HYDROGEL-BASED VIABILITY AND MIGRATION STUDIES OF DENDRITIC CELLS FOR IMMUNOTHERAPEUTIC APPLICATIONS

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Advika Srinivasan

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HYDROGEL-BASED VIABILITY AND MIGRATION STUDIES OF DENDRITIC CELLS FOR IMMUNOTHERAPEUTIC APPLICATIONS

Approved by:

Dr. Julia E. Babensee, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Marian Ackun-Farmmer
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Leslie Chan
School of Biomedical Engineering
Georgia Institute of Technology

Date Approved: [April 29, 2025]

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen-presenting cell
BSA	Bovine serum albumin
cDC	Conventional dendritic cell
cLNs	Cervical lymph nodes
CNS	Central nervous system
DC10	Interleukin-10-treated dendritic cell
DCs	Dendritic cells
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FMO	Fluorescence minus one
FSC	Forward Scatter
G’	Storage modulus
G’’	Loss modulus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank’s Buffered Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS-IL-10	interleukin-10 with free thiol
IACUC	Institutional Animal Care and Use Committee
iDC	Immature dendritic cell
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-4	Interleukin-4
LN	Lymph node

LPS	Lipopolysaccharide
mDC	Mature dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple sclerosis
PBS	Phosphate buffered saline
PEG	Poly (ethylene glycol)
PEG-4MAL	Maleimide-functionalized 4-arm poly (ethylene glycol)
PEG-IL-10	PEG-4MAL-bound-IL-10
PEG-4MAL:HS-IL-10	PEG-4MAL-bound with IL-10 with free thiol
PGN	Peptidoglycan
PI	Propidium iodide
RBC	Red blood cell
RGD	GRGDSPC peptide
SSC	Side scatter
STIM-DC	Stimulated dendritic cell

T1D	Type 1 diabetes
TGF-β	Transforming growth factor-β
TNF-γ	Tumor necrosis factor-α
VD3	1,25-dihydroxyvitamin D3
VPM	GCRDVPMSMRGGDRCG peptide
σ	Standard deviation

SUMMARY

Over the years, seminal research in biomaterials and immunoengineering has enabled the development of advanced therapies to treat cancers, autoimmune diseases, and other possible immune-related pathologies. Dendritic cells (DCs) have emerged as a key focus in pharmaceutical and biological therapeutics due to their ability to bridge the innate and adaptive immunity. More recently, DCs have successfully utilized as cell therapies for transplantation, vaccines, tumor clearance and autoimmunity due to their role in the initiation of adaptive immunity. The discovery of the Biomaterial Adjuvant Effect by Dr. Julia Babensee has opened new avenues of research in the use of biomaterials as stimulators of autoimmunity where the primary focus lies on biomaterials that promote tolerance. While there is a lot of focus in the development of biomaterials for immunostimulatory applications such as vaccines and cancer, there has been far less effort on biomaterials that promote tolerance and regulatory mechanisms.

Therefore, this study investigates the immunomodulatory potential of DCs for disease severity attenuation using a murine experimental autoimmune encephalomyelitis (EAE) preclinical Multiple Sclerosis (MS) model. Dendritic cells treated with interleukin-10 (IL-10) referred as DC10s were transplanted using *in situ* gelling poly (ethylene glycol)- 4 arms maleimide (PEG-4MAL) hydrogels conjugated with the immunosuppressive cytokine, interleukin, IL-10, for target site localization. Poly(ethylene glycol) (PEG) was selected for this study due to its unique attributes, particularly its branched macromer precursor structure, where each arm is conjugated to a maleimide group (PEG-4MAL) and enables the bioconjugation of crosslinkers, adhesive ligands, growth factors and cytokines. The

PEG-4MAL hydrogel provides a protective environment for DCs by shielding them from inflammatory conditions *in vivo* and enhancing their viability post-delivery.

Additionally, the study also investigates the viability and migratory potential of DC10s embedded in the hydrogel system. The viability and migratory status of the hydrogel-delivered DCs will be an important determining factor in the mode by which they exert their immunomodulatory effect. This research demonstrates the success of the biomaterial to promote tolerogenic processes *in vivo*, thereby attenuating disease progression in a preclinical model of MS.

CHAPTER 1. INTRODUCTION

Multiple Sclerosis is an autoimmune disease characterized by the destruction of myelin in the central nervous system, resulting in a variety of symptoms such as vision impairment, muscle weakness, vertigo, memory deficits, and mood changes, eventually resulting in paralysis [1]. An estimated 2.8 million people worldwide are living with MS, with prevalence continuing to rise and incidence predominantly higher in women [2]. Clinically active and progressive MS is treated with drugs such as IFNβ-1α, glatiramer acetate, teriflunomide, dimethyl fumarate, fingolimod, mitoxantrone, alemtuzumab, and natalizumab, Ocrelizumab, Ofatumumab [3]. While these drugs slow MS progression by serving as an immune system decoy, they only reduce the symptom severity and disease progression and are associated with increased risks of infection, depression, and organ damage [4].

Dendritic cells are antigen presenting cells that bridge the innate and adaptive immune system, and they direct antigen-specific immune responses [5]. Given this unique ability, antigen-specific DC-based immunotherapies are being considered to replace immunosuppressive drug therapies to reduce patient risks and maximize therapeutic outcomes by promoting tolerance towards MS-disease associated antigens [6]. Tolerogenic DCs (tolDCs) are known immunomodulators which recruit lymphocytes, to therapeutically treat the progression of MS [7][8]. In cell therapy, DCs have a high efficacy especially when being used for cell transplantation, vaccines, tumor clearance, and autoimmunity. Research in biomedical adjuvant effect with DCs has been pioneered by Dr. Julia Babensee

and elicited subsequent research interests by other investigators in the use of biomaterials for immunostimulatory applications such as vaccine and cancer. However, there has been far less focus on biomaterials that promote tolerance and regulatory mechanisms. Additionally, the efficacy of cell-only therapeutic strategies is constrained by lack of localization to target tissues, due to the limited survival and migratory potential of DCs following systemic delivery [9].

This research thesis therefore sought to contribute towards the ongoing research in the Babensee laboratory, which focuses on developing a biomaterial system to ameliorate autoimmunity by providing an immunomodulatory environment. Here we treat DCs with interleukin-10 (IL-10) (DC10s) and transplant them into a maleimide functionalized 4-arm poly(ethylene glycol) (PEG- 4MAL) hydrogel with thiolated IL-10, which can be used for immune suppressive or tolerogenic applications through the effects on T cells [10]. This biomaterial platform can be utilized to investigate the inhibition of neuroinflammation and to improve immunosuppressive DC therapy for MS through targeted delivery and precise regulation of immune cell phenotypes.

An Encephalomyelitis Autoimmune Experimental (EAE) model was utilized to evaluate the success of the biomaterial system in an autoimmune situation. One of the most commonly used experimental models for MS is EAE due to the similarity in pathogenesis and key factors such as neuroinflammation, peripheral inflammatory infiltrates, demyelination in white matter, and axonal injury and axon degeneration [11]. Many drugs that are currently in use for MS many of the drugs that are in current or imminent use in MS have been developed, tested or validated on the basis of EAE studies [12].

1.1 Research Significance and Innovation

The research utilizes a tunable and multifunctional biomaterial for tolerance induction and immunomodulation by applying immunological and neuropathological concepts. The innovation lies in the design of the biomaterial due to the incorporation of IL10 and use of this platform for delivery of DC10s for induction of tolerance to disease relevant antigens in the EAE model. TolDCs will modulate autoimmune mechanisms by conditioning cognate T cells and other DCs recruited to the gel and injection site at the cervical lymph nodes [13]. It has been reported that the FDA approved PEG does not result in any local or systemic toxicity after degradation along with minimal inflammation, and 96% urine clearance in approximately 24 hours [14]. In general, this tolerogenic biomaterial system serves as a model for cell-delivery scaffolds in immunosuppressive applications, including islet transplantation for the treatment of type 1 diabetes (T1D) and other autoimmune diseases.

CHAPTER 2. SPECIFIC AIMS

2.1 Specific Aim 1

Assess the immunosuppressive nature and viability of DCs embedded in PEG-4MAL: HS-IL- 10 (PEG-4MAL-bound with IL-10 with free thiol) to ameliorate symptoms in a mouse model of MS.

2.1.1 Significance and Approach

This aim focuses on confirming the benefits of biomaterial delivery *in vivo*, which is essential for advancing the clinical translation of DC therapies. Bone marrow-derived dendritic cells were cultured with different treatments at Georgia Tech, as access to laboratory space at Augusta University was limited. The EAE model was induced at Augusta University in collaboration with the Narayanan laboratory, due to their expertise in EAE induction, clinical scoring, and animal care. On day 7 of the DC culture, the cells were transported in a Darwin Chamber which acts as a temperature incubator and maintains 37 °C but does not maintain CO_2 concentration. Experiments first assesses DC viability post-transport to Augusta University, and these results were compared to flow cytometry data obtained prior to transport at Georgia Tech. Following this, DC10s embedded within the biomaterial system were subcutaneously delivered, and their therapeutic potential in ameliorating autoimmune disease was evaluated using the EAE model. These experiments illustrate the importance of viability, targeted delivery and post injection cell support provided by our immunosuppressive biomaterial niche. For each treatment group, clinical scores and body weight were monitored daily over a 28-day period by the Research

Professional at Augusta University, Dr. Liu Fang. Processing immunological and disease relevant tissues such as the lymph node and spleen from EAE mice will provide insights into the mechanism of tolerance that leads to the amelioration of symptoms. We hypothesize that compared to DCs inoculated in suspension, the hydrogel delivered DC10s will significantly improve the EAE scores through the induction of regulatory cells.

2.2 Specific Aim 2

Evaluate the viability of different treatment groups of DCs embedded in PEG-4MAL bound with IL-10 with free thiol at different time points through an *in vitro* study.

2.2.1 Significance and Approach

For the proposed treatment to effectively ameliorate EAE symptoms, it is essential that DCs maintain viability within the biomaterial system following encapsulation, as their presence is critical for promoting immune tolerance and driving therapeutic outcomes. This study aims to evaluate the viability of different treatment groups of DCs embedded in PEG-4MAL:HS-IL-10 hydrogels at multiple time points through an *in vitro* assay. On day 6 of culture, DCs were treated with immunomodulatory agents such as LPS or IL-10 to induce distinct phenotypic profiles, followed by embedding in the hydrogel on day 7 in a 96-well plate format. Viability was assessed through flow cytometry and metabolic activity using the CCK-8 viability assay. This experiment provided insights into the critical early phase post-delivery and would optimize this cell-biomaterial platform for tolerogenic DC therapy in the EAE model. We hypothesize that the DCs embedded in the hydrogel will maintain

both their viability and their induced immunosuppressive phenotype over time proving that the three-dimensional microenvironment supports cell survival.

2.3 Specific Aim 3

Optimize treatment conditions for generating DCs with an immunosuppressive phenotype, distinct migratory capacities and investigate the effect of functional attributes on the therapeutic efficacy of DC delivery using a biomaterial-based system.

2.3.1 Significance and Approach

The objective of this aim is to evaluate the impact of immunosuppressive treatments (IL-10, vitamin D3 (VD3), or dexamethasone (DEX)) followed in some cases with a maturation stimulus (peptidyl glycan or lipopolysaccharide) on the generation of DCs with defined immunosuppressive and migratory versus non-migratory phenotypes. Previous work in the laboratory has focused on optimizing material properties such as degradation kinetics, stiffness, and degradability along with investigating the role of biomaterials as modulators of DC function. These studies have produced promising results in the development of a biomaterial system for DC immunosuppression and function. The experiments outlined in this aim build upon these preliminary studies, to assess the migration of DCs, immunomodulatory and maturation stimulus, and hydrogel release rates. These results will elucidate the migratory abilities of DCs, and the efficacy of DCs to exert immunomodulatory ability directly on host cells. We hypothesize that immature dendritic cells (iDCs) will exhibit limited migratory capacity, whereas DCs treated with maturation stimulus will migrate at a higher rate through the membrane.

CHAPTER 3. LITERATURE REVIEW

3.1 The Role of Dendritic Cells in Immune Responses

3.1.1 Adaptive Immune Responses

The discovery of DCs was credited to Ralph Steinman in 1973 when he discovered a rare cell type in murine spleen cells which was phagocytic with an unprecedented ability to activate naive T cells [14]. They were named after their dendritic extensions, which creates a greater cell surface area to constantly sample antigens. Dendritic Cells are the most efficient, potent, and professional antigen-presenting cells (APCs) of the immune system, whose primary role is to phagocytose antigens, process and present antigen fragments on major histocompatibility complexes (MHCs) to condition other immune cells [15]. Dendritic Cells recognize foreign antigens through specialized receptors on their surface known as "pattern-recognition receptors" such as TLR, C-type lectins (CLR), and intracellular helicases such as retinoic acid-inducible gene-I (RIGI) [16].

They are the primary instigators of adaptive immunity beginning with the activation of naïve T-cells, guiding T cells to become specialized subtypes such as helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$). Helper T cells activate other immune cells, while cytotoxic T cells target infected or cancerous cells. At the same time, B cells recognize antigens directly or through T cell help, becoming antibody-producing plasma cells [17]. Macrophages, B cells, and DCs are all APC, but DCs are consider the more efficient as they are efficient at both processing and presenting exogenous antigens on both MHC I

and MHCII molecules to naïve CD8+ and CD4+ T cells, which initiates the adaptive immune response [18]. Therefore, DCs are important to downstream immune responses for effective immunity in response to antigens or homeostatic tolerance.

3.1.2 Development and Function of Dendritic Cells

Dendritic Cells are referred to as antigen presenting cells that have essential mediators of immunity and tolerance. The derivation of DCs starts from pluripotent hematopoietic stem cells (HSCs) of the bone marrow, which differentiates into conventional/classical DC (cDC) or plasmacytoid DC (pDC) progenitors that circulate around the body. The monocyte and dendritic cell progenitor (MDP) give rise to monocytes and committed DC progenitors (CDPs) in the bone marrow. The CDPs give rise to pre-DCs, which migrate to the lymphoid and non-lymphoid tissues after traveling from the bone-marrow [19]. The life cycle of DCs include differentiation into immature DCs, infiltration into peripheral tissues, sampling the environment for antigen, phenotypic maturation in response to a pathogen, migration to proximal LNs and conditioning effector cells until cell death. Dendritic Cells have an extremely limited ability to proliferate, and it has been estimated that the lifespan of DCs is of 2 weeks once reaching their fully differentiated state [20].

Dendritic cells act as the bridge between innate and adaptive immunity. In their immature state, DCs patrol tissues, collecting antigens via pattern recognition receptors. Upon encountering foreign material, they mature with an increase in MHC and co-stimulatory molecule expression [21]. They also contribute to immune tolerance by promoting regulatory T cell development and preventing harmful reactions against the body's own

tissues [22][23]. Dendritic Cells are directly involved in vaccinations and immunotherapies due to their plastic and robust nature, making them a critical target for both immunoactivation [24] and immunosuppression [25].

3.2 Dendritic Cell Responses to Biomaterial Systems

3.2.1 Biomaterial Adjuvant Effects and Receptor Signalling

Recent advances in biomedical research have been key in the field of tissue engineering and regenerative medicine, with several innovative applications already translated into clinical use. Biomaterials are an essential consideration to achieve optimal effectiveness of immunotherapies through immunomodulation strategies leveraging immune cell-biomaterial interactions [26][27]. Dendritic cells can modify their immune function based on the direct recognition of PAMPs present on or associated with biomaterials via PRRs, which can drive immunoactivation, a response that is highly desirable in vaccine delivery platforms but unfavorable in transplant settings [28][29]. Work in the field has shown that certain biomaterials can act like immune adjuvants presumably by mimicking molecular signals associated with pathogens or damaged tissue or presenting endogenous TLR ligands adsorbed on the biomaterial surface. These materials activate DCs using receptors such as Toll-like receptors, C-type lectin receptors, and the cGAS–STING pathway receptors—prompting DC maturation and cytokine secretion [30]. Materials such as poly(lactic-co-glycolic acid) (PLGA) trigger these pathways through both their surface interactions and internalization. Damage-associated molecular patterns (DAMP) from injured tissue can further amplify immune signaling. Such DAMPs have also been

observed as adsorbed to the biomaterial surface to mediate inflammatory cell interactions [31]. For cancer therapies, activating the cGAS–STING axis is especially valuable, as it enhances $CD8^+$ T cell responses via increased type I interferon production [32]. Since there are far fewer studies investigating tolerance inducing biomaterials, the success of the modular PEG-4MAL biomaterial system which can be engineered to promote tolerance highlights its potential as a versatile platform for maintaining homeostatic immunity in autoimmune disease therapies.

3.2.2 Effect of Biomaterial Mechanical Characteristics on Embedded Cells

Biomaterial selection criteria and scaffold design parameters must be carefully considered since they can promote an anti-inflammatory response or stimulate inflammation [33]. Since the selection of biomaterials can significantly influence DC maturation, with factors such as polymer size, biomaterial shape, and surface charge playing critical roles, due to sensitivity to their physical environment [34][35]. Recent studies have demonstrated the ability of both natural (chitosan, alginate, gelatin) and synthetic (poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG)) biomaterials to modulate immune responses for various immunomodulatory applications.

Dendritic cells exposed to dynamic environments adjust their structure, gene expression, and interactions. This sensitivity allows biomaterials to be designed in a way that guides cell function and healing [36][37]. Mechanical stimulation of DCs leads to increased expression of co-stimulatory molecules such as CD86 and cell death [38]. Physical disturbances to culture plates, and agitation of hydrogel embedded DCs can result in DCs

with activated phenotypes similar to our mature controls. The biophysical and biochemical properties of the biomaterial microenvironment, particularly those mimicking the extracellular matrix (ECM), are essential in shaping cellular behavior - differentiation, viability, morphology, and immunological function through interactions such as cell attachment and spreading [39]. The PEG-4MAL hydrogel used in this study was selected for its unique capacity to be engineered towards promoting immune tolerance, while remaining non-activating to immune cells, which is crucial for tolerogenic DC (tolDC) delivery.

3.3 Biomaterials for Dendritic Cell Delivery

Biomaterials have revolutionized DC-based immunotherapies due to their advantages over traditional methods of delivery since they can be engineered to deliver payloads by modulating particle size, shape, hydrophobicity, degradability, charge and other factors. Biomaterials also have a major role in the design of controlled release systems for the delivery of biomolecules through encapsulation of biological compounds [40].

Conjugation of biomolecules to biomaterials enables control over the rate and location of release in response to various stimuli. Diverse platforms such as nanoparticles, liposomes, and dendrimers can deliver antigens and stimulatory molecules directly to DCs, improving antigen uptake and presentation which is important for cancer vaccines and antiviral responses [41][42].

Injectable scaffolds recruit DCs to the delivery site and establish a localized immune-activating environment through the sustained release of chemokines and antigens create an

immune-activating environment [43][44]. In particular, hydrogel systems have significantly improved the effectiveness of cell therapies and are important in a regenerative capacity since the polymer matrix can serve as a temporary ECM to be remodeled by infiltrating cells in a tissue specific manner while also supporting cell growth and vascularization.

The ability to engineer the biomaterial system, biocompatibility, controlled release and ease of use makes the PEG hydrogel superior to other biomaterials. The hydrogel system has been previously modulated to use with other varieties of cells such as Islet of Langerhans, cardiac progenitors, and skeletal muscle satellite cells [45][46][47]. The PEG-4MAL hydrogel system offers a highly suitable environment for DC delivery, as its tunable mesh size and non-activating properties can maintain the desired DC phenotype while supporting cell viability and facilitating local immune modulation. The ability to engineer the biomaterial system, biocompatibility, controlled release and ease of use makes the PEG hydrogel superior to other biomaterials.

3.4 Dendritic Cell Viability in Biomaterial Systems

Studies on DC-mediated immunity toward biomaterials center on their adjuvant effects in facilitating the adaptive immunity of co-delivered antigens due to their role in innate and adaptive immunity [48]. The effectiveness of DC-based therapies depends on keeping the cells alive and functional within the biomaterial. Chitosan and PLGA are some materials that stimulate DCs, while softer materials such as hyaluronic acid or agarose gels help maintain them in a resting, long-lived state [49][50].

Material flexibility and surface design also influence cell survival. Hydrogels that relax under stress can support DC function and reduce inflammation. Similarly, minimizing protein adsorption on surfaces can prevent unwanted immune reactions [35][51]. Tuning the material to create an environment supportive of cell survival makes PEG hydrogel a highly suitable biomaterial for DC delivery. Ensuring DC viability throughout the treatment course is essential since their ability to exert either pro-inflammatory or immunomodulatory effects depending on the therapeutic goal is linked to their survival and functional integrity within the host environment. For tolerogenic therapies of autoimmune diseases, PEG hydrogel can also be modified with bioactive cues in the form of thiol-conjugated cytokines such as IL-10) or adhesion peptides which further support DC survival and help maintain their immature or tolerogenic phenotype post-implantation [52][53]. Therefore, a biomaterial that enables both the survival and the functional performance of DCs throughout the treatment course is essential for long-term therapeutic efficacy.

3.5 Migration of Dendritic Cells

Dendritic cells exhibit distinct migratory capacities depending on their activation status to initiate an immune response. While iDCs display limited migratory ability and are primarily specialized in sampling both self and foreign antigens, mature DCs (mDCs) upregulate surface molecules essential for migration and antigen presentation, leading to significantly enhanced migratory capabilities [54]. A key mechanism driving DC migration is the interaction between the chemokine receptor CCR7, expressed on mature DCs, and its ligands CCL21 and CCL19, which are found on lymphatic endothelial cells and

lymphoid tissues [55][56][57]. This interaction directs DCs into the lymphatic vessels via a process known as chemotaxis, allowing them to reach draining lymph nodes for T cell priming [58].

Active immunosuppressive and migratory DCs can also be obtained through a TLR ligand post treatment with immunomodulatory agents and this approach has been associated with enhanced *in vivo* therapeutic performance, likely due to improved lymph node homing capacity [59][60][61]. TLR agonists such as lipopolysaccharide (LPS) and peptidoglycan (PGN) increase CCR7 expression on DCs, thereby promoting their migration to lymph nodes, while immunosuppressive cytokines such as TGF-β have been shown to reduce CCR7 expression and inhibit DC migration to lymph nodes [62]. Recent studies show that DC migration also follows daily rhythms and responds to physical cues in the tissue, such as stiffness. These insights offer new opportunities to control immune responses by tuning the mechanical and temporal cues in biomaterials [63][64].

3.6 Experimental Autoimmune Encephalitis (EAE) Model used to demonstrate human Multiple Sclerosis

Experimental autoimmune encephalomyelitis is a well-established animal model used to mimic the inflammation and nerve damage seen in MS. Experimental autoimmune encephalomyelitis is a complex condition in which the interaction between a variety of immunopathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss and gliosis. The use of complete Freund's adjuvant and myelin oligodendrocyte glycoprotein MOG35–55

peptide introduces highly reproducible EAE in mice, where a pertussis toxin treatment increases the blood brain barrier [65][11]. The pathological hallmark in these models is the appearance of large confluent plaques of primary demyelination with partial axonal sparing, similar to the lesions seen in MS patients [66][67].

Other studies have used the EAE model to study the role of the sympathetic nervous system, or to optimize cell-based therapies shown to restore homeostasis in MS patients [54][58]. Dendritic cells are central to this model since they present myelin antigens to naïve T cells, driving them to become harmful T helper 1 and T helper 17 cells. These activated cells enter the CNS and sustain the autoimmune attack [68]. Targeting DC activity in this context is a promising strategy for treating MS.

CHAPTER 4. EFFECTIVENESS OF SUBCUTANEOUS INJECTION OF PEG-IL-10 DELIVERED DENDRITIC CELLS IN AMELIORATING SYMPTOMS IN MURINE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS[1]

4.1 Overview

Recently, antibody therapies have been FDA-approved to ameliorate MS, however these approaches still broadly suppress key components of the immune system [69]. While antibody therapies that target CD20 and deplete B cells are the most common therapeutic strategies, their immune reactions against infections are comprised [70]. Therefore, therapeutic strategies inducing antigen-specific tolerance hold substantial promise in enhancing the quality of life of patients living with autoimmune disorders [71]. Previous studies have shown that tolerogenic DCs secrete cytokines that influence both innate and adaptive immune responses, where IL-10 has shown to have anti-inflammatory properties and induce tolerance [72]. Tolerogenic DCs are capable of conditioning T cells towards a regulatory phenotype and can even inhibit autoreactive effector T cells [73][74]. Thus, the objective of this aim is to demonstrate the effectiveness of PEG-IL10 hydrogel delivered DC10s in ameliorating the symptoms of MS using a EAE model by skewing the autoreactive T cell population towards tolerance through a IL-10 dependent mechanism.

[1] I would like to acknowledge Abigail Damico, a previous graduate student in the Babensee Laboratory for her collaboration and contribution to this experiment. The data of this study is part of a manuscript currently in preparation for submission to the Journal of Biomaterials Research Part A for which we will both be equal first co-authors.

The success of this aim is expected to highlight the role of the biomaterial delivery platform and its ability to promote selective tolerance.

4.2 Materials and Methods

4.2.1 Animals

All animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology or Augusta University, with protocol number: BABENSEE-A100488-1/27/2025. Mice were housed and maintained in the (DAR) facilities at Georgia Institute of Technology of the Physiological Research Laboratory of Augusta University. Procedures conducted at Augusta University were conducted under the approval of the Institutional Animal and Care Use Committee of Augusta University. The animals were 8-12-week-old mice C57BL/6J mice (Jackson Laboratories, Inc.).

4.2.2 Differentiation of Dendritic Cells

Bone marrow was harvested from 8-week-old male C57BL/6J mice, red blood cells (RBCs) were lysed, and the remaining cells were washed twice in phosphate buffered saline (PBS) with 10% fetal bovine serum (FBS). Cells were then cultured ($1.5x10^6$ cells/mL) in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS supplemented with 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), refreshed every three days, for six days. On Day 6, non-adherent cells were removed, and fully differentiated adherent iDCs were released from plates using CellStripper (Corning) for 15 minutes at 37°C/5% CO_2. DCs were then treated with 500

ng/mL IL-10 (DC10s), 50 ng/mL TNF-α and 10 ng/mL IFN-γ (stimulated DCs, STIM-DC), 1 μg/mL LPS (mature DCs, mDC) or no treatment (immature DC, iDC) and cultured (10^6 cells/mL) in 24-well plates for 24 hours at 37°C/5% CO_2.

4.2.3 PEG-4MAL Hydrogel Synthesis

To form hydrogels, the PEG-4MAL macromer (20 kDa; Laysan Bio) was dissolved in PBS containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) [75] The cell-adhesive peptide GRGDSPC (RGD; >95% purity; GenScript) was dissolved (1.0 mM) in PBS containing 10 mM HEPES and added to the PEG-4MAL to produce a solution of RGD-functionalized PEG-4MAL. After 10 minutes of incubation, the protease-degradable cross-linking peptide GCRDVPMSMRGGDRCG (VPM; GenScript) was added (4.0126 mM), and hydrogels were polymerized at 37 °C/5% CO_2 for 5 minutes. For hydrogels incorporating DCs, cells were added to the RGD-functionalized PEG-4MAL solution prior to the addition of cross-linker. DC-laden hydrogels were cultured in DMEM with 10% FBS supplemented with 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20ng/ml Interlukin-4 (IL-4). Samples were maintained in 24-well plates at 37 °C/5% CO_2, refreshing media every 2 days until needed for analysis.

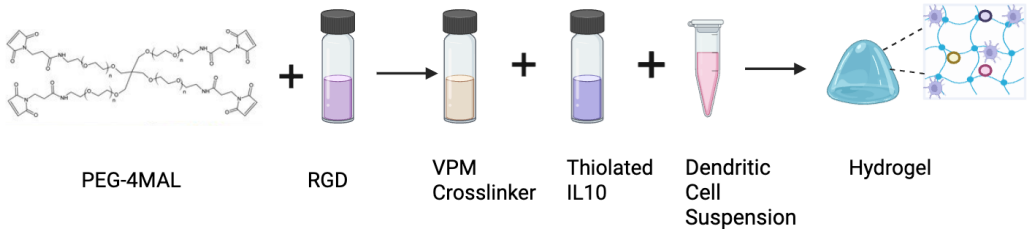

Figure 1: Process of PEG-4MAL: HS-IL10 hydrogel synthesis for DC embedding

4.2.4 Characterization of Hydrogels using Rheology

PEG-4MAL hydrogels were prepared as described in 4.2.3, tethered with IL-10 500ng. Once fully crosslinked, hydrogels were removed and swelled overnight in 1x PBS (pH 7.4) at 4°C. Studies on the viscoelasticity properties were examined on the hydrogels using a rheometer (MCR-302, Anton Paar;CP10-2). Samples were maintained at 37°C while a frequency sweep (100 to 0.1 rad/s) was performed at a constant strain of 1%. Storage modulus (G') and loss modulus (G'') were determined by averaging values acquired within the linear range. PEG-4MAL hydrogels are viscoelastic materials that are primarily treated as linearly elastic based on higher G' values and lower G'' values (loss factor ~0.05 for most samples).

4.2.5 Thiolation of IL10

Lysine residues, the expected sites for thiolation on IL-10 (interlukin-10) we targeted using Traut's reagent (Sigma-Aldrich). Recombinant murine IL-10 (Peprotech) was thiolated with 30 molar excess of Traut's reagent in PBS with 5mM EDTA (Gibco) at pH 8.0 for 1 hour, according to manufacturer's protocol. Protein samples were washed twice by diluting with PBS (pH 8.0) and passing through a 10 kDa cellulose filter (Amicon Ultra; Millipore) at 14,000g.

4.2.6 Conjugation of PEG-4MAL

Conjugation of HS-IL-10 to PEG-4MAL was achieved by reacting both components at a molar ratio of 1000:1 (PEG-4MAL:HS-IL-10) in PBS (pH 7.0) for 30 minutes [76].

4.2.7 Embedding of DCs in Hydrogel

PEG-4MAL hydrogels were prepared as previously described in 4.2.3 with specified modifications to incorporate cells. After 10 minutes of incubating PEG-4MAL and RGD, DCs in 1x PBS containing 10 mM HEPES were added to the hydrogel solution at a concentration of 10^6 cells per 50 µL hydrogel. Immediately following the addition of mDCs, the protease-degradable crosslinking peptide GCRDVPMSMRGGDRCG (VPM;GenScript) was added and hydrogels were polymerized at 37°C/5% CO_2 for 5 minutes. DC laden hydrogels were incubated in the same DMEM-based media with cytokines, as specified previously in 4.2.2.

4.2.8 Injection of DCs embedded in PEG-4MAL:IL10 Hydrogel

Mice were subcutaneously injected with BMDCs embedded within a liquid hydrogel to form a crosslinked gel *in situ*. Mice were injected by the cervical lymph nodes with a total of 100 µL of PEG- 4MAL:HS-IL-10 with embedded DC10s using a 26-guage diameter needle on the scruff of the neck. The needle was inserted between the folds of the skin into the base of the triangle that is formed when traction is applied to the skin overlying the animal's scruff on the back of the neck. The syringe's plunger was retracted to verify that a vacuum is created, and no blood is aspirated. A 27 ½ Gauge needle, the smallest diameter needle possible was used for the PEG-4MAL hydrogel to be delivered. All procedures were done in a sterile area, with sterile techniques.

4.2.9 Induction of EAE

The EAE induction kit (Hooke Laboratories, Lawrence, MA, cat. no. EK-2110) was utilized for chronic EAE induction. On day 0, mice in the EAE group received subcutaneous injections of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide emulsion (200 µL, 200 µg/mouse) along with Complete Freund's Adjuvant (CFA, killed M. tuberculosis H37Ra, 400 µg/µL) in the hind flank. Injections of pertussis toxin (PTX, 100 ng in 50 µL PBS) were administered intraperitoneally to each mouse 2h post-immunization on day 0 and day 1.

4.2.10 Clinical Scoring

Clinical scores are given to mice with EAE based on their level of paralysis, as outlined in Table 1. Neurological deficits were assessed daily using a blind scoring method described in [46]. Animals displaying paralysis on four limbs and/or weight loss more than 15% were sacrificed. Soft food was provided in the cage for paralyzed mice.

Table 1: Clinical Scoring Guidelines

Clinical Score	Description
0	No Disease
1	Flaccid Tail
2	Hind Limb Weakness
3	Hind Limb Paralysis
4	Forelimb Weakness
5	Moribund

4.2.11 Specimen collection

On Day 28, or if euthanasia criteria were met, mice were euthanized using CO_2 for 10 minutes. The PEG-4MAL/lymph node were excised using scissors and placed in a collection solution on ice (4% paraformaldehyde in 1X PBS for flow cytometry samples, 4% formaldehyde in 1X PBS for histology samples). The brain and spinal cord were excised and split in half for different analysis processes and in collection solution depending on analysis. Following removal hydrogels one hydrogel was degraded and then tested using flow cytometry, and the other hydrogel was processed for histological assessment.

4.2.12 Hydrogel Digestion

To recover cells from hydrogels, culture medium was removed, and 1 mL of collagenase II (2 mg/mL; Gibco) was added to each well of PEG-4MAL hydrogels, incorporating DCs. Hydrogels were incubated at 37 °C/5% CO_2 for 15 minutes. The contents of the wells were then pipetted directly into fresh media with 25% FBS to quench collagenase II activity. Cells were collected by centrifugation at 300 x g and prepared for flow cytometry in 4.2.15.

4.2.13 Lymph Node Digestion

To recover cells from lymph nodes 0.2 units of Liberase R1(Roche), DNase 1 (50µg/mL) (Sigma) and 25 mM HEPES was added to conical tubed and incubated at 37 °C for 45 minutes while the contents were mechanically mixed using a pipette every 5 minutes. Once lymph nodes were digested, the solution was immediately pipetted into a tube with 1mL FBS and 3 mL incomplete BMDC media to cease digestion. The cells were strained through

a 70 µm stainer and washed with PBS followed by centrifugation at 400 x g for 5 minutes at 4°C. The cell pellet was resuspended in 2 mL of 1X RBC lysis buffer and incubated for 5 minutes on ice. The cell suspension was washed with 10 mL of PBS at 400 x g for 5 minutes at 4 °C and was prepared for flow cytometry 4.2.15.

4.2.14 Spleen Digestion

To recover cells from the spleen, PBS+ HEPES was removed, and the spleen was placed into a petri dish and 5mL of Hanks Balanced salt solution (HBSS) was added. The spleen was minced into small pieces (~0.2 cm^2) with a scalpel. The spleen was incubated for 20-30 minutes at 37°C with 5 mL of HBSS solution containing Collagenase IV (100 U/mL), DNase I (20 U/mL), and 1% FBS. To stop the reaction, EDTA was added at a concentration of 1mM for 5 minutes. Cells were strained through a 70 µm strainer into a 50mL conical tube, and mashed and pressed through the 70 µm stainer. Cells were washed through the strainer with PBS and collected by centrifugation at 400 x g for 5 minutes. The cell pellet was resuspended with cold RBC (Red blood cell) lysis buffer (Biolegend) and incubated on ice for 5 minutes. The cell suspension was then washed with cold PBS and centrifuged at 400 x g to prepare cells for analysis by flow cytometry in 4.2.15.

4.2.15 Flow Cytometry

Dendritic cells were processed for flow cytometry using a method previously described with minor modifications [47,48]. Cells were washed with PBS and then resuspended in 100 µL of fluorescence-activated cell sorting (FACS) buffer (Hank's Buffered Salt Solution, HBSS; 1% bovine serum albumin, BSA; 1 mM ethylenediaminetetraacetic acid,

EDTA). For DC samples, TruStain FcX (anti mouse CD16/32; BioLegend) was added at the manufacturer's recommended dose, and cells were incubated on ice for 10 minutes. Cells were then stained with fluorescently stained monoclonal antibodies against FITC anti-IA^b (AF6−120.1; BD), APC anti-CD86 (GL-1; BioLegend), BV785 anti- CD11c (N418;BioLegend), or PE anti-PD-L1 (MIH7; BioLegend) at the manufacturer's recommended dose, (1:100 (fluorescein isothiocyanate, FITC), 1:200 for APC, 1:100 CD11c, and 1:200 PE anti-PD-L1), for 30 minutes on ice, protected from light. Cells were washed twice with FACS buffer and analyzed on a BD FACSAria III cytometer. Propidium iodide (PI) was added 5 minutes before analysis to assess viability. DC samples were analyzed based on the gating strategy in Figure 16.

To study the phenotype of DCs used for *in vivo* studies at Augusta University, cells (2–5 × 10^6 cells/ml) were blocked for Fc-receptors (1:200, eBioscience 14-0161-86) for 15 minutes at 4 °C and then incubated with antibodies (Table 1) for 30 minutes at 4°C. All solutions were in fluorescence-activated cell sorting (FACS) buffer (bovine serum albumin (1%, Sigma A4503) and sodium azide (0.01%, Mallinckrodt 195– 3-57) in phosphate buffered saline antibodies were diluted 1:100 (fluorescein isothiocyanate, FITC) or 1:200 for all others besides, CD11c was used in a 1:100, $CD45_{RA}$ 1:800, and IA^B at a 1:400 ratio.

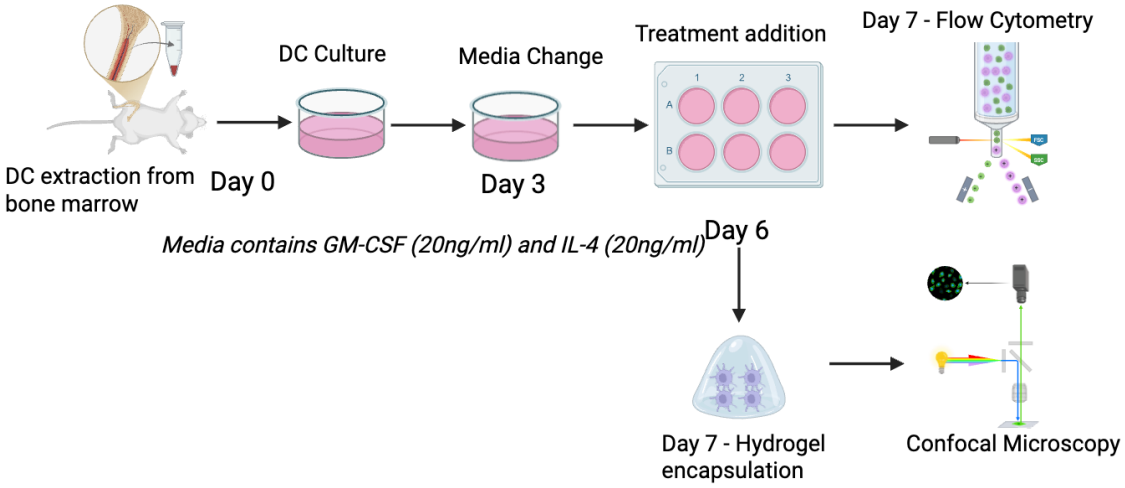

Figure 2: Schematic of BMDC Culture, BMDC Treatment Schedule and analysis techniques

4.2.16 Chrome Alum-Gelatin Slide Preparation

Distilled deionized (DI) water (200 mL) was heated to 60 °C on a hot plate, with a stir rod added. Chrome Alum-Gelatin(1mL/100mL) (Fisher Scientific) was added to DI water, and glass microscopy slides were dipped in solution 3-5 times for 5-10 seconds per dip and were left to drains for 3-5 minutes onto filter paper. Slides were dried in an oven at 60 °C for 3 hours and stored in a dry box to protect from dust.

4.2.17 Histology

Samples were prepared through cryoprotection with sucrose and infiltration with OCT (optimal cutting temperature) compound, starting with a 5% sucrose solution in PBS and working up to a 15% sucrose solution, with an initial vacuum infiltrate for 1 hour, followed by the addition of 0.5mL of 20% sucrose solution, mixed for 3 minutes and vacuum infiltrate for 30 minutes. Following this process hydrogels were moved to 4:1 sucrose:OCT solution

and vacuum infiltrated for 1 hour, following the same steps in a 3:1 solution, 2:1 solution, 1:1 solution, and a final 1:2 sucrose:OCT solution, with vacuum infiltration overnight. OCT was placed on the bottom of the cryomold as well as on top, and the samples were flash frozen in dry ice, and then placed in a -80 °C freezer, until sectioning. Cryostat sections were cut on (Cryostar NX70) at 10 µm thickness at -20 °C and placed on gelatin-chrome alum (0.1% w/v gelatin, .001% w/v chrome potassium sulfate)-treated slides. Slides with sections were stored in -8 °C until staining.

4.2.18 Hematoxylin and Eosin Staining

Samples were stained in 200 µL hematoxylin solution for 3 minutes, washed in running tap water for 15 minutes, and rinsed in distilled water twice for 1 minute each time. Excess liquid was carefully wiped away, and then sample was stained in 400 µL of eosin solution for 1-3 minutes. Sample was dehydrated in absolute alcohols, 80% ethanol, 96%, 100%, 100%. Samples were then cleared in xylene for two changes of 10 minutes each. Dried sections were mounted with Permount (Fisher Scientific).

4.2.19 Masson Trichrome Staining

Samples were stained with Weigert's hematoxylin (StatLab) for 10 minutes and dried without washing. Samples were placed in alcoholic Picric acid for minutes and quickly washed in distilled water for 3-4 seconds. Samples were then stained with acid crimson fuchsin solution for 4 minutes and then washed in distilled water. Samples were placed in phosphomolybdic acid for 10 minutes and dried without washing. Samples were then stained with Masson's anyline blue (Statlab) for 5 minutes and then washed in distilled

water. Samples were then cleared in xylene for two changes of 10 minutes each. Dried sections were mounted with Permount (Fisher Scientific).

4.2.20 Light Microscopy

Mounting media (Fisher Chemical Permount Mounting Medium) was used to add a cover slip and preserve samples for later imaging. Images were obtained using a Biotek Cytation 7/Biostack Slide and Plate Scanner. Whole slides were imaged at 4x magnification. Points of interest were then imaged at 10x magnification, or 20x magnification.

4.2.21 Overall Experimental Approach

Dendritic cells were cultured following 4.2.2 with some modifications. On day 7 of culture DCs were placed into flask and filled with completed BMDC media to lessen the possibility of mechanical activation and arranged in a Darwin Chamber to limited shift of the flasks, through the period of travel needed to be endured. The Darwin Chamber acts as a temperature incubator and maintains 37 °C but does not maintain CO_2 concentration. Half of the cells that were cultured were prepared for flow cytometry, 4.2.15 and analyzed at Augusta University on a comparable machine to the machine used at Georgia Tech (BDAria II), while the other half of the cells cultured were prepared and analyzed at Georgia Tech prior to travel.

For *in vivo* studies, DCs were prepared for travel in the same manner, but were not analyzed for flow cytometry at Augusta University. Dendritic cells treated with IL-10 were embedded in PEG-IL-10 hydrogel, depending on treatment group (Table 2 and Table 3). Hydrogel preparation of reagents was completed at Georgia Tech, the day prior to

travel and pH was measured again in the morning prior to departure to ensure reagents retained pH.

At Augusta University mice were anesthetized with isoflurane and undergo injection of treatment into cLNs, as well as induction of EAE immediately after injection. (4.2.8, 4.2.9). Mice were placed under the care and observation of Research Professional, Dr. Liu Fang over the 28-day timeline, to ensure proper scoring and body weight measurements, and samples were procured from mice of all groups for further analysis on day 28 (4.2.10).

Table 2: Treatment conditions of mice was designated based upon cage assignment at August University

Treatment Group	**Description**	**Number of Mice**
Cage 1	EAE + Hydrogel + DCs	5
Cage 2	EAE + Hydrogel	5
Cage 3	EAE	5
Cage 4	Hydrogel + DCs	5

Table 3: Order of Treatment given per cage. Identification of mice is done through notches in ears, donated by L-left ear, R-right ear, and no denotes no notches in the ear. The number in the front of side ear identification notch denotes the treatment group of mice. EAE only mice in cage 3 were identified through tags in ears, when specific numbers on each tag.

Cage Number	1	2	3	4	5
Cage 1	1L	1No	1R	1LR	1LL
Cage 2	2R	2No	2LL	2LR	2L
Cage 3	739	738	735	736	737
Cage 4	4R	4L	4LR	4No	4LL

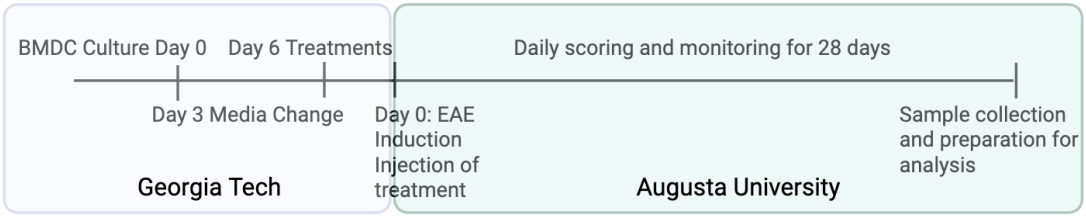

Figure 3: Schematic of Augusta *in vivo* studies preparation workflow

4.2.22 Statistical Analysis

Experimental values were reported as mean and standard deviation for all samples with statistical analysis conducted using one or two-way analysis of variance (ANOVA) coupled with Tukey's pairwise tests using GraphPad (Prism Inc.). All datasets were normally distributed. P-values <0.05 were considered statistically significant. Error bars represent standard deviation.

4.3 Results

4.3.1 Dendritic Cells Viability and Phenotype Post Transportation

Pretreated DCs cultured at Georgia Tech were transported to Augusta University in flasks to be prepared for flow cytometry at the core facility and analyzed on a similar flow cytometer to Georgia Tech instrument of BDAria III. This process was designed to evaluate whether the DCs could retain their phenotype prior to travel and maintain viability through the travel for subsequent *in vivo* experiments with the DCs. Surface marker analysis completed at Augusta University showed similar maturity in DCs as well, as other treatments (IL-10, TNF-α/IFN-γ, VD3, and iDCs) similar to the results observed at Georgia Tech. Propidium iodide (PI) exclusion indicated reduction in the overall cell number post transportation and slightly larger number of dead cells (Figure 4).

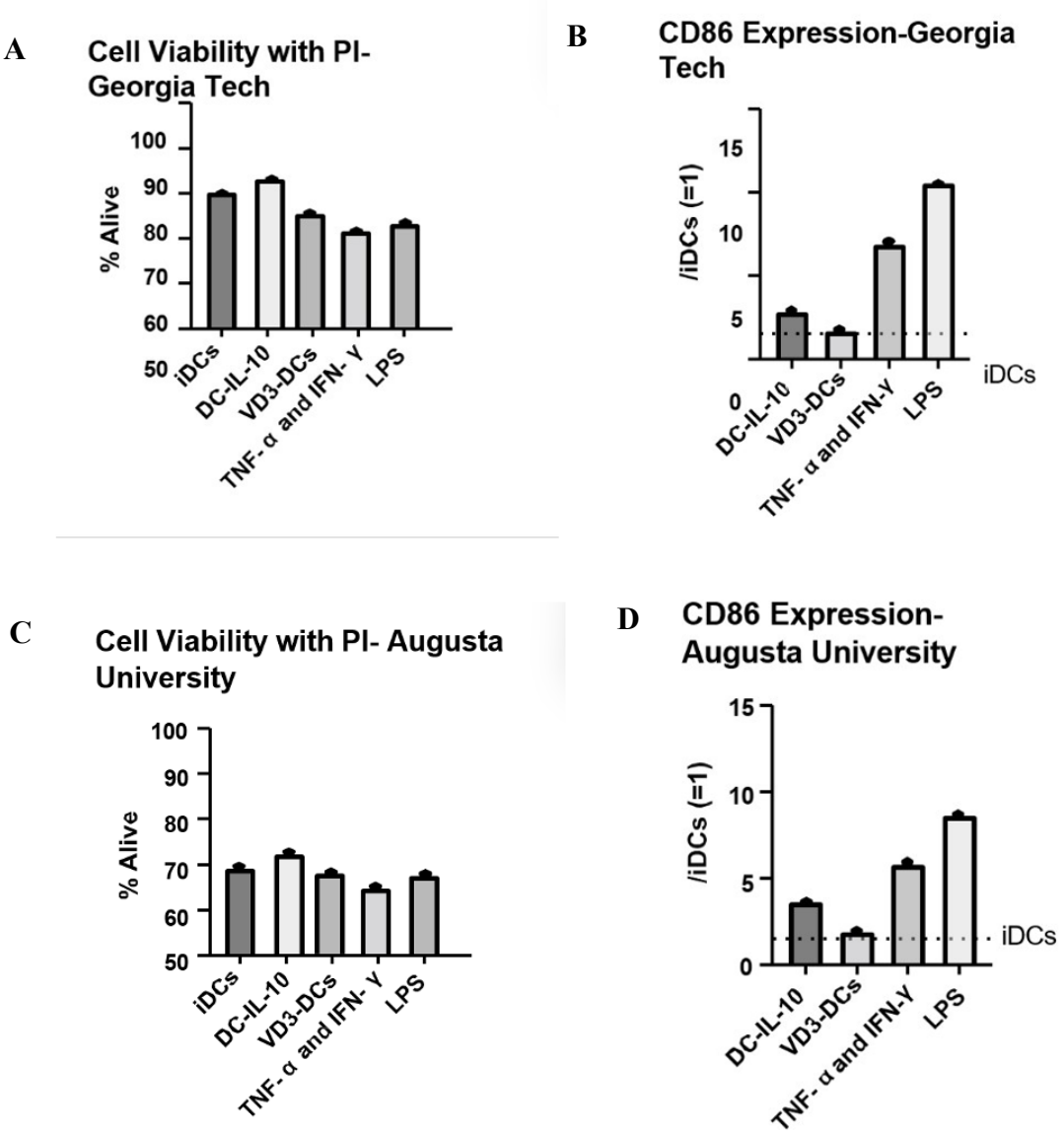

Figure 4: Cell Viability and Phenotype of Pretreated Female BMDCs remained similar before and after travel to Augusta University. Georgia Tech prior to travel- A) Cell Viability through PI exclusion, B) flow cytometry analysis for CD86 expression; at Augusta University C) Cell Viability through PI exclusion, D) flow cytometry analysis for CD86 expression.

4.3.2 Transport Stimulation of Dendritic Cells

Biomarker expression analysis of DCs subjected to simulated transport conditions in a Darwin Chamber at Georgia Tech showed results similar to those of cells that underwent actual transport to Augusta University, as shown in Figure 4. The simulation run at Georgia Tech resulted in a higher percentage of viable cells for DCs derived from both male and female mice, while surface biomarkers remained consistent with those observed prior to travel (Figure 5, Figure 6). These results suggest that although the physical stress of transport slightly impacts cell viability, the overall phenotype of the DCs is preserved.

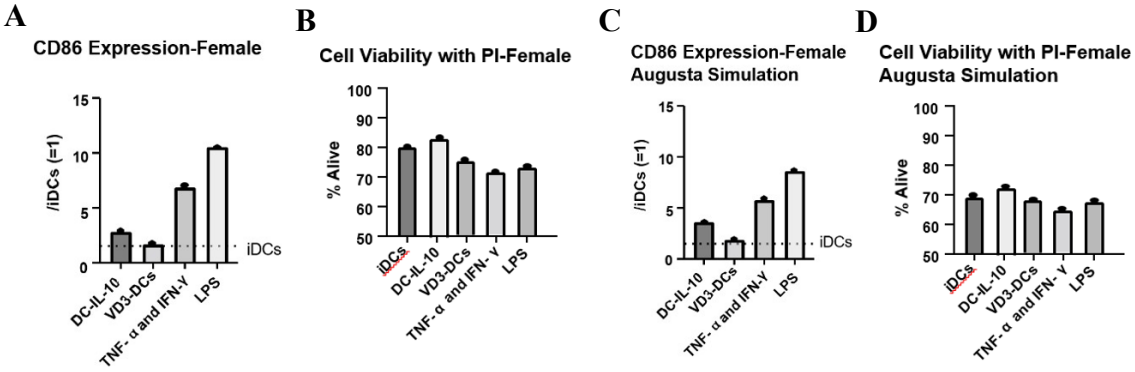

Figure 5: Viability and Phenotype of Pretreated Female BMDCs were unaffected by the transport stimulation.A) CD86 expression of DCs, B) Cell Viability, C) CD86 expression of DCs after 3 hours in stimulation of travel, D) Cell Viability after 3 hours in simulation of travel. Number of experimental replicated, n=3.

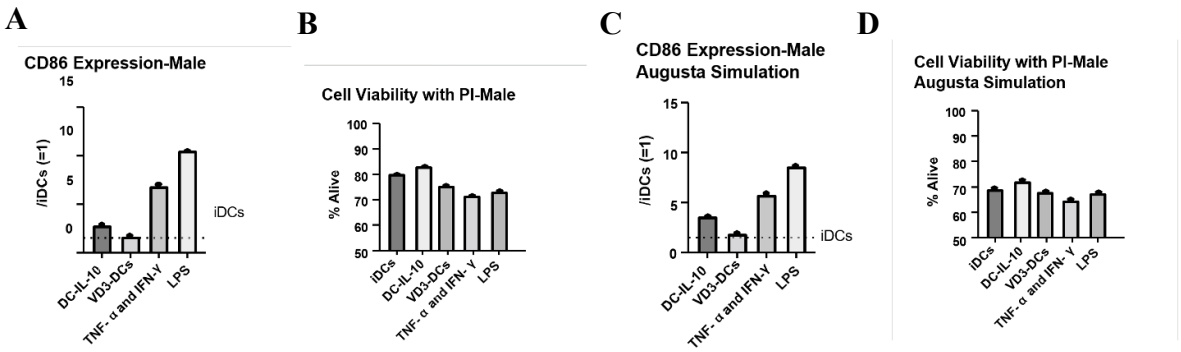

Figure 6: Viability and Phenotype of Pretreated Male BMDCs were unaffected by the transport stimulation. A) CD86 expression of DCs, B) Cell Viability, C) CD86 expression of DCs after 3 hours in stimulation of travel, D) Cell Viability after 3 hours in simulation of travel. Number of experimental replicated, n=3.

4.3.3 Hydrogel delivered DCs injection effects in EAE Induced Mice

Dendritic cells derived at Georgia Tech, pretreated with IL-10 and MOG_{35-55}, were embedded in the hydrogel conjugated with thiolated IL-10 and injected into mice at Augusta University at the time of EAE induction (Prophylactic). EAE scoring and body weight analysis over a period of 28 days showed that the immunosuppressive DCs delivered via the IL-10–laden hydrogel significantly reduced disease severity compared to the hydrogel alone (no DCs). Apart from the lower EAE scores with treatment, symptoms started to appear at a later timepoint than in the EAE model without treatment, indicating the success of the treatment in delaying the onset of disease. Treatment groups maintained a higher body weight throughout the study, consistent with the clinical scoring trends and suggesting an improved health status following treatment. (Figure 7).

While flow cytometry analysis did not reveal substantial differences in surface marker expression between groups due to the late endpoint of 28 days for the EAE model, it was found that the CDllb+ cells accounted for the largest cell population of endogenous

immune cells observed in both the spleen and lymph node/implant region, with a higher percentage in the spleen compared to the lymph node/implant region (Figure 8A and C). Endogenous DCs, identified as CD11c+ cells in the receptive tissues were further analyzed for biomarkers that are markers indicative of distinct DC phenotypes, as well as markers for additional immune cells that may have been recruited to the target tissues by the DCs.

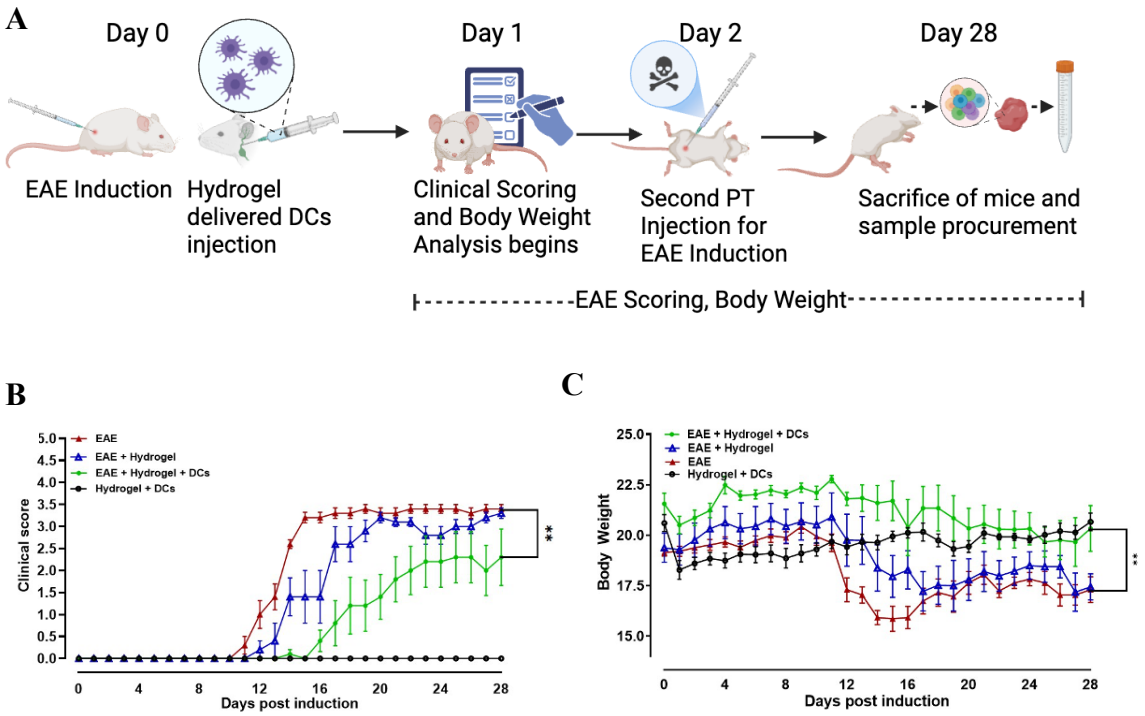

Figure 7: (A) EAE induction and workflow, (B) evaluation of clinical scores, (C) body weight to mice with EAE induced and no treatment (EAE), with EAE induced with hydrogel-IL-10 (no DCs) (EAE + Hydrogel), . with EAE induced with hydrogel-IL-10 with embedded DCs (EAE + Hydrogel + DCs), control was no EAE, just delivery of Hydrogel + DCs. n=5 mice **.

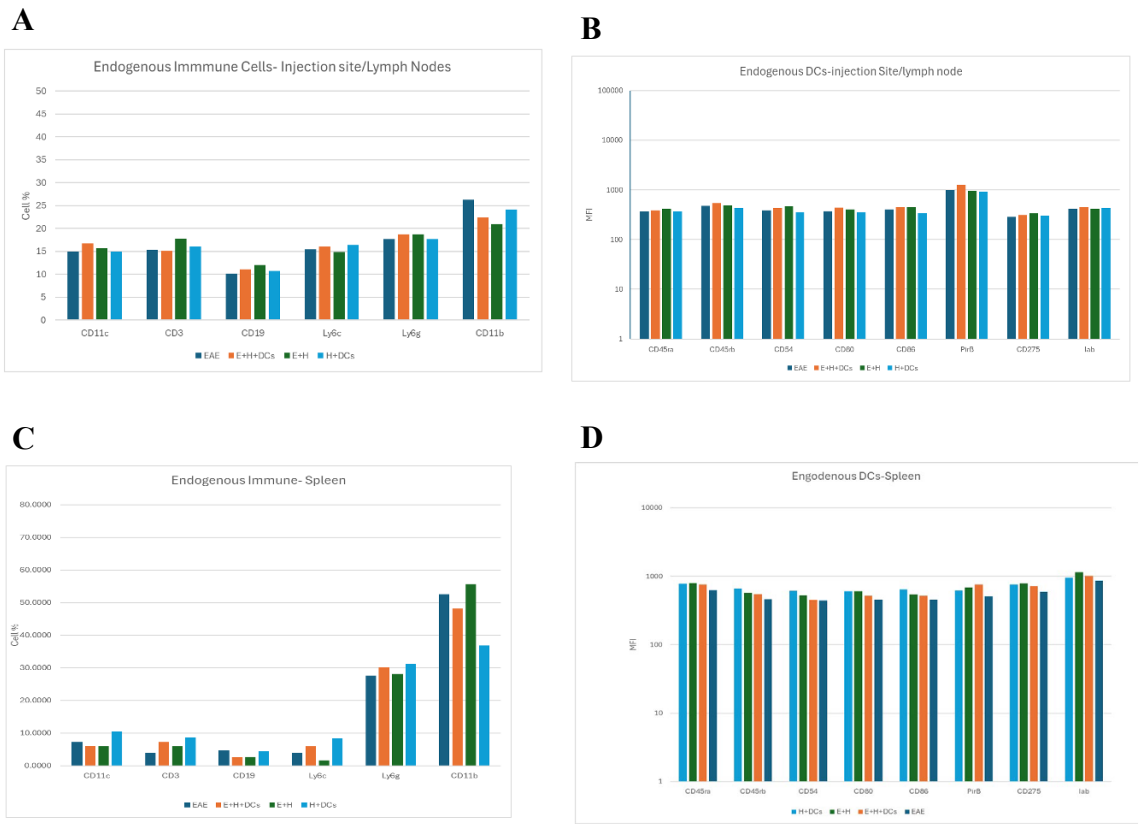

Figure 8: Increased expression of endogenous cells at the hydrogel injetion site and spleen.Injection Site - A) Endogenous immune cell phenotype, B) Endogenous DC surface marker expression level (MFI); Spleen - C) Endogenous immune cell phenotype, D) Endogenous DC surface marker expression level (MFI). Number of replicates, n=5.

4.3.4 Histological Analysis of Hydrogel Injection Site

Characterization of the injection site using H & E staining along with Masson Trichrome, provided some insight into the local tissue response following treatment. Due to the late time point of 28 days, the information was limited. While tissue reactions were clearly observed in the stained sections, no visible remnants of the injected hydrogel polymer were detected. As a result, it is uncertain that the space observed corresponds to the original site of subcutaneous hydrogel injection (Figure 9). Figure 10 provides a clear visualization with and without the DCs in the hydrogel system, wherein the presence of DCs allows a better integration of the biomaterial at the injection site.

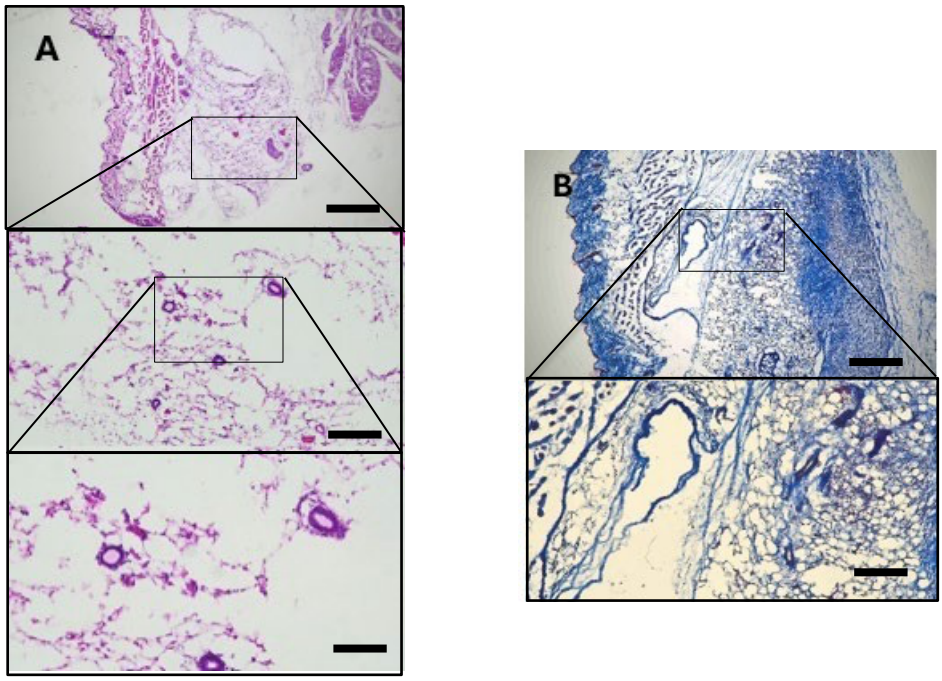

Figure 9: Histological Staining of Injection Site from female mouse treated with DC10s implanted in PEG-4MAL:HS-IL-10 hydrogel shows formation of blood vessels. A) H & E staining of 10 µm thick section at 4x magnification, 10x magnification and 20x magnification, (B) Masson Trichrome staining of 10 µm thick section at 4x magnification and 10x magnification. Scale Bars= 100 µm.

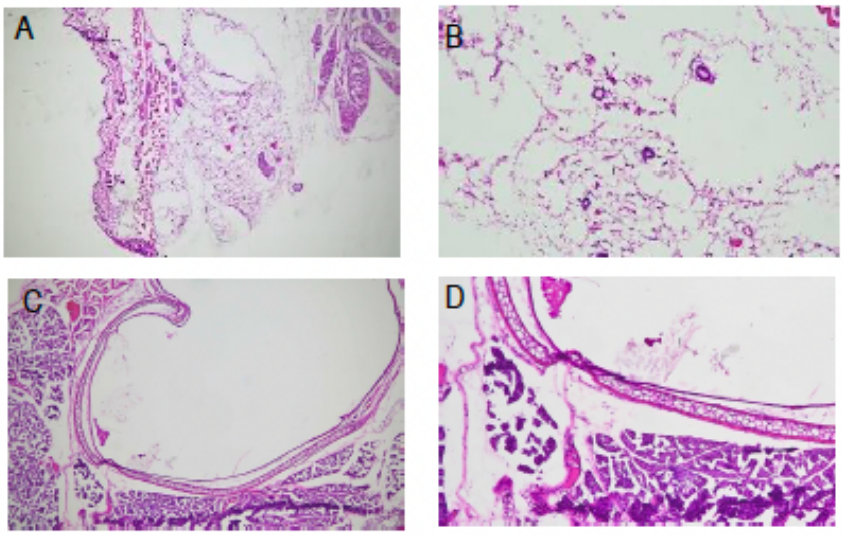

Figure 10: Histological staining of injection site from female mice with induced EAE and treated with hydrogel laden DCs showed better integration compared to only the hydrogel. (A, B) PEG-4MAL:HS-IL-10 hydrogel delivering DC10s; (A) 4xmag. and (B) 10x mag. A control injection site of (C, D) PEG-4MAL:HS-IL-10 hydrogel without DC10s; (C) 4xmag. and (D) 10x mag.

4.4 Discussion

4.4.1 Effect of transport on pretreated Dendritic Cells

Dendritic cells are sensitive environmental disturbances such as mechanical stimulation, temperature fluctuations and some potentially unknown factors which can result in their activation to a mature phenotype. Pretreating BMDCs in culture allows their phenotype to be established prior to transport, which may help them resist activation. The characterization of DCs using flow cytometry is a well-established tool. It was crucial to demonstrate that the DCs could be transported to Augusta University without mechanical maturation of the DCs occurring. The expression levels of sensitive DC maturation marker, CD86, were not significantly changed from starting values at GT to those after the 3-hour trip to Augusta University where the cells were analyzed on site using flow cytometry.

To further investigate this observation, simulated transport experiments were performed following the same steps used for the Augusta University experiments. During these simulations, cells were either driven around or carried for a 3-hour period inside the Darwin Chamber before being prepared for flow cytometry analysis. This method yielded results similar to those observed during the travel to Augusta University, and this was confirmed using both male- and female-derived BMDCs. Therefore, these findings prove that the pretreatment of BMDCs prior to transport appears to minimize activation caused by mechanical stimulation, while maintaining cell viability for subsequent experimentation.

4.4.2 Effect of Hydrogel Delivered DCs on Ameliorating Symptoms of EAE model

The use of EAE models to simulate MS is a well-established and widely accepted approach for evaluating and refining therapeutic strategies While there are different types of EAE models, a chronic model was used in this study to assess treatment efficacy. A previous study carried out at the Babensee Laboratory proved that the delivery proximal to the cervical lymph nodes significantly attenuated paralysis, while delivery to the flank (proximal to the vaccination site) was less effective at the amelioration of EAE symptoms, likely due to less interaction with myelin- and CNS-responsive immune cells [12]. Therefore, localization of DC transplantation near the cervical lymph nodes was employed to enhance therapeutic efficacy and support the translational potential of this experiment.

The use of PEG-4MAL in conjugation with thiolated IL-10 and embedded DC10s was found to ameliorate EAE symptoms and delayed the onset of disease MS symptoms, whereas EAE mice treated with only the PEG-4MAL:IL-10 hydrogel exhibited minimal

improvements in their clinical scores (Figure 7). Due to limitations on the number of animals used per experiment, a control group receiving DC10s without the hydrogel was not included in this study.

4.4.3 Immune Cell Recruitment and Migration in Autoimmune Situation due to Treatment Timepoints

Following the use of the EAE model to evaluate the effectiveness of the biomaterial system on clinical scores, tissues including the injection site, spleen, brain, and spinal cord were collected to assess immune cell recruitment through flow cytometry and histological analysis. Hydrogels and spleens from each treatment group were enzymatically digested for the use in surface marker analysis, but no significant differences were observed between the treatment groups on the day of animal sacrifice. Histological samples were stained with H&E or Masson's Trichrome to visualize the areas of interest, but it was challenging to distinguish between the residual hydrogel material and surrounding lymph nodes. As a result, definitive conclusions regarding immune cell recruitment at the injection site could not be determined from histology alone. Despite these challenges, the treatment demonstrated efficacy in improving EAE scores, suggesting that the hydrogel's sustained release of IL-10 plays a crucial role in maintaining therapeutic levels of IL-10 at the injection site, suppressing local inflammation and promoting immune tolerance. Additionally, tolDCs can work alongside to promote regulatory T cell (Treg) expansion and inhibit autoreactive T cell activation, thereby preventing the initiation of the autoimmune response. Given that the injection site is in proximity to the lymph nodes, IL-10 can be transported via the lymphatic system to the CNS, where it helps suppress

inflammation and protect the myelin sheath. As shown in Chapter 6, DC10s are not very migratory. Hence, it is expected that the delivered DCs embedded in the hydrogel, educate endogenous inflammatory/immune cells to have a subduing effect on the autoimmune disease score.

CHAPTER 5. CHAPTER 5 *IN VITRO* ANALYSIS OF DENDRITIC CELL VIABILITY WITHIN ENGINEERED HYDROGEL ENVIRONMENTS

5.1 Overview

Dendritic cells (DCs) are antigen-presenting cells that are crucial for maintaining self-tolerance, initiating immune responses against pathogens, and patrolling body compartments, which is crucial for effective immunotherapy [77]. While DC-based immunotherapies have shown promise, the field of immunotherapy is rapidly evolving, and the effectiveness of these approaches depend on maintaining cell viability, localization, and phenotype stability post-delivery. Poly(ethylene glycol) (PEG)-based matrices offer a highly tunable and biocompatible platform that can provide mechanical protection and localized retention of DCs at the target site. This ensures not only enhanced DC viability *in vivo* but also facilitates the maintenance of the desired functional phenotype, such as tolerogenicity, which is critical for inducing therapeutic immune modulation.

In this chapter, the viability of DCs with variable stimuli conditions were studied with immunomodulatory stimulus such as IL10 and maturation stimulation such as LPS *in vitro*, as well as the viability of embedded DCs with variable stimuli in PEG-IL-10, and the time differential between embedded DCs vs. unembedded DCs. Additionally, maintaining DC viability is critical for the therapeutic success of DC-based immunomodulation in EAE studies. The ability to interact with endogenous immune cells and present antigens in a tolerogenic context would be lost if DCs undergo premature cell death post-administration. Therefore, high DC viability post-delivery in the inflamed environment of EAE, is essential

to enable sufficient interaction with host immune cells either locally at the implant site or systemically if migration occurs.

5.2 Materials and Methods

5.2.1 Animals

All animal procedures were conducted following procedures described in 4.2.1.

5.2.2 CCK8 (Assay to measure relative cell metabolic activity)

For the hydrogel groups, the existing media was carefully removed without disturbing the hydrogel to assess the viability using an assay giving the metabolic activity of the embedded cells. For each time point, 10 µL of CCK-8 solution was added to each well containing 100 µL of culture medium, and the plate was incubated at 37°C for 15-30 minutes, protected from light. Following incubation, the absorbance was measured at 450 nm using a microplate reader (BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader). The cell metabolic activity was analyzed after subtracting the background calculated from non-cell controls.

5.2.3 Flow Cytometry

Dendritic cells were processed for flow cytometry (Cytek Auora) using a method previously described with minor modifications. Cells were washed with PBS and then stained with Zombie Violet (1:500 in PBS) to analyse viability at the manufacturer's recommended dose. The cells are washed after a 30-minute incubation period with FACS buffer (Hank's Buffered Salt Solution, HBSS; 1% bovine serum albumin, BSA; 1 mM

ethylenediaminetetraacetic acid, EDTA). were blocked for Fc-receptors (1:200, eBioscience 14-0161-86) for 15 minutes at 4 °C and then incubated with antibodies (Table 1) for 30 minutes at 4°C. All solutions apart from Zombie Violet were in fluorescence-activated cell sorting (FACS) buffer (bovine serum albumin (1%, Sigma A4503) and sodium azide (0.01%, Mallinckrodt 195– 3-57) in phosphate buffered saline antibodies were diluted 1:100 (fluorescein isothiocyanate, FITC) or 1:200 for all others besides, CD11c was used in a 1:100, $CD45_{RA}$ 1:800, and IA^{B} at a 1:400 ratio .

5.2.4 Statistical Analysis

Experimental values are reported as mean and standard deviation for all samples with statistical analysis conducted using one-way analysis of variance (ANOVA) coupled with Tukey's pairwise tests using GraphPad (Prism Inc.). All datasets were normally distributed. P-values <0.05 were considered statistically significant. Error bars represent standard deviation.

5.3 Results

5.3.1 Flow Cytometric Analysis of Dendritic Cell Phenotype Prior to Viability Assessment Under Varying Treatment Conditions

Prior to evaluating cell viability under the different treatment conditions, DCs were characterized for their surface marker expression using flow cytometry to confirm their phenotypic profiles. For the LPS treated group, mDC population exhibited elevated expression of CD86, a key co-stimulatory molecule associated with antigen presentation and T cell activation whereas the IL-10 treated group had a significant expression for

PDL1. These results confirmed the DC phenotypic profiles prior to their use in the viability studies at different time points (Figure 11).

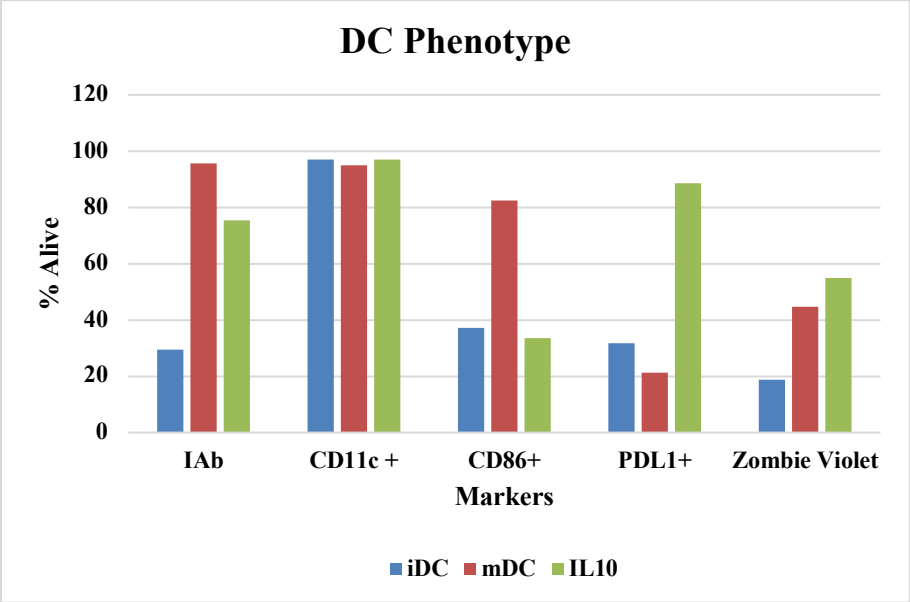

Figure 11: Flow cytometry analysis for key surface markers of DCs proves their phenotype.

5.3.2 In vitro viability assessment of Hydrogel embedded Dendritic Cells under various Treatment Conditions.

The viability of tolDCs embedded within the hydrogel was higher compared to the other treatment groups across all time points. This observation proves that the hydrogel provides a supportive microenvironment that enhances cell survival. These results indicate the successful establishment of the hydrogel-based delivery system for tolDCs and provide valuable insights into the cellular response during the initial days post-injection (Figure 12).

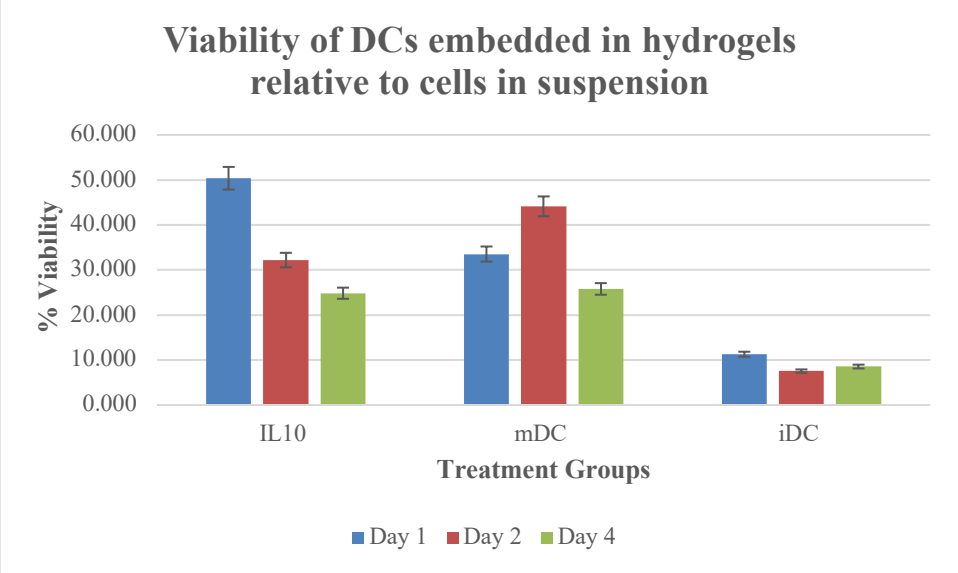

Figure 12: Assessment of metabolic activity using CCK-8 assay confirms better viability of DCs treated with IL10. N=1, T=3.

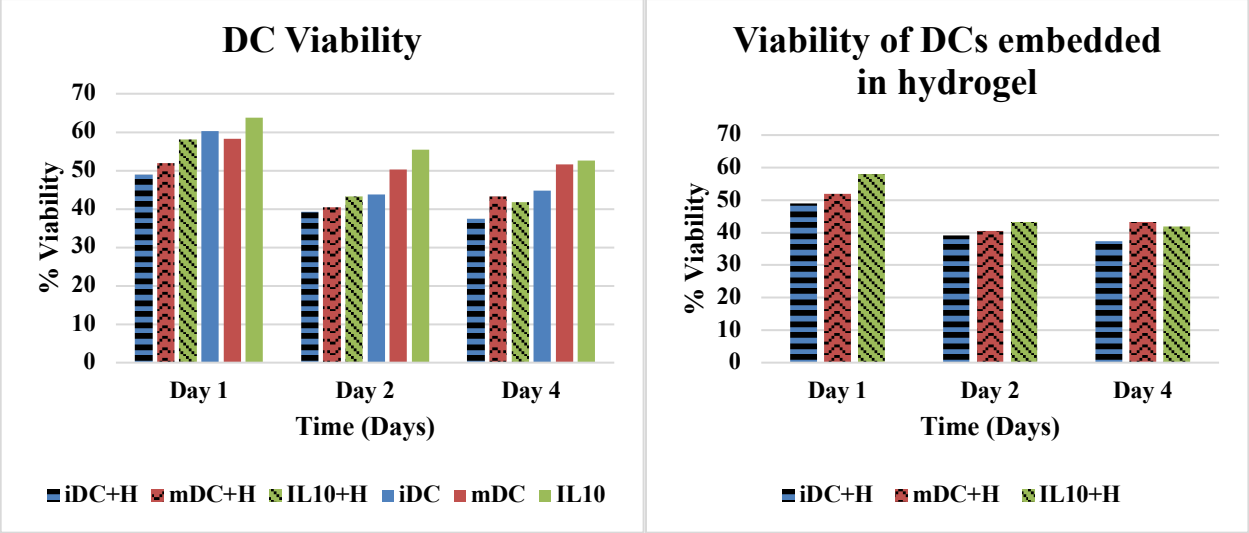

Figure 13: Assessment using Zombie Violet in flow cytometry analysis confirms better viability of DCs treated with IL10. N=1, T=3.

5.4 Discussion

5.4.1 Effect of Treatment on DC Phenotype

The functional outcome of DC-based studies relies entirely on the accurate immunological function of the cells, so confirming the phenotype of the DCs by flow cytometry is essential. In this experiment, pretreatment with IL-10 induced a tolerogenic phenotype characterized by the upregulation of PD-L1 expression. Increased PD-L1 expression on DCs facilitates the suppression of effector T cell activation and promotes regulatory T cell induction, contributing to the overall immunosuppressive environment. Treatment with LPS induced a mature, immunogenic phenotype characterized by high expression of CD86, which enhances the ability of the DCs to present antigen and initiate an adaptive immune response. While CD86 expression was elevated across all groups, including untreated controls, this could potentially be attributed to mechanical stress associated with pipetting, centrifugation, or physical disturbances during culture. It is well established that DCs are highly sensitive to environmental cues, and mechanical stress has been reported to trigger partial activation, which could be the cause of the elevation of CD86 in all groups. Despite this, the clear distinction in PD-L1 levels highlights the successful skewing of DCs toward a tolerogenic state, which is essential for their therapeutic application in the EAE model.

5.4.2 Effect of Hydrogel Environment on Dendritic Cell Viability at Different Timepoints

Growth factors such as GM-CSF and IL-4 are crucial for DC survival *in vitro* since they promote differentiation and long-term viability of DCs whereas DCs *in vivo* face a more complex environment where their survival is influenced by various factors, including

migration patterns and interactions with other immune cells. Pretreatment of DCs with certain cytokines can enhance their viability, ensuring they are better equipped to function once administered [100]. Additionally, embedding DCs in hydrogels provides protection, helping to prevent premature degradation and ensuring sustained delivery to the desired site. In the presence of active inflammation, tolDCs can expand Treg populations and modulate immune responses, which can support their survival and functionality for longer periods than in typical immune responses. This makes tolDCs particularly valuable in therapeutic applications, such as in autoimmune diseases like EAE, where they can help regulate immune responses and potentially improve disease outcomes.

Studying DC viability embedded in a hydrogel *in vitro* can provide valuable insights into DC function in vivo. While the immune system *in vivo* is much more dynamic, exposing DCs to inflammatory signals or interactions with other immune cells in a controlled *in vitro* environment can help capture aspects of the complexity of the *in vivo* microenvironment. Using assays like flow cytometry to assess viability and CCK-8 to measure relative metabolic activity can provide important data on how DCs survive within the hydrogel and can help predict how DCs might behave once delivered *in vivo*, aiding in the design and optimization of therapeutic DC-based strategies.

The viability results obtained from both flow cytometry and through the metabolic activity in CCK8 assay were consistent and supported the overall trend observed in this study wherein DC10s consistently demonstrated higher viability compared to iDCs at all evaluated time points. A gradual decline in viability was observed for all groups over time, this decrease could be attributed to the migration of viable cells out of the hydrogel matrix

rather than cell death alone. Flow cytometry results using Zombie Violet viability dye indicated a relatively higher percentage of dead cells compared to the CCK8 assay. This could be explained by potential background staining or due to the staining non-DC populations since bone marrow-derived cultures contain a heterogeneous mixture of cells and that only a small fraction differentiates into DCs. The sustained survival of DC10s is important to maintain their immunomodulatory phenotype for a longer duration, enabling an effective interaction with endogenous immune cells and sustained secretion of regulatory signals at the site of delivery. This provides a strong explanation for both the delayed onset and the reduced severity of disease symptoms observed in the treated groups.

CHAPTER 6. INVESTIGATION OF MIGRATION OF BONE MARROW DERIVED DENDRITIC CELLS INDUCED BY CCL19

6.1 Overview

Dendritic cells play crucial roles not only in initiating protective immunity against invading pathogens but also in inducing immune tolerance toward harmless antigens and this depends on their proper migration to specific destinations [78]. Since DC migration is fundamental for execution of their functions during maintenance of immune surveillance and disease pathogenesis, a better understanding of DC migration is critical for successful development of immunotherapies [79][80]. Studies have explored the ability of DCs to acquire a phenotype that balances immune suppression with migratory ability when exposed to both immunomodulatory and maturation stimuli [81]. To understand the success behind our treatment in the EAE models, we aimed to study tolDC migration generated *in vitro* using a variety of immunomodulators, such as IL-10 [82-86], DEX [87-91], and VD3 [92-94], while DCs have also been generated *in vitro* using maturation stimuli such as LPS or PGN, as well as mechanical stimulation or environment cues. Mature DCs have been shown to have increased migratory abilities compared to iDCs or tolDCs, highlighting the importance of maturation signals in enhancing their ability to reach target tissues such as draining lymph nodes for effective immune modulation. The objective of this aim is to evaluate the migration of DCs embedded in PEG-IL-10 with variable stimuli combinations outside the hydrogel at different time points – 6, 12 and 24 hours.

6.2 Materials and Methods

6.2.1 Animals

All animal procedures were conducted following procedures described in 4.2.1.

6.2.2 Migration Assay through PEG-IL10

Dendritic cells were cultured following BMDC protocol in 4.2.2 in a 6 well plate. On day 1, 9 wells on DCs were cultured with VD3 (1µL/mL). On day 3, DCs were treated with DEX (20 µL/mL), in 9 wells. Day 6, other treatments were added to wells PGN (5µL/mL), LPS (1µg/mL) or no treatment. Culture inserts were placed in wells, and 1.5 mL of medium was added with or without CCL19 (100ng/mL) (R&D: Recombinant Mouse CCL19/MIP-3β) on day 7. BMDCs were detached using cell stripper (Corning) and washed with 2 mL of PBS and 220 x g for 4 minutes at 22°C. Cells were seeded into PEG-IL10 hydrogels following 4.2.7, and hydrogel reagents were pipetted onto the transwell membrane, for variable times of 6, 12, and 24 hours. Media was carefully removed from cell inserts, and media was carefully aspirated from the bottom of the well plate and washed with 1 mL of PBS. Transwell membranes were dried with 750 µL of cold methanol and incubated for 20 minutes at room temperature to fix the cells. After the removal of methanol, and the membrane was left to air dry for 30 minutes. The transwell membrane was stained with 750 µL of crystal violet (Sigma Aldrich) and incubated for 20 minutes. The transwell membrane was washed 2 times with PBS and imaged using an inverted microscope to count visually the migrated cells on the lower side of the membrane.

6.2.3 Statistical Analysis

Experimental values are reported as mean and standard deviation for all samples with statistical analysis conducted using one-way analysis of variance (ANOVA) coupled with Tukey's pairwise tests using GraphPad (Prism Inc.). All datasets were normally distributed. P-values <0.05 were considered statistically significant. Error bars represent standard deviation.

6.3 Results

6.3.1 Analysis of DC migration out of hydrogels in response to various treatment conditions

The DCs embedded within hydrogels required a longer time to migrate through the transwell membrane compared to the timelines observed in previous studies conducted without hydrogels, where migration occurred within 3 hours. With the presence of the hydrogel, cells only began migrating after 6 hours and the results were similar for 12 and 24 hours with an increase in the percentage of cells migrated for each treatment group. The result suggests that DEX (dexamethasone) treated DCs had the best migration when treated with PGN, or LPS, whereas IL-10 had the lowest percentage of migration, even when treated with a maturation stimulus at all the time points.

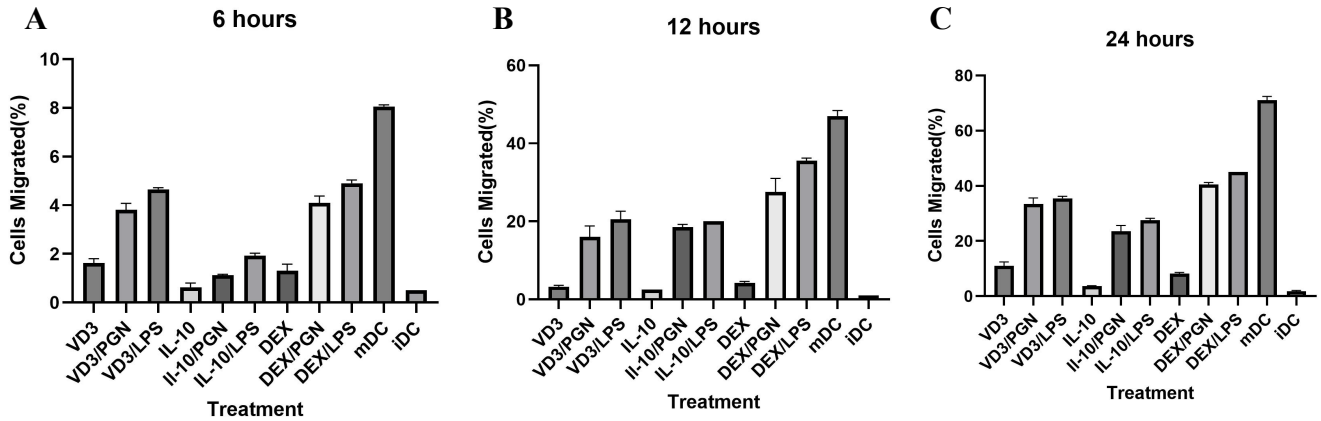

Figure 14: Transwell Migration Assay of DCs embedded in PEG-4MAL:HS- IL-10 shows the best migration capability with cotreatment of DEX and LPS. A) Female BMDCs transwell migration assay with t=6. (B) Female BMDCs transwell migration assay with t=12. (C) Female BMDCs transwell migration assay with t=24. Number of replicates N=1, T=3.

6.4 Discussion

6.4.1 Effect of Maturation Stimulation on Migration of Tolerogenic Dendritic Cells

It is well-established that mDCs migrate more efficiently than iDCs. It was essential to identify a combination of a maturation stimulus and tolerogenic stimulus that can allow for the cells to maintain both characteristics. This is important because the cells must be able to migrate out of the hydrogel and travel throughout the body once injected. This migration capability is a critical design parameter for the hydrogel system, which makes the optimization of tolerogenic and maturation stimuli essential.

To evaluate the migration of DCs out of the hydrogel, tolerogenic stimuli, such as IL-10, was added in DC culture, and cocultured with maturation stimuli, such as PGN. On day 7, a transwell migration assay was performed to compare the effect of these stimuli on the migration of mDCs and iDCs. Since migration out of the hydrogel was slower, migration was analyzed after 6 hours and DCs treated with both DEX and PGN exhibited the highest migration percentage, indicating that the combination of maturation and tolerogenic stimuli was most effective. At both 12 and 24 hours the percentage of cells migrated across the membrane increased compared to earlier time points. Analysis with previous studies showed that, migration of the DCs took a longer period when implanted in the hydrogel but could still reach the same migratory abilities as the hydrogel began to degrade.

Dendritic cells cultured with IL-10, without the maturation stimulus, demonstrated a lower migration percentage, but when combined with maturation stimuli, migration improved. However, this combination still showed a significantly lower migration percentage compared to other maturation-tolerogenic combinations. Despite the limited migration, the observed reduction in EAE clinical scores and delayed disease onset suggests that the DC10s exerted their immunomodulatory effect locally. It is possible that the hydrogel served as a microenvironment that both supported DC10 viability and acted as an immunological niche which recruited endogenous immune cells such as effector T cells, regulatory T cells, or monocytes. However, treatment post-EAE induction or the administration of multiple doses may yield broader effects on the brain and CNS by influencing immune responses more extensively. Labeling lymph nodes for targeted injection could provide a more effective strategy to enhance therapeutic outcomes since it may direct the DCs to areas of the CNS and reverse the damage caused by autoimmune responses. Given that DEX and LPS treatment showed the best DC migration, co-treatment with these factors could be explored to determine whether achieving a balance between tolerance and migration is necessary for treatment success *in vivo*.

CHAPTER 7. CONCLUSIONS AND FUTURE DRECTIONS

7.1 Conclusions

The research described herein highlights the therapeutic potential of the DC10-laden hydrogel system in mitigating symptoms of the EAE model, while also refining the optimization of DC treatment parameters through the combined use of maturation and tolerogenic stimuli during culture. This work builds upon prior research conducted in the Babensee Laboratory, expanding on earlier work involving DC-laden PEG-4MAL injections into the flank and cLNs [12], as well as EAE studies that analyzed body weight and clinical scores. In the current experiment, the conjugation of thiolated IL-10 to the maleimide group in PEG-4MAL via Michael addition enables the transport of tolDCs. IL10 is an anti-inflammatory cytokine which suppresses pathogenic inflammation and promotes tolerance without inducing fibrosis like cytokines like TGF-β [95]. Avoiding fibrosis is important since fibrotic scarring locks the CNS into a state of chronic damage and impedes tissue repair in the axons. Sustained release of IL-10 from hydrogel degradation, triggered by enzymatic activity at the injection site, allows for localized and prolonged immunomodulation, counteracting the autoimmune attack in EAE.

Preliminary studies conducted at Augusta University demonstrated that administration of DC10-laden PEG-4MAL:HS-IL-10 hydrogels at the time of EAE induction alleviated disease symptoms in female mice, as shown by clinical scoring and body weight measurements over a 28-day period. The biomaterial system has shown to prolong the benefits of encapsulation and protect DCs from inflammation, thereby significantly

ameliorating the symptoms of EAE and delaying the onset of disease. The studies focused on feasibility of transporting pretreated DCs and analysis of their viability and phenotype both prior to and post travel, along with clinical scoring of EAE mice with hydrogel treatment. While pretreated DCs retained comparable MFI values after travel, flow cytometry revealed a higher percentage of dead cells in the suspension post-transport. Repeated simulated travel studies produced results consistent with actual transport, and sufficient numbers of DCs survived to be embedded in hydrogels for future experimental use.

In Chapter 5, the viability through Zombie Violet dye and the metabolic activity of DCs through CCK8 assay was evaluated during the first few days following embedding within the hydrogel system. The results demonstrated that IL-10–treated DCs showed improved viability compared to immature iDCs, confirming that IL-10 conditioning enhances cellular tolerance to the embedding process. The decrease in viability over time could be attributed not only to reduced cell survival but also to the potential migration of viable DCs out of the hydrogel. It is important that the IL-10 pretreatment appears to support DC adaptation during the early post-embedding period since this aligns with the improved EAE clinical scores starting from day 7 post-treatment, proving their immunomodulatory function *in vivo*.

In Chapter 6, the migration of DCs out of the hydrogel was modulated and assessed through the manipulation of varying, maturation and tolerogenic stimulus combinations and controls. The findings demonstrate that the addition of a maturation stimulus significantly enhances the migratory capacity of tolerogenic DCs even when embedded within a

hydrogel delivery system, which emphasizes the importance of this balance when designing cell-based therapies for autoimmune diseases.

Therefore, this research has been successful in the development of a tolerogenic biomaterial system for DC therapies in the treatment of disease models with over stimulated immune systems.

7.2 Significance of Findings

The work described here contributes to the scientific community by elucidating the impact of the PEG-4MAL: HS-IL-10 hydrogel delivery system on DC10 viability and migration, thereby ameliorating symptoms with the EAE model and lowering clinical scores. The characterization of surface markers on DCs in the spleen and lymph node provides findings on the therapeutic effects of DCs along with the fate of DCs implanted into an autoimmune situation. The development of the biomaterial system would improve the success of DC therapies and provide an immunosuppressive niche for the treatment of autoimmunity through improved cell viability and migration. While a lot of work focuses on adjuvants to DCs and bioinert materials, the research herein focuses on immunosuppression and transforming the polymer-based biomaterial into a material that is capable of directly inducing tolerance along with targeted cell delivery. These findings broaden the range of possibilities for DC therapies especially in the context of autoimmune disorders such as T1D and hopefully translate into clinical applications.

7.3 Future Directions

The results of this thesis provide a foundation for understanding the cellular and molecular basis of tolerance induction by hydrogel delivered DC10s, but there are many promising next steps to this project that can improve the efficacy of cell therapy. Studies focused on analyzing the cell phenotype during the earlier timepoints of the disease would provide a deeper understanding of the initial immune-modulating events following DC10-laden hydrogel treatment, between the injection and the onset of clinical symptom improvement.

While the current studies focus on co-administration of the treatment at the time of EAE induction, testing different timepoints for DC10-laden hydrogel injection after the onset of EAE symptoms would provide valuable insight into the therapeutic potential. Assessment of EAE models for a longer time frame such as 40- 60 days, would answer questions of a possible remission and relapse. Since demyelination is a characteristic of MS, stains that bind to the myelin such as Luxol Fast Blue (LFB) and myelin basic protein (MBP) allow for a detailed visualization of myelin damage and remyelination in the CNS [96]. Incorporating behavioral assessments such as Open Field Test, Rotarod Test and Wire Grip Test alongside clinical scoring in the EAE model would offer a more comprehensive evaluation of treatment efficacy that may not be captured by standard scoring [97][98][99].

By establishing a baseline of the system there will be a better understanding of fundamental components to the system such as the migration of the cells. Subcutaneous delivery of DC10s targeting the draining cLNs will be followed by histological analysis and flow cytometry to assess cell presence and phenotype at early timepoints such as 4 hours, days 1, 2, 5 and 7. Apart from the cLNs, the migratory capacity of DCs will be evaluated in

secondary organs such as the spleen and brain to understand DC migration and their role in modulating immune responses at distal sites. Studies including the addition of maturation stimulus in coculture to increase the migratory abilities of tolerogenic DCs would ensure sustained immune modulation.

Lastly, future studies should focus on defining the optimal IL-10 dose and its release profile *in vivo* to ensure sustained therapeutic efficacy, which is important for clinical application. The release kinetics of IL-10 from the hydrogel system are tuned to maintain an effective local immunomodulatory environment throughout the treatment course and assessing the degradation profile of the hydrogel will be critical to confirm that the system provides consistent delivery over the period of the study.

APPENDIX A.

A.1 Supplemental to Aim 1

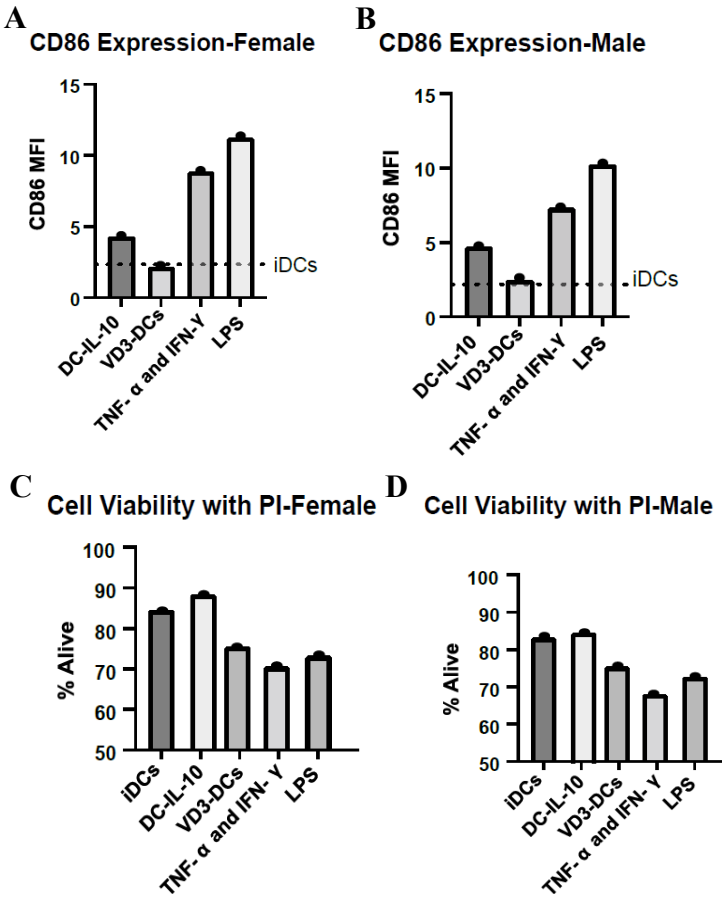

Figure 15: Flow Cytometry Replication Results on BMDC culture for verification of proper cell culture of DC viability and phenotype. CD86 expression in DC assessed by flow cytometry, (A) from female mice, (B) from male mice. Percentage of alive cells assessed through PI exclusion, (from female mice, (D) from Male mice. Number of experimental replicated, n=3.

A.2 Supplemental to Aim 2

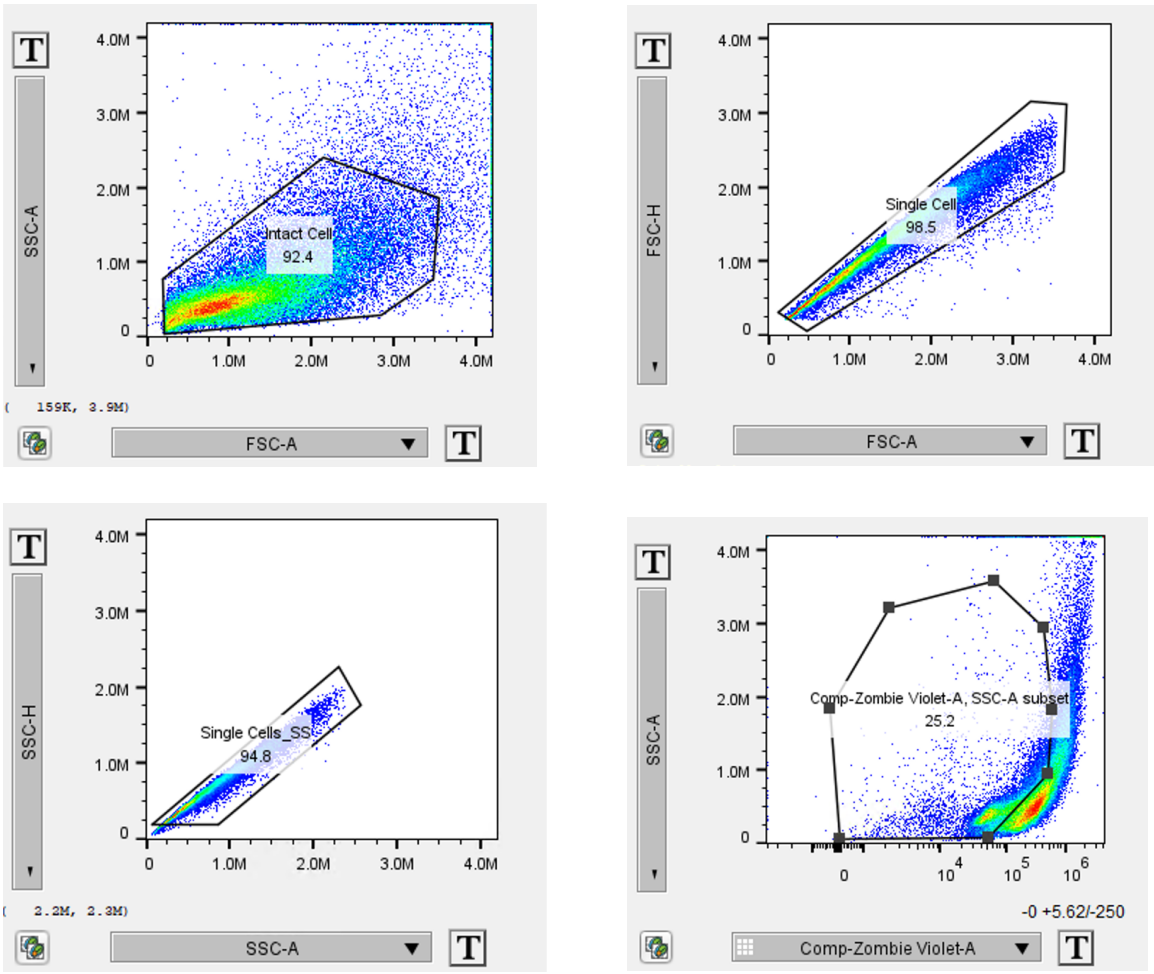

Figure 16: Flow Cytometry Gating Strategy for DCs used for the identification of proper cell culture, and phenotype of cells.

Table 4: DC Viability through CCK8 Assay Significance. Day 1.**p < 0.0001, ***<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test.**

Tukey's multiple comparisons test	Summary	Adjusted P Value
H+ iDC vs. H+ mDC	**	0.004
H+ iDC vs. H+ IL10	****	<0.0001
H+ iDC vs. iDC	****	<0.0001
H+ iDC vs. mDC	****	<0.0001
H+ iDC vs. IL10	****	<0.0001
H+ mDC vs. H+ IL10	ns	0.0942
H+ mDC vs. iDC	****	<0.0001
H+ mDC vs. mDC	****	<0.0001
H+ mDC vs. IL10	****	<0.0001
H+ IL10 vs. iDC	****	<0.0001
H+ IL10 vs. mDC	****	<0.0001
H+ IL10 vs. IL10	****	<0.0001
iDC vs. mDC	ns	>0.9999

iDC vs. IL10	ns	>0.9999

Table 5: DC Viability through CCK8 Assay Significance. Day 2.**p < 0.0001, ***<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test.**

Tukey's multiple comparisons test	Summary	Adjusted P Value
H+ iDC vs. H+ mDC	**	0.0014
H+ iDC vs. H+ IL10	*	0.0162
H+ iDC vs. iDC	****	<0.0001
H+ iDC vs. mDC	****	<0.0001
H+ iDC vs. IL10	****	<0.0001
H+ mDC vs. H+ IL10	ns	0.6631
H+ mDC vs. iDC	****	<0.0001
H+ mDC vs. mDC	****	<0.0001
H+ mDC vs. IL10	****	<0.0001
H+ IL10 vs. iDC	****	<0.0001

H+ IL10 vs. mDC	****	<0.0001
H+ IL10 vs. IL10	****	<0.0001
iDC vs. mDC	ns	0.9873
iDC vs. IL10	ns	0.9992

Table 6: DC Viability through CCK8 Assay Significance. Day 4.**p < 0.0001, ***<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test.**

Tukey's multiple comparisons test	Summary	Adjusted P Value
H+ iDC vs. H+ mDC	ns	0.7514
H+ iDC vs. H+ IL10	ns	0.6576
H+ iDC vs. iDC	***	0.0008
H+ iDC vs. mDC	***	0.0002
H+ iDC vs. IL10	****	<0.0001
H+ mDC vs. H+ IL10	ns	>0.9999
H+ mDC vs. iDC	**	0.0065
H+ mDC vs. mDC	**	0.0015

H+ mDC vs. IL10	***	0.0003
H+ IL10 vs. iDC	**	0.0086
H+ IL10 vs. mDC	**	0.0019
H+ IL10 vs. IL10	***	0.0004
iDC vs. mDC	ns	0.9352
iDC vs. IL10	ns	0.4578

A.3 Supplemental to Aim 3

Table 7: Hydrogel Transwell Migration Assay Experimental Significance T=6 hrs.**p < 0.0001, ***<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test.**

Tukey's multiple comparisons test	Summary	Adjusted P Value
VD3 vs. VD3/PGN	****	<0.0001
VD3 vs. VD3/LPS	****	<0.0001
VD3 vs. IL-10	**	0.0042
VD3 vs. Il-10/PGN	ns	0.2608
VD3 vs. IL-10/LPS	ns	0.8125

VD3 vs. DEX	ns	0.7425
VD3 vs. DEX/PGN	****	<0.0001
VD3 vs. DEX/LPS	****	<0.0001
VD3 vs. mDC	****	<0.0001
VD3 vs. iDC	**	0.0016
VD3/PGN vs. VD3/LPS	*	0.0142
VD3/PGN vs. IL-10	****	<0.0001
VD3/PGN vs. Il-10/PGN	****	<0.0001
VD3/PGN vs. IL-10/LPS	****	<0.0001
VD3/PGN vs. DEX	****	<0.0001
VD3/PGN vs. DEX/PGN	ns	0.8125
VD3/PGN vs. DEX/LPS	**	0.0019
VD3/PGN vs. mDC	****	<0.0001
VD3/PGN vs. iDC	****	<0.0001
VD3/LPS vs. IL-10	****	<0.0001
VD3/LPS vs. Il-10/PGN	****	<0.0001

VD3/LPS vs. IL-10/LPS	****	<0.0001
VD3/LPS vs. DEX	****	<0.0001
VD3/LPS vs. DEX/PGN	ns	0.1772
VD3/LPS vs. DEX/LPS	ns	0.9215
VD3/LPS vs. mDC	****	<0.0001
VD3/LPS vs. iDC	****	<0.0001
IL-10 vs. Il-10/PGN	ns	0.2608
IL-10 vs. IL-10/LPS	***	0.0004
IL-10 vs. DEX	ns	0.0629
IL-10 vs. DEX/PGN	****	<0.0001
IL-10 vs. DEX/LPS	****	<0.0001
IL-10 vs. mDC	****	<0.0001
IL-10 vs. iDC	ns	0.9994
Il-10/PGN vs. IL-10/LPS	*	0.0217
Il-10/PGN vs. DEX	ns	0.9917
Il-10/PGN vs. DEX/PGN	****	<0.0001

Il-10/PGN vs. DEX/LPS	****	<0.0001
Il-10/PGN vs. mDC	****	<0.0001
Il-10/PGN vs. iDC	ns	0.0959
IL-10/LPS vs. DEX	ns	0.0959
IL-10/LPS vs. DEX/PGN	****	<0.0001
IL-10/LPS vs. DEX/LPS	****	<0.0001
IL-10/LPS vs. mDC	****	<0.0001
IL-10/LPS vs. iDC	***	0.0002
DEX vs. DEX/PGN	****	<0.0001
DEX vs. DEX/LPS	****	<0.0001
DEX vs. mDC	****	<0.0001
DEX vs. iDC	*	0.0217
DEX/PGN vs. DEX/LPS	*	0.0217
DEX/PGN vs. mDC	****	<0.0001
DEX/PGN vs. iDC	****	<0.0001
DEX/LPS vs. mDC	****	<0.0001

DEX/LPS vs. iDC	****	<0.0001
mDC vs. iDC	****	<0.0001

Table 8: Hydrogel Transwell Migration Assay Experimental Significance T=12 hrs. ****p < 0.0001, *<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test.**

Tukey's multiple comparisons test	Summary	Adjusted P Value
VD3 vs. VD3/PGN	***	0.0002
VD3 vs. VD3/LPS	****	<0.0001
VD3 vs. IL-10	ns	>0.9999
VD3 vs. Il-10/PGN	****	<0.0001
VD3 vs. IL-10/LPS	****	<0.0001
VD3 vs. DEX	ns	0.9998
VD3 vs. DEX/PGN	****	<0.0001
VD3 vs. DEX/LPS	****	<0.0001
VD3 vs. mDC	****	<0.0001
VD3 vs. iDC	ns	0.9237

VD3/PGN vs. VD3/LPS	ns	0.2662
VD3/PGN vs. IL-10	***	0.0001
VD3/PGN vs. Il-10/PGN	ns	0.8703
VD3/PGN vs. IL-10/LPS	ns	0.3935
VD3/PGN vs. DEX	***	0.0005
VD3/PGN vs. DEX/PGN	***	0.0005
VD3/PGN vs. DEX/LPS	****	<0.0001
VD3/PGN vs. mDC	****	<0.0001
VD3/PGN vs. iDC	****	<0.0001
VD3/LPS vs. IL-10	****	<0.0001
VD3/LPS vs. Il-10/PGN	ns	0.961
VD3/LPS vs. IL-10/LPS	ns	>0.9999
VD3/LPS vs. DEX	****	<0.0001
VD3/LPS vs. DEX/PGN	*	0.0272
VD3/LPS vs. DEX/LPS	****	<0.0001
VD3/LPS vs. mDC	****	<0.0001

VD3/LPS vs. iDC	****	<0.0001
IL-10 vs. Il-10/PGN	****	<0.0001
IL-10 vs. IL-10/LPS	****	<0.0001
IL-10 vs. DEX	ns	0.9834
IL-10 vs. DEX/PGN	****	<0.0001
IL-10 vs. DEX/LPS	****	<0.0001
IL-10 vs. mDC	****	<0.0001
IL-10 vs. iDC	ns	0.9944
Il-10/PGN vs. IL-10/LPS	ns	0.9944
Il-10/PGN vs. DEX	****	<0.0001
Il-10/PGN vs. DEX/PGN	**	0.0044
Il-10/PGN vs. DEX/LPS	****	<0.0001
Il-10/PGN vs. mDC	****	<0.0001
Il-10/PGN vs. iDC	****	<0.0001
IL-10/LPS vs. DEX	****	<0.0001
IL-10/LPS vs. DEX/PGN	*	0.017

IL-10/LPS vs. DEX/LPS	****	<0.0001
IL-10/LPS vs. mDC	****	<0.0001
IL-10/LPS vs. iDC	****	<0.0001
DEX vs. DEX/PGN	****	<0.0001
DEX vs. DEX/LPS	****	<0.0001
DEX vs. mDC	****	<0.0001
DEX vs. iDC	ns	0.6381
DEX/PGN vs. DEX/LPS	*	0.0107
DEX/PGN vs. mDC	****	<0.0001
DEX/PGN vs. iDC	****	<0.0001
DEX/LPS vs. mDC	***	0.0005
DEX/LPS vs. iDC	****	<0.0001
mDC vs. iDC	****	<0.0001

Table 9: Hydrogel Transwell Migration Assay Experimental Significance, T=24hrs. ****p < 0.0001, *<0.001, **0.01 and *p < 0.05 via one-way ANOVA with and Tukey's comparison test**

Tukey's multiple comparisons test	Summary	Adjusted P Value
VD3 vs. VD3/PGN	****	<0.0001
VD3 vs. VD3/LPS	****	<0.0001
VD3 vs. IL-10	**	0.0017
VD3 vs. Il-10/PGN	****	<0.0001
VD3 vs. IL-10/LPS	****	<0.0001
VD3 vs. DEX	ns	0.4529
VD3 vs. DEX/PGN	****	<0.0001
VD3 vs. DEX/LPS	****	<0.0001
VD3 vs. mDC	****	<0.0001
VD3 vs. iDC	***	0.0002
VD3/PGN vs. VD3/LPS	ns	0.7973
VD3/PGN vs. IL-10	****	<0.0001
VD3/PGN vs. Il-10/PGN	***	0.0001

VD3/PGN vs. IL-10/LPS	**	0.0082
VD3/PGN vs. DEX	****	<0.0001
VD3/PGN vs. DEX/PGN	**	0.0024
VD3/PGN vs. DEX/LPS	****	<0.0001
VD3/PGN vs. mDC	****	<0.0001
VD3/PGN vs. iDC	****	<0.0001
VD3/LPS vs. IL-10	****	<0.0001
VD3/LPS vs. Il-10/PGN	****	<0.0001
VD3/LPS vs. IL-10/LPS	***	0.0008
VD3/LPS vs. DEX	****	<0.0001
VD3/LPS vs. DEX/PGN	*	0.0294
VD3/LPS vs. DEX/LPS	***	0.0002
VD3/LPS vs. mDC	****	<0.0001
VD3/LPS vs. iDC	****	<0.0001
IL-10 vs. Il-10/PGN	****	<0.0001
IL-10 vs. IL-10/LPS	****	<0.0001

IL-10 vs. DEX	ns	0.0529
IL-10 vs. DEX/PGN	****	<0.0001
IL-10 vs. DEX/LPS	****	<0.0001
IL-10 vs. mDC	****	<0.0001
IL-10 vs. iDC	ns	0.8177
Il-10/PGN vs. IL-10/LPS	ns	0.1075
Il-10/PGN vs. DEX	****	<0.0001
Il-10/PGN vs. DEX/PGN	****	<0.0001
Il-10/PGN vs. DEX/LPS	****	<0.0001
Il-10/PGN vs. mDC	****	<0.0001
Il-10/PGN vs. iDC	****	<0.0001
IL-10/LPS vs. DEX	****	<0.0001
IL-10/LPS vs. DEX/PGN	****	<0.0001
IL-10/LPS vs. DEX/LPS	****	<0.0001
IL-10/LPS vs. mDC	****	<0.0001
IL-10/LPS vs. iDC	****	<0.0001

DEX vs. DEX/PGN	****	<0.0001
DEX vs. DEX/LPS	****	<0.0001
DEX vs. mDC	****	<0.0001
DEX vs. iDC	**	0.0044
DEX/PGN vs. DEX/LPS	ns	0.0564
DEX/PGN vs. mDC	****	<0.0001
DEX/PGN vs. iDC	****	<0.0001
DEX/LPS vs. mDC	****	<0.0001
DEX/LPS vs. iDC	****	<0.0001
mDC vs. iDC	****	<0.0001

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