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A vaccine-like administration of PLP-PEG-B7AP and MOG-PEG-B7AP to control EAE in relapse-remission and chronic progressive animal models of multiple sclerosis: Bifunctional peptide inhibitors as peptide-based therapeutic vaccines

KEYWORDS: Peptide-based therapeutic vaccines, multiple sclerosis, EAE, autoimmune diseases, bifunctional peptide inhibitor, cytokines.

Abstract Most current therapies for treatment of multiple sclerosis or other autoimmune diseases work by suppressing the general immune response. Normally they treat the symptoms but do not address the underlying change in the immune system balance. In this study, PLP-PEG-B7AP and MOG-PEG-B7AP, new bifunctional peptide inhibitors or BPI molecules, were evaluated for suppressing experimental autoimmune encephalomyelitis (EAE) in relapse-remission (RRMS) and chronic progressive (CPMS) animal models for multiple sclerosis, respectively, when administered in a vaccine-like manner. PLP-PEG-B7AP and MOG-PEG-B7AP are conjugates between an antigenic peptide (PLP139-151 or MOG38-50 peptide) and a B7 antisense peptide (B7AP) derived from a CD28 receptor linked via a pegylated moiety. When administered as a vaccine-like treatment, PLP-PEG-B7AP was effective in suppressing EAE in the RRMS animal model while MOG-PEG-B7AP was effective in suppressing EAE in the CPMS animal model. Both BPI molecules were more effective than their respective parent antigenic peptides alone (i.e., PLP139-151 or MOG38-50). Vaccination with the PLP-PEG-B7AP molecule produced a long-term suppression of pro-inflammatory cytokine IFN-γ.

INTRODUCTION

A breakdown in the host immune system is one of the contributing factors that cause autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes (T1D), and rheumatoid arthritis (RA), amongst others (1-3). In MS, the host immune cells infiltrate the central nervous systems (CNS) and attack the myelin sheath of the neurons to expose the underlying axons, so they cannot translate signals within neurons (4). As a result of this neuronal damage, MS patients suffer from symptoms of neurological disorders such as sensory loss, loss of vision, and varying degrees of paralysis. MS patients can be classified as relapsing-remitting (RRMS), primary progressive (PPMS), secondary progressive (SPMS), and progressive-relapsing (PRMS), depending on the clinical stage of the disease. About 80-90% of MS patients initially exhibit symptoms of RRMS typified by episodes of relapses and remission (5, 6). But as the disease progresses, RRMS is transformed into chronic progressive MS (CPMS) (6, 7). Most of the current therapies for MS mitigate the symptoms of the disease, and they do not cure the underlying causes of myelin-specific neuronal damages. Thus, there is a need to develop a better way to specifically target and control the myelin-specific aberrant immune cells that are involved in MS pathogenesis. One way is to use antigen-specific immunotherapy, which has been widely investigated as a potential strategy for the treatment of autoimmune diseases.

Experimental autoimmune encephalomyelitis (EAE) animal models have been widely used to study MS and develop novel therapies for treating MS. There are several EAE mouse models, which are correlated with RRMS and CPMS. As in MS, the disease in these animal models begins with the host immune cells recognizing the major self-proteins on the myelin sheath, including proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP) (8). Activation of host T cells generally requires two signals (i.e., Signal-1 and Signal-2) (9-11). Signal-1 is delivered through T cell receptors (TCR) on T cells that recognize antigen-loaded major histocompatibility complex class II (Ag/MHC-II) on the antigen-presenting cells (APC). Signal-2 is delivered by a cluster of molecular pairs such as CD28/B7, CTLA-4/B7, and intercellular adhesion molecule-1 (ICAM-1)/leukocyte function-associated antigen-1 (LFA-1) (9-11). A combination of Signal-1 and Signal-2 forms the immunological synapse that is proposed to be necessary for T-cell activation (10).

Previously, we developed novel bifunctional peptide inhibitor (BPI) molecules by conjugating antigenic and costimulatory peptides (i.e., ICAM-1, LFA-1, and CD28 peptides) (12-16).

BPI molecules inhibit the formation of the immunological synapse at the T-cell-APC interface to alter the balance of immune cells from inflammatory to regulatory/suppressor phenotypes (11, 14, 17). This process modulates a subpopulation of T cells in an antigen-specific manner without suppressing the general immune response. BPI molecules were more effective in suppressing EAE compared to the corresponding antigenic peptides alone or physical mixtures of antigenic peptides and cell adhesion peptides (12-16). Recently, PLP-B7AP was designed as a conjugate of $PLP_{139-151}$ and B7AP peptides via an aminocaproate-glycine (Acp-Gly) linker (Table 1) (18). B7AP peptide was derived from CD28, and it binds to B7 protein on APC to block the C28/B7 costimulatory signal (Signal-2). PLP-B7AP has been shown to be effective in suppressing EAE (18).

$PLP_{139-151}$	Ac-HSLGKWLGHPDKF-NH_2
PLP-BPI	Ac-HSLGKWLGHPDKF-(Acp-G-Acp-G-Acp)$_2$-ITDEATDSG-NH_2
PLP-PEG-B7AP	Ac-HSLGKWLGHPDKF-(PEG)$_2$-G-(PEG)$_2$-EFMYPPPYD-NH_2
MOG_{38-50}	Ac-GWYRSPFSRVVH-NH_2
MOG-PEG-B7AP	Ac-GWYRSPFSRVVHL-(PEG)$_2$-G-(PEG)$_2$-EFMYPPPYD-NH_2
Acp = Amino caproic acid; G = Glycine; PEG = Polyethylene glycols	

Table 1. Peptide sequences of BPI molecules.

In this study, PLP-PEG-B7AP and MOG-PEG-B7AP (Table 1) were developed using a polyethylene glycols (PEG) linker to improve solubility and lower the side effects of the BPI molecules (19). Here, PLP-PEG-B7AP or MOG-PEG-B7AP is proposed to bind MHC-II and B7 on APC and disrupt the complete formation of MHC-II/TCR and CD28/B7 clusters (11, 17). As a result, the peptide prevents the activation of inflammatory T cells, thereby inducing tolerance against EAE. PLP-PEG-B7AP was synthesized by conjugating $PLP_{139-151}$ peptide to B7AP peptide from CD28 conjugated via a PEG linker. Similarly, MOG-PEG-B7AP is a conjugate between MOG_{38-50} peptide and B7AP peptide via a PEG linker. The efficacy of PLP-PEG-B7AP was evaluated in the EAE mouse model of relapse remission-remission MS (RRMS), while MOG-PEG-B7AP was evaluated in the EAE mouse model for chronic progressive MS (CPMS). Both molecules were administered subcutaneously (s.c) in a vaccine-like mode in which the peptide solution was injected several days prior to the stimulation of the disease in mice. EAE clinical scores, incidence of disease, and changes in body weight were monitored to evaluate disease progression. Cytokines produced by splenocytes on days 15 and 30 were measured and used to characterize T-cell phenotypes that are inhibited or proliferated as a result of the treatment.

EXPERIMENTAL PROCEDURES

Mice

All protocols for experiments involving SJL/J ($H\text{-}2^s$) and C57BL/6 mice (Charles River, Wilmington, MA) were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Kansas. All animals were housed under specific pathogen-free conditions at the animal care unit (ACU) facility under the supervision of veterinarians at The University of Kansas. The ACU facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Peptide synthesis

Crude peptides that included $PLP_{139-151}$, PLP-BPI, PLP-PEG-B7AP, MOG_{38-50}, and MOG-PEG-B7AP were synthesized by Shanghai Mocell Biotech Co. (Shanghai, China). The crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a semi-preparative C_{18} column with a gradient of solvent A (94.5% H_2O, 5% acetonitrile, 0.1% TFA) and solvent B (100% acetonitrile). The purity of each fraction from semi-preparative HPLC was evaluated using analytical RP-HPLC (C_{18} column), and all pure fractions were pooled and lyophilized. The identity of each purified peptide was confirmed by electrospray ionization mass spectrometry.

Vaccine-like treatment of EAE

PLP-PEG-B7AP in Relapsing-Remitting EAE Model: PLP-PEG-B7AP was injected in a vaccine-like manner, where it was dosed prior to disease induction against PLP-specific autoantigen. In this case, four groups of SJL/J female mice (5–7 weeks, 6 mice/group) received three s.c. injections of either 100 µl of sterile phosphate-buffered saline (PBS) or 100 µl (100 nmol/injection/day) of PLP-PEG-B7AP, PLP-BPI, and PLP peptides on days -11, -8, and -5 prior to induction of disease on day 0. On day 0, the mice were immunized subcutaneously with 50 µl of PLP/CFA emulsion at regions above the shoulder and the flanks (a total of 4 sites). 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was administered intraperitoneally (i.p.) on days 0 and 2. PLP/CFA emulsion was generated from 0.1 ml of 200 µg $PLP_{139-151}$ in PBS and 0.1 ml of complete Freund's adjuvant (CFA) containing killed *mycobacterium tuberculosis* strain H37RA (4 mg/ml final concentration, Difco, Detroit, MI). EAE disease progression was evaluated using a clinical score by the same observer in a blinded fashion using a scale ranging from 0 to 5 as previously described (18, 20). EAE clinical scores were evaluated 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 a second limb, 4 – full paralysis of two limbs, 4.5 – full paralysis of two limbs with partial paralysis of forelimbs, and 5 – moribund or dead. Body weight of each animal was also measured daily and used to determine percent loss in body weight.

MOG-PEG-B7AP in Chronic Progressive EAE Model: Three groups of C57BL/6 mice (4–6 weeks old; 6 mice/group) were treated with three s.c. injections of either 100 µl of PBS or 100 ml (100 nmol/injection/day) of MOG and MOG-PEG-B7AP on days -11, -8, and -5 prior to disease induction on day 0. On day 0, C57BL/6 mice (4-6 weeks old) were immunized in a similar fashion used in PLP-induced EAE except that 200 µg MOG_{38-50} peptide was used instead of $PLP_{139-151,}$ and 400 ng/mouse/injection of pertussis toxin was administered on days 0 and 2. EAE disease progression was evaluated using clinical scores and body weights (18, 20).

In vitro cytokine production assay

In vitro cytokine assays were performed following a protocol similar to what was described previously (12, 18). Briefly, two groups of SJL/J mice (n = 3 per group) were treated with either PBS (100 µl) or PLP-PEG-B7AP (100 nmol/100 µl) on days -11, -8, and -5 prior to EAE induction. EAE was induced by subcutaneous injection of PLP/CFA emulsion and pertussis toxin as described above. Spleens were harvested on day 15 during periods when the disease was most severe as indicated by the highest disease score in the negative control group. The spleens were also harvested on day 30 during periods of disease remission. Splenocytes were isolated by mashing the spleen and running the cells through a cell strainer in serum-free RPMI-1640 medium. ACK lysis buffer (Invitrogen, Carlsbad, CA) was used to remove the red blood cells. Serum-free RPMI-1640 medium (Cellgro, Manassas,

VA) was used to wash the remaining splenocytes. The cells were placed in each well of a 24-well plate at a density of 5 × 10^6 cells/well and primed with PLP (20 µM). After 72 h, the supernatants from each well were collected and stored in a –80°C freezer for up to 78 h until cytokine analysis to measure IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and IFN-γ using a quantitative ELISA-based Q-Plex assay (Quansys Biosciences, Logan, UT).

STATISTICAL ANALYSIS

Statistical analysis was done using one-way analysis of variance followed by Fisher's least significant difference to compare the different parameters, including EAE clinical scores, changes in body weight, and *in vitro* cytokine production. All statistical analyses were performed using SigmaPlot software (Systat Software Inc, San Jose, CA). A *p*-value of less than 0.05 was used as a criterion for statistical significance. All *p*-values of clinical scores and percent body weight changes were determined by comparing the data from day 10 through day 25.

RESULTS

In vivo study 1: Vaccine-like treatment using PLP-PEG-B7AP against EAE in the relapsing-remitting EAE mouse model

In this study, the *in vivo* efficacy studies of PLP-PEG-B7AP were done in a vaccine-like fashion to suppress EAE in the mouse model. The efficacy of PLP-PEG-B7AP was compared to that of a vehicle (PBS) as negative control, PLP, and PLP-BPI as a positive control. The results show that PLP-PEG-B7AP was more efficacious in suppressing the disease compared to PBS ($p < 0.0001$), PLP ($p < 0.0001$), and PLP-BPI ($p < 0.001$) (Figure 1A) when the scores were compared from day 10 to day 25. PLP-PEG-B7AP-treated mice had a maximum average disease score of 0.78 ± 0.08 observed on day 13, which is a much less severe disease score compared to the PBS control groups. PLP-BPI also had a maximum average disease score of 1.08 ± 0.15 observed on day 13. EAE vaccine-like treatment with PLP had no or low efficacy in inhibiting the progression of the disease compared to PBS. PBS- and PLP-treated mice showed a maximum average disease score on day 15 of 2.50 ± 0.27 and 2.00 ± 0.32, respectively. After the peak of the disease, all the mice slowly underwent remission around day 25.

The efficacy of PLP-PEG-B7AP was also evaluated using percent change in body weight of the mice during progression of disease, and the changes in body weight correlate well with disease scores (Figure 1B). Mice in the PBS group had the biggest loss of body weight during the peak of the disease on day 15. PLP-PEG-B7AP- and PLP-BPI-treated mice did not experience dramatic weight loss compared to the PBS group on day 15. The PLP-treated group had the second biggest changes in body weight during the peak of the disease.

In vivo study II: Vaccine-like treatment using MOG-PEG-B7AP against EAE in a chronic EAE mouse model

For *in vivo* study II, the efficacy of MOG-PEG-B7AP vaccine treatment of chronic progressive EAE was evaluated. Its efficacy was compared to that of MOG_{38-50} antigenic peptide and PBS as a negative control. As anticipated, PBS-treated mice developed EAE around day 10, and the symptoms of the disease became severe around day 15 and remained severe throughout the study without remission. PBS-treated mice reached an average maximal disease score of 2.33 ± 0.15 and had about 5% loss in body weight (Figure 2B). In contrast, MOG-PEG-B7AP-treated mice had significantly lower EAE scores compared to the PBS group ($p < 0.0001$). The average maximum score for MOG-PEG-B7AP-treated mice was 0.75 ± 0.22, and most of the mice in this group did not experience any significant weight loss ($p > 0.05$, Figure 2B). MOG-PEG-B7AP can delay the observed disease symptoms; the symptom began to show on day 14 while for the PBS group the symptom was observed starting on day 9. MOG_{38-50} peptide suppressed and delayed the progress of chronic EAE with an average clinical score of about 1.77 ± 0.30 and about 2% loss in body weight (Figure 2B). The disease symptom was observed starting on day 10. The EAE-suppressive activity of MOG_{38-50} was lower than of MOG-PEG-B7AP.

In vitro cytokine production by isolated splenocytes

To understand the mechanism of action of vaccine-like delivery of PLP-PEG-B7AP, splenocytes were isolated and their cytokine production was analyzed using a quantitative ELISA-based Q-Plex TM assay (18). The types and levels of cytokines secreted by the splenocytes can predict the T-cell phenotypes that are proliferated or inhibited by the treatment and should be helpful in understanding how this treatment works. It is anticipated that PLP-PEG-B7AP will shift the immune system from inflammatory to regulatory/suppressor responses. The levels of pro-inflammatory cytokines were compared on days 15 and 30, including IL-6 (Figure 3A), IL-17 (Figure 3B), and IFN-γ (Figure 3C). There was no difference in the levels of IL-6 during disease severity on day 15 between PLP-PEG-B7AP- and PBS-treated mice (Figure 3A); however, there was a significantly lower production of IL-6 on PLP-PEG-B7AP- compared to PBS-treated mice ($p < 0.001$) on day 30 after the remission (Figure 3A). On day 15, IL-17 level was significantly elevated ($p < 0.001$) in PLP-PEG-B7AP-treated mice compared to the PBS control group (Figure 3B); however, the level of IL-17 was significantly lower in PLP-PEG-B7AP-treated mice compared to the PBS control group at day 30 ($p < 0.001$) (Figure 3B). In contrast to IL-17, IFN-γ levels were significantly ($p < 0.001$)

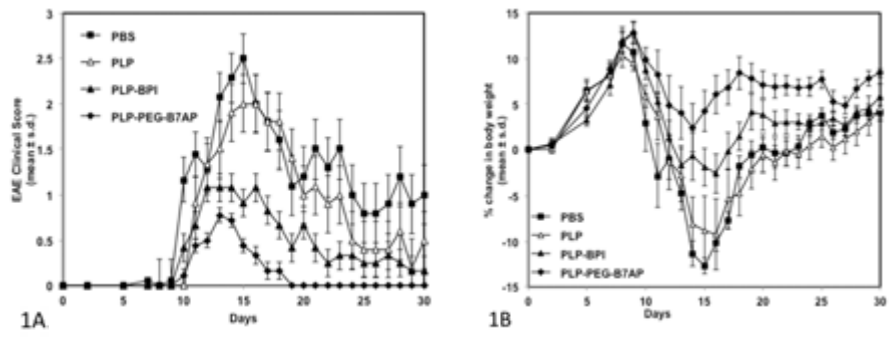

Figure 1. In vivo efficacies of PLP-PEG-B7AP, PLP-BPI, PLP, and PBS in suppressing relapsing-remitting EAE upon vaccination 1 on days –11, –8, and –5 followed by immunization with PLP/CFA on day 0. PLP-PEG-B7AP, PLP-BPI, and PLP peptides were administered at a dose of 100 nmol/100 µl/injection. (A) The disease scores were significantly lower for PLP-PEG-B7AP treated mice than those treated with PLP-BPI ($p < 0.001$), PLP ($p < 0.0001$), and PBS ($p < 0.0001$) treated mice. (B) For percent changes in body weight, PLP-PEG-B7AP-treated mice did not show any significant loss in body weight. PLP-BPI-, PLP-, and PBS-treated mice had approximately 2%, 8%, and 12% loss in body weight, respectively. The statistical analysis was done for data between day 10 and 25.

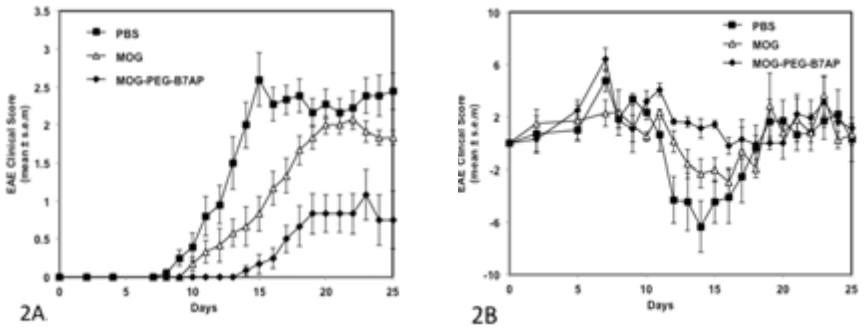

Figure 2. In vivo efficacies of MOG-PEG-B7AP, MOG, and PBS in suppressing chronic EAE upon vaccination on days -11, -8, and -5 followed by immunization with MOG/CFA on day 0. MOG-PEG-B7AP and MOG peptides were administered at a dose of 100 nmol/100 µl/injection. (A) EAE disease scores of mice treated with MOG-PEG-B7AP, MOG, and PBS. EAE disease scores were significantly lower for MOG-PEG-B7AP treated mice than those of MOG ($p < 0.001$) and PBS ($p < 0.0001$) treated mice. (B) Percent changes in body weight of the animals. MOG-PEG-B7AP-treated mice did not show any loss in body weight. MOG- and PBS-treated had approximately 2% and 5% loss in body weight, respectively.

lower in PLP-PEG-B7AP-treated mice compared to the PBS-treated control group on both days 15 and 30 (Figure 3C). As expected, the respective levels inflammatory cytokines IL-17, IFN-γ, and IL-6 were significantly ($p < 0.05$) lower on day 30 than day 15 in both treated and control groups.

There was no statistically significant difference in the levels of regulatory cytokine IL-2 ($p > 0.05$) between PLP-PEG-B7AP- and PBS-treated groups on days 15 or 30 (Figure 4A). Suppressor cytokine IL-5 was produced at higher levels in the PLP-PEG-B7AP group compared to the PBS group on day 15 ($p < 0.05$, Figure 4B), but there was no difference ($p > 0.05$) in the levels IL-5 on day 30. There were decreased productions of IL-4 (Figure 4C) in PLP-PEG-B7AP-treated compared to PBS-treated groups on day 15 ($p < 0.001$) and day 30 ($p < 0.05$). The level of IL-10 was lower in the treated group than the control group on days 15 and 30 ($p < 0.001$), and its level was barely detected on day 30.

DISCUSSION

Previously, PLP-B7AP (Table 1) with an Acp-Gly linker has been shown to be effective in suppressing EAE in the RRMS mouse model when administered as prophylactic treatment (18, 21). PLP-BPI molecules with a PEG linker have been shown to have better solubility and lower side effects (19). It has been shown that PEGylated peptides and proteins have better pharmacokinetic profiles and reduced or no immunogenicity compared to the parent peptides or proteins. This is due to decreased degradation by metabolic enzymes, lower physical aggregation, and lack of recognition by antibodies (21). Therefore, PLP-PEG-B7AP and MOG-PEG-B7AP were designed with a PEG linker to improve their solubility and pharmacokinetic profiles and lower their side effects. Furthermore, this study was also designed to evaluate the efficacy of these BPI molecules when administered as vaccines to suppress EAE in RRMS and CPMS mouse models. The mechanism of action of PLP-PEG-B7AP administered in a "vaccine-like" manner was also evaluated by determining the levels of inflammatory, suppressor, and regulatory cytokines.

EAE clinical scores indicated that PLP-PEG-B7AP was effective in inhibiting EAE in the RRMS mouse model upon vaccine administration, and it is better than PLP-BPI and PLP peptide alone (Figure 1A). Most of the animals treated with PLP-PEG-B7AP only have mild disease symptoms, and some did not show any symptoms at all. The percent loss of body weight of the EAE animals was consistent with the clinical scores (Figure 1B). Although there was a decrease in the percent of body weight on day 15 from day 8, PLP-PEG-B7AP-treated mice have the same body weights as the starting weight at day 0. In contrast, the change in body weights of animals treated with PBS dropped to about 13% from the body weight at day 0. A similar result was observed previously for PLP-B7AP with an Acp-Gly linker when it was administered as a prophylactic and vaccine treatment to suppress EAE in mice (18).

The effect of PLP-PEG-B7AP in the cytokine production on days 15 and 30 was evaluated to determine the T-cell balance (inflammatory and suppressor/regulatory) upon vaccination of the EAE mouse model of RRMS. In the case of an inflammatory response, a greater production of pro-inflammatory cytokines (IL-6, IL-17 and IFN-γ) is expected. If there were an activation of regulatory and suppressor immune response, there would be a greater concentration of regulatory cytokines (IL-2, IL-10) and suppressor cytokines (IL-5 and IL-4) than in the normal condition. The selection of day 15 for detecting cytokine level was based on the time of disease exacerbation, while the selection of day 30 was based on the time of disease remission.

As with inflammatory cytokine, the levels of IFN-γ were lower on PLP-PEG-B7AP-treated than PBS-treated mice in both days 15 and 30, suggesting a long-term suppression of inflammatory immune cells (e.g., Th1 cells) (Figure 3C).

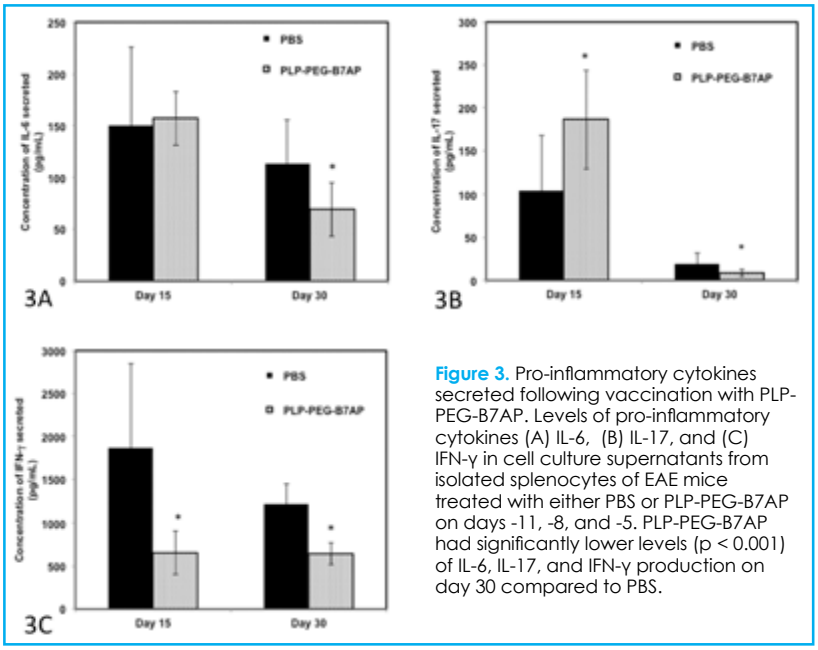

Figure 3. Pro-inflammatory cytokines secreted following vaccination with PLP-PEG-B7AP. Levels of pro-inflammatory cytokines (A) IL-6, (B) IL-17, and (C) IFN-γ in cell culture supernatants from isolated splenocytes of EAE mice treated with either PBS or PLP-PEG-B7AP on days -11, -8, and -5. PLP-PEG-B7AP had significantly lower levels ($p < 0.001$) of IL-6, IL-17, and IFN-γ production on day 30 compared to PBS.

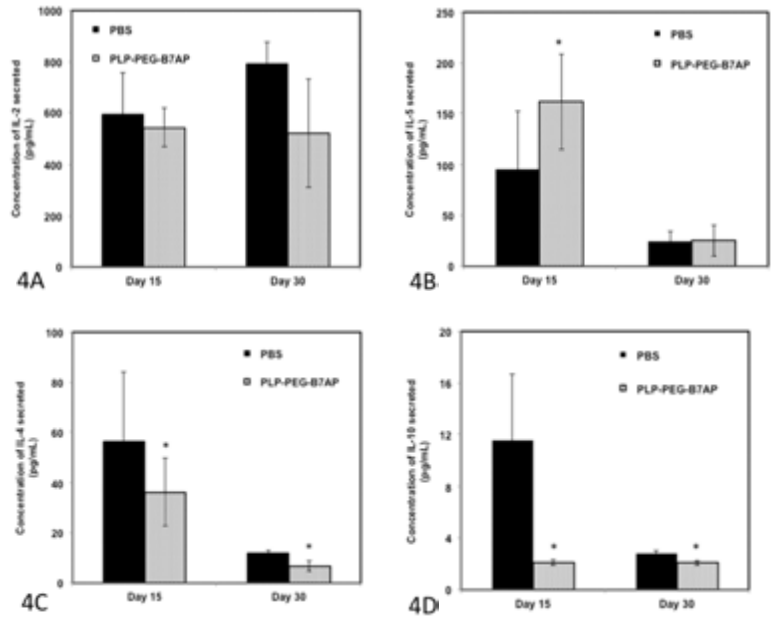

Figure 4. Cytokines associated with regulatory and/or suppressor activity secreted following vaccination with PLP-PEG-B7AP. Concentrations of regulatory/suppressor cytokines (A) IL-2, (B) IL-5, (C) IL-4, and (D) IL-10 in cell culture supernatants from isolated splenocytes of EAE mice treated with either PBS or PLP-PEG-B7AP on days -11, -8, and -5. (A) On both days 15 and 30, there was no difference in the levels IL-2 between PLP-PEG-B7AP- and PBS-treated groups. (B) IL-5 level was higher in the PLP-PEG-B7AP group compared to the PBS group ($p < 0.05$) on day 15, but there was no difference on day 30. (C) The IL-4 levels were significantly lower in PLP-PEG-B7AP-treated compared to PBS-treated groups on day 15 ($p < 0.001$) and day 30 ($p < 0.05$). (D) The levels of IL-10 were lower in the PLP-PEG-B7AP group than in the PBS group control group on days 15 and 30 ($p < 0.001$).

In contrast, the level of IL-17 on PLP-PEG-B7AP-treated mice was higher on day 15 but lower on day 30 compared to PBS-treated mice. This result was different when the mice were treated with PLP-B7AP as a prophylactic on days 4, 7, and 10, where the treatment lowered the IL-17 production on both days 15 and 30 (18). The presence of IL-17 indicated the proliferative state of inflammatory Th17 cells during the disease state. Similarly, treatment of EAE using PLP-B7AP compared to PBS significantly inhibited secretion of IL-6 on day 15, but there was no difference on day 30. However, vaccination using PLP-PEG-B7AP compared to PBS showed no difference in the levels of IL-6 on day 15 but a lower level of IL-6 on day 30. IL-6 has a role in the stimulation of Th1 and Th17 differentiation (22). Lowering both levels of IL-17 and IL-6 on day 30 suggests that it contributes to suppression of Th1 and Th17 differentiation that leads to prevention of disease relapse in peptide-treated animals (Figure 1A). The results suggest that the levels of inflammatory cytokines depend on the timing of administration and on what state of the disease when the cytokine is determined.

There are changes in specific regulatory or suppressor cytokines (i.e., IL-5, IL-4, IL-10) during treatment with PLP-PEG-B7AP compared to PBS on days 15 and 30. PLP-PEG-B7AP-treated mice had an increased level of IL-5 on day 15 but not on day 30. The increase in IL-5 on day 15 suggests that the peptide changes the balance of Th1 to Th2 cells during the exacerbation of the disease. For prophylactic treatment with PLP-B7AP, the increase in IL-5 was observed only on day 30 (18). In this study, vaccination with PLP-PEG-B7AP suppressed the IL-4 on both days 15 and 30; however, IL-4 was increased in prophylactic treatments with PLP-B7AP. The production of IL-10 on day 15 was lower in PLP-PEG-B7AP-treated mice than those treated with PBS. This is in contrast to our previous studies with PLP-BPI molecules; upon prophylactic treatment of EAE with PLP-BPI and PLP-cIBR the animals produced a high level of IL-10 (13, 20). Taken together, the results suggest that time of administration is important in the production of suppressor and regulatory cytokines. In the future, it is necessary to systematically evaluate time-dependent levels of inflammatory and regulatory/suppressor cytokines as a function of mode or schedule of administrations (i.e., vaccine, prophylactic, and treatment). It will be valuable to perform side-by-side comparison of the efficacy and mechanisms of action of PLP-PEG-B7AP, PLP-B7AP and PLP-BPI molecules as a function of schedule of administrations (i.e., vaccine-like, prophylactic, or treatment) to determine the effect of linker and costimulatory signal on the mechanism of action of BPI molecules. Furthermore, the type of infiltrating cells as well as extension of demyelination in the brain after administration of different BPI molecules in a vaccine-like manner should also be determined. The results suggest that the production of suppressor and regulatory cytokines is influenced by the timing of the injection of BPI molecules (i.e., vaccine-like vs. prophylactic).

MOG-PEG-B7AP was used to suppress EAE in the CPMS mouse model as a vaccine treatment, and it was compared to a positive control (MOG_{38-50} peptide) and a PBS negative control. It is clear that MOG-PEG-B7AP has higher efficacy in suppressing chronic EAE than the parent MOG_{38-50} peptide. Previously, MOG-BPI, which contains MOG_{38-50} and LABL peptides, suppressed EAE in the CPMS model better than MOG_{38-50} peptide when administered as prophylactic (12). The current study is the first to indicate that MOG-PEG-B7AP can effectively delay and suppress EAE as a vaccine treatment. As it has been shown previously, the efficacies of BPI molecules are antigenic specific. For example, PLP-BPI molecule is only effective in suppressing EAE in the RRMS mouse model stimulated by PLP peptide in CFA but not in the CPMS model that is stimulated by MOG peptide (12). Similarly, the MOG-BPI is effective in suppressing EAE in the CPMS model but not effective in suppressing EAE in the RRMS model (12). One advantage of this antigenic selectivity is that the BPI molecules can suppress inflammatory immune cells in an antigenic-specific manner without suppressing the general immune responses (11, 17).

It has been shown that peptide-based therapeutic vaccines can prevent autoimmune diseases and have a potential place in combating autoimmune diseases (23-28). Although early clinical data for therapeutic vaccines were rather disappointing, continued research has led to promising results that demonstrate the potential of peptide-based immunotherapy in the treatment of allergy and autoimmune diseases. A 24-month double-blind placebo controlled clinical trial of myelin basic protein peptide in 32 patients with progressive MS showed that treatment with peptide delayed disease progression (29). Furthermore, results from a randomized double-blind phase II clinical trial indicated that early treatment with a peptide derived from heat shock protein (hsp60) inhibited β-cell destruction and maintained insulin production in patients with newly diagnosed type 1 diabetes (30).

Peptide-based immunotherapy involves the use of native peptides or peptides that mimic the naturally processed antigen when bound to MHC-II (27). Effective treatment of autoimmune

diseases with these peptides requires treatment to be initiated early or as soon as possible following definitive disease diagnosis. Studies have shown that a single peptide could prevent insulitis and diabetes in NOD mice when administered at an early stage of the disease (31). However, single peptides were not effective in the treatment of overt diabetes but required administration of a mixture of peptides (31). Mechanistically, peptide antigens induce immune tolerance when administered in soluble form and via a tolerogenic route of administration. At a higher dose antigenic peptides are thought to cause anergy or clonal deletion of antigen-specific cells but induce a regulatory immune response when administered at low doses. Others, however, have demonstrated that both high and low doses administration have featured the induction of T cells with suppressor or regulatory activity (32, 33). Nevertheless, soluble peptides can bind directly to empty MHC-II molecules on the surface of immature dendritic cells and in the absence of costimulatory signals causes naïve T cells to differentiate into regulatory T cells (34).

Despite their ability to induce immune tolerance, antigenic peptides alone have given moderate results. This is presumably due to the mechanism of action of the antigenic peptide that controls only one of the two important signals for T-cell activation. It has been proposed that the activity of the antigenic peptide is due to its binding to empty MHC-II on immature dendritic cells that lack CD80/CD86 (1, 2, 23, 27, 34). Upon recognition of the MHC-II/Antigen complex by TCR on naïve T cells and the absence of CD80/CD86, the naïve T cells differentiate to regulatory T cells (23). The upregulation of regulatory T cells can suppress the proliferation of the inflammatory Th1 and Th17 cells. Thus, the BPI molecules (e.g., PLP-BPI, PLP-PEG-B7AP) can utilize this mechanism to suppress EAE. Because the efficacy of BPI molecules (e.g., PLP-BPI or MOG-BPI) is usually better than the respective parent antigenic peptides (e.g., $PLP_{139-151}$ and MOG_{38-50}), it is proposed that BPI molecules have an additional mechanism of action to induce immune tolerance. The potential second mechanism of action is that the BPI molecules bind simultaneously to both MHC-II and costimulatory signal molecules (i.e., B7 or ICAM-1) on mature dendric cells. This simultaneous binding prevents the formation of the immunological synapse during interaction between T cells and dendritic cells. Blocking the immunological synapse formation prevents the proliferation of inflammatory Th1 and Th17 cells. The overall result is the suppression of the inflammatory immune response to induce immune tolerance and prevent the exacerbation of the autoimmune disease.

BPI molecules have also been designed to treat other autoimmune diseases such as type-1 diabetes (T1D) and rheumatoid arthritis (RA). The GAD-BPI molecule is a conjugate between a peptide from glutamic acid decarboxylase (GAD) peptide and LABL peptide from the I-domain of LFA-1. GAD-BPI suppresses the development of T1D in non-obese diabetes (NOD) mice, and it prevents the infiltration of T cells into the pancreatic islets to destroy the beta-cells that produce insulin (i.e., insulitis) (35). Recently, the collagen II peptide was conjugated to the LABL peptide to make CII-BPI molecules, and the CII-BPI molecules have been shown to effectively suppress RA in the collagen-induced arthritis (CIA) mouse model (36). The results suggest that the formation of BPI molecules effectively delivered the antigenic peptides to immature and mature dendritic cells for their function to alter the commitment of naïve T cells from inflammatory to regulatory/suppressor phenotypes.

In conclusion, PLP-PEG-B7AP and MOG-PEG-B7AP were effective in suppressing EAE in animal models of RRMS and CPMS, respectively, when administered in a vaccine-like manner. The cytokine levels indicate that PLP-PEG-B7AP suppresses the inflammatory cytokine IFN-γ and IL-6 and enhances the production of IL-5. The results suggest that the peptide suppresses EAE by inhibiting the proliferation of inflammatory Th1 cells and shifting the balance towards Th2 cells. Finally, the production of different cytokines depends on the timing of BPI molecule administration and the stage of the autoimmune diseases. In the future, further studies will be carried out to elucidate the potential mechanisms of action of PLP-PEG-B7AP and MOG-PEG-B7AP in suppressing EAE as vaccine, prophylactic, and treatment agents.

ACKNOWLEDGMENTS

We thank the National Institutes of Health (R01AI063002 and R56AI063002) and National Multiple Sclerosis Society for supporting this work. We would like to thank Kristine Bruss for proofreading the manuscript.

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