ABSTRACT

Title of Thesis: **Controlled Delivery of a Glutamate Receptor Modulator to Promote Regulatory T cells and Restrain Autoimmunity**

Joshua M. Gammon, Master of Science, 2015

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Autoimmunity occurs when the immune system incorrectly recognizes and attacks self-molecules. Current therapies involve broad immunosuppressants that are not curative and leave patients immunocompromised. Dendritic cells (DCs) are a target for new therapies because DCs influence the differentiation of immune effector cells. *N*-Phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide (PHCCC), a glutamate receptor enhancer, modulates DC cytokine profiles to polarize T cells toward regulatory phenotypes (T_{REG}) that are protective in multiple sclerosis (MS). However, PHCCC treatment is limited by poor solubility, a short half-life, and toxicity. We hypothesized that controlled delivery of PHCCC from nanoparticles would alter DC function with reduced treatment frequency. PHCCC nanoparticles attenuated DC activation and promoted T_{REGs} while reducing toxicity 30-fold. In

mouse models of MS, these particles delayed disease and reduced severity compared to an equivalent dosing schedule of soluble drug. This outcome demonstrates controlled delivery of metabolic modulators can promote tolerance, suggesting a new route to improve autoimmune therapy.

CONTROLLED DELIVERY OF A GLUTAMATE RECEPTOR MODULATOR TO PROMOTE REGULATORY T CELLS AND RESTRAIN AUTOIMMUNITY

by

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List of Abbreviations

APC- antigen presenting cell

cAMP- cyclic adenosine monophosphate

CFA- complete Freund’s adjuvant

CNS- central nervous system

CSFE- carboxyfluorescein succinimidyl ester

DAPI- 4',6-diamidino-2-phenylindole

DC- Dendritic cell

EAE- Experimental Autoimmune Encephalomyelitis

EDTA- ethylenediaminetetraacetic acid

i.p.- intra-peritoneal

ITE- 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester

LN- lymph node

LPS- lipopolysaccharide

MFI- Mean fluorescent intensity

mGluR- metabotropic glutamate receptor

mGluR4- metabotropic glutamate receptor 4

MP- microparticle

MPA- mycophenolic acid

MS- multiple sclerosis

NP- nanoparticles

PHCCC- N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide

PLGA- Poly(lactic-co-glycolic acid)

PVA- polyvinyl alcohol

s.c.- subcutaneous

TLR- Toll-like receptor

TLRa- TLR agonist

TREG- Regulatory T cell

Chapter 1: Introduction and Background

1.1 Organization of thesis

This thesis describes a new idea for using nanoparticles to control the release of drugs that alter the metabolic function of immune cells to restrain autoimmune disease. The first chapter provides a survey of key background information in the areas of immune function, autoimmunity, and biomaterials. In the second chapter the significance of the thesis work to the field of immunotherapy is discussed. The third chapter describes the experimental methods of the research and the fourth chapter details the experimental results. The fifth chapter contains a discussion of the results, and in the sixth chapter the conclusions of the research are summarized; potential areas for future investigation are also presented.

1.2 Overview of adaptive immunity

The key role of the immune system is to distinguish self-molecules and cells from pathogens and other non-self-molecules (1). In a healthy individual, foreign molecules or cells are recognized as non-self and attacked, while self-cells belonging to the host are ignored by immune system through a phenomenon termed “tolerance” (2). Central tolerance occurs in primary lymphatic organs such as the thymus and bone marrow, deleting immune cells which recognize self-antigens (3). Peripheral tolerance controls self-reactive immune cells that have escaped central tolerance and are present in the peripheral tissues not involved in the initial development and maturation of immune cells (4). When these processes fail, the immune system attacks host tissue in a process called autoimmunity.

The immune response consists of two main arms, called innate and adaptive immunity. Innate immune responses recognize foreign pathogens largely through the sensing of danger signals (5). These danger signals generally consist of molecular patterns broadly conserved across pathogens and not present on host cells. Some examples include bacterial lipopolysaccharide (LPS) and viral RNA (5). Thus, the immune system has evolved to recognize and react to pathogens through the sensing of these danger signals present on foreign pathogens. The innate immune response is not specific to individual pathogens, and responds to multiple pathogens containing danger signals. Although innate responses occurs quickly and provide a first line of defense, these mechanisms are not specific and do not exhibit immunological "memory", responding identically to subsequent infections by the same pathogen.

The adaptive immune response recognizes specific molecules, called antigens that are specific to individual pathogens. The first purpose of adaptive immunity is to initiate a primary response to infection, where lymphocytes called T and B cells recognize and attack pathogens expressing specific antigens (6). The subsequent purpose of adaptive immunity is to establish immunological memory towards previously encountered pathogens, allowing rapid and efficient recall responses to subsequent infection. This recall occurs through the generation of long lived memory cells after primary infection. Memory cells quickly respond and proliferate after recognition of a previously encountered pathogen, providing a pool of effector lymphocytes capable of combating the pathogen (7). This mechanism provides a much more rapid response to pathogens than the primary infection, allowing pathogens to be cleared before they can establish a foothold in the body (8). The

fundamental goal for vaccination is to generate immunologic memory toward specific pathogens and therefore protect the host from infection by these pathogens.

The generation of an adaptive immune response is dependent on an important subset of the immune cells called antigen presenting cells (APCs); APCs can be seen as a bridge between innate and adaptive immunity (6). APCs create this link by processing and presenting antigens to T and B cells in lymph nodes and spleen – immune organs that orchestrate adaptive immunity. When APC presents an antigen to a naïve antigen-inexperienced lymphocyte with the correct co-stimulatory signals, lymphocytes become activated and expand (1). These lymphocytes are specific for the antigen presented by the APCs and then migrate from lymph nodes and spleen to seek out and perform effector functions such as secretion of inflammatory proteins and direct destruction of host-cells displaying antigen they are specific for (e.g., when infected by a virus) (9).

Lymphocytes perform their effector functions through two types of immunity, termed humoral or cellular immunity (6). B cells are the main effector cells providing humoral immunity through the production of antibodies, which among other functions, neutralize extracellular pathogens such as bacteria, enhancing phagocytosis and destruction by macrophages and other innate immune cells (1) **(Figure 1D)**. Cellular immunity is carried out by a class of T cells called $CD8^+$ T cells, which recognize and lyse cells that have been infected with viruses or other intracellular pathogens **(Figure 1D)**. Another class of T cells involved in cellular immunity, called $CD4^+$ or helper T cells, support the adaptive and innate immune response through the secretion of immune signaling proteins called cytokines (10).

Dendritic cells (DCs) are a class of professional APCs, which play a key role in generating adaptive immune response (11, 12). DCs patrol the periphery, where they specialize in phagocytosing particulate matter, including microbes, pathogens and apoptotic or necrotic cells **(Figure 1A, 1B)**. DCs then migrate to lymph nodes and the spleen. At these sites, DCs process and present antigens contained in phagocytosed material to lymphocytes (13) **(Figure 1C)**. As lymphocytes become activated and proliferate, they differentiate into different phenotypes that play distinct roles in adaptive immunity. The phenotype a lymphocyte differentiates to is largely controlled by signals presented and secreted by DCs during interaction with an antigen specific lymphocyte. DCs present costimulatory molecules (e.g., CD40, CD80) on their surface that ligate receptors expressed on lymphocytes to cause robust activation and proliferation. DCs also secrete a variety of cytokines, and the amount and relative levels of different cytokines drive the phenotypic polarization of lymphocytes. IL-12, for example, is important in promoting the differentiation of a subset of helper T cells, called T_H1 cells, which drive a $CD8^+$ T cell mediated cellular immune response (14). In contrast, IL-4 drives helper T cells to differentiate to a phenotype called T_H2, which is important in promoting B cell mediated humoral immunity (14).

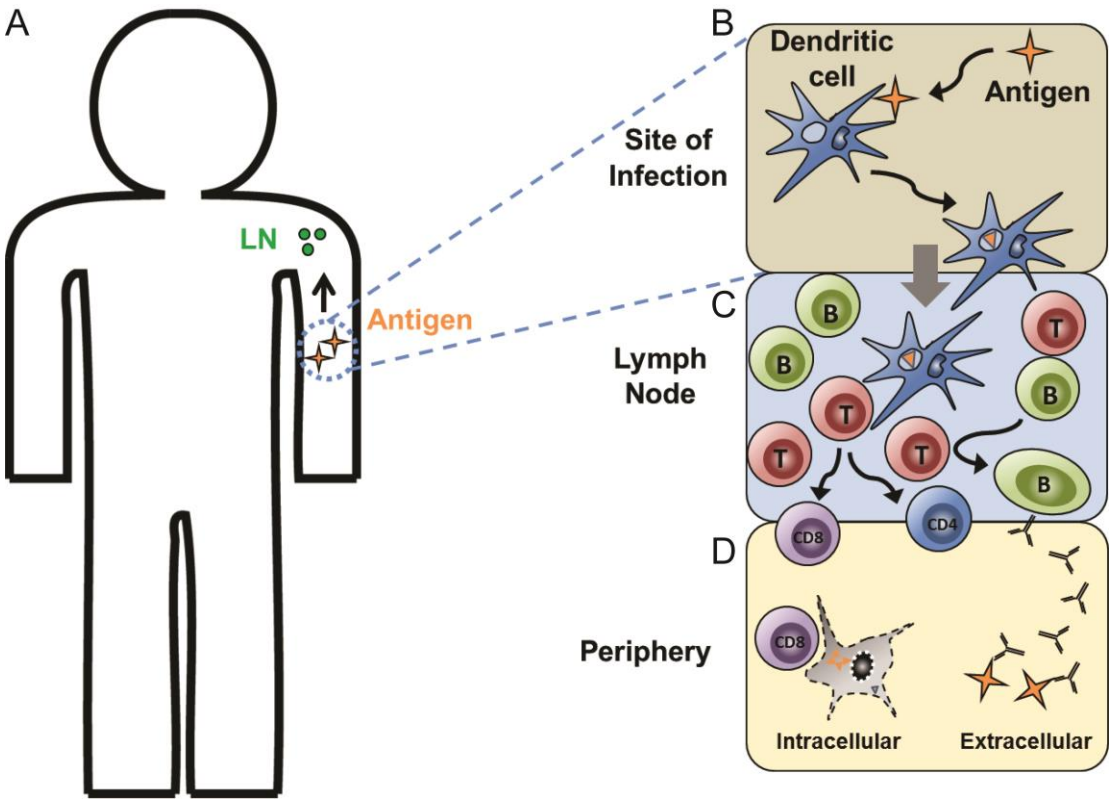

Figure 1. Schematic overview of the generation of adaptive immune response after exposure to an antigen. (A) Antigens present on pathogens are initially in the periphery. (B) DCs patrolling the periphery internalize pathogens and then migrate to lymph nodes. (C) DCs process and present antigens contained on pathogens to T and B cells in lymph nodes. (D) Activated T and B cells enter the periphery to perform cell mediated or humoral immunity upon encounter with the antigen they are specific for.

1.3 Adaptive immunity in autoimmune disease

Autoimmune disease occurs when the adaptive immune system aberrantly recognizes and attacks self-antigens. In terms of public health, some of the most widespread autoimmune diseases are type 1 diabetes, rheumatoid arthritis, lupus and multiple sclerosis (MS). During these diseases, immune cells seek out and attack self-cells expressing these molecules, causing autoinflammation – local infiltration of

autoreactive lymphocytes and cells of the innate immune system, high levels of inflammatory cytokines, and production of autoreactive antibodies (15).

Different phenotypes of lymphocytes play different roles in the pathology of autoimmune disease, with some phenotypes highly inflammatory, and others can actually control autoimmune disease through suppressive or regulatory mechanisms. Of particular importance in many autoimmune disorders are $CD4^+$ T cells, where these cells differentiate into T_H1, T_H17 or T_{REG}. T_H1 and T_H17 cells are highly pathogenic during autoimmunity, secreting inflammatory cytokines such as IFNγ and IL-17 (14). In contrast, T_{REGS} are natural suppressor cells which help regulate immune function after infections are cleared – and as recent intense research efforts have shown – also play a critical role in supporting peripheral tolerance to control inflammation and autoimmunity (2, 16) **(Figure 2A)**.

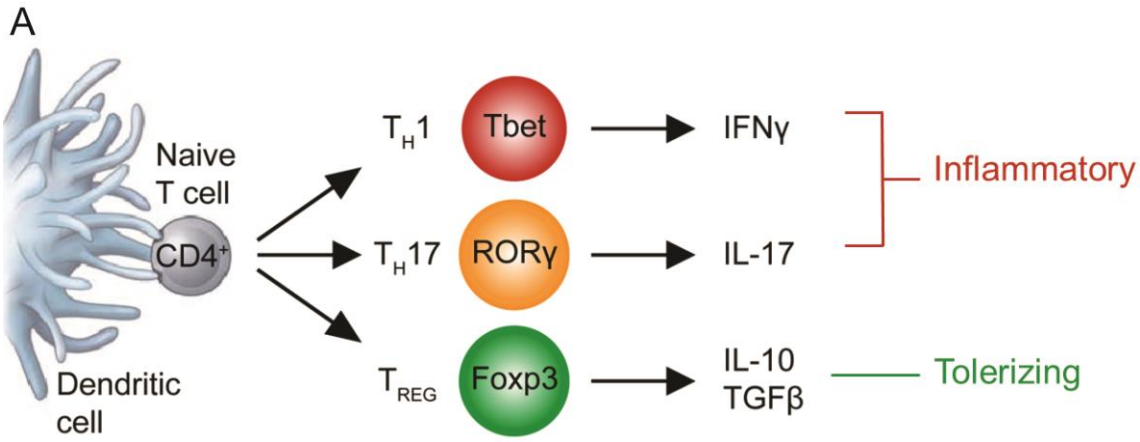

Figure 2. DCs orchestrate adaptive autoimmune responses through the polarization of $CD4^+$ T cells. (A) Graphical depiction of the differentiation of inflammatory T cells (T_H1/T_H17) or regulatory T cells (T_{REG}) after interacting with a DC. Adapted from O'Shea and Paul, *Science*. 2010 (17)

As discussed in **Section 1.2**, DCs play an important role in polarization of $CD4^+$ T cells responses, and therefore are also often involved in autoimmune disease **(Figure 2A)**. The magnitude and phenotype of the immune response, including malfunctions during autoimmunity, is largely driven by the integration of the levels of presentation of self or foreign antigen, and the types and expression levels of activation markers and cytokines produced by DCs and lymphocytes(17). Expression of high levels of activation markers and secretion of inflammatory cytokines by a DC during the interaction with a T cell will drive the polarization of inflammatory phenotypes, while absence of activation markers and presence of regulatory cytokines will polarize T cells toward regulatory phenotypes. IL-6, for example, can promote the differentiation of T_H17 cells, while inhibiting the differentiation of T_{REG} (18). Additionally, as DCs polarize T cells, these cells secrete cytokines (e.g., IFNγ during inflammation; TGFβ during regulation) that exert autocrine effects as well as paracrine effects on other nearby T cells, further amplifying this polarization (14).

1.4 The pathology of multiple sclerosis and current treatments

MS is an autoimmune disorder which occurs when the adaptive immune system attacks myelin, a protein which insulates axons of neurons. MS affects over 2.1 million people and is the most common autoimmune disorder (19). Autoinflammation in MS results in chronic demyelination in the central nervous system (CNS). This demyelination causes disruption in neuronal signaling resulting in severe symptoms such as loss of vision, paralysis, and death (20). The pathology of MS is largely driven by autoreactive T_H1 and T_H17 cells that infiltrate the CNS and cause inflammation and tissue destruction. However, autoimmune reactions toward

myelin can be suppressed by T_{REGS} and other regulatory mechanisms (16, 21, 22). Therefore a common therapeutic strategy for MS is to polarize T cells toward regulatory (T_{REG}) and away from inflammatory phenotypes (T_H1/T_H17).

Treatments for M.S have historically aimed to inhibit the self-reactive immune response and the resulting autoinflammation. Broad immunosuppressants administered systemically, such as steroids or immunosuppressive cytokines, have traditionally been used as treatments (23). More recently monoclonal antibodies have been approved for treatment, which are able to target more specific pathways (24). However, these treatments are not curative, require life-long administration, and still leave patients immunocompromised. A new strategy being investigated is the promotion of myelin-specific tolerance, which would offer the potential to inhibit autoimmune reactions toward myelin while leaving the rest of the immune system functional and able to protect against foreign pathogens (25). As in vaccination and other immunotherapies, these strategies can be further enhanced through better control over the delivery of the immune signals involved in polarizing T cells toward tolerance, ideas discussed below in **Sections 1.4** and **1.5**.

1.5 Use of biomaterials for modulating immune function

Biomaterials offer many favorable properties which can be harnessed to enhance vaccination and immunomodulation (26, 27). Many biomaterials, such as biodegradable polymers (28-30), liposomes (31), and self-assembling complexes (32) can be used to encapsulate and control the delivery of immune signals, including antigens, adjuvants – substances that increase the potency of vaccines – and immunomodulatory drugs. Both hydrophobic (30, 33, 34) and hydrophilic (35-37)

cargos can be encapsulated, providing protection from degradation, controlled and sustained co-delivery, and targeting to specific immune cells or tissues (29, 38-40). APCs specialize in phagocytosing particulate material and readily endocytose microparticles (MPs) or nanoparticles (NPs). Thus these materials allow co-delivery of encapsulated immune signals into the cytosol. This is an important feature for vaccines and immunotherapies because it can enhance the processing and presentation of antigens, ensure that adjuvants and immunomodulators – signals that modify the function of the immune response – reach APCs together, as well as providing control over the type of immune responses developed (e.g., $CD8^+$ vs. $CD4^+$ T cells). Additionally this mechanism provides localization of immunomodulators and adjuvants to APCs, allowing potential dose sparing while reducing systemic exposure and potential toxicities (41).

The level of activation and balance between inflammatory and regulatory cytokines secreted by a DC when it presents an antigen is key in driving the balance between immunity and tolerance toward that specific antigen. This balance between immunity and tolerance is at the core of strategies for vaccination and more specific therapies for autoimmunity, respectively. A common vaccination strategy to safely generate an effective adaptive immune response against a desired pathogen is to use subunit vaccines, formulations consisting only of well-defined antigens isolated from a specific pathogen. These antigens are generally poorly immunogenic, and without stimulatory molecules do not generate robust immune responses. Toll-like receptors (TLRs) are receptors present on cells of the innate immune system which function to detect molecular danger signals as discussed in **Section 1.2** (42). Thus TLR agonists

(TLRas) serve as adjuvants by potently activating DCs in a well-defined manner. These molecules have also been used in combination with antigens to synergistically enhance vaccination (43). However, systemic administration of adjuvants and antigens does not ensure that both components reach DCs together, so DCs may not be activated in the presence of the antigen. In the context of autoimmunity, a therapeutic treatment may conversely involve administering regulatory signals to DCs with the autoantigen, in order to decrease DC activation during the presentation of autoantigens; these effects are known to encourage the promotion of T_{REGs} are specific to the self-antigen (44). To this end, a new materials-based strategy to target multiple immune signals to DCs is to encapsulate and co-deliver the signals in NPs or MPs (34, 45).

The kinetics and concentrations of immune signals during vaccination are key parameters affecting the properties of the resulting immune response (46). Biomaterials offer control over the dosing and persistence of immune signals, and can be harnessed to enhance vaccination and immunotherapies. For example, NPs provide sustained concentrations of cargo as it is released over time. This property can be tuned to better recapitulate kinetics of antigens and danger signals which would occur in response to infection, and therefore generate a more substantial immune response (27).

In addition to delivery of vaccines, biomaterial-mediated controlled delivery of small molecule immunomodulators can enhance immunotherapies (30, 34). Hydrophobic small molecules are a large and important class of immunomodulatory drugs, but are frequently poorly soluble and cannot be delivered in aqueous solvents.

Additionally, hydrophobic small molecule drugs typically have short half lives *in vivo*, and are rapidly cleared from the body after systemic administration. Controlled delivery of immunomodulators can address these limitations, alleviating the need for frequent high dose administrations, which potentially cause toxicities and off target effects (47, 48).

Lymph nodes are key centers for the generation of immune responses (49), and are therefore the site immune signals much reach to modulate the adaptive immune response. Biomaterials offer the ability to target lymph nodes through passive lymphatic drainage (50) or actively target immune signals to lymph nodes. NPs in the range of 20-50nm passively drain through lymphatics to the lymph nodes where they are retained and taken up preferentially by LN-resident DCs and macrophages (45, 51). Larger NPs as well as MPs are phagocytosed by APCs at the site of the injection, and are then trafficked to lymph nodes by these cells (52). In addition to passive targeting, active targeting of NPs to LNs or LN-resident APCs can be achieved through the conjugation of a variety of targeting ligands or receptors (29, 38, 39). These provide strategies to deliver a variety of immune signals, such as antigens, adjuvants and drugs to lymph nodes more effectively to bias how lymphocytes differentiate.

1.6 Application of biomaterials to promote tolerance

Recent insights into the pathology and mechanisms of autoimmune reactions have led to exploration of many potential strategies for autoimmune therapies. A clear consensus exists that some autoreactive immune cells evade central tolerance and escape into the periphery. In healthy hosts, autoimmune reactions do not develop and

are kept in check by peripheral tolerance. In the case of autoimmune diseases, these autoreactive cells overcome peripheral tolerance and drive autoimmune reactions. Strategies for autoimmune therapies generally aim to suppress inflammation, or more recently, to restore peripheral tolerance by 1) eliminating self-reactive immune cells, or 2) generating T_{REGS} that can suppress the function of these cells. Biomaterials can be harnessed to this end through the controlled delivery of regulatory signals (53). Regulatory signals, including small molecule drugs or biologics, may be delivered alone to broadly suppress autoreactive responses with reduced toxicity, or may be co-delivered with self-antigens in an effort to promote T_{REGS} that can specifically inactivate lymphocytes specific for the self-antigens. An additional strategy includes using biomaterials as platforms to change the way self-antigens are processed to promote differentiation of T_{REGS} over inflammatory cells (44).

Biomaterial mediated controlled delivery of a variety of regulatory signals, including small molecule drugs, enzymes, and cytokines has been investigated to restore tolerance. Poly(lactic-co-glycolic acid) (PLGA) NPs encapsulating mycophenolic acid (MPA), which is a potent immunosuppressant, have been used to tolerize the immune system toward allografts in transplantation (30). Soluble treatment of MPA is effective in prolonging allograft survival, but requires frequent high dose injections which often result in toxic side effects. Systemic administration of PLGA NPs encapsulating MPA resulted in accumulation of NPs in spleens and LNs, and NPs were preferentially taken up by APCs. This resulted in upregulation of the inhibitory signal, PD-L1, on DCs, which suppressed the generation of T cell

responses directed against antigens present on the allograft and improved allograft survival.

Biomaterials themselves can also act as a regulatory signal through modulating how APCs interact with and process self-antigens. The pathways through which APCs internalize soluble and particulate material are largely a function of size and can be mediated by a variety of receptors (54). Internalization of apoptotic cells plays an important role in the maintenance of tolerance to self-antigen. As a strategy to mimic this tolerogenic pathway, polystyrene or PLGA MPs conjugated to myelin peptides have been used to restrain autoimmune reactions in mouse models of MS (55). These therapeutic effects are dependent on the uptake of the MPs in the spleen through the MARCO scavenger receptor, which specializes in uptake and clearance of cellular debris (56).

NPs have also been used to co-deliver regulatory signals and self-antigens as a strategy to restore tolerance to specific antigens while leaving the rest of the immune system unaltered (28, 34, 57). Co-delivery of rapamycin and myelin autoantigen with PLGA NPs effectively inhibits autoimmune reactions directed towards myelin in mouse models of MS (34). Rapamycin is a tolerogenic small molecule that inhibits the mTOR pathway, a pathway involved in a variety of cellular division and maintenance processes. Inhibition of this pathway in DCs can modulate their T cell phenotype to promote differentiation of T_{REGS}. NP mediated co-delivery of myelin antigen and rapamycin to DCs caused the generation of T_{REGS} in an antigen specific manner, which suppresses the immune response toward myelin antigen, resulting in amelioration of symptoms of neuroinflammation in animal models of MS. These

recent strategies demonstrating that DCs can be effectively modulated to control the immune response for therapeutic effects in autoimmune disease have motivated the investigation of targeting other DC pathways such as metabolic function. However, the features of biomaterials have not previously been harnessed to support this strategy.

Chapter 2: Project Significance[‡]

2.1 Glutamate signaling to modulate DCs for autoimmune therapy

High levels of glutamate are present in the CNS during autoinflammation in MS patients, leading to a state of excitotoxicity that exacerbates inflammation and depletes oligodendrocytes – the cells responsible for remyelinating neurons (58, 59). The metabotropic glutamate receptor (mGluR) family helps control these effects, resulting in inflammation or protection against excitotoxicity, depending on the relative presence and activity of each mGluR receptor in the CNS during disease (58-61). In particular, metabolism of glutamate through mGluR4 reduces *N*-Methyl-D-aspartate toxicity in cortical neurons and kainate mediated toxicity in oligodendroctyes. (61, 62).

In addition to serving as a neurotransmitter, glutamate also functions as an immunomodulator by interacting with glutamate receptors expressed on the surface of DCs or other immune cells (63). As discussed in **Section 1.3**, DCs play a key role in initiating adaptive immunity by processing and presenting exogenous antigens, or in the case of autoimmune diseases such as MS, self-antigens (e.g., myelin). Presentation of these antigens to a cognate T cell drives antigen-specific proliferation and differentiation. The magnitude and type of T cell response is dictated in part by the activation and inflammatory state of DCs during the formation of an immune synapse with a T cell (12). For example, suppression of DC activation can induce secretion of regulatory cytokines that diminish T cell expansion and shift the phenotypes toward T_{REG}s and away from inflammatory T cells (e.g., T_H17) (64-66).

During inflammation, DCs release glutamate that regulates T cell activation and proliferation by binding glutamate receptors on DCs and T cells (63, 67, 68). The identity and abundance of the mGluRs that are bound by glutamate alters cyclic adenosine monophosphate (cAMP) levels, modulating the balance of inflammatory and regulatory cytokines that direct the resulting immune response. This mechanism has stimulated interest in controlling glutamate receptor signaling as one route to regulate immune cell function in new therapies for MS (68-70). The Di Marco group recently demonstrated that mGluR4 is expressed at high levels on DCs and exerts an immunoregulatory function by showing that DCs with defective mGluR4 signaling preferentially polarize T cells to inflammatory T_H17 phenotypes. This work further revealed that *N*-Phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide (PHCCC), a small molecule positive allosteric modulator of mGluR4, can bias T cell function toward tolerance during autoimmunity in mice (62, 68). Prophylactic treatment of mice with PHCCC during Experimental Autoimmune Encephalomyelitis (EAE) – a mouse model of MS – inhibited clinical symptoms of neuroinflammation by inducing regulatory cytokine profiles in DCs that promoted T_{REGS} and reduced T_H17 cells (10). However, the use of PHCCC is hindered by poor solubility and a short half-life (71), with neurological symptoms and paralysis returning within one day after daily systemic injections were stopped (68).

2.2 Hypothesis and Research Strategy

To address the challenges associated with PHCCC treatment, we hypothesized that controlled release of PHCCC might maintain or improve efficacy while offering less frequent dosing and reduced toxicity. We thus tested if polarization through

sustained release of small molecules that control immune cell metabolism can promote tolerance and restrain autoimmunity. In these studies we employed a well-understood PLGA NP platform to test if controlled release of these drugs would alter DC function, promote regulatory T cells, and more effectively control disease in mouse models of autoimmunity with less frequent dosing and reduced toxicity.

Chapter 3: Methods[‡]

3.1 PHCCC NPs

3.1.1 Synthesis

PLGA NPs encapsulating PHCCC were synthesized by nanoprecipitation. The solvent phase was prepared by dissolving 25mg of PLGA in 1.5mL acetone. For samples loaded with PHCCC, this polymer solution was transferred to a vial containing the appropriate mass of dried PHCCC. For samples loaded instead with DiO, 5µL of 1µM DiO solution was added to the polymer solution. The non-solvent phase consisted of 20mL deionized water with 0-2% polyvinyl alcohol (PVA). The solvent phase was then uniformly injected through a 31g needle under the surface of the non-solvent phase while mixing with a magnetic stir bar. The solvent was evaporated for 3 hours, leaving a suspension of stabilized NPs. The suspension was poured through a 40µm cell strainer then centrifuged for 75 min at 4 °C at 3000g. The supernatant was decanted and the NPs were resuspended in deionized water.

3.1.2 Particle Characterization

To measure PHCCC encapsulation, NPs were dried and dissolved in DMSO. The absorbance was then measured at 300nm on a UV-VIS spectrophotometer and corrected by subtracting the absorbance value measured for empty NPs dissolved at the same mass concentration in Dimethyl-sulfoxide (DMSO). Absorbance values were compared to a PHCCC standard curve to determine a mass concentration.

Particle size distributions were measured by laser diffraction using a Horiba Partica LA 950V2.

To measure release kinetics, known concentrations of PHCCC NPs were incubated at 37 °C in RPMI 1640 media under sink conditions to eliminate release effects arising from saturated drug solution. At each interval PHCCC NPs were centrifuged at 18,000g for 5 minutes, and PHCCC concentration in the supernatants was determined by UV-VIS spectrophotometry as above. Particles were then resuspended in media and returned to incubation at 37 °C. The cumulative PHCCC released was calculated at each time and normalized to the total loading to calculate the percent of PHCCC released.

3.2 In vitro effects of PHCCC NP properties on DCs

3.2.1 Uptake

Isolated DCs were plated in flat bottom 96 well plates and incubated in media with the specified concentrations of DiO NPs for 90 minutes. Cells were washed with PBS to remove free NPs, and then detached using cold, 10mM ethylenediaminetetraacetic acid (EDTA). Detached cells were washed with cold PBS + 1% BSA, then resuspended in 4',6-diamidino-2-phenylindole (DAPI) (5µg/mL) in PBS + 1% BSA. DCs were analyzed by flow cytometry to determine the percentages of cells positive for DiO (particles). For analysis of particle uptake by microscopy, splenic $CD11c^+$ DCs were plated in 35mm dishes and incubated in media with DiO NPs for 90 minutes. Cells were washed with PBS to remove free NPs, then fixed with 4% paraformaldehyde for 15 minutes at 37°C. Cell membranes were stained at room

temperature in the dark for 10 minutes with 5µg/mL wheat germ agglutinin Texas Red conjugate in PBS. The cells were then washed with PBS, labeled with Hoechst nuclear stain diluted to 2µg/mL in PBS. Cells were imaged using a Leica SP5 X laser scanning confocal microscope with a 63X objective.

3.2.2 Reduction in toxicity

To analyze toxicity of soluble PHCCC or PHCCC delivered in NP format, $CD11c^+$ cells isolated from splenocytes ($1x10^5$ cells/well) were stimulated with LPS (1µg/mL) and treated with equivalent concentrations of PHCCC delivered in soluble or NP format. After 18 hours toxicity was analyzed by flow cytometry by staining with DAPI and quantifying the percentage of $DAPI^-$ events. Relative viability was calculated by normalizing the percent of viable cells to the value of the untreated LPS stimulated control. The fold-decrease in toxicity was calculated by dividing the relative viability of cells treated with PHCCC NPs by the value for cells treated with soluble PHCCC at 400µM.

3.3 In vitro DC immunodulation with PHCCC NPs

3.3.1. Effect of PHCCC NPs on DC activation and cytokine secretion

For analysis of DC surface activation markers and cytokine secretion levels, $CD11c^+$ cells isolated from splenocytes ($1x10^5$ cells/well) were stimulated with LPS (1µg/mL) and left untreated or treated with soluble PHCCC (40µM), PHCCC NPs, or empty NPs using equivalent particle doses/masses. For soluble PHCCC controls, PHCCC was dissolved in DMSO to 20mM then diluted to a final concentration of

40μM in media. After 18, 44 and 68 hours, cells and cell culture supernatants were collected. IL-6 and IL-10 concentrations in the supernatants were determined by ELISAs (BD Biosciences). The collected cell pellets were stained as above for viability ($DAPI^-$), CD11c (APC-Cy7) and activation/costimulatory markers (i.e., FITC I-A/I-E, PE CD40, PE-Cy7 CD86, APC CD80). Cells were gated under $CD11c^+/DAPI^-$ negative events.

3.3.2 Effect of PHCCC NPs on DC antigen presentation

For analysis of DC antigen presentation, DCs stimulated with LPS were treated with PHCCC, PHCCC NPs, and empty NPs as in DC activation studies. After 18 hours, cells were incubated for 2 hours with SIINFEKL peptide (5μg/mL), or with MOG_{35-55} (5μg/mL) as an irrelevant peptide control. Cells were then stained for presentation of SIINFEKL via the H-2kb complex using an antibody which binds SIINFEKL when presented in H-2kb. Cells were gated under $CD11c^+/DAPI^-$.

3.4 In vitro effect on PHCCC NPs on T cell polarization

3.4.1 Effect of PHCCC NPs on T cell phenotype

Isolated DCs ($1x10^5$ cells/well) were stimulated with LPS (1μg/mL) and MOG (10μg/mL) and treated with soluble PHCCC (40μM), PHCCC NPs, or empty NPs. After 18 hours, splenic $CD4^+$ T cells isolated from 2D2 mice ($3x10^5$ cells/well) were added to the DC culture. After an additional 2 days, supernatants were collected and IFNγ concentrations were measured by ELISA (BD Bioscience). Cells from these cultures were stained for CD4 and CD25 surface markers, along with FoxP3 and

RORγ transcription factors as markers for T_{REGS} ($CD4^+/CD25^+/FoxP3^+$) and inflammatory T_H17 cells ($CD4^+$/ $ROR\gamma^+$), respectively.

3.4.2 Effect of PHCCC NPs on T cell proliferation

For proliferation studies, splenic $CD4^+$ T cells isolated from 2D2 mice were incubated with carboxyfluorescein succinimidyl ester (CSFE) before addition to DCs treated with LPS, peptide, and particles identically as described in **Section 3.4.1**. The T cells were labeled by resuspension in media at $50x10^6$ cells/mL then incubation in CSFE at a final concentration of 5μM for 5 minutes at room temperature. Cells were washed four times with media, then added to the DC cultures. Cells were collected 72 hours later and analyzed by flow cytometry to determine the extent of T cell proliferation (i.e., CSFE levels among $CD4^+/DAPI^-$ cells). Analysis was performed using FlowJo software (Treestar).

3.5 In vivo effect of PHCCC NPs on restraining autoimmune reactions

3.5.1 Efficacy of PHCCC NPs with different treatment regimens

All animal research and care was carried out in accordance with local, state, and federal regulations and under guidelines approved by the University of Maryland IACUC. EAE was induced in 10 week old C57BL/6 mice as previously described(72, 73). Briefly, mice were immunized with two subcutaneous (*s.c.*) injections of emulsions prepared from complete Freund's adjuvant (CFA) and 100μg of MOG_{35-55} in killed mycobacterium tuberculosis H37Ra (200-500μg). Subsequently, 200ng of

pertussis toxin in 100μL of PBS was injected intra-peritoneal (*i.p.*) on the day of immunization and again the following day.

Soluble PHCCC , PHCCC NPs (each group n=6, dosed with 3 mg/kg with respect to drug dose) were administered *s.c.* at the tailbase every 3 days or every 5 days beginning on the day of EAE induction (day 0). For soluble treatment, PHCCC was dissolved in sesame oil (68) and PHCCC NPs were suspended in deionized water. Empty NPs at the same mass as the PHCCC NP treatments were included as controls. Mice were weighed and scored daily for signs of disease using an accepted clinical pathology scoring scale: 0, no symptoms; 1, limpness in entire tail; 2 weakness in hind legs; 3, paralysis of hind legs or paralysis of one front leg and one hind leg; 4, moribund. Mice were euthanized according to defined endpoints if a score of 4.5 was reached, upon a score of 4.0 for 2 days, or upon 25% weight loss (with respect to initial weight).

3.6 Statistical analysis

Statistical analysis was undergone for *in vitro* and *in vivo* studies conducted utilizing the optimized NP formulation. Student's T test was used when comparing two groups, and one way ANOVA with a Tukey post-hoc analysis was used with comparisons of three of more groups. For EAE studies, unpaired T tests were used to compare mean clinical scores between groups at each study day. P values to determine statistical significance are indicated as*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Error bars in all panels represent mean ± SEM.

Chapter 4: Results[‡]

4.1 Synthesis and physiochemical characterization of PHCCC NPs

PLGA NPs synthesized by nanoprecipitation are a well characterized platform that has been used extensively for the controlled delivery of small molecule drugs. We therefore used this platform to test the new idea that controlled release of the glutamate receptor enhancer, PHCCC, might modulate immune cell function to restrain autoimmune reactions with reduced toxicity and frequency of treatment.

PHCCC was successfully encapsulated in NPs at high levels, with PHCCC loading levels correlated with increasing ratios of drug to polymer input. Loading was optimal at a drug:polymer input ratio of 0.2, which resulted in a drug loading level of 73.2 ± 25.9µg of PHCCC per mg of NPs (**Table 1**). Further increasing the drug to polymer ratio to 0.4 or higher did not result in any further increase in loading. NPs exhibited relatively uniform size distributions, with mean diameters ranging from 114-185nm depending on the drug to polymer ratio (**Figure 3A, 3B**).

Table 1. Properties of PHCCC NPs synthesized with different input ratios of PHCCC to PLGA.

PHCCC:polymer input ratio (mg)	PHCCC input (mg)	Polymer input (mg)	Loading Level (µg PHCCC/mg particle)	Diameter (nm)
0	0	25	N/A	125.2 ± 60.2
0.04	1	25	25.4 ± 12.2	132.9± 36.4
0.12	3	25	44.7 ± 13.3	185.9 ± 33.7
0.2	5	25	73.2 ± 25.9	148.5 ± 47.1
0.4	10	25	78.3 ± 12.9	114.4 ± 42.2

To examine the release kinetics of PHCCC from NPs, particles were incubated in media at 37°C. PHCCC NPs released 66.3% of drug over the first 4 hrs, followed by a slower release rate that accounted for release of 86.3% of total encapsulated drug after 48 hours (**Figure 3C**).

4.2 Effects of NP mediated controlled delivery of PHCCC on DCs

After determining PHCCC NPs could be synthesized in the nm range and released drug over time, we investigated how these general properties would affect the uptake and viability of primary DCs. To test the ability of DCs to internalize NPs, fluorescently-labeled empty NPs were incubated with $CD11c^+$ splenic DCs for 90 minutes. Flow cytometry analysis of these samples revealed a dose dependent uptake of NPs with up to 58.4 ± 2.8% of live, $CD11c^+$ cells positive for NPs (**Figure 3D**). Visualization of cells by confocal microscopy confirmed these results, showing punctate NPs distributed throughout the cytosolic regions of DCs (**Figure 3E**, green signal).

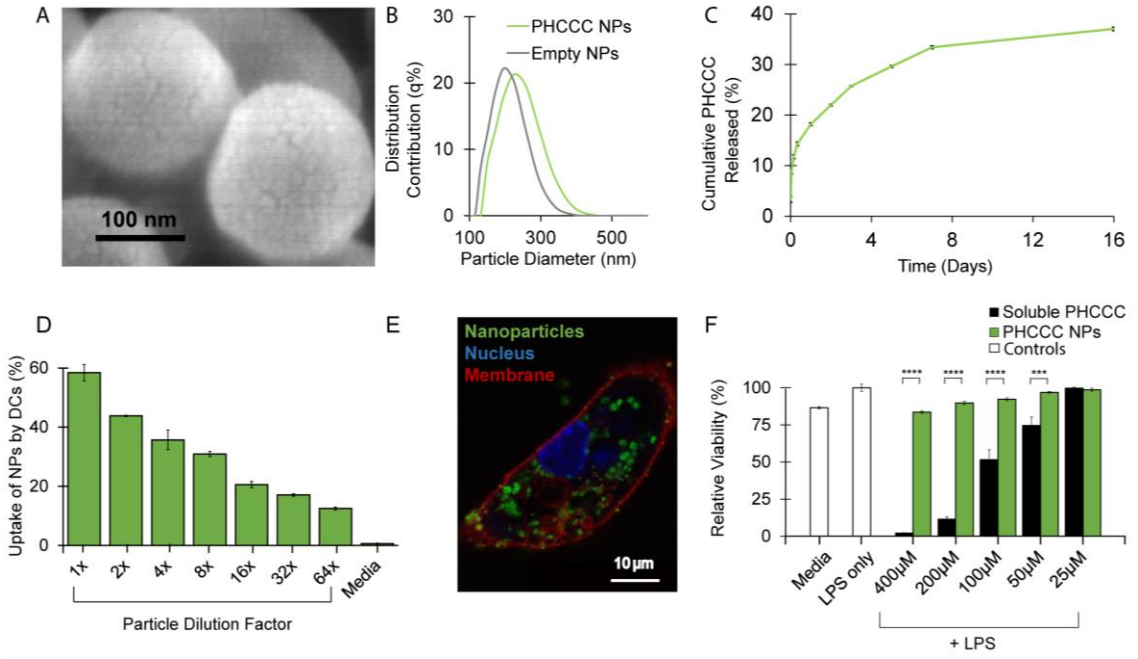

Figure 3. NPs synthesized by nanoprecipitation are internalized by DCs and eliminate toxicity observed from soluble PHCCC. (A) SEM image of PHCCC NPs. (B) Histogram showing size distribution of PHCCC NPs synthesized with 2% PVA and a drug to polymer ratio of 0.2. (C) *In vitro* release kinetics of PHCCC NPs. (D) Primary splenic DCs were incubated for 90 min with fluorescent NPs, and the percent of DCs positive for NP uptake was quantified by flow cytometry. (E) Confocal microscopy image of DCs treated as in (D), demonstrating colocalization of NPs (green) within cells. DCs were stained with a Texas Red wheat germ agglutinin conjugate (red) and Hoechst nuclear stain (blue). (F) Primary splenic DCs were stimulated with LPS (1μg/mL) and treated with PHCCC in soluble or NP form. Viability relative to LPS stimulated cells was quantified by DAPI staining and analysis by flow cytometry. All data were collected in triplicate and are representative of 3 similar experiments.

To assess the toxicity of PHCCC on immune cells, we stimulated DCs with LPS, along with varying doses of PHCCC in soluble form or loaded in NPs. After 18 hours, only 2.2% of cells treated with LPS and soluble PHCCC remained viable relative to cells treated with LPS alone (**Figure 3F**). Strikingly, 83.6% of DCs remained viable following treatment with LPS and an equivalent concentration of PHCCC in NP form, representing a 36-fold reduction in toxicity (**Figure 3F**).

4.3 Immunomodulation of DCS by PHCCC NPs in vitro

After determining that NPs were readily internalized by DCs with minimal toxicity, we investigated the immunomodulatory properties of PHCCC encapsulated in NPs. Optimization studies of the NP formulation were conducted. The effect of NPs synthesized with varying concentrations of PVA stabilizer and different drug:polymer ratios on DC inflammatory cytokine secretion was investigated. This was performed in order to determine an optimal NP formulation which would maximize the effects of encapsulated PHCCC with minimal effects from the empty NP carrier. An optimized formulation was chosen, and the effects of a range of doses of PHCCC NPs synthesized with this formulation were tested inflammatory cytokine secretion, activation marker expression and antigen presentation by DCs.

To determine what range of drug input levels in NPs would alter DC function, DCs were stimulated with LPS and incubated with a fixed dose of PHCCC loaded in NPs that were prepared using different drug:polymer ratios (**Figure 4**, green bars). Empty NP (**Figure 4**, gray bars) controls were prepared at the same mass of NPs (i.e., polymer) used for PHCCC NPs to confirm that the effect of PHCCC NPs was attributable to encapsulated PHCCC. The levels of IL-6 and IL-10 secretion were then measured in the supernatants to determine if PHCCC NPs reduced the inflammatory effects caused by LPS treatment. IL-6 levels generally increased over the 3 day incubation, but at each time point, DCs treated with LPS and a fixed drug dose loaded in PHCCC NPs, caused a significant reduction in IL-6 secretion compared with cells treated with LPS only (**Figure 4A-C**). These reductions were not as large as those observed in samples treated with soluble PHCCC. In contrast, empty

particles generally did not cause a significant change in IL-6 level. However, at empty particle masses equivalent to those used for the lowest drug:polymer ratio (i.e., highest polymer mass), empty NP controls had a modest effect on IL-6 secretion. PHCCC also decreased IL-10 secretion, but the kinetics of this reduction after treatment with soluble PHCCC or PHCCC NPs was delayed compared with the timeline observed for IL-6 (**Figure 4D-F**). The effects of PHCCC NPs were evident at 44 hours and 68 hours, but not at the earlier time point of 18 hours where no differences were observed between empty NPs and PHCCC NPs (**Figure 4D-F**).

A further optimization study was performed by varying the stabilizer (i.e., PVA) concentration for NPs prepared at a drug:polymer ratio of 0.2 (**Figure 4**). This

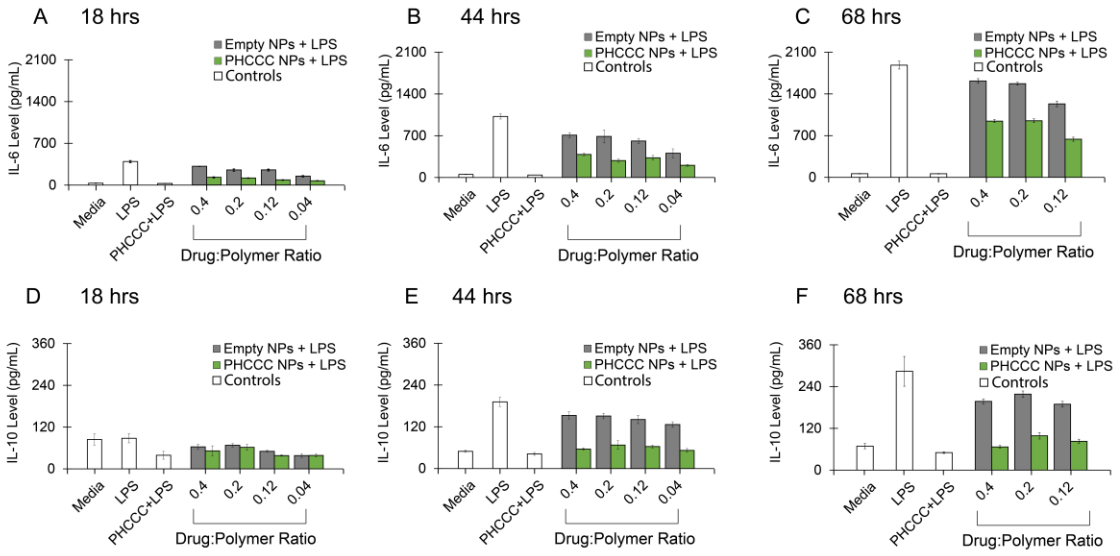

Figure 4. Impact of PHCCC loading in NPs on DC cytokine secretion. $CD11c^{+}$ cells isolated from spleens were stimulated with LPS (1μg/mL) and treated with soluble PHCCC (40μM) or PHCCC NPs (100 μM with respect to drug) synthesized by fixing polymer mass and varying drug input (drug:polymer ratio). Cells treated with equivalent masses of empty NPs were also included as controls. Supernatants were collected and the concentrations of IL-6 (A-C) and IL-10 (D-F) were measured by ELISA at 18 hours (A,D), 44 hours (B,E), and 68 hours (C,F). Samples were prepared in triplicate and data are representative of results from at least 3 similar experiments.

ratio was selected since PHCCC loading was most efficient using this drug input ratio, and minimal background effects were observed from empty particles. In the stabilizer studies, the effect of empty NPs on cytokine secretion was minimized at a PVA concentration of 2% (**Figure 5**). Therefore particles synthesized using these two parameters were chosen for all subsequent studies.

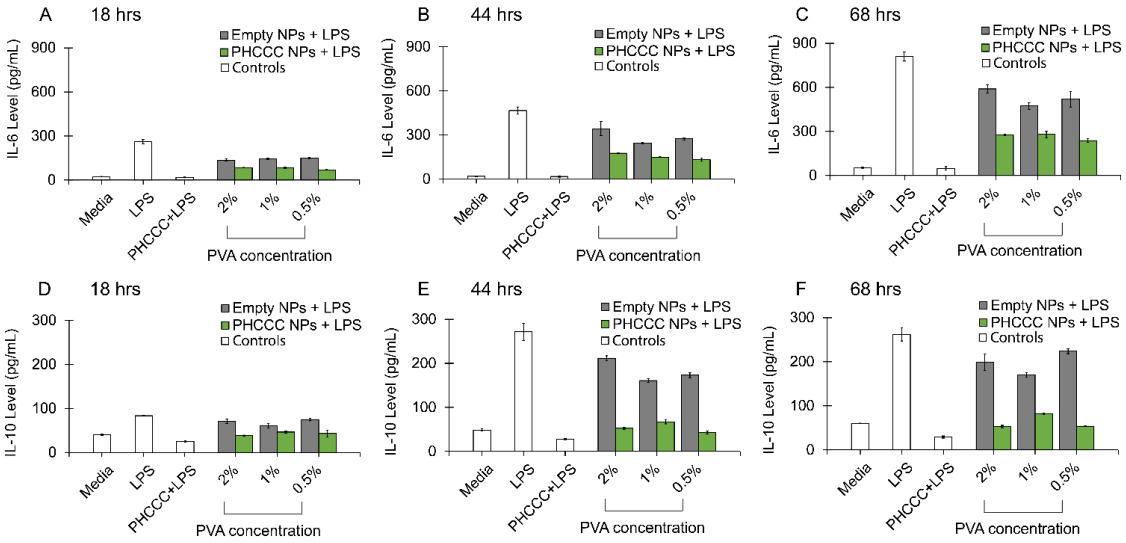

Figure 5. Impact of PVA concentration in PHCCC NPs on DC cytokine secretion. $CD11c^{+}$ splenocytes were stimulated with LPS (1µg/mL) and treated with soluble PHCCC (40µM), PHCCC NPs (100µM) synthesized with different PVA concentrations, or equivalent masses of empty NPs. Supernatants were collected and IL-6 (A-C) and IL-10 (D-F) concentrations were measured by ELISA at 18 hours (A,D), 44 hours (B,E), and 68 hours (C,F).

Using the optimized particle formulation, we next identified a dosing range where PHCCC NPs modulate DC function by stimulating DCs with LPS and treating with a range of PHCCC NP doses. For IL-6, a dose dependent response was observed at each time point, with PHCCC NPs at higher concentrations causing larger

reduction in IL-6 secretion (**Figure 6A**-6**C**). A similar trend was observed at 44 hours and 68 hours for IL-10, but was less clear at the 18 hour time point (**Figure 6D-4F**). Empty NP controls did not significantly suppress IL-6, but caused a modest decrease in IL-10 levels at 44 hours and 68 hours compared with LPS. However, at all three time points PHCCC NPs significantly decreased cytokine secretion compared to empty NPs.

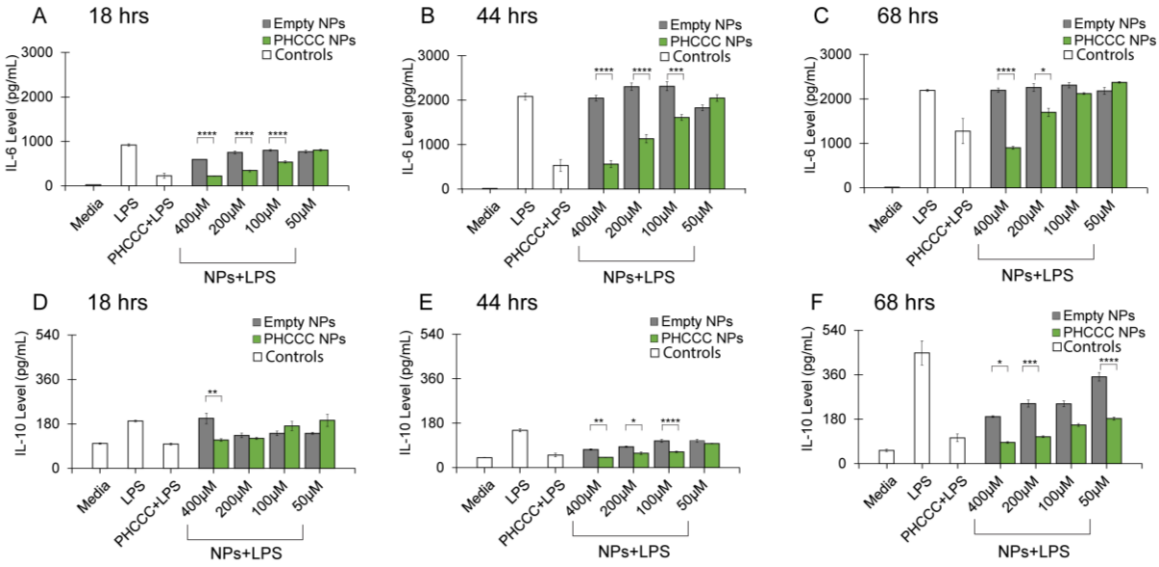

Figure 6. PHCCC NPs alter cytokine secretion by DCs in a dose-dependent manner. $CD11c^{+}$ cells isolated from spleens stimulated with LPS (1μg/mL) were treated with soluble PHCCC (40μM), PHCCC NPs at decreasing doses, or equivalent masses of empty NPs. Supernatants were collected and the concentrations of IL-6 (A-C) and IL-10 (D-F) were measured by ELISA at 18 hours (A,D), 44 hours (B,E), and 68 hours (C,F). Samples were prepared in triplicate and data are representative of results from at least 3 similar experiments.

T cell proliferation and phenotype (e.g., T_H17 vs. T_{REG}) are dependent on the levels and balance of antigen and co-stimulatory signals presented by DCs. To test the hypothesis that PHCCC NPs would decrease stimulatory cues presented by DCs, DCs were stimulated with LPS and left untreated or treated with PHCCC in soluble or NP form. After 18 hours, flow cytometry was used to assess CD40, CD80, and CD86

expression, as well as loading and presentation of a common antigenic peptide by the MHC-I pathway. As shown in **Figure 7A-C** (green bars), PHCCC NPs significantly decreased CD40, CD80 and CD86 expression on DCs relative to DCs cultured only with LPS. These effects were dose dependent and were comparable to the reduction caused by soluble PHCCC. Compared with LPS-treated positive controls, empty NPs did not significantly alter DC activation at any concentration (**Figure 7A-C**, gray bars), though for CD40, a slight reduction in expression was observed at the highest doses.

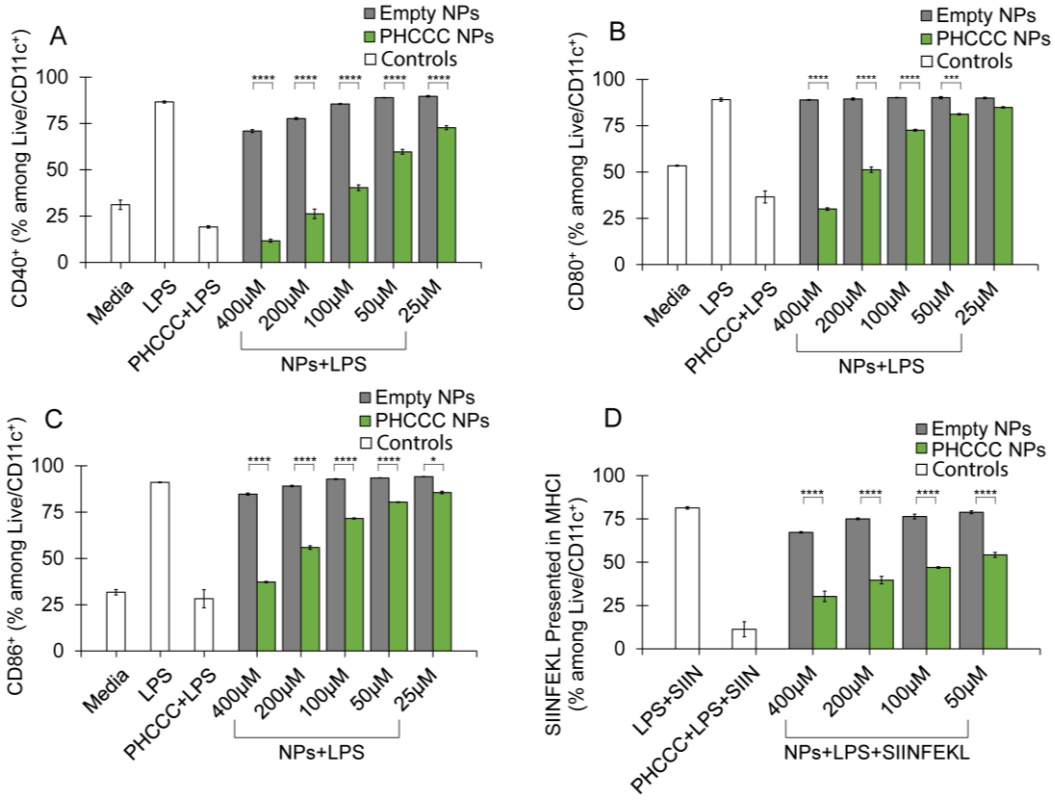

Figure 7. PHCCC NPs reduce DC activation and antigen presentation. $CD11c^+$ splenocytes were stimulated with LPS (1μg/mL) and treated with soluble PHCCC (40μM), PHCCC NPs at decreasing doses, or equivalent masses of empty NPs. After 18 hours cells were collected and the percent of live/$CD11c^+$ cells expressing (A) CD40, (B) CD80, (C) and CD86 were quantified by flow cytometry. (D) $CD11c^+$ splenocytes were stimulated and treated with PHCCC NPs as in (A-C). After 18 hours, SIINFEKL peptide (5μg/mL) was added. Two 2 hours later, flow cytometry was used to analyze cells for the percent of live/$CD11c^+$ cells presenting SIINFEKL in H-2kb. Samples were prepared in triplicate and data are representative of results from at least 3 similar experiments.

To assess the impact of PHCCC NPs on antigen presentation, DCs were incubated with LPS, soluble PHCCC or PHCCC NPs, and SIINFEKL peptide - a common model antigen derived from ovalbumin. After 20 hours, cells were stained with an antibody that binds SIINFEKL only when presented in MHC-I (H-2kb). While 81.3 ± 0.7% of cells treated with LPS and SIINFEKL presented this antigen via MHC-I (**Figure 7D**), treatment with PHCCC NPs reduced SIINFEKL presentation to 30.3% ± 3.0%, depending on NP dose (**Figure 7D**, green bars). The magnitude of this reduction was significant compared with cells lacking PHCCC treatments, but less pronounced than the reduction in SIINFEKL presentation observed in cells treated with soluble PHCCC (11.3 ± 4.3%).

4.4 Polarization of T cells by PHCCC NPs in vitro

As discussed in **Section 1.3**, $CD4^+$ T cells are the main effector cells responsible for the pathology of MS, and the proliferation and differentiation of $CD4^+$ T cells is largely controlled by signals presented by DCs during an interaction with antigen specific T cells. Therefore we investigated whether decreased inflammatory cytokine secretion, activation marker expression and antigen presentation by DCs treated with PHCCC NPs would polarize regulatory T cell responses.

To determine if the effects of PHCCC NPs on DC activation and cytokine profiles alter T cell differentiation, we employed a co-culture model in which DCs from wild-type mice were cultured with splenic $CD4^+$ T cells isolated from transgenic 2D2 mice – a strain in which $CD4^+$ T cell receptors are specific for a myelin autoantigen, MOG peptide (74). DCs were first treated with LPS and MOG, along

with soluble PHCCC, PHCCC NPs, or empty NPs at equivalent polymer masses. After 24 hours, $CD4^+$ 2D2 splenocytes were added to the culture. 72 hours later, cells were collected and T cell proliferation was examined. DCs treated with PHCCC NPs drove proliferation in only 10.8 ± 0.4% of T cells (**Figure 8A,** green bars), a striking decrease compared to 55.7 ± 1.9% and 68.4 ± 0.5% observed in DCs treated with soluble PHCCC or those receiving only the stimulants (i.e., MOG+LPS), respectively (**Figure 8A**). Attenuation of T cell proliferation was dose dependent, but was significant using even at the lowest doses of PHCCC delivered in NP form (25.4 ± 2.2%). Equivalent masses of empty particles did not cause any significant reduction in proliferation compared with cells treated with LPS and MOG. Mean fluorescent intensity (MFI) analysis of these data also confirmed PHCCC NPs significantly reduce proliferation (**Figure 8B**).

Treatment of DCs with PHCCC NPs as in **Section 4.4.2** also polarized T cell phenotype, shifting $CD4^+$ T cells away from inflammatory T_H17 phenotypes ($CD4^+/ROR\gamma^+$) and toward T_{REGS} ($CD4^+/CD25^+/FoxP3^+$) (**Figure 8C, 6D**, green). At the highest dose, T_{REG} levels in wells treated with PHCCC NPs were 5.7 ± 0.5%, compared to 3.3 ± 0.7% in samples treated with MOG and LPS, 4.5 ± 0.6% in samples treated with soluble PHCCC, and 3.6 ± 0.7% in cells treated with equivalent masses of empty particles (**Figure 8C, 6D**). PHCCC NPs also reduced T_H17 levels. In cells treated with MOG and LPS, 82.1 ± 5.3% of $CD4^+$ T cells exhibited a T_H17 phenotype ($CD4^+/ROR\gamma^+$), while soluble PHCCC treatment reduced RORγ frequencies to 67.0 ± 9.9% (**Figure 8E**). PHCCC NPs mediated a dose dependent response, with 58.9 ± 1.9% of cells $CD4^+/ROR\gamma^+$ at the highest particle dose (**Figure**

8E, green). The supernatants from these studies also revealed significant changes in IFNγ production, with PHCCC NPs reducing secretion of IFNγ to 390 ± 77pg/mL compared to 13600 ± 550pg/mL after treatment with MOG and LPS and 8970±1110pg/mL after treatment with soluble PHCCC (**Figure 8F**). Together, these co-culture results indicate that PHCCC NPs reduce antigen specific T cell proliferation and cytokine response, while promoting T_{REGS} and reducing inflammatory (T_H17) phenotypes.

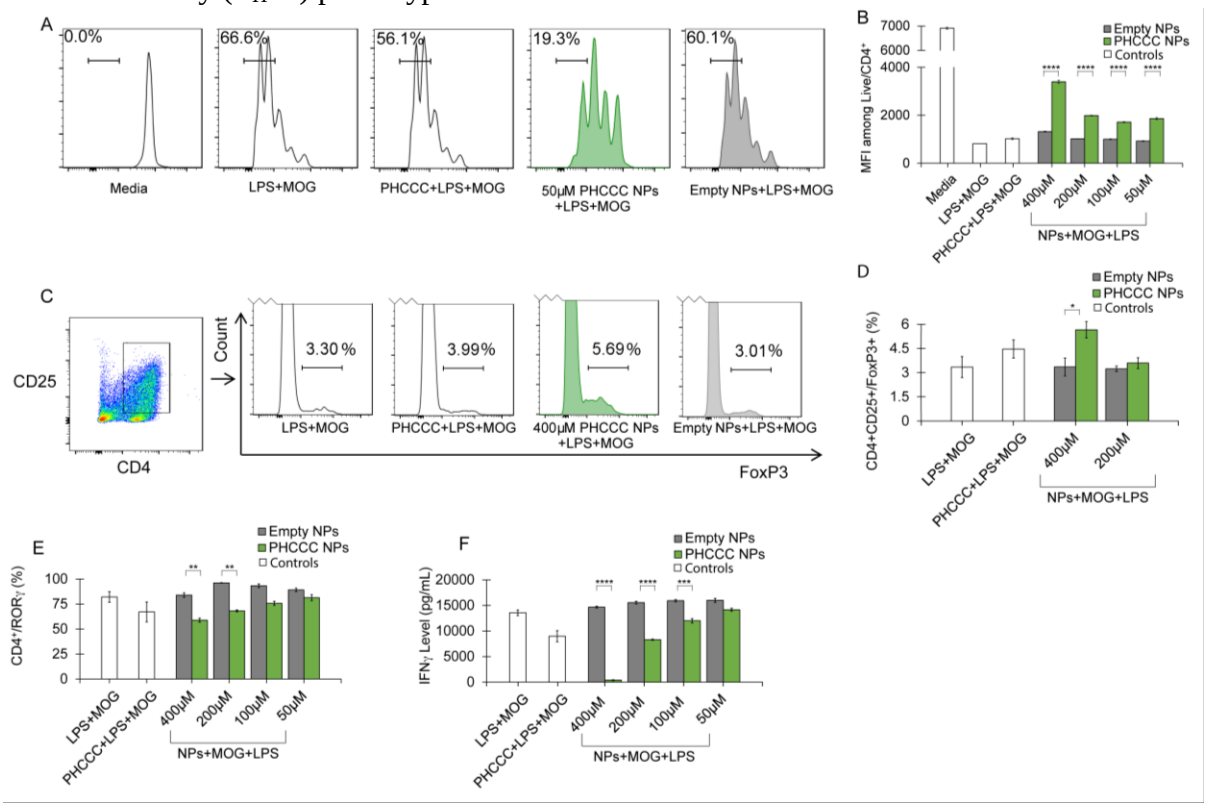

Figure 8. PHCCC NPs restrain T cell proliferation and inflammatory cytokines, promote T_{REGS}, and reduce T_H17 cells. $CD11c^+$ cells isolated from spleens were stimulated with LPS (1μg/mL) in the presence of MOG peptide (10μg/mL), and treated with soluble PHCCC (40μM), PHCCC NPs at decreasing doses, or equivalent masses of empty NPs. After 18 hours, $CD4^+$ splenocytes from 2D2 mice were added to culture. (A) CSFE dilutions of live/$CD4^+$ T cells from representative co-culture samples and (B) mean fluorescent CSFE intensities (MFI). (C) Gating scheme and histograms of FoxP3 expression among $CD4^+$/$CD25^+$ cells (i.e., T_{REG}). (D) Frequencies of T_{REGS} measured for each treatment type. (E) Percentages of $CD4^+$ cells expressing RORγ as an indicator of T_H17 cells. (F) IFNγ in supernatants. For all studies, samples were prepared in triplicate and are representative of at least 3 similar experiments.

4.5 PHCCC NP mediated Inhibition of autoimmune reactions in vivo

We next evaluated whether controlled release of PHCCC from NPs could provide a therapeutic benefit during autoimmunity in mice, but with less frequent or less toxic dosing than required for soluble drug. We tested the effects of PHCCC NPs in the EAE model, which is the gold standard animal model for human MS. EAE is induced by immunizing mice with a high concentration of myelin peptide and a strong inflammatory adjuvant, CFA, resulting in the generation of autoreactive T cells which are reactive against myelin. The blood brain barrier is then opened by injection with pertussis toxin, allowing autoreactive T cells to enter the periphery and attack myelin, causing symptoms of neurodegeneration such as paralysis. A clinical score is assigned to each mouse daily, which is proportional to the severity of symptoms of neuroinflammation. The effectiveness of a therapy for reducing autoimmune reactions can be tested using this model by evaluating the ability of the treatment to inhibit neuroinflammatory symptoms as measured by a reduction in clinical score.

In these studies, mice were induced with EAE, then injected subcutaneously with soluble PHCCC or PHCCC NPs every 5 days starting on the day of EAE induction (day 0) (**Figure 9A**). In this study, mice treated with soluble PHCCC or PHCCC NPs exhibited identical disease progression and severity (**Figure 9B-E**). We next tested a regimen in which mice received soluble PHCCC or PHCCC NPs every three days (**Figure 9F**). During this regimen, PHCCC NPs caused a statistically significant delay in disease onset (**Figure 9G–I**) and decreased disease severity compared to mice treated with soluble PHCCC or untreated mice (**Figure 9J, data**

not shown). In groups treated with soluble PHCCC, 100% of mice developed symptoms by day 13, compared to 50% for groups treated with PHCCC NPs (**Figure 9I**). Additionally, the maximum mean clinical score over the extent of the study was lower in the PHCCC NP treated group, reaching a value of 2.5 compared to a score of 3.2 for mice receiving soluble PHCCC.

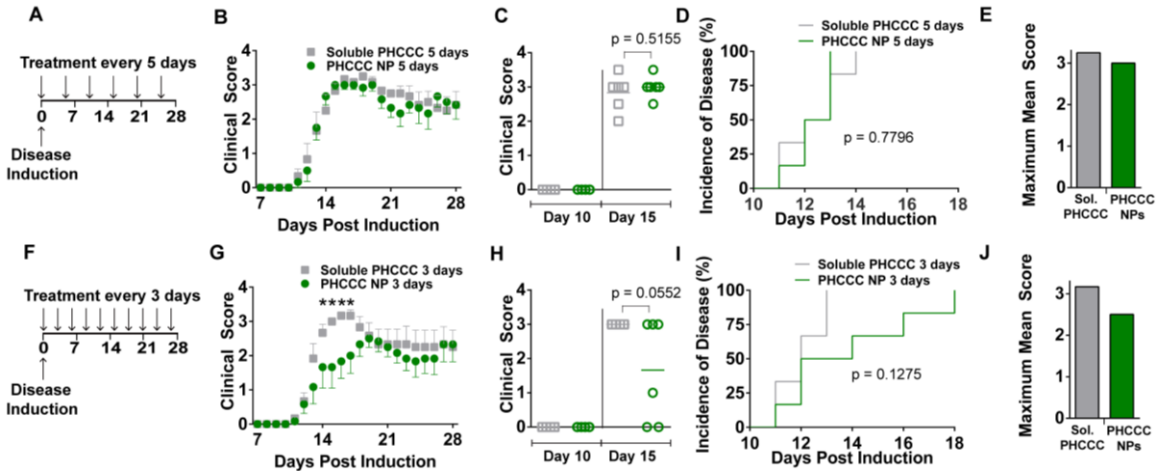

Figure 9. PHCCC NPs delay the onset of EAE and reduce disease severity. Mice were induced with EAE and treated with 3mg/kg soluble PHCCC or PHCCC NPs at (A) five day or (F) three day intervals. Disease pathology was assessed daily using the clinical scale described in the methods. (B) Mean clinical score time course for mice treated using the regimen in (A). (C) Individual clinical scores of mice shown in (B) on day 10 and 15. (D) Incidence of disease (EAE score >0) for the mice in (B). (E) Maximum mean score observed during studies using the regimen in (A). (G) Mean clinical score time course for mice treated using the regimen in (F). (H) Individual clinical scores of mice shown in (G) on day 10 and 15. (I) Incidence of disease (EAE score >0) for the mice in (G). (J) Maximum mean score observed during studies using the regimen in (F). Studies were conducted with groups of 6 mice.

Chapter 5: Discussion[‡]

5.1 Relevance of results for ongoing work in the field

Several recent immunotherapies have explored controlled release to provide better or more targeted control over the function and phenotype of DCs or T cells. Some of the important drugs that have been tested in this area include MPA (75), rapamycin (34, 76), and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (57). The current study is the first investigation of controlled release of small molecule metabolic enhancers to modulate immune cell function and restrain autoimmunity. Since glutamate is expressed at high, toxic levels during autoinflammation (e.g., during MS), increasing the activity of receptors such as mGluR4 that provide a less toxic metabolic pathway to reduce these levels can help address excitotoxicity and neurodegeneration (61). Of even greater relevance to our studies, modulating mGluR4 signaling on DCs can decrease the expression of activation markers and alter cytokine secretion profiles to suppress T_H17-mediated inflammation, while driving T cells toward protective T_{REG} phenotypes (10, 11). Thus, PHCCC has the potential to help address both excitotoxicity (by reducing glutamate levels) and inflammation (by redirecting T cell response). Toward this goal, we investigated the ability of controlled release of PHCCC to improve T cell polarization with less toxic, lower frequency dosing.

5.2 PHCCC NPs reduce toxicity while maintaining immunomodulatory function

During *in vitro* studies, PHCCC NPs exhibited several favorable properties compared with soluble drug. First, PHCCC could be solubilized and slowly released from NPs without need of solvents or other vehicles (e.g., sesame oil). PHCCC delivered in particle form was 36-fold less toxic, with equivalent doses in soluble exhibiting high levels of toxicity compared to PHCCC NPs (**Figure 1F**). Interestingly, PHCCC toxicity was almost completely eliminated when delivered in NP format, approaching the levels measured in unstimulated controls. This observation was true even at the higher concentrations required for PHCCC NPs to match the effectiveness of soluble PHCCC (**Figure 1F**). Although toxicity was absent when cells were treated with PHCCC NPs, clear effects on DCs (e.g., surface markers, cytokine profiles) and T cells were observed, indicating a modulatory function rather than toxicity or cell death.

5.3 Effects of release kinetics on effective dose of PHCCC NPs in vitro

The immunomodulatory effects of soluble PHCCC on DCs were maintained when PHCCC was delivered in NPs, though higher concentrations had to be delivered to achieve the same level of change in cytokine profiles and activation marker expression. This observation may result from the controlled release of PHCCC from NPs. Soluble PHCCC delivery results in immediate availability of all drug, while PHCCC is released more slowly from NPs (**Figure 1C**). DC activation studies lasted

18 hours, and while release studies show that 75% of drug was released over this interval under sink conditions, without the DMSO vehicle used in soluble PHCCC treatments (and media controls), drug release from particles likely achieved a much lower effective solution concentration of free drug in cell culture wells compared to the solution concentration in wells treated with soluble formulations containing DMSO. This idea may also explain why lower concentrations of PHCCC NPs were more effective than soluble drug in promoting T_{REGS}, reducing IFNγ, and limiting T cell proliferation during co-culture studies that continued for 96 hours. For example, 400μM PHCCC NP had a similar effect to 40μM soluble PHCCC on reducing DC activation (**Figure 4A-4C**), while 50μM PHCCC NP caused a larger reduction in T cell proliferation compared to 40μM soluble PHCCC (**Figure 5A,5B**). Soluble drug may be exhausted or degraded over this interval, whereas drug in particles may be more stable. The greater time for metabolism of drug in solution could also allow continued dissolution of PHCCC that is release from particles or that has precipitated.

5.4 Potential benefits from uptake of PHCCC NPs by DCs

Since fluorescent analogs of PHCCC have not been reported, we studied uptake using NPs loaded with a fluorescent lipophilic dye as a model cargo. Our results demonstrate that these NPs are readily internalized by DCs (**Figure 1D,1E**). Thus, in addition to reduced toxicity, NP-mediated delivery may provide a simple method of targeting PHCCC to DCs residing in lymph nodes or spleen since antigen presenting cells are specialized to internalize particulate materials over soluble molecules (18, 19). This possibility is important because DCs present autoantigens to

T cells in lymphoid organs (6, 7, 8). Thus, to polarize T cell function and ameliorate autoimmune responses, it may be beneficial for immunomodulatory drugs acting on DCs to reach the lymphoid organs and persist at sufficient concentrations in these tissues during (self) antigen presentation. Further, soluble delivery results in systemic exposure of drug, while NPs reach lymph nodes through passive drainage and through trafficking by DCs after NP uptake. Using PHCCC NPs that release PHCCC over an extended time could help target LNs and avoid systemic exposure while avoiding continual dosing of soluble drug.

5.5 Observed effects of PLGA NP carriers on DCs and T cells

Although PLGA has traditionally been seen as immunologically inert, recent studies have demonstrated that PLGA can activate DCs and inflammasome pathways, amplify immune responses to toll-like receptor agonists, and cause secretion of inflammatory cytokines (77, 78). Thus, empty NP controls were included in all experiments in order to isolate any intrinsic polymer effects from the encapsulated PHCCC. The highest masses of empty NPs – correlating to the lowest drug to polymer ratios – generally had a slight suppressive effect on cytokine secretion (**Figure 3)**, but minimal effects on DC activation (**Figure 4**) and T cell studies (**Figure 5**). Interestingly, other reports demonstrate a decrease in the expression of DC activation markers when DCs are treated with PLGA particles for 48 hours before stimulation with LPS (79). From a physicochemical standpoint, the slight effects of empty particles observed in our experiments at high concentrations may be driven by acidification of the media from lactic acid byproducts as PLGA degrades (80).

5.6 Expected and observed effects of PHCCC NP modulation of DC cytokine profiles and polarization of T cell phenotype

During adaptive immune response, IL-6 and IL-12 drive proinflammatory function, secreted by DCs to promote T_H17 and T_H1 cells, respectively (81). IL-10 is a polyfunctional cytokine, but often serves as a negative regulator to restrain DC function. Although IL-10 production is a goal of many tolerogenic therapies, PHCCC has been shown to decrease IL-10 production in response to LPS stimulation. The proposed mechanism for this process is drug-mediated reduction of the cAMP triggered by LPS, resulting in a shift away from cytokines that drive T_H17 cells and toward cytokines that promote T_{REG} and T_H1 (10). Therefore PHCCC is a modulator, and not a strict immunosuppressive agent. We observed these effects through a decrease in IL-6 and IL-10 secretion compared to untreated DCs stimulated with LPS (**Figure 2, 3**), but did not observe any differences in IL-12 secretion (data not shown). Interestingly, after LPS stimulation, IL-10 secretion was delayed compared with inflammatory cytokines (**Figure 2D-F, 3D-F**), in agreement with the natural time course for regulation during which IL-10 supports return of immune function to basal homeostatic levels following inflammation or infection events (81).

The effects of PHCCC on DCs described above have an important impact on T cell differentiation and function during autoimmunity. For example, during EAE and MS, disease is driven by subsets of $CD4^+$ helper T cells, but it is unclear whether T_H17 or T_H1 cells induce more severe pathology (81). The Di Marco group has shown that protective effects of PHCCC treatment on EAE coincide with a shift away from T_H17 cells and toward not only T_{REG} but also IFNγ-producing T_H1 cells (10). Our co-

culture studies revealed that treatment with PHCCC NPs shifted T cells away from T_H17 and toward T_{REG} phenotype, while decreasing IFNγ (**Figure 5**). Thus, although we did not observe an increase in T_H1 function (e.g., increased IFNγ), decreased levels of IFNγ associated with delivery of PHCCC NPs could be beneficial in reducing the activity of autoreactive T cells during EAE or other autoimmune diseases (3).

5.7 Effects of release kinetics on in vivo efficacy

In recent studies, soluble PHCCC protected mice from EAE when injected daily in sesame oil as a vehicle. However, symptoms rapidly returned in less than 24 hours when treatment was stopped for even a single day (10). Our studies demonstrate that when PHCCC NPs are administered every three days, these particles delay the onset of EAE and reduce the severity of disease compared with soluble drug (**Figure 6F-J**) or untreated mice **(Figure S3**). In contrast, PHCCC NPs delivered every 5 days had no effect on clinical score compared to untreated mice or mice treated with soluble PHCCC (**Figure 6A-E**). From a pharmacokinetic perspective, one possibility may be that the NPs do not provide a drug concentration in the therapeutic window with 5 day intervals, which based on the release studies (**Figure 1C**), appear to be during a time frame over which the drug release rate is negligible.

Although treatment with PHCCC NPs successfully delayed the onset of neurological symptoms, autoimmunity eventually progressed to an incidence of 100%. This was evident through similar mean clinical scores in both PHCCC NP and soluble groups at the end of the experiment. These results are consistent with findings

from the Di Marco group indicating that PHCCC does not reverse established disease even when soluble PHCCC is administered daily starting one day after the appearance of neurological symptoms (68). Thus, controlled release from PHCCC NPs may initially be sufficient to delay symptoms, but as the concentration of PHCCC wanes over the 3 day interval, myelin-reactive events (e.g., T cell expansion) may increase to cause neurodegeneration that is not reversed even when another dose of PHCCC NPs is administered. This hypothesis could be studied and used to further enhance metabolic control over DC and T cell function by exploring polymeric materials that provide longer term or higher levels of release, through addition of targeting molecules to improve delivery to DCs or lymph nodes, or by inclusion of mGluR4 enhancers with increased potency. An additional strategy to potentially enhance PHCCC mediated protection from autoimmune reactions is to encapsulate and co-deliver myelin with PHCCC in NPs. This may further promote long lived myelin-specific T_{REG} cells which can suppress autoreactive T cells in the periphery, while potentially eliminating the need for continual exposure to PHCCC.

Chapter 6: Conclusions and Future Work[‡]

6.1 Conclusions

In this study we utilized an established nanoprecipitation platform to test the new idea that controlled delivery of a glutamate receptor enhancer could alter immune cell function to improve autoimmune therapy. PHCCC NPs exhibited greatly reduced toxicity, while maintaining the immunomodulatory effects of soluble PHCCC on DCs and T cells *in vitro*. Treatment of mice with PHCCC NPs delayed disease and decreased severity compared to mice treated with soluble PHCCC. More broadly, this report demonstrates that controlled delivery of metabolic modulators that alter immune cell signaling could contribute to new therapeutic options for autoimmunity or inflammatory disease.

6.2 Future Work

6.2.1 Investigating the mechanism of efficacy through metabolic signaling

The work described in **Section 4.3** and **Section 4.4** demonstrated that PHCCC NPs restrained autoimmune reactions by modulating DCs, which in turn polarized a regulatory T cell response. PHCCC NPs inhibited inflammatory cytokine secretion and expression of activation markers mediated by stimulation with the inflammatory signal, LPS, which is a TLR4 agonist. This investigation did not investigate the signaling pathways involved in the effects of the PHCCC NPs. PHCCC is a positive allosteric modulator of mGluR4, which enhances the glutamate signaling through

mGluR4 (62). The proposed mechanism of action is through the mGluR4 signaling-mediated inhibition of cAMP formulation; cAMP is a secondary messenger involved in TLR signaling that is normally upregulated in response to LPS (62). However this study did not directly confirm modulation of the mGluR4 pathway on DCs.

Toward this goal, preliminary studies were done utilizing 8-bromo-cAMP, which is a membrane permeable cAMP analogue. These *in vitro* studies aimed to determine if addition of 8-bromo-cAMP to LPS-stimulated DCs would inhibit the effects of PHCCC treatment. This would be expected since 8-bromo-cAMP can enter the cell and act in place of cAMP as a secondary messenger, bypassing the effects of inhibited cAMP formulation from PHCCC treatment. This mechanism was probed with an initial assay quantifying the ability of PHCCC to inhibit IL-6 secretion of LPS stimulated DCs with or without the presence of 8-bromo-cAMP, using ELISA. DCs stimulated with LPS secreted 1516.2 ± 132.2 pg/mL of IL-6, while soluble PHCCC treatment reduced IL-6 secretion to 1009.5 ± 113.5 pg/mL. Addition of 8-bromo-cAMP inhibited the effects of PHCCC treatment, as samples stimulated with LPS and treated with PHCCC and 8-bromo cAMP secreted levels of IL-6 similar to the LPS stimulated control (**Figure 10A)**.

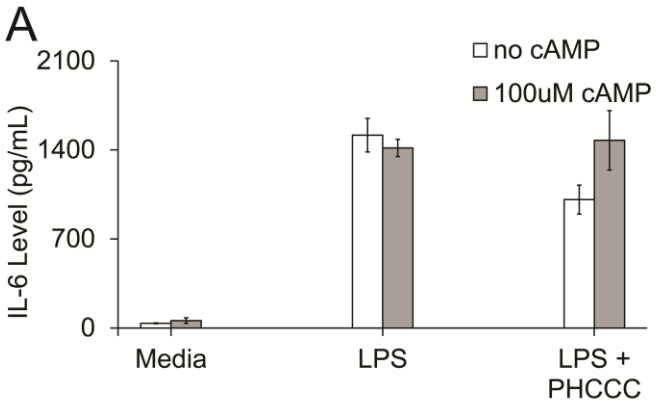

Figure 10. PHCCC signaling in the presence of 8-bromo-cAMP. $CD11c^+$ cells were isolated from spleens, stimulated with LPS (1µg/mL) and treated with soluble PHCCC with or without the addition of 8-br-cAMP (100uM). Supernatants were collected and the concentrations of IL-6 were measured by ELISA at 48 hours

This study was repeated with the inclusion of samples treated with PHCCC NPs or equivalent masses of empty NPs with or without the addition of 8-bromo-cAMP. After 48 hrs of cultures, LPS stimulated DCs secreted 2197.1 ± 108.8 pg/mL of IL-6, and PHCCC NP treatment reduced IL-6 levels to 1667.7 ± 94.5 pg/mL **(Figure 11A)**. Addition of 8-bromo-cAMP inhibited the effects of PHCCC NPs, as samples treated with PHCCC NPs in the presences of 8-bromo-cAMP secreted a similar level of IL-6 compared to the LPS control **(Figure 11A)**.

Additional studies may also use ELISA to investigate total levels of cAMP formulated by DCs after stimulation with LPS with or without PHCCC NP treatment, to confirm that PHCCC NP activity is a result of the inhibition of cAMP formulation. Finally, studies investigating whether PHCCC NPs affect the expression of mGluR4 may be done using western blot.

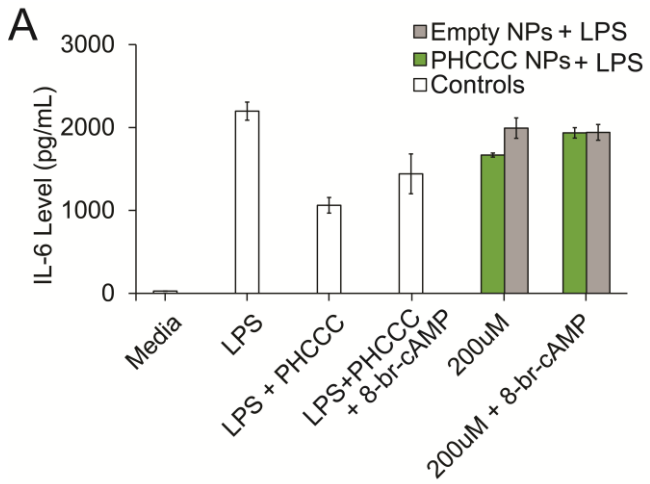

Figure 11. PHCCC NP signaling in the presence of 8-bromo-cAMP. $CD11c^{+}$ cells were isolated from spleens, stimulated with LPS (1µg/mL), treated with soluble PHCCC, PHCCC NP(200µM), or equivalent masses of empty NPs with or without the addition of 8-br-cAMP (100uM). Supernatants were collected and the concentrations of IL-6 were measured by ELISA at 48 hours

6.2.2 Enhancing the degree of tolerance using other biomaterial carriers

Although treatment with PHCCC NPs inhibited the onset of symptoms of neuroinflammation in mice induced with EAE, the protective effects were only achieved when PHCCC NPs were administered every 3 days. A potential strategy to try to enhance the protective effects of PHCCC with even further reduction in treatment frequency may be to utilize alternate biomaterial delivery vehicles to tune the release kinetics of the drug. One hypothesis is that controlled delivery of PHCCC with a more gradual and extended rate of drug release may allow for enhanced efficacy with reduced treatment intervals. Polymers with higher hydrophobicities, such as polycaprolactone or PLGA with higher lactide ratios, generally allow for slower release kinetics of encapsulated cargos, and may be investigated to this end. Alternatively, liposomes have been widely used in the delivery of therapeutics and have been clinically approved as biomaterial carriers for a variety of drug delivery

applications (82). Liposomes can encapsulate hydrophobic small molecules, can be PEGylated for enhanced circulation time and retention in the lymph nodes, and have the advantage of being easily formulated at a variety of sizes from the order of 20nm to the µm range. Additionally, liposomes can easily be functionalized with a variety of targeting ligands (83).

Preliminary studies have been conducted investigating PEGylated liposomes as a potential carrier for the controlled delivery of PHCCC. Liposomes consisted of varying molar ratios of cholesterol, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC) and poly-ethylene glycol conjugated 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000 (DSPE-PEG). Liposomes were formulated by dissolving and drying lipids and PHCCC in chloroform. Lipid films with PHCCC were then hydrated in PBS, and were subjected to sonication and multiple extrusions through a 200µm membrane. Liposomes encapsulating PHCCC were successfully synthesized using 5, 10 and 15 molar percentages of PEG, and a trend showing increased PHCCC loading with increasing molar ratios of DSPE-PEG was observed. PHCCC Liposomes formulated with 15 % DSPE-PEG successful encapsulated 55.3±11 µg PHCCC/mg of liposome **(Table 2)**.

Table 2. Properties of PHCCC liposomes synthesized with different PEG percentages

PEG input (Molar %)	PHCCC input (mg)	Loading Level (µg PHCCC/mg liposome)	Diameter (nm)
15	1	55.3 ± 12.0	126 ± 12.0
10	1	43.5 ± 7.8	132 ± 12.7
5	1	19.7 ± 11.6	145 ± 6.4

Treatment of DCs with PHCCC liposomes after stimulation with LPS for 48 hrs resulted in reduced IL-6 secretion as measured by ELISA. DCs stimulated with LPS and treated with 400µm PHCCC liposomes secreted 239.4 ± 7.0 pg/mL IL-6, compared to 736.9 ± 19.0 pg/mL for untreated LPS stimulated DCs and 1193.2 ± 33.0 pg/mL for LPS stimulated DCs treated with an equivalent mass of empty liposomes **(Figure 12A)**. Future work on this project will investigate effects of PHCCC liposomes on DC activation as well as the ability of PHCCC liposome treated DCs on the generation of a suppressive T cell response.

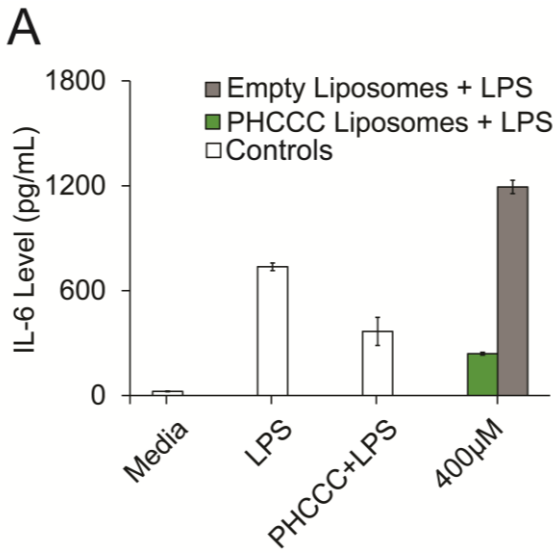

Figure 12. PHCCC Liposomes inhibit inflammatory cytokine secretion by DCs. $CD11c^{+}$ cells were isolated from spleens, stimulated with LPS (1µg/mL), treated with soluble PHCCC, PHCCC Liposomes(400µM), or equivalent masses of empty Liposomes). Supernatants were collected and the concentrations of IL-6 were measured by ELISA at 48 hours

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