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Vaccine-like and Prophylactic Treatments of EAE with Novel I-Domain Antigen Conjugates (IDAC): Targeting Multiple Antigenic Peptides to APC

Barlas Büyüktimkin, Prakash Manikwar, Paul K. Kiptoo, Ahmed H. Badawi, John M. Stewart Jr., and Teruna J. Siahaan*

Department of Pharmaceutical Chemistry, The University of Kansas, Simons Research Laboratories, 2095 Constant Ave., Lawrence, Kansas 66047

Abstract

The objective of this work is to utilize novel I-domain antigenic-peptide conjugates (IDAC) for targeting antigenic peptides to antigen-presenting cells (APC) to simulate tolerance in experimental autoimmune encephalomyelitis (EAE). IDAC-1 and IDAC-3 molecules are conjugates between the I-domain protein and PLP-Cys and Ac-PLP-Cys-NH₂ peptides, respectively, tethered to N-terminus and Lys residues on the I-domain. The hypothesis is that the I-domain protein binds to ICAM-1 and PLP peptide binds to MHC-II on the surface of APC; this binding event inhibits the formation of the immunological synapse at the APC-T-cell interface to alter T-cell differentiation from inflammatory to regulatory phenotypes. Conjugation of peptides to the I-domain did not change the secondary structure of IDAC molecules as determined by circular dichroism spectroscopy. The efficacies of IDAC-1 and -3 were evaluated in EAE mice by administering i.v or s.c. injections of IDAC in a prophylactic or a vaccine-like dosing schedule. IDAC-3 was better than IDAC-1 in suppressing and delaying the onset of EAE when delivered in prophylactic and vaccine-like manners. IDAC-3 also suppressed subsequent relapse of the disease. The production of IL-17 was lowered in the IDAC-33 treated mice compared to those treated with PBS. In contrast, the production of IL-10 was increased, suggesting that there is a shift from inflammatory to regulatory T-cell populations in IDAC-33 treated mice. In conclusion, the I-domain can effectively deliver antigenic peptides in a vaccine-like or prophylactic manner for inducing immunotolerance in the EAE mouse model.

Keywords

multiple sclerosis; EAE; vaccine; peptide/protein conjugates; TCR/MHC-Ag; PLP; I-domain; Multi-antigens; I-domain; Th1; Th17; T-reg; APC

INTRODUCTION

One of the characteristics of multiple sclerosis (MS) is neuron demyelination, which causes the disruption of nerve impulses due to damage to the protective covering of the axons. While the triggers of MS have not been clearly elucidated, several factors such as Epstein-Barr virus (EBV) infection, genetic predisposition, and environmental effects are thought to play key roles in its development.¹⁻⁴ Just as in rheumatoid arthritis (RA) and type-1 diabetes (T1D), MS is a result of activation of a subpopulation of autoreactive T cells that

*To whom correspondence should be addressed: Dr. Teruna J. Siahaan, Department of Pharmaceutical Chemistry, The University of Kansas, Simons Research Laboratories, 2095 Constant Ave., Lawrence, Kansas 66047, Phone: 785-864-7327, Fax: 785-864-5736, siahaan@ku.edu.

demyelinate the nerve fibers in CNS.^{1,4,5} While there is no cure for MS, many of today's therapies, including biologic drugs such as interferons (avonex®, betaseron®), antibodies (tysabri®), and antineoplastics (mitoxantrone) focus on slowing down or altering the disease progression.⁶ One of disadvantages of these current therapies is that they do not target specific immune cells that cause MS but rather suppress the general immune system, leading to potential serious side effects such as inability to fight pathogenic infections.⁷ Therefore, there is a need to investigate a new and more specific way to control a subpopulation of immune cells generated in an autoimmune disease such as MS without suppressing the general immune response.

To solve the problem of non-specific control of immune response, we have developed novel bifunctional peptide inhibitor (BPI) molecules that target antigenic peptides to antigen-presenting cells (APC) to control the activation of a subpopulation of T cells specific to a particular autoimmune disease.^{6,7} In this approach, a cell adhesion peptide was conjugated to an antigenic peptide to make BPI molecules. For example, PLP-BPI is a conjugate between an antigenic peptide from proteolipid protein (PLP₁₋₉₋₁₅₁) and a cell adhesion peptide (LABL) derived from the I-domain of lymphocyte function-associated antigen-1 (LFA-1) tethered together by a linker molecule. PLP-BPI derivatives have been shown to successfully suppress the progression experimental autoimmune encephalomyelitis (EAE) in mice, a model for MS.⁸⁻¹² Different BPI molecules such as GAD-BPI and CII-BPI can effectively suppress T1D and RA, respectively, in animal models.¹³ The hypothesis is that the BPI molecules bind simultaneously to the major histocompatibility complex class II (MHC-II) and intercellular adhesion molecule-1 (ICAM-1) on APC to block immunological synapse formation at the T cell-APC interface.⁶ Blocking the immunological synapse formation changes T-cell differentiation and T-cell balance from inflammatory to regulatory. Unfortunately, BPI molecules can deliver only one antigenic peptide at a time and, thus, cannot provide multiple antigens when antigenic spreading has occurred.^{14,15} Therefore, there is still a need to develop a method to simultaneously deliver multiple antigens to APC to overcome the problem of antigenic spreading in a particular autoimmune disease.

As a proof-of-concept, I-domain-antigen conjugate (IDAC) molecules were developed for delivering multiple antigens to APC with the goal of preventing antigenic spreading during disease relapse and exacerbation. IDAC-1 molecule was synthesized with antigenic peptides from one epitope of PLP (e.g., PLP₁₃₉₋₁₅₁-Cys-OH or PLP-Cys-OH) conjugated to the I-domain protein via multiple lysine residues and the N-terminus; this molecule has been shown to suppress EAE in mice.¹⁶ The hypothesis is that the I-domain protein delivers several antigenic peptides to APC upon binding of the I-domain to domain-1 (D1) of ICAM-1 and PLP peptides to MHC-II molecules on the surface of APC to block the immunological synapse formation. The advantage of IDAC over BPI molecules is that IDAC can simultaneously deliver several different antigenic peptides to MHC-II molecules to prevent antigenic spreading.^{6,17} To improve peptide enzymatic stability and IDAC efficacy, IDAC-3 molecules were synthesized by conjugating Ac-PLP-Cys-NH₂ peptides to the I-domain. The purified IDAC molecules were characterized using mass spectrometry and circular dichroism (CD) spectroscopy. The efficacies of IDAC-1 and -3 molecules were compared by treating EAE mice with IDAC molecules in prophylactic and vaccine-like manners. In both prophylactic and vaccine-like methods, IDAC molecules effectively suppressed EAE compared to treatment with PBS. The cytokine production data suggested that IDAC molecules stimulate the proliferation of regulatory/suppressor cells and suppress the inflammatory cells.

MATERIALS AND METHODS

Animals

The protocols to use live mice have been approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Kansas. Mice (SJL/J female) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and they were housed under specific pathogen-free conditions at the animal facility at The University of Kansas approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Peptide Synthesis

An automated peptide synthesizer (Pioneer; Perceptive Biosystems, Framingham, MA) with 9-fluorenylmethyloxycarbonyl-protected amino acids was used to synthesize all peptides for this study (Table 1). After peptide cleavage from the resin, the crude product was purified using a C18 semi-preparative column in high-performance liquid chromatography (HPLC) as described previously.¹⁰ The identity of the peptides was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI3TOF) mass spectrometry.

I-domain Preparation

The I-domain protein was over-expressed, refolded, and purified as previously described.¹⁷ The identity, purity, and secondary structure of the protein were confirmed by mass spectrometry, SDS-PAGE, and CD, respectively.

Synthesis of IDAC-1 and -3

The method used to synthesize IDAC-3 was similar to that for IDAC-1, except that Ac-PLP-NH₂ was used in IDAC-3.¹⁶ Briefly, the I-domain was modified to the gamma-maleimidobutyramide-I-domain (GMB-I-domain) by reacting the lysine residues with *N*-[γ-maleimidobutyryloxy]-succinimide ester (GMBS). After purification of the GMB-I-domain with size-exclusion chromatography (SEC), the pure product was concentrated and reacted with the thiol group on the Cys residue of PLP-Cys-OH and Ac-PLP-Cys-NH₂ peptides, respectively, at pH 8.5 to make IDAC-1 and IDAC-3. After a one-hour reaction, the pH of the solution was adjusted to 7.4, and the reaction mixture was purified using SEC. The purity, identity, and conformation of IDAC-1 and -3 were determined using SDS-PAGE, mass spectrometry (LC ESI-MS), and CD, respectively.

Induction of EAE and Efficacy Studies

EAE disease in animals (6–8 week-old SJL/J female mice) was stimulated by injecting 200 Lg PLP₁₃₉₋₁₅₁ peptide in a 0.2 mL emulsion containing equal volumes of PBS and complete Freund's adjuvant (CFA) with killed *Mycobacterium tuberculosis* strain H-7RA (Difco, Detroit, MI; final concentration 4 mg/mL) as described previously.⁹ Briefly, 50 LL of PLP/CFA emulsion was administered to regions above the shoulder and the flanks on day 0 followed by injection of 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2. The mice then received either intravenous or subcutaneous injections of IDAC (10 or 26 nmol/injection) or positive control peptides (100 nmol/injection/mouse for Ac-PLP-BPI-NH₂-2 or 50 nmol/injection/mouse for Ac-PLP-cIBR1-NH₂). The prophylactic disease suppression was carried out with subcutaneous or intravenous injections of IDAC molecules on days 4 and 7 or BPI molecules on days 4, 7, and 10. Mice receiving vaccine-like treatment were given subcutaneous injections of IDAC and BPI molecules at 11, 8, and 5 days prior to the induction of disease. As negative controls, mice were treated with PBS, I-domain, and GMB-I-domain. Disease progression was evaluated

by monitoring the change in weight of the mice and clinical scoring based on the severity of nerve damage, ranging from 0 to 5 as described previously.⁹

Determination of Cytokine Levels *In Vitro*

Representative spleens for each group (IDAC-3 or PBS) were harvested from female SJL/J (H-2^S) mice on days 13 and 35. Splenocytes were isolated by gently grinding the spleen in RPMI 1640 medium (10% FBS, 0.05 M BME) in a petri dish. Then, these cells were passed through a 40-micrometer strainer. The cells were centrifuged and the red blood cells were lysed using an ammonium chloride-potassium (ACK) lysis buffer. The remaining white blood cells were washed three times with medium. Splenocytes (5×10^6 cells/mL) were cultured in RPMI medium in the presence or absence of 20 LM PLP peptide. After incubation for 72 h, the supernatants were collected and analyzed for the level of different cytokines with a quantitative ELISA-based Q-Plex™ (Mouse Cytokine Screen, Quansys Biosciences, Logan, UT).

Statistical Analysis

Statistical differences in clinical disease scores among the groups were determined by calculating the average score for each mouse from day 12 to day 17 by one-way analysis of variance followed by Fisher's least significant difference. Statistical differences in body weight among groups were also analyzed in the same fashion, but from day 12 to day 24. Comparison of cytokine concentrations was also performed by one-way analysis of variance. All analyses were performed using StatView (SAS Institute, Cary, NC).

RESULTS

Synthesis and Characterization of IDAC-1 and -3

IDAC-1 and -3 were made by randomly reacting the free amine groups of the I-domain with the N-hydroxysuccinimide group of GMBS to produce GMB-I-domain. The resulting GMB-I-domain was then reacted with the thiol group of the Cys residue on PLP-Cys-OH or Ac-PLP-Cys-NH₂, yielding IDAC-1 and -3, respectively. Next, the resulting mixture was purified using size-exclusion column chromatography, and the eluted fractions were subsequently analyzed using SDS-PAGE (Fig. 1A). The observed multiple bands on SDS-PAGE were due to the varying number of conjugated peptides on the I-domain. The earlier lanes on the gel had bands at a higher molecular weight compared to the later lanes, which corresponded to the order of elution of IDAC from the SEC. Analysis with circular dichroism showed that both IDAC-1 and -3 have spectra similar to that of the I-domain (Fig. 1B). The deconvoluted LC-MS data showed that both IDAC molecules had 0-5 PLP-Cys peptides conjugated per I-domain, with an average of 2.5 PLP-Cys peptides per I-domain molecule (Fig. 1C).

Suppression of EAE by IDAC-1 and -3

To test whether the I-domain and GMB-I-domain had *in vivo* efficacy, three groups of mice were treated with two intravenous injections of the I-domain or GMB-I-domain (26 nmol/injection) as well as PBS on days 4 and 7. Although there was a slight delay in the onset of the disease, neither the I-domain nor GMB-I-domain significantly suppressed the progress of EAE compared to PBS, as determined by the clinical score (Fig. 2A) and change in body weight (Fig. 2B).

In the second study, the efficacies of IDAC-1 and IDAC-3 with uncapped and capped PLP peptides, respectively, were compared upon intravenous injections of 26 nmol/injection on days 4 and 7; the control group was injected with PBS. Clinical scores (Fig. 3A) indicated that both proteins delayed the onset of disease and were significantly better at suppressing

EAE than PBS ($p < 0.0005$, through days 12-17). Furthermore, IDAC-3 was better than IDAC-1 in suppressing EAE ($p < 0.005$, through days 12-17). The body weight change for IDAC-1- and IDAC-3-treated animals supported the clinical score data; two injections of IDAC-1 and -3 were significantly more effective than PBS in suppressing disease (Fig. 3B, $p < 0.05$ through days 12-24). In addition, there were delays in disease incidence in IDAC-1- and IDAC-3-treated animals (data not shown).

After establishing that IDAC-3 was a better candidate to suppress EAE, the third study was aimed at evaluating an alternative route of injection (i.e., subcutaneous or s.c.), dose response to determine therapeutic index, and optimal timing of IDAC-3 injections (Fig. 4). First, IDAC-3 injected s.c. (26 nmol/injection) on days 4 and 7 was significantly more efficacious than PBS in suppressing EAE as shown by clinical scores ($p < 0.005$, through days 12-17; Fig. 4A), change in body weight of the mice ($p < 0.005$, through days 12-24; Fig. 4B), and disease incidence (Fig. 4C). Second, the efficacy of IDAC-3 upon vaccine-like administrations was evaluated in different doses to determine the therapeutic index of the molecule. In this case, the mice received s.c. injections of two different doses of IDAC-3 (26 nmol/injection and 10 nmol/injection), Ac-PLP-BPI-NH₂-2 (100 nmol/injection), and PBS at 11, 8, and 5 days prior to the induction of the disease at day 0. Ac-PLP-BPI-NH₂-2, as positive control, significantly suppressed EAE compared to PBS as reflected in the clinical scores (Fig. 4A; $p < 0.005$, days 12-17) and change in body weight (Fig. 4B; $p < 0.005$, days 12-24). Although IDAC-3 was less potent than Ac-PLP-BPI-NH₂-2, mice treated with a low dose of it (10 nmol/injection) had significantly better clinical scores (Fig. 4A, $p < 0.005$, days 12-17) and body weight changes (Fig. 4B; $p < 0.005$ through days 12-24) than those receiving PBS control. At a high dose (26 nmol/injection), the third injection of IDAC-3 unfortunately caused a toxic effect in two of six mice; thus, the efficacy data were representative of four animals (Fig. 4). Although statistical analysis was not carried out due to the lower number of animals, a high dose (26 nmol/injection) of IDAC-3 seemed to be better than the lower dose (10 nmol/injection) and similar to Ac-PLP-BPI-NH₂-2.

To test the optimal concentration for vaccine-like delivery and its long-term effect in suppressing relapse, IDAC-3 was injected twice via the s.c. route (26 nmol/injection on days -11 and -8) and was compared to three s.c. injections of Ac-PLP-BPI-NH₂-2 (100 nmol/injection; days -11, -8, and -5) and PBS (days -11, -8, -5). Two injections of IDAC-3 (26 nmol) clearly suppressed EAE significantly better than PBS and had a long-term effect in suppressing relapse of the disease as indicated by the clinical scores (Fig. 5A, $p < 0.0001$, days 12-17, and days 45-55). Similarly, Ac-PLP-BPI-NH₂-2 also had a long-term effect in preventing relapse. The changes in body weight and delay in disease onset were monitored for 24 and 35 days, respectively; the changes in body weight in IDAC-3- and Ac-PLP-BPI-NH₂-2-treated mice were significantly lower than those of PBS-treated mice (Fig. 5B, $p < 0.0001$, days 12-24). The delay in disease onset of disease was greater in mice treated with IDAC-3 and PLP-BPI than in those treated with PBS (Fig. 5C).

Cytokine Levels in SJL/J Mice In Vitro

The potential mechanism of action of IDAC-3 was elucidated by comparing the cytokine levels (i.e., IL-2, IL-5, IL-10, IL-12, and IL-17) in splenocytes after two s.c. injections of IDAC-3 (26 nmol/injection) and PBS on days 4 and 7. The levels of cytokines were determined during the peak of disease on day 13 and after EAE went into remission and plateaued on day 35 (Fig. 6). Due to the low detection limit or the lack of statistical significance between IDAC-3 and PBS treatments, the levels of IL-4, IL-6, and IFN γ were inconclusive. IDAC-3-treated animals had fourfold lower IL-17 during disease remission on day 35 compared to the PBS-treated group (Fig. 6A, $p < 0.0001$).

In the PBS-treated group, IL-2 cytokine levels were higher than in the IDAC-3-treated group on day 13 ($p < 0.05$) whereas no significant difference was observed on day 35 (Fig. 6B). Although there was no significant difference in IL-5 levels on day 13, the IL-5 levels on day 35 were significantly higher in the IDAC-3-treated group than in the PBS-treated group (Fig. 6C, $p < 0.0005$). Cytokine levels of IL-10 on day 13 could not be detected; however, once the disease remission plateaued on day 35, the IDAC-3-treated group had significantly higher levels of IL-10 compared to the PBS group (Fig. 6D, $p < 0.05$). In the IDAC-3-treated group, the level of IL-12 was significantly lower than in the PBS-treated group on day 35 (Fig. 6E, $p < 0.005$).

Discussion

The I-domain emerged as a useful carrier protein to target antigenic peptides to suppress EAE. Our current findings indicate that two injections (26 nmol/injection) of IDAC-3 with capped PLP peptide can suppress disease severity more efficiently than IDAC-1 with the uncapped PLP peptide. This corroborates a similar finding from our previous study in which capped Ac-PLP-BPI-NH₂-2 peptide could suppress EAE better than its uncapped counterpart (PLP-BPI).⁸ The pharmacokinetics studies of Ac-PLP-BPI-NH₂-2 in rats showed that the peptide has $t_{1/2}$ around 2-3 h depending on the dose.¹⁰ These results suggested that capped PLP peptide might have higher metabolic stability than the uncapped peptide; in other words, the uncapped peptide is susceptible to exopeptidases (i.e., amino- and carboxy-peptidases). Because SDS-PAGE, CD, and mass spectrometry analyses suggest that IDAC-1 and IDAC-3 are very similar, it is plausible that the major difference in their *in vivo* activity is due to the improved metabolic stability of the peptide and not to the different conformation of the molecule.

IDAC-3 dosed subcutaneously on days 4 and 7 was very effective in suppressing EAE. Our previous studies also indicated that s.c. administration of BPI molecules was more effective than i.v. administration.¹⁸ One advantage of subcutaneous dosing is that IDAC molecules may drain into the lymph nodes to modulate immune cells.^{19,20} The vaccine-like administration (two injections of 26 nmol) of IDAC-3 prior to the induction of disease had a significant long-term effect in suppressing disease relapse after 55 days compared to PBS (Fig. 5), suggesting that IDAC-3 works by tipping the balance of the immune cells from inflammatory to regulatory phenotypes. It also suggested that vaccine-like delivery could alter the balance of immune cells to regulatory cells prior to the stimulation of the disease. As controls, I-domain and the GMB-I-domain produced slight delays of disease onset but no significant suppression of disease; this activity was probably due to the general inhibition of LFA-1/ICAM-1-mediated leukocyte adhesion.¹⁷ In the future, the effect of injections of IDAC-3 and I-domain on the differentiation of immune cells will be evaluated. Because the therapeutic index of IDAC-3 has not been fully determined, the effect of increasing the dose while maintaining the schedule of 2 injections as well as multiple injections (greater than 3 injections) at low doses (less than 10 nmol) by spreading the injection over a longer timespan will also be investigated.

It seems that IDAC-3 is less potent than Ac-PLP-BPI-NH₂-2 at a similar PLP peptide concentration; however, it is difficult to make a direct comparison at this time because the IDAC-3 molecules contain a mixture of conjugates while Ac-PLP-BPI-NH₂-2 is a single molecule. One possible explanation for the lower activity of IDAC-3 is that not all of the conjugates have efficacy or that some conjugates are more efficacious than others. The less potent of the IDAC-3 conjugates could have lower binding properties to ICAM-1 and/or MHC-II due to the location of the PLP peptide on the I-domain. For example, conjugation of PLP peptide near or around the binding site of the I-domain may interfere with binding of the I-domain of the conjugate to ICAM-1. For a certain population of conjugates, the steric

effect could interfere with the binding property of PLP peptides to MHC-II on the surface of APC. Previously, the locations of PLP peptides on the I-domain have been determined by a combination of tryptic digest and mass spectrometry analyses.¹⁶ In the future, we will produce several IDAC molecules with a single specific site of PLP-peptide conjugation on the I-domain to determine the conjugates that produce in vivo efficacy in EAE mice. Furthermore, more thorough studies are needed to compare the efficacy and mechanism of action of IDAC and BPI molecules.

There are several working hypotheses for the mechanism of action of IDAC molecules to produce their efficacy. The data indicate that IDAC molecules suppress the disease by suppressing the proliferation of inflammatory immune cells (i.e., Th17 and Th1) and stimulating the suppressor and regulatory T cells (i.e., Th2 and T-reg). One hypothesis is that the IDAC-3 molecules simultaneously bind to ICAM-1 and MHC-II on the surface of mature dendritic cells and, upon interaction between mature dendritic cells and T cells, the IDAC-3 molecules prevent the formation of the immunological synapse. The prevention of the immunological synapse formation suppresses the activation of inflammatory Th1 and Th17 cells via the anergy process. The proposed colocalization of MHC-II and ICAM-1 by IDAC molecules is based on our previous finding that GAD-BPI can colocalize MHC-II and ICAM-1 on the surface of B cells isolated from NOD mice.¹³ The fluorescence-labeled I-domain has been shown to bind ICAM-1 on the surface of APC (i.e., B cells).¹⁷ Because fluorescence-labeled I-domain is also endocytosed by APC (i.e., B cells),¹⁷ IDAC-3 molecules could also undergo internalization by ICAM-1 followed by cellular processing to produce MHC-II-bound antigenic peptides that can be presented on the surface of APC to naïve T cells. The MHC-peptide complexes can be presented by immature dendritic cells that lack costimulatory signal molecules such as CD80 (B7-1) and CD86 (B7-2). The MHC-II3 peptide complexes presented on immature dendritic cells will be recognized by T-cell receptors (TCR) on naïve T cells. This TCR/MHC-peptide recognition process will be without costimulatory signal B7/CD28 due to the absence of B7 molecules on immature dendritic cells.^{7,21,22} As a result, the naïve T cells will differentiate to T-reg cells that suppress the proliferation of inflammatory cells.^{7,21,22}

As previously stated, BPI molecules were a conjugate between an antigenic peptide and LABL (CD11a₂₃₇₋₂₄₆) peptide derived from the I-domain sequence. In the current study, the I-domain protein was conjugated with multiple peptides from a single epitope (PLP₁₃₉₋₁₅₁). This approach would allow simultaneous delivery of multiple epitopes of PLP, as well as a mixture of epitopes from the proteolipid protein, myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP). Another advantage of I-domain over LABL is that the I-domain contains the metal ion-dependent adhesion site (MIDAS) necessary for divalent cation coordination (Ca²⁺, Mg²⁺, or Mn²⁺) to enhance binding selectivity to ICAM-1 on the cell surface.²³ The expression of ICAM-1 is increased on cell surfaces in autoimmune diseases as well as in other diseases.^{24,25}

During the peak severity of the disease on day 13, splenocytes isolated from the mice treated with PBS and IDAC-3 had similar levels of IL-17 production. However, on day 35, IDAC-3-treated mice were found to have significantly lower levels of IL-17 compared to the PBS group, indicating that IDAC-3 has long-term effects in suppressing EAE. Th17 is a major T cell that has been found to play a large role in the pathogenesis of both EAE and MS.^{5,26-29} Significant suppression of disease, coupled with lower levels of IL-17 on day 35, suggested that two injections of IDAC-3 suppressed the disease by modulating the immune system of the mice and shifting the response from an inflammatory to a regulatory phenotype. The higher level of IL-17 on day 35 in the PBS group may suggest an impending relapse of disease. This correlated with our previous observations in mice treated with Ac-

PLP-BPI-NH₂-2, suggesting the involvement of regulatory cells and the downregulation of Th17.^{8,10}

While IL-10 levels were below detection limits on day 13, higher levels of IL-10 production were observed in mice treated with IDAC-3 compared to PBS on day 35, indicating the potential involvement of regulatory T cells (T-reg cells). Previously, BPI-treated animals also produced a higher level of IL-10 cytokine than did PBS-treated animals.⁹ In addition, IDAC-3 induced IL-5, implying the involvement of Th2 phenotype. A shift away from a Th1-response was observed from the involvement of lower levels of IL-2 in the IDAC-3-treated group compared to the PBS-treated mice. Furthermore, the lower levels of IL-12 in the IDAC-3-treated compared to PBS-treated mice point away from a Th1-immunogenic response.

Although IDAC molecules tip the balance of immune cells from an inflammatory to a regulatory phenotype, the mechanism of action of IDAC molecules has not been fully elucidated. One proposed mechanism of action is that an IDAC molecule can simultaneously bind to ICAM-1 receptor and deliver multiple antigenic peptides to MHC-II receptors on the surface of APC. As a result, IDAC molecules on APC inhibit the formation of the immunological synapse at the interface between T cells and APC, alter the differentiation of naïve T cells to regulatory T cells, and suppress the proliferation of inflammatory Th17 and Th1 cells. This hypothesis is supported by the previous observation that GAD-BPI molecule, a suppressor of type-1 diabetes in non-obese diabetic (NOD) mice, could bind simultaneously to MHC-II and ICAM-1 and colocalize them on the surface of B cells isolated from NOD mice.¹³

Another possible mechanism is that IDAC molecules bind to ICAM-1 receptors on APC followed by ICAM-1-mediated internalization similar to that of I-domain peptides.³⁰ Our previous studies had shown that the I-domain conjugated to fluorophores at the Lys residues could effectively bind to ICAM-1 and enter leukocytes by receptor-mediated endocytosis despite having a heterogeneous mixture.^{17,31} After internalization, there are several possible fates of the IDAC molecules, two of which are presented here. One possibility is that upon internalization the antigenic peptides, while still attached to the I-domain, bind to empty MHC-II molecules as molecular clusters with ICAM-1 molecules. These clusters of MHC-II/IDAC/ICAM-1 as colocalized complexes are recycled to the surface of APC for presentation to naïve T cells for differentiation to regulatory T cells or for presentation to the activated T cells for their suppression because of the inability to form mature a immunological synapse.^{17,32} The second possible fate of IDAC molecules is that they degrade and release the PLP peptides; then, the PLP peptides are loaded onto MHC-II molecules in a Golgi apparatus, and are transported to the cell surface for presentation of antigenic-peptide/MHC-II complexes to T-cell receptors on T cells.³² At the time that the PLP-MHC-II-complex is presented at the surface of APC, the second signal (ICAM-1:LFA-1) necessary for T-cell activation is absent due to ICAM-1 internalization, leading to T-reg differentiation.^{21,33} However, the internalized ICAM-1 has been shown to recycle and resurface and, therefore, the absence of the second signal is transient.³² In the future, the potential mechanisms of action of IDAC molecules will be elucidated.

IDAC-3 is a mixture of conjugation products in which several lysine residues are conjugated with PLP peptide. The conditions of conjugation reaction have been optimized to maintain batch-to-batch reproducibility, as determined by mass spectrometry and CD. Using tryptic digest and mass spectrometry, the sites of peptide conjugation were determined and the number of conjugations was found to be between one and five peptides per I-domain.¹⁶ Because IDAC-3 is a mixture, it is possible that not all of the conjugated products have biological activity to suppress EAE. In the future, several individual lysine residues will be

mutated to cysteine residues (Cys-I-domain) for selective conjugation of peptides to a selected cysteine residue. Thus, the resulting conjugate will be a single conjugate instead of a mixture of conjugates. Then, the efficacy of each conjugated product may be evaluated in the EAE mouse model. This study will provide us with the important conjugation site(s) in the I-domain that produce biological activity.

The toxicity of IDAC molecules could be due to immediate and/or delayed hypersensitivity reactions because of the activation of immune cells upon a second or third exposure to the IDAC molecules. We propose that the IDAC molecules generate the type-I hypersensitivity reaction. In this case, the IDAC molecules bind to B-cell receptors followed by uptake and intracellular processing for antigenic peptide presentation to helper T cells (i.e., Th2 cells) for inducing IL-4 cytokine production. The peptides presented by MHC-II can be from the epitopes of the I-domain or peptides derived from the linked between the I-domain and PLP as foreign antigens. The first injection of IDAC molecules produced IL-4 cytokines that can bind to IL-4 receptors on B cells for their differentiation to plasma cells. The resulting plasma cells produce IgE antibodies specific to the epitopes of the IDAC molecules, and these specific IgE antibodies bind to Fc receptors (i.e., FcεRI) on mast cells. Upon subsequent exposure to the IDAC molecules, the IDAC molecules are recognized by the IDAC-specific IgE molecules on the surface of mast cells to cause the crosslinking of the IgE molecules. The IgE crosslinking upon binding to IDAC molecules stimulates degranulation of mast cells to cause the release of active mediators (i.e., histamine, leukotrienes, and prostaglandins). The release of active mediators can affect many tissues (e.g., smooth muscle cell, small blood vessel, mucous gland) to create the contraction of local and systemic smooth-muscles (i.e., bronchoconstriction and vasodilation) as well as an increase in vasopermeability and mucus production. It has been shown previously that repeated treatments of EAE mice with myelin peptides also generate hypersensitivity reactions. This hypersensitivity reaction can be inhibited by co-administration of anti-IgE antibody along with myelin peptides, suggesting that the mechanism of hypersensitivity is due to generation of antigen-specific IgE or type-I hypersensitivity reaction.³⁴ In the mouse model, delivering antihistamines also suppresses anaphylaxis reaction during treatment of EAE with myelin peptides to further support the proposed type-I hypersensitivity reaction mechanism.³⁵ In the future, we will evaluate the effect of IDAC molecules on the type-I hypersensitivity reaction mechanism and the effect of dose and/or route of administration to lower or eliminate the hypersensitivity reaction.

In conclusion, IDAC-3 effectively inhibited the onset and severity of EAE in mice. The conjugation of multiple copies of a single antigenic epitope to the I-domain of LFA-1 suppressed EAE by shifting the immune response to a regulatory phenotype. IDAC-3 also suppressed the relapse of EAE when delivered in a vaccine-like manner. Cytokine studies suggested that IDAC-3 suppressed disease by shifting the immune balance away from Th17-mediated pathology by increasing the involvement of T-reg cells. Further studies using IDAC molecules will involve optimizing the dose and the dosing strategy to lower toxicity and improve efficacy. Finally, the effect of epitope spreading will be addressed by conjugating other immunodominant epitopes, such as MOG and MBP, to the I-domain.

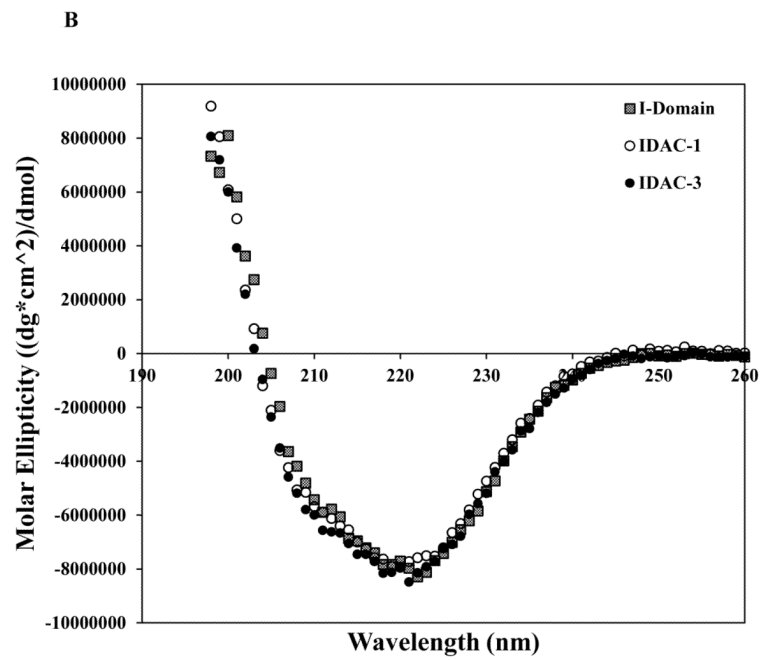
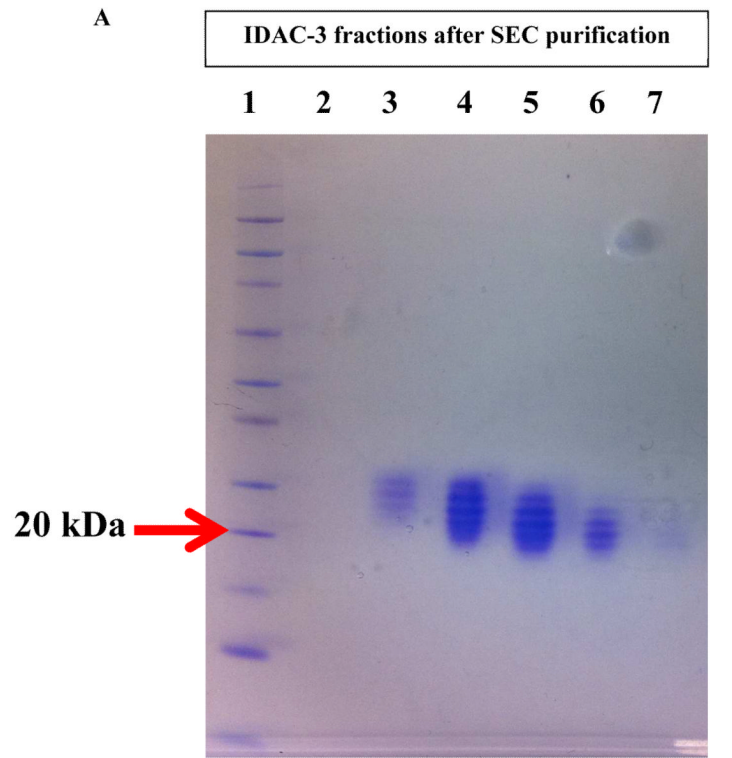
Acknowledgments

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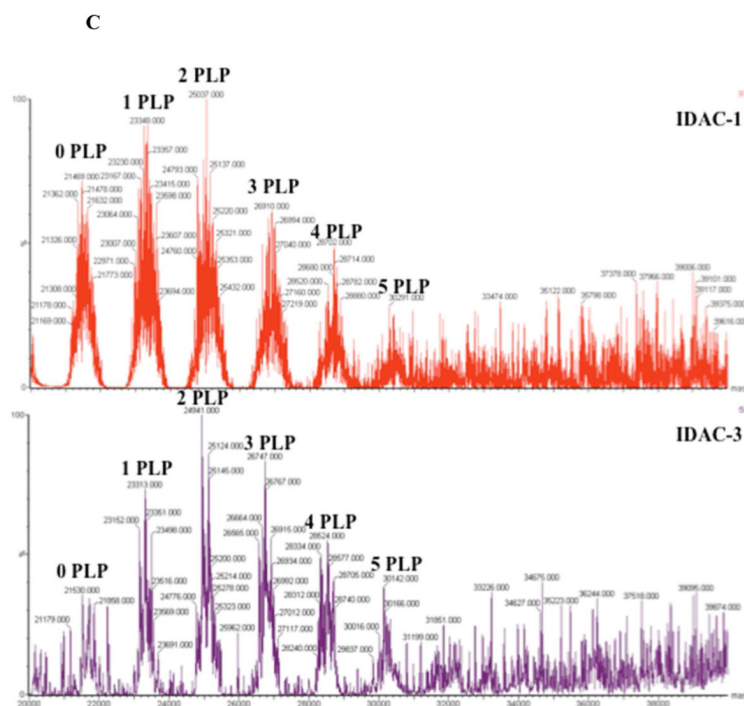


Figure 1. Characterizations of IDAC-1 and -3 were done by (A) SDS-PAGE gel, (B) CD, and (C) mass spectrometry. (A) SDS-PAGE analysis of IDAC molecules after separation using SEC: (lane 1) molecular weight marker, (lane 2) blank, and (lanes 3-7), fractions of conjugated IDAC-3 in the order of elution from SEC. (B) CD spectra of the parent I-domain (square), IDAC-1 (open circle) and IDAC-3 (closed circle). (C) Deconvoluted mass spectra of LC ESI-MS analysis of IDAC-1 and IDAC-3.

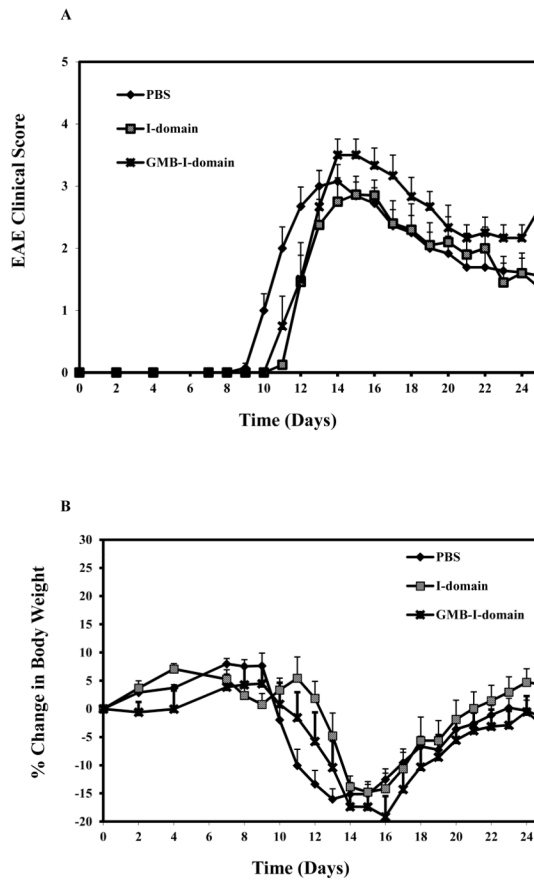


Figure 2.

In vivo activity of I-domain and GMB-I-domain upon i.v. injections of 26 nmol/injection/day on days 4 and 7 in mouse EAE model after immunization with PLP peptide in CFA. Control mice were treated with PBS on days 4, 7, and 10. Disease progression was evaluated using (A) clinical disease scores and (B) change in body weight. The results are expressed as the mean \pm S.E. (n = 6).

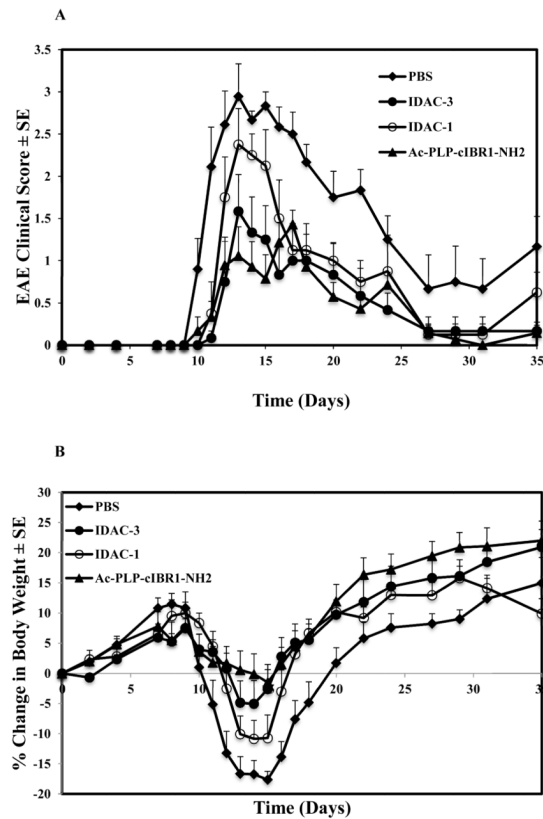


Figure 3.

Comparison of the *in vivo* activity of IDAC-1, IDAC-3, Ac-PLP-cIBR1-NH₂, and PBS in the mouse EAE model using (A) clinical disease scores and (B) change in body weight. After immunization with PLP peptide in CFA, the mice received i.v. injections of 26 nmol/injection/day of IDAC-1 or IDAC-3 on days 4 and 7. For the Ac-PLP-cIBR1-NH₂ treatment group, the mice received i.v. injections of 50 nmol/injection/day of the peptide on days 4, 7, and 10. Control mice were treated with PBS on days 4, 7, and 10. The results are expressed as the mean ± S.E. (n = 6).

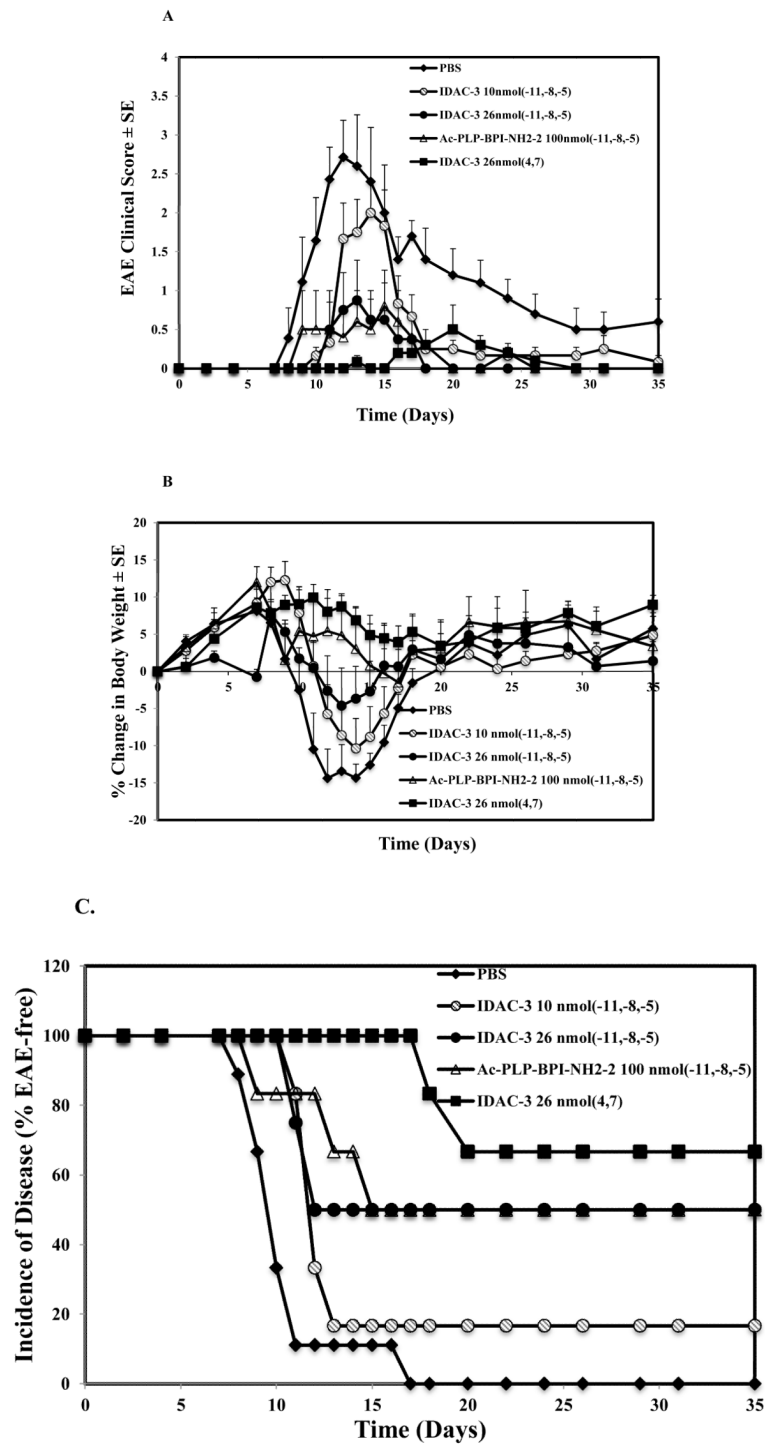


Figure 4. The *in vivo* efficacy of IDAC-3 was compared to that of Ac-PLP-BPI-NH₂-2 and PBS in the mouse EAE model using (A) clinical disease scores, (B) change in body weight, and (C) incidence of disease. The mice received subcutaneous injections of either 10 nmol/injection/day or 26 nmol/injection/day of IDAC-3 on days -11, -8, and -5 prior to immunization on day 0 (vaccine-like administration). A group of mice also received s.c. injections of 26

nmol/injection/day of IDAC-3 on days 4 and 7. The Ac-PLP-BPI-NH₂-2-treatment group received s.c. injections of 100 nmol/injection/day of the peptide on days -11, -8, and -5. The negative control mice were treated with s.c. injections of PBS on days -11, -8, and -5.

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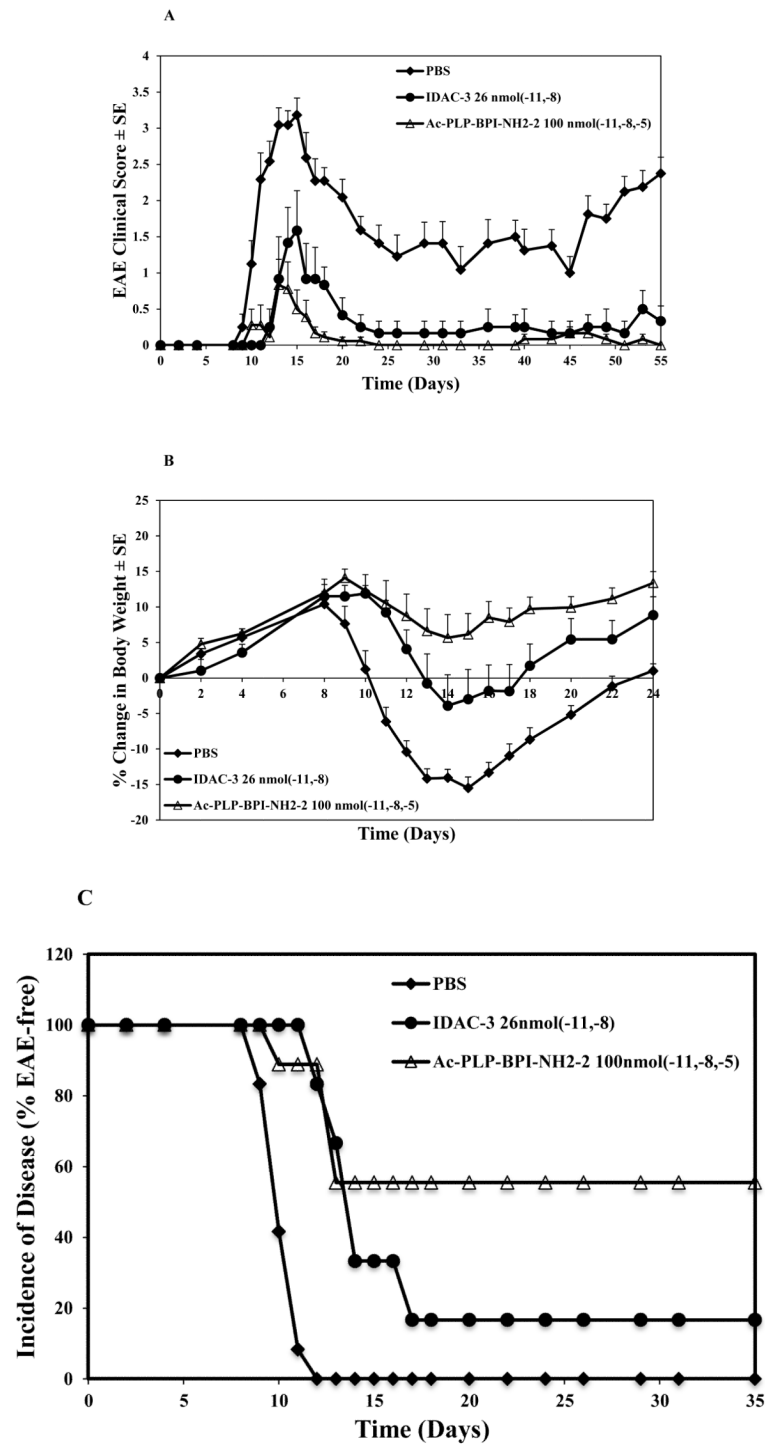


Figure 5. Long-term efficacy evaluation of IDAC-3 and Ac-PLP-BPI-NH₂-2 compared to PBS in the mouse EAE model. The efficacy was evaluated by (A) clinical disease scores, (B) change in body weight, and (C) incidence of disease. After immunization with PLP peptide in CFA, the mice received vaccine-like s.c. injections of 26 nmol/injection/day of IDAC-3 on days -11 and -8. The Ac-PLP-BPI-NH₂-2 treatment group received s.c. injections of 100 nmol/

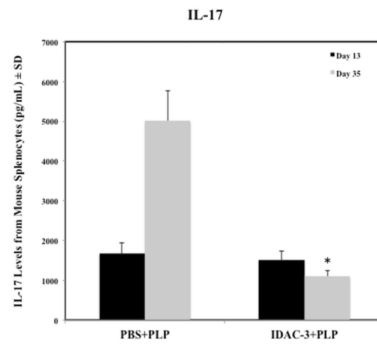
injection/day of the peptide on days -11, -8, and -5. The control mice were treated with PBS on days -11, -8, and -5. The clinical scores were monitored up to day 55 to observe the disease relapse. The results are expressed as the mean \pm S.E. (n = 6).

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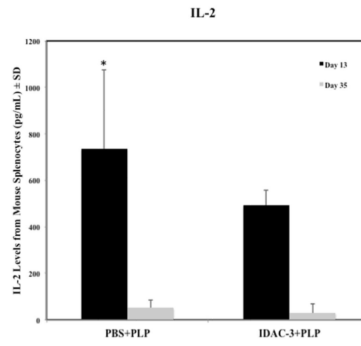
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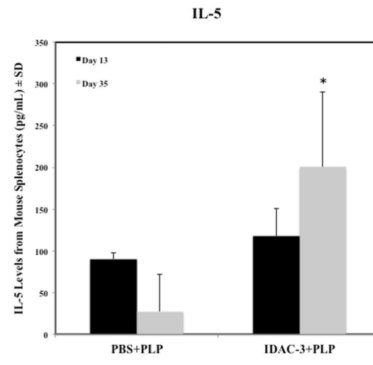
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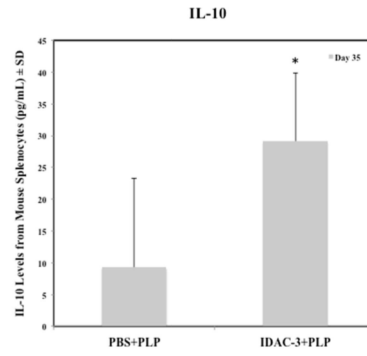
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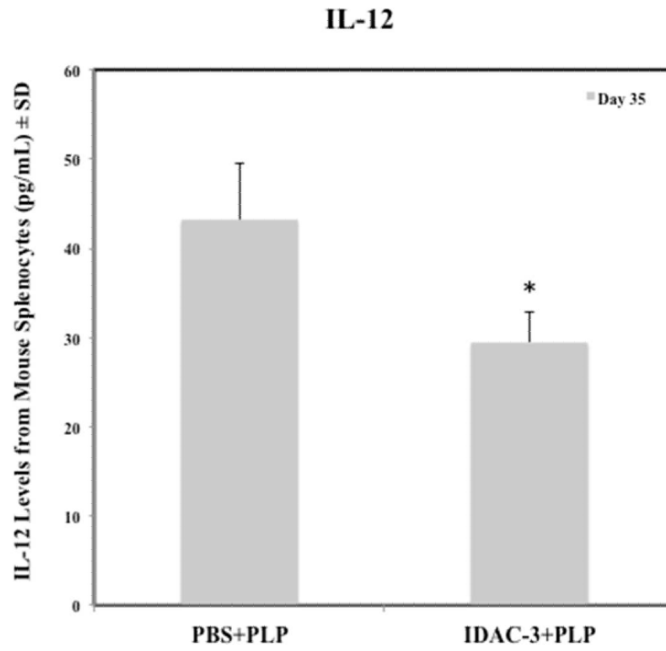


Figure 6.

The effect of IDAC-3 in altering the cytokine production was compared to that of PBS. Mice were treated subcutaneously with PBS and IDAC-3 (26 nmol on days 4 and 7) and levels of different cytokines were determined from the isolated splenocytes of IDAC-3- and PBS-treated animals on days 13 and 35. The evaluated cytokines include (A) IL-17, (B) IL-2, (C) IL-5, (D) IL-10, and (E) IL-12.

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Table 1
The Sequences of Peptides and Proteins Used in the Present Study

Peptide/Protein	Sequence
Ac-PLP-BPI-NH ₂ -2	Ac-HSLGKWLGHDPKF-(AcpGAcpGAcp) ₂ -ITDGEATDSG-NH ₂
Ac-PLP-cIBR1-NH ₂	Ac-HSLGKWLGHDPKF-(AcpGAcpGAcp) ₂ -Cyclo(1,12)-PenPRGGSVLVTGC-NH ₂
IDAC-1	(HSLGKWLGHDPKFC) _n -linker-I-domain
IDAC-3	(Ac-HSLGKWLGHDPKFC-NH ₂) _n -linker-I-domain
GMB-I-domain	[N-(γ -maleimido)-1-oxybutyl] _n -I-domain
I-domain	MGNVDLVFLFDGSMSLQPDFQKILDFMKDVMKK LSNTSYQFAAVQFSTSYKTEFDFSDYVKRKDPDALL KHKHMLLLTNTFGAINYVATEVFREELGARPAT KVLIIITDGEATDSGNIDAAKDIIRYIIGIGKHFQTKES QETLHKFASKPASEFVKILDTEKLDLFTTELQKKIY

Ac = Acetyl and Acp = Aminocaproic acid