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Triple threat : chimeric immunoglobulins for the antagonism, prevention, and treatment of autoimmunity

Kevin L. Legge

To the Graduate Council:

I am submitting herewith a dissertation written by Kevin L. Legge entitled "Triple threat : chimeric immunoglobulins for the antagonism, prevention, and treatment of autoimmunity." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Habib Zaghouani, Major Professor

We have read this dissertation and recommend its acceptance:

Robert Moore, Nicholas Potter, Barry Rouse

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Associate Vice Chancellor and
Dean of The Graduate School

TRIPLE THREAT: CHIMERIC IMMUNOGLOBULINS FOR THE ANTAGONISM, PREVENTION, AND TREATMENT OF AUTOIMMUNITY

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Kevin L. Legge
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DEDICATION

This dissertation is dedicated to my parents, Mr. Clarence G. Legge and Mrs. Sharon M. Legge, my brother Joe Legge, and to all of my family members, teachers, and colleagues that have given me invaluable support and encouragement throughout my education.

Thanks!

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ABSTRACT

Multiple sclerosis is an inflammatory disease that leads to a demyelination of the CNS that is responsible for the symptomatic neurological deficits, including paralysis. A predominance of evidence supports the idea that this autoimmune disease is mediated through $CD4^+$ T cells reactive to myelin antigens, and as such, these T cells have been shown to be at higher levels in MS patients than in healthy individuals. Likewise, work on Experimental Allergic Encephalomyelitis, an animal model for human MS, has shown that immunization with myelin proteins or transfer of activated $CD4^+$ T cells reactive against myelin antigens are sufficient to transfer disease to naive animals.

Therefore this study explores three distinct approaches to target and silence autoreactive T cells within the EAE model of autoimmune disease. Part II investigates the introduction of efficient peptide antagonist ligands to downregulate autoreactive T cells. Part III delves into the consequences of efficient transfer and presentation of self-antigen and altered self-antigen into the fetal thymus and periphery on subsequent autoimmunity and T cell development. Lastly, Part IV explores the outcome of efficient peripheral self-peptide presentation, without induction of co-stimulation thus mimicking peripheral tolerance, and the triggering of anti-inflammatory cytokines to possibly induce anergy of self-reactive T cells and modulate clinically evident autoimmune disease. Each aspect of these studies uses novel Ig-chimeric molecules, which express either self or altered self ligands within the CDR3 region of the heavy chain. These molecules allow for the efficient presentation of the myelin based ligands to T cells and can also induce the production of anti-inflammatory cytokines.

The results indicate that in order for antagonist ligands to down regulate a protein based autoimmune response, which would occur during natural disease, the antagonists must be presented efficiently. Furthermore this study demonstrates that introduction of self but not altered self ligands, within the fetal thymus, alleviates subsequent autoimmunity to that self-epitope in later life. Lastly the results reported herein show that treatment of clinically evident autoimmunity, even involving multiple myelin epitopes, is possible by administering aggregated Ig-myelin chimeras into the periphery during the course of disease.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
Ag	antigen
agg	aggregated
APC	antigen presenting cell
BG	background
BSA	bovine serum albumin
CDR	complementarity-determining region
CFA	complete Freund's adjuvant
CNS	central nervous system
cpm	counts per minute
Ci	Curie
D	dalton
DC	dendritic cell
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FcR	Fc receptor
HA	influenza virus hemagglutinin
HLA	human leukocyte antigens
IFN	interferon
Ig	immunoglobulin

IFA	incomplete Freund's adjuvant
i.p.	intraperitoneal
i.v.	intravenous
IL	interleukin
KLH	keyhole limpet hemocyanin
LN	lymph node
Mϕ	macrophage
MBP	myelin basic protein
MHC	major histocompatibility complex molecule
mAb	monoclonal antibody
MS	multiple sclerosis
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLP	proteolipid protein
PPD	purified protein derivative
rads	radians
r	recombinant
RIA	radioimmunoassay
sol	soluble
SP	spleen
SFU	spot forming units
SD	standard deviation

s.c.	subcutaneous
TAP	transporter associated with antigen presentation
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
U	unit

PART I.

Introduction

Chapter 1. T lymphocytes

T cell development

T cell development and maturation takes place in the thymus via a process in which T cells whose antigen receptors are strongly reactive with self-antigens are deleted by apoptosis (negative selection) (1-4) and cells whose receptors have lower avidity to autoantigens mature, exit the thymus, and participate in peripheral immune surveillance (5-11). Some autoantigens, however, are hidden from the immune system and do not reach or are not produced and processed within the thymus for presentation (12-14). Consequently, T cells expressing receptors specific for these autoantigens will not be negatively selected and, although potentially dangerous, will exit the thymus and migrate to the periphery (15-17).

If the self-antigen is sequestered in an immune privileged tissue (one where the immune system does not normally patrol due to structural barriers) like the CNS with it's blood-brain barrier, the T cells are thought to circulate harmlessly as encounter with antigen does not normally occur. However, if the antigen is available in the periphery a second round of T cell screening, known as peripheral tolerance, will follow to further minimize autoreactivity (18-23). Presumably, peripheral tolerance develops as a consequence of presentation of autoantigen by non-activated antigen presenting cells (APCs) expressing minimal or no costimulatory molecules (5, 24). Events such as viral and bacterial infections or other tissue injuries that trigger exposure of the otherwise hidden self-antigens that are not seen in the periphery or that cause increased release of self-antigens coupled with inflammatory signals that increase the costimulatory molecules on local APCs might activate the circulating T cells (25-28). The results of

such T cell activation might be the escalation of inflammatory reactions and injury of specific tissues and organs (29-31).

T cell antigen recognition and antagonism.

T cells recognize short peptides rather than native antigens associated with major histocompatibility complex (MHC) molecules (32, 33). These peptides are generated from the native antigen subsequent to uptake by antigen presenting cells and processing within acidic vacuoles or endosomes (34). The liberated peptides bind to newly synthesized MHC molecules to form complete functional complexes that then translocate to the surface of the APCs (34). Once at the surface, the peptide-MHC complexes are suitable for recognition by the T cell antigen receptor (TCR) (32-34). Events that typically occur during injurious exposure to antigen (i.e. inflammation) trigger the upregulation of co-stimulatory molecules on APCs (35-36). The engagement of the TCR to the MHC-peptide complex (Signal 1) along with the costimulatory interactions (Signal 2) between the APC and T cell lead to activation, proliferation, and cytokine production of the T cell (Figure 1a, Figure 2a)* (37-39).

Supply of antigen in an adjuvant-free form might not lead to inflammatory signals, and result in peptide presentation without upregulation of the costimulatory molecules on APCs necessary for activation of the T cell (Figure 1b) (13, 40, 41). In a way, a regimen of this kind would simulate peripheral tolerance and inactivate rather than stimulate the T cells (42, 43). Therefore, T cells that engage, via TCR, APCs expressing

* Figures and tables may be found at the end of each part.

MHC-peptide complexes without costimulatory molecules would be turned off and enter a silent state we refer to as anergy (Figure 1b) (37-39).

Recently the molecular basis of peptide binding to MHC and its interaction with the TCR have been elucidated (44, 45). Accordingly, within a peptide, the amino acid residues that mediate binding to the MHC molecule are distinct from those that contact the TCR (46, 47). On the basis of these findings altered peptides (APL) mutated at the TCR contact residues were generated that could bind to MHC molecules and still interact with the TCR (48-50). However, because the overall resulting avidity of interaction is weaker, the T cell does not proliferate or produce cytokines (Figure 2b)(48-50). Furthermore, interaction of T cells with APLs leads to the spoiling of T cell receptors on the cell surface (51). This spoiling is due to incomplete signaling through both the CD3 ζ chain and Ca^{2+} signaling pathways (51-53). Once spoiled, a TCR becomes unresponsive to other ligands. Since T cell activation requires triggering of a finite number of TCRs, spoiling of TCRs by APLs presumably leaves the T cell anergic and with fewer usable receptors than are needed to respond to stimulatory ligands (51). This process is called T cell antagonism and is being considered for treatment of T cell mediated autoimmune diseases.

Chapter 2. Autoimmunity

General information

One of the more striking features of immune development is the ability of the immune system to educate itself not to respond to self-antigen while maintaining a robust response to foreign invaders. This aspect of development is even more remarkable when one considers that during T cell development, T cells are positively selected on self-ligand MHC complexes in the thymus (5-11). Yet, the immune system also takes careful note to eliminate those developing T cells that react too strongly with the self-ligand MHC complexes through a process termed negative selection (1-4).

However, some self-proteins/peptides are not presented in the thymus and therefore are not available to trigger negative selecting events. This lack of elimination allows a few autoreactive T cells to escape the thymus and migrate into peripheral surveillance (15-17). The immune system is ever vigilant, and continues to try and silence these T cells through peripheral tolerance mechanisms, yet events that allow activation of these T cells might allow autoimmunity to develop (18-23).

Several well-known autoimmune diseases (multiple sclerosis (MS), type I diabetes, rheumatoid arthritis) in humans are thought to be mediated, at least in part, by inflammatory autoreactive T cells (29-31). While healthy individuals have T cells capable of responding to self-antigen in their periphery, they normally remain silent. Those persons with active autoimmunity, however, exhibit increased numbers of activated autoreactive T cells (54). The events that initiate the break in self-tolerance for these cells are not yet understood, but superantigens capable of reacting with particular Vβ TCRs (55) and infections that could allow antigen molecular mimicry (56), epitope

spreading (57) or peripheral loosening in peptide crypticity (58), have been proposed as possible triggers for autoreactive T cell activation. As such, a number of studies have suggested that viral (59, 60), bacterial (61-63), and parasitic (64, 65) infections are associated with autoimmune disease. Likewise particular genotypes of MHC or HLA molecules have been shown to be closely linked with increased susceptibility to some autoimmune diseases, suggesting, as would be expected, that the MHC/HLAs ability to bind the processed self-peptides also plays a distinct role in the T cells' ability to see and respond to the self-antigen (66-68). As without self-peptide display, the T cell cannot respond to it.

Multiple Sclerosis and EAE

Multiple sclerosis is a relapsing/remitting or chronic progressive paralytic disease characterized by inflammation and demyelination within the central nervous system (CNS) (69). A large body of evidence indicates that MS is an autoimmune disease mediated by myelin-reactive $CD4^+$-T cells (54,69-73), which secrete inflammatory cytokines such as tumor necrosis factor and IFNγ (74-75). The involvement of myelin proteins and T cells in MS is further supported by the observation that injection of myelin proteolipid protein (PLP) and/or myelin basic protein (MBP) into animals induces myelin-specific inflammatory T cells that populate the CNS and mediate the paralytic disease known as experimental autoimmune encephalomyelitis (EAE) (69, 76-78). In fact, myelin reactive T cells are clonally expanded in the blood and CNS lesions of patients with MS (54).

Clinically, MS patients exhibit paralysis, visual and sensory impairments, as well as other neurologic disorders (69). Histopathologically, MS is characterized by sclerotic lesions of the CNS white matter (75). More specifically, there is a loss of myelin from the perivascular regions, the optic nerves, brain stem, and spinal cord (75). The clinical and histopathological symptoms of EAE resemble those observed in MS, and EAE is considered the best available animal model to study the pathogenesis and treatment of human MS (69). Indeed, the EAE model was born from Louis Pasteur's rabies vaccination experiments of the early 1900's, as some vaccinations led to a monophasic neurological disorder that was later traced to immunization with the spinal cord that the virus had been prepared from (79, 80). Subsequent studies have shown that EAE is inducible in animals as diverse as mice, rats, guinea pigs, rabbits, and monkeys (69) by immunization with at least three of the myelin proteins from the CNS, myelin proteolipid protein (PLP) (76, 77), myelin basic protein (MBP) (78, 81), and myelin oligodendrocyte glycoprotein (MOG) (82, 83) or through adoptive transfer of activated CD4+ T cell clones specific for myelin antigens (84-88).

Murine CD4+ T cells can be classified into at least two phenotypes based upon the cytokines they produce (89). Cells producing IL-2, IFNγ and TNFα are termed as Th1 cells (89, 90). Inversely, cells producing IL-4 and IL-10 are labeled as Th2 cells (89, 90). Encephalitogenic T cells belong to the Th1 subgroup (69), and clinical scores can be altered by regulating the balance between Th1 and Th2 type cytokines or T cells (91). In fact mice deficient for IL-4 (92) and IL-10 (93) exhibit increased susceptibility to EAE, while those treated with neutralizing antibody to TNFα show reduced clinical signs (94).

Treatment of EAE

One initial treatment strategy for autoimmunity utilized the fact that T cells require MHC complexes for peptide recognition, and blocked TCR binding to self-peptide MHC complexes by competitively using up the available MHC, thus not allowing self-peptide presentation (52). Subsequent strategies have modified this approach by mutating just the TCR contact residues from self determinates, to create ligands termed altered peptide ligands (APL) (52, 78, 95). Some such mutations not only block by inhibiting at high concentrations MHC self-peptide presentation, they incompletely signal the TCR and spoil it for subsequent self-ligand MHC signaling (51). In fact this method of TCR spoiling is the major mechanism of APL mediated inhibition of self-reactive T cells, since the amino acid residues responsible in binding the peptide to the MHC are the same as those of the self-peptides and therefore the APL and self-peptide typically have the same binding affinities for the MHC (95).

A second method being employed to inhibit autoreactive T cells is to deviate the prevailing response of the T cells from an inflammatory Th1 response to a protective Th2 response. Indeed, autoreactive T cell clones or hybridomas expressing the cytokines IL-4 or IL-10, as a consequence of transfection or infection, induced recovery from disease when injected into animals with ongoing EAE (96, 97) Likewise, induction of anti-inflammatory immune response to a non-self Ag at the time of disease has been shown to mediate bystander suppression of Th1 self-reactive T cells (98).

Since autoreactive T cells, like all T cells, only recognize peptides within the context of MHC ligands and require such interaction to occur in the presence of co-stimulatory molecule involvement, a third strategy for treatment of autoimmunity might

be the presentation of self-peptide without co-stimulation. In fact, there is good evidence that blockade of costimulation during ongoing disease can lead to amelioration of clinical scores (99-101). However such a strategy of using anti-costimulatory mAb to treat disease leaves the animal in a T cell immunosuppressed state, because the Abs are not specific for the self reactive T cells but rather block costimulation by APCs to all T cell specificities.

Chapter 3. Immunoglobulins as an Antigen Delivery System

Since T cells only recognize antigens within the framework of MHC molecules, the pathway of antigen processing and presentation is important in determining the type of T cell response generated. Proteins, such as viral and cellular proteins, that are synthesized within APCs are ubiquitinated, degraded by the proteasome, shuttled into the endoplasmic reticulum by TAP, and the peptide products are loaded onto MHC class I α chain associated with β_2 microglobulin for subsequent recognition by CD8+ T cells (102). In contrast, proteins that are taken up by fluid phase pinocytosis or by FcR mediated internalization, are degraded in the endosome, and following fusion with MHC class II containing vesicles bind to fresh MHC class II molecules and subsequently are recognized at the cell surface by $CD4^+$ T cells (102, 103). Cognizant recognition of the appropriate MHC-peptide complex by T cells through their TCRs leads to activation of the T cells and development of specific immunity.

Immunoglobulins can enhance T cell recognition of potentially harmful stimuli by binding to and shuttling bacterial or viral proteins to the APC for subsequent processing (102). This is accomplished by the binding of Ig-antigen complexes to the cell surface FcRs found on APCs (104). Since Igs are autologous molecules and therefore should be devoid of harmful side effects and are permissive for expression of antigenic peptides, they have been further explored as a vehicle to shuttle T cell peptides to APCs (105-110). Epitopes for both viruses (106, 108, 109) and parasites (110) have been expressed in the CDR regions of Igs and were able to generate in vivo both humoral and cell-mediated immune responses (109, 110).

A major benefit of the use of chimeric immunoglobulins is their ability to enhance the efficiency of antigen presentation. In vitro, this is exhibited by their increased efficiency relative to free peptide in activation of T cells when used as antigen (110, 111). Such delivery also allows one to overcome the short half-life of free peptide in vivo. Since Ig-chimera presentation can be partially inhibited by blockade of the FcR, it suggests that the Ig-chimeras use the same pathways for antigen presentation as Ig-Ag complexes for the efficient presentation of antigen (109). Indeed, antigenic peptide complexes are recovered from APCs at increased levels following incubation with Ig-chimeras rather than free peptide (111). Furthermore, such inhibition suggests that the Ig-chimeras might be able to use the neonatal FcR to cross the maternal placenta and drag self-peptides from mother to fetus. Cross-linking of Fc receptors (FcRs) on target cells by antigen-antibody complexes has been shown to trigger the production of cytokines (112-114). Moreover, aggregation of Igs confers the effector functions associated with the Fc fragment without the need for complex formation. Therefore Ig-chimeras could be considered as a potential strategy for the efficient introduction of antagonist ligands into an autoimmune setting, the potential transfer of self-antigen and altered self-antigen from mother to fetus for efficient presentation in the fetal thymus and periphery, and used as a means to deliver efficient peptide presentation without inducing co-stimulation to mimic peripheral tolerance and trigger anti-inflammatory cytokines to induce anergy of self reactive T cells in autoimmune diseases.

Chapter 4. Rational and Research Objectives.

While the immune system develops under a tight control that normally eliminates self-reactive T cells, some autoreactive T cells do escape and participate in peripheral immune surveillance (15-17). The research contained herein targets the silencing of those aggressive T cells and amelioration of autoimmune diseases through introduction of self- or altered-self antigen. In an ongoing disease, by nature of the unlimited supply of autoantigen and incessant generation of self-peptide-MHC complexes, continuous T cell proliferation could occur generating a high frequency of aggressive T cells. Therefore, in order for a treatment strategy to be effective, generation of sufficient self-peptide-MHC complexes or altered self-peptide-MHC complexes to anergize or eliminate the large number of aggressive T cells would be needed. Hypothetically, the efficient endocytic presentation of self-peptides would be necessary to achieve such down-regulation.

In order to address the issues of treatment or prevention of active ongoing autoimmune diseases, these studies use the encephalitogenic peptide corresponding to aa residues 139-151 of PLP (PLP1) as an autoantigen and EAE as a disease model system. PLP1 induces a relapsing/remitting EAE in SJL mice when injected in complete Freund's adjuvant (76, 85). The studies reported herein use three distinct approaches to either modulate or ameliorate autoimmune disease through the use of novel Ig-chimera molecules that contain either self-peptides, Ig-PLP1 or Ig-PLP2, or an altered self – peptide, Ig-PLP-LR, derived from modification of the two major TCR contact residues of PLP1.

The first study (Part II.) concentrates on issues concerning the requirements for reversal of active autoimmunity through TCR antagonism, since TCR antagonism is

being considered for inactivation of aggressive T cells and reversal of T cell mediated autoimmune diseases. TCR antagonist peptides have been shown to silence aggressive T cells and reverse experimental allergic encephalomyelitis (EAE) induced with free peptides (78, 95). However, it is not clear whether free antagonist peptides could reverse natural disease where the large quantities of antigen are presumably available for endocytic processing thereby allowing self-peptides to readily gain access to newly synthesized class II MHC molecules. Using an efficient endocytic presentation system, the Ig-chimera, this study explores whether an efficient endocytic presentation of antagonist peptides might be critical for reversal of spontaneous T cell mediated autoimmune diseases where incessant endocytic antigen processing could be responsible for T cell aggressivity.

During thymic development the avidity of T cell-APC interaction dictates the fate of the developing T cell (1-11). Low or moderate avidity drives positive selection (5-11), while higher avidity leads to cell death or negative selection (1-4). It has been recently suggested that peptides degenerate at TCR contact residues provide a low affinity that supports positive selection (11). Furthermore, due to antigen sequestration some autoantigens are not present within the thymus to induce negative selection. However, for unsequestered antigens, studies have also shown that peptides presented by central and peripheral APCs are nearly the same (115, 116). Therefore, how peptides used for thymic T cell development do not activate mature T cells in the periphery remains unresolved. In this study (Part III.) an Ig delivery system that shuttles either a self, which is normally sequestered, or altered self-peptide onto fetal central and peripheral APCs is examined to

determine the consequence of such presentation on T cell development and subsequent EAE induction.

The third study (Part IV.) curtails an attempt to mimic peripheral tolerance to silence T cells after clinical EAE has been exhibited. Administration of free self-peptide without adjuvant, before disease induction or prior to clinical manifestation, has been shown to silence or delay T cell activation and EAE induced by free encephalitogenic peptides (116-124), presumably through peptide presentation without costimulation (125, 126). However, the usefulness of this approach for modulation of autoimmunity following the appearance of clinical signs has been shown to be quite limited and is hampered by the unlimited supply of autoantigen at the injury site and the consequent continuous activation of the self-reactive T cells as well as the short half-life of the peptide in vivo. In addition, since bystander suppression (92) is unlikely to occur, the approach holds little promise for modulation of T cell mediated autoimmunity involving multiple antigens. The limitations on self-antigen presentation in the above strategy presumably might be over come by introducing the antigen by way of an Ig-chimera, which shows an increased efficiency of antigen presentation relative to free peptide (see Part II.). Likewise the introduction of the autologous molecule in PBS should not result in up regulation of costimulatory molecules, therein allowing a strong signal 1 in the absence of a signal 2 and lead to anergy (Figure 1b). Experimental evidence has also shown that cross-linking of Fc receptors on target APCs by aggregated antibody complexes can trigger the production of anti-inflammatory cytokines (112-114). Therefore this study will explore the efficacy of treatment of clinically evident EAE with soluble and aggregated Ig-PLP1.

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Figure 1.

Schematic representation of T cell recognition of peptide

(a) T cells recognize antigen through interaction of their TCR with peptides displayed on the surface of APCs in MHC molecules (Signal 1). However, this interaction is not always sufficient for activation of the T cell, as T cell activation and effector functions normally require a secondary interaction through the costimulatory molecules (Signal 2). (b) In the absence of these costimulatory interactions, T cells become angerized and will not respond to subsequent stimulation with the peptide-MHC complexes on APCs expressing costimulatory molecules.

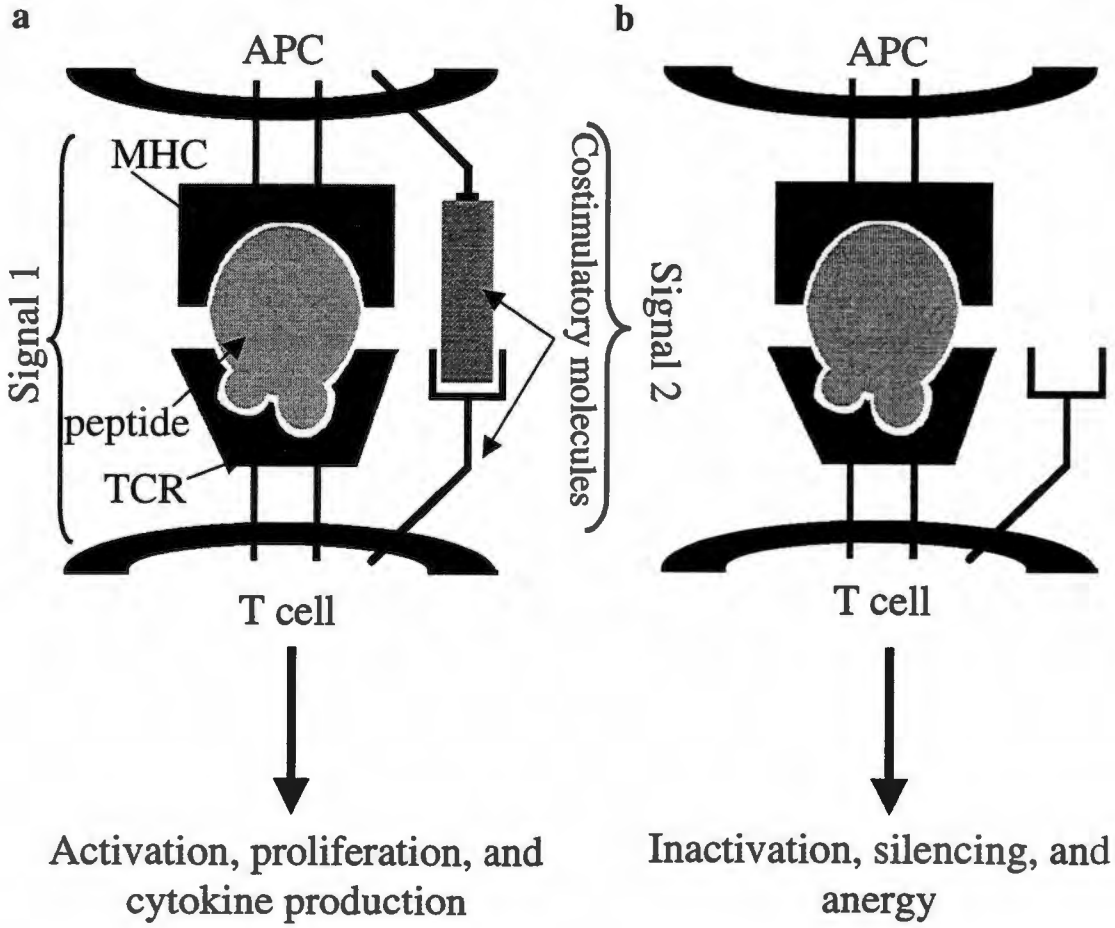
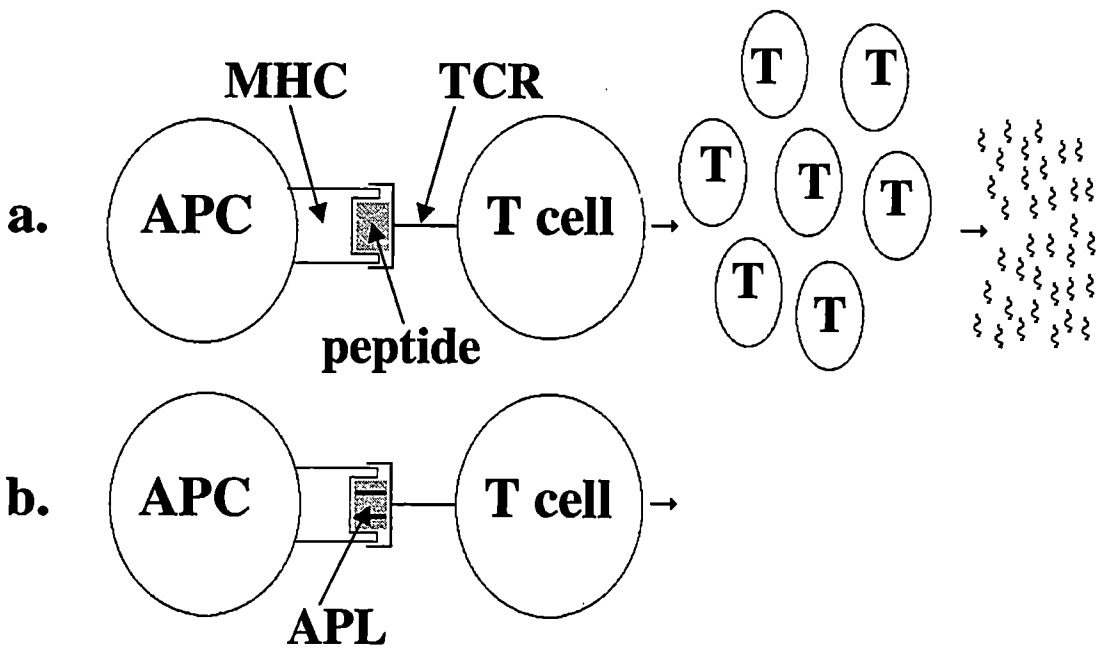

a
APC
MHC
Signal 1
peptide
TCR
T cell
Activation, proliferation, and cytokine production
b
APC
Costimulatory molecules
Signal 2
T cell
Inactivation, silencing, and anergy

Figure 2.

Consequence of T cell antagonism

(a) T cells that see their cognate (agonist) ligand in appropriate MHC molecules on the surface of APCs, respond by proliferating and producing cytokines. (b) T cells that recognize antagonist ligand-MHC complexes, are instead rendered into a state of anergy and do not proliferate or produce cytokines even when subsequently encountering agonist ligand-MHC complexes.


a.
MHC
TCR
APC
T cell
peptide
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b.
APC
T cell
APL

PART II.

Presentation of a T Cell Receptor Antagonist Peptide by Immunoglobulins Ablates Activation of T Cells by a Synthetic Peptide or Proteins Requiring Endocytic Processing.

(Journal of Experimental Medicine, 1997, 185:1043-1053)

Chapter 1. Abstract

TCR antagonism is being considered for inactivation of aggressive T cells and reversal of T cell mediated autoimmune diseases. TCR antagonist peptides silence aggressive T cells and reverse experimental allergic encephalomyelitis (EAE) induced with free peptides. However, it is not clear whether free antagonist peptides could reverse natural disease where the antigen is presumably available for endocytic processing and peptides gain access to newly synthesized class II MHC molecules. Using an efficient endocytic presentation system, it is demonstrated that a proteolipid protein (PLP) TCR antagonist peptide (PLP-LR) presented on an Ig molecule (Ig-PLP-LR) abrogates the activation of T cells stimulated with free encephalitogenic PLP peptide (PLP1), native PLP, or an Ig containing PLP1 peptide (Ig-PLP1). Free PLP-LR abolishes T cell activation when the stimulator is free PLP1 peptide but has no measurable effect when the stimulator is the native PLP or Ig-PLP1. In vivo, Ig-PLP1 induces a T cell response to PLP1 peptide. However, when co-administered with Ig-PLP-LR the response to PLP1 peptide is markedly reduced, while the response to PLP-LR is normal. Free PLP-LR co-administered with Ig-PLP1 has no effect on the T cell response to PLP1. These findings indicate that endocytic presentation of an antagonist peptide by Ig out competes both external and endocytic agonist peptides while free antagonist hinders external but not endocytic agonist peptide. Direct contact with antagonist ligand and/or trans-regulation by PLP-LR specific T cells may be the operative mechanism for Ig-PLP-LR mediated down-regulation of PLP1 specific T cells, in vivo. Efficient endocytic presentation of antagonist peptides, which is the fundamental event for either mechanism may be critical

for reversal of spontaneous T cell mediated autoimmune diseases where incessant endocytic antigen processing could be responsible for T cell aggressivity.

Chapter 2. Introduction

Over the last few years it has become clear that the avidity of T cell-APC interactions dictates thymic learning and tolerance to self antigens (1). Accordingly, high avidity interactions lead to elimination of the T cell whereas low avidity interactions allow for maturation and exit from the thymus (2-4). Although this mechanism is effective in purging the immune system of autoreactivity, T cell precursors endowed with self reactivity could still be generated if the autoantigen is sequestered and does not reach the thymus for presentation, is subjected to thymic crypticity, or is poorly presented (5-7). Superantigens capable of reacting with particular Vβ TCR (8) and events that could set to motion antigen mimicry (9), epitope spreading (10) or peripheral loosening in peptide crypticity (11), may trigger activation of those self-reactive T cells and cause antigen exposure. Continuous supply of autoantigen and abundant generation of TCR ligands may be the mechanism of T cell aggressivity. Multiple sclerosis (MS), type I diabetes, and rheumatoid arthritis, all of which are thought to be T cell mediated autoimmune diseases qualify as examples of a spontaneous break of self tolerance (12-14).

EAE, which is used as an animal model for MS, can be induced in susceptible strains of mice with myelin autoantigens such as PLP and myelin basic protein (MBP)(15). The encephalitogenic activity of these proteins correlates with the presence of peptides which induce in vivo class II restricted encephalitogenic T cells and consequently EAE (15). The peptide corresponding to amino acid (aa) residues 139-151 of PLP (hereafter is referred to PLP1) is encephalitogenic in H-2^s SJL mice (16), and T cell lines specific for PLP1 transfer EAE into naive animals (17). Although the target antigen(s) in human MS is still debatable, the frequency of T cells specific for myelin

proteins are higher in MS patients than in normal subjects (18-19). Therefore, silencing those myelin-reactive T cells may be a logical approach to reverse MS.

Interaction of T cells with altered peptide ligands could have various effects on TCR-mediated effector functions (20). These include induction of cytokine production without proliferation (21), changes in the profile of cytokines produced (22), TCR antagonism which is a cytokine and proliferative unresponsiveness (23-25), and anergy which is a state of cytokine and proliferative unresponsiveness to a subsequent stimulation with the agonist peptide (26). Peptide analogues represent an attractive approach to modulate the effector functions of aggressive T cells and ameliorate autoimmune diseases. Promising success has been achieved in the EAE system where mice induced for EAE with a free MBP encephalitogenic peptide or by transfer of an MBP-specific T cell clone recovered from the disease when they were treated with a peptide analog (27, 28). Similarly, treatment of human T cells specific for MBP with a TCR antagonist peptide modulated their cytokine production profile and increased secretion of TGFβ (22). Reversal of EAE has also been achieved with a TCR antagonist peptide derived from PLP1 peptide (29). Indeed, when the major TCR contact residues within PLP1 were mutated, the resulting peptide analog (hereafter referred to as PLP-LR), although binding to I-A^s equally as well as PLP1, does not activate PLP1-specific T cells. Instead PLP-LR inhibits in vitro activation of the T cells by PLP1. In addition, EAE induced in mice with free PLP1 peptide resolved following treatment with free PLP-LR (29). Since only a few MHC-peptide complexes are available on the surface of APCs and a single complex might be required to serially trigger about 180 TCRs to activate the T cells (30,31), the ratio of antagonist versus agonist ligands on the surface of a given APC

becomes a major factor as to whether injection of free peptide analogues could reverse spontaneous autoimmune disease where the autoantigen could be continuously available. Furthermore, the presentation of auto-antigens may operate through an endocytic pathway loading peptides onto newly synthesized MHC molecules, and generating an insurmountable agonist-MHC target to overcome. Overcoming such obstacles may demand highly effective antagonist systems. One such approach might well be peptide presentation on autologous Ig. Ig can function as a delivery system for T cell peptides (32, 33). A 100-1000 fold increase in T cell activation was observed when a class II restricted peptide from the hemagglutinin of influenza virus was presented on an Ig chimera (34). Similar results were obtained when a class II peptide from λ_2 phage repressor protein was expressed on an IgG1 molecule (35). The increase in T cell activation appears to result from efficient peptide loading onto MHC molecules (36).

In the present report, we asked whether Ig mediated endocytic presentation of an antagonist peptide could out compete high endosomal antigen load and down regulate autoreactive T cells. To this end PLP-LR antagonist peptide was expressed on an Ig molecule and the resulting Ig-PLP-LR chimera was compared with free PLP-LR for antagonism of PLP1-specific T cells. The results indicate that Ig-PLP-LR inactivates PLP1-specific T cells whether the stimulator is PLP1 peptide, native PLP, or even an Ig expressing PLP1 (Ig-PLP1). However, free PLP-LR peptide could not inhibit IL-2 production when the T cells were stimulated with APCs pulsed with Ig-PLP1 or native PLP. In vivo, when Ig-PLP1 was administered to SJL/J mice it induced a strong PLP1-specific T cell response, but when co-administered with Ig-PLP-LR, the response to PLP1 fell to almost background levels. Efficient endocytic presentation of antagonist

peptides may therefore oppose the unlimited and persistent generation of endogenous self peptides which might occur in T cell mediated autoimmune diseases such as MS.

Chapter 3. Materials and Methods

Animals

Six to eight week old SJL/J mice (H-2^s) were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in the university of Tennessee animal facility for the duration of experiments. New Zealand white rabbits were purchased from Myrtle's Rabbitry (Thompson Station, TN).

Antigens

Peptides. All peptides used in these studies were purchased from Research Genetic, Inc (Huntsville, Alabama) and purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of PLP (16). PLP-LR (HSLGKLLGRPDKF) is a mutant form of PLP1 in which Trp144 and His147 were replaced with Leu and Arg, respectively (29). PLP1 and PLP-LR bind equally well to I-A^s class II molecules (29). However, stimulation of T cell hybridomas with PLP1 in the presence of PLP-LR leads to blockade of IL-2 production by these T cells (29). PLP2 peptide (NTWTTCQSIAFPSK) encompasses an encephalitogenic sequence corresponding to aa residues 178-191 of PLP (37). This peptide binds to I-A^s class II molecules and induces EAE in SJL/J mice (37). HA110-120 peptide corresponds to aa residues 110-120 of the hemagglutinin of Influenza virus. HA110-120 binds to I-E^d class II molecules and is used here as a control peptide (34).

Ig-PLP chimeras. PLP1 and PLP-LR peptides were expressed on Ig chimeras that were designated Ig-PLP1 and Ig-PLP-LR, respectively. The genes used to construct these chimeras are those coding for the light (38) and heavy (39) chains of the IgG2b, K anti-

arsonate antibody, 91A3. The procedures for deletion of the heavy chain CDR3 region and replacement with nucleotide sequences coding for PLP1 and PLP-LR are similar to those described for the generation of Ig-NP (40), a chimera carrying a CTL epitope corresponding to aa residues 147-161 of the nucleoprotein of PR8 influenza A virus. Briefly, the 91A3V_H gene was subcloned into the EcoRI site of pUC19 plasmid and used as template DNA in PCR mutagenesis reactions (40) to generate 91A3V_H fragments carrying PLP1 (91A3V_H-PLP1) and PLP-LR (91A3V_H-PLP-LR) sequences in place of CDR3. Nucleotide sequencing analysis indicated that full PLP1 and PLP-LR sequences were inserted in the correct reading frame. The 91A3V_H-PLP1 and 91A3V_H-PLP-LR fragments were then subcloned into the EcoRI site of pSV2-gpt-Cγ2b in front of the exons coding for the constant region of a Balb/c γ2b which generated pSV2-gpt-91A3V_H-PLP1-Cγ2b and pSV2-gpt-91A3V_H-PLP1-LR-Cγ2b plasmids, respectively. These plasmids were then separately cotransfected into the non-Ig producing SP2/0 B myeloma cells with an expression vector carrying the parental 91A3 light chain, pSV2-neo-91A3L (38,40). Transfectants producing Ig chimeras were selected in the presence of geneticin and mycophenolic acid. Transfectants were cloned by limiting dilution, and final clones secreted 1 to 4 μg/ml of Ig-PLP chimeras. All the cloning, sequencing, and purification procedures are similar to those used to generate Ig-NP (40) and Ig-HA (34). Nucleotide sequences and detailed mutagenesis procedures for Ig-PLP1 and Ig-PLP-LR are published elsewhere.

Large scale cultures of transfectants were carried out in DMEM media containing 10% iron enriched calf serum (Intergen, New York). Ig-PLP chimeras were purified from culture supernatant on columns made of rat-anti-mouse kappa chain coupled to CNBr

activated Sepharose 4B (Pharmacia). To avoid cross contamination separate columns were used to purify the chimeras.

PLP. PLP was purified from rat brain according to a previously described procedure (41). Briefly, the brain was homogenized in 2/1 vol/vol chloroform/methanol, and the soluble crude lipid extract separated by filtration through a scintered glass funnel. PLP was then precipitated with acetone, and the pellet redissolved in a mixture of chloroform/methanol/acetic acid and passed through an LH-20-100 Sephadex column (Sigma) to remove residual lipids. Removal of chloroform from the eluates and conversion of PLP into its apoprotein form were carried out simultaneously through gradual addition of water under a gentle stream of nitrogen. Subsequently, extensive dialysis against water was performed to remove residual acetic acid and methanol.

Production of rabbit anti-peptide antibodies

PLP1 and PLP-LR peptides were coupled to KLH and BSA as described (42). Rabbits were immunized with 1 mg peptide-KLH conjugates in CFA and challenged monthly with 1 mg conjugate in IFA until a high antibody titer was reached as described (43). The peptide-BSA conjugates were coupled to sepharose and used to purify anti-peptide antibodies from the rabbit anti-serum.

Radioimmunoassay (RIA)

Capture RIA was used to assess expression of PLP peptides on Ig. Microtiter 96-well plates were coated with rabbit anti-peptide antibodies (5μg/ml) overnight at 4°C and blocked with 2% BSA in PBS for 1 hour at room temperature (RT). The plates were then

washed 3 times with PBS, and graded amounts of Ig-PLP chimeras were added and incubated for 2 hours at RT. After 3 washes with PBS, captured Ig-PLP chimeras were revealed by incubating the plates with 100 x 10^3 cpm/well ^{125}I-labeled rat anti-mouse kappa mAb for 2 hours at 37°C. The plates were then washed 5 times with PBS and counted using an LKB gamma counter.

Cells

PLP1-specific T cell hybridomas 5B6 and 4E3 (29) and the IL-2 dependent HT-2 T helper were obtained from Drs M.B. Lees and V. Kuchroo (The Eunice Kennedy Shriver Center, Waltham, MA). The 5B6 and 4E3 T cells recognize PLP1 in association with I-A^s and produce IL-2 in response to it (29). However, when stimulated with PLP1, then with PLP-LR, they become unable to produce IL-2 (29). The rat anti-mouse kappa chain mAb (HB-58) and the mouse anti-rat kappa light chain mAb (MAR 18.5, TIB 216) were obtained from ATCC. These hybridomas were grown to large scale and purified from culture supernatant on each other. The rat anti-mouse kappa mAb was used to prepare columns on which Ig-PLP chimeras were purified from culture supernatant.

T cell activation assay

Irradiated (3,000 rads) SJL splenocytes (used as APCs) were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with graded concentration of antigens (100 μl/well). After one hour T cell hybridomas (5 X 10^4 cells/well/50 μl) were added and the culture was continued overnight. Activation of the T cells was assessed by measuring production of IL-2 in the culture supernatant. This was done by ^{3}H-thymidine

incorporation using the IL-2 dependent HT-2 cells. Briefly, culture supernatants (100 μl/well) were incubated with HT-2 cells (1x 10^4/100 μl/well) in 96-well flat bottom plates for 24 hours. Subsequently, 1 μCi ^{3}H-thymidine was added per well and the culture continued for an additional 12-14 hours. The cells were then harvested on glass fiber filters and incorporated ^{3}H-thymidine was counted using the trace 96 program and an Inotech β counter. The culture media used to carry out these assays were DMEM supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium puryvate and 50 μg/ml gentamycin sulfate.

Assay for inhibition of T cell activation

Irradiated (3,000 rads) SJL/J splenocytes (used as APCs) were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with the stimulator antigen (optimal dose in 50 μl/well) and graded concentration of inhibitor (100 μl/well) for 1 hour. Subsequently, T cell hybridomas (5 x 10^4 cells/well/50 μl) were added and the culture continued overnight. IL-2 production in the supernatant, which was used as a measure of T cell activation, was determined using HT-2 cells, as above.

Immunization of mice with Ig chimeras and peptides

Immunization with Ig-PLP1. Mice were immunized s.c. in the foot pads and at the base of the limbs and tail with 50 µg of Ig-PLP1 emulsified in a 200 µl mixture 1vol/1vol PBS/CFA. Ten days later the mice were sacrificed by cervical dislocation, the spleens and lymph nodes (axillary, inguinal, popliteal, and sacral) were removed, single cell suspensions prepared, and the T cell responses analyzed as described below.

Co-immunization of mice with Ig-PLP1 and Ig-PLP-LR, Ig-W or PLP-LR peptide. Individual mice from three groups (4 mice per group) were injected s.c. as above with a 200 µl mixture (PBS/CFA, 1vol/1vol) containing 50 µg Ig-PLP1 and 150 µg Ig-PLP-LR; 50 µg Ig-PLP1 and 150 µg Ig-W; or 50 µg Ig-PLP1 and 100 µg PLP-LR peptide. Splenic and lymph node T cell responses were analyzed at day 10 post immunization.

Assays for spleen and lymph node proliferative responses

Lymph node and spleen cells were incubated in 96-well round bottom plates at 4 and 10 x 10^5 cells/100 µl/well, respectively, with 100 µl of stimulator for three days. Subsequently, 1 μCi ^{3}H-thymidine was added per well, and the culture continued for an additional 12-14 hours. The cells were then harvested on glass fiber filters, and incorporated ^{3}H-thymidine counted using the trace 96 program and an Inotech β counter. The stimulators were used at the following concentrations: PLP1, PLP2, and PLP-LR peptides at 15 µg/ml, and PPD at 5 µg/ml. A control of media with no stimulator was included for each mouse and used as background.

Chapter 4. Results

Expression of PLP peptides on Ig molecules.

Two Ig-PLP chimeras designated Ig-PLP1 and Ig-PLP-LR were constructed to include PLP1 and PLP-LR peptide, respectively (Figure 1). In both cases the heavy chain CDR3 loop was deleted and replaced with nucleotide sequences coding for the selected peptide. DNA sequencing analysis indicated insertion of peptide nucleotide sequences in the correct reading frame. In addition, rabbit antibodies to synthetic PLP1 and PLP-LR peptides recognized the chimeras (Figure 2). Indeed, when Ig-PLP1 and Ig-PLP-LR were incubated on plates coated with rabbit anti-PLP1 antibodies, they were captured by these rabbit antibodies and bound ^{125}I-labeled rat anti-mouse kappa chain mAb (Figure 2a). Similarly, both Ig-PLP1 and Ig-PLP-LR were captured by rabbit anti-PLP-LR (Figure 2b). Ig-W, the wild type 91A3 antibody without peptide and an IgM control antibody, did not show significant binding to the rabbit antibodies. Ig-PLP1 bound to both anti-PLP1 and anti-PLP-LR antibodies better than Ig-PLP-LR, indicating that structural differences affected accessibility of the peptides to the rabbit antibodies. The above experiments also indicated that peptide expression on the chimeras did not alter heavy and light chain pairing because the rabbit antibodies bind to the PLP peptide on the heavy chain and the rat anti-kappa binds on the light chain.

Presentation of Ig-PLP chimeras to T cells.

The CDR3 of the 91A3 Ig is permissive for peptide expression, and both class I and class II-restricted epitopes have been efficiently processed and presented to T cells when grafted in place of the D segment (34,40). Ig-PLP1, which includes the PLP1

peptide within CDR3, is also presented to specific T cells (Figure 3). T cell hybridomas 5B6 and 4E3 specific for PLP1 produced IL-2 subsequent to stimulation with APCs pulsed with Ig-PLP1 as they have done when pulsed with PLP1 and native PLP. The negative controls Ig-W, Ig-HA, and PLP2 peptide did not induce the production of IL-2 by the T cells. Both Ig-PLP-LR and PLP-LR peptide do not stimulate 5B6 and 4E3 for production of IL-2 (Figure 3). These results are expected because PLP-LR peptide is known to negate rather than stimulate IL-2 production. However, while these experiments could not show the processing and presentation of Ig-PLP-LR, we have evidence that PLP-LR peptide is released from the chimeras and is presented to the T cells (see below).

Efficient presentation of Ig-PLP1 to T cells.

In spontaneous disease, exposure and continuous endocytic presentation of autoantigen may generate significant levels of MHC-agonist complexes. Ig-PLP1 was constructed for the purpose of establishing a peptide delivery system that could efficiently operate through the endocytic pathway and generate high levels of agonist ligand and as such it provides a relevant system to investigate T cell antagonism in a situation similar to presentation of autoantigens. It is therefore important to determine the efficacy of Ig-PLP1 in peptide delivery and presentation to specific T cells. To this aim, dose response T cell activation assays were performed with free PLP1 peptide, native PLP, and Ig-PLP1. The results shown in figure 4 indicate that the PLP1 T cell epitope was better presented by Ig-PLP1 than by native PLP or by free PLP1 peptide. Although the plateau of IL-2 production was higher when the T cell stimulator is PLP1 synthetic

peptide, the individual half maximal IL-2 production by the T cells required about 100 fold higher of PLP or PLP1 peptide than Ig-PLP1 (Figure 4). The efficacy of Ig-PLP1 in peptide delivery may be related to FcR mediated internalization and access to newly synthesized MHC molecules, as we have previously shown for Ig-HA (34,36), while PLP may internalize by simple fluid phase pinocytosis and PLP1 peptide may bind to empty MHC class II molecules at the cell surface. Overall Ig-PLP1 is efficient in loading PLP1 peptide onto class II molecules within the endosomal compartment.

Inhibition of T cell activation by Ig-PLP-LR.

The potency of Ig-PLP1 chimeras in peptide loading onto class II molecules provides a situation that probably resembles in vivo autoimmune circumstances, where a continuous supply of antigen may allow for abundant generation of self peptides, which could trigger T cell aggressivity. The Ig-PLP1 endocytic presentation system was then used to investigate Ig-PLP-LR for inactivation of PLP1-specific T cells. As shown in figure 5a, when T cells were incubated with APCs in the presence of both PLP1 and Ig-PLP-LR, a specific decrease in IL-2 production occurred as the concentration of Ig-PLP-LR increased. These results are in agreement with a previous report, which showed that efficient endocytic presentation of an antagonist form of hemoglobin out competed an external agonist peptide (44). A similar decline in IL-2 production was evident when the synthetic PLP-LR peptide was used during T cell activation with PLP1 peptide. Antagonistic effects were not observed with the Ig-W chimera or PLP2 peptide used as negative controls (Figure 5a). The half maximal inhibition of IL-2 production (60% control thymidine incorporation) required 0.4 μM Ig-PLP-LR versus 9 μM PLP-LR

peptide indicating a more efficient presentation of, and consequently T cell antagonism by Ig-PLP-LR (Figure 5a).

Further evidence that the chimera is more efficient than the free peptide in T cell antagonism is shown in figure 5b and 5c. Ig-PLP-LR inhibited T cell activation mediated by Ig-PLP1 (Figure 5b) while free PLP-LR did not show any significant antagonism like the negative control PLP2 peptide (Figure 5b). Ig-W, the wild type 91A3 Ig without peptide, showed partial inhibitory activity in Ig-PLP1 mediated T cell activation (Figure 5b). This is likely the result of competition for binding to the FcR on APCs because both Ig-PLP1 and Ig-W share identical IgG2b constant regions. As the concentration of Ig-W increases, less Ig-PLP1 will bind to FcR and internalize into the APCs, resulting in a diminished presentation and IL-2 production. Ig-W had similar inhibitory effects on the presentation of Ig-HA, as did the anti-FcR mAb 2.4G2 (34). Finally, Ig-PLP-LR, but not Ig-W, abolished the activation of T cells by native PLP (Figure 5c). However PLP-LR and the negative control PLP2 peptide did not inhibit PLP mediated T cell activation.

Competition for binding to class II molecules seems not to be the operative mechanism of antagonism at the endocytic level. This conclusion is drawn from the observation that Ig-PLP2, a chimera carrying PLP2 peptide, did not inhibit PLP mediated T cell activation (Figure 6) even though Ig-PLP2 is presented by I-A^s like PLP1.

In vivo antagonism of PLP1-specific T cells by Ig-PLP-LR.

As demonstrated in figure 7, when individual mice were immunized with Ig-PLP1 they developed strong PLP1-specific T cell responses in the lymph nodes (Figure 7a) and even significant proliferation in the spleen (Figure 7b). Consequently, Ig-PLP1, which is

presumably processed in endocytic vacuoles like autoantigens, provides a relevant system to assay the antagonists Ig-PLP-LR and PLP-LR peptide for in vivo T cell antagonism.

The results in figure 8 indicate that co-immunization of mice with Ig-PLP1 and Ig-PLP-LR led to a reduced T cell response to PLP1 when compared to responses obtained in mice injected with the Ig-PLP1/Ig-W mixture. Both lymph node (Figure 8a) and splenic (Figure 8b) T cell responses were markedly reduced as a consequence of co-administration of Ig-PLP-LR with Ig-PLP1.

Because Ig-PLP-LR could induce a T cell response to PLP-LR, lymph node and spleen cells from mice immunized with the Ig-PLP1/Ig-PLP-LR mixture were stimulated in vitro with PLP-LR peptide, and the specific ^{3}H-thymidine incorporation was measured and compared with that of PLP1 specific proliferation. The results depicted in figure 9 indicate that PLP-LR-specific T cells were present in both the lymph nodes (Figure 9a) and spleen (Figure 9b), and the specific proliferation to PLP-LR was 2 to 9 fold higher than the proliferation to PLP1.

Mice co-immunized with Ig-PLP1 and free PLP-LR peptide, showed no evidence for reduction of PLP1-specific responses (Table 1). To minimize the role of individual and experimental intrinsic variability on the overall outcome of the in vivo experiments, the PLP1-specific proliferations were expressed as percent of the individual response to PPD (Table 1). The standardized results clearly indicated a fall in the PLP1-specific response in the mice injected with Ig-PLP1 and Ig-PLP-LR relative to those injected with the Ig-PLP1/Ig-W or Ig-PLP1/ PLP-LR peptide mixtures.

Chapter 5. Discussion

Herein, we designed an endocytic antigen presentation system and evaluated fundamental mechanisms as to whether TCR antagonist peptides could overcome antigens that because of efficient supply and access to endocytic processing could generate high levels of encephalitogenic peptides and therefore MHC-agonist complexes. In this system PLP1 peptide and a TCR antagonist form of it, PLP-LR were expressed on the anti-arsonate antibody, 91A3, and the resulting Ig-PLP1 and Ig-PLP-LR chimeras were utilized to evaluate T cell antagonism in an antigen system requiring endocytic processing as might occur in natural autoimmune diseases. Both Ig-PLP1 and Ig-PLP-LR could be captured by rabbit antibodies to the synthetic peptides and bind rat anti-mouse kappa mAb indicating peptide expression and proper pairing of the heavy and light chains (Figure 2). Ig-PLP1 was presented to T cells in a specific manner indicating that the PLP1 peptide was released from the Ig and bound class II I-A^s molecules (Figure 3). In this case the flanking regions seem to have no interfering effect on the presentation of Ig-PLP1, as has been observed for other T cell peptides expressed on proteins unrelated to their own environment (32-35, 45, 46). The presentation of Ig-PLP1 was 100 fold better than free PLP1 peptide (Figure 4). This observation parallels with results obtained with an IgG1 chimera expressing a T cell peptide from λ_2 phage repressor protein (35) and with Ig-HA (34). The efficacy of Ig-PLP1 in activating specific T cells is probably in part due to efficient internalization via FcR as has previously been seen for Ig-HA (34). Moreover, since Ig-PLP1 is presumably, like Ig-HA, processed in endocytic vacuoles, the released PLP1 peptides access newly synthesized class II molecules, and allow for the formation of significant amounts of peptide-class II complexes (36). Ig-

PLP-LR is also taken up by APCs, processed, and presented to T cells otherwise it would not have inhibited PLP1 mediated T cell stimulation. Indeed, when APCs were incubated with PLP1 peptide in the presence of Ig-PLP-LR there was no activation of the PLP1-specific T cell hybridomas (Figure 5a). Ig-PLP-LR was more potent than free PLP-LR peptide in inhibiting PLP1 mediated T cell activation indicating a better presentation of the peptide when delivered on the Ig-chimera, as was the case for PLP1. These results confirm the observation by Vidal et al (44) showing that efficient endocytic presentation of an antagonist peptide could out compete an external agonist and inhibit IL-2 production by specific T cells.

Furthermore, when the activation of T cells by native PLP and Ig-PLP1 was carried out in the presence of graded concentrations of Ig-PLP-LR, IL-2 production declined as Ig-PLP-LR increased. However, free PLP-LR peptide failed to inhibit T cell activation mediated by native PLP or Ig-PLP1 (Figure 5). A maximum of 50% inhibition in IL-2 production was seen when the activation of T cells by Ig-PLP1 was carried out in the presence of Ig-W (Figure 5b). Ig-PLP1 and Ig-W have an identical heavy chain constant region and use the same FcR to internalize into APCs. Therefore, Ig-W could out compete Ig-PLP1 for internalization and diminish the activation of T cells. Ig-W had a similar effect on the presentation of Ig-HA (34) but had no effect on the activation of T cells by native PLP (Figure 5c).

While free PLP-LR antagonized only activation mediated by free PLP1 peptide, the spectrum of antagonism by Ig-PLP-LR broadened to include antigen requiring endocytic processing such as native PLP and Ig-PLP1 (Table 2). Two lines of evidence indicated that the mechanism responsible for PLP-LR and Ig-PLP-LR mediated

inactivation of T cells was likely to be TCR antagonism rather than blockage of class II molecules. At the extracellular level, PLP2 peptide, which also uses I-A^s class II molecules for presentation (37), did not inhibit the activation of T cells by free PLP1 peptide. At the endocytic level, Ig-PLP2, which is presented by I-A^s, did not antagonize native PLP for the activation of T cells. Competition for binding to class II may take place. However, a living antigen presenting system, such as the one we used, and the design of our experimental approach are not suitable for optimal blockade as demonstrated by the control experiments using PLP2 peptide and Ig-PLP2 chimera. Therefore, one can speculate that TCR engagement with PLP-LR-I-A^s complexes on the surface of APCs antagonizes the cells rather than stimulates them. If we retain this possibility, one may explain the antagonism by Ig-PLP-LR as follows: because of efficient presentation of Ig-PLP-LR in endocytic vacuoles, significant levels of PLP-LR-I-A^s complexes are generated. The amount of complexes on the cell surface would be proportional to the amount of Ig-PLP-LR offered to the APCs. When PLP1 stimulation is carried out in the presence of Ig-PLP-LR, both PLP-LR-I-A^s and PLP1-I-A^s are present on the surface of a given APC and an increase in the concentration of Ig-PLP-LR leads to higher number of PLP-LR-I-A^s complexes. Considering that approximately 8000 TCR have to be engaged in order for a T cell to be activated (47), and that a given complex of peptide-class II can serially engage up to 200 TCRs (31), a T cell is antagonized when TCR engagement with PLP-LR-I-A^s complexes override engagement with PLP1-I-A^s. Overall, because of efficient loading of PLP-LR by Ig-PLP-LR, T cell antagonism is achieved by a higher frequency of serial triggering of TCRs by PLP-LR-I-A^s complexes. This is probably more conceivable when Ig-PLP-LR is engaged in antagonizing native

PLP or Ig-PLP1, all of which are processed in endocytic vacuoles. How could Ig-PLP-LR antagonize PLP1 peptide, a stimulator that may not require processing but rather binds directly to cell surface class II molecules? One possibility is that only a limited number of PLP1-class II complexes could be generated because external PLP1 binds empty class II and/or displaces other peptides from I-A molecules. These conditions may limit the number of complexes that could be available for stimulation. Another possibility is that the natural turnover of cell surface MHC molecules contributes to the shorter stay of complexes formed at the extracellular milieu (class II molecules have been on the cell surface for some time before binding the extracellular peptide), while complexes formed in the endocytic compartment will reside for a normal period of time because they have just been translocated to the cell surface. This may also be the reason why PLP-LR could not antagonize Ig-PLP1 or PLP but did antagonize PLP1 peptide. Considering recent findings that complexes made of MHC-antagonist peptide engage the TCR for a shorter period of time than those made of MHC-agonist peptide (48), we lean toward the possibility that external peptide forms very few complexes that have shorter stays at the cell surface, and that endocytic processing is more effective for the generation of MHC-peptide complexes that could trigger more TCRs because of longer residency at the cell surface. Overall, internalization via FcR of Ig chimeras and efficient endocytic presentation may be responsible for the broad antagonism by Ig-PLP-LR, and the formation of fewer short lived complexes, when the peptide is externally added to the APCs, maybe responsible for the inability of PLP-LR to antagonize the endocytic presentation of PLP and Ig-PLP1. Overall, this demonstrates for the first time that competition between agonist and antagonist at the endocytic level is achievable but this

only occurs when the antagonist peptide is efficiently presented within the endocytic compartment.

In vivo, when Ig-PLP1 was injected subcutaneously in the foot pads and at the base of the limbs and tail, routes that mostly target the response to the lymph nodes, a strong specific T cell response to PLP1 peptide was induced (Figure 7). These results are expected considering that Ig-PLP1 was efficient in presenting the peptide to T cells in vitro (Figure 3) and that Ig-HA has been shown to prime a strong HA-specific T cell response (34). However, interestingly there is a significant PLP1 specific response detected in the spleen, an organ that mostly filters and responds to systemic Ags (Figure 7b). One possibility that can be put forth to explain these results is that Ig-PLP1, because of it's long half life, was able to circulate and reach both the lymphatic and blood circulation and consequently be presented at both systemic and lymphatic sites.

Although Ig-PLP1 was efficiently presented and induced a strong in vivo T cell response, it was possible to antagonize such a response by Ig-PLP-LR (Figure 8). Indeed, when Ig-PLP1 was co-administered to mice with Ig-PLP-LR, the response to PLP1 peptide was markedly reduced. This decline in PLP1 response was specifically induced by Ig-PLP-LR, because when Ig-PLP1 was co-administered with Ig-W instead of Ig-PLP-LR, the response to PLP1 was not affected. Efficient in vivo endocytic presentation of Ig-PLP-LR may be the fundamental basis for the decline in the PLP1-specific response. The failure of PLP-LR peptide to inhibit Ig-PLP1 mediated T cell activation in vitro coupled with the potency of Ig-PLP-LR in antagonizing Ig-PLP1 T cell stimulation supports the belief that Ig-PLP-LR mediated in vivo antagonism may be related to efficient presentation. Moreover, when free PLP-LR peptide was co-administered with

Ig-PLP1 there was no evidence for a decline of the PLP1 response (Table 1). The lack of antagonist effect by free PLP-LR peptide was not due to a net lower amount of injected peptide because the mice were given approximately 34 fold more PLP-LR in the free peptide form than Ig-PLP-LR form (on the basis of a MW of 150,000 d, the 150 μg Ig-PLP-LR given to the mice correspond to 1 nmole of Ig that contains 2 nmoles of PLP-LR peptide, while with a molecular weight of 1,468 daltons, the 100 μg of free PLP-LR peptide correspond to 68 nmoles of peptide). The mechanism by which Ig-PLP-LR reduced the response to PLP1 is not clear. However, knowing that Ig-PLP-LR induced PLP-LR specific T cells (Figure 8) when it was co-administered with Ig-PLP1, it can be speculated that these PLP-LR-specific T cells down-regulate PLP1 specific T cells (49). Although there was induction of a PLP-LR-specific response when free PLP-LR peptide was administered with Ig-PLP1, there was no evident reduction in the proliferative response to PLP1. Further studies are required to identify any qualitative differences among T cells induced by Ig-PLP-LR and those induced by PLP-LR peptide. Another possibility that could explain the reduction in T cell response to PLP1 is in vivo antagonism by PLP-LR-MHC complexes. Ig-PLP1 and Ig-PLP-LR have identical isotypes and could bind the same FcR and internalize into the same APCs. Simultaneous presentation of PLP-LR and PLP1 by the same APCs could, as is seen in the in vitro assays, be responsible for the antagonism of PLP1 specific T cells by Ig-PLP-LR. The striking features associated with this endocytic antagonist system are its high efficacy and its broad spectrum of activity against free peptides and most importantly autoantigens, which require endocytic processing. Indeed, our data demonstrate for the first time that competition between agonist and antagonist is achievable at the endocytic level allowing

the down regulation of autoreactive T cells, in vivo. Efficient endocytic presentation of peptide analogs may operate through mechanisms that could overcome the abundant MHC-agonist complexes generated in spontaneous disease subsequent to the eruption and continuous endocytic presentation of autoantigens.

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Table 1. Ig-PLP-LR but not free PLP-LR peptide mediates T cell antagonism in vivo.

Mouse	Ig-PLP1 co-administered with		
	Ig-W	Ig-PLP-LR	PLP-LR peptide
		PLP1/PPD* (%)	
1	100	28	81
2	95	40	91
3	78	37	93
4	79	25	100

Three groups of mice (4 per group) were respectively, immunized with 50 μg Ig-PLP1 mixed with 150 μg Ig-W; 50 μg Ig-PLP1 mixed with 150 μg Ig-PLP-LR; and 50 μg Ig-PLP1 mixed with 100 μg PLP-LR peptide. After ten days the lymph nodes were removed and 4 x 10^5 cells were stimulated in vitro with 15 μg/ml PLP1 peptide, 15 μg PLP2 peptide, 5 μg/ml PPD, or media without stimulator and assayed for ^{3}H-thymidine incorporation as described in Chapter 3 Materials and Methods. The mean cpm obtained for media without stimulator was used as background.

* The indicated numbers represent percentage values of PLP1-specific proliferation relative to PPD specific proliferation and were estimated as follows: (mean cpm of triplicates obtained with PLP1 stimulation - mean cpm triplicate BG) / (mean cpm of triplicates obtained with PPD - mean cpm triplicate BG) x 100.

The percentage values of PLP2-specific proliferation relative to PPD ranged between 0 and 15% indicating the absence of significant proliferation to PLP2 peptide (used as negative control).

Table 2. Ig-PLP-LR and PLP-LR mediated T cell antagonism in vitro.

	Stimulator		
Antagonist	**PLP1**	**PLP**	**Ig-PLP1**
PLP-LR	+	-	-
Ig-PLP-LR	+	+	+

This summarizes the effect of PLP-LR and Ig-PLP-LR on IL-2 production by PLP1-specific T cell hybridomas when they are stimulated with APCs pulsed with the stimulators Ig-PLP1, PLP1, or native PLP in the presence of PLP-LR or Ig-PLP-LR.

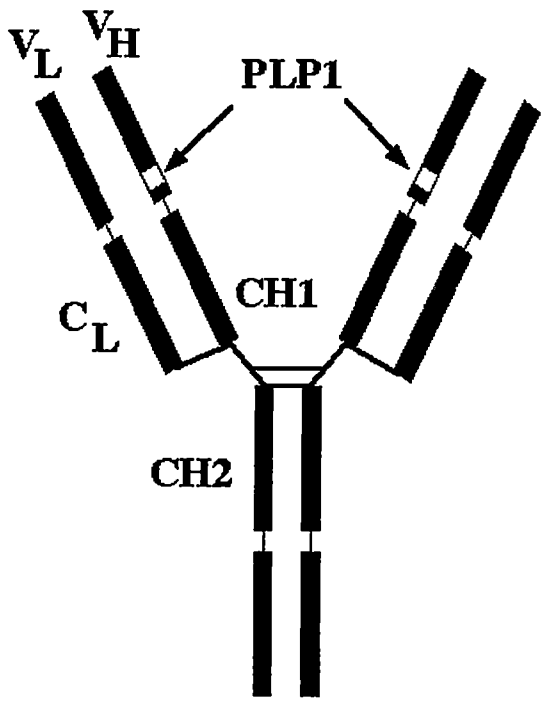
A plus sign indicates inhibition of IL-2 production and therefore antagonism. A minus sign indicates absence of inhibition of IL-2 production and therefore no antagonism.

Figure 1.

Schematic representation of Ig-PLP1 and Ig-PLP-LR.

The CDR3 loop (D segment) of the heavy chain variable region of the anti-arsonate antibody, 91A3, was deleted and replaced with nucleotide sequences that encode PLP1 and PLP-LR peptides. These chimeric V_H genes were then ligated to a gene encoding a Balb/c γ2b constant region to generate a complete chimeric heavy chain. These heavy chains were then cotransfected with parental 91A3 kappa light chain into the non-Ig secreting myeloma cell SP2/0 to generate a complete IgG2b, K chimera carrying PLP1 (Ig-PLP1) and PLP-LR (Ig-PLP-LR) peptide. Other chimeras were also used as controls. These are: Ig-HA, an Ig molecule carrying in place of the D segment the HA110-120 T helper epitope from the HA of influenza virus and differs from Ig-PLP1 and Ig-PLP-LR only by the peptide inserted within CDR3. Ig-W is the product of an unmodified (wild type) 91A3V_H gene, Balb/c γ2b constant region and 91A3 kappa light chain. Therefore, it differs from Ig-PLP1 and Ig-PLP-LR in the CDR3 region, which is the parental D segment. Ig-PLP2 is a chimera that carries within the heavy chain CDR3 loop aa residues 178-191 of PLP.

Ig-PLP1



Ig-PLP-LR

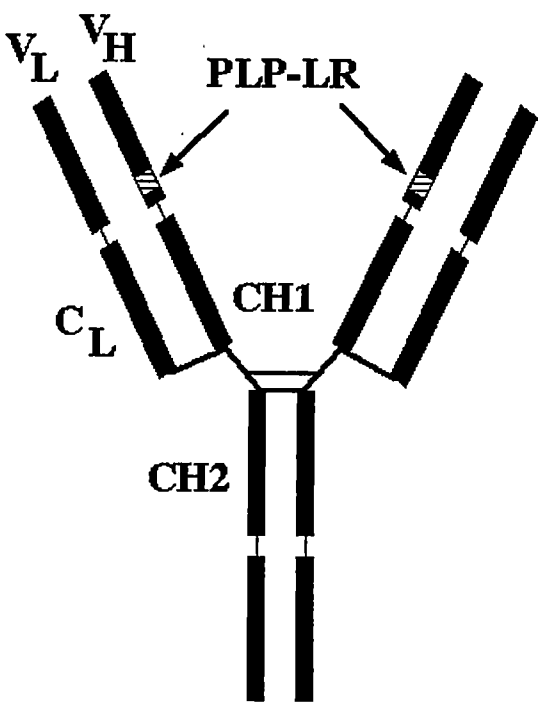


Figure 2.

PLP peptide expression on Ig-PLP1 and Ig-PLP-LR.

Rabbit antibodies to PLP1 and PLP-LR were used in a capture RIA to demonstrate peptide expression on the chimeras. Micotiter plates were coated with affinity purified rabbit antibodies to PLP1 (a) or to PLP-LR (b), blocked with BSA and incubated with graded amounts (27, 9, 3, 1 μg/ml) of Ig-PLP1, Ig-PLP-LR, or negative control Ig-W. Captured Igs were revealed with ^{125}I-labeled rat anti-mouse kappa light chain mAb. Shown are the mean ± SD of triplicates obtained with 27 μg/ml of chimeras.

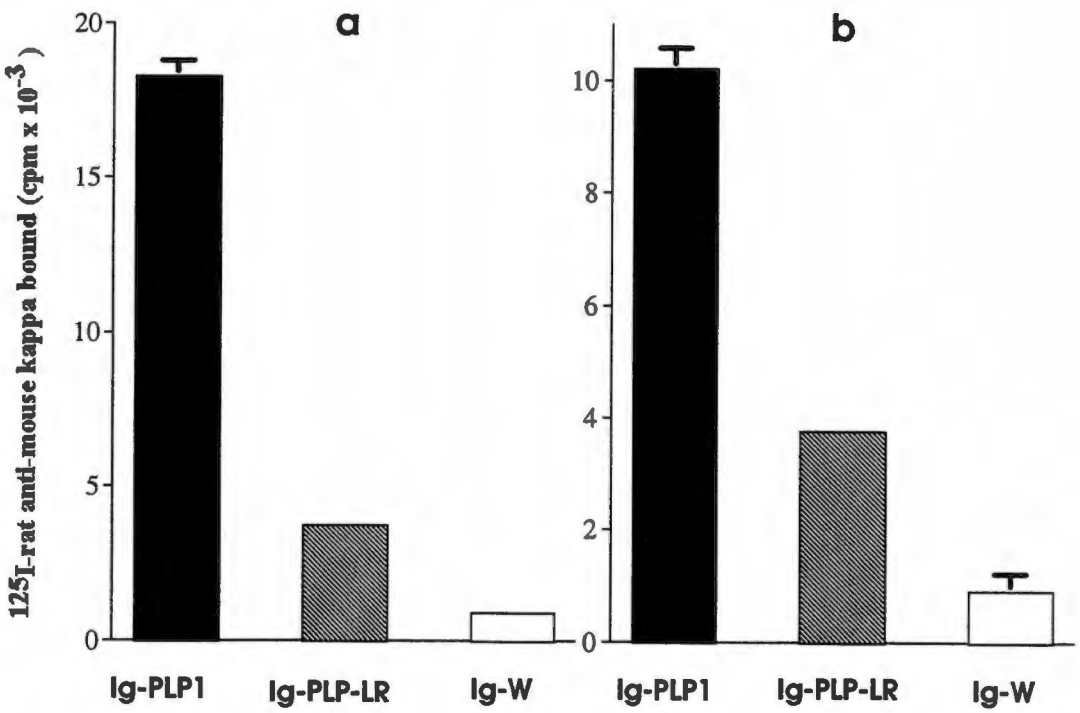

a
b
^{125}I-rat anti-mouse kappa bound (cpm x 10^{-3})
20
15
10
5
0
10
8
6
4
2
0
Ig-PLP1
Ig-PLP-LR
Ig-W
Ig-PLP1
Ig-PLP-LR
Ig-W

Figure 3.

Presentation of Ig-PLP chimeras to PLP1-specific T cell hybridomas.

Ig-PLP1 and Ig-PLP-LR were assayed for presentation to the PLP1-specific T cell hybridomas 4E3 (a) and 5B6 (b) by measurement of IL-2 production as indicated in Chapter 3 Materials and Methods section. Irradiated SJL/J splenocytes (as APCs) were incubated with the indicated antigens and T cells overnight, and IL-2 content of the supernatant measured by ^{3}H-thymidine incorporation using the IL-2 dependent HT-2 cells. The concentration of antigen was 0.1 μM for Ig-PLP1, Ig-PLP-LR, Ig-HA, and Ig-W; 1 μM for PLP1 and PLP2 peptides; and 1.7 μM for PLP. Each value represents the mean ± SD of triplicate wells.

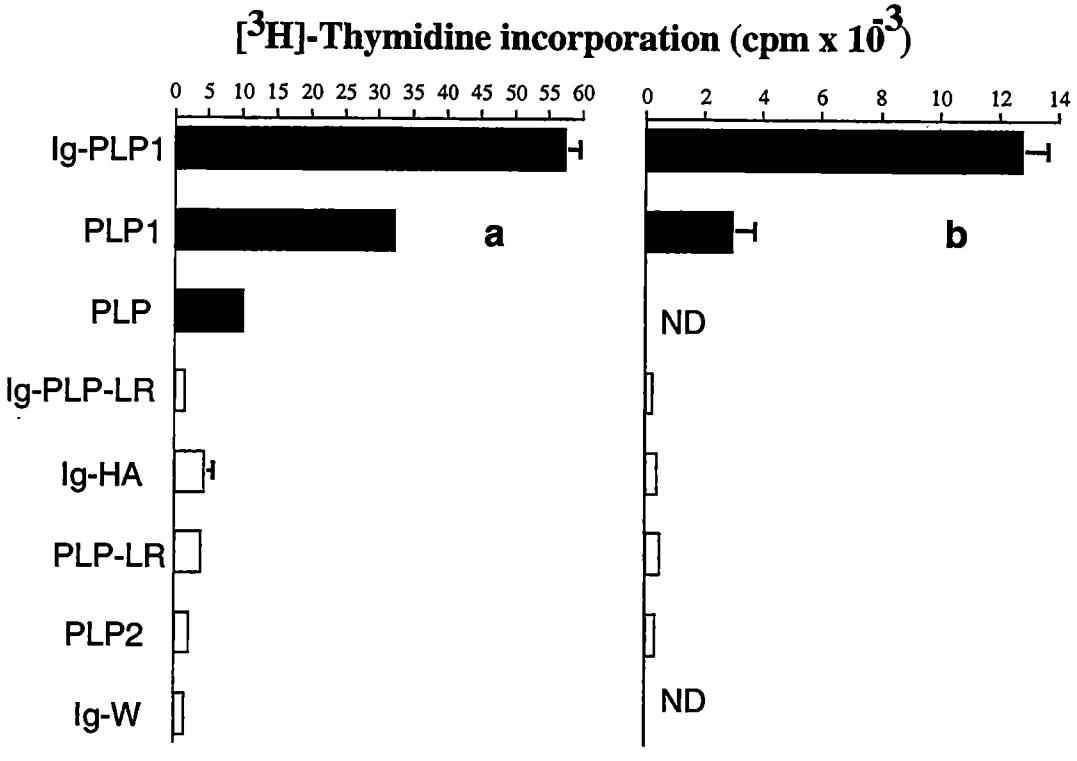

[3H]-Thymidine incorporation (cpm x 10-3)
0 5 10 15 20 25 30 35 40 45 50 55 60
0 2 4 6 8 10 12 14
Ig-PLP1
PLP1
PLP
Ig-PLP-LR
Ig-HA
PLP-LR
PLP2
Ig-W
a
b
ND
ND

Figure 4.

Efficient presentation of Ig-PLP1 to 4E3 T cell hybridoma.

Graded amounts of each antigen were incubated with splenic SJL/J APCs and the PLP1-specific 4E3 T cell hybridoma, and IL-2 production measured by ^{3}H-thymidine incorporation using IL-2 dependent HT-2 cells as described in the legend to figure 2. Each point represents the mean of triplicates. The standard deviation did not exceed 10% of the mean value.

Although the maximal activation varied among the three different stimulators, the individual half maximal activation required less Ig-PLP1 (0.005 μM) than PLP (0.5 μM) or PLP1 peptide (0.6 μM).

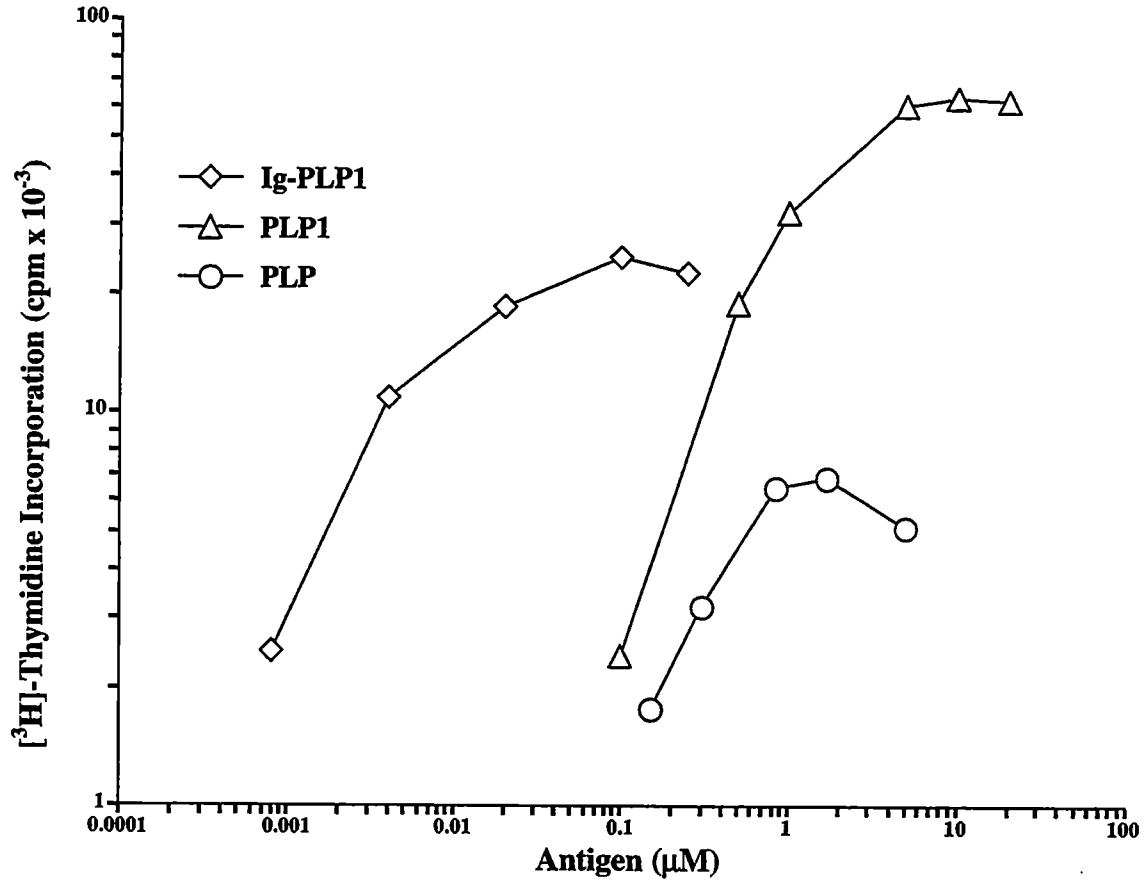

100
10
1
[3H]-Thymidine Incorporation (cpm x 10-3)
Ig-PLP1
PLP1
PLP
0.0001
0.001
0.01
0.1
1
10
100
Antigen (μM)

Figure 5.

Antagonism of PLP1, PLP, and Ig-PLP1 mediated T cell activation by Ig-PLP-LR.

SJL/J splenic APCs were incubated with (a) 1 μM PLP1 peptide, (b) 0.05 μM Ig-PLP1, or (c) 6.8 μM PLP (c) in the presence of graded amounts of antagonists or control antigens and then assayed for activation of the PLP1-specific T cell hybridoma, 4E3, by measuring IL-2 production as described in the Chapter 3 Materials and Methods section. The antagonists were Ig-PLP-LR (squares), PLP-LR (circles), and the controls were Ig-HA (diamonds) and PLP2 (triangles).

The cpm value obtained when the APCs were incubated with the stimulator but no antagonist was used as control ^{3}H-thymidine incorporation. This value was 7,503 ± 1,302 for Ig-PLP1; 31,089 ± 3,860; and 8,268 ± 915 for PLP.

The cpm value obtained when the APCs were incubated with no stimulator and no antagonist was used as background (BG). This value was 1,560 ± 323 for Ig-PLP1; 2,574 ± 290; and 2,127 ± 177 for PLP.

The percent control thymidine incorporation was calculated as follows: [(cpm obtained in the presence of test antagonist) - (BG)] / [(cpm control thymidine incorporation value) - (BG)]

Each point represents the mean of triplicates.

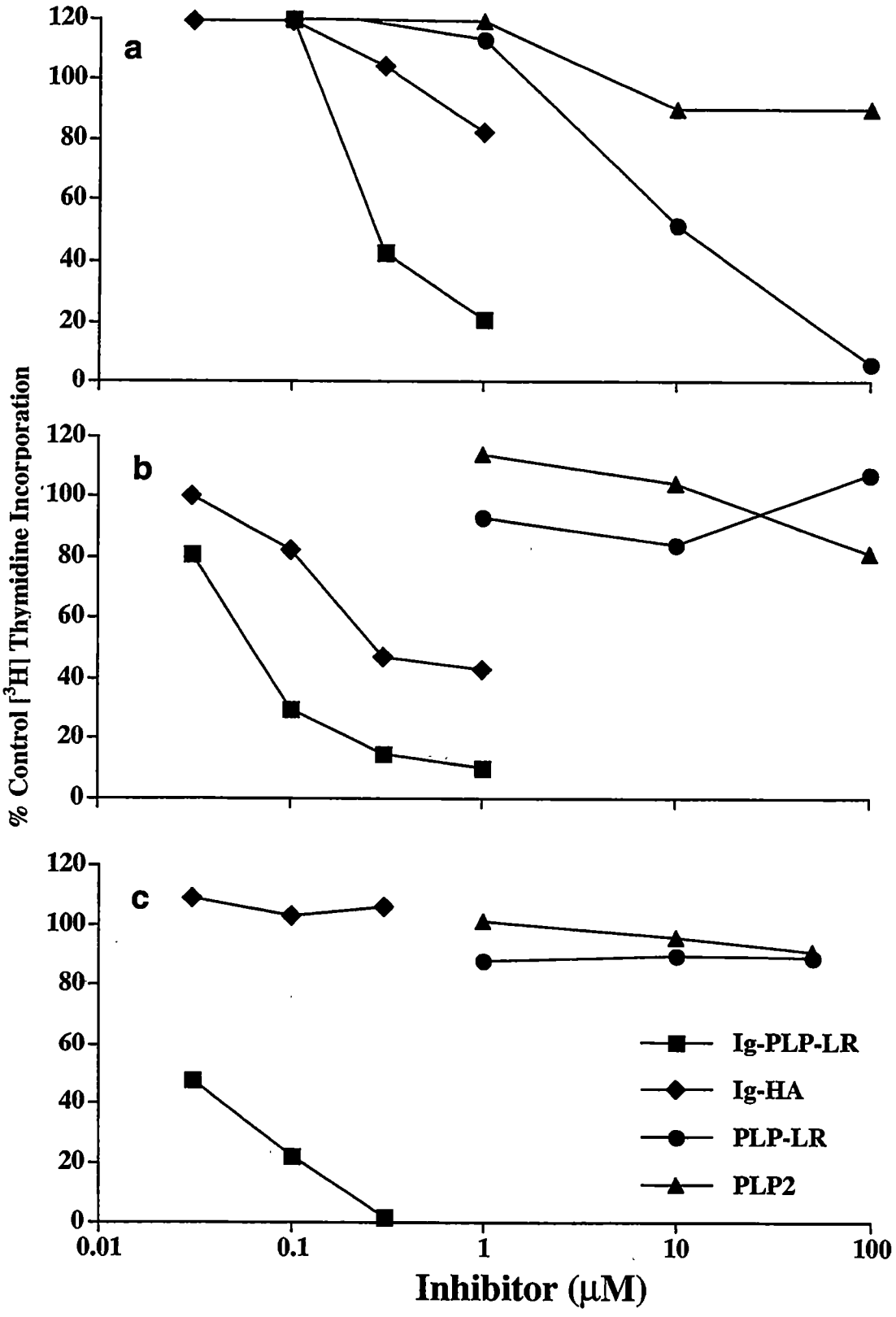

a
b
c
120
100
80
60
40
20
0
% Control [3H] Thymidine Incorporation
0.01
0.1
1
10
100
Inhibitor (μM)
Ig-PLP-LR
Ig-HA
PLP-LR
PLP2

Figure 6.

Competition for binding to class II at the endocytic level is not the mechanism for Ig-PLP-LR mediated antagonism.

SJL/J splenic APCs were incubated with native PLP (6.8 μM) in the presence of 50 μg/ml (0.3 μM) Ig-PLP2, Ig-PLP-LR, or Ig-W and 5 x 10^4 PLP1-specific 4E3 T cells. IL-2 production was assessed by ^{3}H-thymidine incorporation using HT-2 cells as described in the legend to figure 5. The % control ^{3}H-thymidine incorporation was calculated as in figure 5. Each column represents the mean ± SD of triplicates.

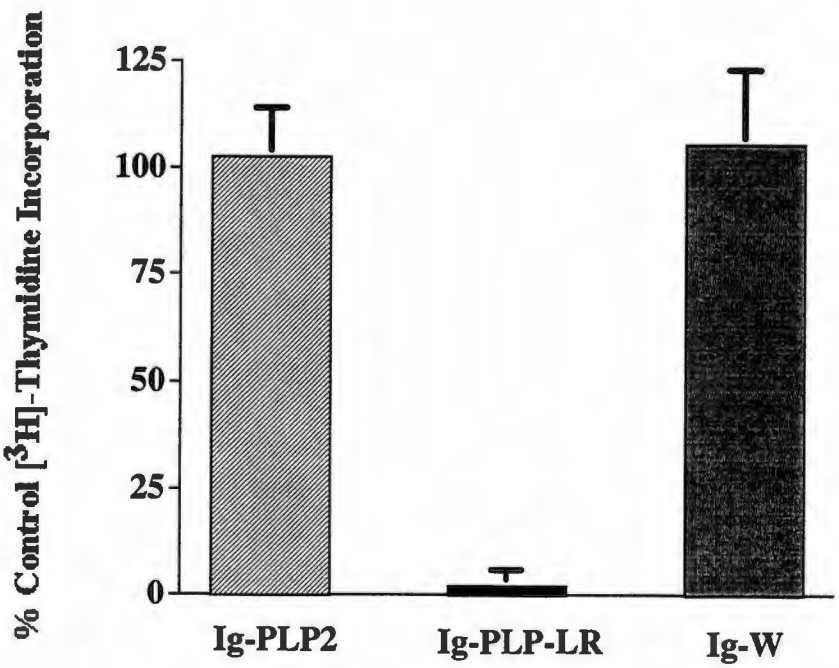

% Control [3H]-Thymidine Incorporation
125
100
75
50
25
0
Ig-PLP2
Ig-PLP-LR
Ig-W

Figure 7.

In vivo priming of PLP1 specific T cells by Ig-PLP1.

Mice were immunized s.c. with 50 μg of Ig-PLP1 in CFA as described in materials and methods, and after 10 days cells from the lymph nodes (a) and spleen (b) were tested for specific proliferation to PLP1. The indicated results are those obtained with 4 x 10^5 lymph node cells/well and 10 x 10^5 spleen cells/well. The stimulators PLP1 and PLP2 were used at 15 μg/ml and PPD was used at 5 μg/ml. Each value represents the mean ± SD of triplicates after deduction of the BG cpms obtained with no stimulator in the media. The cpm values obtained with PPD for each mouse exceeded the cpm values obtained with PLP1 by 20 to 60% dependent upon each mouse. Similar results were obtained when mice were immunized with 150 μg of Ig-PLP1 per mouse.

Note: The fact that some mice show proliferation with PLP2 may be because this peptide is presented by I-A^s, like PLP1, and low affinity cells could bind to it.

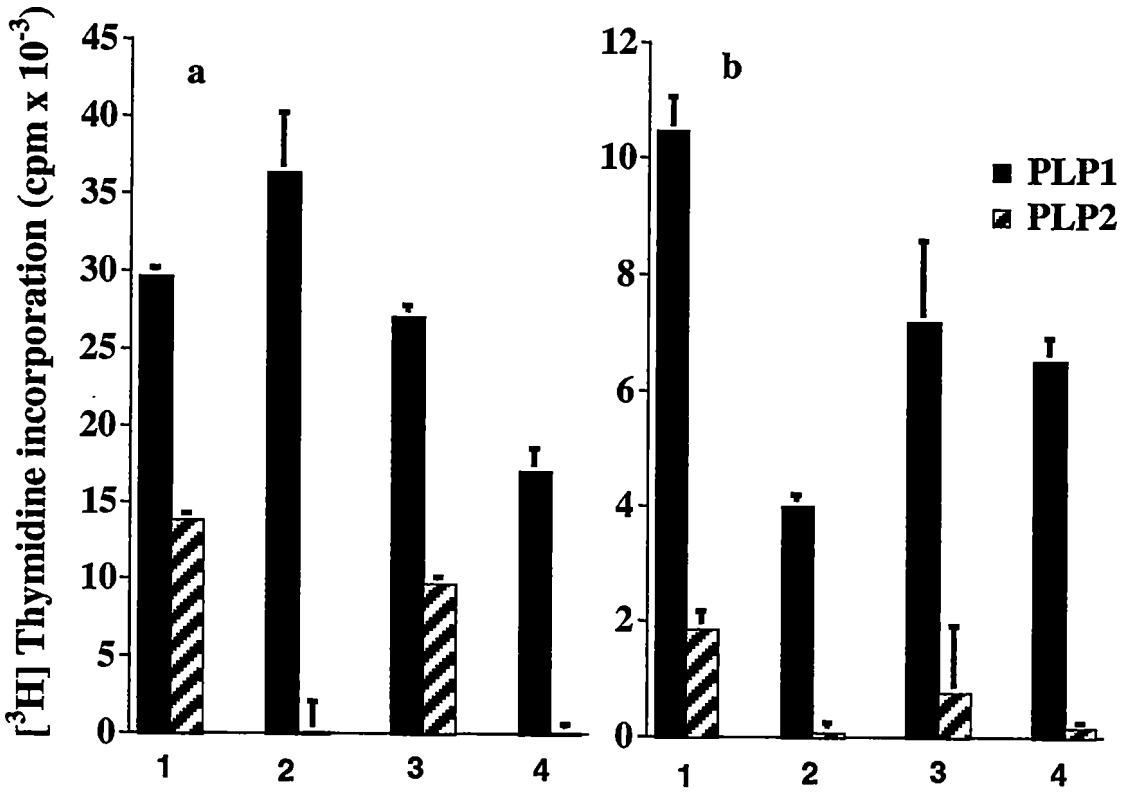

[3H] Thymidine incorporation (cpm x 10-3)
a
b
PLP1
PLP2
45
40
35
30
25
20
15
10
5
0
12
10
8
6
4
2
0
1
2
3
4

Figure 8.

Co-administration of Ig-PLP-LR with Ig-PLP1 reduces the response to PLP1 peptide.

Mice were co-immunized with 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR or 50 μg Ig-PLP1 and 150 μg Ig-W as indicated in Chapter 3 Materials and Methods. The lymph node (a) and splenic (b) proliferative responses to PLP1 peptide were analyzed 10 days later. The lymph node cells were used at 4 x 10^5 cells/well and the spleen cells at 10 x 10^5 cells per well. The stimulators were PLP1 (15 μg/ml), and PPD (5 μg/ml). The indicated results are those obtained with PLP1 peptide and represent the mean ± SD of triplicates after deduction of the BG cpms obtained with no stimulator in the media. The cpm values obtained with PPD were similar in both groups of mice and were 5 to 30% higher than the cpm values obtained with PLP1 in the mice immunized with Ig-PLP1 and Ig-W.

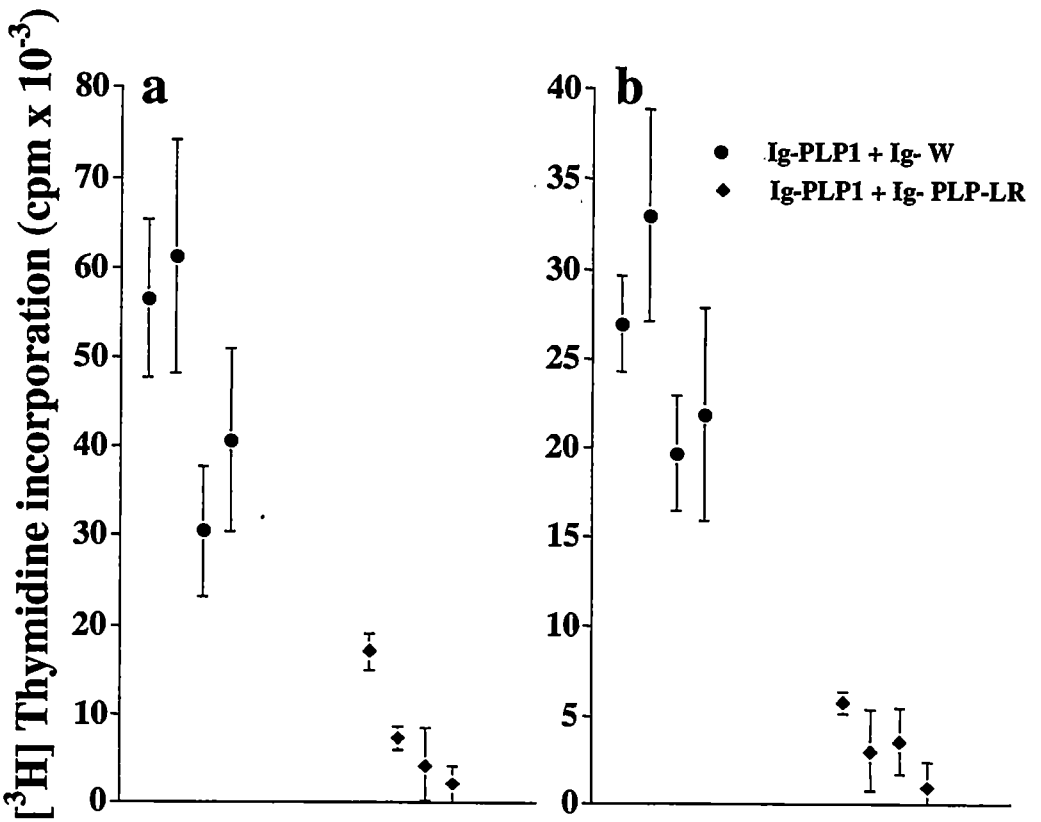
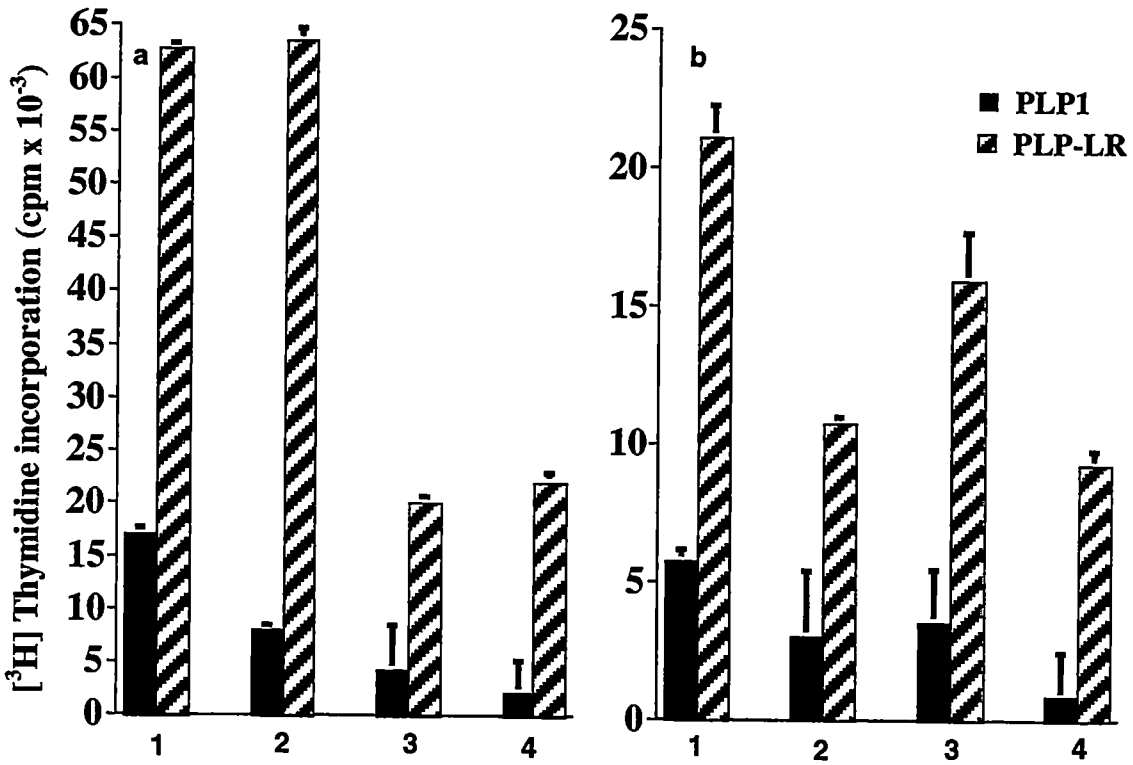

[3H] Thymidine incorporation (cpm x 10-3)
a
b
Ig-PLP1 + Ig- W
Ig-PLP1 + Ig- PLP-LR
80
70
60
50
40
30
20
10
0
40
35
30
25
20
15
10
5
0

Figure 9.

Mice co-injected with Ig-PLP1 and Ig-PLP-LR develop higher proliferative response to PLP-LR than PLP1 peptide.

Day 10 post immunization lymph node (a) and splenic (b) proliferative responses to PLP-LR peptide, in mice that were co-injected with Ig-PLP1 and Ig-PLP-LR and described in figure 8, were measured and shown here along with the responses to PLP1 peptide.


[3H] Thymidine incorporation (cpm x 10-3)
a
b
PLP1
PLP-LR
0
5
10
15
20
25
30
35
40
45
50
55
60
65
1
2
3
4

PART III.

Differential Presentation of an Altered Peptide within Fetal Central and Peripheral Organs Supports an Avidity Model for Thymic T Cell Development and Implies a Peripheral Re-adjustment for Activation.

(Journal of Immunology, 1999, 162: 5738-5746)

Chapter 1. Abstract

Altered self peptides may drive T cell development by providing avidity of interactions low enough to potentiate positive selection but not powerful enough to trigger programmed cell death. Since the peptide repertoire in both central and peripheral organs is nearly the same, interactions of these peptides with T cells in the thymus would have to be different from those taking place in the periphery otherwise T cell development and maturation would result either in autoimmunity or T cell deficiency. Herein, a self and an altered self peptide were delivered to fetuses, and their presentation as well as the consequence of such presentation on T cell development were assessed. The results indicate that the self peptide was presented in both central and peripheral fetal organs and that such presentation abolished T cell responses to both peptides during adult life. However, the altered peptide, although presented in vivo as well as in vitro by splenic cells, was unable to stimulate a specific T cell clone when the presenting cells were of thymic origin and allowed the offspring to be responsive to both peptides. These findings indicate that central and peripheral organs accommodate selection and peripheral survival of T cells by promoting differential altered peptide presentation.

Chapter 2. Introduction

During thymic development a T cell must engage a self-peptide-MHC complex in order to survive and continue maturation (1, 2). This process is known as positive selection (3-5). Subsequently, in order to escape negative selection or programmed cell death, the cell should not engage the self-peptide MHC complex with high avidity (6, 7). For quite some time this selection process appeared paradoxical and reconciliation awaited the postulation of the avidity model of T cell selection which suggested that the interactions operating positive selection are of much lower avidity than those maneuvering negative selection (3, 8-11). This model, although logical, requires a setting that could both provide and sense variable avidity. Recently, it has become clear that the productivity of the biochemical signals that arise from cross linking the TCR are dependent upon the strength of the avidity of interaction between the T cell and APC (8, 9, 11, 12). Therefore, the TCR is not simply an off/on switch but rather a practical sensitive sensor (5, 13). During T cell development, the density of cell surface TCR increases as the T cell progresses in maturation (5, 14). In addition, expression of accessory molecules on both the T cell and APC tends to change as T cells migrate through the thymic tissues (5, 14). These changes may increase the avidity of the T cell-APC interaction and most likely lead to negative selection. Therefore, if the selecting peptide contributes an affinity similar to that of foreign peptides, which activate peripheral T cells, the maturation system would only function to negatively select T cells. Recently, a postulate was put forth suggesting that self peptides, degenerate at the TCR contact residues, could operate T cell thymic development and provide low affinity interactions sufficient for positive selection but moderate enough not to reach the

threshold for negative selection, even when enhanced by accessory molecules (15-18). This postulate, although challenged by a few in vitro studies (19,20), if confirmed could account for the 1% of T cells that succeed in escaping negative selection and exiting the thymus (14). Recent studies have indicated that the peptides presented on central and peripheral APCs are nearly the same (7,21). Accordingly, the T cell selecting peptides present in the thymus would also be present on peripheral APCs (21). How mature peripheral T cells are not activated by those peptides remains an unsolved issue (18, 22). In the present study we designed a delivery system that escorts peptides to both central and peripheral fetal APCs and investigated the consequence of such fetal presentation of self and altered self peptides on the development of T cells and the subsequent response of those cells to challenges with the peptides later in life.

The peptide used in these studies encompasses the amino acid (aa) sequence 139-151 of proteolipid protein (PLP). This peptide (referred to as PLP1) is encephalitogenic and induces experimental autoimmune encephalomyelitis in SJL/J mice (23-25). The altered peptide designated PLP-LR was derived from PLP1 by replacing the TCR contact residues Trp144 and His147 with Leu144 and Arg147, respectively (26). PLP-LR binds to I-A^s class II molecules equally as well as PLP1, but when presented to PLP1-specific T cell clones and hybridomas, induces inactivation (26). PLP1 and PLP-LR were genetically engineered into the heavy chain variable region of Igs, and the resulting Ig-PLP1 and Ig-PLP-LR chimeras were efficiently presented to T cells (27). Ig-PLP-LR, like free PLP-LR, antagonizes T cell lines and hybridomas developed against free PLP1 (27). In vivo Ig-PLP1 and Ig-PLP-LR induce T cells that are cross-reactive with both PLP1 and PLP-LR (28). However, when co-injected into animals there is a down-

regulation of both responses, with more Ig-PLP-LR being required for full down-regulation of PLP1-reactive T cells, and much less Ig-PLP1 being required for inactivation of PLP-LR-reactive T cells (27,28). These results indicated that Ig-PLP1 and Ig-PLP-LR target common naive T cells and that Ig-PLP-LR drives a lower avidity of interaction with these T cells than Ig-PLP1 possibly because of a lower affinity (28). In addition, it has been suggested that a quicker off rate, which reflects low affinity, forms the basis for antagonism by altered peptides, such as PLP-LR (29).

Since Ig-PLP1 and Ig-PLP-LR express a γ2b constant region isotype, they should be able to cross the maternal placenta and drag the peptides from mother to fetus. Fetal presentation of PLP1 and PLP-LR provides a system to investigate the role that peptide affinity plays on T cell development. The results indicate that both Ig-PLP1 and Ig-PLP-LR when injected into pregnant mothers cross the maternal placenta and transfer to the fetus. Thymic APCs of neonates born to mothers that were injected with Ig-PLP1 at day 19 of gestation stimulated a PLP1-specific T cell hybridoma. Moreover, adult offspring born to mothers given Ig-PLP1 on days 16, 17, and 18 of gestation could not mount proliferative and cytokine responses or develop EAE when challenged with either Ig-PLP1 or Ig-PLP-LR. In contrast, thymic APCs from neonates born to mothers that were injected with Ig-PLP-LR could not stimulate a PLP-LR-specific T cell clone. In addition, these offspring developed proliferative and cytokine responses, as well as EAE, when challenged with either chimera as adults. The interpretation we would like to put forth for these results is that PLP1 most likely supports negative selection and ablates responses to both PLP1 and PLP-LR, while the altered peptide, PLP-LR, does not. These in vivo observations favor an avidity model for T cell selection and provide evidence that altered

peptides could support positive selection. In addition, the splenic APCs of neonates born to mothers that were injected with Ig-PLP-LR during gestation, like the splenic APCs of neonate offspring of Ig-PLP1 recipient mothers, stimulate specific T cell clones. These results indicate that the T cells, which were selected in a thymus most likely presenting PLP-LR at levels undetectable by T cell clones, survive in a periphery that supports a much stronger PLP-LR presentation. Therefore, the altered peptide, PLP-LR, supports positive selection and, although presented in the periphery in a manner that provides higher avidity, allows the development and generation of specific T cells.

Chapter 3. Materials and Methods

Animals

SJL/J (H-2^s) mice were bred, housed and maintained in the University of Tennessee animal care facility for the duration of experiments according to the guideline of the University of Tennessee Animal Care Committee. Females were assessed daily for seminal plugs. When found, the female was removed from the breeding cage (labeled as day two of gestation) and allowed to develop possible pregnancy until day 16. On day 16 of gestation, the pregnant females were administered as described below.

Antigens

Peptides. The peptides used in this study were purchased from Res. Genetics (Huntsville, Alabama) and were purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of PLP (22-24). PLP-LR (HSLGKLLGRPDKF) is an altered peptide form of PLP1 in which the TCR contact residues Trp144 and His147 were replaced with Leu and Arg, respectively (25). PLP1 and PLP-LR are known to bind equally well to I-A^s class II molecules (25). PLP2 peptide (NTWTTCQSIAFPSK) encompasses the encephalitogenic aa sequence corresponding to residues 178-191 of PLP. PLP2 also binds to I-A^s MHC class II molecules and induces EAE in SJL/J mice (28).

Ig-PLP chimeras. Ig-PLP1, Ig-PLP-LR, and Ig-PLP2 are chimeras expressing PLP1, PLP-LR, and PLP2 respectively (26). Construction of these chimeras used the genes coding for the light and heavy chains of the anti-arsonate antibody, 91A3, and the procedures for deletion of the heavy chain CDR3 region and replacement with the

nucleotide sequence coding for the selected peptide were previously described (26, 27). Ig-W is the parental Ig not encompassing any PLP peptide and was described elsewhere (26).

Large-scale cultures of transfectants were carried out in DMEM containing 10% iron enriched calf serum (Intergen Corp., Purchase, New York). The Ig-PLP chimeras were subsequently purified on separate rat anti-mouse κ chain Sepharose columns in order to avoid cross-contamination.

Fetal tolerization of mice with Ig-PLP chimeras

Pregnant mothers were injected i.v. with 100μg of Ig-PLP1, Ig-PLP-LR, or Ig-W on days 16, 17, and 18 of gestation, and the offspring used for analysis of immune response or induction of EAE at the age of 6-8 weeks.

Immunization of mice with Ig-chimera and peptides

Immunizations with Ig-PLP chimeras. 6-8-week-old fetal tolerized offspring were immunized s.c. in the footpads and at the base of the limbs and tail with 50μg of Ig-PLP1, Ig-PLP-LR, or Ig-W emulsified in a 200 μl mixture PBS/CFA (vol/vol). After ten days the mice were sacrificed by cervical dislocation, and the spleens and lymph nodes (axillary, lateral axillary, and popliteal) were removed, single cell suspensions were prepared, and the T cell proliferative and cytokine responses were analyzed as described below.

Immunization with PLP peptides. 6-8-week-old fetal tolerized offspring were immunized as above with 100 μg of PLP1 or PLP2 in 200 μl mixture PBS/CFA (vol/vol),

and splenic and lymph node proliferation and cytokine production were analyzed 10 days after immunization.

Assessment of transfer of Ig-PLP1 from mother to fetus

Pregnant mice were given 300 μg of Ig-PLP1 i.v. in the tail vein at day 19 of gestation, and serum from offspring born 2 days later was used for detection of Ig-PLP1 by immunospot assay. Serum (10μl) was deposited on a nitrocellulose membrane and allowed to adsorb for 30 min. at room temperature. The membrane was then blocked with PBS-5% BSA for one hour and then incubated in a PBS-2%BSA solution containing 1 x 10^6 cpm/ml ^{125}I-labeled rabbit antibodies to PLP1 (26). Subsequently, the membrane was washed with 0.05% Tween20 in PBS, dried, and exposed to a Cronex film.

Fetal presentation of Ig-PLP chimeras

To assess fetal presentation of the Ig chimeras, splenic and thymic cells from neonates born to mothers that were injected with 300 μg of Ig-PLP1, Ig-PLP-LR, or Ig-W at day 19 of gestation were tested for stimulation of specific T cell hybridomas or clones. Graded numbers of thymic or splenic cells were irradiated (3,000 rads) and incubated with 5 x 10^4 4E3 PLP1-specific T hybridoma or 2.1H8 cross-reactive T cell clone without the addition of exogenous antigen in a total volume of 200 μl. After 24 hours the supernatant was used for cytokine detection.

For control purposes, thymic and splenic cells from mice born to untreated mothers were used to assess presentation of the chimeras in vitro. In this case, 5 x 10^5 thymic or splenic cells were irradiated (3,000 rads) and incubated with graded amounts of

Ig chimera or free peptide and 5 x 10^4 T cells for 24 hours. Subsequently, cytokine detection in the supernatant was carried out by ELISA.

Assays for spleen and lymph node proliferative responses

Lymph node and splenic cells were incubated in 96-well flat-bottom plates at 4 and 10 x 10^5 cells/100 µl/well, respectively, with 100 µl of stimulator for 3 days. Subsequently, 1 µCi [^{3}H]thymidine was added per well and the incubation continued for an additional 14.5 hours. The cells were then harvested onto glass fiber filters, and the incorporated [^{3}H]thymidine counted using the trace 96 program on an Inotech β counter. The stimulators were used at the defined optimal concentrations of 15µg/ml for PLP1, PLP-LR, and PLP2. A control of media without stimulator was included for each mouse and used as background.

ELISPOT assay

The cytokines produced by lymph node T cells were measured by ELISPOT assay as described (29). Briefly, HA-multiscreen plates (Millipore, Bedford, MA) were coated with 100µl/well 1 M $NaHCO_3$ buffer containing 2 µg/ml capture antibody (PharMingen, San Diego, CA). The capture antibodies were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; and rat anti-mouse IFNγ, R4-6A2. After an overnight incubation at 4° C, the plates were washed 3X with sterile PBS, and then free sites were blocked for 2 hours at 37° C with 100µl/well DMEM culture media containing 10% calf serum. Subsequently, 50µl of media was removed and 5 x 10^5 lymph node cells/50µl/well and stimulator (100 µl) were added. After a 24 hour incubation the plates were washed and

100µl of biotinylated anti-cytokine antibody (1µg/ml) was added. The biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES6-5H4; rat anti-mouse IL-4, BVD6-24G2; and rat anti-mouse IFNγ, XMG1.2 (Pharmingen). Following overnight incubation at 4° C, the plates were washed and 100µl of 2.5 µg/ml avidin-peroxidase (Sigma, St. Louis, MO) was added. After a 1 hour incubation at 37° C, the plates were washed, visualized by addition of 100µl of AEC (3-amino-9-ethylcarbazole, Sigma) in 50µM acetate buffer pH 5.0, allowed to dry, and counted under a dissecting microscope. The stimulators were used at the defined optimal concentrations of 15µg/ml for PLP1, PLP-LR, and PLP2. A control of media without stimulator was included for each mouse and used as background.

ELISA for detection of cytokines

Detection of cytokine in culture supernatant was carried out by ELISA according to Pharmingen's standard protocol. The anti-cytokine antibody pairs used here are those described for ELISPOT. The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Menlo Park, CA) using SoftMAX Pro version 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, and IFNγ were included in all experiments to construct standard curves. The concentration of cytokines in culture supernatants was estimated by extrapolation from the linear portion of the standard curve. The stimulators were used at the defined optimal concentrations of 15µg/ml for PLP1, PLP-LR, and PLP2. A control of media with no stimulator was included for each mouse and used as background.

Measurement of IL-2 by HT-2 cells

Measurement of IL-2 was done by [^{3}H]thymidine incorporation of the IL-2 dependent HT-2 cell line. Briefly, 100µl of culture supernatant was incubated with 1 x10^4 HT-2 cells for 17 hours. One µCi [^{3}H]thymidine was then added per well, and the culture was continued for 12 hours. The cells were then harvested onto glass fiber filters, and the incorporated [^{3}H]thymidine counted using the trace 96 program on an Inotech (Wohlen, Switzerland) β counter.

EAE Induction

6-8-week-old fetal tolerized mice were induced for EAE by s.c. injection in the foot pads and at the base of the limbs and tail with a 200µl IFA/PBS (vol/vol) solution containing 200µg of *M. tuberculosis* H37Ra and 200µg of Ig-PLP1, 200µg of Ig-PLP-LR, or 100µg of free PLP1. Six hours later 5 x 10^9 inactivated *B. pertussis* were given intravenously. After 48 hours a second dose of 5 x 10^9 inactivated *B. pertussis* was administered. Mice were scored daily for clinical signs as follows: 0, no clinical signs; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Chapter 4. Results

Fetal transfer and presentation of Ig-PLP1.

To ascertain that the Ig-PLP chimeras could cross the maternal placenta and transfer from mother to fetus, pregnant mice were injected i.v. with Ig-PLP1 at day 19 of gestation, and the serum of offspring born two days later was tested for the presence of Ig-PLP1. Figure 1 shows that the serum of mice born to mothers that were injected with Ig-PLP1 during pregnancy like the control purified Ig-PLP1 bound rabbit antibodies to PLP1 peptide. This spot blot system was specific because the serum from offspring born to mothers injected with Ig-W instead of Ig-PLP1 during gestation did not bind the rabbit antibodies. These data indicate that Ig-PLP1 crossed the maternal placenta and reached the fetal circulation. Subsequently, it was asked whether the transferred Ig-PLP chimeras could reach the fetal lymphoid organs for presentation. To address this issue, thymic and splenic cells from neonates born to mothers injected with either Ig-PLP1 or Ig-W during gestation were assayed, without the addition of antigen, for the ability to activate the PLP1-specific T cell hybridoma, 4E3. Figure 2 shows that both thymic and splenic cells from mice born to Ig-PLP1 recipient mothers stimulated the production of IL-2 by the 4E3 hybridoma while cells from mice born to Ig-W recipient mothers did not. These data indicate that the transferred Ig-PLP1 was taken up by APCs and that I-A^s-PLP1 complexes were generated in both the central and peripheral fetal lymphoid organs.

Fetal exposure to Ig-PLP1 ablates PLP1-specific T cell responses.

Expression of MHC molecules on fetal APCs begins at day 14 of gestation (30, 31), and T cell receptor rearrangement and expression on T cells follows three days later

(32-35). Therefore, T cell selection commences at day 17 of fetal life. To investigate the consequences of Ig-PLP1 fetal presentation on the development of specific T cells, pregnant mothers were injected with Ig-PLP1 at days 16, 17, and 18 of gestation, and the offspring immunized as adults with either Ig-PLP1 or PLP1 peptide in CFA. Ten days later the lymph node and spleen cells were assessed for proliferative and cytokine responses. Figure 3 shows that fetal presentation of Ig-PLP1 dramatically reduces the lymph node and splenic proliferative responses of adult offspring to immunization with either Ig-PLP1 or free PLP1. In contrast, mice born to mothers that received the parental Ig-W instead of Ig-PLP1 developed specific responses in both lymphoid organs to either immunization. These results indicate that fetal presentation of Ig-PLP1 neither primes specific T cells nor allows the development of memory cells. Rather, the precursor cells are not present and/or incapable of responding to challenge with antigen. Stimulation of cells in the presence of exogenous IL-2 did not restore the proliferative response of cells from offspring born to Ig-PLP1 injected mothers.

At the cytokine level while fetal presentation of Ig-W had no effect on the production of IL-2, Ig-PLP1 partially reduced IL-2 secretion when the offspring were immunized with PLP1 in CFA but completely inhibited cytokine production when the immunization was carried out with Ig-PLP1 (Figure 4). IL-4 was undetectable in all groups of mice. Additionally, while these mice were completely resistant to EAE induction by Ig-PLP1, those born to mothers who received Ig-W during pregnancy developed, like the offspring born to unmanipulated mothers, a standard monophasic EAE (Figure 5a). When free PLP1 peptide was used for disease induction the mice born to mothers that received Ig-W developed a severe clinical relapsing remitting form of

EAE with relapses occurring up to day 100. Those offspring born to mothers who received Ig-PLP1 during gestation developed a milder monophasic form of EAE that did not relapse through day 120 (Figure 5b). Overall, Ig-PLP1 transfers from mother to fetus, and the consequent fetal presentation of PLP1 peptide influences T cell development.

Fetal exposure to Ig-PLP-LR does not inhibit specific T cell responses.

This fetal delivery and presentation strategy was then chosen to investigate the role of TCR-peptide affinity on T cell development in vivo. Altered peptides mutated at the TCR contact residues have a faster off rate than wild type peptides and presumably interact with the TCR with lower affinity (36, 37). PLP-LR, a peptide derived from PLP1 by mutation of TCR contact residues 144W and 147H to 144L and 147R, respectively, binds to I-A^s MHC class II molecules equally as well as PLP1 peptide but generates an altered peptide ligand that antagonizes PLP1-specific T cell lines and hybridomas (25, 26). T cell antagonism most likely is the consequence of low affinity TCR-ligand interactions and TCR spoiling (36-38). Altered peptides, used as tools to modulate affinity, provide useful ligands to investigate the avidity model of T cell selection. Recently, PLP-LR peptide was expressed on an Ig molecule, and the resulting Ig-PLP-LR chimera, like Ig-PLP1, induced T cells that were cross-reactive with both PLP1 and PLP-LR peptides (27). Since Igs can transfer from mother to fetus, PLP1 and PLP-LR, expressed on Igs, could access the developing fetal thymus and provide an in vivo system to study the effect of differential affinity on T cell development. To address this issue, pregnant mice were injected with either Ig-PLP1 or Ig-PLP-LR on days 16, 17, and 18 of gestation, and the offspring born to these mothers were immunized with either Ig-PLP1

or Ig-PLP-LR. Ten days later their proliferative and cytokine responses to stimulation with PLP1 and PLP-LR were assessed. Figure 6 shows that fetal injection of Ig-PLP-LR has little or no down regulatory effect on either lymph node or splenic proliferative responses to a challenge with Ig-PLP1 or Ig-PLP-LR, in fact there is a slight proliferative enhancement when the stimulator is PLP-LR. In contrast, when Ig-PLP1 was injected into the pregnant mothers the offspring's proliferative responses to PLP1 and PLP-LR were markedly reduced, whether the immunogen was Ig-PLP1 or Ig-PLP-LR (Figure 6). Overall, mother sensitization with Ig-PLP1 precludes the responses to both Ig-PLP1 and Ig-PLP-LR, while sensitization with Ig-PLP-LR, the chimera carrying the low affinity peptide, does not significantly reduce the proliferative responses to either chimera. Similar results were obtained at the cytokine production level (Figure 7). While fetal administered Ig-PLP1 abrogated the offsprings' cytokine responses to both Ig-PLP1 and Ig-PLP-LR, fetal injection of Ig-PLP-LR did not modify the IL-2 response, and the amount of cytokine produced in response to either Ig-PLP1 or Ig-PLP-LR was comparable to that obtained in mice born to mothers that were injected with Ig-W during gestation (Figure 7). IL-4 was undetectable in all groups of mice. Fetal presentation of Ig-PLP1 and its consequent effect on T cell development is specific and does not affect the development of other T cell precursors using I-A^s class II molecules for selection and maturation. This conclusion is drawn from the observation that injection of Ig-PLP1 during gestation does not interfere with the proliferative and cytokine responses to PLP2 peptide (Figure 8). Indeed, offspring from mothers that were injected with Ig-PLP1 during gestation developed lymph node and splenic proliferative and cytokine responses to a challenge with PLP2 peptide that were similar to the responses of offspring born to

mothers that received Ig-W instead of Ig-PLP1 during pregnancy. Offspring born to mothers injected with Ig-PLP1 during pregnancy resisted induction of EAE by both Ig-PLP1 and Ig-PLP-LR (Figure 9). In contrast, offspring born to mothers that received Ig-PLP-LR during pregnancy developed clinical signs of EAE when induced with either Ig-PLP1 or Ig-PLP-LR.

Differential presentation of Ig-PLP1 and Ig-PLP-LR in peripheral and central fetal organs.

Since Ig-PLP1 was presented by fetal thymic APCs in vivo and neither proliferative/cytokine response nor EAE could develop subsequent to challenge with either Ig-PLP1 or Ig-PLP-LR, it is likely that PLP1/PLP-LR specific T cell precursors were incapacitated or negatively selected during development. Anergy is most likely not responsible for the Ig-PLP1 mediated suppression of proliferative response because offspring born to Ig-PLP1 treated mothers do not restore their proliferative response when peptide stimulation is carried out in the presence of IL-2. The absence of a down regulatory effect on T cell development by Ig-PLP-LR could suggest that Ig-PLP-LR was not presented by fetal APCs or that presentation took place, but the generated ligand could not drive negative selection. To investigate this issue a readout system for Ig-PLP-LR fetal presentation was needed. Because PLP-LR and Ig-PLP-LR are antagonists for PLP1-specific hybridomas we needed a PLP-LR specific T cell clone to assess the in vivo presentation of Ig-PLP-LR. To this aim mice were immunized with Ig-PLP-LR and the lymph node cells were cycled through stimulation/resting until reactivity with PLP-LR peptide was apparent and a line was established. Subsequently, the line was cloned by

limiting dilution and wells positive for cell growth were tested for proliferation to PLP-LR and PLP1. One clone, designated 2.1H8, proliferated to Ig-PLP1, Ig-PLP-LR, PLP1, and PLP-LR and produced IL-4 in response to these stimulators (Figure 10). This production of IL-4 is antigen specific and does not occur when 2.1H8 is stimulated with Ig-PLP2 or PLP2 peptide, antigens that are also presented by I-A^s class II molecules like PLP1 and PLP-LR peptides. 2.1H8 was then used to assess fetal presentation of Ig-PLP-LR. Mice were injected with Ig-PLP-LR or Ig-PLP1 on day 19 of pregnancy, and the thymic and splenic cells from offspring born on day 21 were assayed for stimulation of the 2.1H8 T cell clone. Figure 11a indicates that thymic APCs from offspring born to Ig-PLP1 recipient mothers induced IL-4 production by the 2.1H8 clone, while thymic APCs from offspring born to Ig-PLP-LR recipient mothers did not stimulate IL-4 production. In contrast, splenic cells from both types of offspring stimulated IL-4 production by the 2.1H8 clone (Figure 11b). Finally, while neonatal thymic APCs incubated in vitro with Ig-PLP1 stimulated the 2.1H8 clone, those incubated with Ig-PLP-LR did not induce IL-4 production by the 2.1H8 cells (Figure 11c). In contrast, neonatal splenic APCs whether incubated with Ig-PLP1 or Ig-PLP-LR stimulated the 2.1H8 T cell clone (Figure 11d).

Chapter 5. Discussion

Ig-PLP1 and Ig-PLP-LR chimeras, injected into pregnant mothers, cross the maternal placenta and transfer to the fetuses (Figure 1 and 11). Ig-PLP1 was presented both in the fetal thymus and spleen to an extent that was detectable by T cell clones and hybridomas (Figure 2 and 11). However, although Ig-PLP-LR was transferred from mother to fetus as suggested by its presentation by splenic APCs to a specific T cell clone, it was most likely presented in the thymus in a manner that could not be detected by the T cell clone (Figure 11). In vitro, while Ig-PLP1 stimulated the PLP1/PLP-LR-specific T cell clone when presented by neonatal splenic or thymic APCs, Ig-PLP-LR stimulated the T cell clone only when the APCs were of splenic origin (Figure 11). These observations indicate that presentation of altered (or low affinity) peptides by fetal thymic APCs is less potent than that of peripheral presentation. It is worth noting that the 2.1H8 T cells are more sensitive to stimulation with PLP-LR or Ig-PLP-LR than PLP1 or Ig-PLP1 (Figure 10). The inability of the thymic APCs to stimulate 2.1H8 when Ig-PLP-LR is used as antigen could be related to a diminished internalization and/or processing of Ig-PLP-LR or to an inadequate expression of costimulatory molecules on fetal thymic APCs. Either of these events combined with the lower affinity of PLP-LR peptide would promote low avidity interactions between T cells and APCs. Ig-PLP1 would be subject to the same events, but because the peptide's affinity is optimal the overall avidity would still be sufficient for activation of the 2.1H8 clone. The undetectable in vivo thymic presentation of PLP-LR versus PLP1 may be responsible for the discrepancy of the responses in mice recipient of the Ig-PLP1 fetal tolerization procedure versus those that received Ig-PLP-LR. Indeed, offspring born to mothers that were injected with Ig-PLP1

during gestation presented PLP1 in the thymus as well as the spleen during fetal development but were unable to mount responses or develop EAE when challenged as adults with either Ig-PLP1 or Ig-PLP-LR (Figure 3, 4, and 5). The residual proliferative and cytokine responses observed in these mice when they were challenged with free PLP1 peptide instead of Ig-PLP1 could be related to a difference in the T cell repertoire generated by free peptide versus Ig-PLP1 (Figure 3 and 4). This statement is supported by the data illustrated in Figure 5, which demonstrates that free PLP1 induces EAE with a relapsing remitting pattern of paralysis different from the monophasic pattern induced by Ig-PLP1. Consequently, fetal tolerization with Ig-PLP1 had a partial effect on the T cell response that led to a decrease in disease severity and absence of relapses when the mice were induced for EAE with free peptide (Figure 5b). In contrast, offspring born to mothers that were recipient of Ig-PLP-LR during pregnancy presented PLP-LR peptide in the spleen but their thymic APCs were unable the stimulate the 2.1H8 clone (Figure 11). Moreover, these mice responded to both peptides when challenged with Ig-PLP1 or Ig-PLP-LR (Figure 7 and 8) and developed EAE when induced with either Ig chimera (Figure 9). The effect of the Ig-chimeras on T cell development is antigen specific because fetal tolerization with Ig-PLP1 did not affect the T cell response to immunization with PLP2, an irrelevant peptide that, like PLP1 and PLP-LR, uses I-A^s class II molecules for presentation and stimulation of T cells (Figure 8).

The puzzle that remains to be solved involves the issue of peripheral presentation of Ig-PLP-LR at a level that activates a mature T cell clone. The question of how PLP-LR-specific T cell precursors, positively selected in the thymus, are not activated by the strong peripheral presentation of Ig-PLP-LR remains unanswered. One possibility is that

T cells, once exiting the thymus, are subject to a second round of selection (i.e. peripheral tolerance) (39-42) and that only those cells which readjust their activation avidity to a level higher than the avidity provided by peripheral presentation survive (43,44). As mentioned above because the avidity of interaction between T cells and APCs only increases as the T cell makes its way through the thymic tissues to the peripheral circulation, if readjustment in the avidity of activation does not occur the system will probably either eliminate T cells exiting the thymus or evolve to promote autoimmune disease. Knowing that the T cell receptor is not subject to mutation, that the T cell may not re-rearrange the TCR in the periphery, and that most T cell responses to peptides are not restricted to a particular V-gene usage the postulate of readjustment in the avidity required for activation becomes more attractive (21, 44, 45). In addition, T cells still develop in transgenic mice expressing a single TCR and low levels of the corresponding antigen (45). However, when the presentation of antigen was enhanced to generate an avidity that surpasses the readjusted threshold the T cells became activated (21, 45).

Figure 12 shows a schematic diagram that illustrates arbitrary levels of avidity for the different developmental stages of the T cell. The diagram reflects a coordination of events with the level of avidity. In the thymus, cells expressing a TCR that is unable to engage a MHC-peptide complex will die by neglect (5,46). However, if the TCR can mediate an interaction with thymic APCs with an avidity below the threshold for negative selection then the cell will be positively selected. For instance, thymic APCs from fetuses' that received Ig-PLP-LR could not stimulate 2.1H8 clones. Consequently, positive selection had taken place, and T cell responses to PLP-LR were obtained when the mice were challenged with Ig-PLP-LR. In vitro, splenic APCs pulsed with Ig-PLP-LR

did stimulate the T cell clone, while thymic APCs did not. Therefore, although we have no direct evidence for thymic presentation of Ig-PLP-LR, we think that it was processed and that the peptide was loaded onto MHC molecules, but the resulting complexes, because of instability, supported an avidity that was too low to stimulate the 2.1H8 T cell clone (47). If the avidity of interaction between the thymocyte and APCs was high enough to stimulate the mature 2.1H8 T cell clone then negative selection should occur. This is probably the case for Ig-PLP1 which was able to stimulate the 2.1H8 T cell clone when fetal APCs were of thymic origin, yet neither proliferative nor cytokine responses occurred when offspring recipient of the Ig-PLP1 fetal procedure were challenged with either Ig-PLP1 or Ig-PLP-LR. Since Ig-PLP-LR was presented in the fetal spleen and the mice developed normal responses when challenged as adults with either chimera, the T cells must have re-adjusted their avidity of activation to a level higher than that provided by the peripheral presentation of Ig-PLP-LR (which activates the mature 2.1H8 T cell clone). Finally, the avidity required for activation should be higher than the threshold for negative selection, as fetuses presenting Ig-PLP-LR in the fetal spleen were able to develop T cell responses when challenged with Ig-PLP-LR as adults. Meanwhile, Ig-PLP1 generated a fetal thymic presentation avidity capable of activating mature T cells, but could not subsequently immunize the mice because the T cells were negatively selected. Overall, this strategy provides a system that confirms that altered peptides can mediate T cell selection in vivo and sheds light on the necessity for peripheral re-adjustment of the presentation avidity. When used with TCR transgenic cells, this in vivo strategy will permit investigation of T cell selection and peripheral readjustment avidity at the single cell level.

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Figure 1.

Transfer of Ig-PLP1 from mother to fetus.

Pregnant mothers were given 300 μg of Ig-PLP1 or Ig-W i.v. on day 19 of gestation, and serum from offspring born on day 21 was used for detection of Ig-PLP1 by an immunospot assay. Serum (10μl) was deposited on a nitrocellulose membrane and bound Ig-PLP1 detected by exposure of the membrane to ^{125}I-labeled anti-PLP1 peptide antibody as described in Chapter 3 Materials and Methods. (a) Serum from neonates born to a mother recipient of Ig-PLP1 and (b) serum from offspring born to a mother that was injected with Ig-W. The spots labeled c and d are controls obtained by deposit of 1 μg of affinity purified Ig-PLP1 and Ig-W, respectively.

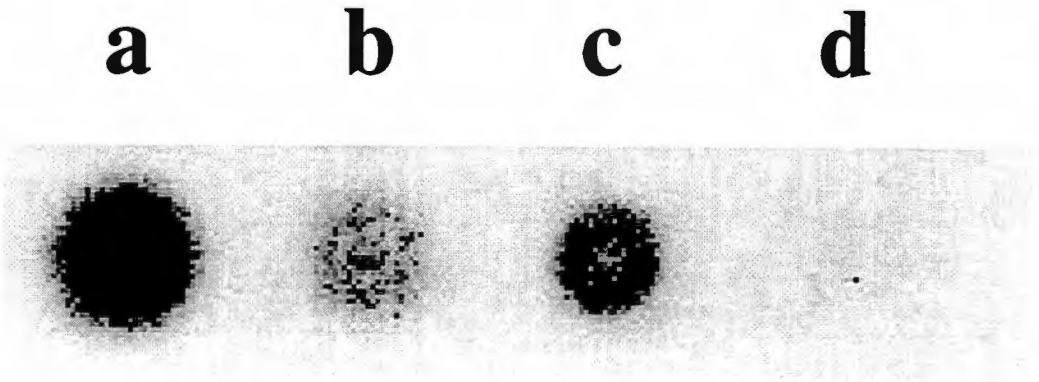

a
b
c
d

Figure 2.

Fetally transferred Ig-PLP1 is presented by both thymic and splenic APCs.

Pregnant mothers were given 300 μg of Ig-PLP1 or control Ig-W on day 19 of gestation and thymic (a) and splenic (b) cells of the offspring born on day 21 were irradiated and assayed for activation of the PLP1-specific T cell hybridoma, 4E3. IL-2 production, used as a measure of T cell stimulation, was assessed by incubation of the supernatant with the IL-2 dependent T cell line, HT-2 and subsequent measurement of proliferation by [^{3}H]thymidine incorporation. Each point represents the mean ± SD of triplicates.

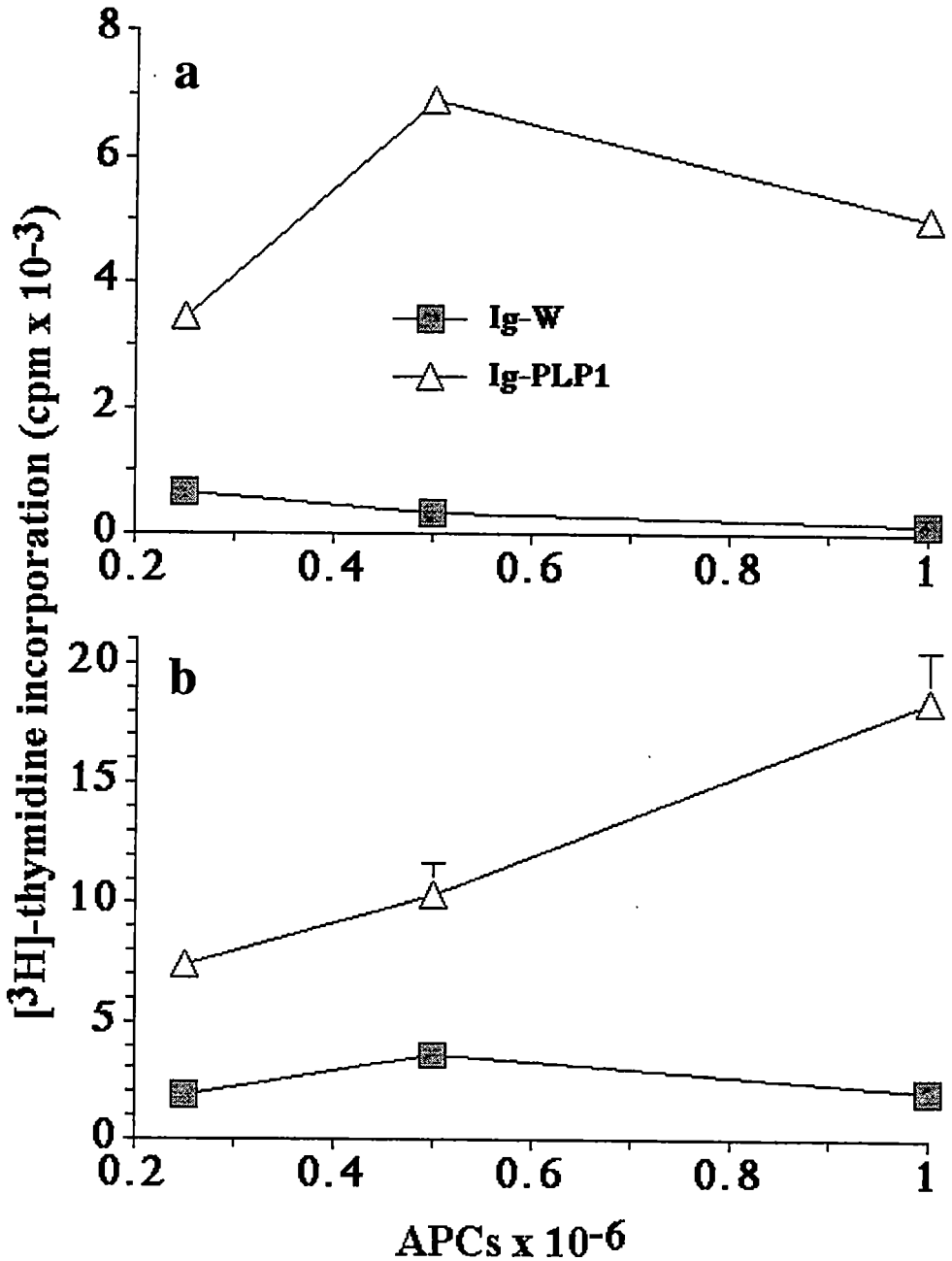

a
b
Ig-W
Ig-PLP1
[3H]-thymidine incorporation (cpm x 10-3)
APCs x 10-6
0
2
4
6
8
0.2
0.4
0.6
0.8
1
5
10
15
20

Figure 3.

Exposure to Ig-PLP1 during fetal development ablates response to PLP1 peptide as an adult.

Pregnant mothers were injected with a 200 µl saline solution containing 100 µg of Ig-PLP1 or control Ig-W (Tolerogen) on days 16, 17, and 18 of gestation. Adult offspring (6-8-week-old) were challenged with 50 µg of Ig-PLP1, 50 µg of Ig-W, or 100 µg of PLP1 (Immunogen) in CFA as described in Chapter 3: Materials and Methods. Ten days later the lymph node (a and c) and splenic (b and d) proliferative responses to PLP1 (filled bars) and PLP2 (hatched bars) were assessed. Lymph node cells were used at 4 x 10^5 cells/well and splenic cells at 1 x 10^6 cells/well. The stimulators PLP1 and PLP2 were used at the defined optimal concentration of 15µg/ml. Each bar represents the mean ± SD of 5 individually tested mice.

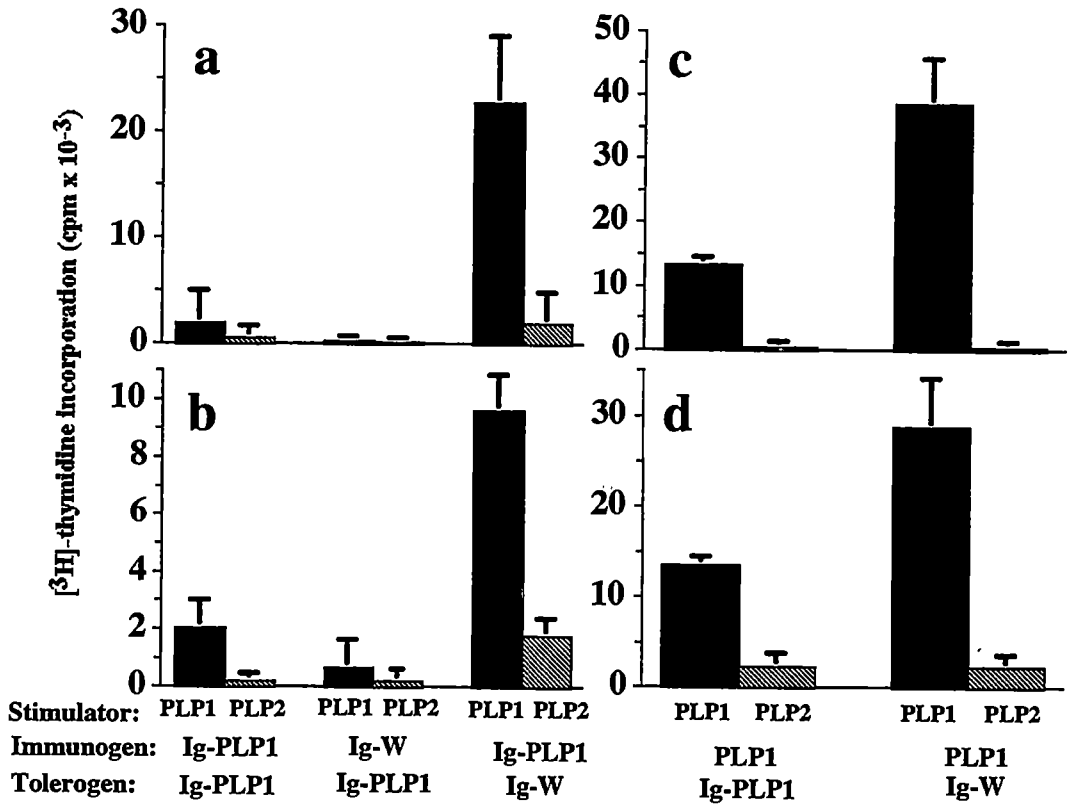

a
b
c
d
[3H]-thymidine incorporation (cpm x 10-3)
30
20
10
0
10
8
6
4
2
0
50
40
30
20
10
0
30
20
10
0
Stimulator: PLP1 PLP2 PLP1 PLP2 PLP1 PLP2 PLP1 PLP2 PLP1 PLP2
Immunogen: Ig-PLP1 Ig-W Ig-PLP1 PLP1 PLP1
Tolerogen: Ig-PLP1 Ig-PLP1 Ig-W Ig-PLP1 Ig-W

Figure 4.

Fetal exposure to Ig-PLP1 ablates IL-2 responsiveness to PLP1 during adult life.

Offspring born to mothers that were injected as per figure 3 with either Ig-PLP1 or Ig-W (Tolerogen) during pregnancy were challenged with either chimeras or PLP1 peptide (Immunogen) as adults. Ten days later lymph node (a and c) and splenic (b and d) cells were stimulated with either PLP1 (filled bars) or PLP2 (hatched bars) peptide (Stimulator). IL-2 production was measured by ELISPOT for the lymph node cells and by ELISA for the splenic cells as described in the Chapter 3 Materials and Methods section. Each bar represents the mean ± SD of 5 individually tested mice.

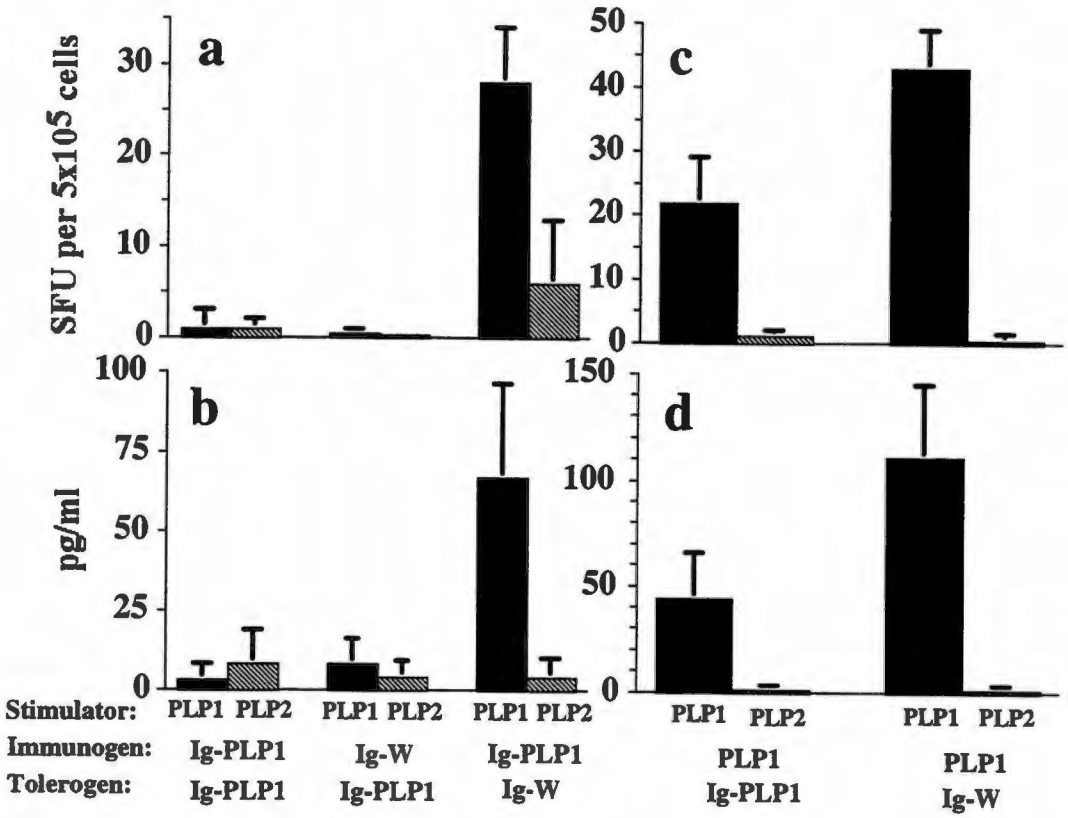

SFU per 5x10^5 cells
a
c
b
d
pg/ml
Stimulator:
Immunogen:
Tolerogen:
PLP1 PLP2
Ig-PLP1
Ig-PLP1
Ig-W
Ig-PLP1
Ig-PLP1
PLP1
Ig-PLP1
PLP1
Ig-W

Figure 5.

Fetal exposure to injected Ig-PLP1 ablates Ig-PLP1 mediated specific EAE.

Pregnant mothers were injected with 100 μg of Ig-PLP1 (open triangles), control Ig-W (closed squares), or saline (closed triangles) on days 16, 17, and 18 of gestation, and adult offspring were induced for EAE with 200μg Ig-PLP1 (a) or 100μg of PLP1 peptide (b) as described in Chapter 3 Materials and Methods. Each point represents the mean clinical score of 8 mice.

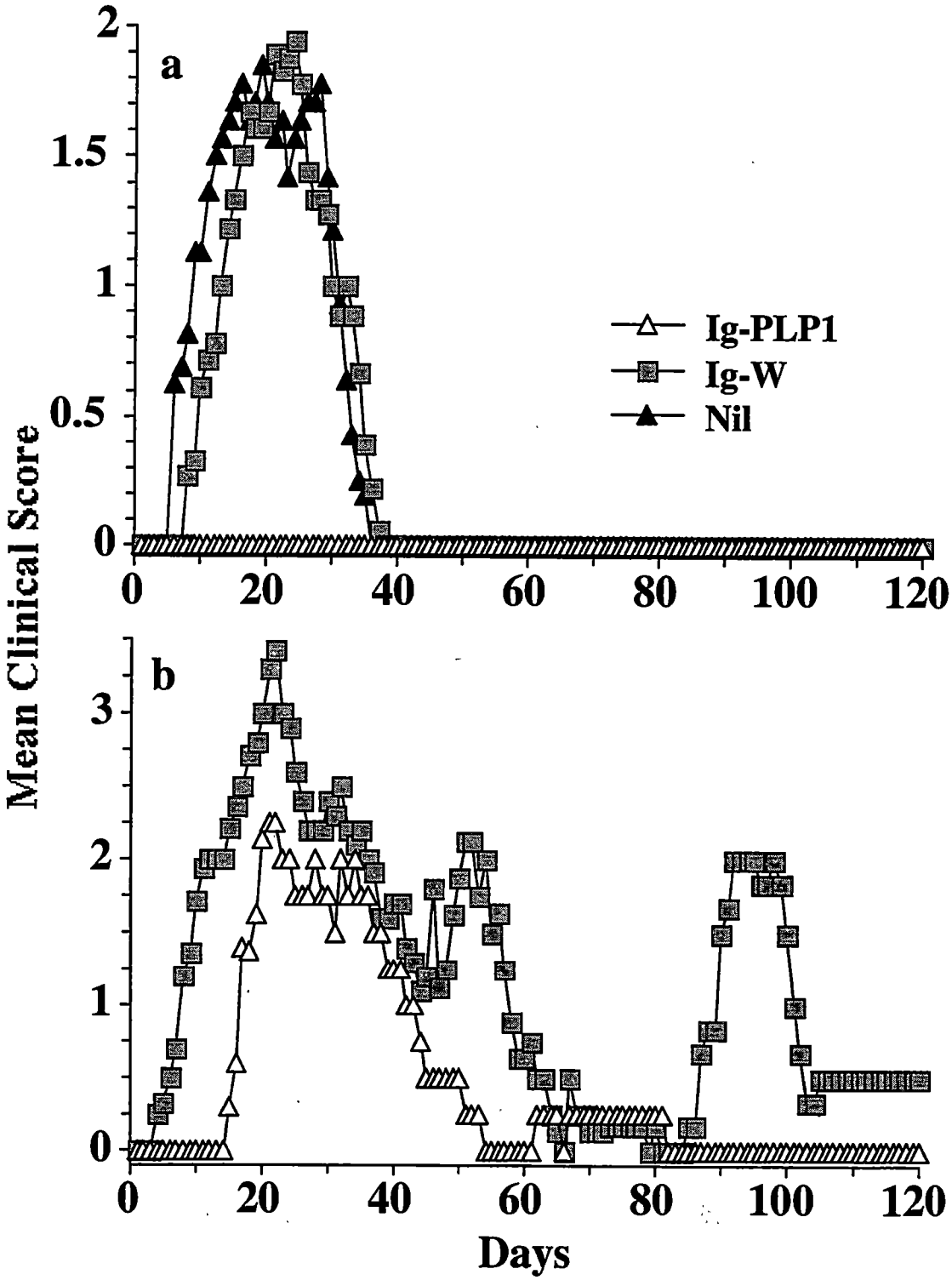

a
b
Ig-PLP1
Ig-W
Nil
Mean Clinical Score
Days
0
0.5
1
1.5
2
3
0
20
40
60
80
100
120

Figure 6.

Fetal exposure to Ig-PLP-LR does not affect proliferative responses to challenge with Ig-PLP1 or Ig-PLP-LR.

Pregnant mothers were injected with 100 μg of Ig-PLP1, Ig-PLP-LR, or control Ig-W (Tolerogen) on days 16, 17, and 18 of gestation, and adult offspring (6-8 week old) were challenged with 50 μg of Ig-PLP1 or Ig-PLP-LR (Immunogen) in CFA as described in Chapter 3 Materials and Methods. After 10 days the lymph node (a) and splenic (b) proliferative responses to the stimulators PLP1 (closed bars) and PLP-LR (open bars) were assessed. Each bar represents the mean ± SD of 5 individually tested mice.

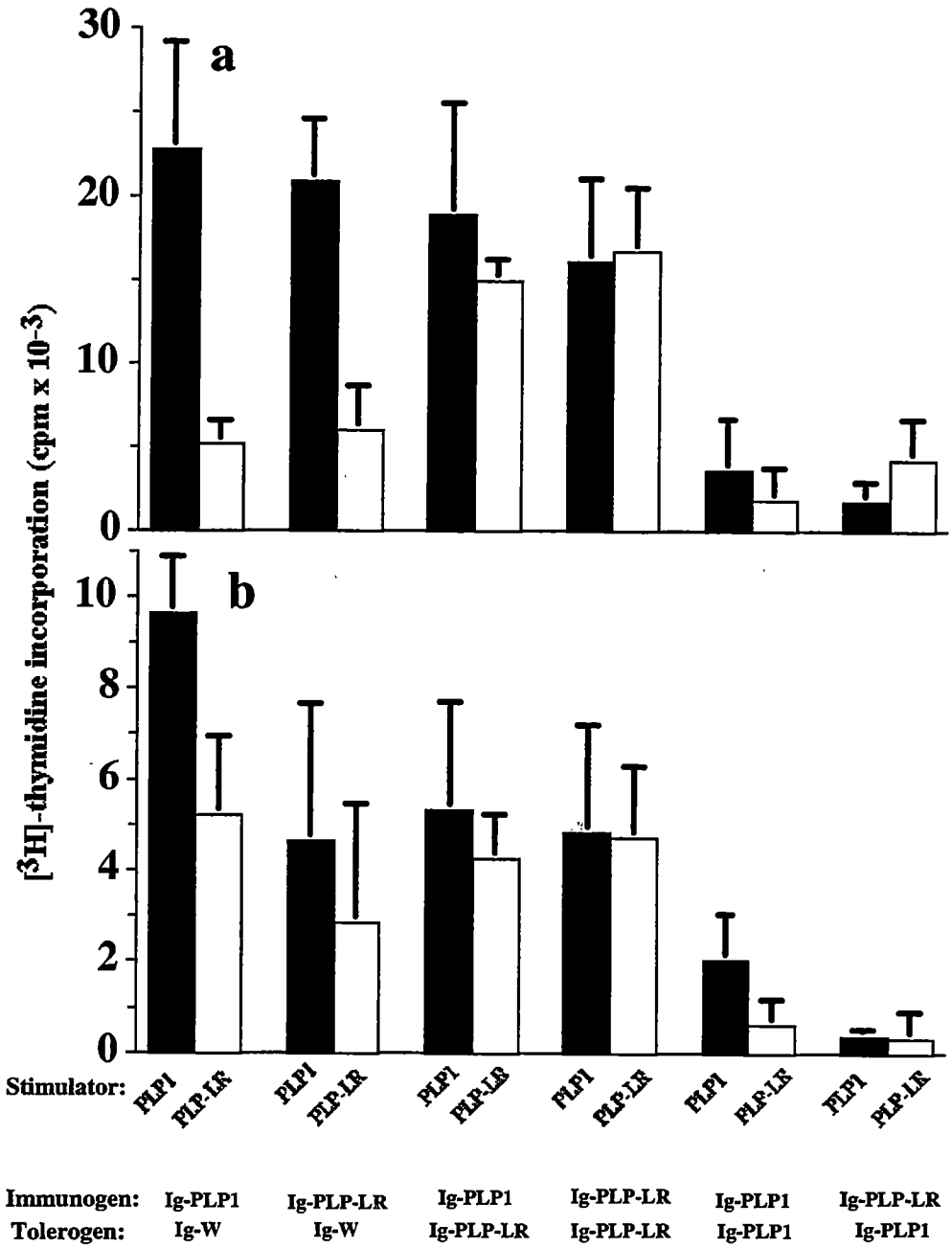

30
a
20
10
0
10
b
8
6
4
2
0
[3H]-thymidine incorporation (cpm x 10-3)
Stimulator:
PLP1
PLP-LR
PLP1
PLP-LR
PLP1
PLP-LR
PLP1
PLP-LR
PLP1
PLP-LR
PLP1
PLP-LR
Immunogen:
Ig-PLP1
Ig-PLP-LR
Ig-PLP1
Ig-PLP-LR
Ig-PLP1
Ig-PLP-LR
Tolerogen:
Ig-W
Ig-W
Ig-PLP-LR
Ig-PLP-LR
Ig-PLP1
Ig-PLP1

Figure 7.

Fetal exposure to Ig-PLP-LR does not alter the production of IL-2 in response to challenge with Ig-PLP1 or Ig-PLP-LR.

IL-2 production by lymph node cells in response to stimulation with PLP1 (closed bars) and PLP-LR (open bars) of the mice described in figure 6 was assessed by ELISPOT as described in Chapter 3 Materials and Methods. Each bar represents the mean ± SD of 5 individually tested mice.

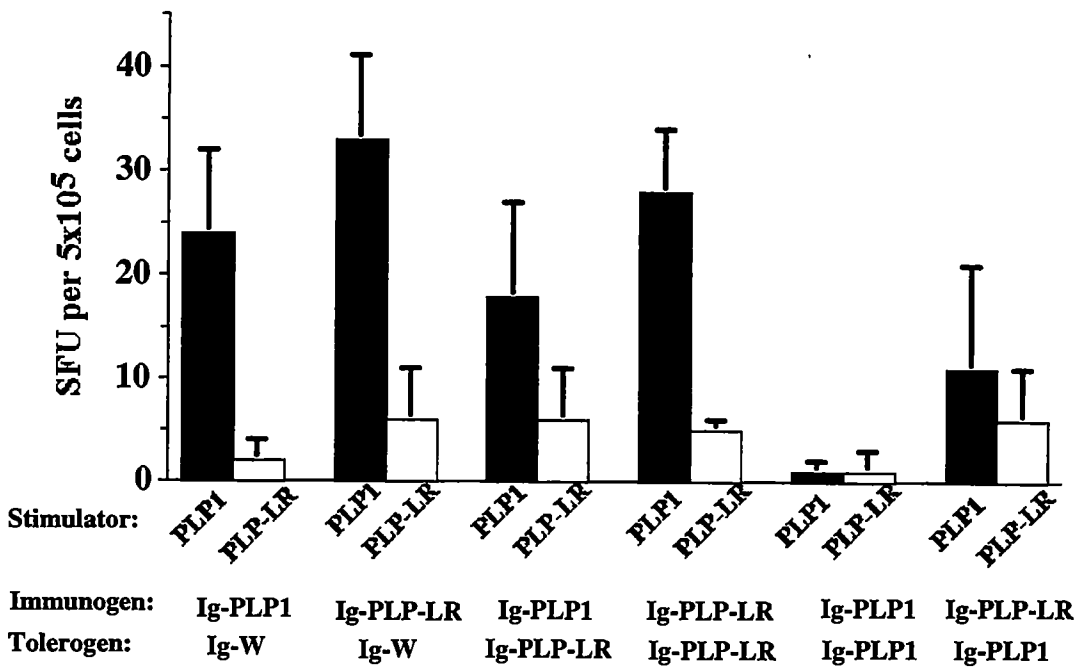

SFU per 5x10^5 cells
40
30
20
10
0
Stimulator:
PLP1
PLP-LR
Immunogen:
Ig-PLP1
Ig-PLP-LR
Ig-PLP1
Ig-PLP-LR
Ig-PLP1
Ig-PLP-LR
Tolerogen:
Ig-W
Ig-W
Ig-PLP-LR
Ig-PLP-LR
Ig-PLP1
Ig-PLP1

Figure 8.

Fetal exposure to Ig-PLP1 does not affect responses to PLP2.

Pregnant mothers were injected with 100 μg of Ig-PLP1 or control Ig-W (Tolerogen) on days 16, 17, and 18 of gestation and 6-8-week-old offspring were challenged with 100 μg of PLP2 (Immunogen) in CFA as described in Chapter 3 Materials and Methods. After 10 days, the lymph node (a) and splenic (b) proliferative responses to the stimulators PLP1 (closed bars) and PLP2 (hatched bars) were assessed by [^{3}H]thymidine incorporation as described in Chapter 3 Materials and Methods. IL-2 production in the lymph node (c) and spleen (d) was measured by ELISPOT and ELISA, respectively. The lymph node cells were used at 4 x 10^5 cells/well (a) and 5 x 10^5 cells/well (b), and splenic cells (b and d) were used at 1 x 10^6 cells/well. The stimulators PLP1 and PLP2 were used at the defined optimal 15μg/ml concentration.

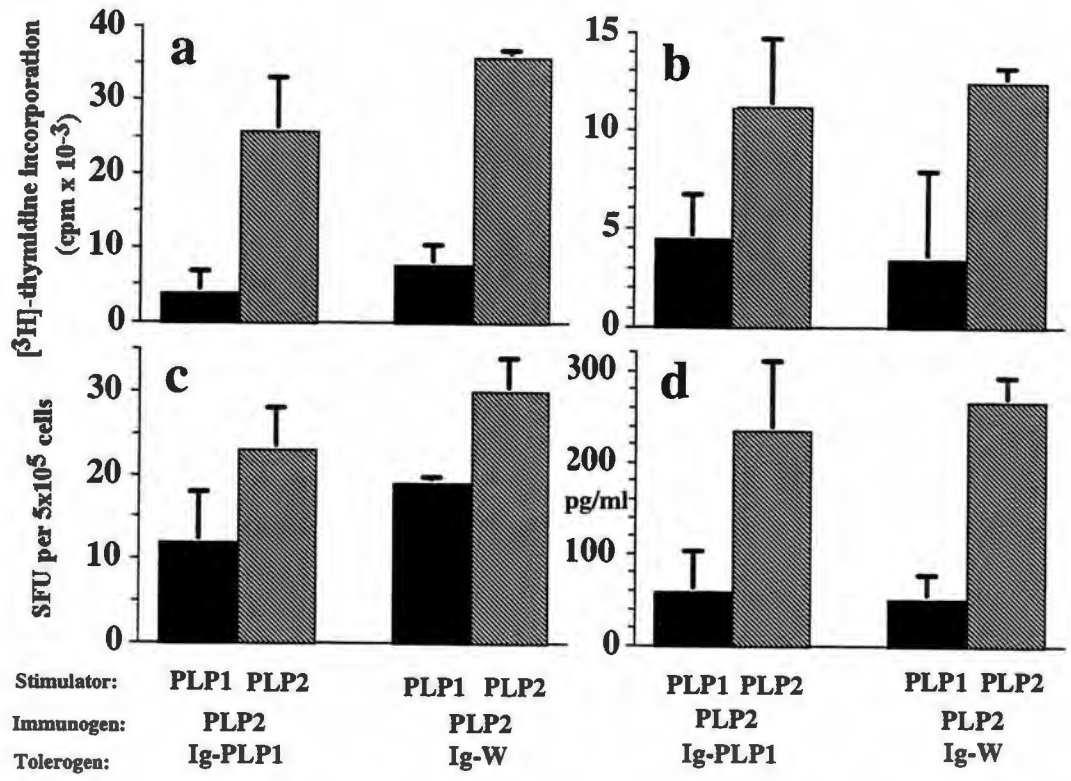

a
b
c
d
[3H]-thymidine incorporation (cpm x 10-3)
SFU per 5x10^5 cells
pg/ml
40
30
20
10
0
15
10
5
0
300
200
100
Stimulator: PLP1 PLP2 PLP1 PLP2 PLP1 PLP2 PLP1 PLP2
Immunogen: PLP2 PLP2 PLP2 PLP2
Tolerogen: Ig-PLP1 Ig-W Ig-PLP1 Ig-W

Figure 9.

Fetal exposure to Ig-PLP-LR does not ablate Ig-PLP1 or Ig-PLP-LR mediated EAE.

Pregnant mothers were injected with 100 μg of Ig-PLP1 (triangles), Ig-PLP-LR (circles), or saline (squares) on days 16, 17, and 18 of gestation, and adult offspring (6-8 week old) were induced for EAE with 200μg of Ig-PLP1 (a) or Ig-PLP-LR (b) as described in Chapter 3 Materials and Methods. The mice were scored daily for disease for 120 days. Each point represents the mean clinical score of 7 mice.

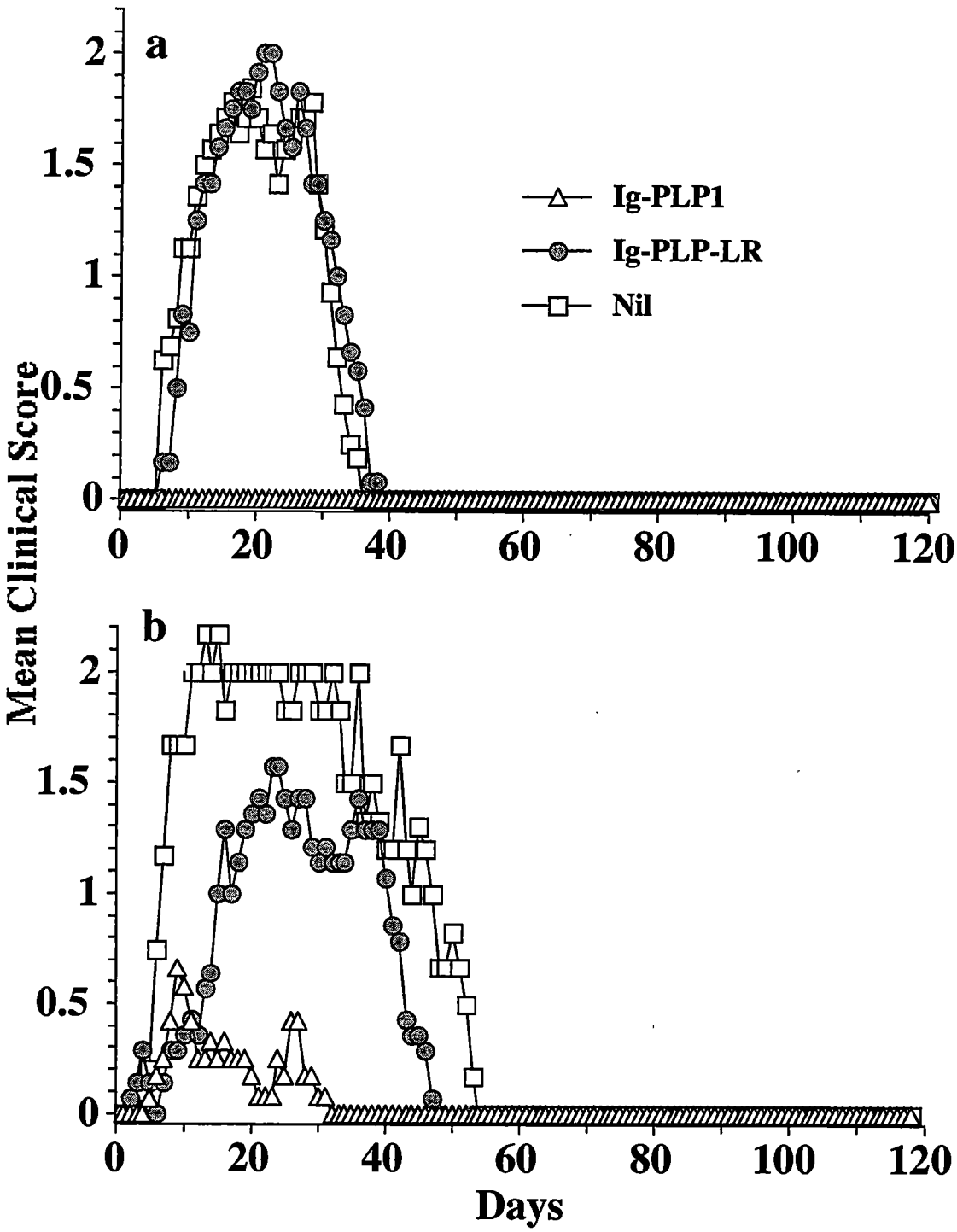

a
b
Ig-PLP1
Ig-PLP-LR
Nil
Mean Clinical Score
Days
0
0.5
1
1.5
2
0
20
40
60
80
100
120

Figure 10.

Specificity of the 2.1H8 T cell clone.

Adult SJL/J mice were immunized s.c. with 50 μg Ig-PLP-LR in CFA. Ten days later the lymph nodes were removed, and the cells (5 x 10^6) were stimulated with PLP-LR peptide (15μg/ml). After 5 days blasts were separated on a histopaque gradient, and the cells were restimulated with peptide and fresh irradiated (3,000 rads) APCs. Ten days later the cells were washed, resuspended in media containing 10% T-STIM (Collaborative Research, Boston, MA), and rested for 7 days. After 3 cycles of stimulation/resting the cells were cloned by limiting dilution (0.3 cells/well), and the clones screened for reactivity to PLP-LR by proliferation. Positive clones were then tested for cytokine production by ELISA, and clone 2.1H8, which produced IL-4 but not IFNγ or IL-2 was retained. The T cell clone was then tested against various antigens to assess its fine specificity. Cells (5 x 10^4/well) were incubated with irradiated (3,000 rads) splenic APCs (5 x 10^5/well) and graded amounts of antigen for 24 hours. Subsequently, the supernatant was separated from the cells and used for detection of IL-4 by ELISA. Each point represents the mean of triplicate wells.

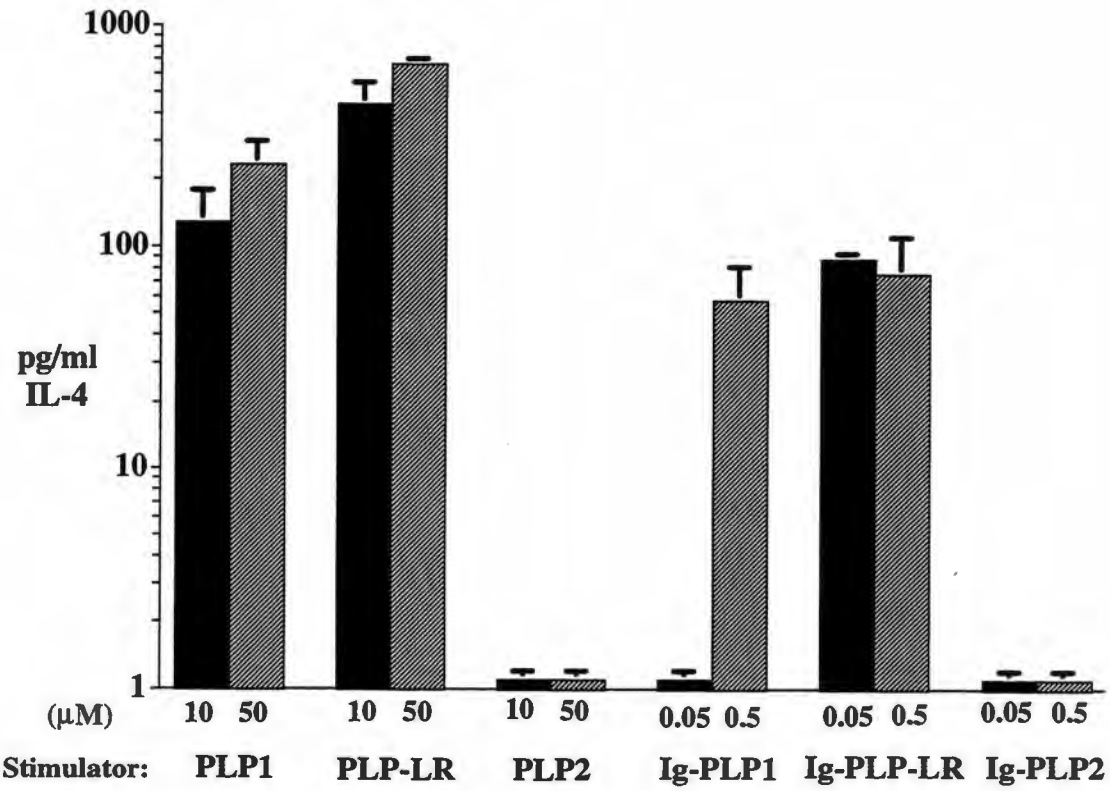

1000
100
10
1
pg/ml
IL-4
(μM)
10 50
10 50
10 50
0.05 0.5
0.05 0.5
0.05 0.5
Stimulator:
PLP1
PLP-LR
PLP2
Ig-PLP1
Ig-PLP-LR
Ig-PLP2

Figure 11.

Discrepancy between central and peripheral fetal presentation of Ig-PLP-LR.

Thymic (a) and splenic (b) cells from a pool of 4 neonates born to mothers injected with 300 μg Ig-PLP1 (triangles), Ig-PLP-LR (circles), or control Ig-W (diamonds) were incubated with 2.1H8 T cells (5 x 10^4) without addition of exogenous antigen. After 24 hours the supernatant was separated from the cells and used for detection of IL-4 by ELISA. For control purposes, 5 x 10^5 thymic (c) and splenic (d) cells, from neonates born to mothers that were injected with saline instead of the Ig chimeras, were cultured with 2.1H8 T cells (5 x 10^4) in the presence of graded amounts of exogenous Ig-PLP1 (triangles), Ig-PLP-LR (circles), or control Ig-W (diamonds). After a 24 hour incubation, IL-4 production was measured by ELISA. Each point represents the mean ± SD of triplicates.

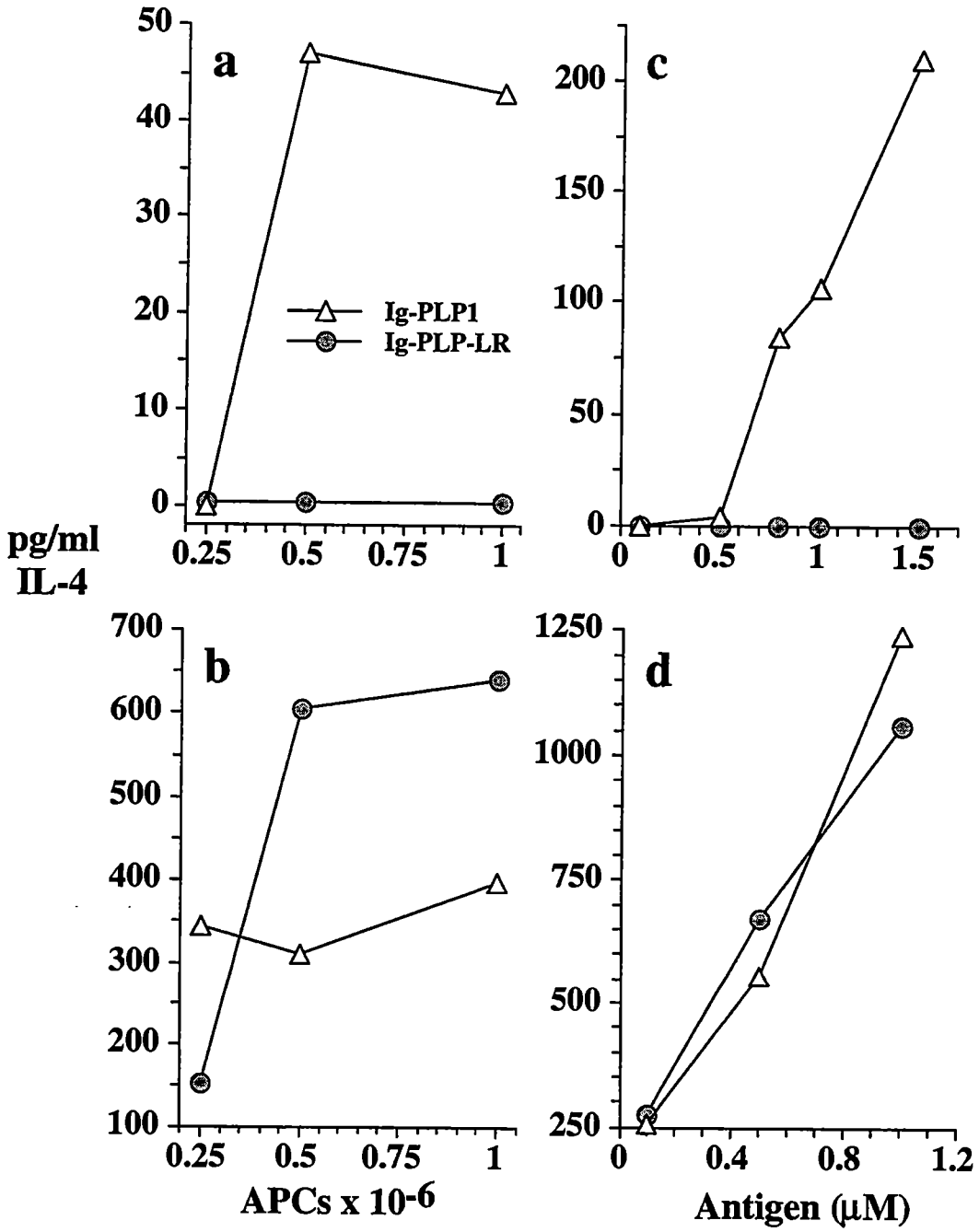
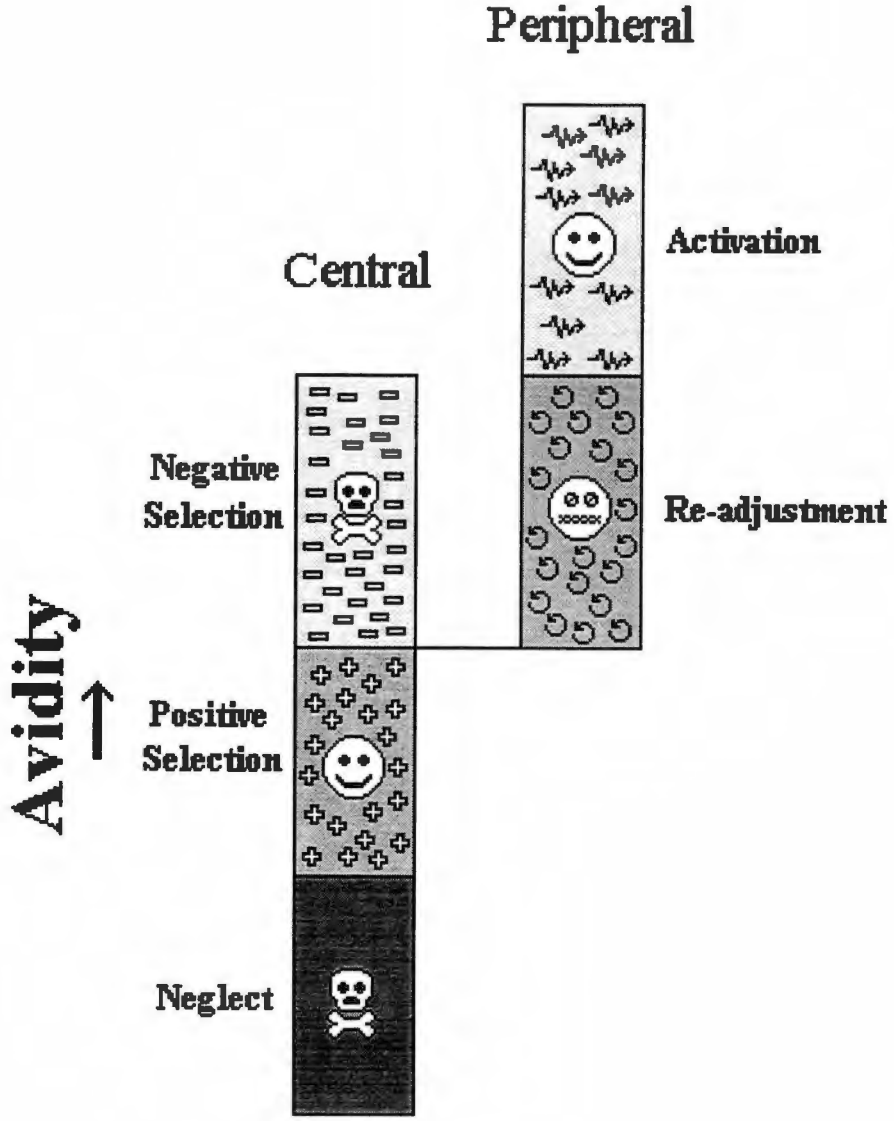

a
b
c
d
pg/ml
IL-4
Ig-PLP1
Ig-PLP-LR
APCs x 10-6
Antigen (μM)
50
40
30
20
10
0
0.25
0.5
0.75
1
200
150
100
50
0
0.5
1
1.5
700
600
500
400
300
200
100
1250
1000
750
500
250
0.4
0.8
1.2

Figure12.

Central and peripheral T cell development: dependence on TCR-ligand avidity at both stages.


Peripheral
Central
Activation
Negative
Selection
Re-adjustment
Avidity
Positive
Selection
Neglect

Part IV.

Coupling of Peripheral Tolerance to Endogenous IL-10 Promotes Effective Modulation of Myelin-Activated T cells and Ameliorates Experimental Allergic Encephalomyelitis.

(Journal of Experimental Medicine, 2000, 191: 2039-2051)

Chapter 1. Abstract

A number of immune-based approaches are being considered for modulation of inflammatory T cells and amelioration of autoimmune diseases. The most recent strategies include simulation of peripheral self-tolerance by injection of adjuvant free antigen, local delivery of cytokines by genetically altered T cells, and interference with the function of costimulatory molecules. Although promising results have been obtained from these studies, which define mechanisms of T cell modulation efficacy, practicality, and toxicity concerns remain unsolved, thereby, justifying further investigations to define alternatives for effective down-regulation of aggressive T cells. In prior studies we demonstrated that Ig-PLP1, an immunoglobulin (Ig) chimera carrying the encephalitogenic PLP1 peptide corresponding to amino acid (aa) sequence 139-151 of proteolipid protein (PLP), is presented to T cells ≈100 fold better than free PLP1. Herein, we demonstrate that aggregation endows Ig-PLP1 with an additional feature, namely, induction of IL-10 production by macrophages and dendritic cells, both of which are antigen presenting cells (APCs). These functions synergize in vivo and drive effective modulation of autoimmunity. Indeed, it is shown that animals with ongoing active experimental allergic encephalomyelitis (EAE) dramatically reduce the severity of their paralysis when treated with adjuvant free aggregated (agg) Ig-PLP1. Moreover, IL-10 displays bystander antagonism on unrelated autoreactive T cells allowing for reversal of disease involving multiple epitopes. Therefore, aggregated Ig-PLP1 likely brings together a peripheral T cell tolerance mechanism emanating from peptide presentation by APCs expressing suboptimal costimulatory molecules and IL-10 bystander suppression to drive

a dual-modal T cell modulation system effective for reversal of autoimmunity involving several epitopes and diverse T cell specificities.

Chapter 2. Introduction

During development, T cells whose antigen receptors are devoid of self reactivity exit the thymus, and participate in immune surveillance, while those bearing receptors endowed with self reactivity are negatively selected and deleted by programmed cell death (1). This process of T cell screening and selection, known as central tolerance, requires the antigen to be available in the thymus in sufficient quantities and in a form presentable by MHC molecules (2-5). Although central tolerance exerts a tight control on the shaping of the T cell repertoire, some self-reactive T cells still escape the thymus and migrate to the periphery (6-8). If the antigen is available in the periphery a second round of T cell screening, known as peripheral tolerance, will follow to further minimize autoreactivity (3, 9-13). Presumably, peripheral tolerance develops as a consequence of presentation of autoantigen by non-activated APCs expressing minimal or no costimulatory molecules (3, 14).

For sequestered autoantigens that are not available for presentation in either the thymus or the periphery, the corresponding T cells will circulate harmlessly. However, events that trigger exposure of those autoantigens, which are usually accompanied by conditions favorable for activation of local APCs, lead to an optimal presentation to and activation of the circulating T cells (15-18). The results of this T cell activation may be the escalation of inflammatory reactions and injury of specific tissues and organs (19-21). Supply of antigen in an adjuvant free form might not stimulate the expression of costimulatory molecules on APCs and thereby drive an antigen presentation inadequate for T cell activation (3,22,23). Prior studies have in fact indicated that this approach modulates autoreactive T cells, and promotes recovery from illness (24-26). However, the

usefulness of this approach for modulation of autoimmunity is hampered by the unlimited availability of autoantigen at the injury site and the consequent continuous activation of the self-reactive T cells. In addition, since bystander suppression is unlikely to occur, the approach holds little promise for modulation of T cell-mediated autoimmunity involving multiple antigens. To overcome these issues, an in vitro approach that uses plasmid (27) and viral (28, 29) vector driven modulatory cytokines was adopted. Indeed, autoreactive T cell clones or hybridomas expressing the cytokines IL-4 or IL-10, as a consequence of transfection or infection, induced recovery from disease when injected into animals with ongoing EAE (27, 28). This is a promising approach and bodes well with the development of practical strategies that could combine both peripheral tolerance and cytokine antagonism to combat autoimmunity.

It has previously been shown that peptide delivery on Igs increases presentation by 100-1000 fold relative to free peptide (30, 31). It is also known that cross-linking of Fc receptors (FcRs) on target cells by antigen-antibody complexes can trigger the production of cytokines (32-34). Moreover, aggregation of Igs confers the effector functions associated with the Fc fragment without the need for complex formation (35, 36). Herein, the encephalitogenic PLP1 peptide was genetically engineered into an Ig molecule (30), and the resulting Ig-PLP1 chimera was aggregated and assayed for modulation of autoreactive T cells and amelioration of active EAE. The results show that aggregated (agg) Ig-PLP1 induced IL-10 secretion by both macrophages and dendritic cells but not B cells. In vitro, APCs incubated with agg Ig-PLP1 presented PLP1 to specific T cells. However, because of the IL-10 secreted by the presenting APCs, IFNγ production by the T cells was impaired. In vivo, when soluble (sol) Ig-PLP1 was injected

into mice with ongoing EAE the severity of disease was slightly reduced. However, when mice were given agg Ig-PLP1 full recovery was achieved. Moreover, agg Ig-PLP1 was able to modulate disease induced either by an encephalitogenic peptide, other than PLP1, or CNS homogenate. Neutralization of endogenous IL-10 by injection of anti-IL-10 antibody during administration of agg Ig-PLP1 restored disease severity. Therefore, agg Ig-PLP1 triggers IL-10 production by APCs, drives inadequate peripheral presentation of PLP1, and couples both events to modulate autoimmunity involving diverse T cell specificities.

Chapter 3. Materials and Methods

Animals

SJL/J (H-2^s) mice were purchased from Harlan-Sprague-Dawley (Frederick, MD), bred, and maintained in the University of Tennessee animal care facility for the duration of the experiments.

Antigens

Peptides. The peptides used in this study were purchased from Research Genetics (Huntsville, AL) and were HPLC purified to > 90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses amino acid (aa) residues 139-151 of PLP and is encephalitogenic in SJL/J mice (37). PLP2 peptide (NTWTTCQSIAFPSK), encompassing aa 178-191 of PLP, is likewise encephalitogenic in SJL/J (38). MBP3 peptide (VHFFKNIVTPRTP) corresponding to aa residues 87-99 of myelin basic protein (MBP) is also I-A^S restricted and induces EAE in SJL/J mice (39). HA peptide, an I-E^d-restricted epitope (31), corresponding to aa residues 110-120 of influenza virus hemagglutinin (HA) was used for negative control purposes.

CNS homogenate. Fifty frozen unstripped rat brains (Pelfreez Biologicals, Rodgers, AK) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS. CNS homogenate was stored at –20°C.

Ig-PLP chimeras. The Ig-PLP1 and Ig-PLP2 chimeras harbor, within the heavy chain CDR3 region, PLP1 and PLP2, respectively, and were previously described (30, 40, 41). Ig-W is the parental IgG2b anti-arsonate antibody, 91A3 not encompassing any PLP peptide and was described elsewhere (30). Large-scale cultures of Ig-W, Ig-PLP1,

and Ig-PLP2 transfectants were preformed in DMEM containing 10% serum supreme (BioWhittaker, Walkersville, MD) and purified on separate rat anti-mouse κ chain sepharose columns to avoid cross-contamination. Subsequently the Ig-chimeras were dialyzed against PBS and concentrated on collodion membranes (Schleicher & Schuall, Keene, NH). The chimeras were aggregated by precipitation with 50%-saturated $(NH_4)_2SO_4$ as described (42). Briefly, filtered 100% saturated $(NH_4)_2SO_4$ was added at an equal volume to the sol Ig-chimera preparation. The mixture was incubated at 24°C for 1 hour with gentle agitation every 20 minutes. Subsequently, the samples were spun down at 10,000 rpms and the pellet resuspended at 1mg/ml in PBS. Electrophoresis on a 10% acrylamide gel indicated that the sol Ig-chimera entered the gel and migrated around 160 kD. However, the agg Ig-chimera did not enter the gel. Knowing that we applied the equivalent of 2 μg of agg Ig-chimera and that the sensitivity of the technology is 0.1 μg, we concluded that at least 95% of the agg Ig-chimera preparation is in an aggregate form.

Induction of EAE

6-8 week old mice were induced for EAE by s.c. injection in the footpads and at the base of the limbs and tail with a 200μl IFA/PBS (vol/vol) solution containing the autoantigen and 200μg Mycobacterium tuberculosis H37Ra. Six hours later the mice were given i.v. 5 x 10^9 inactivated *Bordetella pertussis* (Bioport, Lansing, MI). A second injection of *B. pertussis* was given after 48 hours. Subsequently, the mice were scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or

death. In some experiments purified pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) was used instead of whole *B. pertussis* organism.

Treatment of EAE with Ig-PLP1

Mice induced for EAE with PLP1, PLP2, a mixture of PLP1+PLP2, or CNS homogenate began receiving treatment with Ig-PLP1 after loss of tail tone, which occurs regularly between days 6 and 8 post disease induction. Treatment injections were given i.p. in PBS on days 9, 13, and 17.

Histopathology

Mice treated with agg Ig-PLP1 or agg Ig-W were sacrificed at the peak of the initial phase of disease (day 28 post disease induction), and the brain and spinal cord were removed, fixed with formalin, and embedded in paraffin. Serial cross-sections (6μm) from the cerebellum, cerebrum, and lumbar cord were cut and stained with hematoxylin-eosin (H&E). Perivascular clusters containing at least 20 mononuclear cells were counted as an inflammatory focus.

T Cells

TCC-PLP1-1B10. Adult SJL mice were immunized s.c with 100μg PLP1 peptide in CFA, and 10 days later the draining lymph nodes were removed and the cells (5×10^6 cells/ml) stimulated with PLP1 (15μg/ml). After 5 days the blasts were separated on a Histopaque gradient (Sigma, St. Louis, MO), and then restimulated with peptide and fresh irradiated (3000 rads) syngenic APCs. Ten days later, the cells were washed,

resuspended in media containing 10% T-Stim (Collaborative Research, Boston, MA) and rested for 7 days. After three cycles of stimulation/resting, the cells were cloned by limiting dilution (1 cell/3 wells) and positives were subjected to a second round of limiting dilution cloning. Subsequently, one clone, designated TCC-PLP1-1B10, was selected for these studies.

Isolation of Macrophages, Dendritic cells, and B cells

Macrophages. Macrophages were obtained from the peritoneal cells of mice injected with thioglycolate broth as previously described (43). Briefly, 2 ml of thioglycolate broth was injected i.p., and after 5 days the macrophages were removed by washing the peritoneal cavity with 8 ml of HBSS 4μM EDTA. Macrophage purity was ≥ 93% as determined by FACS® analysis using antibody to F4/80 marker.

Dendritic cells. Dendritic cells were purified from SJL/J spleen according to the standard collagenase/ differential adherence method (44). Cell purity was ≥ 94% as determined by FACS® analysis using antibody to the 33D1 marker.

B cells. SJL/J splenocytes were panned on plates coated with rat anti-mouse κ (1mg/ml) for 15 minutes at 25°C. Non-adherent cells were washed out with PBS. B cells were then dissociated from the plate by incubation with lidocaine HCl (0.8mg/ml) followed by vigorous pipeting. Cell purity was ≥ 90% as determined by FACS® analysis for expression of B220 marker.

Proliferation assays

SJL/J splenocytes (10 x 10^5cells/well/100µl) were pulsed with graded amounts of antigen on round-bottom 96-well plates for 4 hours, pelleted, fixed with 1% paraformaldehyde for 15 minutes washed, and transferred to a fresh 96-well plate. TCC-PLP1-1B10 cells (0.5 x 10^5cells/well/100µl) were then added and incubated for 3 days. Subsequently, 1µCi [^{3}H] thymidine was added per well, and the incubation continued for an additional 14.5 hours. The cells were then harvested on glass fiber filters, and incorporated [^{3}H] thymidine counted using an Inotech β counter (Wohlen, Switzerland).

Cytokine detection

ELISA. ELISA was done according to PharMingen's standard protocol. The capture Abs were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; rat anti-mouse IFNγ, R4-6A2; rat anti-mouse IL-10, JES5-2A5; and rat anti-mouse IL-5, TRFK5. The biotinylated anti-cytokine Abs were rat anti-mouse IL-2, JES6-5H4; rat anti-mouse IL-4, BVD6-24G2; rat anti-mouse IFNγ, XMG1.2; rat anti-mouse IL-10, JES5-16E3; and rat anti-mouse IL-5, TRFK4. ELISA for the detection of active TGFβ was preformed using the human $TGF\beta_1$ DuoSet kit (Genzyme, Cambridge, MA) according to the manufacture's instructions. Bound ligand was revealed using the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaitherburg, MA). Assays were read on a SpectraMAX 340 counter. Graded amounts of recombinant mouse IL-2, IL-4, IFNγ, IL-10, IL-5, and TGFβ were included in all experiments for

construction of standard curves. The cytokine concentration in culture supernatants was estimated by extrapolation from the linear portion of the standard curve.

ELISPOT. ELISPOT assays were used to measure the cytokines produced by lymph node T cells upon stimulation with antigen as described (41). Briefly, lymph node cells (5 x 10^5 cells/100µl/well) and the antigen (100 µl/well) were incubated in HA-multiscreen plates (Millipore, Bedford, MA) coated with capture antibody for 24 hours. Bound cytokines were revealed with peroxidase and anti-cytokine antibodies. The anti-cytokine antibody pairs used here were those described for the ELISA technique. Spots were counted under a dissecting microscope.

Stimulation of cytokine production by TCC-PLP1-1B10

Stimulation was performed with both irradiated and fixed APCs. In one case, SJL/J splenocytes were irradiated (3000 rads) and plated at (5 x 10^5 cells/well/50µl) with graded concentrations of antigens (100µl/well). After 1 hour, TCC-PLP1 1B10 cells (0.5 x 10^5cells/well/50µl) were added and the culture incubated for 24 hours. For fixed APCs, SJL/J splenocytes (10 x 10^5cells/well/100µl) were pulsed with graded amounts of antigen, fixed with 1% paraformaldehyde and incubated with TCC-PLP1 1B10 cells (0.5 x 10^5 cells/well/100µl) for 24 hours. Detection and quantification of cytokines was then assessed by ELISA from 100µl of culture supernatant.

Chapter 4. Results

Soluble Ig-PLP1 reduces paralytic severity and suppresses clinical relapses in mice with ongoing EAE.

Prevention and treatment of active autoimmune disease have been achieved by injection of adjuvant free autoantigens or peptides (24-26, 45-49). However, repetitive injections of the autoantigens are required and the disease rebounds when the supply of antigen is discontinued (48). One approach that may overcome these setbacks and modulate active disease is the delivery of the self-peptide on Igs. Igs have long half-lives and grant the peptides access to newly synthesized MHC molecules (31,50), which could lead to efficient peptide loading onto MHC molecules (50) over an extended period of time. To test the Ig delivery system for treatment of active autoimmunity, SJL/J mice were induced for EAE with free PLP1 peptide, and when the clinical signs of disease became apparent the animals were given 3 injections of sol Ig-PLP1 in saline at 4 days intervals and assessed for reduction in disease severity. Control mice were given sol Ig-W, the parental Ig without any PLP1 peptide. The results illustrated in figure 1 show that mice treated with the sol Ig-W had an initial severe phase of paralysis with a mean maximal score of 3.7 ± 0.5 and displayed relapses throughout the 120 day period of examination. The mice treated with sol Ig-PLP1, however, had a reduced severity of paralysis at the initial phase of disease with a mean maximal score of 2.5 ± 0.3 ($p < 0.005$) and fully recovered by day 42. Mice treated with 10-fold excess of free PLP1 peptide had a slight reduction in the severity of paralysis at the initial phase of disease (mean maximal clinical score 3.0 ± 0.2) but never recovered and underwent relapses throughout the entire 120-day observation period (Figure 1).

Aggregated Ig-PLP1 displays higher efficacy than soluble Ig-PLP1 in reversing active EAE.

Binding of antigen-antibody complexes to Fc receptors (FcRs) on target cells induces the production of cytokines (34,51,52). IL-10, produced by macrophages upon exposure to antigen-antibody complexes, exerts antagonist effects on IL-12 production and reverses pro-inflammatory responses (34,52). Similar to complexation of antibodies with antigen, aggregation confers to Igs the effector functions associated with the Fc fragment (35, 36). These include the binding of complement and cross-linking of FcRs. We then reasoned that aggregation of Ig-PLP1 should be able to cross-link FcRs on APCs and drive both cytokine production and efficient loading of PLP1 peptide onto MHC molecules. If this hypothesis proved accurate and IL-10 was among the cytokines produced by APCs, agg Ig-PLP1 should be much more efficient than sol Ig-PLP1 for modulation of active EAE. To investigate this issue mice were induced for EAE with free PLP1 peptide and when signs of clinical EAE became apparent they were given 3 injections of 300 μg agg Ig-PLP1 at 4-day intervals and assessed for signs of paralysis. Control mice were treated with agg Ig-W instead of agg Ig-PLP1. As can be seen in figure 2a, the initial phase of paralytic disease severity was reduced from a mean maximum score of 3.3 ± 0.3 in agg Ig-W treated animals to 1.1 ± 0.5 ($p<0.001$) in the agg Ig-PLP1 recipient mice. In addition, the animals fully recovered within 9 days of completion of the treatment and never relapsed throughout the entire 120-day observation period while agg Ig-W treated mice never recovered and showed relapses throughout the entire period of clinical assessment. The effectiveness of agg Ig-PLP1 is also apparent

when the paralytic clinical signs of agg Ig-PLP1 treated animals were compared to those of animals injected with sol Ig-PLP1 ($p<0.001$) (Figure 2b). Indeed, the mean maximum clinical score was much lower and the recovery faster. Histologic examination of the cerebellum at the peak of disease indicated a lower number of foci and a reduced number of infiltrating mononuclear cells per foci in the mice treated with agg Ig-PLP1 versus those given agg Ig-W (Figure 3). Moreover, when serial histologic cross sections were prepared from both the brain and spinal cord and the mean foci per cross section estimated, there was a two to three fold reduction in the number of foci in agg Ig-PLP1 treated mice versus mice recipient of agg Ig-W (Table I). Furthermore, the foci in agg Ig-PLP1 treated mice had less infiltrating mononuclear cells than those of agg Ig-W treated mice (agg Ig-W: 73 ± 39, agg Ig-PLP1: 32 ± 14, $p<0.005$)

Aggregated Ig-PLP1 induces the production of IL-10 by APCs and down regulates IFNγ secretion by specific T cells in vitro.

To delineate the mechanism underlying the effective modulation of EAE by agg Ig-PLP1, we sought to investigate whether agg Ig-PLP1 stimulates the production of IL-10 by APCs and whether such IL-10 would display an inhibitory function on T cells engaged in the recognition of the PLP1 peptide presented by IL-10 producing APCs. To this end, naïve splenocytes were incubated with sol or agg Ig chimeras, and the supernatants were used for IL-10 detection. As indicated in figure 4a, agg Ig-PLP1, Ig-PLP2, and Ig-W chimeras stimulated the production of IL-10 by splenic cells in a dose-dependent manner. The soluble forms of the chimeras did not induce detectable levels of IL-10. Furthermore, we decided to investigate whether cells known to function as

professional APCs are able to produce IL-10 upon incubation with agg Ig chimeras. To address this issue, thioglycolate-induced peritoneal macrophages and splenic B and dendritic cells were isolated and tested for IL-10 production upon incubation with agg Ig-PLP1. Figure 4b indicates that macrophages and dendritic cells, but not B cells, produce IL-10 upon incubation with agg Ig-PLP1. Mouse IgM was unable to stimulate IL-10 production by any of the APCs tested. These results indicate that agg Ig-PLP1 crosslinks FcγR and induces the production of IL-10 by APCs. Furthermore, pre-incubation of APCs with soluble mouse IgG inhibited agg Ig-PLP1-induced IL-10 production. These results indicate that the IL-10 produced by the APCs was due to crosslinking of FcγR rather than to contamination with endotoxin.

To investigate the effect that APC-derived IL-10 might have on T cells specifically engaged with the APCs through antigen presentation, a PLP1-specific Th0 clone able to produce both type I and type II cytokines upon peptide stimulation was used. This clone, designated TCC-PLP1-1B10, proliferates upon incubation with paraformaldehyde-fixed splenic APCs that were previously pulsed with free PLP1 peptide or agg Ig-PLP1 (Figure 5a). TCC-PLP1-1B10 did not show significant proliferation when the APCs were pulsed with the negative control PLP2 or agg Ig-PLP2. When tested for cytokine production upon incubation with non-fixed splenic APCs and free PLP1 peptide, TCC-PLP1-1B10 produced significant amounts of IL-2, IL-4, and IFNγ (Figure 5b, c, and d). All three cytokines were also detected when agg Ig-PLP1 was used for stimulation (Figure 5b, c, and d). However, IL-10 was detectable at significant levels when the stimulator was agg Ig-PLP1 but not free PLP1 (Figure 5e). Since agg Ig-PLP1 induces IL-10 production by macrophages and dendritic cells, it is likely that the

IL-10 seen in the T cell cytokine assessment assay was the product of splenic APCs rather than TCC-PLP1-1B10. In fact, this statement is confirmed by the observation that IL-10 was undetectable when APCs, pulsed with agg Ig-PLP1, were washed and fixed with paraformaldehyde prior to incubation with TCC-PLP1-1B10 (Figure 6). The other striking observation from the T cell cytokine assessment assay was that the production of IFNγ seemed to be decreased as IL-10 production by APCs increased (Figure 5c and e). To investigate this issue further, an extended range of Ig-PLP1 concentrations were used for stimulation of bulk and purified APCs, and IL-10 and IFNγ production were assessed simultaneously from the same tissue culture well. The results presented in figure 7 clearly indicate that the IL-10 secreted by the APCs antagonizes the production of IFNγ by the T cells. Indeed, when the stimulation assay was preformed using splenocytes, purified DCs, or enriched peritoneal macrophages as APCs (all of which produce IL-10 upon incubation with agg Ig-PLP1, Figure 4), IFNγ production by the T cells decreased dramatically and became undetectable as the production of IL-10 by APCs increased (Figure 7a, b, and c). However, when B cells were used as APCs, which do not produce IL-10 upon incubation with agg Ig-PLP1 (Figure 4b), the secretion of IFNγ by T cells was not affected (Figure 7d). Overall, these results indicate that agg Ig-PLP1 triggers IL-10 production by the presenting APCs (DC and Mϕ) and that such IL-10 antagonizes the production of IFNγ by the T cells.

Synergy between endogenous IL-10 and peripheral tolerance for in vivo modulation of aggressive T cells.

Systemic antigen given to animals without adjuvant usually drives tolerance operating through antigen presentation by peripheral APCs expressing minimal or no costimulatory molecules (3, 22, 23). Incubation of purified macrophages or dendritic cells with sol or agg Ig-PLP1, which allows for efficient loading of peptide onto MHC class II molecules, does not lead to up-regulation of B7-1, B7-2 or CD40. Furthermore, since agg Ig-PLP1 causes the production of IL-10 by APCs (Figure 4), it is likely that IL-10, as has previously been shown (53, 54), inhibits up-regulation of costimulatory molecules on APCs.

Since IL-10 has been defined to antagonize Th1 cytokines (55) and possibly interfere with inflammatory functions, we postulated that the effectiveness of agg Ig-PLP1 in T cell modulation and reversal of disease lies on inadequate peptide presentation by APCs expressing minimal costimulatory molecules and the inhibitory function of IL-10 produced by such APCs. To test this hypothesis, mice were induced for EAE with PLP1 peptide and when the signs of paralysis became apparent the mice were given agg Ig-PLP1 together with anti-IL-10 antibody and assessed for reduction in disease severity. The results presented in figure 8a indicate that the severity of paralysis was restored when in vivo IL-10 was neutralized by the anti-IL-10 antibody. In fact, mice treated with agg Ig-PLP1 alone had a mean maximal clinical score of 1.1 ± 0.5 while the mice injected with both agg Ig-PLP1 and anti-IL-10 antibody had a score of 3.0 ± 0.3 which is comparable to the 3.3 ± 0.3 ($p>0.23$) score seen in mice treated with agg Ig-W. Furthermore, control mice given agg Ig-PLP1 together with rat IgG, instead of anti-IL-10 antibody, did not restore disease severity and had a mean maximal score of 1.6 ± 0.2. Injection of anti-IL-10 antibody together with agg Ig-W neither reduced nor exacerbated

the severity of disease. These results indicate that agg Ig-PLP1 induced IL-10 plays a significant role in controlling disease severity and that for the effects of IL-10 to occur a specific interaction between APCs and the target T cells is required. In support of this statement is the observation that treatment with sol Ig-PLP1 plus exogenous IL-10 reduces the severity of paralysis to the same extent as agg Ig-PLP1 (Figure 8b). Soluble Ig-PLP1, which does not induce detectable levels of IL-10, ameliorates the disease slightly with a mean maximal score of 2.5 ± 0.3 while sol Ig-PLP1, together with exogenous IL-10, further reduces the disease to a mean maximal clinical score of 1.1 ± 0.3 which is comparable to the 1.1 ± 0.5 score obtained with mice treated with agg Ig-PLP1. Furthermore, for endogenous IL-10 to modulate the disease, a physical bridging of the APCs to the T cells seems to be required. This conclusion is drawn from the observation that treatment of diseased mice with a mixture of agg Ig-W and free PLP1 peptide, instead of agg Ig-PLP1, did not reduce the severity of disease (Figure 9). Overall, effective T cell down-regulation requires physical interaction between IL-10 producing APCs, and the target pathogenic T cell. The likely explanation for this requirement is that IL-10, as a paracrine cytokine, needs to be in close proximity to T cells in order to achieve antagonism.

Treatment with aggregated Ig-PLP1 decreases the clinical severity of active EAE induced by multiple epitopes.

IL-10 produced by APCs as a result of agg Ig-PLP1-mediated FcR crosslinking may antagonize specific T cells engaged to the PLP1-MHC ligand on the APCs as well as neighboring T cells with unrelated specificity. This phenomenon known as bystander

suppression has proven effective in IL-4 (56) and IL-10 (57) settings. One way to find out if bystander suppression could be ascribed to the IL-10 in our experimental system is to induce EAE with a mixture of epitopes and test if treatment with agg Ig-PLP1 could modulate unrelated autoreactive T cells and ameliorate the disease. This experiment was carried out and the results presented in figure 10a show that mice with ongoing EAE induced by a mixture of PLP1 and PLP2 peptides, manifested reduced severity of paralysis and fully recovered by day 33 post disease induction after treatment with agg Ig-PLP1 while animals treated with agg Ig-W had severe paralysis and did not recover from the disease during the 50 day period of clinical assessment. Therefore, endogenous IL-10 may have displayed down-regulatory effects on PLP2-specific T cells. Induction of disease with PLP2 peptide should expose whole PLP and drive spreading and activation of PLP1-specific T cells (17, 58). In this case, injection of agg Ig-PLP1 should bridge IL-10 producing APCs to PLP1-specific T cells and promote bystander suppression of these cells as well as neighboring PLP2-specific T cells. To address this issue mice were induced for EAE with PLP2 peptide and when signs of paralysis became apparent they were treated with agg Ig-PLP1. Figure 10b shows that although the initial phase of paralysis in these mice is only slightly milder than untreated mice, the animals quickly recovered by day 26 and, unlike the untreated mice, did not relapse for the remaining period of clinical assessment. These results strengthen the notion of bystander suppression and suggest that epitope spreading offers an opportunity to modulate disease at a later stage of paralysis.

To further explore the broadness of this approach in T cell down-regulation, we tested agg Ig-PLP1 for modulation of disease induced with CNS homogenate, which

incorporates a full range of myelin autoantigens. The results presented in figure 11 clearly indicate that mice injected with agg Ig-PLP1 had mild signs of paralysis in the initial phase of paralysis and fully recovered by day 24 post disease induction without any relapses for the 60 day period of clinical assessment. Control mice treated with agg Ig-W, instead of agg Ig-PLP1, had a disease pattern similar to that of untreated animals (Figure 11). These results indicate that the down-regulatory function of agg Ig-PLP1 extends both to intra- and intermolecular epitopes and suppresses diverse T cell specificities.

Exposure to IL-10 seems to be the likely mechanism underlying down-regulation and suppression of pathogenic myelin-specific T cells. The source of IL-10, as demonstrated in figures 4 and 6, is APCs such as dendritic cells and macrophages. However, the broadened effectiveness and the endurance of T cell modulation in this setting raises the question of whether the bystander suppression was due to antagonism of the pathogenic T cells by APCs' IL-10 or to down-regulation by regulatory T cells generated under the effect of such IL-10 (59). The rational for this statement derives from previous studies suggesting that IL-10 enables naïve T cells to develop into regulatory cells (59) that could produce IL-10, IL-5 or TGFβ and inhibit the function of pathogenic T cells thereby sustaining suppression (46, 60-63). To address this issue, the lymph node T cells from mice, which were recovering from CNS-induced paralysis subsequent to treatment with agg Ig-PLP1, were stimulated with antigen and tested for proliferation and production of cytokines markers of regulatory T cells. The results presented in figure 12 show that 2 days after the final injection of agg Ig chimeras proliferation to myelin peptides was significant in the mice treated with the control Ig-W but at background

levels for those recipient of agg Ig-PLP1. Similarly, while the mice injected with agg Ig-W had significant amounts of IL-2 and IFNγ, those treated with agg Ig-PLP1 had neither Th1 nor Th2 type cytokines and did not produce IL-10, IL-5, or TGFβ. Similar results were obtained when the mice were tested at day 9 after completion of the treatment regimen. Furthermore, splenic T cells and cells harvested from the peritoneum showed a similar pattern of responses. Overall, these results suggest that the typical proliferative and cytokine responses trademark of regulatory T cells are undetectable in this particular setting of systemic treatment of active autoimmunity.

Chapter 5. Discussion

Previous studies have demonstrated that delivery of peptides on Igs increases presentation to T cells in vitro by 100-1000 fold relative to free peptide (30, 31), and induces stronger T cell responses in vivo (31, 64). Internalization of Ig-peptide chimeras into APCs via FcRs and access of the attached peptide to newly synthesized MHC class II molecules are most likely responsible for such effective presentation (31,50). The efficacy of peptide delivery by Igs seems to extend to peripheral APCs expressing minimal or no costimulatory molecules since injection of the Ig-PLP1 chimera without adjuvant into diseased mice modulates PLP1-specific pathogenic T cells and ameliorates EAE (Figure 1). This statement is supported by the finding that 200 nmoles of PLP1 in the form of free peptide reduced the severity of disease only slightly and the animals never recovered, but 20 nmoles of peptide in the form of sol Ig-PLP1 reduced the severity of the initial phase of disease and most of the animals fully recovered by day 42 (Figure 1). The effectiveness of Ig-PLP1 in disease reversal was more dramatic when the chimera was given to mice in an aggregated form (Figure 2 and 3, and Table I). The explanation we wish to put forth for this observation is that agg Ig-PLP1 displays an antagonistic function against T cells at two levels. On the one hand since Ig-PLP1 was injected without any adjuvant it is likely that presentation was carried out mostly by peripheral APCs not expressing optimal costimulatory molecules thereby leading to inactivation of the T cells. In fact incubation of purified dendritic cells and macrophages with sol or agg Ig-PLP1 did not induce up-regulation of B7 or CD40 costimulatory molecules. On the other hand, the presenting APCs produce IL-10 that further antagonizes the T cells engaged to them through PLP1 peptide. The evidence for this dual-modal mechanism for

T cell turn-off derives from two essential observations. First, soluble adjuvant free Ig-PLP1 which does not induce detectable levels of IL-10 (Figure 4a) modulates the disease with significantly fewer copies of peptide than free PLP1 peptide (Figure 1). Second, agg Ig-PLP1 which induces IL-10 production by macrophages and dendritic cells (Figure 4b) enhances T cell antagonism, as the mice display a significant reduction in the frequency and size of inflammatory foci both in the brain and spinal cord (Figure 3 and Table 1), manifest very mild clinical signs of EAE, and fully recover from paralysis by day 25 post disease induction (Figure 2). The contribution of IL-10 to T cell modulation and disease amelioration is supported by both in vitro and in vivo data. Indeed, the TCC-PLP1-1B10 T cell clone, which proliferates and produces both type I and II cytokines upon incubation with free PLP1 (Figure 5) significantly reduced IFNγ production when agg Ig-PLP1 was used for stimulation (Figure 5). This is well illustrated in figure 7, which shows that the production of IFNγ by T cells is inversely proportional to the IL-10 production by dendritic cells and macrophages within the same cell culture. In vivo, neutralization of APCs' IL-10 by anti-IL10 antibodies during treatment with agg Ig-PLP1 restores the severity of disease to scores comparable with Ig-W treatment (Figure 8a). Furthermore, the combination of rIL-10 and sol Ig-PLP1 reduces disease severity to the same extent as agg Ig-PLP1 (Figure 8b). All together, these results suggest that effective control of EAE requires synergy of antagonistic functions against T cells and points out the need for a multi-modal strategy to contain autoimmunity. In fact, systemic injection of IL-10 has proven inefficient for modulation of autoimmunity (65), and only over expression of IL-10 by means of transgenic manipulation (66), targeted delivery of the cytokine by means of engineered T cells (27), or viral gene transfer (29) has proven

effective for control of T cell-mediated autoimmunity. The agg Ig-peptide approach brings together a peripheral tolerance-like mechanism and the antagonism activity of IL-10 to strengthen the modulatory functions against aggressive T cells. Furthermore, since IL-10 exerts its down-regulatory function in a paracrine fashion it may counteract neighboring Th1 cells regardless of antigen specificity. This behavior, known as bystander suppression (56, 57), seems to contribute to disease modulation by agg Ig-PLP1. Indeed, mice induced for EAE with a mixture of PLP1 and PLP2 peptides recovered from paralysis when they were treated with agg Ig-PLP1 (Figure 10). The likely explanation for these results is that IL-10 produced by the APCs exerts an antagonist effect on neighboring T cells specific for PLP2 peptide thereby modulating disease involving two epitopes. Moreover, when the disease was initiated with PLP2 peptide, the treatment with agg Ig-PLP1 allowed paralysis to peak to a significant clinical score but the recovery was expeditious and relapses did not occur. This pattern could suggest that IL-10-mediated bystander suppression had to await exposure of PLP and generation of PLP1-specific T cells to focus the presenting APCs and their IL-10 on the pathogenic T cells. This is two fold important for the usefulness of this approach for treatment of autoimmunity. First, using this strategy for intervention at the initial phase of disease will likely modulate paralysis since the target T cells should become available to focus the APCs and their IL-10 on neighboring pathogenic T cells regardless of their specificity. Second, since epitope spreading seems to follow a sequential order and sustain specific relapses (58) one could devise a regimen to target T cell modulation at later phases in the relapses. The data presented in figure 11, showing that agg Ig-PLP1 modulates disease induced with CNS homogenate, argues for a broad effectiveness of the

approach and supports the notion that bystander suppression offers a strategy for intervention at various stages of the disease.

At the mechanistic level, the in vitro data demonstrate that while IL-10 down-regulates the Th1 type cytokine IFNγ, produced by the TCC-PLP1-1B10 Th0 clone, production of the Th2 cytokine IL-4 was not affected (Figure 5). This suggests that IL-10 antagonism partially affects the specific functions of the T cell rather than driving full inactivation or death of the cells. Whether, IL-10 exerts a similar activity on differentiated Th1 cells in vivo remains to be investigated. On a speculative basis, if IL-10 affects IL-12 synthesis by the APCs a complex regulatory mechanism could be triggered around T cell-APC interactions possibly via CD40-CD40L resulting in down-regulation of Th1 function and suppression of IFNγ production (67-69).

On the other hand, one could speculate that APCs' IL-10 may promote the development of regulatory T cells (46, 59-63), which in turn would help in down regulation of the pathogenic T cells. In fact, this would provide a good argument for the broad and sustainable bystander suppression since regulatory T cells could produce suppressive cytokines such as IL-10 and TGFβ (61, 63). The results presented in figure 12, however, provided no support for this speculation and neither proliferation, IL-10, IL-5, nor TGFβ were obtained subsequent to treatment with agg Ig-PLP1. Although high concentrations of peptide were used for in vitro stimulation and cells from various tissues have been assayed for production of IL-10, IL-5, and TGFβ, this does not provide a definitive exclusion of regulatory cells. A few decades ago Jerne (70) postulated that the immune system keeps itself in check by sustaining an equilibrium of interactions among its components. Pathogenic T cells could be kept harmless by a tight control from

regulatory T cells. A perturbation of these interactions would lead to oscillations in the levels of each component. At the time of recovery, the regulatory T cells may have returned to minimal levels below the sensitivity of our assays.

Overall, agg Ig-PLP1 promotes efficient peptide loading onto MHC molecules and couples a peripheral tolerance-like mechanism and directed IL-10 bystander suppression to counteract aggressive T cells and modulate complex autoimmunity involving multiple epitopes. One should emphasize that the effectiveness of the approach and its bystander suppression likely lies on the physical link of the Ig vehicle to the peptide, which serves to bridge the APCs to the T cell and focus IL-10 on the neighboring pathogenic lymphocytes. This dual-model approach, which proves to be efficient in this autoimmune setting where a high precursor frequency probably entertains the severe paralysis (71), may prove feasible for treatment of human MS, which likely involves a number of myelin autoantigens (19, 72).

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Table I. Treatment with agg Ig-PLP1 ablates clinical and histologic EAE.

	Clinical EAE	Histologic EAE	
Treatment	Mean Maximum Severity	Cerebrum	Lumbar spinal cord
		Foci/cross-section	
agg Ig-W	3.3± 0.3	11.7± 2.1	18.5± 3.3
agg Ig-PLP1	1.1± 0.5	6.5± 0.5	6.9± 1.1

6-8 wk old mice were induced for EAE with PLP1 as described in *Materials and Methods*, and then subsequently treated with 300μg agg Ig-PLP1 or agg Ig-W on days 9, 13, and 17 post disease induction and scored daily for clinical disease. The mean maximum severity was determined by averaging the maximal clinical score obtained from each mouse within a group. In order to determine histological disease, brains and spinal cords were removed from mice on day 28 post disease induction (peak of disease), fixed in formalin, paraffin embedded, serially cross-sectioned at 6μm, and then stained with hemotoxylin-eosin (H&E). An inflammatory foci represents a minimum of 20 mononucular cells/perivascular cluster. Statistical (T test) comparison of agg Ig-W and agg Ig-PLP1 yielded $p < 0.001$.

Figure 1.

Inhibition of paralytic relapses by treatment with sol Ig-PLP1.

Groups of 6-8 week old SJL/J mice were induced for EAE with 100μg PLP1 peptide, and then treated i.p. with 500μg sol Ig-PLP1, 500μg sol Ig-W, or 100μg free PLP1 peptide in PBS on days 9, 13, and 17 post disease induction. Each point represents the mean clinical score of 8 mice. These results are representative of 2 independent experiments.

On the basis of 150 kD molecular weight for Ig-PLP1 and 1.5 kD for free PLP1 we estimated that the 300 μg free PLP1 given during the three injections correspond to ≈ 200 nmoles of PLP1 peptide, and the 1500 μg sol Ig-PLP1 encompasses ≈ 20 nmoles PLP1 peptide. Therefore, when free PLP1 is used for treatment the mice were given ≈10-fold more peptide than for treatment with sol Ig-PLP1.

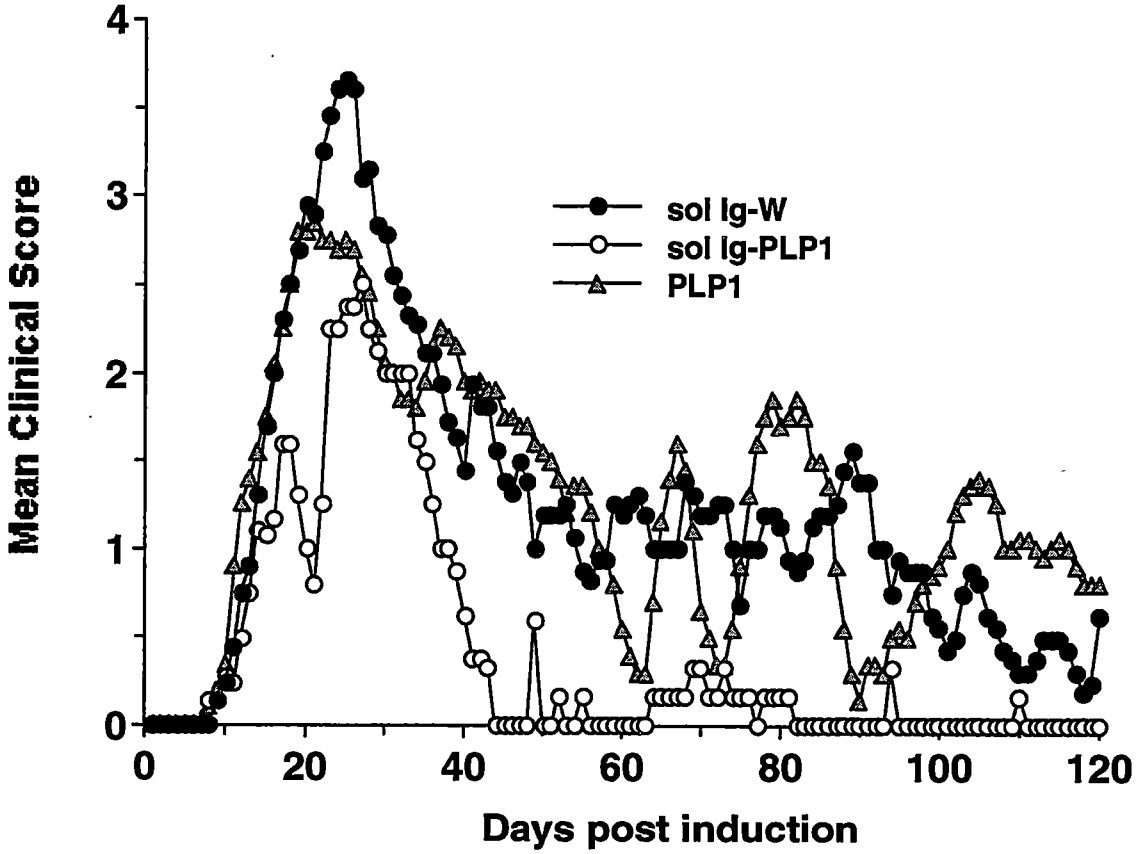

Mean Clinical Score
4
3
2
1
0
0
20
40
60
80
100
120
Days post induction
sol Ig-W
sol Ig-PLP1
PLP1

Figure 2.

Effective amelioration of EAE by agg Ig-PLP1.

(a) Groups of mice (8 per group) were induced for EAE with 100μg PLP1 and then treated with 300μg of agg Ig-PLP1 or agg Ig-W in PBS on days 9, 13, and 17 post disease induction. (b) Direct comparison of the disease course of PLP1 peptide induced EAE following treatment with sol Ig-PLP1 (from figure 1) vs. agg Ig-PLP1 (from figure 2a). Each point represents the mean clinical score of 8 mice. These results are representative of 3 independent experiments.

The 900 μg agg Ig-PLP1 given to mice contains ≈12 nmoles PLP1. This amount is ≈ 17 fold lower than the 200 nmoles given as free PLP1 yet disease modulation was more effective.

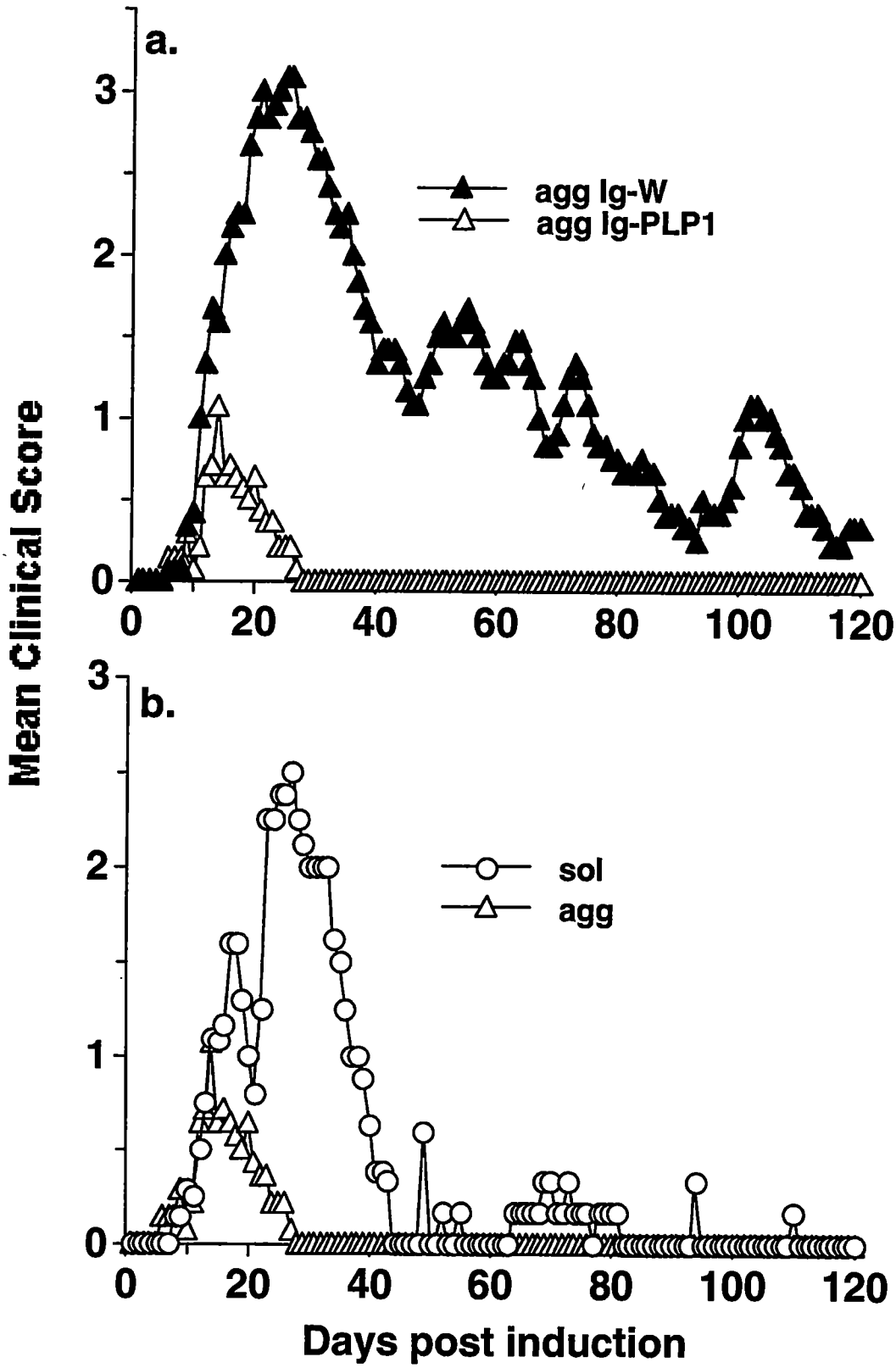
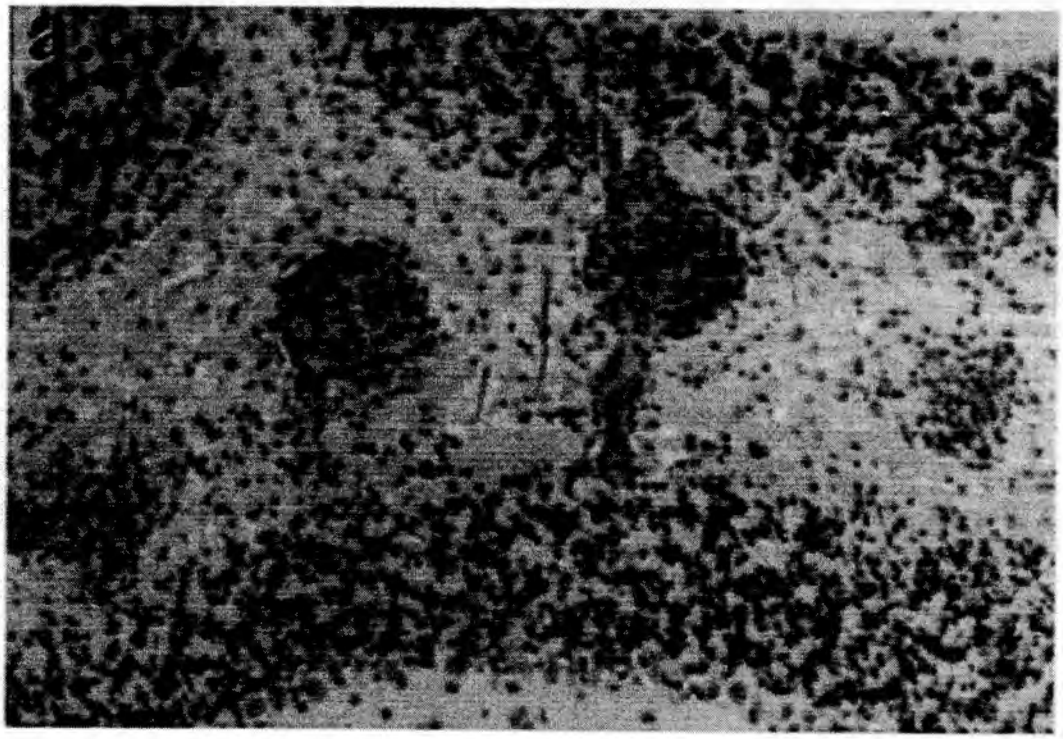

a.
b.
agg Ig-W
agg Ig-PLP1
sol
agg
Mean Clinical Score
Days post induction
0
1
2
3
0
20
40
60
80
100
120

Figure 3.

Agg Ig-PLP1 significantly reduces the number of inflammatory foci within the CNS.

Serial cross-sections were prepared at the peak of disease (day 28) from the cerebellum of mice treated with agg Ig-W (a) and agg Ig-PLP1 (b) and described in figure 2. The brains were fixed with formalin, embedded in paraffin, and stained with hematoxylin-eosin (H&E) as described in Chapter 3 Materials and Methods. Magnification = 40X.



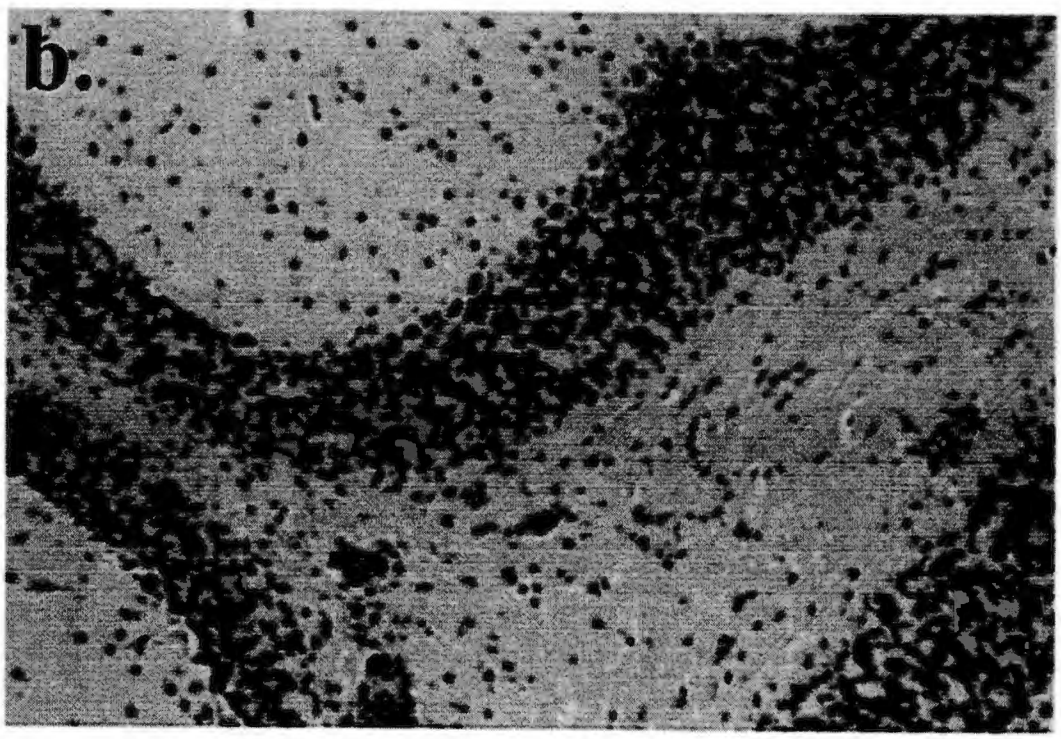

b.

Figure 4.

Agg Ig-PLP chimeras induce the production of IL-10 by APCs.

(a) Irradiated (3000 rads) SJL/J splenocytes (5 x 10^5cells/well) were incubated with graded amounts of sol Ig-PLP1, agg Ig-PLP1, agg Ig-W, sol Ig-PLP2, or agg Ig-PLP2 for 24 hours, and the supernatant used to quantitate IL-10 production by ELISA. (b) Irradiated (3000 rads) B cells (2x10^5cells/well), macrophages (0.2x 10^5cells/well), and dendritic cells (0.2x 10^5cells/well) were incubated with graded amounts of agg Ig-PLP1 (open symbols) or mouse IgM (closed symbols) for 24 hours, and cell culture supernatant was used to measure IL-10 production. Each point represents the mean of triplicate wells. These data are representative of 4 independent experiments.

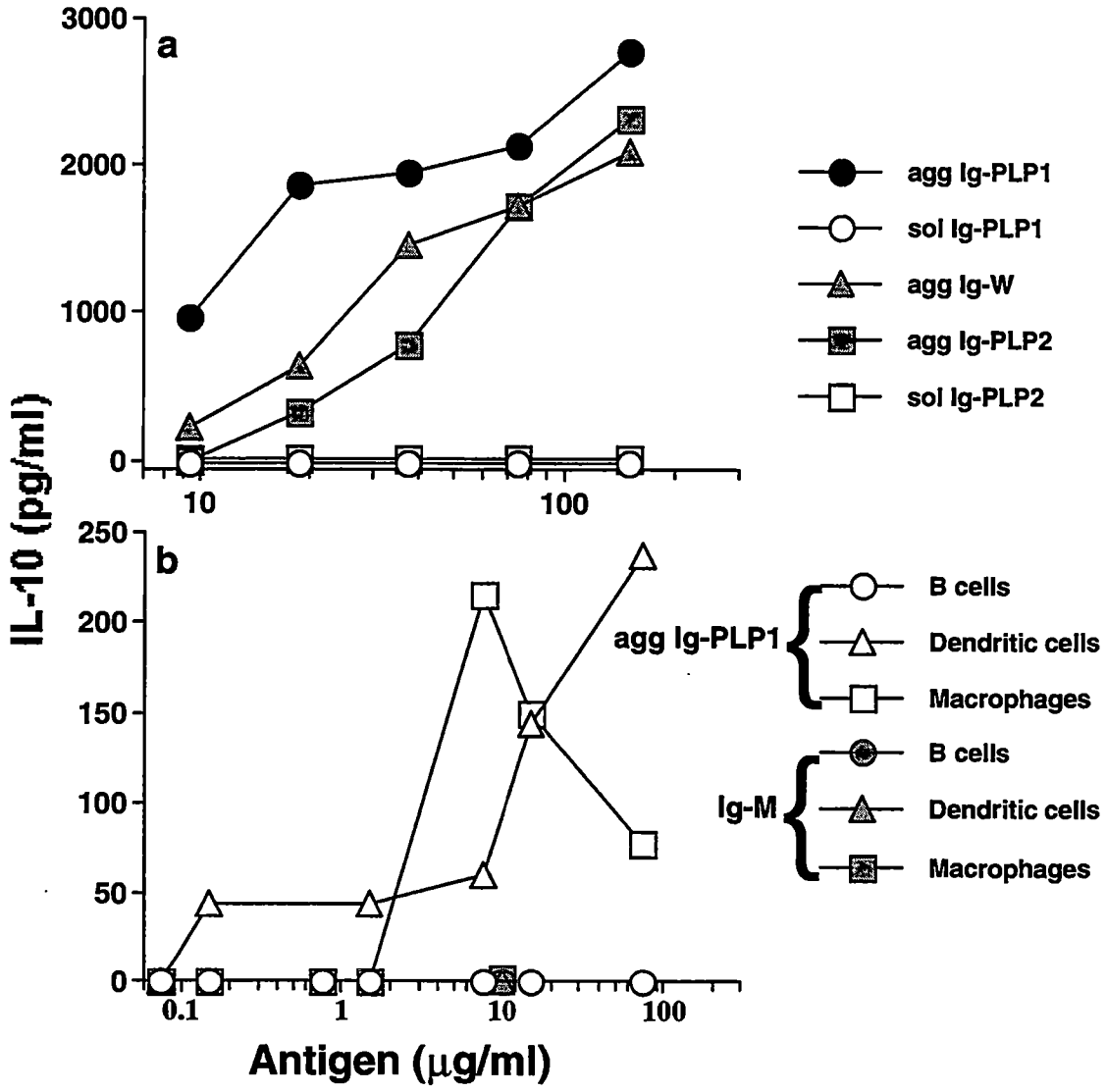

a
3000
2000
1000
0
10
100
agg Ig-PLP1
sol Ig-PLP1
agg Ig-W
agg Ig-PLP2
sol Ig-PLP2
b
250
200
150
100
50
0
0.1
1
10
100
IL-10 (pg/ml)
Antigen (μg/ml)
agg Ig-PLP1
B cells
Dendritic cells
Macrophages
Ig-M
B cells
Dendritic cells
Macrophages

Figure 5.

Specificity and cytokine response of a Th_0 T cell clone to agg Ig-PLP1.

(a) Shows the specificity of the TCC-PLP1-1B10 T cell clone. SJL/J splenocytes ($10.0x10^5$cells/well) were pulsed for 4 hours with graded amounts of free PLP1, free PLP2, agg Ig-PLP1, or agg Ig-PLP2 and then fixed with paraformaldehyde as described in Chapter 3 Materials and Methods. Subsequently, TCC-PLP1-1B10 cells ($0.5x10^5$cells/well) were added to the wells and proliferation assessed by [^{3}H]thymidine incorporation. Panels (b) through (e) show the pattern of cytokine production by TCC-PLP1-1B10 upon stimulation with PLP1 versus agg Ig-PLP1. Irradiated (3000 rads) SJL/J splenocytes (5 x 10^5cells/well) were incubated with graded amounts of PLP1 peptide (closed circles) or agg Ig-PLP1 (open circles) for 1 hour after which TCC-PLP1-1B10 cells (0.5x 10^5cells/well) were added and the incubation continued for an additional 24 hours. Cytokine production was measured by ELISA from 100µl of culture supernatant. Each point represents the mean of triplicate wells.

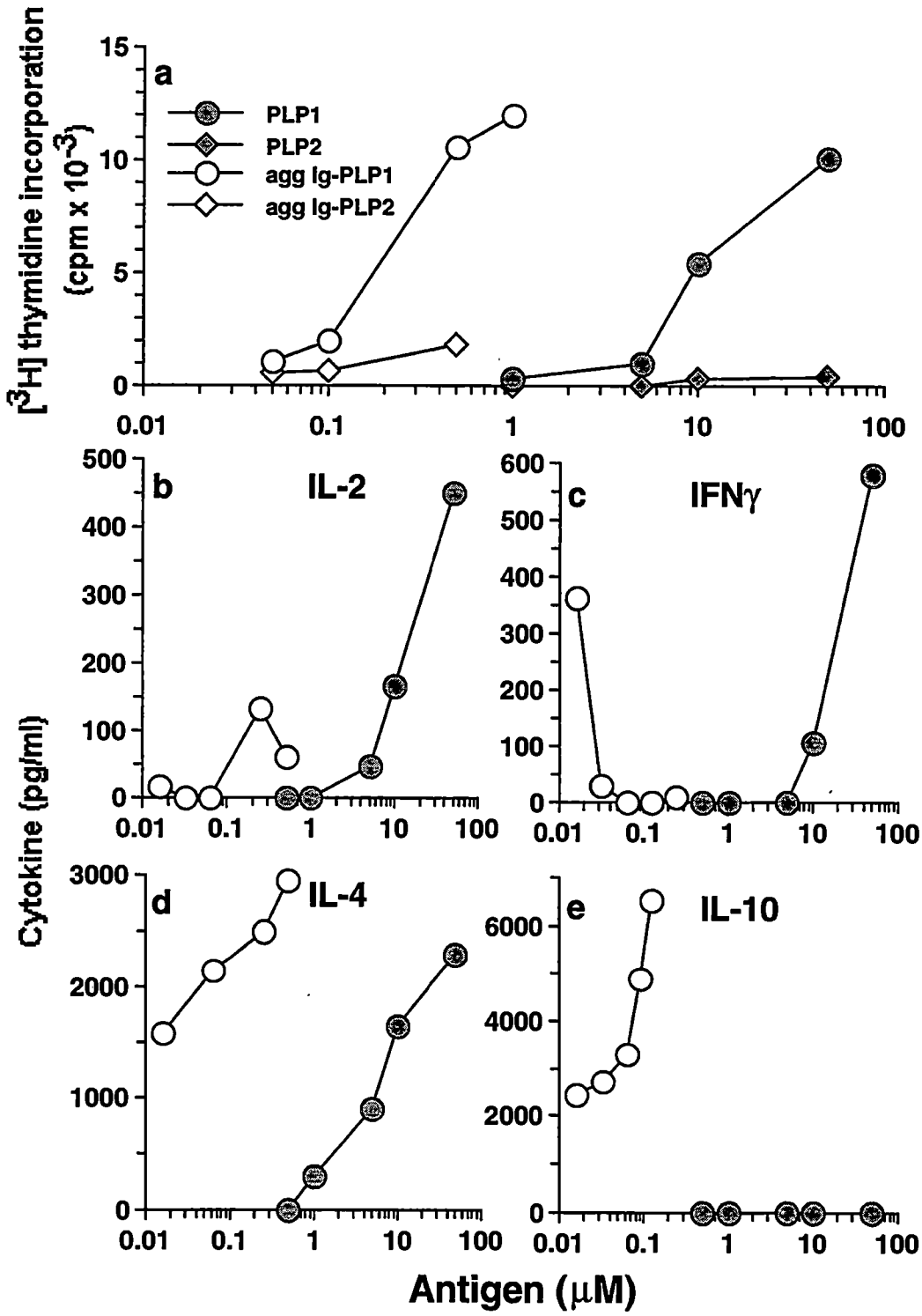

[3H] thymidine incorporation (cpm x 10-3)
a
PLP1
PLP2
agg Ig-PLP1
agg Ig-PLP2
15
10
5
0
0.01
0.1
1
10
100
b
IL-2
500
400
300
200
100
0
c
IFNγ
600
500
400
300
200
100
0
Cytokine (pg/ml)
d
IL-4
3000
2000
1000
0
e
IL-10
6000
4000
2000
0
Antigen (μM)

Figure 6.

Agg Ig-PLP1 does not induce IL-10 production by the TCC-PLP1-1B10 T cell clone.

Fixed and live APCs were used to identify the source of IL-10 in T cell activation by agg Ig-PLP1. In the fixed APCs assay, SJL/J splenocytes (10 x 10^5cells/well) were pulsed with graded amounts of agg Ig-PLP1 for 4 hours, washed extensively, and fixed with paraformaldehyde. In the live APCs assay, irradiated (3000 rads) SJL/J splenocytes (5 x 10^5cells/well) were mixed with graded amounts of agg Ig-PLP1 and incubated for 1 hour. Subsequently, TCC-PLP1-1B10 cells (0.5x 10^5cells/well) were added to both assays and the incubation continued for an additional 24 hours. IL-10 production was measured by ELISA from 100µl of culture supernatant. Each point represents the mean of triplicate wells.

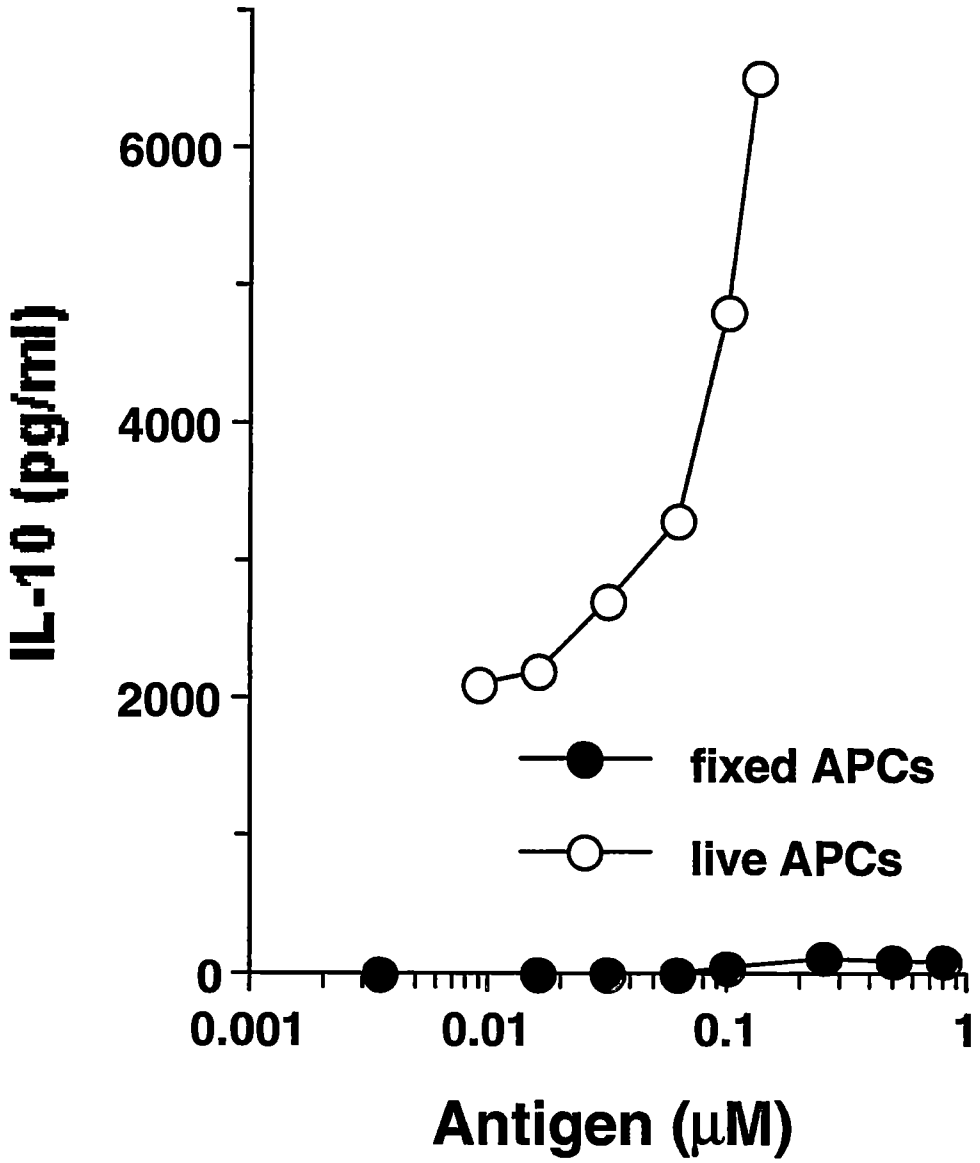

IL-10 (pg/ml)
6000
4000
2000
0
0.001
0.01
0.1
1
Antigen (μM)
fixed APCs
live APCs

Figure 7.

APCs' IL-10 induced by agg Ig-PLP1 antagonizes IFNγ production by T cells.

Irradiated (3000 rads) SJL/J splenocytes (5 x 10^5 cells/well), dendritic cells (0.2 x10^5 cells/well), macrophages (0.2 x 10^5 cells/well), or B cells (2 x 10^5 cells/well) were incubated with graded amounts of agg Ig-PLP1 and after 1 hour TCC-PLP1-1B10 cells (0.5x 10^5cells/well) were added and the incubation was continued for an additional 24 hours. IFNγ and IL-10 production in the same culture well was measured by ELISA. Each point represents the mean of triplicate wells.

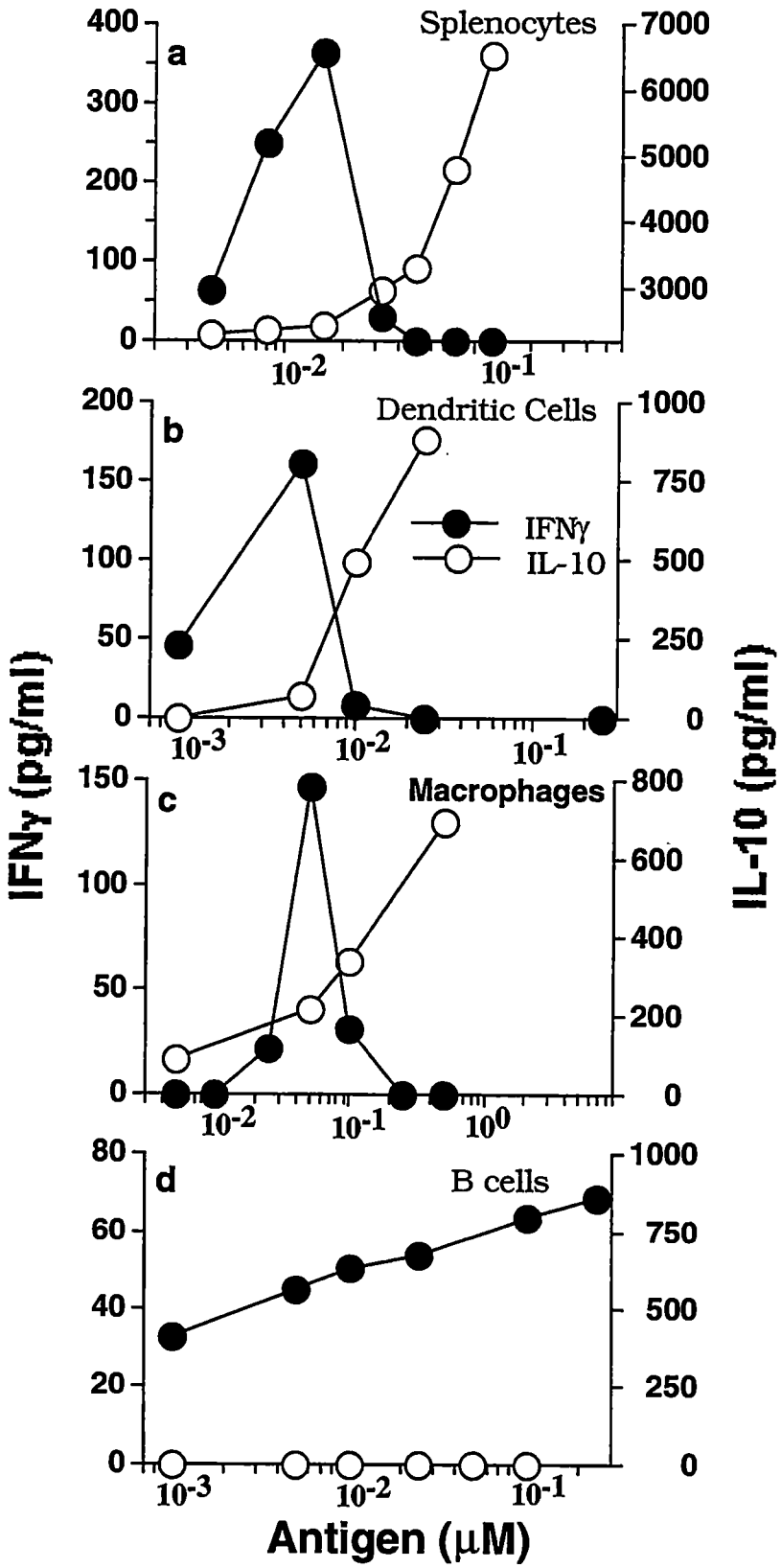

a
Splenocytes
400
300
200
100
0
7000
6000
5000
4000
3000
10-2
10-1
b
Dendritic Cells
200
150
100
50
0
1000
750
500
250
0
IFNγ
IL-10
10-3
10-2
10-1
c
Macrophages
150
100
50
0
800
600
400
200
0
10-2
10-1
100
d
B cells
80
60
40
20
0
1000
750
500
250
0
10-3
10-2
10-1
IFNγ (pg/ml)
IL-10 (pg/ml)
Antigen (μM)

Figure 8.

Neutralization of Ig-PLP1-induced endogenous IL-10 restores severity of paralysis.

(a) SJL/J mice (8 per group) were induced for EAE with 100μg PLP1, and on days 9, 13 and 17 were given i.p. in PBS 300μg agg Ig-PLP1 (agg Ig-PLP1); 300μg agg Ig-PLP1 + 500μg Rat anti-mouse IL-10 antibody, 2A5 (agg Ig-PLP1 + anti-IL-10); 300μg agg Ig-PLP1 + 500 μg Rat IgG (agg Ig-PLP1 + Rat IgG); 300μg agg Ig-W (agg Ig-W); or 300μg agg Ig-W + 500μg Rat anti-mouse IL-10 antibody, 2A5 (agg Ig-W + anti-IL-10). All the injections were done i.p. in PBS. (b) Shows that exogenous IL-10 synergizes with sol Ig-PLP1 to reduce the severity of EAE. Groups of mice (8 per group) were induced for EAE with 100μg PLP1 and on days 9, 13, and 17 were given i.p. in PBS 300μg sol Ig-PLP1 (sol Ig-PLP1); 300μg agg Ig-PLP1 (agg Ig-PLP1); 300μg sol Ig-PLP1 + 400 U rIL10 (sol Ig-PLP1 + IL-10); or 300μg agg Ig-W (agg Ig-W).

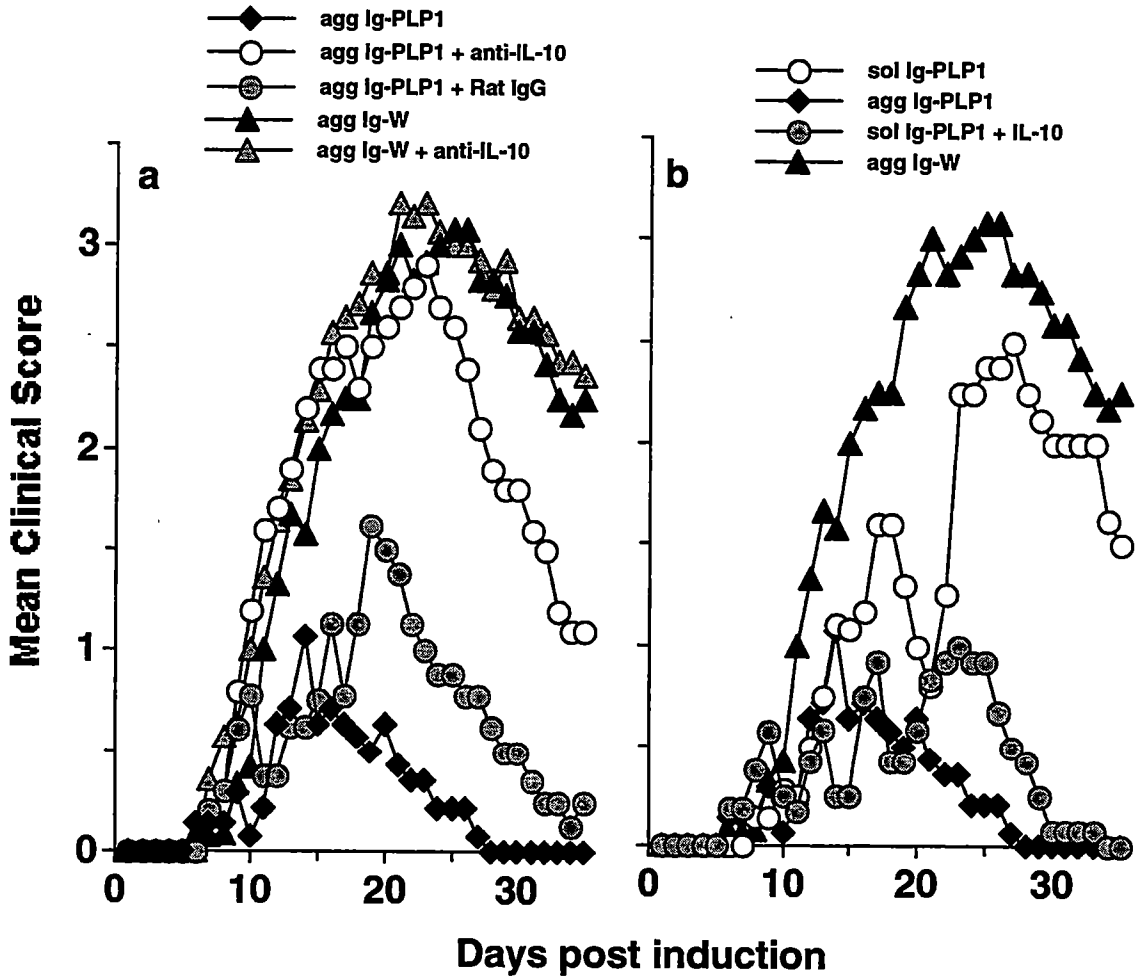

agg Ig-PLP1
agg Ig-PLP1 + anti-IL-10
agg Ig-PLP1 + Rat IgG
agg Ig-W
agg Ig-W + anti-IL-10
a
b
sol Ig-PLP1
agg Ig-PLP1
sol Ig-PLP1 + IL-10
agg Ig-W
Mean Clinical Score
0
1
2
3
0
10
20
30
Days post induction

Figure 9.

Requirement for a physical linkage of the Ig vehicle to PLP1 peptide for amelioration of EAE.

Groups of mice (8 per group) were induced for EAE with 100μg PLP1 and then treated with 300μg of agg Ig-PLP1 (agg Ig-PLP1), 300μg agg Ig-W (agg Ig-W), or 300μg agg Ig-W + 100μg PLP1 (agg Ig-W + PLP1) in PBS on days 9, 13, and 17 post disease induction. The onset of disease was at day 7 in these experimental groups.

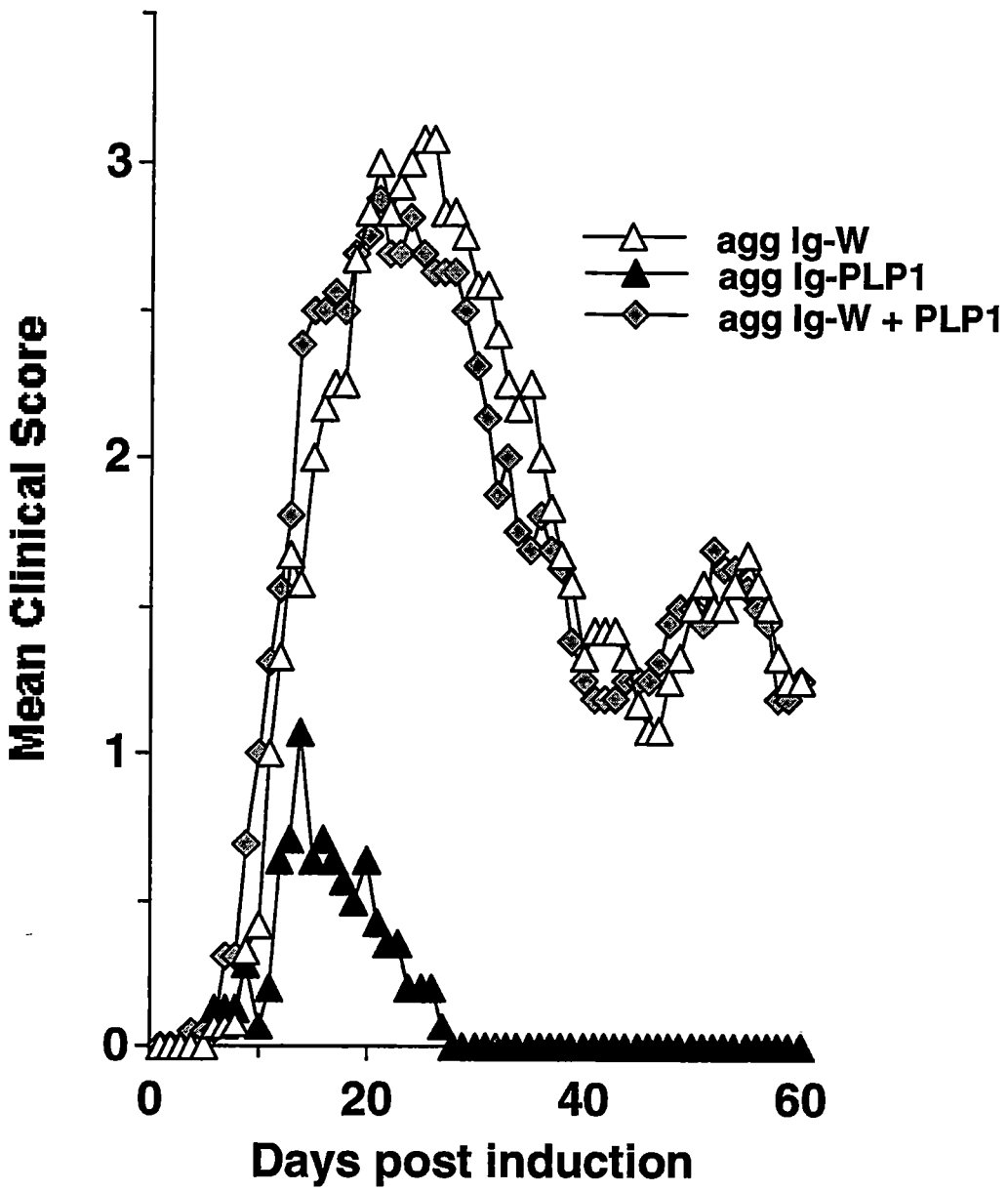

Mean Clinical Score
3
2
1
0
0
20
40
60
Days post induction
agg Ig-W
agg Ig-PLP1
agg Ig-W + PLP1

Figure 10.

Aggregated Ig-PLP1 modulates disease involving intramolecular epitopes.

(a) Groups of SJL/J mice (8 per group) were induced for EAE with a mixture of 100μg PLP1 and 100μg PLP2 and on days 9, 13, and 17 treated with 300μg agg Ig-PLP1 or agg Ig-W per injection. All treatments were i.p. in PBS. The onset of disease was at day 7 in these experimental groups. Each point represents the mean clinical score of 8 mice. (b) Groups of SJL/J mice (8 per group) were induced for EAE with 100μg PLP2 and on days 9, 13, and 17 treated i.p. with 300μg agg Ig-PLP1 per injection. A group of untreated mice (NIL) was included for comparison purposes.

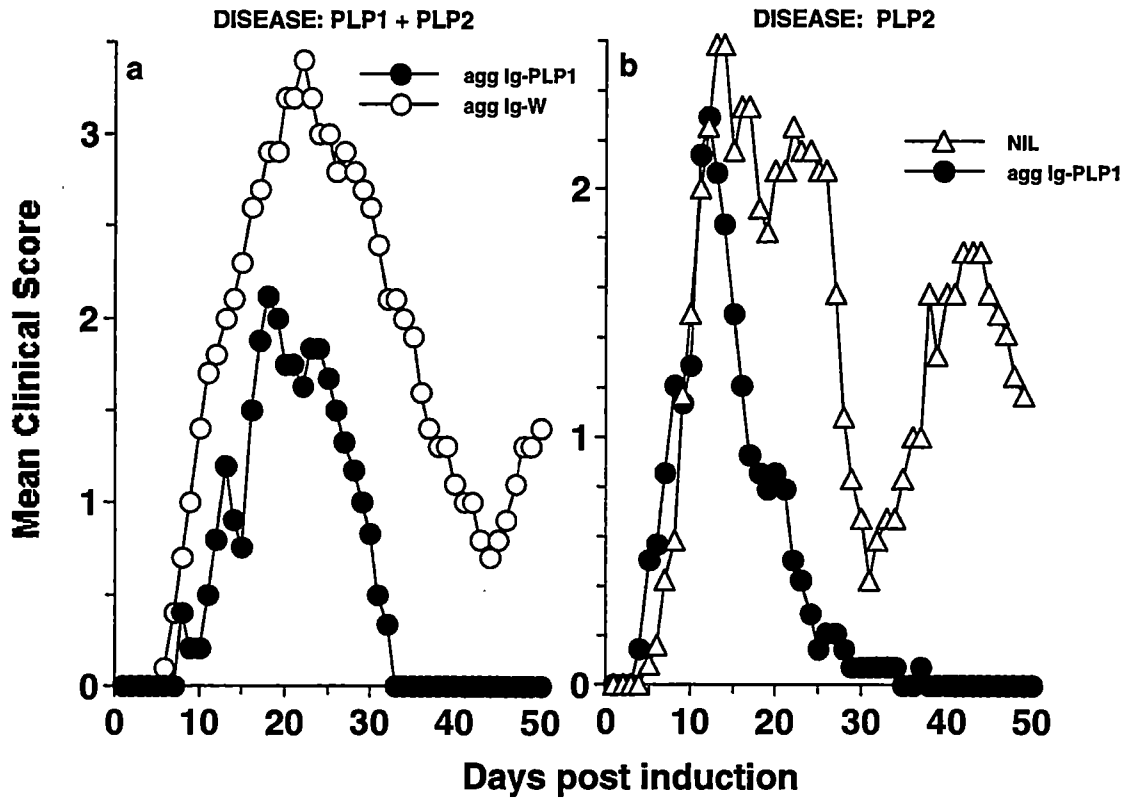

DISEASE: PLP1 + PLP2
a
agg Ig-PLP1
agg Ig-W
DISEASE: PLP2
b
NIL
agg Ig-PLP1
Mean Clinical Score
Days post induction
0
1
2
3
0
10
20
30
40
50

Figure 11.

Agg Ig-PLP1 modulates disease involving intra and intermolecular epitopes.

Groups of SJL/J mice (9 per group) were induced for EAE with 6 mg of CNS homogenate and on days 9, 13, and 17 treated i.p. with 300μg agg Ig-PLP1 or agg Ig-W per injection. A group of untreated mice (NIL) was included for comparison purposes.

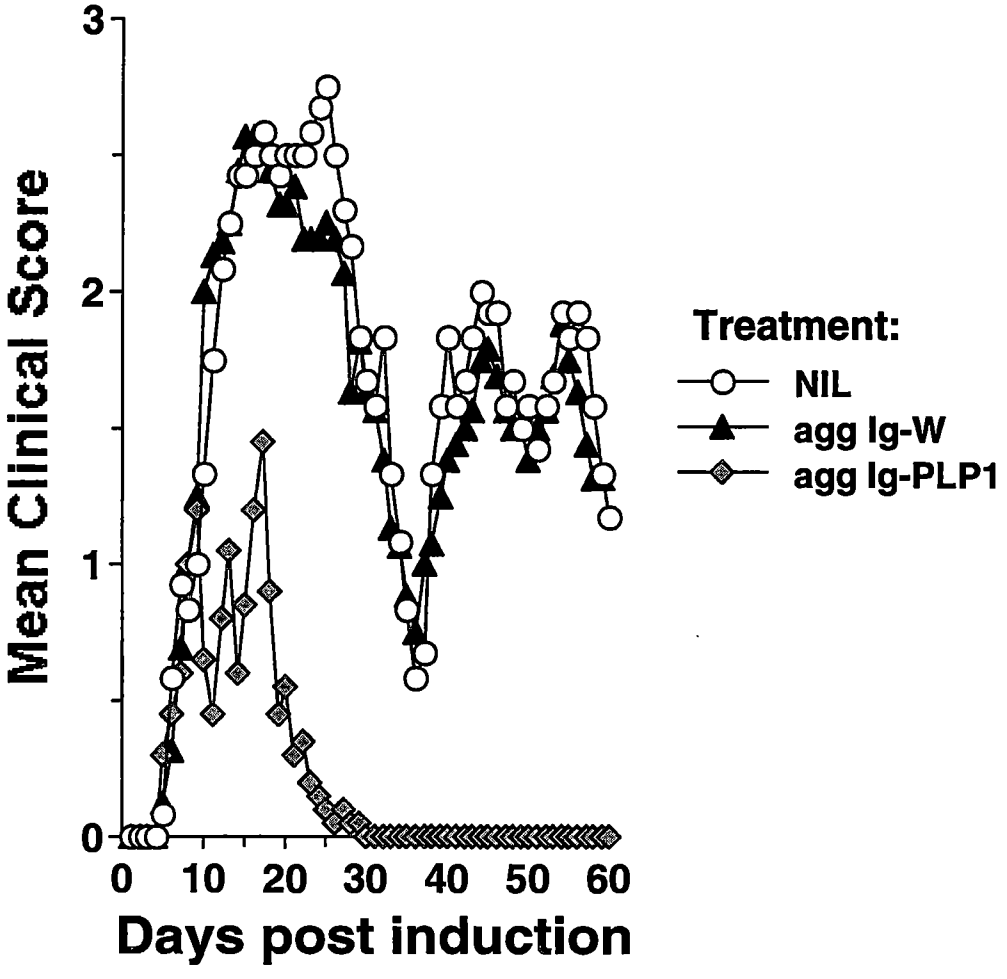
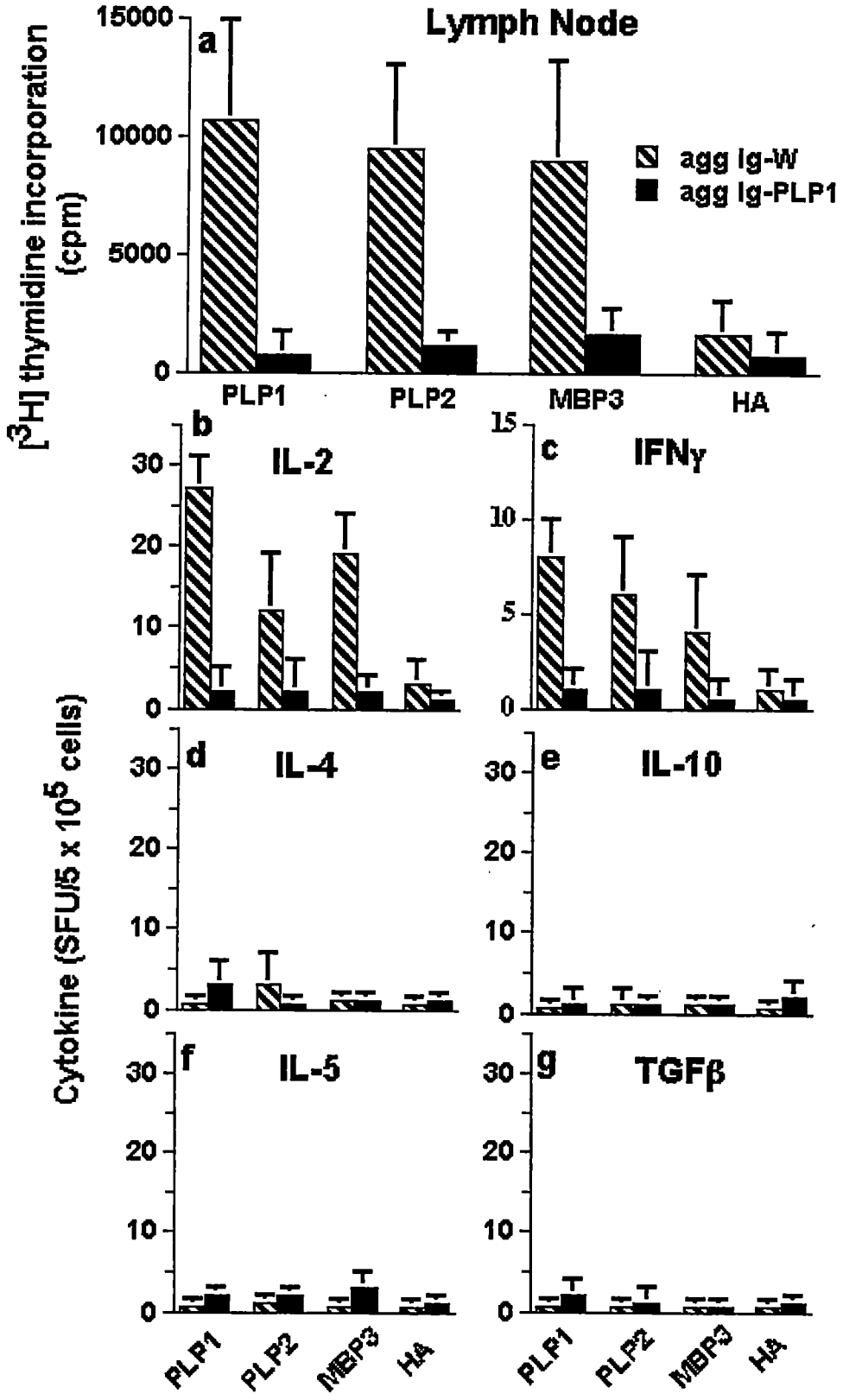

Mean Clinical Score
3
2
1
0
0
10
20
30
40
50
60
Days post induction
Treatment:
NIL
agg Ig-W
agg Ig-PLP1

Figure 12.

Treatment of CNS homogenate-induced EAE with aggregated Ig-PLP1 suppress proliferative and cytokine responses to diverse myelin epitopes.

Mice (6 per group) were induced for EAE with CNS homogenate and then treated with agg Ig-W (hatched bars) or agg Ig-PLP1 (closed bars) on days 9, 13, and 17 as described in figure 11. Two days after completion of the treatment regimen, the lymph nodes (axillary, lateral axillary, and popliteal) were harvested, and the cells (4 x 10^5 cells/100μl/well) were stimulated with 100μl/well of antigen. (a) Cell proliferation was assessed three days later using [^{3}H]thymidine incorporation assay. (b-g) Cytokine responses were analyzed after 24 hours of incubation with antigen by ELISPOT using 5 x 10^5 cells per well. The antigens were used at the defined optimal concentrations of 15μg/ml for PLP1, PLP2, and HA and 30μg/ml for MBP3. Control wells of media without addition of antigen were included and used as background. Each bar represents the mean ± standard deviation of 6 individually tested mice.

Similar results were observed when the in vitro stimulators were used with two times higher concentrations. Also, the pattern of proliferation and cytokine production was similar when the splenic and peritoneal cells from these animals were analyzed.


Lymph Node
a
15000
10000
5000
0
[3H] thymidine incorporation (cpm)
agg Ig-W
agg Ig-PLP1
PLP1
PLP2
MBP3
HA
b
IL-2
c
IFNγ
d
IL-4
e
IL-10
f
IL-5
g
TGFβ
Cytokine (SFU/5 x 10^5 cells)
30
20
10
0
15
5

PART IV.

Concluding Remarks

The overall goal of this work was to down-modulate the ability of autoreactive T cells to mediate EAE, as a method of studying possible MS treatment strategies. This down-modulation is being accomplished through the use of altered peptide ligands to turn off an ongoing disease, fetal thymic expression of wild type ligands to allow for negative selection or deviation of the developing autoreactive T cells, and presentation of the wild type ligand in the periphery in the absence of costimulation coupled with IL-10 production to halt and silence clinically evident disease.

Any attempt to regulate T cell responses in vivo with APLs during MS would require the efficient presentation of the APLs to counteract the constant supply of the agonist ligand that is readily available throughout the now immune compromised CNS. To study this type of response, these studies used a model of EAE that is mediated through reactivity to amino acid residues 139-151 of PLP (PLP1). This peptide has been shown to be encephalitogenic and capable of mediating EAE in SJL/J H-2^s mice. While free peptide antagonists have shown the ability to silence T cells induced by free peptide in EAE, it was unclear whether they would be sufficient as a treatment in counteracting the constant supply of antigen during a natural autoimmune disease. This is due to the fact that the administered peptide APL would replace peptides in the MHC on the surface of antigen presenting cells (APC). Due to the finite life span of MHC on the cell surface, these complexes would be short lived when compared to the agonist ligands being constantly generated endocytically, loaded internally onto MHC, and then translocated to the cell surface. In order to create an efficient delivery system for antagonist peptides (Part II.), an antagonist form (PLP-LR) of PLP1, in which amino acid residues Trp144 and His147 have been replaced with Leu and Arg, respectively, was expressed, through

PCR mutagenesis, in the CDR3 region of the anti-arsonate antibody, 91A3. The resulting immunoglobulin (Ig), referred to as Ig-PLP-LR, and its corresponding agonist form Ig-PLP1 were then used to assay the ability of endocytic antagonist ligands to down-regulate endocytically presented agonist ligands. The Ig-PLP1 molecule was presented to autoreactive T cells 100 fold better than free PLP1 peptide, thereby demonstrating the efficient presentation of the Ig-chimera system. Interestingly, the antagonist Ig-PLP-LR was able to abrogate the activation of T cells stimulated with the free PLP1 peptide, the endocytically processed Ig-PLP1, or the endocytically processed native PLP protein. However, free PLP-LR peptide abolishes T cell activation only when T cells were stimulated by free PLP1, but not native PLP protein or Ig-PLP1. In vivo, this endocytic antagonism was also exhibited since reactivity to PLP1 is inhibited when SJL/J mice are co-challenged with Ig-PLP1 and Ig-PLP-LR. These findings show that the endocytic presentation of an antagonist peptide by an Ig outcompetes both external and endocytic agonist peptides, whereas free antagonist peptide hinders external but not endocytic agonist peptide. Furthermore, these findings suggest that the effective treatment of autoimmune diseases with APLs will require an efficient endocytic presentation system, such as an Ig-peptide-chimera.

In order to better understand this in vivo down-regulation, mice were challenged with Ig-PLP1 or Ig-PLP1 + Ig-PLP-LR in PBS/CFA. Interestingly, despite a lack of cross-reactivity in single free peptide immunizations, Ig-PLP1 or Ig-PLP-LR injections led to good cross-reactive responses against both the agonist, PLP1, and antagonist, PLP-LR peptides (data not shown). When the two chimeras were co-immunized there was a down-regulation in T cell response by both proliferation and cytokine production to PLP1

peptide. This effect was not seen when the Ig-PLP1 chimera was co-immunized with Ig-W, the wildtype Ig expressing no PLP peptide, or Ig-PLP2, an Ig-chimera encompassing amino acid residues 178-191 of PLP which is also presented by I-A^s like PLP1 and PLP-LR, thereby showing the specificity of the down-regulation. This is evidence that, in fact, an Ig-chimera based antagonist can exert a cross-regulation on the agonist response in vivo even when the agonist ligand is presented through the endocytic pathway.

The second strategy for down-regulating autoreactive T cells and ameliorating EAE used in these studies was to introduce the agonist ligand into the thymus during fetal development in order to obtain negative selection of specific T cells (Part III.). This early fetal entry was necessary in order to effect all T cells specific for a given peptide, as later entry might leave a hole through which some T cells would remain unaffected and capable of triggering disease. This hole would be created because MHC expression in mice starts on day 14 of gestation, with TCR complexes and thymic education beginning on day 16. The Ig-peptide-chimera provides an effective solution to deliver the agonist ligand to the developing fetus, as Ig molecules are known to readily transfer from mother to fetus during gestation. In order to test for transfer and presentation of an agonist autoreactive ligand by Ig-peptide-chimera to the fetal mouse, Ig-PLP1 was given i.v. to pregnant mothers. After birth, both the thymus and spleen of pups from mothers administered Ig-PLP1 showed the ability to stimulate a PLP1 specific hybridoma (4E3), while cells from pups born to mothers administered the control Ig-W showed no ability to stimulate 4E3. These results show that the agonist ligand is transferred and that MHC-PLP1 complexes are present on the cell surface of each tissue only when Ig-PLP1 is used. To assay the consequences of such fetal thymic presentation, mice were tolerized via

administration to pregnant mothers of Ig-PLP1 or Ig-W on days 16, 17, and 18 of gestation. After birth the pups were allowed to develop normally and at 6-8 weeks of age challenged with of Ig-PLP1 or Ig-W in PBS/CFA. After challenge, the draining lymph nodes and spleens were removed from the individual mice and assayed for in vitro reactivity to PLP1 or PLP2. When Ig-PLP1 tolerized mice were challenged with PLP1 or Ig-PLP1 as adults there was an ablation of response to the PLP1 epitope, demonstrated by the reduced proliferation and IL-2 production in both the lymph nodes and spleen. This ablation did not occur when Ig-W was used for tolerization. Furthermore, Ig-PLP1 tolerization did not effect the response of mice challenged with PLP2 peptide. This illustrates that the down-regulation was specific to the PLP1 tolerizing epitope. Furthermore, cells from Ig-PLP1 tolerized individuals were unable to regain their ability to react with PLP1 in the presence of exogenously added IL-2, suggesting that the mode of down-regulation is not driven by anergy.

To test the consequences of this down-regulation of PLP1 reactive cells, mice that had been tolerized with Ig-PLP1 or Ig-W were assayed for susceptibility to EAE. Additionally, mice tolerized with Ig-PLP-LR under the same protocol were tested, as the PLP-LR ligand could possibly yield a lower avidity of interaction and not lead to negative selection of PLP1 reactive cells in the thymus. Only mice tolerized with Ig-PLP1 are resistant to EAE mediated through Ig-PLP1. Ig-W and Ig-PLP-LR tolerization elicited no effect on the outcome of clinical disease. Due to the cross-reactive nature of Ig-PLP-LR, the studies allowed the effects of the various tolerization procedures on Ig-PLP-LR mediated EAE to be assayed. Only mice tolerized with the strong agonist Ig-PLP1 exhibited ablation of disease. These results suggest that the presence of the agonist

PLP1 epitope in the thymus leads to negative selection of autoreactive T cells, while the antagonist PLP-LR's avidity may not be strong enough to support apoptosis. These results lend great credence to the avidity model of positive and negative selection, over the differential distribution (i.e. different type of ligands on different cell types) model. This reasoning is based upon the hypothesis that MHC-peptide complexes generated from the Ig-chimeras should be universally expressed because of their use of Fc receptors, which should be constitutively expressed in the thymus, for internalization. Overall, this strategy of preventive tolerization yields protection against subsequent specific autoimmume induction, and could have far reaching effects on the prevention of human autoimmune diseases such as MS, diabetes, and rheumatoid arthritis.

The third approach (Part IV.) for down-regulating autoreactive T cells and ameliorating EAE used in these studies was to administer Ig-chimeras in PBS i.p. following the appearance of clinical paralysis. It was anticipated that this type of treatment might mimic peripheral tolerance and lead to a decrease in clinical severity, due to efficient introduction of self-antigen without the adjuvant needed to upregulate the costimulatory molecules on APCs required by T cells to become activated. The results showed that treatment of free PLP1 peptide induced disease with soluble Ig-PLP1 did in fact lessen clinical severity during the initial phase of disease and prevented relapses. However, treatment with an aggregated form of Ig-PLP1 completely ablated all clinical paralysis and relapses. Furthermore, histologically, agg Ig-PLP1 treatment reduced both the numbers of inflammatory foci and cells per foci in the CNS when compared to mice treated with the control agg Ig-W.

Previous research has demonstrated that aggregation of Igs can lead to crosslinking of FcRs on the cell surface of APCs and production of anti-inflammatory cytokines. Therefore agg Ig-chimeras were assayed for the ability to induce anti-inflammatory cytokines in various APC populations. The results illustrated that agg Ig-chimeras induced IL-10 production from macrophages and dendritic cells but not from B cells. Furthermore such IL-10 regulated the ability of a PLP1-specific T cell clone to make IFNγ in response to PLP1 and was responsible for the increased efficiency in treatment of disease with agg Ig-PLP1 as removal of IL-10 by administration of an anti-IL-10 mAb at the time of treatment restored disease severity to levels seen in sol Ig-PLP1 treated mice.

Since IL-10 might confer a bystander ability to treat epitopes other than the one contained within the Ig-chimera, agg Ig-PLP1 was assayed for treatment of disease involving multiple disease inducing epitopes. In fact agg Ig-PLP1 was able to down modulate disease induced by PLP1+ PLP2 peptides, PLP2 peptide alone, and even CNS homogenate. Overall these results suggest that clinical treatment of multiple epitope autoimmunity might be possible by treatment with agg Ig-self epitope chimeras through a mechanism that mimics peripheral tolerance coupled with IL-10 mediated bystander suppression.

In summary, this study was geared toward the ablation of autoreactive responses in EAE, the animal model for human MS. It approached this goal through three methods: (1) the possible use of efficient antagonist ligands to turn off an ongoing disease, (2) the purging or deviation of autoreactive T cells during fetal development in order to alleviate the threat of disease at a later time point, and (3) the treatment of multi-epitope disease

following clinical evidence by mimicking peripheral tolerance coupled to IL-10 mediated bystander suppression. Each strategy has yielded promising results that might transfer into the clinical treatment of humans with MS or other T cell mediated autoimmune diseases.

Vita

Kevin L. Legge was born in Columbus, Indiana on August 4, 1972. During his childhood he moved to Hendersonville, TN, where in 1990 he graduated from Hendersonville High School. Following graduation, Kevin enrolled at the University of Tennessee at Knoxville where he studied Microbiology and worked as a research trainee in the laboratory of Dr. Dwayne Savage. In 1994, Kevin completed a Bachelors of Sciences degree with a major in Microbiology and a minor in Biochemistry. For the next 1.5 years, Kevin was employed as a research technician in the laboratory of Dr. Habib Zaghouani. In the fall of 1996, Kevin enrolled in the graduate school of Microbiology at the University of Tennessee, Knoxville were he has been pursuing research toward alleviating autoimmune disease. Kevin is currently seeking a Ph.D. in Microbiology, scheduled for completion in the summer of 2000.