Optimizing the Treatment of Experimental Autoimmune Encephalomyelitis via Pulmonary Delivery of Soluble Antigen Arrays

By

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ABSTRACT

Soluble antigen arrays (SAgAs) were used to treat experimental autoimmune encephalomyelitis (EAE), which is a mouse model for multiple sclerosis (MS). SAgAs offer a targeted therapy for MS, which is not present in current therapies. The different routes of administration, injection volume, dosing amount, and dosing schedule were explored to find that pulmonary instillation of SAgAs at 50 µL on a 200 nMol PLP basis on days 4, 7, and 10 of the study are most efficacious. With the next study, the different components of SAgAs (hyaluronic acid, PLP, LABL, and bifunctional peptide inhibitor) were also investigated via pulmonary delivery to find that PLP and SAgAs decreased the symptoms of EAE the most. PLP, HA-PLP, and SAgAs were further explored either via subcutaneous injection or pulmonary instillation to find that HA-PLP and SAgAs decreased disease progression in mice with EAE. Cytokine panels were also used to determine if tolerance was induced in these mice via the measurements of pro- and anti-inflammatory cytokines. Lastly, lung histology was explored to find signs of inflammation. Overall, SAgAs are found to be very efficacious in treating mice via PI and the presence of PLP makes a big difference. Other methods like the presence of regulatory T cells need to be used to find additional signs of tolerance induction in future studies.

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TABLE OF CONTENTS

Chapter 1:

Outline

The overall goal of this thesis is to find the optimal way to deliver Soluble Antigen Arrays (SAgAs) to mice with experimental autoimmune encephalomyelitis (EAE) to ameliorate disease. EAE is the mouse model of multiple sclerosis, which is a neurodegenerative disease caused by demyelination of neurons. While there are many existing treatments for multiple sclerosis, they lack specificity in targeting just immune cells that cause disease. SAgAs were developed to target the immune cells that target proteolipid protein ($PLP_{139-151}$), which is one of the major epitopes on myelin sheath. The additional peptide co-delivered with PLP is LABL, which binds to the cell surface receptor ICAM-1. Evidence from our lab and from collaborators with Professor Siahaan suggest SAgAs can block immune cell interaction, but only when both PLP and LABL are co-delivered. Since this therapy has a specific antigen epitopes ($PLP_{139-151}$), the hope is to decrease immunological activity of PLP-specific cells, but not induce systemic immune suppression.

Chapter 2 addresses the background of multiple sclerosis, existing treatments, the murine model EAE, and the proposed SAgA treatment. The co-delivery of PLP and LABL and how the sites of delivery may affect the immune system is discussed, offering a comparison to traditional ASIT. The three studies that were run in this dissertation are reviewed briefly: (1) varying schedule, dose, amount, and routes of administration of SAgAs; (2) delivering the components of SAgA via the most effective route of administration and observing the disease progression via cytokine profiles; and (3) investigating the necessity of delivering PLP as an array and co-delivering LABL with PLP as an array.

In Chapter 3, the dose schedule, dose amount, dose volume, and routes of delivery of SAgA were varied to see which was the most effective in decreasing EAE disease symptoms. Historically, treatments were given on days 4, 7, and 10 of the study, but in this study, the idea of treating only on one of the days was explored. SAgAs have

also usually been dosed at 200 nMol PLP, but a 50 nMol PLP dose was also given to see if a full dose is necessary. The concentration of SAgA was increased 5-fold to see if a 20 µL injection would be comparable to the 100 µL injection that is usually given. Lastly, the routes of administration were also varied from the typical subcutaneous injections given between the mouse shoulder blades to subcutaneous injections given between the hind quarters, intramuscular injections given in the upper limbs of the mouse, intramuscular injections given in the lower limbs of the mouse, intraperitoneal injections, intravenous tail vein injections, and pulmonary instillation.

Chapter 4 focused on delivering the components of SAgA via the optimized treatment regimen, including the best route of delivery. SAgAs are made up of hyaluronic acid, LABL, and PLP, so these were the three main controls given to the mice. Additionally, there is a small molecule version of the SAgA, called a bifunctional peptide inhibitor (BPI), that was also given as treatment to the mice. BPI contains PLP and LABL, connected via a short linker. Instead of being presented on an array, like the SAgAs, BPI is a smaller molecule version for comparison. Also, since SAgA was developed from studies on BPI, it was used as a control to see if SAgAs still ameliorate disease as well as BPI when given via a different route of delivery. A cytokine panel was used to see how the splenocytes of the treated reacted to PLP.

The results discussed in chapter 4 gave the impetus for the study design in chapter 5. While SAgA decreased EAE scores the most, PLP alone was also seen to be slightly effective. As a result, this study explored the necessity of co-delivering PLP and LABL. PLP alone was also presented in an array using the same hyaluronic acid backbone (HA-PLP). Lastly, PLP, HA-PLP, and SAgA were dosed via the route of delivery typically used for studies preceding chapter 3 and compared the route of delivery that this dissertation identified to be the most optimal. Thus, the study presented in chapter 5 explores the effect that PLP can have in ameliorating disease. Another

cytokine panel was used at the end of the study, where both pro-inflammatory and anti-inflammatory cytokines were presented to see what kind of effects the various treatments had on the splenocytes of the mice.

Chapter 6 offers concluding remarks of the thesis. Interpretations from each study are summarized, as well as future directions for this project. These remarks can be used to gain a further understanding of treating EAE with SAgAs.

Overall, this dissertation will provide a better understanding of EAE progression, and what components of SAgA are necessary in a treatment to ameliorate disease. The optimal route of administration will provide insight into a possible mechanism of disease. Finally, the necessity of PLP presented on an array and being co-delivered with LABL in treating mice will also be further questioned.

Chapter 2:

Introduction

Multiple Sclerosis (MS) is an autoimmune disease where the myelin sheath is broken down by immune cells. MS-patients experience varying degrees of physiological and neurological symptoms. Loss of vision is reported as one of the first manifestations, but as the disease progresses, symptoms also intensify to loss of balance, paralysis, muscle spasms, loss of brain function, etc[1]. While there are existing treatments that can decrease symptoms, the disease will still progress over time. Instead of treating the disease symptoms, another approach is to induce immune tolerance.

In order to study possible treatments, a murine model of MS named experimental autoimmune encephalomyelitis (EAE) is often utilized. EAE is induced in female SJL mice because there is a high T cell count in SJL mice[2]. There are four subtypes of MS: relapsing-remitting, secondary progressive, primary progressive, and progressive relapsing (Figure 1)[3]. In most cases, MS starts as a relapsing-remitting subtype, where there are periods of remission and then periods of relapse, but the baseline never returns to a point where no symptoms are felt. As the disease progresses, there are fewer periods of relapse as the disease symptoms increase, which is the progressive-relapsing subtype[4]. At the peak of the disease, there are no periods of relapse and the disease just continues progressing, which is the primary progressive subtype. This progression from relapsing-remitting MS to primary progressive MS is known as the secondary progressive subtype of MS. About 85% of patients with MS have relapsing-remitting MS since this subtype lasts for the longest period of time before disease progresses[5].

To reflect relapsing-remitting MS in the animal model, proteolipid protein ($PLP_{139-151}$), one of the three main epitopes on myelin sheath, is used to induce disease[6]. The use of other epitopes can induce different subtypes of MS. At the end of the month-long PLP-induced EAE study, the animals return to baseline, followed by one period of relapse. EAE induced with myelin oligodendrocyte glycoprotein (MOG) causes

symptoms similar to primary progressive MS[7]. At the end of the MOG-EAE study, mice are at the peak of disease, with no sign of improvement (Table 1).

2.1 Existing Therapies for MS

Disease modifying treatments to help alleviate symptoms of MS include: natalizumab (Tysabri®), glatiramer acetate (Copaxone®), fingolimod (Gilyena®), and interferon beta (Avonex®, Rebif®, and Cinnovex®). Natalizumab is the only monoclonal antibody currently available to treat MS. It binds to the α1β2 integrin on the blood brain barrier to inhibit monocytes from passing into the brain. As a result, there is a chance for the brain to be infected with the JC virus. The JC virus causes progressive multifocal leukoencephalopathy in an immunosuppressed patient, which is characterized by inflammation of the white matter of the brain. Patients must now be screened for the JC virus before natalizumab can be administered[8]. Another treatment, glatiramer acetate, is made up of four amino acids (EAKY) that are most commonly found in the proteins that make up myelin sheath. It was initially tested as an antigen to induce EAE, but it was later found to ameliorate the disease instead, and it is now used as a therapeutic. Initially, glatiramer acetate was thought to act as a competitive inhibitor against myelin sheath, but the latest data indicates that it shifts the Th1 response to an anti-inflammatory Th2 response[9]. Fingolimod acts very differently in comparison to natalizumab and glatiramer acetate. It sequesters lymphocytes in lymph nodes, so T cell expansion is less likely to occur and less T cells migrate into the periphery to attack the neurological tissue[10]. Interferon beta balances both pro- and anti-inflammatory cytokines, thus reducing neuronal inflammation (Table 2)[9].

All of the existing treatments have been screened through the EAE murine model. In this induced disease model, the mice are vaccinated with an emulsion containing PLP and incomplete Freund's adjuvant. EAE was initially used to portray demyelinating diseases, but has transformed into the main animal model used for

multiple sclerosis[3]. Spontaneous EAE models do exist, but they are difficult to track in animal studies when it is unclear when to start treatment and when disease actually manifests itself in the mice. Another criticism of EAE is that once these treatments are given in humans, there can be unexpected immunogenic responses. Also, since EAE is given via a vaccination and MS is spontaneous, the mechanism of the disease differs, meaning that treatments given in mice with EAE may work for that specific mechanism of disease, but not for MS.

2.2 Introducing SAgAs to Treat EAE

Existing treatments lessen the symptoms over time, but do not induce immune tolerance. A novel therapeutic has been introduced in the Berkland lab, called soluble antigen array (SAgA)[11]. SAgAs are composed of hyaluronic acid (HA), $PLP_{139-151}$, and LABL. HA is a biodegradable polymer that is already present in the extracellular matrix and in lymph. $PLP_{139-151}$ is the antigenic peptide epitope on myelin sheath used to induce disease. LABL is a peptide derived from leukocyte function-associated antigen-1 (LFA-1) and is an intercellular adhesion molecule-1 (ICAM-1) ligand. LABL is a part of the I domain of LFA-1, which is what binds with domain 1 of ICAM-1[12]. The LFA-1/ICAM-1 interaction allows T cells and dendritic cells to adhere to one another and form an immunological synapse. SAgAs were designed to co-deliver $PLP_{139-151}$ and LABL in the format of an array to block the immunological synapse between a dendritic cell that presents myelin sheath as 'non-self' and the cognate T cell to inhibit T cell expansion. This allows for a targeted approach to decrease immunological activity in the case of autoimmune diseases. SAgA technology was based on a smaller version called bifunctional peptide inhibitor (BPI), which was developed in the Siahaan lab[12]. BPI is composed of LABL and PLP, with a linker in between. Since T cell receptors are known to cluster during an immunological synapse, SAgAs were designed to block multiple

receptors with one molecule. Additionally, the larger molecule was hypothesized to have a longer half-life in the body.

When the SAgAs were designed, a concept known as 'Dintzis rules' was followed to favor a tolerogenic response (Table 3)[13]. Dintzis rules indicate that a soluble, flexible polymer that is <100 kDa with antigen spacing of 2 – 7 nm with ~5 antigens/kDa must be utilized to induce tolerance. A linear polymer with ~10 antigenic PLP epitopes was used. SAgAs contain HA, which is a soluble flexible polymer. There were also two sizes of SAgAs used in early experiments, 30 kD and 50 kD SAgA[11]. Both of the sizes of SAgA decreased clinical scores of EAE, but the 30 kD SAgA delayed the onset of the disease by a few days. In addition, there are two peptides on SAgA as previous data had shown that co-delivery of PLP and LABL decreased the symptoms of EAE. Instead of blanket immunosuppression, SAgAs may offer a more targeted treatment for EAE, and ultimately MS.

2.3 SAgA Treatment as Antigen Specific Immunotherapy to Induce Tolerance

One school of thought in developing the SAgA treatment is to follow a similar methodology used to induce tolerance to allergens. In antigen-specific immunotherapy (ASIT), patients are exposed to the allergen starting at low quantities, increasing over time[14]. The goal is to decrease IgE response to the allergen, thus, inducing tolerance to this antigen over the course of many months or years. Thus, the overall goal of SAgA treatment was to shift cytokine and cellular responses towards tolerance and away from inflammatory markers associated with PLP-induced EAE. Basically, the body is being reprogrammed to avoid an immunogenic response against PLP by using an optimized SAgA treatment regimen.

2.3.1 ASIT Routes of Administration

ASIT typically uses subcutaneous injections of allergen. Intravenous and intranodal injections in ASIT can increase anaphylaxis events, as the intravenously

injected allergens are more likely to passively diffuse systemically and the intranodal injections may be too high of a targeted dose, inducing anaphylaxis shock[14]. Intranodal delivery is attractive, because it has been shown to take a shorter period of time to induce tolerance to the allergen. Other mucosal delivery routes are also being considered, since mucosal surfaces have been hypothesized to induce a tolerogenic phenotype[15]. The most common mucosal delivery routes investigated are sublingual immunotherapy and oral immunotherapy. While the sublingual route can be applied to many different types of allergens, the oral route is thought to be best for food allergens. Patient compliance may also be higher as it is much easier to take a pill instead of having someone administer an injection[16].

2.3.2 ASIT Dosing Regimen

The amount of time allergy therapy requires is a huge factor in patient compliance as well. Subcutaneous ASIT, for example, can take up to 3 years to complete the therapy, whereas clinical studies suggest that timeline can be shortened down to 6-8 months with intranodal delivery. Intranodal delivery is, however, more invasive, so that could decrease patient compliance. Sublingual immunotherapy has reported a shorter timeline (14 months), and it is less likely to induce anaphylaxis in comparison to an injection. While a timeline for effective oral ASIT has yet to be identified, reports suggest decreased anaphylaxis compared to injections[14,16].

2.3.3 SAgA Route of Administration

The potential for ASIT in MS is still being debated, but one school of thought is that MS is a systemic disease in early stages and localizes to the lymph nodes close to the central nervous system (CNS) in later stages[17]. In order to find an effective delivery route of SAgAs, various routes of administration have been explored. In this dissertation, SAgAs were delivered subcutaneously and intramuscularly, both near the CNS and away from the CNS to determine the optimal route of administration. Additionally, SAgAs

were delivered intravenously and intraperitoneally to study systemic delivery and delivery to lymph nodes away from the CNS, respectively. Subcutaneous delivery allows for slow absorption into the body, compared to immediate systemic delivery of the therapeutic via intravenous delivery. On the other hand, intramuscular delivery may favor a depot at the injection site, potentially decreasing the chance of anaphylaxis.

If SAgAs are most potent via delivery to the lymph nodes, various mechanisms of transport should be considered (Figure 2). HA is known to travel to the lymph nodes when injected in the mouse footpad, as shown by Bagby, et al[18]. In this study, 70-kD HA seemed to have the longest residence time in the draining lymph node, which is why 80-kD SAgA was designed by our lab. Since SAgAs contain peptides as well, the behavior may differ from that of HA. Another size restriction to consider is that mentioned by Rao et al[19]. If a molecule has a hydrodynamic radius less than 10 nm, it may tend to absorb into the circulatory system. If between 10 nm to 50 nm, the molecule should travel to the lymph nodes, and when the molecules are greater than 50 nm in size, they tend to stay at the injection site. Subsequently, our lab discovered 50-kD SAgA was about 10 nm in size and 80-kD SAgA were larger. In vivo results published previously also showed that the smaller SAgA decreased EAE symptoms, thus the 50-kD SAgA was chosen in this dissertation[11].

One last route of delivery that we considered was pulmonary instillation (PI) of SAgAs in mice with EAE. PI of SAgAs was motivated by the potential for mucosal tolerization and the recent 'Hub and Spoke' model, which says that T cells are given instructions on where to go (i.e. are licensed) in the lung before they are sent out to the periphery[20,21]. Delivery of a therapeutic to the lung may allow for maximal interaction with autoreactive T cells, according to this model. Whether this route will induce tolerance is still unknown.

2.4 Conclusions

This dissertation concentrates on finding the optimal route and regimen to deliver SAgAs. Approaches for treating allergies were hypothesized to be a guide for treating autoimmune diseases. Both seek to induce tolerance to the antigen, whether it be in the form of an allergy shot or via SAgAs to treat EAE. The typical route of delivery for ASIT is a subcutaneous injection, but other routes like intranodal, sublingual, and oral are being explored. In order to find the optimal SAgA route of administration, various parenteral routes (subcutaneous, intramuscular, intravenous, and intraperitoneal) and pulmonary instillation were considered. The dose schedule, dose, and injection volume were also systematically explored. The most effective route of administration was then used to explore the mechanism of action by comparing the individual components to SAgA molecules. Lastly, the necessity of co-delivery of PLP and LABL and delivery of these peptides with HA was explored to help deduce a mechanism of action.

Table 1. Subtypes of multiple sclerosis and EAE.

Multiple Sclerosis Subtype	Antigen Used	Rodent Type	Strain	Reference
relapsing-remitting	Proteolipod Protein (PLP 139-151)	Mouse	SJL/J	6 4 22
relapsing-remitting	adoptive transfer of Myelin Basic Protein	Mouse	SJL/J	7
relapsing-remitting	Proteolipod Protein (PLP 139-151)	Mouse	PLP-specific Transgenic mice	7
primary progressive	Myelin Oligodendrocyte Glycoprotein (MOG 35-55)	Mouse	transgenic OGE	23
primary progressive	Myelin Oligodendrocyte Glycoprotein (MOG 35-55)	Mouse	C57BL/6	7 24 22
primary progressive	Myelin Oligodendrocyte Glycoprotein (MOG 35-55)	Mouse	transgenic IL-32a mice	25
primary progressive	Myelin Oligodendrocyte Glycoprotein (MOG 35-55)	Mouse	transgenic CRP mice	26
primary progressive	Myelin Oligodendrocyte Glycoprotein (MOG 35-55)	Mouse	TCR transgenic mice	27
secondary progressive	synegic spinal cord homogenate	Mouse	Biozzi ABH	28
relapsing-remitting	Guinea Pig Spinal Cord Homogenate	Rat	Lewis	29 30

Table 2. Existing treatments of multiple sclerosis.

Existing Treatment	Mechanism of Action	Expected Site of Action
Natalizumab	Binds to α1β2 integrin on the blood brain barrier to inhibit monocytes from crossing into the brain	Blood brain barrier
Glatiramer acetate	Shifts Th1 response to anti-inflammatory Th2 response	Periphery
Fingolimod	Sequesters T cells to the lymph node so they cannot leave	Lymph node
Interferon-β	Balances pro- and anti-inflammatory cytokines	Periphery

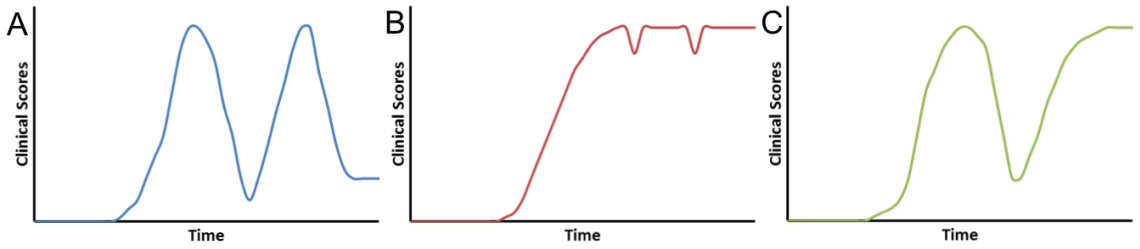
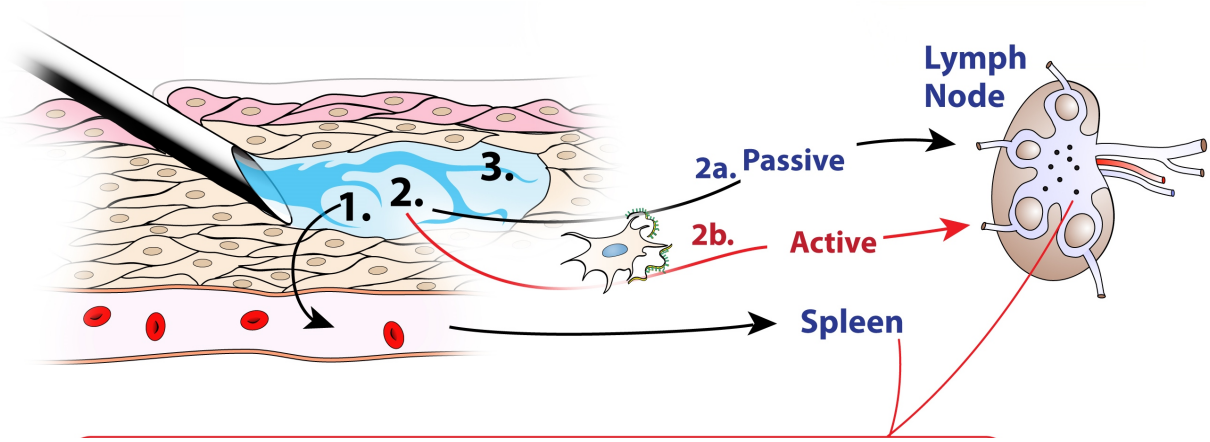


Figure 1. Score profiles of the different types of multiple sclerosis. (A) Relapsing-remitting MS. (B) Primary progressive MS. (C) Secondary progressive MS.

Table 3. Dintzis rules for immune response.

Polymer properties	Immunogenic SAgA	Tolerogenic
Mw	>100 kDa	<100 kDa
Antigen Density/kDa	>10	~5
Antigen Spacing	2-7 nm	2-7 nm
Structure	Rigid	Flexible
Solubility	Poorly soluble	Highly soluble

Figure 2. Depending on the size, SAgAs will either (1) absorb into the blood, (2) drain to the lymph nodes, or (3) remain at the injection site.



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Chapter 3:

Routes of Administration and Dose Optimization of Soluble Antigen Arrays in Mice with Experimental Autoimmune Encephalomyelitis

3.1 Introduction

Multiple sclerosis (MS), an autoimmune disease that destroys the myelin sheath, impacts the patient both physically and mentally[1]. Loss of vision, varying degrees of paralysis, loss of balance, and lack of coordination constitute the most common physical symptoms. Mental symptoms include slurring of speech and cognitive difficulties. The onset of disease typically occurs in young adults, but the disease continues into adulthood. Current therapeutic interventions can mitigate symptoms but none address the underlying immune response specific to offending myelin autoantigens.

MS can be classified into the following types: (1) secondary progressive, (2) primary progressive, (3) progressive relapsing, and (4) relapsing remitting. Because relapsing remitting MS (RRMS) affects 80% of patients with MS, current treatments have focused on this form of the disease[2]. In RRMS, the symptoms manifest periodically. In a period of relapse, the patient is acutely aware of the symptoms, but feels them to a lesser degree during periods of remission. Over time, symptoms are continually perceived even during periods of remission, a development indicative of progressive RRMS.

Not much is known about the cause of MS in humans. One theory asserts that MS is a systemic disease in its early stages, and in the later stages MS is localized to the lymph nodes located close to the spinal cord[3]. As a result, designing a treatment that can be active at the lymph nodes may be important. Another theory suggests that T cells are licensed (programmed with a tropism for the cervical lymph nodes) in the lungs before being sent to the CNS, alluding that an important site of intervention may be in the lungs, instead of the cervical lymph nodes[4,5].

The animal model typically used to mimic MS is EAE, where young mice are given adjuvant along with specific epitopes of PLP (proteolipid protein) or other antigens from proteins that make up myelin sheath[6-8]. In this PLP-induced EAE model, one cycle

of MS-like symptoms is followed by remission over a 25-day period. Clinical scores are assigned by the degree of paralysis and balance. Other symptoms usually present in MS, such as cognitive ability, are difficult to measure in mice. The severity of EAE is measured by the clinical scores given to each mouse, with an increase in score indicating an increasing degree of paralysis, ascending from the tail to the hind-limbs and then the fore-limbs as the disease worsens.

The available treatments for MS are known to slow the disease progression and to manage specific symptoms. In early stages of the illness, disease-modifying treatments like interferon-β, glatiramer acetate, mitoxantrone, and natalizumab are prescribed. Natalizumab, also known as Tysabri®, inhibits transit of lymphocytes across the blood-brain barrier. In the later stages of the disease, very low dose cancer chemotherapeutics may be used to suppress the immune system while minimizing systemic toxicity[9]. Another approved treatment, fingolimod (Gilenya®), sequesters lymphocytes within lymph nodes, thereby restricting further destruction of the myelin sheath[10]. This leaves the patient open to opportunistic infections, however, as is the case with global immunosuppressors. Designing therapies that specifically target lymphocytes associated with MS or that induce tolerance to autoantigens by inhibiting costimulation during autoantigen recognition may further stymie the progression of the disease.

Our lab has developed Soluble Antigen Arrays (SAgAs), composed of a hyaluronic acid (HA) backbone with grafted autoantigen (e.g. PLP epitope) and a second peptide, such as an ICAM-1 ligand (LABL) (Table 3.1)[11]. The size of SAgAs can be designed to drain with interstitial fluid and fit through the pores of lymphatic vessels, which range between 10 nm and 100 nm[12]. At least three factors affect SAgA drainage and, ultimately, absorption: the site of injection, diffusion of the SAgA, and local vascular and lymphatic network. After subcutaneous injection, for example, HA can drain to lymph

nodes and certain molecular weights of HA can have a long retention time[13]. By varying the route of administration (site of injection and the type of injection), we investigated which route offers the best efficacy. SAgA treatment alleviates the clinical symptoms of EAE, yet the mechanism of action is still unclear[11]. Here, variations in dose schedule, SAgA dose, injection volume, and different routes of administration were tested to improve understanding of the clinical mechanism. Intramuscular (IM), subcutaneous (SC), intraperitoneal (IP), intravenous (IV), and pulmonary instillation (PI) routes of administration were studied.

3.2 Materials and Methods

3.2.1 Peptide Synthesis. Peptides were made using an automated solid phase peptide synthesizer (Pioneer; Perceptive Biosystems, Framingham, MA). 9-florenylmethyloxycarbonyl-protected amino acids were used to synthesize both $PLP_{139-151}$ and LABL. In order to conjugate these peptides onto HA, an aminooxy (Ao) group was added to the N-terminus of PLP and LABL in the peptide synthesizer. Peptides were cleaved off of the resin using trifluoroacetic acid (TFA) and scavengers.

The peptides were then purified using a C18 semipreparative column (Higgins Analytical, Proto200, 5 µm, 200 Å, 250x20mm) with reversed-phase high performance liquid chromatography (HPLC). A gradient method was used to purify the peptides, with an aqueous mobile phase A (94.9% d.d. H_2O, 5% acetonitrile, and 0.1% TFA) and an organic mobile phase B (99.9% acetonitrile and 0.1% TFA). The purity of the peptides was analyzed using HPLC on a C18 analytical column (Higgins Analytical, Proto200, 5 µm, 200 Å, 250x4.6mm) with the same mobile phases and similar gradient program. The molecular weight of the peptides was identified using an electrospray ionization time-of-flight mass spectrometer (**Supplemental Figure 3.1**).

3.2.2 SAgA Synthesis. After the peptides were purified, they were then conjugated onto HA. A 2-mg/mL solution of HA (16.9 kDa, 16 kDa, Lifecore Biomedical, Chaska, MN)

was made using 20 mM acetate buffer at pH 5.5. AoLABL and AoPLP were mixed together in equal molar proportions, then added to the 2-mg/mL HA solution at a ratio of 1 aminooxy-peptide to 2 monomers of HA. The reaction continued for 24 hours, and then the reaction was dialyzed (MW 3500, 6000-8000) against purified water for 24 hours. Samples were frozen and dried using a lyophilizer.

SAgAs were further analyzed to check peptide content. The SAgAs were dissolved in 0.1-N HCl at pH 1 for at least 4 hours to cleave the peptides. Using a calibration curve of free AoLABL and AoPLP at various concentrations, the amount of peptides in SAgAs were determined via reversed-phase HPLC on a C18 analytical column.

3.2.3 Dynamic Light Scattering (DLS). The size distribution of SAgAs were determined using a Brookhaven Zeta-PALS at various concentrations (0.5 – 10 mg/mL) in PBS after they were filtered through a 0.45 μm PVDF membrane. Light scattering was detected at 90° and using a laser operated at 658 nm. The hydrodynamic radius (R_h) was determined from the Stokes-Einstein equation: $R_h=k_bT/6\pi\eta D_T$, where k_b is the Boltzmann's constant, T is temperature (K), η is the solvent viscosity, and D_T is the translational diffusion coefficient. The particle size was derived using the Stokes-Einstein equation and the autocorrelation function using a non-negatively constrained least squares (NNLS) deconvolution algorithm.

3.2.4 EAE Model. The 6-8 week old female SJL/J mice for the Route of Administration study were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The 3-week old female SJL/J mice for the dosing study were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). The mice were housed in a pathogen-free facility at the University of Kansas approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures using live animals

have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas.

On Day 0 of the study, the SJL/J mice were induced with EAE through four 50-µL injections of an emulsion containing Incomplete Freund's Adjuvant Oil (BD Difco Adjuvants, Franklin Lakes, NJ), *Mycobacterium tuberculosis* (BD Difco Adjuvants, Franklin Lakes, NJ), and 200 nMol of PLP per animal. The SC injections were given above each of the shoulder blades and the rear haunches of the mouse. Each animal was also given a 100-µL IP injection of 200 ng of pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) on Days 0 and 2. The mice were weighed on each day of the 25-day study and were given a clinical score ranging between 0 and 5 from Day 7 to the end of the study. The clinical score increases with disease progression. Scores increased in correlation with the level of paralysis, starting with the tail and advancing to the head. The following clinical score scale was used: 0 – no symptoms of disease were seen, 1 – limp tail and waddling gait, 2 – partial hind leg paralysis, 3 – paraplegia (complete hind leg paralysis), 4 – partial front leg paralysis, 5 – moribund or complete front leg paralysis. The mice were then treated on Days 4, 7, and 10 with 100 µL of SAgA (containing 200 nMol of PLP) on each treatment day, unless otherwise stated. There were 6 mice in each group, with three mice housed together per cage. All the statistical analysis was done on Prism GraphPad 5 software, using ANOVA analysis.

3.2.5 Changing the Dose Schedule, Volume, and Amount of SAgA Treatment. EAE mice were treated on only one of the three days mentioned (day 4, 7, or 10). Days 4 and 7 was chosen as a treatment day to monitor disease progression if treatment occurred prior to symptoms and day 10 was chosen to track the disease severity if treated after the symptoms were visible. SAgA was still given on a 200-nMol PLP basis using 100 uL per injection. Two other variables were changed in the study, the amount of SAgA given and the volume of the injection. One group of mice was treated on a 50-nMol PLP basis

of SAgA (100 uL per injection) given on all three treatment days. Another group of mice was treated on a 200-nMol PLP basis of SAgA using 20 uL per injection given on all three treatment days. All of these injections were given in the Upper SC site. The same SAgA was used for each animal study and the PLP:LABL ratio (1:1) was kept the same in each study.

As a negative control, a group that only received phosphate buffered saline (PBS) as a treatment was included in each study. A group that received a SC SAgA injection in the upper back was also included as a positive control in each study since treatment route was used in previous[11,14].

3.2.6 Route of Administration EAE Study. The administration routes initially explored were IM, SC, and IP. More sites were then added to test whether clinical scores may improve if administering SAgAs near the upper lymph nodes, close to the spinal cord. As a result, the following injection sites were used: IP, Upper IM, Lower IM, Upper SC, and Lower SC (Figure 3.1).

In a second component of the study, IV and pulmonary routes were also compared to the Upper SC delivery of SAgAs. A tail vein IV injection site was chosen to measure what the effect systemic delivery of SAgA would produce. SAgAs were given at a 200-nMol PLP basis at an injection volume of 100 µL. For PI, SAgAs were given at a 200 nMol PLP basis, but the injection volume was decreased to 50 µL due to the limitation in volume that can be safely delivered to lungs. Administration positive and negative controls were included for both studies as mentioned above.

For PI administration, each animal was anesthetized with inhaled isoflurane in an induction chamber. After the mouse was fully anesthetized, the mouse was positioned with its back on a dosing board at approximately a 60° angle to a supine position using a thin wire to suspend them by their incisor teeth with a nose cone being used to maintain anesthesia. The mouth was opened and the tongue was gently pulled out and to the

side of the mouth. A laryngoscope was then positioned to depress the tongue and see the cords at the top of the trachea and 50 uL of solution was administered at the top of the trachea. The tongue was withheld for 3 breaths at which time the mouse was maintained under anesthesia for an additional 3 minutes on the dosing board. The mouse was then removed from the dosing board and held vertically until she began to recover from the anesthesia.

3.3 Results

3.3.1 Peptide Conjugation Efficiency. Before any of the SAgAs were injected into animals, the amount of peptide per SAgA was confirmed. For the first EAE study exploring the dosing schedule, amount and volume, approximately 9 LABL molecules and 8 PLP molecules were in SAgAs. **Table 3.1** reports the characteristics of SAgAs that were used for the remaining two routes of administration studies. Using these data, a 200-nMol PLP dose was calculated for each SAgA treatment. The average molecular weight of the SAgA, the approximate number of PLP and LABL molecules on one 16 kDa HA chain, and the final ratio of PLP:LABL in SAgA were calculated using reverse phase HPLC data. (Table 3.1)

3.3.2 DLS. Figure 3.1 shows the hydrodynamic radius of SAgAs ranged between 3 – 10 nm. At concentrations at 1 mg/mL and lower, SAgA size ranged between 5 – 10 nm. SAgAs were injected at a maximum of 10 mg/mL, which falls in the size range of 3 – 10 nm. A range of sizes was expected for SAgAs since the starting HA exhibits a size distribution and the number of peptides associated with each HA strand may vary. According to studies tracking passive tissue drainage of HA, SAgAs at the higher end of the size distribution (~10 nm) would be expected to infiltrate the lymphatic system. At the lower size range (~3-5 nm), SAgAs would be more likely to enter the systemic circulation. The size of SAgAs is also small enough to bind immune cells and be actively transported to secondary lymphoid organs.

3.3.3 EAE Study of Dosing Schedule, Amount, and Volume. The SAgA dosing schedule has been determined from work by Siahaan et al. using their bifunctional peptide inhibitor (BPI), which is a small molecule version of SAgAs that contains PLP and LABL with a linker in between. BPI ameliorated disease best when given on days 4, 7, and 10, at a concentration of 100 nMol[15-18]. In early studies, SAgAs were injected on a 100-nMol PLP basis, but after a small dosing study, it was found that a 200-nMol PLP basis decreased clinical scores of EAE mice more than the 100-nMol dose[11,14].

Here, the treatment schedule was changed to a single SC injection on only one of the three treatment days (day 4, 7, or 10 of the study). Treating only on day 7 seemed to decrease the disease symptoms the most when compared to either the PBS-treated group or the group that received treatment on all three days. Differences in clinical score, however, were not significantly different (Figure 3.2A). The incidence of disease graph is another way to view the clinical scoring of animals. A score of 1.5 indicates that the animal experiences full paralysis of the tail with partial paralysis of hind-limbs. As a result, 1.5 is a critical score to determine if the disease affects mouse movement or not. The incidence of disease for variation of dosing schedule (Figure 3.2B) indicated, however, that only half of the group dosed on day 7 had a score of 1.5 or higher.

In another study, the dose was decreased from 200 nMol PLP to 50 nMol PLP. As mentioned above, the SAgA dose was calculated based on the amount of conjugated PLP antigen administered. Decreasing the dose reduced efficacy, even if the SC treatment was dosed on all three days. The clinical scores (Figure 3.2C) and incidence of disease (Figure 3.2D) resembled that of the PBS control group more closely than the group that was treated at the 200-nMol dose on all three days.

Lastly, the total volume of the SC injection was reduced from 100 µL to 20 µL, while maintaining the 200-nMol PLP dose. The clinical score data (Figure 3.2E) showed no difference compared to either of the control groups (PBS or SAgA treatment using

100 µL injections). The incidence of disease (Figure 3.2F), however, suggested lowering the injection volume reduced efficacy in a majority of the animals, even when the dose injected did not change. The significant difference in clinical scores between the 20 µL and the 100 µL injection suggested a larger injection volume may improve SAgA exposure (e.g. local drainage or systemic absorption).

3.3.4 EAE Study of Routes of Administration. Next, the 200-nMol dose in 100 µL was injected into different sires: upper SC (as before), lower SC, upper IM, lower IM, and IP (**Figure 3.3**). In exploring the different routes of administration, each of the treatments was found to be statistically significant ($p<0.05$) from the PBS control group during the peak of disease, days 12-17 (Figure 3.4A). In this study, no difference was apparent between different SAgA routes of administration. Both the clinical score data and the weights of the animals revealed differences between the PBS control group and the various treatment groups. The incidence of disease graph (Figure 3.4B), however, suggested the upper IM group had the lowest number of animals with a disease score of 1 or higher.

A subsequent study compared upper SC, IV, and PI routes of administration. The SC and IV group scores (**Figure 3.4C**) and weights (**Supplemental Figure 3.2E**) were similar to one another and were, once again, significantly improved compared to the PBS group ($p<0.05$) during the peak of disease. The PI group maintained a clinical score of zero and showed the best maintenance of weight throughout the study. In the incidence of disease profiles (**Figure 3.4D**), no mice in the PI group had a clinical score of one or higher, further supporting pulmonary instillation of SAgAs provided the best amelioration of disease.

3.4 Discussion

In vaccine treatments, IM injections are usually given to produce an immune response. In contrast, SC injections are typically used for hyposensitization of patients

(e.g. allergy shots). Subcutaneous injections are more likely to diffuse from the injection site, where as intramuscular injections may form a depot, depending on the properties of the injected material. Since transport to the lymph nodes was thought to be vital for SAgAs, the SC route was chosen as it allows for both active transport and passive diffusion to the lymph nodes. Bagby et al. have found that injecting HA subcutaneously in a mouse footpad results in HA draining to lymph nodes[13]. Since SAgAs contain HA and are delivered subcutaneously, it is possible that they can also be transported to the lymph nodes, either by passively draining with interstitial fluid or by active transport by binding immune cells. Additionally, SC injection of SAgA was previously shown to decrease EAE scores, so dose schedule, amount, and volume were explored by this route first[11,14].

3.4.1 EAE Study of Dosing Schedule, Amount, and Volume. Treating on one of the three treatment days or injecting SAgA at a lower concentration generally caused the disease symptoms to increase compared to treating on days 4, 7, and 10 with 200 nMol PLP conjugated to SAgA. Treatment on day 4 did not allow for the disease to fully manifest, since 7 – 10 days are required for the immune response to occur[19]. Treatment on day 10 allowed for the symptoms to be apparent, which means that the immune system has already damaged the CNS (e.g., spinal cord). The incidence of disease graph having clinical score greater than 1.5 supports the argument that day 7 had the lowest incidence of disease, however clinical scores were similar for all days.

Arguably, day 10 would be the most similar to a clinical setting with a human subject. While considering the incidence of disease trends, administering treatment after symptoms have persisted for a few days seems not as effective as treating earlier. For full effectiveness, it may be important to treat before the symptoms are felt, with enough time for the body to process an immunological response. For example, natalizumab treatment regimen is given on a monthly schedule, prior to symptom presentation, which

would be similar to early treatment using SAgA$^{20\text{-}23}$. These similar mechanisms suggest SAgAs may be interacting with immune cells and somehow inhibiting trafficking, sequestering these cells in lymphoid tissue, or triggering anergy.

SAgAs were not as effective when the overall dose was decreased from 200 nMol to 50 nMol PLP. All six of the mice had a clinical score greater than 1.5 just one day after the PBS-treated group reached that level. While the onset of disease was slightly delayed, symptoms progressed very quickly once present. Going forward, a 200-nMol PLP dose was deemed necessary for SC delivery to be effective.

To test the last dosing variable, SAgA concentration was increased to deliver 200 nMol of PLP in 20 µL instead of 100 µL. The injected material was hypothesized to remain more local and not spread under the skin to the same extent as in the typical 100 µL injection groups. While the onset of disease was delayed, the majority of the animals did get sick. The smaller volume injected did decrease the clinical score, but it was not as effective as the larger volume injection even though the dose was the same. In conclusion, the 100 µL SC injection of SAgA allowed for better amelioration of disease perhaps because of improved local distribution and exposure, compared to the lower volume injection. The larger volume injection was observed to spread under the skin to a greater extent than the smaller volume injection. As a result, the injection is much more likely to spread itself over a large surface area, giving SAgAs an increased likelihood of absorption or binding to peripheral immune cells for active transport. It is reasonable to suspect the higher volume injection may have even hydrostatically perfused more SAgA into lymphatic ducts, since lymph node clearance is driven by a pressure differential between the lymphatic ducts and tissue space.

3.4.2 EAE Study of Route of Administration. Since changes in dose and injection volume changed response, the route of administration was hypothesized to have an important effect. EAE mice were treated using the most effective regimen (200 nMol

PLP, 100 µL injection days 4, 7, 10) by injecting upper SC, lower SC, upper IM, lower IM, or IP. All of the routes of administration significantly improved the disease state compared to the PBS group, but the treatment groups did not differ from each other (Figure 3.4A). Comparing the severity of disease in individual animals showed no animals in the upper IM group had a disease score of 1.5 or higher, whereas 5 out of 6 animals in the upper SC group had a score of 1.5 or higher at the peak of disease (Figure 3.4B). Additionally, all of the mice in the PBS control group had a score of 1.5 or higher by day 13.

SAgAs were originally designed to transport to the lymph nodes, through either passive drainage or active transport. Passive drainage allows SAgAs to diffuse with interstitial fluid to the lymph vessels, then drain to the appropriate lymph nodes. During active transport, peripheral antigen presenting cells such as macrophages or dendritic cells may carry SAgAs to the lymph nodes (e.g., after recognizing PLP antigen[24]). Intravenous delivery of SAgAs was used to compare results from upper and lower SC, upper and lower IM, and IP injections. Similar efficacy via different routes of administration chosen for this study would suggest SAgAs may act systemically to ameliorate EAE. This is supported by DLS data that indicates the size distribution of SAgAs would include some SAgA molecules that may be small enough to enter the circulation and some SAgA molecules large enough to favor passive drainage via the lymph vessels.

IV administration using the same SAgA dose injection volume and schedule was compared to upper SC administration. The IV route provided similar disease amelioration and this group provided a key benchmark for comparing other routes of administration (**Figure 3.4C**). Since there was no difference in scores between the IV and the SC groups, it is probable that the SC injection is also delivering SAgAs systemically. Small, twenty-gram mice were injected with 100 µL, perhaps increasing the

likelihood that SAgAs absorb into circulation instead of creating a depot. In the incidence of disease graph (**Figure 3.4D**), the SC and IV treatment groups both had over half of the mice that were affected by the disease. This complements the clinical score data for the SC and IV treatment groups, supporting the notion that subcutaneous injection may lead to systemic delivery of SAgA. SAgAs may transport to the spleen when delivered systemically, which may allow for the SAgAs to interact with B and T cells. Further studies would be needed to support or disprove this hypothesis.

Finally, another route of administration was explored in this study, pulmonary instillation. In the 'Hub and Spoke' model of the immune system, T cells are licensed in the lung before they are distributed throughout the body[25]. In the case of multiple sclerosis, the lung is the hub and the CNS tissue is the spoke. Odoardi et al also determined that T cells acquire the properties needed to migrate to other parts of the body in the lung[4]. Delivering SAgAs to the lungs may allow multiple sclerosis-specific T cells to interact with SAgAs. An exact mechanism between T cells and SAgAs is still being explored, but delivering SAgAs to the lungs proved to significantly decrease EAE disease symptoms. After PI of SAgAs, mice showed disease scores of zero throughout the course of the 25-day study. In comparison to other routes of delivery (e.g., SC and IV), pulmonary delivery provided the lowest clinical scores, the best maintenance of weight, and no animals were affected by disease (**Figures 3.4C and 3.4D**).

The increased efficacy of PI of SAgAs compared to subcutaneous or intravenous injection may also be explained by some of the physiological differences in transport. Several different transport processes may occur after PI. Passive transport routes include diffusion of SAgAs through the lung epithelial tissue, followed by absorption into the lymphatic network or into circulation. Xie et al, determined that pulmonary instillation of an HA conjugate allowed the majority of the drug to stay within the lung, where it could be targeting the surrounding lymph nodes[26]. It is possible that the effect of SAgAs

after PI administration is more pronounced than SC or IM because of the shorter pathways from the lung to the lymph node, thus leading to higher dose or better timing during the pre-symptomatic stage of immune response.

Another possibility to consider is that SAgA degradation and diffusion of components is possible, but our previous studies have shown neither free peptides nor HA (alone or in combination) have a therapeutic effect[11]. Active processes of mucocillary clearance and macrophage clearance may also affect SAgA transport. Mucous is transported by cilia located in the conducting airways and is deposited in the oropharynx and swallowed. The potency of SAgAs administered PI suggest minimal mucociliary clearance. Macrophage clearance typically occurs in the lower airways including terminal bronchioles and alveoli. Macrophages then transport phagocytosed cargo to the lymph nodes for processing. Lymph nodes are key to the efficacy of immune therapies, since that is a primary site of immune cell priming.

Observations from SC and IV routes of administration do not support a systemic mechanism of SAgA efficacy of after PI. Although, it is possible increased PI efficacy is linked to avoiding fist pass metabolism, which could diminish SAgA effect after IV or SC administration. Furthermore, it is plausible the lung may act as a SAgA depot after PI, since macrophage clearance can take days, providing an extended duration of action compared to the other administration routes. Perhaps the most plausible explanation is that pulmonary instillation may offer a direct avenue to the lymphatic system, either passively or via active transport or may somehow bind and inhibit T cells migrating through the lungs to be licensed for trafficking to the CNS. Additional studies will be required to elucidate these possible mechanisms.

3.5 Conclusion

SAgA treatment schedule, dose, injection volume, and routes of administration were systemically explored to treat EAE. Timing the SAgAs dose was important,

suggesting it must follow the schedule of a typical natalizumab dosing, before symptoms are fully realized. Decreasing SAgA dose to 50 nMol (i.e. 50 nMol of PLP delivered) administered on days 4, 7, 10 was not as effective as 200 nMol. Decreasing the volume of the dose to 20 µL meant increasing the concentration 5 fold and SAgAs were slightly less efficacious at the lower volume, suggesting the lower volume, higher concentration had limited exposure.

Exploring routes of administration provided new insight into possible mechanisms of action. The initial goal was to deliver SAgAs to lymph nodes. The first set of administration sites (upper and lower SC, upper and lower IM, and IP) was chosen with this goal in mind. Previous studies suggested a portion of SAgAs injected at the upper SC site would drain to the cervical lymph nodes, which drain the CNS and are implicated in EAE. Delivering SAgAs via the SC route was similar to delivering IV suggesting all peripheral injections may be acting via systemic exposure. The most surprising result was that the pulmonary delivery of SAgAs decreased the clinical scores with no mice exhibiting symptoms throughout the study. Applying the 'Hub and Spoke' theory to pulmonary delivery, it is possible that delivering SAgAs to the lungs allows maximized interaction with the immune system, either within the lung or via local lymphatics.

To further explore mechanisms of SAgA efficacy, the dosing schedule, amount, and volume studies could be repeated via the pulmonary instillation route of administration. Further studies that track the distribution of SAgA throughout the mouse after pulmonary instillation would provide useful insight to the trafficking of SAgA molecules. Additionally, in vitro work is under way to determine target cells by screening SAgAs in EAE splenocytes and measuring cytokine responses to help explain the efficacy observed in EAE mice.

$SAgA_{PLP:LABL}$ used in EAE Studies	Calculated MW	Number of PLP Peptides per Scaffold	Number of LABL Peptides per Scaffold	Final Ratio (PLP:LABL)
Dosing Schedule, Amount, Volume	39605	8.84	8.27	1.069:1
Route of Administration (Upper and Lower SC, Upper and Lower IM, IP)	34269	6.42	6.85	0.94:1
Route of Administration (SC, IV, PI)	34788	8.19	5.53	1.48:1

Table 3.1. Using pH 1.0 solution, the peptides were hydrolyzed from HA to indirectly quantify the amount of AoPLP (circle) and AoLABL (triangle) grafted to SAgA.

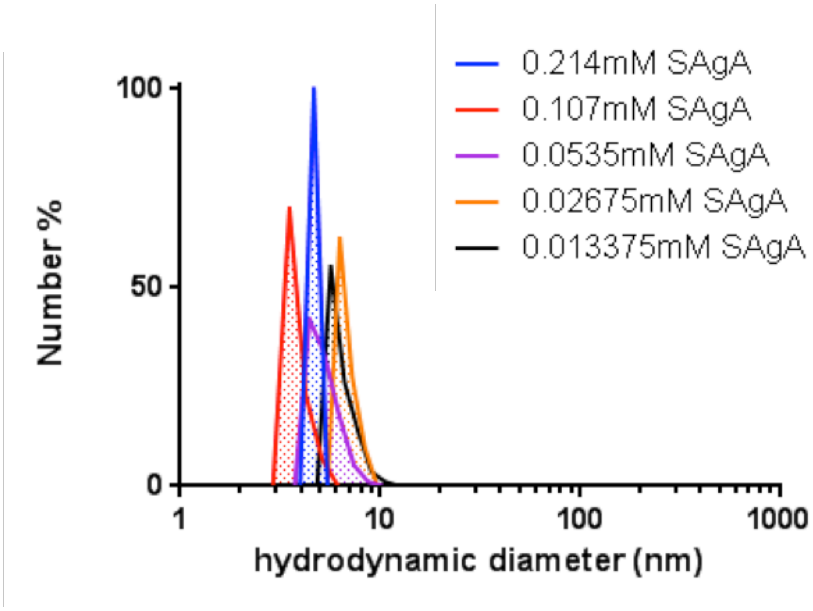

Figure 3.1. DLS measurements show various concentrations of SAgAs to measure between 3 – 10 nm in hydrodynamic radius.

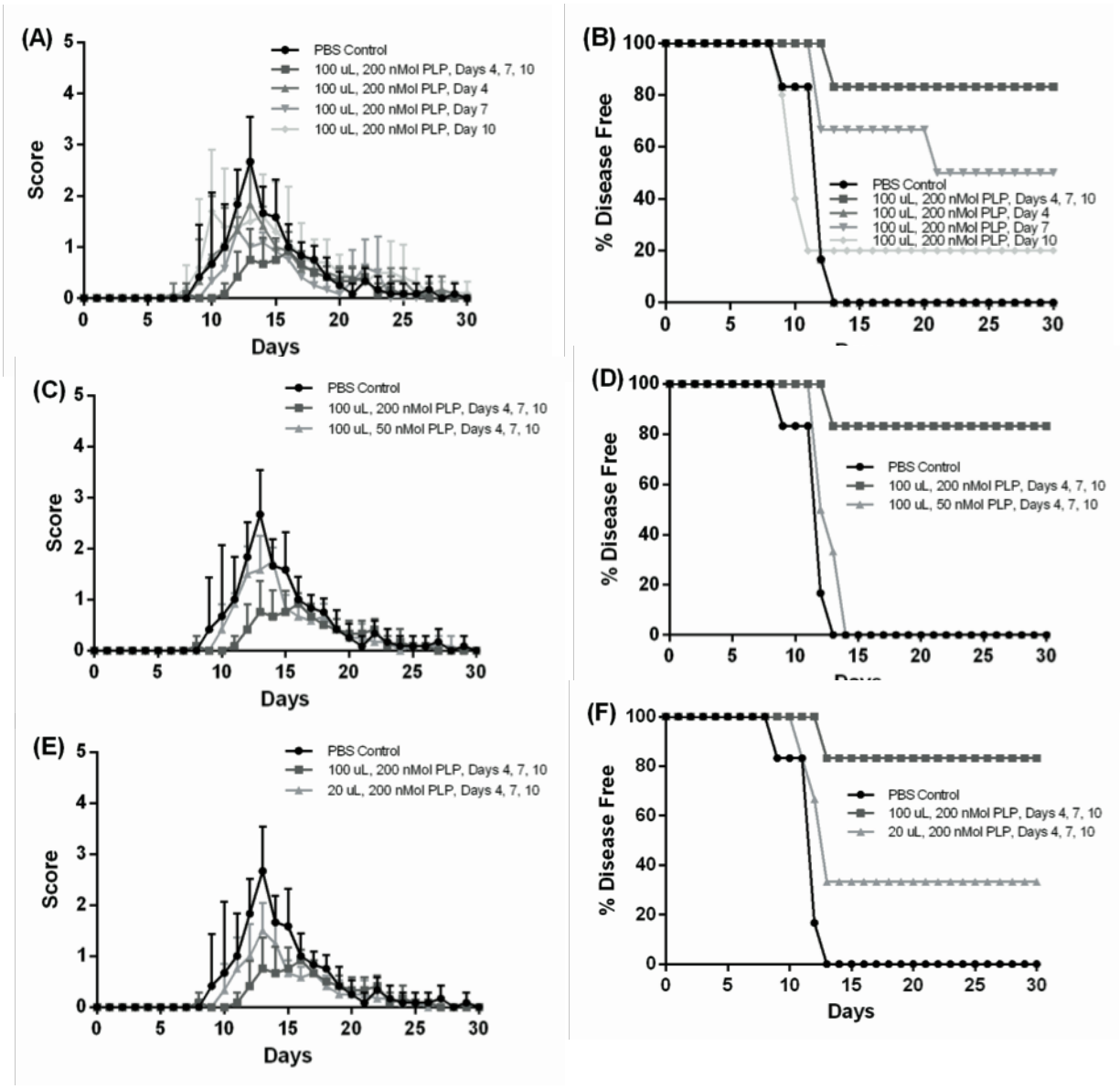

Figure 3.2. Clinical score and incidence of disease data for dosing schedule, dosing amount and dosing volume. For the incidence of disease data, a disease score of 1.5 or higher shows that the animal is affected by the disease. (A) Dosing schedule was changed to one of the three treatment days. There is significance ($p<0.05$) on Days 13 and 15 of the study between the PBS control group and the group that was treated only on Day 7. (B) The incidence of disease for the dosing schedule study shows that treating on Day 7 results in the lowest incidence of disease. (C) Dosing amount clinical scores

indicated that decreasing the dose decreases the effectiveness of the treatment. (D) The incidence of disease for the dosing amount study showed that decreasing the dose increased the number of animals affected by the disease. (E) The dosing volume clinical scores showed that the lower volume injection is not as effective in comparison to the 100 uL injection. (F) The incidence of disease for dosing volume shows that the lower injection volume had a 50% greater incidence of disease.

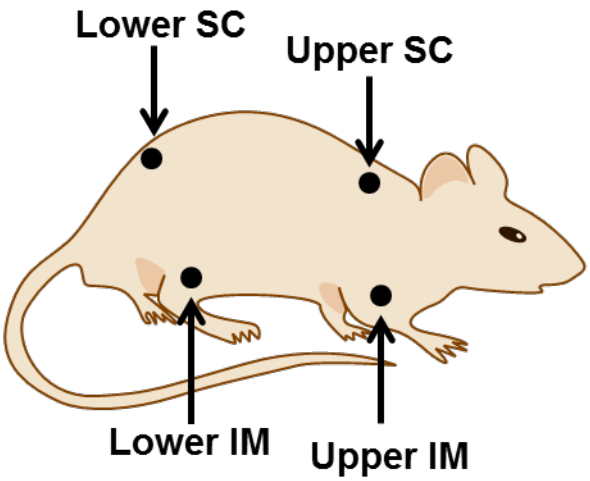

Figure 3.3. Injection sites for route of administration studies.

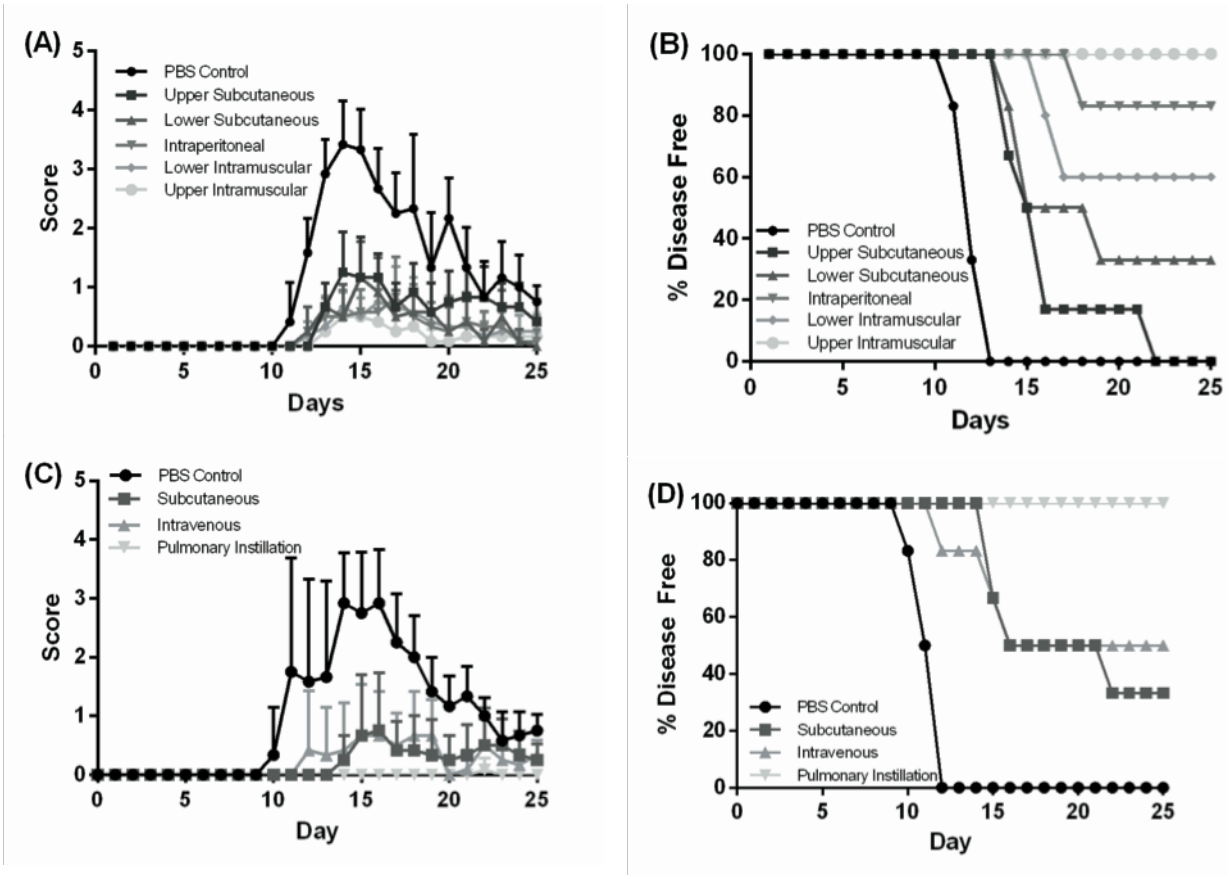

Figure 3.4. Route of administration study clinical score (A) and incidence of disease (B) data. (A) There is no difference between different routes of administration, but there is a significant difference ($p<0.05$) between the PBS-treated group and all other SAgA-treated groups on Days 12-18. (B) Incidence of disease plots show animals with a disease score of at least 1.5 affected by the disease. The upper IM group was the least affected and the PBS-treated and upper SC groups were the most affected. **(C)** The pulmonary instillation group had disease scores of 0 throughout the peak of disease. **(D)** No animals in the pulmonary instillation group had a score of 1.5 or higher.

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Chapter 4:

Pulmonary Administration of Soluble Antigen Arrays (SAgAs) is Superior to Antigen in Treatment of Experimental Autoimmune Encephalomyelitis

4.1 Introduction

Inducing antigen specific immune tolerance is an emerging avenue of treatment of autoimmune diseases[1]. The classical approach of antigen specific immunotherapy (ASIT) has been to desensitize the immune system through a series of subcutaneous injections. This approach has generally been applied to foreign antigens (e.g., hyposensitization in the form of "allergy shots"). The treatment regimen, however, requires injections over multiple years and disease improvement is often modest[1-5]. Similar strategies have been investigated for desensitizing patients to autoantigens including proinsulin peptide for type-1 diabetes[6], splicesomal peptide for Lupus[7], and a pathogenic peptide for rheumatoid arthritis[8]. Alternative delivery approaches have appeared as strategies to improve efficacy of ASIT. Recently, new products including Ragwitek® and Grastek® utilize sublingual administration to desensitize the immune system to foreign antigens through mucosal surfaces. Treating mucosal surfaces, such as nasal passages or lungs, may also improve performance or reduce the timeline required for ASIT, a concept that may also extrapolate to autoimmune diseases[2,5,9].

Traditional ASIT induces tolerance of allergens over the course of treatment. As a result, the immune system is slowly desensitized, avoiding an immune response against said antigen. Because the immune system cannot differentiate between self and non-self in autoimmune diseases, an autoimmune response occurs. Thus, it may be possible to apply ASIT approaches to educate the immune system to tolerate the self-antigen associated with autoimmune diseases. One particular autoimmune disease this concept has been applied to is Multiple Sclerosis.

Multiple Sclerosis (MS) is an autoimmune disease, where immune cells attack and degrade the myelin sheath surrounding neurons, thus interrupting the transmission of electrical signals[3,4,10,11]. Many of the current therapies approved for MS focus on treating inflammation or neurological symptoms such as loss of balance, degrees of

paralysis, and a loss of coordination. Recently, researchers have aimed to correct the underlying immune response using ASIT. If this autoimmunity is uncorrected, patients can experience progressive cycles of remission and relapse, where periodic symptoms are followed by remission, but the patient does not fully recover to the previous baseline. There are different forms of this disease: secondary progressive, primary progressive, progressive relapsing, and relapsing remitting, which affects a majority of patients, approximately 80%[4,10,11]. Experimental autoimmune encephalomyelitis (EAE), the murine model of relapsing and remitting MS, is induced by a proteolipid protein epitope ($PLP_{139-151}$) and serves as a useful model for studying ASIT approaches to autoimmune diseases[3,4,12].

We explored ASIT using pulmonary delivery of multivalent soluble antigen arrays (SAgAs), which are composed of a hyaluronic acid (HA) polymer backbone with grafted myelin sheath epitope $PLP_{139-151}$, and a grafted peptide inhibitor (LABL) of cell surface receptor intracellular cell-adhesion molecule-1 (ICAM-1)[3]. Previous work has shown that pulmonary delivery of SAgAs had increased efficacy compared to subcutaneous and intravenous delivery[4]. In this chapter, SAgAs, its components (HA, LABL, and PLP), and a bifunctional peptide inhibitor (BPI) containing LABL and PLP were compared when delivered to the lungs of mice. Clinical scores and cytokine responses were compared to elucidate mechanisms of inducing immune tolerance in mice with EAE.

4.2 Materials and methods

4.2.1 Materials

Hyaluronic acid (HA) molecular weight 16 kDa was purchased from Lifecore Biomedical (Chaska, Minnesota). Aminooxy-LABL (AoLABL) and Aminooxy-PLP (AoPLP) peptides were obtained from PolyPeptide, Inc. (San Diego, CA). Incomplete Freund's Adjuvant Oil and Mycobacterium tuberculosis were obtained from BD Difco Adjuvants (Franklin Lakes, New Jersey) and pertussis toxin was purchased from List

Biological Laboratories, Inc. (Campbell, California). The mouse laryngoscope was purchased from Penn-Century (Wyndmoor, Pennsylvania). All water used was deionized (DI) water from a Labconco Pro PS system. All other chemicals and materials including 3500 Da molecular weight cutoff dialysis tubing, bent fine dissecting forceps, glacial acetic acid, sodium acetate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, and phosphate buffered saline were purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

4.2.2 Soluble Antigen Array (SAgA) Synthesis

The purified peptides were conjugated onto HA. In 20-mM acetate buffer pH 5.5, a concentration of 2 mg/mL of 16.9-kDa HA was made. AoLABL and AoPLP peptides were added in equal molar proportions, then were added to the 2-mg/mL HA solution using a ratio of 2 HA monomers to 1 Ao peptide. The reaction was allowed to proceed for 24 hours at room temperature. After 24 hours, the reaction mixture was added to 3500-Da molecular weight cutoff dialysis tubing using DI water as the dialysate for 24 hours with the dialysate being changed every 6 hours. Samples were then frozen and lyophilized for 72 hours at a condenser temperature of -72 °C and at a vacuum of <300 millitorr (VirTis Feezemobile-12XL, The Virtis Company, NY).

The peptide loading of SAgAs was analyzed using reverse phase high performance liquid chromatography (RP-HPLC) and size exclusion chromatography (SEC). SAgAs were dissolved in 0.1-M HCl pH 1.0 for 4 hours to hydrolyze peptides from the HA backbone. With the HPLC, a calibration curve of free peptide was employed to determine the amount of peptide that was conjugated to the HA for SAgAs, which was compared to the relative size ascertained from SEC. The HPLC analysis was performed using a Waters 2487 dual absorbance detector and a Waters 2796 bioseparation module. A gradient separation was performed with a mobile phase A comprised of 94.9% DI water, 5% acetonitrile, and 0.1% trifluoroacetic acid and mobile

phase B comprised of 99.9% acetonitrile and 0.1% trifluoroacetic acid. The gradient was 100% A from 0 to 15 minutes, 85% A at 15 minutes, 83% A at 20 minutes, 80% A at 33 minutes, 75% A at 42 minutes, 30% A at 43 minutes, and 100% A at 46 minutes until 60 minutes with a total flow rate of 1 mL/min. A C18 Higgins Analytical Proto200, 5 µm, 200 Å, 250 × 4.6 mm^2 column was used with an injection volume of 30 µL using samples with a concentration of 1 mg/mL with wavelength detection at 220 nm.

SEC was employed to determine the increase in size of SAgA from HA due to the conjugation of peptides to HA. The system used included a Waters e2695 separation module, Waters 2414 refractive index detector, and Waters 2489 UV/Vis detector with two columns in series: a PL Aquagel-OH 60 Analytical SEC (300 x 7.5 mm) then a PL Aquagel-OH 40 Analytical SEC (300 x 7.5 mm). The mobile phase employed for SEC was of 0.1-M ammonium acetate with 0.136M sodium chloride at pH 5 using an isocratic system. Samples injected were 80 µL at a concentration of 5 mg/mL with a total flow rate of 0.5 mL/min flow. Samples were dissolved in the mobile phase at a concentration of 5 mg/ml and chromatograms were analyzed using EMPower 3.

The hydrodynamic radius of the samples was determined using ZetaPALS. The samples were all at a concentration of 1 mg/mL and then dissolved in filtered phosphate buffered saline (PBS). Each of the samples was filtered once again immediately before the readings were taken. Three readings were taken per sample and then averaged for the values seen in the results (**Table 4.1**).

4.2.3 Animals

SJL/J mice (female, 4 weeks old) were supplied by Harlan Laboratories (Indianapolis, IN) for the study. Mice were approximately 13-16 g and were weighed throughout the course of the experiment. The mice were housed in a pathogen-free facility at the University of Kansas approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice were maintained on a 12 hour

light/dark cycle and were fed standard rodent pellets with no dietary restrictions or withheld food and water was always available. All animal experiments were approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

4.2.4 Induction of Experimental Autoimmune Encephalomyelitis (EAE) and Time Course of Study

Each SJL/J mouse had EAE induced on study day 0 with 4 injections of an emulsion of Incomplete Freund's Adjuvant Oil, Mycobacterium tuberculosis, and 200 nM of PLP. Each of the 4 injections was 50 µL given subcutaneously: 2 above the shoulder blades and 2 above the rear hip haunches. Each SJL/J mouse had 100 µL of 200 ng pertussis toxin injected intraperitoneal on day 0 as well as day 2. There were 6 mice in each of the groups split into 2 cages of 3 mice.

Over the course of the 25-day study, each mouse was weighed and given a clinical score from study day 7 to 25. The clinical scoring ranged from 0 to 5 with half integer values and a higher score correlated to an increase in disease progression. The advancement of the scoring was as follows: 0 corresponded to no symptoms of disease; 1 with a limp tail and waddling gait; 2 with partial leg paralysis and increased alteration of gait; 3 with paraplegia or complete hind leg paralysis; 4 with partial front leg paralysis; and 5 with complete front leg paralysis or moribund. Half integer values corresponded with disease between scoring levels[3,4].

4.2.5 Treatment Schedule and Pulmonary Instillation of Compounds

Treatment was administered on study days 4, 7, and 10. Before treatment, each of the study compounds was dissolved in sterile PBS. The study groups were PBS, 16 kDa HA, LABL, PLP, SAgA, and bifunctional peptide inhibitor (BPI). Each animal was administered 50 µL of each compound corresponding to 200 nM with the exception of BPI, which was 150 nM administered via pulmonary instillation as described in details

below[3,4]. All the statistical analyses were performed on Prism GraphPad 5 software using ANOVA analysis. Tukey post-hoc analysis was applied to the data.

For lung administration via pulmonary instillation, each animal was anesthetized with 2% isoflurane in oxygen in an induction chamber for approximately 4 minutes. After the mouse was fully anesthetized, the mouse was positioned in dorsal recumbency using a dosing board at approximately 60° to a supine position suspended by incisor teeth using a thin wire. A nose cone was used to maintain anesthesia. The mouth was opened and the tongue was gently pulled out and to the side of the mouth. A laryngoscope was then positioned to depress the tongue and visualize the vocal cords at the top of the trachea. A 50 µL solution of HA was then pipetted at the top of the trachea. The tongue was withheld for at least 3 breaths, after which time the mouse was maintained under anesthesia for an additional 3 minutes on the dosing board. The mouse was then removed from the dosing board and allowed to recover from the anesthesia by being held vertically until movement was regained[13].

4.2.6 Collection and Culturing of Splenocytes

The mice were euthanized via isoflurane overdose. Spleens were harvested under sterile conditions and were immediately transferred to a Falcon tube with 5 mL sterile RPMI media with 1% Penicillin-Streptomycin (P/S). Within 30 minutes, the spleens were mashed under sterile conditions using a wire mesh in a petri dish and the rubber stopper of a 1-mL syringe plunger. A 5-mL pipette was used to transfer the cell solution to a 15-mL Falcon tube. The splenocytes were centrifuged at 3500 rpm for 4.5 minutes. The old media was decanted and the cells were resuspended in 1 mL of RPMI with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin.

A fresh solution of Gey's Lysis solution was made and 3.5 mL was added to the splenocytes. The cells were set on ice for 3.5 minutes to lyse the red blood cells. Then, 10 mL RPMI with 10% FBS and 1% P/S was added to the cells. They were centrifuged

once again at 3500 rpm for 4.5 minutes. The media was decanted and the cells were resuspended in 5 mL of fresh RPMI with 10% FBS and 1% P/S. The cells were counted using a hemocytometer and were plated at 5 x 10^6 cells per well at 500 µL per well in a 24-well plate. The splenocytes were incubated for 120 hours before the supernatant containing cytokines was collected and resazurin assay was conducted.

4.2.7 Determination of Cytokine Profiles by ELISA

ELISAs from R and D Systems (Minneapolis, MN) were used to determine the following cytokine levels (IFN-γ, TNF-α, IL-2, IL-6, IL-10, and IL-17). A 100-µL aliquot of the collected supernatant was used for each of the ELISAs except for IFN-γ and IL-17, where the samples were diluted 2:5 using RPMI media with 10% FBS and 1% P/S. The ELISA was read using a SpectraMax plate reader at an absorbance of 450 nm. The protocol for each of the ELISAs was provided by R and D Systems.

4.2.8 Lung Histology

Mice were euthanized as described previously. An incision was made in the ribcage to expose the trachea and lungs. A 10% formalin solution was injected into the trachea down into the lungs via a syringe until inflation was observed via visual inspection, which typically required 3 mL. The lungs were tied off using nylon sutures to keep the lungs inflated with 10% formalin and were suspended in 10% formalin in a 50 mL test tube. Enough formalin was used to cover the lungs and the test tube was inverted to maintain the lungs in formalin. After 24 hours, the remaining tissue was removed from the lungs, leaving just the lungs, which were then split into right and left and were placed in a histology cassette in 70% ethanol. After 72 hours, the 70% ethanol was replaced to remove excess red blood cells from solution.

For histology, the mice lungs were sectioned and submitted for formalin fixation. Briefly, the specimens were grossed and submitted in plastic cassettes. The cassettes then underwent additional processing within a Tissue-tek VIP Tissue Processor, at a

temperature of 10° - 40° C. The tissue went through further formalin fixation followed by 70% alcohol, 95% alcohol, 100% alcohol, and xylene. The tissue was then embedded in paraffin wax at 62° C.

The paraffin embedded tissue was then sectioned with a microtome at four microns per section. These slides containing both the paraffin and tissue were then placed into an oven to remove excess paraffin wax. After the slides and tissue were cooled, they were stained with hematoxylin and eosin. A cover slip was placed over the stained tissue samples allowing the tissue to be evaluated.

4.3 Results

4.3.1 Characterization of SAgAs

Reverse phase chromatography was utilized to determine the amount of peptide conjugated to HA. Based on the RP-HPLC results, there were approximately 5 to 6 PLP peptides and 7 to 8 LABL peptides conjugated per polymer chain. By determining the number of peptides conjugated, the approximate average molecular weight was calculated using both SEC and HPLC results (**Table 4.1**). The ZetaPALS readings for hydrodynamic radius are also reported in **Table 4.1**.

4.3.2 Co-Delivery of Peptides Offers Improved Therapeutic Efficacy

The co-delivery of peptides using SAgAs was necessary for ameliorating EAE symptoms. The mice were treated on study days 4, 7, and 10, as was previously established in earlier publications. The PBS group had high clinical scores that were similar to the group treated with LABL (**Figure 4.1**). Both groups had scores that increased as of day 10 with the PBS group having the highest scores between days 12-15 and peaking on 14, while LABL peaked slightly earlier at day 12. Scores for mice administered PBS or LABL remained high throughout the course of the study, ending with scores of approximately 1.0. The HA group had a slightly delayed onset of disease as disease was observed at day 11, peaking on day 14, and decreasing to near baseline

values by day 18. The PLP group had similar scoring to the BPI group with scores remaining around 0.5 after disease onset. The SAgA group had low scores throughout the length of the study with scoring ranging between approximately 0 and 0.25, but were not statistically different from PLP and BPI.

The area under the curve (AUC) from clinical scores in **Figure 4.1** provides a holistic view of the disease state of EAE mice (**Figure 4.2**). The PBS group had the highest AUC of approximately 20 with LABL being similar. HA showed a decreased AUC compared to LABL and PBS but was higher compared to both PLP and BPI treatments. BPI and PLP were quite similar, while SAgA had the lowest AUC value, although not statistically lower than the BPI treatment.

The incidence of disease was also determined (**Figure 4.3**). Mice are considered 'diseased' when progressing to a score of 1.0 or higher, corresponding to symptoms affecting the rear legs as well as the tail. The PBS, HA, and LABL groups had a rapid progression to disease. All HA and PBS-treated animals were sick by day 14 and all LABL-treated animals by day 17. The PLP group had only 1 animal remain disease free throughout the course of the study. The BPI group had 4 of 6 animals remain disease free, while the SAgA group had 5 of the 6 animals remain disease free.

Changes in the weights of animals were also assessed throughout the study (**Figure 4.4**). The weight of the animal decreases around the onset of disease due to decreased mobility of the animal and general decrease of health. Typically, animal weight begins to decrease around day 10 and as the disease resolves, weight will gradually increase. The PBS, LABL, and HA groups had similar decreases in weight starting on day 10 with the greatest decrease in weight occurring on days 14 and 15, corresponding to peak of disease. All three groups had similar weights by the end of the study, recovering to levels similar to the starting weights. The BPI and PLP groups, again, were quite similar with decreases in weight from day 11 to 18 but with less weight

loss compared to PBS, HA, and LABL groups. The SAgA group had almost continuous weight gain throughout the course of the study ending with ~10% weight gain on average.

4.3.3 Cytokine Profiles from Splenocytes

Splenocytes were harvested from treated and control animals at the end of the study. Splenocytes were exposed to media with or without PLP and the cytokine response was determined. IFN-γ levels were significantly higher in splenocytes derived from animals treated with SAgAs compared to animals that were dosed with the individual components (**Figure 4.5**). IL-6 and IL-17 levels were significantly higher in splenocytes from animals treated with SAgAs compared to animals that received only PLP antigen. IL-2 levels were lower in animals treated with PLP, SAgA, and BPI compared to animals dosed with PBS, HA, or LABL. IL-10 and TNF-α levels in the SAgA and BPI groups trended slightly higher than other groups, but there was no statistical significance.

4.3.4 Lung Histology

Lung histology highlighted some intriguing trends but it was difficult to draw firm conclusions based on the heterogeneity of the results within and between groups (**Figures 4.6 and 4.7**). Inflammation from the lungs was scored by differentiating into three distinct categories of mild, moderate, and severe inflammation, as described in Zhornitsky et al[14]. The treatment groups of PBS, LABL, and HA had a wide range of scoring with animals ranging from no inflammation (0) and mild focal inflammation (1) to severe diffuse inflammation with granulomas (6). Due to the wide range of scores, a quantitative analysis showed no statistical difference (**Figure 4.7**). The treatment groups of SAgA and BPI had lower cumulative scores across the groups with maximum lung inflammation of 2 still qualifying as mild. The remaining group of PLP was more similar

to the SAgA and BPI groups with a lower average and with most of the animals having 0 or 1.

4.4 Discussion

4.4.1 The Lungs and Immune Tolerance

Antigen-specific immunotherapies are traditionally administered via subcutaneous injections. Injections of antigens can potentially transport by two mechanisms: (i) via diffusion from the injection site to regional lymph nodes or systemic circulation or (ii) via active cellular transport to lymph nodes[1-5]. Mucosal delivery, including buccal (e.g. Grastek®), nasal, and pulmonary delivery, offers a convenient route of administration and recent studies have suggested the potential for improved tolerization compared to subcutaneous injection. Mucosal surfaces serve as the primary barrier to entry and are constantly inundated with foreign pathogens. These surfaces have their own mucosa-associated lymphoid tissue, which includes specialized epithelial cells that can take up antigens and direct the antigens to antigen presenting cells to elicit an immune response[2,5,9,15]. More recently, however, additional immunological mechanisms of the lungs have emerged.

4.4.2 The 'Hub and Spoke' Hypothesis of Immune Cell Licensing in the Lungs

Recent research has suggested the lungs may have a unique role in the immune system. For example, the 'Hub and Spoke' hypothesis suggests immune cells traffic through the lungs ('hub') acquiring information required to migrate to different parts of the body. Once these immune cells are imbued with this information ('licensed'), they can pass through to the particular 'spoke', such as the brain for MS, pancreas for type-1 diabetes, and the large intestine for irritable bowel disease[16]. In support of this, a recent publication showed that activated immune cells specific to CNS antigens did not elicit CNS symptoms until 5 days after injection when given intravenously. If these same cells were delivered directly into the CNS, no symptoms were observed. If administered

directly into the lungs, however, these cells elicited CNS symptoms after approximately 1 day[17,18].

The 'Hub and Spoke' hypothesis appears to be especially important for T cells, dictating migration to a particular location in the body by modifying transcription and translation of integrin molecules necessary for homing to target tissues. Inhibitors of tissue-specific integrins have proven to be successful as therapeutics (e.g. TYSABRI®). Thus, interfering with immune cells during licensing in the lungs of EAE mice may be a viable therapeutic approach. Researchers are currently elucidating the immune cell populations passing through the lungs and are tracking cells to determine how these cells affect disease. Modification of the immune response may hinge on the ability of cells to migrate through the lungs and to tissue compartments. Thus, the lungs provide an intriguing route of ASIT administration to skew immune responses throughout the body[16-18].

4.4.3 Potential Immune Modulation Mechanism for SAgAs

Pulmonary delivery of SAgA components indicated the need for co-delivery of the PLP antigen epitope with LABL peptide to reverse EAE in mice. HA has some inherent immune activity as it can bind to CD44 on immune cells yet no therapeutic efficacy was observed with the delivery of HA. The delivery of LABL also did not change the disease state suggesting blocking ICAM-1, a known mediator of immune cell adhesion and co-stimulation, was not therapeutic. It is possible that it was quickly eliminated from the lung tissue via clearance or absorption or that LABL was digested quickly by peptidases in the lungs. Treating with PLP alone had increased efficacy based on the AUC of the clinical scores, but most EAE mice treated with PLP became sick. Animals treated with PLP had a delayed onset of disease and exhibited decreased scores compared to HA and LABL hinting that PLP may function as conventional ASIT. If this is the case, repeated, escalating doses may hyposensitize EAE mice to PLP. Ultimately, the co-

delivery of the peptides practically eliminated disease as seen with both BPI and SAgA suggesting that efficacy depends on co-incident delivery of both PLP and LABL.

4.4.4 Cellular and Cytokine Response in EAE with SAgA Treatment

Cytokine profiles provide some insight regarding possible mechanisms of action for SAgA therapy. Some cytokines have been classically considered to be pro-inflammatory or anti-inflammatory but with further study the picture becomes more complex. For example, IFN-γ has traditionally been identified as a deleterious inflammatory response when elevated in autoimmune diseases. New studies, however, have shown that local, elevated levels of IFN-γ may not always correspond to inflammatory responses.

Cytokine profiles differ kinetically throughout the course of the disease and spatially depending on the tissue compartment. Circulating cytokines will differ from cytokines gathered from splenocytes, which are also different from cytokines expressed in affected tissues (e.g. MS brain lesions). We observed a pronounced IFN-γ response in splenocytes from mice that received SAgA treatment[19]. Splenocytes were collected at day 25, during remission of EAE, thus, PLP-specific T cells may have returned to the spleen, which caused the spike in IFN-γ levels. In related studies, Kobayashi et al. reported the levels of IFN-γ seem to be significantly higher in the group of mice treated with PLP-BPI in comparison to the PBS group. The levels of IFN-γ were significantly higher in mice treated with SAgAs than that of mice treated with PBS, 16kD HA, PLP, and LABL. Following the trends seen with Kobayashi, it is possible that the increased levels of IFN-γ from splenocytes corresponded to improved treatment efficacy.

IL-6 responses from splenocytes were also high in mice treated with SAgA. IL-6 may cause EAE to progress, suppressing Fox3P+ Treg cells, causing a pro-inflammatory response[20]. Similar to the rationale for IFN-γ, IL-6-producing cells may

have returned to the spleen during remission, instead of being present in the CNS. A similar rationale may apply to the observed increase in IL-17.

The IL-2, IL-10, and TNF-α results in **Figure 4.5** were not statistically different. IL-2 is known for differentiation of T cells, and is typically pro-inflammatory, as shown by Saskida, et al[21], suggesting this cytokine should increase as did IFN-γ. But, no change was seen in these cytokines, as has been shown in literature[3,19]. All three cytokine levels, however, seem to be similar to previous studies[22].

One major reason for the variation in cytokine levels reported in literature is because of comparisons made between differing time points of the study. Cytokines in the spleen will differ at the point before disease symptoms were prominent, at the peak of disease, and at the end of the study, where symptoms had decreased. Assays of cytokines secreted from splenocytes also use time points from 0 hours to 120 hours after starting the primary splenocyte culture. Future studies should compare cytokine levels in different tissue samples over the course of the study, as well as compare cytokines collected at varying time points after the primary splenocyte culture is established.

Some of the results may be explained by looking at potential immune interactions of SAgAs compared to earlier studies of BPI treatment. Like SAgA, BPI is also composed of the PLP peptide and the LABL peptide, but with a short linker connecting the two peptides. When antigen presenting cells such as dendritic cells interact with T cells, they form an immunological synapse where the cell surface receptors coalesce into a distinct pattern[23,24]. It was previously shown that a BPI molecule called GAD-BPI with glutamic acid decarboxylate (GAD) peptide antigen could effectively colocalize both MHC-II and ICAM-1 on the surface of antigen presenting cells (APCs) from NOD mice compared to a mixture of GAD peptide and LABL peptide[25]. The results suggested that GAD-BPI molecule could bind simultaneously to MHC-II and ICAM-1 on the surface of APCs. As a result, the BPI molecule prevented the aggregation of TCR/MHC-II-Ag

complex from LFA-1/ICAM-1 complex to form clusters for the immunological synapse at the interface between T cell and APC[26]. Blocking the formation of the immunological synapse can alter the differentiation of subpopulation of T cells.

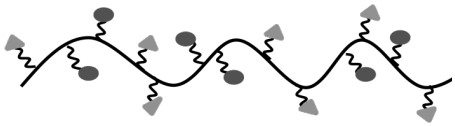
Besides possible cytokine and cellular mechanisms, transport of SAgA molecules may be substantially different than BPI. Previous work on SAgAs reported a range of physical sizes as determined by the HA polymer size and the amount of peptide conjugated. The size of SAgA constructs has typically ranged from 3-10 nm[4]. This size range may facilitate access to different physiological compartments as the largest of these, 10 nm, can potentially be excluded from absorption into systemic circulation and passively drain to the lymphatics[27-29]. It is plausible SAgA molecules reduced lesions in the lungs as a result of longer persistence. Alternatively, active binding to immune cells and transport to secondary lymphoid organs may be a likely mechanism of SAgA efficacy, which could favor smaller, more mobile SAgAs or BPI. Future studies aim to define these potential mechanisms when comparing SAgAs to conventional ASIT.

4.5 Conclusion

Restoring immune tolerance in autoimmune diseases may one day reverse the root cause of disease. ASIT has been used for decades to desensitize patients to allergens, but similar approaches applied to autoimmune diseases have yet to achieve robust clinical success. Traditional allergy desensitization strategies have employed injections, while new approaches have achieved immune tolerance by applying ASIT to mucosal membranes (e.g. Grastek® and Ragwitek®), suggesting the lung may also be a viable target tissue. SAgA molecules exhibiting grafted antigen and a grafted inhibitor of immune cell adhesion may amplify the effect of traditional ASIT. SAgAs with grafted PLP and LABL delivered into the lungs of EAE mice showed increased efficacy based on clinical scoring, weight gain, and incidence of disease compared to the individual components. The cytokine response of splenocytes indicated a shift towards increased

levels of IFN-γ when treated with SAgA compared to the individual components and elevated IL-6 and IL-17 with SAgA compared to PLP. Cytokines, such as IL-10, IL-2, and TNF-α showed no significant difference and yet co-delivery of PLP and LABL using SAgAs or BPI provided a potent ASIT when delivered to the lungs and the distinct mechanisms of these therapeutics merits further exploration.

Table 4.1: Characterization of treatments.

Sample		Molar Ratio of LABL:PLP:HA	Approximate MW (kDa)	D_h in PBS (nm)
Hyaluronic Acid (HA)		0:0:1	16	2.4 ± 1.0
Proteolipid Protein ($PLP_{139-151}$)		0:10:0	1.5	N/A
LABL		10:0:0	1.0	N/A
Soluble Antigen Array (SAgA)		7.5:5.8:1	33	3.7 ± 1.6
Bifunctional Peptide Inhibitor (BPI)		1:1:0	3.0	N/A

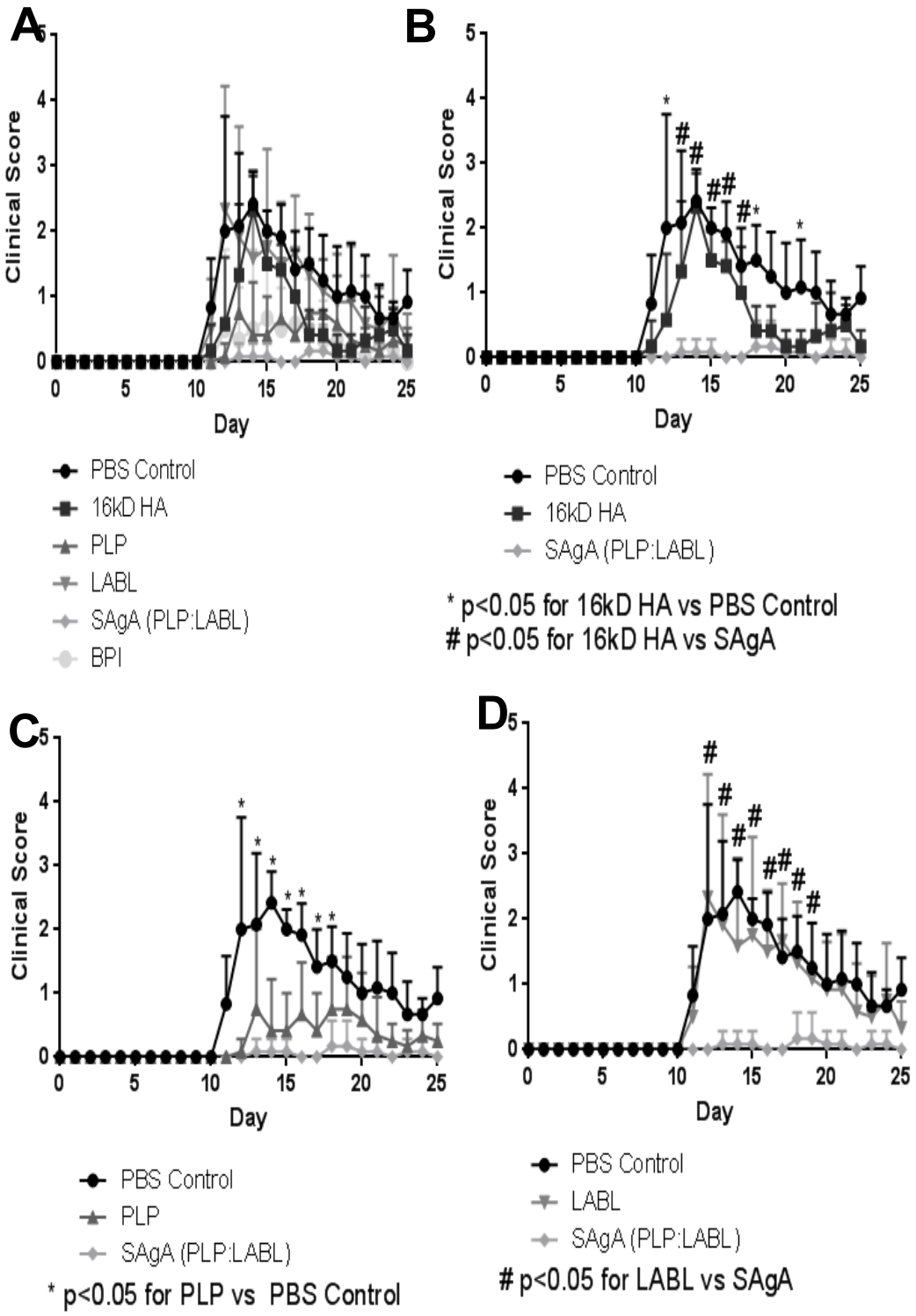


Figure 4.1: Clinical scores throughout the study from each of the 6 groups: PBS control, HA, LABL, PLP, SAgA, and BPI. Panel A depicts all 6 groups; Panel B shows PBS, HA, and SAgA; Panel C represents PBS, PLP, and SAgA; and Panel D depicts PBS, LABL,

and SAgA. Each point is the average of 6 animals with the standard deviation within each group.

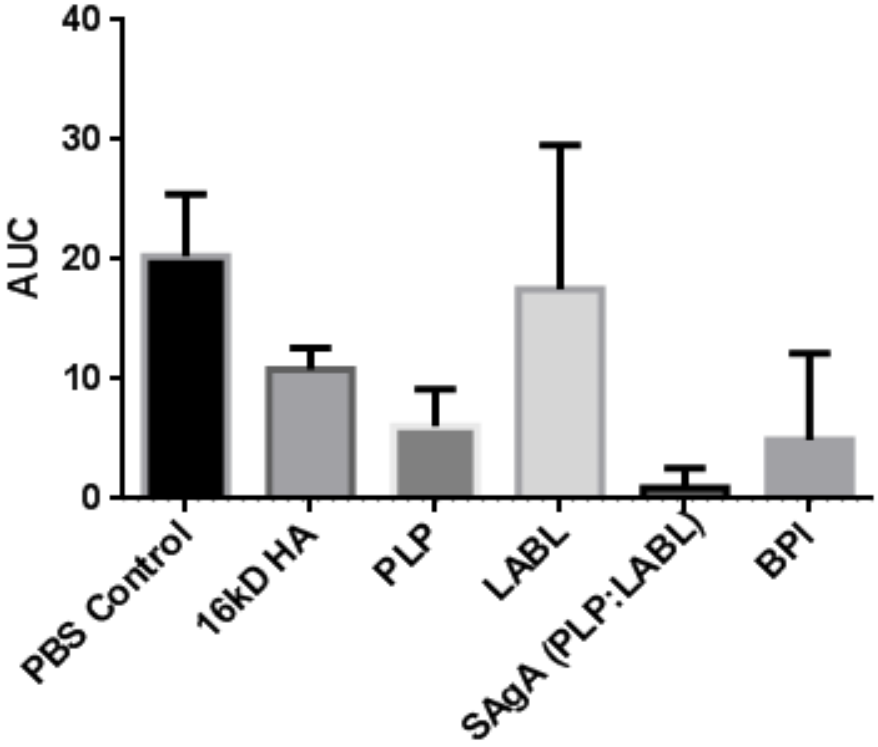


Figure 4.2: Area under the curve (AUC) of clinical scores throughout the study from each of the 6 groups. Each group is the aggregate total of the scores from the 6 animals.

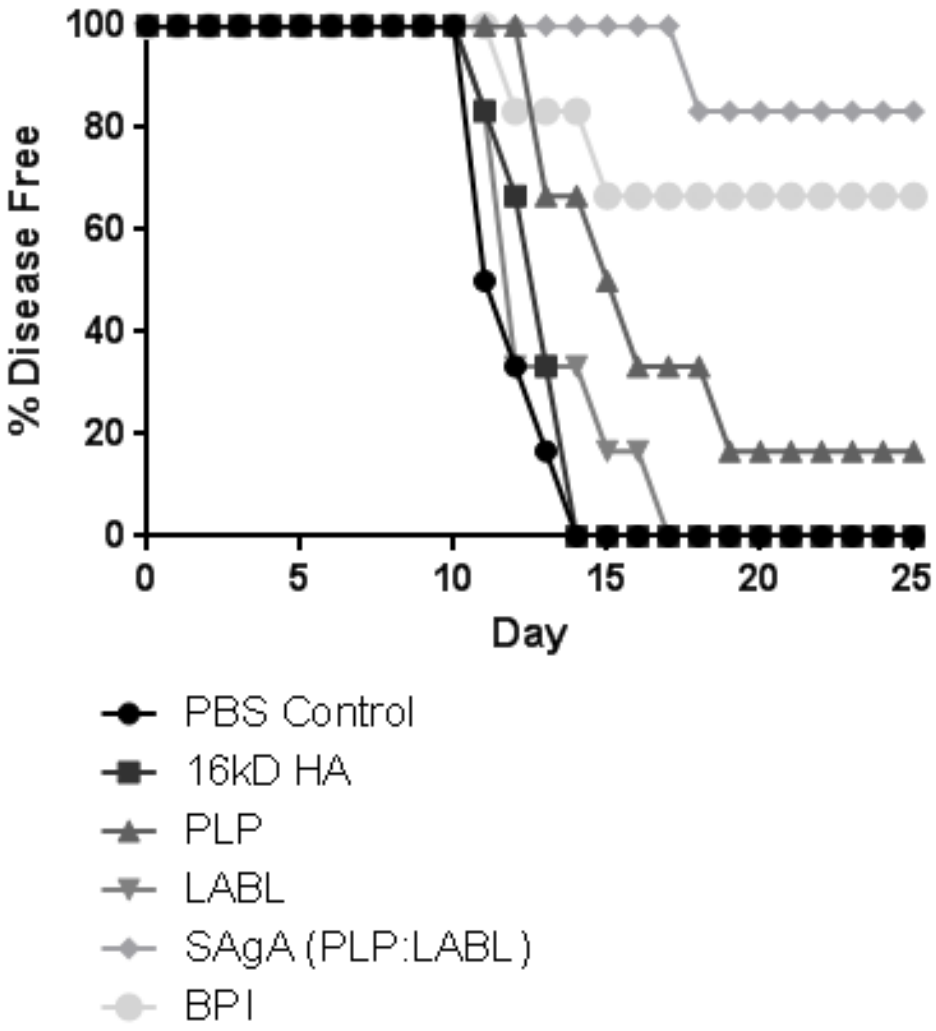


Figure 4.3: Incidence of disease, represented by a clinical score of 1.0, throughout the study from each of the 6 groups (PBS control, HA, LABL, PLP, SAgA, and BPI). Panel A depicts all 6 groups; Panel B shows PBS, HA, and SAgA; Panel C represents PBS, PLP, and SAgA; and Panel D depicts PBS, LABL, and SAgA. Each point is the average of 6 animals with the standard deviation within each group.

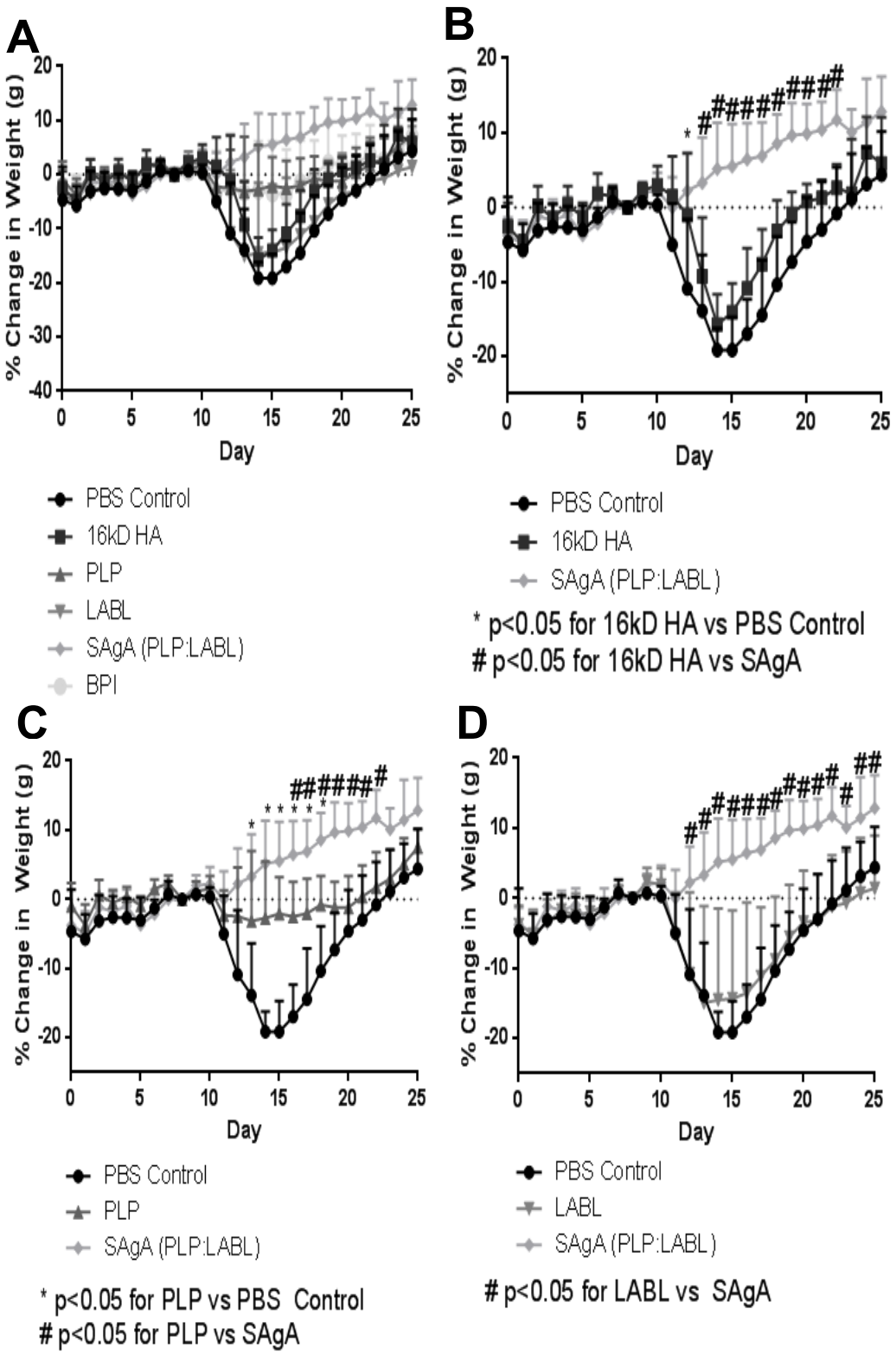


Figure 4.4: Change in weight based on normalization to the initial weight on Day 0 throughout the study with all 6 groups (PBS control, HA, LABL, PLP, SAgA, and BPI). Panel A depicts all 6 groups; Panel B shows PBS, HA, and SAgA; Panel C represents

PBS, PLP, and SAgA; and Panel D depicts PBS, LABL, and SAgA. Each point is the average of 6 animals with the standard deviation within each group.

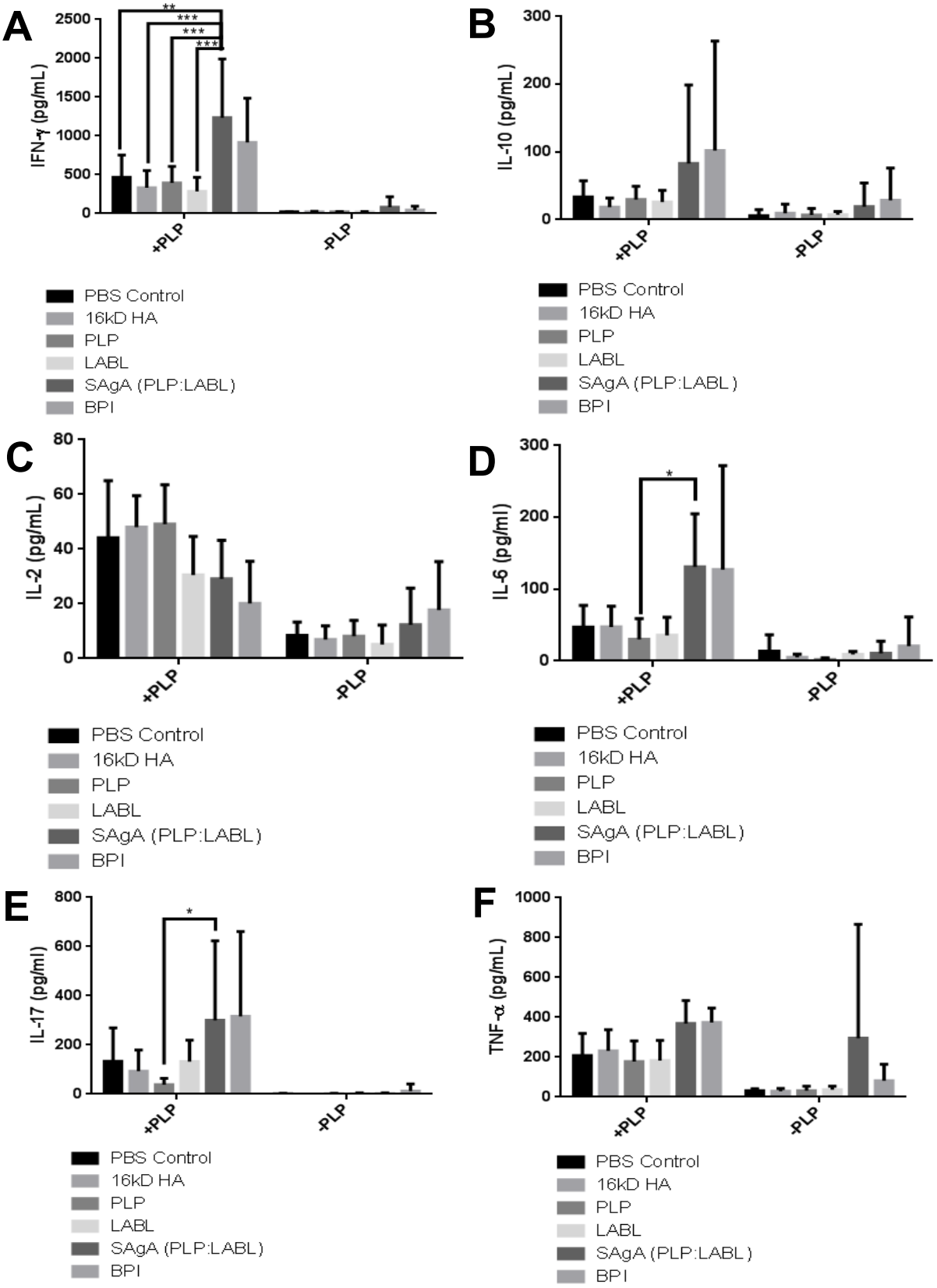


Figure 4.5: Cytokine profiles with IFN-γ in Panel A, IL-6 in Panel B, IL-2 in Panel C, and IL-10 in Panel D.

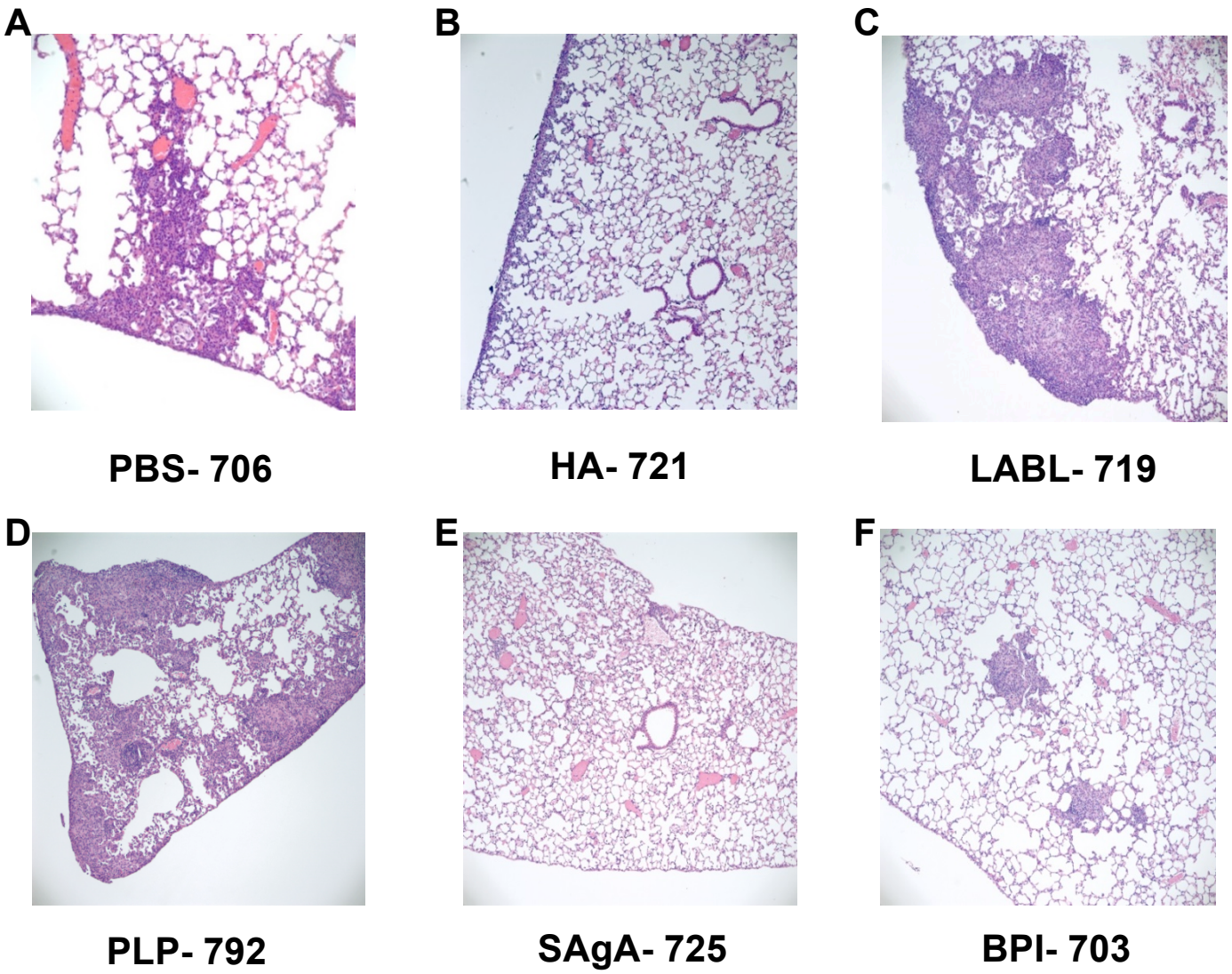


Figure 4.6: Histology of lungs from a mouse from each group. Each image was taken at 100X magnification and was performed after staining and fixing of tissue. Panel A is mouse 706 from the PBS group and shows severe inflammation; B is mouse 721 from the HA group and shows mild inflammation; C is mouse 719 from the LABL group and shows moderate inflammation; D is mouse 792 from the PLP group and shows moderate inflammation; E is mouse 725 from the SAgA group and shows no inflammation; F is mouse 703 from the BPI group and shows mild inflammation.

Table 4.2. Lung histology descriptions and scores. H denotes histiocytes and L denotes lymphocytes with letter coming first being the major cell population. Scores range between 0 and 6, with the higher scores indicating a higher level of inflammation.

	Mouse Number	*Description of Inflammation*	*Granuloma*	*Immune Cells*	*Score*
PBS	704	Mild, focal	No	L and H	1
	706	Severe, nodular and diffuse	Yes	H and L neutrophils	6
	794	No inflammation	No	NA	0
	796	Mild	Yes	H and L	2
	797	Mild, focal	Yes	H	1
	800	Moderate	Yes	H and L	3
16 kD HA	700	Mild, focal	Yes	H and L	1
	710	No inflammation	NA	NA	0
	720	Mild, focal	No	L	1
	721	Mild, focal	No	L	1
	762	Severe, focal and diffuse	Yes	H and L	6
	793	Mild, focal	No	L and H	1
PLP	679	Minimal to mild	No	L	1
	709	Mild, focal	No	L	1
	711	Mild, focal	Yes	H and L	1
	792	Moderate, focal	Yes	H and L	3
	795	No inflammation	NA	NA	0
LABL	701	Mild, focal	Yes	H and L	1
	708	Mild, focal	Yes	H and L	1
	719	Moderate, focal and diffuse	Yes	H and L	4

	723	No inflammation	NA	NA	0
	761	Mild, focal	Yes	H and L	1
	765	Severe, focal and diffuse	Yes	H and L	6
	609	Mild, focal	No	L	1
	713	Mild, focal	Yes	H and L	1
	717	Mild, focal	Yes	H and L	1
SAgA	725	No inflammation	NA	NA	0
	763	Mild, focal	Yes	H and L	1
	799	Mild, focal	Yes	H and L	1
	703	Mild	Yes	H and L	2
	707	Mild, focal	Yes	H and L	1
	712	No inflammation	NA	NA	0
BPI	724	No inflammation	NA	NA	0
	764	Mild, focal	Yes	H and L	1
	798	Mild, focal	Yes	H and L	1

Treatment	Days of Significance for Clinical Scores	Days of Significance for Weights
PBS vs 16 kD HA	12,18,21	12
PBS vs PLP	12-17	13-18
PBS vs LABL	ns	ns
PBS vs SAgA	12-18,20-25	12-23
PBS vs BPI	12-16,18-25	13-19
16 kD HA vs PLP	14,15	14,15
16 kD HA vs LABL	12,18	12
16 kD HA vs SAgA	13-17	13-22
16 kD HA vs BPI	13,14,16	14,15
PLP vs LABL	12-15,17	13-17
PLP vs SAgA	ns	16-22
PLP vs BPI	ns	ns
LABL vs SAgA	12-19	12-25
LABL vs BPI	12-18	13-17
SAgA vs BPI	ns	15,16

Supplementary Table 4.1. Two-way ANOVA results comparing all of the groups' scores and weights to each other.

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Chapter 5:

Subcutaneous or Pulmonary Delivery of Soluble Antigen Arrays to Mitigate Experimental Autoimmune Encephalomyelitis

5.1 Introduction

Antigen-specific immune therapy (ASIT) is an emerging approach for treating autoimmune diseases. The idea of inducing immune tolerance originates from classic approaches to ASIT, such as allergy shots[1]. Unfortunately, allergy shots often have modest efficacy and require long treatment schedules. Introducing antigen to mucosal surfaces may improve efficacy and decrease the amount of time required to induce tolerance in the case of ASIT. For example, comparing subcutaneous injections to sublingual delivery of allergens, the amount of time to induce tolerance is decreased from 3-4 years of injections to 14 months with sublingual therapy[2]. Since the end goal for both ASIT and treatment of autoimmune disease is to induce tolerance, similar methodologies may be effective. In fact, we previously reported improved efficacy when Soluble Antigen Array (SAgA) were applied to the lungs of mice with experimental autoimmune encephalomyelitis (EAE), which is a murine model of multiple sclerosis (MS).

In the autoimmune disease MS, the immune system identifies myelin sheath as 'non-self.' The resulting neurological symptoms stem from myelin damage, resulting in interruptions in signal transduction through axons. One particularly relevant EAE model depicts relapsing-remitting MS, since 88% of people with MS have this particular subtype[3]. In order to portray the relapsing-remitting MS subtype of MS, $PLP_{139-151}$ is the epitope used to induce EAE. The same epitope is then used to tolerize the immune system against EAE. Thus, PLP is one of the three components of SAgA, the other two being LABL and a hyaluorinic acid (HA) backbone to which the peptides are connected. LABL binds to ICAM-1, which disrupts interaction between two immune cells[4].

Previous papers have suggested SAgAs delivered to the lungs via pulmonary instillation (PI) may be an effective EAE therapy but it was unclear why the lungs improved efficacy[5]. The lung may be involved with the immune mechanism or may

provide improved delivery of SAgAs. For example, the 'Hub and Spoke' model states that the lung acts as a hub that instructs T cells where to go. In the case of EAE, T cells would migrate through the lungs before being deployed to the vasculature of the CNS (the spoke)[6,7]. Additionally, the lungs present a large mucosal surface that may contain elements that promote tolerance of inhaled antigen[8]. Finally, the lung periphery provides access to the circulatory system if SAgAs act systemically. Pulmonary delivery significantly decreased EAE clinical scores of SAgA or PLP via PI was reported, suggesting the antigenic epitope was a key component driving tolerance. To explore this further, in this study, mice were either given PLP, SAgA, or HA-PLP, which presents PLP as an array. Additionally, mice were given these treatments either via a subcutaneous injection or as applied to the mucosal surface in the lungs via PI. Clinical scores, mouse weights, cytokine profiles, and lung histology results were compared.

5.2 Materials and Methods

5.2.1 Murine EAE Model

The animals used were female SJL mice at about 4 weeks old at the beginning of the study (Harlan Laboratories, Inc., Indianapolis, IN). The mice were housed in the animal facility at the University of Kansas under regulations set by the Association for Assessment and Accreditation of Laboratory Care and Institutional and Animal Care and Use Committee. On day 0 of the study, mice were subcutaneously injected with a 200 µL of emulsion containing incomplete Freund's Adjuvant (BD Difco Adjuvants, Franklin Lakes, NJ), *Mycobacterium tuberculosis* capsid (BD Difco Adjuvants, Franklin Lakes, NJ), and 200 nMol $PLP_{139-151}$ per animal. They were also given 100 µL of 200 ng of pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) via intraperitoneal injection on days 0 and 2. All treatments were given on days 4, 7, and 10 of the study. The mice were weighed every day of the 26-day study and given clinical scores ranging from 0 – 5 starting from day 7. A clinical score of 0 showed no disease symptoms; 1 –

tail weakness; 2 – hind leg weakness and partial paralysis; 3 – complete paralysis of hind legs (paraplegia); 4 – partial front leg paralysis; and 5 – moribund or complete front leg paralysis.

A group of 6 mice were treated with subcutaneous delivery of phosphate buffered saline (PBS), 200 nMol PLP, HA-PLP, or SAgA (Table 5.1). Similarly, mice were treated via pulmonary instillation of 200 nMol PLP, HA-PLP, or SAgA. HA-PLP and SAgA were given on a 200 nMol PLP-basis. The subcutaneous injections were at a volume of 100 µL and the pulmonary instillation was at a volume of 50 µL. In pulmonary instillation, each mouse was put under 3% isoflurane in an induction chamber. When the animal passed the pinch test, the mouse was suspended on the back via the front teeth, on a board at a 60° angle. The tongue was pulled aside using forceps and a laryngoscope was used to visualize the trachea and 50 µL of treatment was dispelled to the lungs and the mouse was kept at the 60° angle for another minute to allow the liquid to fully enter the lung the mouse was monitored while coming out of anesthesia for irregular breathing. When the mouse was fully awake and breathing has returned to normal, the mouse was returned to its cage.

5.2.2 SAgA Production and Analysis

Hyaluronic acid, 16.9 kD in size, (LifeCore Biomedical, Chaska, MN) was hydrated at 2 mg/mL in pH 5.5 acetate buffer. $PLP_{139-151}$ and LABL (PolyPeptide, Inc., San Diego, CA) were added in a ratio of 4 HA monomer repeats to 1 $PLP_{139-151}$ to 1 LABL. The pH was adjusted to 5.5 with 0.1-N NaOH and 0.1-N HCl. The reaction was left to mix at 400 rpm for 24 hours. The reaction was dialyzed with a membrane molecular weight cutoff of 6,000-8,000 Da against purified water for 24 hours. The dialysis water was changed every 6 hours, then the reaction mixture was frozen down at -80 °C and lyophilized for 3 days.

The SAgAs were dissolved at 1 mg/mL using 0.1-N HCl to be compared against varying concentrations of $PLP_{139-151}$ and LABL (0.0625 – 1 mg/mL, using mobile phase A) via RP-HPLC using a C18 Higgins Analytical PROTO200 5 µm, 200 Å, 250 x 4.6 mm^2 column. Mobile Phase A was made of 95% water, 5% acetonitrile, and 0.1% trifluoroacetic acid. Mobile Phase B was made of 100% acetonitrile and 0.1% trifluoroacetic acid. A gradient used was 100% A from 0 to 15 minutes, 85% A at 15 minutes, 83% A at 20 minutes, 80% A at 33 minutes, 75% A at 42 minutes, 30% A at 43 minutes, and 100% A at 46 minutes until 55 minutes with a total flow rate of 1 mL/min. An injection volume of 50 µL was used and detection occurred at 220 nm.

The hydrodynamic radius of SAgAs was determined using dynamic light scattering via ZetaPALS. The samples were made up at a concentration of 1 mg/mL in filtered (0.45-µm PVDF) PBS. The samples were filtered once again immediately before they were read. Three readings were taken per sample and the average and standard deviation were reported.

5.2.3 Splenocyte Collection

Spleens were collected under sterile conditions within a minute after isoflurane overdose of mice and were immediately placed in RPMI media with 1% Penicillin-Streptomycin (P/S) on ice. The spleens were pushed through a wire mesh using the rubber end of a 1-mL syringe and a petri dish. The cell mixture was transferred to a 15-mL Falcon tube using a 5-mL pipette. The cells were centrifuged for 4.5 minutes at 3500 rpm. After the media was decanted, 1 mL media with serum (RPMI media with 10% fetal bovine serum and 1% P/S) and 3.5 mL Gey's lysis solution was added. The cells were then incubated on ice for 3.5 minutes. Ten mL of media with serum was added, and then they were centrifuged again at 3500 rpm for 4.5 minutes. Five mL media with serum was added and cells were counted using a trypan blue stain and a hemocytometer. The

splenocytes were plated at 10^6 cells/mL with and without 25 μM PLP. The supernatant was collected at 120 hours and analyzed for cytokines.

5.2.4 ELISAs of Cytokines IFN-γ, IL-2, IL-6, IL-10, IL-17, and TNF-α

The cytokine panels included IFN-γ, IL-2, IL-6, IL-10, IL-17, and TNF-α using kits from R and D Systems. A 100-μL aliquot of the supernatant collected from splenocytes at 120 hours was used for IL-2, IL-6, IL-10 and TNF-α. The samples for IFN-γ and IL-17 were diluted 2.5-fold using PBS for final results. The plates were read using a SpectraMax plate reader at an absorbance of 450 nm and 540 nm. The protocol used was designated from R and D Systems.

5.2.5 Lung Histology

After the spleens were excised, the lungs were perfused with 10% buffered formalin and the trachea was tied off using surgical string. The lungs were stored in 10% buffered formalin for 72 hours, then the formalin was exchanged for 70% ethanol for another 72 hours. The surgical string was cut off before the tissue was placed in cassettes in 70% ethanol. The samples were submitted to the pathology department (KIDDRC) at the University of Kansas – Medical Center for embedding in paraffin wax, sectioning, and staining with H&E.

5.3 Results

5.3.1 Characteristics of Test Articles

The characteristics of PLP, HA-PLP, and SAgA were similar to those previously reported (Table 5.1)[4,5,9]. HPLC was used to quantify peptides released from HA at low pH, which was then used to calculate the number or peptides per HA chain. HPLC was also emplyed to estimate the approximate molecular weights. Finally, DLS provided information of the hydrodynamic radii of the different test articles.

5.3.2 EAE Study

Each of the mice was compared to their weights at day 8, which is the point in the study when the mice weighed the most. The percent change in weights was compared for each of the treatments (PLP, HA-PLP, SAgA) given subcutaneously (SC), or given via pulmonary instillation (PI). When PLP was injected SC, the decrease in weight occurred at an earlier time, but the profiles looked very similar. This later onset of weight loss was also seen in the groups where HA-PLP or SAgA (LABL:PLP) were given via PI. When comparing individual treatments, SC delivery led to a greater loss in weight compared to PI. Mice treated with PLP alone had the greatest weight loss. On the other hand, both HA-PLP and SAgA treatments exhibited similar weight profiles, whether given subcutaneously or via PI (Figure 5.1).

Clinical scores trended similarly to weight loss. PI of PLP, HA-PLP, or SAgA (LABL:PLP) seemed to induce lower scores and a later onset of disease in the case of HA-PLP and SAgA treatments. While there was not a difference between any of the subcutaneously treated mice, the treatments of HA-PLP and SAgA appeared to have clinical score profiles that trended lower (Figure 5.2).

The incidence of disease graph showed how many animals in a group of six were affected by the disease, meaning they had a clinical score of 1.5 or higher. Both of the groups treated via PI with HA-PLP or SAgA had more disease-free animals. Mice treated subcutaneously with SAgA or via PI of PLP had just one disease-free animal out of six. Mice in the remaining groups all progressed to scores above 1.5 (Figure 5.3).

Another way to provide an overview of the extent of disease is to take the area under the curve (AUC) of the clinical scores, as shown in figure 5.4. Compared to the PBS-treated control group, the groups treated with subcutaneous HA-PLP, PI HA-PLP, and PI SAgA exhibited the lowest AUCs. Additionally, PI SAgA was lower than SC SAgA, but not significantly lower than HA-PLP given SC or via PI.

5.3.3 Cytokine Panels

After the primary splenocytes were incubated with and without PLP, the following cytokine levels were measured (Figure 5.5): IFN-γ, IL-2, IL-6, IL-10, IL-17, and TNF-α. While there are no significant differences in cytokine levels in IFN-γ, IL-6, and IL10, there were differences in IL-2, IL-17 and TNF-α. IL-2 levels were highest in the group that was treated with PI PLP alone, even compared to the PBS control group. IL-17 levels were also high in the PI PLP group compared to all other treatments, but were not statistically significant when compared to the PBS control group. TNF-α levels, on the other hand, were decreased in the SC HA-PLP group, in comparison to the PBS control group.

5.3.4 Lung Histology

Histological images of the lungs showed that there were sporadic events in the luminal space and tissue thickening. In the mice treated via subcutaneous injection, PLP and SAgA had similar lung histology as the PBS control. The lungs of mice treated via PI presented some evidence of thickening of tissue denoted by dense regions of pink and purple staining. This thickening of the tissue was attributed to inflammation. The mice given PLP via PI showed less thickening of tissue than mice treated with HA-PLP and SAgA via PI. It should be noted, however, that stains indicative of inflammation were sparse and comparisons subjective.

5.4. Discussion

5.4.1 EAE Study

This study compared different molecular formats of antigen epitope delivery via the SC route or via PI. $PLP_{139-151}$ was used to induce the murine model of relapse remitting EAE. Then, ASIT was performed by delivering this same PLP epitope alone, PLP grafted to HA, or as PLP and LABL grafted to HA (SAgA) (Table 5.1). PI of HA-PLP and SAgA caused the least decrease in weights during the peak of disease. The PBS control group showed the greatest weight loss, as expected[4,5,9]. The same trends were

observed in clinical scores. The groups treated with HA-PLP and SAgA via PI exhibited significantly lower clinical scores compared to the PBS control group. When considering the incidence of disease, the PI HA-PLP and PI SAgA groups also had the most disease-free animals (clinical score lower than 1.5). The area under the curve of clinical scores plot, however, showed that the mice treated subcutaneously with HA-PLP also had low overall disease. Thus, data suggested PLP must be delivered on an array to be effective, but PI delivery led to faster and more complete correction.

5.4.2 Cytokine Panels

The cytokines tested in this paper included IFN-γ, IL-2, IL-6, IL-10, IL-17, and TNF-α. IL-10 was the only anti-inflammatory cytokine assessed. The remaining cytokines are typically considered to be pro-inflammatory. Immune responses can be highly complex, however, depending on the number of cytokines tested, tissue assayed, and timing. IFN-γ, IL-6, and IL-10 showed no significant differences. This could be because the spleens were taken at the end of the study, when the PLP-specific T cells would hypothetically be sequestered to the spleen. In this case, splenocytes from animals responding to treatment are still likely to produce high levels of these cytokines, similar to animals exhibiting disease.

IL-2, IL-17, and TNF-α levels expressed significant differences (Figure 5.5). IL-2, IL-17, and TNF-α are traditionally identified with pro-inflammatory cytokines, but IL-17 has been found to act in conjunction with IL-23 to have a regulatory effect. Additionally, IL-2 is known to be represent T cell differentiation, which can make it either a pro- or anti-inflammatory cytokine depending on if regulatory T cells are also differentiating. The PI PLP treated mice had the highest levels of IL-2, but whether this is pro- or anti-inflammatory is yet to be determined. According to Badawi et al., an increase in IL-2 levels seen at a period of remission in EAE may indicate proliferation of regulatory T cells, thus contributing to immune tolerance[10-13]. The levels of IL-2 for the remaining

groups were similar to each other, which suggests limited T cell differentiation, perhaps due to a limited pool of effector cells responding to PLP.

TNF-α is known to induce systemic inflammation, and, thus, is a pro-inflammatory cytokine marker[14,15]. Since TNF-α was significantly reduced in the SC HA-PLP group compared to the PBS control group, it may indicate that this treatment induced tolerance. There was no significant difference with the rest of the groups, but the overall trends within the PI groups may suggest that PLP delivered on an array and co-delivered with LABL may decrease TNF-α levels.

IL-17 levels were high in the splenocytes of the mice treated with PLP via PI. The lowest IL-17 response occurred in the SC SAgA group. In previous studies done by Siahaan, et al., a decrease in IL-17 levels was observed when splenocytes were harvested on day 35. The clinical scores were also low at this time, indicative of remission[10,16,17]. We also observed a decrease in IL-17 levels as EAE mice approached remission. The decrease in IL-17 during remission suggested animals treated with SC SAgA group have fewer Th17 cells, which are thought to play a significant role in the pathogenesis of EAE[18,19].

The increase in IL-2 levels of the splenocytes from mice receiving PLP via PI indicated that PLP plays a role in inducing tolerance of PLP-induced EAE. The splenocytes from the SC SAgA treated mice, however, depicted a decrease in both TNF-α and IL-17 levels. Thus, it is likely that subcutaneously delivered SAgA may have also induced tolerance. Additionally, cytokines can work in conjunction with one another to be either pro- or anti-inflammatory. IL-2 in conjunction with IL-10 can behave in an anti-inflammatory manner, whereas IL-2 with IL-17 may indicate pro-inflammation[20,21]. Since IL-10 levels were not increased in the splenocytes of mice treated with PLP via PI, it may be unlikely that IL-2 was acting in conjunction with IL-10 or cytokine differences were not detectable due to the small population of antigen-specific effector cells. IL-2 and IL-17

levels in splenocytes of mice treated with PLP via PI were both increased, indicating that these two cytokines could work in conjunction with each other to be pro-inflammatory. It is still unclear if these levels are coming from antigen-specific effector cells or the remaining mixed population of the splenocytes.

5.4.3 Lung Histology

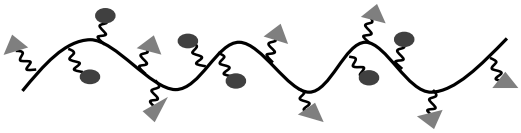
The lung tissue was studied as a result of some signs of inflammation observed previously. Here, the effect of delivering various ASITs via PI or SC injection was assessed. Gross observations suggested there were only very slight differences in histology of the lungs of the different treatment groups. Mice treated SC appeared to have more luminal space while the PI treated mice presented more thickening of lung tissue, which is indicative of inflammation[22]. Since the mice treated via PI had treatment directly applied to the lungs, it is possible that the lung tissue was irritated. In general, stains appeared to be more frequent and more pronounced in groups treated via PI compared to SC injections. The results were subjective, but supported further exploration to determine the immunological mechanism of immunogenicity in the lungs.

5.5. Conclusions

Clinical scores of mice with EAE indicated that HA-PLP and SAgA delivered via PI yielded optimal therapy. The weight loss data supported clinical scores, suggesting PI of HA-PLP and SAgA were the most optimized treatments. On the other hand, cytokine levels derived from mouse splenocytes during disease remission seemed to show that mice treated with PLP via PI and mice treated with SAgA subcutaneously may promote immune tolerance. Since many cytokines can either behave as pro- or anti-inflammatory, future studies should consider a broader set of assays including analysis of IL-23 and flow cytometry to determine effector cell populations. Lastly, the lung histology results suggested inflammation was increased with mice that were treated via PI, however, interpreting histological staining was subjective. Additional studies of lung

epithelial cells and pulmonary lymph nodes will help clarify if immunological mechanisms in the lungs play an important role in the treatment of EAE.

Table 5.1. Depiction of all of the treatments given to mice with EAE.

Sample		Molar Ratio of LABL:PLP:HA	Approximate MW (kDa)	D_h in PBS (nm)
Proteolipid Protein ($PLP_{139-151}$)		0:10:0	1.5	N/A
LABL		N/A	1.0	N/A
HA-PLP		0:24.8:1	55	5.9 ± 1.1
Soluble Antigen Array (SAgA)		24.3:13.5:1	63	6.6 ± 0.5

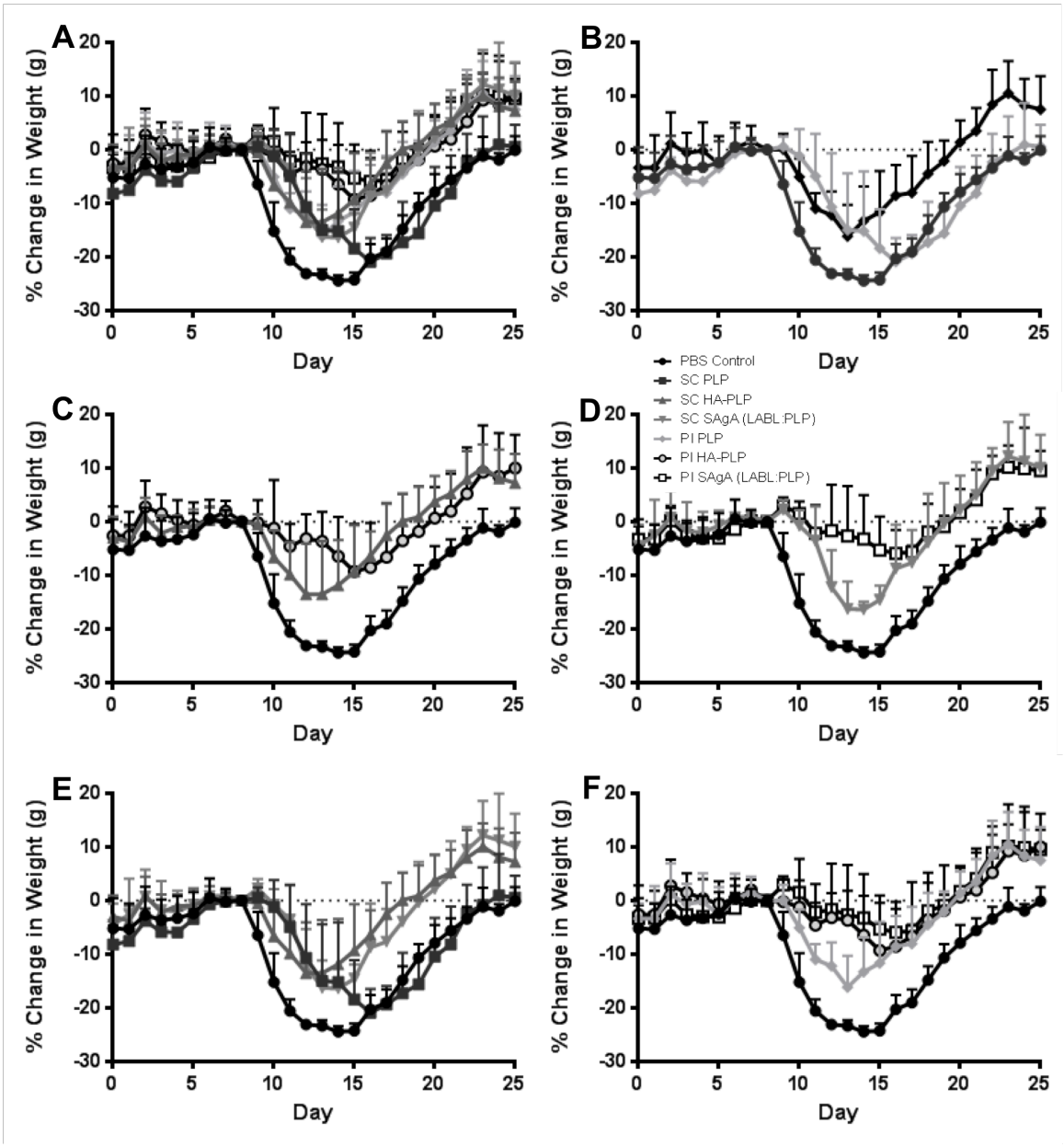


Figure 5.1. Change in weight of all animals as compared to day 8. (A) Overall summary of all groups. (B) Comparison of SC PLP and PI PLP. (C) Comparison of SC HA-PLP and PI HA-PLP. (D) Comparison of SC SAgA (LABL:PLP) and PI SAgA (LABL:PLP). (E) Comparison of SC PLP, SC HA-PLP, and SC SAgA (LABL:PLP). (F) Comparison of PI PLP, PI HA-PLP, and PI SAgA (LABL:PLP). Only top half of error bars are shown, which represent the standard deviation. There are 6 mice per group.

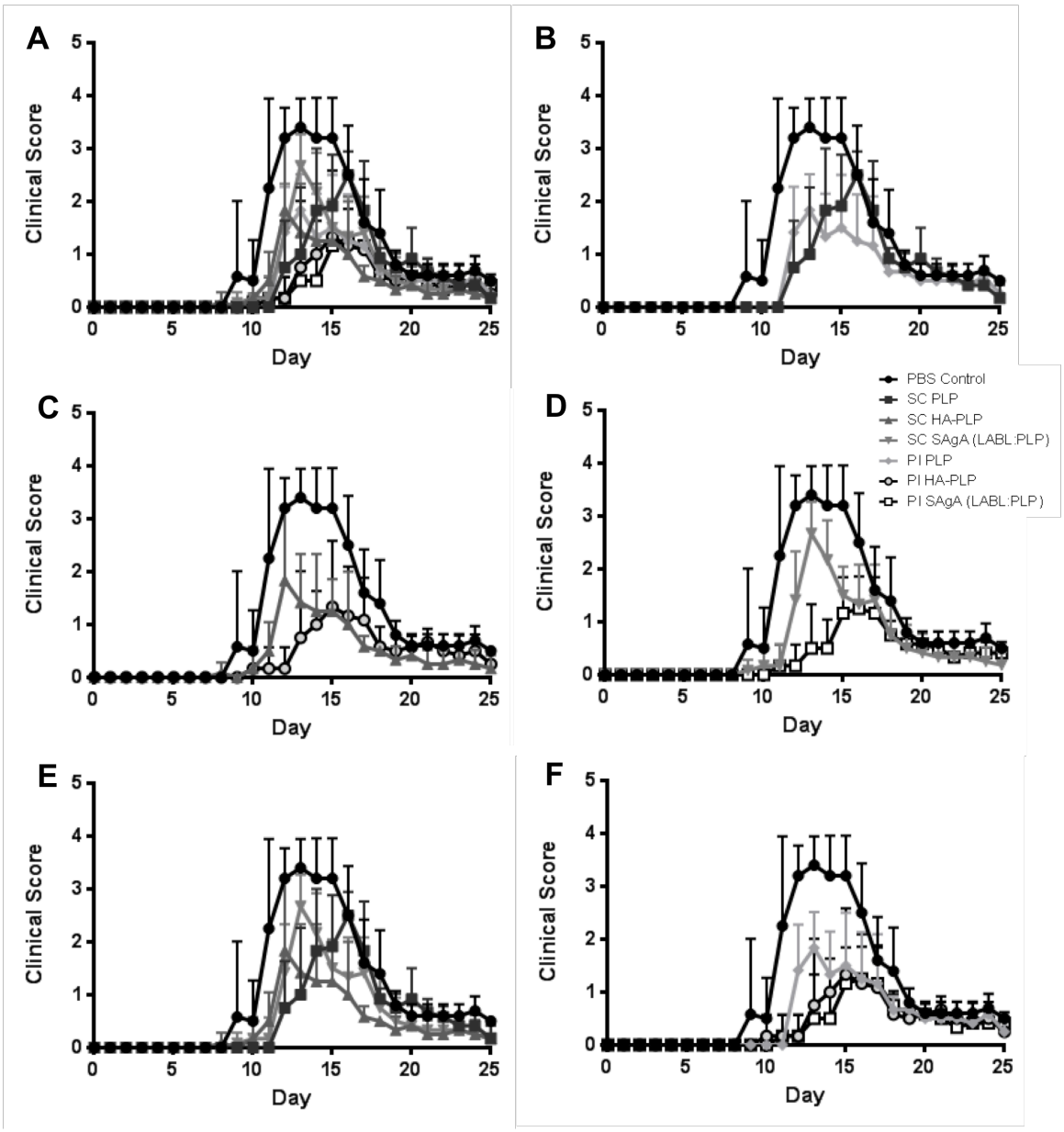


Figure 5.2. Clinical scores of animals throughout the EAE study. (A) Overall summary of all groups. (B) Comparison of SC PLP and PI PLP. (C) Comparison of SC HA-PLP and PI HA-PLP. (D) Comparison of SC SAgA (LABL:PLP) and PI SAgA (LABL:PLP). (E) Comparison of SC PLP, SC HA-PLP, and SC SAgA (LABL:PLP). (F) Comparison of PI PLP, PI HA-PLP, and PI SAgA (LABL:PLP). Only top half of error bars are shown, which represent the standard deviation. There are 6 mice per group.

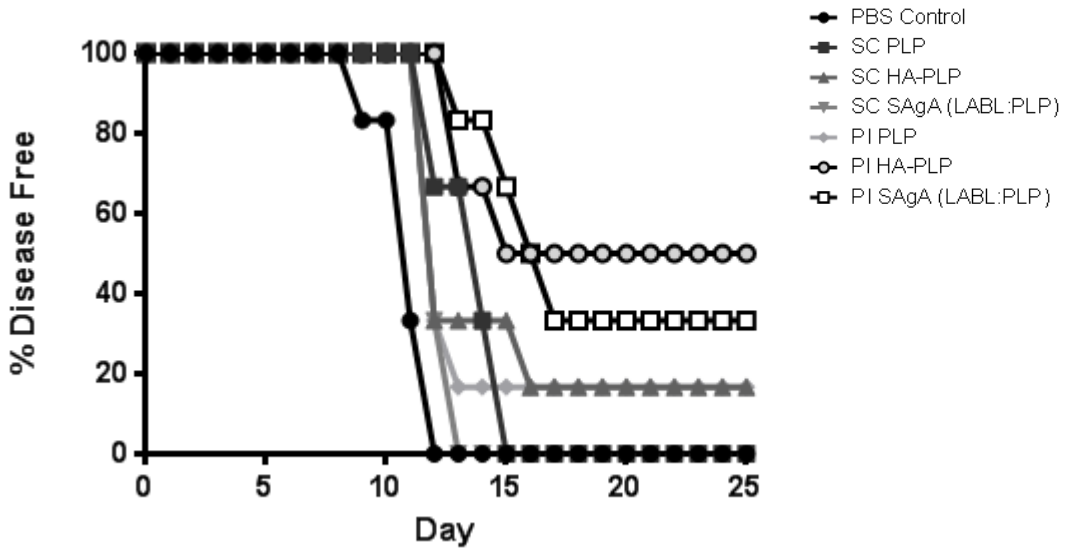


Figure 5.3. Incidence of disease showing how many animals remained disease free (scored 1.5 or higher).

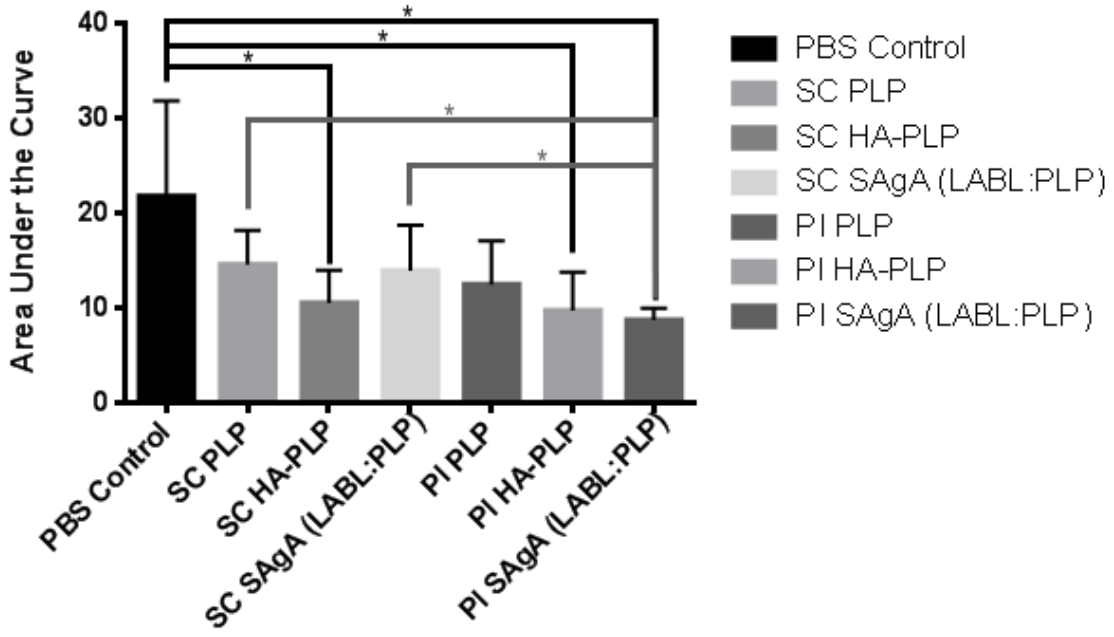


Figure 5.4. Average area under the curve of the clinical scores of each of the mice. Only top half of error bars are shown, which represent the standard deviation. There are 6 mice per group.

* $p<0.05$

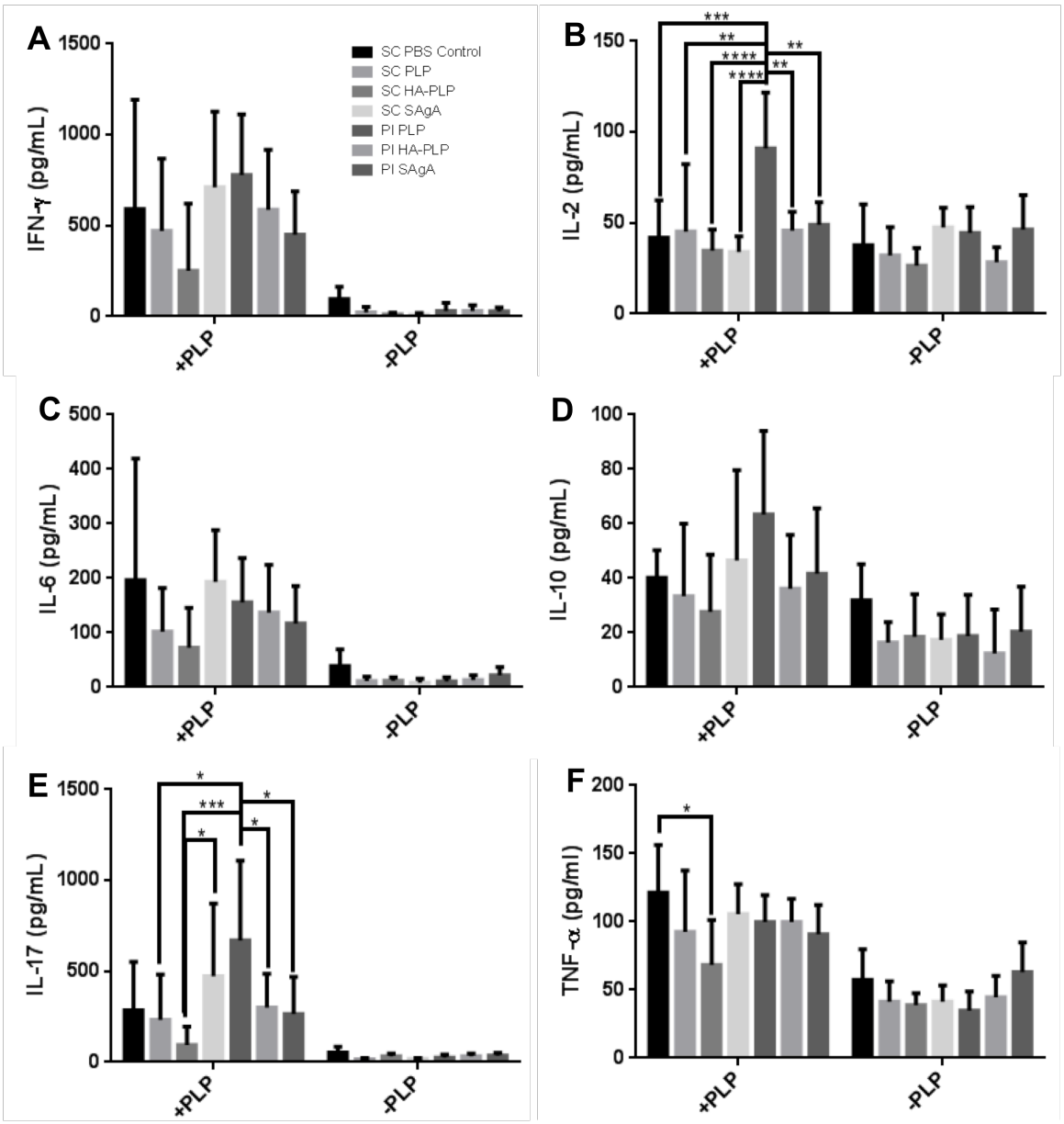


Figure 5.5. Cytokines responses of splenocytes when reintroduced to PLP and without PLP. Cytokines explored are (A) IFN-γ, (B) IL-2, (C) IL-6, (D) IL-10, (E) IL-17, and (F) TNF-α. Only top half of error bars are shown, which represent the standard deviation. There are 6 mice per group.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Treatment	Days of Significance for Clinical Scores	Days of Significance for Weights
PBS Control vs SC PLP	11-15	10-12
PBS Control vs SC HA-PLP	11-18	11-24
PBS Control vs SC SAgA	11, 12, 14-16	10-12, 15-25
PBS Control vs PI PLP	11-16	10-12, 14-18, 22-24
PBS Control vs PI HA-PLP	11-16	10-18, 23-25
PBS Control vs PI SAgA	11-16	10--25
SC PLP vs SC HA-PLP	12, 16, 17	15-23
SC PLP vs SC SAgA	13, 16	16-25
SC PLP vs PI PLP	13, 16	16-23
SC PLP vs PI HA-PLP	14, 16	13, 15-20, 23, 25
SC PLP vs PI SAgA	14, 16	12-23
SC HA-PLP vs SC SAgA	13, 14, 17	ns
SC HA-PLP vs PI PLP	ns	ns
SC HA-PLP vs PI HA-PLP	12	12, 13
SC HA-PLP vs PI SAgA	12, 13	12, 13
SC SAgA vs PI PLP	13, 14	ns
SC SAgA vs PI HA-PLP	12-14	12-14
SC SAgA vs PI SAgA	12-14	12-15
PI PLP vs PI HA-PLP	12, 13	12, 13
PI PLP vs PI SAgA	12-14	12-14
PI HA-PLP vs PI SAgA	ns	ns

Supplemental Figure 1. Statistics for clinical scores and change in weights data.

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Chapter 6:

Conclusions and Future Directions

6.1 Conclusions

The optimal way to deliver SAgAs was explored in this thesis. SAgAs were developed to include a targeting portion to the therapy, instead of global immunosuppression, which occurs with all other existing treatments. The co-delivery of PLP and LABL has been found to be effective with BPI, so this technology was used to display these peptides in an array, as there is evidence that receptors tend to cluster on cells. The three major studies explored in this work are listed here: (1) finding the optimal method in which to administer SAgA by varying dose schedule, dose, amount, and routes of administration; (2) further exploring the components of SAgA via pulmonary delivery and observing the cytokine profiles of splenocytes re-introduced to the antigenic PLP; and (3) studying the necessity to deliver PLP as an array and to co-deliver PLP with LABL.

In chapter 3, SAgA treatment schedule, dose, injection volume, and routes of administration were explored in order to ameliorate EAE symptoms. SAgA treatment schedule must be such that the dose must be given before symptoms are fully realized. Decreasing the dose of SAgA to 50 nMol PLP was not as effective as the typical 200 nMol dose. Decreasing the injection volume to 20 µL was not as efficacious as the 100 µL injection volume, as the lower volume likely had limited exposure.

Routes of administration were explored as well to show that pulmonary instillation decreased clinical scores the most. While the initial goal was to find a way to deliver SAgAs to the lymph nodes, no significant differences were seen with the first set of administration sites (upper and lower SC, upper and lower IM, and IP), as they all showed similar decreases in clinical scores. Additionally, delivering SAgAs IV was also similar to the upper SC injection, suggesting that the upper SC injection was also delivering SAgAs systemically. Since pulmonary instillation was the best route of administration of SAgAs, a ‘Hub and Spoke’ model of the lungs was used to explain this

further. In this model, T cells were licensed in the lung, so it is possible that the pulmonary delivery allowed SAgAs to interact with the T cells more either within the lung or via the lymphatics of the lung.

In chapter 4, the individual components of SAgA were further explored via pulmonary instillation. SAgAs were once again found to decrease the clinical scores the most and also showed weight gain. When PLP was also delivered via PI, the clinical scores were decreased, but there was still weight loss in the mice. As a result, the cytokine levels were studied to determine if immune tolerance was achieved. When the splenocytes of mice treated with SAgA were reintroduced to PLP, the cytokine responses showed a significant increase in IFN-γ, IL-6, and IL-17 compared to mice treated with PLP. Since there were no differences in TNF-α, IL-2, or IL-10, but there were differences in vivo, there may need to be additional testing done. A lot of the comparisons made to literature showed that the time point at which the splenocytes are taken (during remission or relapse), the time point at which the cytokines are collected after reintroduction to PLP in vitro (48 hours, 96 hours, 120 hours), and the tissues harvested will give varying cytokine responses. Since it was still difficult to determine if tolerance induction occurred with PLP-treated mice or SAgA-treated mice, further exploration of the effects of PLP, HA-PLP, and SAgA was carried out in chapter 5. The lungs of mice that were treated via SC and PI were also compared via histology.

The clinical scores indicated that HA-PLP and SAgA treatments delivered via PI decreased scores the most. The change in weight data supported the clinical scores, signifying that PI of HA-PLP and SAgA were the optimal treatments. The cytokine results, on the other hand, showed that mice treated with PLP via PI and SAgA treated SC may induce tolerance. This is based on the increased IL-2 levels for the splenocytes of mice treated with PLP via PI and the decreased TNF-α and IL-17 levels in the splenocytes of mice treated with SAgA SC. Lung histology, on the other hand, depicted

that the mice treated via PI showed signs of increased inflammation, which could be because the lung tissue interacted directly with the treatments. There needs to be further research done to study the result of multiple cytokines interacting with one another, considering individual cytokines that may be either pro- or anti-inflammatory can be the opposite when expressed in conjunction with another cytokine. Such is the case with the interaction between IL-2 and IL-10. IL-2 was known to be a pro-inflammatory cytokine, but IL-10 is an anti-inflammatory cytokine. In conjunction, IL-2 and IL-10 work together to increase regulatory T cells, so they seem to work in an anti-inflammatory manner. On the other hand, IL-2 and IL-17 are known to indicate an increase in Th17 cells, which are known to be pro-inflammatory. In this study, since there was no increase in IL-10, it is unlikely that IL-2 was working in conjunction with IL-10 to decrease inflammation. On the other hand, there was an increase in IL-2 and IL-17 levels of mice treated with PLP via PI, so there may be indications of inflammation, as a result.

6.2 Future Directions

To further explore the mechanism of action of SAgAs, there should be a study trafficking SAgAs from the lungs. Some preliminary studies were done in trying to track a conjugate of similar molecular weight HA using a near IR dye, IR-820 (**Figure 6.1**). HA-IR820 was injected in the upper SC position of the mouse, behind the shoulder blades. IR-820 was chosen because it is outside the range of skin autofluorescence. The drawback of using this method was that a clear draining was never found. Since the injection drained over the axillary lymph nodes, it was difficult to view the signal coming from the lymph nodes themselves. Additionally, since the new route of administration to explore is pulmonary instillation, the dye cannot be used anymore, since the fluorescence intensity would be very low as the signal from IR-820 cannot penetrate through the lungs, the rib cage, and the epidermal layer. Radio-labeled HA has been further explored in work done by Christopher Kuehl. Radio-labeled SAgA is an option,

but each of the separate components would also have to be labeled so they can individually be tracked through the body of the mouse. This work would be a separate dissertation all on its own.

Additional future in vitro work can be done to help determine whether the primary splenocytes of treated mice would still be pro-inflammatory when re-introduced to PLP. This in vitro work was carried out in the chapters 4 and 5. Lastly, to fully optimize the delivery of SAgAs, it would be imperative to study SAgA treatment schedule, amount, and injection volume via pulmonary instillation.

While in vitro work was completed in chapter 4, there were many differences found between this study and what is found in literature. No one protocol is present to standardize the testing for inducing tolerance. Ideally, a protocol should be established in order to be able to compare splenocyte cytokine levels across different labs in various parts of the world. The splenocytes should be taken at one time point, the cytokines should be ready at one time point, and corresponding tissues should be taken. Lungs should also be re-examined to confirm what the histology results have shown for this study.

Chapter 5 included more experiments with splenocytes and lung histology, but there is more information that other cytokines and a combination of cytokines can provide. So far, we have found a link between IL-2 and IL-17, but IL-23 should also be studied considering it is another cytokine that works in conjunction with IL-2 as a pro-inflammatory cytokine in multiple sclerosis. Others in the group are working toward finding the effector cell populations via antibody labeling and labeling with fluorescent PLP in these mixed splenocyte populations. Using this method to identify effector cells and ELISpot as an analytical tool may indicate other signs of tolerance induction in EAE. Additionally, the pulmonary lymph nodes and lung tissue should be further assayed to determine signs of inflammation. ELISpot can be developed to determine the different

cytokines expressed for these tissues as there will be far fewer cells from the lungs and lymph nodes in comparison to the spleen. More assays need to be run to properly identify if tolerance has been induced in mice with EAE.

SAgAs must still be further explored by changing these aspects of the molecule: peptide density, peptide ratio (LABL:PLP ratio), and size of backbone. The existing chemistry does not allow for a the differences in peptide density and ratio to be controllable, but click chemistry has been implemented in making “clickable” SAgAs with the hope that the molecular dynamics of SAgA can be further explored by fellow Berkland lab members. All three must be tested in vivo, but studying the binding kinetics of labeled “clickable” SAgAs with varied peptide density and ratio against Raji B cells or splenocytes will be interesting to compare to the data the Berkland lab has accumulated with existing SAgAs.

The use of clinical scores for EAE studies is very subjective and other ways of analyzing the level of paralysis may also be useful for the future. The use of MRI to find the number of lesions present in brain and spinal cord tissue would be closer to what is used in clinical settings with humans to determine the progression of the disease. Furthermore, a balance beam test including a harness to hold up mice with increased paralysis could also be implemented in better assessing the level of paralysis. These assessments could hopefully pinpoint the level of disease progression in later studies to better discriminate which changes in treatment will be most effective.

In continuing to bridge the gap between treating mice and humans, pulmonary instillation will need to be improved upon. There can be many problems with trying to have a human inhale liquid. A possibility is to make SAgAs into a dry, inhalable product, but much more research needs to be done to optimize a way to make SAgAs inhalable. Lastly, SAgAs can also be expanded to treat other autoimmune diseases once the disease-specific antigens and peptides are found. The applications for a tolerance-

inducing treatment for autoimmune diseases, including multiple sclerosis, using SAgAs are endless.

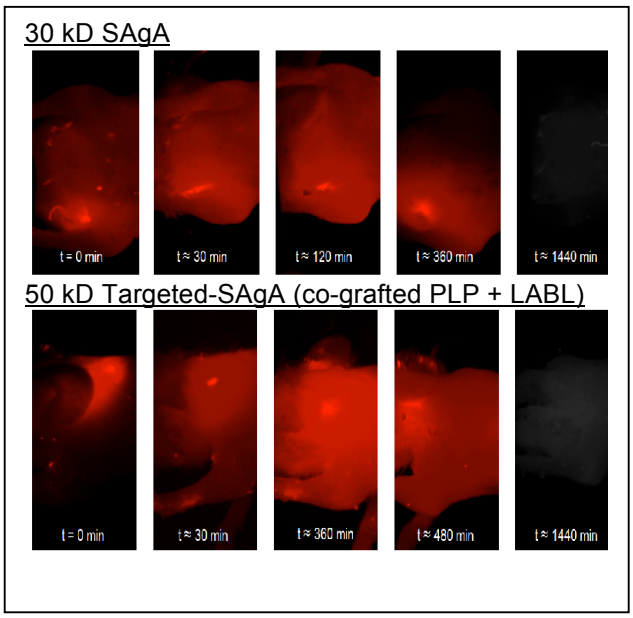

Figure 6.1. *In vivo* imaging of 30 kD HA and 50 kD SAgA with IR-820. The mouse ear and forelimb can be seen in most images. Darkened images means complete drainage.