Co-Delivery of Immunomodulators and Autoantigen as Antigen-Specific Immunotherapy for the Treatment of a Murine Model of Multiple Sclerosis

By

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Abstract

Multiple Sclerosis (MS) is an autoimmune disease characterized by the breakdown of immune tolerance towards autoantigen in the myelin sheath surrounding the neurons. Current therapies for MS and other autoimmune diseases focus on treating the symptoms and not the cause of the disease. A major setback in improving current therapeutics for autoimmunity is the lack of antigen specificity. Successful antigen-specific immunotherapy (ASIT) would allow for improved treatment of autoimmune diseases. This thesis investigates the creation of ASIT for autoimmunity through the co-delivery of an immunomodulator and autoantigen. First, immunomodulatory peptides targeting the B7 costimulatory pathway and cell adhesion were tested for their ability to suppress a murine model of MS, experimental autoimmune encephalomyelitis (EAE), utilizing a co-delivery vehicle developed in our lab, soluble antigen arrays. Peptides targeting different surface markers were all found to suppress EAE when co-delivered with autoantigen, demonstrating the ability of different immunomodulators to create effective ASIT. Expanding upon the idea of combinational ASIT, eleven different small molecules immunomodulators were screened for properties indicative of autoimmune suppression in an antigen-specific splenocyte system. This screen revealed that several compounds, most notably dexamethasone, had the ability to skew the antigen-specific immune response towards autoimmune suppression. The ability of dexamethasone to act as an effective immunomodulator in ASIT for autoimmunity was confirmed *in vivo* in the treatment of EAE. Co-delivery of dexamethasone and autoantigen in an oil-in-water emulsion, incomplete Freund's adjuvant, was found to suppress EAE and shift the immune response. Overall, the results presented dissertation provide evidence for the successful creation of ASIT for autoimmunity by combining immunomodulator and autoantigen.

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Chapter I: Introduction

1. Introduction to Antigen-Specific Immunotherapy

Antigen-specific immunotherapy (ASIT) has been used in the clinic for over a century to induce antigen-specific immune responses. Vaccines were the first modern successful antigen-specific immunotherapies, utilizing disease-causing antigens in order to induce prophylactic protective immune responses against specific foreign pathogens. Treatments with specific allergy-inducing antigens have also been useful for the induction of antigen-specific immune tolerance for allergy desensitization. Clinical treatment of autoimmune diseases, however, still relies primarily on global immune suppression through the use of potent small molecule immunomodulators. Within the last decade, scientists have begun to explore combinations of immunomodulators and autoantigens in the hope of creating effective ASIT for the treatment of autoimmune diseases; a strategy that could substantially improve clinical outcomes without compromising the entire immune system.

One of the most successful strategies in ASIT for inducing immune tolerance has been the use of hyposensitization therapy in the treatment of allergies. Hyposensitization therapy has been used since the early 1900s as a means to desensitize patients to specific allergens [1]. In the seminal papers published by Noon [2] and Freeman [3] in 1911, pollen extracts were injected subcutaneously using an increasing-dose schedule in order to relieve symptoms from grass pollen allergy and hay fever [1]. The current ‘gold standard’ for hyposensitization therapy is surprisingly similar to these techniques described over a century ago [1]. Although hyposensitization for allergies has been effective in many cases, several disadvantages have yet to be remedied. The dosing schedule is often difficult for patients to complete due to the frequency and length of the therapy [1]. The majority of hyposensitization therapy is given via subcutaneous injections and needs to be administered by a trained professional over a period of

years [1, 4]. Sublingual ASIT may ultimately increase treatment convenience; however, the most important consideration, safety, may remain an issue [4]. Unfortunately, in some cases, hyposensitization therapy can become life threatening as anaphylaxis can occur following treatment of severe allergies, reinforcing the requirement for administration by a trained professional in a clinical setting [1]. Additionally, the mechanisms whereby hyposensitization therapy induces therapeutic immune tolerance or anaphylactic shock are still not completely understood [5].

Using approaches similar to allergen immunotherapies, ASIT for the treatment of autoimmune diseases using only disease-causing autoantigen has been explored with minimal clinical success. Although these therapies often work in animal models; translation to humans has not shown the same level of efficacy [6-8]. Efforts to induce tolerance in autoimmune patients often use repeat administration of low doses of autoantigen or altered peptide ligands, but thus far, these approaches have suffered from poor long term clinical effectiveness and variable outcomes [6, 9, 10].

Most of the currently approved autoimmune therapies are immunomodulators; the majority of these immunomodulators non-specifically cause immune suppression (i.e., immunosuppressants). As our understanding of immunology has improved, many therapeutic molecules once thought to act as specific immunosuppressants have recently been shown to have multiple mechanisms of action with numerous downstream effects. For example, rapamycin (Sirolimus) has traditionally been considered an immunosuppressant drug; however, recently it has been discovered that the mammalian target of rapamycin (mTOR) pathway is essential in maintaining the balance between tolerance and inflammation [11]. Immunomodulation in the treatment of autoimmunity, therefore, extends far beyond immunosuppression and can involve shifting the immune response towards tolerance through a variety of mechanisms (**Table 1**). Unfortunately, the lack of antigen-

specificity in immunomodulation can lead to undesired side-effects and potentially increase the risk of opportunistic infections in patients taking these immunosuppressive therapies.

One promising strategy in the creation of ASIT for autoimmunity is combination therapy of antigen and immunomodulator. This strategy mimics the successful "antigen-adjuvant" model used in the creation of vaccines. Adjuvants are immunomodulators used in vaccines to enhance the antigen-specific immune response, increasing the potency of the vaccine. Applying this paradigm for treating autoimmune disease, the combination of antigen and immunomodulator may be able to direct the immune response toward tolerance to autoantigen.

This review highlights recent work combining immunomodulators with autoantigen either by co-administration or co-delivery to induce tolerance in autoimmune disease. We present a thorough background on the immunological processes involved in autoimmunity and tolerance, along with an examination of currently approved therapies. Recent experimental work utilizing co-administration and co-delivery techniques, combining antigen and immunomodulator, have shown exciting new promise in autoimmune therapy. ASIT combination therapies have also shown promise in the clinic. With the recent advances in ASIT, the potential to induce antigen-specific tolerance to treat, prevent, or possibly cure a subset of autoimmune diseases in humans may be on the horizon.

2. Introduction to Autoimmune Diseases

2.1. Immune tolerance and Regulatory Responses

The protective response of the immune system is deeply rooted in the selective recognition of foreign substances, or non-self-antigens, and the absence of a reaction to native antigens; the latter can be defined as immunological self-tolerance. The loss of this tolerance to self-antigens may

result in an immune response directed towards ‘self’ and is defined as an autoimmune response. While the origin of many autoimmune diseases still remains unclear, it is thought that a lapse in tolerance to autoantigens is a key step in the progression of the autoimmune response [12]. In order to understand how autoimmune diseases may develop in an individual, it is important to first assess the ways in which the body maintains tolerance toward autoantigens. The processes through which the immune system attempts to achieve and maintain tolerance toward autoantigens can be classified into two categories: central and peripheral.

Central tolerance involves the presentation of autoantigen to T-cells and B-cells in the thymus and bone marrow. This process is commonly referred to as negative selection and includes inducing apoptosis in developing lymphocytes which may recognize autoantigens or preventing their expansion and release into systemic circulation. Inevitably, some lymphocytes that recognize autoantigens are able to bypass the mechanism of central tolerance [13]. Fortunately, the immune system contains a variety of mechanisms to prevent activation of these potentially auto-reactive lymphocytes in peripheral tissue, known as peripheral tolerance. These mechanisms include physical separation of auto-reactive T-cells from cells presenting autoantigens via the major histocompatibility complex (MHC) [14, 15]. Naïve T-cells are contained primarily to lymphoid peripheral tissues and blood, and as a result their encounters with autoantigen presentation by non-lymphoid tissue cells are limited in healthy individuals [14]. In addition to antigen presentation via MHC restriction, T-cell activation requires the presence of surface expressed secondary context signaling (co-stimulatory) receptors, examples include CD80 (B7-1), CD86 (B7-2), CD40L, CD70, OX40L, and many others [16, 17]. Failure to provide the appropriate stimulatory context signals may result in functional inactivation of the lymphocyte, known as anergy. Besides these co-stimulatory signals, secondary context receptors exist which are capable of inducing anergy in T-

cells, also known as ‘co-inhibitory’ receptors, and include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) [16]. Ligation of these receptors has been shown to inhibit T-cell activation [18]. Conversely, mice lacking the co-inhibitory receptor CTLA-4 develop lymphoproliferative disorders leading to death, suggesting a highly dependent regulatory component of these receptors [19]. The combination of these factors help support peripheral tolerance to maintain T-cell-dependent self-tolerance.

The immune system also regulates antigen presentation in order to control peripheral T-cell responses. In the periphery, antigen presenting cells (APCs), particularly dendritic cells (DCs), are major contributors to the initiation and regulation of downstream immune responses. In addition to antigen uptake, processing, and presentation capability, DCs express a variety of co-stimulatory and co-inhibitory receptors and are responsive to their local external environment. For example, DCs can respond to signals elicited by pattern recognition receptors (PRRs) binding pathogen associated molecular patterns (PAMPs) [20]. Encounters with many PAMPs can result in up-regulation of co-stimulatory surface receptors, overexpression of MHC, and secretion of inflammatory cytokines; a microenvironment that can stimulate activation of naïve T-cells [20, 21]. It is important to note that in the absence of these inflammatory signals, immature DCs can present antigen and induce tolerance in naïve T-cells, providing another means for regulation of autoimmune responses [14].

A third mechanism of peripheral tolerance is the presence of regulatory T-cells (Tregs). Tregs suppress immune responses in an antigen-specific manner through cytokine secretion, metabolic disruption, and alteration of DC function [22]. It has been shown that secretion of cytokines such as interleukin (IL)-10, transforming growth factor (TGF)-β, and IL-35 play a role in the suppression of immune response; however, the importance of these cytokines in the overall

function of Tregs is still a point of debate [22]. Furthermore, it is hypothesized that Treg populations are capable of inducing apoptosis through deprivation of IL-2, a pro-inflammatory cytokine, due to the high expression of CD25, although the mechanisms are still not yet understood [22]. In addition to the previously mentioned influences on the local environment, it is believed that regulatory T-cells also act to alter the function of DCs upon antigen-MHC recognition and CTLA-4 ligation. Studies indicate that Tregs are capable of up-regulating the expression of indoleamine 2, 3-dioxygenase (IDO) in DCs, an enzyme that has been found to limit the inflammatory response and induce a tolerogenic response [23]. Additionally, studies have indicated that Treg interactions with DCs may downregulate the expression of B7 (CD80/CD86) limiting DC function in activating T-cells [22].

2.2 Immunology of Autoimmunity

In general, autoimmune diseases develop upon failure of the numerous regulatory pathways mentioned previously; however, ongoing studies are continuously evaluating and exploring new mechanisms whereby self-tolerance is disrupted. Breakdown of tolerance toward autoantigen is often thought to be a result of both genetic and environmental risk factors, including exposure to infection by particular pathogens [24]. Multiple hypotheses have been generated to explain the downstream processes by which immune responses against autoantigen may occur upon exposure to an infectious pathogen including molecular mimicry of endogenous protein antigens, epitope spreading, and bystander activation; however, the exact mechanisms whereby autoimmune disease develops are still not well understood [24].

2.3 Autoimmune Diseases

There are currently over 80 autoimmune diseases identified by the National Institute of Allergy and Infectious Diseases (NIAID) affecting an estimated 20 million Americans [25]. Some of the

most common autoimmune diseases include type 1 diabetes (T1D), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease, psoriasis, and multiple sclerosis (MS). The discovery of a disease causing antigen or epitope is vital to the development of ASIT for autoimmunity; however, identifying such antigens is not a simple task, particularly in systemic autoimmune diseases such as SLE, for which there may be multiple antigenic targets [26]. The majority of current research in ASIT is focused on RA, T1D, and MS as they all have robust animal models, allowing for a greater understanding of autoimmune pathogenesis and the identification of disease-causing autoantigens.

RA is typified by infiltration of the synovium by $CD4^+$ T-cells, B-cells, and macrophages resulting in inflammation in joints. In recent years, the focus of RA pathogenesis has shifted to the study of autoantibodies including anti-IgG rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs), as these autoantibodies have been found to reliably predict disease progression [27]. Further research is required to determine the relevance of these autoantibodies and others to subsets of RA patients and disease progression.

Recently, disease-specific targets for the treatment of T1D have also been identified including preproinsulin (PPI), glutamic acid decarboxylase (GAD65), and heat shock protein 60 (HSP60) [28]. T1D involves the destruction of insulin-producing pancreatic β-cells, resulting in the loss of the body's ability to produce insulin and failure to control blood glucose levels. As such, clinical studies are commonly performed in early onset T1D patients in order to retain β-cell function and provide the greatest benefit to the patient. Each of these antigens has been identified to play a role in the non-obese diabetic (NOD) mouse model for T1D and have recently been explored in clinical trials for antigen-specific therapies [29-31].

Similarly, potential disease causing autoantigens have been identified in MS including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin associated glycoprotein (MAG) [32]. MS is characterized by inflammation of the central nervous system (CNS) due to immune cell mediated degradation of myelin proteins, resulting in neurological complications. In the most common form of MS, symptoms follow a relapsing-remitting form, and these symptoms may vary from one relapse period to another depending upon the location of the CNS inflammation [33]. A commonly employed animal model for MS is experimental autoimmune encephalomyelitis (EAE), which is usually induced in healthy mice by vaccination with MBP, MOG, or PLP autoantigens, allowing for insight into the pathogenesis of demyelinating autoimmune diseases [34].

3 Current therapies for autoimmunity

Although autoimmune diseases are diverse in both cause and progression, most of the current therapies fall into a few distinct categories; general immunosuppressants, mobility and transport inhibitors, immune cell activation inhibitors, and antigen mimics (shown in **Figure 1**). The downfall of the majority of current autoimmune therapies is the lack of antigen specificity. Many therapies inhibit or modify the global immune response hindering the patient's ability to fight off foreign pathogens. In order to decrease unwanted side effects and increase efficacy, treatments that induce antigen-specific tolerance are needed for autoimmune diseases. Recent advances in combinational ASIT may hold the key to improved therapeutics, and will be discussed in a later section.

3.1 General Immunosuppressants

Autoimmune diseases have traditionally used immunosuppressant medications that globally suppress the immune response. Immunosuppressants are highly effective for many patients and therefore remain the current "gold standard" of autoimmunity treatment [35]. In many immunosuppressant therapies the benefits can be counterbalanced by toxicity or severe adverse events. In fact, current treatments for the autoimmune disease RA fail in up to 50% of patients due to adverse side effects [36].

Immunosuppressants used in autoimmune treatment consist of both small molecules and biologics, such as proteins and antibodies, and can elicit their effect through several different mechanisms. Many anti-inflammatory compounds act by inhibition of immune cell proliferation, such as lefunomide (Arava) for RA and teriflunomide (Aubagio) for MS, which block synthesis of DNA necessary for cell division [37]. Chemotherapeutics including mitoxantrone (Novantrone) and methotrexate have also been used in treating autoimmunity due to their ability to inhibit DNA synthesis [37, 38]. Inhibition of cellular proliferation inhibits the rapid expansion of autoreactive immune cells that can cause tissue damage and further inflammation thereby reducing disease symptoms.

Another common mechanism whereby immunosuppressant drugs act is via control of the cytokine response. Cytokines act as soluble messengers of the immune system; creating inflammatory or tolerogenic responses depending on the type and quantity of cytokines that are secreted in the local microenvironment. Autoimmune therapies have tried to leverage the complexity of the cytokine response by inhibiting the production and action of pro-inflammatory cytokines. Small molecule immunosuppressant compounds such as ciclosporin, used in the treatment of RA and T1D, act by disrupting the pathway by which the pro-inflammatory cytokine

IL-2 is produced [38]. Additionally, several different biologics inhibiting cytokine binding are approved for use to treat RA including tocilizumab (Actemra) and etancercept (Enbrel), which inhibit pro-inflammatory cytokine signaling by IL-6 and tumor necrosis factor (TNF)-α, respectively [38, 39]. In the treatment of MS, interferon (IFN)-β therapy has been shown to decrease IFN-γ production through induction of neutralizing antibodies which help to decrease relapse rates in relapsing-remitting MS [37]. Although cytokine-targeted therapies have had successes in the clinic, the fact remains that cytokines are important in protection against invading pathogens, thus disruption of cytokine production or action can increase susceptibility to infection [12].

The mechanisms for many immunosuppressants currently used to treat autoimmunity are not well understood. Glucocorticoids, mainly prednisone and prednisolone, are commonly given to patients with SLE and RA. These drugs have been shown to have numerous pleiotropic immunosuppressant effects, but may act somewhat by their ability to reduce the expression of cellular receptors needed for robust immune responses [40]. Dimethyl fumarate (Tecfidera) was approved by the FDA for treatment of MS in 2013 and is believed to work by preventing oxidative stress via activation of the Nrf2 transcriptional pathway; however, its influence on the immune response is still debated [37, 41].

Unfortunately, a common theme among all immunosuppressants is their lack of specificity. These therapies must often be used long-term in order to suppress the immune response to self and do not cure the underlying disease condition, but rather mitigate symptoms by reducing tissue damage and inflammation. Due to their long term use and lack of specificity, severe toxicity issues associated with global immunosuppression are common [38, 42].

3.2 Mobility and Transport Inhibitors

Autoimmune diseases require the mobility of auto-reactive immune cells or antibodies to migrate to their site of action. Mobility and transport inhibitors attempt to prevent this process. Similar to general anti-inflammatory molecules, severe side effects are often associated with these therapies, as they can restrict the movement of immune cells that are necessary to fight off foreign pathogens. One such therapy, natalizumab (Tysabri), is a humanized antibody targeting vascular cell adhesion molecule-1 (VCAM-1) for the treatment of MS. Natalizumab reduces leukocyte trafficking across the blood brain barrier by inhibiting binding to the necessary cell adhesion molecules, thereby decreasing the number of auto-reactive T-cells in the CNS tissue [43]. Unfortunately, soon after it was approved by the FDA in 2004, natalizumab was found to be associated with an increased incidence of progressive multifocal myeloencephalopathy (PML), a fatal viral disease of the CNS [44]. It was found that there were several risk factors associated with PML, most notably the presence of JC virus antibodies in MS patients. Upon implementation of PML risk mitigation strategies, including testing for JC virus antibodies before beginning therapy, natalizumab was reapproved in 2006 for MS patients un-responsive to other therapies [44]. Another mobility blocking therapy, efalizumab (Raptiva), an anti-LFA-1 antibody, met with a similar fate as natalizumab. Efalizumab was found to reduce the severity of chronic psoriasis, an autoimmune disease of the skin, but it was withdrawn from clinical use in all cases after several PML cases in patients [16].

Another FDA approved drug, fingolimod (Gilenya) is a small molecule mobility inhibitor used for the treatment of MS. Fingolimod acts by internalization of S1P-receptors on immune cells to prevent them from egressing from lymph nodes and trafficking to the CNS [37]. Unlike natalizumab and efalizumab, fingolimod has not been shown to result in PML and can therefore

be used in patients that test positive for the JC virus. Nevertheless, mobility and transport inhibitors are often not prescribed until an MS patient presents with aggressive disease and CNS lesions [37].

3.3 Immune cell activiation inhibitors

As previously discussed, both antigen presentation and a co-stimulatory context signal are needed to activate immune cells in an antigen-specific immune response. Recent evidence suggests that a change or lack of co-stimulation can prevent immune activation and even skew the response towards tolerance [16, 45, 46]. Due to the importance of co-stimulation in directing the antigen-specific immune response, several co-stimulatory pathways have been investigated in the induction of tolerance and treatment of autoimmunity.

The B7 (CD80/86) signaling pathway is one of the most well-characterized co-stimulatory pathways in T-cell activation and has therefore been a major target in T-cell mediated autoimmune diseases. The B7 pathway consists of two main molecular interactions, B7:CD28 binding leading to immune-stimulation and B7: CTLA-4 binding leading to immunosuppression or tolerance to the presented antigen [47]. Since CTLA-4 acts as a co-inhibitory signal in T-cell activation it has been the key focus in targeting the B7 pathway for autoimmune therapy. Three immunomodulatory biologics approved by the FDA in the past 10 years either target or are derived from CTLA-4; with two primarily used in the treatment of autoimmunity [48]. Abatacept (Orencia), a CTLA-4 IgG1 fusion protein, was the first biologic targeting the B7 pathway approved to treat autoimmunity. It was initially approved to treat RA in 2005, and is currently under investigation in the treatment of other T-cell mediated autoimmune diseases including T1D, psoriasis, and SLE [48]. Another CTLA-4 IgG1 fusion protein, belatacept (Nulojix), was created to improve binding affinity to B7 as compared to abatacept. Belatacept

was approved to treat organ transplant rejection in 2011 and is currently in clinical trials for the treatment of RA and T1D [48]. Although these B7 pathway inhibitors show promise in the treatment of autoimmunity, they are not antigen-specific in their immune modulation.

Several other therapies target cell surface markers involved in activation of the immune response. Alemtuzumab (Lemtrada) is an anti-CD52 antibody approved for the treatment of MS [37, 39]. CD52 is found on a variety of immune cells and, although its exact function is still unknown, it is believed to be involved in co-stimulation as its cross-linking leads in T-cell activation [37]. Another antibody targeting T-cell receptors, daclizumab (Zenapex) is approved to prevent organ transplant rejection and is currently under investigation as a treatment for MS. Daclizumab binds to CD25, which is expressed on activated T-cells and Tregs. Ongoing phase III clinical studies indicate that in addition to blocking T-cell activation, daclizumab also works to expand regulatory natural killer (NK) cells to treat MS [49].

In addition to targeting T-cell activation, with our increasing understanding of the role of B-cells in autoimmunity, there has been investigation into the use of B-cell targeted therapies in the treatment of autoimmune diseases such as RA, MS, and SLE. Rituximab (Rituxan), a chimeric IgG1 anti-CD20 monoclonal antibody, is often administered alongside methotrexate to RA patients who are unresponsive to more common treatments such as anti-TNF agents [50]. Rituximab has also been successful in clinical trials investigating the effectiveness of B-cell depletion in the treatment of MS [51] and SLE [52]. Recently, other human antibodies targeting CD20 such as ocrelizumab, veltuzumab, ofatumumab, and TRU015 have been clinically investigated for treatment of autoimmune diseases [53].

3.4 Antigen Mimics

Use of antigen mimics, or “decoys”, is a strategy aimed at inducing an antigen-specific immune response while avoiding potential anaphlaxysis that may be associated with the native antigen. Insulin and insulin analogues used in the treatment of T1D are some of the most widely used antigen mimics for autoimmune therapy; however, until just recently insulin was considered a hormonal therapy that had little to no effect on the immune response. Recent evidence suggesting that insulin is the initiating antigen in the development of T1D has led researchers to revisit insulin therapy [54]. Although better understanding of the immune response offers the potential to enhance T1D treatment, so far clinical trials have failed to improve upon current insulin therapy [54].

Another form of antigen mimics, altered peptide ligands (APLs), are created through substituting different amino acid for those in the antigenic epitope. APLs of antigenic epitopes in MBP with varying affinity for MHC class II molecules have been synthesized and studied for the induction of immune tolerance to treat EAE. Results indicate a correlation between APL affinity for MHC class II molecules and EAE disease prevention, with APLs of higher affinity displaying a shift in cytokine secretion toward IL-10 and greater suppression of T-cell proliferation [55]. Due to the heterogeneity of the antigen-specific T-cell populations involved in an autoimmune response, it may be necessary to design an APL capable of inducing tolerance across a wide range of T-cell receptor (TCR) affinities in order to produce a lasting effect [56, 57].

Glatiramer acetate (Copaxone) is an altered polymeric version of the MS-associated antigen MBP. Many immunomodulatory mechanisms have been proposed for glatiramer acetate including competitive binding to MHC class II molecules, a shift toward a T-helper type 2

immune response, and TCR antagonism in MBP-specific T-cells [57, 58]. The multiple mechanism of action would suggest that glatiramer acetate may act through both antigen-specific and non-specific pathways to alter autoimmune responses; however, further studies are required to determine the relevance of each of these mechanisms.

3.5 Current combination therapies for autoimmune disease

In many cases, combinations of drugs from the therapeutic categories discussed previously are used in order to enhance efficacy. In RA, a small molecule immunosuppressant, methotrexate, is often prescribed with TNF-α inhibitor therapy in order to achieve a synergistic effect [38]. This synergistic effect is not found in all combinations of therapies utilizing two biologics. For example, a TNF-α inhibitor and co-stimulation inhibitor, abatacept, did not achieve additional clinical benefits in the treatment of RA, but rather caused toxicity from immunosuppression complications [38]. Although this combination approach has shown promise, it is still missing the antigen specificity needed to reduce side effects and increase long term efficacy.

4 Combination strategies for ASIT in autoimmunity

With the clinical failure of many antigen-only therapies for autoimmunity, recent research has focused on combination therapy containing antigen and immunomodulator to enhance efficacy. Combination therapy can be accomplished by either co-administration (dosing in a similar time-frame, often via the same route) or co-delivery (utilizing a vehicle to physically or chemically keep the antigen and immunomodulatory in close physical proximity) (**Table 2** and **Figure 2**). By applying the “antigen-adjuvant” combination paradigm of vaccines to the

treatment autoimmunity, it may be possible skew the immune response towards antigen-specific tolerance.

4.1 Co-Administration

Many of the initial studies done with antigen and immunomodulators in the early to mid-2000s utilized co-administration to create ASIT combination therapy (**Table 3**). Dosing antigen and immunomodulator together without a co-delivery vehicle offers the flexibility of delivering the compounds via different routes. Also, the lack of a vehicle needed to co-encapsulate or connect the components may be more economically feasible and allow for ease of manufacturing and formulation; factors that may help accelerate the transition into to the clinic. Using co-administration in ASIT also has the disadvantage of producing similar side-effects as many current therapies; since when the antigen and immunomodulator are separated the immunomodulator may produce a general immunosuppressive response rather than an antigen-specific response.

4.1.1 Co-Administration with small molecule immunosuppressants

Small molecule immunosuppressants are commonly prescribed for autoimmune disease treatment. In order to reduce global immunosuppression, recent studies have investigated the co-injection of autoantigen, or DNA encoding autoantigen, simultaneously with a small molecule immunosuppressant. Kang and colleagues pioneered the use of the term ‘tolerogenic adjuvant’ in their 2008 paper involving the co-administration of dexamethasone and autoantigen to induce antigen-specific tolerance in a model of autoimmunity [59]. Co-injection of dexamethasone and OVA resulted in long-term antigen-specific tolerance as well as the proliferation of OVA-specific regulatory T cells. Similar antigen-specific tolerogenic responses were also seen using a T1D murine model [59]. In a subsequent paper, Kang and colleagues demonstrated that co-

injection of a different ‘tolerogenic adjuvant’, the immunosuppressant FK-506 (Tacrolimus), with a plasmid DNA encoding autoantigen, rather than the antigen itself, also results in expansion of Tregs and suppression of autoimmunity in mice [60].

4.1.2 Co-Administration with Biological Molecules

Unlike the monoclonal antibodies that dominate the clinically-approved biologics for autoimmunity, the majority of co-administration research for ASIT has focused on the use of plasmid DNA as the biological delivery platform. Co-administration of plasmid DNA encoding autoantigen and a plasmid containing immunomodulatory gene have been studied by several research groups. The injection of two plasmids, with autoantigen on one and immunomodulator on the other, classify these studies as co-administration rather than co-delivery.

In 2001, Garren and colleagues published a paper examining DNA vaccination using two plasmids, one encoded with interleukin (IL)-4, a cytokine associated with immunosuppression in MS, and the second encoded with a MS-associated autoantigen [61]. The co-vaccination strategy was tested in EAE mice with both PLP and MOG-induced models. In both models, co-administration of IL-4 and autoantigen encoding plasmids was found to suppress EAE disease compared to treatment with each gene individually. Interestingly, the MOG and IL-4 DNA vaccination was able to reverse established disease when given after EAE symptoms were present [61]. In a similar study, Glinka and colleagues investigated the use of DNA vaccination to co-administer autoantigen and a co-stimulation blocker for the treatment of NOD mice [62]. The study used a plasmid encoding for a fusion construct of PPI and GAD65 for induction of autoantigen expression, along with a plasmid encoding a mutant B7 molecule known to bind CTLA-4 and block co-stimulation during T-cell activation. This approach was successful in

decreasing disease symptoms and stimulating the tolerogenic response following co-administration of the plasmids [62].

Although co-administration of DNA has been successful in animal models, it is difficult to control dosage kinetics and gene expression, limiting its clinical potential. The combination of protein immunomodulators with antigens has gained interest for easier clinical translation. In a recent study, MOG_{35-55} and the tolerogenic cytokine IL-10 were encapsulated into separate PLGA nanoparticles for the treatment of EAE [63]. Both prophylactic and therapeutic treatment regimens co-administering particles containing MOG and particles containing IL-10 significantly suppressed EAE symptoms [63]. Using a similar strategy, Lewis and colleagues created PLGA microparticles each containing a single component; insulin B autoantigen, GM-CSF, vitamin D3, or TGF-β1. When these 4 different microparticles were mixed at a 1:1:1:1 ratio they were found to significantly prevent the incidence of T1D in NOD mice [64]. These successful experimental studies suggest the feasibility of a prophylactic or therapeutic co-administration platform to treat autoimmune disease.

4.1.3 Drawbacks of Co-Administration

While the co-administration approach has shown potential in animal models of autoimmunity, the lack of a formulation keeping the autoantigen and immunomodulator in the same microenvironment opens the door for non-specific immunosuppression or complete lack of efficacy upon separation of the components following dose administration. In fact, not delivering antigen and immunomodulator together both temporally and spatially can result in induction of an inflammatory response rather than a tolerogenic response. In a recent study, it was found that autoantigen co-administered with rapamycin, a small molecule

immunosuppressant, resulted in expansion of autoantigen-specific T-cells and inhibition of Tregs, the opposite of the desired tolerogenic response [65].

4.2 Co-delivery

Unlike co-administration, co-delivery ensures that the antigen and immunomodulator are delivered at the same time and presented in the same environment to auto-reactive immune cells (**Table 2**). Many investigators have examined the effect of delivering encapsulated immunomodulators or autoantigen alone for autoimmune therapy [66-70]; however, only recently have studies focused on the effect of co-delivering these components via a variety of different delivery vehicles (**Table 4** and **Figure 2**).

4.2.1 Co-Delivery with small-molecule immunosuppressants

Building upon previous literature using co-administration of autoantigen and small-molecule immunosuppressant, several research groups have investigated the possibility of co-delivering these components. Various delivery vehicles have been employed with results that suggest dosing compounds together both spatially and temporally may enhance treatment efficacy. Liposomes, dextran microparticles, and gold and PLGA nanoparticles have all been used in recent studies in order to co-deliver autoantigen and immunosuppressant for ASIT in animal models of autoimmunity [65, 71-73]. In each of these examples, the two components were either co-encapsulated or co-adsorbed to the delivery vehicle to ensure simultaneous delivery of the components to immune cells [65, 71-73].

Peine and colleagues extrapolated work by Kang and colleagues by co-encapsulating autoantigen and dexamethasone in microparticles [59, 71]. Dexamethasone was co-encapsulated into acid-sensitive acetylated dextran microparticles with the MS-antigen MOG and used in the treatment of EAE. The co-delivery of the components significantly decreased clinical disease

score as compared to mixtures of dexamethasone and MOG, demonstrating the importance of delivering both components concurrently to immune cells [71].

Yeste and colleagues investigated co-delivery of MOG and the small molecule immunosuppressant 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) [72]. Both MOG and ITE were co-loaded onto the outside of gold nanoparticles and were found to induce tolerogenic DCs and FoxP3+ Tregs in naïve primary cells. The nanoparticles significantly suppressed EAE disease symptoms as compared to the components given without use of the co-delivery vehicle [72]. In a unique experiment, Yeste and colleagues also demonstrated that their co-delivery system was effective even after epitope spreading, by utilizing two different epitopes of autoantigen to suppress EAE [72]. Using a similar approach, Maldonado and colleagues delivered both autoantigen and rapamycin co-encapsulated in PLGA nanoparticles to treat a number of autoimmune models [65]. The antigen-rapamycin nanoparticles were able to induce antigen-specific tolerance in EAE, in a model of hypersensitivity, and in a model of the genetic disease hemophilia [65]. In both studies encapsulating the immunosuppressant alone was found to suppress disease; however, the autoantigen-containing nanoparticle did not [65, 72].

In addition to the possible use of different types of delivery vehicles to produce antigen-specific tolerance, Capini and colleagues demonstrated that different immunosuppressant drugs could also be effective [73]. Their study examined the effects of three different NF-κB inhibitors; curcumin, quercetin, and Bay11-7082. When co-encapsulated into liposomes with autoantigen each of the three compounds were able to induce antigen-specific Treg responses and decrease disease severity in a mouse model of RA, antigen-induced inflammatory arthritis (AIA) [73].

4.2.2 Co-Delivery with Peptides

Peptides targeting immune cell adhesion or co-stimulation molecules have been conjugated with autoantigen epitopes to enable co-delivery. Siahaan and colleagues have published a number of papers on bi-functional peptide inhibitors (BPI) that link a peptide autoantigen with an immune cell inhibitor targeting the cell adhesion molecule ICAM-1. BPIs have suppressed disease in animal models of both T1D and MS [74, 75]. The originally developed BPI co-delivery vehicle contained the MS epitope $PLP_{139-151}$ linked to the peptide LABL, derived from α_L integrin, for the treatment of EAE. This unique ASIT was found to significantly decrease the severity of EAE disease as compared to each peptide alone or mixed [75, 76]. Interestingly, the BPI decreased the rate of anaphylaxis in mice as compared to PLP alone, suggesting that autoimmune treatments containing immunomodulators may offer improved safety as compared to autoantigen alone [75].

Building off of Siahaan's work, Berkland and colleagues have published several papers on a multivalent approach known as a Soluble Antigen Arrays (SAgAs). SAgAs consist of antigenic peptides and immunomodulator peptides that are co-delivered via a hyaluronic acid backbone. Peptides inhibiting cell adhesion (via ICAM-1) and the B7 (CD80/CD86) pathway have shown efficacy in EAE when co-delivered with MS antigen using SAgAs [77].

4.2.3 Co-Delivery with Biological Molecules

As previously discussed, DNA vaccines have successfully co-administered two plasmids, separate autoantigen and immunomodulator, to treat autoimmunity. In attempt to improve upon this technique, autoantigen and immunomodulator were encoded on a single plasmid. A couple of recent studies investigated this strategy for the treatment of TID in NOD mice with plasmids containing GAD65 and a secondary molecule, the cytokine IL-4 or the pro-apoptotic protein

BAX, respectively [78, 79]. In both studies, the plasmid containing both autoantigen and immunomodulator were able to prevent and suppress disease [78, 79].

Another study utilized the co-delivery of a plasmid encoding the B and T lymphocyte attenuator protein (BTLA) and MOG antigen to pre-treat DCs before using those DCs to treat EAE mice [80]. These pre-treated DCs were found to decrease the severity of EAE when injected prophylactically; however, this approach may be too complex for clinical application [80].

5 Clinical Trials of ASIT for Autoimmunity

5.1 Antigen-only ASIT Clinical Trials

Similar to allergy hyposensitization, the introduction of a disease-causing autoantigen to a patient with autoimmunity can result in undesired and potentially life threatening adverse events [81, 82]. A Phase II clinical study of an altered peptide ligand of MBP, an antigen associated with MS, had to be halted due to three of the eight patients suffering worsening symptoms, resulting in an increase in CNS lesions up to 2.4 times the amount before therapy and leaving one patient unable to walk. In other cases, administration of a slightly altered autoantigen to treat autoimmunity did not directly aggravate the disease but instead resulted in an allergic response to the antigen [82]. Fortunately, in the majority of clinical trials these adverse events were avoided; however, ASIT did not result in any benefit compared to placebo [83].

5.2 Combination ASIT Clinical Trials

Recently, trials of combinations of antigen and immunomodulator for ASIT have shown promise. In several clinical studies for MS, the FDA approved drug glatiramer acetate (GA) was used as a mimic of the disease-associated antigen MBP and was co-administered with

immunomodulators to study the effects of combination therapy. In one study improved success in both decreasing CNS symptoms and lowering the risk of disease relapse was seen in combination of GA and the antibiotic minocycline, as compared to GA alone [84]. In another study, GA and natalizumab co-therapy was found to be safe and suppressed CNS lesions in MS compared to GA alone [85]. Unfortunately, the majority of combination trials with GA did not contain a control group with immunomodulator alone; however, in comparing therapy outcomes to those seen with natalizumab it was found that the combination therapy did not improve efficacy [85]. A few studies have had success with a modified dosing schedule, where patients undergo short term use of immunosuppressant therapy, either with mitoxantrone or methylprednisolone, with GA before starting on GA alone [86, 87]. In addition to improving clinical outcomes compared to GA alone, it was also found that the short-term therapy limited the adverse effects associated with long-term immunosuppressive use [86].

Although ASIT using only antigen has been successful in allergies, recent clinical trials with co-administration of an immunomodulator have been found to be more effective than allergen alone. In several clinical studies omalizumab (Xolair), an anti-IgE antibody, was added to allergen hyposensitization therapy in the hope of decreasing the chance of dangerous side-effects, such as anaphylaxis [88]. This combination therapy was found to increase efficacy as compared to the allergy-associated antigen alone [88, 89].

A slight variation of the use of ‘tolerogenic adjuvants’ in ASIT for autoimmunity is the success of traditional vaccine adjuvants co-administered with antigen for improved allergy hyposensitization therapy and ASIT for asthma [90]. When using traditional adjuvants for autoimmune therapy, the key to creating a successful ASIT for autoimmunity may reside in finding the appropriate patient population. Attempts to co-administer a T1D antigen GAD65

with a traditional vaccine adjuvant, alum, met with limited success [31]. Upon further trials, it was found that this treatment was successful in suppressing T1D but only in children and adolescents with recent onset of the disease [30]. In another successful clinical study, human B-chain of insulin was given to patients with incomplete Freund's adjuvant. Although the focus of this study was safety, it was also found that patients had a robust antigen-specific Treg response even two years after finishing the treatment [91]. With the success of immunomodulator co-administration in recent literature, it is possible that the addition of an immunosuppressant to traditional adjuvants combined with antigen may allow for even broader patient efficacy.

6 Challenges for the Future of ASIT for Autoimmunity

6.1 Human Translation of Pre-Clinical Successes

Although there are promising results in animal models of autoimmune diseases, most successes in these models have not been translatable to humans. One of the most promising methods for ASIT autoimmunity that had success in animal models was the administration of oral antigen to treat MS, T1D, or RA. When attempted in humans, no therapeutic benefits were found [7, 8]. While it is difficult to interpret these negative findings, discrepancies in immune tolerance and autoimmunity between humans and animal models, such as mice, have been noted. These include differential expression of Treg markers such as FoxP3, variations in the balance of leukocyte subsets, dysregulation of central tolerance such as thymic selection, and different roles played by cells that produce IL-17, among others [92]. Directly linked to these immunological differences, the development of the disease in animals is often unrelated to that in humans. Often animal models of autoimmunity require induction with an immunogenic antigen, such as in the majority of EAE models of MS [93]. A few disease models do exist where the

autoimmune disease can occur spontaneously such as NOD and some versions of EAE, which in some instances may offer better understanding of the human disease than inducible models [92, 93].

Acute animal models of autoimmunity also may not be predictive for the treatment of chronic human immune disorders. The majority of animal studies conducted treat the disease before symptoms appear; whereas human therapies will mostly be given years after onset of the pathogenic process [8, 93]. Many animal studies are terminated too early to see any long term issues that may arise. For example, only 7% of all studies with NOD mice are followed up beyond 32 weeks, which does not reflect the lifelong duration of T1D in humans [92]. Additionally, the complete disease causing mechanisms are not completely understood in humans. For example, it has been hypothesized from studies of identical twins, that while there is a genetic component to many autoimmune disorders there is also additional "environmental" components that affect the disease that are not reflected in highly controlled pre-clinical studies and may lead to less than perfect applicability to human trials [94].

Treatment safety and tolerability, which is immensely important in human therapies, is also often overlooked or difficult to assess in animal models [8]. Some safety issues may only arise in humans, and using cells from human donors in combination with animal models may help prevent toxic compounds from reaching the clinic [92]. For example, the production of a cytokine storm in humans using a CD28 agonist was not foreseen using animal models [92].

Nevertheless, these animal models have helped make important discoveries in the treatment of human autoimmunity. The EAE model of MS has helped identify four recently approved therapies; glatiramer acetate (Copaxone), mitoxantrone (Novantrone), natalizumab (Tysabri), and fingolimod (Gilenya) [7]. Improved animal models and better understanding of the

immunology of human autoimmunity may increase the clinical success of experimental therapies.

6.2 Antigen Identification and Epitope Spreading

For many autoimmune diseases the animal model is not the rate-limiting step to developing ASIT; rather the immunodominant disease-causing antigen(s) may not be identified. SLE, for example, can manifest symptoms in many different organs and the disease-causing autoantigen may vary greatly between SLE patients [95]. Even diseases that have relatively well-characterized disease-causing antigens, such as MS, a single antigen for ASIT can be difficult to determine due to epitope spreading [96]. A few recent trials in MS have shown promise by using multiple antigens to elicit the antigen-specific response; however, they are still in the early stages of human testing [97, 98].

In allergy hyposensitization therapy, diagnostics, such as the skin prick test, determine the most important allergen in specific patients [99]. If this type of 'personalized medicine' could be applied to autoimmunity, it may greatly improve outcomes. The analysis of peripheral blood for immunodominant autoantigens may allow for ASIT to be tailored to the individual patient, increasing the possibility for therapeutic success [92]. Emerging diagnostic practices such as component resolved testing, high-throughput antibody repertoire analysis, and indirect T-cell recognition assays may improve the ability to determine the correct autoantigen for personalized ASIT [100-102].

Autoimmune diseases with only one immunodominant antigen, including myasthenia gravis and neuromyelitis optica, may provide better targets for ASIT [103]. Recent studies using an antibody against the disease-causing antigen in neuromyelitis optica, aquaporin 4 have shown success in animals and will soon enter clinical trials [104].

6.3 Immunomodulator Optimization

While antigen(s) for ASIT are defined by the disease, there are a wide-array of immunomodulators to choose from when exploring combination therapy. To date, the immunomodulator chosen for co-administration or co-delivery with antigen has been ad hoc at best. The majority of studies focus on a single immunomodulator in combination therapy for ASIT. A few recent studies have attempted to determine the best tolerance-inducing immunomodulator by measuring the induction of Tregs by various small molecule immunosuppressants [105, 106]. However, a successful immunomodulator in one autoimmune disease may not be appropriate for another, and therefore immunomodulator screening may need to occur on a disease by disease basis. Additionally, recent successes of combinations of immunomodulators for autoimmune treatment may indicate that multiple immunomodulators may be more effective for the induction of antigen-specific tolerance [107, 108].

Recent clinical successes have been achieved utilizing traditional adjuvants as immunomodulators in ASIT [30, 91]. Unfortunately, the viability of this method for the treatment of autoimmunity is still hotly debated as conflicting studies have shown that combinations of traditional adjuvants and antigen can both induce and treat autoimmune disease in rodent models. Immunologists have only recently started unraveling mechanisms such as immune cell ‘exhaustion’ in autoimmunity and immune tolerance pathways in cancer, both of which may have direct implications for ASIT combination therapy [109, 110].

6.4 Co-Delivery Vehicle

Co-delivery adds an extra layer of complexity to the creation of ASIT for autoimmunity due to the need for the correct delivery vehicle, yet mounting evidence suggests co-delivery may do more to enhance the antigen-specific tolerance than co-administration of separate components.

The determination of the correct co-delivery vehicle is important to ensure both antigen and immunomodulator interact with the immune response at the same time and in the same space. Numerous vehicles for co-delivery were utilized for combination therapy in ASIT reviewed here, including microparticles, nanoparticles, liposomes, direct chemical linkage, multivalent presentation on polymers, and plasmid DNA (**Figure 2**) [65, 71-75, 77-79]. Each of these approaches offers its own unique benefits, challenges, and potential.

The particulate delivery systems; (e.g. microparticles, nanoparticles, and liposomes) most directly mimic the delivery systems currently employed in vaccines. Vaccines commonly utilize aluminum salts, which are particulate in nature, to deliver the antigen of interest [111]. Recent studies with antigen conjugated to micro- and nanoparticles have successfully suppressed disease in EAE [112, 113]. These particulate systems are often intended to be immunologically inert; however, the material, size, and shape of the particles can promote immune responses [114, 115]. Particulate delivery systems are unique in that the antigen and immunomodulator may be on the surface of the particle, encapsulated, or both. Particles with surface conjugated antigens may be preferred when targeting B-cells [115]. Surface antigens may also target T-cells when displayed in the context of MHC [116]. Alternatively, encapsulation of antigen is often used when uptake by APCs is preferred as much higher concentrations of antigen per particle can be achieved with encapsulation [115]. Encapsulation of antigen and/or immunomodulator can also improve pharmacokinetic properties; for example, encapsulation of antigen can decrease rapid dilution and clearance that is associated with many injected biologics [115].

Multivalency may also influence the immune response, as it has been shown that the valency and the size of multivalent scaffolds play an important role in immunomodulation [114]. Dintzis and colleagues developed a number of ‘rules’ exploring the effect of multivalency on the

immunogenicity or tolerogenic-properties of linear polymeric delivery systems [114, 117]. They found that polymers with a molecular weight greater than 100 kDa and a valency greater than 20 compounds per polymer were more immunogenic, while systems under 100 kDa tended to be more tolerogenic [114]. Both particulate systems with surface-bound materials and linear polymers displaying antigens have utilized multivalency as an approach to ASIT combination therapy for autoimmunity [72, 77].

Plasmid DNA delivery systems have also been investigated for combination therapy, as both antigen and immunomodulator can be encoded onto a single plasmid [78, 79]. Antigen-specific treatments utilizing DNA have been shown to have benefits over whole protein or peptide antigens such as increased intercellular persistence due to stable expression from transduced genes [118]. Recent clinical trials utilizing a plasmid DNA encoding proinsulin demonstrated positive results in antigen-specific tolerance in T1D patients [119].

Finally, a very unique delivery system of utilizing cells themselves as delivery vehicles for ASIT has emerged with the potential to induce antigen-specific tolerance in autoimmunity. In studies spanning several decades, Miller and colleagues have shown that chemically coupling antigen to apoptotic cells, can be used to induce antigen-specific tolerance [120, 121]. Antigens coupled to apoptotic splenocytes, peripheral blood leukocytes, or erythrocytes have had positive results in animal models of autoimmunity [121-123]. Additionally, these antigen-coupled cells have been tested in humans, and shown promising results in a Phase 1 clinical trial [124]. Another innovation utilizes cells treated with ASIT *ex vivo*. In these systems, DCs are obtained from the bone marrow of genetically similar animals and treated with antigen and immunomodulator [80, 125]. The cells treated with the combination therapy are then injected into the autoimmune animal model to induce tolerance [80, 125]. These studies benefit from

utilizing a delivery system capable of removing the 'middle-man' of cellular uptake by APCs and co-delivery of immunomodulator, since cells are treated *ex vivo*. Unfortunately, cell-based methods for ASIT are still relatively young and experimental. Furthermore, the complexity of these systems may create difficulty in wide spread clinical application due to challenges associated with manufacturing, high cost, and patient accessibility [124].

6.5 Route of Administration

Recently published studies in animals have used a variety of different routes of administration including intravenous (IV), intramuscular (IM), and subcutaneous (SC), with over-arching success. When translating these therapies to humans and larger animal models, the route of administration will certainly play an import role in clinical outcomes. The route of administration dictates the barriers the therapy will face before reaching the site of action. For example, oral therapies must migrate through the GI track and often undergo first-pass metabolism in the liver before entering circulation, whereas IV therapies bypass these barriers. The route of administration in animals may not be translatable to humans, such as the use of oral antigen administration for autoimmunity that was found to have minimal clinical efficacy [7, 8].

Many of the ASIT strategies utilize the interaction of immune cells in the lymphatic system in order to skew the immune response towards tolerance. It has been demonstrated that efficient delivery of vaccine components to the lymph nodes is critical to mounting an effective antigen-specific response [126]. By optimizing delivery vehicle size, drainage to lymph nodes has been achieved from multiple different injection sites [127]. Nanoparticles ranging in size from 10 nm to 200 nm have been found to drain to the lymph nodes following injection [127]. SC delivery has been effective in both passive drainage and active transport by peripheral macrophages from the site of injection to the lymph node [114]. IM injection may be more likely to utilize active

transport as immune cells are often recruited to the depot at the injection site [114]. A unique route of administration, intranodal injection, bypasses the transport step. Intranodal administration in allergy hyposensitization has been shown to safely promote antigen-specific tolerance while reducing dose size by up to 1000x the dose delivered via conventional routes [128].

Allergy hyposensitization strategies have explored sublingual, intranasal, and oral routes of administration [1]. Sublingual treatment has yielded the greatest success as it increases convenience while maintaining the efficacy of the traditional SC therapy [4]. Recently, three sublingual hyposensitization therapies have been approved by the FDA to treat grass and ragweed allergies [129]. Historically, intranasal administration of hyposensitization therapy had suffered from a high number of local adverse events [130]. A new approach utilizing strips coated with dust-mite allergens reported positive outcomes in a recent clinical trial [131]. Oral hyposensitization to food allergens has also had some clinical success; however, there is still concern about serious adverse reactions, which could be addressed via combination therapy strategies proposed here [130].

7 Conclusion

ASIT has long been the cornerstone of vaccines, arguably one of the most important healthcare related-inventions. Mechanisms based on prototypical vaccine design have been effectively adapted for producing antigen-specific tolerance for allergies (i.e., hyposensitization therapy); however, the application towards clinical advancement of effective experimental ASIT therapeutics to treat autoimmune disease(s) has not been as successful. As outlined above, the approach of vaccines, which utilize both antigen and immunomodulator (i.e., adjuvant), may

hold the key to developing successful ASIT for autoimmune disorders and potentially to improve current hyposensitization therapies. Researchers have seen promising results in an array of experimental models of autoimmunity by both co-administration and/or co-delivery of autoantigen and immunomodulator as an enhanced ASIT treatment. Future work should emphasize the effects of each component alone and together in combination therapies to enhance our understanding of the mechanisms by which tolerance is induced. As these strategies and experimental therapies evolve and move into the clinic, the outcomes of these studies may vastly change the way that autoimmune therapy is approached, especially with the potential to increase efficacy, diminish side effects, and reduce the lengthy dosing schedule of current hyposensitization therapy. With several recent successful proof-of-principle studies, there is increased hope that ASIT combination therapy may hold the potential to cure the disease, rather than just treat and/or prevent disease symptoms.

Table 1. Mechanisms of action for ASIT to treat autoimmunity.

Mechanism of Action	Drug Example
Cell Cycle Interference	
Prevent Cell Division	lefunomide
Inhibition of DNA Synthesis	methotrexate
Controlling Pro-Inflammatory Cytokines	
Prevent Cytokine Production	ciclosporin
Inhibit Receptor Binding	tocilizumab
Induction of Neutralizing Antibodies	interferon-β
Inhibiting Transport of Auto-reactive Cells	
Preventing Cell-adhesion	natalizumab
Trap Cells in Lymphatics	finglomod
Inhibiting T-cell Activation	
Blocking B7 Co-stimulation	abatacept
Blocking other Co-stimulation Pathways	alemtuzumab
Competitive Inhibition of MHC Binding	glatiramer acetate
B-cell Depletion	
Antibody-Dependent Cell Cytotoxicity	rituximab
Other Proposed Mechanism to Improve Therapies	
Auto-antibody Deletion	
Ex Vivo Antigen-specific Immune Cell Activation	
Anergy of Auto-reactive cells	
Inducing Regulatory Cell Proliferation	
Antigen-specific Interruption of T-cell Activation	
Inducing Antigen Presentation with Co-inhibitory Signals	

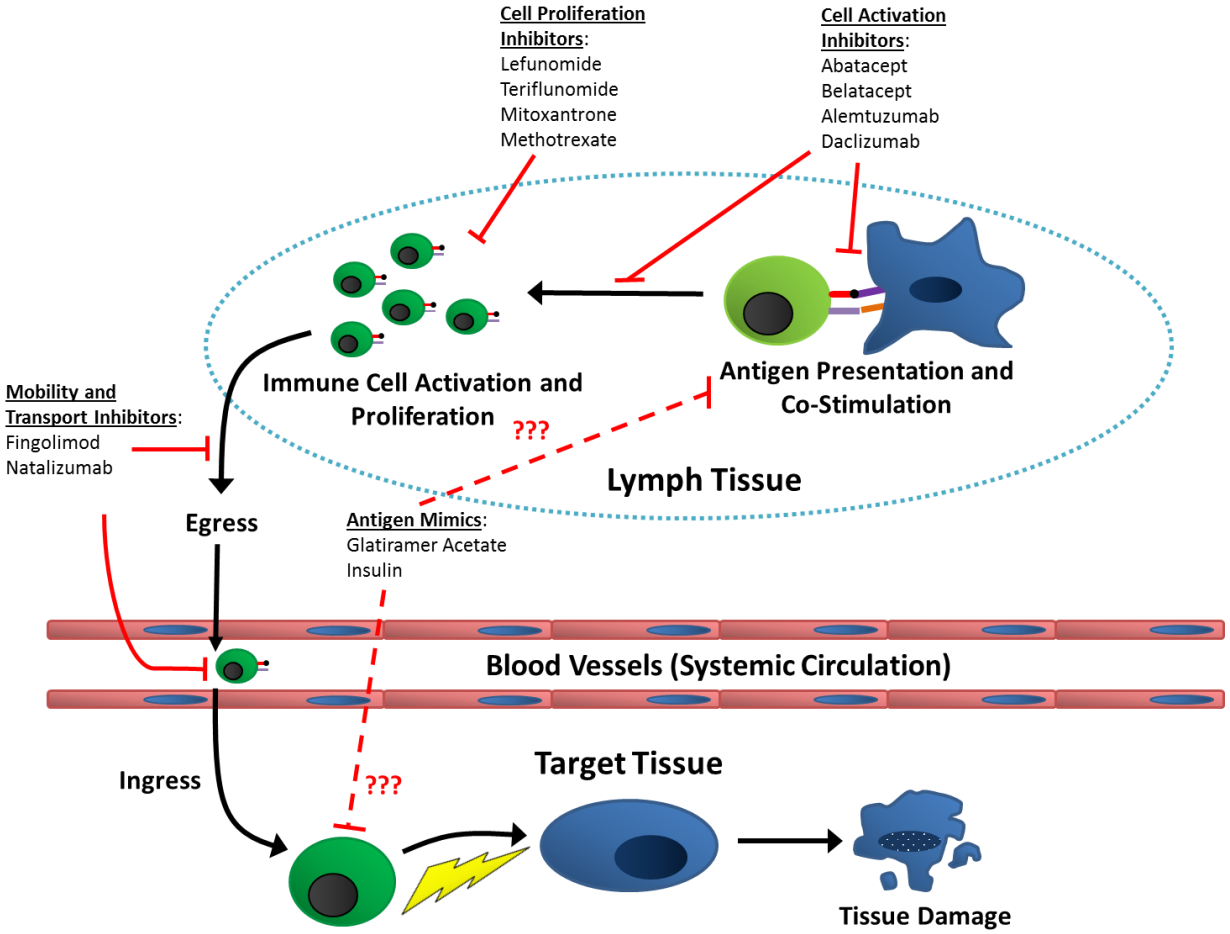

Figure 1. Current therapies for autoimmunity fall into distinct categories; including immunosuppressants, mobility inhibitors, co-stimulation and cell activation inhibitors, and antigen mimics. These therapies act throughout the immune response, in the lymph tissue, in systemic circulation, and in the diseased tissue to suppress autoimmune disease symptoms. Therapies often act at several locations and the mechanism of action of many of them, particularly those that fall under the category of antigen mimics, are not well understood.

Table 2. Definitions of Types of ASIT Delivery

Term	Definition	Example
Mono-therapy	Single component therapy	Immunomodulatory drug alone, or antigen alone
Combination Therapy	Multiple components given together in either the same time and/or same space	Encompasses both co-administration and co-delivery
Co-Administration	Multiple components given together at the same time but not in the same space	Injection of antigen and immunomodulatory drug together but not held together either chemically or physically
Co-Delivery	Multiple components given together in the same time and same space	Antigen and immunomodulatory drug are linked, co-encapsulated, or held together another way either chemically or physically

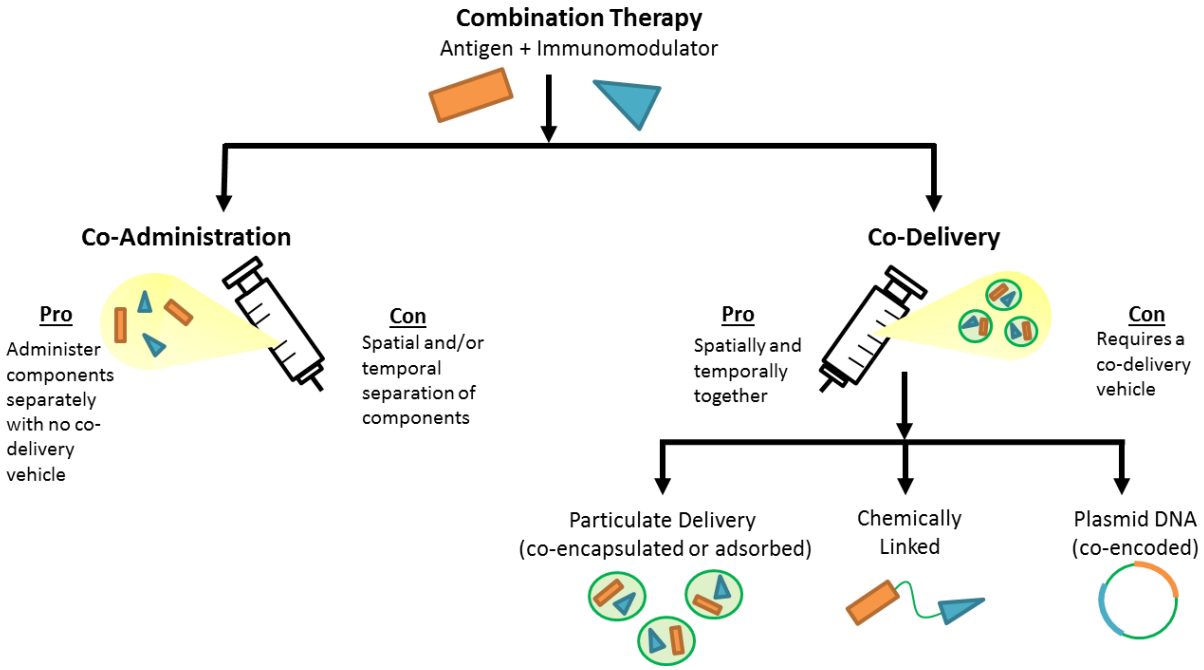

Figure 2. Combination therapy for ASIT can be accomplished by either co-administration or co-delivery of antigen and immunomodulator. With the increasing diversity of antigen and immunomodulatory molecules that could be used for ASIT, each independent combination therapy will have to be rationally designed to fit appropriate formulation parameters. Several experimental technologies exist to temporally and/or spatially link antigen with immunomodulatory molecules. These include 1) co-administration or formulation of independent components into a single injection solution and 2) co-delivery or physical linkage of the antigen and immunomodulatory molecule. Both methods have shown positive ASIT data; however, long term clinical benefit has not been established each of these ASIT formulation approaches.

Table 3. Co-Administration Examples in ASIT Combination Therapy

Immunomodulator	Antigen	Disease Model	Reference
Dexamethasone	$OVA_{323-339}$ peptide	Allergy	[59]
Dexamethasone	Insulin-derived B:9-23 peptide	T1D	[59]
FK-506	plasmid encoding MOG_{35-55} peptide	MS	[60]
plasmid encoding IL-4	plasmid encoding $PLP_{139-151}$ peptide	MS	[61]
plasmid encoding IL-4	plasmid encoding MOG protein	MS	[61]
plasmid encoding mutant B7-1 (B7-1wa)	fusion plasmid of PPIns-GAD65 proteins	T1D	[62]
recombinant IL-10	MOG_{35-55} peptide	MS	[63]
GM-CSF, vitamin D3, and TGF-β1	Insulin-derived B:9-23 peptide	T1D	[64]

Table 4. Co-Delivery Examples in ASIT Combination Therapy

Immunomodulator	Antigen(s)	Disease Model	Co-Delivery Vehicle	Reference
Dexamethasone	MOG_{35-55} peptide	MS	acetylated dextran microparticles, co-encapsulated	[71]
ITE	MOG_{35-55}, $PLP_{139-151}$, and PLP_{18-191} peptides	MS	gold nanoparticles loaded on surface and stabilized by PEG	[72]
Rapamycin	OVA protein and $OVA_{323-339}$ peptide	Allergy	PLGA nanoparticles, co-encapsulated	[65]
Rapamycin	$PLP_{139-151}$ peptide	MS	PLGA nanoparticles, co-encapsulated	[65]
Rapamycin	$FVIII_{74-89}$, $FVIII_{1723-1737}$, $FVIII_{2191-2210}$ peptides	Hemophilia	PLGA nanoparticles, co-encapsulated	[65]
NF-κB inhibitor (curcumin, quercetin, or Bay 11-7082)	OVA protein	Allergy	co-encapsulated in liposomes	[73]
Curcumin	methylated BSA protein	RA	co-encapsulated in liposomes	[73]
LABL peptide ($CD11a_{237-247}$)	$GAD65_{208-217}$ peptide	T1D	linked via spacer peptide	[74]
LABL peptide ($CD11a_{237-246}$)	$PLP_{139-151}$ peptide	MS	linked via spacer peptide	[75]
LABL peptide ($CD11a_{237-246}$)	$PLP_{139-151}$ peptide	MS	mulivalently linked to same hyaluronic acid polymer backbone	[132]
B7 pathway targeting peptide (B7AP, CD80-CAP, or sF2)	$PLP_{139-151}$ peptide	MS	mulivalently linked to same hyaluronic acid polymer backbone	[77]
plasmid encoding IL-4	plasmid encoding GAD65-IgG Fc fusion protein	T1D	encoded on same plasmid	[78]
plasmid encoding BAX	plasmid encoding GAD65	T1D	encoded on same plasmid	[79]
plasmid encoding BTLA	TAT_{49-57} MOG_{35-55} peptide	MS	self-assembled nanoparticles of peptide and plasmid; used to treat DC that were injected into mice	[80]

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

1. Introduction

Multiple sclerosis (MS) is a neurodegenerative disease characterized by an inflammatory reaction against proteins in the central nervous system (CNS) leading to nervous system dysfunction and paralysis [1-3]. Several FDA-approved therapies exist to treat patients with MS; however, these therapies do not cure the disease, but rather are designed to slow disease progression and manage symptoms [4-6]. Additionally, several of these therapies are associated with severe adverse events, compelling a need for safer and more efficacious therapies to treat patients with MS and other autoimmune disorders [4, 6, 7].

Autoimmune diseases, such as MS, result from the breakdown of mechanisms controlling immune tolerance and the subsequent failure of the host immune system to distinguish self from non-self antigens [8, 9]. In MS, auto-reactive T-cells are thought to escape endogenous immune tolerance mechanisms, inflicting subsequent damage to the myelin sheath and leading to neural degeneration [1, 8, 10]. Classical immunology dictates that two signals are required for T-cell activation by professional antigen presenting cells (pAPCs); an antigen specific signal (signal 1) and a "context" or immunomodulatory signal (signal 2, co-stimulatory/inhibitory) that ultimately dictates the resulting immune response [8, 11, 12]. Co-delivery of both antigen and a co-stimulatory context signal activates an antigen-specific adaptive immune response, whereas delivery of a co-inhibitory context signal or absence of either signal can render an anergic response (i.e. no response) and is believed to be a main mechanism of peripheral immune tolerance [8, 9, 13]. Thus, co-delivery of a synthetic co-inhibitory context signal and autoantigen may be a suitable pharmacological template to restore immune tolerance and treat various autoimmune disorders [13-16].

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

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

autoimmune therapies. Several previous studies have shown that co-delivering autoantigen and immune inhibitory peptide can inhibit autoimmune disease in animal models [22-28].

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

2. Materials and Methods

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

2.2 Conjugation of aminooxy peptides to HA polymer. Single step grafting of aminooxy

peptides to 16 kDa hyaluronic acid (HA) was performed as described previously [24]. Briefly, HA was dissolved in 20 mM acetate buffered solution (pH 5.5 ± 0.1) and each aminooxy reactive peptide was added simultaneously. After addition of the peptides, the reaction solution was adjusted to pH 5.5 ± 0.1 and stirred at 500 RPM using a magnetic stir bar for 16 hr at room temperature. The samples were then transferred to dialysis bags (MWCO 3500 Da, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and dialyzed against 2 liters of deionized water for 24 hours, with dialysis water exchanged every 6 hours to remove unreacted peptides and residual buffer. After dialysis, the dialysate was frozen at -70 °C and lyophilized.

2.3 Characterization of SAgAs by High Performance Liquid Chromatography. The amount of PLP and context signal- binding peptides conjugated to HA for each of the SAgAs was determined using digestion in acidic medium (4 hours at pH 2.0) and then subsequently analyzed by HPLC as previously reported [24]. For analysis of $SAgA_{PLP:sF2}$, following peptide cleavage the solution was centrifuged for 5 min at 13,000 rpm and the aggregates were dissolved in DMSO before running both the pellet and supernatant on HPLC. The HPLC consisted of SCL-20A SHIMADZU system controller, LC-10AT VP SHIMADZU liquid chromatograph, SIL-10A XL SHIMADZU auto-injector set at 50 µL injection volumes, DGU-14A SHIMADZU degasser, sample cooler, and SPD-10A SHIMADZU UV-Vis detector (220 nm). The HPLC-UV system was controlled by a personal computer equipped with SHIMADZU class VP Software. All separations were carried out using a Vydac® 179 HPLC Protein and Peptide C18 column. Gradient elution was carried out to determine the amount of PLP and context signal peptide at constant flow of 1 mL/min, from 100% A to 35% A (corresponding to 0% B to 65% B) over 50 min, followed by an isocratic

elution at 75% B for 3 min. Mobile phase compositions were (A) acetronitrile-water (5:95) with 0.1% TFA and (B) 100% acetonitrile with 0.1% trifluoroacetic acid (TFA).

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

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

2.6 Induction of EAE and Therapeutic Study. SJL/J (H-2s) female mice, 4 – 6 weeks old, were purchased from Harlan Laboratory and housed under specified, pathogen-free conditions at The University of Kansas. All protocols involving live mice were approved by The University of Kansas Institutional Animal Care and Use Committee. The PLP-induced model of EAE was used to mimic relapsing-remitting multiple sclerosis [29]. Mice were immunized subcutaneously with 200 mg of $PLP_{139–151}$ in a 0.2 mL emulsion composed of equal volumes of phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA)

containing killed Mycobacterium tuberculosis strain H37RA (final concentration of 4 mg/mL; Difco). The $PLP_{139–151}$/CFA was administered to regions above the shoulders and the flanks (total of four sites; 50 µL at each injection site). In addition, 200 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was injected (100 µL) intraperitoneally on the day of immunization (day 0) and 2 days post-immunization. To inhibit disease, mice received subcutaneous injections (100 µL) at the nape of the neck of each SAgA sample or vehicle (sterile PBS), on days 4, 7, and 10. SAgA dose was based on delivering 200nmol PLP peptide as calculated by HPLC. Treatments of HA were administered at a dose equivalent of the SAgAs, 29 nMol. Disease progression was evaluated blindly by the same observer using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund. Mice were euthanized if they were found to have a clinical score of 4 or above. Body weight was also measured daily and is expressed as a percent weight loss calculated from day 8 (peak weight prior to EAE-disease). The total duration of the EAE study was 26 days, from day 0 to day 25.

2.7 Splenocyte Isolation and ex vivo treatment of splenocytes. Mouse spleens were resected 25 days post immunization, passed through a wire mesh using the rubber end of a sterile 1 mL syringe plunger, and collected in 5 mL of RPMI 1640 media. The crude cellular extract was then centrifuged at 1,100 x g for 5 minutes and the resulting cell pellet was resuspended in 3.5 ml of 1x Gey's lysis solution and place on ice for 3.5 minutes to lyse splenic red blood cells. The lysis reaction was stopped by the addition of 10.5 ml RPMI 1640 media containing 10% FBS and centrifuged at 1,100 x g for 5 minutes. The remaining cell pellet

was resuspended in fresh media (RPMI 1640 media containing 10% FBS and 1% Penicillin-Streptomycin) and seeded in 96-well cell culture plates at a cell density of 1x10^6 cells/well in a final volume of 100 µl. Splenocytes were then immediately stimulated with 25 µM PLP or vehicle (RPMI media). Stimulated cell cultures were incubated for 120 hours at 37°C in a CO_2 (5%) incubator. Cell culture supernatant levels of cytokines were determined by a commercially available bead array ELISA kit and supporting FlowCytomix ™ software (ebiosciences, San Diego, CA).

2.8 Statistical analysis. Statistical evaluation of data was performed using a one-way or two-way analysis of variance (ANOVA) as experimentally appropriate, followed by Fisher's least significant difference post-hoc test. The criteria for statistical significance was set at $p<0.05$. All analyses were performed using GraphPad Software (GraphPad Software Inc.).

3. Results

3.1. Synthesis and Characterization of Soluble Antigen Arrays

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

High performance liquid chromatography (HPLC) was used to determine the amount of peptide conjugated for each synthesized SAgA and the number of peptides conjugated per HA

molecule was determined (**Table 2**). Further analysis by gel permeation chromatography (GPC) was used to confirm the final molecular weight of the SAgAs upon peptide conjugation (**Table 3**). Final conjugate molecular weights of approximately 37 to 47 kDa estimated using these two methods were overall in agreement; however, the molecular weight of $SAgA_{PLP:sF2}$ found by GPC was approximately double that originally estimated by HPLC suggesting possible aggregation.

Micro-flow imaging (MFI) analysis of $SAgA_{PLP:sF2}$ revealed the presence of particles, confirming formation of $SAgA_{PLP:sF2}$ aggregates in aqueous solutions (**Figure 1**). Upon observation of the aggregation and poor solubility, $SAgA_{PLP:sF2}$ was solubilized in DMSO following peptide cleavage from the HA backbone. Subsequent analysis of $SAgA_{PLP:sF2}$ by HPLC confirmed a similar conjugation efficiency compared to the other SAgAs, with approximately 7 to 11 peptides per HA backbone of both autoantigen and B7-binding peptide (**Tables 2 and 3**).

3.2. Suppression of EAE by Soluble Antigen Arrays

Soluble Antigen Arrays (SAgAs) co-grafted with autoantigen (PLP) and B7-binding peptide were evaluated in EAE to determine their effect on disease progression. Similar to previous studies, the positive control $SAgA_{PLP:LABL}$ suppressed EAE as indicated by clinical score and weight loss (**Figure 2**) [24, 25]. Additionally, HA alone did not effect EAE disease scores as compared to the negative control, PBS. Interestingly, compared to PBS all B7-targeted SAgAs were able suppress the symptoms of EAE as evaluated by clinical disease score and weight loss (**Figure 2**). All SAgAs also reduced the incidence of disease compared to mice treated with PBS or HA alone (**Figure 3**). No statistical differences in clinical score, weight loss, or incidence of

disease were found between the SAgAs containing different context signal peptides. The data suggests that co-delivery of B7-binding peptides and autoantigen can protect against EAE.

3.3. SAgAs containing B7AP peptide reduced pro-inflammatory cytokine production

To analyze the ability of immune cells in the periphery to respond to re-challenge with PLP, the cytokines GM-CSF, IFN-γ, TNF-α, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17 were measured from splenocytes taken from animals treated with B7-targeted SAgAs or controls (**Figure 4**). Splenocytes were harvested on Day 25 and were cultured directly in the presence of PLP (25 μM) to re-stimulate the immune cells. GM-CSF and IL-2, but not IFN-γ, TNF-α, IL-1α, IL-4, IL-5, IL-6, IL-10, or IL-17, increased in PLP-stimulated splenocytes isolated from PBS-treated EAE mice (**Figure 4**). Levels of IL-10 were below the limit of detection for all treatment groups using this assay (data not shown). Interestingly, the PLP-dependent induction of the pro-inflammatory cytokines IL-2 and GM-CSF was reduced in EAE splenocytes from mice treated with $SAgA_{PLP:B7AP}$ as compared to mice given PBS. Decreased levels of GM-CSF were also found in PLP-stimulated EAE splenocytes from mice treated with $SAgA_{PLP:sF2}$ or with HA as compared to the mice treated with PBS. In EAE mice treated with $SAgA_{PLP:sF2}$, basal levels of IL-6 were not detectable. The data suggested each independent SAgA differentially modulated the cytokine response in splenocytes isolated from EAE mice.

4. Discussion

The B7 signaling pathway has emerged as an important target for immune modulation, with several experimental biologic therapeutics entering clinical trials [17]. For autoimmune diseases, the ability to suppress unwanted immunity by blocking co-stimulation during antigen presentation has the potential to regulate T-cell activation and inhibit subsequent T-cell-

dependent tissue damage [13, 17]. Addition of antigen specificity to such an approach may reduce undesired side effects associated with global immunosuppression that accompanies many of the current immunomodulatory therapies available [4-7]. Indeed, several groups have begun investigating antigen specific immunotherapies to treat autoimmune disorders [22-28, 32-36].

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

The B7 pathway can deliver either a co-stimulatory or co-inhibitory signal during antigen presentation depending on the combinations of surface receptors engaged [12]. In order to investigate the application of SAgAs targeting the B7 pathway we selected three peptides (B7AP, CD80-CAP, sF2), which had been previously reported to bind B7 (Table 1). Two peptides used in this study, B7AP and CD80-CAP, have been shown to inhibit inflammatory immune response in several rodent autoimmune models [18-20, 30, 37]. In contrast, the peptide mimic of CTLA-4 (sF2) was originally selected for this study as a positive control to enhance the immune response by blocking the B7:CTLA-4 regulatory interactions [31]. The reported mechanisms of these peptides led us to hypothesize that $SAgA_{PLP:B7AP}$ and $SAgA_{PLP:CD80\text{-}CAP}$ would suppress EAE disease severity, while $SAgA_{PLP:sF2}$ would increase disease severity in EAE. Surprisingly, all B7-targeted SAgAs, including $SAgA_{PLP:sF2}$, inhibited disease. Other reports,

however, help explain this result. CTLA-4-IgG and soluble CTLA-4 have been shown to have inhibitory functions in several *in vivo* models, suggesting that delivery of a multivalent CTLA-4 mimetic peptide (i.e. $SAgA_{PLP:sF2}$) may actually suppress pro-inflammatory immune responses [38, 39]. Additionally, current immunological models suggest that "over-stimulation" of T-cells may induce anergy and be a mechanism of both peripheral and central tolerance [40]. Delivering multivalent SAgAs that achieve simultaneous high local concentrations of antigen and context signal (i.e. multiple copies of each peptide per molecule) may overwhelm sensitive immune cells and subsequently induce anergy.

In addition, all B7-targeted SAgAs were shown to have similar clinical efficacy as $SAgA_{PLP:LABL}$, which has be shown to inhibit the symptoms EAE in previous studies [24, 25]. Although the SAgAs may be equivalent in the suppression of EAE symptoms, the B7-targeted SAgAs offer an advantage over their ICAM-1-targeted counterparts. Unlike the molecular signals of the B7 pathway, which are only found on either pAPCs or T-cells, ICAM-1 is found on immune cells, endothelial cells, and even in soluble form in the blood [8]. Additionally, previous studies have demonstrated that the conjugation of only PLP to HA does not result in EAE suppression [25]. The importance of the context signal peptide for the suppression of EAE suggests that optimization this peptide may enhance SAgA efficacy. While $SAgA_{PLP:LABL}$ has not demonstrated any off-target effects in previous studies, the specificity of the B7-targeting SAgAs may result in more effective delivery of the SAgA to their immune cell targets.

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

contribute to increased disease severity in EAE [1, 41]. Additionally, $SAgA_{PLP:B7AP,}$ $SAgA_{PLP:sF2,}$ and HA treatments inhibited PLP-dependent GM-CSF cytokine expression, a cytokine suggested to play a role in EAE disease progression [8, 42]. HA alone did not suppress disease symptoms compared to PBS, indicating that reduction of GM-CSF cytokine levels may not be an accurate indicator of *in vivo* efficacy.

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

5. Conclusion

The B7 signaling pathway is a promising target for the treatment of EAE using SAgA technology. Both B7-targeted and ICAM-1-targeted SAgAs were found to suppress clinical disease symptoms as compared to the negative controls. Complexity in cytokine suppression

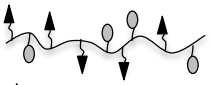
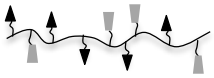
indicates that although all SAgAs resulted in similar *in vivo* clinical efficacy, they may act through different immunological mechanisms at the cellular level. These results suggest that targeting other surface receptors on immune cells may be a beneficial therapeutic option for the treatment of autoimmune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

Table 1. B7-Targeted Peptide Sequences

Peptide[a]	Molecular Mimic	Peptide Sequence[b]	Proposed Interactions Blocked	References
B7AP	CD28	Ao-GGGEFMYPPPYLD	B7/CD28	[18, 30]
CD80-CAP	CD28	Ao-GGGMQPPGC	B7/CD28 and B7/CTLA-4	[19, 20]
sF2	CTLA-4	Ao-TEAGAAGCRGVGVAFIGSCVFG [cyclized]	B7/CTLA-4	[31]

[a] Each peptide was created to mimic a signaling molecule in the B7 pathway and has been previously shown to block B7 pathway signaling interactions.
[b] Ao indicates the N-terminal aminooxy addition for covalent grafting onto hyaluronic acid.

Table 2. SAgA Peptide Conjugation

Soluble Antigen Array		PLP Concentration (nMol)	Context Signal Peptide Concentration (nMol)	Number of Peptides per HA polymer (PLP: Context Signal Peptide)
$SAgA_{PLP:LABL}$		205	277	9:11
$SAgA_{PLP:B7AP}$		170	223	7:09
$SAgA_{PLP:CD80\text{-}CAP}$		233	220	9:08
$SAgA_{PLP:sF2}$*		212	152	10:7*

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

*Indicates that the SAgA was solubilized in DMSO following acidic peptide cleavage.

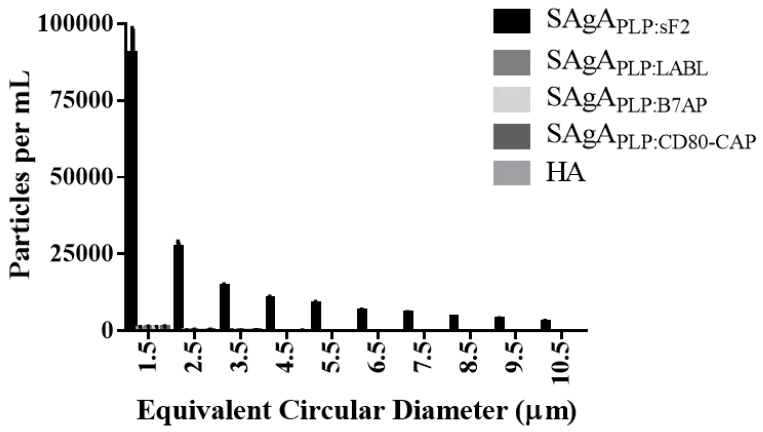
Table 3. SAgA Molecular Weight Calculation by both HPLC and GPC

Soluble Antigen Array	MW calculated from peptide conjugations determined by HPLC (kDa)	MW calculated from GPC data (kDa)[a]
$SAgA_{PLP:LABL}$	42	44
$SAgA_{PLP:B7AP}$	42	41
$SAgA_{PLP:CD80\text{-}CAP}$	37	42
$SAgA_{PLP:sF2}$*	47*	45

[a] The molecular weight of each SAgA was calculated from GPC data as compared to a pullulan polymer standard curve. Results shown are an average from triplicate injections of a single batch preparation. All samples had a RSD$\leq$ 0.01.

*Indicates that the SAgA was solubilized in DMSO following acidic peptide cleavage.

A



B

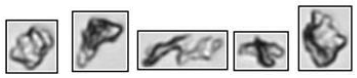
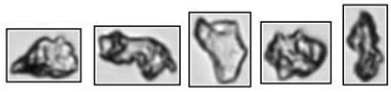
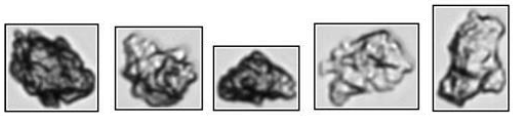
Equivalent Circular Diameter (µm)	Representative Particles
25 to 40µm	
40 to 50µm	
50 to 70µm	

Figure 1. ICAM-1 and B7-targeted SAgAs were analyzed for subvisible particulate formation at a concentration of 0.1 mg/ml in PBS using mico-flow imaging (MFI). A) Concentrations of particles of equivalent circular diameters from 1.5 µm to 10.5 µm are shown for $SAgA_{PLP:sF2,}$ $SAgA_{PLP:LABL}$, $SAgA_{PLP:B7AP}$, $SAgA_{PLP:CD80-CAP}$, and HA alone. B) Representative images of insoluble aggregates from 25 to 70 µm equivalent circular diameter are shown. (Data presented as mean ± S.D. n=3 independent samples)

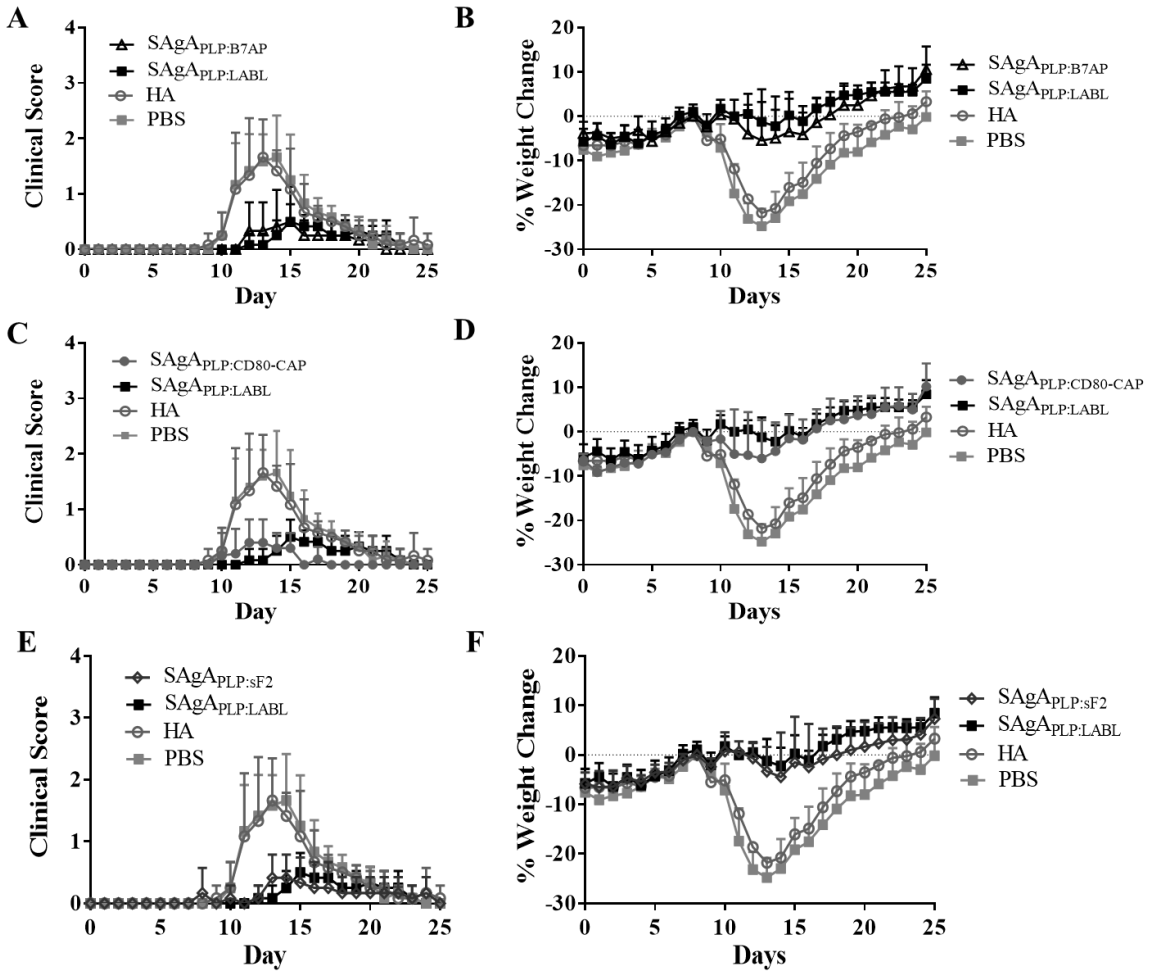


Figure 2. ICAM-1 and B7-targeted SAgAs were found to decrease both clinical score and maintain weight in EAE mice. EAE was induced on day zero, and mice were treated on days four, seven, and ten with a dose of SAgA equivalent to 200 nMol PLP. Treatments of HA were administered at a dose equivalent of the SAgAs, 29 nMol. Clinical disease score and percent weight change were analyzed for groups treated with (A-B) $SAgA_{PLP:B7AP}$, (C-D) $SAgA_{PLP:CD80-CAP}$, and (E-F) $SAgA_{PLP:sF2}$. All SAgAs significantly suppressed clinical disease score as compared to PBS on Days 11 to 15 and as compared to HA on Days 11 to 14. Also, all SAgAs suppressed weight loss as compared to PBS on Days 11 to 18 and as compared to HA on Days 12 to 16. There were no statistical differences in disease suppression as indicated by both clinical score and weight loss between SAgAs containing different B7-binding peptides. Additionally, there were no statistical differences between PBS and HA control groups. (Data are presented as mean ± S.D. n=6 mice per group, $p<0.05$)

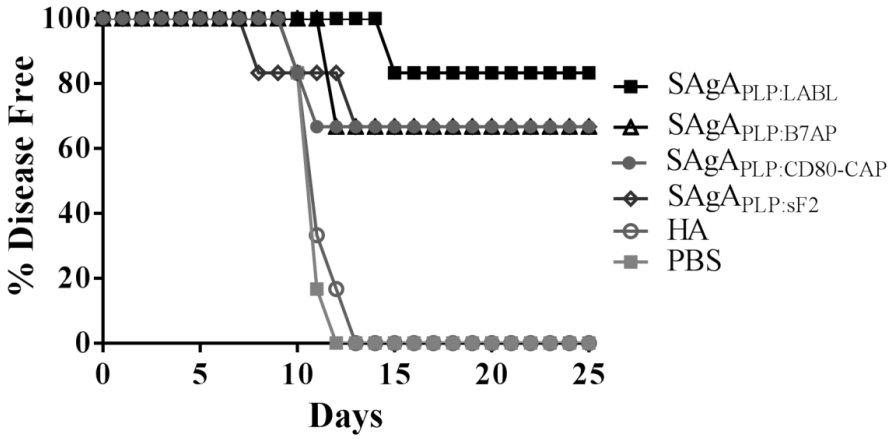


Figure 3. ICAM-1 and B7-targeted SAgAs were found to reduce disease incidence in EAE mice. EAE was induced on day zero, and mice were treated on days four, seven, and ten with a dose of SAgA equivaent to 200 nMol PLP. Treatments of HA were administered at a dose equivalent of the SAgAs, 29 nMol. EAE disease incidence was evaluated such that disease free animals maintained a clinical score <1. In all the SAgA treatment groups, over half of the animals remained disease free over the course of the study, while in both negative control groups all animals became diseased. (n=6 mice per group)

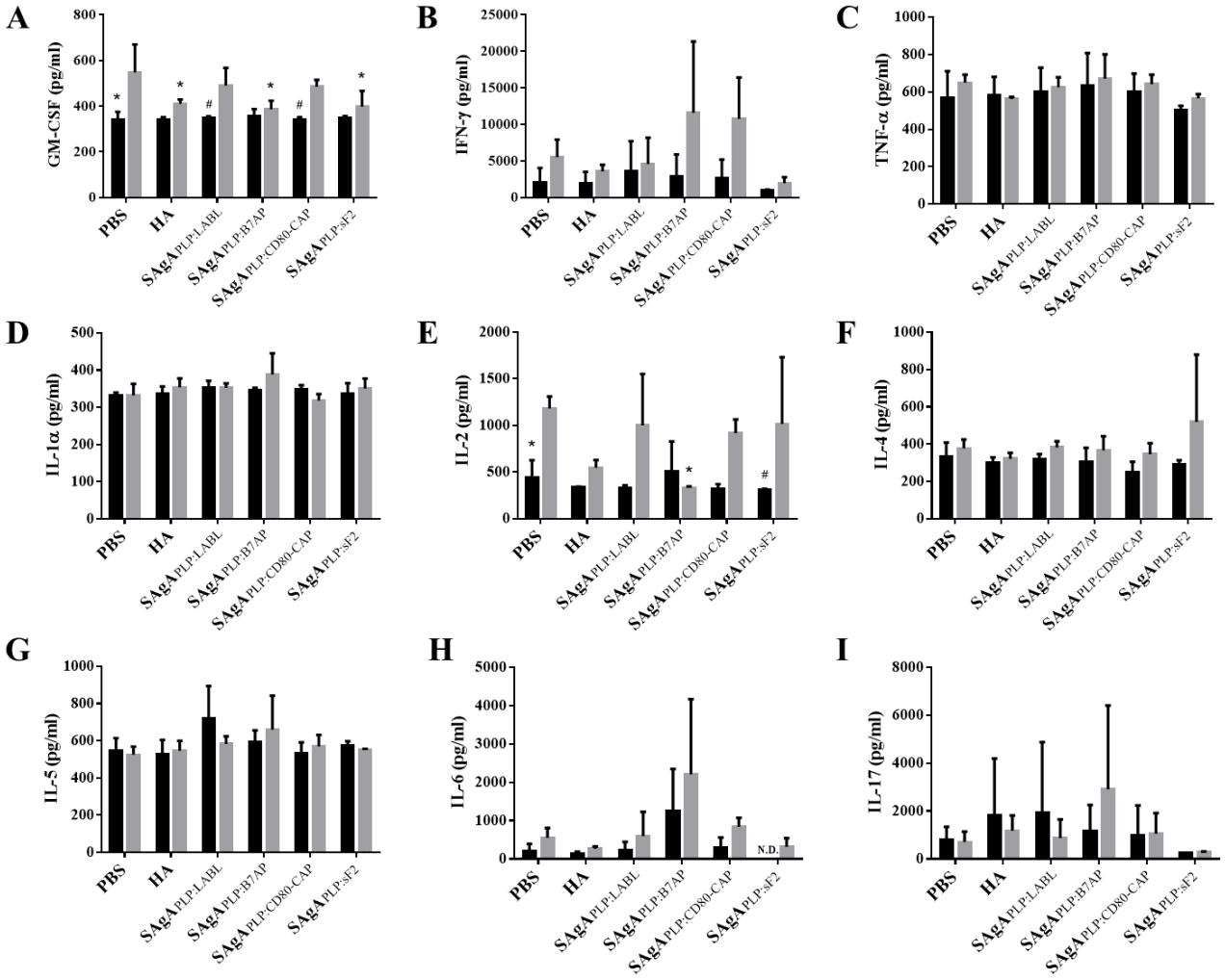


Figure 4. The cytokines GM-CSF, IFN-γ, TNF-α, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17 were measured from splenocytes taken on Day 25 from animals treated with B7-targeted SAgAs or controls. The splenocytes were re-stimulated with 25 µM PLP or vehicle (media) *in vitro* for 120 hours. Supernatant levels of the cytokines (A) GM-CSF, (B) IFN-γ, (C) TNF-α, (D) IL-1α, (E) IL-2, (F) IL-4, (G) IL-6, (H) IL-6, and (I) IL-17 were determined. Cytokine expression of IL-2 and GM-CSF was significantly reduced in splenocytes isolates from EAE mice treated with B7-targeted SAgAs. (Data are presented as mean ± S.D. of independent splenocyte populations isolated from 3 mice, gray bars indicate stimulated with 25 µM PLP, black bars indicate vehicle or media control, * indicates $p<0.05$ as compared to PBS-treated *in vivo* and stimulated *in vitro* with 25 µM PLP, and # indicates $p<0.05$ as compared to the same *in vivo* treatment with 25 µM PLP stimulation *in vitro*.)

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Chapter III: Screening immunomodulators to skew the antigen specific immune response in an autoimmune system

1. Introduction

Autoimmune diseases occur due to the breakdown of immune tolerance towards autoantigens, resulting in the inability of the immune response to distinguish self from non-self. Current treatments for autoimmune diseases are often unsuccessful in stopping or reversing disease progression, and often utilize broadly active immunosuppressants. For example, the immunosuppressant FK-506 (Tacrolimus) has been used for the treatment of several autoimmune diseases including psoriasis, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (MS); all of which have very different immune pathologies [1-6]. While immunosuppressants have been effective in many patients and are currently the 'gold standard' therapy, their benefits are often counterbalanced by toxicities or adverse side-effects [7, 8].

Antigen-specific immunotherapy has long been the 'holy grail' of autoimmune treatments. The ability to suppress the immune response against autoantigens without non-specific global immunosuppression would allow for disease treatment without hindering the ability of the patient's immune system to fight off foreign or opportunistic pathogens. Although different in desired immunological outcome, vaccines are the quintessential example of the potential impact of guiding antigen-specific immune responses [9, 10]. In order to elicit an immune response to a specific antigen, vaccines must often be combined with a secondary signal, or adjuvant, to direct and enhance the antigen-specific immune response [10]. An adjuvant may be defined as a substance capable of enhancing the potency, quality, or longevity of an antigen-specific immune response [11]. While traditional adjuvants have been used to induce protective immune responses against foreign pathogens, it may be possible to skew the response in the opposite direction, towards antigen-specific tolerance by what may be referred to as a 'tolerogenic adjuvant' [12].

Unlike vaccines, antigen-specific immunotherapies (ASIT) applied to allergies or autoimmune diseases have typically utilized the disease-causing antigen without an adjuvant. The use of ASIT to induce tolerance to a particular antigen has been around for over a century in the form of allergen hyposensitization therapy, in which small doses of allergen are administered over an extended period of time to induce immune tolerance [13]. Similar methods have been investigated for autoimmunity where the disease-causing autoantigen is administered in an effort to create autoantigen-specific tolerance. Unfortunately, the success of autoantigen-only ASIT applied to autoimmunity has been modest [9, 14-16]. Attempts to create ASIT have also utilized traditional adjuvants to boost the immune response; however, the efficacy of this strategy has been limited [17, 18]. Recent studies have suggested that combining autoantigen with an immunosuppressant may substantially improve ASIT for autoimmunity by creating a tolerogenic antigen-specific immune response [12, 19-24].

Over the last decade a novel tolerance-induction therapy has emerged in which immunosuppressants (hence forth referred to as immunomodulators) are used in a similar mechanism as traditional adjuvants to enhance immune tolerance to disease-causing autoantigens in autoimmunity. Recent studies indicate that antigen-specific immune tolerance may be achieved by administration of a combination of a disease-causing autoantigen and an immunomodulator [12, 19-24]. Immunomodulators used in this context have been referred to as ‘tolerogenic adjuvants’ in order to describe their ability to direct the immune response to an antigen [19, 25-27]. The majority of these studies have been conducted *ad hoc*, often on only one compound at a time. We observed the need for a screening method to compare these immunomodulators and their ability to skew antigen-specific immune responses towards tolerance.

Current drug screening in the pharmaceutical industry often utilizes systems containing a single target molecule or cell type in order to perform high-throughput analysis [28]. The use of these simple systems can allow for predictions of specific drug target interactions, but has largely been unsuccessful at predicting the result on complex cellular interactions, such as the immune response [29]. The diversity of factors effecting the immune response in treatment of diseases such as autoimmunity may benefit from a more complex screening system. Recent progress has been made in the area of 'physiological screening', where full tissues are used to better understand drug effects of diverse cellular systems [30]. Techniques including whole organ culture, organotypic culture (use of heterogeneous cell cultures to mimic *in vivo* environments), three-dimensional culture, and primary cell cultures have been investigated in order to better mimic the complexity of an *in vivo* system in an *in vitro* environment for improved drug screening [29].

Primary splenocytes in particular are a promising system with which to perform drug screening. As the largest secondary lymphoid organ, the spleen contains a complex immune cell population that migrates to and from the organ as cells travel throughout the body [31]. In the context of the treatment of autoimmune diseases, the spleen is also important as it assists in peripheral tolerance to autoantigens [31]. Splenic antigen presenting cells promote peripheral tolerance through uptake and identification of apoptotic debris as non-immunogenic [31]. The spleen has also been shown to be the primary site for interaction between regulatory T-cells and auto-reactive T-cells in the treatment of a murine model of the autoimmune disease multiple sclerosis (MS), known as experimental autoimmune encephalomyelitis (EAE) [32].

The use of a primary cell system such as splenocytes offers many benefits over immortalized cell systems, especially in the ability to utilize disease models that closely mimic the human

response. EAE has been used in the discovery of several FDA approved drugs for MS, including glatiramir acetate (Copaxone), miroxantrone (Novantrone), natalizumab (Tysabri), and fingolimod (Gilenya) [33]. The version of EAE examined in the following study utilized an epitope from a myelin sheath protein, proteolipid protein (PLP), known to be one of the many disease-causing autoantigens in human MS [34]. EAE induced by $PLP_{139-151}$ mimics the relapsing-remitting form of MS, which affects approximately 80% of MS patients [35, 36]. Although in both MS and EAE the majority of disease progression occurs in the CNS, throughout the course of the disease antigen-specific immune cells return to lymphoid organs to expand the immune response such that antigen-specific cells can be found in the spleen [37, 38]. The use of antigen-educated EAE splenocytes offers a complex antigen-specific environment to facilitate drug screening while minimizing animal use.

Many investigations performed on splenocytes obtained from EAE mice have indicated the potential to discern the antigen-specific immune response *ex vivo*. Rechallenge with PLP *ex vivo* has facilitated examination of immune response following *in vivo* treatment [38, 39]. Studies utilizing *ex vivo* rechallenge with antigen have also improved understanding of epitope spreading in EAE [40, 41]. Although EAE splenocytes have been widely used as an antigen-specific *ex vivo* system, there is no record of their use as a screening system for potential therapeutics. Alternatively, a few other studies have reported *in vitro* screening of immunomodulators to induce tolerance; however, these studies have not used cells obtained from an autoimmune model system [42, 43]. Here, we utilized antigen-specific splenocytes obtained from EAE mice to screen immunomodulators for their ability to skew the antigen-specific immune response in an autoimmune system. Fifteen compounds, including four controls (**Table 1**) and eleven

immunomodulator therapies (**Table 2**) were added during autoantigen rechallenge to determine if splenocyte response could be skewed towards markers of immune tolerance.

2. Materials and Methods

2.1 Materials. The peptide antigen, PLP139-151(HSLGKWLGHPDKF) was obtained from PolyPeptide Laboratories (Torrance, CA). For EAE induction incomplete Freund's adjuvant (IFA) and killed Mycobacterium tuberculosis strain H37RA were purchased from Difco (Sparks, MD), and pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Several immunomodulators were purchased from Sigma-Aldrich (St. Louis, MO), including dexamethasone, simvastatin, andrographolide, dimethyl fumarate, monomethyl fumarate, and lipopolysaccharide (LPS) from E. coli. Rapamycin, FK-506, and ibrutinib were obtained from LC Laboratories (Woburn, MA). Pure curcumin was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Imiquimod (4-amino-1-isobutyl-1H-imidazo[4,5-c]quinoline) was purchased from Accela ChemBio Inc (San Diego, CA). Pam2CSK4 was purchased from Tocris Bioscience (Avonmouth, Bristol, United Kingdom). Mammalian TGF-β1(from HEK293 Cells) was obtained from PeproTech (Rocky Hill, NJ). For imaging and flow cytometry studies, Alexa Fluor 488- conjugated anti-mouse CD3, Alexa Fluor 647-conjugated anti-mouse CD22, and R-phycoerythrin-conjugated anti-mouse CD4 antibodies and their respective isotype controls were purchased from Biolegend (San Diego, CA). All other chemicals and reagents were analytical grade and used as received.

2.2 Induction of EAE. SJL/J female mice, 4 – 5 weeks old, were purchased from Envigo (Harlan) Laboratories and housed under specified, pathogen-free conditions at The

University of Kansas. All protocols involving live mice were approved by The University of Kansas Institutional Animal Care and Use Committee. Mice were immunized subcutaneously with 200 μg of $PLP_{139–151}$ in a 0.2 mL emulsion composed of equal volumes of phosphate-buffered saline (PBS) and incomplete Freund's adjuvant (IFA) containing killed *Mycobacterium tuberculosis* strain H37RA at a final concentration of 4 mg/mL. The $PLP_{139–151}$/CFA was administered to regions above the shoulders and the flanks (total of four sites; 50 μL at each injection site). In addition, 100 μL of pertussis toxin (200 ng) was injected intraperitoneally on the day of immunization (day 0) and 2 days post-immunization. Disease progression was evaluated using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund. Body weight was also measured daily.

2.3 Splenocyte isolation and *ex vivo* treatment. Mouse spleens were resected from EAE mice at peak of disease (11-13 days post immunization) and cultured as previously described [44]. Briefly, the spleens were first passed through a wire mesh using the rubber end of a sterile 1 mL syringe plunger, and collected in 5 mL of RPMI 1640 media. The crude cellular extract was then centrifuged and the resulting cell pellet was resuspended in 3.5 ml of 1x Gey's lysis solution and placed on ice for 3.5 minutes to lyse splenic red blood cells. The lysis reaction was stopped by the addition of 10 ml RPMI 1640 media containing 10% FBS and centrifuged at 1,100 x g for 5 minutes. The remaining cell pellet was resuspended in fresh media (RPMI 1640 media containing 10% FBS and 1% Penicillin-Streptomycin) and seeded in 24-well cell culture plates at a cell density of $5x10^6$ cells/well ($4x10^6$ cells/well for **Figure**

1 only) in a final volume of 1 ml. Splenocytes were plated as biological replicates, with spleens of similar disease severity pooled together as needed. The cells were immediately treated with the immunomodulatory compound of interest, both with and without 25 µM PLP. Stimulated cell cultures were incubated for 120 hours at 37°C in a CO_2 (5%) incubator.

2.4 Measurement of cytokines and cellular metabolism. Supernatants of cell cultures were collected after 120 hour incubation post spleen harvest. Secreted TNF-α, IL-2, IFN-γ, and IL-10 were detected by individual ELISA assay kits (R&D Systems). Cell metabolism was determined by a resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay. Briefly, resazurin (75 µmol/L final) was added to splenocyte cultures and incubated for 3 hours. Metabolic reductive capacity was determined by a change in fluorescence (excitation 560/emission 590). Background fluorescence was determined in RPMI media and was subtracted from each experimental read. All fluorescent readings were performed using a Spectramax M5 (Molecular Devices) plate reader.

2.5 Fluorescent staining. For imaging and flow cytometry experiments, immediately after isolation splenocytes were seeded in 12-well cell culture plates at a cell density of $7.5x10^6$ cells/well in a final volume of 1.5mL. Treatments were then given at the concentration of interest, both with and without 25 µM PLP. Stimulated cell cultures were incubated for 120 hours at 37°C in a CO_2 (5%) incubator. At 120hrs, cells were fixed using 4% paraformaldehyde fixation buffer for 15 minutes on ice. Following fixation, splenocytes were stained with the desired antibodies. Briefly, $1x10^6$ cells were washed with 1mL of wash buffer (RPMI 1640 media containing 5% FBS) before centrifugation and resuspension in 50 µL block buffer containing 20ug/ml TruStain fcX (anti-mouse CD16/32 antibody,

Biolegend) in wash buffer. Cells were incubated on ice for 25 minutes before adding the desired antibodies or isotype controls in 50 µL at 2x the manufacturer recommended concentration for 1 hour. Finally, cells were washed three times and the nuclei were stained with Hoechst 3342 (Life Technologies, Eugene, OR).

2.6 Imaging and flow cytometry. Cell imaging was conducted in a 96-well glass bottom plate (In Vitro Scientific, Sunnyvale, CA) using an Olympus IX81 inverted epiflourescent microscope using 60x magnification. Variations in light intensity were corrected using corresponding isotype controls in Slidebook 6 Software. For flow cytometry analysis 50,000 cells per sample were detected using a BD FACSFusion cytometer. Data was analyzed using FlowJo Software.

2.7 Statistical analysis. Statistical evaluation of data was performed using a two-way analysis of variance (ANOVA), followed by Tukey and Sidak multiple comparison tests. For figures composed of multiple splenocyte isolation experiments, data is presented as fold change from vehicle and was normalized to the vehicle controls (0.1% DMSO) with no antigen for each splenocyte isolation experiment. IC50 values were determined using a 4PL-sigmodal function. A single outlier data point was removed from rapamycin and dexamethasone concentration curves (**Figure 4**) following analysis via the Grubbs' Outlier Test. The criteria for statistical significance was set at $p<0.05$. All analyses were performed using GraphPad Software (GraphPad Software Inc.).

3. Results

3.1. Characterization of an Antigen-Educated System Capable of Demonstrating Immune Response

In order to create a robust screening methodology, the model disease system of EAE splenocytes was thoroughly tested. First, the antigen-specific immune response to proteolipid peptide ($PLP_{139-151}$) was analyzed by comparing splenocytes obtained from both healthy mice and mice that had developed EAE symptoms (day 11-13 post-disease induction). To observe antigen-specific immune responses, healthy and EAE splenocytes were rechallenged with 25 μM PLP and analyzed for cellular metabolic activity and cytokine response. Cellular metabolic activity was found to significantly increase in EAE splenocytes as compared to healthy splenocytes, both with and without rechallenge (**Figure 1A**). Splenocytes obtained from healthy mice did not demonstrate any significant differences with and without rechallenge with antigen (**Figure 1**). Additionally, healthy splenocytes rechallenged with PLP produced a significantly lower cytokine response in all cytokines measured as compared to EAE rechallenged with PLP (**Figure 1B-E**). For all cytokines measured, EAE splenocytes had significantly higher levels when rechallenged with 25 μM PLP compared to no rechallenge (**Figure 1B-E**). The length of PLP rechallenge was also determined in EAE splenocytes to identify a time point that offered the most discernable differences for cellular metabolic activity and cytokine responses (**Figure 2**).

Several controls were analyzed in EAE splenocytes to establish the robustness of the screening system, including three immunogenic controls, imiquimod, Pam2CSK4, and lipopolysaccharide (LPS), and a tolerogenic control, TGF-β (**Table 1**). Our vehicle control, 0.1% DMSO, was also compared to the effect of media alone to confirm that the vehicle did not influence the immune response. All cell metabolic activity and cytokine results were normalized to the response to the vehicle control, 0.1% DMSO with no PLP, for each *ex vivo* experiment.

Cell metabolic activity with 25 μM PLP was shown to significantly increase upon imiquimod treatment with PLP rechallenge, Pam2CSK4 treatment without rechallenge, and significantly decrease with TGF-β treatment both with and without rechallenge as compared to the vehicle (**Figure 3A**). Pam2CSK4 was also found to increase IFN-γ upon rechallenge with antigen as compared to vehicle (**Figure 3C**). Multiple cytokines were found to significantly increase upon LPS treatment as compared to vehicle, both with PLP rechallenge (TNF-α, IFN-γ, IL-10) and without rechallenge (TNF-α, IL-2, IL-10) (**Figure 3B-E**). Overall these results align with previously reported immune effects of the control compounds, verifying the ability of the *ex vivo* splenocyte system to predict immune responses.

3.2. Evaluation of Cytokine Response to Immunomodulators

Once it was confirmed that the EAE splenocyte screening system produced expected outcomes with control treatments, eleven immunomodulatory compounds were analyzed (**Table 2**). As elaborated upon in the Discussion section, a dose of 1 nM was chosen to screen the immunomodulators in the splenocyte system. For each immunomodulator treatment the cellular metabolic activity and cytokine response both with and without PLP antigen rechallenge were measured and compared to the vehicle control (**Figure 4**). Additionally, the immune response of each immunomodulator was compared to the immunostimulant control of LPS (**Figure 5**). Analysis of cellular metabolic activity indicated that the screen contained both compounds that increased and compounds that decreased cell metabolism at the 1 nM dose. Rapamycin was found to decrease metabolic activity both with and without PLP rechallenge, while dexamethasone only decreased metabolism without rechallenge as compared to vehicle (**Figure 4A**). Alternatively, curcumin and acetylsalicylic acid increased metabolism upon PLP rechallenge as compared to both vehicle and LPS controls (**Figure 4A & Figure 5A**).

The cytokine response to each compound during rechallenge with 25 μM PLP was analyzed by measuring TNF-α, IFN-γ, IL-2, and IL-10. The majority of immunomodulator treatments resulted in increased TNF-α levels with 25 μM PLP as compared to the same treatment without antigen rechallenge (**Figure 4B**). The three compounds that did not significantly increase TNF-α upon antigen rechallenge as compared to no rechallenge, dexamethasone, FK-506, and dimethyl fumarate, also significantly decreased TNF-α as compared to the vehicle control (**Figure 4B**). Dexamethasone and FK-506 were also found to decrease IFN-γ levels as compared to vehicle with PLP rechallenge, as did rapamycin and ibrutinib (**Figure 4C**). Curcumin was found to increase IFN-γ both upon antigen rechallenge and as compared to the vehicle control (**Figure 4C**). Levels of TNF-α and IFN-γ were both shown to significantly decrease upon treatment with all immunomodulators as compared to LPS control (**Figure 5B-C**).

Additionally, dexamethasone was found to increase IL-2 levels both upon rechallenge with 25 μM PLP and as compared to the vehicle control (**Figure 4D**). Finally, the cytokine IL-10 was analyzed due to its association with the anti-inflammatory response.[45] Only dexamethasone was shown to increase IL-10 levels both upon rechallenge with antigen and as compared to the vehicle control (**Figure 4E**).

Although our screen at 1 nM was successful, we wanted to confirm that this was the most effective dose for two of the compounds with promising immune responses and low IC50 values, dexamethasone and rapamycin. Five different concentrations of the compounds near the experimentally determined IC50 (**Table 3**) were tested and the resulting cytokine responses were measured (**Figure 6**). While both rapamycin and dexamethasone decreased cellular metabolic activity at 1 nM as compared to the vehicle control (**Figure 4A**), the cytokine responses at these concentrations were not found to follow the same trend. In fact, 1 nM of dexamethasone

increased both IL-2 and IL-10 as compared to vehicle with PLP rechallenge, while lower treatment concentrations did not (**Figure 6C-D**). Additionally, no significant decreases in cytokine levels were shown with 1 nM rapamycin treatment (**Figure 6**).

3.3. Assesment of Cell Population Changes with Immunomodulators

Several compounds which exhibited interesting effects on cell metabolism and cytokine response were further analyzed for changes in splenocyte cell populations, including dexamethasone, rapamycin, ibrutinib, dimethyl fumarate and the controls TGF-β, Pam2CSK4, and LPS. Changes in the population of T-cells and B-cells with respect to the whole splenocyte population were evaluated by flow cytometry and observed by fluorescent microscopy (**Figures 7 and 8**). The dendritic cell (DC) population was also investigated; however, levels were not detectable (data not shown).

The overall T-cell population, as indicated by CD3+ cells, was found to decrease upon dexamethasone and rapamycin treatments as compared to LPS without antigen rechallenge (**Figure 7A**). In contrast to T-cell changes, both ibrutinib and TGF-β treatments were found to decrease B-cell populations upon antigen challenge as compared to LPS with PLP rechallenge (**Figure 7B**). Additionally, the population of T-helper cells characterized by both CD3 and CD4 surface markers was measured. Both dexamethasone and rapamycin were found to decrease the percent of CD3+CD4+ T-cells without PLP rechallenge as compared to LPS (**Figure 7C, D**). No statistical differences were found between the immunomodulator treatments and the vehicle control.

Finally, the ratio of CD3+ T-cells to CD22+ B-cells was evaluated upon treatment with several compounds. Dexamethasone was found to increase the CD3+ to CD22+ ratio upon

rechallenge with antigen as compared to LPS (**Figure 8A**). This effect may also be visually observed in fluorescent imaging of the cell populations following treatments with vehicle (0.1% DMSO), dexamethasone, or LPS (**Figure 8B**).

4. Discussion

Several published studies have reported antigen rechallenge experiments using EAE splenocytes and a few other studies reported *in vitro* screening of immunomodulators to induce tolerance [38, 39, 42, 43]. To our knowledge this is the first paper to combine these two techniques in order to enhance discovery of treatments of EAE capable of skewing the antigen-specific immune response towards markers of immune tolerance. Several reports using EAE splenocytes *ex vivo* have suggested utilizing these cells may improve immunological relatability to the *in vivo* model, as studies have found corresponding immune responses both *in vivo* and *ex vivo* [22, 44]. Specifically, the studies presented here utilized a mixed cell system found to contain diverse populations of B-cells (CD22+), T-cells (CD3+), and T-helper cells (CD3+CD4+) to better mimic the immunological environment *in vivo* (**Figures 7 and 8**). As compared to an *in vivo* study, the *ex vivo* screen utilized fewer animals and material to demonstrate immune responses. Overall, the complex immune environment of the spleen may serve as a bridge between *in vitro* immortalized cell lines and *in vivo* studies for drug screening.

In order to evaluate the immune response to various treatments in the EAE splenocytes system, cellular assays were needed to measure both metabolic activity and cytokines responses. Cellular metabolic activity was primarily utilized as a measure of cell viability to ensure the compounds tested were not cytotoxic at the concentrations analyzed. Four different cytokines, TNF-α, IFN-γ, IL-2, and IL-10, were used to compare the immune response to various

treatments as each of these cytokines has been shown to have important impacts on EAE, both in disease development and suppression. Pro-inflammatory cytokines TNF-α and IFN-γ were measured as both have been found to indicate EAE pathogenesis [34, 46] and are characteristic of T-helper type 1 (Th1) activation, which is believed to be the primary cell type in development of EAE [45, 47]. The cytokine IL-2 plays a particularly interesting role in autoimmune pathology and treatment, as it has been indicated in both inflammatory T-cell and regulatory T-cell (Treg) responses [45, 48]. Although increased concentrations of IL-2 have been found in MS patients indicating a pro-inflammatory activity, IL-2 has recently been shown to be necessary for the production of memory Tregs [48-51]. Finally, the cytokine IL-10 was chosen as a marker of immunosuppression and tolerance. IL-10 has been shown to suppress the activity of effector T-cells and promote the expansion of Tregs in both EAE and MS [52]. Each assay was used to both establish the EAE splenocyte system as an *ex vivo* screening tool and to evaluate the immune response to various treatments.

During development of the *ex vivo* screening assay using EAE splenocytes, we first confirmed the presence of an antigen-specific response *ex vivo*. When comparing the immune response upon antigen rechallenge, EAE splenocytes were found to produce significantly higher cytokine responses upon PLP rechallenge as compared to no rechallenge (**Figure 1**). Healthy splenocytes did not produce an antigen-specific response for any of the cytokines measured (**Figure 1**). Additionally, cytokine levels with PLP rechallenge were significantly increased in EAE mice as compared to healthy mice (**Figure 1**). These results demonstrate the importance of an antigen-educated system such as EAE splenocytes to produce an antigen-specific immune response *ex vivo*.

Following demonstration of antigen-specific responses in EAE splenocytes, several controls were tested in order to confirm the robustness of the *ex vivo* screening system (**Figure 3**). The controls chosen all had well established immune responses, both pro-inflammatory (imiquimod, Pam2CSK, and LPS) and anti-inflammatory (TGF-β) responses (**Table 1**). LPS treatment is well known to increase pro-inflammatory cytokine production, including TNF-α and IFN-γ [45]. IL-2 is most likely increased due to inflammatory T-cell proliferation following LPS activation, although this was only observed without antigen rechallenge (**Figure 3D**). Although IL-10 is generally considered an anti-inflammatory cytokine, LPS treatment of EAE splenocytes increased IL-10 (**Figure 3E**). LPS had been shown in literature to drastically increase IL-10 levels in cells that have been 'over-stimulated' in order to control excessive immune activation, as may be the case when stimulating splenocytes with both PLP antigen and LPS [53, 54]. Pam2CSK4 stimulates the immune response through toll like receptors in a similar mechanism to LPS [55] and therefore is associated with many similar pro-inflammatory responses, such as the increase in IFN-γ as compared to vehicle (**Figure 3C**).

The concentration at which to screen small molecule immunomodulators was then determined through analysis of successful *in vivo* studies in literature and known IC50 concentrations (**Table 3**). The compounds selected have all demonstrated tolerogenic potential in literature at *in vitro* doses ranging from 0.1 nM to 2.5 mM (**Table 2**). For screening the compounds in our splenocyte system, a concentration was determined to best mimic the immunomodulator concentration that would be experienced by immune cells in secondary lymphoid organs following *in vivo* treatment while preventing significant cytotoxicity. Literature examining the *in vivo* doses of these immunomodulators reported effective tolerogenic doses ranging from 0.08 to 2.0 mM, and often utilized particulate delivery systems such as

microparticles or liposomes given subcutaneously [22, 24, 56]. The micromolar dosing of immunomodulator subcutaneously in particles has been found to correlate with ≤0.1% of total dose trafficking to secondary lymphoid organs such as the spleen [57, 58]. The reported *in vivo* doses and delivery vehicle distribution would suggest approximately 0.08 to 2 nM dose reaches splenocytes. Immunomodulator dose to these cells is important as the majority of antigen-specific cell activation occurs in secondary lymphoid organs [31, 45].

In addition, many of these compounds cause global immunosuppression at high concentrations, suggesting they should be given at lower doses to induce immune tolerance [59, 60]. In order to prevent possible global immunosuppression and cellular cytotoxicity, the IC50 for cellular metabolic activity was found in literature and in our splenocyte system for several compounds (**Table 3**). Previous studies and knowledge of the IC50 concentrations led us to explore a dose of 1 nM. Finally, further investigation of the dosing range of dexamethasone and rapamycin supported the selection of a 1 nM dose (**Figure 6**).

Once the EAE splenocyte system was established as being antigen-specific, having reproducible immune responses, and an appropriate concentration was determined for small molecule immunomodulators, eleven compounds were screened for their ability to skew the antigen-specific immune response (**Table 2**). Cell metabolism, cytokine response, and the change in effector cell populations with different treatments were investigated. Several compounds demonstrated unique immune responses in the splenocytes, indicative of both successful and unsuccessful tolerogenic potential. Dexamethasone, rapamycin, FK-506, ibrutinib, and dimethyl fumarate each demonstrated somewhat unique responses indicating a tolerogenic effect. The other compounds screened (propargylglycine, simvastatin, andrographolide, curcumin, acetylsalicylic acid, and monomethyl fumarate) either had minimal

differences as compared to the vehicle control, or resulted in a response that did not indicate immune tolerance.

Overall, dexamethasone presented the most significantly different responses as compared to the vehicle control in all cytokines measured (**Figure 4**). Dexamethasone also decreased both CD3+ and CD3+CD4+ T-cell populations as compared to LPS (**Figure 7**). Significant decreases in both TNF-α and IFN-γ indicated the ability of dexamethasone to maintain immunosuppression even upon rechallenge with antigen (**Figure 4 B, C**). Dexamethasone also increased both IL-2 and IL-10 with PLP rechallenge and therefore potentially promoted an antigen-specific regulatory response (**Figure 4 D, E**). These findings are consistent with previous studies, which have shown dexamethasone can increase tolerogenic cytokines and regulatory cell populations [12, 42, 61, 62]. Additionally, dexamethasone treatment decreased both CD3+ T-cell and CD3+CD4+ T-cell populations as compared to the immunostimulant LPS without antigen rechallenge (**Figure 7 A, C, D**). With the increased IL-2, the population of T-cells would be expected to increase with dexamethasone treatment; however, it has been previously shown in literature that the presence of both dexamethasone and IL-2 in EAE shifts the T-cell population towards CD4+ Tregs while decreasing immunogenic CD4+ T-cells at a higher capacity, resulting in a net decrease in the CD4+ T-cell population [62, 63]. In contrast to the overall T-cell decreases, the ratio of CD3+ T-cells to CD22+ B-cells was found to increase upon treatment with dexamethasone as compared to LPS with PLP rechallenge (**Figure 8**). Although historically considered to be a T-cell mediated disease, recently B-cells have been shown to play an important role in EAE and MS pathogenesis [64, 65]. The importance of B-cell populations in EAE was demonstrated in our results by the known tolerogenic cytokine TGF-β specifically decreasing the percent of CD22+ B-cells in splenocytes as compared to LPS (**Figure 7B**). The

depletion of B-cells has also been shown to be effective in treating MS, with several therapies targeting CD20+ B-cells (Rituximab, Ocrelizumab, and Ofatumumab) demonstrating efficacy in humans [66-68]. Overall, dexamethasone was the most effective at skewing the antigen-specific immune response of the compounds screened at a dose of 1 nM, and will be more deeply investigated in future studies.

In addition to dexamethasone, the immunomodulators rapamycin, FK-506, ibrutinib, and dimethyl fumarate were all found to have effects on cell metabolism and cytokines indicative of antigen-specific immunomodulation and tolerance (**Figure 4**). FK-506 was both found to decrease TNF-α and IFN-γ as compared to vehicle upon rechallenge with antigen (**Figure 4 B, C**). These results consistent with the tolerogenic abilities FK-506 has demonstrated in literature, including the ability to expand Treg populations while decreasing immunogenic T-cell populations such as T-helper type 17 (Th17) cells [19]. Additionally, rapamycin, dimethyl fumarate, and ibrutinib were each found to decrease one pro-inflammatory cytokine, either TNF-α or IFN-γ (**Figure 4 B, C**).

Rapamycin was found to not only decrease pro-inflammatory cytokines as compared to vehicle, but also resulted in significant decreases in both CD3+ and CD3+CD4+ T-cell populations as compared to LPS (**Figure 7A, C**). While these findings point towards strong anti-inflammatory activity, the decreased cellular metabolic activity at the same concentration (**Figure 4A**) may point to a mechanism based on cytotoxicity rather than immune modulation. Both measured and literature IC50 values for rapamycin fell below the screening concentration of 1 nM (**Table 3**). It is possible that either cell death or quiescence was occurring at this concentration. When alternative rapamycin concentrations were studied, higher levels of TNF-α and IL-2 were found at 0.01 nM (**Figure 6A-B**), as further evidence that cytotoxicity at 1 nM

may be leading to decreased pro-inflammatory cytokine response. Rapamycin has previously been shown in literature to promote Treg expansion; however, it is possible that the decrease in cellular metabolic activity at 1 nM (**Figures 4A**) may have limited the ability for this proliferation to occur [69-71]. These results indicated that although 1 nM was a reasonable concentration for an initial screen, further analysis of compounds with low IC50s, such as rapamycin, may benefit from screening at a lower dose to fully understand their tolerogenic potential.

Several of the other immunodulators screened, including propargylglycine, simvastatin, andrographolide, and monomethyl fumarate, resulted in no significant change in metabolism or cytokine response as compared to the vehicle control (**Figure 4**). Additionally, the compounds curcumin and acetylsalicylic acid seemed to produce metabolic and cytokine responses, which opposed their tolerogenic potential reported in literature [72, 73]. Curcumin and acetylsalicylic acid were both found to increase cellular metabolic activity compared to vehicle and LPS controls with antigen rechallenge (**Figure 4A and Figure 3A**). Curcumin was also found to display further pro-inflammatory characteristics by increasing IFN-γ levels as compared to vehicle (**Figure 4C**). The lack of immunosuppression by curcumin may be due to the 1 nM dose, as previous studies have shown that *in vitro* doses of 1000 nM or greater are needed for immunosuppressive effects, and 25,000 nM or greater for inducing tolerogenic responses [72, 74].

5. Conclusion

In this chapter we demonstrated that splenocytes from EAE mice can be used as an effective *ex vivo* screening system for evaluating the antigen-specific immune response in an autoimmune

model. EAE splenocytes demonstrated antigen-specific immune responses through rechallenge with PLP antigen, and therefore offered a mechanism by which to screen immunomodulators for their ability to skew the antigen-specific immune response. Of the eleven immunomodulators investigated, only dexamethasone increased tolerogenic cytokines, decreased pro-inflammatory cytokines, and decreased CD3+ and CD3+CD4+ T-cell populations at a relevant dose. Further studies into other immunological markers and *in vivo* effects of dexamethasone are needed to confirm the ability to induce antigen-specific tolerance upon co-delivery with autoantigen. Additionally, further investigation is needed to determine if less potent compounds, such as curcumin and acetylsalicylic acid, have potential to skew the antigen specific immune response at higher concentrations. Nevertheless, these *ex vivo* studies provide a foundation for the investigation of immunomodulators for use in antigen-specific treatment of autoimmunity, which may be extrapolated to other animal models of autoimmune diseases or perhaps even human samples.

Table 1. Controls with known Immune Response Used for Validation of Screen

Control Compound	Target	Immune Response
Transforming Growth Factor (TGF)- β	TGF-β receptor	Produces a regulatory response by inducing Treg and tolerogenic DC production and suppression of inflammation [45, 75, 76]
Imiquimod	TLR7	Immunostimulation and pro-inflammatory cytokine production [77]
Lipopolysaccharide (LPS)	TLR4	Immunostimulation and pro-inflammatory cytokine production [45]
Pam2CSK4	TLR2 & TLR 6	Immunostimulation and pro-inflammatory cytokine production [55]

Table 2. Immunomodulators Screened in EAE Splenocytes

Compound	Target	Proposed Tolerogenic Functions	Dosing range to induce tolerance *in vitro* from Literature (nM)
Dexamethasone	Glucocorticoid receptor	Induces Treg and tolerogenic DC proliferation [12, 42, 61, 62, 78]	0.1 to 1000 [61, 62, 78-80]
Rapamycin	mTOR pathway	Suppresses IL-2 production, induces Treg proliferation [42, 43, 69, 81-83]	10.9 to 100 [69, 70, 81]
FK-506	Calcineurin	Suppresses IL-2 production, decreases Th17 response, induces Treg and tolerogenic DC proliferation [19, 84, 85]	0.124 to 12.4 [84-86]
Simvastatin	HMG CoA Reductase	Induces IDO expression, induces Treg proliferation, induces production of IL-10 and TGF-β [42, 87]	4,800 to 10,000 [42, 87]
Andrographolide	NF-κB	Induces tolerogenic DC proliferation [88]	10,000 [88]
Proparglyglycine	Cystathionine-γ-lyase	Suppresses IL-12 and IFN-γ production [89]	Not reported *in vitro*
Curcumin	unknown	Induces tolerogenic DC proliferation and IL-10 production [72, 74]	50,000 [72, 74]
Acetylsalicylic Acid	Cyclooxygenase 1,2	Induces Treg and tolerogenic DC proliferation [73, 90]	2,500,000 [73, 91]
Ibrutinib	Burton's tyrosine kinase	Inhibits APC migration, suppresses pro-inflammatory cytokine production [92]	Not reported *in vitro*
Dimethyl fumarate	Nrf2 via monomethyl fumarate metabolite	Decreases oxidative stress and pro-inflammatory cytokine production, induces Treg proliferation [93-95]	Not reported *in vitro*
Monomethyl fumarate	Nrf2	Decreases oxidative stress, protects inflamed blood-brain barrier [93, 94, 96]	45,000 to 90,000 [96]

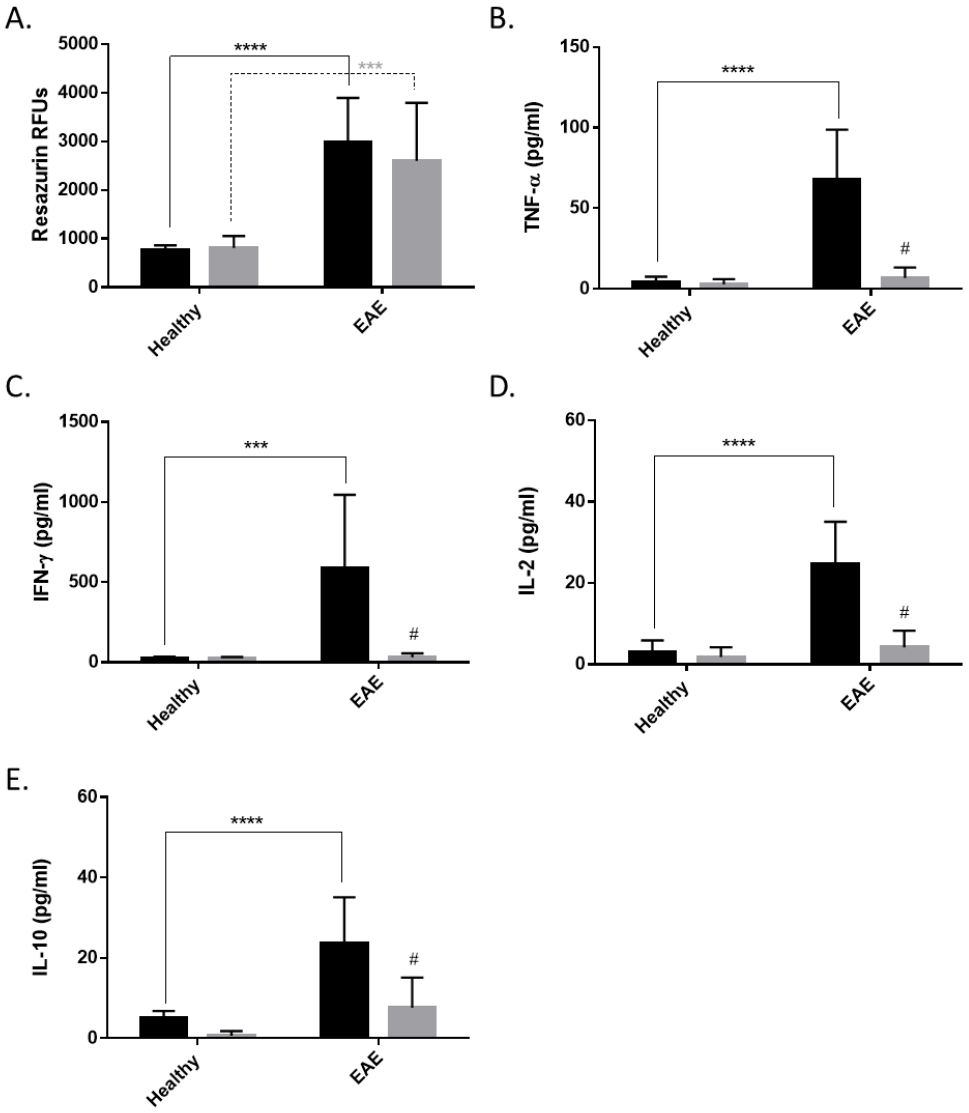

Figure 1: Splenocytes were harvested from healthy and EAE mice and rechallenged with (black bars) or without (gray bars) 25 μM PLP antigen for 120 hours. A.) Cell metabolism via resazurin and supernatant cytokine levels of B.) TNF-α, C.) IFN-γ, D.) IL-2, E.) IL-10 were determined for healthy splenocytes and EAE splenocytes. (n=7 per group, * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ for healthy versus EAE splenocytes with comparisons to 25 μM PLP in black and no PLP in gray, # $p<0.05$ for 25 μM PLP versus no PLP, Black bars indicate with 25 μM PLP and Gray bars indicate no PLP rechallenge, RFUs are relative fluorescence units)

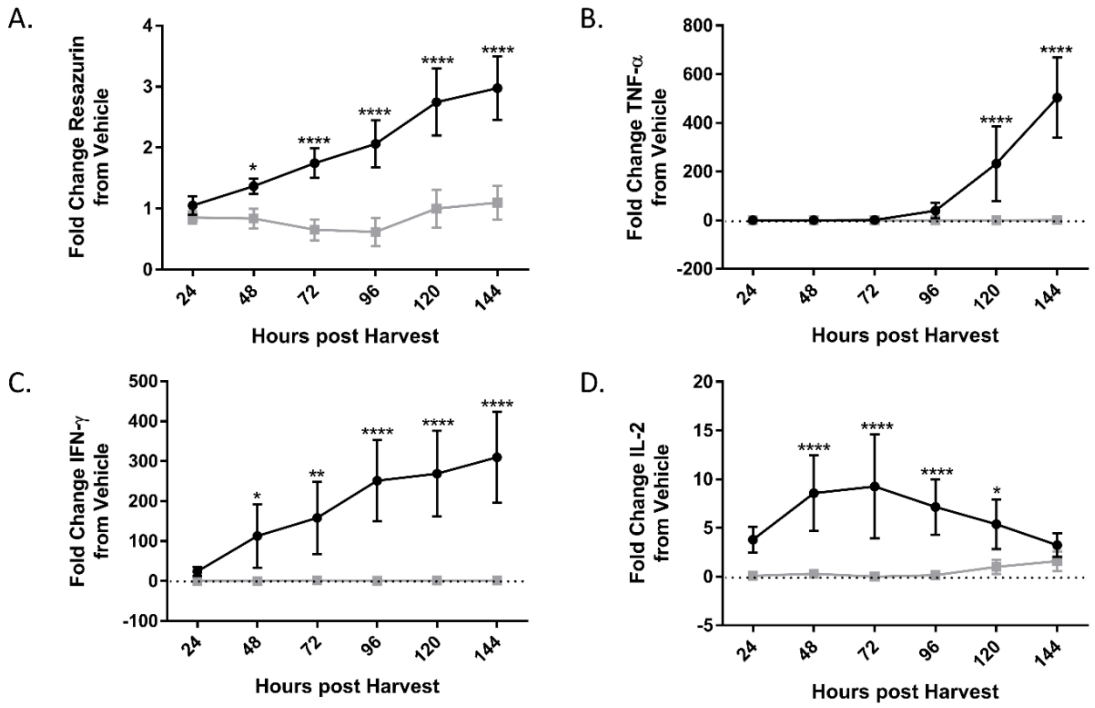

Figure 2: Splenocytes were harvest from EAE mice and treated with and without 25 μM PLP rechallenge for 24, 48, 72, 96, 120, or 144 hours. A.) Cell metabolism via resazurin and supernatant cytokine levels of B.) TNF-α, C.) IFN-γ, and D.) IL-2 were determined. All data was normalized to 120 hours without PLP rechallenge. The cytokine IL-10 was measured but not detectable at 120 hours without rechallenge, so data is not shown. ($n \geq 5$ per group; * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ 25 μM PLP versus no PLP rechallenge for each time-point, Black lines indicate with 25 μM PLP and Gray lines indicate no PLP rechallenge)

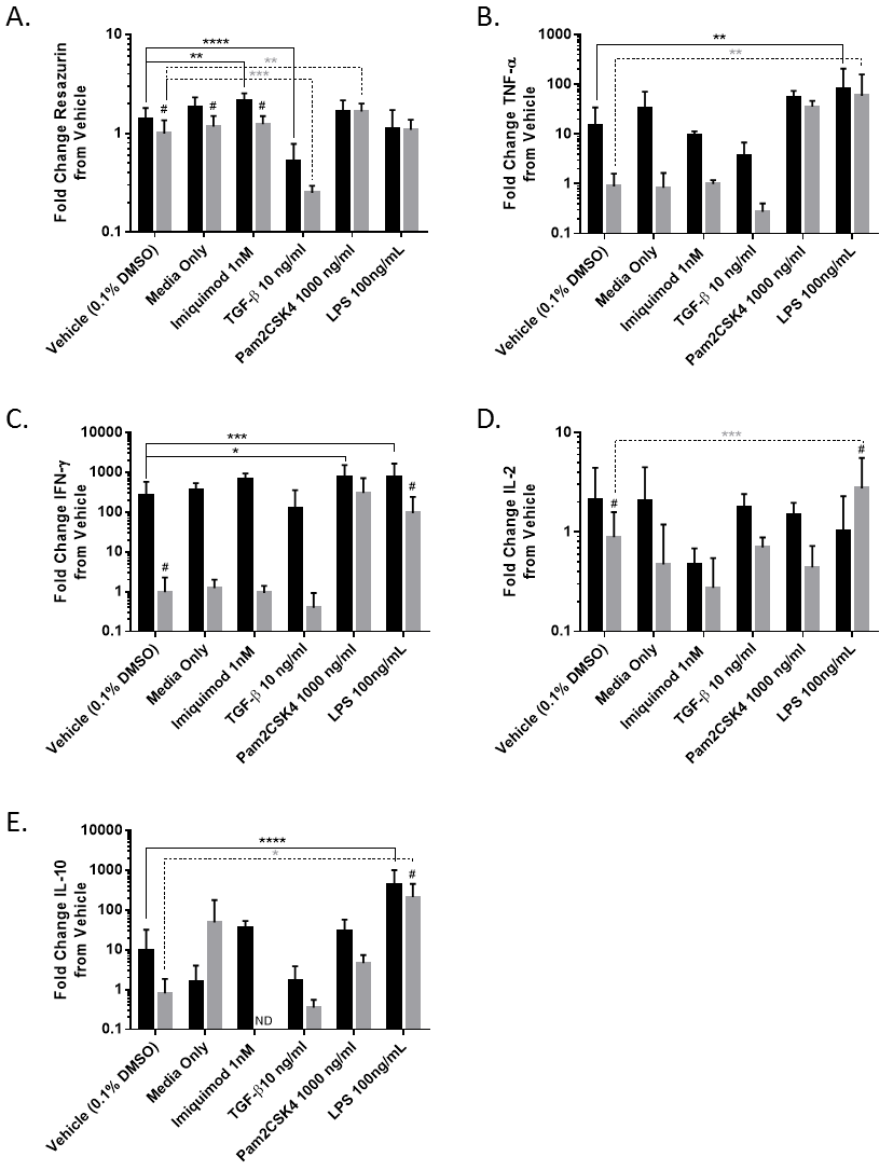

Figure 3. Splenocytes were harvested from EAE mice and treated with a control compound for 120 hours, with or without 25 µM PLP antigen rechallenge. A.) Cell metabolism and supernatant cytokine levels of B.) TNF-α, C.) IFN-γ, D.) IL-2 and E.) IL-10 were determined. All data was normalized to vehicle control without PLP rechallenge. (n≥5 per group; * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ for treatment group versus vehicle with comparisons to 25 µM PLP in black and no PLP in gray, # $p<0.05$ for 25 µM PLP versus no PLP, ND indicates cytokine levels were not detectable, Black bars indicate with 25 µM PLP and Gray bars indicate no PLP rechallenge)

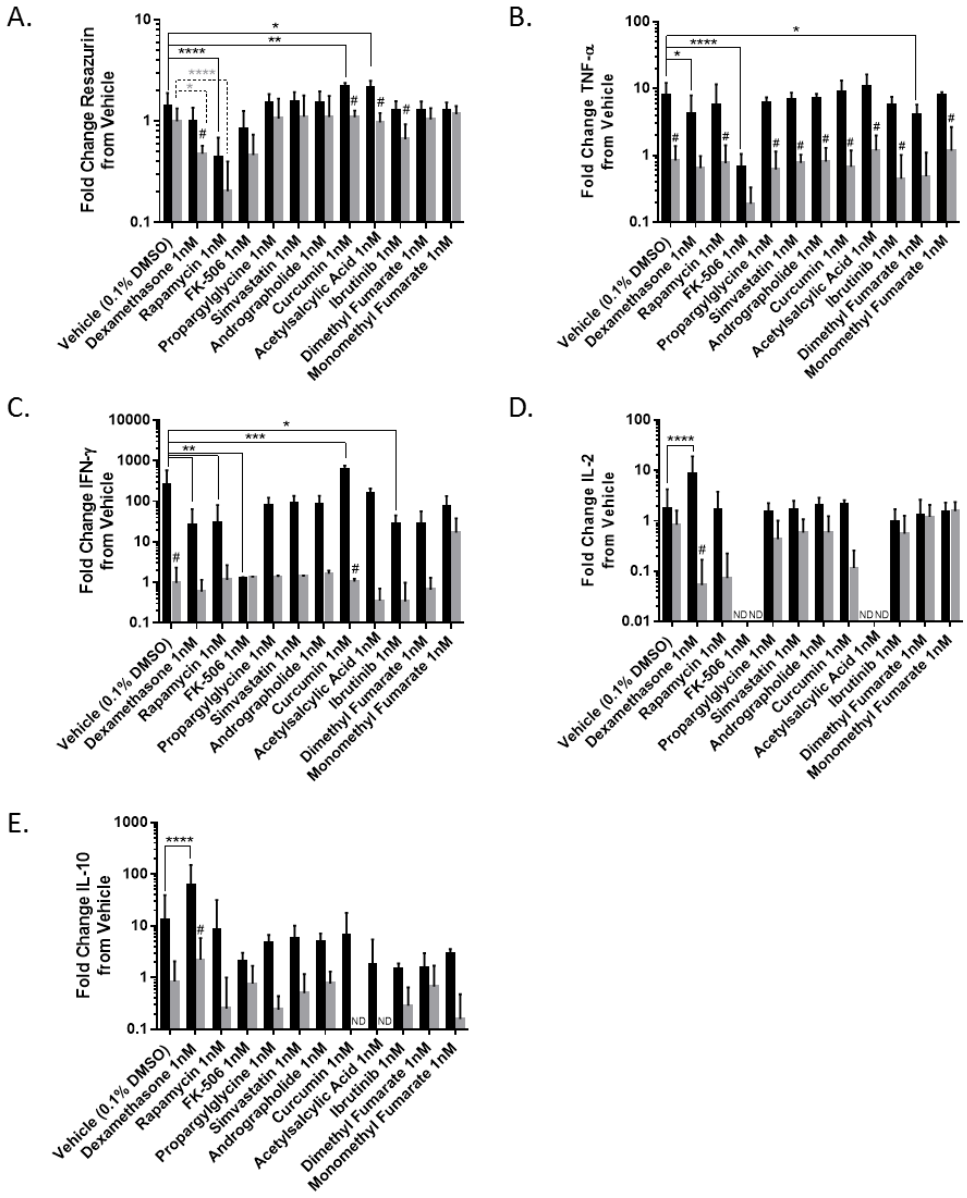

Figure 4. Splenocytes were harvested from EAE mice and treated with either vehicle or an immunomodulatory compound at 1 nM for 120 hours, with or without 25 μM PLP antigen rechallenge. A.) Cell metabolism and supernatant cytokine levels of B.) TNF-α, C.) IFN-γ, D.) IL-2 and E.) IL-10 were determined. All data was normalized to vehicle control without PLP rechallenge. ($n \geq 5$ per group; * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ for treatment group versus vehicle with comparisons to 25 μM PLP in black and no PLP in gray, # $p<0.05$ for 25 μM PLP versus no PLP, ND indicates cytokine levels were not detectable, Black bars indicate with 25 μM PLP and Gray bars indicate no PLP rechallenge)

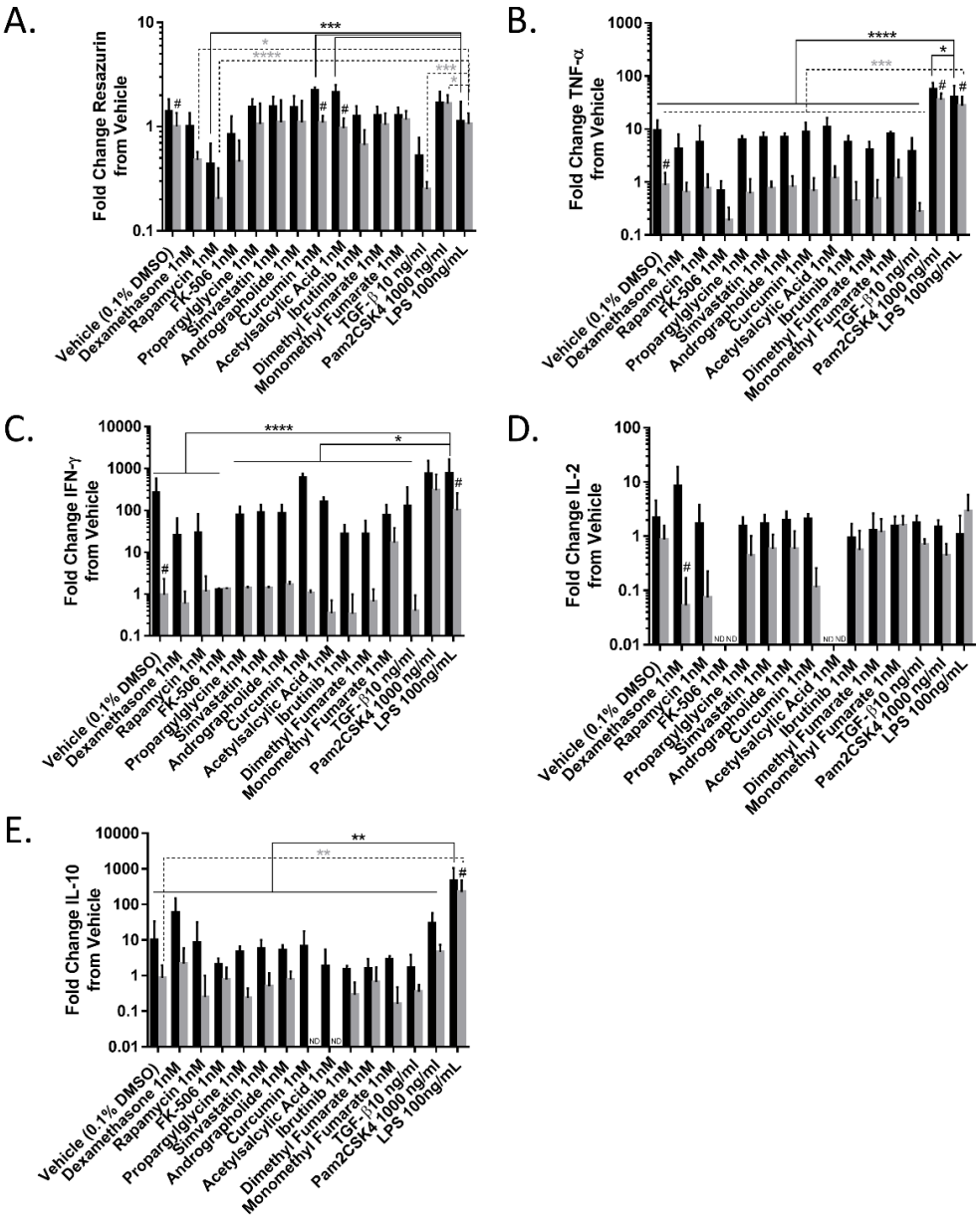

Figure 5. Splenocytes were harvested from EAE mice and treated with either vehicle or an immunomodulator at 1 nM for 120 hours, with or without 25 μM PLP antigen rechallenge. A.) Cell metabolism and supernatant cytokine levels of B.) TNF-α, C.) IFN-γ, D.) IL-2 and E.) IL-10 were determined. All data was normalized to vehicle control without PLP rechallenge. (n≥5 per group; * $p<0.05$ ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ for treatment group versus LPS control with comparisons to 25 μM PLP in black and no PLP in gray, # $p<0.05$ for 25 μM PLP versus no PLP, ND indicates cytokine levels were not detectable, Black bars indicate with 25 μM PLP and Gray bars indicate no PLP rechallenge)

Table 3. Molecular weight and IC50 range, both from literature and experimentally determined for immunomodulatory compounds screened.

Compound	**Molecular Weight**	**IC50 Range from Literature in nM** ***(Experimentally determined IC50 in EAE Splenocytes in nM)***
Dexamethasone	392.5	4 to 1,000 *(5.0)* [97-99]
Rapamycin	914.2	0.2 to 0.5 *(0.04)* [100]
FK-506	804.02	0.1 to 8.6 *(0.2)* [100-103]
Simvastatin	418.6	170 to 35,300 *(100)* [104, 105]
Andrographolide	350.45	1,000 to 6,000 (2,000) [106-108]
Propargylglycine	113.11	>8.84 x 10^10 *(>100,000)* [109]
Curcumin	368.38	5,500 to 11,600 [110]
Acetylsalcylic Acid	180.16	100,000 [111]
Ibrutinib	440.50	2.0 to 6,400 [92, 112-114]
Dimethyl fumarate	144.13	2,300 to 2,500 [93, 115]
Monomethyl fumarate	130.10	See dimethyl fumarate

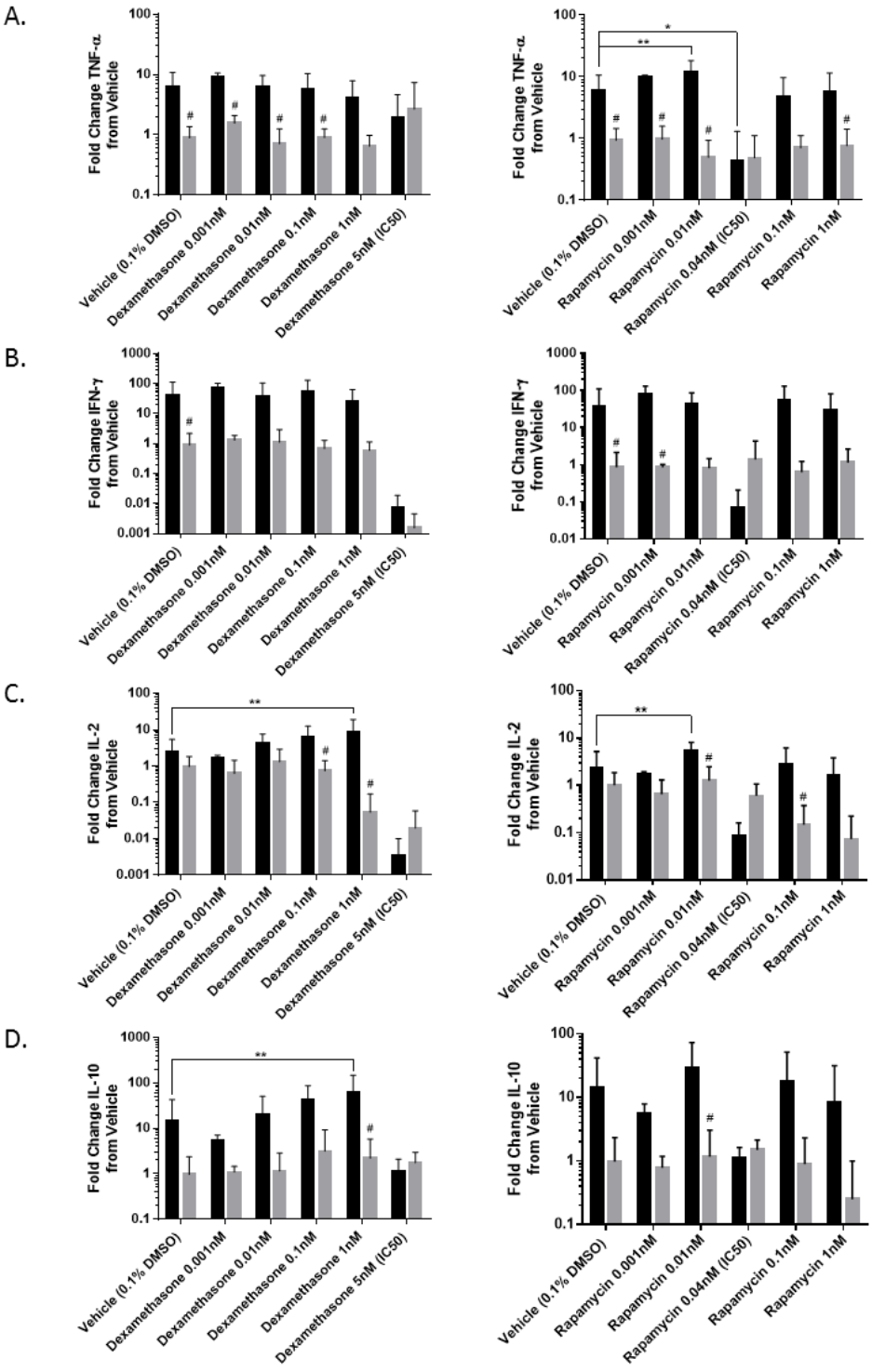

Figure 6. Splenocytes were harvest from EAE mice and treated with different concentrations of dexamethasone and rapamycin for 120 hours, with or without 25 µM PLP rechallenge.

Concentrations around the IC50 (5 nM-0.001 nM) were analyzed and supernatant cytokine levels of A.) TNF-α, B.) IFN-γ, C.) IL-2, and D.) IL-10 were determined. All data was normalized to vehicle control without PLP rechallenge. ($n \geq 5$ per group; * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ for different treatment concentrations versus vehicle with comparisons to 25 μM PLP in black and no PLP in gray, # $p<0.05$ for 25 μM PLP versus no PLP, Black bars indicate with 25 μM PLP and Gray bars indicate no PLP rechallenge)

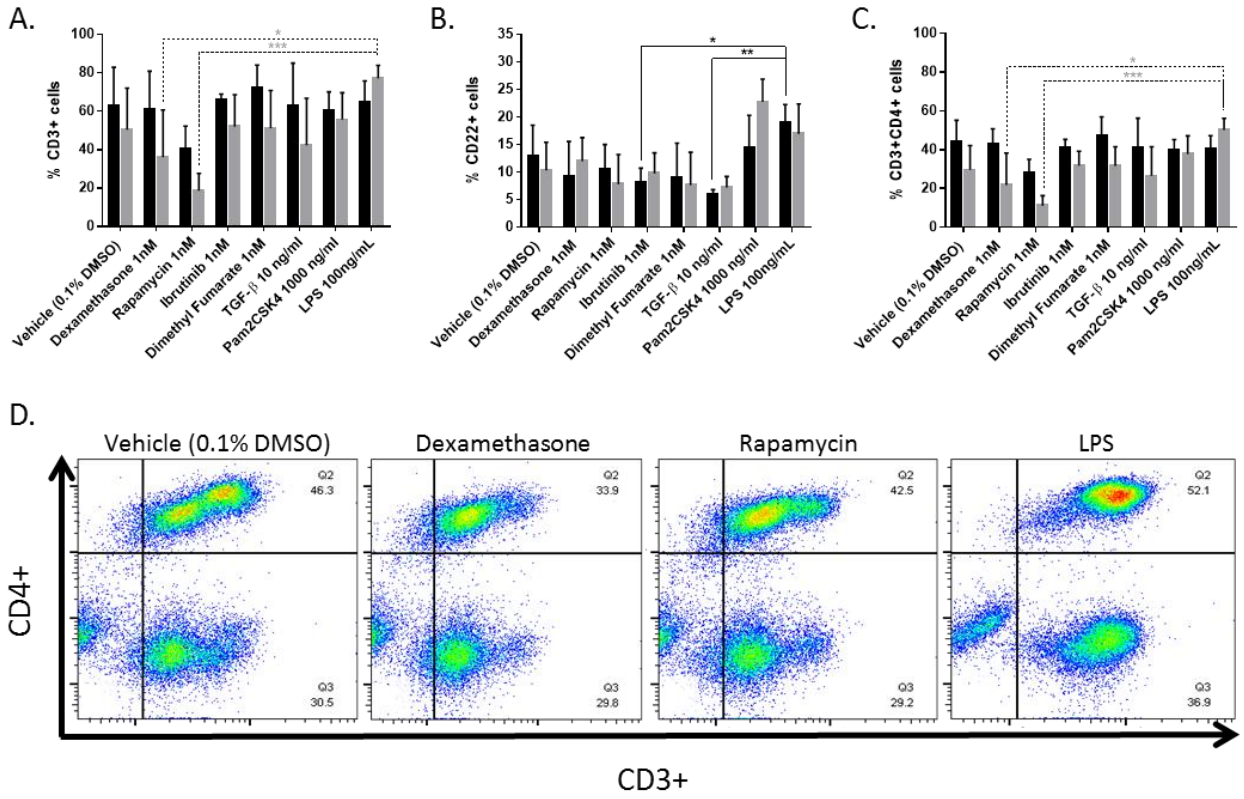

Figure 7. Splenocytes were harvested from EAE mice and treated with vehicle, an immunomodulator at 1 nM, or a control compound for 120 hours, with or without 25 µM PLP antigen rechallenge. The treated cells were then stained with antibodies for CD3 (Alexa Fluor 488), CD22 (Alexa Fluor 647), and CD4 (R-phycoerythrin) and analyzed by flow cytometry. Cell populations were determined for A.) T-cells (CD3+), B.) B-cells (CD22+), and C.) T-helper cells (CD3+CD4+) as a percentage of the total cell population. D.) Representative flow cytometry data demonstrates the CD3+CD4+ population trends observed in the splenocytes. (n=4 per group; * $p<0.05$ **$p<0.01$, ***$p<0.001$, **** $p<0.0001$ for different treatments versus LPS control with comparisons to 25 µM PLP in black and no PLP in gray, # $p<0.05$ for 25 µM PLP versus no PLP, Black bars indicate with 25 µM PLP and Gray bars indicate no PLP rechallenge)

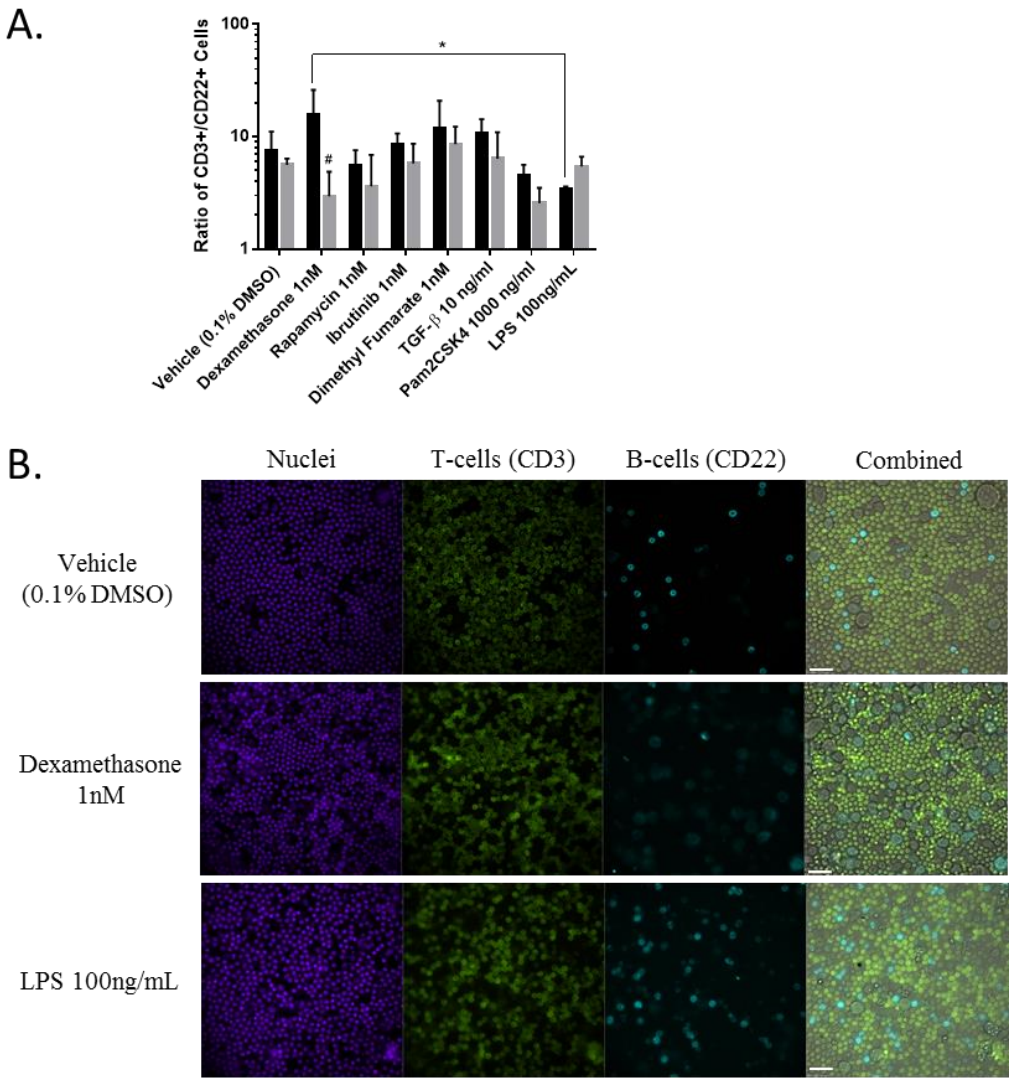

Figure 8. Splenocytes were harvested from EAE mice and treated with either vehicle, an immunomodulator at 1 nM, or a control compound for 120 hours, with or without 25 µM PLP antigen rechallenge. The treated cells were then stained with antibodies for CD3 and CD22. The ratios of T-cells (CD3+) to B-cells (CD22+) for each treatment was determined by A.) flow cytometry. B.) Representative images are shown from vehicle (0.1% DMSO), dexamethasone, and LPS treatment groups all rechallenged with 25 µM PLP. Images indicate cell nuclei (Hoechst), T-cells (green, Alexa Fluor 488), and B-cells (blue, Alexa Fluor 647). (n=4 per group, * $p<0.05$ for different treatments versus LPS control with comparisons to 25 µM PLP in black and no PLP in gray, Black bars indicate with 25 µM PLP and Gray bars indicate no PLP, Scale bar= 25 µm)

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Chapter IV: Co-Delivery of Autoantigen and Dexamethasone in Incomplete Freund's Adjuvant Ameliorates Experimental Autoimmune Encephalomyelitis

1. Introduction

The current therapies for autoimmune diseases, including multiple sclerosis (MS), are often unsuccessful in stopping or reversing disease progression and may lead to non-specific immunosuppression [1, 2]. Effective antigen-specific immunotherapy (ASIT) has the potential to suppress the immune response in regards to a specific autoantigen, and therefore would not hinder the ability of the patient's immune system to fight off foreign pathogens. Unfortunately, attempts to create ASIT for autoimmunity using only autoantigen have largely been unsuccessful [3-5]. In recent years, the idea of combining autoantigen and immunomodulators has emerged as a way to improve upon ASIT for autoimmunity [6]. Promising research has indicated that co-delivery of a small molecule immunomodulator with an autoantigen can lead to the creation of antigen-specific treatment of autoimmune disease [7-11].

Co-delivery of autoantigen and immunomodulator has been successful in animal models of autoimmunity [7-14]. These promising results may be due to the importance of having the components combined both spatially and temporally to elicit the appropriate antigen-specific immune response. In fact, traditional vaccines have utilized co-delivery of antigen and immunomodulator (i.e., adjuvant) to successfully induce a protective antigen-specific response [15]. One hypothesized mechanism responsible for the success of co-delivery is the ability to mimic the natural two-signal paradigm of antigen-specific immunity, wherein concurrent antigen and a secondary signal (co-stimulatory or co-inhibitory) are needed to elicit an antigen-specific immune response [15, 16]. In this regard, several studies have explored co-delivery of autoantigen and immunomodulator that allow for the controlled release of one or both components, increasing the exposure of immune cells to antigen and/or immunomodulator,

which is known to contribute to efficacy of antigen-specific responses in autoimmune models [7-11, 15].

The potential to mimic vaccine mechanisms, not only through antigen and adjuvant co-delivery but also through induction of humoral or T-helper type-2 (Th2) immunity, has shown efficacy in the treatment of T-helper type-1 (Th1) related autoimmune diseases. Both MS and the murine model of the disease, experimental autoimmune encephalomyelitis (EAE), are believed to be primarily Th1 and Th17-mediated [17, 18]. Utilizing an adjuvant initially developed for protective vaccination against foreign pathogens, incomplete Freud's adjuvant (IFA), studies have shown a shift from a Th1/Th17 to a Th2/humoral response to autoantigen resulting in the suppression of autoimmune disease in animals [19, 20]. IFA is composed of 85% mineral oil (paraffin oil) and 15% emulsifier (mannide monooleate), and a 1:1 emulsion of IFA with an aqueous solution containing antigen has been shown to enhance the antigen-specific immune response in animals [16, 21, 22]. Additionally, IFA emulsions both alone and in combination with autoantigen have demonstrated the capacity to mitigate symptoms of autoimmune disease [19, 20, 23-28]. A recent clinical trial has also shown the ability of IFA to deliver autoantigen for the treatment of type 1 diabetes in humans [29].

Recently our lab has found that IFA has the ability to co-deliver dexamethasone (DEX) and water-soluble antigen without increasing pro-inflammatory responses in dendritic cells (Antunez et al, Manuscript in Progress). Although traditionally thought of as a general immunosuppressant, DEX has been shown to be an effective immunomodulator with the ability to decrease the Th1 response, enhance the Th2 response, and even skew the immune system towards a regulatory response [30-34]. Our lab has verified these results in a screen of eleven

immunomodulators in antigen-specific splenocytes obtained from EAE mice, and determined that DEX is capable of suppressing pro-inflammatory Th1-related cytokines, increasing regulatory cytokines, and decreasing the overall T-cell response (Northrup et al, Submitted Manuscript). Other groups have tested the co-administration or co-delivery of dexamethasone and autoantigen in animal models of autoimmunity with successful outcomes [9, 35].

We investigated the co-delivery of DEX and proteolipid protein epitope ($PLP_{139-151}$), the antigen used to induce EAE, with an IFA emulsion. Additionally, all individual components of the co-delivery system and their possible combinations were investigated (**Table 1**) for the treatment of EAE. To our knowledge, this is the first study demonstrating the use of IFA for co-delivery of an immunomodulator and autoantigen for autoimmune therapy. Our approach seeks to expand upon current knowledge of co-delivery in ASIT immunotherapy, the unique immune effects of IFA, and the immunomodulatory mechanisms of DEX in order to create an antigen-specific therapy for autoimmunity.

2. Materials and Methods

2.9 Materials. The peptide antigen, $PLP_{139-151}$, was obtained from PolyPeptide Laboratories (Torrance, CA). For EAE induction incomplete Freund's adjuvant (IFA) and killed *Mycobacterium tuberculosis* strain H37RA were purchased from Difco (Sparks, MD), and pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Dexamethasone (DEX) was purchased from Sigma-Aldrich (St. Louis, MO). For flow cytometry studies, Alexa Fluor 488- conjugated anti-mouse CD3, Alexa Fluor 647-conjugated anti-mouse CD19, and Pacific Blue-conjugated anti-mouse CD11c, and their respective isotype controls were purchased from Biolegend (San Diego, CA). All other

chemicals and reagents were analytical grade and used as received.

2.10 Preparation of IFA emulsion. All IFA emulsions were created with a 1:1 volumetric ratio of IFA to phosphate buffered saline (PBS). Before emulsification, the PLP peptide was solubilized in PBS at 2x the final concentration of 2000 µM. Dexamethasone was first dissolved in DMSO and added to the PBS phase before emulsification such that the final amount of DMSO was less than 0.5% (the maximum concentration recommended by ICH Guideline, Impurities: Guideline for Residual Solvents, Q3C (R5), 2011) and the final concentration of DEX was 20 µg/ml. The IFA and PBS phases were then emulsified using a 20 gage micro-emulsifying needle (stainless steel, 20G X 2-7/8" with reinforcing bar, Cadence Science, Inc.) using two 6 cc plastic syringes, and the emulsion was passed through the needle 15 times. For *in vivo* treatments, IFA emulsions were vortexed (speed 10, Mini Vortexer, Fisher Scientific) for 3 min immediately prior to injection.

2.11 Characterization of PLP and DEX Release. For the release characterization, 4 mL of the IFA emulsion was placed into regenerated cellulose dialysis bags with 6,000-8,000 MWCO (30µm wall thickness, Fisherbrand Dialysis Tubing) which was then placed into 100 mL of PBS in a glass beaker. Alternatively, PLP and DEX were also placed in PBS at the same concentration as in the final emulsion, and release was characterized via the same procedure. All release studies were kept at 37ºC on an incubator shaker (79 rpm, Excella E24 Incubator Shaker, New Brunswick Scientific), and 1 mL samples were taken at specific time points; with 1 mL of PBS re-added at each time point. Characterization of the PLP and DEX concentrations released from the dialysis bags were performed using gradient reverse-phase HPLC (Waters 2796 Bioseparations Module, Waters Corp) on a C4 analytical column (Waters, XBridge Protein BEH column, 300 Å, 3.5µm, 4.6 mm x 150 mm, 10-500 K).

Samples were eluted using mobile phases A (100% water with 0.1% trifuoroacetic acid (TFA)) and B (100% acetonitrile with 0.1% TFA) with a gradient of 100% A to 20% A over 25 min at a constant flow of 1 ml/min. Mobile phase compositions were (A) 100% water with 0.1% TFA and (B) 100% acetonitrile with 0.1% trifluoroacetic acid (TFA). PLP was detected at 220nm and DEX was detected at 240nm with a dual wavelength absorbance detector (Waters 2487 Dual λ Absorbance Detector, Waters Corp). Data was collected and processed using Empower 3 Software (Waters Corp).

2.12 Induction of EAE and Therapeutic Study. Single step SJL/J female mice, 4 – 5 weeks old, were purchased from Envigo Laboratories and housed under specified, pathogen-free conditions at The University of Kansas. All protocols involving live mice were approved by The University of Kansas Institutional Animal Care and Use Committee. Mice were immunized subcutaneously with 200 µg of $PLP_{139–151}$ in a 0.2 mL emulsion composed of equal volumes of PBS and IFA containing killed *Mycobacterium tuberculosis* strain H37RA at a final concentration of 4 mg/mL. The $PLP_{139–151}$/CFA was administered to regions above the shoulders and the flanks (total of four sites; 50 µL at each injection site). In addition, 100 µL of pertussis toxin (200 ng) was injected intraperitoneally on the day of immunization (day 0) and 2 days post-immunization. Each treatment group contained six mice, except for the group with PLP treatment, which only contained five mice due to an unexpected death before disease induction. Mice received 100 µL subcutaneous injections at the nape of the neck on days 4, 7, and 10 of the study. All DEX-containing treatment groups were given at 20 µg DEX per injection and all PLP-containing treatments groups were given 200 nmol PLP per injection. Disease progression was evaluated using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete

paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund. Body weight was also measured daily.

2.13 Splenocyte Isolation and *ex vivo* treatment. Mouse spleens were resected from EAE mice on Day 25 following disease-induction and cultured as previously described [13]. Briefly, the spleens were first passed through a wire mesh using the rubber end of a sterile 1 mL syringe plunger, and collected in 5 mL of RPMI 1640 media. The crude cellular extract was then centrifuged and the resulting cell pellet was resuspended in 3.5 ml of 1x Gey's lysis solution and placed on ice for 3.5 minutes to lyse splenic red blood cells. The lysis reaction was stopped by the addition of 10 ml RPMI 1640 media containing 10% FBS and centrifuged at 1,100 x g for 5 minutes. The remaining cell pellet was resuspended in fresh media (RPMI 1640 media containing 10% FBS and 1% Penicillin-Streptomycin) and seeded in 96-well cell culture plates at a cell density of $1x10^6$ cells/well in a final volume of 200 µl. The cells were immediately rechallenged with and without 25 µM PLP antigen. Stimulated cell cultures were incubated for 120 hours at 37°C in a CO_2 (5%) incubator.

2.14 Measurement of Cytokines. Supernatants of cell cultures were collected after 120 hour incubation post day 25 spleen harvest. Secreted cytokines; GM-CSF, TNF-α, IL-2, IFN-γ, IL-10, IL-17, IL-15, and IL-23 were detected using a U-Plex assay kit according to manufacturer instructions (Meso Scale Discovery). Briefly, each U-Plex was coated with 50 µL of the multiplex coating solution containing linker and biotinylated capture antibody combinations for each cytokine and incubated on a shaker at 700 rpm for 1 hour at room temperature. Following washing each well 3 times with 150 µL PBS containing 0.05% Tween 20, 25 µL of diluent and 25µL of sample was added to each well and incubated again

for 1 hour on a shaker at room temperature. Detection antibody was added at 50 µL/well and incubated for 1 hour. Finally, each assay plate was read using the QuickPlex multiplex plate reader (Meso Scale Discovery).

2.15 Measurement of Cellular Metabolism. Cell metabolism was determined by a resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay. Briefly, resazurin (75 µmol/L final) was added to splenocyte cultures and incubated for 3 hours. Metabolic reductive capacity was determined by a change in fluorescence (excitation 560/emission 590). Background fluorescence was determined in RPMI media and was subtracted from each experimental read. All fluorescent readings were performed using a Spectramax M5 (Molecular Devices) plate reader.

2.16 Fluorescent staining and Flow Cytometry. Immediately after isolation, splenocytes were seeded in 12-well cell culture plates at a cell density of $5x10^6$ cells/well in a final volume of 1.5mL. Treatments were then given at the concentration of interest, both with and without 25 µM PLP. Stimulated cell cultures were incubated for 120 hours at 37°C in a CO_2 (5%) incubator. At 120hrs, splenocytes were stained with the desired antibodies. Briefly, $1x10^6$ cells were washed with 1mL of wash buffer (RPMI 1640 media containing 5% FBS) before centrifugation and resuspension in 50 µL block buffer containing 20ug/ml TruStain fcX (anti-mouse CD16/32 antibody, Biolegend) in wash buffer. Cells were incubated on ice for 25 minutes before adding the desired antibodies or isotype controls in 50 µL at 2x the manufacturer recommended concentration for 1 hour. For flow cytometry analysis, 40,000 cells per sample were detected using a BD FACSFusion cytometer. Data was analyzed using FlowJo Software.

2.17 Detection of PLP-Specific Antibodies by ELISpot. 96-well plates (Immunlon 2HB)

were coated with 50 µg/ml PLP in 100 µl of PBS and incubated overnight at 4°C. The PLP coating solution was discarded and the plate was blocked with PBS containing 0.5% FBS for 1 hour prior to addition of splenocytes. Immediately after their isolation, splenocytes were seeded onto the coated 96-well plate at a cell density of $1x10^6$ cells per well in a final volume of 100 µL. The splenocyte cultures were incubated for 48 hours at 37°C in a CO_2 (5%) incubator. Following incubation, each plate was washed 4x with PBS containing 0.05% Tween 20 for 2 min, followed by washing 2x with PBS for 2 min. 100 µL of horseradish peroxidase (HRP) conjugated anti-mouse IgG at a concentration of 1 µg/mL was added to each well and incubated for 1 hour at 37°C. Following a second wash step, identical to previously described, a buffer containing TrueBlue Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc) and agarose (1:1 ratio) was heated in a water bath to 56°C and 100 µL was added to each well using reverse pipetting to avoid bubbles. Plates were incubated overnight at 4°C before reading on the CTL Ultimate S6 ImmunoSpot Analyzer (Cellular Technology Limited). Analysis of spots was done using CTL ImmunoSpot software (Cellular Technology Limited.

2.18 Statistical analysis. Statistical evaluation of data was performed using a one-way or two-way analysis of variance (ANOVA) as experimentally appropriate, followed by Tukey and Sidak multiple comparison tests. Trends were described following individual t-tests and linear regression analysis. A single outlier was removed from the cytokine analysis of the IFA with Dex and PLP group (**Figure 5**) following outlier confirmation via the Grubb' Outlier Test. The criteria for statistical significance for all analyses was set at $p<0.05$. The majority of analyses were performed using GraphPad Software (GraphPad Software Inc.) and linear regression analysis was performed using MATLAB (The MathWorks, Inc).

3. Results

3.1. Characterization of IFA Emulsion containing PLP and Dex

IFA oil was combined at a 1:1 ratio with phosphate buffered saline (PBS) containing PLP and DEX in order to create the IFA emulsion for co-delivery of autoantigen and immunomodulator. The release of both PLP and DEX from either the IFA emulsion or PBS was measured by placing each formulation into a dialysis membrane (6,000-8,000 molecular weight cutoff) and tracking the release into sink conditions of PBS at 37ºC. PLP and DEX were found to be fully released from PBS within the membrane after 24 hours and 10 hours, respectively (**Figure 1).** DEX within the IFA emulsion was not fully released until approximately 120 hours (**Figure 1A**). PLP was much slower to release from the IFA emulsion, with less than 10% of the total PLP in the formulation being released within 192 hours (**Figure 1B**).

3.2. Treatments containing PLP delivered in IFA Suppress EAE Symptoms

IFA emulsion containing PLP and DEX, along with each combination of the components and a PBS control, were tested in mice with EAE (**Table 1**). Treatments were given subcutaneously between the shoulder blades on Days 4, 7, and 10 of the study (as shown on **Figure 2A-B** and **Figure 3A-B**). By day 12, half of the mice in the PBS control group had died. Due to these deaths, and the corresponding disease score of 5, the PBS was statistically significant from all other treatments, including the IFA vehicle control, on at least one day of the study (**Figure 2A-B**). Additionally, due to the high error in clinical score associated with the days in which the PBS mice died (Days 11-12), the IFA is shown as an alternative control compared to all other treatments (**Figure 2**). Weight changes were also monitored throughout the study and are shown as a percent change as compared to day 8, the last day with no disease symptoms (**Figure 3**). Although the death of the three PBS treated mice contributed to a significant increase in clinical

score, the weight change was unaffected. In fact, there was no statistical difference between IFA and PBS treatment groups in terms of weight change (**Figure 3A**).

In order to determine the benefits offered by co-delivery of autoantigen and immunomodulator, co-delivery of IFA with DEX + PLP was also compared to the clinical score and change in weight from each treatment group (**Figures 2 & 3**). IFA with DEX + PLP significantly decreased clinical score and weight change as compared to PBS on three days of the study (**Figure 2B & Figure 3B**). Interestingly, as compared to IFA alone, IFA with DEX + PLP decreased the both clinical score and weight change during days before, during, and after peak disease (as determined by peak clinical scores and maximum weight change, see **Table 1**) (**Figure 2C & Figure 3C**). IFA with DEX + PLP also significantly suppressed disease symptoms on at least one day during the study as compared to DEX only, IFA with DEX, and DEX + PLP (**Figure 2D, F, H**). Treatments that were not statistically different than IFA with DEX + PLP; both PLP and IFA with PLP, decreased clinical scores as compared to IFA only (**Figure 2 E, G**). All of these treatments (PLP, IFA with PLP, and IFA with DEX + PLP) also significantly decreased the peak clinical score as compared to the PBS control (**Figure 4A**). Interestingly, both IFA with DEX + PLP and IFA with PLP suppressed EAE symptoms to a similar degree. The suppression by both treatments containing PLP in IFA is most clearly observed by examining the area under the curve for clinical score and peak clinical disease score (**Figure 4B**). Surprisingly, this same trend was not seen in weight change data, as IFA with PLP actually had significantly more weight loss as compared to IFA with DEX + PLP around the peak of disease (**Figure 3G**).

3.3. Dexamethasone Shifts Cytokine Profiles Away from Inflammation

Following disease remission, on day 25 of the EAE study splenocytes were collected from the mice and were rechallenged *in vitro* with and without PLP antigen. Cellular metabolic activity and cytokine responses were measured at 120 hours post-rechallenge (**Figure 5**). Metabolic activity was found to significantly increase with DEX treatment as compared to IFA only, IFA with DEX, and IFA with DEX + PLP without rechallenge and increased metabolic activity both with and without rechallenge as compared to PLP treatment (**Figure 5A**). DEX + PLP was also found to increase metabolic activity via measurement of resazurin as compared to PLP only with antigen rechallenge (**Figure 5A**).

Rechallenge with PLP was found to significantly increase the cytokine levels of GM-CSF, IFN-γ, TNF-α, IL-6, and IL-4 for the majority of treatment groups and increased levels of IL-10, IL-17, IL-23, and IL-15 in some of the treatment groups (**Figure 5**). Significant differences between the treatment groups were found in the cytokine response of GM-CSF, IFN-γ, IL-6, IL-4, and IL-23; however, differences were only found for samples with PLP rechallenge. Treatments containing DEX tended to decrease pro-inflammatory cytokines including GM-CSF, IFN-γ, and IL-23 as compared to other treatment groups upon rechallenge with PLP (**Figure 5B, C, J**). Both IFA and PLP only treatments were found to significantly increase the pro-inflammatory GM-CSF as compared to IFA with DEX and IFA with DEX + PLP (**Figure 5B**). IL-23 also significantly increased due to treatment with PLP, this time compared to DEX and IFA with DEX (**Figure 5J**). Surprisingly, the general pro-inflammatory cytokine IFN-γ was shown to increase with IFA with PLP, a treatment that was effective at suppressing clinical score *in vivo*. IFA with PLP significantly increased levels of IFN-γ as compared to treatments of IFA

only, DEX + PLP, and IFA with DEX + PLP (**Figure 5C**). IL-6 was shown to be significantly increased with DEX treatment as compared to IFA with DEX and IFA with DEX + PLP (**Figure 5F**). Unlike the majority of cytokines analyzed, IL-4 significantly increased with PBS treatment as compared to all other treatment groups (**Figure 5I**).

3.4. Effective in vivo Therapies Increase B-cell-related Responses in Splenocytes

In addition to cytokine responses, changes in cell populations in the splenocytes obtained from the different *in vivo* treatment groups were examined both with and without PLP antigen rechallenge (**Figure 6**). The population of T-cells, as identified by CD3, was found to be statistically similar for all treatment groups, both with and without rechallenge (**Figure 6A**). CD11c, which is a common marker for dendritic cells (DCs), was found to be increased both with and without antigen rechallenge for IFA with DEX + PLP and with antigen for IFA with PLP (**Figure 6B**). B-cells, as identified by CD19, were found to be significantly increased in the IFA with DEX+ PLP treatment group (**Figure 6C, F**). CD19+CD11c+cells were also analyzed, as these cells have been shown to act as autoimmune associated B-cells (ABCs) [36, 37]. CD19+CD11c+ population was significantly increased for the IFA with DEX + PLP treatment group without antigen rechallenge (**Figure 6D**). The ABC population in the IFA with DEX + PLP group was significantly higher than all other treatment groups as a percent of the total splenocytes; however, the ABCs were not increased as a percentage of the B-cell (CD19+) population (**Figure 6E**).

Autoantibody production was also measured using ELISPot to determine the number of cells producing PLP-specific antibodies in the spleen following each *in vivo* treatment. Similar to the increased presence of B-cells demonstrated by flow cytometry, ELISpot showed significantly

higher anti-PLP antibody producing cells in splenocytes from mice treated by IFA with DEX + PLP as compared to other treatment groups (**Figure 7**).

4. Discussion

The combination of autoantigen and immunomodulators has recently emerged as a potentially effective ASIT strategy for the treatment of autoimmunity [6]. In this study, IFA was investigated as a co-delivery vehicle in order to treat EAE with a combination of DEX and PLP. Our studies demonstrate that controlled release of DEX and PLP from IFA may provide optimal efficacy in suppressing EAE. In particular, the release of PLP into solution was greatly slowed when it was emulsified into IFA (**Figure 1B**) and controlled release decreased disease severity in therapies containing PLP in an IFA emulsion (**Figures 2 & 4B**). IFA has been previously indicated to have significant immunosuppressive properties alone [26]; however, we did not observe this phenomenon. IFA suppressed disease symptoms as compared to the PBS control on only one day, which was seen for all other treatments (**Figure 2A**). When used as a delivery vehicle for autoantigen or immunomodulator, IFA containing PLP resulted in the most pronounced disease suppression (**Figures 2 & 4B**). These results were surprising in that co-delivery of DEX and PLP in IFA did not suppress disease clinical scores to any noticeable extent as compared to IFA with only PLP (**Figure 2G and Figure 4B**). The slow release of PLP from the IFA emulsion may be extending the exposure of autoantigen to antigen-presenting cells, allowing the therapy to influence the immune system long after the injections. Previous studies in animal models of autoimmunity have seen similar phenomena, where the controlled release of

autoantigen increases disease suppression both with and without an immunomodulatory signal [7, 8, 11, 38-40].

We also observed that treatments containing PLP, even without controlled release, significantly decreased peak clinical score (**Figure 4A**) and seemed to delay disease onset (**Figure 4C**). Treatment with an appropriate dose of autoantigen has often been reported to decrease autoimmune symptoms in animals, but has not been successfully translated to the clinic [3, 41]. The clinical efficacy of this treatment was also contradictory to the cytokine response observed in splenocytes obtained on day 25 of the study. Treating EAE mice with PLP alone was shown to promote pro-inflammatory cytokine production by significantly increasing GM-CSF and IL-23, and slightly increasing IFN-γ and IL-2 although not to a significant degree compared to other treatments (**Figure 5**). The correlation of the poor efficacy of autoantigen alone in humans with the increased pro-inflammatory cytokine production by PLP treatment emphasizes the importance of analyzing the immune response in addition to clinical symptoms in EAE.

The *in vivo* data indicated the importance of controlled release of autoantigen in the creation of effective ASIT, while the addition of an immunomodulator such as DEX had little effect on clinical symptoms (**Figure 2 & 3**). When analyzing the immune response more closely, it was found that DEX does play an important role in shifting the cytokine profile away from pro-inflammatory Th1/Th17 responses (**Figure 5**). Decreased pro-inflammatory cytokine levels were in direct contrast with an increase in cellular metabolic activity upon DEX treatment, therefore the suppression of pro-inflammatory cytokine by DEX may be even more significant when considering the results on a per metabolizing cell basis (**Figure 5A**). DEX was also shown

to increase IL-6 (**Figure 5F**); however, this cytokine can be indicative of both pro-inflammatory (Th17) and regulatory (Treg) immune responses depending on the presence of other cytokine responses [42, 43]. The increase in IL-6 coupled with a decrease in IL-23 and GM-CSF, along with no change in IL-17, suggests that DEX is not producing a Th17 response and therefore IL-6 production may not be indicative of inflammation [42, 43]. Another cytokine demonstrating unexpected results was IL-4, which was shown to decrease with all therapies, even IFA alone (**Figure 5I**); however, the role of IL-4 in the treatment of EAE has been debated [44].

Interesting immunological trends due to DEX treatment were also observed in the CD11c+ dendritic cell (DC) population upon rechallenge with PLP antigen (**Figure 6B**). IFA with DEX and IFA with DEX + PLP significantly increased the population of DCs compared to the majority of treatments, and DEX + PLP increased DCs as compared to PLP only treatment. The increase of DCs with treatments containing DEX is directly reverse of the trend for DEX to decrease GM-CSF (**Figure 5B**). GM-CSF has been known to be essential for the generation of inflammatory DCs from monocytes in the spleen, and plays a crucial role in EAE pathogenesis [45]. The inversely proportional trend of GM-CSF and CD11c+ DCs following treatments with DEX may indicate that the DCs being produced are not pro-inflammatory, and may actually be immature or even tolerogenic [46, 47]. CD11c+ DCs have been shown to act as tolerogenic DCs in the spleen [46]. Also, the treatment of DCs with DEX has previously been shown to produce higher levels of tolerogenic DCs [45, 48-50]. Additionally, although not statistically significant, a trend in the decrease of CD3+ T-cells with the addition of DEX was also noted (**Figure 6A**).

Previous studies co-administering IFA and autoantigen have shown that a shift towards a Th2/humoral immune response allows for the suppression of Th1/Th17-mediated autoimmune

diseases [19, 20]. Our study supported this hypothesis by demonstrating increased autoantibody production and increase B-cell populations in splenocytes from mice treated with either IFA with PLP or IFA with DEX + PLP (**Figures 6 & 7**). Although IFA with DEX + PLP increased the percent of autoimmune associated B-cells (ABCs) in the entire splenocyte population, this trend appeared to be associated with an increase in B-cells overall rather than an increase in the proportion of B-cells that are ABCs (**Figure 6D, E**). Both B-cell and ABC populations were found to significantly decrease upon rechallenge with antigen. Unresponsiveness to antigen rechallenge may indicate that many of these cells are anergic and therefore not contributing to EAE progression [51, 52].

In measuring PLP-specific autoantibody producing B-cells via ELISpot, we discovered that the treatments that suppressed clinical symptoms of EAE had much higher levels of PLP-specific antibodies (**Figure 7**). Increased production of antibody following effective treatment with IFA and autoantigen has been observed before in both human autoimmunity and animal models [20, 29]. This increase in antibody production against autoantigen has been found to directly correspond to skewing the immune response towards a Th2/humoral response resulting in suppression of Th1/Th17-mediated autoimmunity [20]. Our results demonstrating both an increase in the B-cell population and PLP-specific antibody production with IFA with DEX + PLP treatment of EAE strongly support the hypothesis that the clinical disease suppression is due to a shift towards a Th2/humoral immune response.

5. Conclusion

As the combination of autoantigen and immunomodulator gains more traction as an effective autoimmune therapy, it is important to understand how each component contributes to the desired outcome. By investigating all possible treatment combinations of DEX and PLP using IFA as a delivery vehicle, we were able to identify some interesting trends in the treatment of EAE. Although co-delivery of DEX and PLP was hypothesized to be most effective, it was found that IFA with PLP was just as efficacious at ameliorating disease symptoms *in vivo*. Upon closer analysis of the immunological responses, it was found that DEX plays an important role in decreasing pro-inflammatory cytokines and possibly increasing tolerogenic DCs. Additionally, the co-delivery treatment of IFA with PLP and DEX was shown to increase humoral responses through increased B-cell and antibody production. A shift towards a humoral response is associated with a therapeutic efficacy in the treatment of EAE and MS. Overall, this data indicates that co-delivery of PLP autoantigen and DEX in an IFA emulsion is effective in the treatment of a murine autoimmune model.

Table 1. *in vivo* Treatment Groups

Treatment Groups	Mice Alive at end of Study	Mice without Severe Disease Symptoms (score < 2)	Day of Peak Disease Score (Mean ± SD)[a]	Day of Maximum % Weight Change (Mean ± SD)[a]
PBS	3/6	1/6	12.3 ± 1.0	11.8 ± 1.3
IFA	6/6	0/6	13.3 ± 0.5	12.0 ± 1.3
DEX	6/6	0/6	12.2 ± 1.5	12.0 ± 0.9
PLP	5/5	1/5	14.6 ± 4.7	15.0 ± 4.5
IFA with DEX	6/6	1/6	12.5 ± 1.4	12.5 ± 1.4
IFA with PLP	6/6	6/6	11.3 ± 1.4	11.7 ± 0.5
DEX + PLP	6/6	2/6	13.0 ± 1.1	13.7 ± 0.8
IFA with DEX + PLP	6/6	6/6	13.2 ± 1.9	13.3 ± 1.6

[a] Not statistically different between treatment groups

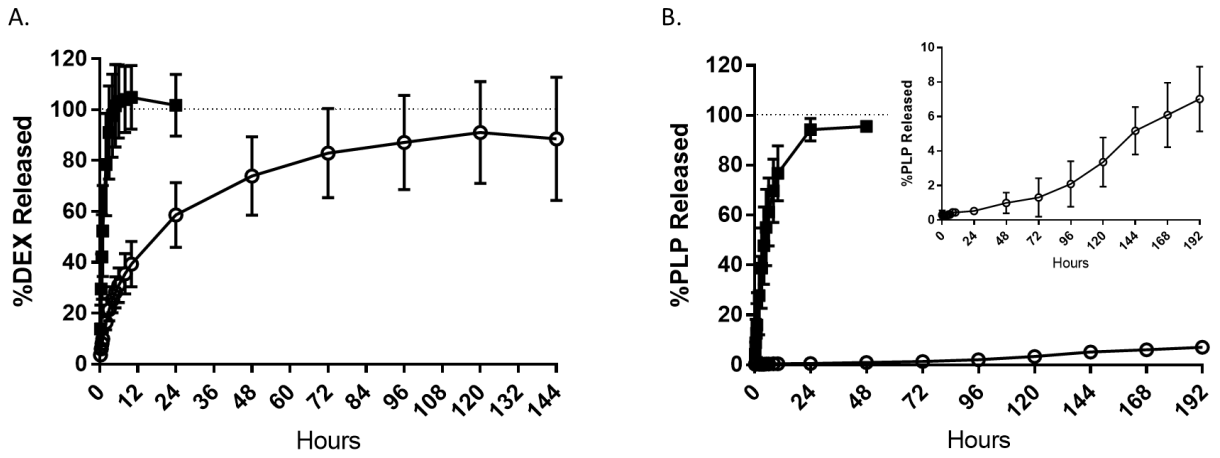

Figure 1. Release at 37ºC of A.) DEX and B.) PLP from IFA emulsion (open circle) and PBS (black square) formulation within dialysis bag in sink conditions of PBS. Concentrations determined at each time point via RP-HPLC. Data shown as a percentage of total DEX or PLP in formulation. (n=3 per formulation)

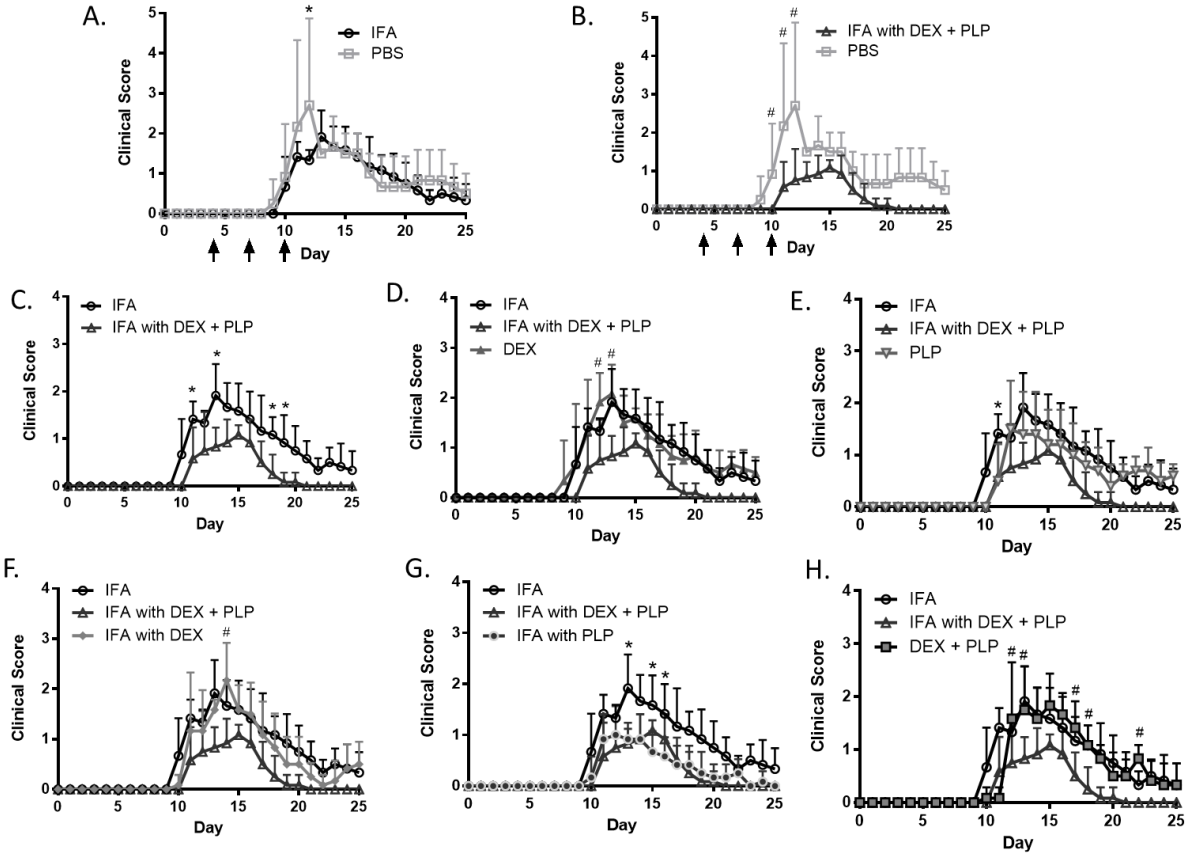

Figure 2. Clinical disease scores in EAE mice given components of co-delivery treatment, IFA with DEX + PLP. Disease score with PBS control treatment compared to A.) IFA and B.) IFA with DEX + PLP. IFA and IFA with DEX + PLP treatments were compared to C.) each other, D.) DEX treatment, E.) PLP treatment, F.) IFA with DEX, G.) IFA with PLP and H.) DEX + PLP. All treatments were given on Days 4, 7, and 10 (black arrows). (n = 5-6 mice per group, * $p < 0.05$ as compared to IFA, # $p<0.05$ as compared to IFA with DEX + PLP).

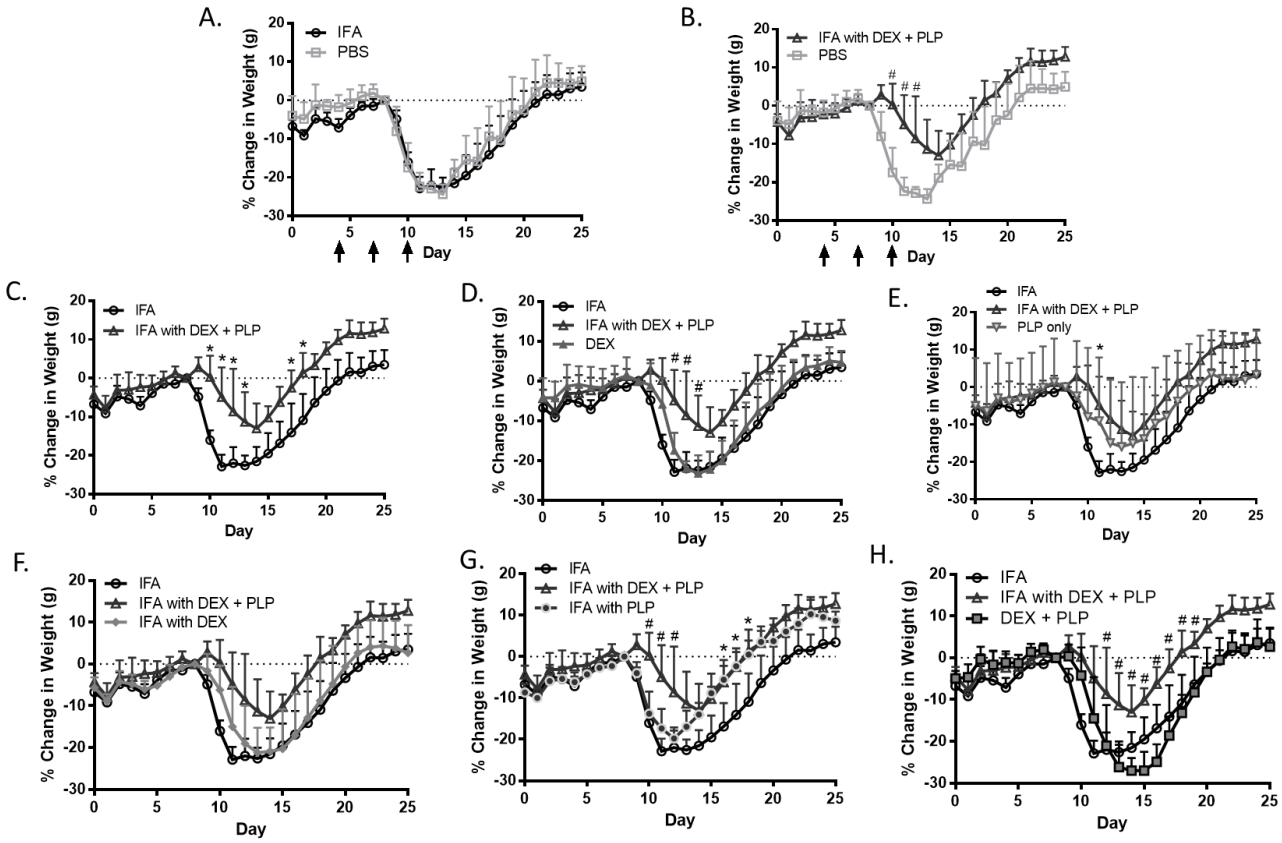

Figure 3. Percent change in weight as compared to day 8 (last day before symptoms) in EAE mice given components of co-delivery treatment, IFA with DEX + PLP. Percent weight change with PBS control treatment compared to A.) IFA and B.) IFA with DEX + PLP. IFA and IFA with DEX + PLP treatments were compared to C.) each other, D.) DEX treatment, E.) PLP treatment, F.) IFA with DEX, G.) IFA with PLP and H.) DEX + PLP. All treatments were given on Days 4, 7, and 10 (black arrows). (n = 5-6 mice per group, * $p < 0.05$ as compared to IFA, # $p<0.05$ as compared to IFA with DEX + PLP).

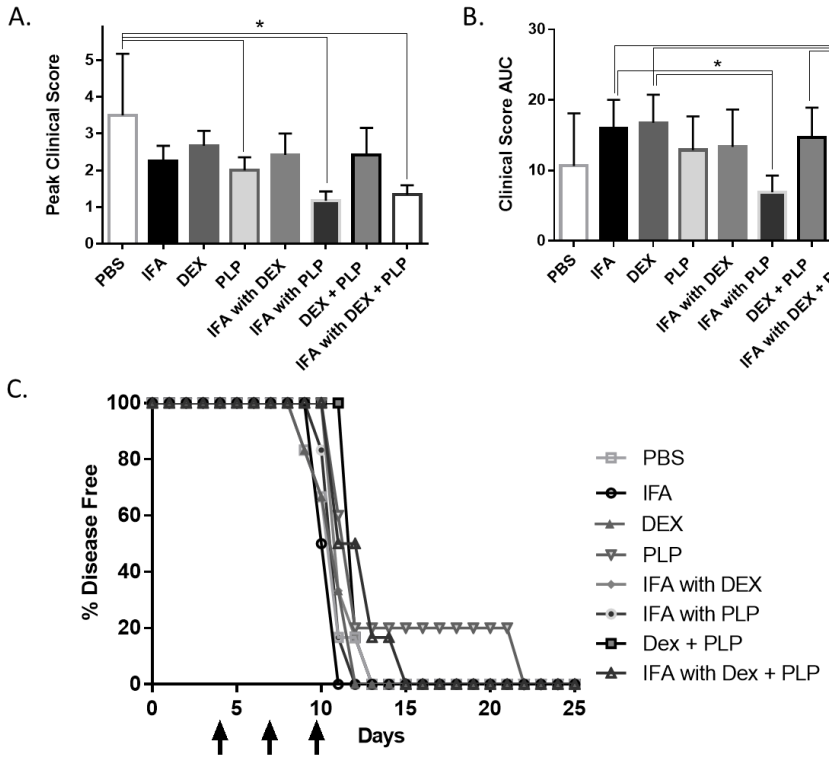

Figure 4. The effect of *in vivo* treatments on EAE was also analyzed by comparing the A.) peak clinical score, B.) area under the curve for clinical scores, C.) and the incidence of disease. EAE disease incidence was evaluated such that disease free animals maintained a clinical score < 1. (n = 5-6 mice per group, * $p < 0.05$ comparing different treatment groups)

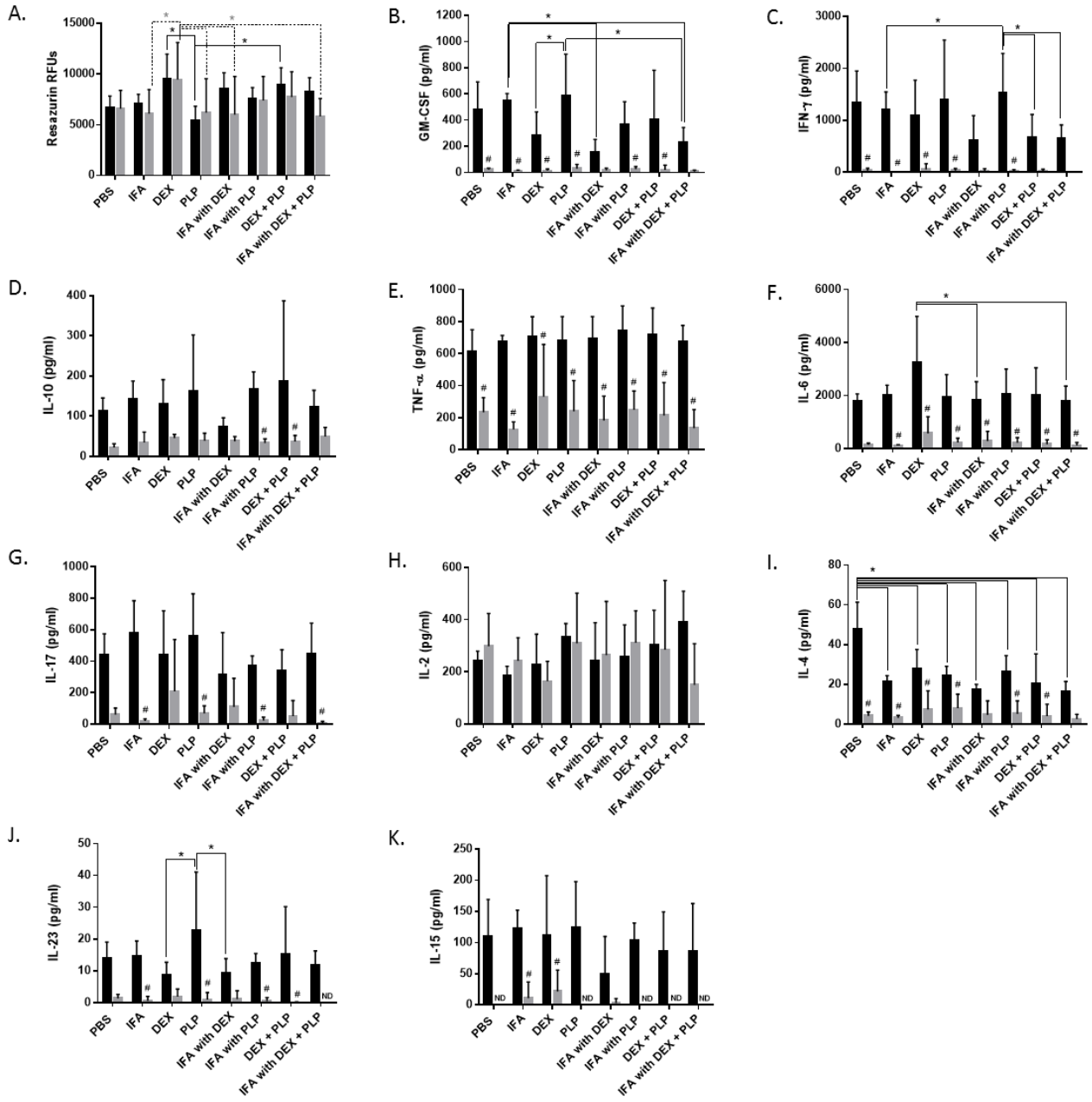

Figure 5. Splenocytes were harvested on day 25 from EAE mice treated *in vivo* with components of co-delivery treatment, IFA with DEX + PLP. Splenocytes were incubated for 120 hours with or without 25 µM PLP rechallenge. A.) Cell metabolism and supernatant cytokine levels of B.) GM-CSF, C.) IFN-γ, D.) IL-10, E.) TNF-α, F.) IL-6, G.) IL-17, H.) IL-2, I.) IL-4, J.) IL-23, and K.) IL-15 were determined. (n= 5-6 per group, * $p < 0.05$ comparing different treatment groups, # $p<0.05$ for 25 µM PLP versus no PLP for the same treatment, ND indicates cytokine levels were not detectable, Black bars indicate 25 µM PLP and Gray bars indicate no PLP rechallenge)

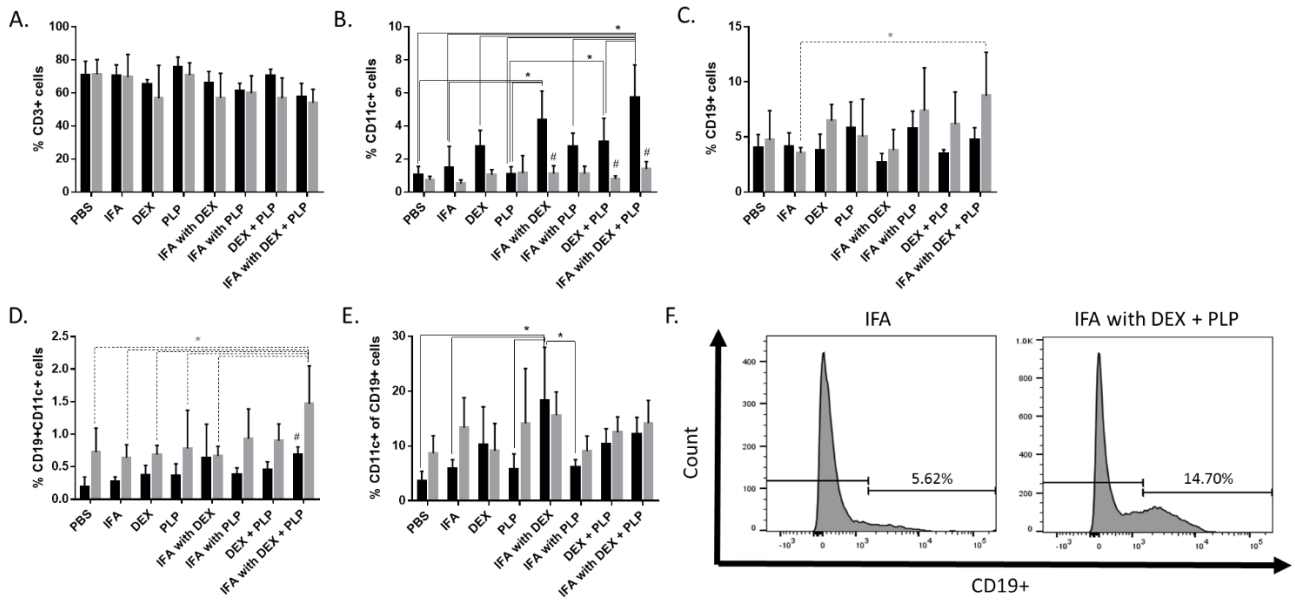

Figure 6. Splenocytes were harvested on day 25 from EAE mice treated *in vivo* with components of co-delivery treatment, IFA with DEX + PLP. Splenocytes were incubated for 120 hours with or without 25 µM PLP rechallenge. The cells were then stained with antibodies for CD3 (Alexa Fluor 488), CD19 (Alexa Fluor 647), and CD11c (Pacific Blue) and analyzed by flow cytometry. Cell populations were determined for A.) T-cells (CD3+), B.) Dendritic Cells (CD11c+), C.) B-cells (CD19+) and D.) autoimmune associated B-cells (ABCs, CD19+CD11c+) as a percentage of the total cell population. E.) The percentage of CD11c+ cells out of the CD19+ cell population was also determined. F.) Gating for CD19+ B-cells is shown for both IFA and IFA with DEX + PLP, without antigen rechallenge. (n= 3-5 per group, * $p < 0.05$ comparing different treatment groups, # $p<0.05$ for 25 µM PLP versus no PLP for the same treatment, Black bars indicate 25 µM PLP and Gray bars indicate no PLP rechallenge)

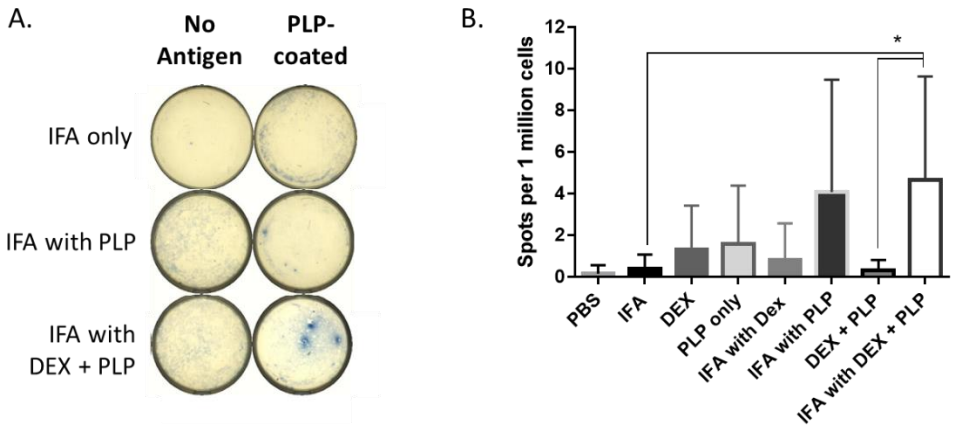

Figure 7. Splenocytes were harvested on day 25 from EAE mice treated *in vivo* with components of co-delivery treatment, IFA with DEX + PLP, and were plated on wells coated with or without PLP antigen for 48 hours. Spots associated with B-cells producing PLP-specific antibodies were detected using HRP anti-IgG and TrueBlue stain, and imaged with a CTL ImmunoSpot Analyzer. A.) Representative wells both with and without antigen coating are shown. B.) The number of spots per 1 million cells were compared for different treatment groups. (n= 5- 6 per group in duplicate wells, * $p < 0.05$ comparing different treatment groups)

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Chapter V: Conclusions and Future Work

1. Conclusions

Antigen-specific immunotherapy (ASIT) has long been the cornerstone of vaccines, arguably one of the most important healthcare related-inventions. The approach of vaccines, to utilize both antigen and immunomodulator (i.e., adjuvant), may hold the key to developing successful ASIT for autoimmunity. As reviewed in chapter 1 and elaborated on in chapters 2, 3, and 4; promising results in experimental models of autoimmunity have demonstrated the efficacy of co-delivery of autoantigen and immunomodulator as ASIT for autoimmunity. With the positive results in these proof-of-concept studies there is increased hope that the combination of autoantigen and immunomodulator may hold the key to the creation of successful ASIT to treat, prevent, and possibly cure autoimmune disease.

The B7 signaling pathway is a promising immunomodulator target for the treatment of EAE. In chapter 2, co-delivery of B7-targeted peptides and autoantigen with a novel vehicle, a soluble antigen array (SAgA) was found to suppress clinical disease symptoms of EAE as compared to the negative controls. SAgAs co-delivering PLP autoantigen with each of the three different peptides targeting the B7 pathway or LABL, which targets ICAM-1, suppressed disease symptoms to a similar degree. Although the clinical effects of the SAgAs were comparable, the cytokine responses in splenocytes harvested from the *in vivo*-treated mice demonstrated different immunological mechanisms. Most notably the peptide mimic of CD28, B7AP, decreased levels of the pro-inflammatory cytokines GM-CSF and IL-2, while the other SAgA treatments did not. Another interesting finding was that the particulate nature of the SAgA with sF2, a peptide mimic of CTLA-4, did not change the ability to suppress EAE. Combined, these results suggest that multiple immunomodulatory signals are capable of EAE suppression when co-delivered

with autoantigen, and different physical properties of the delivery vehicle (soluble or colloidal) can still be effective.

In chapter 3, we demonstrated that splenocytes from EAE mice can be used as an antigen-specific *in vitro* screening system for evaluating the immune response in an autoimmune model. EAE splenocytes demonstrated antigen-specific immune responses through rechallenge with PLP autoantigen, and therefore offered a mechanism by which to screen immunomodulators for their ability to skew the antigen-specific immune response towards tolerance. Eleven small molecule immunomodulators were investigated for their potential to mitigate the antigen-specific immune response; however, only dexamethasone demonstrated tolerogenic responses. Dexamethasone was found to increase tolerogenic cytokines, decrease pro-inflammatory cytokines, and decreased CD3+ and CD3+CD4+ T-cell populations at a relevant dose.

Chapter 4 elaborated on the work conducted in chapter 3, as dexamethasone was co-delivered with autoantigen (PLP) *in vivo* for the treatment of EAE. Using an IFA emulsion as a co-delivery vehicle, it was found that the controlled release of autoantigen was important for the suppression of clinical disease symptoms. Co-delivery using IFA also shifted the immune response towards a humoral response by increasing B-cell populations. Additionally, analysis of the immune response via cytokines revealed that dexamethasone was important for shifting the immune response away from inflammation. Overall, this data indicated that the co-delivery of PLP and dexamethasone with an oil-and-water emulsion is effective in treating a murine autoimmune model, and with further optimization the potential to be successful in humans.

2. Future Work

Although these studies demonstrate the potential of co-delivering autoantigen and immunomodulator to treat autoimmunity, there is much work still to be done. Future studies

should focus on optimizing each of the components in this therapy; the immunomodulator, the co-delivery vehicle, and possibly the autoantigen. A better understanding of how each component acts alone and in combination will enhance our understanding of the mechanisms by which antigen-specific immune tolerance can be induced in combinational ASIT.

Chapters 2 and 3 both focused primarily on optimization of the immunomodulator, and demonstrated that both B7-targeted peptides and small molecules such as dexamethasone may suppress autoimmunity. Although both types of immunomodulators were effective, they act through very different mechanisms. B7-targeted peptides are believed to bind proteins in the B7 co-stimulatory pathway on the cell surface, whereas dexamethasone acts via an intracellular glucocorticoid receptor [1]. It is possible that the antigen-specific *ex vivo* screening system used in chapter 3 could be used to compare the tolerogenic effects of compounds that work extracellularly, such as the peptides described in chapter 2. Many co-stimulatory pathways exist beyond the B7 pathway such as PD-1/PD-1L, CD40/CD40-L, ICOS/ICOS-L, and many others that could potentially be targeted by peptides [2]. A closer examination of these pathways and their potential to induce tolerance may lead to the discovery of an even more effective immunomodulatory peptide than those targeting the B7-pathway. Additionally, even for peptides that act on the same pathway, further investigation into their mechanisms of action may lead to more insight towards immunomodulator optimization. For example, the sF2 peptide studied in chapter 2 was originally created to enhance T-cell proliferation and possibly exacerbate T-cell mediated diseases such as EAE [3]. The contradictory results found in chapter 2, that co-delivery of this peptide with autoantigen suppresses EAE, should be further investigated to better understand the immunological mechanisms leading to EAE disease suppression.

The *ex vivo* screen of immunomodulators described in chapter 3 indicated that dexamethasone had the most tolerogenic potential; however, that screen may be further optimized to better examine the production of an antigen-specific regulatory immune response. Regulatory cell types, such as regulatory T-cells, B-cell, and DCs, are all important in the creation of antigen-specific tolerance that would be needed to reverse or cure autoimmunity. The presence of these cell types upon treatment with immunomodulators would lead to greater certainty of immunomodulator efficacy *in vivo*. Regulatory T-cells (Tregs) are the most well studied regulatory cell population, and can be characterized by the surface markers CD3, CD4, and CD25 and by the intercellular protein FoxP3 [4, 5]. The presence of these cells even with the induction of immune tolerance is extremely low and often difficult to detect in a large splenocyte population, therefore the separation and concentration of the T-cell subpopulation before analysis may help enhance detection. Both regulatory B-cells (Bregs) and tolerogenic DCs (tolDCs) are more difficult to detect and, due to their relatively recent discovery, there is not a consensus on the surface markers that designate these cell types [6, 7]. These cell types can be defined by the proteins or enzymes they produce; IL-10 for Bregs and indoleamine 2,3-dioxygenase (IDO) for tolDCs [6, 7]. Similar to with Tregs, the separation of subpopulations (B-cells and DCs) prior to analysis rather than analysis on all splenocytes may lead to greater ability to detect these populations.

In addition to the immunomodulator, the co-delivery vehicle is essential for the production of an effective ASIT for autoimmunity. As reviewed in chapter 1 several different vehicles have been studied for the co-delivery of immunomodulator and autoantigen, including microparticles, nanoparticles, liposomes, multivalent polymeric arrays, direct chemical linkage, and in chapter 4 an oil-in-water emulsion was studied. This dissertation has demonstrated that both hyaluronic

acid-based soluble antigen arrays (SAgAs) and the oil-in-water emulsion of incomplete freund's adjuvant (IFA) can be efficacious at co-delivering autoantigen and immunomodulator for the treatment of EAE. Neither of these delivery systems has been approved for use in humans, therefore investigations of currently approved vehicles may allow for expedited creation of successful ASIT to treat human autoimmunity. Hyaluronic acid, the backbone of SAgAs, has been approved in many products on the market providing a clear path to SAgA approval [8]. On the other hand, IFA has been around for decades and although it has been used in clinical trials [9, 10] it has never been approved in a human therapeutic. A major reason that it has not been approved is the propensity for IFA to cause severe inflammation or even granulomas at the site of injection [11]. Although IFA is not approved for human use, several oil-in-water emulsions are approved in the US or EU [12]. Additionally, several approved vaccine adjuvants are oil-in-water emulsions such as MF59 and AS03[13].

One interesting FDA-approved oil-based system is the tocopherol (tocol) family, including vitamin E and its derivatives. Tocols have natural anti-oxidative and anti-inflammatory properties; therefore, a tocol-based emulsion may act as both a co-delivery vehicle and contribute to efficacy of an ASIT for autoimmunity [14]. Vitamin E and tocol esters such as α-tocopherol-polyethyleneglycol-1000-succinate (TPGS) have been used in the creation of oil-in-water emulsions [14, 15]. Development of a vitamin E/ TPGS-based emulsion is currently underway in the Berkland laboratory, and future work with this delivery system is an exciting next step in the creation of ASIT for autoimmunity.

Finally, the development of ASIT by combining autoantigen and immunomodulator can be applied to many different autoimmune diseases by changing the autoantigen. In addition to MS, rheumatoid arthritis and type 1 diabetes also have clearly defined autoantigen targets and

corresponding animal models [16, 17]. Future work in applying similar strategies for co-delivery of immunomodulator with autoantigens in other autoimmune diseases would bolster the amount of evidence in favor of this emerging trend in ASIT. Overall, with continued research and optimization of the immunomodulator, co-delivery vehicle, and autoantigen, this combinational approach towards ASIT for autoimmunity may prove key in the treatment and even cure of autoimmune diseases.

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