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Co-Delivery of Autoantigen and B7 Pathway Modulators Suppresses Experimental Autoimmune Encephalomyelitis

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Abstract. Autoimmune diseases such as multiple sclerosis (MS) are characterized by the breakdown of immune tolerance to autoantigens. Targeting surface receptors on immune cells offers a unique strategy for reprogramming immune responses in autoimmune diseases. The B7 signaling pathway was targeted using adaptations of soluble antigen array (SAgA) technology achieved by covalently linking B7-binding peptides and disease causing autoantigen (proteolipid peptide (PLP)) to hyaluronic acid (HA). We hypothesized that co-delivery of a B7-binding peptide and autoantigen would suppress experimental autoimmune encephalomyelitis (EAE), a murine model of MS. Three independent B7-targeted SAgAs were created containing peptides to either inhibit or potentially stimulate the B7 signaling pathway. Surprisingly, all SAgAs were found to suppress EAE disease symptoms. Altered cytokine expression was observed in primary splenocytes isolated from SAgA-treated mice, indicating that SAgAs with different B7-binding peptides may suppress EAE through different immunological mechanisms. This antigen-specific immunotherapy using SAgAs can successfully suppress EAE through co-delivery of autoantigen and peptides targeting with the B7 signaling pathway.

KEY WORDS: antigen-specific immunotherapy; B7/CD28:CTLA-4 co-stimulatory pathway; experimental autoimmune encephalomyelitis (EAE); proteolipid peptide; soluble antigen array.

INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease characterized by an inflammatory reaction against proteins in the central nervous system (CNS) leading to nervous system

dysfunction and paralysis (1–3). Several FDA-approved therapies exist to treat patients with MS; however, these therapies do not cure the disease but rather are designed to slow disease progression and manage symptoms (4–6). Additionally, several of these therapies are associated with severe adverse events, compelling a need for safer and more efficacious therapies to treat patients with MS and other autoimmune disorders (4,6,7).

Autoimmune diseases, such as MS, result from the breakdown of mechanisms controlling immune tolerance and the subsequent failure of the host immune system to distinguish self from non-self antigens (8,9). In MS, autoreactive T cells are thought to escape endogenous immune tolerance mechanisms, inflicting subsequent damage to the myelin sheath and leading to neurodegeneration (1,8,10). Classical immunology dictates that two signals are required for T cell activation by professional antigen-presenting cells (pAPCs), an antigen-specific signal (signal 1) and a “context” signal (signal 2, co-stimulatory/inhibitory) that ultimately dictates the resulting immune response (8,11,12). Co-delivery of both antigen and a co-stimulatory context signal activates an antigen-specific adaptive immune response, whereas delivery of a co-inhibitory context signal or

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ABBREVIATIONS: HA, Hyaluronic acid; SAgA, Soluble antigen array; PLP, Proteolipid peptide; B7AP, B7 antisense peptide; CD80-CAP, CD80 competitive antagonist peptide; SAgA_{PLP-LABL}, Soluble antigen array co-grafted with PLP and LABL peptides; SAgA_{PLP-B7AP}, Soluble antigen array co-grafted with PLP and B7AP peptides; SAgA_{PLP-CD80-CAP}, Soluble antigen array co-grafted with PLP and CD80-CAP peptides; SAgA_{PLP-sF2}, Soluble antigen array co-grafted with PLP and sF2 peptides.

absence of either signal can render an anergic response (*i.e.*, no response) and is believed to be a main mechanism of peripheral immune tolerance (8,9,13). Thus, co-delivery of a synthetic co-inhibitory context signal and autoantigen may be a suitable pharmacological template to restore immune tolerance and treat various autoimmune disorders (13–16).

To mount appropriate immune responses, the immune system uses several cell surface signaling proteins such as those in the B7 (CD80/CD86) pathway, CD40, PD-1 (CD279), ICOS (CD278), and others (9,11,12). The B7 signaling pathway is one of the most well-characterized co-stimulatory pathways in T cell activation (9,11,12). B7, found on pAPCs, has the ability to either activate T cells through binding with CD28 (co-stimulatory function) or to inhibit T cell activation and promote tolerance upon binding CTLA-4 (co-inhibitory function) (9,11,12). The possibility to modulate the B7 signaling pathway has become an alluring avenue for the treatment of autoimmune disease, including MS. Over the past decade, two recombinant fusion proteins targeting this co-stimulatory pathway through the use of portions of the CTLA-4 molecule fused to IgG, Abatacept (Orencia, Bristol-Myers-Squibb) and Belatacept (Nulojix, Bristol-Myers-Squibb), have been approved for the treatment of rheumatoid arthritis (RA) and kidney transplant rejection, respectively (17).

Unlike antibody-derived therapeutics that can be immunogenic, small peptide therapies are less likely to be immunogenic and offer an alternative method of inhibiting activation of self-reactive T cells and pAPCs (14). Methods employing peptides targeting different aspects of the B7 signaling pathway have been tested in animal models of RA, MS, and allograft rejection with positive results (18–21). These peptide therapies are thought to work by blocking delivery of the co-stimulatory signal from the pAPC, resulting in T cell anergy and promotion of tolerance to treat autoimmunity (14). Therapies only targeting the B7 pathway share the same drawback as most current therapies for autoimmune disease as they are prone to non-specific suppression of the immune system rendering the patient more susceptible to opportunistic infections (13,14). The creation of an antigen-specific treatment capable of suppressing the immune response by co-delivery of autoantigen and immune inhibitor may improve the safety and efficacy of autoimmune therapies. Several previous studies have shown that co-delivering autoantigen and immune inhibitory peptide can inhibit autoimmune disease in animal models (22–28).

In this study, we tested whether co-delivering a B7-binding peptide and disease-specific autoantigen simultaneously would inhibit the autoimmune disease experimental autoimmune encephalomyelitis (EAE), a murine model of relapsing-remitting MS (29). We grafted to hyaluronic acid both a myelin sheath antigenic peptide (PLP_{139–151}) and peptides capable of binding B7 signaling pathway surface receptors by following previously reported procedures (15,24,25). The resulting soluble antigen arrays (SAGAs) act as a carrier to co-deliver multiple copies of the autoantigen and a peptide from the B7 pathway. We investigated the clinical outcomes in EAE mice treated with these novel SAGA molecules and further characterized the cytokine

expression in primary splenocytes isolated from SAGA-treated EAE mice.

MATERIALS AND METHODS

Materials. All peptides (PLP_{139–151}, LABL, B7AP, CD80-CAP1, sF2 [cyclized]; Table 1) were obtained from PolyPeptide Laboratories (Torrance, CA) and were synthesized with N-terminal aminooxy functional groups. Hyaluronic acid (HA), with an average molecular weight of 16.0 kDa, was purchased from Lifecore Biomedical (Chaska, MN). Analytical-grade acetonitrile and synthesis-grade trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Waltham, MA). Research-grade sodium acetate and acetic acid were purchased from Sigma (St. Louis, MO). Complete Freund's adjuvant (CFA) and killed *Mycobacterium tuberculosis* strain H37RA were purchased from Difco (Sparks, MD). Water was provided by a Labconco (Kansas City, KS) Water PRO PS ultrapure water purification unit.

Conjugation of Aminooxy Peptides to HA Polymer. Single-step grafting of aminooxy peptides to 16 kDa HA was performed as described previously (24). Briefly, HA was dissolved in 20 mM acetate-buffered solution (pH 5.5±0.1) and each aminooxy reactive peptide was added simultaneously. After addition of the peptides, the reaction solution was adjusted to pH 5.5±0.1 and stirred at 500 rpm using a magnetic stir bar for 16 h at room temperature. The samples were then transferred to dialysis bags (MWCO 3,500 Da, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and dialyzed against 2 L of deionized water for 24 h, with dialysis water exchanged every 6 h to remove unreacted peptides and residual buffer. After dialysis, the dialysate was frozen at –70°C and lyophilized.

Characterization of SAGAs by High-Performance Liquid Chromatography. The amount of PLP and context signal-binding peptides conjugated to HA for each of the SAGAs was determined using digestion in acidic medium (4 h at pH 2.0) and then subsequently analyzed by HPLC as previously reported (24). For analysis of SAGA_{PLP:sF2}, following peptide cleavage, the solution was centrifuged for 5 min at 13,000 rpm and the aggregates were dissolved in DMSO before running both the pellet and supernatant on HPLC. The HPLC consisted of SCL-20A Shimadzu system controller, LC-10AT VP Shimadzu liquid chromatograph, SIL-10A XL Shimadzu auto-injector set at 50-μL injection volumes, DGU-14A Shimadzu degasser, sample cooler, and SPD-10A Shimadzu UV-vis detector (220 nm). The HPLC-UV system was controlled by a personal computer equipped with Shimadzu class VP Software. All separations were carried out using a Vydac® 179 HPLC Protein and Peptide C18 column. Gradient elution was carried out to determine the amount of PLP and context signal peptide at constant flow of 1 mL/min, from 100% mobile

Table I. B7-Targeted Peptide Sequences

Peptide ^a	Molecular mimic	Peptide sequence ^b	Proposed interactions blocked	References
B7AP	CD28	Ao-GGGEFMYPPPYLD	B7/CD28	18, 30
CD80-CAP	CD28	Ao-GGGMQPPGC	B7/CD28 and B7/CTLA-4	19, 20
sF2	CTLA-4	Ao-TEAGAAGCRGVGVAFIGSCVFG [cyclized]	B7/CTLA-4	31

^a Each peptide was created to mimic a signaling molecule in the B7 pathway and has been previously shown to block B7 pathway signaling interactions

^b Ao indicates the N-terminal aminooxy addition for covalent grafting onto hyaluronic acid

phase A to 35% A (corresponding to 0% B to 65% B) over 50 min, followed by an isocratic elution at 75% B for 3 min. Mobile phase compositions were (A) acetonitrile-water (5:95) with 0.1% TFA and (B) 100% acetonitrile with 0.1% trifluoroacetic acid (TFA).

Characterization of SAgAs by Gel Permeation Chromatography. The molecular weight of each SAgA was determined using a Shimadzu HPLC system with a refractive index detector previously described (15). Samples were separated by utilizing a tandem column setup of an Agilent PL aquagel-OH 60 and Agilent PL aquagel-OH 40 column in series. Columns were heated to 45°C using an Eldex CH-150 heating box. All pullulan standards and SAgAs were separated in a mobile phase of 0.1 M ammonium acetate with 0.25 M NaCl, pH 5.0, at a flow rate of 0.6 mL/min for 45 min.

Characterization of SAgAs by MFI. To detect particulates formed from SAgA aggregation, microflow imaging (MFI) with a DPA-4200 flow microscope (Protein Simple, Santa Clara, CA) was used to capture digital images of subvisible particles with equivalent circular diameters from 1.5 to 100 μ m. Each SAgA and HA control were dissolved in 0.1 mg/mL PBS, and approximately 0.6 mL of each sample was analyzed. Particle statistics were generated using MFI View Analysis Suite (MVAS) version 1.3 (Protein Simple, Santa Clara, CA).

Induction of EAE and Therapeutic Study. SJL/J (H-2s) female mice, 4–6 weeks old, were purchased from Harlan Laboratory and housed under specified, pathogen-free conditions at the University of Kansas. All protocols involving live mice were approved by the University of Kansas Institutional Animal Care and Use Committee. The PLP-induced model of EAE was used to mimic relapsing-remitting multiple sclerosis (29). Mice were immunized subcutaneously with 200 μ g of PLP_{139–151} in a 0.2 mL emulsion composed of equal volumes of phosphate-buffered saline (PBS) and CFA containing killed *Mycobacterium tuberculosis* strain H37RA (final concentration of 4 mg/mL; Difco). The PLP_{139–151}/CFA was administered to regions above the shoulders and the flanks (total of four sites; 50 μ L at each injection site). In addition, 100 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was injected (100 μ L) on two occasions,

intraperitoneally on the day of immunization (day 0) and 2 days post-immunization. To inhibit disease, mice received subcutaneous injections (100 μ L) at the nape of the neck of each SAgA sample or vehicle (sterile PBS), on days 4, 7, and 10. SAgA dose was based on delivering 200 nmol PLP peptide as calculated by HPLC. Treatments of HA were administered at a dose equivalent of the SAgAs, 29 nmol. Disease progression was evaluated using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund. Mice were euthanized if they were found to have a clinical score of 4 or above. Body weight was also measured daily and is expressed as a percent weight loss calculated from day 8 (peak weight prior to EAE disease). The total duration of the EAE study was 26 days, from day 0 to day 25.

Splenocyte Isolation and Ex Vivo Treatment of Splenocytes. Mouse spleens were resected 25 days post-immunization, passed through a wire mesh using the rubber end of a sterile 1 mL syringe plunger, and collected in 5 mL of RPMI 1640 media. The crude cellular extract was then centrifuged at 1,100 $\times$ g for 5 min, and the resulting cell pellet was resuspended in 3.5 mL of 1 $\times$ Gey's lysis solution and place on ice for 3.5 min to lyse splenic red blood cells. The lysis reaction was stopped by the addition of 10.5 mL of RPMI 1640 media containing 10% FBS and centrifuged at 1,100 $\times$ g for 5 min. The remaining cell pellet was resuspended in fresh media (RPMI 1640 media containing 2.05 mM L-glutamine, 10% FBS, and 1% penicillin-streptomycin) and seeded in 96-well cell culture plates at a cell density of 1 $\times$ 10⁶ cells/well in a final volume of 100 μ L. Splenocytes were then immediately stimulated with 25 μ M PLP or vehicle (RPMI media). Stimulated cell cultures were incubated for 120 h at 37°C in a CO₂ (5%) incubator. Cell culture supernatant cytokine levels were determined by a commercially available bead array ELISA kit and supporting FlowCytomix™ software (eBiosciences, San Diego, CA).

Statistical Analysis. Statistical evaluation of data was performed using a one-way or two-way analysis of variance (ANOVA) as experimentally appropriate,

followed by Fisher’s least significant difference post hoc test. The criteria for statistical significance was set at $p<0.05$. All analyses were performed using GraphPad Software (GraphPad Software Inc.).

RESULTS

Synthesis and Characterization of Soluble Antigen Arrays

Peptides selected for use in this study were previously shown to interact with and modulate the B7 signaling pathway (Table I) (18–20,30,31). SAgAs targeting the B7 pathway were created by co-grafting to hyaluronic acid polymers (HA) both autoantigen (PLP_{139–151}) and B7-binding peptides (B7AP, CD80-CAP, or sF2) as previously reported (15,24,25). For a positive control, an ICAM-1-targeted SAgA (SAgA_{PLP:LABL}) was created containing autoantigen (PLP_{139–151}) and the cell adhesion peptide LABL derived from leukocyte function associated antigen-1 (LFA-1), which has previously shown efficacy in EAE (24,25).

HPLC was used to determine the amount of peptide conjugated for each synthesized SAgA, and the number of peptides conjugated per HA molecule was determined (Table II). Further analysis by gel permeation chromatography (GPC) was used to confirm the final molecular weight of the SAgAs upon peptide conjugation (Table III) by comparison to pullulan standards (Supplemental Figure 1). Final conjugate molecular weights of approximately 37 to 47 kDa estimated using these two methods were overall in agreement; however,

the molecular weight of SAgA_{PLP:sF2} found by GPC was approximately double that originally estimated by HPLC (data not shown), suggesting possible aggregation.

MFI analysis of SAgA_{PLP:sF2} revealed the presence of particles, confirming formation of SAgA_{PLP:sF2} aggregates in aqueous solutions (Fig. 1, Supplemental Figure 2). Upon observation of the aggregation and poor solubility, SAgA_{PLP:sF2} was solubilized in DMSO following peptide cleavage from the HA backbone. Subsequent analysis of SAgA_{PLP:sF2} by HPLC confirmed a similar conjugation efficiency compared to the other SAgAs, with approximately 7 to 11 peptides per HA backbone of both autoantigen and B7-binding peptide (Tables II and III).

Suppression of EAE by SAgAs

SAgAs co-grafted with autoantigen (PLP) and B7-binding peptide were evaluated in EAE to determine their effect on disease progression. Similar to previous studies, the positive control SAgA_{PLP:LABL} suppressed EAE as indicated by clinical score and weight loss (Fig. 2) (24,25). Additionally, HA alone did not affect EAE disease scores as compared to the negative control, PBS. Interestingly, compared to PBS, all B7-targeted SAgAs were able suppress the symptoms of EAE as evaluated by clinical disease score and weight loss (Fig. 2). All SAgAs also reduced the incidence of disease compared to mice treated with PBS or HA alone (Supplemental Figure 3). No statistical differences in clinical score, weight loss, or incidence of disease were found between the SAgAs containing different context signal peptides. The data suggests that co-delivery of B7-binding peptides and autoantigen can protect against EAE.

Table II. SAgA Peptide Conjugation

Soluble Antigen Array		PLP Concentration (nMol)	Context Signal Peptide Concentration (nMol)	Number of Peptides per HA polymer (PLP: Context Signal Peptide)
SAgA _{PLP:LABL}		205	277	9:11
SAgA _{PLP:B7AP}		170	223	7:9
SAgA _{PLP:CD80-CAP}		233	220	9:8
SAgA _{PLP:sF2} ^a		212	152	10:7 ^a

Each SAgA molecule was analyzed by HPLC following acidic cleavage of peptides from the 16 kDa HA backbone. The peptide concentrations were calculated based on HPLC analysis of 1 mg of SAgA. Results shown are an average from triplicate injections of a single batch preparation. All samples had a RSD ≤ 0.01

^aIndicates that the SAgA was solubilized in DMSO following acidic peptide cleavage

Table III. SAgA Molecular Weight Calculated by Both HPLC and GPC

Soluble antigen array	MW calculated from peptide conjugations determined by HPLC (kDa)	MW calculated from GPC data (kDa) ^a
SAgA _{PLP:LABL}	42	44
SAgA _{PLP:B7AP}	42	41
SAgA _{PLP:CD80-CAP}	37	42
SAgA _{PLP:sF2} ^b	47 ^b	45

^a The molecular weight of each SAgA was calculated from GPC data as compared to a pullulan polymer standard curve (Supplemental Figure 1). Results shown are an average from triplicate injections of a single batch preparation. All samples had a RSD ≤ 0.01

^b Indicates that the SAgA was solubilized in DMSO following acidic peptide cleavage

SAgAs Containing B7AP Peptide Reduced Pro-Inflammatory Cytokine Production

To analyze the ability of immune cells in the periphery to respond to re-challenge with PLP, the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin (IL)-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17 were measured from splenocytes taken from animals treated with B7-targeted SAgAs or controls (Fig. 3). Splenocytes were harvested on day 25 and were cultured directly in the presence of PLP (25 μ M) to re-stimulate the immune cells. GM-CSF and IL-2, but not IFN- γ , TNF- α , IL-1 α , IL-4, IL-5, IL-6, IL-10, or IL-17, increased in PLP-stimulated splenocytes isolated from PBS-treated EAE mice (Fig. 3). Levels of IL-10 were below the limit of detection for all treatment groups using this assay (data not shown). Interestingly, the PLP-

dependent induction of the pro-inflammatory cytokines IL-2 and GM-CSF was reduced in EAE splenocytes from mice treated with SAgA_{PLP:B7AP} as compared to mice given PBS (Fig. 3a, e). Decreased levels of GM-CSF were also found in PLP-stimulated EAE splenocytes from mice treated with SAgA_{PLP:sF2} or with HA as compared to the mice treated with PBS (Fig. 3a). In EAE mice treated with SAgA_{PLP:sF2}, basal levels of IL-6 were not detectable (Fig. 3h). The data suggested each independent SAgA differentially modulated the cytokine response in splenocytes isolated from EAE mice.

DISCUSSION

The B7 signaling pathway has emerged as an important target for immune modulation, with several experimental biologic therapeutics entering clinical trials (17). For autoimmune diseases, the ability to suppress unwanted immunity by blocking co-stimulation during antigen presentation has the potential to regulate T cell activation and inhibit subsequent T cell-dependent tissue damage (13,17). Addition of antigen specificity to such an approach may reduce undesired side effects associated with global immunosuppression that accompanies many of the current immunomodulatory therapies available (4–7). Indeed, several groups have begun investigating antigen-specific immunotherapies to treat autoimmune disorders (22–28,32–36).

Previously, a bifunctional peptide inhibitor (BPI) demonstrated the importance of co-delivering both antigenic peptide and a peptide inhibiting T cell activation by blocking immune cell adhesion (22,23,26–28). Applying this co-delivery approach of autoantigen and peptide inhibitor to a multivalent delivery vehicle (SAgA_{PLP:LABL}), *i.e.*, multiple copies of peptide per therapeutic molecule, has also suppressed EAE (24,25). In this study, multivalent SAgA technology was used as a foundation to synthesize new SAgAs (SAgA_{PLP:B7AP}, SAgA_{PLP:CD80-CAP}, SAgA_{PLP:sF2}) that target the B7 signaling pathway and co-deliver PLP autoantigen for the treatment of EAE.

The B7 pathway can deliver either a co-stimulatory or co-inhibitory signal during antigen presentation depending on the combinations of surface receptors engaged (12). In order to investigate the application of SAgAs targeting the B7 pathway, we selected three peptides (B7AP, CD80-CAP, sF2), which had been previously reported to bind B7 (Table I). Two peptides used in this study, B7AP and CD80-CAP, have been shown to inhibit inflammatory immune response in several rodent autoimmune models (18–20,30,37). In contrast, the peptide mimic of CTLA-4 (sF2) was originally selected for this study as a positive control to enhance the immune response by blocking the B7:CTLA-4 regulatory interactions (31). The reported mechanisms of these peptides led us to hypothesize that SAgA_{PLP:B7AP} and SAgA_{PLP:CD80-CAP} would suppress EAE disease severity, while SAgA_{PLP:sF2} would increase disease severity in EAE. Surprisingly, all B7-targeted SAgAs, including SAgA_{PLP:sF2}, inhibited disease. Other reports, however, help explain this result. CTLA-4-IgG and soluble CTLA-4 have been shown to have inhibitory functions in several *in vivo* models, suggesting that

Fig. 1. ICAM-1 and B7-targeted SAgAs were analyzed for subvisible particulate formation at a concentration of 0.1 mg/mL in PBS using microflow imaging (MFI). Concentrations of particles of equivalent circular diameters from 1.5 to 10.5 μ m are shown for SAgA_{PLP:sF2}, SAgA_{PLP:LABL}, SAgA_{PLP:B7AP}, SAgA_{PLP:CD80-CAP} and HA alone. Data presented as mean $\pm$ SD ($n=3$ independent samples)

Fig. 2. ICAM-1 and B7-targeted SAgAs were found to decrease both clinical score and maintain weight in EAE mice. EAE was induced on day 0, and mice were treated on days 4, 7, and 10 with a dose of SAgA equivalent to 200 nmol PLP. Treatments of HA were administered at a dose equivalent of the SAgAs, 29 nmol. Clinical disease score and percent weight change were analyzed for groups treated with **a, b** SAgA_{PLP:B7AP}, **c, d** SAgA_{PLP:CD80-CAP}, and **e, f** SAgA_{PLP:sF2}. All SAgAs significantly suppressed clinical disease score as compared to PBS on days 11 to 15 and as compared to HA on days 11 to 14. Also, all SAgAs suppressed weight loss as compared to PBS on days 11 to 18 and as compared to HA on days 12 to 16. There were no statistical differences in disease suppression as indicated by both clinical score and weight loss between SAgAs containing different B7-binding peptides. Additionally, there were no statistical differences between PBS and HA control groups. Data are presented as mean $\pm$ SD ($n=6$ mice per group, $p<0.05$)

delivery of a multivalent CTLA-4 mimetic peptide (*i.e.*, SAgA_{PLP:sF2}) may actually suppress pro-inflammatory immune responses (38,39). Additionally, current immunological models suggest that “overstimulation” of T cells may induce anergy and be a mechanism of both peripheral and central tolerance (40). Delivering multivalent SAgAs that achieve simultaneous high local concentrations of antigen and context signal (*i.e.*, multiple copies of each peptide per molecule) may overwhelm sensitive immune cells and subsequently induce anergy.

In addition, all B7-targeted SAgAs were shown to have similar clinical efficacy as SAgA_{PLP:LABEL}, which has been shown to inhibit the symptoms of EAE in previous studies (24,25). Although the SAgAs may be equivalent in the suppression of EAE symptoms, the B7-targeted SAgAs offer an advantage over their ICAM-1-targeted counterparts. Unlike the molecular signals of the B7 pathway, which are only found on either pAPCs or T

cells, ICAM-1 is found on immune cells, endothelial cells, and even in soluble form in the blood (8). Additionally, previous studies have demonstrated that the conjugation of only PLP to HA does not result in EAE suppression (25). The importance of the context signal peptide for the suppression of EAE suggests that optimization of this peptide may enhance SAgA efficacy. While SAgA_{PLP:LABEL} has not demonstrated any off-target effects in previous studies, the specificity of the B7-targeting SAgAs may result in more effective delivery of the SAgA to their immune cell targets.

Although all SAgAs reduced clinical disease similarly, several differences between each of the unique SAgA molecules were noted in splenocytes that were re-challenged with PLP. Interestingly, only splenocytes isolated from mice treated with SAgA_{PLP:B7AP} had reduced levels of PLP-dependent IL-2 expression compared to PBS, a cytokine that has been shown to contribute to

Fig. 3. The cytokines GM-CSF, IFN- γ , TNF- α , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17 were measured from splenocytes taken on day 25 from animals treated with B7-targeted SAgAs or controls. The splenocytes were re-stimulated with 25 μ M PLP or vehicle (media) *in vitro* for 120 h. Supernatant levels of the cytokines **a** GM-CSF, **b** IFN- γ , **c** TNF- α , **d** IL-1 α , **e** IL-2, **f** IL-4, **g** IL-6, **h** IL-6, and **i** IL-17 were determined. Cytokine expression of IL-2 and GM-CSF was significantly reduced in splenocytes isolated from EAE mice treated with B7-targeted SAgAs. Data are presented as mean $\pm$ SD of independent splenocyte populations isolated from three mice; gray bars indicate stimulated with 25 μ M PLP; black bars indicate vehicle (media) control; asterisk indicates $p < 0.05$ as compared to PBS-treated *in vivo* and stimulated *in vitro* with 25 μ M PLP; and number sign indicates $p < 0.05$ as compared to the same *in vivo* treatment with 25 μ M PLP stimulation *in vitro*.

increased disease severity in EAE (1,41). Additionally, SAgA_{PLP:B7AP}, SAgA_{PLP:sF2}, and HA treatments inhibited PLP-dependent GM-CSF cytokine expression, a cytokine suggested to play a role in EAE disease progression

(8,42). HA alone did not suppress disease symptoms compared to PBS, indicating that reduction of GM-CSF cytokine levels may not be an accurate indicator of *in vivo* efficacy.

Fig. 3. (continued)

Furthermore, SAgA_{PLP:sF2} was the only SAgA shown to form aggregates in a physiological buffer. Particulate adjuvants used in traditional prophylactic vaccines elicit their immune effect in part through recruitment of pAPCs to the site of injection and by stimulating a local pro-inflammatory immune response at the site of antigen recognition (43,44). In contrast, clinical treatment of allergies induces tolerogenic immune responses through a series of injections of soluble antigens in the absence of adjuvant (*i.e.*, particles), which can bypass the local pro-inflammatory response (16). Indeed, SAgA_{PLP:sF2} aggregates may form a depot at the site of injection, which may cause local inflammation and recruitment of cells. The suppression of EAE by SAgA_{PLP:sF2} may suggest that formulation of non-soluble (colloidal) treatments for co-delivery of antigen and context signal may also be a viable strategy for development of tolerogenic autoimmune therapies. When taken together, the cytokine and particulate data suggest that the different B7-targeted SAgAs protect against EAE disease progression by slightly different immunological mechanisms.

CONCLUSION

The B7 signaling pathway is a promising target for the treatment of EAE using SAgA technology. Both B7-targeted

and ICAM-1-targeted SAgAs were found to suppress clinical disease symptoms as compared to the negative controls. Complexity in cytokine suppression indicates that although all SAgAs resulted in similar *in vivo* clinical efficacy, they may act through different immunological mechanisms at the cellular level. These results suggest that targeting other surface receptors on immune cells may be a beneficial therapeutic option for the treatment of autoimmune disorders. Furthermore, SAgA technology provides an easily adaptable platform to test a diverse library of multivalent peptide therapies targeting immune cell surface receptors and antigenic epitopes. Expansion and customization of SAgA-based therapeutics may enable extrapolation of this antigen-specific immunotherapy to other autoimmune diseases.

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REFERENCES

- Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol*. 2010;162(1):1–11.
- Ransohoff RM, Brown MA. Innate immunity in the central nervous system. *J Clin Invest*. 2012;122(4):1164–71.
- Amor S, Puentes F, Baker D, Van Der Valk P. Inflammation in neurodegenerative diseases. *Immunology*. 2010;129(2):154–69.
- Feldmann M, Steinman L. Design of effective immunotherapy for human autoimmunity. *Nature*. 2005;435(7042):612–9.
- Bates D. Treatment effects of immunomodulatory therapies at different stages of multiple sclerosis in short-term trials. *Neurology*. 2011;76:S14–25.
- Miller SD, Turley DM, Podojil JR. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol*. 2007;7(9):665–77.
- Langer-Gould A, Steinman L. Progressive multifocal leukoencephalopathy and multiple sclerosis: lessons from natalizumab. *Curr Neurol Neurosci Rep*. 2006;6(3):253–8.
- Kindt TJ, Goldsby RA, Osborne BA, Kuby J. *Kuby immunology*. 6th ed. New York: W.H. Freeman and Company; 2007.
- Gonsette RE. Self-tolerance in multiple sclerosis. *Acta Neurol Belg*. 2012;112(2):133–40.
- Barnett MH, Sutton I. The pathology of multiple sclerosis: a paradigm shift. *Curr Opin Neurol*. 2006;19(3):242–7.
- Bromley SK, Iaboni A, Davis SJ, Whitty A, Green JM, Shaw AS, *et al.* The immunological synapse and CD28-CD80 interactions. *Nat Immunol*. 2001;2(12):1159–66.
- Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002;2(2):116–26.
- Chittasupho C, Siahaan TJ, Vines CM, Berkland C. Auto-immune therapies targeting costimulation and emerging trends in multivalent therapeutics. *Ther Deliv*. 2011;2(7):873–89.
- Badawi AH, Siahaan TJ. Immune modulating peptides for the treatment and suppression of multiple sclerosis. *Clin Immunol*. 2012;144(2):127–38.
- Chittasupho C, Sestak J, Shannon L, Siahaan TJ, Vines CM, Berkland C. Hyaluronic acid graft polymers displaying peptide antigen modulate dendritic cell response in vitro. *Mol Pharm*. 2013;11(1):367–73. doi: 10.1021/mp4003909.
- Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol*. 2010;22(5):609–15.
- Yao S, Zhu Y, Chen L. Advances in targeting cell surface signalling molecules for immune modulation. *Nat Rev Drug Discov*. 2013;12(2):130–46.
- Chen J, He Q, Zhang R, Chu Y, Wang Y, Liu Q, *et al.* Allogenic donor splenocytes pretreated with antisense peptide against B7 prolong cardiac allograft survival. *Clin Exp Immunol*. 2004;138(2):245–50.
- Srinivasan M, Lu D, Eri R, Brand DD, Haque A, Blum JS. CD80 binding polyproline helical peptide inhibits T cell activation. *J Biol Chem*. 2005;280(11):10149–55.
- Dudhgaonkar SP, Janardhanam SB, Kodumudi KN, Srinivasan M. CD80 blockade enhance glucocorticoid-induced leucine zipper expression and suppress experimental autoimmune encephalomyelitis. *J Immunol*. 2009;183(11):7505–13.
- Srinivasan M, Gienapp IE, Stuckman SS, Rogers CJ, Jewell SD, Kaumaya PT, *et al.* Suppression of experimental autoimmune encephalomyelitis using peptide mimics of CD28. *J Immunol*. 2002;169(4):2180–8.
- Kobayashi N, Kobayashi H, Gu L, Malefyt T, Siahaan TJ. Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor. *J Pharmacol Exp Ther*. 2007;322(2):879–86.
- Murray JS, Oney S, Page JE, Kratochvil-Stava A, Hu Y, Makagiansar IT, *et al.* Suppression of type 1 diabetes in NOD mice by bifunctional peptide inhibitor: modulation of the immunological synapse formation. *Chem Biol Drug Des*. 2007;70(3):227–36.
- Sestak J, Mullins M, Northrup L, Thati S, Forrest ML, Siahaan TJ, *et al.* Single-step grafting of aminoxy-peptides to hyaluronan: a simple approach to multifunctional therapeutics for experimental autoimmune encephalomyelitis. *J Control Release*. 2013;168(3):334–40.
- Sestak JO, Sullivan BP, Thati S, Northrup L, Hartwell B, Antunez L, *et al.* Codelivery of antigen and an immune cell adhesion inhibitor is necessary for efficacy of soluble antigen arrays in experimental autoimmune encephalomyelitis. *Mol Ther Methods Clin Dev*. 2014. doi:10.1038/mtm.2014.8.
- Manikwar P, Kiptoo P, Badawi AH, Büyüktimkin B, Siahaan TJ. Antigen-specific blocking of CD4-Specific immunological synapse formation using BPI and current therapies for autoimmune diseases. *Med Res Rev*. 2012;32(4):727–64.
- Badawi AH, Kiptoo P, Wang W-T, Choi I-Y, Lee P, Vines CM, *et al.* Suppression of EAE and prevention of blood-brain barrier breakdown after vaccination with novel bifunctional peptide inhibitor. *Neuropharmacology*. 2012;62(4):1874–81.
- Ridwan R, Kiptoo P, Kobayashi N, Weir S, Hughes M, Williams T, *et al.* Antigen-specific suppression of experimental autoimmune encephalomyelitis by a novel bifunctional peptide inhibitor: structure optimization and pharmacokinetics. *J Pharmacol Exp Ther*. 2010;332(3):1136–45.
- Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol*. 2011;164(4):1079–106.
- Yao YF, Zhou YM, Xiang JB, Gu XD, Cai D. Inhibition of arterial allograft intimal hyperplasia using recipient dendritic cells pretreated with B7 antisense peptide. *Clin Dev Immunol*. 2012;2012:892687.
- Fukumoto T, Torigoe N, Kawabata S, Murakami M, Uede T, Nishi T, *et al.* Peptide mimics of the CTLA4-binding domain stimulate T-cell proliferation. *Nat Biotechnol*. 1998;16(3):267–70.
- Lutterotti A, Yousef S, Sputtek A, Stürner KH, Stellmann JP, Breiden P, *et al.* Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci Transl Med*. 2013;5(188):188ra75.
- Bar-Or A, Vollmer T, Antel J, Arnold DL, Bodner CA, Campagnolo D, *et al.* Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial. *Arch Neurol*. 2007;64(10):1407–15.
- Kang Y, Xu L, Wang B, Chen A, Zheng G. Cutting edge: immunosuppressant as adjuvant for tolerogenic immunization. *J Immunol*. 2008;180(8):5172–6.
- Kang Y, Zhao J, Liu Y, Chen A, Zheng G, Yu Y, *et al.* FK506 as an adjuvant of tolerogenic DNA vaccination for the prevention of experimental autoimmune encephalomyelitis. *J Gene Med*. 2009;11(11):1064–70.
- Cousens LP, Su Y, McClaine E, Li X, Terry F, Smith R, *et al.* Application of IgG-derived natural Treg epitopes (IgG Tregitopes) to antigen-specific tolerance induction in a murine model of type 1 diabetes. *J Diabetes Res*. 2013;2013:621693.
- Eri R, Kodumudi KN, Summerlin DJ, Srinivasan M. Suppression of colon inflammation by CD80 blockade: evaluation in two murine models of inflammatory bowel disease. *Inflamm Bowel Dis*. 2008;14(4):458–70.
- Deppong CM, Bricker TL, Rannals BD, Van Rooijen N, Hsieh CS, Green JM. CTLA4Ig inhibits effector T cells through regulatory T cells and TGF-beta. *J Immunol*. 2013;191(6):3082–9.
- Ward FJ, Dahal LN, Wijesekera SK, Abdul-Jawad SK, Kaewarpai T, Xu H, *et al.* The soluble isoform of CTLA-4 as a regulator of T-cell responses. *Eur J Immunol*. 2013;43(5):1274–85.

40. Yamamoto T, Hattori M, Yoshida T. Induction of T-cell activation or anergy determined by the combination of intensity and duration of T-cell receptor stimulation, and sequential induction in an individual cell. *Immunology*. 2007;121(3):383–91.
41. Petitto JM, Streit WJ, Huang Z, Butfiloski E, Schiffenbauer J. Interleukin-2 gene deletion produces a robust reduction in susceptibility to experimental autoimmune encephalomyelitis in C57BL/6 mice. *Neurosci Lett*. 2000;285(1):66–70.
42. Rodgers JM, Miller SD. Cytokine control of inflammation and repair in the pathology of multiple sclerosis. *Yale J Biol Med*. 2012;85(4):447–68.
43. Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. *Curr Opin Immunol*. 2009;21(1):23–9.
44. Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev*. 2008;60(8):915–28.