DEVELOPMENT OF PEPTIDES TO TARGET ANTIGEN PRESENTING CELLS FOR CONTROLLING THE IMMUNE RESPONSE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

By

Ahmed Hassan Badawi

B.S., Pharmaceutical Science, University of Missouri - Kansas City, 2007

M.S., Pharmaceutical Chemistry, University of Kansas, 2009

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Dissertation Committee:

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Chairperson   Teruna J. Siahaan

_____

Cory J. Berkland

_____

Jeffrey P. Krise

_____

David B. Volkin

_____

Krzysztof Kuczera

Date Defended: November 18, 2011

The Dissertation Committee for Ahmed Hassan Badawi

certifies that this is the approved version of the following dissertation:

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Development of peptides to target antigen presenting cells for controlling the immune response in experimental autoimmune encephalomyelitis

Ahmed Hassan Badawi

The University of Kansas, 2011

Experimental autoimmune encephalomyelitis (EAE) is an animal model for the human disease multiple sclerosis (MS). In EAE and MS, the immune system recognizes proteins of the myelin sheath as antigenic, and an inflammatory reaction is initiated within the central nervous system (CNS), leading to demyelination of the axons. Current therapies for the treatment of MS are generally non-specific and weaken the global immune system, thus making the individual susceptible to opportunistic infections. The objective of this project is to develop peptides that target myelin-specific antigen presenting cells (APC) in order to modulate the immune response towards the myelin sheath. Bifunctional peptide inhibitors (BPI) are molecules composed of an antigenic peptide and an adhesion peptide that are designed to target the major histocompatibility class-II molecule and adhesion receptors, respectively, on the surface of APC. The simultaneous binding to both receptors on the APC is proposed to hinder the delivery of activation signals to T cells and, therefore, attenuate the inflammatory T cell response. In this study, PLP-BPI, a well-studied BPI molecule, was tested as a peptide vaccine in preventing the onset of EAE as well as for its role in providing protection against blood-brain barrier breakdown during disease. Next, a novel BPI molecule known as PLP-B7AP, which targets costimulatory molecules, was developed and tested for the first time in suppressing EAE. Finally, to provide protection against the diverse pool of antigenic proteins of the myelin sheath, BPI molecules targeting other myelin

antigens as well as a multivalent BPI molecule were developed. These novel peptides have consistently demonstrated a shift towards an immuno-tolerant state accompanied by significant suppression of EAE.

In the name of God, the Most Gracious, the Most Merciful

Dedicated to:

My parents, Hassan Badawi and Samaa El-Alfy

My sisters, Noran, Yomna, and Yasmin

My soul mate, Nadine Aboul-Magd

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CHAPTER 1

Immune modulating peptides for the treatment and suppression of multiple sclerosis

1.1 MULTPLE SCLEROSIS

1.1.1 Disease Introduction

Multiple sclerosis (MS) is the most common immune-mediated disease of the central nervous system. It is characterized by severe demyelination, axonal injury, lesion formation in the brain and spinal cord, blood-brain barrier (BBB) opening, and inflammatory immune cell infiltration.[1] MS is a very heterogeneous disease with very diverse pathological and clinical manifestations. Some of the clinical symptoms include loss of balance and coordination, visual and sensory impairment, fatigue, and cognitive difficulties.[2] The pathogenesis of the disease is not well understood, and there are a multitude of factors that may cause the onset of this disease. Genetic factors may play a major role, and it has been shown that a particular class-II allele of the major histocompatibility complex (MHC) may increase the risk for developing MS.[3,4] Other studies have indicated a correlation between pathogenic infections and the development of the disease. This is believed to be caused by a phenomenon known as molecular mimicry or bystander activation.[5] Some links have been made between various different viruses to MS, such as the Epstein-Barr virus[6,7] and varicella zoster virus,[8] as well as bacterial pathogens such as chlamydia pneumoniae.[9-11] However, there is no direct evidence of the link between pathogenic infections and MS. Currently, the most widely accepted hypothesis is that MS is an autoimmune disease that affects genetically pre-disposed individuals afflicted with an environmental pathogen.[12]

Diagnosis of MS is complicated and unfortunately the majority of the current treatments are non-specific. The most common diagnostic tool for MS is magnetic resonance imaging (MRI). MRI has become a very important tool in diagnosis and monitoring of disease progression and is crucial for devising proper treatment plans. It is used to look for white matter

lesion formation, particularly in the pons and the cerebellum,[13] and the development of "black holes" that are a hallmark of severe demyelination and axonal damage.[14] There are currently eight FDA-approved therapies for the treatment of MS. Four forms of IFN-β therapies are being used for treatment, but their mechanism of action remains unknown.[15] It is believed that they work primarily by inducing an anti-inflammatory response.[16] Another commonly used therapeutic agent is glatiramer acetate (Copaxone), which is a polymer made up of a random mixture of four amino acids (alanine, glutamic acid, lysine, and tyrosine).[17] The proposed mechanism of action of Copaxone is the diversion of the T cell response from type-1 (T_H1) to type-2 helper (T_H2) T cells. Mitoxantrone is an alternative drug that works primarily by inhibiting the proliferation of immune cells.[16] A monoclonal antibody (mAb) called natalizumab (Tysabri) is also being used to treat MS; it binds the α4β1 integrin[18] to inhibit the migration of lymphocytes into the BBB, thus preventing the infiltration of immune cells into the central nervous systems (CNS). Fingolimod (Gilenya), which prevents lymphocytes from exiting the lymph nodes and keeping them at the periphery so they cannot reach the CNS, is the latest FDA-approved drug.[19]

1.1.2. Cellular Mechanisms and Role of Cytokines

The body has protective mechanisms in the thymus to prevent and eliminate any autoreactive T cells by a process known as central tolerance.[20] If autoreactive T cells fail to become tolerant by resident antigen presenting cells (APC) in the thymus, they can escape to the periphery, thus making the individual susceptible for the development of an autoimmune disease. However, the body has back-up protective peripheral-tolerance mechanisms to prevent these autoreactive T cells from proliferating and attacking self-components.[21] In the case of MS, it is

proposed that both the central and peripheral tolerance mechanisms fail to induce tolerance or anergy to myelin-specific T cells. Furthermore, under yet unknown conditions, these myelin-reactive T cells can cross the BBB to enter the CNS via adhesion molecule interactions.[22,23] Once in the CNS, these T cells become re-activated by resident APC such as microglia, macrophages, and dendritic cells (DC) and induce an inflammatory response in the CNS.[20,24] DC play a crucial but contradictory role in the body; they are important both for maintaining peripheral tolerance and inducing an immunogenic response. It has been reported that DC can pick up myelin proteins and present them to T cells in the periphery.[25-27] DC have a strong presence in the inflammatory lesions of MS patients[28] and thus are key players in the reactivation of autoreactive T cells in the CNS.[29] In addition, DC have been implicated in epitope spreading.[30] The contribution of B cells to the development and progress of MS is not very clear. However, studies from phase II clinical trials in MS patients indicate that B cells have a role in the pathogenesis of disease,[31] and myelin-specific antibodies have been found in the cerebrospinal fluid (CSF) of MS patients.[32]

In the past, MS was believed to be solely a CD4$^+$ T_H1 disease; recently, evidence has strongly suggested that CD4$^+$ type-17 T cells (T_H17) have a key role in its pathogenesis.[33] The contribution of T_H17 and/or T_H1 cells to the disease has not been fully elucidated, but the balance between these two T cell subsets has an important role in determining the location of the lesions within the brain.[24] MS is traditionally thought to be purely a CD4$^+$-mediated disease with little appreciation of the contribution of CD8$^+$ T cells. Myelin-specific CD8$^+$ T cells have been found in greater amounts in the lesions of MS patients but not healthy individuals;[34-36] this is unlike myelin-specific CD4$^+$ T cells, which are found in both MS and healthy individuals.[37] In addition, the depletion of CD4$^+$ cells has no affect on disease progression, but depletion of both CD4$^+$ and

CD8^{+} T cells has beneficial effects.[38] Like CD4^{+}, CD8^{+} T cells are activated in the periphery and can cross the BBB under inflammatory conditions. Activation of CD8^{+} in the periphery is accomplished through cross-presentation, which means APC that do not synthesize myelin proteins can present antigens to CD8^{+} T cells in the context of the MHC-I molecule.[39] Activation in the CNS occurs via resident APC, and it still remains unclear which types of APC are involved.[24] CD8^{+} T cells exert their effector function in the CNS through the production of soluble inflammatory mediators as well as direct cell lysis.[24,34,35] Therefore, contributions from both CD4^{+} and CD8^{+} are probably important in the development and pathogenesis of disease, and the different involvement of both T cells is proposed to be the reason behind the broad heterogeneity of the disease.[24] Autoreactive T cells can recognize several proteins of the myelin sheath as antigenic. The most common antigenic proteins in MS patients are myelin basic protein (MBP); myelin proteolipid protein (PLP), which makes up 50% of total myelin protein; and myelin oligodendrocyte glycoprotein (MOG), which is found on the outside of myelin sheath.[5] Identifying these autoantigens has become important for developing antigen-specific therapies as well as for induction of the disease in animal models for studying MS.

Currently, the widely accepted model for T-cell activation and induction of an inflammatory response is the "two-signal" model.[40,41] The model proposes that two signals, an antigen-specific and a "danger" signal, must be delivered to T cells by APC such as an activated or mature DC (mDC). The maturation of an immature DC (iDC) is triggered by the phagocytosis of an insoluble antigen.[42,43] Next, the antigen is broken down into small peptides, processed, and presented by the APC to a T cell via the MHC-II molecule; this is known as Signal 1. During the maturation process, the phenotype of DC changes and expresses costimulatory molecules and adhesion molecules on its surface (**Fig. 1.1**). The presence of the
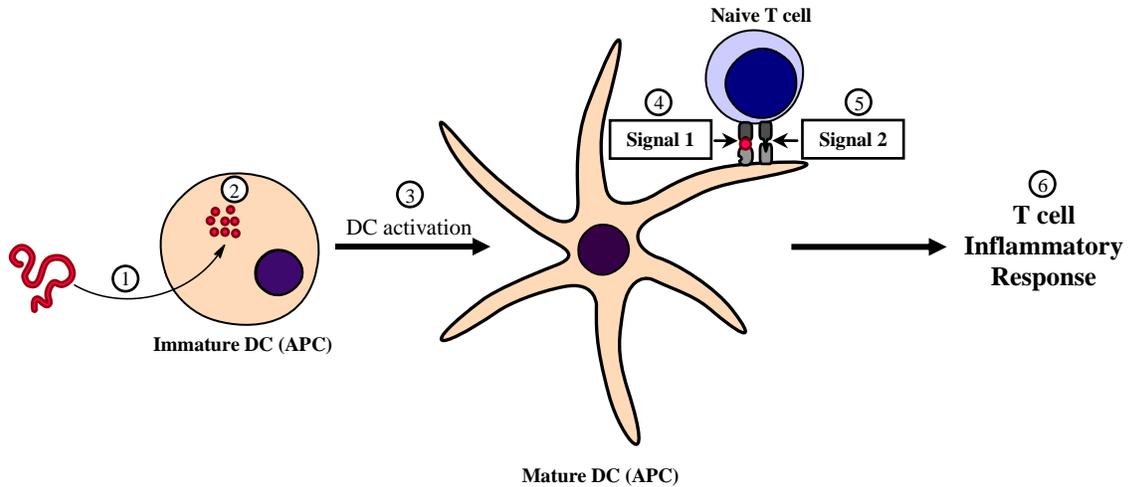
Figure 1.1 Activation of a T cell inflammatory response. 1) A steady-state APC such as an iDC internalizes an insoluble antigen. 2) The antigen is then processed and broken down into immuno-dominant epitopes that can be presented by the MHC-II molecule. 3) Internalization and processing of the antigen triggers the activation of the DC, thus forming a mDC. 4) Presentation of the antigen in the context of the MHC-II molecule is known as Signal 1. 5) mDC expresses costimulatory molecules (e.g. B7/CD28) which deliver Signal 2. 6) The presentation of an antigen in presence of costimulatory signals activates an inflammatory response towards that antigen.

costimulatory molecules, also known as Signal 2, informs the T cell of "danger" and, thus, the T cell differentiates into a pro-inflammatory phenotype to initiate an inflammatory response. One of the most important costimulatory signals is delivered via the B7/CD28 protein interaction and is a positive or activation signal.[44,45] This interaction is vital for the activation of T cells in MS and its animal model, experimental autoimmune encephalomyelitis (EAE). Another costimulatory signal is sent via the B7/CTLA-4 interaction, which is known as an inhibitory (negative) signal to suppress T cell activation.[46] Other well-studied costimulatory molecules for T-cell activation include CD40/CD40L[47] and cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-1 (LFA-1).[48] Following the delivery of Signal 1 and 2, a phenomenon known as the immunological synapse (IS) must take place to complete the activation of T cells.[49,50] The formation of the IS involves the translocation between Signal 1 molecules (TCR/MHC-II-Ag complex) and the adhesion molecules (ICAM-1/LFA-1 complex). Because it is believed that the formation of IS is vital for the activation of T cells, IS could be an important target for developing therapeutics aimed at suppressing the immune response.

All the immune responses involved in the pathogenesis and treatment of MS are mediated via a complex network of cytokines. During steady-state conditions (i.e., homeostasis), there is a balance between pro- and anti-inflammatory cytokines. In MS, inflammatory cytokines are responsible for the pathogenesis of the disease in the periphery as well as within the CNS. The function of each cytokine has not been fully elucidated due to the dynamic network and complex nature of the cytokine milieu. For the development and progression of disease, the pro-inflammatory cytokines are key players. There are several cytokines involved in the inflammatory response, particularly T_H1 cytokines such as IL-12, IFN-γ, and TNF-α as well as

T_H17 cytokines such as IL-23 and IL-17.[24] The exact contribution of each of these cytokines remains unclear and difficult to sort out. The involvement of IL-12 and IFN-γ was established by their heightened expression in the CNS and CSF of MS patients with increased clinical activity.[51] In addition, the roles of TNF-α and IFN-γ were determined when peripheral blood mononuclear cells (PBMC) isolated from MS patients secreted significant amounts of them.[52-57] IL-17 transcripts were found in CNS lesions of MS patients, thus indicating a major role of IL-17 in disease pathogenesis.[33] Immunotolerance is believed to be maintained by a group of suppressor (T_H2) and regulatory T cells (T_{reg}) that produce anti-inflammatory cytokines such as IL-2, IL-4, and IL-10.[58] During the disease state, it has been reported that PBMC isolated from MS patients secrete no or low amounts of the anti-inflammatory cytokines.[58] Moreover, during ongoing disease there is a shift towards the production of pro-inflammatory cytokines. Therefore, a major strategy for treating an inflammatory disease like MS is shifting the balance towards the production of anti-inflammatory cytokines such as the ones secreted by T_{reg} and T_H2 cells (**Fig. 1.2**).

1.1.3 Experimental Autoimmune Encephalomyelitis

The EAE animal model is used to study the underlying disease pathogenesis of MS and develop new therapies. EAE can be induced either by adoptive transfer of myelin-specific T cells or by the administration of a CNS homogenate or specific myelin proteins/peptides in the presence of an adjuvant such as complete Freund's adjuvant (CFA).[59] More recently, spontaneous models of EAE have also been developed.[60] The EAE model mimics MS in several ways such as the development of multiple CNS lesions, destruction of the myelin sheath, and the breakdown of the BBB. Similarly to MS, various immune cells are involved in the disease
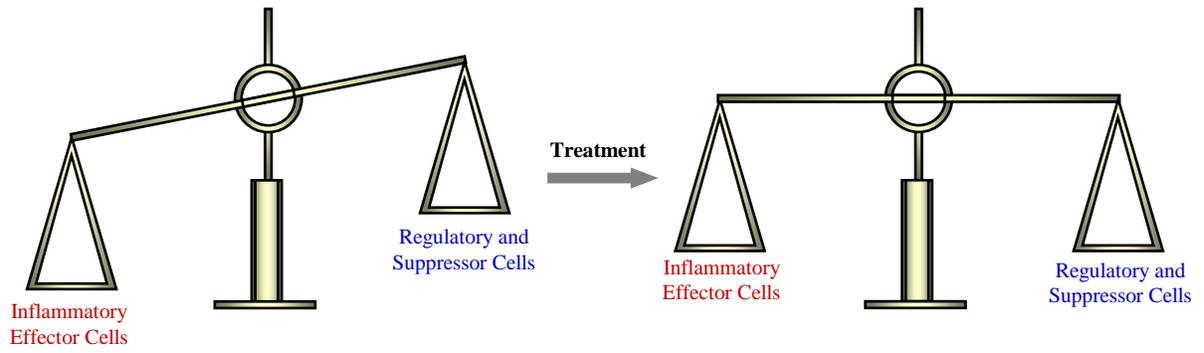
Figure 1.2 During MS and EAE, the inflammatory immune response overcomes regulatory functions. Immune modulating treatments should restore tolerance by promoting a shift in the balance towards regulatory and suppressor immune responses.

pathogenesis. Macrophages, microglia cells, DC, B-cell antibodies, and both CD4$^+$ and CD8$^+$ have vital roles in the development of the inflammatory response and tissue destruction.[61] The model has been very useful in studying the mode of action of four therapies currently on the market for the treatment of MS such as glatiramer acetate (Copaxone),[62] mitoxantrone,[63] natalizumab (Tysabri),[64] and, most recently, fingolimod (Gilenya).[65-69] It is important to recognize the limitations of the animal model as most successes in that model did not translate to humans.[70] In addition, many of the adverse side effects observed in clinical trials, from therapies initially tested in EAE, could not have been predicted from the animal model.[71] No one model of EAE mimics the heterogeneous pathology of MS and, therefore, more work must be done in order to more closely mimic the human disease. Nevertheless, EAE played a key role in understanding many pathogenic aspects of the disease and led to the development of four important MS therapeutics; thus its contribution in the past must not be undermined. For these reasons, the EAE animal model is continuously being used to test and develop new therapies for MS.

1.2 PEPTIDE TREATMENTS FOR MS

Most of the current therapies for MS do not regulate specific immune cells and they normally suppress the general immune response, which leads to many adverse side effects from opportunistic infections. Thus, there is a need to develop therapeutic agents that specifically control the myelin-reactive immune response for maintaining host capability to protect against foreign pathogens provided by the general immune response. Peptides are excellent specific inhibitors of protein-protein interactions and, therefore, are valuable specific modulators of protein-mediated signaling of the immune system. In this section, many of the current myelin-

specific peptides being tested for the treatment of MS will be discussed. In addition, important advances in the development of non-specific peptides that have efficacy in the EAE animal model will be discussed.

1.2.1. Antigenic Peptides

Specific immunotherapy (SIT) has been used for about a century to induce tolerance for the treatment of allergies such as hay fever[72] and, more recently, seasonal allergic rhinitis,[73] asthma,[74] bee venom,[75] peanut,[76] cow milk,[77] and birch pollen.[78] The strategy behind allergen- or antigen-SIT is to administer the antigenic protein/peptide in a proper dose to modulate the immune response and reduce the immunogenicity towards a particular allergen/antigen.[79] The goal of SIT is to induce T cell anergy, activate T_{reg}, or promote a shift from a T_H1 phenotype to T_H2 phenotype.[80] Translating this strategy for inducing tolerance to treat autoimmune diseases has been the focus of many research groups. In this section, the successes of antigenic-SIT in the MS animal model and difficulties in applying the technology to humans will be discussed. In addition, some of the mechanistic aspects of this therapy will be discussed.

Tolerance induction via the mucosal route has been studied extensively in the EAE model. There are numerous studies showing that oral administration of myelin proteins or peptides is an effective way for inducing tolerance, by causing either T cell clonal anergy or induction of the regulatory immune response. It is reported that this depends on the dose of the administered antigen.[81-83] The attractive aspect of the oral route is that it mimics naturally induced tolerance to ingested antigens (with the exception of food allergies), in addition to its ease of administration. Studies reporting suppression of disease with whole proteins has been reported[84,85] and, more importantly, there are numerous studies showing that induction of

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tolerance to suppress EAE can be achieved using small protein fragments and peptides. In one study, MBP fragments (1-37, 44-89, and 90-170) suppressed the disease significantly.[86] The oral administration of guinea pig-MBP$_{68-88}$ suppressed rat-MBP$_{68-88}$-induced EAE in Lewis rats.[87] Other reports showed that MBP and MBP peptide suppressed PLP-induced EAE, suggesting that bystander suppression is possible via the oral route.[88] Lastly, another study showed that feeding animals with PLP$_{139-151}$ peptide induced T-cell clonal anergy and prevented the onset of EAE.[89] Unfortunately, the success in the EAE animal model could not be translated to MS patients. One phase-III clinical trial conducted to test the efficacy of orally administered bovine-myelin containing MBP and PLP showed no significant difference between the treatment and placebo groups [reviewed in ref. 90]. Thus, even though studies conducted in humans have proven that administration of antigen via the oral route is a safe method, no studies have reported any significant benefit so far. The other mucosal route used to deliver antigens is nasal administration. Studies using MBP whole protein,[91,92] MBP peptides,[93] and a mixture of myelin peptides (PLP$_{139-151}$, MBP$_{1-11}$, MBP$_{89-101}$)[94] have induced peripheral tolerance and prevented the onset of EAE but, similar to the oral route, no significant benefit in humans has been reported.

Other routes that have been more successful in attenuating MS and EAE were intravenous (i.v.) and transdermal administration. There have been several reports indicating the successful suppression of EAE after i.v. administration of MOG (41-60) and MBP peptides[95] and whole MBP.[96,97] When the MBP$_{82-98}$ peptide fragment was tested in MS patients, it generally reduced anti-MBP antibodies and significantly delayed the progression of disease in a particular sub-group of MS patients with the HLA haplotype DR2/DR4.[98] Another study indicated that i.v. administration of MBP$_{85-96}$, but not intrathecal or subcutaneous administration, led to undetectable amounts of MBP autoantibodies in the CSF for several months post-treatment.[99]

More recently, transdermal delivery of myelin antigens has shown some clinical benefit following the success observed in EAE. MBP_{Ac1-11}[100] and whole MBP[101] delivered transdermally protected mice from developing EAE. A small study conducted in patients diagnosed with relapsing-remitting MS was performed to test the immunological modulation caused by a mixture of three peptides (MBP_{85-99}, MOG_{35-55}, and $PLP_{139-151}$) via an adhesive skin patch. Myelin-specific T cell responses were completely eliminated after only four months of treatment.[102] In addition, there was an up-regulation in the production of IL-10 and a down-regulation of TGF-β and IFN-γ in the MS patients, indicating a shift towards an immunotolerant state. These results are promising and may show clinical efficacy if tested on a larger scale. So far, translating efficacy from the EAE animal model to MS treatment has proven to be a difficult task. This is probably due to the complexity and heterogeneity of human autoimmune diseases. Many factors must be considered when trying to apply antigenic-SIT for the treatment of human autoimmune disease such as dosing amount and frequency, route of administration, and specificity of antigens administered.

As described previously, the inflammatory response is initiated by a mDC due to exposure to an insoluble antigen. The uptake and processing of an insoluble antigen leads to the activation of a DC and the presentation of the antigen in presence of costimulatory molecules, thus inducing an inflammatory response. The immunological basis for antigenic-peptide therapy is that when the peptide is given in a soluble state, it binds directly to empty MHC-II molecules on the surface of iDC.[103] Because iDC do not have surface costimulatory molecules, the presentation of antigen by MHC-II on iDC in the absence of costimulatory signal(s) causes the naïve T cells to differentiate to regulatory T cells after their interactions with antigen-presenting iDC.[43] Activation and proliferation of T_{reg} cells influences the balance of the immune response

to restore tolerance by shifting from an effector T cell response (T_H1) to an immune-suppressor (T_H2) or an immune-regulatory response (**Fig. 1.3**).

1.2.2 Altered Peptide Ligands

Altered peptide ligands (APL) are another group of peptides that are proposed to cause antigen-specific immunosuppression. These are molecules that are similar in sequence to native peptides with one or more amino acid modification(s) and can bind to MHC-II molecules and engage with the TCR to alter or inhibit the delivery of signal to the T cell. Thus, these molecules act as antagonists to produce T cell anergy or as partial agonists to produce incomplete activation of T cells. Incomplete activation of T cells will cause a shift from a pro-inflammatory T cell response (T_H1 and T_H17) to a regulatory/suppressor T cell response (T_H2 and T_H3).[104] APL with sequence modifications in MBP_{1-9},[105] MBP_{87-99},[106,107] and $PLP_{139-151}$[108-112] have been shown to attenuate disease in the EAE model. In phase I clinical trial, an APL from MBP_{83-99} showed a T_H2 bias and produced anti-inflammatory cytokines; the peptide was well-tolerated by the patients in this trial.[113] However, when this APL was tested in two separate phase II clinical trials, there was no significant clinical benefit seen in treated patients.[114,115] In one of the clinical trials, there was no difference observed in the small group of treated patients and the study was terminated due to adverse side effects from the treatment; in addition 3 of 8 patients experienced exacerbations of disease.[114] In the other clinical trial, hypersensitivity reactions were also present and no clinical differences between the APL-treated and the placebo groups were observed, albeit there was a reduction in the number and volume of gadolinium-enhanced CNS lesions.[115]

Naive T cell

Signal 1

Signal 2 absent

T cell Regulatory Response

**Immature DC (APC)**

Immature DC (APC)
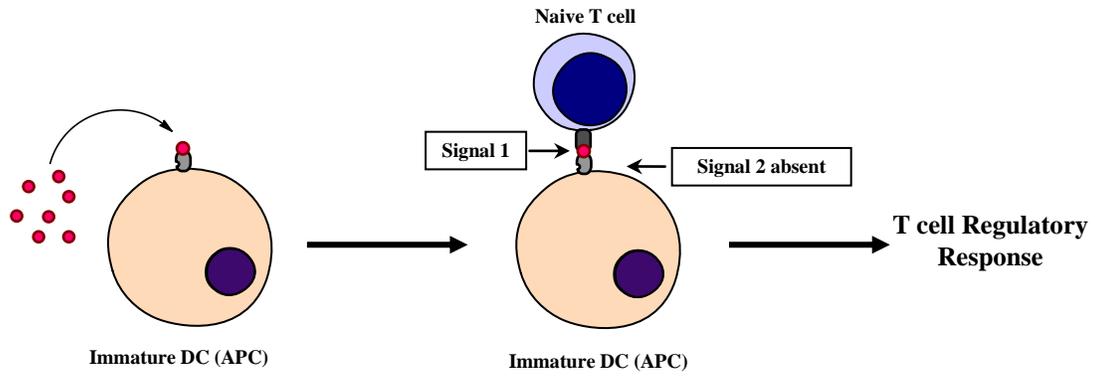
Figure 1.3 Administration of soluble peptide antigens induces a T cell regulatory immune response. Soluble peptides can bind directly to empty MHC-II molecules on the surface of iDC avoiding internalization and processing of the antigen. Presentation of the antigen in absence of Signal 2 by an iDC leads to an antigen-specific regulatory response.

Glatiramer acetate (Copaxone) is a random polymer of four amino acids (poly(YEAK)$_n$) that has been shown to modulate the immune response by competing with MBP epitopes for MHC binding as well as causing TCR antagonism.[116] Therefore, it is the only APL on the market for the treatment of MS. Following the success of Copaxone, similar molecules have been developed and tested in the EAE model. A poly(EYYK)$_4$ peptide that was developed to bind to the MHC-II binding pocket was shown to inhibit EAE in Lewis rats.[117] Other molecules such as poly(FYAK)$_n$ and poly(VWAK)$_n$ also ameliorated both MBP$_{85-99}$- and PLP$_{139-152}$-induced EAE in mice.[118,119] However, one study indicated that Copaxone has no beneficial effects on disease progression and the risk of developing relapses; and therefore, its clinical use may be questionable.[120] It should also be noted that the efficacy observed from these short amino acid polymers in the animal model may not be translatable to humans.

1.2.3 Bifunctional Peptide Inhibitors

Our group has developed a novel group of bifunctional peptide inhibitors (BPI), which target APC and are proposed to selectively inhibit an immunogenic response towards a specific antigen. BPI molecules are composed of an antigenic peptide covalently linked to an adhesion peptide.[121] It is proposed that the antigenic peptide fragment of the BPI molecule binds to MHC-II molecules and the adhesion peptide binds simultaneously to an adhesion protein on the surface of the APC. The linker is made up of either aminocaproic acid or polyethelene glycol (PEG) to ensure simultaneous binding of the antigenic peptide portion as well as the adhesion peptide (LABL) to their respective receptors on the surface of the APC. The original length of the linker was estimated upon docking of the antigenic peptide and LABL peptide to X-ray structures of MHC-II[122] and ICAM-1, respectively.[123-125] As mentioned earlier, a step necessary for the

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activation of a pro-inflammatory T cell response is the formation of the immunological synapse, which occurs at the interface of APC and T cells and is the translocation of Signal 1 and adhesion proteins.[40,41,49,50] The hypothesis is that BPI molecules bind to both MHC-II (Signal 1) and ICAM-1 (adhesion protein) on the surface of APC to tether both molecules and prevent the formation of the immunological synapse, thus altering the differentiation and proliferation of T cells from an inflammatory to a regulatory phenotype.

Several BPI molecules consisting of various antigens and adhesion peptides have been developed for the suppression of autoimmune diseases in animal models. A GAD-BPI molecule composed of $GAD_{208-217}$ and LABL peptides suppressed Type-1 diabetes in the non-obese diabetes mouse model.[126] GAD-BPI significantly suppressed insulitis and lowered blood glucose levels compared to control. Currently, CII-BPI composed of a collagen-II antigenic peptide ($CII_{256-270}$, $CII_{707-721}$, or $CII_{1237-1249}$) conjugated to LABL peptide attenuated clinical signs of rheumatoid arthritis in the collagen-II-induced model (unpublished data). More importantly, PLP-BPI, composed of $PLP_{139-151}$ conjugated to LABL, was the first BPI molecule to suppress EAE and modulate the immune response by increasing the proliferation of TGF-β-, IL-4-, and IL-10-producing $CD4^+CD25^+$ T cells, indicating a shift towards a suppressor and regulatory immune response.[127-129] Other studies with PLP-BPI showed that it can also suppress disease when injected three times (s.c.), or when dosed in a controlled release fashion.[130] Current studies prove that PLP-BPI is effective when administered prior to induction of disease, or even after the appearance of clinical signs. Recently, PLP-cIBR, which contains cIBR7 peptide from the D1 domain of ICAM-1, was shown to be more potent than the parent PLP-BPI. A new MOG-BPI molecule composed of MOG_{38-50} can suppress MOG-induced EAE in the mouse model. Finally, a multivalent BPI molecule composed of both MOG_{38-50} and $PLP_{139-151}$ has been shown to

suppress disease significantly in both MOG_{38-50}- and $PLP_{139-151}$-induced EAE. The value of the multivalent BPI molecule is that it can suppress disease regardless of the inciting antigen as well as attenuate new antigenic responses created by epitope spreading.

In summary, BPI molecules have excellent efficacy in suppressing EAE and other autoimmune diseases in animal models. Current studies indicate that BPI molecules down-regulate the production of pro-inflammatory cytokines and increase the production of regulatory cytokines. These results suggest that BPI molecules promote a shift towards a regulatory and suppressor immune response. However, more studies need to be done to elucidate the mechanisms of action of BPI molecules.

1.2.4 Other Peptides

A novel group of non-antigen-specific peptide inhibitors which bind to B7 on the surface of T cells and prevent the delivery of the costimulatory signal are derived from the sequence of the CD28 costimulatory protein on the surface of APC.[44,45] The presentation of an antigen in the absence of a costimulatory signal will lead to T cell anergy, therefore inhibiting the inflammatory response (**Fig. 1.4**). Peptides derived from the conserved region of CD28 containing the motif MYPPPY bind to B7 and have suppressed EAE in B10.PL mice.[131] A similar but shorter peptide that showed efficacy in prolonging cardiac allograft rejection[132] was tested in our lab, and results indicated significant suppression of $PLP_{139-151}$-induced EAE in SJL/J mice (unpublished data).

Another approach to suppressing the immune response is targeting the CD4 molecule on the surface of $CD4^+$ T cells. $CD4^+$ T cells are known to have a key role in the pathogenesis of disease and, therefore, preventing their activation would be a valuable target for attenuating any

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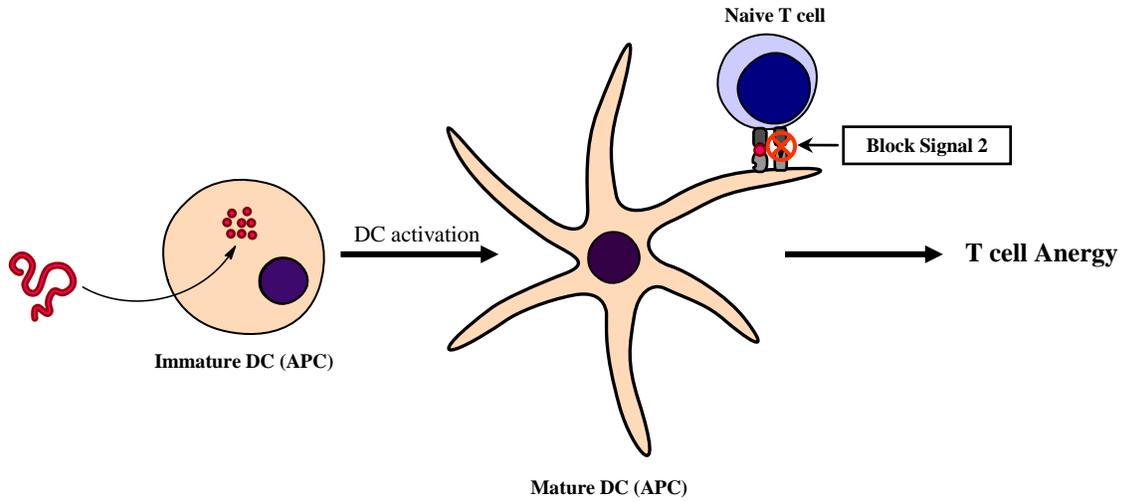
Figure 1.4 Presentation of antigen with Signal 2 blockade causes improper activation of T cells thus leading to T cell anergy.

CD4$^+$-mediated immune response such as in MS. A cyclic peptide complementary to the CDR3-like region of CD4[133] and another peptide designed based on the D1-CC' loop region[134] were developed and found to suppress EAE effectively. Another immunomodulatory peptide known as RDP58 inhibits T$_H$1 cytokines[135] as well as upregulates heme-oxygenase-1.[136,137] It has been shown that heme-oxyhenase-1 has a protective role in EAE;[138,139] therefore, when combined with the inhibition of T$_H$1 cytokines, RDP58 significantly lowered the incidence of EAE in Lewis rats.[140]

Recently, new peptides have been developed for the treatment of MS by evaluating them in EAE animal models. First, IIIM1 is a 9-amino acid peptide derived from histone H2A$_{36-44}$ that possesses anti-inflammatory activity and suppressed MOG- and PLP-induced EAE.[141,142] When administered orally, this peptide reduced the production of pro-inflammatory cytokines such as IL-17, IFN-γ, IL12, and IL-23 and promoted T$_{reg}$ cell proliferation accompanied by an increase in TGF-β and IL-10 production. Secondly, four peptides that bind to the first two extracellular loops (ECL1 and ECL2) of the CC chemokine receptor 5 (CCR5) have been shown to significantly reduce the infiltration of monocytes and lymphocytes into the spinal cord and attenuated EAE in mice.[143] CCR5 has been shown to contribute significantly to the pathogenesis of disease by its role in the activation and migration of leukocytes.[144] Peptides targeting CCR5 have a mechanism of action similar to that of Tysabri, a monoclonal antibody used for the treatment of MS.[145] Thirdly, glucocorticoid-induced leucine zipper- (GILZ) peptides that bind to nuclear factor-kappa B (NF-κB) can modulate T-cell activation and induce an anti-inflammatory immune response to suppress the progression of EAE in mice.[146] GILZ peptides were derived from the binding sequence of GILZ to the p65 subunit of NF-κB.[146] GILZ-peptides inhibit the function of NF-κB and suppress the activation of inflammatory cytokines.[147] Finally, it has

recently been proposed that treatment of MS can be achieved by modulating toll-like receptors (TLR) because TLR play an integral part in the development of MS and EAE.[148-151] Gambuzza et al. described different types of TLR that are involved in progression of MS and EAE and illustrated several peptides that modulate TLR and can potentially suppress disease.[151]

1.3 SAFETY CONCERNS

A major safety concern involving antigen or antigen-derived therapies is the risk of developing anaphylaxis, which is a severe hypersensitivity reaction. Two clinical trials with an APL were terminated due to hypersensitivity reactions that developed in the patients.[114,115] An anaphylactic reaction can occur from the initial burst of immune cell activation and proliferation accompanied by a storm of cytokine release. The generally accepted mechanism for induction of anaphylaxis is due to the release of inflammatory mediators that are triggered by cross-linking of IgE molecules bound to FcεRI on mast cells. This can lead to life-threatening symptoms such as tissue edema, leukocyte recruitment, excessive mucous production, and bronchoconstriction.[152,153] Anaphylaxis has been observed in numerous EAE models after treatment with myelin peptides,[154,155] but when the peptides were administered in combination with an anti-IgE antibody, onset of anaphylaxis was inhibited. The route of administering the peptides plays a major role in mitigating the risk of developing hypersensitivity reactions. It is thought that i.v. injections have the greatest risk for developing anaphylaxis since the antigen becomes accessible to the systemic circulation immediately. S.c. and intradermal injections are believed to have a lower incidence of anaphylaxis, and mucosal administration is the safest.[104] It should be noted, however that induction of EAE by priming with myelin peptide in the presence of CFA leads to the production of IgE molecules,[156] thus fostering a hypersensitivity response.

This is in contrast to what occurs in MS patients, in which there is production of IgG antibodies.[99] To prevent side effects, Wraith et al. suggested that antigenic peptides could be delivered in a fashion similar to the way that allergens are delivered for the treatment of allergies.[79] In this case, the antigenic peptide should be administered by gradually increasing the dose to avoid rapid induction of anergy or activation of T_{reg} that leads to side effects.

1.4 CONCLUSION

MS pathogenesis is very complex, involving many different branches of the immune system, and still remains to be fully elucidated. Current treatments for MS are generally non-specific, leading to suppression of the general immune response to fight pathogenic infections. Therefore, there is a need to develop more antigen-specific treatments that avoid this general suppression. Recently, antigen-specific treatments such as antigenic peptides, APL, and bifunctional peptide inhibitors have been very successful in suppressing EAE in animal models. Unfortunately, many of these successes in animal models have not been yet translated to humans in treating MS; this is partly due to the generation of hypersensitivity reactions upon treatment with the antigenic peptides. In addition, the mechanisms of action of antigenic peptides and their derivatives in suppressing autoimmune diseases such as EAE and MS are not yet fully understood. Thus, more research needs to be done to elucidate their mechanisms of action and delineate why these antigenic peptides and their derivatives induce side effects such as hypersensitivity reactions. It has been shown that the method of delivery and dosing schedule could reduce side effects. In the future, studies performed to develop novel delivery methods and dosing schedules of antigenic peptide therapies will be carried out to improve the efficacy and safety profiles of peptide therapies for MS.

1.5 REFERENCES

1   Lassmann, H. Classification of demyelinating diseases at the interface between etiology and pathogenesis. *Curr Opin Neurol* **14**, 253-258 (2001).

2   McFarland, H. F. & Martin, R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* **8**, 913-919 (2007).

3   Compston, A. The genetic epidemiology of multiple sclerosis. *Philos Trans R Soc Lond B Biol Sci* **354**, 1623-1634 (1999).

4   Lincoln, M. R. *et al.* A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. *Nat Genet* **37**, 1108-1112 (2005).

5   Sospedra, M. & Martin, R. Immunology of multiple sclerosis. *Annu Rev Immunol* **23**, 683-747 (2005).

6   Pender, M. P. Does Epstein-Barr virus infection in the brain drive the development of multiple sclerosis? *Brain* **132**, 3196-3198 (2009).

7   Bagert, B. A. Epstein-Barr virus in multiple sclerosis. *Curr Neurol Neurosci Rep* **9**, 405-410 (2009).

8   Gilden, D. H. Infectious causes of multiple sclerosis. *Lancet Neurol* **4**, 195-202 (2005).

9   Gaydos, C. A. Chlamydia pneumoniae and its proposed link to multiple sclerosis: to be or not to be? *Neurology* **56**, 1126-1127 (2001).

10   Jacobson, S. & Cross, A. Association of Chlamydia pneumoniae and multiple sclerosis: stage two? *Neurology* **56**, 1128-1129 (2001).

11   Yao, S. Y., Ljunggren-Rose, A., Stratton, C. W., Mitchell, W. M. & Sriram, S. Regulation by IFN-beta of inducible nitric oxide synthase and interleukin-12/p40 in

murine macrophages cultured in the presence of Chlamydia pneumoniae antigens. *J Interferon Cytokine Res* **21**, 137-146 (2001).

12   Kenealy, S. J., Pericak-Vance, M. A. & Haines, J. L. The genetic epidemiology of multiple sclerosis. *J Neuroimmunol* **143**, 7-12 (2003).

13   Ormerod, I. E. *et al.* The role of NMR imaging in the assessment of multiple sclerosis and isolated neurological lesions. A quantitative study. *Brain* **110 (Pt 6)**, 1579-1616 (1987).

14   van Walderveen, M. A. *et al.* Histopathologic correlate of hypointense lesions on T1-weighted spin-echo MRI in multiple sclerosis. *Neurology* **50**, 1282-1288 (1998).

15   Yong, V. W., Chabot, S., Stuve, O. & Williams, G. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. *Neurology* **51**, 682-689 (1998).

16   Derwenskus, J. Current disease-modifying treatment of multiple sclerosis. *Mt Sinai J Med* **78**, 161-175 (2011).

17   Arnon, R. The development of Cop 1 (Copaxone), an innovative drug for the treatment of multiple sclerosis: personal reflections. *Immunol Lett* **50**, 1-15 (1996).

18   Ransohoff, R. M. Natalizumab for multiple sclerosis. *N Engl J Med* **356**, 2622-2629 (2007).

19   Horga, A., Castillo, J. & Montalban, X. Fingolimod for relapsing multiple sclerosis: an update. *Expert Opin Pharmacother* **11**, 1183-1196 (2010).

20   Stoeckle, C. & Tolosa, E. Antigen processing and presentation in multiple sclerosis. *Results Probl Cell Differ* **51**, 149-172 (2010).

21   Goodnow, C. C., Sprent, J., Fazekas de St Groth, B. & Vinuesa, C. G. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* **435**, 590-597 (2005).

22   Butcher, E. C. & Picker, L. J. Lymphocyte homing and homeostasis. *Science* **272**, 60-66 (1996).

23   Ransohoff, R. M., Kivisakk, P. & Kidd, G. Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol* **3**, 569-581 (2003).

24   Goverman, J. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* **9**, 393-407 (2009).

25   Marty, M. C. *et al.* The myelin basic protein gene is expressed in differentiated blood cell lineages and in hemopoietic progenitors. *Proc Natl Acad Sci U S A* **99**, 8856-8861 (2002).

26   Karman, J., Ling, C., Sandor, M. & Fabry, Z. Initiation of immune responses in brain is promoted by local dendritic cells. *J Immunol* **173**, 2353-2361 (2004).

27   Hatterer, E., Touret, M., Belin, M. F., Honnorat, J. & Nataf, S. Cerebrospinal fluid dendritic cells infiltrate the brain parenchyma and target the cervical lymph nodes under neuroinflammatory conditions. *PLoS One* **3**, e3321 (2008).

28   Serafini, B. *et al.* Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. *J Neuropathol Exp Neurol* **65**, 124-14 (2006).

29   Greter, M. *et al.* Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat Med* **11**, 328-334 (2005).

30   McMahon, E. J., Bailey, S. L., Castenada, C. V., Waldner, H. & Miller, S. D. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med* **11**, 335-339 (2005).

31   Hauser, S. L. *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* **358**, 676-688 (2008).

32   Kabat, E. A., Freedman, D. A. & et al. A study of the crystalline albumin, gamma globulin and total protein in the cerebrospinal fluid of 100 cases of multiple sclerosis and in other diseases. *Am J Med Sci* **219**, 55-64 (1950).

33   Lock, C. *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* **8**, 500-508 (2002).

34   Goverman, J., Perchellet, A. & Huseby, E. S. The role of CD8(+) T cells in multiple sclerosis and its animal models. *Curr Drug Targets Inflamm Allergy* **4**, 239-245 (2005).

35   Friese, M. A. & Fugger, L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? *Brain* **128**, 1747-1763 (2005).

36   Junker, A. *et al.* Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain* **130**, 2789-2799 (2007).

37   Crawford, M. P. *et al.* High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood* **103**, 4222-4231 (2004).

38   Coles, A. J. *et al.* The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy. *J Neurol* **253**, 98-108 (2006).

39   Perchellet, A., Brabb, T. & Goverman, J. M. Crosspresentation by nonhematopoietic and direct presentation by hematopoietic cells induce central tolerance to myelin basic protein. *Proc Natl Acad Sci U S A* **105**, 14040-14045 (2008).

40   Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).

41   Tseng, S. Y. & Dustin, M. L. T-cell activation: a multidimensional signaling network. *Curr Opin Cell Biol* **14**, 575-580 (2002).

42   Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* **192**, 1213-1222 (2000).

43   Larche, M. & Wraith, D. C. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med* **11**, S69-76 (2005).

44   Jenkins, M. K. & Johnson, J. G. Molecules involved in T-cell costimulation. *Curr Opin Immunol* **5**, 361-367 (1993).

45   June, C. H., Bluestone, J. A., Nadler, L. M. & Thompson, C. B. The B7 and CD28 receptor families. *Immunol Today* **15**, 321-331 (1994).

46   McCoy, K. D. & Le Gros, G. The role of CTLA-4 in the regulation of T cell immune responses. *Immunol Cell Biol* **77**, 1-10 (1999).

47   Schonbeck, U. & Libby, P. The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci* **58**, 4-43 (2001).

48   Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med* **181**, 577-584 (1995).

49   Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-1542 (2002).

50   van der Merwe, P. A. Formation and function of the immunological synapse. *Curr Opin Immunol* **14**, 293-298 (2002).

51   Gutcher, I. & Becher, B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest* **117**, 1119-1127 (2007).

52   Cannella, B. & Raine, C. S. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* **37**, 424-435 (1995).

53   Hirsch, R. L., Panitch, H. S. & Johnson, K. P. Lymphocytes from multiple sclerosis patients produce elevated levels of gamma interferon in vitro. *J Clin Immunol* **5**, 386-389 (1985).

54   Maimone, D., Reder, A. T. & Gregory, S. T cell lymphokine-induced secretion of cytokines by monocytes from patients with multiple sclerosis. *Cell Immunol* **146**, 96-106 (1993).

55   Merrill, J. E., Strom, S. R., Ellison, G. W. & Myers, L. W. In vitro study of mediators of inflammation in multiple sclerosis. *J Clin Immunol* **9**, 84-96 (1989).

56   Selmaj, K., Raine, C. S., Cannella, B. & Brosnan, C. F. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J Clin Invest* **87**, 949-954 (1991).

57   Ozenci, V., Kouwenhoven, M., Huang, Y. M., Kivisakk, P. & Link, H. Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-alpha)- and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-beta) treatment. *Clin Exp Immunol* **120**, 147-153 (2000).

58   Mouzaki, A., Tselios, T., Papathanassopoulos, P., Matsoukas, I. & Chatzantoni, K. Immunotherapy for multiple sclerosis: basic insights for new clinical strategies. *Curr Neurovasc Res* **1**, 325-340 (2004).

59   Racke, M. K. Experimental autoimmune encephalomyelitis (EAE). *Curr Protoc Neurosci* **Chapter 9**, Unit9 7 (2001).

60   Waldner, H., Whitters, M. J., Sobel, R. A., Collins, M. & Kuchroo, V. K. Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic for the

myelin proteolipid protein-specific T cell receptor. *Proc Natl Acad Sci U S A* **97**, 3412-3417 (2000).

61   Gold, R., Linington, C. & Lassmann, H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* **129**, 1953-1971 (2006).

62   Teitelbaum, D., Meshorer, A., Hirshfeld, T., Arnon, R. & Sela, M. Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur J Immunol* **1**, 242-248 (1971).

63   Lublin, F. D., Lavasa, M., Viti, C. & Knobler, R. L. Suppression of acute and relapsing experimental allergic encephalomyelitis with mitoxantrone. *Clin Immunol Immunopathol* **45**, 122-128 (1987).

64   Yednock, T. A. *et al.* Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* **356**, 63-66 (1992).

65   Chiba, K. *et al.* Fingolimod (FTY720), sphingosine 1-phosphate receptor modulator, shows superior efficacy as compared with interferon-beta in mouse experimental autoimmune encephalomyelitis. *Int Immunopharmacol* **11**, 366-372 (2011).

66   Chun, J. & Hartung, H. P. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. *Clin Neuropharmacol* **33**, 91-101 (2010).

67   Papadopoulos, D. *et al.* FTY720 ameliorates MOG-induced experimental autoimmune encephalomyelitis by suppressing both cellular and humoral immune responses. *J Neurosci Res* **88**, 346-359 (2010).

68   Kataoka, H. *et al.* FTY720, sphingosine 1-phosphate receptor modulator, ameliorates experimental autoimmune encephalomyelitis by inhibition of T cell infiltration. *Cell Mol Immunol* **2**, 439-448 (2005).

69   Fujino, M. *et al.* Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. *J Pharmacol Exp Ther* **305**, 70-77 (2003).

70   Baker, D., Gerritsen, W., Rundle, J. & Amor, S. Critical appraisal of animal models of multiple sclerosis. *Mult Scler* **17**, 647-657 (2011).

71   Baxter, A. G. The origin and application of experimental autoimmune encephalomyelitis. *Nat Rev Immunol* **7**, 904-912 (2007).

72   Noon, L. Prophylactic inoculation against hay fever. Historical document. *Ann Allergy* **18**, 287-291 (1960).

73   Durham, S. R. *et al.* Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* **341**, 468-475 (1999).

74   Bousquet, J. *et al.* Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. American academy of Allergy, Asthma and Immunology. *Ann Allergy Asthma Immunol* **81**, 401-405 (1998).

75   Akdis, C. A. *et al.* Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* **98**, 1676-1683 (1996).

76   Jones, S. M. *et al.* Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* **124**, 292-300, 300 e291-297 (2009).

77   Skripak, J. M. *et al.* A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. *J Allergy Clin Immunol* **122**, 1154-1160 (2008).

78   Mobs, C. *et al.* Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* **184**, 2194-2203 (2010).

79   Sabatos-Peyton, C. A., Verhagen, J. & Wraith, D. C. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol* **22**, 609-615 (2010).

80   McDevitt, H. Specific antigen vaccination to treat autoimmune disease. *Proc Natl Acad Sci U S A* **101 Suppl 2**, 14627-14630 (2004).

81   Friedman, A. & Weiner, H. L. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci U S A* **91**, 6688-6692 (1994).

82   Faria, A. M. & Weiner, H. L. Oral tolerance: mechanisms and therapeutic applications. *Adv Immunol* **73**, 153-264 (1999).

83   Weiner, H. L. *et al.* Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* **12**, 809-837 (1994).

84   Bitar, D. M. & Whitacre, C. C. Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *Cell Immunol* **112**, 364-370 (1988).

85   Meyer, A. L., Benson, J. M., Gienapp, I. E., Cox, K. L. & Whitacre, C. C. Suppression of murine chronic relapsing experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *J Immunol* **157**, 4230-4238 (1996).

86   Higgins, P. J. & Weiner, H. L. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J Immunol* **140**, 440-445 (1988).

87   Javed, N. H., Gienapp, I. E., Cox, K. L. & Whitacre, C. C. Exquisite peptide specificity of oral tolerance in experimental autoimmune encephalomyelitis. *J Immunol* **155**, 1599-1605 (1995).

88   al-Sabbagh, A., Miller, A., Santos, L. M. & Weiner, H. L. Antigen-driven tissue-specific suppression following oral tolerance: orally administered myelin basic protein suppresses proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL mouse. *Eur J Immunol* **24**, 2104-2109 (1994).

89   Karpus, W. J., Kennedy, K. J., Smith, W. S. & Miller, S. D. Inhibition of relapsing experimental autoimmune encephalomyelitis in SJL mice by feeding the immunodominant PLP139-151 peptide. *J Neurosci Res* **45**, 410-423 (1996).

90   Faria, A. M. & Weiner, H. L. Oral tolerance. *Immunol Rev* **206**, 232-259 (2005).

91   Bai, X. F. *et al.* Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. *J Neuroimmunol* **80**, 65-75 (1997).

92   Bai, X. F. *et al.* Complexities of applying nasal tolerance induction as a therapy for ongoing relapsing experimental autoimmune encephalomyelitis (EAE) in DA rats. *Clin Exp Immunol* **111**, 205-210 (1998).

93   Metzler, B. & Wraith, D. C. Mucosal tolerance in a murine model of experimental autoimmune encephalomyelitis. *Ann N Y Acad Sci* **778**, 228-242 (1996).

94   Anderton, S. M. & Wraith, D. C. Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. *Eur J Immunol* **28**, 1251-1261 (1998).

95   Leadbetter, E. A. *et al.* Experimental autoimmune encephalomyelitis induced with a combination of myelin basic protein and myelin oligodendrocyte glycoprotein is

ameliorated by administration of a single myelin basic protein peptide. *J Immunol* **161**, 504-512 (1998).

96   Staykova, M. A., Simmons, R. D. & Willenborg, D. O. Infusion of soluble myelin basic protein protects long-term against induction of experimental autoimmune encephalomyelitis. *Immunol Cell Biol* **75**, 54-64 (1997).

97   Gaur, A., Wiers, B., Liu, A., Rothbard, J. & Fathman, C. G. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science* **258**, 1491-1494 (1992).

98   Warren, K. G., Catz, I., Ferenczi, L. Z. & Krantz, M. J. Intravenous synthetic peptide MBP8298 delayed disease progression in an HLA Class II-defined cohort of patients with progressive multiple sclerosis: results of a 24-month double-blind placebo-controlled clinical trial and 5 years of follow-up treatment. *Eur J Neurol* **13**, 887-895 (2006).

99   Warren, K. G., Catz, I. & Wucherpfennig, K. W. Tolerance induction to myelin basic protein by intravenous synthetic peptides containing epitope P85 VVHFFKNIVTP96 in chronic progressive multiple sclerosis. *J Neurol Sci* **152**, 31-38 (1997).

100   Bynoe, M. S., Evans, J. T., Viret, C. & Janeway, C. A., Jr. Epicutaneous immunization with autoantigenic peptides induces T suppressor cells that prevent experimental allergic encephalomyelitis. *Immunity* **19**, 317-328 (2003).

101   Szczepanik, M., Tutaj, M., Bryniarski, K. & Dittel, B. N. Epicutaneously induced TGF-beta-dependent tolerance inhibits experimental autoimmune encephalomyelitis. *J Neuroimmunol* **164**, 105-114 (2005).

102   Jurynczyk, M. *et al.* Immune regulation of multiple sclerosis by transdermally applied myelin peptides. *Ann Neurol* **68**, 593-601 (2010).

103   Santambrogio, L., Sato, A. K., Fischer, F. R., Dorf, M. E. & Stern, L. J. Abundant empty class II MHC molecules on the surface of immature dendritic cells. *Proc Natl Acad Sci U S A* **96**, 15050-15055 (1999).

104   Miller, S. D., Turley, D. M. & Podojil, J. R. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol* **7**, 665-677 (2007).

105   Kumar, V., Urban, J. L., Horvath, S. J. & Hood, L. Amino acid variations at a single residue in an autoimmune peptide profoundly affect its properties: T-cell activation, major histocompatibility complex binding, and ability to block experimental allergic encephalomyelitis. *Proc Natl Acad Sci U S A* **87**, 1337-1341 (1990).

106   Karin, N., Mitchell, D. J., Brocke, S., Ling, N. & Steinman, L. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J Exp Med* **180**, 2227-2237 (1994).

107   Gaur, A. *et al.* Amelioration of relapsing experimental autoimmune encephalomyelitis with altered myelin basic protein peptides involves different cellular mechanisms. *J Neuroimmunol* **74**, 149-158 (1997).

108   Nicholson, L. B., Murtaza, A., Hafler, B. P., Sette, A. & Kuchroo, V. K. A T cell receptor antagonist peptide induces T cells that mediate bystander suppression and prevent autoimmune encephalomyelitis induced with multiple myelin antigens. *Proc Natl Acad Sci U S A* **94**, 9279-9284 (1997).

109   Young, D. A. *et al.* IL-4, IL-10, IL-13, and TGF-beta from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *J Immunol* **164**, 3563-3572 (2000).

110   Kuchroo, V. K. *et al.* A single TCR antagonist peptide inhibits experimental allergic encephalomyelitis mediated by a diverse T cell repertoire. *J Immunol* **153**, 3326-3336 (1994).

111   Franco, A. *et al.* T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis. *Eur J Immunol* **24**, 940-946 (1994).

112   Nicholson, L. B., Greer, J. M., Sobel, R. A., Lees, M. B. & Kuchroo, V. K. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* **3**, 397-405 (1995).

113   Crowe, P. D., Qin, Y., Conlon, P. J. & Antel, J. P. NBI-5788, an altered MBP83-99 peptide, induces a T-helper 2-like immune response in multiple sclerosis patients. *Ann Neurol* **48**, 758-765 (2000).

114   Bielekova, B. *et al.* Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* **6**, 1167-1175 (2000).

115   Kappos, L. *et al.* Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med* **6**, 1176-1182 (2000).

116   Aharoni, R., Teitelbaum, D., Arnon, R. & Sela, M. Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking. *Proc Natl Acad Sci U S A* **96**, 634-639 (1999).

117   Ruiz, P. J. *et al.* Immunomodulation of experimental autoimmune encephalomyelitis with ordered peptides based on MHC-TCR binding motifs. *J Immunol* **167**, 2688-2693 (2001).

118   Illes, Z. *et al.* Modified amino acid copolymers suppress myelin basic protein 85-99-induced encephalomyelitis in humanized mice through different effects on T cells. *Proc Natl Acad Sci U S A* **101**, 11749-11754 (2004).

119   Stern, J. N. *et al.* Peptide 15-mers of defined sequence that substitute for random amino acid copolymers in amelioration of experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **102**, 1620-1625 (2005).

120   Munari, L., Lovati, R. & Boiko, A. Therapy with glatiramer acetate for multiple sclerosis. *Cochrane Database Syst Rev*, CD004678 (2004).

121   Manikwar, P., Kiptoo, P., Badawi, A. H., Buyuktimkin, B. & Siahaan, T. J. Antigen-specific blocking of CD4-specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev* (2011).

122   Corper, A. L. *et al.* A structural framework for deciphering the link between I-Ag7 and autoimmune diabetes. *Science* **288**, 505-511 (2000).

123   Xu, C. R., Yusuf-Makagiansar, H., Hu, Y., Jois, S. D. & Siahaan, T. J. Structural and ICAM-1-docking properties of a cyclic peptide from the I-domain of LFA-1: an inhibitor of ICAM-1/LFA- 1-mediated T-cell adhesion. *J Biomol Struct Dyn* **19**, 789-799 (2002).

124   Casasnovas, J. M., Stehle, T., Liu, J. H., Wang, J. H. & Springer, T. A. A dimeric crystal structure for the N-terminal two domains of intercellular adhesion molecule-1. *Proc Natl Acad Sci U S A* **95**, 4134-4139 (1998).

125   Bella, J., Kolatkar, P. R., Marlor, C. W., Greve, J. M. & Rossmann, M. G. The structure of the two amino-terminal domains of human ICAM-1 suggests how it functions as a

rhinovirus receptor and as an LFA-1 integrin ligand. *Proc Natl Acad Sci U S A* **95**, 4140-4145 (1998).

126   Murray, J. S. *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des* **70**, 227-236 (2007).

127   Kobayashi, N., Kobayashi, H., Gu, L., Malefyt, T. & Siahaan, T. J. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* **322**, 879-886 (2007).

128   Kobayashi, N. *et al.* Prophylactic and therapeutic suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *Clin Immunol* **129**, 69-79 (2008).

129   Ridwan, R. *et al.* Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor: structure optimization and pharmacokinetics. *J Pharmacol Exp Ther* **332**, 1136-1145 (2010).

130   Zhao, H., Kiptoo, P., Williams, T. D., Siahaan, T. J. & Topp, E. M. Immune response to controlled release of immunomodulating peptides in a murine experimental autoimmune encephalomyelitis (EAE) model. *J Control Release* **141**, 145-152 (2010).

131   Srinivasan, M. *et al.* Suppression of experimental autoimmune encephalomyelitis using peptide mimics of CD28. *J Immunol* **169**, 2180-2188 (2002).

132   Chen, J. *et al.* Allogenic donor splenocytes pretreated with antisense peptide against B7 prolong cardiac allograft survival. *Clin Exp Immunol* **138**, 245-250 (2004).

133   Marini, J. C., Jameson, B. A., Lublin, F. D. & Korngold, R. A CD4-CDR3 peptide analog inhibits both primary and secondary autoreactive CD4+ T cell responses in experimental allergic encephalomyelitis. *J Immunol* **157**, 3706-3715 (1996).

134   Edling, A. E., Choksi, S., Huang, Z. & Korngold, R. Effect of a cyclic heptapeptide based on the human CD4 domain 1 CC' loop region on murine experimental allergic encephalomyelitis: inhibition of both primary and secondary responses. *J Neuroimmunol* **112**, 115-128 (2001).

135   Iyer, S., Lahana, R. & Buelow, R. Rational design and development of RDP58. *Curr Pharm Des* **8**, 2217-2229 (2002).

136   Cuturi, M. C. *et al.* RDP1258, a new rationally designed immunosuppressive peptide, prolongs allograft survival in rats: analysis of its mechanism of action. *Mol Med* **5**, 820-832 (1999).

137   Iyer, S. *et al.* Characterization and biological significance of immunosuppressive peptide D2702.75-84(E --> V) binding protein. Isolation of heme oxygenase-1. *J Biol Chem* **273**, 2692-2697 (1998).

138   Liu, Y. *et al.* Heme oxygenase-1 plays an important protective role in experimental autoimmune encephalomyelitis. *Neuroreport* **12**, 1841-1845 (2001).

139   Liu, Y. *et al.* Bilirubin as a potent antioxidant suppresses experimental autoimmune encephalomyelitis: implications for the role of oxidative stress in the development of multiple sclerosis. *J Neuroimmunol* **139**, 27-35 (2003).

140   DeVry, C. G. *et al.* RDP58, a novel immunomodulatory peptide, ameliorates clinical signs of disease in the Lewis rat model of acute experimental autoimmune encephalomyelitis. *J Neuroimmunol* **152**, 33-43 (2004).

141 Brodsky, B., Erlanger-Rosengarten, A., Proscura, E., Shapira, E. & Wormser, U. From topical antidote against skin irritants to a novel counter-irritating and anti-inflammatory peptide. *Toxicol Appl Pharmacol* **229**, 342-350 (2008).

142 Shapira, E. *et al.* Amelioration of experimental autoimmune encephalitis by novel peptides: involvement of T regulatory cells. *J Autoimmun* **35**, 98-106 (2010).

143 Zheng, H. M., Jiang, Y., Wang, J. R., Gong, X. L. & Guo, B. Y. Mimic peptides bonding specifically with the first and second extracellular loops of the CC chemokine receptor 5 derived from a phage display peptide library are potent inhibitors of experimental autoimmune encephalomyelitis. *Inflamm Res* **60**, 759-767 (2011).

144 Hamann, I., Zipp, F. & Infante-Duarte, C. Therapeutic targeting of chemokine signaling in Multiple Sclerosis. *J Neurol Sci* **274**, 31-38 (2008).

145 Bielekova, B. & Becker, B. L. Monoclonal antibodies in MS: mechanisms of action. *Neurology* **74 Suppl 1**, S31-40 (2010).

146 Srinivasan, M. & Janardhanam, S. Novel p65 binding GILZ peptide suppresses experimental autoimmune encephalomyelitis. *J Biol Chem* (2011).

147 Ayroldi, E. *et al.* Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* **98**, 743-753 (2001).

148 Marta, M. Toll-like receptors in multiple sclerosis mouse experimental models. *Ann N Y Acad Sci* **1173**, 458-462 (2009).

149 Weiner, H. L. Multiple sclerosis is an inflammatory T-cell-mediated autoimmune disease. *Arch Neurol* **61**, 1613-1615 (2004).

150 Racke, M. K. & Drew, P. D. Toll-like receptors in multiple sclerosis. *Curr Top Microbiol Immunol* **336**, 155-168 (2009).

151   Gambuzza, M. *et al.* Targeting Toll-like receptors: Emerging therapeutics for multiple sclerosis management. *J Neuroimmunol* (2011).

152   Bochner, B. S. & Lichtenstein, L. M. Anaphylaxis. *N Engl J Med* **324**, 1785-1790 (1991).

153   Martin, T. R., Galli, S. J., Katona, I. M. & Drazen, J. M. Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J Clin Invest* **83**, 1375-1383 (1989).

154   Smith, C. E., Eagar, T. N., Strominger, J. L. & Miller, S. D. Differential induction of IgE-mediated anaphylaxis after soluble vs. cell-bound tolerogenic peptide therapy of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **102**, 9595-9600 (2005).

155   Pedotti, R. *et al.* An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat Immunol* **2**, 216-222 (2001).

156   Katz, D. H., Bargatze, R. F., Bogowitz, C. A. & Katz, L. R. Regulation of IgE antibody production by serum molecules. IV. Complete Freund's adjuvant induces both enhancing and suppressive activities detectable in the serum of low and high responder mice. *J Immunol* **122**, 2184-2190 (1979).

CHAPTER 2

Suppression of EAE and prevention of blood-brain barrier breakdown after vaccination

with novel bifunctional peptide inhibitor

2.1 INTRODUCTION

Multiple sclerosis (MS) is a neurological disease in which the body's immune system recognizes protein fragments of the myelin sheath as antigenic and initiates an inflammatory response in the central nervous system (CNS). This immune response leads to breakdown in the BBB integrity and demyelination of neurons.[1] Major proteins that make up the myelin sheath are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), which can be recognized as antigens by $CD4^+$ T cells, and most likely promote neurodegenerative diseases such as MS. Activation of T cells takes place after the delivery of two signals by antigen-presenting cells (APC).[2,3] The first signal (Signal 1) is the interaction between the T-cell receptor (TCR) and the antigen-loaded major histocompatibility complex class-II (MHC-II). The second signal (Signal 2) is provided by costimulatory molecules (CD28/B-7),[4,5] and is strengthened by the interaction between adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-1 (LFA-1).[6] Novel bifunctional peptide inhibitor (BPI) molecules are composed of an antigenic peptide fragment covalently conjugated to an adhesion molecule fragment.[7] BPI molecules were designed based on a proposed mechanism that they can bind simultaneously to the MHC-II and adhesion molecules on the surface of APC such as dendritic cells (DC). This simultaneous binding is proposed to prevent proper delivery of signals through the immunological synapse and, therefore, hinder the induction of a specific immune response. Consequently, only a subpopulation of T cells that recognize the antigenic peptide fragment in the BPI would be affected and, thus, tolerance toward that particular antigen would be restored.

In order to study the etiology of MS as well as to develop new therapies for the disease, the EAE animal model can be used. EAE animals undergo a pathogenesis very similar to MS,

including inflammation of the CNS and breakdown of the BBB. EAE is an inflammatory disease characterized by the differentiation and proliferation of type-17 helper T cells (T_H17)[8] and type-1 helper T cells (T_H1).[9] To suppress or prevent disease, several therapies have been designed to down-regulate these pro-inflammatory T cells and promote the activation of the regulatory and suppressor immune response by promoting the differentiation and proliferation of regulatory T cells (T_{reg})[10] and type-2 helper T cells (T_H2).[11] Many potential therapies for MS (i.e., peptides and small molecules) are being evaluated in EAE animal models after induction of disease with antigen(s). Previous studies have shown that BPI molecules suppressed EAE in mice upon BPI treatment during disease induction or after the initial progress of the disease.[7,12] In the current study, the *in vivo* efficacy after vaccination of the mice with peptides, i.e., before induction of disease, was evaluated. We hypothesized that the vaccination with Ac-PLP-BPI-NH$_2$ (PLP-BPI) would stimulate a regulatory or tolerogenic response in mice; therefore, when EAE was induced with antigen in the presence of adjuvant, the disease would not develop due to priming of the regulatory response of the immune system by PLP-BPI.

The severe CNS inflammation in MS and EAE leads to BBB breakdown and CNS lymphocyte infiltration.[13] It is not yet clear whether the breakdown of the BBB is a secondary effect of the disease or an initiator of the disease. A therapy such as Tysabri has been shown to prevent lymphocyte infiltration into the brain.[14] Studies suggest a link between the initiation of inflammation and activation of leukocytes leading to the breakdown of the BBB.[15,16] Therefore, we propose that blocking the induction of the pro-inflammatory T cells can prevent the disruption of the BBB after induction of EAE.

PLP-BPI is a well-characterized BPI molecule capable of suppressing EAE in mice when administered after disease stimulation. PLP-BPI is composed of the antigenic peptide PLP$_{139-151}$

(PLP)[17] covalently conjugated to the ICAM-1 ligand, LABL peptide (derived from LFA-1)[18-21] via a stable linker composed of glycine and aminocaproic acid (**Fig. 2.1**). In this study, we tested the novel use of PLP-BPI as a peptide vaccine. The *in vivo* efficacy of PLP-BPI was evaluated and the effect of PLP-BPI treatment on preventing breakdown of the BBB was determined. BBB permeation of Gd-DTPA was quantified using MRI in normal mice (no EAE), phosphate-buffered saline (PBS)-treated EAE mice and PLP-BPI-treated mice. The brain deposition of Gd-DTPA was determined using contrast enhanced T1-weighted MRI. Scans were performed on various brain regions and enhancement of signal before and after Gd-DTPA injection was imaged and quantified. Finally, the immune-modulation mechanisms were elucidated by determining the cytokine production of splenocytes that were isolated from PBS- and PLP-BPI-treated mice.

2.2 MATERIALS AND METHODS

2.2.1 Mice

All protocols for experiments involving SJL/J (H-2^s) (Charles River, Wilmington, MA) were approved by the University's Institutional Animal Care and Use Committee. The mice were housed under specific pathogen-free conditions at a facility at the University of Kansas, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2.2 Peptide Synthesis

PLP$_{139-151}$ (HSLGKWLGHPDKF) and PLP-BPI (Ac-HSLGKWLGHPDKF-(AcpGAcpGAcp)$_2$-ITDGEATDSG-NH$_2$, Ac being an acetyl group and Acp being ε-
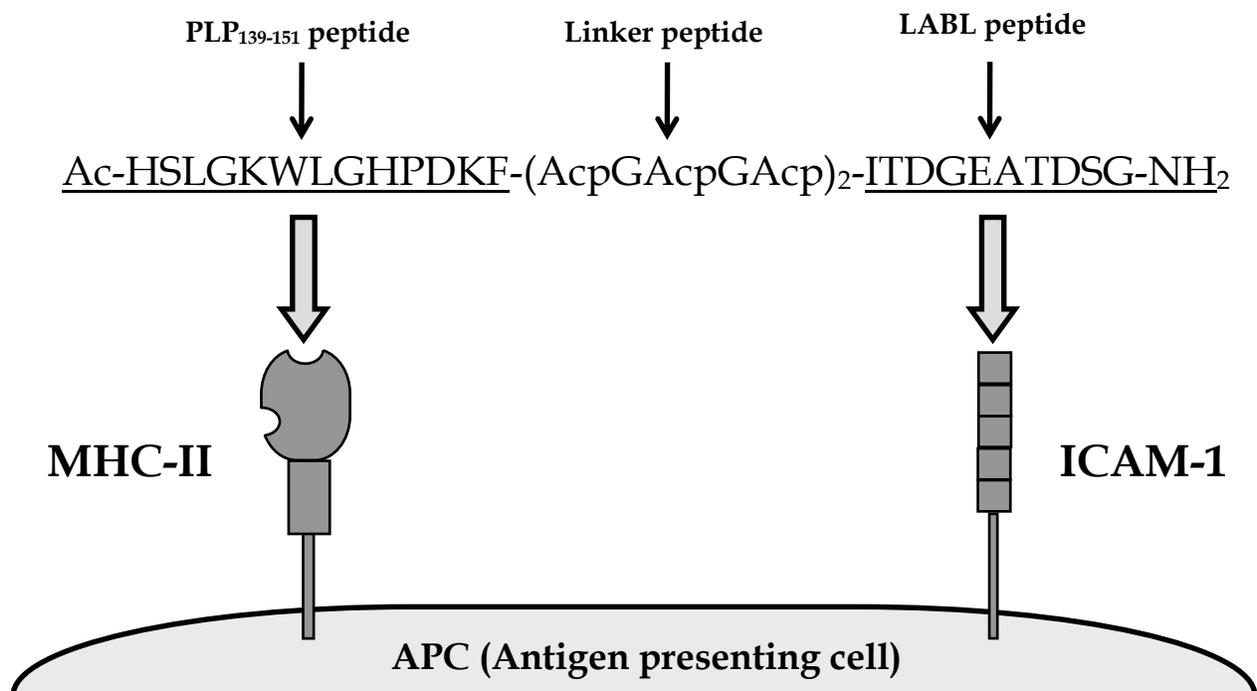
Figure 2.1 Sequence and target receptors of PLP-BPI. PLP-BPI is a linear 33-amino acid peptide, which is composed of the antigenic peptide, $PLP_{139-151}$ and the ICAM-1 binding peptide, LABL, which is derived from the α-subunit of LFA-1 ($CD11a_{237-246}$). Both peptides are covalently conjugated to each other via a linker composed of ε-aminocaproic acid and glycine. The N- and C- termini of the peptide are capped by acetylation and amidation, respectively. The hypothesis is that the $PLP_{139-151}$ portion will bind to MHC-II ($I-A^s$) and LABL will simultaneously bind to ICAM-1 on the surface of the APC.

aminocaproic acid) were synthesized with 9-fluorenylmethyloxy-carbonyl-protected amino acid chemistry on an appropriate PEG-PS™ resin (Applied Biosystems, Foster City, CA) using an automated peptide synthesis system (Pioneer™:PerSeptive Biosystems, Framingham, MA). Cleavage of the peptides from the resin and removal of the protecting groups from the side-chain were carried out using 90% TFA with 10% scavenger reagents (1,2-ethane dithiol (3%), anisole (2%), and thioanisole (5%)). The crude peptides were purified by reversed-phase HPLC using a semi-preparative C18 column with a gradient of solvent A (95%/5% = H_2O (0.1% TFA)/acetonitrile) and solvent B (100% acetonitrile). The purity of the peptides was analyzed by HPLC using an analytical C18 column. The identity of the synthesized peptide was confirmed by electrospray ionization mass spectrometry.

2.2.3 Induction and Treatment of EAE

SJL/J female mice (5–7 weeks old) were immunized subcutaneously (s.c.) with 200 μg PLP in a 0.2 ml emulsion comprised of equal volumes of PBS and complete Freund's adjuvant (CFA) containing killed mycobacterium tuberculosis strain H37RA at a final concentration of 4 mg/ml (Difco, Detroit, MI). The PLP/CFA emulsion was administered to regions above the shoulder and the flanks (total of 4 sites; 50 μl at each injection site). In addition, 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally (i.p.) on the day of immunization (day 0) and 48 h post-immunization. The clinical scores that reflect the disease progression were determined by the same observer in a blinded fashion using a scale ranging from 0 to 5 as follows: 0 - no clinical symptoms, 1 - limp tail or waddling gait with tail tonicity; 2 - waddling gait with limp tail (ataxia); 2.5 - ataxia with partial paralysis of one limb; 3 - full paralysis of one limb; 3.5 - full paralysis of one limb with partial paralysis of the second

limb; 4 - full paralysis of two limbs; 4.5 - full paralysis of two limbs with partial paralysis of forelimbs; 5 - moribund or dead. Body weight was also measured daily.

For the vaccination study, the mice received three s.c. injections of either 100 μl vehicle (PBS) or 100 μl of treatment peptides (100 nmol/injection/day) 11, 8, and 5 days prior to induction of disease on day 0. PLP-BPI's potency was compared to a negative control (PBS) and a positive control (PLP).

2.2.4 *In Vitro* Cytokine Production Assay

In vitro cytokine assays were performed following a protocol similar to that reported previously.[22] SJL/J mice were treated with PBS (100 μl) and PLP-BPI (100 nmol/100 μl/injection) on days −11, −8, and −5 followed by injection of PLP/CFA and pertussis toxin as described in section 2.2.3 to induce EAE. Spleens were isolated from three PLP-BPI- and PBS-treated mice on the day of maximum disease (i.e., day 15). Single cell suspensions of splenocytes were harvested by gently mashing the spleen through a cell strainer using the rubber end of a 1-ml syringe in a petri dish containing serum-free RPMI-1640 supplemented with 10% fetal bovine serum, 100 U penicillin/100 μg streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. Red blood cells were lysed using ACK lysis buffer (Invitrogen). The remaining splenocytes were then washed three times with serum-free RPMI-160 medium (Cellgro). The cells were then primed with PLP (20 μM) in a 24-well plate (5×10^6 cells/well). Supernatants of cell cultures were collected for cytokine detection 72 hours later and stored in a −80°C freezer until analysis. Secreted IL-2, IL-4, IL-5, IL-6, IL-17, and IFN-γ were measured by quantitative ELISA-based Q-Plex^{TM} assay (Quansys Biosciences, Logan, UT).

2.2.5 MRI Scans

To evaluate the effect of PLP-BPI treatment on the breakdown of the BBB, three different groups of animals were used. The first group consisted of five normal SJL/J mice with no EAE induction. The second and third groups of mice were treated with PBS and PLP-BPI, respectively, on days −11, −8, and −5, followed by induction of EAE with PLP/CFA on day 0 as described in section 2.2.3.

In vivo MRI scans were performed using contrast enhanced T1-weighted imaging to determine the extent of BBB breakdown at the highest peak of the disease at 15 days after induction of EAE. The contrast agent, Gd-DTPA (Magnevist, Bayer HealthCare, Leverkusen, Germany), was delivered via an i.p. catheter, which enabled us to acquire images before and after Gd-DTPA infusion under an identical experimental setup. For MRI experiments, the animals were anesthetized using 3 % isoflurane initially followed by 1-2 % isoflurane in a gas mixture of air and oxygen (ratio = 1:1). Before MRI scans were performed, an i.p. catheter (Insyte Autogard, 22 GA, 0.9 × 25 mm, Becton Dickinson, Sparks, MD) was inserted in to the animal's peritoneal cavity and secured with tapes.

All MR imaging was performed with a 9.4 T horizontal bore spectrometer equipped with a Varian INOVA console (Varian Inc., Palo Alto, CA) and gradient coils (40 G/cm, 250 µs) of 12 cm in diameter (Magnex Scientific, Abingdon, UK). The animal was positioned supine in an acrylic sled with its head held steady by using a nose cone for anesthesia delivery. The animal's body temperature was monitored by a rectal temperature sensor (Cole-Palmer, Vernon Hills, IL) and maintained at 37 ± 0.5°C using a blanket with warm water circulation. The animal's respiratory rate was monitored using a respiration pillow (SA instruments, Stony Brook, NY). A quadrature RF surface coil was placed on top of the animal's head to acquire T1-weighted spin-

echo MR images before and after a bolus infusion of Gd-DTPA (0.6 mmol/kg body weight). The imaging parameters were TE/TR = 12.5/600 ms, matrix size = 256 × 256, field of view = 20 × 20 mm, slice thickness = 0.5 mm, and number of averages = 2. The corresponding nominal image resolution was $78 \times 78 \times 500 \ \mu m^3$. The timing of the post-contrast administration MRI scan was set to 20 min to allow uptake of Gd-DTPA into the blood from the i.p. injection and deposition in the brain.

MR data analysis was performed by calculating the percent signal enhancement due to Gd-DTPA deposition in the brain using the following equation: $([v_1-v_0]/v_0)$, where v_1 is the MR signal after Gd-DTPA injection, and v_0 is the MR signal before Gd-DTPA injection. The percent signal enhancement was measured in six regions of interest (ROI) of the brain, including spinal cord, brain stem, cerebellum, hippocampus, cortex, and striatum.

2.2.6 Statistical Analysis

Statistical analysis was done using one-way analysis of variance followed by Fisher's least significance difference to compare the different parameters, including EAE clinical scores, change in body weights, *in vitro* cytokine production, and percent MR signal enhancement. All statistical analyses were performed using StatView software (SAS Institute, Inc., Cary, NC). A *p*-value of less than 0.05 was used as the criterion for statistical significance.

2.3 RESULTS

2.3.1 Suppression of EAE by PLP-BPI

It has been shown previously that PLP-BPI molecules have better efficacy in suppressing EAE than PLP peptide when injected intravenously (i.v.)[12,23] on days 4, 7, 10, and 14 or injected

s.c.[24] on days 4, 7, and 10 after disease stimulation using a PLP/CFA emulsion on day 0. Here, the efficacy of PLP-BPI as a peptide vaccine was evaluated following s.c. injections of PLP-BPI on days −11, −8, and −5 prior to stimulation on day 0 with PLP/CFA emulsion.

Disease severity was measured using a standard disease scoring protocol (**Fig. 2.2A**), which ranges from 0 to 5. The first sign of EAE was evident in the PBS- and PLP-treated mice on days 11–12. The peak of the disease was between days 14 and 17 with a maximum average disease score of 2.30 ± 0.43 for PBS-treated and 1.50 ± 0.31 for PLP-treated mice ($n = 12$). After the peak of the disease, the mice slowly went into remission. Most of the PLP-BPI-treated mice were disease-free with only one mouse having slight weakness in its tail, this resulted in a maximum average disease score of 0.08 ± 0.08 ($n = 12$). Therefore, PLP-BPI was able to significantly suppress EAE compared to PBS ($p < 0.0001$) and PLP ($p < 0.0001$). The efficacy of PLP-BPI was also evaluated using percent change in body weight of the mice. The PBS-treated mice showed a significant loss of body weight (approximately 15%) when compared to the PLP-BPI-treated mice ($p < 0.0001$). The PLP-treated group showed about 10% loss of body weight which was also significantly lower than the PLP-BPI-treated mice ($p < 0.001$). The PLP-BPI-treated group showed no loss in body weight (**Fig. 2.2B**). A summary of the results is shown in **Table 2.1**.

2.3.2 *In Vitro* Cytokine Production

To examine the cytokines induced by PLP-BPI and better understand the immune response, splenocytes from PLP-BPI-treated and PBS-treated mice were isolated on day 15. The type of immune cell differentiation upon treatment can be determined by evaluating the
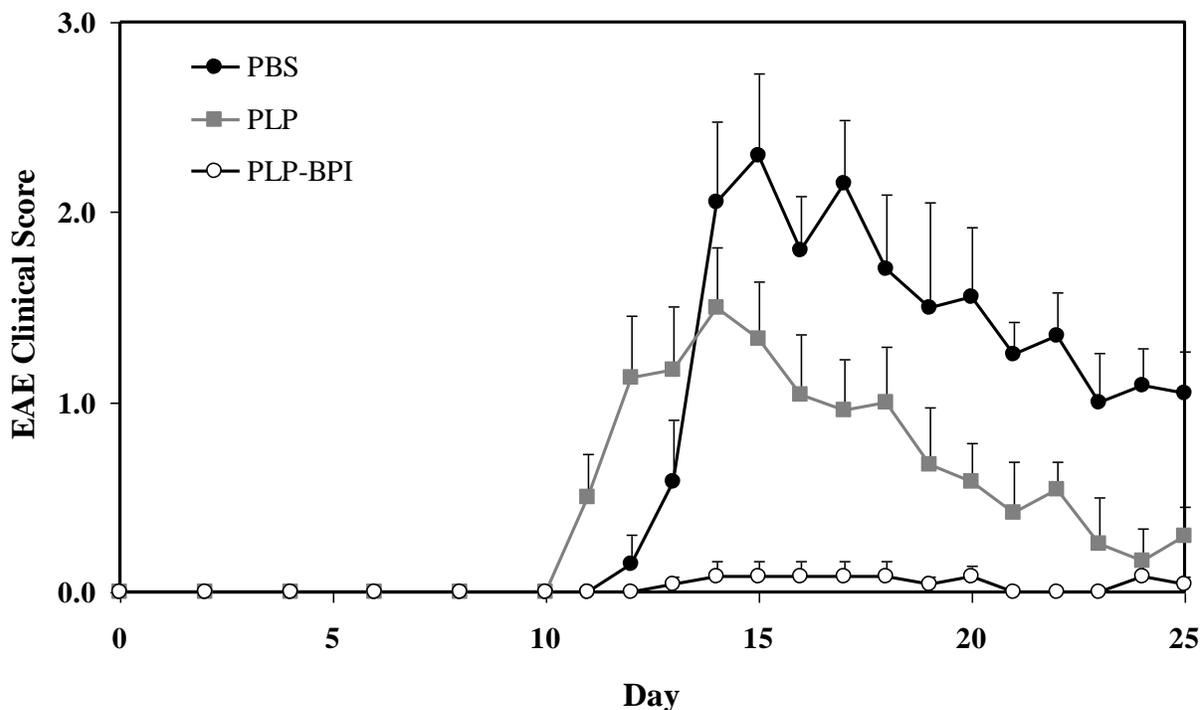
Figure 2.2A *In vivo* efficacies of PLP-BPI and PLP in suppressing EAE in the mouse model upon vaccination with peptides and immunization with PLP/CFA on day 0. PBS-treated mice received subcutaneous injections of 100 μl PBS on days −11, −8, and −5. PLP-BPI- and PLP-treated mice received 100 nmol/100 μl PBS on days −11, −8, and −5. Results are expressed as the mean clinical score ± SEM (n = 12). EAE scores from all PLP-BPI treated mice were significantly lower than those of PBS- and PLP-treated mice (p < 0.0001). For statistical analysis, data points from days 10 to 25 were used.
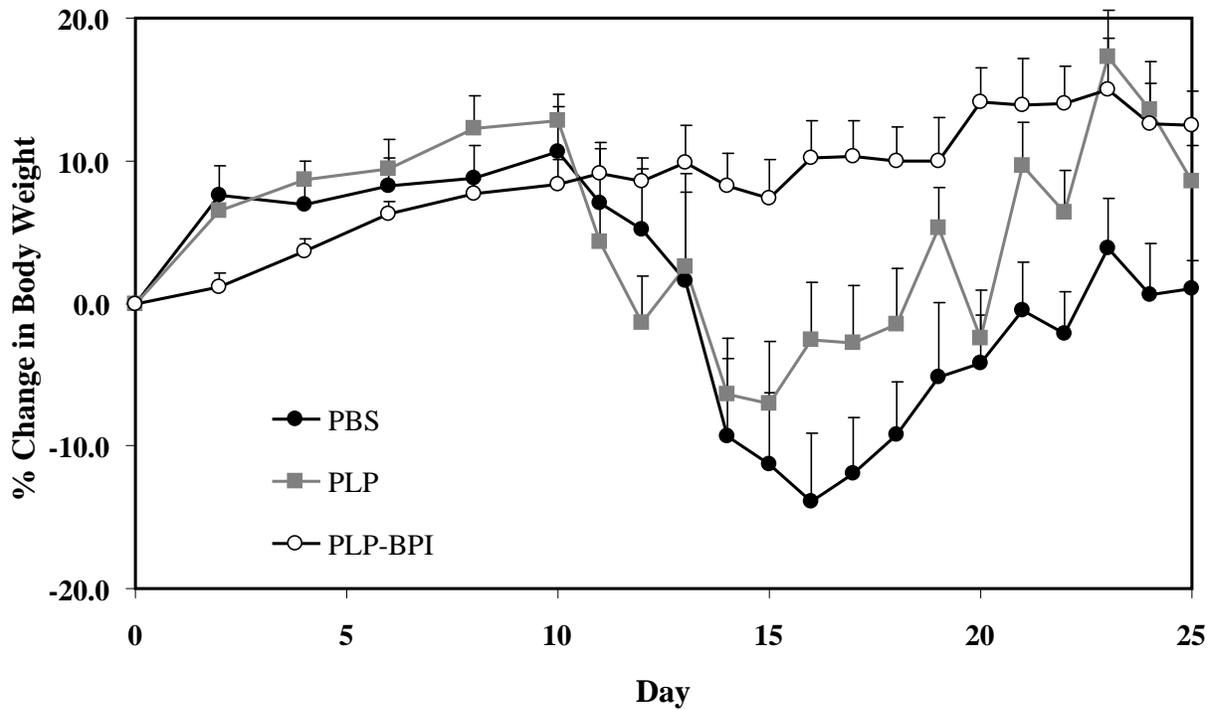
Figure 2.2B *In vivo* efficacies of PLP-BPI and PLP in suppressing EAE in the mouse model upon vaccination with peptides and immunization with PLP/CFA on day 0. PBS-treated mice received subcutaneous injections of 100 μl PBS on days −11, −8, and −5. PLP-BPI- and PLP-treated mice received 100 nmol/100 μl PBS on days −11, −8, and −5. Results are expressed as the mean % change in body weight ± SEM ($n = 12$). Loss of body weight was also significantly lower in PLP-BPI-treated mice compared to those treated with PBS ($p < 0.0001$) and PLP ($p < 0.001$). For statistical analysis, data points from days 10 to 25 were used.

Group	Dose[a]	Incidence of disease[b]	Mean maximal score $\pm$ SEM[c]
PBS	100 µl/mouse on days −11, −8, and −5	12/12	2.30 ± 0.43
PLP	100 nmol/mouse on days −11, −8, and −5	12/12	1.50 ± 0.31
PLP-BPI	100 nmol/mouse on days −11, −8, and −5	1/12	0.08 ± 0.08

Table 2.1 Summary of *in vivo* results.

[a]All injections were administered subcateneously.

[b]Incidence of disease was defined as a disease score of 1 or higher.

[c]Mean maximal disease scores were expressed as mean ± SEM ($n = 12$).

cytokines secreted from the splenocyte culture. Although this method will not provide an exact concentration of cytokines in systemic circulation, it will provide relative levels of cytokines produced by cells in the mice following different treatments. If there is a general inflammatory response, one would expect to see a greater production of pro-inflammatory cytokines (IL-6, IL-17 and IFN-γ). If there were an activation of the regulatory and suppressor immune response, there would be a greater concentration of regulatory (IL-2)[25] and suppressor cytokines (IL-4 and IL-5).

EAE is believed to be predominantly a T_H17- and T_H1-mediated disease; therefore, the *in vitro* production of cytokine markers for both types of inflammatory T cells was evaluated. In these studies, resident APC served to activate the T cells that had been isolated from mice at the height of the disease in a recall assay, using PLP as the target antigen. Following a 72-hour incubation with PLP, splenocytes isolated from PBS-treated mice produced approximately three times higher IL-17 than PLP-BPI-treated mice, indicating a greater presence of T_H17 cells in PBS-treated than in PLP-BPI-treated mice (**Fig. 2.3A**, $p < 0.0001$). There was a significant increase in the production of IFN-γ as well (**Fig. 2.3B**, $p < 0.05$), but only a slight increase in the production of IL-6 that was not significantly different (**Fig. 2.3C**, $p > 0.05$) in the culture supernatants isolated from the PBS-treated mice than that of PLP-BPI-treated mice. From these results, we concluded that PLP-BPI down-regulated the T_H1 phenotype.

The next step was to evaluate the role of PLP-BPI in skewing the response toward regulatory (T_{reg}) and suppressor (T_H2) T cell phenotypes. In the recall assay, splenocytes from PLP-BPI-treated mice produced significantly higher IL-2 (**Fig. 2.4A**, $p < 0.0001$) than those from PBS treated mice, indicating that PLP-BPI may promote T_{reg} differentiation.[26,27] More
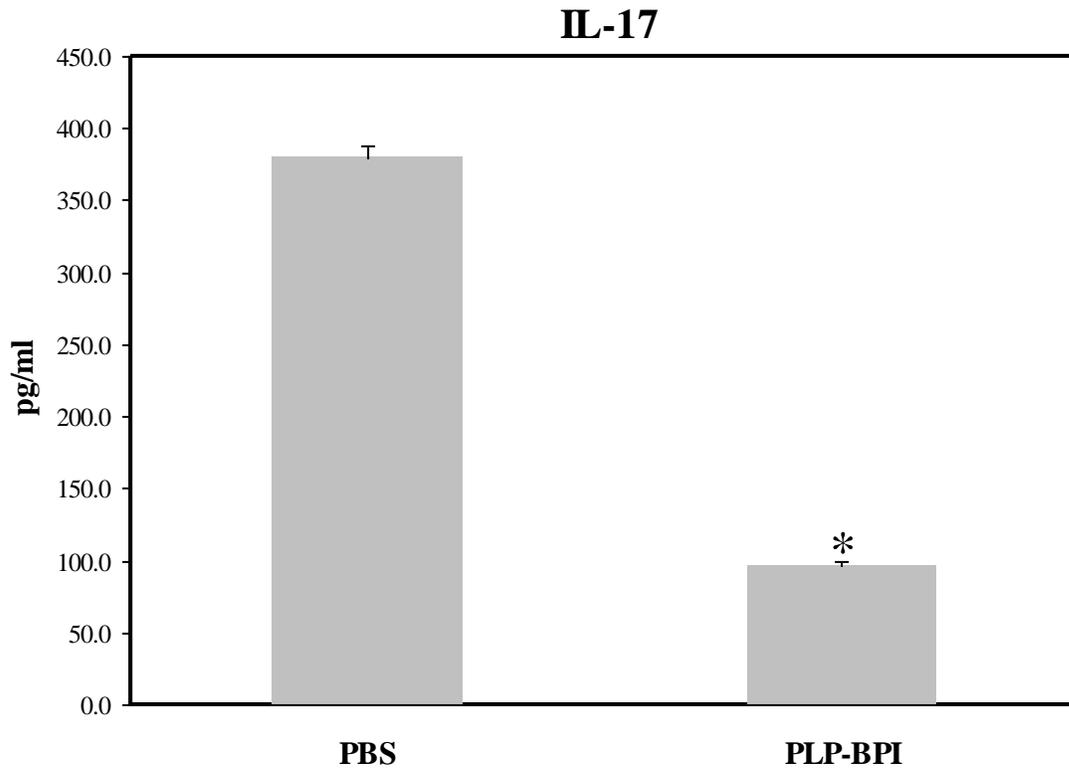
Figure 2.3A Concentrations of the pro-inflammatory cytokine, IL-17, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice that were treated with either PBS or PLP-BPI on days -11, -8, and -5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with PLP$_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.0001$).

IFN-γ
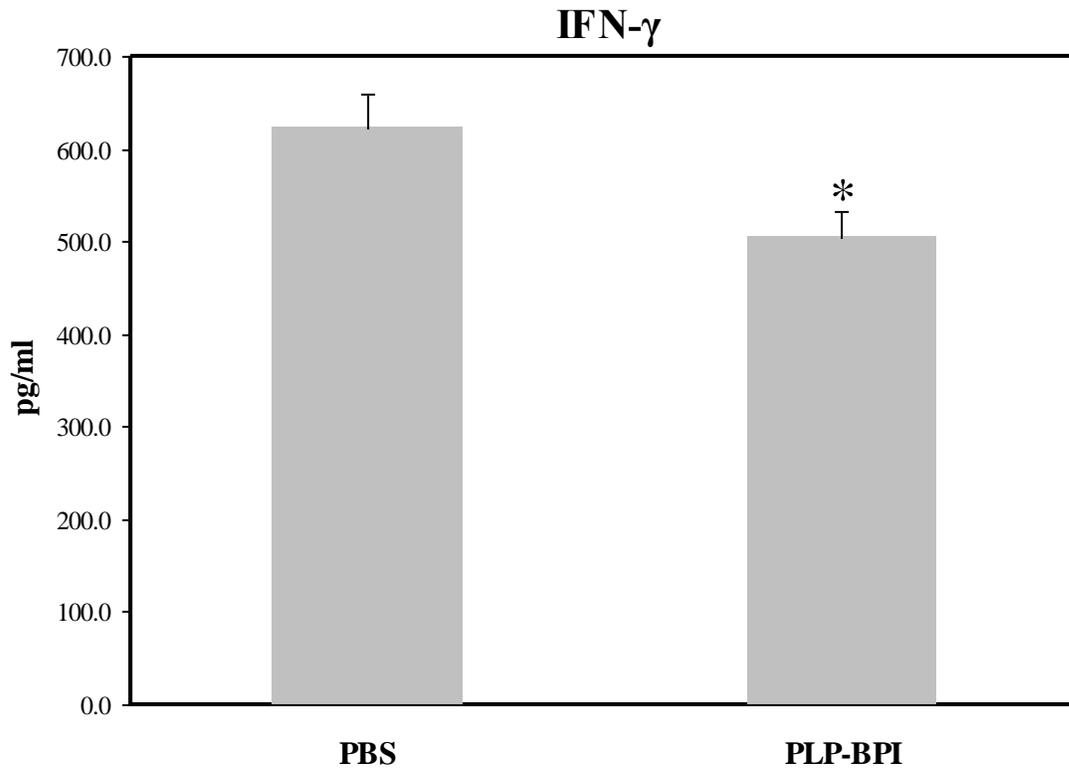
Figure 2.3B Concentrations of the pro-inflammatory cytokine, IFN-γ, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice that were treated with either PBS or PLP-BPI on days -11, -8, and -5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with $PLP_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.05$).
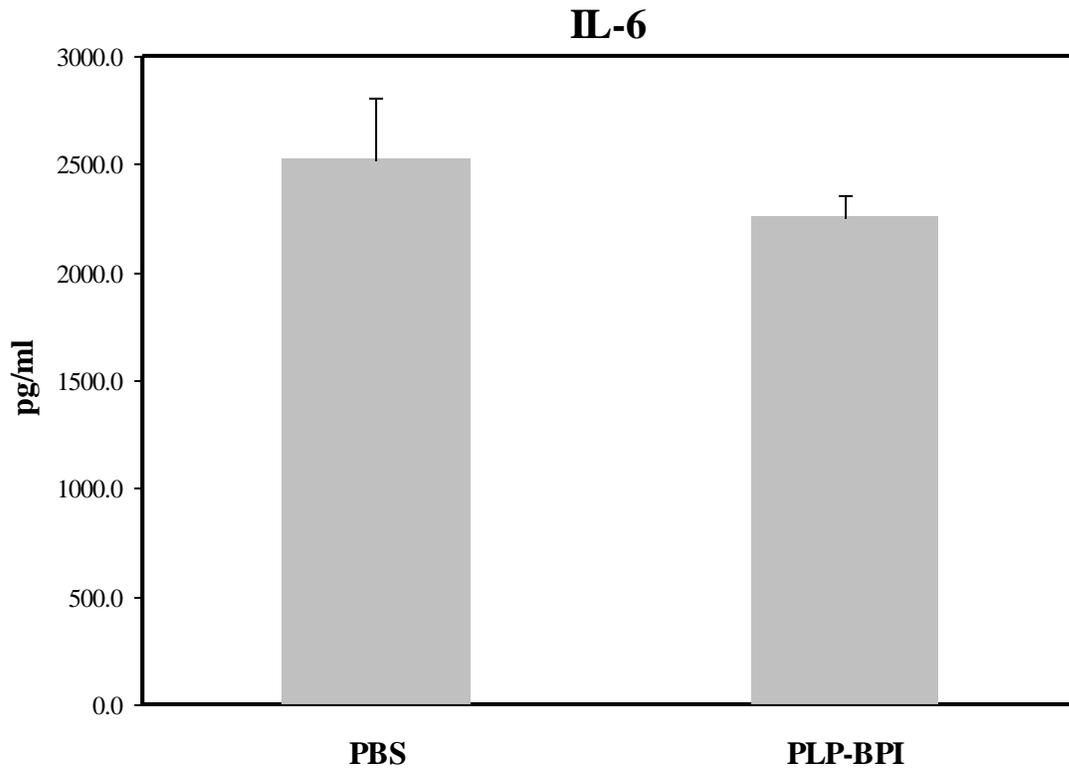
Figure 2.3C Concentrations of the pro-inflammatory cytokine, IL-6, from the cell culture supernatant ($p > 0.05$). Splenocytes were isolated from the spleens of EAE-induced mice that were treated with either PBS or PLP-BPI on days −11, −8, and −5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with PLP$_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection.

IL-2
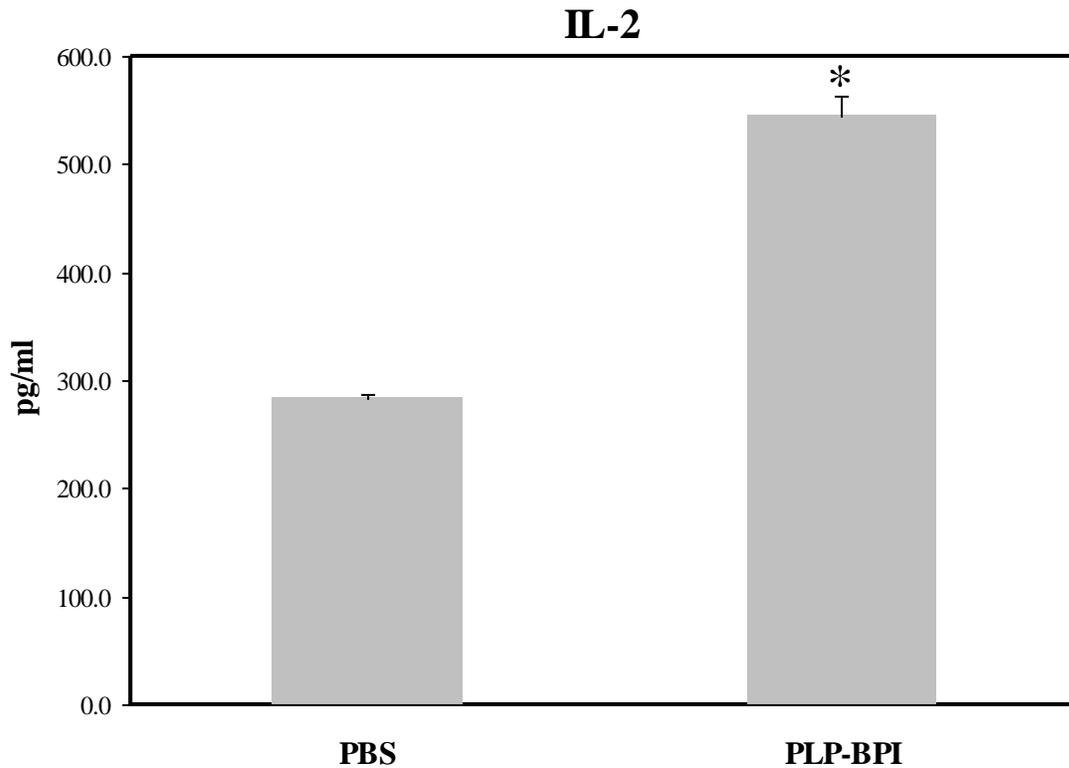
Figure 2.4A Concentrations of the regulatory cytokine, IL-2, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE- induced mice that were treated with either PBS or PLP-BPI on days −11, −8, and −5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with $PLP_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.0001$).

remarkably, splenocytes from PLP-BPI-treated mice produced significantly higher levels of IL-4 (**Fig. 2.4B**, $p < 0.0001$) and IL-5 (**Fig. 2.4C**, $p < 0.001$) when compared to PBS-treated mice. Our observation suggests that PLP-BPI promoted T_H2 differentiation and proliferation.

2.3.3 MRI Data

Cytokine data demonstrated that injection of PLP-BPI subcutaneously on days 11, 8, and 5 prior to induction of disease promoted the regulatory response and lowered the inflammatory response. It is believed that CNS inflammation leads to breakdown of the BBB. We proposed that PLP-BPI increased the regulatory immune response and suppressed the activation of inflammatory immune response, which would prevent the breakdown of the BBB. We used MRI following injection of Gd-DTPA to monitor the breakdown of the BBB. The PBS-treated mice developed EAE and showed high enhancement of Gd-DTPA signal in most brain regions compared to normal mice (negative control); this result indicated that the BBB of PBS-treated mice was compromised. In contrast, there was no obvious enhancement in Gd-DTPA signal in the brain of PLP-BPI-treated mice compared to normal mice without disease induction (**Fig. 2.5**). These result indicated that there was no BBB breakdown in PLP-BPI-treated mice. The quantitative enhancement of Gd-DTPA signals in different regions of the brain from all three groups of mice is shown in **Fig. 2.6**. There was a consistent trend of signal enhancement in all regions of the brains of PBS-treated mice. However, different regions of the brain of PLP-BPI-treated mice had signals similar to those of normal mice. Taken together, from these results we concluded that PLP-BPI can prevent the breakdown of the BBB by suppressing the inflammatory immune response, which is likely due to the generation of regulatory and suppressor cells following vaccination.

IL-4
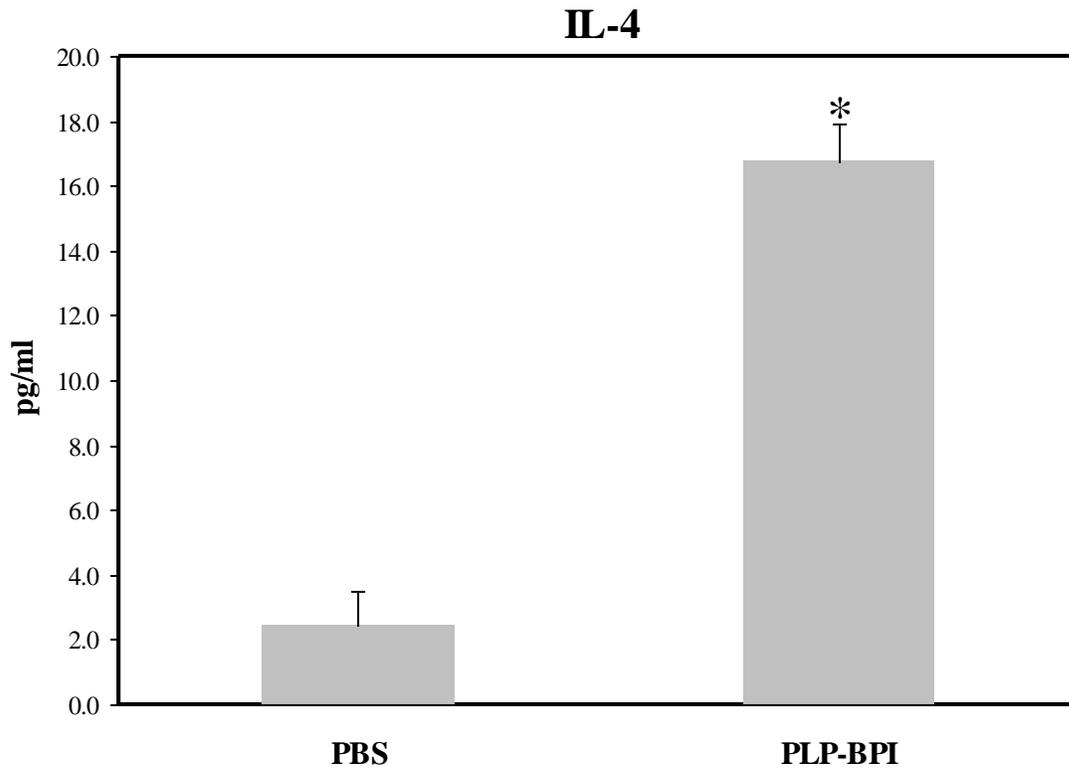
Figure 2.4B Concentrations of the suppressor cytokine, IL-4, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE- induced mice that were treated with either PBS or PLP-BPI on days −11, −8, and −5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with PLP$_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p <$ 0.0001).

60

IL-5
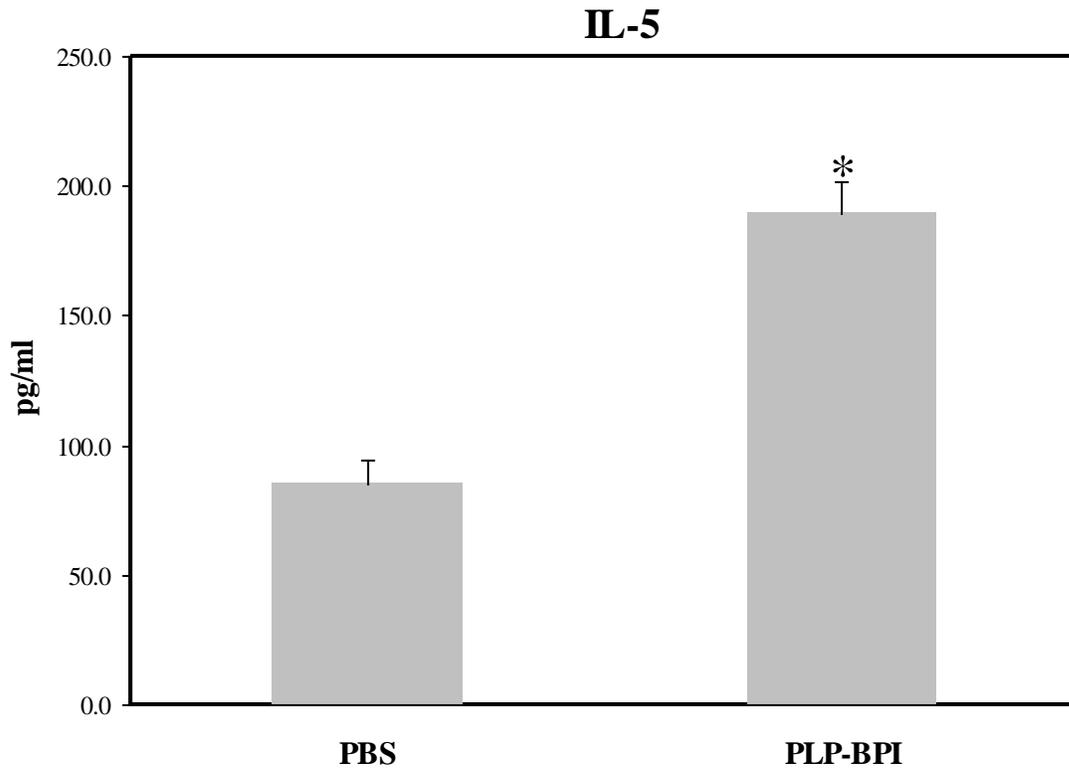
**Figure 2.4C** Concentrations of the suppressor cytokine, IL-5, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE- induced mice that were treated with either PBS or PLP-BPI on days −11, −8, and −5. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with PLP$_{139\text{-}151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p <$ 0.001).
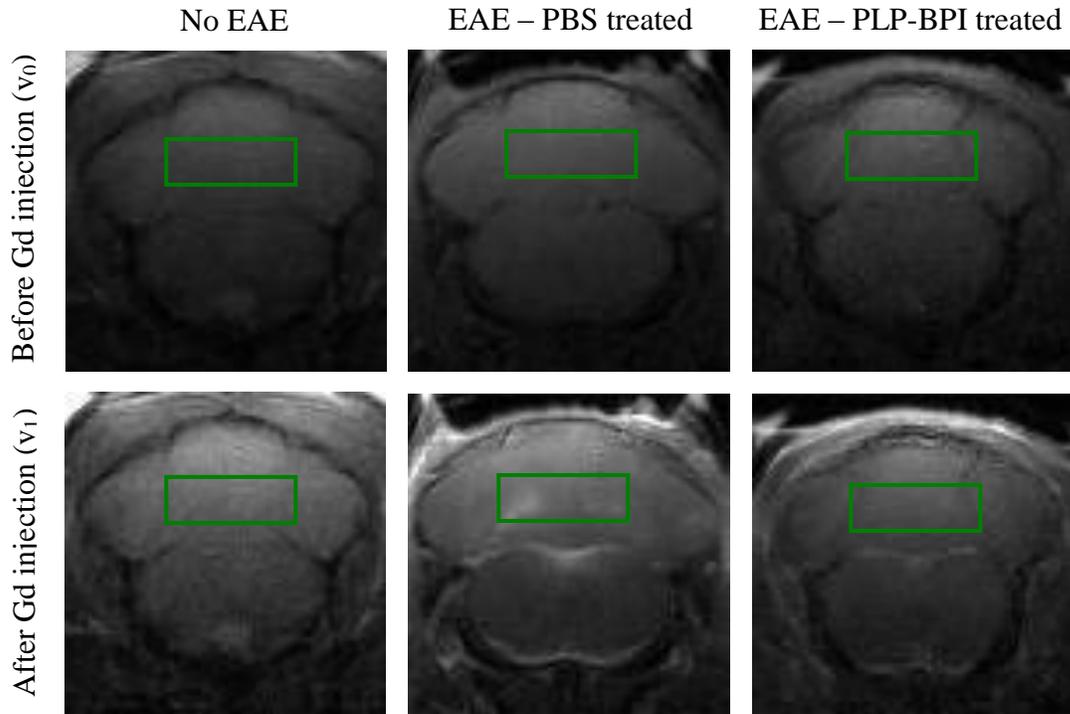
Figure 2.5 Representative scans of the cerebellum (highlighted with box) and brainstem of three different groups of mice ($n = 5$/group). The first group consisted of normal SJL mice with no EAE induced. The second and third groups were mice treated with either PBS or PLP-BPI on days −11, −8, and −5 and immunized to develop EAE on day 0. Each mouse was scanned before (v_0) and after (v_1) an i.p. bolus injection of Gd-DTPA contrast agent. There is obvious enhancement in signal within the cerebellum (ROI) of mice treated with PBS, but no obvious enhancement in signal in normal mice and PLP-BPI-treated mice.
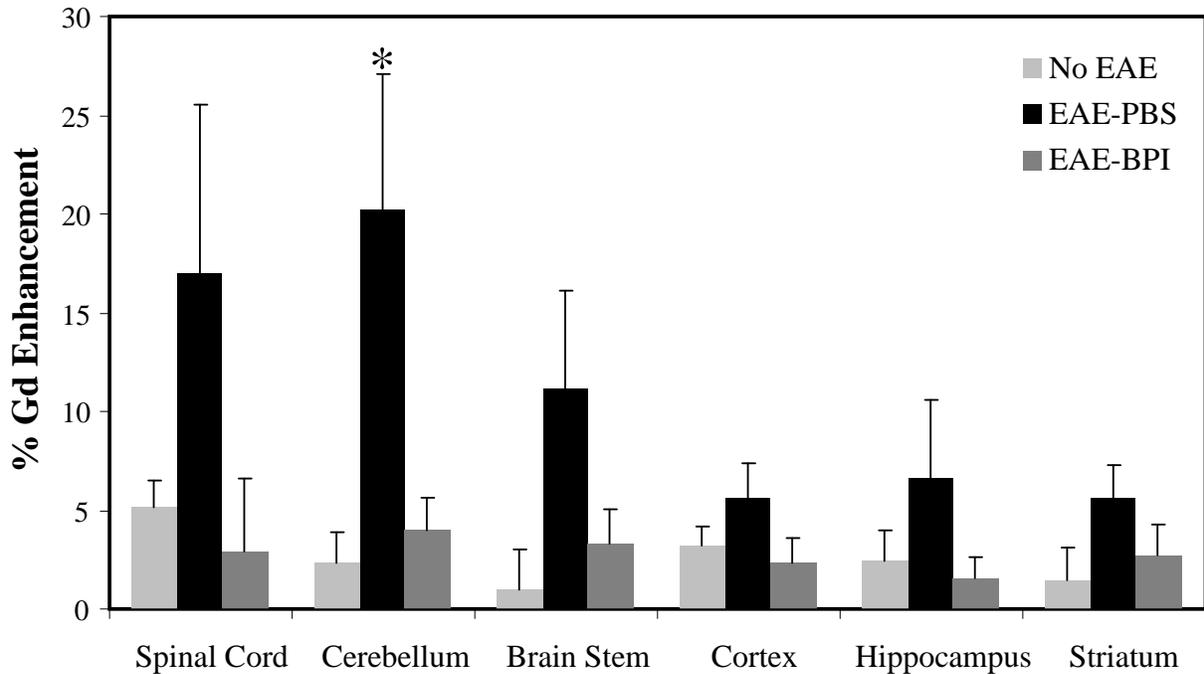
Figure 2.6 Quantitative signal enhancement using Gd-DTPA. Each mouse ($n = 5$ per group) was scanned before (v_0) and after (v_1) an i.p. bolus injection of the Gd-DTPA contrast agent. The percentage was calculated from the ratio of signal enhancement using the equation $[v_1-v_0]/v_0$. The signal enhancement within the ROI can be correlated to the breakdown of the BBB. All the regions of the brain had greater enhancement of the signal within the ROI in the PBS-treated mice than in the normal mice (no EAE induced) and PLP-BPI treated mice. The cerebellum was the only region in which there was a statistically significant difference between groups. Normal control and PLP-BPI-treated mice had a significantly lower signal enhancement within the ROI of the cerebellum (*$p < 0.05$).

2.4 DISCUSSION

Antigenic peptides have recently found an application for the treatment of allergic and autoimmune diseases in a procedure known as soluble antigen-specific immunotherapy (SIT). Wraith and colleagues proposed a potential mechanism of action for the way that soluble antigenic peptides modulate the immune response.[28] Empty MHC-II molecules on the surface of APC such as DC are capable of being loaded by exogenous peptides.[29] Therefore, it is proposed by Wraith and colleagues that soluble antigenic peptides can bind directly to the empty MHC-II molecules on the surface of immature DC (iDC) without being internalized and processed, thus leading to the induction of a regulatory response by promoting a T_{reg} phenotype. In this case, the induction of the regulatory T cell response leads to restored tolerance for specific antigens in the treatment of autoimmune diseases such as MS,[30,31] rheumatoid arthritis (RA),[32] and type-1 diabetes (T1D).[33]

Bifunctional peptide inhibitors (BPI) have been shown to suppress EAE,[7] T1D,[33] and RA (unpublished data) and are consistently more efficient at suppressing disease than the corresponding parent antigenic peptides. We hypothesize that the antigenic peptide and the adhesion peptide of the BPI molecule bind to MHC-II and ICAM-1 molecules, respectively, on the surface of APC. This simultaneous binding will interfere with the maintenance of the functional immunological synapse at the APC-T cell interface, a step necessary for full activation of an immunogenic response.[34,35] Alternatively, the two molecules on the surface of the APC are clustered and internalized. Without the appropriate delivery of signals, the cells fail to induce an inflammatory response and instead promote the differentiation of naïve T cells toward regulatory and/or suppressor T-cell phenotypes. A third possible mechanism is that the antigenic peptide fragment of the BPI molecule could bind directly to the empty MHC-II molecules on the surface

of iDC and act similarly to the antigenic peptide on its own. If this is the case, binding of a naïve T cell to an APC would induce the differentiation to regulatory T cells as with the administration of soluble antigenic peptide alone. However, the mechanism of action of BPI molecules remains to be fully elucidated.

Previously, PLP-BPI has been administered in solution i.v.[12] and s.c. as well as in a controlled-release fashion using nanoparticles.[24] It was shown that s.c. administration was more effective than i.v. administration of PLP-BPI. In addition, increasing the length of the linker as well as capping the N- and C- termini proved to enhance the *in vivo* efficacy of the peptide.[23] In these studies, PLP-BPI prevented the onset of disease significantly when the peptide was administered after the induction of disease. In addition, mice treated with PLP-BPI after the onset of disease went into remission faster than those treated with PBS.

In our proposed mechanism of action of PLP-BPI, in which the peptide induces the regulatory response, injection of PLP-BPI prior to induction of disease should protect the mouse from developing severe EAE. Therefore, our study was designed to investigate the effects of PLP-BPI and PLP on the disease progression when these peptides were administered prior to disease induction. It is interesting that three subcutaneous injections of PLP-BPI at 11, 8, and 5 days prior to induction of disease effectively suppressed the development of EAE compared to PLP peptide and PBS. The superior efficacy of PLP-BPI relative to PLP is consistent with what we found previously when injecting the peptides after induction of disease.[12,23,24] Unlike in previous studies[23] in which 43.8 - 45.5% of the mice developed anaphylaxis, only 8.33% (1 out of 12) developed anaphylaxis when PLP-BPI is injected as a peptide vaccine, thus adding another advantage to vaccination. Administration of PLP-BPI several days prior to induction of disease proves to us that mechanistically it works by modulating the immune system and not

simply acting as a protein/peptide blocker since we know that *in vivo* half-life of PLP-BPI is approximately 2 hours.[36] Although five days elapsed between the last injection of peptide and disease induction, the effect of PLP-BPI persists after the peptide is eliminated from the systemic circulation. It is still not completely clear why PLP-BPI has better efficacy than PLP, but it is possibly due to the presence of LABL peptide on PLP-BPI, which could improve peptide binding to DC in addition to hindering the development of the immunological synapse. Further investigations need to be carried out for elucidating the difference in the mechanisms of action between PLP-BPI and PLP.

EAE is a demyelinating disease that mimics the inflammatory disease MS, which is driven by the induction of pro-inflammatory T cells such as T_H17 and T_H1. Restoring tolerance to specific myelin sheath antigens has become the most important strategy for the treatment of EAE and MS. Establishing the immuno-tolerant state has become possible due to the production of cytokines that probably promote the development of regulatory (T_{reg}) and suppressor T cells (T_H2) cells. Our cytokine studies indicated that splenocytes from PLP-BPI-treated mice produced a lower level of IL-17, which would lead to a decreased population of T_H17 cells when compared to PBS-treated mice. There is some controversy on the importance of IL-17 in the pathogenesis of EAE[37] since it's been reported that the absence of IL-17 has not affected the progression of the disease.[38] However, T_H17 cells have been shown to have a vital role in the development of EAE[8,39] and the IL-17 role is not yet completely clear as a key marker for T_H17 cells. In the current study, we also reported that PLP-BPI-treated mice had reduced levels of the T_H1 cytokines as well, as indicated by the decrease in secretion of IL-6 and IFN-γ. The next step was to evaluate whether PLP-BPI induced expression of cytokines that promote T_{reg} and T_H2 cells, both of which have been associated with the amelioration of EAE. IL-2 levels were

significantly greater in PLP-BPI-treated mice than in PBS-treated mice; this strongly suggests that there was skewing of the immune response toward the production of T_{reg} cells. In addition, PLP-BPI led to a prominent shift toward a T_H2 profile as reflected by the increased production of IL-4 and IL-5.

Inflammation and leukocyte recruitment to the CNS during MS and EAE are known to cause BBB breakdown.[40,41] The cellular infiltration is initiated by the interaction of LFA-1 on leukocytes and ICAM-1 on the surface of vascular endothelial cells of the brain (i.e., BBB).[42] It has been shown that T_H1[43] and T_H17[44] cells may enter the CNS during EAE. The production of inflammatory cytokines is believed to lead to BBB impairment.[40] Administration of PLP-BPI led to a reduction in the levels of inflammatory cytokines and, therefore, it is proposed that administration of PLP-BPI prevents the destruction of the BBB in EAE-induced mice due to its ability to modulate the immune response. This idea is supported by our observations from the MRI studies in determining the extent of the breakdown of the BBB of PLP-BPI- and PBS-treated mice compared to normal mice. The breakdown of the BBB can be assessed by determining the amount of Gd-DTPA that was deposited in the brain upon its permeation through the BBB. This is very important because the breakdown of the BBB is believed to precede cellular infiltration, which leads to destruction of the myelin sheath. It is remarkable that PLP-BPI-treated mice had similar amounts of Gd-DTPA brain distributed to all brain regions compared to normal healthy mice. In contrast, there was greater Gd-DTPA deposition in different brain regions of PBS-treated mice compared to PLP-BPI-treated and healthy mice. We observed a significant enhancement of signal in the cerebellum of EAE mice treated with PBS compared to that in PLP-BPI-treated mice ($p < 0.05$). We also observed a difference ($p = 0.06$) in the enhancement of signal in the spinal cord between diseased mice and PLP-BPI-treated mice.

The great protection from BBB breakdown in these two regions is particularly important; it has been demonstrated that the cerebellum and the spinal cord are equally the most susceptible regions for breakdown in this EAE model.[45] It is evident that the integrity of the BBB of PLP-BPI-mice is maintained to the same degree as in healthy mice, and PBS-treated mice have a breach of the BBB. Thus, PLP-BPI functions prior to the breakdown of the BBB, possibly by suppressing the activation of immune cells prior to their infiltration into the brain. Additionally, PLP-BPI could prevent the infiltration of immune cells by blocking ICAM-1/LFA-1-mediated immune cell adhesion to the vascular endothelial cells of the BBB.

In conclusion, PLP-BPI, when administered as a peptide vaccine, suppresses EAE. Administration of PLP-BPI prior to induction of disease led to expression of regulatory and suppressor immune cytokines that mediate suppression of the inflammatory immune responses as shown by the *in vitro* cytokine production study. The inhibition of the inflammatory response by PLP-BPI prevented the breakdown of the BBB in EAE-induced mice. Further studies are necessary to elucidate the mechanisms of action of PLP-BPI in comparison to those of PLP peptide alone. In the future, we plan to study the effect of a long-term effect of PLP-BPI as a vaccine.

2.5 REFERENCES

1   Lassmann, H. Classification of demyelinating diseases at the interface between etiology and pathogenesis. *Curr Opin Neurol* **14**, 253-258 (2001).

2   Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).

3   Tseng, S. Y. & Dustin, M. L. T-cell activation: a multidimensional signaling network. *Curr Opin Cell Biol* **14**, 575-580 (2002).

4   Jenkins, M. K. & Johnson, J. G. Molecules involved in T-cell costimulation. *Curr Opin Immunol* **5**, 361-367 (1993).

5   June, C. H., Bluestone, J. A., Nadler, L. M. & Thompson, C. B. The B7 and CD28 receptor families. *Immunol Today* **15**, 321-331 (1994).

6   Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med* **181**, 577-584 (1995).

7   Manikwar, P., Kiptoo, P., Badawi, A. H., Buyuktimkin, B. & Siahaan, T. J. Antigen-specific blocking of CD4-specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev*, doi:10.1002/med.20243 (2011).

8   Zepp, J., Wu, L. & Li, X. IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. *Trends Immunol* **32**, 232-239 (2011).

9   Hedegaard, C. J. *et al.* T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. *Immunology* **125**, 161-169 (2008).

10   Wraith, D. C. Therapeutic peptide vaccines for treatment of autoimmune diseases. *Immunol Lett* **122**, 134-136 (2009).

11   Liblau, R. S., Singer, S. M. & McDevitt, H. O. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol Today* **16**, 34-38 (1995).

12   Kobayashi, N., Kobayashi, H., Gu, L., Malefyt, T. & Siahaan, T. J. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* **322**, 879-886, (2007).

13   Floris, S. *et al.* Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: a quantitative MRI study. *Brain* **127**, 616-627 (2004).

14   Bielekova, B. & Becker, B. L. Monoclonal antibodies in MS: mechanisms of action. *Neurology* **74 Suppl 1**, S31-40 (2010).

15   Minagar, A. & Alexander, J. S. Blood-brain barrier disruption in multiple sclerosis. *Mult Scler* **9**, 540-549 (2003).

16   Sun, D. *et al.* Role of chemokines, neuronal projections, and the blood-brain barrier in the enhancement of cerebral EAE following focal brain damage. *J Neuropathol Exp Neurol* **59**, 1031-1043 (2000).

17   Tuohy, V. K., Lu, Z., Sobel, R. A., Laursen, R. A. & Lees, M. B. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J Immunol* **142**, 1523-1527 (1989).

18   Yusuf-Makagiansar, H., Makagiansar, I. T. & Siahaan, T. J. Inhibition of the adherence of T-lymphocytes to epithelial cells by a cyclic peptide derived from inserted domain of lymphocyte function-associated antigen-1. *Inflammation* **25**, 203-214 (2001).

19   Yusuf-Makagiansar, H. *et al.* Sequence recognition of alpha-LFA-1-derived peptides by ICAM-1 cell receptors: inhibitors of T-cell adhesion. *Chem Biol Drug Des* **70**, 237-246 (2007).

20   Tibbetts, S. A. *et al.* Peptides derived from ICAM-1 and LFA-1 modulate T cell adhesion and immune function in a mixed lymphocyte culture. *Transplantation* **68**, 685-692 (1999).

21   Tibbetts, S. A., Seetharama Jois, D., Siahaan, T. J., Benedict, S. H. & Chan, M. A. Linear and cyclic LFA-1 and ICAM-1 peptides inhibit T cell adhesion and function. *Peptides* **21**, 1161-1167 (2000).

22   Youssef, S. *et al.* The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* **420**, 78-84 (2002).

23   Kobayashi, N. *et al.* Prophylactic and therapeutic suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *Clin Immunol* **129**, 69-79 (2008).

24   Zhao, H., Kiptoo, P., Williams, T. D., Siahaan, T. J. & Topp, E. M. Immune response to controlled release of immunomodulating peptides in a murine experimental autoimmune encephalomyelitis (EAE) model. *J Control Release* **141**, 145-152 (2010).

25   Malek, T. R. The main function of IL-2 is to promote the development of T regulatory cells. *J Leukoc Biol* **74**, 961-965 (2003).

26   D'Cruz, L. M. & Klein, L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* **6**, 1152-1159 (2005).

27   Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* **6**, 1142-1151 (2005).

28   Larche, M. & Wraith, D. C. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med* **11**, S69-76 (2005).

29   Santambrogio, L., Sato, A. K., Fischer, F. R., Dorf, M. E. & Stern, L. J. Abundant empty class II MHC molecules on the surface of immature dendritic cells. *Proc Natl Acad Sci U S A* **96**, 15050-15055 (1999).

30   Bai, X. F. *et al.* Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. *J Neuroimmunol* **80**, 65-75 (1997).

31   Metzler, B. & Wraith, D. C. Mucosal tolerance in a murine model of experimental autoimmune encephalomyelitis. *Ann N Y Acad Sci* **778**, 228-242 (1996).

32   Meyer, O. Oral immunomodulation therapy in rheumatoid arthritis. *Joint Bone Spine* **67**, 384-392 (2000).

33   Murray, J. S. *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des* **70**, 227-236 (2007).

34   Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-1542 (2002).

35   van der Merwe, P. A. Formation and function of the immunological synapse. *Curr Opin Immunol* **14**, 293-298 (2002).

36   Ridwan, R. *et al.* Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor: structure optimization and pharmacokinetics. *J Pharmacol Exp Ther* **332**, 1136-1145 (2010).

37   Haak, S., Gyulveszi, G. & Becher, B. Th17 cells in autoimmune disease: changing the verdict. *Immunotherapy* **1**, 199-203 (2009).

38   Haak, S. *et al.* IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J Clin Invest* **119**, 61-69 (2009).

39   Cao, W. *et al.* Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. *Immunity* **35**, 273-284 (2011).

40   Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* **7**, 41-53 (2006).

41   Huber, J. D. *et al.* Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am J Physiol Heart Circ Physiol* **280**, H1241-1248 (2001).

42   Prendergast, C. T. & Anderton, S. M. Immune cell entry to central nervous system--current understanding and prospective therapeutic targets. *Endocr Metab Immune Disord Drug Targets* **9**, 315-327 (2009).

43   O'Connor, R. A. *et al.* Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* **181**, 3750-3754 (2008).

44   Reboldi, A. *et al.* C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* **10**, 514-523 (2009).

45   Tonra, J. R. *et al.* Comparison of the timing of acute blood-brain barrier breakdown to rabbit immunoglobulin G in the cerebellum and spinal cord of mice with experimental autoimmune encephalomyelitis. *J Comp Neurol* **430**, 131-144 (2001).

CHAPTER 3

Development of bifunctional peptide inhibitors to target the costimulatory molecules

B7/CD28 for the treatment of experimental autoimmune encephalomyelitis

3.1 INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease caused by the onset of an inflammatory response in the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics some disease characteristics of MS, including CNS inflammation, lesion formation, blood-brain barrier breakdown and demyelination.[1] Therefore, EAE is a widely used model to study novel therapies for MS. For both MS and EAE, it is believed that the inflammatory response is primarily due to the activation of $CD4^+$ T cells. For the induction of a $CD4^+$ T cell inflammatory response, two signals must be delivered to the T cell via an antigen-presenting cells (APC)—an antigen-specific signal and a costimulatory signal.[2,3]

The antigen-specific signal, known as Signal 1, is delivered via the interaction of the T cell receptor (TCR) on the surface of a T cell and an antigen-loaded major histocompatibility complex class-II (MHC-II) molecule on the surface of an APC. The costimulatory signal, also known as Signal 2, is produced by interaction of various molecules on the surface of T cells and APC. The most important costimulatory signal is generated by the interaction between CD28 on $CD4^+$ T cells and its coreceptor B7 on the surface of APC.[4,5] It has been shown that the B7/CD28 interaction is crucial for the progression of MS and EAE. CD28 binds to B7 via a conserved extracellular region characterized by the residues MYPPPY.[6-8] This region has become very important for the design of therapies that target the B7/CD28 interaction. Another molecule that interacts with B7 is CTLA-4, but, unlike CD28, CTLA-4 is important for inhibiting the stimulation of T cells.[9] This interaction has also become important for designing therapies that can imitate the inhibitory signal for the suppression of inflammatory responses. Other well-studied costimulatory signals include the CD40/CD40L interaction[10] as well as a set

of adhesion molecules interactions between the T cells and APC. Adhesion molecules are believed to strengthen the connection between both cells and, therefore, enhance the delivery of signals from the APC to the T cells. The most important pair of adhesion molecules is the intercellular adhesion molecule-1 (ICAM-1) on the APC and leukocyte function-associated antigen-1 (LFA-1) on the T cell.[11]

Several therapies have been developed in an attempt to allow delivery of only an antigen-specific signal (Signal 1) in the absence of costimulatory signals (Signal 2). This has been achieved either by blocking Signal 2[12,13] or using fixed APC primed with antigen.[14,15] Blocking Signal 2 or the absence of Signal 2 can induce anergy in T cells and lead to long-term tolerance towards a specific antigen.[16,17] Using antisense technology, a peptide known as B7AP was derived from the sequence of the CD28 protein, which contains the conserved region, MYPPPY.[18] B7AP was designed by Xiong and colleagues, and it has been reported that the peptide binds to B7 and specifically blocks the B7-CD28 interactions without affecting B7-CTLA-4 interactions. This peptide was used in prolonging allograft rejection in mice. A peptide similar to B7AP but longer was also developed to target B7 and was tested in reducing the severity of EAE.[19] Unfortunately, solely blocking B7-CD28 interactions may cause general immunosuppression because there is no antigenic selectivity of the peptide. Therefore, there is a need to alter the differentiation and proliferation of immune cells in an antigenic-specific manner for controlling immune response in autoimmune diseases.

In this study, B7AP was conjugated with an antigenic peptide ($PLP_{139-151}$) from proteolipid protein (PLP) to form a novel bifunctional peptide inhibitor[20] (BPI) called PLP-B7AP (**Fig. 3.1**). The hypothesis is that the PLP peptide portion of PLP-B7AP binds to empty MHC-II molecules on the surface of APC and the B7AP portion binds to B7, also on the surface of APC.

PLP₁₃₉₋₁₅₁ peptide **Linker peptide** **B7AP peptide**

Ac-HSLGKWLGHPDKF-(AcpGAcpGAcp)$_2$-EFMYPPPYLD-NH$_2$

MHC-II

B7

APC (Antigen presenting cell)
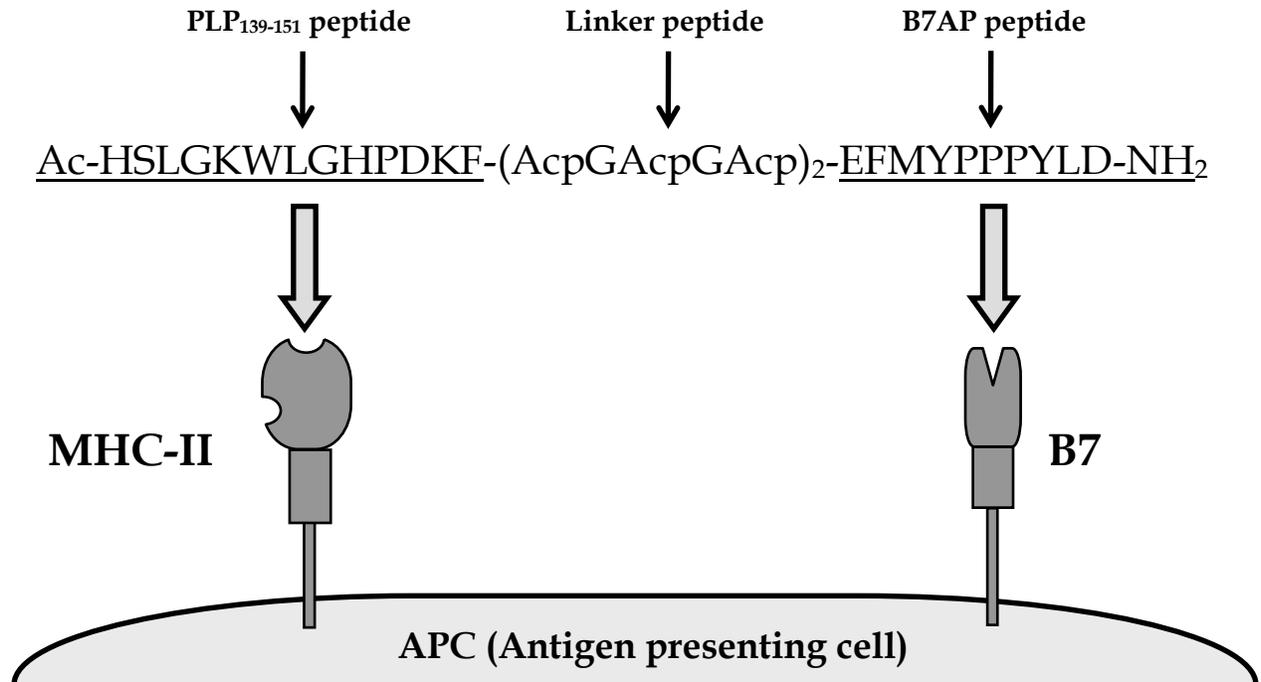
Figure 3.1 Sequence and target receptors of PLP-B7AP. PLP-B7AP is a linear 33-amino acid peptide, which is composed of the antigenic peptide, PLP$_{139\text{-}151}$, and the B7 binding peptide, B7AP, which is derived from the conserved region of the CD28 molecule. Both peptides are covalently conjugated to each other via a linker composed of ε-aminocaproic acid and glycine. The N- and C- termini of the peptide are capped by acetylation and amidation, respectively. The hypothesis is that the PLP$_{139\text{-}151}$ portion will bind to MHC-II (I-A^s) and B7AP will simultaneously bind to B7 on the surface of the APC.

This binding process prevents the formation of complete Signal 1 and Signal 2 and alters the commitment of T cells from inflammatory to regulatory cells, suppressing the progression of autoimmune diseases. PLP-B7AP is proposed to generate long-term tolerance towards a specific antigen without suppressing the general immune response. To test this proposed hypothesis, PLP-B7AP's efficacy was determined and compared to several control peptides in suppressing EAE in the mouse model. The effect of PLP-B7AP peptide on cytokine production was evaluated to understand the potential mechanism of action of PLP-B7AP in suppressing EAE.

3.2 MATERIALS AND METHODS

3.2.1 Mice

All protocols for experiments involving SJL/J (H-2^s) (Charles River, Wilmington, MA) were approved by the University's Institutional Animal Care and Use Committee. The mice were housed under specific pathogen-free conditions at a facility at the University of Kansas, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

3.2.2 Peptide Synthesis

Peptides used in the present study are listed in **Table 3.1**. The peptides were synthesized with 9-fluorenylmethyloxy-carbonyl-protected amino acid chemistry on an appropriate PEG-PS™ resin (Applied Biosystems, Foster City, CA) using an automated peptide synthesis system (Pioneer™:PerSeptive Biosystems, Framingham, MA). Cleavage of the peptides from the resin and removal of the protecting groups from the side-chain were carried out using 90% TFA with 10% scavenger reagents (1,2-ethane dithiol (3%), anisole (2%), and thioanisole (5%)). The crude peptides were purified by reversed-phase HPLC using a semi-preparative C18 column with a

Peptide	Sequence
PLP$_{139-151}$	HSLGKWLGHPDKF
B7AP	Ac-EFMYPPPYLD-NH$_2$
PLP-B7AP	Ac-HSLGKWLGHPDKF-(AcpGAcpGAcp)$_2$-EFMYPPPYLD-NH$_2$
PLP-BPI	Ac-HSLGKWLGHPDKF-(AcpGAcpGAcp)$_2$-ITDGEATDSG -NH$_2$

Table 3.1: List of peptides used in the present study

Acp in the linker represents ε-aminocaproic acid. Ac- represents the acetyl-capped N-terminus of the peptide. -NH$_2$ represents the amide-capped C-terminus of the peptide.

gradient of solvent A (95%/5% = H_2O (0.1% TFA)/acetonitrile) and solvent B (100% acetonitrile). The purity of the peptides was analyzed by HPLC using an analytical C18 column. The identity of the synthesized peptide was confirmed by electrospray ionization mass spectrometry.

3.2.3 Induction of EAE and Clinical Evaluation

SJL/J female mice (5–7 weeks old) were immunized subcutaneously (s.c.) with 200 μg PLP in a 0.2 ml emulsion comprised of equal volumes of phosphate-buffered saline (PBS) solution and complete Freund's adjuvant (CFA) containing killed mycobacterium tuberculosis strain H37RA at a final concentration of 4 mg/ml (Difco, Detroit, MI). The PLP/CFA emulsion was administered to regions above the shoulder and the flanks (total of 4 sites; 50 μl at each injection site). In addition, 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally (i.p.) on the day of immunization (day 0) and 48 h post-immunization. The clinical scores that reflect the disease progression were determined by the same observer in a blinded fashion using a scale ranging from 0 to 5 as follows: 0 - no clinical symptoms, 1 - limp tail or waddling gait with tail tonicity; 2 - waddling gait with limp tail (ataxia); 2.5 - ataxia with partial paralysis of one limb; 3 - full paralysis of one limb; 3.5 - full paralysis of one limb with partial paralysis of the second limb; 4 - full paralysis of two limbs; 4.5 - full paralysis of two limbs with partial paralysis of forelimbs; 5 - moribund or dead. Body weight was also measured daily.

3.2.4 *In Vivo* Peptide Treatments

Study I: This study was performed to test the *in vivo* efficacy of PLP-B7AP in suppressing EAE. Mice were immunized on day 0 in order to develop EAE as described in section 3.2.3. Each mouse received s.c. injections of PLP-B7AP at a concentration of 100 nmol/100 μl/injection (in PBS) on days 4, 7, and 10. The efficacy of PLP-B7AP was compared to that of the vehicle (PBS), 100 nmol/100 μl of PLP, 100 nmol/100 μl of B7AP, and a equal mixture of PLP and B7AP (100 nmol each diluted in 100 μl PBS). The efficacy of each peptide was evaluated by monitoring the clinical score and the change in body weight over a period of 25 days.

Study II: The purpose of this study was to evaluate the potency of PLP-B7AP at a lower dose and lower frequency of injections. EAE was induced on day 0. The first group of mice received s.c. injections of PLP-B7AP at a concentration of 50 nmol/100 μl (in PBS) on days 4, 7 and 10, and its efficacy was compared to that of the negative control (100 μl PBS) and positive control (50 nmol/100 μl of PLP-BPI). In addition another group of mice was treated with only one s.c. injection (100 nmol/100 μl) of PLP-B7AP on day 4. The potency of each treatment was evaluated using the clinical score and the change in body weight over a period of 25 days.

Study III: The efficacy of PLP-B7AP in a vaccine-like treatment was also evaluated, i.e. administration of peptide prior to induction of disease. In this study, the mice received three s.c. injections of PLP-B7AP (100 nmol/100 μl) on days −11, −8 and −5, and EAE was induced on day 0. The efficacy of PLP-B7AP after administration prior to EAE induction was compared to that of the negative control (100 μl PBS). The efficacy of the peptide as a vaccine was evaluated by monitoring the clinical score and change in body weight over a period of 25 days.

3.2.5 *In Vitro* Cytokine Production Assay

In vitro cytokine assays were performed following a protocol similar to that reported previously.[21] EAE was induced in SJL/J mice by injection of PLP/CFA and pertussis toxin as described in section 3.2.3, and mice were treated with either PBS (100 μl) or PLP-B7AP (100 nmol/100 μl/injection) on days 4, 7, and 10. Spleens were isolated from three PLP-B7AP- and PBS-treated mice on the day of maximum disease (i.e., day 15) and day of remission (day 30). Single cell suspensions of splenocytes were harvested by gently mashing the spleen through a cell strainer using the rubber end of a 1-ml syringe in a petri dish containing serum-free RPMI-1640 supplemented with 10% fetal bovine serum, 100 U penicillin/100 μg streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. Red blood cells were lysed using ACK lysis buffer (Invitrogen). The remaining splenocytes were then washed three times with serum-free RPMI-160 medium (Cellgro). The cells were then primed with PLP (20 μM) in a 24-well plate (5×10^6 cells/well). Supernatants of cell cultures were collected for cytokine detection 72 hours later and stored in a –80°C freezer until analysis. Secreted IL-2, IL-4, IL-5, IL-6, and IL-17 were measured by quantitative ELISA-based Q-Plex[TM] assay (Quansys Biosciences, Logan, UT).

3.2.6 Statistical Analysis

Statistical analysis was done using one-way analysis of variance followed by Fisher's least significance difference to compare the different parameters, including EAE clinical scores, change in body weights, and *in vitro* cytokine production. All statistical analyses were performed using StatView software (SAS Institute, Inc., Cary, NC). A *p*-value of less than 0.05 was used as the criterion for statistical significance.

3.3 RESULTS

3.3.1 Study I: Suppression of EAE by PLP-B7AP

For *in vivo* study I, PLP-B7AP's efficacy in suppressing EAE was evaluated for the first time. Three injections were administered s.c. on days 4, 7, and 10. Its efficacy was compared to that of the vehicle (PBS) negative control as well as PLP, B7AP and an unconjugated mixture of PLP and B7AP. Each peptide was administered at a concentration of 100 nmol/100 µl in PBS solution. The clinical score results (**Fig. 3.2A**) indicated that PLP-B7AP suppressed the disease completely with 100% of the mice disease-free ($p < 0.0001$ when compared to PBS). All the PBS-treated mice exhibited severe signs of EAE, which peaked at day 13 with a maximal disease score of 3.5. The PLP-treated mice also exhibited severe signs of EAE but it was still significantly less than the PBS-treated group ($p < 0.05$) with a maximal disease score of 2.6. In addition, the B7AP peptide suppressed disease significantly when compared to PBS ($p < 0.0001$) with a maximal score of 1.5, but it was still not as effective as PLP-B7AP ($p < 0.0001$). Finally, to test the importance of the covalent linker connecting PLP to B7AP, the unconjugated mixture was also tested. It was found that it suppressed the disease slightly better than B7AP, but it was not significantly different ($p > 0.05$) and was less effective than PLP-B7AP ($p < 0.0001$). The loss in body weight correlated well with what was reported from the clinical scores (**Fig. 3.2B**) except that the group treated with the PLP and B7AP mixture had significantly lower loss in body weight compared to the B7AP-treated mice ($p < 0.001$). The PBS-treated mice lost approximately 27.04% of their body weight during the peak of the disease, while the PLP- and B7AP-treated mice lost 19.05% and 13.95% of their body weight, respectively. The mice treated with the unconjugated mixture of PLP and B7AP had a very small loss in body weight with a maximum of 4.91%, while the PLP-B7AP-treated mice mostly exhibited a gain in body weight with a maximal loss of 1.6%.
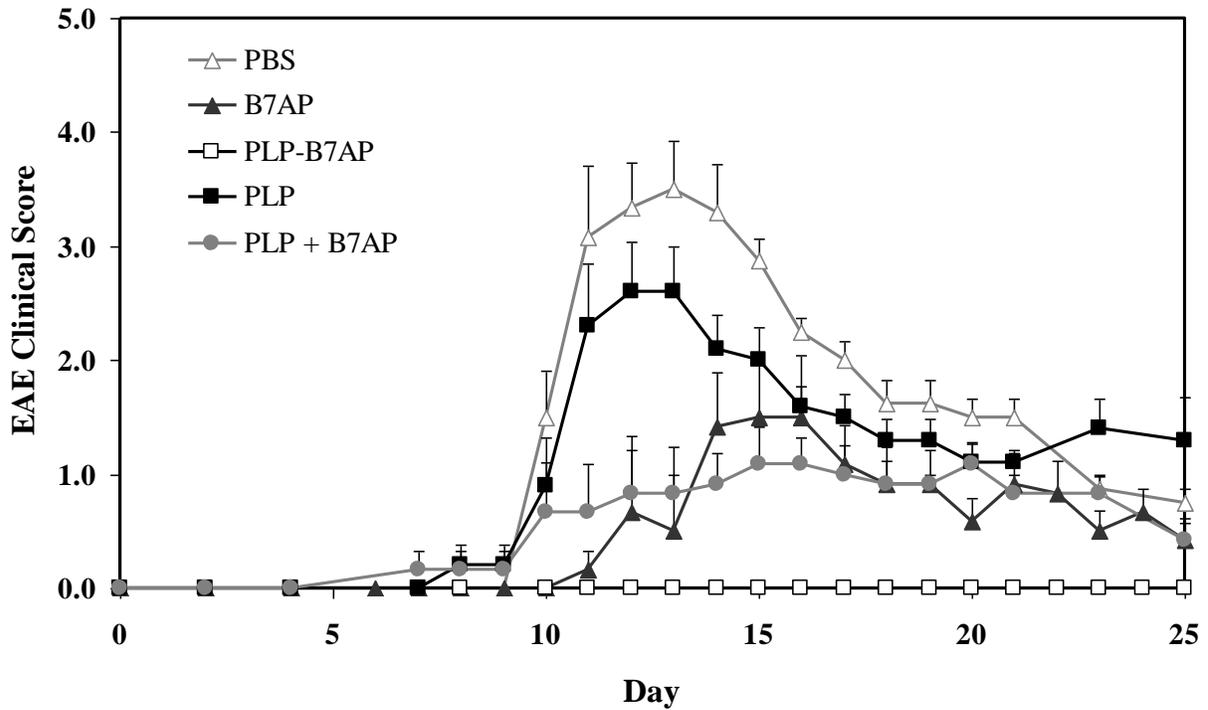
Figure 3.2A *In vivo* efficacies of PLP-B7AP and all the controls in suppressing EAE in the mouse model upon treatment with peptides and immunization with PLP/CFA on day 0. PBS-treated mice received s.c. injections of 100 μl PBS on days 4, 7, and 10. PLP-, B7AP-, PLP + B7AP mixture- and PLP-B7AP-treated mice received 100 nmol/100 μl PBS on days 4, 7, and 10 (s.c.). The efficacy of each peptide was determined by clinical disease score of EAE. Results are expressed as the mean clinical score ± SEM ($n = 6$ for all and $n = 8$ for PLP-B7AP).
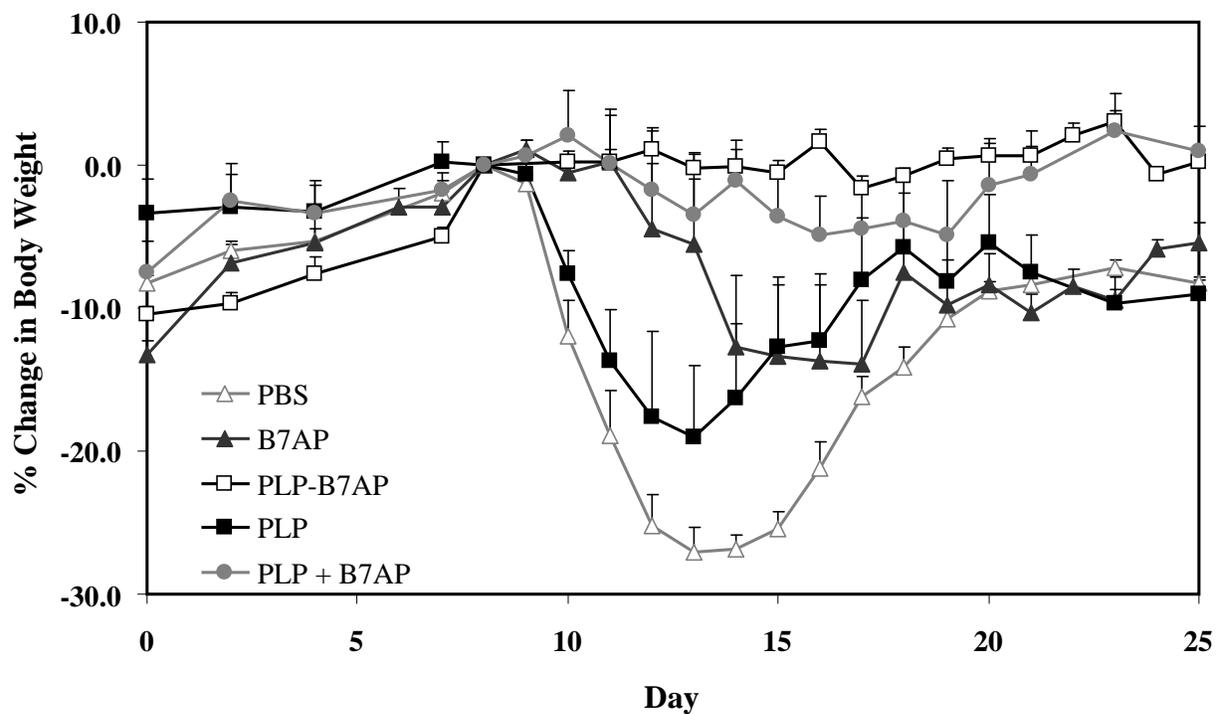
Figure 3.2B *In vivo* efficacies of PLP-B7AP and all the controls in suppressing EAE in the mouse model upon treatment with peptides and immunization with PLP/CFA on day 0. PBS-treated mice received s.c. injections of 100 μl PBS on days 4, 7, and 10. PLP-, B7AP-, PLP + B7AP mixture- and PLP-B7AP-treated mice received 100 nmol/100 μl PBS on days 4, 7, and 10 (s.c.). The efficacy of each peptide was determined by percent change in body weight, relative to day 8 (day of disease onset). Results are expressed as the mean % change in body weight ± SEM ($n = 6$ for all and $n = 8$ for PLP-B7AP).

3.3.2 Study II: Potency and Dose Dependency of PLP-B7AP

In the second *in vivo* study, the potency of PLP-B7AP was evaluated by administering only one injection of PLP-B7AP; in addition, its dose dependency was evaluated and compared to that of a previously well-studied BPI molecule, PLP-BPI.[22-24] The dose-dependency study was achieved by administering half the dose (i.e., 50 nmol/100 µl in PBS). After one s.c. injection, PLP-B7AP delayed the onset of disease, and more, importantly suppressed disease significantly when compared to the PBS-treated mice ($p < 0.0001$). The PLP-B7AP-treated mice eventually reached a maximum clinical score of 1.2 (**Fig. 3.3A**) with a maximum loss of body weight of 12.22% (**Fig. 3.3B**). To test dose-dependency, the efficacy of one injection (50 nmol/100 µl) of PLP-B7AP was compared to that of one injection of PLP-BPI (50 nmol/100 µl). The clinical score (**Fig. 3.3A**) results indicated that there was no significant difference between the PLP-B7AP- and the PLP-BPI-treated mice ($p > 0.05$). The average maximum clinical score reached for the PLP-B7AP- and the PLP-BPI-treated mice were 0.33 and 0.3, respectively. Both treatments suppressed disease significantly when compared to the PBS-treated mice ($p < 0.0001$). However, there was a significant difference in the loss of body weight (**Fig. 3.3B**) between the PLP-B7AP- and PLP-BPI-treated mice ($p < 0.05$). PLP-BPI treated mice lost a small amount of body weight, reaching a maximum of 4.36%, while the PLP-B7AP had a significantly healthier gain in body weight when compared to the PLP-BPI-treated mice.

3.3.3 Study III: Vaccination with PLP-B7AP

For the final *in vivo* study, the hypothesis is that BPI molecules act by promoting the regulatory immune response towards the specific antigen in the BPI molecules. Therefore, 100 nmol/injection of PLP-B7AP was injected on days −11, −8, and −5 prior to induction of disease.
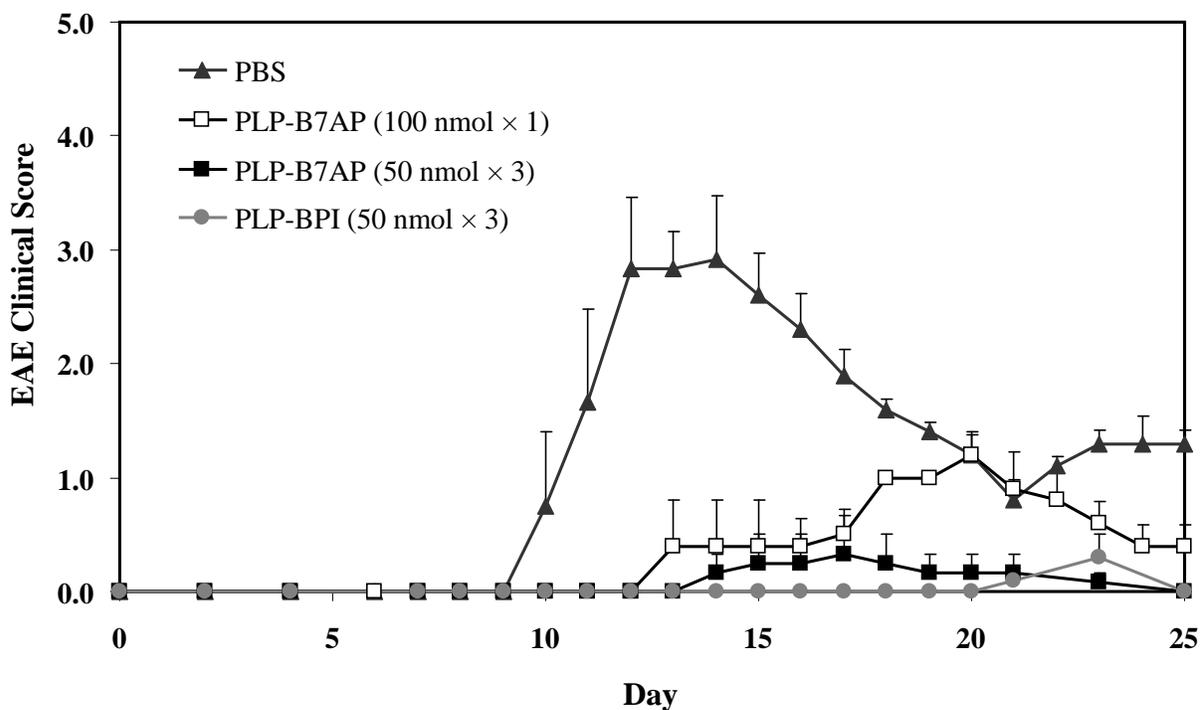
Figure 3.3A *In vivo* evaluation of PLP-B7AP's potency in suppressing EAE in the mouse model upon treatment with peptide and immunization with PLP/CFA on day 0. PBS-treated mice received s.c. injections of 100 μl PBS on days 4, 7, and 10. One group received one s.c. dose of 100 nmol/100 μl PBS on days 4 of PLP-B7AP. The remaining groups received three s.c. injections of PLP-B7AP and the positive control, PLP-BPI, at a dose of 50 nmol/100 μl PBS on days 4, 7, and 10. The efficacy and potency of the peptide were determined by clinical disease score of EAE. Results are expressed as the mean clinical score $\pm$ SEM ($n = 6$).
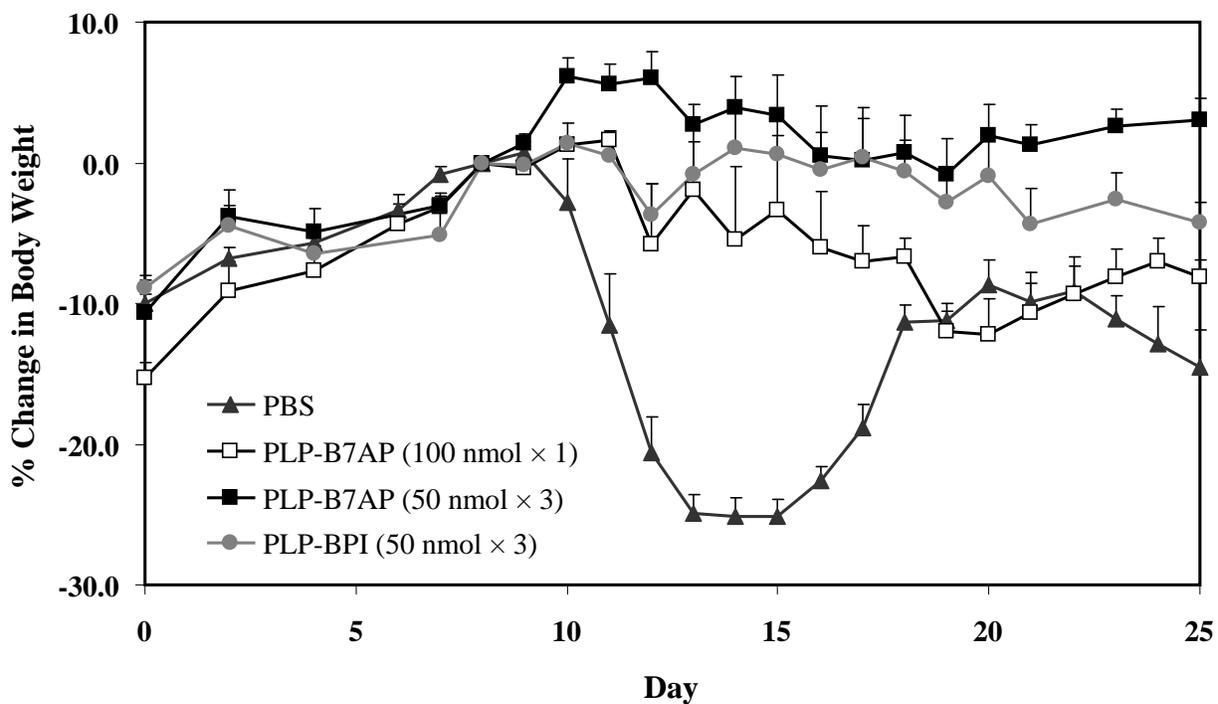
Figure 3.3B *In vivo* evaluation of PLP-B7AP's potency in suppressing EAE in the mouse model upon treatment with peptide and immunization with PLP/CFA on day 0. PBS-treated mice received s.c. injections of 100 µl PBS on days 4, 7, and 10. One group received one s.c. dose of 100 nmol/100 µl PBS on days 4 of PLP-B7AP. The remaining groups received three s.c. injections of PLP-B7AP and the positive control, PLP-BPI, at a dose of 50 nmol/100 µl PBS on days 4, 7, and 10. The efficacy and potency of the peptide were determined by percent change in body weight, relative to day 8 (day of disease onset). Results are expressed as the mean % change in body weight ± SEM (*n* = 6).

According to the clinical score, PLP-B7AP suppressed EAE completely in a fashion similar to when it was injected after induction of disease, while the PBS-treated mice reached a maximum clinical score of 2.56 (**Fig. 3.4A**). The change in body weight of the mice confirmed the results observed by the clinical score, since only a gain in body weight in the PLP-B7AP-treated mice was observed and the PBS-treated mice lost a maximum of 19.37% (**Fig. 3.4B**).

3.3.4 *In Vitro* Cytokine Production

To better understand the mechanism of action of PLP-B7AP, splenocytes were isolated and their cytokine production determined using quantitative ELISA-based Q-Plex^{TM} assay. This method unfortunately does not determine exact concentrations of circulating cytokines, but will provide information regarding the general immune response in the body. If there is a general inflammatory response, it would be expected to see more of a T_H17 and T_H1 phenotype, both being crucial players in the progression of EAE. To treat EAE, therapies need to promote the regulatory and suppressor T cell phenotypes (i.e., more production of T_{reg} and T_H2). The prevalent phenotype of T cells can be determined by the cytokines produced by the splenocytes. If there is a higher population of T_H1 and T_H17 cells, there will be a greater production of their respective cytokines, IL-6 and IL-17. If T_{reg} (IL-2) and T_H2 (IL-4 and IL-5) cells are more prevalent, then there will be a greater production of cytokine markers.

Splenocytes were isolated on days 15 and 30, which correspond to the day of most severe disease and the day when EAE is in remission, respectively, and their cytokine production was measured to determine the T cell phenotype present. At the day of maximum disease score (day 15), there was a significant drop in the production of IL-17 in the PLP-B7AP-treated mice compared to the PBS-treated mice ($p < 0.0001$). At day 30, PLP-B7AP-treated mice produced a
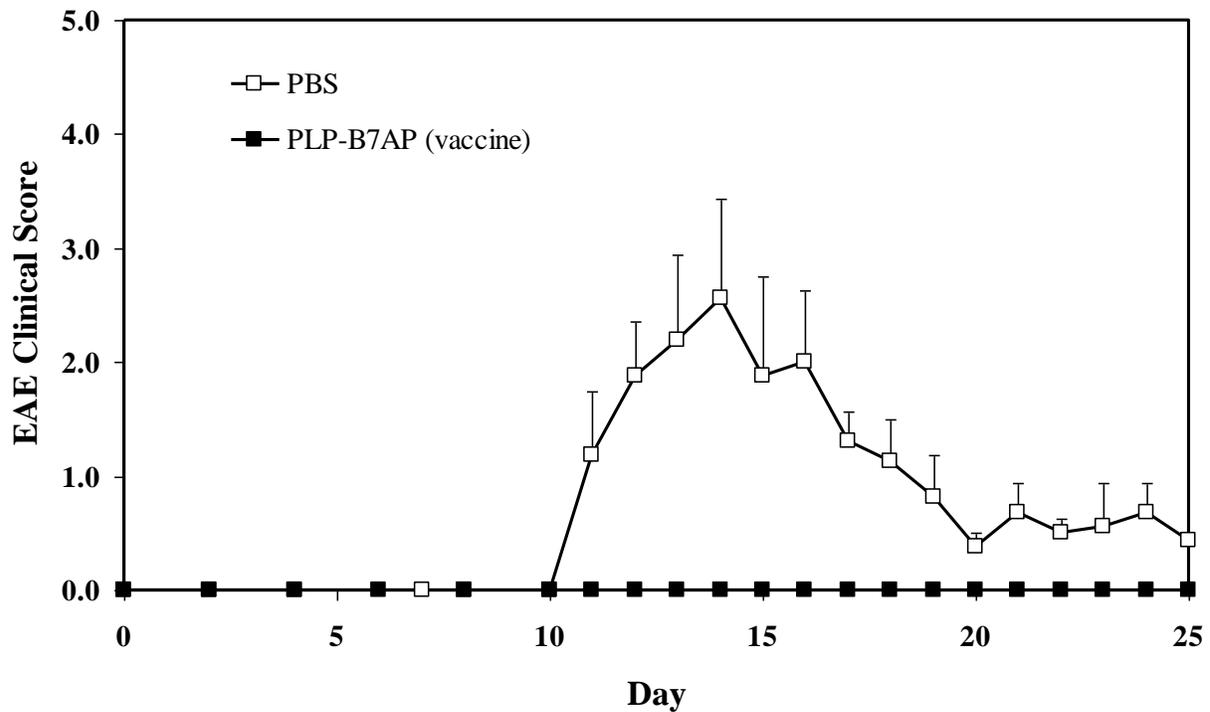
Figure 3.4A Evaluation of PLP-B7AP's *in vivo* efficacy as a vaccine in suppressing EAE. PBS-treated mice received s.c. injections of 100 μl PBS on days −11, −8, and −5. The PLP-B7AP group was vaccinated on days −11, −8, and −5. Each mouse received a s.c. injection of 100 nmol/100 μl PBS. The efficacy of the peptide was determined by clinical disease score of EAE. Results are expressed as the mean clinical score ± SEM ($n = 6$).
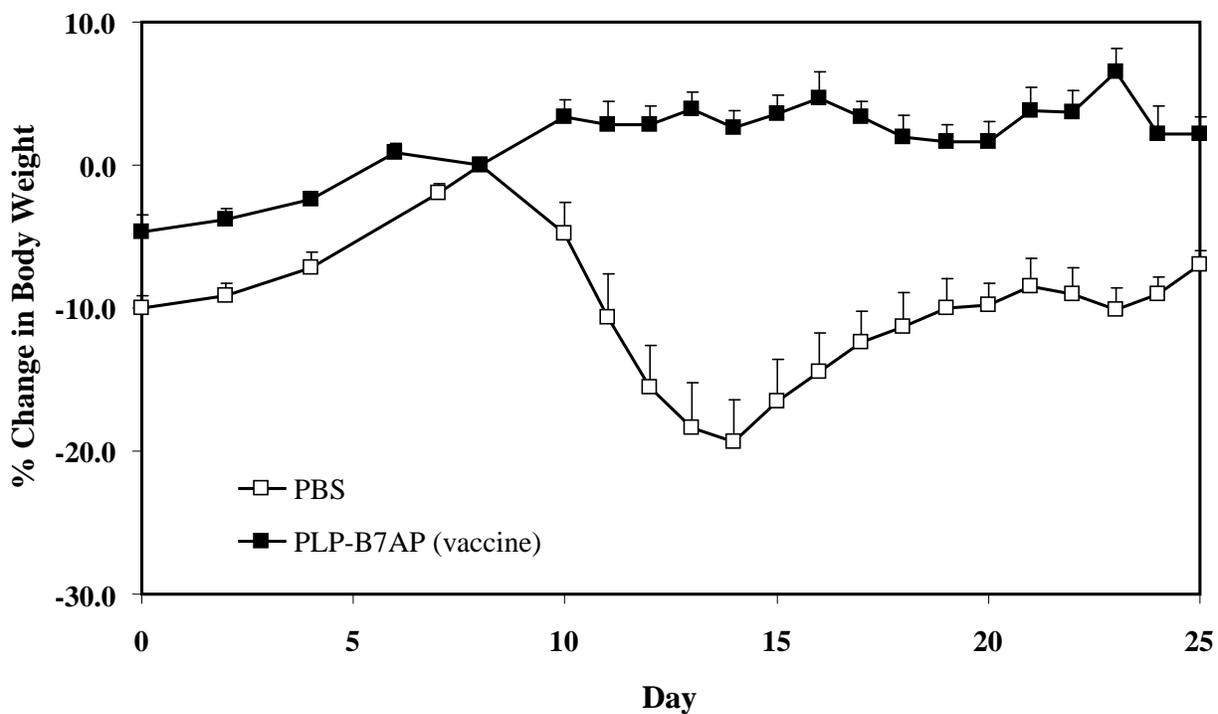
Figure 3.4B Evaluation of PLP-B7AP's *in vivo* efficacy as a vaccine in suppressing EAE. PBS-treated mice received s.c. injections of 100 μl PBS on days −11, −8, and −5. The PLP-B7AP group was vaccinated on days −11, −8, and −5. Each mouse received a s.c. injection of 100 nmol/100 μl PBS. The efficacy of the peptide was determined by percent change in body weight, relative to day 8 (day of disease onset). Results are expressed as the mean % change in body weight ± SEM ($n = 6$).

much lower amount of IL-17 compared to the PBS-treated mice, but not to the extent of day 15 ($p < 0.0001$). This is probably due to the fact that, at this stage, the disease is much weaker. Another pro-inflammatory cytokine tested was IL-6, which is a T_H1 marker. On day 15, the production of IL-6 in the PLP-B7AP-treated mice was lower compared to that of PBS-treated mice ($p = 0.058$). At day 30, there was no difference in the production of IL-6 for the PBS- and PLP-B7AP-treated mice. Therefore, the results indicated that there was a significant shift away from the T_H17 phenotype for both days (**Fig. 3.5A**). However, there was only a significant shift away from T_H1 phenotype only during the day of maximum disease but not after disease remission (**Fig. 3.5B**), suggesting that the shift in T_H1 balance is most needed during the development of the disease.

To monitor whether PLP-B7AP influences the regulatory/suppressor immune response, production of anti-inflammatory cytokines was measured. It has been reported that IL-2 has a main role in the development of T_{reg}; therefore, IL-2 production was monitored upon treatment of mice with PLP-B7AP. On both days 15 and 30, there was an increase in the production of IL-2 by the splenocytes from PLP-B7AP-treated mice when compared to that of PBS-treated mice (**Fig. 3.6A**, $p < 0.0001$ for day 15 and $p = 0.06$ for day 30). For the key T_H2 cytokine markers (i.e., IL-4 and IL-5), PLP-B7AP-treated mice produced a significantly greater amount of IL-4 on both days 15 and 30 (**Fig. 3.6B**, $p < 0.0001$) compared to PBS-treated mice. However, the production of IL-5 was only significantly higher on day 30 for PLP-B7AP-treated mice compared to PBS-treated mice ($p < 0.001$); there was no observable difference in IL-5 production on day 15 for either PLP-B7AP- or PBS-treated mice (**Fig. 3.6C**). These results indicated that there was a shift towards the T_{reg} and T_H2 phenotype when the mice were treated with PLP-B7AP.
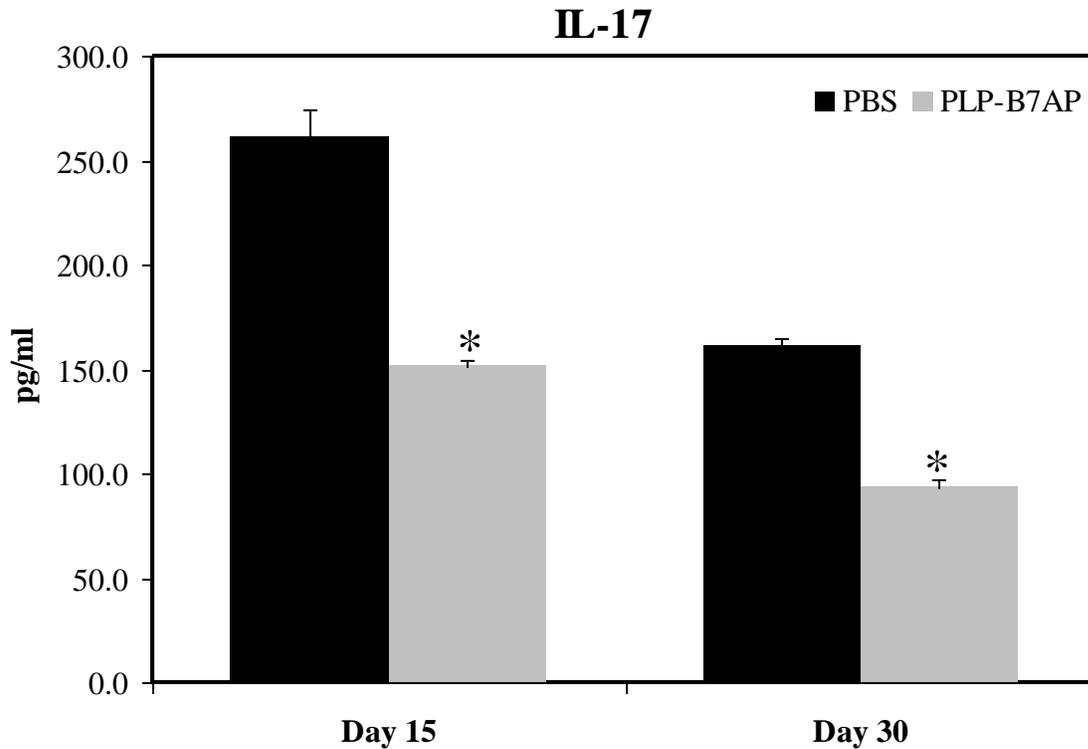
Figure 3.5A Concentrations of the pro-inflammatory cytokine, IL-17, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice on either day 15 or 30. Each mouse was treated with either PBS or PLP-B7AP on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated in vitro with PLP$_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.0001$).

IL-6
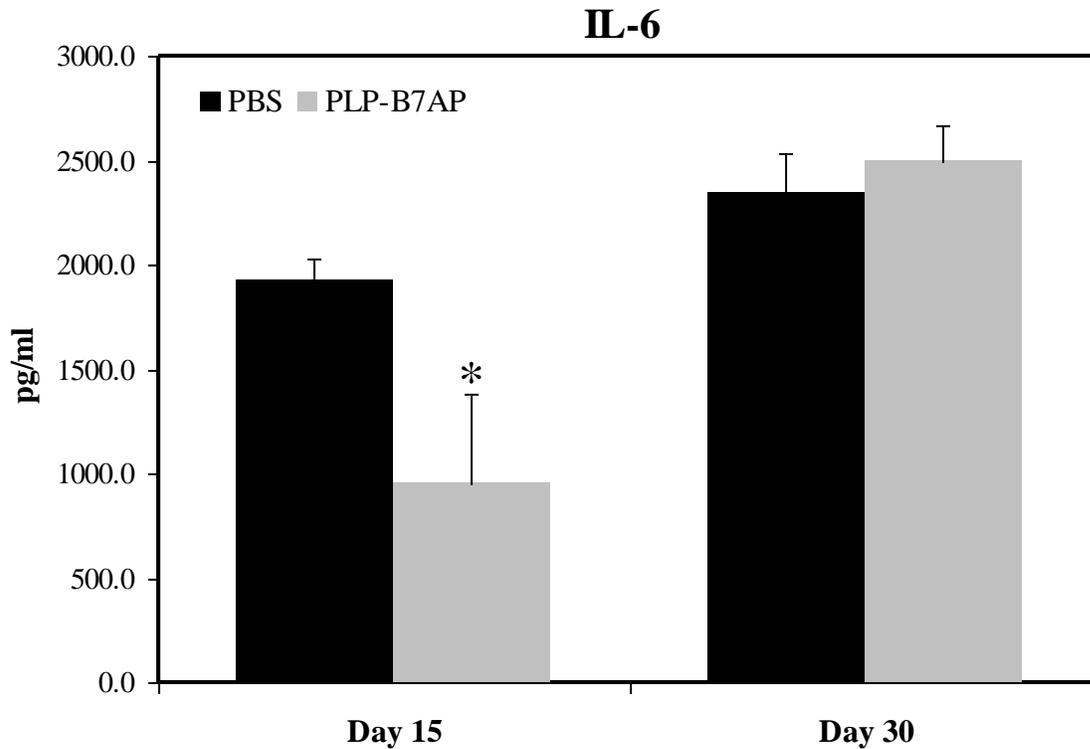
Figure 3.5B Concentrations of the pro-inflammatory cytokine, IL-6, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice on either day 15 or 30. Each mouse was treated with either PBS or PLP-B7AP on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated in vitro with $PLP_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.05$).
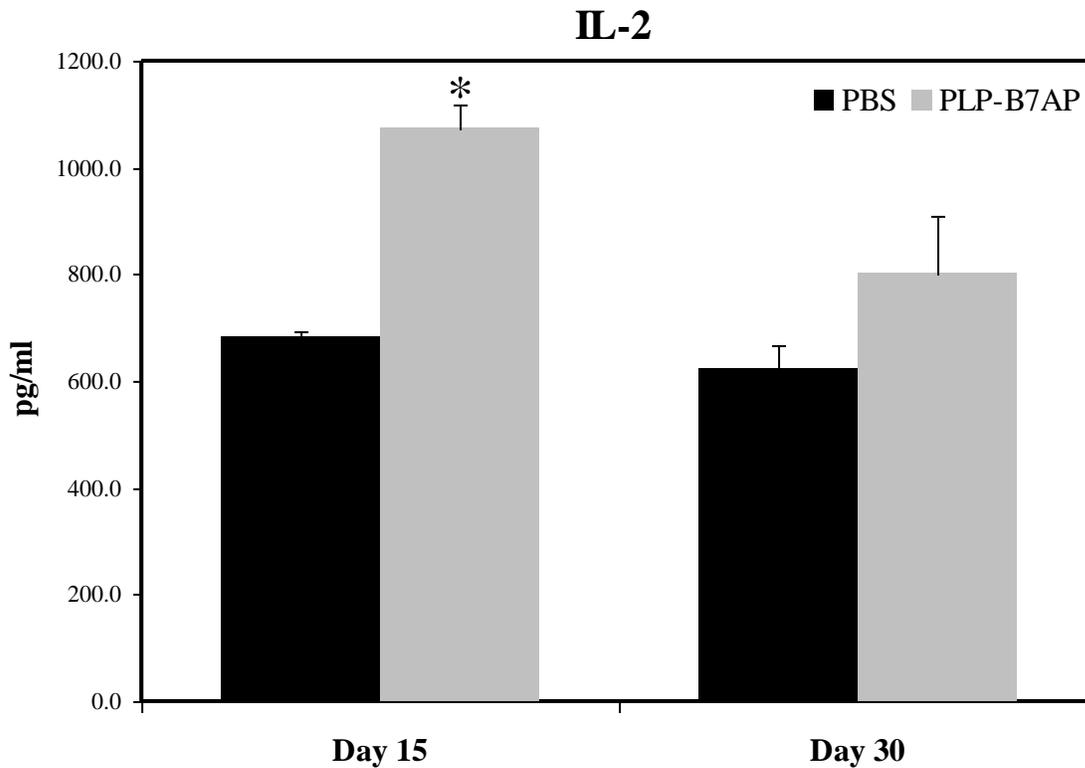
Figure 3.6A Concentrations of the regulatory cytokine, IL-2, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice on either day 15 or 30. Each mouse was treated with either PBS or PLP-B7AP on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated in vitro with PLP$_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.0001$).

IL-4
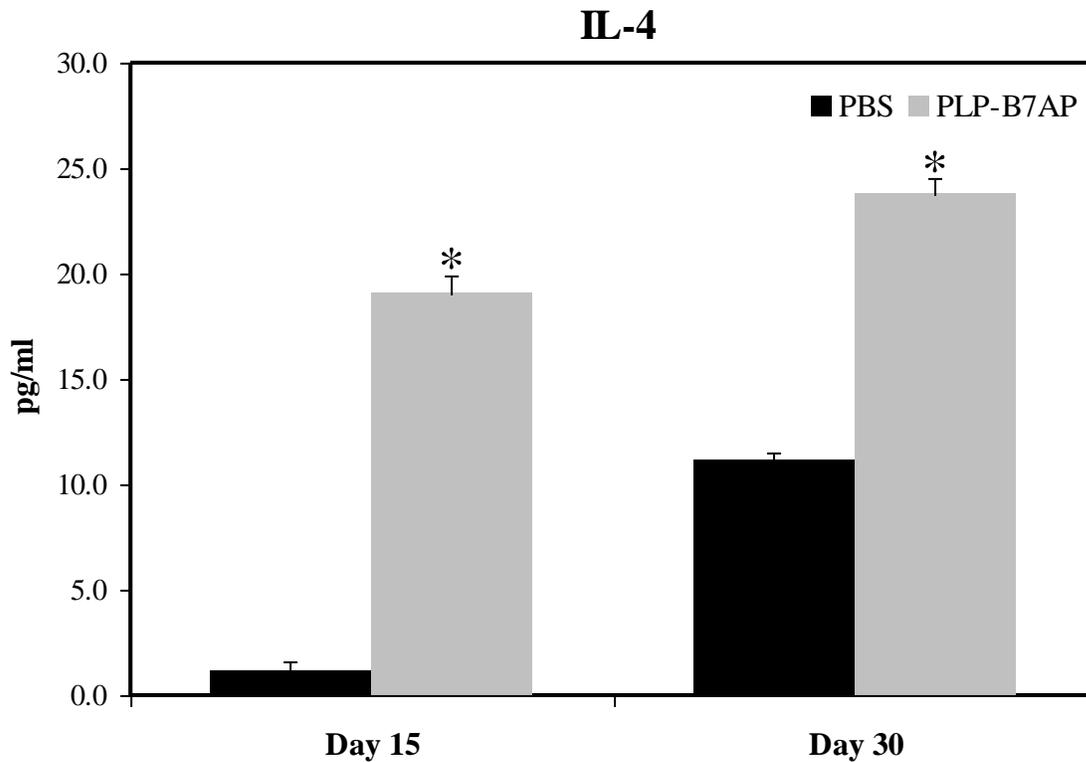
Figure 3.6B Concentrations of the suppressor cytokine, IL-4, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice on either day 15 or 30. Each mouse was treated with either PBS or PLP-B7AP on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated in vitro with $PLP_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.0001$).
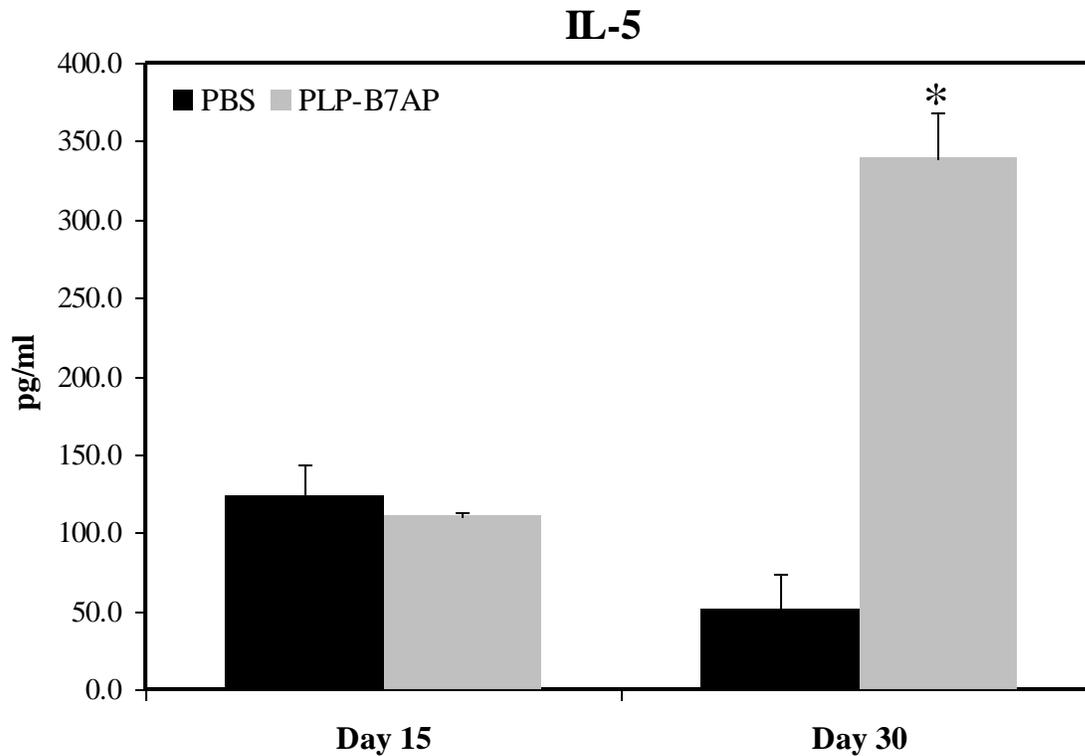
Figure 3.6C Concentrations of the suppressor cytokine, IL-5, from the cell culture supernatant. Splenocytes were isolated from the spleens of EAE-induced mice on either day 15 or 30. Each mouse was treated with either PBS or PLP-B7AP on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated in vitro with $PLP_{139-151}$ and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.001$)..

3.4 DISCUSSION

PLP-B7AP is a novel compound, which statistically has similar efficacy to that of the previously known as PLP-BPI;[22-24] all the *in vivo* results from the present study are summarized in **Table 3.2**. PLP-B7AP was significantly better compared to PLP and B7AP in delaying the onset and suppressing EAE. More importantly, PLP-B7AP treatment was more effective than that of a mixture of unconjugated PLP and B7AP, indicating that the conjugation has an important role in efficacy. In addition, lower dose administrations of PLP-B7AP could provide information on the lowest limit efficacy dose in the therapeutic index of PLP-B7AP. It was interesting to find that, when PLP-B7AP was injected prior to induction of disease (vaccine treatment), EAE was suppressed completely as seen by the clinical score and normal increase in body weight. It is well known that peptides usually have a short half-life and short residence time in the systemic circulation; however, PLP-B7AP was still effective when delivered in a vaccine dose schedule where the last injection was 5 days before disease stimulation (day 0). This result suggests that PLP-B7AP could stimulate the regulatory immune response (i.e., an increased production of T_{reg}) prior to disease stimulation. Thus, when EAE was induced, T_{reg} responded by downregulating any T_H1- and T_H17-mediated inflammatory response towards the antigen and, therefore, prevented the onset of the disease.

Here, the efficacy of a novel PLP-B7AP molecule is reported for the first time. The B7AP portion of the peptide was derived from CD28,[18] which was proposed to bind B7 and block the activation of B7/CD28 costimulatory signal.[4,5] Another important molecule called CTLA-4 also binds to B7, and the signal generated by CTAL-4/B7 inhibits T-cell activation or negative signal,[9] therefore, this signal is important for downregulating an unwanted inflammatory response in MS or EAE. However, due to the relatively fast binding kinetics of B7

Group	Dose[a]	Incidence of disease[b]	Mean maximal score ± SEM
In vivo **Study I**			
PBS	100 µl/mouse on days 4, 7, and 10	6/6	3.50 ± 0.43
PLP	100 nmol/mouse on days 4, 7, and 10	6/6	2.60 ± 0.44
B7AP	100 nmol/mouse on days 4, 7, and 10	5/6	1.50 ± 0.55
PLP + B7AP	100 nmol/mouse on days 4, 7, and 10	6/6	1.08 ± 0.33
PLP-B7AP	100 nmol/mouse on days 4, 7, and 10	0/8	0.00 ± 0.00
In vivo **Study II**			
PBS	100 µl/mouse on days 4, 7, and 10	6/6	2.92 ± 0.55
PLP-B7AP	100 nmol/mouse on day 4	6/6	1.20 ± 0.20
PLP-B7AP	50 nmol/mouse on days 4, 7, and 10	1/6	0.33 ± 0.33
PLP-BPI	50 nmol/mouse on days 4, 7, and 10	1/6	0.30 ± 0.20
In vivo **Study III**			
PBS	100 µl/mouse on days −11, −8, and −5	6/6	2.56 ± 0.88
PLP-B7AP	100 nmol/mouse on days −11, −8, and −5	0/6	0.00 ± 0.00

Table 3.2: Summary of *in vivo* studies

[a]All injections were administered subcutaneously.

[b]Incidence of disease was defined as a disease score of 1 or higher.

and CTLA-4[25] and the high avidity of this interaction,[26,27] it was proposed that B7AP will not affect this interaction.[18] Therefore, B7AP selectively inhibits the CD28/B7 signal while not affecting the CTLA-4/B7 signal. The other major portion of the peptide is PLP, which binds to the MHC-II molecule on the surface of APC. Finally, the third portion of the peptide is the covalent linker, connecting PLP and B7AP. The linker is a vital part of the peptide and may provide two advantages. The first advantage of linking the two peptides is that it allows the blockade of Signal 2 only in T cells that recognize the PLP portion of the peptide as antigenic, leading to specific immunomodulation. It has been known that specificity is a major problem in most therapies aimed at attenuating the immune response. Lack of specific immunosuppression, the individual can become susceptible to opportunistic infections due to suppression of the general immune response, which is the case with most current therapeutic agents. The second proposed advantage is that the two peptide fragments can bind to their respective receptors on the surface of the APC, thus tethering them together and preventing the formation of the immunological synapse. This may alter the differentiation of T cells from inflammatory to regulatory cells.

Previously, several BPI molecules have been developed to target other receptors on the surface of APC such as the adhesion molecules ICAM-1 and LFA-1.[20,22-24] Both of these adhesion molecules have crucial roles for the activation of T cells, especially after formation of the immunological synapse.[3,28,29] The mechanism of action of these peptides is not yet well understood, but it is hypothesized that they act by hindering the formation of the immunological synapse. In the present study, there is no difference ($p > 0.05$) in efficacy between PLP-B7AP and PLP-BPI, which target B7 and ICAM-1, respectively. This could be due to the fact that both molecules are expressed on the surface of APC and are upregulated when the APC are activated.

Another important mechanism, which may explain the similar efficacies, could be solely due to the antigenic peptide portion, with the adhesion peptide and B7-peptide acting as targeting molecules. Antigenic peptides have become very useful for the treatment of autoimmune and allergic diseases. The proposed mechanism of action for soluble antigenic peptides is that they bind directly to empty MHC-II molecules on the surface of naive APC such as immature dendritic cells[30] (iDC). The presentation of an antigen by an iDC without any antigen processing and in the absence of a co-stimulatory signal is believed to lead to differentiation of naïve T cells to regulatory T cells. Therefore, another proposed mechanism of action is that BPI molecules can bind to iDC resulting in activation of the regulatory immune response and inducing antigen-specific tolerance.

Although the mechanism of action of BPI molecules has not been fully understood, the results of the cytokine profiles from the current study and other studies provide clues on how these peptides modulate the immune response. In the current study, the results indicate strongly that treatment with PLP-B7AP lowers the production of IL-17 on days 15 and 30. This most likely indicates a down-regulation of T_H17 cells, a crucial player in the pathogenesis of EAE.[31,32] These results correlate with previous studies using other BPI molecules[33]. In addition, treatment with PLP-B7AP lowered the production of a pro-inflammatory cytokine IL-6 on day 15. There was also a significant increase in the production of IL-2 and IL-4 on days 15 and 30 and IL-5 on day 30 only, indicating a shift away from the inflammatory T_H1 phenotype to an immunosuppressor/regulator T_H2/T_{reg} phenotype. Although the mechanism of PLP-B7AP is still not well elucidated, it is clear that it can modulate the immune response by shifting the balance away from the inflammatory state to an immunotolerant state.

While there is still some skepticism concerning the use of animal models to test therapies for MS,[34] several of the current drug candidates in clinical trials were initially investigated using animal models. It is true that the disease pathogenesis of EAE and MS are different, but the underlying mechanisms and resulting symptoms are similar. Both diseases exhibit an inflammatory response initiated by $CD4^+$ T cells. Once the knowledge of how to modulate the immune response to specific proteins in animal models has been developed, it may become possible to translate this to humans. The potential application for this specific immunomodulation is enormous. Clinical trials are already underway to try to block Signal 2 molecules for the treatment of autoimmune disease[35,36] and allograft rejection.[37,38] Unfortunately, solely blocking Signal 2 has proven to be dangerous, and many therapies have failed due to the onset of severe side effects from general immunosuppression. In the present study, a novel peptide which specifically targets T cells that recognize PLP as antigenic while simultaneously blocking the delivery of Signal 2, has been developed and may be used one day for antigen specific immunosuppression. This form of treatment has been very successful in the mouse model of EAE and can potentially be a safe way for the attenuation of the immunogenic response in MS without suppressing the general immune response. Moreover, other antigens can be conjugated to these Signal 2 blockers in order to treat epitope spreading problems seen in MS. More importantly, this technology can potentially be translated for the treatment of any other form of autoimmune or allergic disease in which the antigens are known. Our lab has developed other BPI molecules, which are composed of other antigenic peptides and were used for the treatment of other autoimmune disease models such as the non-obese diabetes model for type-I diabetes[39] and the collagen-induced arthritis model for rheumatoid arthritis (unpublished data), as well as using other myelin sheath antigens to treat EAE induced by other epitopes. Once there is

a better understanding of the mechanism of action of these peptides, the same strategies may be employed for the treatment of human immune diseases.

In conclusion, PLP-B7AP, when administered after or before induction of the disease, has been shown to be effective in suppressing EAE. Even though the study of PLP-B7AP in the EAE animal model may not translate directly into humans, this study could improve our understanding of how to effectively modulate the immune response in an antigenic-specific manner. The hope is that BPI molecules such as PLP-B7AP could be use in treating autoimmune diseases without suppressing the general immune response.

3.5 REFERENCES

1   Lassmann, H. Classification of demyelinating diseases at the interface between etiology and pathogenesis. *Curr Opin Neurol* **14**, 253-258 (2001).

2   Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).

3   Tseng, S. Y. & Dustin, M. L. T-cell activation: a multidimensional signaling network. *Curr Opin Cell Biol* **14**, 575-580 (2002).

4   Jenkins, M. K. & Johnson, J. G. Molecules involved in T-cell costimulation. *Curr Opin Immunol* **5**, 361-367 (1993).

5   June, C. H., Bluestone, J. A., Nadler, L. M. & Thompson, C. B. The B7 and CD28 receptor families. *Immunol Today* **15**, 321-331 (1994).

6   Peach, R. J. *et al.* Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. *J Exp Med* **180**, 2049-2058 (1994).

7   Truneh, A. *et al.* Differential recognition by CD28 of its cognate counter receptors CD80 (B7.1) and B70 (B7.2): analysis by site directed mutagenesis. *Mol Immunol* **33**, 321-334 (1996).

8   Bajorath, J., Metzler, W. J. & Linsley, P. S. Molecular modeling of CD28 and three-dimensional analysis of residue conservation in the CD28/CD152 family. *J Mol Graph Model* **15**, 135-139, 108-111 (1997).

9   McCoy, K. D. & Le Gros, G. The role of CTLA-4 in the regulation of T cell immune responses. *Immunol Cell Biol* **77**, 1-10 (1999).

10   Schonbeck, U. & Libby, P. The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci* **58**, 4-43 (2001).

11   Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med* **181**, 577-584 (1995).

12   Khoury, S., Sayegh, M. H. & Turka, L. A. Blocking costimulatory signals to induce transplantation tolerance and prevent autoimmune disease. *Int Rev Immunol* **18**, 185-199 (1999).

13   Podojil, J. R. & Miller, S. D. Molecular mechanisms of T-cell receptor and costimulatory molecule ligation/blockade in autoimmune disease therapy. *Immunol Rev* **229**, 337-355 (2009).

14   Wetzig, R., Hanson, D. G., Miller, S. D. & Claman, H. N. Binding of ovalbumin to mouse spleen cells with and without carbodiimide. *J Immunol Methods* **28**, 361-368 (1979).

15   Jenkins, M. K. & Schwartz, R. H. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* **165**, 302-319 (1987).

16   Jenkins, M. K. *et al.* Induction and maintenance of anergy in mature T cells. *Adv Exp Med Biol* **292**, 167-176 (1991).

17   Miller, S. D., Turley, D. M. & Podojil, J. R. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol* **7**, 665-677 (2007).

18   Chen, J. *et al.* Allogenic donor splenocytes pretreated with antisense peptide against B7 prolong cardiac allograft survival. *Clin Exp Immunol* **138**, 245-250 (2004).

19   Srinivasan, M. *et al.* Suppression of experimental autoimmune encephalomyelitis using peptide mimics of CD28. *J Immunol* **169**, 2180-2188 (2002).

20   Manikwar, P., Kiptoo, P., Badawi, A. H., Buyuktimkin, B. & Siahaan, T. J. Antigen-specific blocking of CD4-specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev* (2011).

21   Youssef, S. *et al.* The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* **420**, 78-84 (2002).

22   Kobayashi, N. *et al.* Prophylactic and therapeutic suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *Clin Immunol* **129**, 69-79 (2008).

23   Kobayashi, N., Kobayashi, H., Gu, L., Malefyt, T. & Siahaan, T. J. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* **322**, 879-886 (2007).

24   Zhao, H., Kiptoo, P., Williams, T. D., Siahaan, T. J. & Topp, E. M. Immune response to controlled release of immunomodulating peptides in a murine experimental autoimmune encephalomyelitis (EAE) model. *J Control Release* **141**, 145-152 (2010).

25   van der Merwe, P. A., Bodian, D. L., Daenke, S., Linsley, P. & Davis, S. J. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med* **185**, 393-403 (1997).

26   Linsley, P. S. *et al.* Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* **1**, 793-801 (1994).

27   Greene, J. L. *et al.* Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions. *J Biol Chem* **271**, 26762-26771 (1996).

28   van der Merwe, P. A. Formation and function of the immunological synapse. *Curr Opin Immunol* **14**, 293-298 (2002).

29   Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-1542 (2002).

30   Wraith, D. C. Therapeutic peptide vaccines for treatment of autoimmune diseases. *Immunol Lett* **122**, 134-136 (2009).

31   Cao, W. *et al.* Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. *Immunity* **35**, 273-284 (2011).

32   Zepp, J., Wu, L. & Li, X. IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. *Trends Immunol* **32**, 232-239 (2011).

33   Ridwan, R. *et al.* Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor: structure optimization and pharmacokinetics. *J Pharmacol Exp Ther* **332**, 1136-1145 (2010).

34   Baker, D., Gerritsen, W., Rundle, J. & Amor, S. Critical appraisal of animal models of multiple sclerosis. *Mult Scler* **17**, 647-657 (2011).

35   NIAMS. BG9588 (Anti-CD40L Antibody) to Treat Lupus Nephritis. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000-[cited 2011 Oct 14]. Available from: http://clinicaltrials.gov/ct2/show/NCT00001789?

term=BG9588+%28Anti-CD40L+Antibody%29+to+Treat+Lupus+Nephritis&rank=1 NLM Identifier: NCT00001789.

36   University of California, S. F. T1DM Immunotherapy Using CD4+CD127lo/-CD25+ Polyclonal Tregs. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Oct 14]. Available from: http://clinicaltrials. gov/ct2/show/NCT01210664?term=T1DM+Immunotherapy+Using+CD4%2BCD127lo %2F-CD25%2B+Polyclonal+Tregs&rank=1 NLM Identifier: NCT01210664.

37   Institute, D.-F. C. Bone Marrow Transplantation With Specially Treated Bone Marrow in Treating Patients With Hematologic Cancer That Has Not Responded to Previous Therapy. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Oct 14]. Available from: http://clinicaltrials.gov/ct2/show/ NCT00005988?term=Bone+Marrow+Transplantation+With+Specially+Treated+Bone+ Marrow + in + Treating + Patients + With + Hematologic + Cancer + That&rank = 1 NLM Identifier: NCT00005988.

38   NIDDK. Study Comparing the Safety of BG9588 (Anti-CD40L Antibody) Against Standard Treatment in Kidney Transplantation. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Oct 14]. Available from: http://clinicaltrials.gov/ct2/show/NCT00001857?term=Study+Comparing+the+Safety+of +BG9588+%28Anti-CD40L+Antibody%29+Against+Standard+Treatment+in+Kidney+ Transplantation&rank=1 NLM Identifier: NCT00001857.

39   Murray, J. S. *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des* **70**, 227-236 (2007).

CHAPTER 4

Suppression of MOG- and PLP-induced experimental autoimmune encephalomyelitis

using a novel multivalent bifunctional peptide inhibitor

4.1 INTRODUCTION

Multiple sclerosis (MS) is an immune mediated neurodegenerative disease of the central nervous system (CNS). The pathogenesis of MS has not yet been fully elucidated; however, it is categorized as a CD4^{+} T-cell-mediated autoimmune disease.[1,2] It is thought that there is a breakdown in the recognition of self versus non-self, and that the immune system starts recognizing protein components of the myelin sheath as antigens. Major immunodominant proteins of the myelin sheath are myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP). During disease, T cells can recognize epitopes of these proteins and initiate an inflammatory immune response towards them, leading to tissue damage. The debris from the broken down tissue leads to epitope spreading and thus, resulting in new tissue components becoming antigenic.[3]

An animal model known as experimental autoimmune encephalomyelitis (EAE) is often used to study MS. This model mimics some of the pathological features of MS such as CNS inflammation, lesion formation, blood-brain barrier (BBB) breakdown and the presence of myelin-specific CD4^{+} T cells.[4,5] In addition, the animal model can be used to study the epitope spreading process.[6] Unlike MS, however, EAE has an initiating antigen that can be controlled by the injection of the encephalotogenic peptide in the presence of complete Freund's adjuvant (CFA). This is a powerful tool because therapies can be developed to specifically suppress the immune response to these antigens. Antigen-specific immunotherapy has become widely investigated recently with the aim of inducing tolerance to specific antigens; therefore, it attenuates the inflammatory response. Previously in our lab, bifunctional peptide inhibitors (BPI) composed of antigenic peptides conjugated to adhesion peptides have been developed and have successfully suppressed EAE.[7-11] BPI molecules contain a specific antigen (i.e., PLP) and have

been predicted to suppress EAE induced by a specific antigen (i.e., PLP). Therefore, they will not be useful for suppressing EAE generated by a different antigen (i.e., MOG or MBP). In addition, specific antigenic-peptide modulation may not solve the problem of epitope spreading when the disease is in the late stage. Therefore, a new kind of BPI molecule known as multivalent BPI (MVB) has been designed with more than one antigen. The goal is that the MVB molecule will modulate the immune response to suppress the disease regardless of the inciting antigen, thus solving the problem of epitope spreading and making this strategy more applicable for translation into a MS therapy.

In EAE and MS, the activation of inflammatory $CD4^+$ T cells is mediated by two signals that are delivered from antigen-presenting cells (APC) to T cells.[12,13] The first signal (Signal 1) is the antigen presentation by the major histocompatibility complex class-II (MHC-II) molecule, which is recognized by the T-cell receptor (TCR). The second signal (Signal 2) is made up of costimulatory and adhesion molecules on both APC and T cells. After interactions of molecular pairs form both signals, a process known as signal translocation occurs to form the immunological synapse that leads to activation of a sub-population of antigenic-specific T cells.[14,15] The hypothesis is that BPI molecules are designed to simultaneously target Signal 1 and adhesion molecules on the surface of APC to hinder the formation of the immunological synapse, which will prevent the activation of the inflammatory T cells that specifically recognize the antigenic portion of the BPI molecule. MVB molecules are composed of more than one antigenic peptide, and can therefore bind to different MHC-II molecules on the same or different APC. Thus, the inflammatory response towards more than one antigen is prevented.

In this study, an MVB comprised of MOG_{38-50} and $PLP_{139-151}$ ($MVB_{MOG/PLP}$) was developed and evaluated for suppressing both forms of EAE. As controls, MOG-BPI and PLP-

BPI were evaluated for cross-reactivity in MOG-induced and PLP-induced EAE. In this case, the efficacy of MOG-BPI was evaluated in PLP-induced EAE, and the efficacy of PLP-BPI was evaluated in MOG-induced EAE. Finally, some mechanistic aspects of MVB were elucidated by determining the cytokines produced by splenocytes of the EAE animals upon treatment.

4.2 MATERIALS AND METHODS

4.2.1 Mice

All protocols for experiments involving SJL/J (H-2^s) (Charles River, Wilmington, MA) and C57BL/6 (Jackson Laboratory, Bar Harbor, ME) were approved by the University's Institutional Animal Care and Use Committee. The mice were housed under specific pathogen-free conditions at a facility at the University of Kansas, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

4.2.2 Peptide Synthesis

Peptides used in this study are listed in **Table 4.1**. 9-Fluorenylmethyloxy-carbonyl-protected amino acid chemistry was used to synthesize all peptides, utilizing an appropriate PEG-PS™ resin (Applied Biosystems, Foster City, CA) in an automated peptide synthesis system (Pioneer™:PerSeptive Biosystems, Framingham, MA). The peptides were cleaved from the resin, and removal of the protecting groups from the side-chain was accomplished with 90% TFA with 10% scavenger reagents (1,2-ethane dithiol (3%), anisole (2%), and thioanisole (5%)). The crude products were purified by reversed-phase HPLC using a semi-preparative C18 column with a gradient of solvent A (95%/5% = H$_2$O (0.1% TFA)/acetonitrile) and solvent B (100% acetonitrile). Analytical HPLC with a C18 column was used to determine the purity of each

Peptide	Sequence
$PLP_{139-151}$ (PLP)	HSLGKWLGHPDKF
MOG_{38-50} (MOG)	GWYRSPFSRVVHL
PLP-BPI	Ac-HSLGKWLGHPDKF-$(AcpGAcpGAcp)_2$-ITDGEATDSG-NH_2
MOG-BPI	Ac-GWYRSPFSRVVHL-XGX-ITDGEATDSG-NH_2
$MVB_{MOG/PLP}$	Ac-GWYRSPFSRVVHL-XGX-ITDGEATDSG-XGX-HSLGKWLGHPDKF-NH_2

Table 4.1: List of peptides used in the present study

Acp in the linker represents ε-aminocaproic acid. Ac- represents the acetyl-capped N-terminus of the peptide. -NH_2 represents the amide-capped C-terminus of the peptide. X represents polyethyleneglycol-3.

peptide. The identity of each synthesized peptide was confirmed by electrospray ionization mass spectrometry.

4.2.3 Induction of EAE and Clinical Evaluation

For the PLP-induced EAE, SJL/J female mice (5–7 weeks old) were immunized subcutaneously (s.c.) with 200 μg of $PLP_{139-151}$ peptide in a 0.2 ml emulsion comprised of equal volumes of phosphate-buffered saline (PBS) solution and CFA containing killed mycobacterium tuberculosis strain H37RA at a final concentration of 4 mg/ml (Difco, Detroit, MI). The PLP/CFA emulsion was administered to regions above the shoulder and the flanks (total of 4 sites; 50 μl at each injection site). In addition, 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally (i.p.) on the day of immunization (day 0) and 48 h post-immunization.

For the MOG-induced EAE, C57BL/6 mice (4–6 weeks old) were immunized in a fashion similar to that mentioned above, except that 200 μg of MOG_{38-50} peptide was used and 400 ng/mouse/injection of pertussis toxin was administered on days 0 and 2. The clinical scores that reflect the disease progression were determined by the same observer in a blinded fashion using a scale ranging from 0 to 5 as follows: 0 - no clinical symptoms, 1 - limp tail or waddling gait with tail tonicity; 2 - waddling gait with limp tail (ataxia); 2.5 - ataxia with partial paralysis of one limb; 3 - full paralysis of one limb; 3.5 - full paralysis of one limb with partial paralysis of the second limb; 4 - full paralysis of two limbs; 4.5 - full paralysis of two limbs with partial paralysis of forelimbs; 5 - moribund or dead. Body weight was also measured daily.

4.2.4 *In vivo* Peptide Treatments

Study I - Cross reactivity of MOG-BPI and PLP-BPI: This study was performed to study the *in vivo* cross-reactivity of MOG-BPI and PLP-BPI in suppressing EAE. This was achieved upon induction of the disease with one antigen followed by treating the animals with a BPI molecule containing another antigen. As positive controls, the *in vivo* efficacies of MOG-BPI and PLP-BPI were evaluated to suppress MOG- and PLP-induced EAE, respectively. Induction of the disease was performed on day 0 as described in section 4.2.3. In the MOG-induced EAE, each mouse received s.c. injections of PLP-BPI and MOG-BPI at a concentration of 100 nmol/100 µl/injection (in PBS) on days 4, 7, and 10. The efficacies of both PLP-BPI and MOG-BPI were compared to that of the vehicle (PBS). In the PLP-induced EAE, MOG-BPI was administered s.c. at a concentration of 100 nmol/100 µl/injection (in PBS) on days 4, 7, and 10. The efficacy of each peptide was evaluated by monitoring the clinical score and the change in body weight over a period of 25 days.

Study II - *In vivo* efficacy of novel MVB$_{MOG/PLP}$ in MOG-induced EAE: The purpose of this study was to evaluate the *in vivo* efficacy of the novel MVB$_{MOG/PLP}$ in suppressing MOG-induced EAE. Mice were immunized with MOG/CFA on day 0 as described in section 4.2.3. The first group of mice received three s.c. injections of MVB$_{MOG/PLP}$ at a concentration of 100 nmol/100 µl (in PBS) on days 4, 7, and 10, and its efficacy was compared to those of the vehicle (100 µl PBS) and positive controls, MOG (100 nmol/100 µl) and MOG-BPI (100 nmol/100 µl). The negative (PBS) control and the positive control were each injected three times on days 4, 7, and 10. The efficacy of each treatment was evaluated using the clinical score and the change in body weight over a period of 25 days.

Study III - *In vivo* efficacy of novel MVB$_{MOG/PLP}$ in PLP-induced EAE: The efficacy of MVB$_{MOG/PLP}$ was also evaluated in PLP-induced EAE. All mice were immunized with

PLP/CFA on day 0 as described in section 4.2.3. One group of mice received three s.c. injections of $MVB_{MOG/PLP}$ at a concentration of 100 nmol/100 µl on days 4, 7, and 10; another group received 100 µl of vehicle (PBS) s.c. on the same days. The efficacy of the peptide was evaluated by monitoring the clinical score and change in body weight over a period of 25 days.

4.2.5 *In vitro* Inflammatory Cytokine Production Assay

In vitro cytokine assays were performed following a protocol similar to that reported previously.[16] Cytokines produced from MOG-induced C57BL/6 mice treated with MOG-BPI and $MVB_{MOG/PLP}$ were measured and compared to that from PBS-treated mice. EAE was induced by injection of MOG/CFA and pertussis toxin as described in section 4.2.3 and mice were treated with PBS (100 µl), MOG-BPI (100 nmol/100 µl/injection) or $MVB_{MOG/PLP}$ (100 nmol/100 µl/injection) on days 4, 7, and 10. Spleens were isolated from three mice from each group on day 30. Single cell suspensions of splenocytes were harvested by gently mashing the spleen through a cell strainer using the rubber end of a 1-ml syringe in a petri dish containing serum-free RPMI-1640 supplemented with 10% fetal bovine serum, 100 U penicillin/100 µg streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol. Red blood cells were lysed using ACK lysis buffer (Invitrogen). The remaining splenocytes were then washed three times with serum-free RPMI-160 medium (Cellgro). The cells were then primed with PLP (20 µM) in a 24-well plate (5×10^6 cells/well). Supernatants of cell cultures were collected for cytokine detection 72 hours later and stored in a $-80°C$ freezer until analysis. Secreted IL-6 and IFN-γ were measured by quantitative ELISA-based Q-Plex[TM] assay (Quansys Biosciences, Logan, UT).

4.2.6 Splenocyte Proliferation Assay

A proliferation assay was conducted in SJL/J mice in order to evaluate the cross-reactivity between MOG and PLP. This was accomplished by isolating splenocytes from three PLP-induced EAE mice per group on day 30 as described in section 4.2.5. Splenocytes were isolated from four different groups. One group was from mice that had no EAE induced. The next three groups were splenocytes harvested from mice treated with PBS (100 μl), PLP-BPI (100 nmol/100 μl/injection) or $MVB_{MOG/PLP}$ (100 nmol/100 μl/injection) on days 4, 7, and 10. The cells were cultured and stimulated with PLP (2 μM), MOG (2 μM) or concanavalin A (positive control). Cells were cultured in a 96-well plate (2×10^5 cells/100 μl/well) for 72 hours. Cultures were then pulsed overnight with 1 μCi of [^{3}H] thymidine per well. Cells were harvested onto glass fiber filters using the FilterMate Harvester (PerkinElmer), and the unincorporated [^{3}H] thymidine was removed by multiple washes according to the procedure recommended by the manufacturers. The incorporated radioactivity was then counted using a β-scintillation counter (Microbeta Trilux, PerkinElmer).

4.2.7 Statistical Analysis

Statistical analysis was done using one-way analysis of variance followed by Fisher's least significance difference to compare the different parameters, including *in vitro* cytokine production, splenocyte proliferation, and values from days 10 – 25 (unless otherwise stated) for EAE clinical scores and change in body weights,. All statistical analyses were performed using StatView software (SAS Institute, Inc., Cary, NC). A *p*-value of less than 0.05 was used as the criterion for statistical significance.

4.3 RESULTS

4.3.1 Study I - Cross Reactivity of MOG-BPI and PLP-BPI

MOG-BPI's efficacy in suppressing MOG-induced EAE was evaluated for the first time. To test whether there is cross-reactivity between the different antigens, PLP-BPI was tested for suppressing MOG-induced EAE. The PBS-treated mice developed very severe EAE with a high average clinical score of 4.0 and 22.15% loss in body weight compared to the day of onset of disease. The best suppression was observed in the MOG-BPI-treated mice, with a significant difference in clinical scores and change in body weights on days 15–25 compared to that of the PLP-BPI-treated group ($p < 0.01$ for clinical score and $p < 0.0001$ for change in body weight) and the PBS-treated group ($p < 0.0001$ for clinical score and change in body weight). PLP-BPI significantly suppressed MOG-induced EAE on days 15–25 compared to PBS as indicated by clinical score ($p < 0.01$) and change in body weight ($p < 0.001$) (**Fig. 4.1A** and **Fig 4.1B**).

For the second part of this study, the efficacy of MOG-BPI was evaluated in suppressing PLP-induced EAE. The PBS-treated mice exhibited signs of EAE with a maximal clinical score of 1.67 and a maximal loss in body weight of 13.2%. The MOG-BPI-treated mice showed similar signs of EAE with a maximal clinical score of 1.5 and a maximal loss in body weight of 11.69%. There were no significant differences in clinical score ($p > 0.05$) between the PBS-treated mice and the MOG-BPI treated mice (**Fig. 4.1C** and **Fig. 4.1D**).

4.3.2 Study II - *In vivo* Efficacy of Novel MVB$_{MOG/PLP}$ in MOG-Induced EAE

For this study, the efficacy of the novel MVB$_{MOG/PLP}$ peptide was tested in suppressing MOG-induced EAE and was compared to that in PBS-, MOG-, and MOG-BPI-treated mice. As expected, PBS-treated mice developed severe EAE with a maximal clinical score of 3.58 and a 24.14% loss in the body weight. The greatest suppression of disease was observed in mice
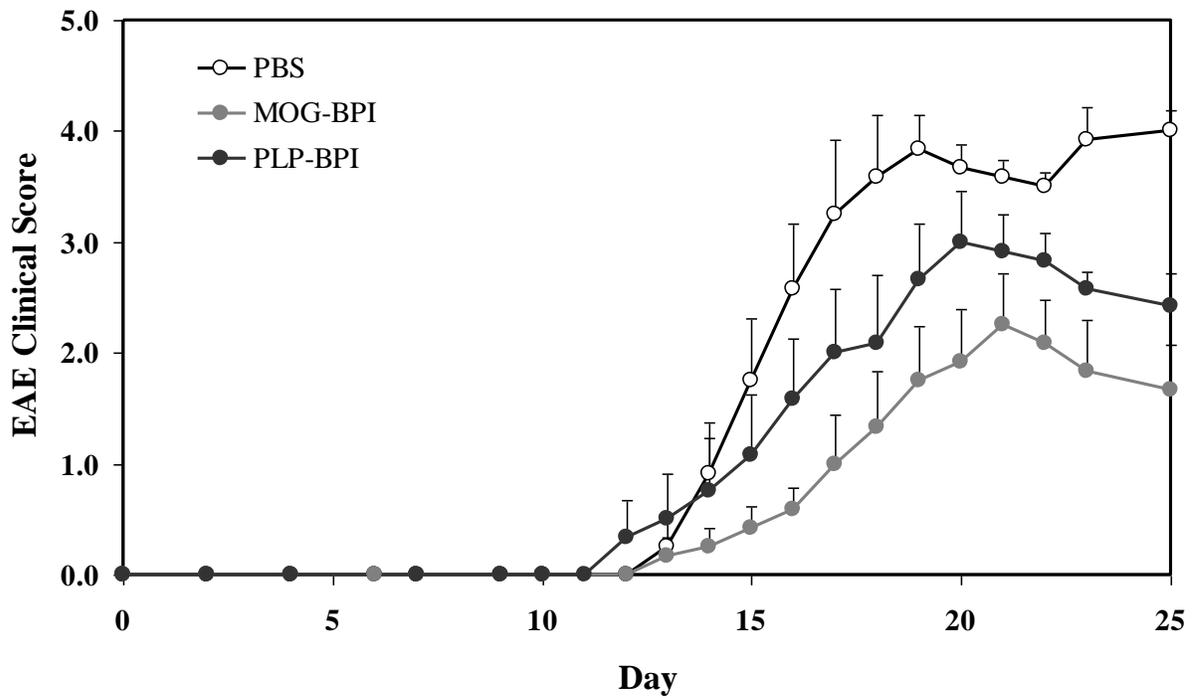
Figure 4.1A *In vivo* cross-reactivity of MOG-BPI and PLP-BPI. PBS (100 μl) and peptides (100 nmol/100 μl) were administered s.c. on days 4, 7, and 10. MOG-BPI- and PLP-BPI-treated mice were compared to PBS-treated mice in the MOG-induced EAE model and efficacy was determined by clinical disease score of EAE. Results are expressed as the mean clinical score ± SEM ($n = 6$).
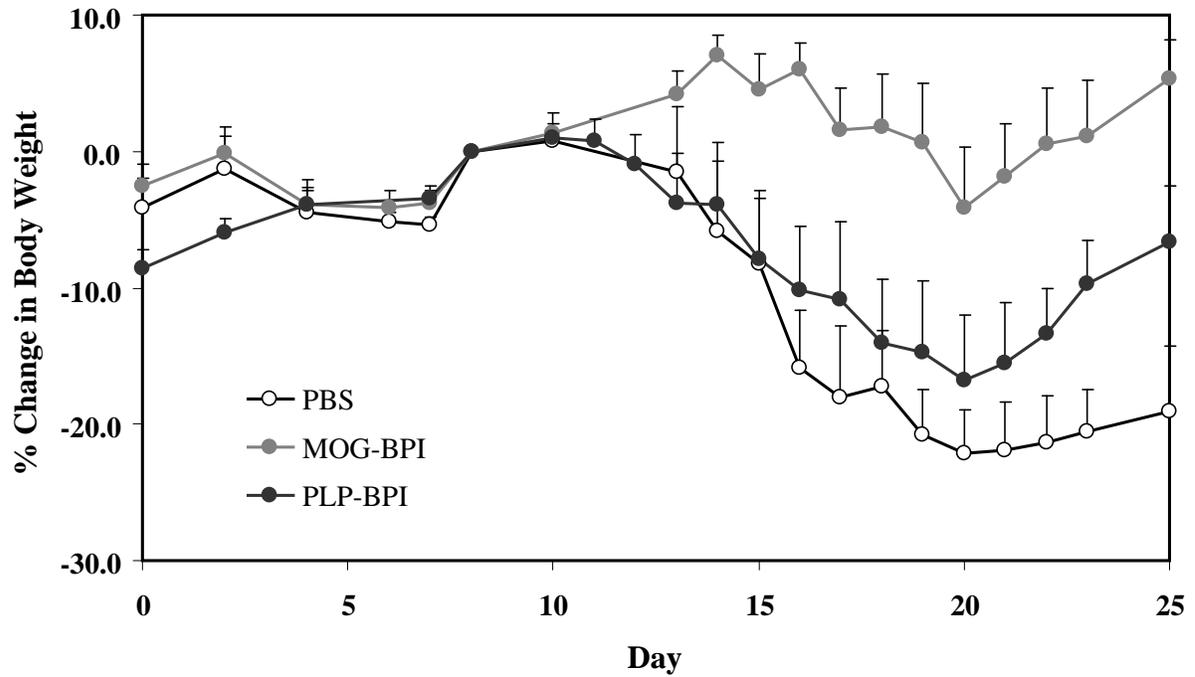
Figure 4.1B *In vivo* cross-reactivity of MOG-BPI and PLP-BPI. PBS (100 μl) and peptides (100 nmol/100 μl) were administered s.c. on days 4, 7, and 10. MOG-BPI- and PLP-BPI-treated mice were compared to PBS-treated mice in the MOG-induced EAE model and efficacy was determined by percent change in body weight. Results are expressed as the mean % change in body weight ± SEM (*n* = 6).
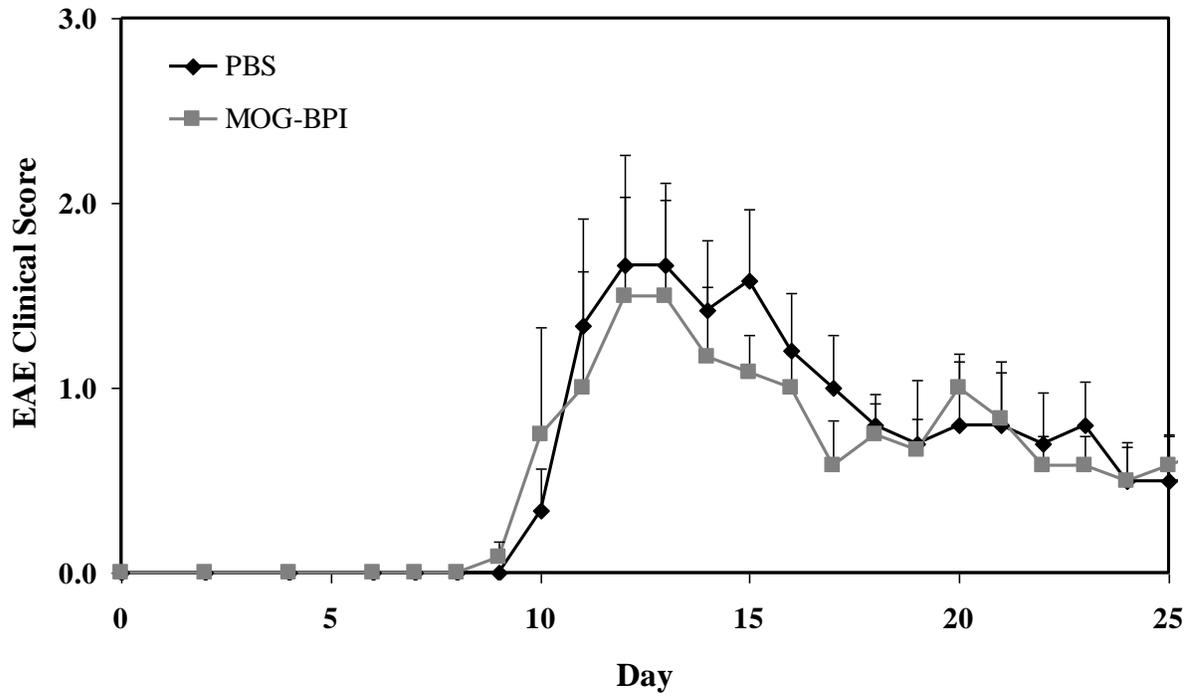
Figure 4.1C *In vivo* cross-reactivity of MOG-BPI and PLP-BPI. PBS (100 μl) and peptides (100 nmol/100 μl) were administered s.c. on days 4, 7, and 10. The efficacy of MOG-BPI in suppressing disease in PLP-induced EAE mice was determined using the clinical disease score of EAE. Results are expressed as the mean clinical score ± SEM ($n = 6$).
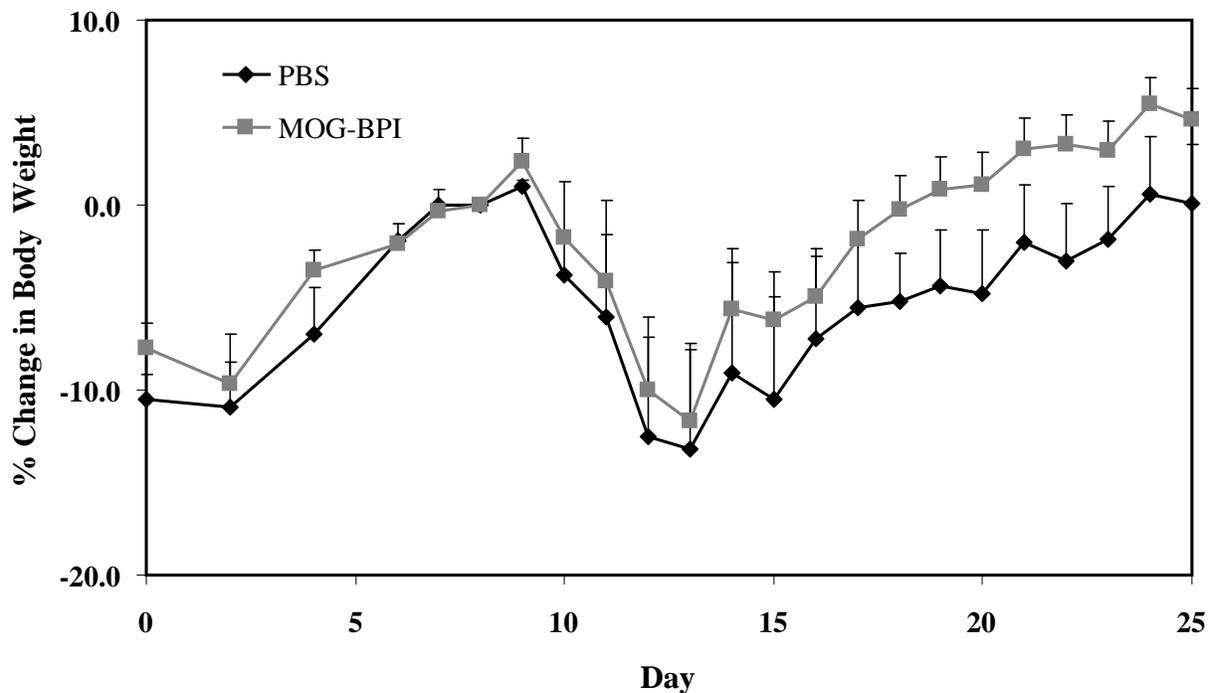
Figure 4.1D *In vivo* cross-reactivity of MOG-BPI and PLP-BPI. PBS (100 μl) and peptides (100 nmol/100 μl) were administered s.c. on days 4, 7, and 10. The efficacy of MOG-BPI in suppressing disease in PLP-induced EAE mice was determined using the percent change in body weight. Results are expressed as the mean % change in body weight $\pm$ SEM ($n = 6$).

treated with the novel MVB$_{MOG/PLP}$. Clinical scores from days 15–25 showed significant suppression in the MVB$_{MOG/PLP}$-treated mice compared to PBS-, MOG-, and MOG-BPI-treated mice (**Fig. 4.2A**, $p < 0.0001$). The MVB$_{MOG/PLP}$-treated group reached only a maximal disease score of 0.88 and 5.15% loss in body weight. Similarly, the change in body weight of MVB$_{MOG/PLP}$-treated mice on days 15–25 showed significant differences compared to those of PBS-, MOG-, and MOG-BPI- treated mice (**Fig. 4.2B**, $p < 0.0001$). Treatment with MOG-BPI delayed onset of disease by four days. The clinical scores and loss in body weight indicated that it suppressed disease significantly compared to the PBS group ($p < 0.0001$). Clinical scores indicated that MOG-BPI was significantly more efficacious than MOG peptide ($p < 0.05$, days 15–25); however, there was no significant difference in the change of body weight between MOG-BPI- and MOG-treated mice. Compared to PBS-treated mice, the MOG-treated mice showed significant suppression of disease ($p < 0.0001$) with only 10.88% loss in body and a maximum average clinical score of 2.42.

4.3.3 Study III - *In vivo* Efficacy of Novel MVB$_{MOG/PLP}$ in PLP-Induced EAE

The purpose of this study was to evaluate whether our novel peptide's efficacy in MOG-induced EAE can be translated to PLP-induced EAE. Therefore, mice were given three s.c. injections of MVB$_{MOG/PLP}$, and its efficacy was compared to that of PBS. According to clinical score data, MVB$_{MOG/PLP}$ suppressed disease significantly ($p < 0.0001$) while the PBS-treated mice reached a maximal score of 1.67 and MVB$_{MOG/PLP}$–treated mice reached a maximum of 0.58 (**Fig. 4.3A**). The changes in body weight correlates exactly with the observed differences in the clinical scores ($p < 0.001$), with PBS-treated mice losing 13.20% of their body weight and MVB$_{MOG/PLP}$–treated mice losing only a maximum of 4.66% (**Fig. 4.3B**).
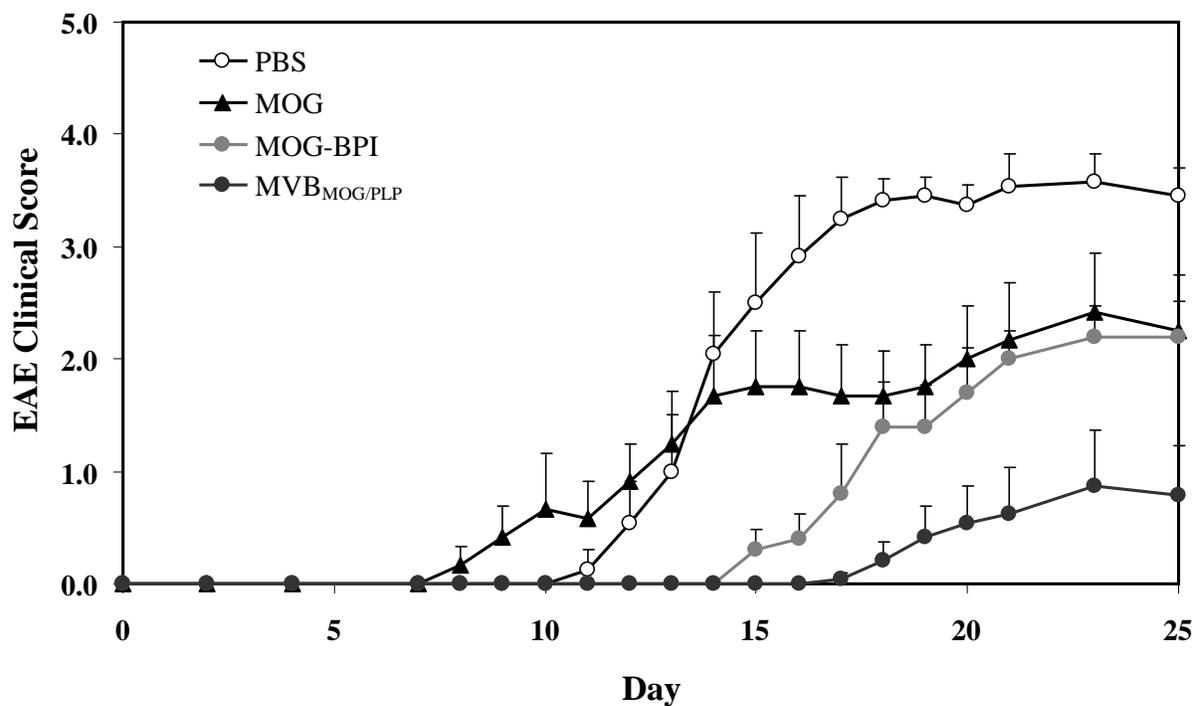
Figure 4.2A *In vivo* efficacies of MVB$_{MOG/PLP}$ and all the controls in suppressing MOG-induced EAE. PBS-treated mice (n = 12) received s.c. injections of 100 μl PBS on days 4, 7, and 10. MOG- (n = 6), MOG-BPI- (n = 5), and MVB$_{MOG/PLP}$- (n = 12) treated mice received 100 nmol/100 μl PBS on days 4, 7, and 10 (s.c.). The efficacy of each peptide was determined by clinical disease score of EAE. Results are expressed as the mean clinical score ± SEM.
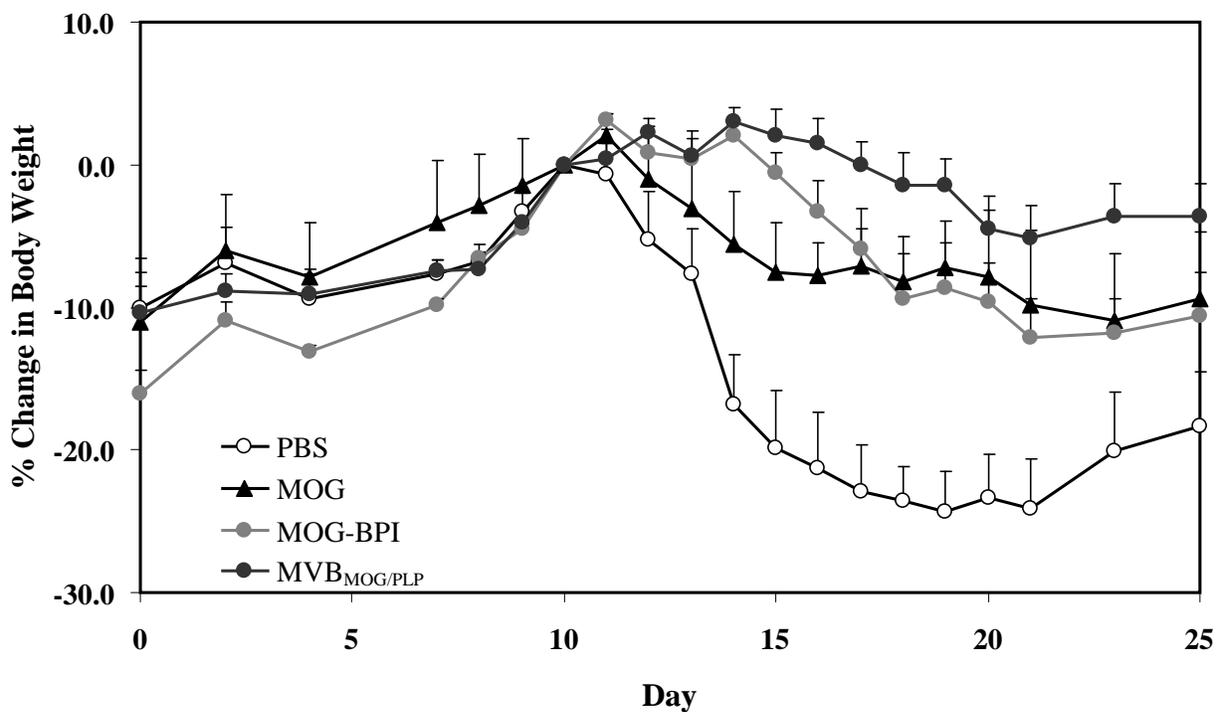
Figure 4.2B *In vivo* efficacies of $MVB_{MOG/PLP}$ and all the controls in suppressing MOG-induced EAE. PBS-treated mice ($n = 12$) received s.c. injections of 100 μl PBS on days 4, 7, and 10. MOG- ($n = 6$), MOG-BPI- ($n = 5$), and $MVB_{MOG/PLP}$- ($n = 12$) treated mice received 100 nmol/100 μl PBS on days 4, 7, and 10 (s.c.). The efficacy of each peptide was determined by percent change in body weight. Results are expressed as the mean % change in body weight $\pm$ SEM.
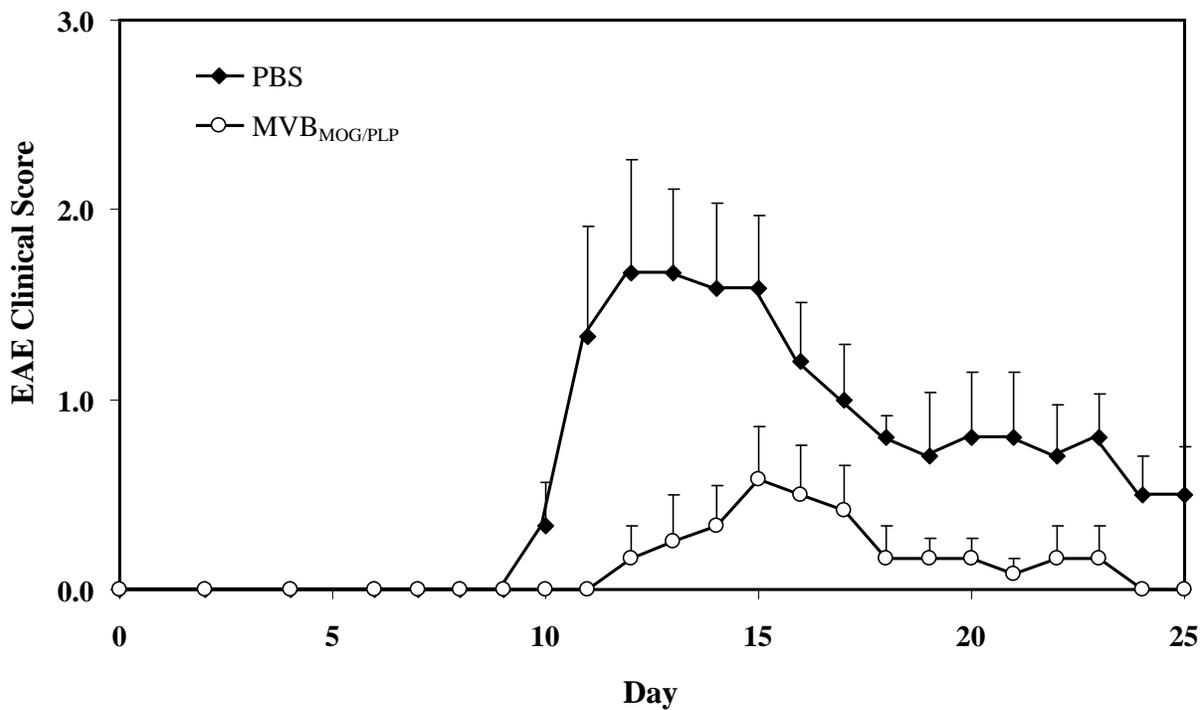
Figure 4.3A *In vivo* efficacy of MVB$_{MOG/PLP}$ in suppressing PLP-induced EAE. MVB$_{MOG/PLP}$-treated mice ($n = 6$) received three s.c. injections at a concentration of 100 nmol/100 μl on days 4, 7, and 10 and its efficacy was compared to PBS-treated mice ($n = 6$) treated in a similar fashion. Progress of the disease was monitored following clinical disease score of. Results are expressed as the mean clinical score ± SEM.
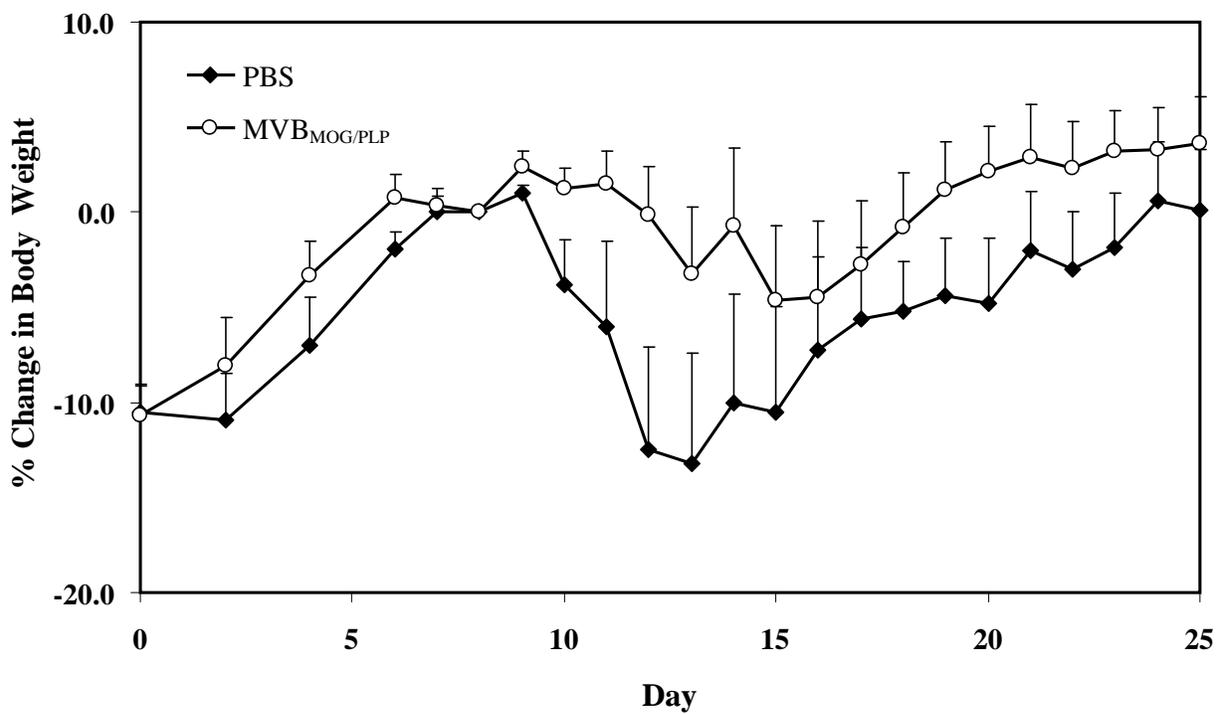
Figure 4.3B *In vivo* efficacy of MVB$_{MOG/PLP}$ in suppressing PLP-induced EAE. MVB$_{MOG/PLP}$-treated mice ($n = 6$) received three s.c. injections at a concentration of 100 nmol/100 μl on days 4, 7, and 10 and its efficacy was compared to PBS-treated mice ($n = 6$) treated in a similar fashion. Progress of the disease was monitored following percent change in body weight. Results are expressed as the mean % change in body weight $\pm$ SEM.

4.3.4 *In vitro* Inflammatory Cytokine Production

Splenocytes from treated mice were isolated on day 30 and their cytokine production was measured. EAE is an inflammatory disease characterized by high proliferation of inflammatory T_H1 cells. *In vitro* cytokine studies can be used to indirectly measure how strong the inflammatory response is in the mouse. The two key cytokine markers for T_H1 cells are IL-6 (**Fig. 4.4A**) and IFN-γ (**Fig. 4.4B**). The production of these pro-inflammatory cytokines was lowest in the $MVB_{MOG/PLP}$-treated mice and was significantly different compared to those treated with PBS ($p < 0.01$). MOG-BPI reduced IL-6 production and significantly suppressed IFN-γ ($p < 0.01$). $MVB_{MOG/PLP}$-treated mice had significantly suppressed production of IL-6 compared to MOG-BPI-treated mice ($p < 0.05$) but only a small difference in IFN-γ.

4.3.5 Splenocyte Proliferation

A proliferation assay was performed to evaluate the extent of responsiveness of splenocytes from the different treatment groups to *in vitro* antigen stimulation. In addition, the cross-reactivity of the antigens was studied. Splenocytes were isolated from PLP-immunized SJL/J mice, and their proliferation was measured in non-immunized mice (no EAE) and immunized mice treated with PBS, PLP-BPI, and $MVB_{MOG/PLP}$. It was observed that the responsiveness of PLP-BPI-treated mice was lower than in the PBS group when the splenocytes were stimulated with PLP ($p < 0.05$). However, $MVB_{MOG/PLP}$ had the least responsiveness when exposed to PLP. Finally, when the splenocytes were stimulated with MOG, there was no difference in proliferation compared to the splenocytes stimulated with medium. Therefore, the results indicated no cross-reactivity between PLP and MOG in PLP-induced EAE (**Fig. 4.5**).
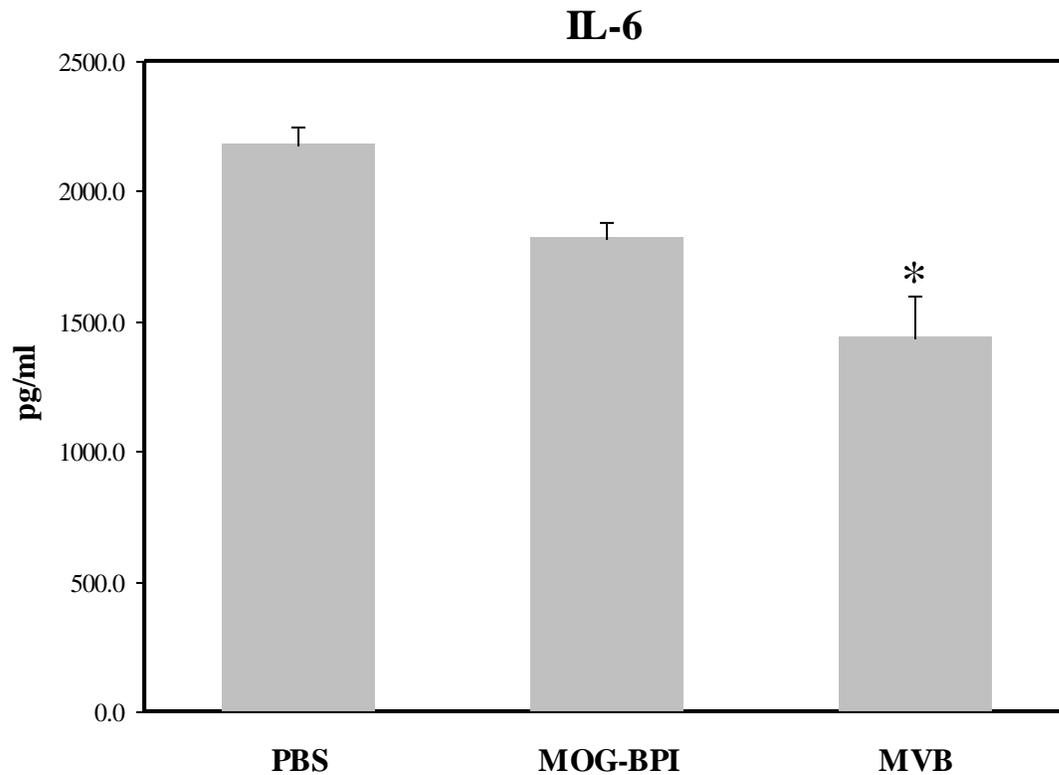
Figure 4.4A Concentrations of the pro-inflammatory cytokine, IL-6, from the cell culture supernatant. Splenocytes were isolated from the spleens of MOG-induced EAE mice on day 30. The three different groups consisted of PBS-, MOG-BPI-, or $MVB_{MOG/PLP}$- treated mice on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with $PLP_{139-151}$, and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.01$).
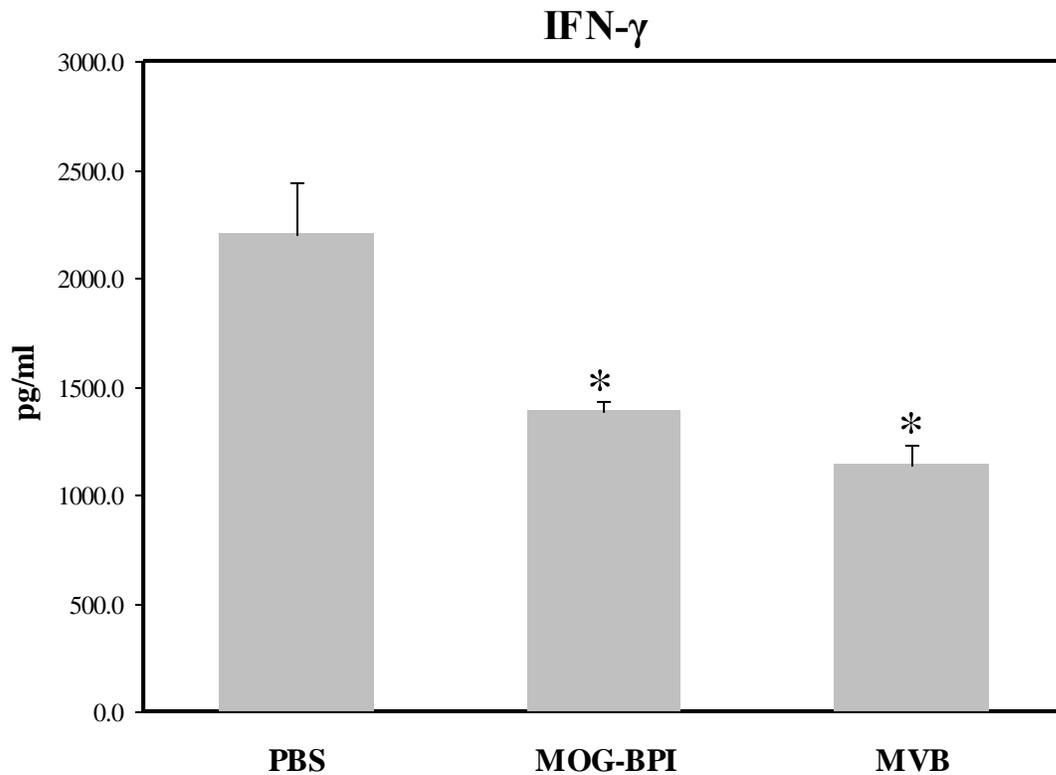
Figure 4.4B Concentrations of the pro-inflammatory cytokine, IFN-γ, from the cell culture supernatant. Splenocytes were isolated from the spleens of MOG-induced EAE mice on day 30. The three different groups consisted of PBS-, MOG-BPI-, or $MVB_{MOG/PLP}$- treated mice on days 4, 7, and 10. The pooled splenocytes ($n = 3$ mice) were stimulated *in vitro* with $PLP_{139-151}$, and supernatant was isolated 72 hours later for cytokine detection (*$p < 0.01$).
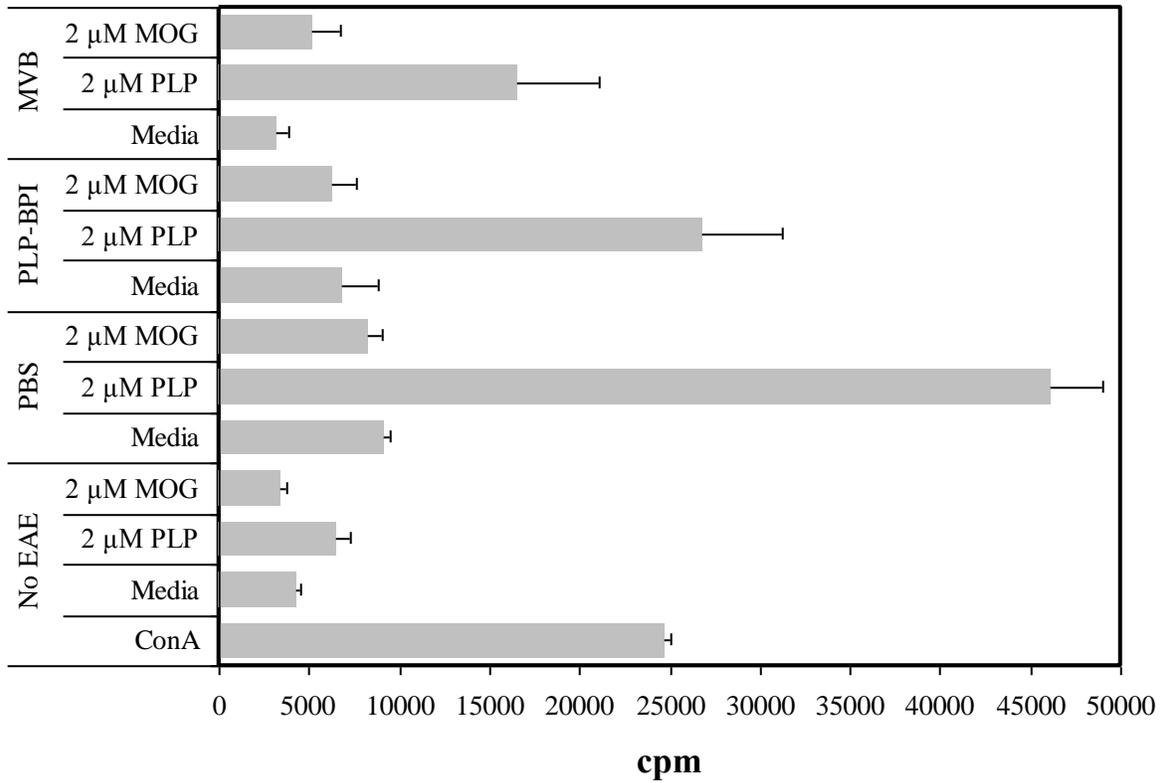
Figure 4.5 Incorporation of [³H] thymidine in splenocytes isolated from PLP-induced EAE mice on day 30. EAE-free mice were stimulated with ConA, medium, PLP (2μM), or MOG (2μM) and the remaining treated groups were stimulated with only medium, PLP (2 μM), and MOG (2μM). The three treatment groups consisted of PBS-, PLP-BPI-, or MVB$_{MOG/PLP}$- treated mice on days 4, 7, and 10. Each group ($n = 3$) was pulsed with [³H] thymidine 72 hours after stimulation and incorporated radioactivity was measured the following day.

4.4 DISCUSSION

BPI molecules have been developed to target many different antigens responsible for the pathogenesis of autoimmune diseases. PLP-containing BPI molecules have been studied extensively for the suppression of PLP-induced EAE.[8-11] GAD-BPI was developed and was successful in suppressing disease in the non-obese diabetic mouse model.[17] CII-BPI molecules composed of collagen-II antigenic peptides have been shown to suppress collagen-II-induced rheumatoid arthritis animal model (unpublished data). Our first goal for this study was to investigate whether BPI molecules composed of other myelin sheath epitopes involved in EAE and MS can be developed to suppress disease. Previously, only PLP-containing BPI molecules were tested. In this study, a novel BPI molecule composed of the MOG antigen (MOG-BPI) was developed and tested in suppressing EAE. While BPI molecules have demonstrated superior efficacy thus far, there is a major limitation to their application. BPI molecules are antigen specific and, therefore, will suppress only the immune response towards other antigens involved in the progression of disease. In reality, MS patients have been shown to respond to many different epitopes,[18-22] and single antigen-specific immunosuppression could have a disadvantage. Thus, there is a need to expand this technology to target multiple antigens. For the first time, a multivalent BPI molecule has been developed and its efficacy has been tested in two separate EAE models.

In vivo data from the PLP-induced model showed that there was no cross-reactivity between MOG-BPI and PLP-BPI, and this was well correlated with the proliferation assay data showing that there was no response in splenocytes stimulated with MOG (**Table 4.2**). However, in the MOG-induced model, significant suppression of disease was observed with PLP-BPI, thus suggesting some cross-reactivity in this model. We propose that the cross-reactivity in the

Group[a]	Induction antigen	Incidence of disease[b]	Mean maximal score ± SEM
In vivo **Study I**			
PBS	MOG	6/6	4.00 ± 0.18
PLP-BPI	MOG	6/6	3.00 ± 0.47
MOG-BPI	MOG	6/6	2.25 ± 0.46
PBS	PLP	6/6	1.67 ± 0.59
MOG-BPI	PLP	6/6	1.50 ± 0.53
In vivo **Study II**			
PBS	MOG	12/12	3.58 ± 0.24
MOG	MOG	5/6	2.42 ± 0.52
MOG-BPI	MOG	5/5	2.20 ± 0.31
MVB$_{MOG/PLP}$	MOG	4/12	0.88 ± 0.49
In vivo **Study III**			
PBS	PLP	6/6	1.67 ± 0.59
MVB$_{MOG/PLP}$	PLP	3/6	0.58 ± 0.27

Table 4.2: Summary of *in vivo* studies

[a]All injections were administered s.c. at 100 nmol (100 µl PBS) on days 4, 7, and 10.

[b]Incidence of disease was defined as a disease score of 1 or higher.

MOG-induced EAE is caused by epitope spreading due to the severity of this chronic model. Epitope spreading is believed to occur when there is major tissue damage, and debris from the destroyed tissue is taken up, processed, and presented by immune cells, leading to inflammatory response towards new epitopes.[23] In the next step, a novel multi-antigenic peptide, $MVB_{MOG/PLP}$, was tested in both PLP- and MOG-induced EAE. $MVB_{MOG/PLP}$ suppressed the disease in both models. Interestingly, MVB suppressed disease better than MOG-BPI and PLP-BPI in MOG-induced EAE, suggesting that there is an additive effect of potency when both antigenic peptides are conjugated in one molecule (i.e., MVB). This further enhances the proposal that there is some cross-reactivity due to epitope spreading in the MOG model.

A key factor in the progression of MS and EAE is a phenomenon known as epitope spreading.[6] During the course of the disease, T cells develop immunogenicity to new myelin proteins. This causes further destruction that leads to chronic tissue damage of the myelin sheath but, more importantly, it creates more difficulties in developing antigen-specific therapies. Epitope spreading can occur intramolecularly in which some mice develop autoreactivity towards MBP_{35-47}, MBP_{81-100}, and $MBP_{121-140}$ over time when MBP_{1-11} is the initiating antigen.[24] In addition, there is evidence of intermolecular epitope spreading where during the course of the disease, proliferative responses towards PLP were present in MBP-induced mice.[25] There are no reports in the literature of epitope spreading between MOG_{38-50} and $PLP_{139-151}$, but we believe that the efficacy of PLP-BPI in the MOG-induced model and the additive efficacy of $MVB_{MOG/PLP}$ are possibly due to epitope spreading. We propose that $MVB_{MOG/PLP}$ suppressed the T cell response towards MOG as well as PLP. This strategy of multi-antigenic immunosuppression has been employed previously. Multi-antigenic peptide therapies made of up four different antigens ($PLP_{139-151}$, $PLP_{178-191}$, MBP_{84-104}, and MOG_{92-106}) fixed to splenic APC

have been developed and have shown efficacy in suppressing EAE induced by all four antigens.[26]

BPI molecules are made up of two main components covalently linked to each other.[7] The first component is the antigenic peptide and the second is the adhesion peptide. It is proposed that the antigenic peptide portion binds to the MHC-II molecule on the surface of APC, and the adhesion peptide, which is derived from the LFA-1 protein, binds to ICAM-1, also on the surface of APC. Traditionally, we used a linker composed of aminocaproic acid and glycine to link both peptide components in BPI molecules. For the synthesis of MOG-BPI and MVB$_{MOG/PLP}$, a polyethylene glycol-3 (PEG$_3$) linker was used to improve solubility. Previously, we saw no significant difference in the use of PEG$_3$ or aminocaproic acid as a linker. We propose that, due to the presence of the covalent linker, the BPI molecule tethers the loaded MHC-II molecule and ICAM-1, thus hindering the formation of the immunological synapse. This prevents the initiation of an inflammatory response to the specific antigen recognized by the MHC-II molecule. With the MVB, the idea has been expanded to incorporate more than one antigenic peptide. It is proposed that each antigenic peptide will be recognized by its respective MHC-II molecule and will suppress the inflammatory response to that antigen.

MS and EAE are characterized by severe inflammation with high levels of IL-6[27] and IFN-γ[28] found in the CNS. Moreover, their role in the pathogenesis of disease is significant. In addition to recruitment of other inflammatory immune cells, IL-6 is a mediator in increasing the permeability of the BBB[29,30] and IFN-γ has been reported to prevent remyelination of the CNS.[31] Therefore, in order to treat or suppress the disease, therapies aimed at reducing the inflammatory response are vital. Currently in the market, glatiramer acetate, a drug used for the treatment of MS, works by diverting the immune response away from the T$_H$1 phenotype.[32,33] The cytokine

data from the present study demonstrated that when MOG-BPI and MVB$_{MOG/PLP}$ were administered to mice with MOG-induced EAE, the inflammatory cytokines IL-6 and IFN-γ, both T_H1 markers, were suppressed significantly by both peptides. This suggests a down-regulation of the T_H1, thus shifting the balance away from the inflammatory response. In addition, the proliferation assay from PLP-induced mice showed that PLP-BPI and MVB$_{MOG/PLP}$ significantly lowered the PLP-responsive population of splenocytes.

In conclusion, developing molecules that can target more than one epitope is critical for making BPI technology more applicable for MS. In human disease, the identity of the inciting antigen is not usually known. In addition, since disease is not diagnosed early, other antigens of the myelin sheath become targeted by immune cells due to epitope spreading. Therefore, molecules that can target many antigens are critically important for the treatment of MS or EAE irrespective of the inciting antigens or antigenic spread. Thus far, we have developed a peptide that is composed of two antigenic peptides. For future studies, we would like to expand this peptide to incorporate other immunodominant antigens to suppress disease initiated by other antigens as well as to avoid the problem of intra- and inter-molecular epitope spreading.

4.5 REFERENCES

1   Bielekova, B. *et al.* Preferential expansion of autoreactive T lymphocytes from the memory T-cell pool by IL-7. *J Neuroimmunol* **100**, 115-123 (1999).

2   Zhang, J. *et al.* Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **179**, 973-984 (1994).

3   Vanderlugt, C. J. & Miller, S. D. Epitope spreading. *Curr Opin Immunol* **8**, 831-836 (1996).

4   Voskuhl, R. R. *et al.* T helper 1 (Th1) functional phenotype of human myelin basic protein-specific T lymphocytes. *Autoimmunity* **15**, 137-143 (1993).

5   Lassmann, H. Classification of demyelinating diseases at the interface between etiology and pathogenesis. *Curr Opin Neurol* **14**, 253-258 (2001).

6   Tuohy, V. K. & Kinkel, R. P. Epitope spreading: a mechanism for progression of autoimmune disease. *Arch Immunol Ther Exp (Warsz)* **48**, 347-351 (2000).

7   Manikwar, P., Kiptoo, P., Badawi, A. H., Buyuktimkin, B. & Siahaan, T. J. Antigen-specific blocking of CD4-specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev* (2011).

8   Kobayashi, N. *et al.* Prophylactic and therapeutic suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *Clin Immunol* **129**, 69-79 (2008).

9   Kobayashi, N., Kobayashi, H., Gu, L., Malefyt, T. & Siahaan, T. J. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* **322**, 879-886 (2007).

10   Zhao, H., Kiptoo, P., Williams, T. D., Siahaan, T. J. & Topp, E. M. Immune response to controlled release of immunomodulating peptides in a murine experimental autoimmune encephalomyelitis (EAE) model. *J Control Release* **141**, 145-152 (2010).

11   Ridwan, R. *et al.* Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor: structure optimization and pharmacokinetics. *J Pharmacol Exp Ther* **332**, 1136-1145 (2010).

12   Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).

13   Tseng, S. Y. & Dustin, M. L. T-cell activation: a multidimensional signaling network. *Curr Opin Cell Biol* **14**, 575-580 (2002).

14   van der Merwe, P. A. Formation and function of the immunological synapse. *Curr Opin Immunol* **14**, 293-298 (2002).

15   Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-1542 (2002).

16   Youssef, S. *et al.* The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* **420**, 78-84 (2002).

17   Murray, J. S. *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des* **70**, 227-236 (2007).

18   Tuohy, V. K., Yu, M., Weinstock-Guttman, B. & Kinkel, R. P. Diversity and plasticity of self recognition during the development of multiple sclerosis. *J Clin Invest* **99**, 1682-1690 (1997).

19   Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A. & Kinkel, R. P. Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *J Exp Med* **189**, 1033-1042 (1999).

20   Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A. & Kinkel, P. R. Regression and spreading of self-recognition during the development of autoimmune demyelinating disease. *J Autoimmun* **13**, 11-20 (1999).

21   Goebels, N. *et al.* Repertoire dynamics of autoreactive T cells in multiple sclerosis patients and healthy subjects: epitope spreading versus clonal persistence. *Brain* **123 Pt 3**, 508-518 (2000).

22   Muraro, P. A. *et al.* Molecular tracking of antigen-specific T cell clones in neurological immune-mediated disorders. *Brain* **126**, 20-31 (2003).

23   Lehmann, P. V., Sercarz, E. E., Forsthuber, T., Dayan, C. M. & Gammon, G. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol Today* **14**, 203-208 (1993).

24   Lehmann, P. V., Forsthuber, T., Miller, A. & Sercarz, E. E. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **358**, 155-157 (1992).

25   Perry, L. L., Barzaga-Gilbert, E. & Trotter, J. L. T cell sensitization to proteolipid protein in myelin basic protein-induced relapsing experimental allergic encephalomyelitis. *J Neuroimmunol* **33**, 7-15 (1991).

26   Smith, C. E. & Miller, S. D. Multi-peptide coupled-cell tolerance ameliorates ongoing relapsing EAE associated with multiple pathogenic autoreactivities. *J Autoimmun* **27**, 218-231 (2006).

27   Maimone, D., Guazzi, G. C. & Annunziata, P. IL-6 detection in multiple sclerosis brain. *J Neurol Sci* **146**, 59-65 (1997).

28   Kebir, H. *et al.* Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol* **66**, 390-402 (2009).

29   Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* **7**, 41-53 (2006).

30   Prendergast, C. T. & Anderton, S. M. Immune cell entry to central nervous system--current understanding and prospective therapeutic targets. *Endocr Metab Immune Disord Drug Targets* **9**, 315-327 (2009).

31   Lin, W. *et al.* Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress. *Brain* **129**, 1306-1318 (2006).

32   Dhib-Jalbut, S. Glatiramer acetate (Copaxone) therapy for multiple sclerosis. *Pharmacol Ther* **98**, 245-255 (2003).

33   Neuhaus, O., Farina, C., Wekerle, H. & Hohlfeld, R. Mechanisms of action of glatiramer acetate in multiple sclerosis. *Neurology* **56**, 702-708 (2001).

CHAPTER 5

Summary, conclusions, and future directions

5.1 SUMMARY AND CONCLUSIONS

The objective of this project was to develop antigen-specific peptides for the treatment of experimental autoimmune encephalomyelitis (EAE), an animal model of the human disease multiple sclerosis (MS). The disease is thought to be established when myelin-reactive T cells that have escaped both central and peripheral tolerance mechanisms cross the blood-brain barrier (BBB) and elicit an inflammatory response with in the central nervous system (CNS).[1] The majority of the current therapies do not specifically target myelin-reactive T cells but target the general immune system, thus weakening the global defense mechanisms and generating adverse side effects primarily due to opportunistic infections. There is, therefore, a need to develop therapies that target only the myelin-specific immune cells involved in the CNS inflammatory response.

Peptides are potentially valuable therapeutics for MS due to their ability to specifically modulate protein-protein interactions, their ease of preparation, and their relatively high safety index. Much research has been conducted to develop peptides as specific and non-specific immune-modulators. Antigenic-specific immunotherapy (antigenic-SIT) has emerged as a promising way to specifically modulate the immune response.[2] The success of antigenic-SIT in EAE and the hope for its success in MS is rooted in the advancements in allergen-SIT, which has been used for many years to treat allergic diseases.[2] Both forms of SIT exert their efficacy by restoring tolerance to a specific antigen/allergen. The induction of an inflammatory response is achieved via the activation of antigen-presenting cells (APC), which occurs when the APC take up an insoluble antigen, break it down, and process it.[3,4] This leads to the presentation of the antigen on the surface of the APC in the context of the major histocompatibilty complex class-II (MHC-II) molecule, as well as increased expression of costimulatory (B7/CD28) and adhesion

molecules (intercellular adhesion molecule-1 (ICAM-1)/leukocyte function-associated antigen-1 (LFA-1)) on its surface. Administration of soluble antigens is believed to work by bypassing the activation of APC and directly binding to the surface of unactivated APC (e.g., immature DC).[3,5] In our laboratory, we developed novel bifunctional peptide inhibitors (BPI) that are composed of an antigenic peptide covalently linked to an adhesion peptide.[6-8] We propose that the BPI molecules simultaneously target MHC-II and adhesion molecules on the surface of APC. Thus, BPI molecules can specifically modulate the activation of T cells that recognize the antigenic peptide portion.

During MS and EAE, T cells can breach the BBB to attack the myelin sheath of the CNS.[9] This breach is believed to occur due to the breakdown of the BBB during the disease state, which is proposed to be due to the activation of a CNS-specific inflammatory response.[10,11] In the past, it has been shown that BPI molecules can induce a proliferation of regulatory cells and inhibit an inflammatory immune response in EAE mice. In this project, the *in vivo* efficacy of PLP-BPI (acetylated-PLP$_{139-151}$ conjugated to amidated-LABL (CD11a$_{237-246}$)) as a peptide vaccine was evaluated, as well its ability to prevent BBB breakdown. Administration of PLP-BPI at 5, 8, and 11 days prior to induction of disease suppressed EAE significantly. In addition, *in vitro* cytokine studies showed that PLP-BPI treatment promoted a shift towards regulatory and suppressor phenotypes. This is indicated by decreased production of pro-inflammatory cytokines (IL-6, IL-17, IFN-γ) and increased production of anti-inflammatory cytokines (IL-2, IL-4, and IL-5). More importantly, using gadolinium- (Gd) enhanced magnetic resonance imaging (MRI), it was possible to evaluate whether PLP-BPI provided any protection from the breakdown of the BBB. Signal enhancement in specific regions of the brain and spinal cord signifies deposition of Gd within the brain and thus a leaky BBB. The data from the MRI study indicated that PLP-BPI

treated mice had the least signal enhancement within the regions of the brain when compared to sick mice treated with PBS. This result indicated that PLP-BPI-treated mice had an intact BBB.

Traditionally, BPI molecules have all been designed to incorporate an antigenic peptide conjugated to an adhesion peptide, thus targeting MHC-II and adhesion molecules on the surface of APC. However, it is known that the costimulatory interaction of B7/CD28 plays a very important role in the activation of T cells.[12,13] Therefore, PLP-B7AP was developed and evaluated for suppressing EAE; PLP-B7AP is composed of $PLP_{139-151}$ and a CD28-derived peptide (B7AP).[14] PLP peptide, B7AP, and an unconjugated mixture of both peptides showed significant suppression of EAE when administered three times subcutaneously (s.c.) after induction of disease; however, PLP-B7AP (100 nmol) had the greatest suppression, with 100% of the mice being disease-free. In another *in vivo* study, PLP-B7AP showed remarkable efficacy with significant attenuation of the disease when it was administered either once (100 nmol) or three times at half the dose (50 nmol). The final *in vivo* study indicated that PLP-B7AP was very effective when administered prior to induction of disease (i.e., a vaccine treatment). Secreted cytokines were measured from splenocytes isolated from the day of maximum disease and a day during remission. Similar to previous studies conducted with PLP-BPI, the results showed that PLP-B7AP treatment induced an increased production of anti-inflammatory cytokines and a lowered production of pro-inflammatory cytokines.

During the pathogenesis of MS and EAE, the T cells elicit an inflammatory response towards the protein of the myelin sheath. Two of the major immunodominant proteins of the myelin sheath include myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP).[15] A process known as epitope spreading will trigger T cells to become responsive to other antigens within the same protein (intramolecular) or to other proteins of the myelin

sheath (intermolecular), thus causing further destruction of the target organ.[16] In addition, during the onset of MS, the inciting antigen is not known. Therefore, peptides composed of multiple antigens were developed to overcome these two problems. For the first time, a multivalent BPI (MVB) molecule composed of MOG_{38-50} and $PLP_{139-151}$ was synthesized and tested in EAE. It was reported that $MVB_{MOG/PLP}$ was able to significantly suppress MOG_{38-50}- and $PLP_{139-151}$-induced EAE; this was accompanied by decreased production of pro-inflammatory cytokines (IL-6 and IFN-γ). In addition, *in vivo* studies reported that PLP-BPI is effective in suppressing MOG_{38-50}-induced EAE, indicating that some cross-reactivity is present in this model, which could be due to epitope spreading in the MOG model. The cross-reactivity was confirmed when $MVB_{MOG/PLP}$ was more effective than either PLP-BPI or MOG-BPI (MOG_{38-50} conjugated to LABL) in suppressing MOG_{38-50}-induced EAE.

In conclusion, BPI molecules have consistently proven to be effective immuno-modulators in all the different models they have been tested in. Proliferation assays from current and previous studies indicated that BPI molecules target cells specific for myelin antigens. In the next section, future studies will be discussed that are designed to elucidate the mechanism of action of BPI molecules as well as improve their molecular composition to broaden the antigenic targets. This will hopefully make BPI molecules a promising candidate to be tested for the treatment of MS.

5.2 FUTURE DIRECTIONS

5.2.1 Studying CNS Cellular Infiltration and Histopathology

The breakdown that occurs in the BBB during disease is believed to be due inflammatory mediators as well as leukocyte recruitment to the CNS.[17,18] Our studies have already shown that

PLP-BPI can suppress the induction of the inflammatory response as well as maintain the integrity of the BBB. The next step is to evaluate whether PLP-BPI prevents the infiltration of immune cells across the BBB. Leukocyte recruitment to the CNS is initiated due to the inflammatory response;[9] therefore, suppression of this response using PLP-BPI may inhibit leukocyte recruitment to the brain and spinal cord. Cellular infiltration can be monitored in numerous ways. In the past, ultra-small particles of iron oxide (USPIO) were utilized to detect the presence of monocytes in the brain using MRI.[9] Another method to detect cellular infiltration in the brain would be to isolate brains of mice during different stages of disease, obtain slices of the brain and spinal cord, and then stain them it for different types of leukocytes. In addition, histopathology studies can be conducted on brain and spinal cord slices to detect lesions as well as areas of demyelination within the CNS. In the future, BPI molecules can be tested to see if treatment can reduce the amount and volume of lesions during disease.

5.2.2 Exploring Mechanistic Aspects of BPI Molecules

Currently, the major proposed mechanism of action for BPI molecules is simultaneous binding to the MHC-II and adhesion molecules on the surface of APC, thus blocking their translocation to form the immunological synapse. Preventing the immunological synapse leads to partial activation of T cells, therefore causing the naïve T cell to differentiate into a regulatory/suppressor phenotype. The only clue thus far that this occurs is from a previous experiment conducted with GAD-BPI which showed that, in the presence of the peptide, there was co-localization of both the MHC-II and ICAM-1.[19] Obviously, more elaborate molecular and cellular studies must be performed to further elucidate the mechanism. The formation of the immunological synapse has been viewed using fluorescence microscopy by labeling the T cell

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receptor and LFA-1.[20] Therefore, microscopy studies should be conducted in the presence and absence of BPI molecules to visualize and quantify whether these peptides hinder the formation of the immunological synapse.

In the present experiments, *in vitro* cytokine detection was performed on either the day of maximum disease (day 15 post induction) or the day of remission (day 30). To better understand how the BPI molecules modulate the immune response, cytokine detection needs to be performed on more days. Other important days for detecting cytokines would be immediately after induction of disease, after peptide administration, and when the disease is approaching remission. In addition, since *in vitro* cytokine studies determine only the amount of cytokines relative to each other and not exact concentrations, other more sensitive techniques may be used in the future to detect cytokines. This can be achieved by isolating the blood of mice and using enzyme-linked immunosorbent spot (ELISPOT) assays to determine the concentration of cytokines. Lastly, since EAE and MS are diseases of the CNS, determining the cytokine concentrations in the brain and spinal cord would be optimal. This can be accomplished by isolating the brain and spinal cord of the mice and extracting the RNA. Quantitative polymerase chain reactions can be performed to determine the types of cytokines by identifying the RNA transcripts present.

5.2.3 Expanding Antigenic Diversity of Multivalent BPI Molecules

In the present study, a MVB that is composed of two antigens (MOG_{38-50} and $PLP_{139-151}$) has already been developed and was successful at suppressing both MOG_{38-50}- and $PLP_{139-151}$-induced EAE. Other immunodominant epitopes must be identified and incorporated in the development of bigger MVB in order to expand the antigenic targets of the peptide. This may

lead to eradicating problems due to the inability of identifying the inciting antigen in MS as well as the problem created by epitope spreading. MVB molecules can be developed to contain multiple epitopes and can be tested in suppressing disease induced by either a mixture of antigens or by each individual antigen. There are numerous antigenic peptides already identified that can be incorporated into the MVB molecule. Some of the antigenic peptides that have already shown efficacy in suppressing EAE include MBP (1-11, and 85-99), MOG (35-55, and 92-106), and PLP (139-151, and 178-191).[21-23] Novel conjugation chemistry must be developed to be able to conjugate multiple antigens to adhesion peptides such as LABL. Another approach would be to use the parent protein of LABL, the I-domain. I-domain antigenic conjugates (IDAC) have already been developed in our lab and can conjugate up to five antigens. Therefore, using this technology, an IDAC molecule containing up to five different antigens can be used for broader suppression of disease.

5.3 REFERENCES

1   McFarland, H. F. & Martin, R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8, 913-919 (2007).

2   Sabatos-Peyton, C. A., Verhagen, J. & Wraith, D. C. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol* 22, 609-615 (2010).

3   Larche, M. & Wraith, D. C. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med* 11, S69-76 (2005).

4   Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192, 1213-1222 (2000).

5   Santambrogio, L., Sato, A. K., Fischer, F. R., Dorf, M. E. & Stern, L. J. Abundant empty class II MHC molecules on the surface of immature dendritic cells. *Proc Natl Acad Sci U S A* 96, 15050-15055 (1999).

6   Manikwar, P., Kiptoo, P., Badawi, A. H., Buyuktimkin, B. & Siahaan, T. J. Antigen-specific blocking of CD4-specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev* (2011).

7   Kobayashi, N., Kobayashi, H., Gu, L., Malefyt, T. & Siahaan, T. J. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther* 322, 879-886 (2007).

8   Kobayashi, N. *et al.* Prophylactic and therapeutic suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *Clin Immunol* 129, 69-79 (2008).

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9   Floris, S. *et al.* Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: a quantitative MRI study. *Brain* 127, 616-627 (2004).

10   Minagar, A. & Alexander, J. S. Blood-brain barrier disruption in multiple sclerosis. *Mult Scler* 9, 540-549 (2003).

11   Sun, D. *et al.* Role of chemokines, neuronal projections, and the blood-brain barrier in the enhancement of cerebral EAE following focal brain damage. *J Neuropathol Exp Neurol* 59, 1031-1043 (2000).

12   June, C. H., Bluestone, J. A., Nadler, L. M. & Thompson, C. B. The B7 and CD28 receptor families. *Immunol Today* 15, 321-331 (1994).

13   Jenkins, M. K. & Johnson, J. G. Molecules involved in T-cell costimulation. *Curr Opin Immunol* 5, 361-367 (1993).

14   Chen, J. *et al.* Allogenic donor splenocytes pretreated with antisense peptide against B7 prolong cardiac allograft survival. *Clin Exp Immunol* 138, 245-250 (2004).

15   Sospedra, M. & Martin, R. Immunology of multiple sclerosis. *Annu Rev Immunol* 23, 683-747 (2005).

16   Tuohy, V. K. & Kinkel, R. P. Epitope spreading: a mechanism for progression of autoimmune disease. *Arch Immunol Ther Exp (Warsz)* 48, 347-351 (2000).

17   Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* 7, 41-53 (2006).

18   Huber, J. D. *et al.* Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am J Physiol Heart Circ Physiol* 280, H1241-1248 (2001).

19   Murray, J. S. *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des* 70, 227-236 (2007).

20   Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* 295, 1539-1542 (2002).

21   Anderton, S. M. & Wraith, D. C. Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. *Eur J Immunol* 28, 1251-1261 (1998).

22   Smith, C. E. & Miller, S. D. Multi-peptide coupled-cell tolerance ameliorates ongoing relapsing EAE associated with multiple pathogenic autoreactivities. *J Autoimmun* 27, 218-231 (2006).

23   Jurynczyk, M. *et al.* Immune regulation of multiple sclerosis by transdermally applied myelin peptides. *Ann Neurol* 68, 593-601 (2010).