Ig-MBP3 or Ig-W in saline. When the mice reached seven weeks of age they were immunized with 200 µg free MBP3 peptide in 200 µl CFA/PBS (vol/vol) s.c. in the foot pads and at the base of the limbs. Ten days later the mice were sacrificed and the lymph node (LN) (0.4×10^6 cells/well) and spleen (SP) (1×10^6 cells/well) cells were stimulated with 30 µg/ml of free MBP3 or PLP2 for 72 hours. IL-10 production was measured by ELISA in the spleen (a) and by ELISPOT in the lymph node (b). The indicated values represent the mean ± SD of eight individually tested mice.

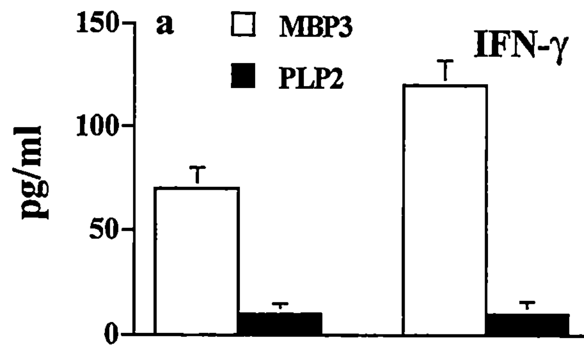


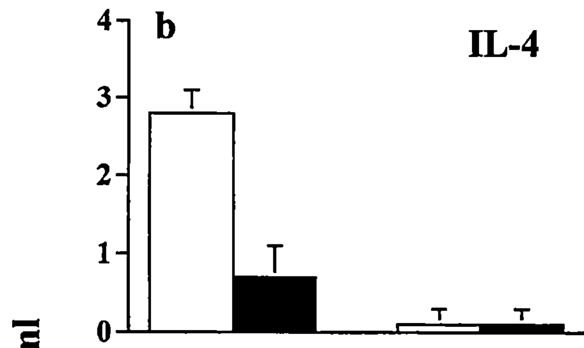
40
30
20
10
0
a
SP
MBP3
PLP2
ng/ml
75
50
25
0
b
LN
SFU per 5 x 10⁵ cells
Tolerogen:
Ig-MBP3
Ig-W
Immunogen:
MBP3
MBP3

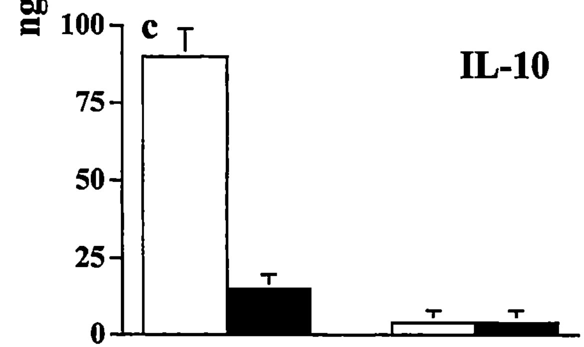
Figure 8.

The splenic IL-10 in Ig-MBP3 tolerized mice is produced by T cells, not APCs

Newborn mice (8 per group) were injected i.p. within 24 hours after birth with either 100 μg of Ig-MBP3 or Ig-W in saline. When the mice reached seven weeks of age they were immunized with 200 μg free MBP3 peptide in 200 μl CFA/PBS (vol/vol) s.c in the foot pads and at the base of the limbs. Ten days later the mice were sacrificed and the spleen was homogenized to a single cell suspension and passed over a nylon wool column to enrich for T cells. Purified T cells (1×10^5 cells/well) were then stimulated for 6 days with APCs that were previously pulsed with either 30 μg/ml of free MBP3 or PLP2 peptide and treated with mitomycin C (50 μg/ml for 30 min). Subsequently, the production of IFN-γ (a), IL-4 (b), and IL-10 (c) in the supernatant was analyzed by ELISA. The indicated values represent the mean ± SD of eight individually tested mice. APCs or T cells alone did not produce any significant cytokine.







Tolerogen:	Ig-MBP3	Ig-W
Immunogen:	MBP3	MBP3

Figure 9.

Neonatal injection of Ig-MBP3 induces bystander suppression of diverse T cell specificities

Newborn mice (8 per group) were injected i.p. within 24 hours after birth with either 100 μg of Ig-MBP3 or Ig-W in saline. When the mice reached seven weeks of age they were immunized with a combination of 200 μg MBP3 and 100 μg PLP2 peptides in 200 μl CFA/PBS (vol/vol) s.c. in the foot pads and at the base of the limbs. Ten days later the mice were sacrificed, and the spleen (1 x 10^6 cells/well) cells were stimulated with 30 μg/ml MBP3, PLP2, or PLP1. Proliferation (a) was measured after 3 days of stimulation, the cytokines IL-4 (b) and IFN-γ (c) after 24 hours of stimulation, and IL-10 (d) after 72 hours of stimulation. The indicated values represent the mean ± SD of eight individually tested mice

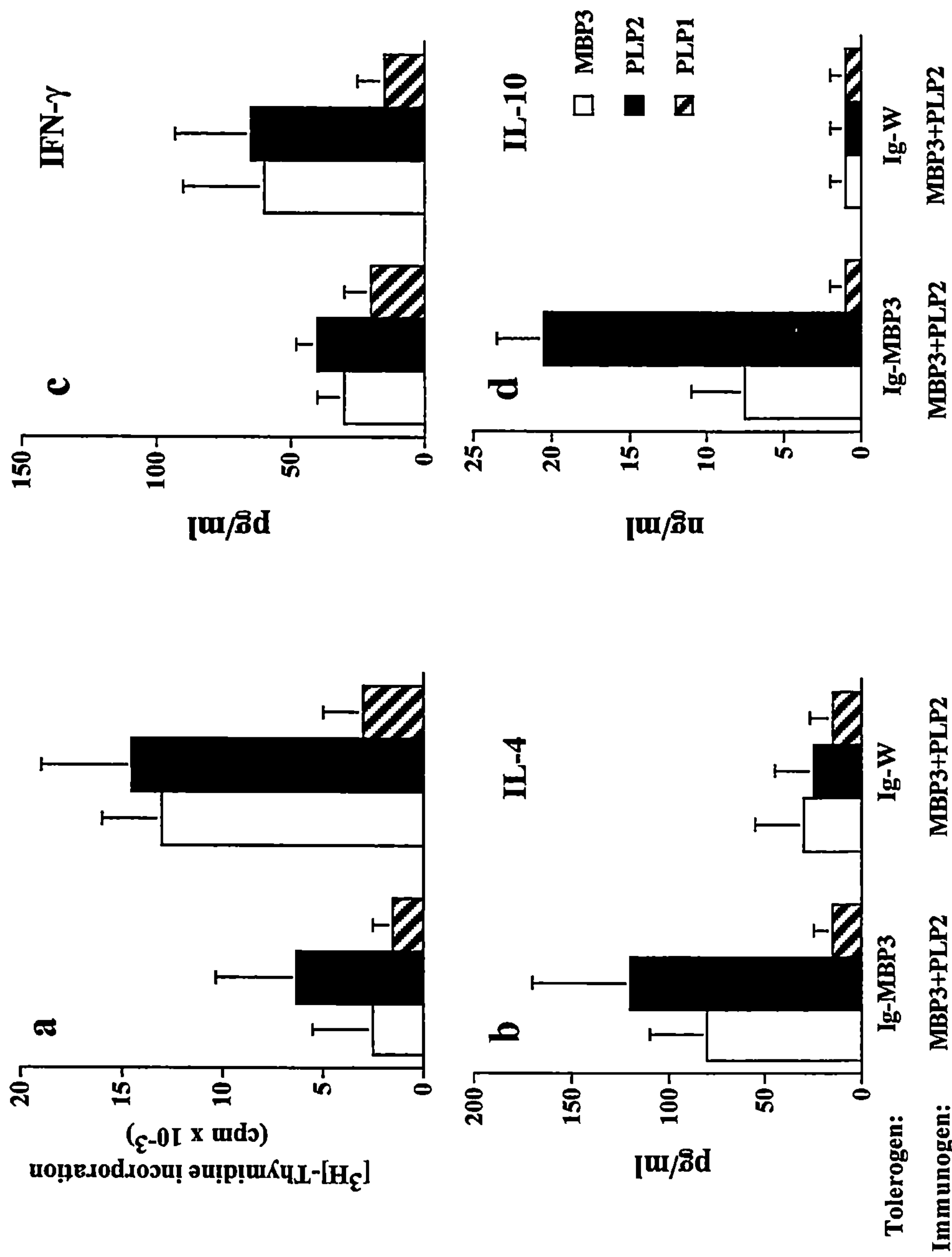

a
b
c
d
[3H]-Thymidine incorporation (cpm x 10-3)
pg/ml
ng/ml
IL-4
IFN-γ
IL-10
MBP3
PLP2
PLP1
Tolerogen:
Immunogen:
Ig-MBP3
Ig-W
MBP3+PLP2
MBP3+PLP2

Figure 10.

Requirement of splenic IL-10 for bystander suppression of EAE involving diverse T cell specificities

Newborn mice were injected within 24 hours of birth with 100 μg of Ig-MBP3 in saline. When they reached seven weeks of age, a group of mice was injected i.p. with 1 mg/mouse of affinity purified JES5-2A5 anti-IL-10 antibody in 1 ml PBS. A second group of mice was injected with 1 mg/mouse of Rat IgG in 1 ml PBS to serve as a control. On the next day all mice were induced for EAE with a combination of free MBP3 and PLP2 peptides as described in the materials and methods. Five days after disease induction the mice were given a second injection of 1 mg/mouse of JES5-2A5 or rat IgG. The mice were scored daily for signs of paralysis. For comparison purposes the clinical scores of a group of mice that were injected with Ig-MBP3 at birth but which did not receive any antibody treatment (Nil) were included.

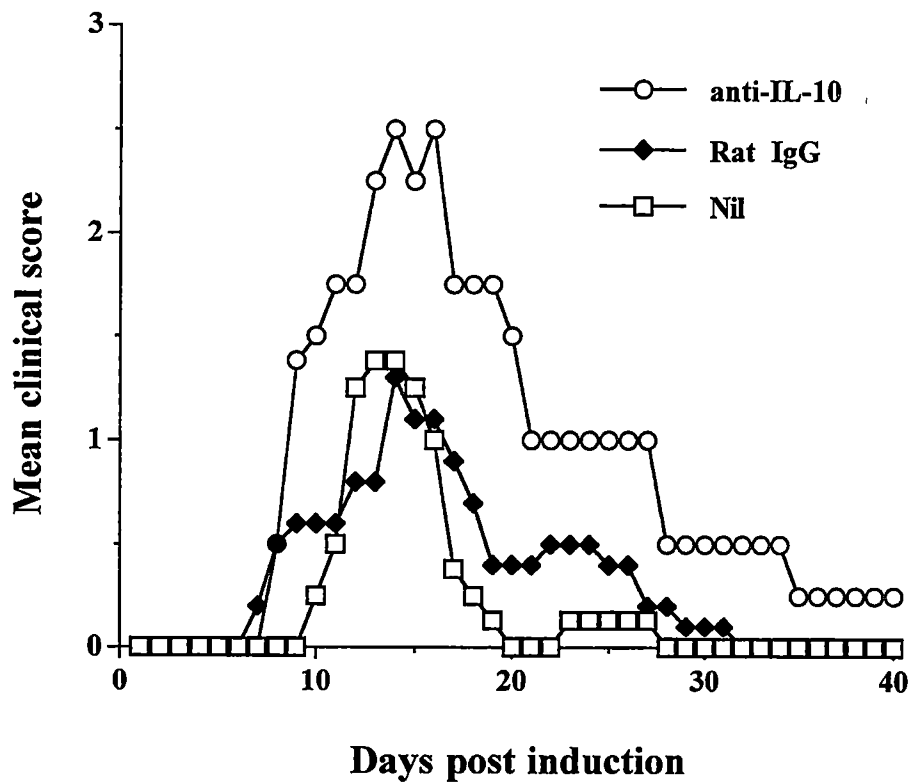

3
2
1
0
Mean clinical score
anti-IL-10
Rat IgG
Nil
0
10
20
30
40
Days post induction

Table I. Reduction of the Clinical Severity of EAE in Ig-MBP3 Tolerized Neonates[a]

Tolerogen (μg)	Incidence	Day of Onset[b]	Mean Maximal Disease Severity[c]	Mortality (%)
A. MBP3 + PLP2 Induced EAE				
Nil	7/7	8.4±0 5	2.6±0.2	0
Ig-W/Sln (100)	5/5	8 2±0.5	2 7±0.2	0
Ig-MBP3/Sln (100)	10/10	11±0.7	1.4±0 9[d]	0
B. MBP3 + PLP1 Induced EAE				
Nil	6/6	12.5±0.8	3.7±1 0	33
Ig-W/Sln (100)	5/5	12±1 2	4 0±1.3	60
Ig-MBP3/Sln (100)	6/6	13±2.5	2.2±0.2[d]	0

[a] The data illustrated in this table were gathered from the mice described in figure 5. Briefly, groups of newborn mice (5-10 per group) were injected with 100 μg of Ig-MBP3 or Ig-W in saline on the day of birth and were induced for EAE with either 200 μg free MBP3 and 100 μg free PLP2 in CFA or 200 μg free MBP3 and 100 μg free PLP1 in CFA when they reached seven weeks of age.
[b] Mean ± SD of the day of disease onset
[c] Mean ± SD of the maximal clinical scores
[d] This score is statistically significant ($p<0.05$) when compared with either the mean score of the Ig-W/Sln injected mice or the Nil group.

VITA

Christopher Dean Pack was born on January 28, 1973 in Knoxville, Tennessee. He grew up in North Knoxville, where he graduated as the salutatorian of Halls High School in 1991. He then accepted an academic scholarship to attend the University of Tennessee, obtaining the Bachelor of Science degree with a major in microbiology and a minor in biochemistry in the fall of 1996. As an undergraduate he was inducted into Phi Beta Kappa, and began volunteer work in Dr. Habib Zaghouani's lab. He would go on to receive the Lisa Kahn Memorial Award for excellence in undergraduate research. After graduation he worked as a technician in the lab of Dr. Zaghouani for one year, where he became profoundly interested in the field of immunology. Chris then entered the graduate program in microbiology at the same university, continuing his research on immune regulation in Dr. Zaghouani's lab. He plans to obtain a Master of Science degree in August 2000. Chris intends to continue his education towards a Ph.D degree at the University of Tennessee in the lab of Dr. Barry T. Rouse. There he will be exploring the ability of heat shock protein/viral peptide complexes to induce a protective immune response against herpes simplex virus.