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Organ specific modulation of autoreactive T cells by neonatal exposure to antigens

Booki Min

To the Graduate Council:

I am submitting herewith a dissertation written by Booki Min entitled "Organ specific modulation of autoreactive T cells by neonatal exposure to antigens." 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 N. Moore, Rouse, Saluson

Accepted for the Council:

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Vice Provost and Dean of the Graduate School

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

To the Graduate Council:
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Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

ORGAN SPECIFIC MODULATION OF AUTOREACTIVE T CELLS BY NEONATAL EXPOSURE TO ANTIGENS

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

Booki Min
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DEDICATION

This dissertation is dedicated to my parents, Mr. Byung-Hoon Min and Mrs. Won-Ryang Bae, my parents-in-law, Mr. Soo-chul Kim and Mrs. So-Young Kim, and all my family members, all of who have given me invaluable support, advice, encouragement, and the educational opportunity.

I especially dedicate this dissertation to my wife, Sohee. She has been an excellent wife and good friend to me. I would not have been able to finish this work without her. I thank her for trusting and supporting me during last 5 years. Whenever I was having a hard time, she was always by me. I thank you and love you.

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ABSTRACT

During T cell development within the thymus the immune system has developed several regulatory checkpoints to ensure the elimination of T cells endowed with antigen receptors specific for self antigens. Although such a tight regulation exists, some autoreactive T cells escape and undergo the maturation process. The activation of those autoreactive T cells leads to the development of autoimmune diseases.

The neonatal period has been considered as a window during which the encounter with antigens instructs not to develop immune responses, but rather, induces tolerance. Therefore, introduction with defined autoantigens into neonates could be an attractive strategy to induce neonatal tolerance, preventing the development of autoimmunity. The necessity of incomplete Freund's adjuvant (IFA), however, hampers its clinical application.

It has been shown that immunoglobulin (Ig) can be used as an antigenic delivery system, and such delivery was efficient by increasing peptide presentation by 100-1000 fold. Furthermore, Igs are persistent for a long time in vivo. Herein, we hypothesized that Ig-mediated peptide delivery into neonates may replace the requirement of IFA, thereby inducing neonatal tolerance.

Following the introductory literature overview, part II describes the expression of a dominant encephalitogenic peptide (PLP 139-151, PLP1) derived from proteolipid protein (PLP) in place of CDR3 region of Ig molecule. PLP is a known autoantigen for experimental allergic encephalomyelitis (EAE), an animal model for human multiple sclerosis (MS). The results indicate that the peptide was incorporated into the variable region in a correct reading frame. Furthermore, peptide presentation by chimeric Ig

molecule was antigen specific and had great efficiency in T cell stimulation by 100-1000 fold.

In the following part III it was shown that neonatal injection with resulting Ig-PLP1 conferred a resistance to EAE without the presence of IFA. The mechanism underlying such resistance included IL-4-driven lymph node deviation, and IFNγ-dependent splenic anergy restorable by exogenous IFNγ or IL-12.

Part IV showed that neonatal tolerance induced by Ig-PLP1 was unique and differentially regulated by the dose of antigen, the presence of adjuvant, and the number of injections. The results indicate that the dose of Ig-PLP1 displayed a quantitative variation in the outcome of tolerance, whereas adjuvant and the number of injections had a rather qualitative effect on the neonatal tolerance.

Part V demonstrated the cellular mechanism for splenic T cell anergy. The results show that splenic cells from Ig-PLP1 tolerized mice displayed an anergic phenotype by the inability to upregulate IL-2 receptor α chain (CD25), which is responsible for responsiveness to IL-2. Furthermore, this defect in CD25 expression by splenic cells was shown to be critical in order to maintain long-lasting persistence of Ig-PLP1 mediated neonatal tolerance.

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PART I. INTRODUCTION

Chapter 1. T Cell Tolerance

General information

The immune system has evolved a property to discriminate between self antigens and non-self antigens. Thus, lymphocytes are able to recognize and mount immune responses to vast arrays of foreign antigens, but remain unresponsive to self antigens, an immunological unresponsiveness known as tolerance. Tolerance to self antigens (self-tolerance) is a key regulatory mechanism that prevents immune system from responding to self antigens. Failure of self-tolerance results in the development of autoimmune diseases.

Induction of self-tolerance is an actively acquired process by which lymphocytes are educated not to react to self antigens during lymphocyte development (1, 2). In the case of T cells specificity is determined by that of T cell receptor (TCR). Lymphoid progenitors derived from bone marrow stem cells migrate into a generative lymphoid organ (thymus) and undergo maturation. During this process the T cell repertoire is diversified by several mechanisms including TCR gene rearrangement and N-region diversification (3). The consequence of such diversification is the generation of a diverse T cell repertoire whose specificity covers many pathogens. However, it also generates T cells bearing nonfunctional TCR, as well as TCR specific for self antigens. Therefore developing T cells go through a series of testing steps which will ensure that T cells expressing useless or harmful antigen receptors do not mature and exit to periphery.

T cells that recognize self antigens are deleted during a development process referred to as negative selection (1, 4). However, recognition of self antigens bound on

self major histocompatibility complex (MHC) molecules also contributes to positive selection, permitting the maturation of T cells (1, 5, 6). This paradox is generally explained by the model where affinity of the TCR to self-peptide-MHC complexes determines whether positive or negative selection occurs (7, 8). Specifically, T cells whose TCR has a low but sufficient affinity to self peptides presented within the thymus are positively selected and continue maturation, while T cells bearing TCR with high affinity to self peptides will be eliminated by negative selection. Although elimination of autoreactive T cells is tightly regulated within the thymus, some autoreactive T cells might escape negative selection, continue maturation, and exit to periphery. It has been reported that autoreactive T cells are found in the periphery of healthy individuals (9). Those cells, however, maintain their inactive harmless status.

Unlike B cells, T cells recognize only short antigenic peptides bound on MHC molecules on the surface of antigen presenting cells (APCs) through the TCR (10, 11). However, recognition of MHC-peptide complex by TCR (signal 1) does not transmit a sufficient signal to activate T cells (12, 13). In order to fully activate T cells they require an additional signal through accessory molecule (or costimulatory molecule), which will provide signal 2. The absence of signal 2 during antigen stimulation is known to result in state of T cell inactivation called anergy (13, 14). A number of studies have shown that anergy is the mechanism for inactivation of peripheral autoreactive T cells (15, 16). Since only professional APCs are able to provide signal 2, most autoreactive T cells specific for self peptides found on peripheral tissues will not be activated due to the absence of signal 2 (17). Therefore, maintenance of self tolerance is a highly orchestrated process through out the whole immune system by preventing maturation of developing autoreactive T

cells and maintaining their inactive status in the periphery.

Neonatal tolerance

A few decades ago it was demonstrated that injection of allogeneic splenic cells into newborn mice on the day of birth rendered the mice tolerant to skin grafts from the same allogeneic donor (18). Ever since, the neonatal stage has been considered as a window during which an initial encounter with antigen would instruct the immune system to tolerate the antigen and to display no aggressivity during subsequent encounters with the antigen. Subsequently, induction of neonatal tolerance was extended to soluble antigens and peptides and similar passivity towards the antigen was observed (19-21). The initial interpretation of these observations leaned toward T cell deletion and/or inactivation as the principal mechanism for such tolerance (19, 21). Further investigations, however, detected specific reactivity to alloantigens upon graft implant into tolerant mice that were recipient of the alloantigen on the day of birth (22). Analysis of this reactivity revealed that the T cells responding to the alloantigen produced Th2 type cytokines instead of the usual type 1 profile (22-24). These observations suggested that neonatal injection of antigen could prime rather than delete or inactivate T cells and promote an immune deviation that might affect the effector function usually associated with graft implant. The search for T cell responses in mice neonatally tolerized with peptides was rapidly extended to organs such as the spleen where the detected T cells displayed deviated phenotypes (25). Furthermore, a number of studies using microbial and other antigens have shown that the outcome as well as the mechanism of neonatal tolerance depends on the dose of antigen (26), the form of antigen (27), the adjuvant the

antigen is administered with (28), the APC presenting the antigen (29), and the availability of the antigen in vivo (30). One aspect of neonatal tolerance that could be advantageous for both fundamental and hopefully health related matters is the induction of immunity that tolerates the antigen. This phenomenon may provide a tool to investigate and understand a mechanism by which autoaggressive T cells can be calmed or modulated and could evolve as a strategy to vaccinate against prevalent autoimmune diseases.

Chapter 2. Autoimmune Diseases

General information

The destruction of the body's own tissues by the immune system leads to the development of autoimmune diseases. Due to great advances in the understanding of how the immune system recognizes antigens and performs its functions, evidence that the autoimmune disease is the result of the unwanted activation of immune system specific for self antigens has increased. It is known that most of autoreactive T cells are eliminated by thymic selection during development, and some of those who escaped such selection process are responsible for the development of autoimmune diseases. Since it has been shown that peripheral autoreactive T cells remain inactive in normal circumstances, current studies are aimed at understanding the mechanism by which harmful autoreactive T cells become activated.

Among the known autoimmune diseases in humans, multiple sclerosis (MS), type I diabetes, and rheumatoid arthritis (RA) are well characterized inflammatory diseases mediated by T cells specific for self antigens within nervous system, pancreas, and joints, respectively (31-33). Although it is known that activation of those self reactive T cells results in the autoimmune diseases, initial triggering events that lead to their pathogenesis still remains unclear. However, development of animal models for those diseases ensued, and intensive investigations began to reveal the important factors involved in their mechanism of pathogenesis.

First, particular genotypes of MHC molecules have been shown to be associated with a number of autoimmune diseases (34). Among them, type I diabetes is the one of

the autoimmune diseases showing strong association with specific MHC genotypes (35). Earlier studies indicated that expression of certain alleles renders susceptibility or resistance to disease (36, 37). They have further demonstrated that certain amino acid difference on the MHC II β chain could determine susceptibility or resistance to diabetes in humans or in a mouse model (38, 39). Differences in susceptibility were partly explained by recent data showing that resistant alleles are protective by thymic negative selection of pathogenic T cells or by thymic positive selection of suppressive T cells for pathogenicity (40, 41). Others also proposed the critical role of MHC molecules in autoimmunity as alteration of thymic selection (42). In other words, high affinity self-reactive T cells escape selection in the thymus as a result of the poor self peptide-binding properties of the disease-associated MHC class II molecules (43).

Second, microbial infection has been proposed as an important pathogenic mechanism for autoimmune diseases. Due to sequence homology between host self antigens and microbial antigens, infection with certain microbes might activate silent peripheral autoreactive T cells (known as molecular mimicry) (44). A number of studies have shown that infections with viruses (45), bacteria (46, 47), or parasites (48, 49) are highly associated with autoimmune diseases in humans. Indeed, some peptides from a certain virus have been shown to activate myelin antigen specific T cells, causing experimental allergic encephalomyelitis (EAE) (50-52). These data implicate an important link between microbial infection and initial triggering of autoimmune pathogenesis.

Treatment of autoimmune diseases becomes another important concern in this area. First, earlier studies demonstrated that blockade of TCR-MHC interaction by

nonpathogenic competitor peptides could prevent autoimmune diseases (53). The recent finding of altered peptide ligand (APL), an analogue of immunogenic peptides where TCR contact residues have been manipulated, prompted a novel approach to treat autoimmune diseases (54). Indeed, the usage of pathogenic peptide analogues has been shown to downregulate or prevent the diseases (55-57). Second, induction of peptide specific tolerance by oral administration with peptides was another significant approach to treat autoimmune diseases (58). Third, since Th1 type cytokines play a critical role in most of the T cell mediated organ specific autoimmune disease, alteration of cytokine milieu into anti-inflammatory Th2 type has been another common way to downregulate pathogenic autoreactive T cells (59). Finally, blockade of positive signals or triggering of negative signals during T cell activation was proven to be effective in the control of autoimmune diseases (60-62). Various approaches to treat autoimmune diseases are discussed in detail on the following section, experimental allergic encephalomyelitis (model system).

Taken together, many experimental advances into the pathogenesis and treatment of autoimmune diseases have been accumulated. However, target antigens for autoreactive T cells are not known or are still debated in some disease models. Investigation into the identification of unknown antigens and the in vivo cellular interactions triggering autoimmune diseases must be done in order to fully understand the pathogenesis of such diseases, allowing us to formulate an effective strategy for treatment and further prevention.

Experimental allergic encephalomyelitis (model system)

Experimental allergic encephalomyelitis (EAE) is a T cell mediated inflammatory disease within the central nervous system (CNS) resulting in the development of neurologic disorders such as paralysis (63). Histopathologically, EAE is accompanied by cell infiltration, mainly concentrated around microvessels of the CNS, and demyelination (64, 65). The clinical and histopathologic features of EAE resemble those of human multiple sclerosis (MS) (66). Therefore, EAE is an animal model under extensive investigation in order to understand the pathogenesis of MS. Many experimental evidences indicate that both EAE and MS are autoimmune diseases mediated by Th1 type CD4 T cells specific for myelin proteins (31, 63, 67). Target antigens identified to date include myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) (67-71). Experimentally, EAE can be induced by active immunization with myelin proteins or myelin derived peptides in susceptible strains of mice (68-70). Alternatively, EAE can be also induced by adoptive transfer with myelin antigen specific CD4 T cells into naive animals (72-74).

Most important clinical characteristics of EAE involve a relapsing-remitting pattern of disease. The relapsing-remitting course of paralysis is characterized by a moderate to severe initial acute phase of disease followed by recovery and one or more relapses (67). The immunologic mechanism for this chronic relapse is still unclear. However, recent studies have suggested that acquired recognition of new antigenic determinants, a process known as epitope spreading, is involved in relapse and disease progression (75, 76). Interestingly, recent study further demonstrated that induction of

peptide specific tolerance to spreading encephalitogenic determinants prevents further progression of EAE (77).

The role of cytokines in the pathogenesis of EAE has been extensively studied. Murine T helper cells can be categorized as either Th1 or Th2 cells, depending on their cytokine profile (78). Th1 cells secrete IL-2, TNF-α, and IFN-γ, whereas Th2 cells produce IL-4 and IL-10 (78, 79). These two subsets of T cells counterregulate each other. IL-12 promotes development of Th1 cells and antagonizes Th2 differentiation. Conversely, IL-10 inhibits the production of Th1 cytokines (78-80). In EAE, encephalitogenic self-reactive T cells belong to the proinflammatory Th1 subsets. In fact, studies on the pathogenesis of EAE demonstrated that T cells producing Th1 cytokines are involved in disease progression, showing the correlation of Th1 and Th2 cytokines with the disease progression and recovery, respectively (81). In support of this notion, it has been demonstrated that mice deficient for IL-4 or IL-10 are more susceptible and develop more severe EAE (82, 83). Furthermore, treatment with antibodies to IL-12 or TNF has shown to inhibit EAE (84, 85). Therefore, modulation of the cytokine environment during disease induction could be used as a powerful tool to regulate the pathogenicity of EAE and treat ongoing disease.

Alteration of costimulation in EAE pathogenesis has been also addressed in recent studies. Of the known costimulatory molecules, the B7 family expressed on APCs appears to be the most critical for T cell activation (86). The family of B7 molecules includes B7-1 (CD80), B7-2 (CD86), and recently identified B7H-1 (86, 87). Interaction between B7 molecules and the ligands on T cells delivers a positive or negative signal for T cell activation through B7 ligands, CD28 or CTLA-4 on T cells, respectively (86, 88,

89). CD28-mediated signal has been shown to induce IL-2 production by T cells and prevent anergy (88, 90), and more recently, to enhance activation signal by inactivating negative regulator (91). In contrast, CTLA-4 was recently demonstrated to inhibit phosphorylation pathway by binding to TCR ζ chain, thereby inhibiting T cell activation (92). By using blocking monoclonal Ab or knockout mice, targeting of the CD28 molecule has been shown to suppress EAE efficiently (93). Furthermore, blocking the CTLA-4 molecule accelerates disease (62). As for blocking B7 molecules, the results are more complicated. The blockade of B7 interaction has been demonstrated to suppress or enhance EAE, depending on the B7 molecule and encephalitogenic peptide (94-97). In fact, B7.1 has been shown to play a major role in PLP induced EAE pathogenesis (94). However, in MOG induced EAE, B7.1 and B7.2 have an overlapping function, since mice deficient in either molecule developed comparable disease to the wild type control (98). Recently, it was also demonstrated that B7.2 appeared to play a critical role in EAE pathogenesis in different strain of mice (99). Since all the results are various depending on strains, epitope, etc., it will require more investigation to understand the exact role of costimulation in EAE pathogenesis. However, it is becoming clear that B7 mediated costimulation is critical in EAE, and its modulation casts a potential possibility in the development of strategies for the treatment of T cell mediated autoimmune diseases.

The recognition by T cells of peptides bound to MHC molecules has been well established (10, 11). Many recent studies suggested that the specificity of TCR recognition is determined by a few peptide residues, and have focused on the nature of TCR contact sites (54, 100, 101). Therefore, mutation of TCR contact residues by single amino acid substitution have led us to identify "APL" in various antigenic system (54).

Depending on the nature of APLs, stimulation of peptide specific T cells with various APLs results in partial activation (102), anergy (103), and antagonism (104). In EAE, some of the encephalitogenic peptide structures based on TCR contact have been identified (105, 106). In addition, it was demonstrated these APLs could prevent EAE by deviating immune responses to protective Th2 type responses (56, 57) or inducing regulatory T cells (107). Since recent study starts to unveil the molecular mechanism for APL-induced T cell modulation (108), it becomes promising strategy to apply autoimmune diseases in human.

Oral administration of soluble antigens was shown to induce antigen specific peripheral tolerance (109). Especially, the use of myelin antigens led to suppression of experimental autoimmune encephalomyelitis (110). As for the mechanism of such oral tolerance, several studies have demonstrated that induction of anergy (111, 112) or clonal deletion (58) is major mechanism depending on antigen dosage. Furthermore, IL-10 and TGF-β were shown to play a pivotal role in oral tolerance (58).

Chapter 3. Neonatal Tolerance and Autoimmunity

Since neonatal exposure to antigen educates the immune system not to react the same antigen by mechanisms such as clonal deletion (19, 21), anergy (16), and immune deviation (22-25), induction of neonatal tolerance could be a powerful tool for treating autoimmune diseases. In fact, it has been shown that injection of pathogenic peptides into neonates was able to suppress the development of autoimmune diseases of different models such as EAE (19, 20, 27), diabetes (113), and arthritis (114). However, those tolerogenic peptides need to be emulsified in incomplete Freund's adjuvant (IFA) in order to induce tolerance. Injection of peptide without IFA does not appear to have an effect on immune responses (24), although slow and repetitive intravenous injection of soluble antigen have shown to induce tolerance in adult animals (115). Presumably, IFA could contribute to slow release of peptide for an extended time. IFA might also cause production of cytokines influential for T cell development and differentiation. Indeed, IFA has been shown to induce certain cytokine production and guide Th2 type T cell development and isotype switching (116, 117). However, in some studies, IFA was shown to induce arthritis in the rat (118). Therefore, the requirement of IFA to induce neonatal tolerance becomes a big hurdle to overcome for its clinical application.

Chapter 4. Immunoglobulin as Antigen Delivery System

It is well established that T cells recognize short peptides in association with MHC molecules (10, 11). In addition, the pathway by which an antigenic peptide is generated is an important factor in the determination of the type of MHC molecules on which the peptide is associated, and further, the type of T cells to be activated. Proteins that are synthesized within the APCs are processed in the intracellular compartments, generating peptides in association with MHC class I molecules, which are then recognized by CD8 T cells (119). In contrast, proteins that are taken up by APCs are degraded in the endosomal compartment, leading to generation of peptides in association with MHC class II molecules, and recognition by CD4 T cells (119). The recognition of peptides by cognate T cells results in the activation of T cells and development of specific immunity. It was suggested that the magnitude of immune responses was affected by several factors such as antigenic nature, amounts, route of entry, the presence of adjuvant, cytokines, and the type of APCs, etc. This is of great importance to develop an efficient vaccine strategy against known nominal antigens.

Immunoglobulins (Igs) have been utilized for this strategy, especially as a vehicle for T cell epitopes (120-122). Because Igs are autologous molecules and permissive for expression of foreign peptides without interfering its structure, delivery of antigen through the form of Igs could overcome the short half-life of native antigens or the limitation of side-effects caused by adjuvant containing bacterial products. In fact, a number of groups have employed this approach by inserting antigenic epitopes onto Ig through molecular engineering.

Antigenic epitopes from viruses (121, 123, 124) or parasites (125) have been

expressed in the complementarity-determining regions (CDRs) of Igs. Engineered Igs were able to induce humoral and cell-mediated immune responses in vivo (124, 125). Furthermore, presentation by engineered Igs was much more efficient in the activation of peptide specific T cells than free peptide (126, 127). Since Ig-peptide induced T cell activation is inhibited by blockade of Fc receptor (FcR), it was suggested that chimeric Ig-peptide complex is efficiently internalized via FcR into APCs, degraded within the endosomes, and liberated peptides are loaded onto newly synthesized MHC molecules (124). Indeed, incorporated peptides were recovered in higher amounts when APCs were incubated with chimeric-Ig molecules compared to free peptide (127). Therefore, chimeric Igs could be used to deliver antigen to the immune system and considered as a potential strategy for effective vaccine development.

Chapter 5. Rationale and Research Objectives

Escape of autoreactive T cells from tightly regulated developmental checkpoints occurs in normal healthy individuals. Although additional mechanism to maintain those peripheral autoreactive T cells in inactive status exists, accidental activation of those cells in the periphery by unknown mechanisms could be a potential risk for development of autoimmune diseases. Since it is known that recognition of antigens during the neonatal period induces tolerance rather than activation of antigen specific T cells upon subsequent encounter with same antigen later in life, induction of neonatal tolerance against possible self antigens by introducing such antigens into neonates could be an attractive strategy to prevent such harmful autoimmune diseases.

For a number of known T cell mediated autoimmune diseases, years of studies have identified different self peptides that are involved in such diseases. The current experimental system in which neonatal tolerance is induced by injecting autoantigens requires IFA. Presumably, the slow release of emulsified peptides and the induction of cytokines contribute to the mechanism of IFA induced neonatal tolerance. Although the use of IFA is an effective way to induce neonatal tolerance in an experimental system, it bears some limitations in the application to a human system. Furthermore, evidence of IFA causing arthritis makes this strategy harder to employ for preventing human diseases.

It has been shown that peptide antigens can be incorporated within the Ig structure by molecular engineering, and immune responses specific for such antigens can be induced by introducing such Igs into animals. Furthermore, Igs have a long half-life in vivo and induce cytokine production upon binding to FcR. Since characteristics of Igs are similar to those of IFA, we hypothesized that the introduction of autoantigen in the form

of Igs might abrogate the requirement of IFA to induce neonatal tolerance. Therefore, this study was designed to test such a novel method for neonatal tolerance using EAE as a model, thereby preventing autoimmune disease and uncovering the underlying mechanism for such tolerance.

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PART II. Construction of Ig-Chimera

(International Reviews of Immunology, In Press)

Chapter 1. Abstract

The encephalitogenic epitopes from proteolipid protein (PLP) responsible for pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model for human multiple sclerosis (MS), were expressed in the context of immunoglobulin (Ig). The resulting chimeras were shown to activate antigen specific T cell hybridomas, indicating that Ig-PLP chimeras are taken up by antigen presenting cells (APCs) and processed. Interestingly, peptide presentation by Ig-PLP chimeras increased by 100 fold when compared to free peptides. Furthermore, in vivo immunization with Ig-PLP chimeras induced PLP peptide specific T cell responses both in the spleen and lymph nodes. These results suggest that peptide delivery in the context of Igs has considerable potential, and Ig-PLP chimeras might provide a attractive strategy to develop vaccine against autoimmune diseases such as MS.

Chapter 2. Introduction

It has been previously reported that microbial peptides were successfully expressed on immunoglobulins (Igs) and their presentation was increased by 100-1000 fold relative to free peptides (1, 2). These peptide-Ig chimeras have proven to be effective for induction of anti-microbial responses and vaccination against infectious diseases (3-5). Herein, the Ig delivery system was used for insertion of self-peptides derived from defined autoantigens from T cell mediated autoimmune diseases such as experimental allergic encephalomyelitis (EAE), an animal model for human multiple sclerosis (MS).

To achieve this the heavy chain CDR3 loop was deleted and replaced with separate nucleotide sequences encoding each dominant encephalitogenic peptides of proteolipid protein (PLP), aa 139-151 and 178-191 (hereafter referred to as PLP1 and PLP2, respectively). The resulting Ig-PLP1 and Ig-PLP2 molecules expressed PLP1 and PLP2 peptide within CDR3 region in a correct reading frame. Ig-PLP chimeras were able to stimulate T cell hybridomas in a peptide specific manner. Consistent with a previous study, Ig-PLP chimera mediated T cell activation was much more efficient than free peptide. Furthermore, immunization with Ig-PLP chimeras induced peptide specific T cell responses in both the lymph node and spleen.

Chapter 3. Materials and Methods

Mice

6-8-week-old SJL/J mice (H-2s) were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in our animal facility for the duration of experiments.

Peptides

All peptides used in these studies were purchased from Research Genetics (Huntsville, AL) and purified by HPLC to > 90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of proteolipid protein (PLP). PLP2 peptide (NTWTTCQSIAFPSK) encompasses an encephalitogenic sequence corresponding to aa residues 178-191 of PLP (6). Both peptides bind to I-As class II molecules and induce EAE in SJL/J mice (7).

Ig-PLP chimeras

PLP1 and PLP2 peptides were expressed on Ig chimeras that were designated Ig-PLP1 and Ig-PLP2, respectively, as previously reported (3). In brief, Ig genes encoding the $91A_3$ anti-arsonate antibody were used as template. Sequences encoding the CDR3 region were deleted and replaced with nucleotide sequences encoding PLP1 or PLP2 peptide by standard PCR mutagenesis. Mutated Ig heavy chain genes were subcloned into expression vector pSV2, and subsequently transfected along with a plasmid harboring the parental light chain gene $91A_3L$ into non Ig-secreting SP2/0 cells by electroporation. The resulting transfectomas were screened by the culture in selection media, G418-MA, and

tested for the production of complete Ig molecule using radioimmunoassay (RIA). The positive transfectoma was further cloned by limiting dilution. Ig-W, the parental Ig not encompassing any PLP peptide, was described elsewhere (3). Purification of Ig-PLP chimeras was carried out on columns of rat anti-mouse κ mAb coupled to Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ).

T cell activation assay

In order to test Ig-PLP chimera-mediated T cell activation, T cell hybridomas specific for either PLP1 or PLP2 peptide were generated. Lymph node cells from the mice immunized with either PLP1 or PLP2 peptide in complete Freund's adjuvant (CFA) were fused with TCR αβ-negative thymoma BW1100. The resulting T cell hybridoma was tested for cytokine production in stimulation with PLP1 or PLP2 peptide and positive hybridomas were further cloned by limiting dilution. TCH-PLP1-#79 and TCH-PLP2-1A7 are the hybridomas specific for PLP1 and PLP2 peptide, respectively, and secrete IFNγ in peptide specific manner.

For testing T cell activation by Ig-PLP chimeras, 5×10^5 irradiated syngenic splenocytes were incubated with various antigens for 1h at 37°C. Subsequently, 5×10^4 T cell hybridoma cells were added to the well. After 24 hours the culture supernatant was taken and measured for cytokine production by enzyme linked immunosorbent assay (ELISA) according to PharMingen (San Diego, CA) protocol.

Immunization and in vitro stimulation assay

Groups of mice were immunized subcutaneously in the footpads and base of limbs with 50 μg of Ig-PLP1 or Ig-PLP2 emulsified in CFA (1:1 vol/vol). Ten days later the lymph node and spleen cells were taken and in vitro stimulated with 15 μg/ml of peptide or Ig-PLP chimeras for 3 days. Subsequently, 1μCi [^{3}H]thymidine was added to each well, and the culture was continued for additional 14.5 h. Plates were then harvested and the incorporated [^{3}H]thymidine was measured using the trace 96 program and an Inotech β counter (Wohlen, Switzerland). A control of media without stimulator was included for each mouse and used as background.

Chapter 4. Results

Two self-peptides designated PLP1 and PLP2, corresponding to proteolipid protein (PLP) amino acid sequences 139-151 and 178-191, respectively, were used to generate peptide-Ig chimeras. Both PLP1 and PLP2 peptides are presented to T cells in association with MHC class II I-A^s molecules and induce EAE in susceptible strains of mice, SJL/J (6, 7). PLP1 and PLP2 peptides were expressed on Ig chimeras that were designated as Ig-PLP1 and Ig-PLP2, respectively. The genes used to construct these chimeras are those coding for the light (8) and heavy (9) chains of the IgG_{2b}, anti-arsonate antibody, 91A3. The $91A_3V_H$ gene was subcloned into pUC19 plasmid and used as a template DNA in PCR mutagenesis reactions to delete the D segment and insert instead nucleotide sequences for PLP1 or PLP2 (Figure 1)*. These procedures generated $91A_3V_H$ fragments carrying PLP1 ($91A_3V_H$-PLP1) or PLP2 ($91A_3V_H$-PLP2) sequences in place of the CDR3 region. Nucleotide sequencing analysis indicated that full PLP1 and PLP2 sequences were inserted in the correct reading frame as the flanking regions around the PLP sequences were identical to those surrounding the D segment within the unmutated $91A_3V_H$ gene (Figure 2). As can be seen in figure 3, the $91A_3V_H$-PLP1 and $91A_3V_H$-PLP2 fragments were then subcloned into pSV2-gpt-C2b in front of the exons coding for the constant region of a Balb/c γ2b which generated pSV2-gpt-$91A_3V_H$-PLP1-Cγ2b and pSV2-gpt-$91A_3V_H$-PLP2-Cγ2b plasmids, respectively. These plasmids were then separately co-transfected into the non-Ig producing SP2/0 B myeloma cells with an expression vector carrying the parental $91A_3$ light chain, pSV-neo-$91A_3$L (8) (Figure 3).

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

Transfectants producing Ig chimeras were selected in the presence of geneticin and mycophenolic acid and cloned by limiting dilution. The final clones secreted 2-4μg/ml of Ig-PLP chimeras. Figure 4 illustrates a schematic representation of Ig-PLP1 and Ig-PLP2 showing the peptides embodied within the heavy chain variable region.

Ig-PLP1 and Ig-PLP2 were affinity chromatography purified from the supernatant of large scale cultures of transfectants and were assayed for presentation of the PLP peptides to T cells in vitro and for induction of peptide-specific T cells in vivo. The T cells used to test the Ig-PLP chimeras for presentation were hybridomas obtained by fusing PLP-specific short term T cell lines with the αβ-T cell receptor (αβ-TCR)-negative lymphoma BW1100. Two of these hybridomas, TCH-PLP1-#79 and TCH-PLP2-1A7, specific for PLP1 and PLP2 peptides, respectively, were chosen for this purpose. As can be seen in figure 5a, Ig-PLP1, like PLP1 peptide, stimulated TCH-PLP1-#79 for IFNγ production. This presentation was specific since Ig-PLP2 and PLP2 peptide, which also bind I-A^s, did not stimulate the T cells. Likewise, Ig-PLP2, like PLP2 peptide, induced IFNγ production by TCH-PLP2-1A7, but Ig-PLP1 and PLP1 peptide did not (Figure 5b). The activation of the T cells by the Ig-PLP chimeras was not related to non-specific activation by the Ig backbone because Ig-W, the parental molecule not encompassing any PLP peptide, did not activate the T cells (Figure 5). More strikingly, both Ig-PLP chimeras were presented much more efficiently than the free peptides, confirming previous observation that Igs present peptide 100-1000 fold better than free peptide (1).

In vivo, both Ig-PLP1 and Ig-PLP2 were immunogenic and induced proliferative responses in both the lymph node and spleen (Figure 6). Indeed, lymph node as well as

splenic T cells from mice that were immunized with Ig-PLP1 showed peptide-specific proliferative responses to free PLP1 (Fig. 6a and 6b) or Ig-PLP1 (Fig 6c and 6d), like cells from mice that were immunized with free PLP1 peptide (not shown). These cells did not proliferate when stimulated with Ig-PLP2 or PLP2 peptide, indicating the specificity of the responses. Similarly, Ig-PLP2 was also immunogenic and induced lymph node and splenic T cells that proliferate upon stimulation with PLP2 (Fig. 6a and 6b) of Ig-PLP2 (Fig. 6c and 6d), but not to stimulation with either PLP1 or Ig-PLP1. These results indicate that Ig-PLP1 and Ig-PLP2 are presented in vivo and induce specific T cells responses. Overall, self-peptides can be efficiently presented by Igs both in vitro and in vivo.

Chapter 5. Discussion

T cells recognize short antigenic peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). The pathways by which such peptides are generated determine not only the type of antigen presenting-MHC molecules but also the type of cognant T cells. Peptides originating from proteins in the cytoplasm gain access to class I MHC molecules and are recognized by CD8+ cytotoxic T lymphocytes (10). In contrast, peptides from proteins in acidic vacuoles are bound on MHC class II molecules and presented to CD4+ helper T lymphocytes (10). The magnitude of immune responses induced by antigens is influenced by several factors, including amount and nature of antigens, route of entry, etc. Therefore, efficiency in antigen presentation may play a critical role in the induction of immune responses. Furthermore, this is of great importance in the development of vaccines.

In a previous study it was shown that Ig-chimera expressing an antigenic epitope from a virus was able to stimulate antigen specific T cells in more efficient manner compared to free peptide (1, 11). Furthermore, when introduced to animals, Ig-chimeras prime virus specific T cell responses (1). A subsequent study also demonstrated that low concentration of Ig-chimeras generated a significantly high number of peptide-MHC complexes on the surface of APCs. Since such Ig-chimera mediated T cell activation is inhibited by blockade of Fc receptor (FcR), it is believed that Ig-chimeras are efficiently internalized into APCs via FcR (1). Thus, those internalized complexes are degraded within the acidic vacuoles and liberated peptides gain access to newly synthesized MHC molecules. This receptor mediated efficient internalization/processing would lead to generation of a high number of peptide complexes on the surface (2).

In the present study we chose dominant encephalitogenic peptides from myelin antigens which are known as target antigens in EAE. Ig-PLP1, expressing the dominant encephalitogenic PLP1 peptide, was able to in vitro stimulate a T cell hybridoma in an antigen specific manner. Furthermore, immunization of mice with Ig-PLP1 also induced significant T cell responses in both the lymph node and spleen. These results suggest that Ig-PLP chimeras could prime and activate T cells both in vitro and in vivo. In addition, presentation by Ig-PLP chimeras was much more efficient than that by free peptide, as significant production of IFNγ by T cell hybridomas was observed even in lower concentration of antigen.

Overall, delivery of antigenic peptide in the context of Ig is an effective approach to induce immune responses and may be a promising strategy for the development of vaccines. Since peptides from PLP are responsible for the induction of autoimmune diseases, Ig-PLP chimeras could be used as the tool for the treatment or prevention of autoimmune diseases such as EAE or MS.

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Figure 1.

Insertion of encephalitogenic peptide sequences derived from PLP in place of the D segment within the $91A_3H$ heavy chain.

5.5Kb EcoRI fragment from anti-arsonate antibody $91A_3H$ gene was used as template for replacing D segment with sequences encoding PLP peptides by PCR mutagenesis. PCR products was ligated by arficially generated AvaI site and digested with NcoI and ApaI restriction enzyme. The resulting fragment was subcloned into pUC19 vector to analyze whether insertion was in the correct frame.

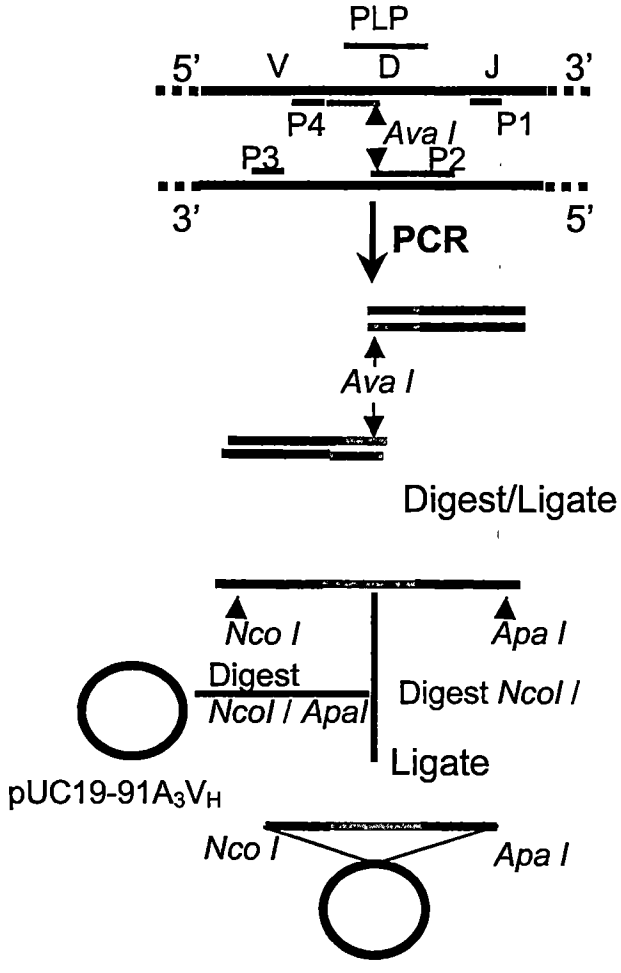

PLP
5'
V
D
J
3'
P4
Ava I
P1
P3
P2
3'
5'
PCR
Ava I
Digest/Ligate
Nco I
Apa I
Digest
NcoI / ApaI
Digest NcoI /
Ligate
pUC19-91A3VH
Nco I
Apa I

Figure 2.

Sequence analysis of wild type and mutated heavy chain gene.

Comparison of nucleotide sequences of the nucleotide sequences of the wild type $91A_3V_H$ gene to those of the chimeric $91A_3V_H$-PLP1 and $91A_3V_H$-PLP2 indicates that nucleotide sequences encoding full PLP1 and PLP2 were inserted in the correct frame in place of the D segment.

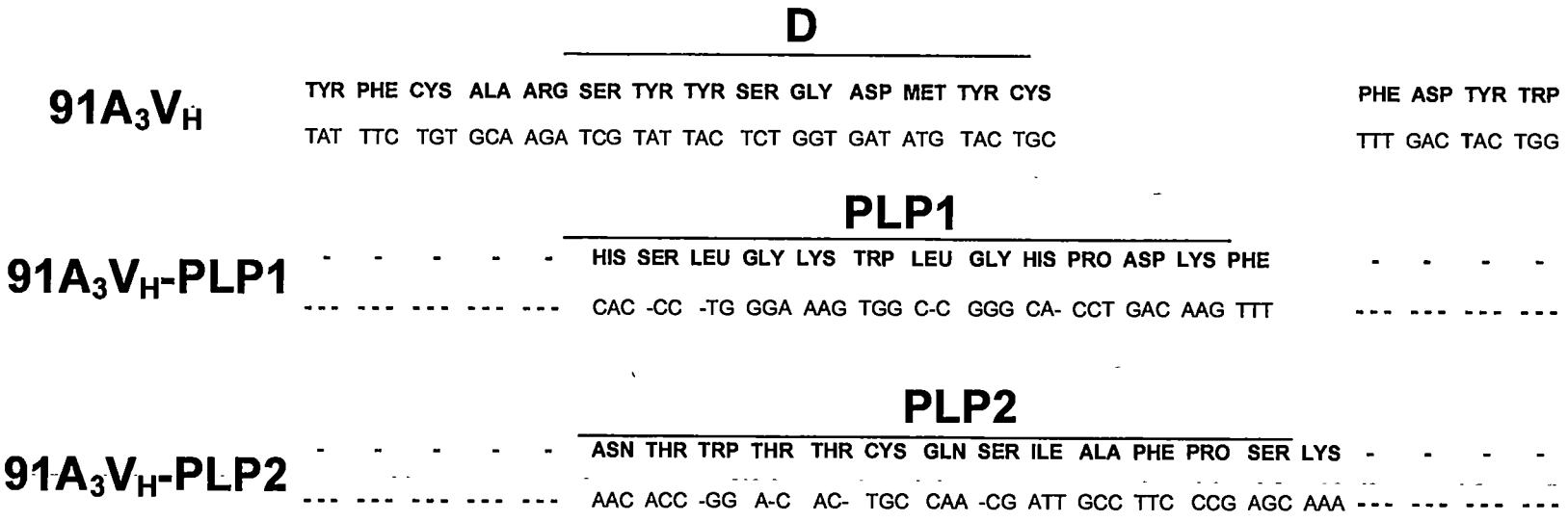

D
91A3VH
TYR PHE CYS ALA ARG SER TYR TYR SER GLY ASP MET TYR CYS
PHE ASP TYR TRP
TAT TTC TGT GCA AGA TCG TAT TAC TCT GGT GAT ATG TAC TGC
TTT GAC TAC TGG
PLP1
91A3VH-PLP1
- - - - - HIS SER LEU GLY LYS TRP LEU GLY HIS PRO ASP LYS PHE - - - -
--- --- --- --- --- CAC -CC -TG GGA AAG TGG C-C GGG CA- CCT GAC AAG TTT --- --- --- ---
PLP2
91A3VH-PLP2
- - - - - ASN THR TRP THR THR CYS GLN SER ILE ALA PHE PRO SER LYS - - - -
--- --- --- --- --- AAC ACC -GG A-C AC- TGC CAA -CG ATT GCC TTC CCG AGC AAA --- --- --- ---

Figure 3.

Generation of transfectoma producing Ig-PLP1.

Mutated heavy chain genes expressing PLP1 or PLP2 sequences were cotransfected along with the plasmid encoding the parental light chain $91A_3L$ into the non Ig-secreting SP2/0 cells. Transfectomas were screened for the secretion of Ig-PLP1 by radioimmunoassay, and positive transfectomas were further cloned by limiting dilution. Purification of Ig-PLP1 was performed by affinity chromatography coupled to rat anti-mouse κ mAb from the culture supernatant of transfectoma.

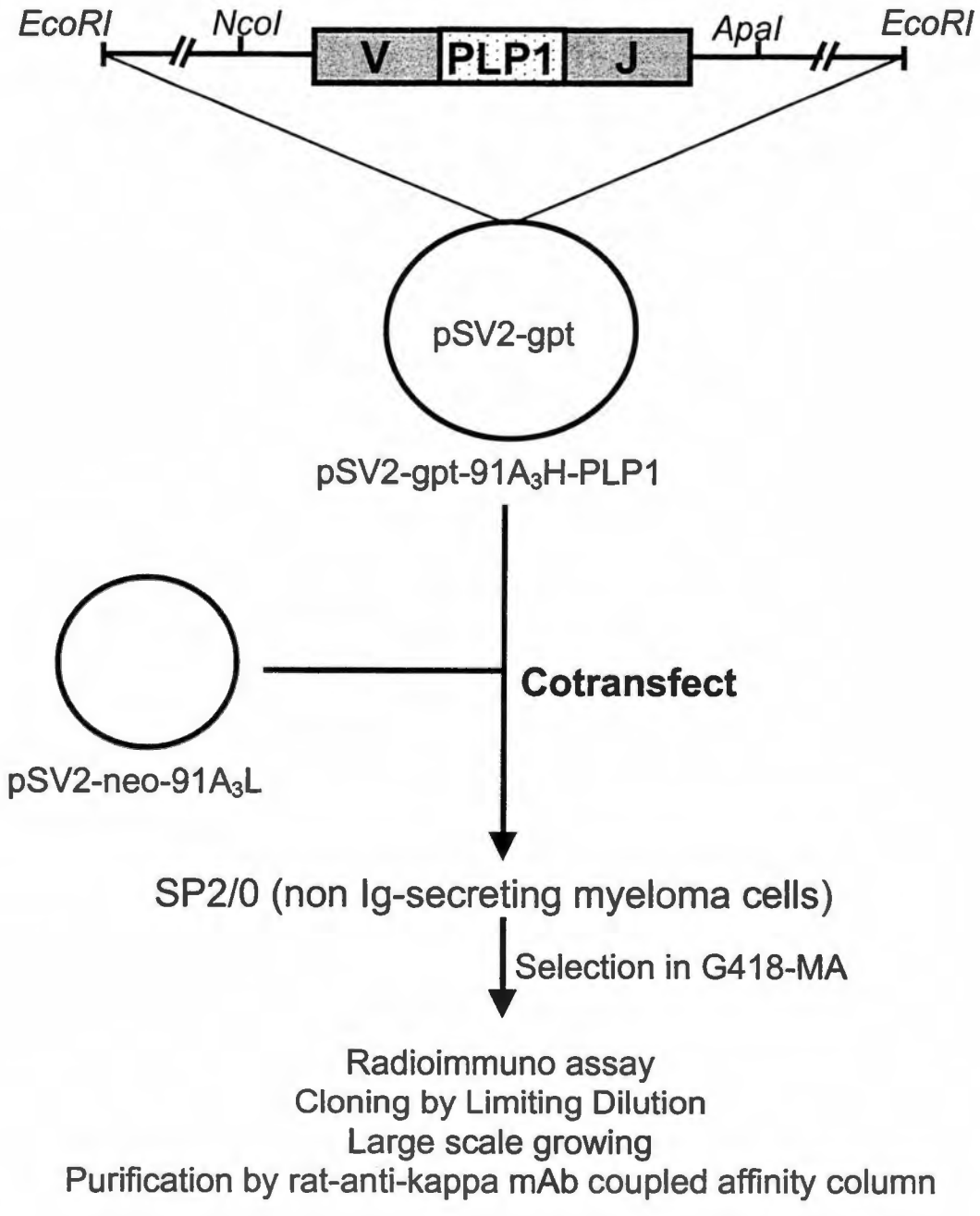

EcoRI
NcoI
V
PLP1
J
ApaI
EcoRI
pSV2-gpt
pSV2-gpt-91A3H-PLP1
pSV2-neo-91A3L
Cotransfect
SP2/0 (non Ig-secreting myeloma cells)
Selection in G418-MA
Radioimmuno assay
Cloning by Limiting Dilution
Large scale growing
Purification by rat-anti-kappa mAb coupled affinity column

Figure 4.

Schematic representation of Ig-PLP constructs.

Ig-W, the parental molecule, is shown with the D segment within the heavy chain variable region illustrated as an open box. Ig-PLP1 shows PLP1 peptide (striped and dotted box) in place of the D segment and Ig-PLP2 encompasses PLP2 (striped box) instead of the D segment.

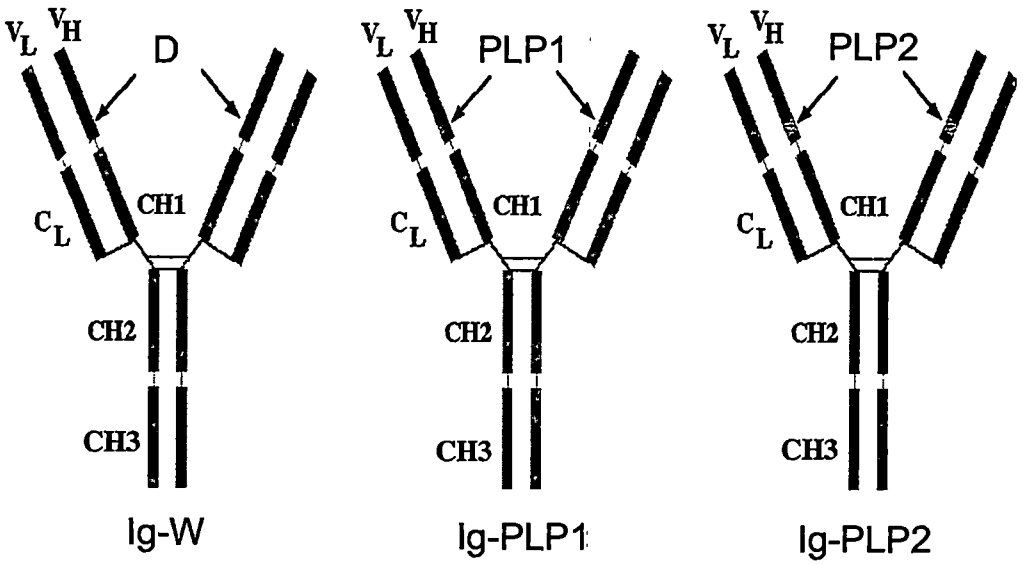

V_L V_H
D
C_L
CH1
CH2
CH3
Ig-W
V_L V_H
PLP1
C_L
CH1
CH2
CH3
Ig-PLP1
V_L V_H
PLP2
C_L
CH1
CH2
CH3
Ig-PLP2

Figure 5.

In vitro presentation of Ig-PLP1 constructs.

T cell hybridomas specific for PLP1, #79 (a) and PLP2, 1A7 (b) were in vitro stimulated with irradiated syngenic splenic APCs in the presence of various antigens for 24 h as described in Materials and Methods. IFNγ production from the supernatant was measured by ELISA.

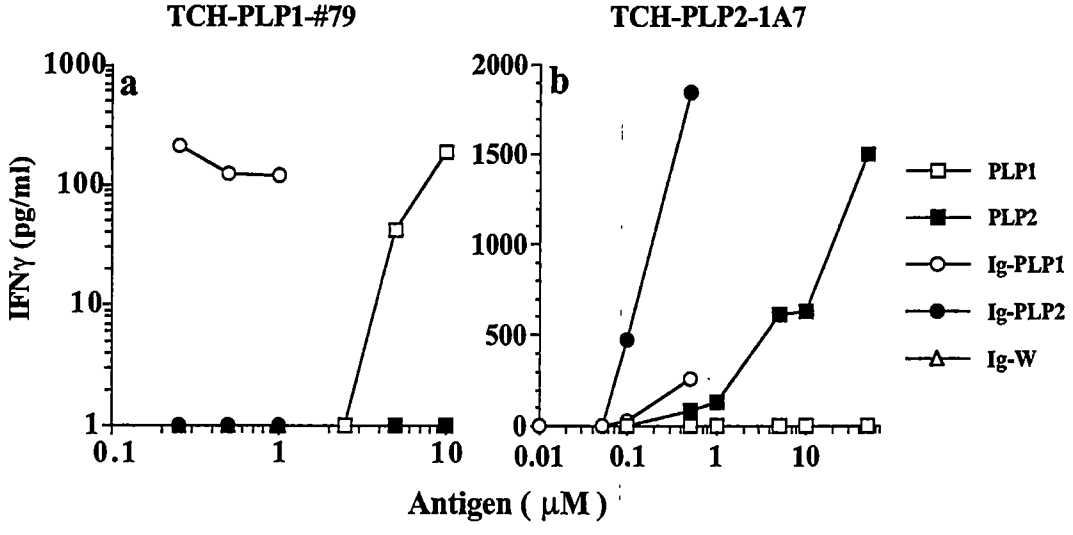
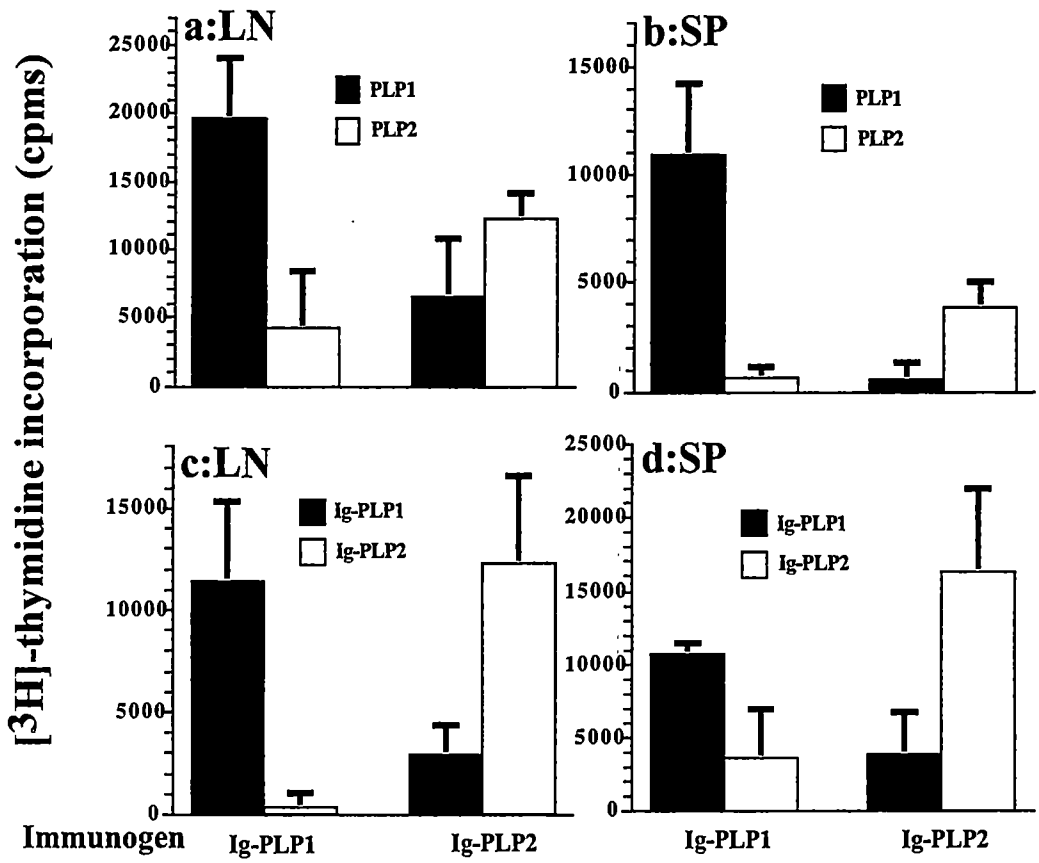

TCH-PLP1-#79
TCH-PLP2-1A7
a
b
IFNγ (pg/ml)
1000
100
10
1
2000
1500
1000
500
0
0.1
1
10
0.01
Antigen (μM)
PLP1
PLP2
Ig-PLP1
Ig-PLP2
Ig-W

Figure 6.

Immunogenecity of the Ig-PLP chimeras.

Groups of SJL/J mice were immunized s.c. in the foot pads and the base of limbs with either Ig-PLP1 or Ig-PLP2 in CFA. Ten days later draining lymph nodes (LN) and spleens (SP) were removed, single cell resuspensions were prepared and stimulated in vitro with PLP1 and PLP2 (a and b) or Ig-PLP1 or Ig-PLP2 (c and d) for 3 days. Subsequently, proliferation was measured by [^{3}H]thymidine incorporation. A control of media without stimulator was included for each mouse and used as background. Values represent the mean ± SD of four individually tested mice.


a:LN
b:SP
c:LN
d:SP
PLP1
PLP2
Ig-PLP1
Ig-PLP2
[3H]-thymidine incorporation (cpms)
Immunogen
Ig-PLP1
Ig-PLP2
0
5000
10000
15000
20000
25000

PART III

Neonatal Exposure to a Self Peptide-Ig Chimera Circumvents the Use of Adjuvant and Confers Resistance to Autoimmune Disease by a Novel Mechanism Involving IL-4 Lymph Node Deviation and IFNγ-Mediated Splenic Anergy.

(Journal of Experimental Medicine, 1998, 188:2007)

Chapter 1. Abstract

Induction of neonatal T cell tolerance to soluble antigens requires the use of incomplete Freund's adjuvant (IFA). The side effects that could be associated with IFA and the ill-defined mechanism underlying neonatal tolerance are setbacks for this otherwise attractive strategy for prevention of T cell-mediated autoimmune diseases. Presumably, IFA contributes a slow antigen release and induction of cytokines influential in T cell differentiation. Igs have long half-lives and could induce cytokine secretion by binding to Fc receptors on target cells. Our hypothesis was that peptide delivery by Igs may circumvent the use of IFA and induce neonatal tolerance that could confer resistance to autoimmunity. To address this issue we used the proteolipid protein (PLP) sequence 139-151 (thereafter referred to as PLP1) which is encephalitogenic and induces experimental autoimmune encephalomyelitis (EAE) in SJL/J mice. PLP1 was expressed on an Ig, and the resulting Ig-PLP1 chimera when injected in saline into newborn mice confers resistance to EAE induction later in life. Mice injected with Ig-PLP1 at birth and challenged as adults with PLP1 developed T cell proliferation in the lymph node but not in the spleen while control mice injected with Ig-W, the parental Ig not including PLP1, developed T cell responses in both lymphoid organs. The lymph node T cells from Ig-PLP1 recipient mice were deviated and produced IL-4 instead of IL-2, while the spleen cells, although non-proliferative, produced IL-2 but not IFNγ. Exogenous IFNγ, as well as IL-12, restored splenic proliferation in an antigen specific manner. IL-12 rescued T cells continued to secrete IL-2 and regained the ability to produce IFNγ. In vivo, administration of anti-IL4 antibody or IL-12 restored disease severity. Therefore, adjuvant free induced neonatal tolerance prevents autoimmunity by an organ specific

regulation of T cells that involves both immune deviation and a new form of cytokine dependent T cell anergy.

Chapter 2. Introduction

Nearly half a century ago, it was shown that mice injected at birth with splenic cells from an allogeneic donor were later able to accept grafts from the same donor (1). Ever since, the neonatal period has been considered as a critical window during which an initial contact with antigen will instruct the immune system to ignore such an antigen and not respond to it during a subsequent encounter.

Soon after the discovery that T cells recognize rather short antigenic fragments, tolerization regimens using peptides in IFA were defined that induced clonal unresponsiveness and mediated neonatal tolerance (2,3). Recent developments, however, indicated that antigen injection during the neonatal stage can immunize rather than suppress (4,5). In allogeneic systems it was discovered that graft acceptance by neonatally tolerized animals was due to the development of functionally deviated T cells producing Th2 type cytokines instead of the usual Th1 cytokines produced by T cells of non-tolerized mice (6,7). These Th2 cells are unable to support development of the cytolytic T lymphocytes required for graft rejection (8). Similarly, neonatal injection of a self peptide in IFA, which protected mice from autoimmune disease induction, was found to operate through clonal unresponsiveness in the lymph nodes accompanied with induction of deviated Th2 cells in the spleen (9). The notion that neonatal injection of antigen can immunize is now well accepted, and evidence has accumulated indicating that factors such as the type of antigen presenting cells (APCs) (5, 10), the adjuvant into which the antigen is emulsified (9), the dose of antigen (11), and the availability of antigen in vivo (12) could engender various outcomes ranging from inactivation of T cells to the priming of an immune response. Therefore, in the face of this susceptibility to

regulation, it is important to further investigate the outcome of neonatal antigen injection, particularly to self antigens, since neonatal tolerance could provide a potential strategy for prevention of autoimmunity.

IFA, which has been required for tolerance induction by soluble proteins and peptides, may exert adjuvanticity by contributing a slow release of antigen and inducing cytokine production by APCs (13). However, the use of IFA may generate side effects and trigger oil induced arthritis (14).

Immunoglobulins (Igs) are autologous molecules permissive for peptide expression and can function as a delivery system for T cell epitopes (15-18). Internalization of Igs into APCs via Fc receptors (FcR) grants the incorporated peptide access to newly synthesized major histocompatibility (MHC) class II molecules, leading to efficient peptide presentation and T cell activation (19). For instance, when the PLP139-151 peptide sequence (hereafter referred to PLP1) was expressed on an Ig molecule the peptide's presentation was increased by 100 fold (20). Furthermore, the Ig-PLP1 chimera was a potent inducer of PLP1-specific T cell responses both in the spleen and lymph nodes (20,21). Since Igs are long-lived molecules in vivo, presentation could persist for a long period of time. In addition by binding FcR on APCs, Igs can trigger production of cytokines (22, 23). These functions may provide the Ig delivery system adjuvant properties that could circumvent the use of IFA for tolerance induction and prevention of autoimmunity. Herein, we report that Ig-PLP1 injected into newborn mice in saline confers resistance to EAE induction by a novel mechanism defined by IL-4 driven lymph node deviation and an unusual IFNγ-mediated splenic proliferative unresponsiveness.

Chapter 3. Materials and Methods

Mice

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

Peptides and Ig-peptide chimeras

Peptides. All peptides used in these studies were purchased from Research Genetics, Inc. (Huntsville, AL) and purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of PLP (24, 25). PLP1 peptide is presented to T cells in association with I-A^s MHC class II molecules. PLP2 peptide (NTWTTCQSIAFPSK) encompasses an encephalitogenic sequence corresponding to aa residues 178-191 of PLP (26). This peptide is also presented by I-A^s MHC class II molecules and induces EAE in SJL/J mice.

Ig-PLP chimeras. Expression of PLP1 peptide on Ig-PLP1 was previously described (20). Ig-W, the parental Ig not encompassing PLP1 peptide, was described elsewhere (27). The genes used to construct these chimeras are those 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 nucleotide sequences coding for PLP1 are similar to those described for the generation of Ig-NP (27). Both Ig-W and Ig-PLP1 are of IgG2b, γ isotypes, and Ig-PLP1 differs from Ig-W only by encompassing PLP1 peptide. Both chimeras were expressed in the non-Ig secreting myeloma B cell line SP2/0 (20). Large scale cultures of transfectants were carried out in DMEM media containing 10% iron enriched calf serum (Intergen, NY). The Ig chimeras were purified from culture supernatant on columns made of rat-anti-mouse kappa chain coupled to CNBr activated Sepharose 4B (Pharmacia, Piscataway, NJ). To avoid cross contamination separate columns were used to purify the chimeras.

Neonatal injections and adult immunizations

Neonatal injections of Ig chimeras were done intraperitoneal (i.p.) in 50 to 100 µl saline. When the mice reached 7 weeks of age they were subjected to immunization with peptide in CFA or to EAE induction.

Immunization of adult mice with peptide in CFA was carried out subcutaneously (s.c.) in the foot pads and at the base of the limbs and tail. The peptide was emulsified in a 200 µl mixture v/v PBS/CFA. Ten days later the mice were sacrificed by cervical dislocation, the spleens and lymph nodes (axillary, inguinal, popliteal, and sacral) were removed for analysis of proliferative and cytokine responses

Induction of EAE

EAE was induced by subcutaneous injection in the foot pads and at the base of the limbs with a 200 µl IFA/PBS (vol/vol) solution containing 100 µg free PLP1 peptide and 200 µg*M. tuberculosis* H37Ra. Six hours later 5 x 10^9 inactivated *B. pertussis* were given intravenously. After 48 hours another 5 x 10^9 inactivated *B. pertussis* were given to the mice. 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.

Assay of neonatal splenic and thymic cells for Ig-PLP1 presentation

Newborn mice were injected i.p. with 100 µg Ig-PLP1 or Ig-W in 100 µl saline, and two days later the spleen and thymus were removed, single cell suspensions were prepared, irradiated (3000 rads), and assayed for stimulation of the PLP1-specific T cell hybridoma 4E3 (28) without addition of exogenous peptide. Briefly, graded numbers of splenic or thymic cells were incubated with 5 x 10^4 4E3 cells, and after 24 hours the supernatant was removed and IL-2 production, used as a measure of T cell activation, was determined. [^{3}H]Thymidine incorporation upon incubation of the supernatant with the IL-2-dependent HT-2 cell line was used for detection of IL-2 as described (20).

Lymph node and spleen T cell proliferation

Lymph node and spleen 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 three days.

Subsequently, 1 μCi [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 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 stimulators were used at the following concentrations: PLP1 and PLP2 at 15 μg/ml. A control media with no stimulator was included for each mouse and used as background.

Assays for restoration of splenic T cell proliferation with exogenous cytokines.

Spleen cells from mice that were injected at birth with Ig-PLP1 and immunized as adults with PLP1 in CFA were incubated in 96-well flat bottom plates at 10 x 10^5 cells/100 μl/well with 100 μl media containing the stimulator peptide and the exogenous cytokine (IFNγ or IL-12) for three days. Subsequently, 1 μCi [^{3}H]thymidine was added per well, and the assay was continued as above.

Cytokine restoration of splenic T cell proliferation was also carried out in the presence of anti-CD4, anti-I-A^s or isotype matched mab. The rat IgG2b anti-CD4 mab, GK1.5, was used at 5μg/ml and the mouse IgG2b anti-I-$A^{k,r,f,s}$ mab, 10-2.16, was used at 100μg/ml. Both hybridomas were obtained from ATCC and antibodies were affinity chromatography purified from culture supernatant on anti-isotypic antibodies coupled to sepharose.

Measurement of cytokines by ELISA

Spleen cells were incubated in 96 well round-bottom plates at 10 x 10^5 cells/100μl/well with 100μl of stimulator for 24 hours. Cytokine production was

measured by ELISA according to Pharmingen's instructions using 100 µl of culture supernatant. Capture antibodies were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; rat anti-mouse IFNγ, R4-6A2; and rat anti-mouse IL-10, JES5-2A5. Biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES6-5H4; rat anti-mouse IL-4, BVD6-24G2; rat anti-mouse IFNγ, XMG1.2; and rat anti-mouse IL-10, JES5-16E3. All cytokines and anti-cytokine antibodies used in these studies were purchased from PharMingen (San Diego, CA). The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Sunnyvale, CA) using SoftMAX PRO version 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, IFNγ, and IL-10 were included in all experiments in order to construct standard curves. The concentration of cytokines in culture supernatants was estimated by extrapolation from the linear portion of the standard curve.

Measurement of cytokines by ELISPOT assay

ELISPOT assay was used to measure cytokines produced by lymph node T cells during antigen stimulation as described (9). HA-multiscreen plates (Millipore, Bedford, MA) were coated with 100 µl/well 1M $NaHCO_3$ buffer containing 2µg/ml capture antibody. After an overnight incubation at 4°C the plates were washed three times with sterile PBS and free sites were saturated with DMEM culture media containing 10% fetal calf serum for 2 hours at 37°C. Subsequently, the blocking media was removed and 5 x 10^5 lymph node cells/100µl/well were added along with 100 µl of antigen and incubated for 24 hours at 37°C in a 7% CO_2 humidified chamber. The plates were then washed and

100 µl of biotinylated anti-cytokine antibody (1µg/ml) was added. The anti-cytokine antibody pairs used here were those described for the ELISA technique. Following overnight incubation at 4°C the plates were washed and 100 µl of avidin-peroxidase (2.5 µg/ml) was added. The plates were then incubated for 1 hour at 37°C. Subsequently, spots were visualized by adding 100µl of substrate (3-amino-9-ethylcarbazole, from Sigma, St Louis, MO) in 50 mM acetate buffer pH 5.0, and counted under a dissecting microscope.

Chapter 4. Results

Neonatal injection of Ig-PLP1 circumvents the use of adjuvant and confers resistance to EAE induction.

The nucleotide sequence coding for PLP1 peptide was genetically inserted into the variable region of an Ig heavy chain gene, and the resulting gene chimera was transfected together with the gene encoding the parental light chain into the non-Ig secreting myeloma B cell line, SP2/0 (20). Selection with the appropriate drugs allowed for the generation of transfectants producing complete Ig molecules with PLP1 peptide embodied within the heavy chain CDR3 region. Affinity chromatography purified Ig-PLP1 was efficiently presented to PLP1-specific T cell hybridomas (20) and induced in vivo T cell responses in both the lymph nodes and spleen that were of Th1 type and produced both IL-2 and IFNγ (20, 21).

To assay for induction of neonatal tolerance one day old mice were injected with Ig-PLP1 in saline, and 7 weeks later were challenged with a disease inducing regimen of free PLP1 peptide and scored daily for paralysis. As can be seen in figure 1A, the mice were resistant to induction of paralysis and developed mild monophasic clinical signs of EAE. The mean maximal disease severity was 2.7 ± 0.5 with 0% mortality rate. However, mice that were injected with Ig-W, the parental wild type Ig not encompassing any PLP peptide (27), developed severe clinical signs of EAE with a 4.2 ± 0.9 mean maximal disease severity accompanied by a 40% mortality. The surviving mice from the group that received Ig-W at birth exhibited a relapsing and remitting disease that persisted to day 100, while animals that were injected with Ig-PLP1 did not display any relapses after recovery from the initial mild disease (Figure 1B).

Mice injected at birth with Ig-PLP1 in saline and challenged with PLP1 peptide in CFA at seven weeks of age developed lymph node but not splenic proliferative T cell responses.

To investigate the mechanism underlying tolerance induction in newborn mice we first determined if Ig-PLP1 is presented by neonatal APCs in vivo. Mice were injected with Ig-PLP1 at birth, and 2 days later thymic and splenic cells were irradiated and assayed for stimulation of the PLP1-specific T cell hybridoma 4E3 (28) without addition of exogenous antigen. Figure 2 shows that both thymic and splenic cells from mice that were injected with Ig-PLP1 stimulated the 4E3 hybridoma, while cells from mice that received Ig-W, instead of Ig-PLP1, did not. These results indicate that Ig-PLP1 was taken up and processed by neonatal APCs and that PLP1/I-A^s complexes were displayed on the surface of these APCs. Next, we investigated the consequences of such neonatal Ig-PLP1 presentation on the outcome of a later challenge with PLP1 peptide. Newborn mice were injected at birth with either Ig-PLP1 or the control Ig-W, challenged with PLP1 peptide in CFA when they reached 7 weeks of age, and then examined for proliferative responses in both lymph node and spleen at day 10 post challenge. The results in figure 3 indicate that those animals, which received Ig-PLP1 at birth, developed proliferative responses to PLP1 peptide in the lymph node but not the spleen while mice that were injected with Ig-W developed proliferative responses in both lymphoid organs. Neither group responded to the negative control PLP2 peptide, corresponding to aa residues 178-191 of PLP (26). Therefore, exposure to Ig-PLP1 during the neonatal stage affects the response to a

challenge with PLP1 peptide and leads to lymph node proliferation and splenic unresponsiveness.

Injection of free PLP1 peptide in saline at birth had no effect on the outcome of a challenge with PLP1 at 7 weeks of age. Indeed, when newborn mice were injected with PLP1 peptide in saline and challenged at seven weeks of age with PLP1 in CFA both the lymph node and spleen developed T cell proliferative responses (Figure 4). These responses were comparable to those obtained in mice that were not subject to any injection at birth but immunized with PLP1 peptide in CFA at 7 weeks of age. In contrast, mice injected at birth with free PLP1 peptide emulsified in IFA and challenged with PLP1 peptide in CFA at the age of 7 weeks developed T cell proliferation in the spleen but were unresponsive in the lymph node (Figure 4). The slight reduction in the splenic response in mice recipient of PLP1/IFA at birth versus those injected with PLP1/saline is not statistically significant (unpaired student t test analysis, $p>0.1$). The results obtained with PLP1/IFA injection are in good agreement with data reported for other peptides (9). Consequently, injection of Ig-PLP1 in saline at birth displays an organ specific regulation of the T cell that is different from the regulation induced by injection of free peptide in IFA or saline (Figure 4).

Newborn mice injected with Ig-PLP1 at birth develop a lymph node deviation and an IFNγ-mediated splenic anergy upon challenge with PLP1 peptide in CFA during adult life.

Examination of the cytokine production in the lymph node and spleen of mice injected at birth with Ig-PLP1 and challenged at 7 weeks of age with PLP1 peptide in

CFA revealed yet another unexpected result. As can be seen in figure 5, the lymph node T cells from the Ig-W recipient group produced IL-2 but not IL-4 or IFNγ, while T cells from the Ig-PLP1 recipient group produced IL-4 instead of IL-2. This cytokine production was specific for PLP1 peptide, since PLP2 was unable to stimulate the cells for cytokine production. In the spleen the Ig-W group produced both IL-2 and IFNγ upon stimulation with PLP1, while cells from the Ig-PLP1 group, although non-proliferative, produced IL-2 and dropped IFNγ to undetectable levels (Figure 6). In vitro stimulation with PLP2 peptide had no effect on cytokine production. IL-4 was undetectable with either stimulation in both groups of mice (Figure 6). IL-10 was undetectable in all groups of mice (not shown).

Restoration of splenic T cell proliferation and IFNγ production by exogenous IL-12.

Because the splenic cells from mice that received Ig-PLP1 at birth produced IL-2 but could not proliferate or secrete IFNγ upon stimulation with PLP1 peptide, we reasoned that the defect in proliferation might be related to the deficiency in IFNγ. To address this issue mice that received Ig-PLP1 at birth were challenged with PLP1 peptide in CFA, and the splenic cells were in vitro stimulated with PLP1 peptide in the presence of exogenous IFNγ or IL-12 (an inducer of IFNγ) and assayed for proliferation. As can be seen in figure 7A, exogenous IFNγ restored proliferation of the spleen cells upon stimulation with PLP1 peptide. The restoration of proliferation is antigen specific, since addition of exogenous IFNγ did not restore proliferation when in vitro stimulation was carried out with PLP2 peptide. Furthermore, IL-12 was also able to restore splenic proliferation (Figure 7B). IL-12 restoration of proliferation also proved to be antigen

specific and did not develop when in vitro stimulation was carried out with PLP2 peptide. The restoration of T cell proliferation by IL-12 was completely inhibited when the in vitro stimulation was carried out in the presence of 5 μg/ml anti-CD4 mab, GK 1.5 indicating that the proliferating cells are CD4-T cells (not shown). In addition, the anti-I-A^s mab, 10-2.16 inhibited IL-12 restoration of proliferation indicating the requirement for peptide presentation (not shown).

While IFNγ restored splenic T cell proliferation did not induce IL-4 production, the production of IL-2 was slightly reduced in comparison to stimulation without cytokine addition (Table 1A). In addition, exogenous IL-12, which restored proliferation but slightly reduced the amount of IL-2, restored production of IFNγ by the splenic T cells (Table 1B). Restoration of IFNγ production was antigen specific because it required stimulation with PLP1 and did not occur when PLP2 was used for stimulation.

Restoration of disease severity in Ig-PLP1 tolerized mice by administration of anti-IL-4 antibody or rIL-12.

To evaluate the contribution of lymph node IL-4 and splenic anergy to the resistance against disease induction mice, neonatally tolerized with Ig-PLP1, were subjected to EAE induction while exposed to anti-IL-4 mab or rIL-12. As can be seen in figure 8 administration of 11B11 rat anti-mouse IL-4 mab restored the severity of EAE to a level comparable to that obtained in the susceptible mice neonatally injected with Ig-W. The control group injected with rat IgG instead of 11B11 mab, like the Ig-PLP1 neonatally tolerized mice that were not given anti-IL-4 during disease induction, did not restore paralysis. The mice treated with anti-IL4 mab had a 4.0 ± 0.9 mean maximal

disease severity, a score comparable to the 4.2 ± 0.9 obtained with the Ig-W tolerized mice, while those treated with the rat IgG instead of 11B11 mab had a mean severity of 2.7 ± 0.2 which is comparable to the 2.7 ± 0.5 of the mice tolerized with Ig-PLP1 but not treated with anti-IL4. The rate of mortality was 40% in the anti-IL4 treated mice and 0% in those treated with the rat IgG. Overall, in vivo neutralization of IL-4 restores severe EAE.

Administration of IL-12 also restores disease severity in Ig-PLP1 neonatally tolerized mice (Figure 9). Indeed, mice injected with Ig-PLP1 during the neonatal stage and exposed to rIL-12 during disease induction developed a pattern of paralysis much more severe than mice that did not receive rIL-12. In fact, the severity followed a profile similar to that of the susceptible mice recipient of Ig-W during the neonatal stage. The mean maximal disease severity in these mice was 3.8 ± 0.8 and the rate of mortality was 29%. These values are significantly higher than the 2.7 ± 0.5 mean maximal disease severity and 0% death obtained in mice that were not administered with rIL-12

Interestingly, the spleen cells from mice tolerized with Ig-PLP1 at birth and immunized with PLP1 peptide at adult life re-acquired proliferative capabilities when the animals were given rIL-12 during peptide immunization (Figure 10). Indeed, the splenic T cell proliferative responses in these mice were significantly higher than those obtained in the mice which were not treated with rIL-12. In addition, the proliferation was optimal as it was only slightly higher than the proliferation of cells provided rIL-12 in vitro only and moderately lower than the proliferation of cells exposed to rIL-12 both in vivo and in vitro (Figure 10).

Chapter 5. Discussion

Ig-PLP1, an Ig molecule expressing the encephalitogenic sequence 139-151 of PLP, injected in saline into newborn mice was efficiently presented by neonatal thymic and splenic APCs in vivo (Figure 2). Consequently, the mice showed resistance to EAE induction later in life (Figure 1). Examination of the T cell responses to PLP1 in mice that received Ig-PLP1 at birth and challenged with PLP1 at seven weeks of age indicated a strong peptide-specific proliferative response in the lymph node but a markedly reduced splenic proliferation (Figure 3). Control animals injected at birth with Ig-W, the parental Ig not encompassing PLP1 peptide, developed proliferative responses in both lymphoid organs (Figure 3). In the lymph node there was a cytokine deviation from IL-2, in Ig-W recipient mice, to IL-4 in Ig-PLP1 recipient mice (Figure 5). In the spleen, while the control mice injected with Ig-W at birth produced both IL-2 and IFNγ, Ig-PLP1 recipient mice, despite the absence of T cell proliferation produced IL-2 but IFNγ was undetectable (Figure 6). The non-proliferative splenic T cells were able to recover from this status and proliferated when stimulated with PLP1 peptide in the presence of IFNγ or IL-12 (Figure 7). The restoration of proliferation by exogenous cytokines was inhibited by anti-CD4 and anti-MHC class II antibodies indicating that the target cells are CD4-positive T cells requiring peptide presentation for re-acquisition of the proliferative status. In addition, these splenic T cells, once recovered, became able to produce IFNγ (Table 1). In vivo, when the Ig-PLP1 tolerized mice were given anti-IL-4 mab during disease induction, the severity of EAE was restored (Figure 8). Similarly administration of rIL-12 during peptide immunization or disease induction restored both splenic proliferation (Figure 10) and disease severity (Figure 9). A summary of these results is illustrated in table 2. The

overall conclusion that could be drawn from these data is that the placing of a peptide in the context of an Ig for delivery to the neonatal immune system circumvents the use of IFA to confer resistance to disease induction later in life. Moreover, the results reveal a new mechanism operating neonatal tolerance. As indicated in table 2, the lymph node T cells were deviated and produced IL-4 instead of IL-2, and the splenic T cells, although non-proliferative, produced IL-2 and regained the ability to proliferate when provided IFNγ or IL-12. We wish to define this phenomenon as IFNγ-mediated anergy.

T cell deviation has previously been associated with neonatal tolerance in allogeneic (6, 7), viral (11), as well as peptide/IFA (9) antigen systems. In the Ig-PLP1 model there is a unique organ specific T cell regulation characterized by a deviation in the lymph node and an unusual IFNγ-mediated anergy in the spleen. In fact when free PLP1 peptide was injected in saline at birth the response to challenge with PLP1 in CFA was normal (Figure 4). Moreover, when free PLP1 peptide was injected into newborn mice in IFA, a challenge with PLP1 in CFA induced significant proliferation in the spleen but the lymph node was unresponsive (Figure 4). Similar results were reported in an MBP/IFA model where the proliferative splenic T cells were deviated and produced Th2 type cytokines (9). The lymph node IL-4 may play an important role in the resistance to disease induction. This statement is supported by the observation that neutralization of IL-4 by the administration of anti-IL-4 antibody restores the severity of disease. In addition, an IL-4-mediated bystander effect (29) may have been responsible for the suppression of epitope spreading and related relapses (30,31) in the Ig-PLP1 tolerized mice.

The second striking observation is that the splenic T cells of mice injected with Ig-PLP1 at birth and challenged with PLP1 peptide as adults could not proliferate (Figure 3B) or produce IFNγ (Figure 6C). However, there was production of IL-2 (Figure 6A). Moreover, these cells regained the ability to proliferate when supplied with IFNγ or IL-12, a cytokine defined as an inducer of IFNγ (Figure 7) (32, 33). This phenomenon may qualify for the term anergy, with the distinction that the T cells produce IL-2 but are unable to produce IFNγ. Therefore, we propose the term IFNγ-mediated anergy to differentiate it from standard T cell anergy (34). The fact that the T cells produce IFNγ when they regain proliferative capacity upon supply of IL-12 further justifies the involvement of IFNγ in this form of unresponsiveness. Moreover, since administration of rIL-12 was able to restore both in vivo splenic T cell proliferation and disease severity, it maybe that bystander re-activation of these cells is responsible for the residual EAE observed in Ig-PLP1 tolerized mice. Also, the question as to the factors involved in the initiation and perpetuation of these altered cellular responses mediating resistance to disease induction remains unanswered. On a speculative basis it could be reasoned that neonatal presentation of Ig-PLP1 involves specific APCs that strongly attach Ig-PLP1 and coordinately regulate expression of costimulatory molecules and/or cytokine production. Under these circumstances, the T cell-APC interactions and the local cytokine environment could be affected, leading to the generation of T cells for which differentiation is still susceptible to regulation. During challenge with antigen these cells would be subject to organ specific regulation. Alternatively, neonatal presentation of Ig-PLP1 could be generating T cells deficient in IFNγ production. During antigen challenge those restimulated in regional lymph nodes would be subject to the effects of CFA and

possibly default to the Th2 pathway, while those restimulated systemically remain dependent on IFNγ for proliferation and differentiation.

The deficiency in IFNγ and the dependency of splenic T cell proliferation on such a cytokine constitute another puzzle in these observations. IL-12, a key cytokine for the differentiation of T cells into effector Th1 cells (35, 36) restored proliferation and IFNγ production by splenic T cells (Figure 7B and Table 1). It could be possible that the splenic T cells, although producing the growth factor IL-2, are defective in the biochemical signals required for differentiation and consequent IFNγ production.

Overall, neonatal injection of antigen, generally thought of as a strategy for induction of unresponsiveness appears to function for immunization in this Ig-peptide model as well as in other systems (4,5,9). In addition, since immune deviation provides the possibility for bystander T cell down-regulation (29,37), the Ig-peptide strategy may evolve as a means of vaccination without the need for adjuvant against autoimmune diseases involving multiple antigens.

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Table 1.

Restoration of IFNγ production by stimulation of splenic cells from Ig-PLP1 recipient mice with PLP1 peptide in the presence of exogenous IL-12*

exogenous cytokine	stimulator peptide	cytokine production		
		IFNγ	IL-2	IL-4
U/ml			pg/ml‡	
A. IFNγ				
0	PLP1	≤16	322 ± 80	≤16
1	PLP1	ND†	244 ± 74	20 ±17
10	PLP1	ND	216 ± 60	≤16
10	PLP2	ND	≤16	≤16
B. IL-12				
0	PLP1	≤16	322 ± 80	≤16
1	PLP1	675 ± 198	242 ± 60	31 ± 24
10	PLP1	654 ± 227	231 ± 60	30 ± 25
10	PLP2	99 ± 78	≤16	≤16

Table 1 (Continued)

* Six newborn mice were injected with 100 μg Ig-PLP1 in PBS at birth and challenged with 100 μg PLP1 peptide in CFA at the age of 7 weeks. Ten days later the mice were sacrificed and the spleen cells (1×10^6 cells/well) were in vitro stimulated with PLP1 or PLP2 peptide (15μg/ml) in the presence of the indicated concentration of IFNγ (A) or IL-12 (B). After 24 hour incubation cytokine production was measured in the supernatant by ELISA as described in Figure 6.

‡ Mean ± SD of six individually tested mice.

† not done

Statistical analysis using unpaired student t test indicated that the slight reduction in IL-2 production when the cells were stimulated with PLP1 in the presence of cytokines versus stimulation in the absence of cytokines is not significant ($p>0.05$ in all groups). It is likely that reactivated proliferative T cells reabsorb some IL-2.

Table 2.
Summary of the lymph node and splenic responses to PLP1 peptide in mice recipient of Ig-PLP1 or Ig-W at birth.

Tolerogen at birth	Proliferation	Cytokine production	Response status
A. Splenic response			
Ig-W	yes	IL-2, IFNγ	standard
Ig-PLP1	No, but can be restored by exogenous IFNγ	only IL-2, but IFNγ production can be restored by or IL-12 in vitro exogenous IL-12 in vitro or IL-12 in vivo	unusual IFNγ-mediated anergy
B. Lymph node response			
Ig-W	yes	IL-2	standard
Ig-PLP1	yes	IL-4	IL-4 driven T cell deviation

Figure 1.

SJL/J mice injected with Ig-PLP1 at birth resist induction of EAE during adult life.

Newborn mice (10 mice per group) were injected with 100 μg of affinity chromatography purified Ig-PLP1 (filled triangle) or Ig-W (open circles) in saline within 24 hours of birth and were induced for EAE with free PLP1 peptide at 7 weeks of age as described in Materials and Methods. Mice were scored daily for signs of paralysis for 100 days. Panel A shows the daily mean clinical score of all mice, and panel B shows the daily mean score of only the surviving animals. While all mice in both groups developed signs of paralysis, 40% of the mice that were injected with Ig-W at birth died of severe disease. Death did not occur in mice injected with Ig-PLP1 at birth. The mean maximal disease severity was 4.2 ± 0.9 in the mice recipient of Ig-W at birth and 2.7 ± 0.5 in the Ig-PLP1 group.

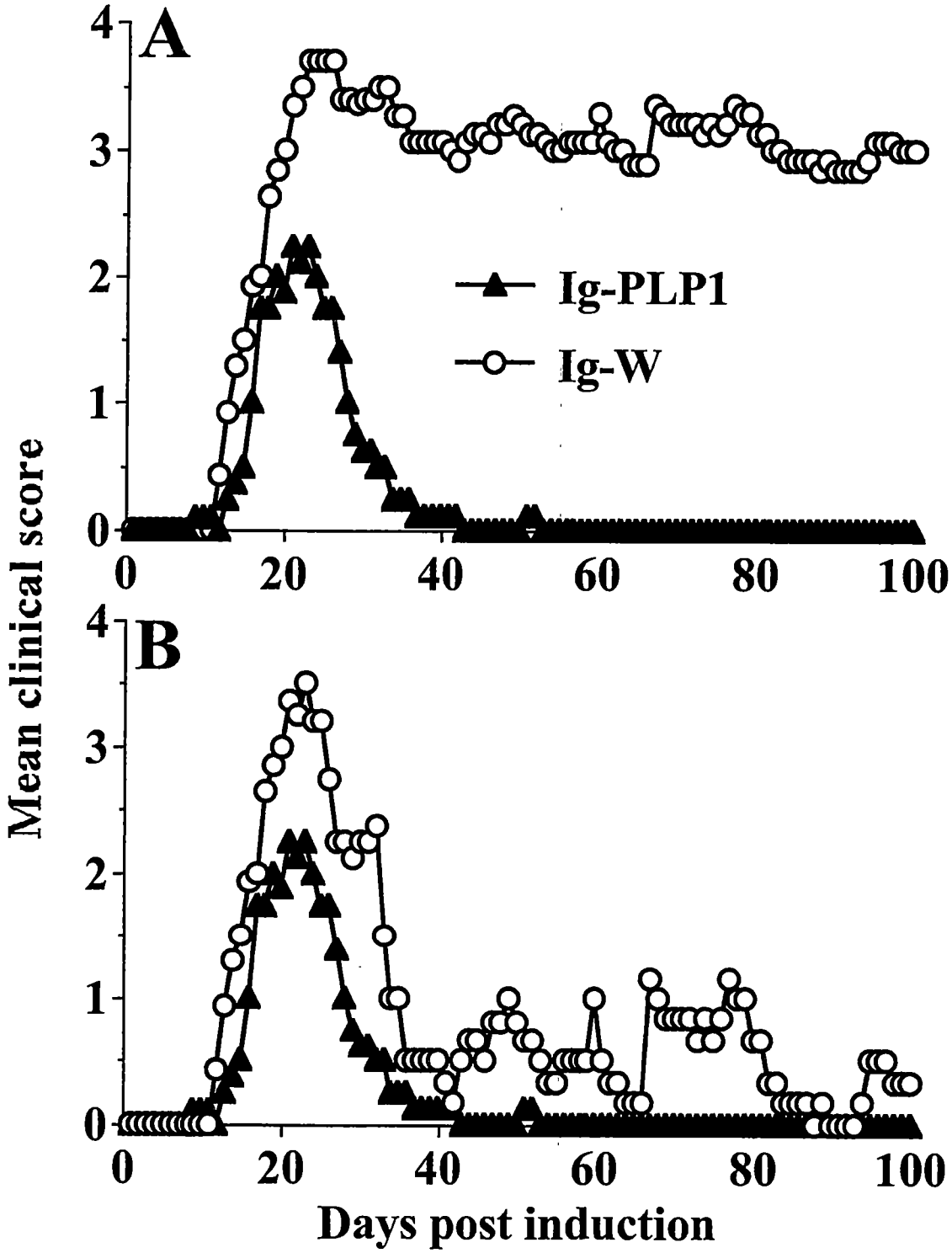

A
B
Ig-PLP1
Ig-W
Mean clinical score
Days post induction
0
1
2
3
4
0
20
40
60
80
100

Figure 2.

In vivo presentation of Ig-PLP1 by neonatal thymic and splenic antigen presenting cells.

Newborn mice (3 per group) were injected with 100 μg Ig-PLP1 (filled triangles) or Ig-W (open circles) in saline within 24 hours of birth, and in vivo presentation of Ig-PLP1 was allowed for two days. The mice were then sacrificed, and pooled thymic (A) and splenic (B) cells were irradiated and used as APCs for stimulation of the PLP1-specific T cell hybridoma 4E3 (28). IL-2 production in the supernatant, which was used as a measure of T cell activation, was determined using the IL-2 dependent HT-2 cell line as described in Materials and Methods. The indicated cpms represent the mean ± SD of triplicates.

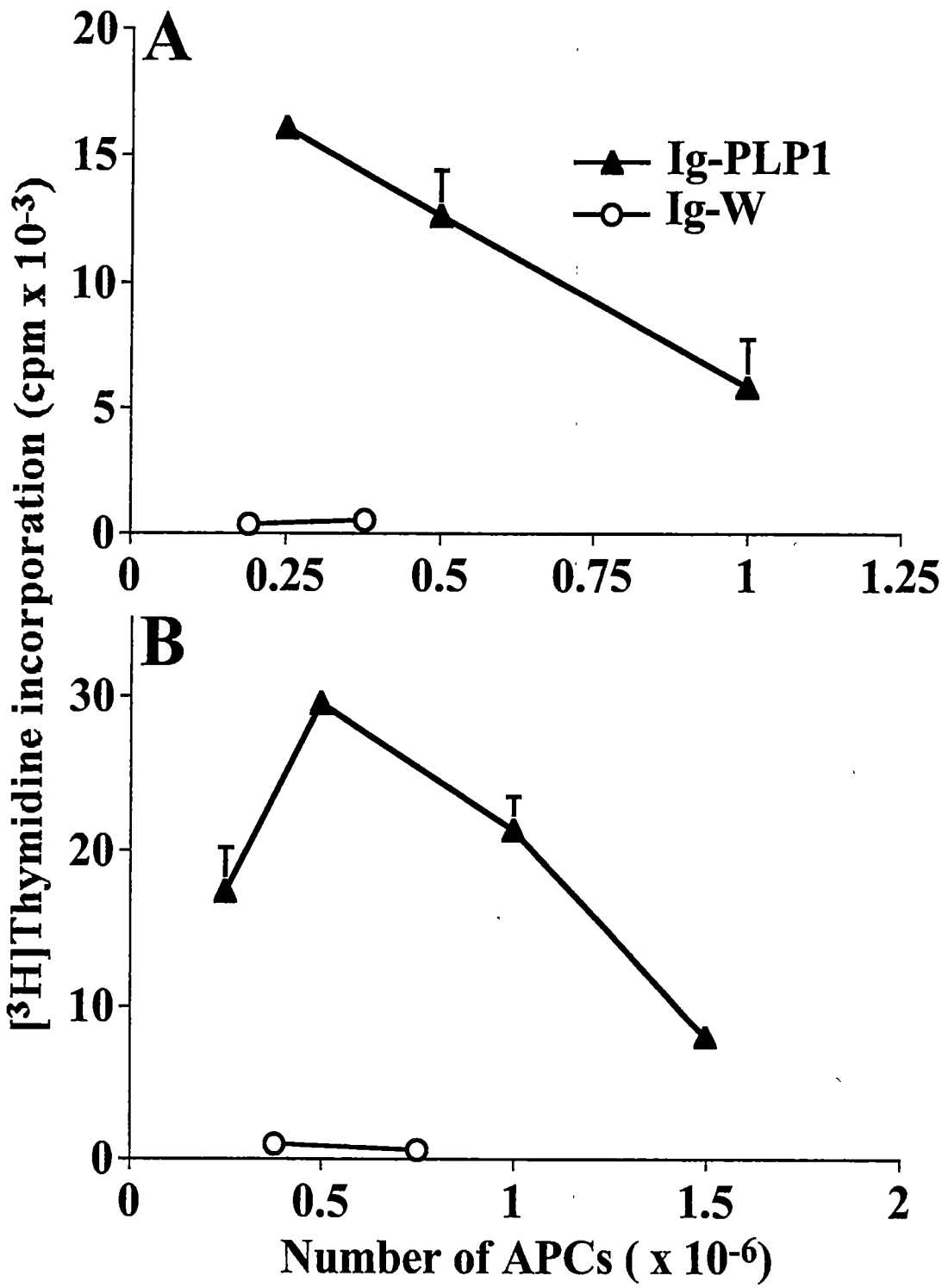

A
B
Ig-PLP1
Ig-W
[3H]Thymidine incorporation (cpm x 10-3)
Number of APCs (x 10-6)
20
15
10
5
0
0
0.25
0.5
0.75
1
1.25
30
20
10
0
0.5
1
1.5
2

Figure 3.

Reduced splenic proliferative T cell response in mice injected with Ig-PLP1 at birth.

Newborn mice (8 per group) were injected intraperitoneal (i.p.) within 24 hours of birth with 100 μg Ig-PLP1 or Ig-W in saline. When the mice reached 7 weeks of age they were immunized with 100 μg free PLP1 peptide in 200 μl CFA/PBS (vol/vol) s.c. in the foot pads and at the base of the limbs and tail. Ten days later the mice were sacrificed, and (A) the lymph node (0.4×10^6 cells/well) and (B) the splenic (1×10^6 cells/well) cells were in vitro stimulated for four days with 15 μg/ml of free PLP1 or PLP2, a negative control peptide corresponding to the encephalitogenic sequence 178-191 of PLP (26). One μCi/well of [^{3}H]thymidine was added during the last 14.5 hours of stimulation, and proliferation was measured using an Inotech β-counter and the trace 96 Inotech program. The indicated cpms represent the mean ± SD of triplicate wells for individually tested mice. The mean cpm ± SD of lymph node proliferative response of all Ig-PLP1 and Ig-W recipient mice was 34,812 ± 7,508 and 37,026 ± 10,333, respectively. The mean splenic proliferative response was 3,300 ± 3,400 for the Ig-PLP1 recipient group and 14,892 ± 4,769 for the Ig-W recipient group.

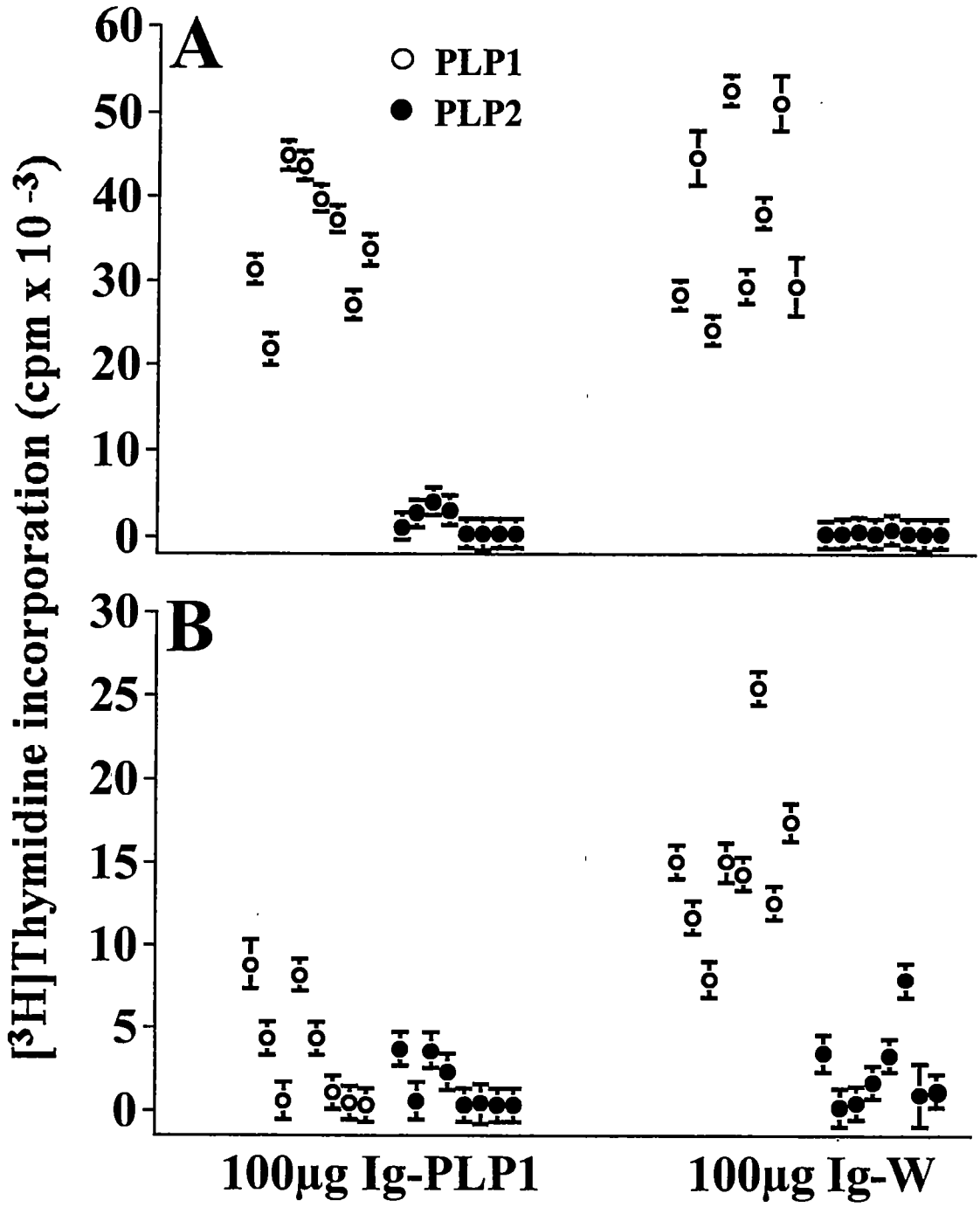

A
B
PLP1
PLP2
[3H]Thymidine incorporation (cpm x 10 -3)
60
50
40
30
20
10
0
30
25
20
15
10
5
0
100µg Ig-PLP1
100µg Ig-W

Figure 4.

Neonatal injection of free PLP1 peptide in saline or IFA has a different effect than Ig-PLP1/saline on the proliferative T cell responses to a challenge with PLP1 in CFA.

Three groups of newborn mice (7 per group) were injected at birth i.p. with 100μg PLP1 peptide in 100μl saline (PLP1/Sln), 100μg PLP1 peptide in 100μl PBS/IFA (vol/vol) (PLP1/IFA), and 100μg Ig-PLP1 in 100 μl saline (Ig-PLP1/Sln) and challenged with 100μg PLP1 in CFA as in figure 3. Ten days later the mice were sacrificed and lymph node (A) and splenic (B) proliferative responses were analyzed by [^{3}H]thymidine incorporation as described in figure 3. A control group of mice that was not injected at birth (None) but immunized as adults with PLP1 in CFA was included for comparison purposes. The bars represent the mean cpm ± SD of seven individually tested mice.

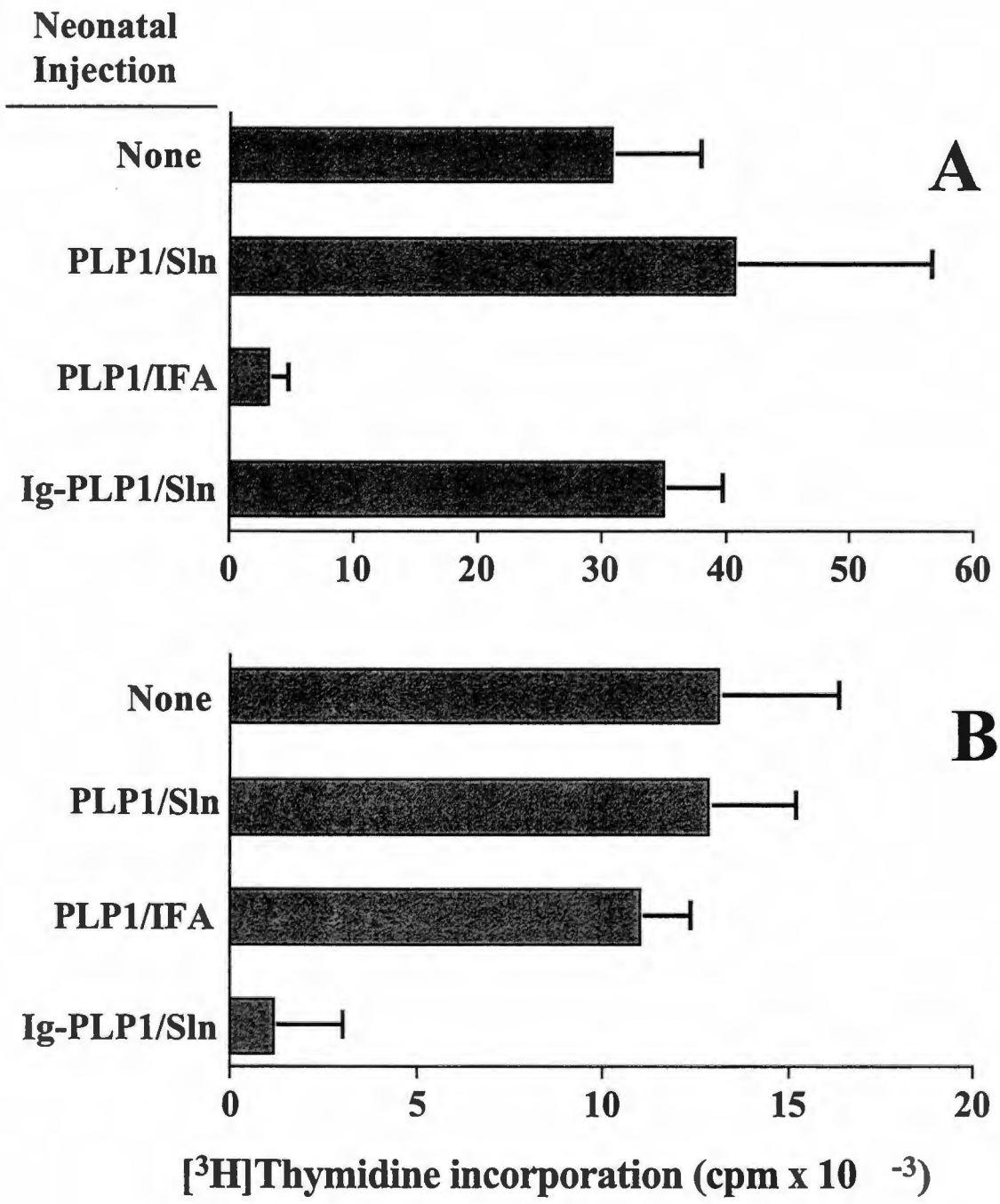

Neonatal Injection
None
PLP1/Sln
PLP1/IFA
Ig-PLP1/Sln
0
10
20
30
40
50
60
A
None
PLP1/Sln
PLP1/IFA
Ig-PLP1/Sln
0
5
10
15
20
B
[3H]Thymidine incorporation (cpm x 10 -3)

Figure 5.

Lymph node T cell deviation in mice recipient of Ig-PLP1 at birth.

Newborn mice (8 per group) were injected intraperitoneal (i.p.) within 24 hours of birth with 100 μg Ig-PLP1 or Ig-W in saline. When the mice reached 7 weeks of age they were immunized with 100 μg free PLP1 peptide in 200 μl CFA/PBS (vol/vol) s.c. in the foot pads and at the base of the limbs and tail. Ten days later the mice were sacrificed, and the lymph node cells (0.4×10^6 cells/well) were in vitro stimulated with free PLP1 or PLP2 (15 μg/ml) for 24 hours. The production of IL-2 (A), IL-4 (B), and IFNγ (C) was measured by ELISPOT as described in the Materials and Methods section using PharMingen anti-cytokine antibody pairs. The indicated values (spot forming units) represent the mean ± SD of 8 individually tested mice.

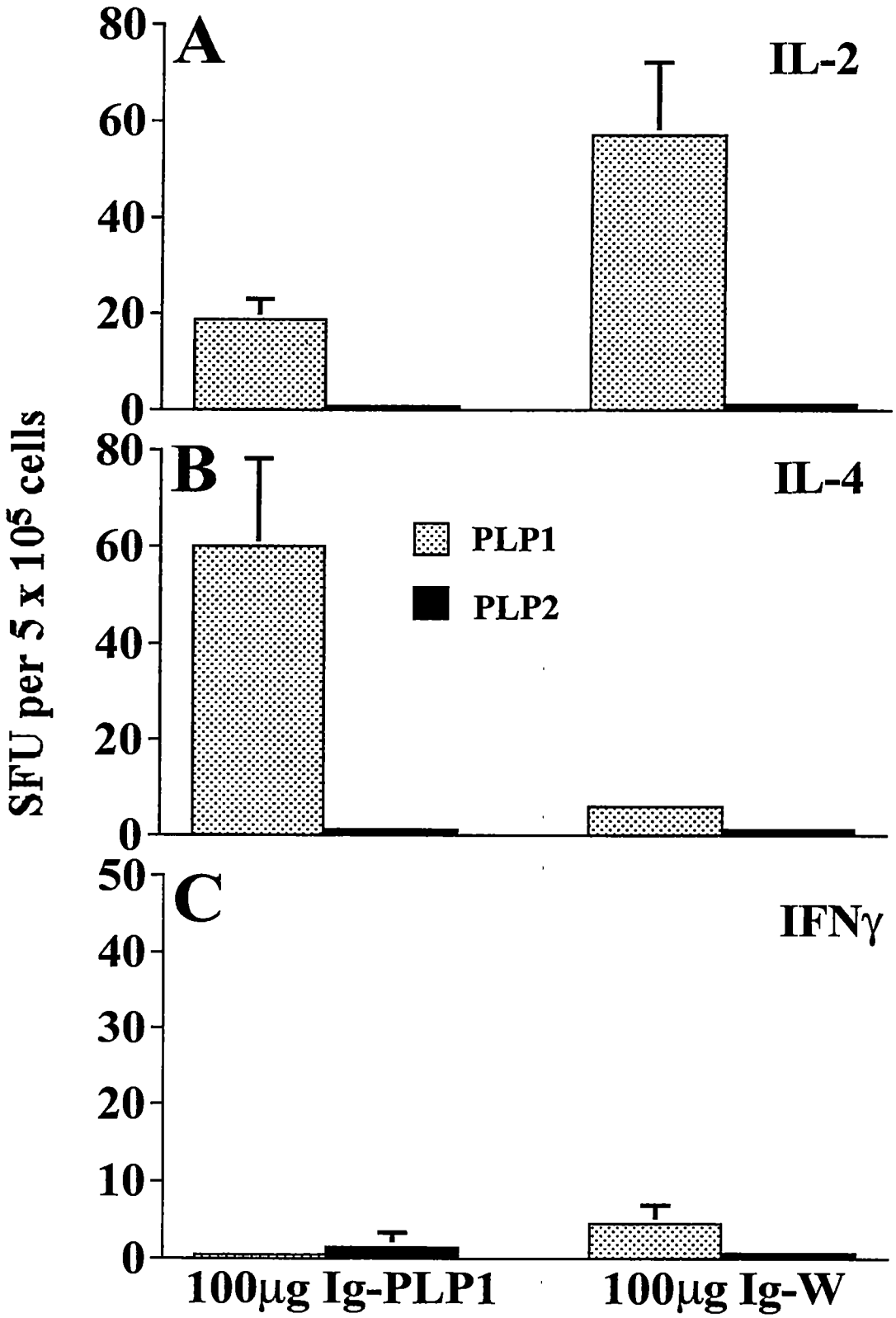

A
IL-2
B
IL-4
C
IFNγ
PLP1
PLP2
SFU per 5 x 10^5 cells
80
60
40
20
0
50
30
10
100μg Ig-PLP1
100μg Ig-W

Figure 6.

Production of IL-2 but not IFNγ by non-proliferative splenic T cells from mice injected with Ig-PLP1 on the day of birth.

Splenic cells (1 x 10^6 cells/well) from the mice described in figure 5 were in vitro stimulated with free PLP1 or PLP2 (15 μg/ml) for 24 hours, and the production of IL-2 (A), IL-4 (B), and IFNγ (C) in the supernatant was measured by ELISA using anti-cytokine antibody pairs from PharMingen according to the manufacturer's instructions. The indicated amounts of cytokine represent the mean ± SD of 8 individually tested mice.

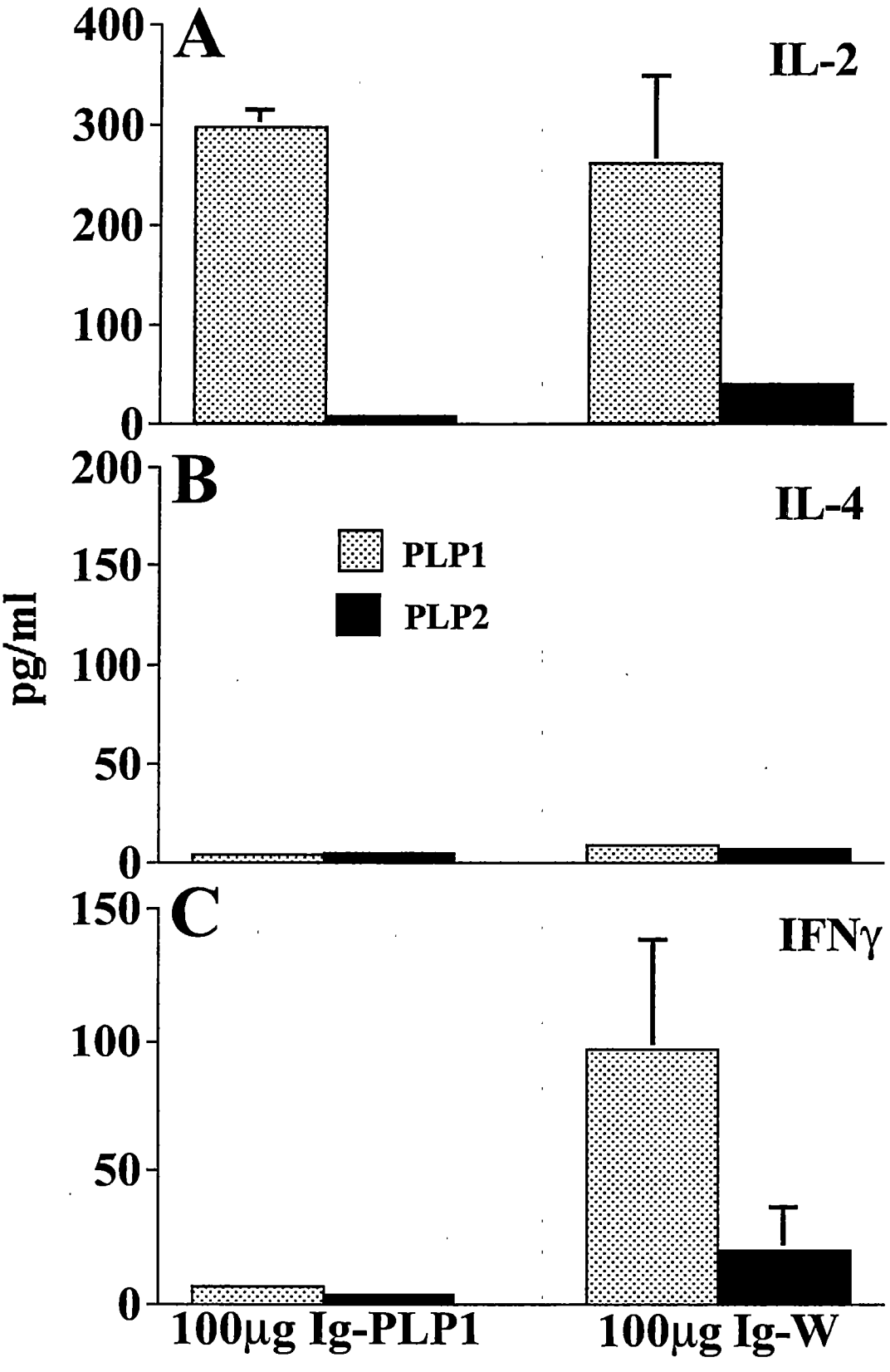

A
IL-2
400
300
200
100
0
B
IL-4
200
150
100
50
0
PLP1
PLP2
pg/ml
C
IFNγ
150
100
50
0
100μg Ig-PLP1
100μg Ig-W

Figure 7.

Cytokine mediated restoration of splenic T cell proliferation in mice injected with Ig-PLP1 at birth.

A group of 5 newborn mice was injected i.p. with 100 μg of Ig-PLP1 and immunized with 100 μg PLP1 peptide in CFA at 7 weeks of age, as in figure 3. Ten days later the splenic cells (1 x 10^6 cells/well) were in vitro stimulated with free PLP1 peptide (15 μg/ml) in the presence of 100 units/ml IFNγ (A) or 10 units/ml IL-12 (B), and [^{3}H]thymidine incorporation was measured as in figure 3. Cells from each mouse were stimulated with PLP1 peptide without addition of exogenous cytokines (dotted bars), with PLP1 peptide in the presence of cytokine (hatched bars), or with PLP2 peptide in the presence of cytokine (black bars). The indicated cpms for each mouse represent the mean ± SD of triplicate wells.

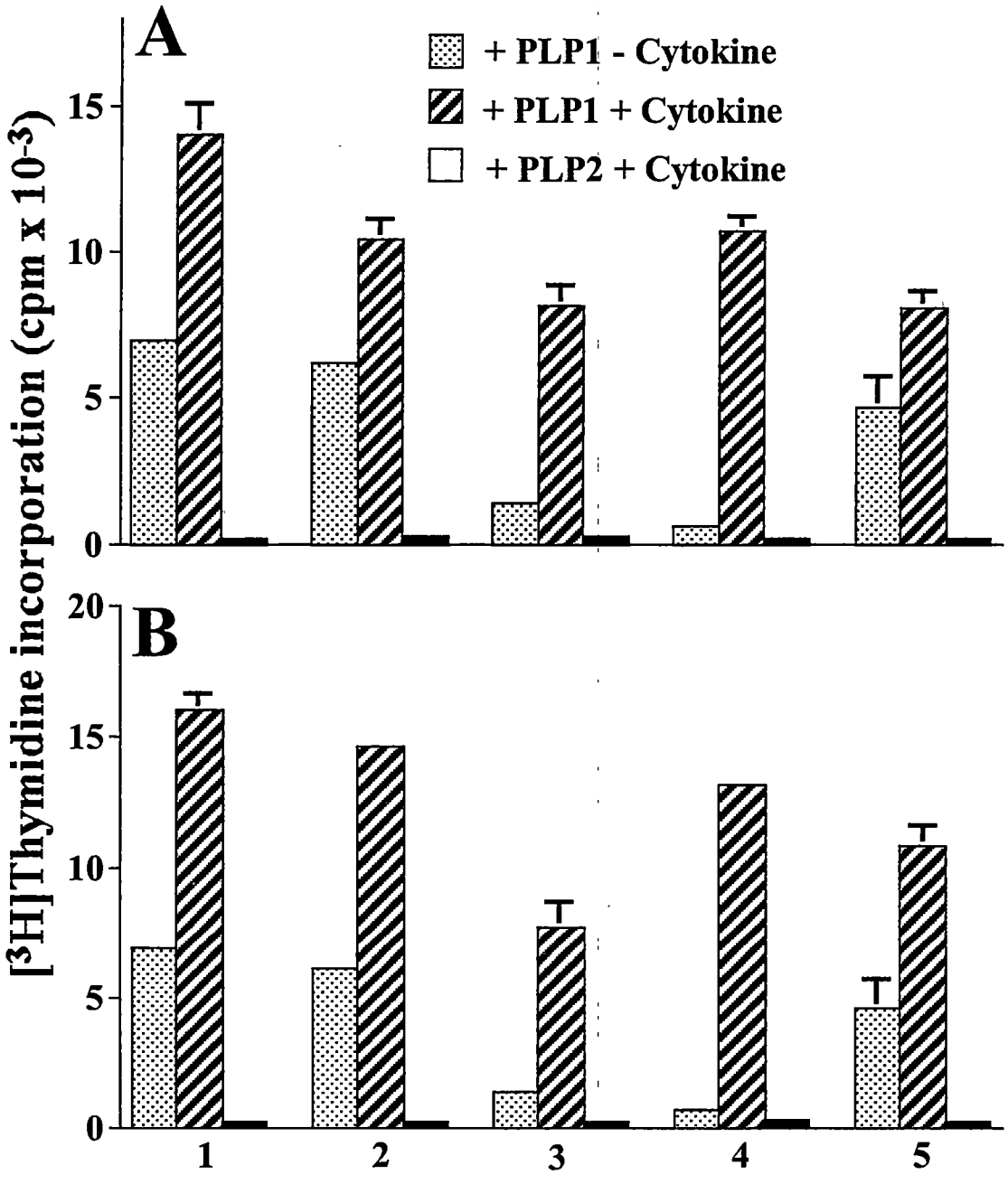

A
B
+ PLP1 - Cytokine
+ PLP1 + Cytokine
+ PLP2 + Cytokine
[3H]Thymidine incorporation (cpm x 10-3)
0
5
10
15
20
1
2
3
4
5

Figure 8.

Restoration of EAE in Ig-PLP1 tolerized mice by administration of anti-IL-4 antibody.

Newborn mice were injected i.p within 24 hours of birth with 100 μg Ig-PLP1 in saline. When they reached 7 weeks of age, a group of 7 mice was injected i.p. with 1 mg/mouse of affinity purified 11B11 anti-IL-4 antibody in 500 μl of PBS (open squares). A second group of 5 mice was injected with 1 mg/mouse of rat IgG in 500 μl PBS (open diamonds) to serve as control. On the next day all mice were induced for EAE with PLP1 peptide as described in figure 1. Five days post disease induction the mice were given a second injection (i.p.) of 1mg/mouse of 11B11 or rat IgG. The mice were scored daily for signs of paralysis. For comparison purposes the clinical scores of the mice described in figure 1 which were tolerized with Ig-PLP1 (filled triangles) or Ig-W (open circles) at birth and induced for EAE with PLP1 peptide at 7 weeks of age were included.

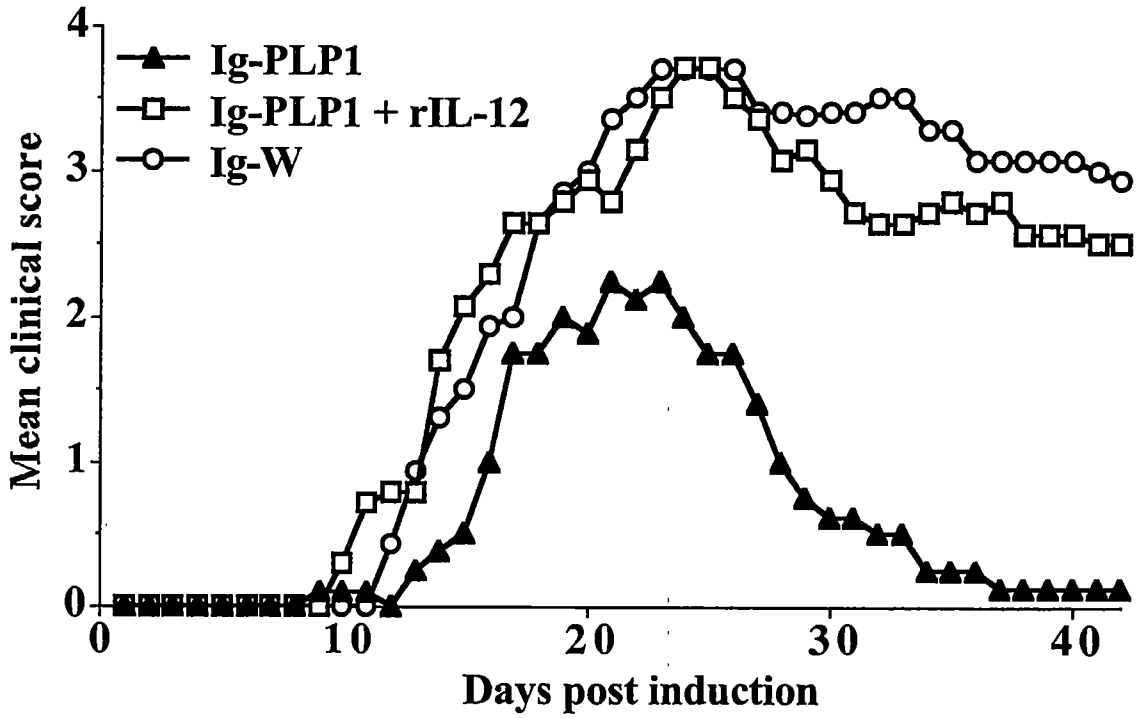

Ig-PLP1
Ig-PLP1 + rIL-12
Ig-W
Mean clinical score
Days post induction
0
1
2
3
4
0
10
20
30
40

Figure 9.

Restoration of EAE in Ig-PLP1 tolerized mice by administration of rIL-12.

Neonates (7 per group) were injected i.p within 24 hours of birth with 100 μg Ig-PLP1 in saline, and when the mice reached 7 weeks of age they were induced for EAE with PLP1 peptide as described in figure 1. Four hours post disease induction the mice were injected i.p. with 500 ng/mouse of rIL-12 (PharMingen). Additional i.p. injections of rIL-12 (500 ng/mouse) were carried out on days 2, 4, and 7 post disease induction. These mice (open squares) were scored daily for signs of paralysis. For comparison purposes the clinical scores of the mice described in figure 1 which were tolerized with Ig-PLP1 (filled triangles) or Ig-W (open circles) at birth and induced for EAE with PLP1 peptide at 7 weeks of age were included.

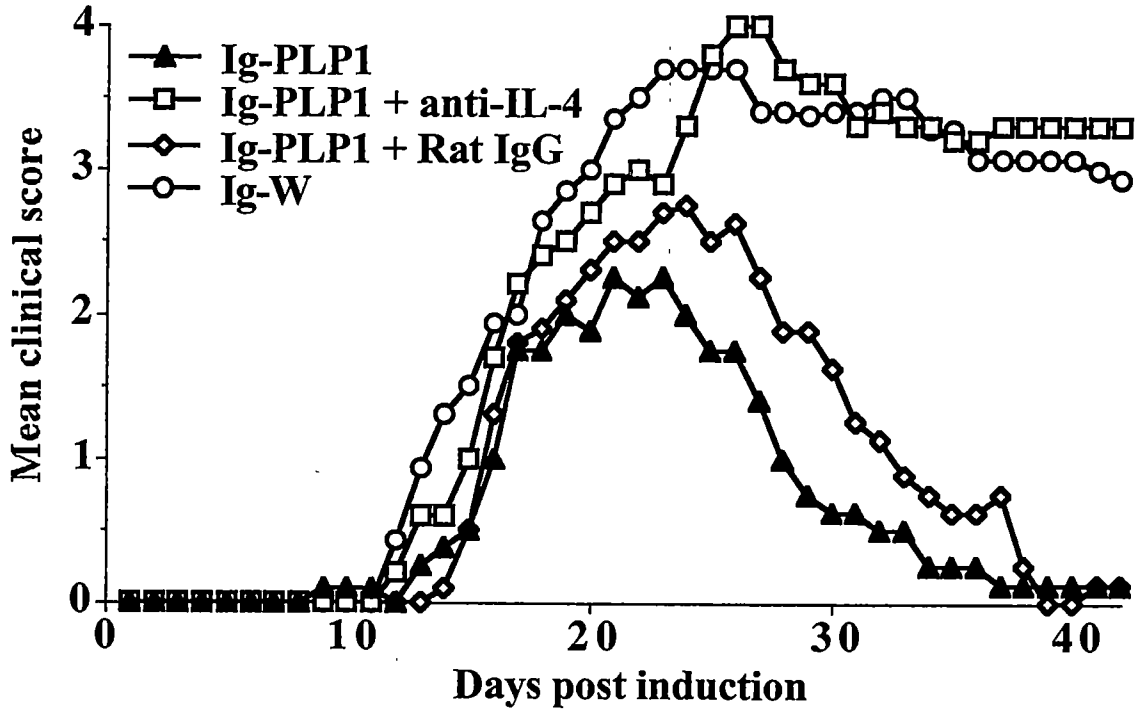
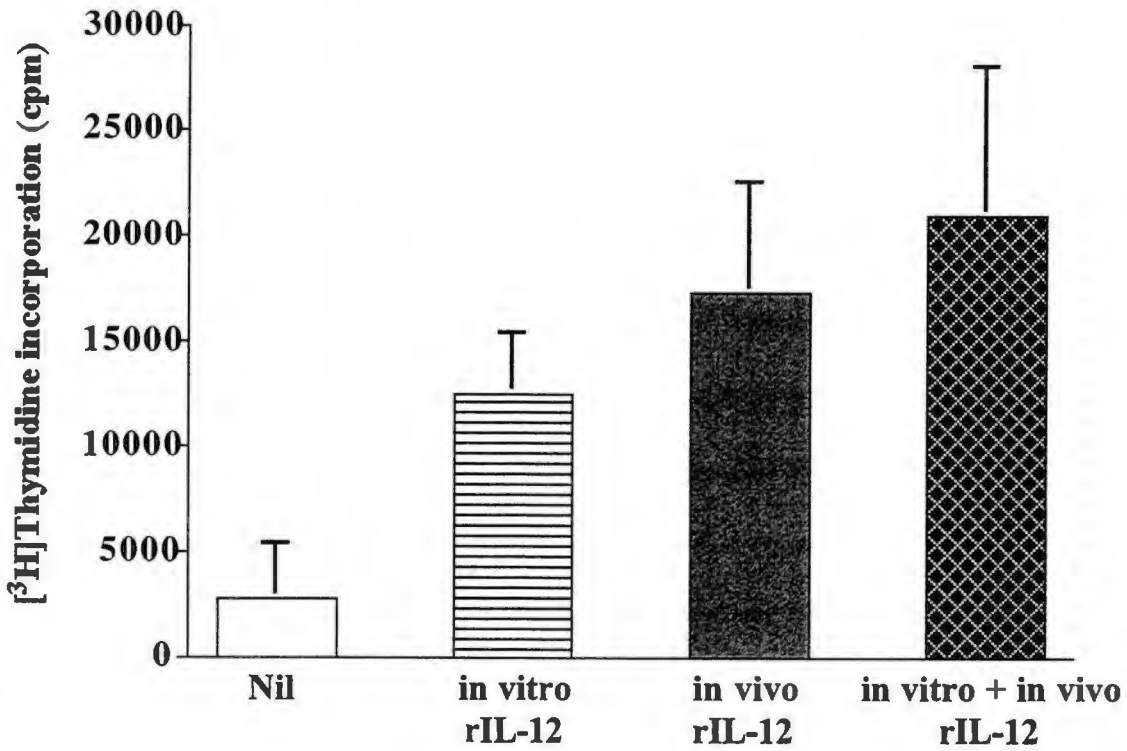

Ig-PLP1
Ig-PLP1 + anti-IL-4
Ig-PLP1 + Rat IgG
Ig-W
Mean clinical score
Days post induction
0
1
2
3
4
0
10
20
30
40

Figure 10.

Restoration of splenic proliferation in Ig-PLP1 tolerized mice by administration of IL-12.

Neonates (7 per group) were injected i.p within 24 hours of birth with 100 μg Ig-PLP1 in saline and when the mice reached 7 weeks of age they were immunized s.c. with 100 μg PLP1 peptide in CFA as described in figure 3. Four hours post immunization the mice were injected i.p. with 500 ng/mouse of rIL-12. Additional i.p. injections of rIL-12 (500 ng/mouse) were carried out on days 2, 4, and 7 post immunization. On day 10 the mice were sacrificed, and the spleen cells (1 x 10^6/well) were stimulated with PLP1 peptide in the presence (hatched bar) or absence (filled bar) of rIL-12 (10 units/well) and proliferation was measured as described in Materials and Methods. For comparison purposes spleen cells from mice that did not receive rIL-12 in vivo were stimulated with PLP1 peptide in the presence (striped bar) or absence (open bar) of rIL-12 (10 units/well). Each bar represents the mean ± SD of seven individually tested mice.


[3H]Thymidine incorporation (cpm)
30000
25000
20000
15000
10000
5000
0
Nil
in vitro rIL-12
in vivo rIL-12
in vitro + in vivo rIL-12

PART IV.

Differential Control of Neonatal Tolerance by Antigen Dose Versus Extended Exposure and Adjuvant

(Cellular Immunology, In Press)

Chapter 1. Abstract

Ig-PLP1, an immunoglobulin (Ig) chimera carrying the encephalitogenic proteolipid protein (PLP) sequence 139-151 (PLP1), induces neonatal tolerance in mice and confers resistance to experimental allergic encephalomyelitis (EAE) without the need for incomplete Freund's adjuvant (IFA). The mechanism underlying such tolerance involves organ specific T cell regulation characterized by lymph node deviation and unusual IFNγ-dependent splenic anergy. This form of T cell modulation may prove useful for prevention of autoimmunity. However, since the neonatal period is susceptible to regulation, further investigations are necessary to define parameters required to establish regimens suitable for optimal protection against disease. To this end studies were carried out to investigate the effect that IFA, the dose of Ig-PLP1, and the number of Ig-PLP1 injections may have on Ig-PLP1-mediated neonatal tolerance and protection against disease. Herein it is reported that as little as 1μg of Ig-PLP1 supports IFNγ-dependent splenic anergy but lymph node deviation was minimal. However, a 10μg dose drives a resistance to EAE similar to the 100μg regimen. However, when a two-injection regimen was applied, resistance to disease was observed but the mechanism manifested proliferative and cytokine unresponsiveness in both lymphoid organs. Furthermore, when IFA was used with Ig-PLP1, a suppressive mechanism similar to the two-injection regimen was observed. Therefore, the dose of Ig-PLP1 displays a quantitative influence while the number of injections of Ig-PLP1 and presence of IFA rather drive qualitative influences on such tolerance.

Chapter 2. Introduction

Tissue graft studies have indicated that mice recipient of allogeneic spleen cells on the day of birth accept grafts from the same donor later in life while mice that did not receive the splenic cells rejected the tissue grafts (1). Since then the neonatal immune system has been considered as a window during which exposure to antigen instructs the immune system to tolerate such antigen during subsequent encounter. This approach for inducing tolerance against antigen is being considered for inactivation of autoreactive T cells and modulation of T cell mediated autoimmunity (2-5). Recent studies, however, have indicated that neonatal induced tolerance does not necessarily reflect an absence of immunity (6-9). In fact, it has been shown that an immune response can develop in animals that were exposed to antigen at the neonatal stage (6-9). However, the effector functions associated with such a response are usually not targeted for elimination of the antigen (6, 8, 10-13). For example, when mice were injected with allogeneic splenic cells on the day of birth and reinjected with the same alloantigen later in life, they develop Th2 type responses instead of the usual Th1 type (11, 12). These cells do not produce the IL-2 necessary for the development of the cytolytic T lymphocytes (CTLs) required for elimination of the alloantigen (14). Similarly, a high virus inoculum on the day of birth promotes the development of Th2 type immune responses that are unable to drive the development of CTLs and clear viral infection later in life (8). For autoantigens, inactivation of T cells was first thought to be the leading mechanism for controlling the development of autoimmunity (3, 15). However, recent studies have revealed that exposure of the neonatal immune system to autoantigen leads to functional alteration in the cytokine production profile by the T cells rather than their inactivation or elimination

(6, 9-13). In fact our own studies using Igs as a delivery system for a self peptide have found that neonatal tolerance can be operated by a multimodal mechanism involving an organ specific regulation of T cells (16). In these studies when mice were given Ig-PLP1 in saline on the day of birth and then challenged with PLP1 peptide at the age of 7 weeks they developed a proliferative response to PLP1 peptide in the lymph node but the splenic T cells were non-proliferative (16). When T cells from both organs were tested for cytokine production the lymph node T cells produced IL-4 instead of IL-2, and the non-proliferative splenic T cells produced IL-2 but were unable to secrete the IFNγ usually seen in mice not tolerized at the neonatal stage. However, when these cells were provided with IL-12 they regained the ability to proliferate and produce IFNγ (16). Moreover, the mice recipient of Ig-PLP1 on the day of birth resisted disease induction later in adult life (16). Therefore, the overall list of mechanisms believed to operate neonatal tolerance extends to include T cell deletion (3), inactivation (15), deviation (6, 8-13), and most recently a novel form of cytokine dependent anergy (16). This novel form of immunity seems to tolerate a second encounter of autoantigen rather than trigger a damaging autoimmune reaction (17, 18). The outcome of neonatal exposure to antigen as well as the mechanism operating neonatal tolerance has been shown to depend upon the type of APC presenting the antigen (7), the availability of antigen in vivo (19), the dose of antigen (8, 20, 21), the number of injections of antigen (22), and the adjuvant with which the antigen is administered (6). In these studies we investigated if the dose of Ig-PLP1, the number of injection of Ig-PLP1, and the presence of IFA would influence the multimodal organ specific mechanism underlying Ig-PLP1 driven neonatal tolerance. The results indicate that while protection from disease induction was obtained with all

regimens, the mechanism was differentially affected. The antigen dose induced quantitative variation while the presence of adjuvant and the number of injections led to qualitative differences in the mechanism of Ig-PLP1 mediated tolerance.

Chapter 3. Materials and Methods

Mice

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

Antigens

Peptides. All peptides used in this study were purchased from Research Genetics Inc. (Huntsville, AL) and purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses an encephalitogenic sequence corresponding to amino acid residues 139-151 of PLP (23). PLP2 peptide (NTWTTCQSIAFPSK) encompasses an encephalitogenic sequence corresponding to amino acid residues 178-191 of PLP (24). Both peptides are presented to T cells in association with I-A^s MHC class II molecules and induce EAE in SJL/J mice (24, 25).

Ig-PLP Chimeras. Ig-PLP1 is a chimera expressing PLP1 peptide (26). Construction of Ig-PLP1 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 PLP1 was previously described (26). Ig-W, the parental Ig not encompassing any PLP peptide was described elsewhere

(27). Large scale cultures of transfectoma cells were carried out in DMEM culture media containing 10% iron-enriched calf serum (BioWhittaker, Walkersville, MD). Purification of Ig-PLP1 and Ig-W was carried out on columns of rat anti-mouse kappa mAb coupled to sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ).

Neonatal injections of tolerogen and adult immunizations with peptide

Neonatal injections of either free peptide or Ig chimeras were done intraperitoneally (i.p.) in 100µl saline or 100µl IFA/PBS (vol/vol) within 24 hours after birth. When the mice reached 7 weeks of age, they were subjected to immunization with peptide to analyze their proliferative and cytokine responses. The immunization of adult mice with 100µg PLP1 in 200µl PBS/CFA (vol/vol) was carried out subcutaneously (s.c.) in the food pads and at the base of the limbs.

Induction of EAE

EAE was induced by s.c. injection in the foot pads and at the base of the limbs with 200µl IFA/PBS (vol/vol) solution containing 100µg free PLP1 peptide and 200µg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Six hours later 5 x 10^9 inactivated *Bordetella pertussis* (Bioport Corp, Lansing, MI) were given intravenously (i.v.). A second injection of *B. pertussis* was given to the mice within 48 hours. Mice were then scored daily for clinical signs of EAE as follows: 0, no clinical sign; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Proliferation assays

Lymph node (axillary, lateral axillary, and popliteal) and spleen cells were incubated in 96-well plates at 4 x 10^5 and 10 x 10^5 cells/100μl per well, respectively, with 100μl of stimulator for 3 days. PLP1 and PLP2 peptides were used at the predefined optimal dose of 15μg/ml (16). In some experiment the stimulation with peptide was carried out in the presence of 10 units/ml of either IL-2 or IL-12. Subsequently, 1μCi [^{3}H]thymidine (ICN Pharmaceuticals Inc., Costa Mesa, CA) was added per well, and the culture was continued for an additional 14.5 hours. The cells were then harvested and incorporated [^{3}H]thymidine was measured using the trace 96 program and an Inotech β counter (Wohlen, Switzerland). A control of media without stimulator was included for each mouse and used as background. All the results presented in the figures represent cpms of test samples from which the background was deducted.

ELISA assay

Cytokine production by spleen cells was measured as previously described (16). Briefly, 10 x 10^5 cells/100μl/well were incubated with 100μl of stimulator for 24 hours, and cytokine production from culture supernatant was measured by ELISA according to PharMingen's (San Diego, CA) instructions. The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Sunnyvale, CA) using SoftMAX PRO 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, and IFNγ (PharMingen) were included in all experiments to construct standard curves. The concentration of cytokines in culture supernatant was estimated by extrapolation from the linear portion of the standard curve. All anti-cytokine antibodies used in these studies were purchased from

PharMingen. Capture antibodies were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; and rat anti-mouse IFNγ, R4-6A2. Biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES5-5H4; rat anti-mouse IL-4, BVD6-24G2; and rat anti-mouse IFNγ, XMG1.2.

ELISPOT assay

ELISPOT assay was used to measure cytokine produced by lymph node T cells during antigen stimulation as previously described (16). Briefly, 5 x 10^5 cells/100μl/well along with 100μl of stimulator were added onto HA-multiscreen plates (Millipore, Bedford, MA) precoated with capture antibody. After 24 hours of incubation, the plates were washed and subsequently incubated with biotinylated anti-cytokine antibody overnight at 4°C. Following incubation with avidin-peroxidase (Sigma, St. Louis, MO) for 1 hour at 37°C, spots were visualized by adding substrate (3-amino-9-ethylcarbazole, Sigma) and counted under a dissecting microscope. The capture and biotinylated anti-cytokine antibodies used for ELISPOT were the same as those used for ELISA.

Chapter 4. Results

Prior studies have indicated that mice injected with 100 μg Ig-PLP1 in saline on the day of birth and immunized with 100 μg free PLP1 peptide in CFA at the age of 7 weeks developed T cell proliferative responses to PLP1 in the lymph node but the splenic T cells were unresponsive (16). Moreover, the proliferative lymph node T cells were deviated and produced IL-4 instead of IL-2 while the splenic T cells, although non-proliferative and unable to produce IFNγ, secreted significant amounts of IL-2 (16). Since the outcome of neonatal tolerance has previously been shown to depend upon the dose of tolerogen (8, 20, 21), we devised experiments to find out whether injection of lower doses of Ig-PLP1 on the day of birth would induce tolerance similar to that obtained with the 100 μg Ig-PLP1. To this end newborn mice were given 1, 10, or 100 μg of Ig-PLP1 in saline on the day of birth, immunized with 100 μg PLP1 peptide at the age of 7 weeks, and then assessed for their proliferative and cytokine responses. As can be seen in figure 1a, a dose of 1 μg Ig-PLP1 given at birth allowed for the development of a significant but not optimal proliferative response in the lymph node. The injection of 10 μg Ig-PLP1, however, led to a response that is comparable to that obtained in mice that were injected with 100 μg Ig-PLP1 or mice recipient of the 100 μg of control Ig-W molecule, the parental Ig not encompassing any PLP peptide. These responses were PLP1 specific since in vitro stimulation with PLP2, a negative control peptide presented by I-A^S class II molecules like PLP1, failed to drive significant T cell proliferation. In the spleen, however, both 1 and 10 μg doses of Ig-PLP1 significantly reduced T cell proliferation almost to the same extent as 100 μg Ig-PLP1 (Figure 1b). Tolerization with 100 μg Ig-W

allowed for significant proliferation (Figure 1b). At the cytokine level IL-2 production in the lymph node decreased as the dose of tolerogen increased (Figure 2a). However, IL-4 production was augmented as the dose of tolerogen elevated (Figure 2c). No IFNγ production was observed in the lymph node (Figure 2e). In Ig-W tolerized mice, IL-2 was produced but neither IL-4 nor IFNγ was detectable. These results confirm our previous observation that tolerization with Ig-PLP1 drives lymph node deviation (16) and also demonstrate that such deviation is dependent upon the dose of Ig-PLP1 given on the day of birth. In the spleen, all 3 doses of Ig-PLP1 allowed for production of IL-2 at levels similar to those produced by cells from mice that were injected with Ig-W at birth (Figure 2b). Therefore unlike the lymph node, the dose of Ig-PLP1 had no influence on the IL-2 production in the spleen since the variations were not statistically significant (*t* test: $p>0.1$). Splenic IL-4 was undetectable in either group of mice (Figure 2d). However, IFNγ production was compromised at all the doses tested in the Ig-PLP1 tolerized mice while cells from the mice recipient of Ig-W at birth produced significant IFNγ (Figure 2f). Furthermore, the non-proliferative splenic cells from the mice tolerized with either 1 or 10 μg Ig-PLP1 like cells from mice recipient of 100 μg Ig-PLP1 regained proliferative responsiveness when peptide stimulation was carried out in the presence of IL-12. Indeed in the absence of IL-12 the mean cpm was 4,702 ± 2,165 for mice recipient of 1μg Ig-PLP1 dose, 4,397 ± 3,129 for those recipient of 10 μg, and 2,330 ± 1,759 for animals injected with 100 μg regimen. However when peptide stimulation was carried out in the presence of IL-12 the mean cpm rose to 10,933 ± 2,711; 12,713 ± 2,002; and 11,960 ± 1,275 respectively. These results indicate that both 1 and 10 μg Ig-PLP1 regimens induce splenic anergy like the 100 μg Ig-PLP1 dose. Since lymph node deviation

decreased proportional to the dose of neonatal tolerogen we sought to evaluate the effect of such variability on the resistance to EAE induction. To this end mice recipient of 1 or 10 μg Ig-PLP1 tolerization regimen on the day of birth were induced for EAE at the age of 7 weeks and assessed for sign of paralysis over a period of 3 months. As can be seen in figure 3, the mice recipient of either 1 or 10 μg Ig-PLP1 developed mild clinical signs of paralysis during the first phase of disease and fully recovered by day 50 without showing any relapses. Although this reflect a significant protection against EAE compared to Ig-W regimen, the profile of clinical score seems to be more effective as the dose of tolerogen increased. Indeed in both groups the onset of disease was 3 days earlier relative to the 100 μg Ig-PLP1 group, the initial phase of disease was more severe and full recovery was delayed by about one week (Table 1). These results along with the observation that lower dose of tolerogen was less effective in the induction of lymph node deviation indicate that the dose of Ig-PLP1 has quantitative rather than qualitative influence on the mechanism and disease outcome of Ig-PLP1-mediated neonatal tolerance.

The adjuvant with which the antigen is administered has been shown to influence the outcome of neonatal tolerance (6, 21). Moreover, we have previously shown that free PLP1 peptide in saline does not induce neonatal tolerance (28). However, when given in IFA free PLP1 peptide was able to mediate neonatal tolerance and protect against EAE (28). Therefore to investigate whether adjuvant influences Ig-PLP1-mediated tolerance we gave newborn mice 100 μg of Ig-PLP1 emulsified in IFA, and at the age of 7 weeks assessed their proliferative and cytokine responses to immunization with PLP1/CFA. Figure 4 shows that mice recipient of this Ig-PLP1/IFA regimen did not develop

proliferative responses in the lymph node or spleen. However, the mice recipient of Ig-PLP1/Sln regimen developed proliferation in the lymph node, but the spleen was unresponsive (Figure 4). At the cytokine level, neither IFNγ nor IL-4 was produced in either organ from the mice tolerized with Ig-PLP1 in IFA, while mice recipient of Ig-PLP1 in saline produced significant amounts of IL-4 in the lymph node (Figure 5). As for IL-2 no significant production was seen in the lymph node of either group while splenic IL-2 was detectable in mice tolerized with Ig-PLP1 in IFA but too a much lower extent than in mice recipient of Ig-PLP1 in saline (Figure 5a and d). Therefore Ig-PLP1/IFA regimen abrogated proliferation and IL-2 production in both lymphoid organs and did not drive IL-4 production in the lymph node. Moreover neither splenic nor lymph node T cells regained the ability to proliferate when stimulated in the presence of IL-2. Indeed none of the 6 mice tested had significant increase in proliferation upon stimulation with PLP1 alone versus stimulation with PLP1 and 10 units/ml IL-2 (not shown). Also, IL-12 which restored proliferation of splenic T cells of Ig-PLP1/Sln recipient mice was unable to promote proliferative responsiveness of splenic T cells from Ig-PLP1/IFA recipient animals (not shown). These observations suggest that anergy is unlikely as a mechanism for the proliferative and cytokine unresponsiveness induced by Ig-PLP1/IFA. Subsequently, we tested the Ig-PLP1/IFA regimen for resistance to disease induction. The results in figure 6 indicate that the Ig-PLP1/IFA regimen conferred protection against EAE induction. Moreover the resistance against the disease was slightly more effective as the mean maximal disease severity was reduced to 2.0 ± 0.6 compared to 2.4 ± 0.2 in mice recipient of Ig-PLP1 in saline and the mice recovered earlier from disease (Table 2). Therefore these results indicate that the presence of IFA in neonatal Ig-PLP1 injection

can alter the adult immune response to PLP1 and confer resistance to EAE with a mechanism different from Ig-PLP1 in saline.

Since the number of injections of antigen has been shown to influence the outcome of neonatal tolerance (22), we investigated if two injections of Ig-PLP1 in saline would drive a tolerance similar to that of a single injection regimen. As can be seen in figure 7 when the mice were given two injections of 100μg Ig-PLP1, day 1 and 7 after birth, and then immunized with PLP1/CFA at the age of 7 weeks, they failed to develop proliferative responses in both the lymph node and spleen. As shown earlier mice recipient of a single injection of Ig-PLP1 on the day of birth responded to PLP1 in the lymph node but not in the spleen. Mice that received two injections of Ig-W instead of Ig-PLP1 developed proliferative responses in both the lymph node and the spleen (Figure 7). At the cytokine level, neither IFNγ nor IL-4 was produced at significant levels in the lymph node or spleen of the mice recipient of two injections of Ig-PLP1 (Figure 8). However, some residual IL-2 was detected in both lymphoid organs (Figure 8). In contrast, Ig-W recipient mice produced significant IL-2 in both the lymph node and spleen and IFNγ only in the spleen. As has previously been shown mice recipient of a single injection of Ig-PLP1 in saline produced significant IL-4 in the lymph node but IL-2 in the spleen (16). Moreover the lymph node and splenic T cells from mice recipient of 2 injections of Ig-PLP1 were unable to regain proliferative responsiveness when the stimulation with peptide was carried out in the presence of IL-2 (not shown). Similarly, the supply of exogenous IL-12 during antigen stimulation while restored proliferation of the splenic T cells of mice recipient of one Ig-PLP1/Sln injection did not restore proliferative responsiveness of the splenic T cells of the mice recipient of 2 Ig-PLP1/Sln

injections (not shown). Finally, the two Ig-PLP/Sln injection regimen showed a protective pattern against EAE similar to the pattern obtained when the mice were injected with a single dose of Ig-PLP1 in saline (Figure 9). However, two injections of Ig-PLP1 during neonatal period induced a form of neonatal tolerance different from the one injection regimen. Since both responses and protection from disease seems similar to Ig-PLP1/IFA regimen, the number of injections may simulate the effect of adjuvant. These results suggest that the number of antigen injection during neonatal period could induce qualitatively different form of neonatal tolerance.

Chapter 5. Discussion

Prior studies from this laboratory have indicated that Ig-PLP1 obviated the use of adjuvant and induced neonatal tolerance operated by T cell lymph node deviation and an unusual cytokine-dependent splenic anergy (16). Specifically, newborn mice given 100 μg Ig-PLP1 in saline on the day of birth and immunized with PLP1 in CFA at the age of 6 to 8 weeks developed T cell proliferation in the lymph node but the spleen was unresponsive (16). Moreover, the lymph node T cells were deviated and produced IL-4 instead of IL-2, and the splenic T cells, although non-proliferative and unable to produce IFNγ, secreted significant amounts of IL-2 (16). Surprisingly, exogenous INFγ or IL-12 restored proliferation and IFNγ production by the anergic splenic T cells (16). The consequence of this organ regulated responses was tolerance to PLP1 peptide and protection of the mice against EAE.

To gain insight into the neonatal immune system and to further explore this novel form of tolerant yet protective immunity we sought to investigate additional parameters known to influence neonatal tolerance. The studies presented in this report demonstrate that varying doses of tolerogen although display similar pattern of organ specific regulation of T cells quantitative differences in the proliferative and cytokine responses were observed. Indeed mice given 1 or 10 μg Ig-PLP1 on the day of birth did not develop proliferative responses in the spleen, similar to those recipient of 100 μg Ig-PLP1 (Figure 1b). However, the level of lymph node proliferation increased proportional to the dose of Ig-PLP1 given at birth (Figure 1a). Furthermore, splenic IFNγ remained at background levels regardless of the dose of Ig-PLP1 given at birth, while IL-2 production was significant with all three doses of tolerization (Figure 2b and f). In addition the T

cells from all three groups regained the ability to proliferate when peptide stimulation was carried out in the presence of IL-12. In the lymph node, however, IL-4 production rose from undetectable in the mice given 1 μg Ig-PLP1 at birth to statistically significant levels when given 100 μg Ig-PLP1 (Figure 2c). IL-2 production, which was significant with all tolerization doses, decreased as the dose of Ig-PLP1 increased (Figure 2a). This decrease is likely to be due to a reabsorption of the IL-2 by the T cells as proliferation optimizes. Therefore, the overall conclusion is that different doses of Ig-PLP1 tolerogen operate neonatal tolerance likely using similar mechanisms but a quantitative variation was observed that gradually optimized as the dose of Ig-PLP1 increased. This conclusion is supported by the slight decrease in the effectiveness of protection from disease in the mice recipient of 1 or 10 μg Ig-PLP1 regimen when compared to those injected with 100 μg Ig-PLP1 (Figure 3 and table 1). One interpretation for these results could be that the avidity of T cell-APC interaction, that is dependent upon the dose of injected Ig-PLP1, may exert quantitative rather than qualitative control on the mechanism of Ig-PLP1 driven neonatal tolerance (29). In another system an increase in virus inoculum has diverted the mechanism of neonatal tolerance from a Th1 into a Th2 response (8). In oral tolerance models low dose antigen promoted the induction of regulatory T cells while high dose antigen led to T cell deletion (30, 31). Herein, a 2-log decrease in the amount of Ig-PLP1 reduced the strength of the response but the mechanism was not diverted. Since 100 μg Ig-PLP1 represents a dose of 66 mg Ig-PLP1 per kg of body weight (considering that the average weight of a newborn SJL mouse is $\approx 1.5 \pm 0.2$ g and that the molecular weight of Ig-PLP1 is 150 kd) corresponding to $\approx$ 1 μmole free PLP1/kg, a peptide dose that certainly exceeds the epitope content in the high virus inoculum, it is

unlikely that factors other than avidity control Ig-PLP1 mediated T cells tolerance. Previously, we also speculated that binding of Ig-PLP1 to FcRs on target APCs may trigger the production of cytokines by the APCs and control the outcome of T cell APCs interaction (16). We have evidence that Ig-PLP1 does induce the production of both IL-10 and IL-6 by APCs (unpublished data). Therefore if the hypothesis holds true, the dose of Ig-PLP1 could control the amount of cytokine produced by the APCs and quantitatively affect the mechanism of neonatal tolerance.

As for the residual EAE seen in the tolerized mice it may be that newly migrant T cells accumulates without being exposed to the tolerogen and support such mild residual EAE. The mice recover from the disease possibly due to low frequency of T cells that accumulated in the periphery. Alternatively, since the splenic T cells are susceptible for restoration by IFNγ or IL-12 bystander reactivation of these cells could occur and drive the observed residual paralysis.

IFA has been shown to induce the production of cytokines influential for T cell-APC interaction (32, 33). This may be a contributing factor for the requirement of IFA by soluble proteins and peptide for induction of neonatal tolerance (2-6). In fact free PLP1 peptide that was unable to drive tolerance when given to newborn mice without adjuvant could induce neonatal tolerance and confer resistance to EAE when emulsified in IFA (28). Surprisingly, the mechanism operating such tolerance involved proliferation in the spleen and unresponsiveness in the lymph node, and this is in good agreement with previous observations (6). Moreover, although IL-4 was undetectable for the proliferative splenic T cells in our system, others have demonstrated that IL-5, another Th2 type cytokine, could be produced by splenic T cells of peptide/IFA tolerized mice (6). IFA

also had influence on Ig-PLP1 mediated neonatal tolerance and mice given Ig-PLP1 in IFA on the day of birth did not develop proliferative responses either in the lymph node or the spleen when they were immunized with PLP1/CFA at adult age (Figure 4). However, although IL-4 and IFNγ were undetectable in both lymphoid organs, there was low but significant production of IL-2 (Figure 5). These results suggest that IFA and Ig-PLP1 may cooperate to suppress the T cell responses (Figure 4 and 5) and increase the effectiveness of protection against the disease (Figure 6, table 2) but display complementary downregulation. This is supported by the fact that both lymphoid organs become unresponsive under the Ig-PLP1/IFA regimen, while the lymph node is proliferative and deviated under Ig-PLP1/saline and the spleen is responsive under the PLP1/IFA regimen. Supply of IL-2 did not restore proliferation in either population. Moreover, the splenic T cells were not restorable by exogenous IL-12. These observations argue against anergy as a mechanism of T cell unresponsiveness.

Similar observations were also made when the animals were tolerized with two injections of Ig-PLP1 in saline. Indeed unlike a single dose of Ig-PLP1 on day 1, two injections on day 1 and 7 abrogated proliferative responses in both lymphoid organs (Figure 7). Furthermore, both IL-4 and IFNγ were undetectable but there were low levels of IL-2 (Figure 8). Also, IL-2 had no effect on the proliferative unresponsiveness in both the lymph node and spleen. IL-12 did not restore proliferation of the splenic T cells. Again, the 2 injection regimen does not seem to employ anergy for proliferative and cytokine unresponsiveness. The 2 injection regimen like the Ig-PLP1/IFA tolerization conferred resistance to EAE (Figure 6 and 9). The explanation we wish to put forth for these observations is that the double injection and the long life span of Ig-PLP1 in vivo

may support peptide supply for an extended period of time and provide a chronic exposure of the T cells to PLP1 peptide, thereby driving inactivation of the T cells (34, 35). IFA, which also contributes a slow release of antigen, may have affected Ig-PLP1 mediated tolerance by supporting a longer exposure to antigen.

Overall, the dose of Ig-PLP1 seems to have a quantitative effect on Ig-PLP1 mediated tolerance but IFA and double injection of Ig-PLP1 may promote different effect on neonatal T cells leading rather to a qualitative influence versus the single dose Ig-PLP1/Sln regimen.

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Table 1.

The severity of clinical EAE is dependent upon the dose of neonatal tolerogen[§]

Tolerogen (μg)	Incidence	Day of Onset[*]	Mean Maximal Disease Severity[†]	Mortality (%)	Day of Recovery[‡]
Ig-W/Sln					
(100)	7/7	13 ± 0.5	4.2 ± 0.8	42	>100
Ig-PLP1/Sln					
(100)	8/8	16 ± 1.0	2.4 ± 0.2[¶]	0	42 ± 1
(10)	6/6	13 ± 1.0	3.1 ± 0.4[¶]	0	49 ± 7
(1)	6/6	13 ± 2.0	3.2 ± 0.6[¶]	0	47 ± 2

Table 1 (Continued)

§ The data illustrated in this table were gathered from the mice described in figure 3. Briefly, groups of newborn mice (6 to 8 per group) were injected with 1, 10, or 100μg of Ig-PLP1 in saline on the day of birth and were induced for EAE with 100 μg free PLP1 in CFA when they reached 7 weeks of age. The mice were scored daily for signs of EAE as described in *Materials and Methods*. A group of mice injected with 100 μg Ig-W in saline at birth was also induced for EAE at the age of 6-8 weeks and included as control.

* Mean ± SD of the day on disease onset

† Mean ± SD of the maximal clinical scores

‡ Mice were considered recovered when their clinical score is < 0.5 for at least 5 days

¶ This score is statistically significant ($P < 0.05$) when compared with the mean score of Ig-W/Sln tolerized mice.

Table 2.

Mice tolerized with Ig-PLP1/IFA or 2 injections of Ig-PLP1/Sln display a similar pattern of resistance to EAE as mice tolerized with one injection Ig-PLP1/Sln[§].

Tolerogen	Incidence	Day of Onset[*]	Mean Clinical Score[†]	Mortality (%)	Day of Recovery[‡]
Nil	6/6	9 ± 1.4	3.9 ± 0.9	50	>80
Ig-PLP1/Sln	8/8	16 ± 1.0	2.4 ± 0.2[¶]	0	42 ± 1.0
Ig-PLP1/IFA	8/8	12 ± 1.7	2.0 ± 0.6[¶]	0	30 ± 6.2

Table 2 (Continued)

[§]The data illustrated in this table were gathered from the mice described in figure 6. Briefly, groups of newborn mice (6 to 8 per group) were injected i.p. on the day of birth with 100 μg Ig-PLP1 in saline (Ig-PLP1/Sln) or 100 μg Ig-PLP1 in IFA (Ig-PLP1/IFA) and induced for EAE with 100 μg PLP1 in CFA when they reached 7 weeks of age. Subsequently, the mice were scored daily for signs of paralysis as described in *Materials and Methods*. A group of mice that were not tolerized at birth (Nil) but were induced for EAE at the age of 7 week was included as control.

* Mean ± SD of the day on disease onset

† Mean ± SD of the maximal clinical scores

‡ Mice were considered recovered when their clinical score is < 0.5 for at least 5 days

¶ This score is statistically significant ($P < 0.05$) when compared with the mean score of non-tolerized (Nil) mice.

Figure 1.

The dose of neonatal tolerogen exerts differential influence on lymph node versus splenic T cell proliferative responses.

Groups of one day old mice (4 to 8 per group) were injected intraperitoneally (i.p.) with 100 μg Ig-W (open symbols) or 1, 10, or 100μg Ig-PLP1 (filled symbols) in saline, and at the age of 7 weeks challenged subcutaneously (s.c.) with 100 μg free PLP1 in CFA. Ten days later lymph node (a) and splenic cells (b) were in vitro stimulated for 3 days with 15μg/ml PLP1 (circles) or PLP2 (squares) peptide and proliferative responses were measured by thymidine incorporation as described in *Materials and Methods*. The indicated cpms represent the mean ± SD of individually tested mice.

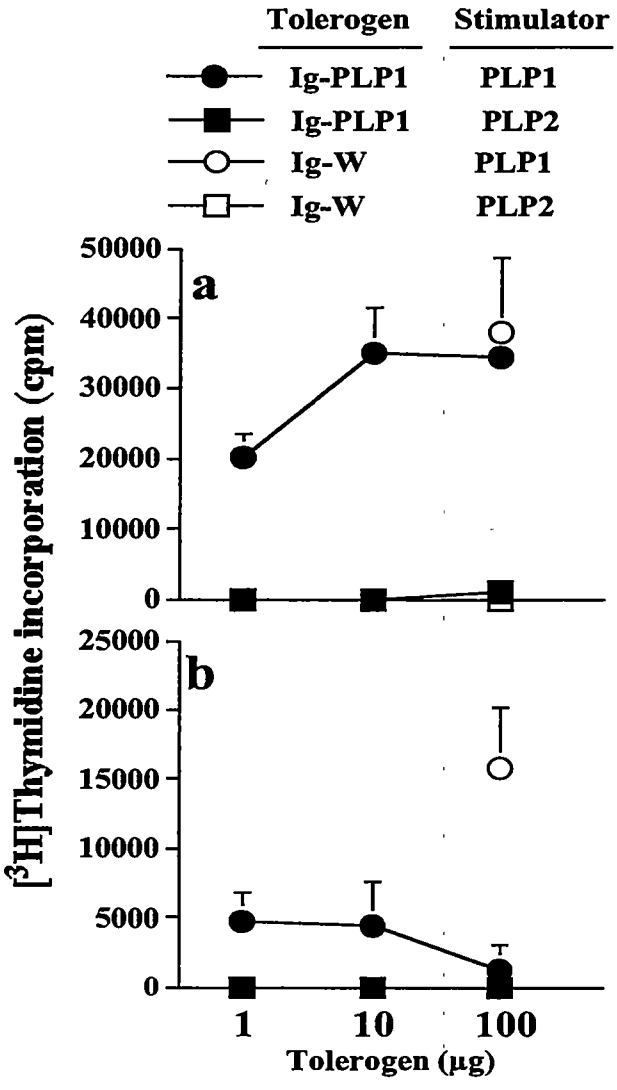

Tolerogen
Stimulator
Ig-PLP1
PLP1
Ig-PLP1
PLP2
Ig-W
PLP1
Ig-W
PLP2
a
b
50000
40000
30000
20000
10000
0
25000
20000
15000
10000
5000
0
1
10
100
Tolerogen (µg)
[3H]Thymidine incorporation (cpm)

Figure 2.

The dose of neonatal tolerogen drives differential cytokine regulation in lymph node and splenic T cells.

Lymph node (upper panels) and spleen (lower panels) cells from the mice tolerized with Ig-PLP1 (filled circles) or Ig-W (open circles) and described in the legend to figure 1 were in vitro stimulated with 15μg/ml PLP1 for 24 hours and tested for production of IL-2 (a and b), IL-4 (c and d), and IFNγ (e and f). Cytokine detection used ELISPOT assay for lymph node T cells and ELISA for splenic T cells as described in *Materials and Methods.* The indicated values (SFU, spot forming units and pg/ml) represent the mean ± SD of individually tested mice. No significant cytokine production was found when the cells were stimulated in vitro with PLP2 peptide instead of PLP1 peptide (not shown).

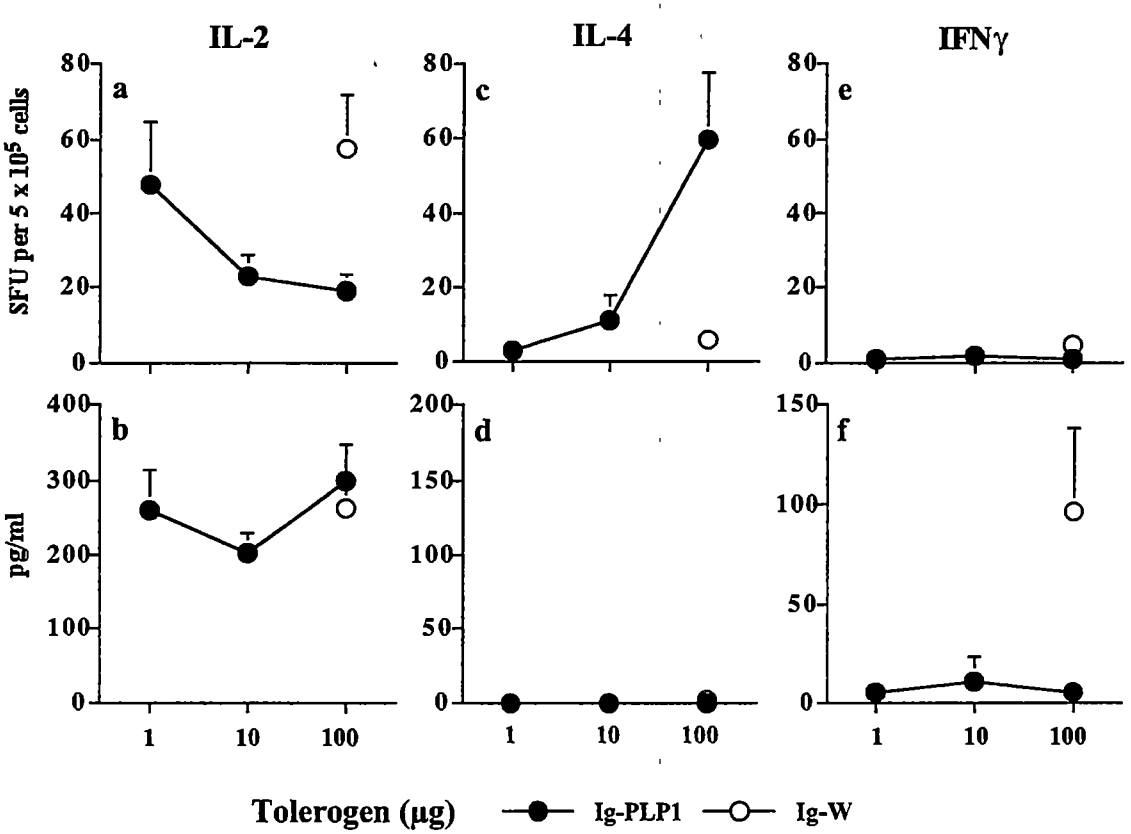

IL-2
IL-4
IFNγ
SFU per 5 x 10^5 cells
pg/ml
a
b
c
d
e
f
80
60
40
20
0
400
300
200
100
150
50
1
10
100
Tolerogen (µg)
Ig-PLP1
Ig-W

Figure 3.

Protection against disease induction is dependent on the dose of neonatal tolerogen.

Groups of newborn mice (6 to 8 per group) were injected with 1 (△), 10 (▲), or 100μg (▲) of Ig-PLP1 in saline on the day of birth and were induced for EAE with 100 μg free PLP1 in CFA when they reached 7 weeks of age. The mice were scored daily for signs of EAE as described in *Materials and Methods*. A group of mice injected with 100μg Ig-W in saline at birth was also induced for EAE at the age of 7 weeks and included as control (○). Each point represents the mean clinical score of all tested mice. Although all mice in all 3 groups developed signs of EAE, 42% of the Ig-W tolerized mice died of severe disease. Death did not occur in the two other groups. Note that full recovery from paralysis was achieved in the 100 μg Ig-PLP1 group earlier than in the 10 μg Ig-PLP1 tolerization group. Furthermore, 17% of the mice recipient of the 10 μg Ig-PLP1 tolerization group showed relapses but none of the mice recipient of the 100μg Ig-PLP1 regimen developed relapses.

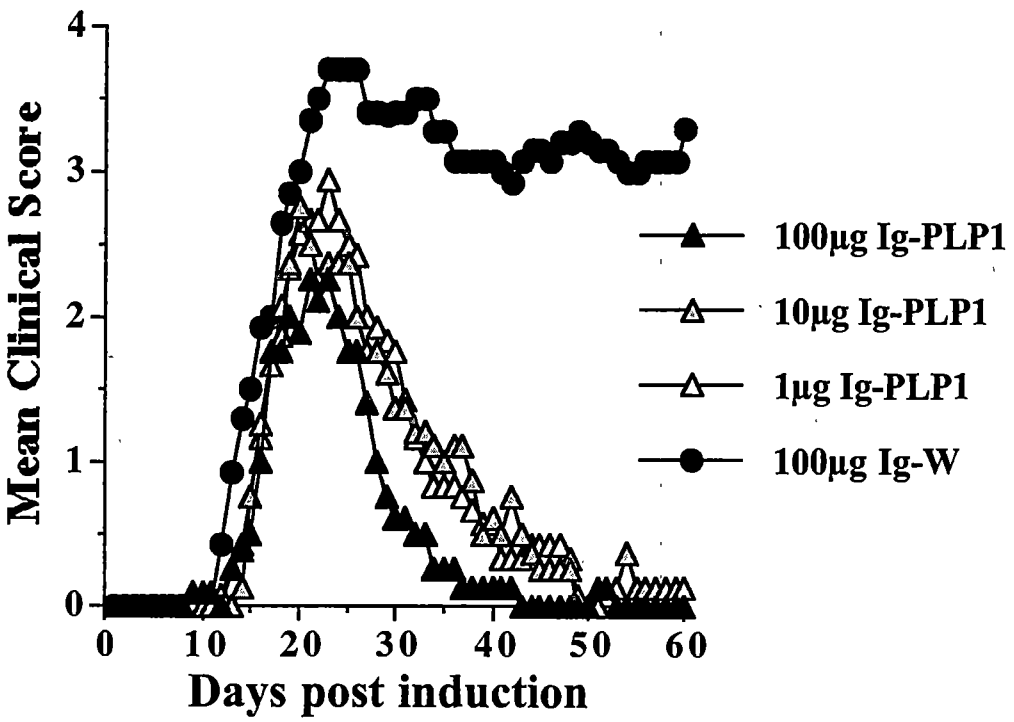

Mean Clinical Score
4
3
2
1
0
0
10
20
30
40
50
60
Days post induction
100µg Ig-PLP1
10µg Ig-PLP1
1µg Ig-PLP1
100µg Ig-W

Figure 4.

Neonatal injection of Ig-PLP1/IFA induces proliferative unresponsiveness in both lymph node and spleen.

Groups of newborn mice (8 per group) were injected i.p. at birth with 100μg Ig-PLP1 in saline (Ig-PLP1/Sln) or in IFA (Ig-PLP1/IFA) and immunized with 100 μg PLP1 in CFA when they reached 7 weeks of age. Ten days later lymph node (a) and spleen (b) cells were stimulated with 15μg/ml PLP1 and their proliferative responses were measured by thymidine incorporation as described in *Materials and Methods*. The indicated cpms represent the mean ± SD of individually tested mice. No significant proliferation was found when the cells were stimulated with PLP2 peptide (not shown).

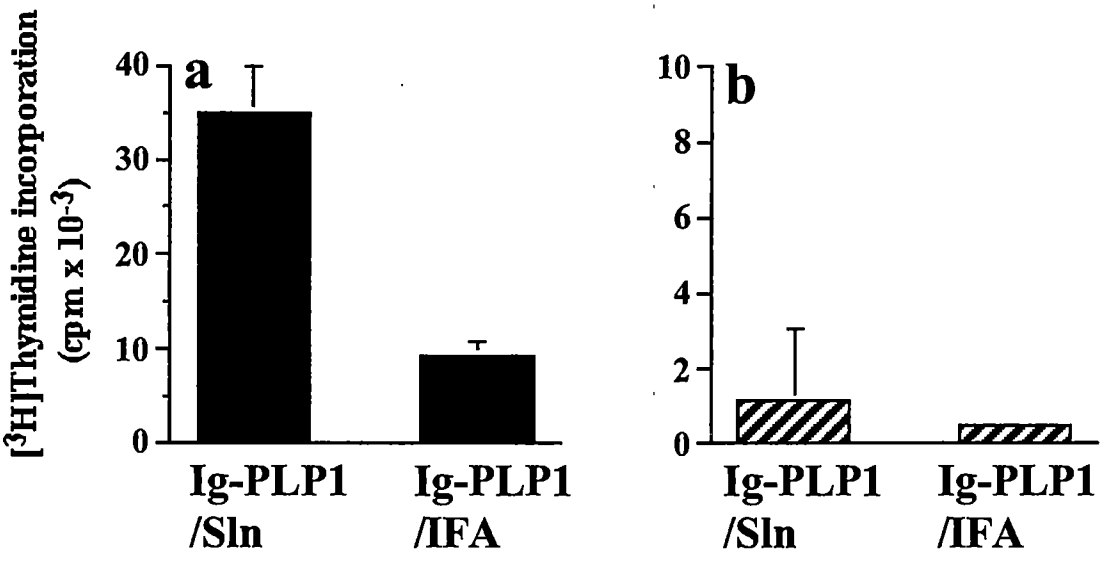

[3H]Thymidine incorporation
(cpm x 10-3)
40
30
20
10
0
a
Ig-PLP1
/Sln
Ig-PLP1
/IFA
10
8
6
4
2
0
b
Ig-PLP1
/Sln
Ig-PLP1
/IFA

Figure 5.

Neonatal injection of Ig-PLP1/IFA abolishes the production of inflammatory cytokines in both lymph node and spleen.

Groups of newborn mice (8 per group) were injected i.p. at birth with 100μg Ig-PLP1 in saline (Ig-PLP1/Sln) or in IFA (Ig-PLP1/IFA), and at the age of 7 weeks immunized with 100 μg free PLP1 in CFA. Ten days later the lymph node (left panels) and splenic (right panels) cells were stimulated with 15μg/ml PLP1 and production of IL-2 (a and d), IL-4 (b and e), and IFNγ (c and f) were measured. Cytokine detection was carried out using ELISPOT assay for lymph node and ELISA assay for splenic cells as described in *Materials and Methods*. The indicated values (SFU and pg/ml) represent the mean ± SD of individually tested mice. No significant cytokine production was found when the cells were stimulated with PLP2 peptide instead of PLP1 (not shown).

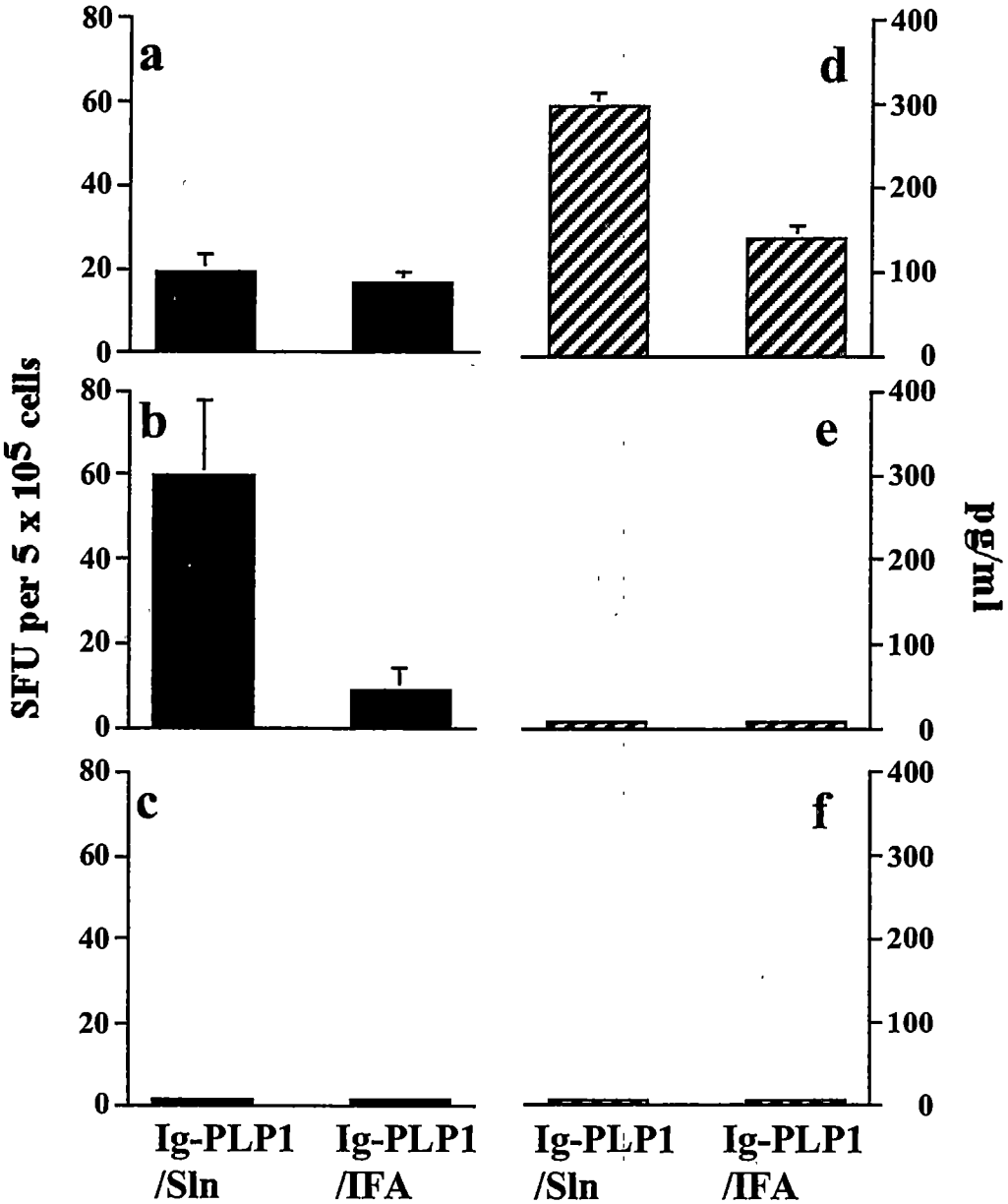

a
b
c
d
e
f
80
60
40
20
0
400
300
200
100
0
SFU per 5 x 10^5 cells
pg/ml
Ig-PLP1 /Sln
Ig-PLP1 /IFA
Ig-PLP1 /Sln
Ig-PLP1 /IFA

Figure 6.

Neonatal injection of Ig-PLP1/IFA confers resistance to EAE induction.

Groups of newborn (6 to 8 per group) mice were injected i.p. with 100μg Ig-PLP1 in saline (Ig-PLP1/Sln, ▲) or in IFA (Ig-PLP1/IFA, ♦) on the day of birth and induced for EAE with 100 μg PLP1 in CFA when they reached 7 weeks of age. Subsequently, the mice were scored daily for signs of paralysis as described in *Materials and Methods*. A group of mice that were not tolerized at birth (Nil, ○) was induced for EAE and included as control. Each point represents the mean clinical score of all tested mice. Note that none of the mice injected with Ig-PLP1/Sln or Ig-PLP1/IFA died from disease while 50% of the Nil mice died in the initial phase of EAE.

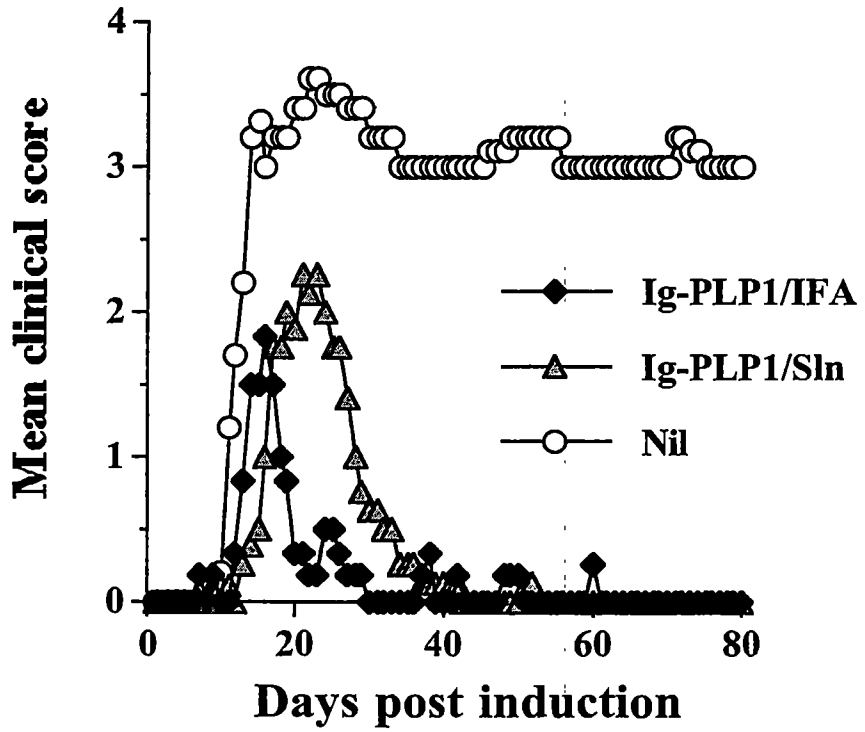

Mean clinical score
4
3
2
1
0
0
20
40
60
80
Days post induction
Ig-PLP1/IFA
Ig-PLP1/Sln
Nil

Figure 7.

Two injections of Ig-PLP1/Sln induce lymph node and spleen proliferative unresponsiveness.

Newborn mice (5 per group) were injected i.p. with 100μg Ig-PLP1 in saline on the day of birth (1X Ig-PLP1/Sln); 100μg Ig-PLP1 in saline on the day of birth and at the age of 7 days (2X Ig-PLP1/Sln); or 100 μg Ig-W in saline on the day birth and at the age of 7 days (2X Ig-W/Sln), and immunized s.c. with 100μg PLP1 in CFA when they reached 7 weeks of age. Lymph node (a) and splenic (b) proliferative responses to stimulation with 15 μg PLP1 peptide were measured by thymidine incorporation as described in *Materials and Methods*. The indicated cpms represent the mean ± SD of individually tested mice. No significant proliferation was seen when the cells were stimulated in vitro with PLP2 peptide instead of PLP1 peptide (not shown).

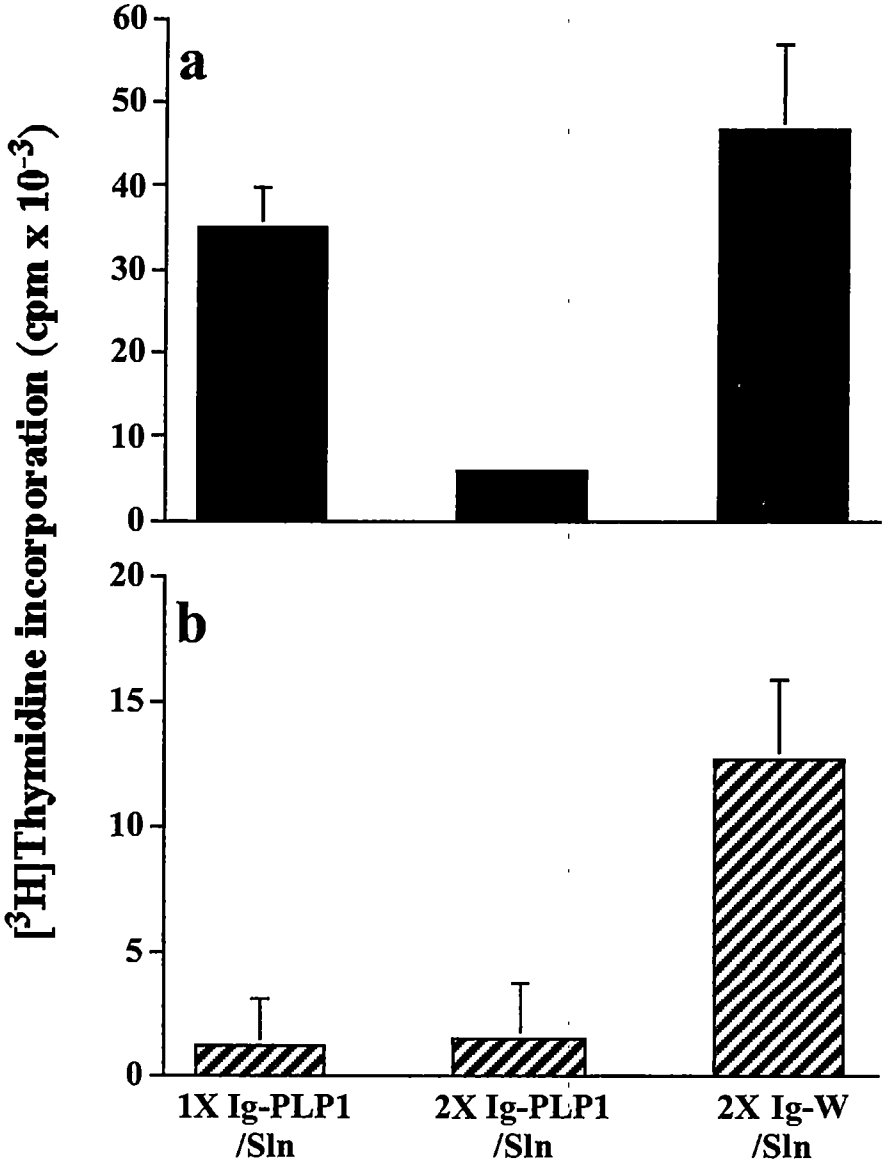

[3H]Thymidine incorporation (cpm x 10-3)
a
b
60
50
40
30
20
10
0
20
15
10
5
0
1X Ig-PLP1 /Sln
2X Ig-PLP1 /Sln
2X Ig-W /Sln

Figure 8.

Two injections of Ig-PLP1/Sln abolish cytokine production in both the lymph node and spleen.

Newborn mice (5 per group) were injected i.p. with 100μg Ig-PLP1 in saline on the day of birth (1X Ig-PLP1/Sln); 100μg Ig-PLP1 in saline on the day of birth and at the age of 7 days (2X Ig-PLP1/Sln); or 100 μg Ig-W in saline on the day birth and at the age of 7 days (2X Ig-W/Sln) and immunized s.c. with 100μg PLP1 in CFA when they reached 7 weeks of age. Ten days later the lymph node (left panels) and splenic (right panels) cells were stimulated with 15 μg/ml PLP1 peptide and assessed for IL-2 (a and d), IL-4 (b and e), and INFγ (c and f) production. Cytokines were detected using ELISPOT assay for lymph node and ELISA for splenic cells. The indicated values (SFU and pg/ml) represent the mean ± SD of individually tested mice. No significant cytokine production was seen when the cells were stimulated with PLP2 peptide instead of PLP1 (not shown)

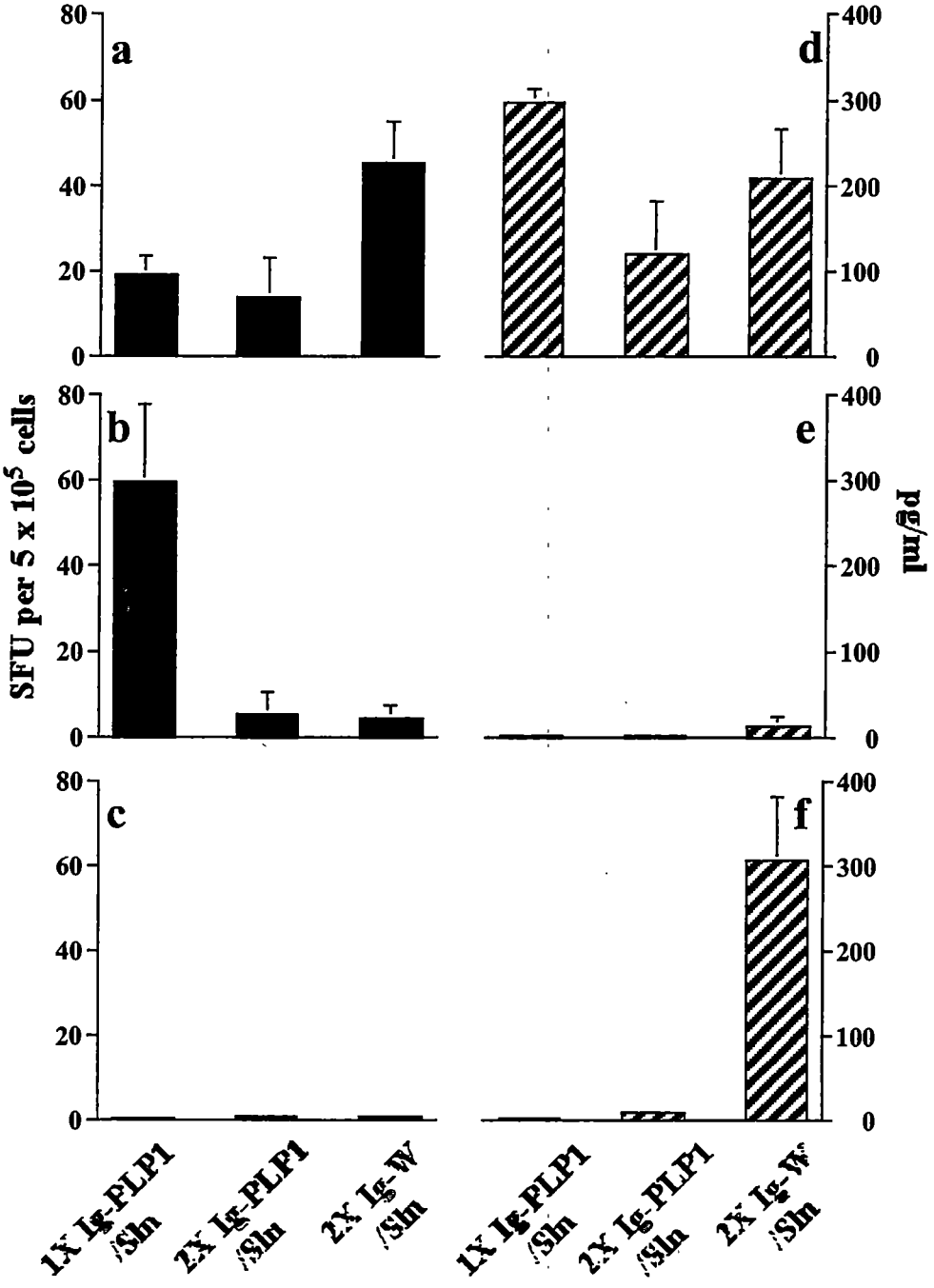
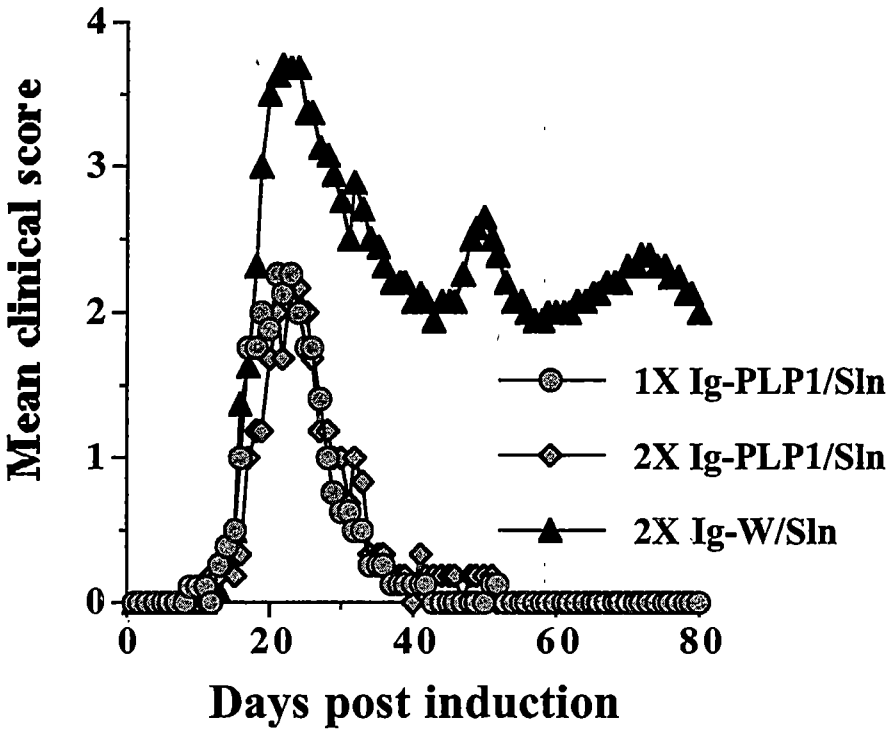

a
b
c
d
e
f
SFU per 5 x 10^5 cells
pg/ml
80
60
40
20
0
400
300
200
100
0
1X Ig-PLP1 /Sln
2X Ig-PLP1 /Sln
2X Ig-W /Sln
1X Ig-PLP1 /Sln
2X Ig-PLP1 /Sln
2X Ig-W /Sln

Figure 9.

Two injections of Ig-PLP1/Sln confer resistance to EAE induction.

Newborn mice (5 per group) were injected i.p. with 100μg Ig-PLP1 in saline on the day of birth (1X Ig-PLP1/Sln, ◎); 100μg Ig-PLP1 in saline on the day of birth and at the age of 7 days (2X Ig-PLP1/Sln, ◈); or 100 μg Ig-W in saline on the day birth and at the age of 7 days (2X Ig-W/Sln, ▲), and induced for EAE with 100 μg PLP1 peptide when they reached 7 weeks of age. The mice were then scored daily for signs of paralysis as described in *Materials and Methods*. Each point represents the mean ± SD of clinical scores of all mice tested. While mice recipient of Ig-W twice had a 40% rate of death the mice recipient of a single or double injection had no mortality.


Mean clinical score
4
3
2
1
0
0
20
40
60
80
Days post induction
1X Ig-PLP1/Sln
2X Ig-PLP1/Sln
2X Ig-W/Sln

PART V.

Defective Up-Regulation of Functional IL-2 Receptor Underlies Interferon-Gamma Mediated Neonatal T Cell Anergy

(Submitted for publication)

Chapter 1. Abstract

Recently a novel form of neonatal induced T cell anergy restorable by IFNγ or IL-12 was reported. T cells of this phenotype do not proliferate or produce IFNγ upon antigen stimulation yet secrete significant amounts of IL-2. Exogenous IFNγ or IL-12 restores both proliferation and IFNγ production in an antigen specific manner. Herein, we demonstrate that defective up-regulation of IL-2Rα chain required for formation of functional IL-2R is responsible for the inability of the T cells to utilize their own IL-2 for proliferation and differentiation. Exogenous IL-12 restores IL-2Rα chain expression, which facilitates IL-2 absorption and drives both proliferation and IFNγ production. However, blockade of IL-12-restored IL-2Rα chain reinstates T cell anergy. The inability of anergic T cells to up-regulate IL-2Rα chain expression is persistent and parallels with the long-lasting neonatal tolerance. These findings indicate that neonatal exposure to antigen can selectively interfere with the regulation of IL-2R expression leading to defective T cell proliferation and differentiation that culminates in a novel form of T cell anergy dependent upon IFNγ rather than IL-2.

Chapter 2. Introduction

Prior studies from our laboratory have defined a novel form of neonatal induced T cell anergy, restorable by IFNγ or IL-12 (1), and is different from the standard IL-2-dependent T cell anergy (2). Upon stimulation with antigen T cells of this phenotype cannot proliferate or secrete IFNγ yet produce significant amounts of IL-2 (1). Specifically, mice given the immunoglobulin (Ig) chimera Ig-PLP1 (3), expressing the encephalitogenic proteolipid protein (PLP) sequence 139-151 (referred to as PLP1) on the day of birth and challenged with a pathogenic regimen of PLP1 at the age of seven weeks develop deviated T cells in the lymph node and IFNγ-dependent anergic T cells in the spleen (1). Both deviated and anergic T cells contribute to protection of the mice against experimental allergic encephalomyelitis (EAE) (1). Furthermore, the anergic splenic T cells regain the ability to proliferate and produce IFNγ when supplied with IFNγ or the IFNγ inducer, IL-12, during stimulation with PLP1 peptide (1). Moreover, in vivo reactivation of these cells with IL-12 restores susceptibility to EAE (1). This form of splenic T cell anergy was unique to the Ig-PLP1 chimera and did not develop in mice that were exposed to free PLP1 peptide (1, 4). Since the splenic T cell anergy is in part responsible for resistance to EAE induction (1), it is important to dissect the mechanism underlying this neonatal T cell regulation in order to gain insight on T cell modulation and prevention of autoimmunity. The puzzling issue regarding these T cells is their inability to proliferate despite the availability of the autocrine growth factor IL-2, which promotes both T cell proliferation and differentiation (5). The postulate put forth for this observation was that the splenic anergic T cells were defective in up-regulation of functional IL-2 receptor (IL-2R) expression and were therefore unable to absorb and

utilize their own IL-2 to proliferate and differentiate into IFNγ producing T cells. The rationale for such a hypothesis derived from the observation that IL-12 restoration of the anergic T cells, which promoted strong proliferation, always reduced rather than increased IL-2 production, suggesting that the T cells reabsorb some of their own IL-2 during restoration of proliferation.

IL-2 receptor is a trimolecular complex comprised of constitutively expressed signaling β and γ chains (5, 6) and an inducibly expressed IL-2 binding α chain that regulates affinity for IL-2 (7). Unlike human IL-2 receptor, the murine heterodimeric IL-2Rβγ does not have any affinity for IL-2, and expression of IL-2Rα is crucial for the formation of a functional high affinity receptor (7). IL-2Rα chain, also known as CD25, is not expressed on resting T cells but is transiently up-regulated upon activation of T cells and is considered as an early activation marker for T cells (8). Although the involvement of IL-2R in T cell activation, growth, and differentiation has mostly been analyzed in conjunction with IL-2, the signaling pathways for IL-2 and IL-2R expression diverge downstream of the TCR signaling pathway and the two elements may differentially control cell survival, expansion, and differentiation (8, 9).

The studies reported herein indicate that the anergic splenic T cells that arise subsequent to neonatal exposure to Ig-PLP1 are unable to up-regulate expression of IL-2Rα upon stimulation with antigen. Consequently, the re-absorption of IL-2 is ineffective, and the T cells do not proliferate or differentiate into IFNγ producing cells. The inability of these cells to express IL-2Rα is long lasting and parallels with the persistence of in vivo T cell tolerance. These results suggest that expression of functional

IL-2R is developmentally regulated and contributes to neonatal tolerance by interfering with cell proliferation and differentiation.

Chapter 3. Materials and Methods

Mice

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

Peptides and Ig-PLP chimeras

All peptides used in these studies were purchased from Research Genetics, Inc (Huntsville, AL) and purified by HPLC to >90% purity. The encephalitogenic PLP1 (HSLGKWLGHPDKF) and PLP2 (NTWTTCQSIAFPSK) peptides encompass PLP sequences 139-151 (10) and 178-191 (11), respectively. Both peptides are presented to T cells in association with I-A^s MHC class II molecules and induce EAE in SJL/J mice (10, 11). For construction of Ig-PLP1 the complementarity determining region 3 (CDR3) of the 91A3 anti-arsonate antibody heavy chain variable region was deleted and replaced with a nucleotide sequence encoding PLP1 peptide. This chimeric heavy chain was then co-transfected into the non-Ig producing SP2/0 myeloma cell line with the parental 91A3 light chain to generate a complete Ig-PLP1 chimera (3). Ig-W, the parental IgG_{2b} not encompassing PLP1 peptide, has been described elsewhere (12) and was used as negative control.

Neonatal tolerization with Ig-PLP1 and immunization of adult mice with PLP1 peptide

Newborn mice were injected i.p. with 100μg Ig-PLP1 or Ig-W in 100μl saline within 24 hours after birth, and when the mice reached 7 weeks of age they were immunized s.c. with 100μg PLP1 peptide emulsified in 200 μl PBS/CFA (vol/vol). Ten days later the mice were sacrificed, and their spleens were removed for analysis of proliferation, cytokine production, and IL-2R expression.

Cell purification

Mice tolerized with Ig-PLP1 or Ig-W at birth were immunized with PLP1 peptide in CFA at 7 weeks of age. Ten days later the spleens were removed and a single cell suspension was prepared. After lysis of red blood cells with NH_4Cl-Tris, cells were further purified for T cells and APCs. For T cell purification, cells were passed through a nylon wool column. By FACS analysis, the cells were ≈ 90% T cells. For T cell-depleted APCs, the cells were treated with anti-CD3 (2C11, 1μg/ml) and incubated with rabbit complement for 45 min at 37°C. Treated cells were then separated on dense BSA (Intergen, Purchase, NY). Both populations obtained above were incubated together with PLP1 for 3 days. Proliferation was measured by [^{3}H]thymidine incorporation as described below.

Proliferation assay

Spleen cells were incubated in 96-well flat-bottomed plates at 10 x 10^5 cells/100µl/well with 100µl of stimulator for 3 d. Subsequently, 1µCi [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 hours. The cells were then harvested onto glass fiber filters, and incorporated [^{3}H]thymidine was counted using the trace 96 program and an Inotech β counter (Wohlen, Switzerland). The stimulators, PLP1 or PLP2, were used at 15µg/ml, and a control of media with no stimulator was included for each mouse and used as background. For restoration of proliferation with rIL-12, the stimulation was carried out in the presence of 10 units/ml of mouse rIL-12 (PharMingen, San Diego, CA) as previously described (1). For testing of inhibition of IL-12-mediated restoration of proliferation, the stimulation was carried out in the presence of 10µg/ml anti-mouse IL-2 (S4B6, rat IgG_{2a}) or anti-mouse CD25 (PC61, rat IgG_1) antibodies (PharMingen). Rat IgG used as isotype control was purchased from Sigma (St. Louis, MO).

ELISA

Spleen cells were incubated in 96-well round-bottom plates at 10 x 10^5 cells/100µl/well with 100µl of stimulator for 24 h. Cytokine production was measured by ELISA according to PharMingen's instructions using 100µl of culture supernatant (1). The capture anti-cytokine antibodies used were rat anti-mouse IL-2, JES6-1A12 and rat anti-mouse IFN-γ, R4-6A2. The biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES6-5H4 and rat anti-mouse IFN-γ, XMG1.2. The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Sunnyvale, CA) using SoftMAX PRO version 1.2.0 software. Graded amounts of recombinant mouse IL-2 and IFNγ were

included in all experiments in order to construct standard curves. The concentration of cytokines in culture supernatant was estimated by extrapolation from the linear portion of the standard curve.

Flow cytometry

Splenic cells from mice tolerized with Ig-PLP1 at birth and immunized with PLP1 peptide at the age of 7 weeks were in vitro stimulated with PLP1. Subsequently, expression of IL-2 receptor α chain (CD25) was measured by FACS™ analysis. Briefly, incubated splenic cells were treated with NH_4Cl-Tris for red blood cell lysis and immediately fixed with 2% formaldehyde. Cells were then incubated with anti-FcγR mAb (2.4G2) to block Fc receptors and labeled with fluorochrome-labeled mAb for 30 min at 4°C. Flow cytometry was done with a FACScan™ (Becton Dickinson, San Jose, CA) and the data were analyzed with CellQuest™ software (Becton Dickinson). Anti-CD25-PE (3C7), anti-CD4-FITC (RM4-5) and isotype matched controls were purchased from PharMingen.

Detection of IL-2Rα chain mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from splenic cells using TRIZOL (Gibco BRL, Grand Island, NY). RT-PCR was carried out using the Access RT-PCR system (Promega, Madison, WI) and consisted of a first strand cDNA synthesis step followed by 40 PCR amplification cycles (94°C, 30 second denaturation; 68°C, 1 minute annealing; and 72°C, 2 minute polymerization). The primers used were; 5'-CAGACATGCAGAAGCCAACA

C-3' and 5'-GGTGAGCCCGCTCAGGAGGA-3' for CD25, and 5'-GTGGGGCGCCCC AGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGAT-3' for β-actin (13). All oligonucleotides were purchased from Life Technologies (Rockville, MD).

Chapter 4. Results

Neonatal exposure to Ig-PLP1 induces an IFNγ-dependent defect in splenic T cell responses.

We have previously shown that neonatal exposure to Ig-PLP1 conferred resistance to EAE induction by a mechanism involving T cell lymph node deviation and an IFNγ-dependent splenic T cell anergy (1). Specifically, mice that were injected with Ig-PLP1 in saline on the day of birth and challenged with free PLP1 in CFA at the age of 7 weeks developed lymph node T cells that produced IL-4 instead of the usual IL-2 in response to PLP1 peptide. The spleen, however, developed T cells that were unable to proliferate or produce IFNγ in response to PLP1 stimulation yet still secreted significant amounts of IL-2 (1; Figure 1). Exogenous IFNγ as well as the IFNγ-inducer, IL-12, restored splenic T cell proliferation, and such restoration was antigen specific as stimulation with PLP2 peptide, instead of PLP1, did not drive T cell proliferation (Figure 1a). Moreover, IL-12 restored T cells regained the ability to produce IFNγ in an antigen-dependent fashion (Figure 1b). It is worth noting that restoration of proliferation by either IL-12 or IFNγ reduces rather than increases IL-2 in the culture supernatant (Figure 1c).

To begin investigation of the mechanism underlying this form of T cell anergy and to evaluate the contribution of T cells versus APCs to the proliferative defect, we performed swap experiments between purified T cells and APCs from animals that were injected with Ig-PLP1 on the day of birth and immunized with PLP1 in CFA at 7 weeks of age and those from mice that were injected with Ig-W on the day of birth and also immunized with PLP1 peptide at the age of 7 weeks. As can be seen in figure 2, when the T cells originated from Ig-W tolerized mice proliferation occurred whether the APCs

were from Ig-W or Ig-PLP1 tolerized mice. However, when the T cells originated from Ig-PLP1 recipient mice the proliferation was defective irrespective of the origin of APCs. These results suggest that APCs have minimal or no contribution to the defective proliferation observed in PLP1-specific splenic T cells from Ig-PLP1 tolerized mice.

Requirement of IL-2 for restoration of splenic T cell proliferation by IL-12.

Since the proliferative defect seems to originate and operate at the level of T cells we directed our attention to the behavior of the T cells upon restoration with cytokine. The one noticeable observation that arose from this analysis was the reduction in the amount of IL-2 in the supernatant of cytokine-restored versus anergic T cells (Figure 1c). In fact T cells driven for proliferation by IFNγ or IL-12 consistently had less IL-2 in the supernatant than the non-proliferative anergic T cells (Figure 1c). This observation raised the possibility that during cytokine-mediated restoration of proliferation the T cells may consume or reabsorb some of their own IL-2 in order to proliferate. This was indeed the case as neutralization of IL-2 by anti-IL-2 antibody blocked IL-12-mediated restoration of splenic T cell proliferation and IFNγ production (Figure 3). Isotype control rat IgG antibodies were unable to inhibit such restoration. These results suggest that the splenic T cells require IL-2 to proliferate and raise the possibility that the anergy of the splenic T cells could be due to a lack of functional IL-2 receptor which is necessary to allow reabsorption of autocrine IL-2 and promote proliferation and differentiation.

Defective up-regulation of IL-2Rα chain expression on anergic splenic T cells.

IL-2 receptor consists of α, β and γ chains (5). Among these, the α chain (CD25, p55) is expressed only on activated T cells and plays a critical role in IL-2 responsiveness (7, 8, 14). To investigate expression of IL-2Rα chain on the splenic anergic T cells we performed both RT-PCR and cell surface staining analysis to measure IL-2Rα chain mRNA and protein levels, respectively. As can be seen in figure 4a, proliferative T cells from mice injected with Ig-W on the day of birth and immunized with PLP1 peptide at the age of 7 weeks expressed significant IL-2Rα chain mRNA. Furthermore, stimulation with PLP1 peptide significantly up-regulated IL-2Rα chain mRNA. Evaluation of band intensity using the NIH image analysis program revealed that the band intensity for IL-2Rα chain DNA was 21% of the β-actin band intensity for the unstimulated T cells of Ig-W recipient mice. This ratio increased to 53% when the cells were stimulated with PLP1 peptide. However, T cells from Ig-PLP1 tolerized mice had marginal IL-2Rα chain mRNA which was not significantly up-regulated by stimulation with PLP1 peptide (Figure 4a). Only the addition of IL-12 during T cell stimulation significantly upregulated IL-2Rα chain mRNA expression (Figure 4a). The percent β-actin band intensity of IL-2Rα chain was <1% when the T cells were unstimulated, 2% when they were stimulated with PLP1 peptide, and increased to 24% when the cells were stimulated with PLP1 peptide and IL-12. Moreover, when graded amounts of mRNA were used in RT-PCR, DNA amplification increased proportional to mRNA input, indicating a specific correlation between the amount of mRNA and the reverse transcription and amplification (not shown). Although these results could suggest that the splenic anergic T cells from Ig-PLP1 recipient mice are defective in expression of IL-2Rα chain, they do not exclude

the possibility that IL-2Rα chain mRNA was at a minimal level due to a lower number of non-proliferative PLP1-specific T cells among the total splenic T cells. To address this issue FACS analysis to detect receptor expression at the single cell level was performed. As shown in figure 4b, splenic T cells from Ig-W recipient mice when stimulated with PLP1 peptide expressed IL-2Rα chain on the cell surface as staining with anti-CD25 antibody on CD4-gated T cells shifted relative to staining with the isotype control. However, there was no shift on CD25 staining relative to isotype control in the case of splenic T cells from Ig-PLP1 tolerized mice. Cell surface staining for CD25 was also performed following 48 and 72 hour stimulation with peptide, yet no IL-2Rα chain was detected on the surface of the anergic splenic CD4 T cells (not shown). However, when the cells were stimulated with PLP1 peptide in the presence of IL-12 a significant up-regulation of cell surface IL-2Rα chain expression was observed after 72 h of stimulation (not shown). These results suggest that anergic splenic T cells from Ig-PLP1 tolerized mice, which do not proliferate or produce IFNγ upon stimulation with PLP1 peptide, are unable to up-regulate IL-2Rα chain expression unless they are provided with exogenous IL-12. This is in good agreement with a previous report showing that IL-12 can upregulate IL-2Rα chain expression (15).

Requirement for IL-2Rα chain expression in IL-12 driven antigen-specific restoration of proliferation and IFNγ production.

Since IL-2 is produced by the anergic PLP1-specific T cells and seems to be required for proliferation (Figure 3) and T cells from Ig-PLP1 tolerized mice fail to up-regulate IL-2Rα chain in response to PLP1 peptide (Figure 4), it is likely that lack of

functional IL-2R expression on PLP1-specific T cells is responsible for the inability of the T cells to utilize IL-2, thereby leading to a failure in T cell proliferation and IFNγ production. Blockade of the IL-2/IL-2R interaction has been shown to suppress T cell proliferation (16). Furthermore, in human T cells, loss of IL-2Rα chain resulted in diminished proliferation and IFNγ secretion (17). As can be seen in figure 5, blockade of IL-12-restored IL-2Rα chain by anti-CD25 antibody prevented the absorption of IL-2 by T cells and inhibited restoration of both proliferation and IFNγ production. Specifically, when anergic splenic T cells were stimulated with PLP1 and IL-12 they proliferated and produced IFNγ, but addition of anti-CD25 antibody to the culture abolished the restoration of both proliferation and INFγ production (Figure 5). In contrast, the addition of the isotype control, rat IgG, instead of anti-CD25 antibody allowed for IL-2 absorption and did not inhibit restoration of proliferation or IFNγ production. It is worth mentioning that the restoration of proliferation by IL-12 reduces the IL-2 content in the supernatant, suggesting that the reabsorption of IL-2 is necessary for proliferation and differentiation.

The defect in IL-2Rα chain expression is long-lasting and parallels with the persistence of in vivo T cell tolerance.

If IL-2Rα chain expression is responsible for the defect in T cell proliferation and IFNγ production, it should parallel with the status of T cell anergy in vivo. To address this issue mice were injected with Ig-PLP1 on the day of birth, and one group of mice per week was challenged with PLP1 in CFA starting at the age of 8 weeks. The splenic T cells were then tested for IL-2Rα chain expression, proliferation, and cytokine production. As can be seen in figure 6, the mice remained unable to mount splenic

proliferative responses or produce IFNγ upon immunization with PLP1 peptide up to week 20 (Figure 6a and b). IL-2 production, however, was significant (Figure 6b). At week 28 the mice regained the ability to mount splenic proliferative T cells responses and to produce significant amounts of IFNγ. IL-2Rα chain expression followed a similar pattern and showed weak expression on week 20 but significantly increased by week 28 to a level of 7.92% among all CD4-T cells (Figure 6c). These results suggest that IL-2Rα chain plays a critical role in the maintenance of Ig-PLP1 mediated neonatal tolerance.

Chapter 5. Discussion

The results presented herein demonstrate that the splenic T cells arising in response to PLP1 peptide in mice that were exposed to Ig-PLP1 on the day of birth are unable to up-regulate IL-2Rα chain expression upon stimulation with antigen. Consequently, IL-2 absorption could not occur likely leading to defective proliferation and IFNγ production. These observations agree well with prior reports demonstrating that T cells from IL-2Rα chain deficient mice, which could not absorb IL-2, were unable to enter the cell cycle and differentiate in response to antigen stimulation (18). Furthermore, IL-12, which has been shown to enhance IL-2Rα chain expression on Th1 cells (15), up-regulates expression of functional IL-2R and restores both proliferation and IFNγ production by the splenic T cells. In murine T cells, IL-2βγ heterodimer, which is constitutively expressed, has no binding affinity to IL-2 (7, 19). IL-2Rα chain is inducibly expressed on activated T cells and is critical for formation of functional IL-2R (7, 14). Therefore, the lack of IL-2Rα chain on the splenic T cells is sufficient to drive mal-absorption of IL-2 and promote proliferative unresponsiveness. Although the argument could be made that weak expression of IL-2Rα chain expression is the consequence of proliferative unresponsiveness, the observation that blockade of IL-12 restored IL-2Rα chain by anti-CD25 antibody reinstates T cells anergy tapers this argument. In fact this observation implies, as has been suggested by others (17), that defective up-regulation of IL-2Rα chain expression is likely responsible for the proliferative unresponsiveness and deficiency in IFNγ production. Moreover, the results presented in figure 6, demonstrating that lack of expression of IL-2Rα chain is long

lasting and parallels with persistence of proliferative and IFNγ unresponsiveness, favor the contribution of IL-2R to this form of neonatally induced IFNγ-mediated T cell anergy.

IL-2 and its receptor have been implicated in T cell survival (20, 21), growth and differentiation (22, 23), and activation-induced death (24). T cells from mice with targeted inactivation of the genes for IL-2 or IL-2R show markedly reduced proliferative responses upon stimulation with antigen or even polyclonal activators (6, 25). Although these observations establish a tight link between IL-2 and its receptor and suggest that TCR-mediated signaling activates both IL-2 and IL-2R genes to drive cell growth and differentiation, there is evidence indicating that the intracellular signaling pathways activating IL-2 and IL-2Rα chain expression diverge downstream of TCR signaling (8, 9). Thus IL-2 and IL-2R activation pathways may be influenced by different factors within the cells (5). For instance, TCR engagement has been shown to be sufficient for IL-2Rα chain expression, whereas costimulatory signals are required for IL-2 production (8, 26). Therefore, it may be that the splenic anergic T cells are defective in the generation of signals required for expression of functional IL-2R.

Regulation of IL-2Rα chain has been shown to play a major role in tolerance induction to renal allografts (27) and in T cell mitogenesis (28). In the present study, neonatal TCR stimulation by Ig-PLP1 may promote differential regulation of IL-2 and its receptor. This regulation may result in the generation of anergic T cells which are capable of producing IL-2 but are unable to express the functional IL-2R required for reabsorption of IL-2. This phenotype may have been responsible for the proliferative unresponsiveness and the lack of differentiation of the splenic T cells. How neonatal

exposure to Ig-PLP1 drives developmental control of IL-2R is not clear. However, on a speculative basis it is possible that Ig-PLP1, by binding FcR for internalization into APCs (29, 30), stimulates the production of cytokines and/or regulates the expression of costimulatory molecules thereby influencing T cell differentiation. Furthermore, it is not clear why neonatal injection of Ig-PLP1 induces cytokine dependent T cell anergy only in the spleen. The CFA injected around the lymph nodes during immunization at adult life may play a role in such organ specific regulation of T cells.

Overall, these observations implicate IL-2Rα chain in neonatal tolerance and reveal a developmental control of IL-2Rα chain expression and its influence on T cell proliferation and differentiation.

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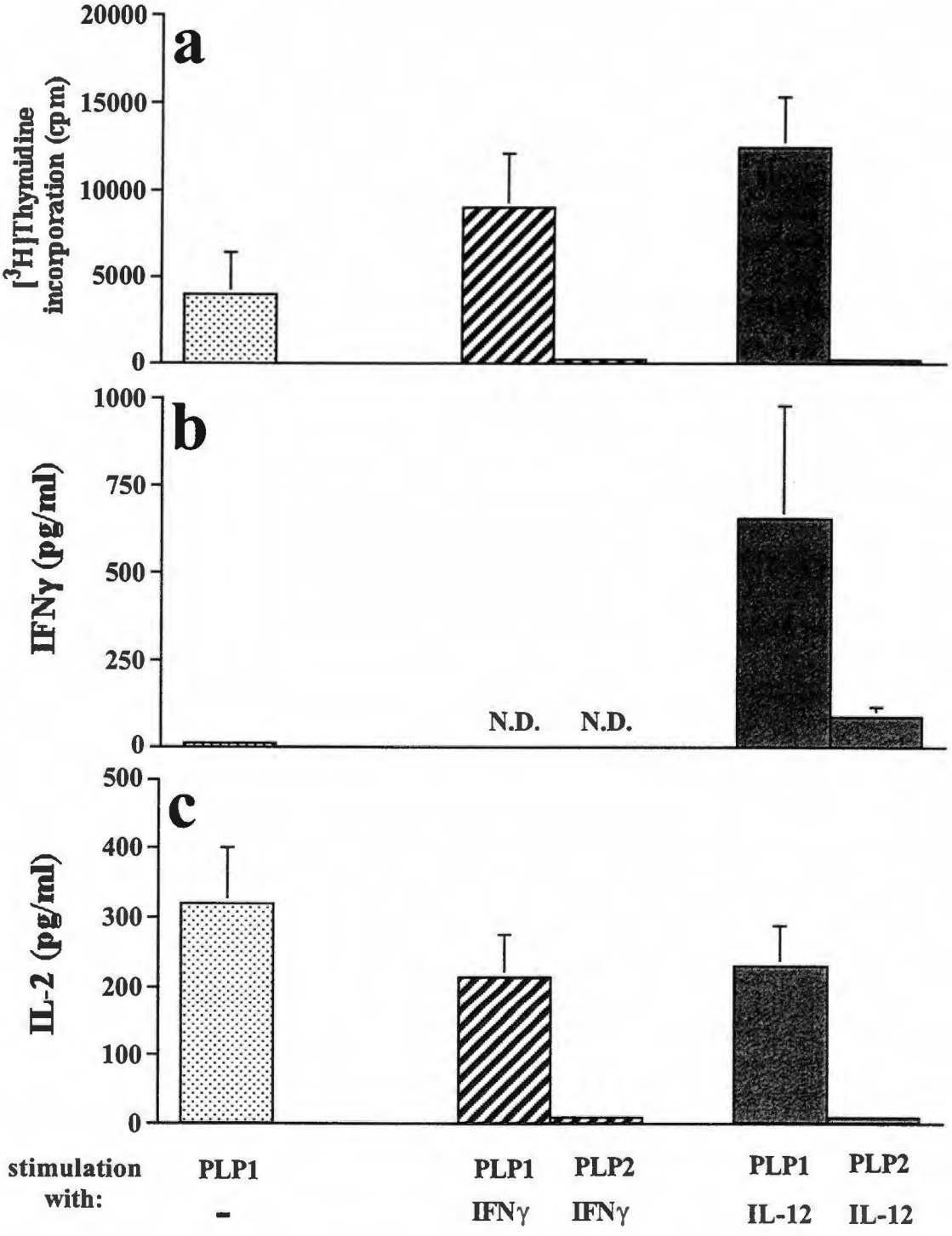
Figure 1.

The splenic T cells of Ig-PLP1 tolerized mice require IFNγ or IL-12 to restore proliferation and IFNγ production.

Groups of newborn mice (4-6 per group) were injected i.p. with Ig-PLP1 in saline on the day of birth and immunized s.c. with PLP1 in CFA when they were 7 weeks of age. Ten days later spleen cells (1 x 10^6 cells/well) were in vitro stimulated with 15μg/ml PLP1 or PLP2 peptide in the presence or absence of 10 units/ml of cytokine, and proliferation (a), IFNγ (b), and IL-2 (c) production were measured as described in Methods. The indicated values (cpm and pg/ml) represent the mean ± SD of individually tested mice. N.D., not determined. The results are representative of 3 experiments.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[3H]Thymidine incorporation (cpm)
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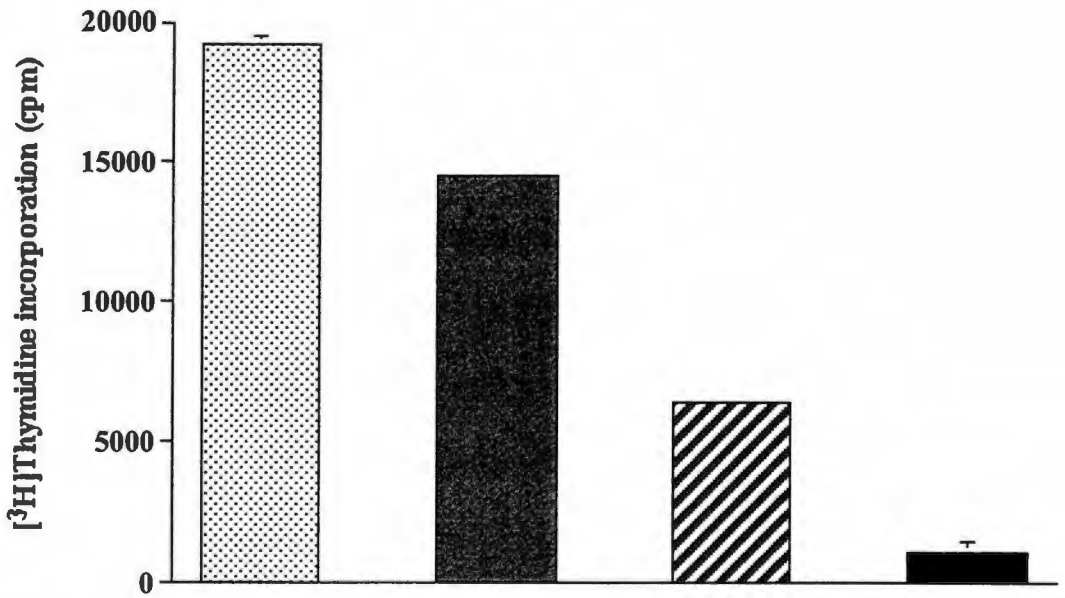
15000
10000
5000
0
b
IFNγ (pg/ml)
1000
750
500
250
0
N.D.
N.D.
c
IL-2 (pg/ml)
500
400
300
200
100
0
stimulation with:
PLP1
-
PLP1
IFNγ
PLP2
IFNγ
PLP1
IL-12
PLP2
IL-12

Figure 2.

Minimal contribution of APCs to splenic T cell anergy.

Groups of newborn mice (4 per group) were injected i.p. on the day of birth with either Ig-PLP1 or Ig-W in saline and were challenged with PLP1 peptide in CFA when they reached 7 weeks of age. Ten days later splenic T cells from both groups were isolated and assayed for proliferation upon stimulation with PLP1 peptide and purified APCs obtained from the spleen of Ig-PLP1 and Ig-W tolerized mice. The source of T cells and APCs is indicated below each bar. T cells (1×10^5 cells/well) and APCs (2×10^5 cells/well) were incubated with 15 μg/ml PLP1 peptide for three days and proliferation was measured by [^{3}H]thymidine incorporation as described in Methods. In vitro stimulation with PLP2 peptide was included as a negative control and no significant response to PLP2 was observed (data not shown). The data represent the mean ± SD of triplicate wells and are representative of two experiments.

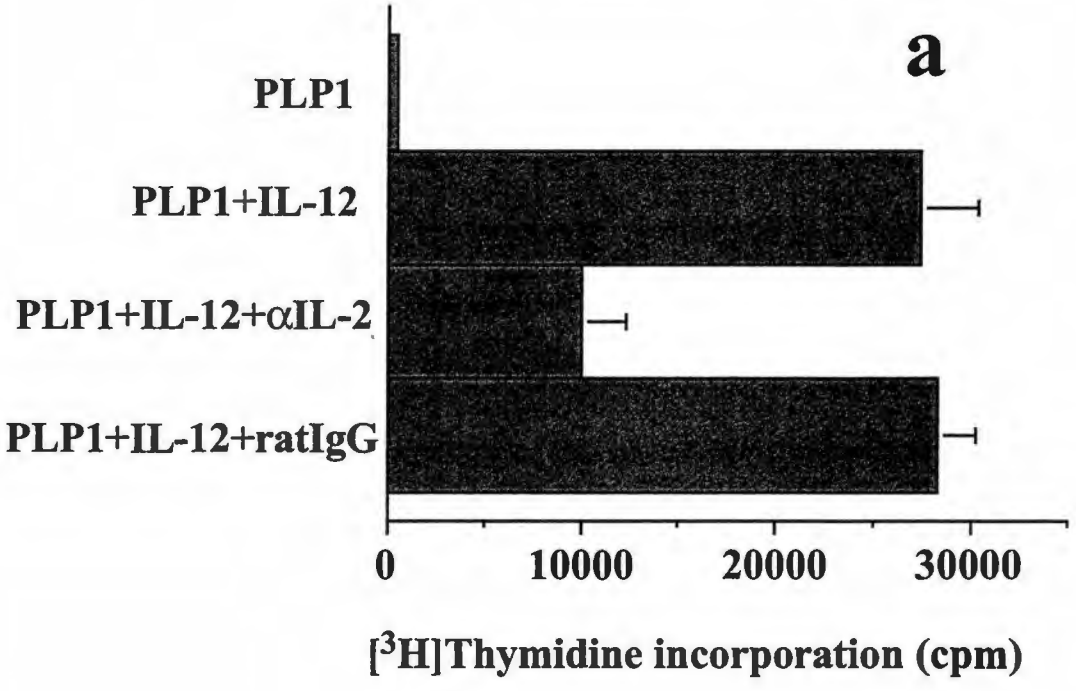


T cells	**Ig-W**	**Ig-W**	**Ig-PLP1**	**Ig-PLP1**
APCs	**Ig-W**	**Ig-PLP1**	**Ig-W**	**Ig-PLP1**

Figure 3.

Restoration of T cell proliferation and IFNγ production by IL-12 is IL-2 dependent.

Groups of 8 newborn mice were injected i.p. with 100μg Ig-PLP1 in saline on the day of birth and challenged with PLP1 in CFA at the age of 7 weeks. Ten days later their splenic T cells were tested for IL-12-mediated restoration of proliferation (a) and IFNγ production (b) in the presence of neutralizing anti-IL-2 antibody. The spleen cells (1×10^6 cells/well) were incubated with PLP1 peptide (PLP1); PLP1 + anti-IL-2 antibody (PLP1 + _IL-2); PLP1 + rIL-12 (PLP1 + IL-12); PLP1 + rIL-12 + anti-IL-2 antibody (PLP1 + IL-12 + _IL-2); or PLP1 + rIL-12 + rat IgG (PLP1 + IL-12 + rat IgG) and IFNγ production was measured by ELISA from culture supernatant after 24 hours. A duplicate set of cells were incubated with the stimulators for 3 days and following [^{3}H]thymidine (1μCi/well) addition the culture was continued for an additional 14.5 hours. Proliferation was then measured as described in Methods. PLP1 peptide was used at 15 μg/ml, IL-12 at 10 units/ml, anti-IL-2 antibody at 10 μg/ml, and rat IgG at 10 μg/ml. The data represent the mean ± SD of individually tested mice. The results are representative of 2 experiments.


a
PLP1
PLP1+IL-12
PLP1+IL-12+αIL-2
PLP1+IL-12+ratIgG
0
10000
20000
30000
[3H]Thymidine incorporation (cpm)

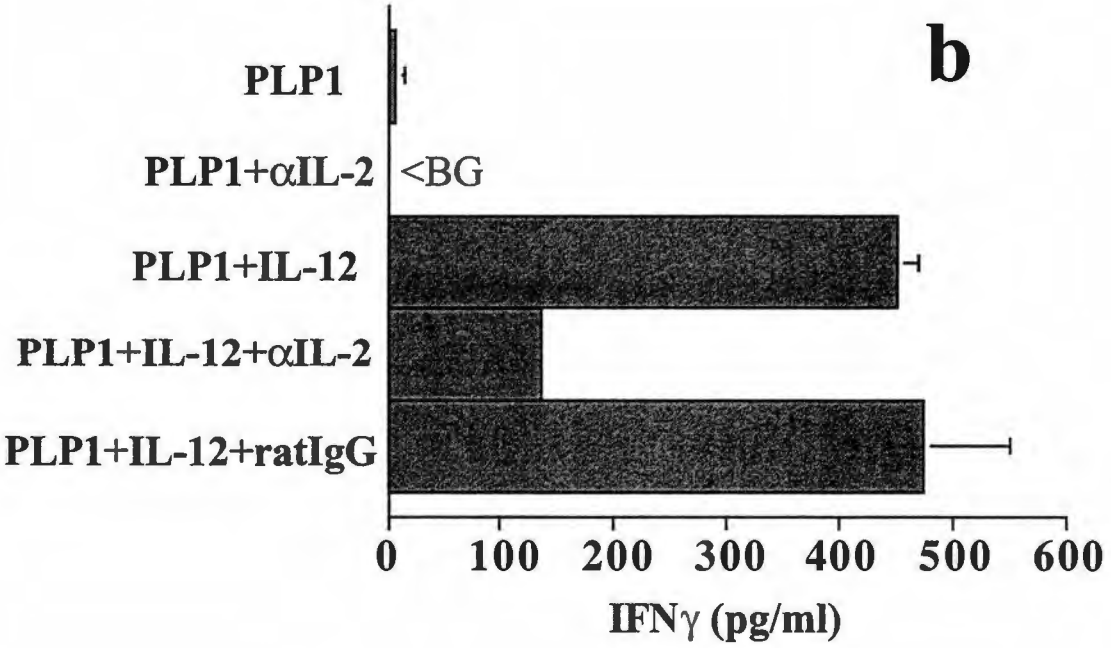

b
PLP1
PLP1+αIL-2
<BG
PLP1+IL-12
PLP1+IL-12+αIL-2
PLP1+IL-12+ratIgG
0
100
200
300
400
500
600
IFNγ (pg/ml)

Figure 4.

IL-2Rα chain mRNA is weakly expressed by anergic splenic T cells but is upregulated upon stimulation with peptide and IL-12.

(a) Splenic cells (5 x 10^6 cells/ml) from mice that were tolerized with either Ig-W or Ig-PLP1 at birth and immunized with PLP1 in CFA at 7 weeks of age were stimulated with 15 μg/ml PLP1 peptide in the presence or absence of IL-12 (10 units/ml) for 24 hours. Total RNA was extracted from 5 x 10^6 cells as described in Methods, and 100 ng from each stimulation was subjected to first strand synthesis by reverse transcription and DNA amplification by PCR using IL-2R α chain (CD25) and β-actin specific primers. Amplified DNA was then run on a 1% agarose gel. The illustrated bands correspond to the expected molecular weight of CD25 and β-actin.

(b) Splenic cells (1 x 10^6) from mice neonatally tolerized with Ig-PLP1 or Ig-W and immunized with PLP1 at adult life were in vitro stimulated with PLP1 peptide for 24 hours, and double stained with anti-CD4 + anti-CD25 antibody or anti-CD4 + rat IgG_{2b} and analyzed by FACS. Shown are histograms of IL-2R α chain expression on CD4 gated T cells from mice recipient of Ig-PLP1 or Ig-W on the day of birth. The histogram (Isotype control) of cells from Ig-W tolerized mice that were double stained with anti-CD4 and rat IgG_{2b}, gated on CD4 and analyzed for staining with rat IgG_{2b} is also shown. Cells from mice recipient of Ig-PLP1 on the day of birth showed a similar pattern when double stained with anti-CD4 antibody and rat IgG_{2b}.

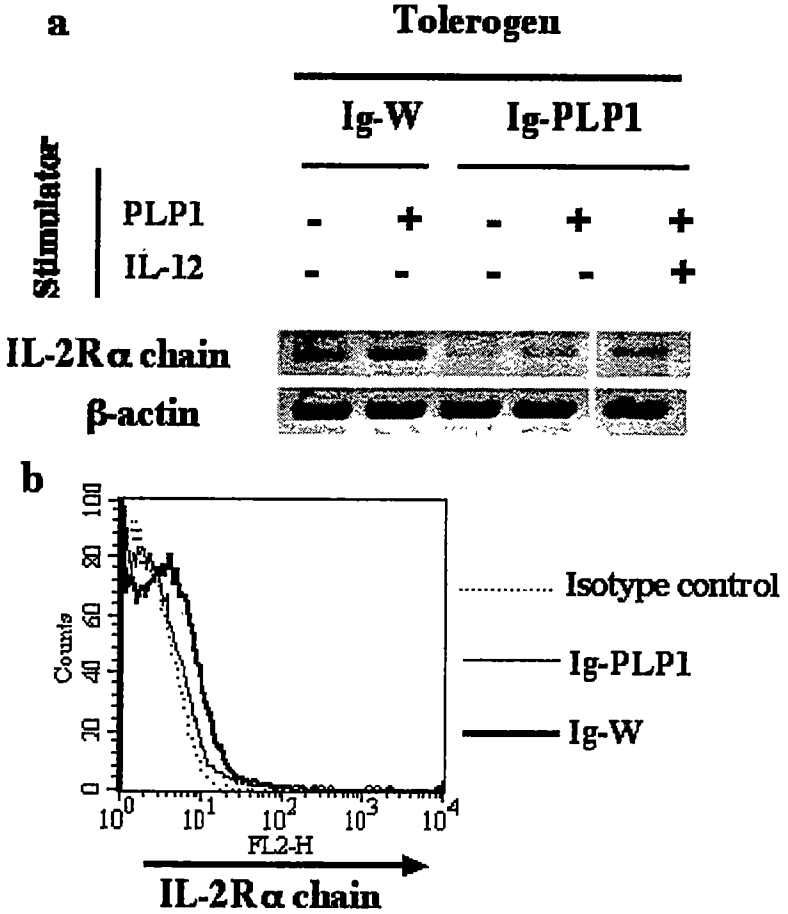

a
Tolerogen
Ig-W
Ig-PLP1
Stimulator
PLP1
IL-12
- + - + +
- - - - +
IL-2Rα chain
β-actin
b
Counts
FL2-H
IL-2Rα chain
Isotype control
Ig-PLP1
Ig-W

Figure 5.

Blockade of IL-12-restored IL-2Rα chain reinstates T cell anergy.

Splenic cells (1 x 10^6/well) from mice that were tolerized with Ig-PLP1/saline on the day of birth and challenged with PLP1/CFA at the age of 7 weeks were stimulated with 15 μg/ml PLP1 peptide + 10 units/ml IL-12 in the presence of anti-CD25 antibody (+PLP1 +IL-12 + αCD25) or control rat IgG (+PLP1 +IL-12 + rat IgG) and assayed for proliferation (a), IFNγ (b), and IL-2 (c). Cells stimulated with PLP1 alone (+PLP1) or PLP1 and IL-12 (+PLP1 + IL-12) were included for control purpose. Anti-CD25 antibody and rat IgG were used at 10 μg/ml. Proliferation was measured by [^{3}H]thymidine incorporation, and cytokines were measured by ELISA as described in Methods. The indicated values (cpm and pg/ml) represent the mean ± SD of 4 individually tested mice. The results presented here are representative of two separate experiments.

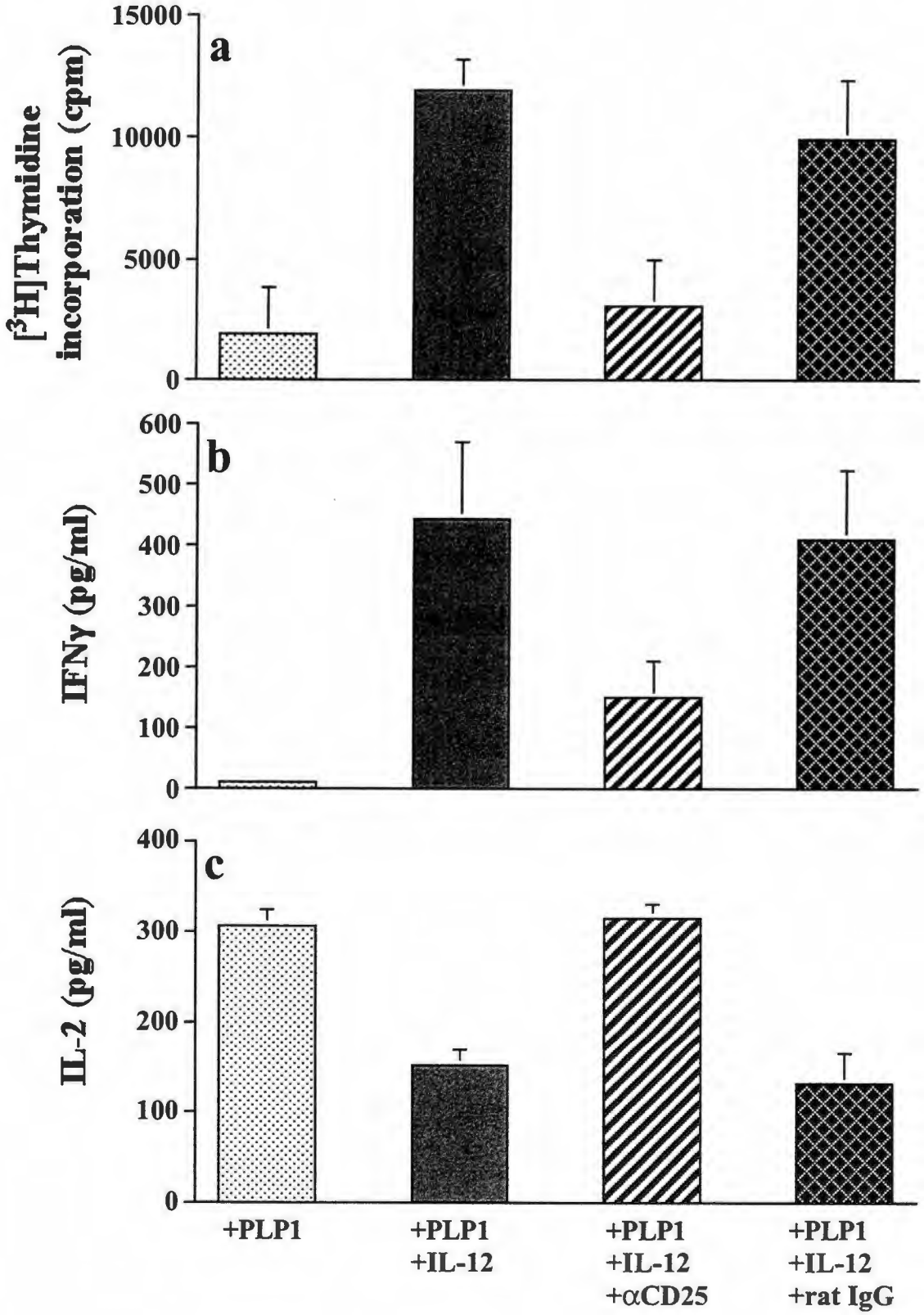
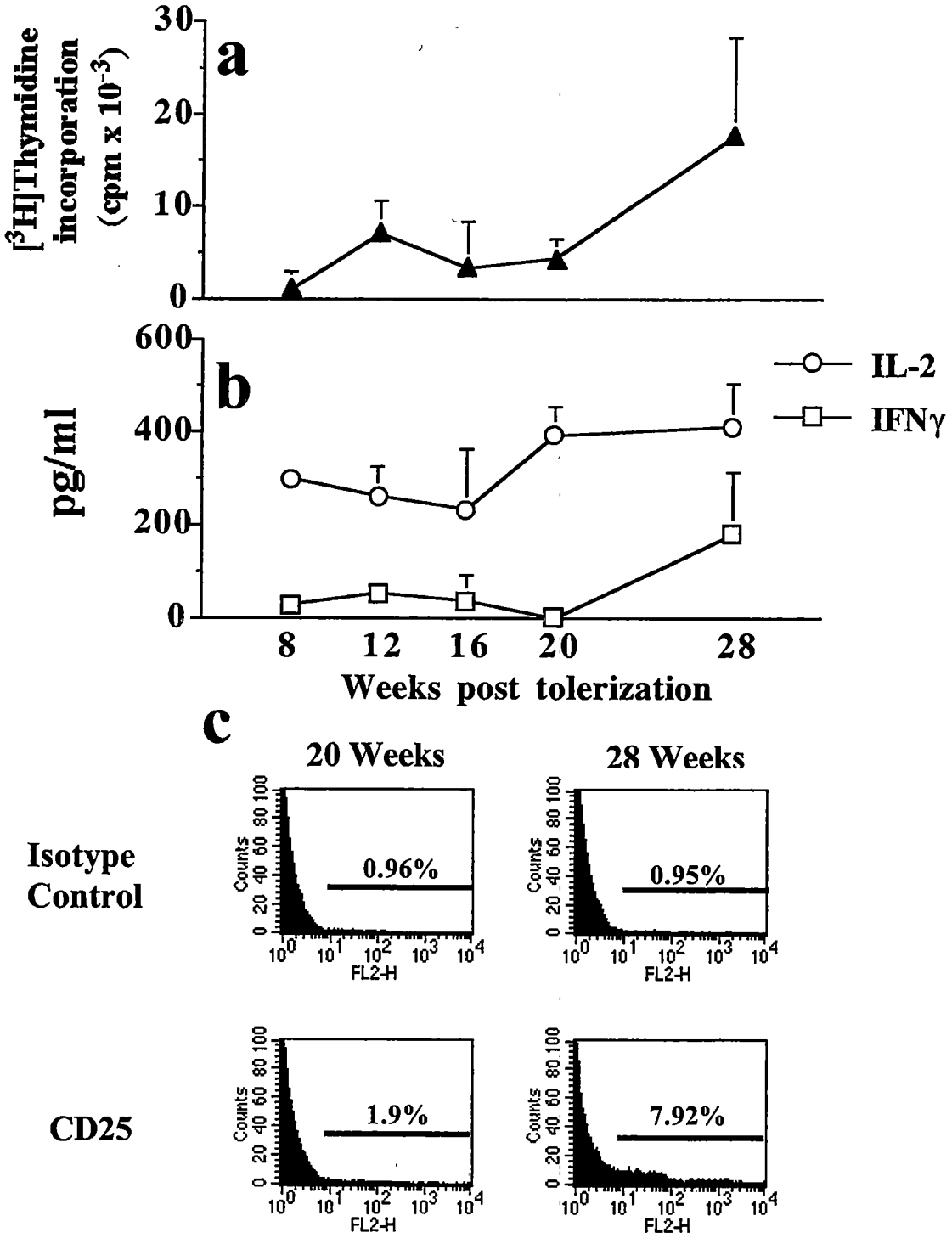

a
[3H]Thymidine incorporation (cpm)
15000
10000
5000
0
b
IFNγ (pg/ml)
600
500
400
300
200
100
0
c
IL-2 (pg/ml)
400
300
200
100
0
+PLP1
+PLP1 +IL-12
+PLP1 +IL-12 +αCD25
+PLP1 +IL-12 +rat IgG

Figure 6.

Weak expression of IL-2Rα chain is long lasting and parallels with T cell tolerance.

(a) Groups of mice (8 per group) were injected i.p. at birth with 100μg Ig-PLP1 in saline and immunized at the indicated weeks with 100μg PLP1 in CFA. Ten days after immunization the mice were sacrificed and splenic cells were in vitro stimulated with PLP1 peptide (15μg/ml) and tested for proliferation (a), IL-2 and IFNγ production (b), and cell surface expression of IL-2Rα chain (c). Proliferation was measured by [^{3}H]thymidine incorporation, and cytokines were assessed by ELISA as described in Methods. Stimulation with PLP2 peptide was included as a negative control in all experiments and no significant response to PLP2 was found (data not shown). The indicated cpm and pg/ml values represent the mean ± SD of individually tested mice. For cell surface analysis of IL-2Rα chain, the cells were double stained with anti-CD4 and anti-CD25 antibodies or anti-CD4 antibody and rat IgG_{2b} and analyzed for CD25 expression by FACS as described in Methods. The histograms were generated by gating on CD4 expression and determining the staining with anti-CD25 antibody or rat IgG_{2b} for isotype control. The results shown in (c) are those obtained at 20 weeks, where T cell tolerance was still apparent, and at 28 weeks, where recovery from tolerance was significant.


a
[3H]Thymidine incorporation (cpm x 10-3)
30
20
10
0
b
pg/ml
600
400
200
0
IL-2
IFNγ
8
12
16
20
28
Weeks post tolerization
c
20 Weeks
28 Weeks
Isotype Control
CD25
0.96%
0.95%
1.9%
7.92%
Counts
FL2-H

PART VI. Concluding Remarks

Recognition of antigens by the immune system during early life induces tolerance rather than immunity. In recent decades it has been well demonstrated that induction of neonatal tolerance leads to immunologic unresponsiveness upon encounter with same antigen, for which various mechanistic explanations have been proposed. Induction of neonatal tolerance in the prevention of autoimmune diseases has been an attractive strategy for clinical application. However, practical limitation and its ill-defined mechanism have been a hurdle for immunologists to overcome.

This study was designed to explore a novel approach for the induction of neonatal tolerance using an Ig molecule as an antigen delivery vehicle. Previous studies have demonstrated the potential of Ig-mediated antigenic delivery and its possibility as a vaccine development. Herein, antigen delivery during the neonatal stage by Igs was tested for the induction of neonatal tolerance in experimental allergic encephalomyelitis (EAE).

To this aim, the encephalitogenic epitope (aa 139-151 of PLP1) from proteolipid protein (PLP), a well defined target autoantigen in EAE, was expressed on the Ig heavy chain in place of the CDR3 region (Ig-PLP1). When Ig-PLP1 was injected into neonates, the recipient mice were resistant to the induction of EAE later in life. The tolerance induced by neonatal injection of Ig-PLP1 was operated by an organ specific immune regulation characterized by lymph node deviation and splenic T cell anergy. Of note, Ig-PLP1 mediated neonatal tolerance does not require the presence of adjuvant, which is key component for tolerance induction using peptide antigens. Due to its efficient peptide presentation and long half-life, it is believed that these characteristics of Ig-PLP1 injected during the early life might induce such a unique neonatal tolerance. Further investigations

were performed to understand the unusual anergic phenotype of splenic T cells found in Ig-PLP1 tolerized mice. It has proven that splenic T cells were unable to upregulate IL-2 receptor α chain, which is a critical component to form a functional cytokine receptor. Exogenous IL-12 restored splenic proliferation and IFNγ production via upregulating IL-2 receptor α chain. Importantly, inability of upregulating functional IL-2 receptor was critical for long persistence of Ig-PLP1 mediated neonatal tolerance.

However, the exact mechanism of IFNγ-mediated splenic T cell anergy is still unknown. Future investigations must be directed to elucidate the cellular events responsible for such immune regulation during the neonatal period.

Vita

Booki Min was born in the southern part of Korea on February 11, 1969. He entered the College of Veterinary Medicine, Seoul National University in 1987. During his college years, he joined the Army service for 30 months as a medic. He then earned a D. V. M. degree in 1994. Right after graduation he worked as a research assistant in the Department of Virology in the Veterinary Research Institute in Korea for a year where he performed a vaccine development research against bovine herpesviruses. In the fall of 1995 he entered the graduate school of Microbiology at the University of Tennessee. He worked on the induction of neonatal tolerance in the EAE model. He is currently seeking a Ph.D. degree in Microbiology, scheduled for completion in the spring of 2000. Booki will be starting his postdoctoral research at Dr. William E. Paul's lab in the National Institutes of Health in July of 2000.