# IMMUNOMODULATION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS USING PEG-4MAL HYDROGEL WITH DENDRITIC CELLS: EFFECTS OF VARIABLE STIMULI ON CELL MATURATION

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Abigail G. Damico

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# IMMUNOMODULATION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS USING PEG-4MAL HYDROGEL WITH DENDRITIC CELLS: EFFECTS OF VARIABLE STIMULI ON CELL MATURATION

Approved by:

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

Dr. Edward Botchwey
School of Biomedical Engineering
*Georgia Institute of Technology*

Dr. Ankur Singh
School of Mechanical Engineering
*Georgia Institute of Technology*

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# Table of Contents

# LIST OF TABLES

# LIST OF FIGURES

# 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-α | tumor necrosis factor-α |
| VD3 | 1,25-dihydroxyvitamin D3 |
| VPM | GCRDVPMSMRGGDRCG peptide |
| σ | standard deviation |

# SUMMARY

Over the recent decades, research in biomaterials and immunoengineering has been investigated at a higher rate than previous years in science. Due to this there has been a surge of development in advanced therapies to treat cancers, autoimmune diseases, and other possible immune-related pathologies. Dendritic cells (DCs) have been at the forefront of pharmaceutical and biological therapeutics, as a bridge between the innate and adaptive immune system. In cell therapy, DCs have a high efficacy especially when being used for cell transplantation, vaccines, tumor clearance, and autoimmunity. Dr. Julia Babensee has pioneered research in biomaterial adjuvant effect with DCs, which was a novel discovery and elicited subsequent research interests by other investigators in the use of biomaterial stimulation of autoimmunity, focusing on biomaterials that promote tolerance.

This research thesis therefore sought to contribute towards the ongoing research in the Babensee lab, which focuses on developing a biomaterial system to ameliorate autoimmunity, with the biomaterial system being applied to the condition of Multiple Sclerosis. The biomaterial system is combined of poly (ethylene glycol)- 4 arms maleimide (PEG-4MAL) hydrogels conjugated with the immunosuppressive cytokine, interleukin, IL-10, which is injectable, *in situ* cross linkable and degradable system for localized delivery of immunosuppressive DCs.

Therefore, it is important to understand how IL-10, and other treatments may affect the migration of DCs, through the hydrogel, and through the body. With mature DCs (mDCs) known to have better migration abilities, the use of a maturation stimulus may be needed as an addition of tolerogenic DCs.

Poly (ethylene glycol) is being used in this study due to its unique attributes, such as a branched macromer precursor, with each arm being conjugated to a maleimide group (PEG-4MAL) which allows for the bioconjugation of crosslinkers, adhesive ligands, growth factors and cytokines. The hydrogels allow for the DCs to be protected from inflammation that may occur and enhance the cell viability *in vivo*.

The amount of IL-10 incorporated in the hydrogel has been shown to maintain incorporated DC viability. Based on preliminary results previously conducted in the laboratory, hydrogels loaded with IL-10 and encapsulated DC10s were shown to have an effect of the clinical score and body weight of mice compared to mice without any treatment [2]. The benefit of the biomaterial system was apparent from the decrease in clinical scores, as well as histological studies. In the current studies, we address the potential of the biomaterial system to ameliorate EAE symptoms, and potential stimulation of pretreated DCs obstacles. Henceforth, these studies focused on characterizing the late-stage immune cell response post injection in an autoimmune situation, and procedures to follow for comparable results to previous studies, as well as optimal maturation and immunosuppressive stimulus for migration of DCs.

# CHPAPTER 1. INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune inflammatory disease that causes demyelination of the neurons in the central nervous system (CNS), that affects 1.2 million people globally with annual cost beings as much as $54,244 per patient [3, 4]. MS can lead to the loss of motor control, cognitive deficits, and eventual paralysis. There is currently no cure for MS, which is the most common cause of chronic neurological disability that usually begins at the prime of people's adult working lives with only treatments that aim to slow the progression of neurodegeneration through inhibition of neuroinflammation[5]. The progression and severity of MS has been shown to be dependent on the balance between the number of and functional capacity of the inflammatory autoreactive T cells and regulatory T cells.

Dendritic cells (DCs) bridge the innate and adaptive immune system, and they direct antigen-specific immune responses. Delivering DCs, which are known immunomodulators, one can recruit lymphocytes, to therapeutically treat the progression of MS. However, cell-only approaches are limited in their efficacy, as the approach lacks localization of the cells at the site due to DCs lack of longevity and migratory abilities when systemically delivered [6]. By using a biomaterial systems unique advantages are offered for autoimmune amelioration by providing an immunomodulatory environment. By treating DCs with interleukin-10 (IL-10) (DC10s) and transplanting them into PEG-4MAL hydrogel with thiolated IL-10, the DCs can be delivered and used for immune suppressive or tolerogenic applications through the effects on T cells [1]. This biomaterial system can be used for further investigation into the inhibition of neuroinflammation for the treatment of MS to improve immunosuppressive DC therapy through targeted delivery and regulation of immune cell phenotype.

Researchers have used an Encephalomyelitis Autoimmune Experimental model to study several aspects of human multiple sclerosis (MS), including the key factors of: neuroinflammation, peripheral inflammatory infiltrates, demyelination in white matter, and axonal injury and axon degeneration [2, 7]. The EAE model is characterized by progressive paralysis, followed by a type of recovery: full or partial, which can then turn to a relapse in disease, or chronic disease, which depends on the antigen used [8]

## 1.1 Research Significance and Innovation

The biomaterial approach used herein has a high potential to provide an immunological breakthrough in immunomodulation therapies, using encapsulated tolerogenic dendritic cells. The unique aspect used here include designing a biomaterial system that incorporates IL-10, and DC10s that induce tolerance through the tolerogenic DCs, through conditioning T cells, macrophages, and other dendritic cells being recruit to the hydrogel injection site at the cervical lymph nodes (cLNs). The use of FDA approved PEG have been reported to not have any local or systemic toxicity when degradation occurs, with minimal inflammation, and 96% urine clearance in approximately 24 hours [9]. In general, this biomaterial system that has tolerogenic properties, has been used as a model for cell-delivery scaffolds for immunosuppressive applications, such as the use in islet transplantation for the treatment of type 1 diabetes(T1D), or other autoimmune related diseases. The use of an EAE model allows for the biomaterial system to be tested in an autoimmune situation, and test the limits of the system, and see benefits the system has on the disease score.

# CHAPTER 2. SPECIFIC AIMS

Research objective: The main research thesis objective was to understand the immunosuppressive nature of the DCs *in vivo*, and their *in vitro* migration. Research experimental design focused on the differing murine model treatment groups, and the cells immunosuppressive status. These factors control the clinical scores of the mice as well as the body weight. Additionally, immune characterization is fundamental in determining properties such as maturation status of DCs, which is key for migration of the DCs. Furthermore, experiments aimed at characterizing hydrogels post-injection into mice were evaluated, through H&E staining, Masson Trichome stain. All of these are key indicators for an appropriate treatment for multiple sclerosis through the incorporation of immunosuppressive DCs.

## 2.1 Specific Aim

To understand the immunosuppressive nature of DCs encapsulated in PEG-4MAL:HS-IL-10 (PEG-4MAL-bound with IL-10 with free thiol) for the treatment of multiple sclerosis as well as the viability of pretreated DCs after travel. Treatment groups were dictated by either encapsulating DC10s in the PEG-4MAL:HS-IL-10, or injecting hydrogel without DCs into the EAE murine model. Pretreated DCs were shown to have a comparable viability to pretreated DCs that had not endured travel and the treatment of DC10s, PEG-4MAL:HS-IL-10 showed significant results compared to mice with no treatment, and the hydrogel on its own also showed significant results part way through the experimental timelines.

### *2.1.1 Significance and Approach*

This aim sought to investigate the biomaterial system using a murine mouse model of multiple sclerosis, as well as DC viability as they can be activated through mechanical stimulation, which may be caused by travel. Bone-marrow derived dendritic cells were cultured at Georgia Tech, due to limited access to lab space at Augusta University, while the EAE model was induced at Augusta University, in collaboration with the Narayanan lab, due to their expertise in the EAE model including induction, as well as scoring and caring for mice. Dendritic cells treated with immunosuppressive stimulates or maturation stimulate and on day 7 DCs were placed into flask and filled with completed BMDC media to lessen the possibility of mechanical stimulation and arranged in a Darwin Chamber to limited shift of the flasks. 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 and analyzed at Augusta University on a comparable machine to the machine used at Georgia Tech, while the other half of the cells cultured were prepared and analyzed at Georgia Tech prior to travel. The PEG hydrogel was modified with cell-adhesive peptide RGD, and degradable peptide crosslinker VPM to facilitate DC with interaction with the hydrogel, and the host remodeling for immune cell trafficking in and out of the hydrogel and into the mouse. Poly (ethylene glycol)- 4 maleimide reagents were prepared and pHed at Georgia Tech prior to travel and placed in the Darwin Chamber with DCs for travel prior to EAE induction.

At Augusta University, EAE induction was completed simultaneously injection, depending on the experimental group. For the following 28 days the clinical scoring and body weight of mice was checked daily, by Augusta University collaborators, specifically postdoctoral student, Dr. Liu Fang. The biomarkers of cells were analyzed after hydrogel, lymph node, and spleen removal through flow cytometry, and lymph node sections were analyzed through cryosections and histological staining.

## 2.2 Specific Aim 2

To determine the optimal treatment regime to derive DCs which are immunosuppressive and either migratory or non-migratory and investigate the importance of this cell phenotype attribute on biomaterial system efficacy.

### *2.2.1 Significance and Approach*

This objective aims at determining which immunosuppressive treatment (IL-10, vitamin D3 (VD3), or dexamethasone (DEX)) and maturation stimulus (peptidyl glycan or lipopolysaccharide) conditions the DCs to the proper phenotype for immunosuppressive and migratory vs. immunosuppressive and non-migratory cells. Previous efforts by the lab have been dedicated in studying material degradation, stiffness, as well as degradability, while also studying materials that act as adjuvants to DCs [1, 10 , 11] . These studies have produced promising results in the development of a biomaterial system for DC immunosuppression and function. Experiments here build on laboratory preliminary studies conducted to assess the migration of DCs, immunomodulatory and maturation stimulus, and hydrogel release rates. The results obtained from this aim will elucidate the migratory abilities of DCs, and the efficacy of DCs to exert immunomodulatory ability directly on host cells.

The hypothesis for this aim is that immature dendritic cells will not migrate well, while 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 Development and Function of Dendritic Cells*

Dendritic Cells (DCs) are referred to as antigen presenting cells that have essential mediators of immunity and tolerance. The name of the cells is based upon their dendritic-like extensions, which gives DCs are structural advantage, as it creates a greater cell surface area for enhanced antigen sampling. The development of DCs starts with hematopoietic progenitor cells in the bone marrow, and then differentiates into two major classes: conventional/classical DC (cDC) or plasmacytoid DC (pDC) progenitors [12].

Both monocyte and DC lineages originate from the same progenitor, called the monocyte and dendritic cell progenitor (MDP). The two cell types differentiate when MDPs give rise to committed Dc progenitors (CDPs) and monocytes in the bone marrow. The CDPs give rise to pre-DCs, which end in the lymphoid and non-lymphoid tissues after traveling from the bone-marrow. The differences in DCs come from their cell-surface markers, anatomic location, and function which determine the fate of the T cells they activate [13, 14]. DCs are the bridge between the innate and adaptive immunity, and generally follow a life cycle that involves differentiation into immature DCs(iDCs), infiltration into peripheral tissues, sampling the environment for antigens, phenotypic maturation in response to a pathogen, migration to proximal LNs, and conditioning effector cells until death [15, 16]. DCs have mostly been used in applications of vaccines and immunotherapies as they have a plastic and robust nature to tailor the immune system for immunosuppression and immunoactivation when related to areas if autoimmunity therapies.

### *3.1.2 Immune Responses by DCs*

Ralph Steinman was celebrated for the identification of DCs in 1973 and awarded the Nobel Prize in 2011 for the discovery, that DCs were an important part of the humoral immune response [17]. Anitgen-presenting cells (APCs) have a primary purpose of phagocytose of proximal antigens, process, and present antigens on major histocompatibility complexes (MHC) to effect other immune cells such as T cells, B cells, and other immune cell types, which is characterized by their unique capacity to initiate primary immune responses and stimulate naive T cells [18]. 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 [19].

Natural killer (NK) cells and DCs play vital roles in antiviral immunity through shaping the quality of adaptive immune responses to viruses, and once their function is jeopardized, advanced immune issues are observed [20].

Through exposure to activation signals: pathogen-associated molecular patterns (PAMPs) and damaged-associated molecular patterns (DAMPs) *in situ* the resting phenotypes of DCs known as the immature state are activated through their surface and intracellular receptors [21]. Downregulation of antigen-capture activity with increased expression of surface MHC class II molecules and enhanced antigen processing and presentation shows the activation of DCs, which results in increased levels of chemokine receptors [22].

## 3.2 Dendritic Cell Responses to Biomaterial Systems

### *3.2.1 Biomaterial Adjuvant Effect and Receptor Signalling*

Biomedical polymers research has recently been key in the field of tissue and regenerative medicine with groundbreaking studies and novel applications on the market. Biomaterial immunomodulation strategies today leverage on immune cell-biomaterial interactions for optimal effectiveness of immunotherapies. Dendritic cells are known to adjust their immune function, based on direct recognition of PAMPs on biomaterials through PRRs [23], which results in immunoactivation in various circumstances such as in vaccination or in transplantation, with the former being desirable. Biomaterials are also known to induce host a complex biological reaction called foreign body response (FBR) [24]. This response is characterized by local inflammation (acute and chronic) with the formation of a fibrotic capsule around implant [25]. Biomaterials can regulate dendritic cell adhesion through integrin-dependent mechanisms, which can impact the DC function and phenotype. Immature DCs can detect antigens through expressing a various array of Toll-like receptors (TLRs) signaling pathways, which is characterized by low levels of MHC I and II [26]. After interacting with pathogens, iDCs are activated to a mature DC (mDC) phenotype which migrate to the lymph nodes stimulate antigen specific CD4 or CD8 naïve T cells, to initiate specific immune responses accordingly [27].

### *3.2.2 Effect of Biomaterial Mechanical Characteristics on Embedded Cells*

The selection and biomaterial processing can significantly impact the DC maturation process, with considerations of the polymer size, biomaterial shape and charge [28]. The function and viability of DCs can be significantly impacted by biomaterial selection, considering the mechanical properties. These properties can modulate embedded DCs during the delivering of DCs in three-dimensional biomaterial scaffold, as seen in recent studies using both natural (chitosan, alginate, and gelatin) and synthetic (poly (lactic-co-glycolic acid) PLGA, poly (ethylene glycol) (PEG)) biomaterials for a variety of immunomodulating applications and system's delivery. The PEG-4MAL biomaterial system used in this thesis, has been shown to be able to be engineered to promote tolerance and be non-activating towards immune cells. The larger mesh size of the PEG-4MAL hydrogels allow for the diffusion of more growth factors and facilitated better cell adhesion compared to a smaller mesh size, which in turn increases cell viability and function [29]. The mesh size of PEG-4MAL hydrogels is directly dictated by the polymer weight percentage, which decreases with increases polymer weight percentage [29]. Aggressive handling of DC cultures, such as physical disturbances to culture plates, and agitation of hydrogel embedded DCs can result in DCs with activated phenotypes, like controls as recently shown [30]. Lastly, the biophysical and biochemical properties of the biomaterial environment are keys in the cellular differentiation, viability, morphology, and function which is mediated by the extracellular matrix (ECM) interactions, such as cellular attachment and spreading [31]. It should be noted that for immunosuppressive, tolerogenic DCs, a non-activating biomaterial needs to be used to embed the DCs to be delivered as the cells need to be non-activated/immature for them to maintain a tolerogenic or immunosuppressive phenotype.

### 3.3 Dendritic Cell Delivery in Biomaterials

The use of a biomaterial system to deliver DCs has advantages over traditional methods of delivery. Biomaterials can serve as scaffolds for cell and tissue regeneration, as they have highly tunable properties, such as surface modality, porosity, and surface charge. Biomaterials also enable access to specific compartments of the immune system, to locally deliver small-molecule or protein-based drugs.

Dendritic Cells interact with biomaterials through pattern recognition receptors, by recognizing “biomaterial-associated molecular patterns” and from this they generate a battery of immune responses. For instance, the use of dexamethasone(DEX) , which is in the corticosteroid family is known to have anti-inflammatory effects [32] . Whereas peptidoglycan (PGN) is a pattern recognition receptor (PRR) antagonist, which is a maturation factor that is known to enhance lymph node migration of tolerogenic DCs [32].

### 3.4 Migration of Dendritic Cells

Dendritic cells have been shown to have different migratory abilities depending on their activation status. Immature DCs have a limited migratory ability, while mDCs have been shown to have increased migratory abilities. Immature DCs specialize in the sampling of foreign and self-antigens using antigen acquisition, which includes receptor dependent endocytosis and micropinocytosis [33]. The interaction of CC-chemokine ligand receptor 7 (CCR7) with ligand CC-chemokine ligand CC21(CCL21), which are found on migrating DCs and lymphatic endothelial cells respectively, which guides the DCs into the lumen of initial lymphatic vessels through a process called haptotaxis [34].

Alternatively active immunosuppressive and migratory DCs can also be obtained through a TLR ligand after immunosuppressant treatment, which has been correlated with improved *in vivo* performance which is likely due to enhancement of LN homing capabilities [35]. TLR agonists such as LPS(lipopolysaccharides), and

PGN(peptidoglycan) are known to increase DC migration to LNs through increasing expression of CCR7 [36-38]. Proinflammatory cytokines such as TNF-α have also been shown to increase DC migration, whereas immunosuppressive cytokines such as TGF-β have been shown to inhibit DC migration to LNs through lowering CCR7 expression [39].

## 3.5 Experimental Autoimmune Encephalitis (EAE) Model used to Demonstrate Human Multiple Sclerosis

The use of the EAE murine model has been used to demonstrate key pathologic features of human multiple sclerosis, and the progression of paralysis which is seen in clinical MS patients. Different models can be used based upon the antigen used, these models are known as chronic or relapsing and remitting [8]. For example, this murine model has been used to study the hypothesis that systemic inflammation accruing in autoimmune diseases can generate a disease-specific environment able to alter the functionality of tolerogenic DCs (tolDCs) [40]. While other studies have used this model to study the role of the sympathetic nervous system, or to optimize cell-based therapies shown to restore homeostasis in MS patients[41, 42]. The EAE murine model provides information of the little understood pathways and immune responses that occur in multiple sclerosis patients.

# CHAPTER 4. AMMELIOTATION OF EXPERIMENTAL ENCHEPELOMYLITIS MURINE MODEL SYMPTOMS WITH SUBCUTANEOUS INJECTION OF PEG-IL-10 WITH EMBEDDED DC10S

## 4.1 Overview

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 in DCs [43]. Thus, the methods described herein aimed to conduct *in vivo* studies to characterize the effect of the PEG-IL10 biomaterial system with implanted DCs on the amelioration of EAE symptoms.

## 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) [43, 44] 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 min 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 min. For hydrogels incorporating DCs, cells were added to the RGD-functionalized PEG-4MAL solution prior to the addition of cross-linker [43]. 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.

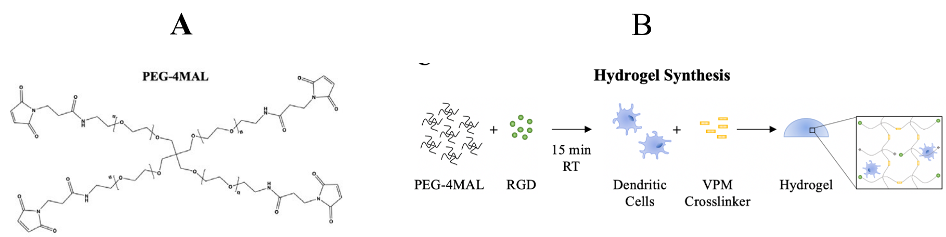


**Figure 1:PEG-4MAL hydrogel overview and synthetic schematic. A) PEG-MAL chemical structure, B) process of hydrogel synthesis [1]**

### *4.2.4 Characterization of Hydrogels-Rheology*

PEG-4MAL hydrogels were prepared as described in 3.2.4, 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 IL-10*

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 to 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 [45]. To visualize the PEGylated IL-10, reacted protein was immediately loaded onto polyacrylamide gels (4−12% Bis-Tris;Invitrogen) and placed into a Novex Mini-Cell (Invitrogen) which was run under nondenaturing conditions.

### *4.2.7 Embedding of DCs in Hydrogels*

PEG-4MAL hydrogels were prepared as previously described in 3.2.4 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 min. 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 PEG-4MAL

Mice will be subcutaneously injected with BMDCs encapsulated within a liquid hydrogel to form a crosslinked gel *in situ*. Mice will be injected with a total of 100 μL of PEG-4MAL:HS-IL-10 with encapsulated DC10s using a 26-guage diameter needle. Administration of the injections will be on the scruff of the neck. The needle will be 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 will be retracted to verify that a vacuum is created, and no blood is aspirated. The smallest diameter needle possible will be used for the PEG-4MAL hydrogel to be delivered. All procedures will be 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). 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. Neurological deficits were assessed daily using a blind scoring method describe in [46]. 0, no disease; complete loss of tail tonicity; 2, partial hind limb paralysis (uneven gate of hind limb); 3, complete hind limb paralysis; 4, complete hind and forelimb paralysis; and 5, moribund or dead. 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.

### *4.2.10 Collection of Specimen*

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 will be degraded and then tested using flow cytometry, and the other hydrogel will be tested using histology procedure.

### *4.2.11 Hydrogel Digestion*

To recover cells from hydrogels, culture medium was removed, and 1 mL of collagenase II (2 mg/mL; Gibco) [43, Patterson, 2010 #5694, Darling, 2016 #5700]was added to each well of PEG-4MAL hydrogels, incorporating DCs. Hydrogels were incubated at 37 °C/5% $CO_2$ for 15 min. 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 4.2.14.

### *4.2.12 Spleen Digestion*

To recover cells from the spleen, PBS+ HEPES was removed, and the spleen was placed into a petri dish. 5mL of Hanks Balanced salt solution (HBSS) was added, and 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. EDTA was added at a concentration of 1mM for 5 minutes, to the solution after time had to stop the reaction. 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. Wash the cell suspension with cold PBS and collect final cell pellet through centrifugation at 400 x g and prepare cells for flow cytometry in 4.2.14.

### 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 conicals and incubated at 37 °C for 45 minutes. Every 5 minutes digestion was mechanically mixed using a pipette. Once lymph nodes are digested, digestion solution is immediately pipetted into a tube with 1mL FBS and 3 mL incomplete BMDC media to cease digestion. Strain cells through 70 µm stainer, and wash with PBS. Centrifuge at 400 x g for 5 min at 4°C. Resuspend in 2 mL 1X RBC lysis buffer and incubate for 5 min. on ice. Wash with 10 mL of PBS at 400 x g for 5 min. at 4 °C and proceed with flow cytometry 3.2.10.

### 4.2.14 *Flow Cytometry*

Dendritic cells were processed for flow cytometry using a method previously described with minor modifications [26, 47]. 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 min. 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 min 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 min before analysis to assess viability. DC samples were analyzed based on the gating strategy in Figure A.15

Cells used for *in vivo studies* ($2–5 \times 10^6$ cells/ml) were blocked for Fc-receptors (1:200, eBioscience 14-0161-86) for 15 min at 4 °C and then incubated with antibodies (Table 1) for 30 min 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 .

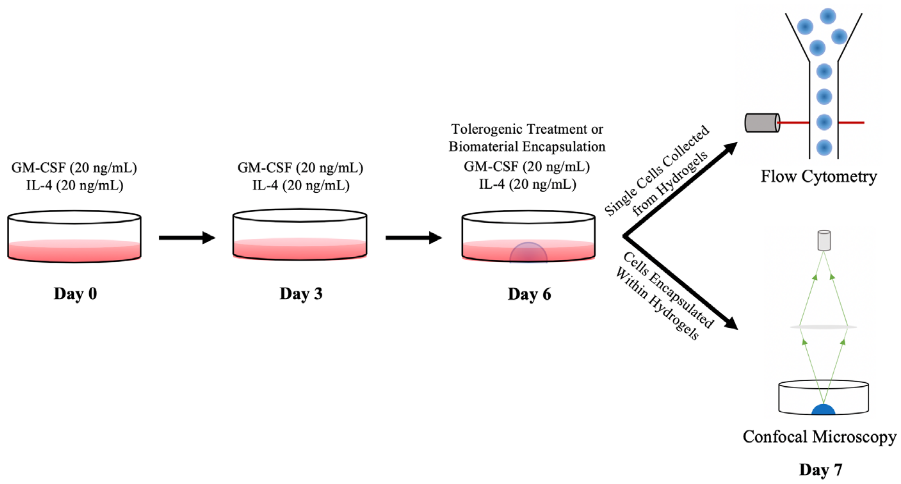


**Figure 2: Schematic of BMDC culture, BMDC treatment schedule, and analysis techniques**

*4.2.15 Chrome Alum-Gelatin Slide Preparation*

200 mL of DI water 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.

*4.2.16 Histology*

Samples were prepared through cryoprotection with sucrose and infiltration with OCT 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.

### *4.2.17 Hematoxylin and Eosin Staining*

Samples were stained in 200 µL hematoxylin solution for 3 min, 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 are then cleared in xylene for two changes of 10 minutes each. Dried sections were mounted with Permount (Fisher Scientific).

### *4.2.18 Masson Trichrome Staining*

Samples were stained with Weigert's hematoxylin (StatLab) for 10 minutes and dried without washing. Placed in alcoholic Picric acid for minutes, and quickly washed in distilled water for 3-4 seconds. Samples are then stained with acid crimson fuchsin solution for 4 minutes, and then washed in distilled water. Samples are placed in phosphomolybdic acid for 10 minutes and dried without washing. Samples are then stained with Masson's anyline blue (Statlab) for 5 minutes and then washed in distilled water. Samples are then cleared in xylene for two changes of 10 minutes each. Dried sections were mounted with Permount (Fisher Scientific).

### *4.2.19 Confocal 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.

### *4.2.20 Overall Experimental Approach for Augusta University studies*

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 stimulation 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, 4.2.14, 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 encapsulated into PEG-IL-10 hydrogel, depending on treatment group (Table 1 and Table 2). Hydrogel preparation of reagents was completed at Georgia Tech, the day prior to travel, following methods above, and pHed 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 postdoctoral student Dr. Liu Fang over the 28-day timeline, to ensure proper scoring and body weight measurements, and on day 28 samples were procured from mice of all groups for further analysis (4.2.10).

**Table 1: Treatment Conditions of mice. Designated based up cage assignment as Augusta 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 2: 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 front of side ear identification denotes the group mice were in. 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 |

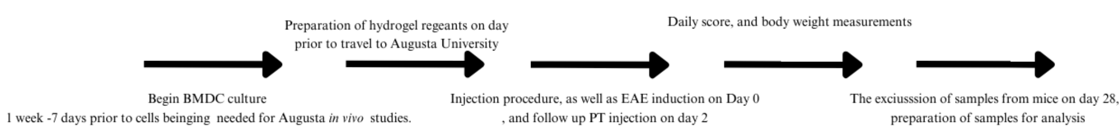


**Figure 3: Schematic of Augusta *in vivo* studies preparation workflow**

#### *4.2.21 Statistical Analysis*

Experimental values are 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 Retain Viability and Phenotype After Transported to Augusta University*

Beginning with the transportation of pretreated DCs cultured at Georgia Tech to Augusta University in flasks to be prepared for flow cytometry and analyzed on a comparable flow cytometer to Georgia Tech instrument of BDAria III. This was to ensure that the DCs could retain their phenotype prior to travel, as well as enough DCs remained viable through travel, to allow for further *in vivo* experiments with the DCs. Based on previous biomarker/surface marker analysis results of DCs at Georgia Tech, surface marker analysis completed at Augusta University showed comparable maturity in DCs as well, as other treatments (IL-10, TNF-α/IFN-γ, VD3, and iDCs). Based upon PI (propidium iodide) exclusion overall cell number in transport cells was shown to be lower than cells analyzed without travel and transported cells had a slightly larger number of cells that had died (Figure 4).

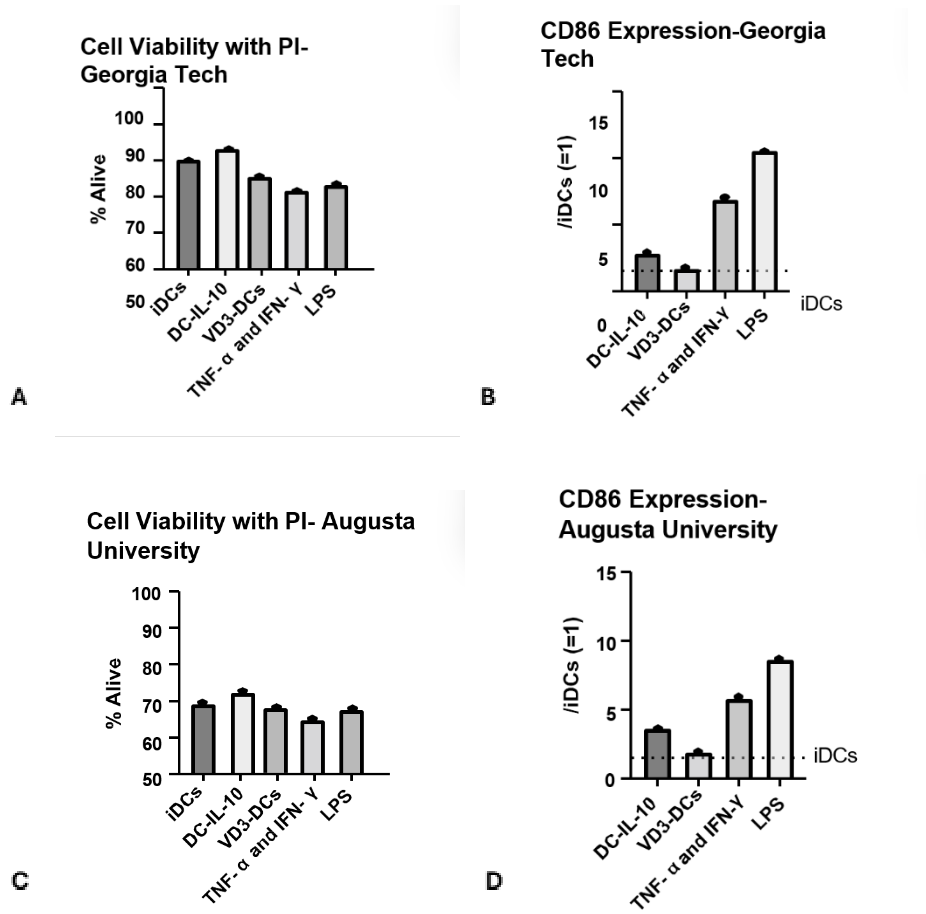


**Figure 4: Cell Viability and Phenotype of Pretreated Female BMDCs. Percentage of alive ells through PI exclusion, A) assessed at Georgia Tech prior to travel, B) assessed at Augusta University after 3 hours of travel. CD86 expression in DCs assessed by flow cytometry, C) at Georgia Tech prior to travel, D) at Augusta University after 3 hours of travel.**

### *4.3.2 Augusta Transport Simulation of Dendritic Cells*

Biomarker expression shown in simulation of the transport of cells to Augusta University in a Darwin Chamber was shown to give similar results to cells that made the trip shown in Figure 4. The simulation run at Georgia Tech had a higher percentage of alive cells in both cell that originated from male and female mice, while other biomarkers remained in the same range as results from cells that were analyzed prior to travel (Figure 5, Figure 6).

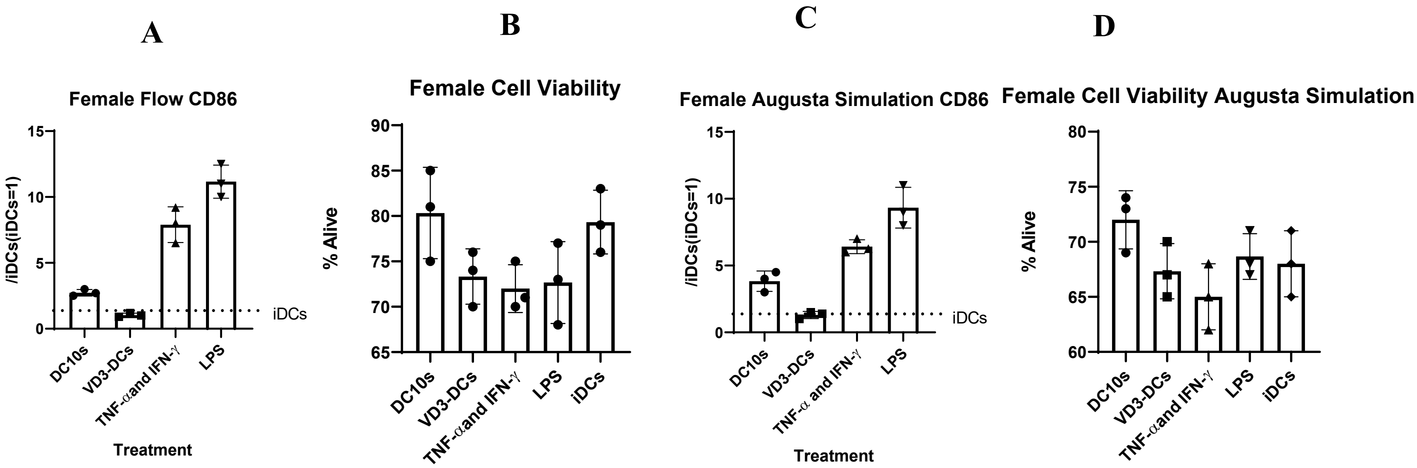


**Figure 5: Viability and Phenotype of Pretreat Female BMDCs. A) CD86 expression in DC assessed by flow cytometry cells after 3 hours in simulation of travel. B) percentage of alive cells after 3 hours in simulation of travel. C) CD86 expression in DC assessed by flow cytometry immediately. D) Percentage of alive cells immediately assessed. Number of experimental replicated, n=3.**

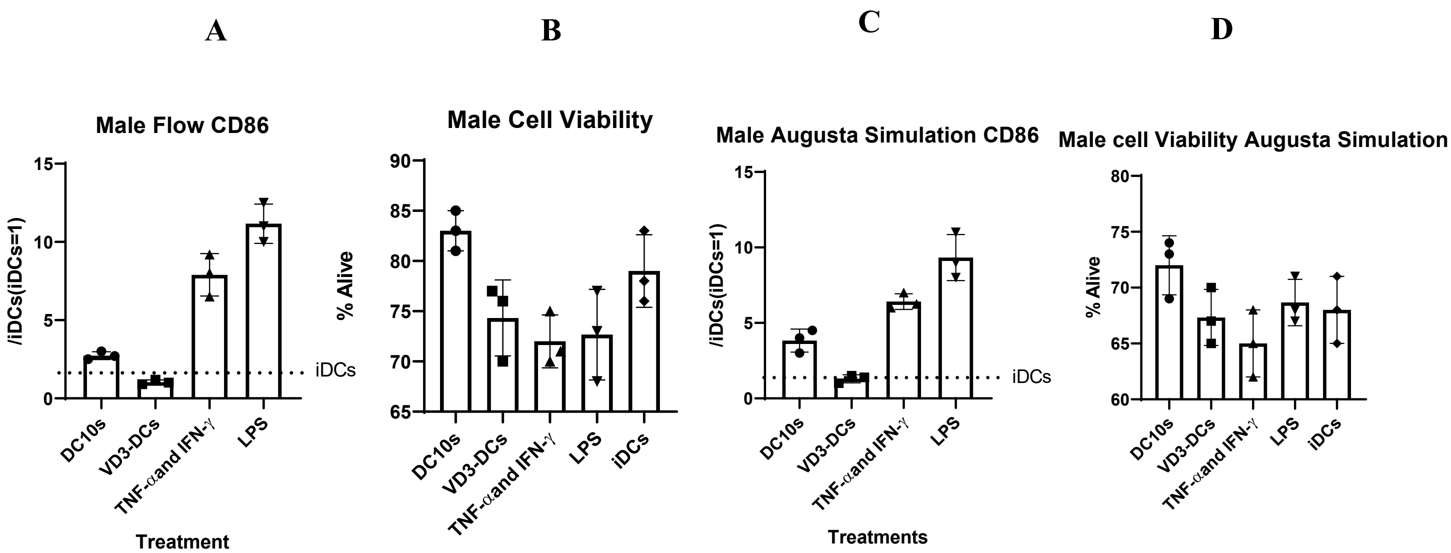


**Figure 6: Viability and Phenotype of Pretreat Male BMDCs. A) CD86 expression in DC assessed by flow cytometry cells after 3 hours in simulation of travel. B) percentage of alive cells after 3 hours in simulation of travel. C) CD86 expression in DC assessed by flow cytometry immediately. D) Percentage of alive cells immediately assessed. Number of experimental replicated, n=3.**

### *4.3.3 DC Transplantation into EAE Mice*

Dendritic cells derived at Georgia Tech that were pretreated with IL-10 and $MOG_{35-55}$ were encapsulated in hydrogel conjugated with thiolated Il-10 and injected into mice. The immunosuppressive DCs in the IL-10 hydrogel significantly lowered the EAE disease score, whereas treatment with just hydrogel had a slight effect on the EAE disease score (Figure 7). The late endpoint of the experiment of 28 days for the EAE murine model did not allow for many significant differences in surface markers to be observed through flow cytometry, but did shown that CDllb+ cells accounted for the largest cell population if endogenous immune cells observed in both the spleen and lymph node region, with a much larger percentage in the spleen, than the lymph nodes (Figure 8A and C). CD11c+ cells found in the receptive tissues are endogenous DCs, as CD11c+ is a marker for DCs, found in receptive tissues were further analyzed for biomarkers that are markers for the differing phenotypes of DCs, as well as expression markers of other immune cells that DCs may recruit to the area.

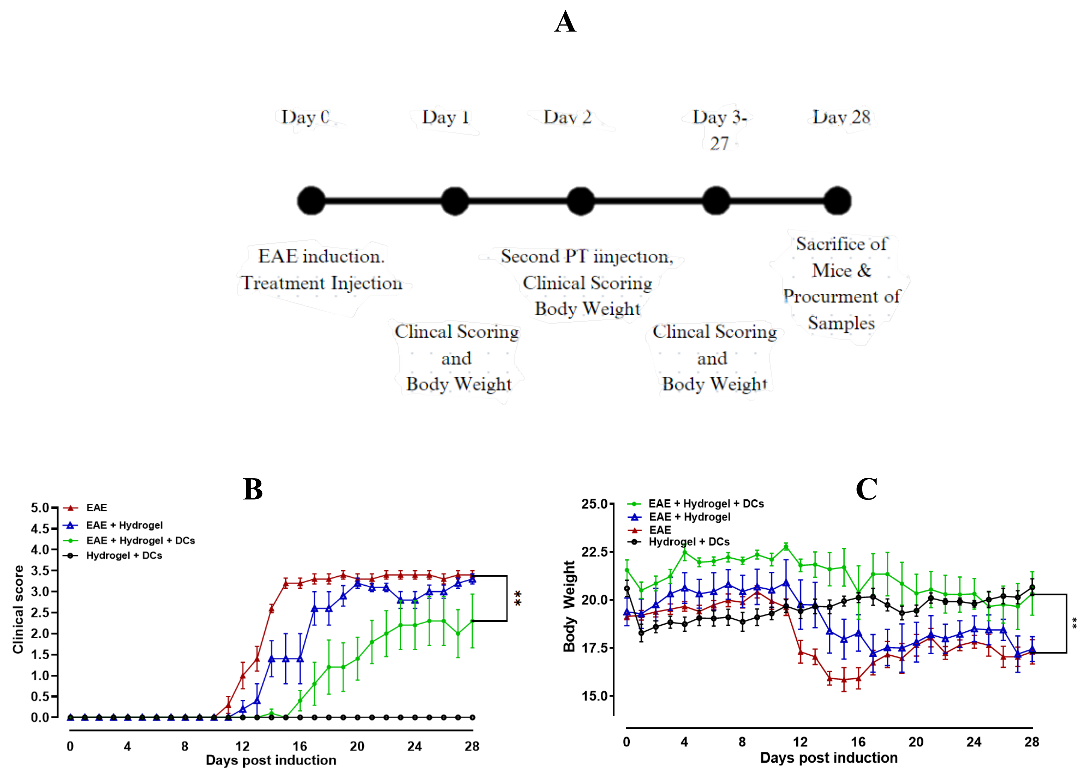


**Figure 7: EAE induction and evaluation of clinical scores. (A) Schematic representation of time points of EAE induction and DC-laden hydrogel injection and controls. (B) Animals were evaluated every day, and the clinical scores were recorded according to a 0-5 scale (see 4.2.9) upon delivery of DC10s implanted on hydrogel to cLNs. (C) Body weight of animals over 28-day period after DC10-laden hydrogels were delivered to the cLNs, and EAE induction. N=5 per group. No animals died or were sacrificed due to weight loss or EAE progression. Data is presented as mean +/- SEM. **P<0.05 via TWO-WAY ANOVA.**

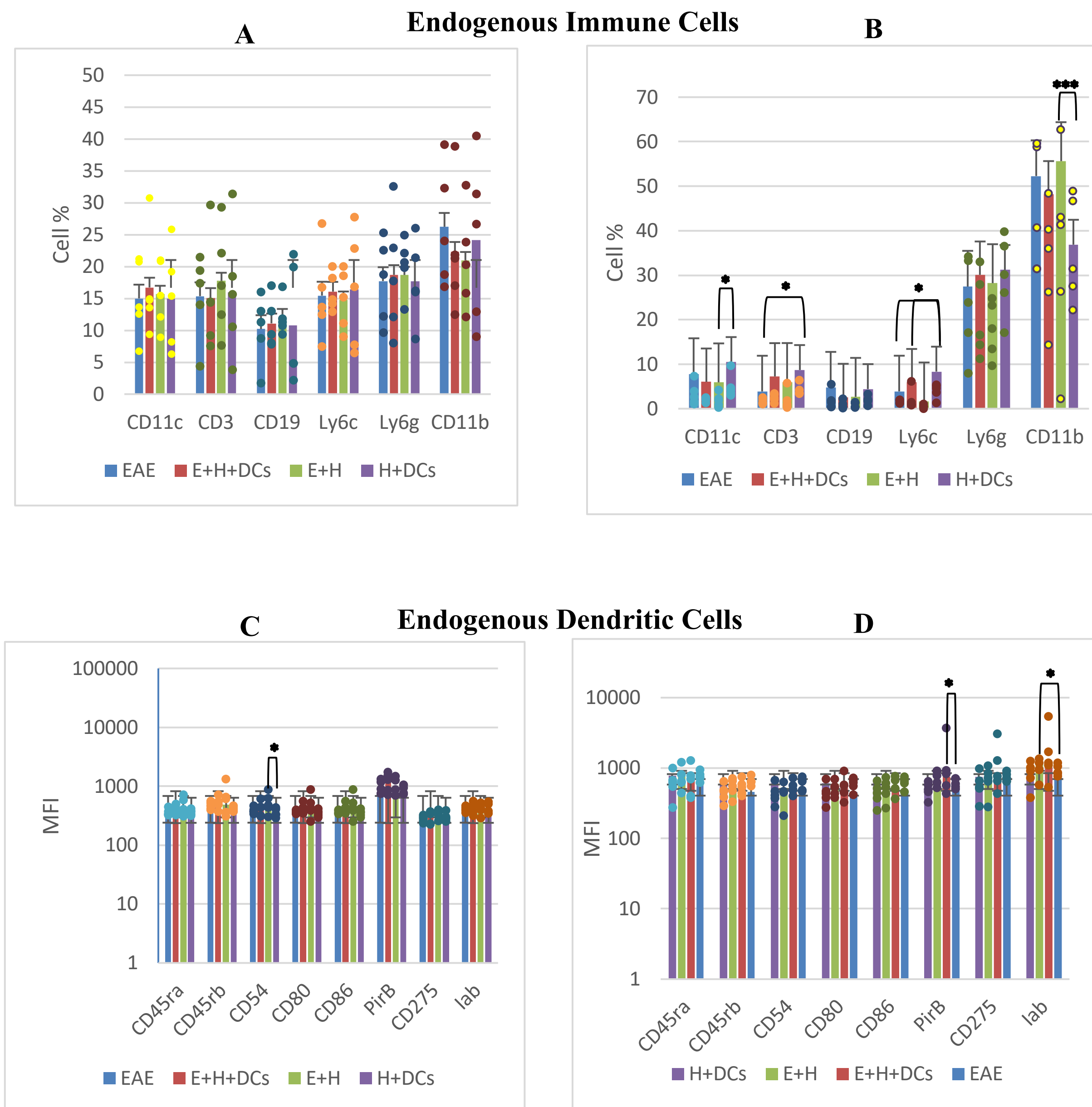


**Figure 8: Endogenous immune cell phenotype after DC10 delivery. Endogenous immune cell presence 28 days after injection (A) hydrogel injection site (B) spleen. Endogenous dendritic cell (DC) surface marker expression level (MFI) (C) hydrogel injection site (D) spleen. Number of replicates, n=5. Data is presented as mean +/- SEM. *p<0.1, ***p<0.01 via ONE-WAY ANOVA**

### *4.3.4 Histological Analysis of Implantation Site*

The characterization of the injection site by using H & E staining as well as Masson Trichrome, gave some insight to what may be going on at the injection site, but due to the late time point of 28 days, the information is limited. Tissue reactions are visible in the sections after staining, but corresponding remnants of the where the polymer was injected cannot be seen. Due to this we cannot be certain that the space observed is the area where the subcutaneous injection of hydrogel occurred (Figure 9).

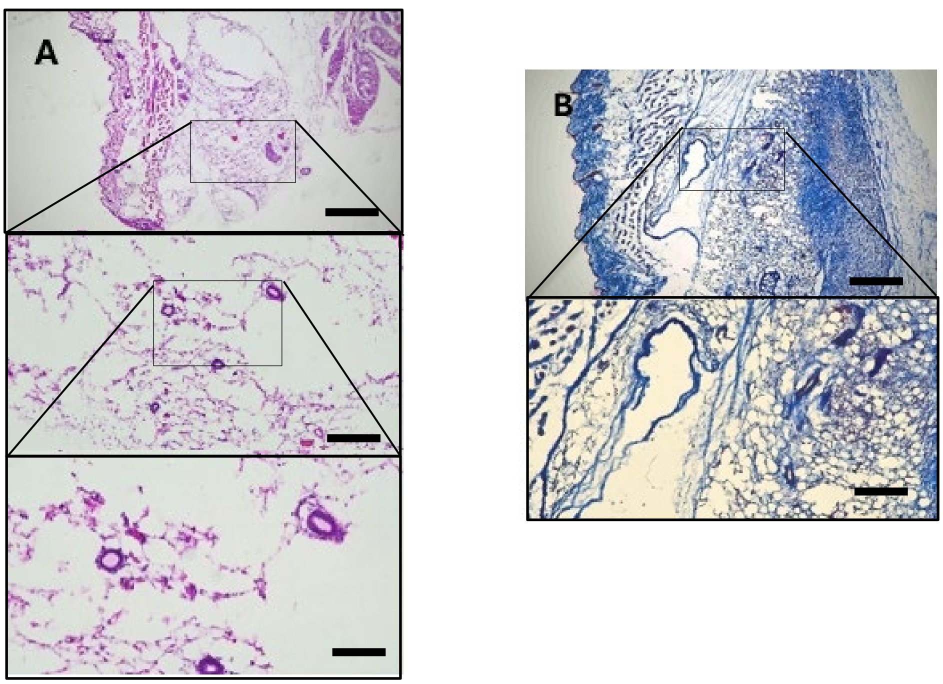


**Figure 9: Histological Staining of Lymph Node and Hydrogel Sample from Female Mouse treated with EAE model, and DC10s implanted in PEG-4MAL:HS-IL-10 hydrogel. (A) H & E staining of 10 µm thick section at 4x magnification, 10x magnification and 20x magnification. (B) Masson Trichrome staining of 10 µm thick section at 4x magnification and 10x magnification. Scale Bars= 100 µm**

## 4.4 Discussion

### *4.4.1 Transport of Pretreated Dendritic Cells does not Cause Mechanical Stimulation*

Dendritic cells are super-sensitive cells which can be activated to a mature phenotype with environmental disturbances such as mechanical stimulation, temperature fluctuations and some potentially unknown factors. The use of pretreatment in culture with BMDCs allows for the DCs to have a phenotype be presented on the cells before travel, which may have resulted in DCs resisting activation during travel. 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 with 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 result simulations of the travel to Augusta University were done by following the same preparation steps as the preparation for Augusta University experiments. Cells were driven around or carried for the 3-hour period in the Darwin Chamber and then prepared for flow cytometry and analyzed. This method proved to provide similar results to the actual travel to Augusta University, when done with both male BMDCs, and female BMDCs.

Based upon this investigation, the use of pretreating the BMDCs before travel to Augusta University limits the activation of DCs through mechanical stimulation, and cells remain viable for use in further experimentation.

### *4.4.2 Effect of PEG-4MAL:IL10 Hydrogel on Ameliorating Symptoms of EAE model*

The use of an EAE model to simulate multiple sclerosis is a well-established tool, that allows for potential treatments to be tested and modified for further investigation. The EAE model has multiple courses that it can take, such as relapsing and remitting, and long term or a chronic model which was used in this study. The use of PEG-4MAL in conjugation with thiolated IL-10 and embedded DC10s was found to ameliorate EAE symptoms, whereas EAE mice only treated with the PEG-4MAL:IL-10 hydrogel and no DCs showed minimal improvement in EAE clinical scores, (Figure 7).

One concern is that DC10s injected only were not used as a control in this study due to constraints with animal numbers per experiment.

### *4.4.3 Late Time points at Hydrogel Injection Site give Limited Information on Immune Cell Recruitment and Migration in Autoimmune Situation*

After the use of an EAE model to show the effectiveness of the biomaterial system of EAE clinical scores, the injection site, spleen, brain and spinal cord were collected to assess the immune cell recruitment to the specified area using flow cytometry and histological staining. Hydrogels and spleens from each treatment group were either digested for the use in surface marker analysis, not shown to have a significant difference between the treatment groups, at the day of animal sacrifice. Histological samples were stained with H& E or Masson's Trichrome, at the areas of interest but lacked definitive information of what may have been the hydrogel previously, and what areas were the lymph node, giving little information to the recruitment of immune cells to the area.

# CHAPTER 5. EFFECT OF VARIABLE STIMULI ON CCR7-INDUCED BMDC MIGRATION ASSAY

## 5.1 Overview

Tolerogenic DCs have been generated *in vitro* using a variety of immunomodulators, such as IL-10 [48-52], DEX [53-57], and VD3 [58-60], while DCs have also been generated *in vitro* using maturation stimuli such as LPS or PGN, as well as mechanical stimulation, or other changes in the environment of the DCs. Mature DCs have been shown to have increased migratory abilities compared to iDCs or tolDCs[11].

In this chapter, we describe the migration of DCs with variable stimuli combinations, as well as the migration of embedded DCs with variable stimuli in PEG-IL-10, and the time differential between embedded DCs vs. unembedded DCs.

## 5.2 Materials and Methods

### *5.2.1 Animals*

All animal procedures were conducted following procedures described in 4.2.1.

### *5.2.2 Transwell Migration Assay*

Dendritic cell migration was evaluated using a previously published method with some modifications [11, 23]. 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_(µL/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). BMDCs were detached using cell stripper (Corning) and washed with 2 mL of PBS and 220 x g for 4 min at 22°C. Cells were seeded into cell culture inserts at a concentration of 1 X $10^6$ cells/mL, and 1 mL per insert and incubated at 37°C/5% $CO_2$, for 3 hours. Media was carefully removed from cell inserts, and media was carefully aspirated from the bottom of the well plate. 1 mL of PBS pipetted into well plate. Transwell membranes were dried with 750 µL of cold methanol and incubated for 20 minutes at room temperature to dehydrate cells. After 20 minutes methanol is removed, and membrane is 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 is washed 2 times with PBS and imaged using an inverted microscope to count the migrated cells on the lower side of the membrane.

### *5.2.3 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_(µL/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). BMDCs were detached using cell stripper (Corning) and washed with 2 mL of PBS and 220 x g for 4 min 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. 1 mL of PBS pipetted into well plate. Transwell membranes were dried with 750 µL of cold methanol and incubated for 20 minutes at room temperature to dehydrate cells. After 20 minutes methanol is removed, and membrane is 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 is washed 2 times with PBS and imaged using an inverted microscope to count the migrated cells on the lower side of the membrane.

### *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 Transwell Migration Assay*

The migration of tolerogenic stimulus with LPS showed the best migration results for all treatments, and PGN (peptidoglycan) treated cells as had improved migration compared to DCs treated with only immunosuppressive stimulus, on both male and female mice. Cells treated with the same immunosuppressive stimulus, but differing maturation stimulus were not statistically significant from each other (Table A.4 and Table A.5). This 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 cocultured with a maturation stimulus.

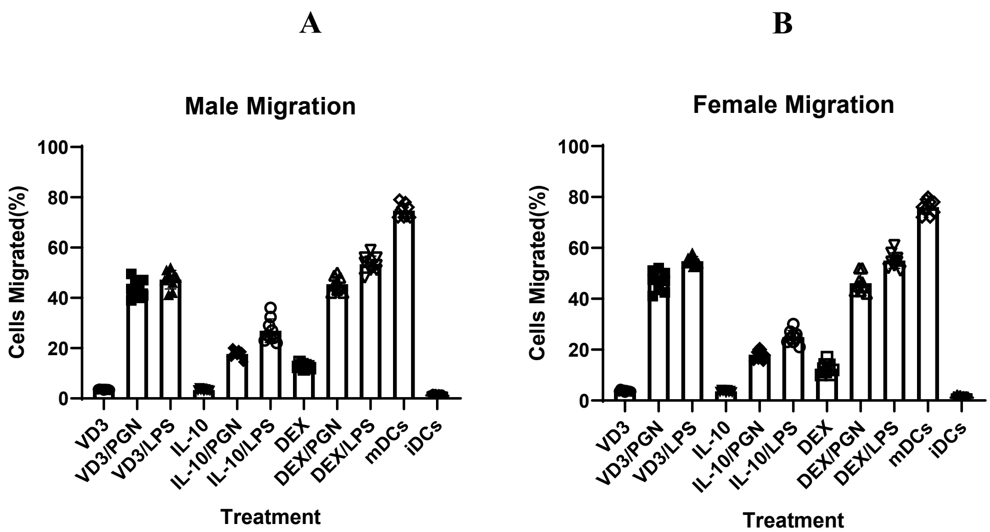


**Figure 10: Transwell Migration Assay of Dendritic Cells with differing tolerogenic and maturation stimulus in culture. (A) Male BMDCs transwell migration assay. (B) Female BMDCs transwell migration assay. Number of replicates, n=3, m=3, t=3 hrs**

### *5.3.2 Transwell Migration Assay Through Hydrogels*

The DCs embedded in hydrogels took much longer to migrate through the transwell membrane, and out of the hydrogel, with the original time for previous experiments being 3 hours. From the cells migrating out of the hydrogel after 3 hours there were no cells that had migrated through the membrane. After 6 hours cells had begun to migrate through the membrane, following a similar pattern to experiments without the hydrogels. The analysis done of experiments that were 12 hours and 24 hours long gave similar results to the 6 hours, with an increase in percentage of cells migrated for each treatment group, but the same groups having higher percentages as shown by comparison through Tukey tests having similar significance between treatments (Table A.6 and Table A.7). After 24 hours the percent migrated gives results like previous migration experiments without the hydrogel (Table A.8).

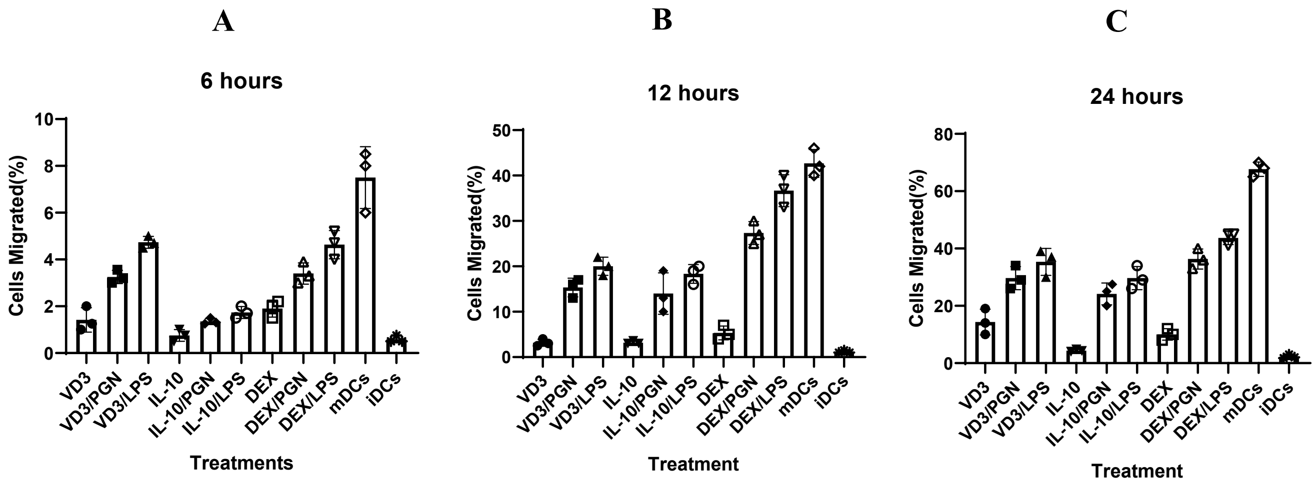


**Figure 11: Transwell Migration Assay of Dendritic Cells embedded in PEG-4MAL:HS-IL-10 with differing tolerogenic and maturation stimulus in culture. (A) Male BMDCs transwell migration assay with t=6. (B) Male BMDCs transwell migration assay with t=12. (C) Male BMDCs transwell migration assay with t=24. Number of replicates, n=1, m=3, t=3 hrs.**

## 5.4 Discussion

### *5.4.1 Effect of Maturation Stimulus of on Migration of Tolerogenic Dendritic Cells*

It is known that mDCs migrate better than iDCs. It was crucial to find a combination of a maturation stimulus and tolerogenic stimulus that can allow for the cells to maintain both characteristics, as once injected they will have to be able to migrate out of the hydrogel, as well as through the body. This is a key design parameter within the hydrogel system, which makes it essential to find the optimal combination of tolerogenic and maturation stimuli.

To assess the migration of DCs, tolerogenic stimuli, such as IL-10, was added in DC culture, and cocultured with maturation stimuli, such as PGN, and on day 7 a transwell migration assay was run so the effect of the stimuli could be compared to DC controls of mDCs, and iDCs. Dendritic cell migration was analyzed after 3 hours and DCs treated with DEX and PGN showed the highest percentage of DCs migrated with both a maturation stimulus and tolerogenic stimuli. Dendritic cells cultured with IL-10 showed a lower percentage of migrated cells without maturation stimulus, than with maturation stimulus, but compared to other combinations of immunosuppressive stimuli with maturation stimuli it has a significantly lower migration percentage. While experiments discussed in CHAPTER 4 in DC10s embedded in hydrogel, future experiments will explore different immunosuppressive stimuli, and variable combination treatments *in vivo.*

### *5.4.2 Dendritic cells implanted in PEG-IL10 Retains Migration Abilities*

*In vivo* the DC10s are implanted into PEG-IL10 and must be able to migrate out of the hydrogel as it degrades and be able to recruit other immune cell types to fight against the EAE symptoms, and progression. A potential concern is that not enough DCs being able to migrate out of the hydrogel and through the immune system. To test the migratory ability of the DCs, cells were treated with variable combinations of maturation stimulus and tolerogenic stimulus in culture and then implanted into PEG-IL10 hydrogels. At 3-, 6-, 12- and 24-hours cells were analyzed for the percentage of cells that had migrated across the membrane to the bottom side from the hydrogel that was polymerized on top of the membrane. At both 12 and 24 hours the percentage of cells migrated across the membrane has increased since pervious time points and were comparable to the cells migration when not implanted in a hydrogel. While the migration of the DCs took a longer period when implanted that DC could still reach the same migratory abilities as the hydrogel began to degrade.

Moreso, the extended period of release/migration of the DCs could show to be useful *in vivo* as other immune cells will be able to be recruited over a longer time.

# CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

## 6.1 Conclusions

The research described herein solidifies the potential of the DC10-laden hydrogel system to ameliorate symptoms of the EAE model as well as optimization of parameters for the treatment of DCs, through the combination of a maturation stimulus and a tolerogenic stimulus in culture. This work builds on previous research conducted in the Babensee lab and has shown earlier timepoints of DC-laden hydrogel injection into the flank and cLNs, as well as EAE studies done concerning only body weight and clinical score. Studies focused on feasibility of travel with pretreated DCs and analysis of DCs both prior to travel and after travel, as well as clinical scores of EAE mice with hydrogel treatment. The pretreated DCs were shown to maintain MFI values after travel, but flow cytometry results exhibited a higher percentage of dead cells within the suspension. Repeated simulation studies of travel showed similar results to actual travel with the DCs, but results showed that enough DCs survived to be implanted to hydrogels for future experiments. In section 3.3.3, preliminary studies of DC transplantation in an EAE model were conducted at Augusta University and showed that the treatment of DC10- laden PEG-4MAL:HS-IL-10 hydrogel ameliorated EAE symptoms in female mice when given simultaneously with EAE induction, shown through clinical scoring, as well as body weight, over the 28-day period. Therefore, overall experimental findings support the research hypothesis that the biomaterials systems implanted with DC10s can ameliorate EAE symptoms.

In section, 5.3.1 and 5.3.2 the migration of DCs were modulated and assessed through the manipulation of varying, maturation and tolerogenic stimulus pairings and DC controls.

Varying the maturation stimulus did not have a significant effect on cells treated with the same immunosuppressive , as just the addition of the maturation stimulus to the tolerogenic cells for the migration of the DCs as evaluated by visual quantification(Table A2, Table 3). Therefore, overall experimental findings support the research hypothesis that adding a maturation stimulus to tolerogenic cells will increase migration of DC both implanted in a hydrogel, and not implanted in a hydrogel.

### 6.2 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 with DC10s on ameliorating symptoms with the experimental autoimmune encephalomyelitis model and lowering clinical scores. Through characterization of surface markers on DCs in the spleen, as well as the lymph node provides findings on the therapeutic effects of DCs, and the fate of DCs implanted into an autoimmune situation. Additionally, the studies described here about dendritic cell migration provide fundamental findings on developing a biomaterial delivery system capable of having therapeutic effects, and the potential fate of DCs being implanted. These results are pivotal for future aspects of this research, as it presents potential for subsequent *in vivo* models, and other DC therapies to treat autoimmune diseases, such as type 1 diabetes(T1D), through clinical applications as well as in science.

### 6.3 Future Directions

The work provides a foundation for future collaborations with Augusta University and for the understanding of the initial immunological changes that may be occurring in the EAE model after treatment injection. The results of this thesis verify that previous results can be reproduced with new collaborations and obstacles. There are many promising next steps to this project, including studies focused on the earlier timepoints of the disease, and the immunological differences between treatment groups, as well as doing similar studies in normal(non-EAE) mice to examine the design parameters of the hydrogel system without autoimmune effects.

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. The delivering of DC10s subcutaneously to the cLNs and preforming histology and flow cytometry to assess the presence of CD86 as well as other previously used biomarkers. Samples shall be extracted at early timepoints such as days 1,3, 5, and 7, and possibly later timepoints if needed. The migratory ability of DCs will also be evaluated though the testing organs such as the spleen, and brain to address further questions of the therapeutic ability of the DCs, as well as possible addition of maturation stimulus in coculture, to increase the migratory abilities of tolerogenic DCs.

Additional studies will be conducted to evaluate the levels of IL-10, and potential toxicity of IL-10 concentrations in mice. Whereas previous projects have focused on loading amounts and optimizing the cell-biomaterial system in view of functionality, other physiological performance parameters may be impacted *in vivo*.

Lastly, the effectiveness of PEG-4MAL hydrogel system ameliorating symptoms with in the EAE model shall be evaluated for longer time points, such as 40- 60 days, to answer questions of a possible remission and relapse, or a second injection, of EAE injection as well as treatment, to see if their clinical scores will be changed. Studies will also be done using a therapeutic treatment, when disease onset begins, to better understand potential benefits of the hydrogel system if delivered later in disease progression.

# APPENDIX A.

## Supplemental to Aim 1

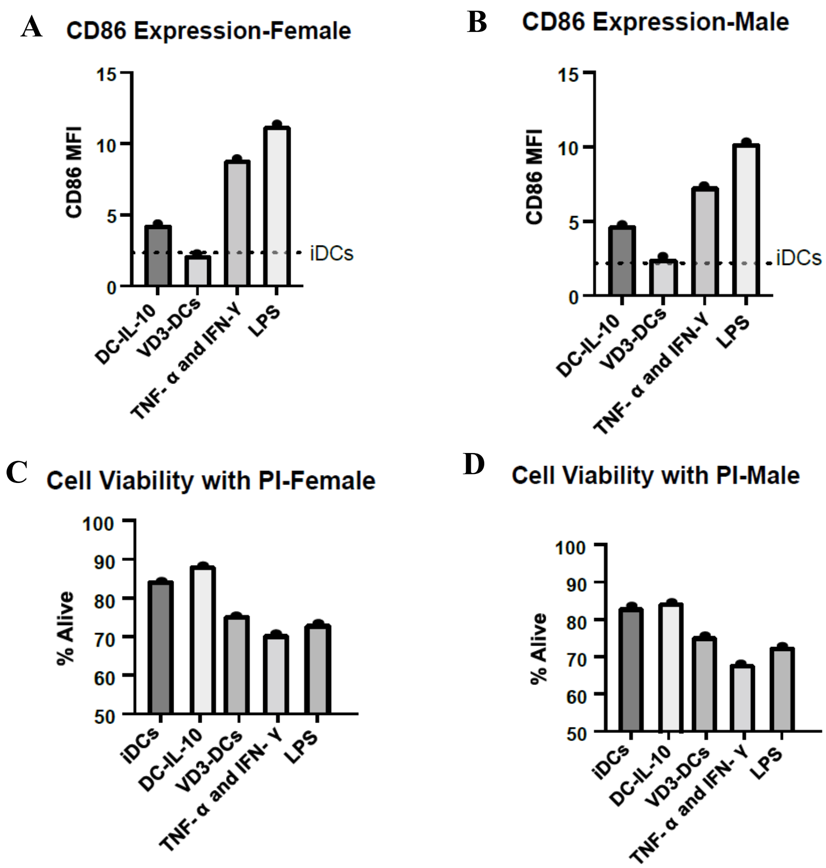


**Figure A.12: Flow Cytometry Replication Results on BMDC culture for verification of proper cell culture of DC viability and phenotype. CD86 expression in DC assessed by flow cytometry, (A) from female mice, (B) from male mice. Percentage of alive cells assessed through PI exclusion, (from female mice, (D) from Male mice. Number of experimental replicated, n=3.**

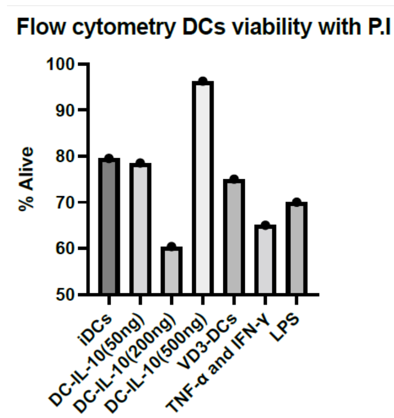


**Figure A.13: DC viability with variable concentration of IL-10. Replication of previous work in Babensee Lab to ensure proper BMDC culture, and cell viability with variable treatments, and IL-10 concentrations added in culture. Cell viability was assessed through PI exclusion using flow cytometry.**

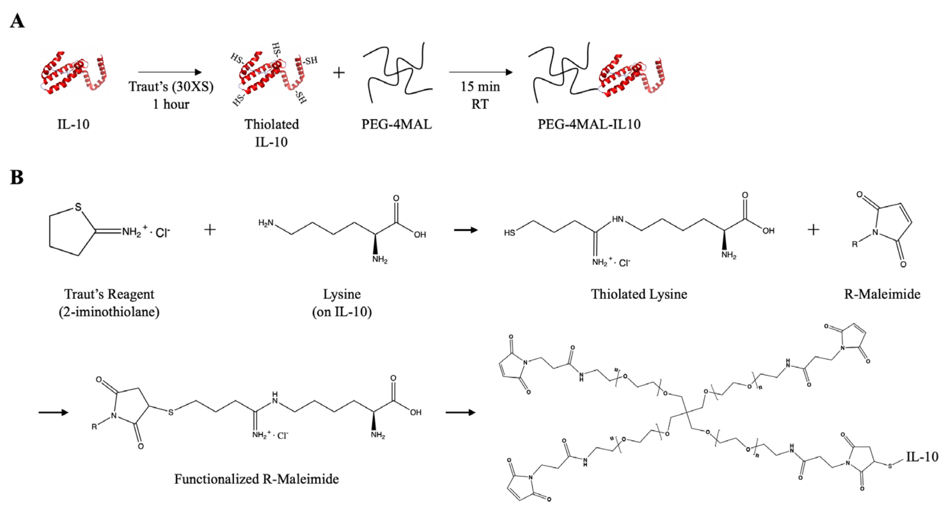


**Figure A.14: Schematic of functionalizing IL-10 onto the backbones of PEG-4MAL macromers. (A) visualization of tethering IL-10 to PEG-4MAL and (B) chemical structures and process of thiolation and PEGylation**

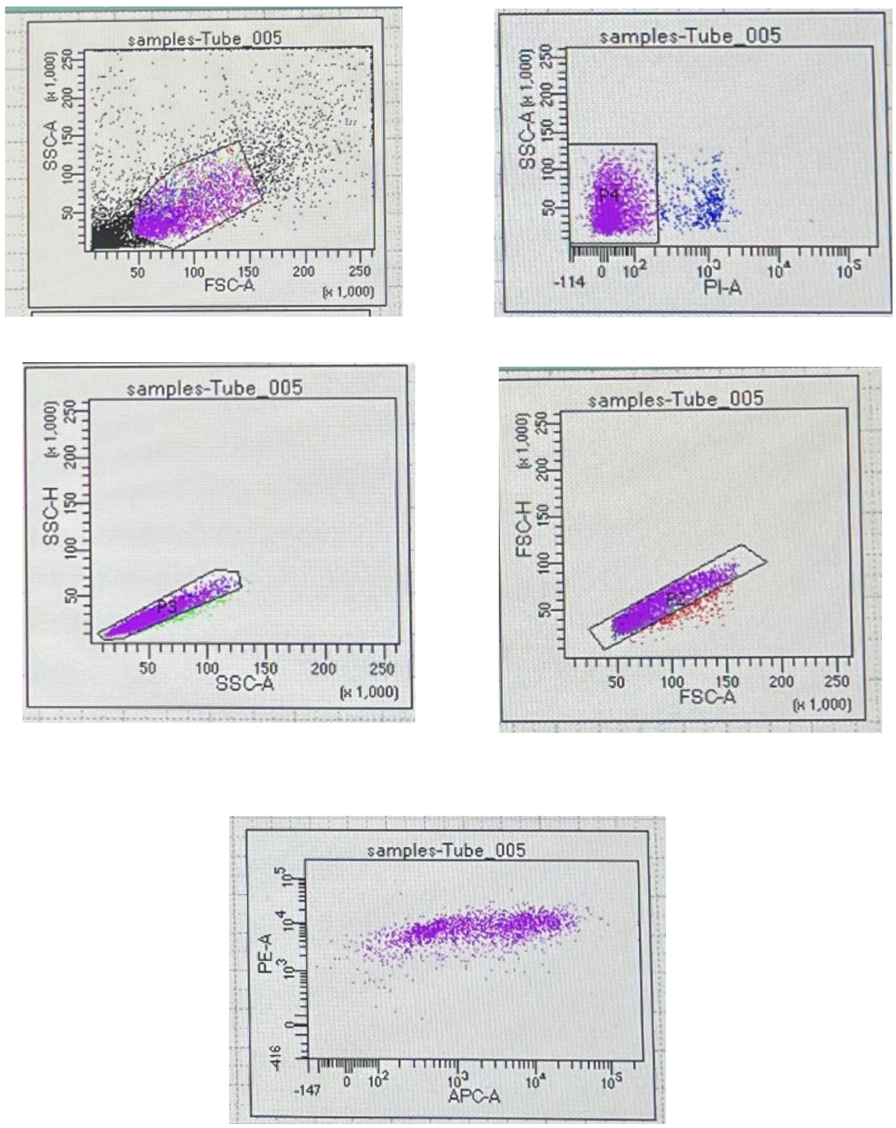


**Figure A.15: Flow Cytometry Gating Strategy for Dendritic Cells. Used for the identification of proper cell culture, and phenotype of cells.**

**Table A.3: Fluorophore Staining for Flow Cytometry Markers. Used in flow cytometry analysis done in CHAPTER 4 and CHAPTER 5.**

| Biomarker-Antibody | Application |
|---|---|
| CD11c-BV-785 | Marker for Dendritic cells |
| PDL1-PE | Marker for tolerogenic Dendritic cells |
| CD86-APC | Marker for mature Dendritic cells |
| CD11c | Activation marker for monocytes/macrophages |
| CD275 | Marker for APCs, B cells, monocytes, macrophages and DCs |
| CD45ra | Expressed on B cells and low on peripheral T cells |
| CD45rb | Expressed in higher density on peripheral B cells, T cytotoxic/suppressor cells |
| CD54 | Marker for T & B cells, macrophages, monocytes, granulocytes and DCs |
| CD80 | Expressed on DCs, monocytes and macrophages |

**Table A.3. continued**

| | |
|---|---|
| Ly6c | Macrophages |
| Ly6g | Bone Marrow Leukocytes |
| $Ia^b$ | MCHII alloantigen |
| PirB | Binds to various MCHI molecules |

**Supplemental to Aim 2**

**Table A.4: Female Transwell Migration Assay Experimental Significant. ****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 | ns | >0.9999 |
| VD3 vs. IL-10/PGN | **** | <0.0001 |
| VD3 vs. IL-10/LPS | **** | <0.0001 |
| VD3 vs. DEX | **** | <0.0001 |
| VD3 vs. DEX/PGN | **** | <0.0001 |
| VD3 vs. DEX/LPS | **** | <0.0001 |
| VD3 vs. iDCs | ns | 0.5359 |

**Table A.4 continued**

| | | |
|---|---|---|
| VD3 vs. mDCs | **** | <0.0001 |
| VD3/PGN vs. VD3/LPS | **** | <0.0001 |
| 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 | >0.9999 |
| VD3/PGN vs. DEX/LPS | **** | <0.0001 |
| VD3/PGN vs. iDCs | **** | <0.0001 |
| VD3/PGN vs. mDCs | **** | <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 |

**Table A.4 continued**

| | | |
|---|---|---|
| VD3/LPS vs. DEX | **** | <0.0001 |
| VD3/LPS vs. DEX/PGN | **** | <0.0001 |
| VD3/LPS vs. DEX/LPS | ns | >0.9999 |
| VD3/LPS vs. iDCs | **** | <0.0001 |
| VD3/LPS vs. mDCs | **** | <0.0001 |
| IL-10 vs. IL-10/PGN | **** | <0.0001 |
| IL-10 vs. IL-10/LPS | **** | <0.0001 |
| IL-10 vs. DEX | **** | <0.0001 |
| IL-10 vs. DEX/PGN | **** | <0.0001 |
| IL-10 vs. DEX/LPS | **** | <0.0001 |
| IL-10 vs. iDCs | ns | 0.6548 |
| IL-10 vs. mDCs | **** | <0.0001 |
| IL-10/PGN vs. IL-10/LPS | **** | <0.0001 |

**Table A.4 continued**

| | | |
|---|---|---|
| IL-10/PGN vs. DEX | *** | 0.0004 |
| IL-10/PGN vs. DEX/PGN | **** | <0.0001 |
| IL-10/PGN vs. DEX/LPS | **** | <0.0001 |
| IL-10/PGN vs. iDCs | **** | <0.0001 |
| IL-10/PGN vs. mDCs | **** | <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. iDCs | **** | <0.0001 |
| IL-10/LPS vs. mDCs | **** | <0.0001 |
| DEX vs. DEX/PGN | **** | <0.0001 |
| DEX vs. DEX/LPS | **** | <0.0001 |
| DEX vs. iDCs | **** | <0.0001 |
| DEX vs. mDCs | **** | <0.0001 |

**Table A.4 continued**

| | | |
|---|---|---|
| DEX/PGN vs. DEX/LPS | **** | <0.0001 |
| DEX/PGN vs. iDCs | **** | <0.0001 |
| DEX/PGN vs. mDCs | **** | <0.0001 |
| DEX/LPS vs. iDCs | **** | <0.0001 |
| DEX/LPS vs. mDCs | **** | <0.0001 |
| iDCs vs. mDCs | **** | <0.0001 |

**Table A.5: Male Transwell Migration Assay Experimental Significance. ****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 | ns | >0.9999 |
| VD3 vs. IL-10/PGN | **** | <0.0001 |
| VD3 vs. IL-10/LPS | **** | <0.0001 |
| VD3 vs. DEX | **** | <0.0001 |
| VD3 vs. DEX/PGN | **** | <0.0001 |
| VD3 vs. DEX/LPS | **** | <0.0001 |
| VD3 vs. iDCs | ns | 0.7244 |

**Table A.5 continued**

| | | |
|---|---|---|
| VD3 vs. mDCs | **** | <0.0001 |
| VD3/PGN vs. VD3/LPS | ns | 0.1873 |
| 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 | 0.9592 |
| VD3/PGN vs. DEX/LPS | **** | <0.0001 |
| VD3/PGN vs. iDCs | **** | <0.0001 |
| VD3/PGN vs. mDCs | **** | <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 |

**Table A.5 continued**

| | | |
|---|---|---|
| VD3/LPS vs. DEX | **** | <0.0001 |
| VD3/LPS vs. DEX/PGN | ns | 0.937 |
| VD3/LPS vs. DEX/LPS | *** | 0.0003 |
| VD3/LPS vs. iDCs | **** | <0.0001 |
| VD3/LPS vs. mDCs | **** | <0.0001 |
| IL-10 vs. IL-10/PGN | **** | <0.0001 |
| IL-10 vs. IL-10/LPS | **** | <0.0001 |
| IL-10 vs. DEX | **** | <0.0001 |
| IL-10 vs. DEX/PGN | **** | <0.0001 |
| IL-10 vs. DEX/LPS | **** | <0.0001 |
| IL-10 vs. iDCs | ns | 0.8606 |
| IL-10 vs. mDCs | **** | <0.0001 |
| IL-10/PGN vs. IL-10/LPS | **** | <0.0001 |
| IL-10/PGN vs. DEX | ** | 0.0083 |

**Table A.5 continued**

| | | |
|---|---|---|
| IL-10/PGN vs. DEX/PGN | **** | <0.0001 |
| IL-10/PGN vs. DEX/LPS | **** | <0.0001 |
| IL-10/PGN vs. iDCs | **** | <0.0001 |
| IL-10/PGN vs. mDCs | **** | <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. iDCs | **** | <0.0001 |
| IL-10/LPS vs. mDCs | **** | <0.0001 |
| DEX vs. DEX/PGN | **** | <0.0001 |
| DEX vs. DEX/LPS | **** | <0.0001 |
| DEX vs. iDCs | **** | <0.0001 |
| DEX vs. mDCs | **** | <0.0001 |
| DEX/PGN vs. DEX/LPS | **** | <0.0001 |

**Table A.5 continued**

| | | |
|---|---|---|
| DEX/PGN vs. iDCs | **** | <0.0001 |
| DEX/PGN vs. mDCs | **** | <0.0001 |
| DEX/LPS vs. iDCs | **** | <0.0001 |
| DEX/LPS vs. mDCs | **** | <0.0001 |
| iDCs vs. mDCs | **** | <0.0001 |

**Table A.6: 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 | * | 0.0106 |
| VD3 vs. VD3/LPS | **** | <0.0001 |
| VD3 vs. IL-10 | ns | 0.8845 |
| VD3 vs. IL-10/PGN | ns | >0.9999 |
| VD3 vs. IL-10/LPS | ns | 0.9994 |
| VD3 vs. DEX | ns | 0.9843 |
| VD3 vs. DEX/PGN | ** | 0.0049 |
| VD3 vs. DEX/LPS | **** | <0.0001 |
| VD3 vs. mDCs | **** | <0.0001 |
| VD3 vs. iDCs | ns | 0.685 |

**Table A.6 continued**

| | | |
|---|---|---|
| VD3/PGN vs. VD3/LPS | ns | 0.0644 |
| VD3/PGN vs. IL-10 | *** | 0.0003 |
| VD3/PGN vs. IL-10/PGN | ** | 0.0074 |
| VD3/PGN vs. IL-10/LPS | ns | 0.0531 |
| VD3/PGN vs. DEX | ns | 0.1158 |
| VD3/PGN vs. DEX/PGN | ns | >0.9999 |
| VD3/PGN vs. DEX/LPS | ns | 0.1027 |
| VD3/PGN vs. mDCs | **** | <0.0001 |
| VD3/PGN vs. iDCs | *** | 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 | 0.1266 |

**Table A.6 continued**

| | | |
|---|---|---|
| VD3/LPS vs. DEX/LPS | ns | >0.9999 |
| VD3/LPS vs. mDCs | **** | <0.0001 |
| VD3/LPS vs. iDCs | **** | <0.0001 |
| IL-10 vs. IL-10/PGN | ns | 0.9358 |
| IL-10 vs. IL-10/LPS | ns | 0.4703 |
| IL-10 vs. DEX | ns | 0.2684 |
| IL-10 vs. DEX/PGN | *** | 0.0001 |
| IL-10 vs. DEX/LPS | **** | <0.0001 |
| IL-10 vs. mDCs | **** | <0.0001 |
| IL-10 vs. iDCs | ns | >0.9999 |
| IL-10/PGN vs. IL-10/LPS | ns | 0.9973 |
| IL-10/PGN vs. DEX | ns | 0.9624 |
| IL-10/PGN vs. DEX/PGN | ** | 0.0034 |
| IL-10/PGN vs. DEX/LPS | **** | <0.0001 |

**Table A.6 continued**

| | | |
|---|---|---|
| IL-10/PGN vs. mDCs | **** | <0.0001 |
| IL-10/PGN vs. iDCs | ns | 0.7746 |
| IL-10/LPS vs. DEX | ns | >0.9999 |
| IL-10/LPS vs. DEX/PGN | * | 0.0256 |
| IL-10/LPS vs. DEX/LPS | **** | <0.0001 |
| IL-10/LPS vs. mDCs | **** | <0.0001 |
| IL-10/LPS vs. iDCs | ns | 0.2684 |
| DEX vs. DEX/PGN | ns | 0.0585 |
| DEX vs. DEX/LPS | **** | <0.0001 |
| DEX vs. mDCs | **** | <0.0001 |
| DEX vs. iDCs | ns | 0.1362 |
| DEX/PGN vs. DEX/LPS | ns | 0.1936 |
| DEX/PGN vs. mDCs | **** | <0.0001 |
| DEX/PGN vs. iDCs | **** | <0.0001 |

**Table A.6 continued**

| | | |
|---|---|---|
| DEX/LPS vs. mDCs | **** | <0.0001 |
| DEX/LPS vs. iDCs | **** | <0.0001 |
| mDCs vs. iDCs | **** | <0.0001 |

**Table A.7: 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 | *** | 0.0007 |
| VD3 vs. IL-10/LPS | **** | <0.0001 |

**Table A.7 continued**

| | | |
|---|---|---|
| VD3 vs. DEX | ns | 0.9874 |
| VD3 vs. DEX/PGN | **** | <0.0001 |
| VD3 vs. DEX/LPS | **** | <0.0001 |
| VD3 vs. mDCs | **** | <0.0001 |
| VD3 vs. iDCs | ns | 0.993 |
| VD3/PGN vs. VD3/LPS | ns | 0.4345 |
| VD3/PGN vs. IL-10 | *** | 0.0002 |
| VD3/PGN vs. IL-10/PGN | ns | 0.9998 |
| VD3/PGN vs. IL-10/LPS | ns | 0.8999 |
| VD3/PGN vs. DEX | ** | 0.0019 |
| VD3/PGN vs. DEX/PGN | *** | 0.0002 |
| VD3/PGN vs. DEX/LPS | **** | <0.0001 |
| VD3/PGN vs. mDCs | **** | <0.0001 |
| VD3/PGN vs. iDCs | **** | <0.0001 |

**Table A.7 continued**

| | | |
|---|---|---|
| VD3/LPS vs. IL-10 | **** | <0.0001 |
| VD3/LPS vs. IL-10/PGN | ns | 0.1476 |
| VD3/LPS vs. IL-10/LPS | ns | 0.9983 |
| VD3/LPS vs. DEX | **** | <0.0001 |
| VD3/LPS vs. DEX/PGN | * | 0.0383 |
| VD3/LPS vs. DEX/LPS | **** | <0.0001 |
| VD3/LPS vs. mDCs | **** | <0.0001 |
| VD3/LPS vs. iDCs | **** | <0.0001 |
| IL-10 vs. IL-10/PGN | *** | 0.0007 |
| IL-10 vs. IL-10/LPS | **** | <0.0001 |
| IL-10 vs. DEX | ns | 0.9887 |
| IL-10 vs. DEX/PGN | **** | <0.0001 |
| IL-10 vs. DEX/LPS | **** | <0.0001 |
| IL-10 vs. mDCs | **** | <0.0001 |

**Table A.7 continued**

| | | |
|---|---|---|
| IL-10 vs. iDCs | ns | 0.9921 |
| IL-10/PGN vs. IL-10/LPS | ns | 0.5351 |
| IL-10/PGN vs. DEX | ** | 0.0087 |
| IL-10/PGN vs. DEX/PGN | **** | <0.0001 |
| IL-10/PGN vs. DEX/LPS | **** | <0.0001 |
| IL-10/PGN vs. mDCs | **** | <0.0001 |
| IL-10/PGN vs. iDCs | **** | <0.0001 |
| IL-10/LPS vs. DEX | **** | <0.0001 |
| IL-10/LPS vs. DEX/PGN | ** | 0.0059 |
| IL-10/LPS vs. DEX/LPS | **** | <0.0001 |
| IL-10/LPS vs. mDCs | **** | <0.0001 |
| IL-10/LPS vs. iDCs | **** | <0.0001 |
| DEX vs. DEX/PGN | **** | <0.0001 |
| DEX vs. DEX/LPS | **** | <0.0001 |

**Table A.7 continued**

| | | |
|---|---|---|
| DEX vs. mDCs | **** | <0.0001 |
| DEX vs. iDCs | ns | 0.5872 |
| DEX/PGN vs. DEX/LPS | ** | 0.004 |
| DEX/PGN vs. mDCs | **** | <0.0001 |
| DEX/PGN vs. iDCs | **** | <0.0001 |
| DEX/LPS vs. mDCs | ns | 0.1476 |
| DEX/LPS vs. iDCs | **** | <0.0001 |
| mDCs vs. iDCs | **** | <0.0001 |

**Table A.8: 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 | *** | 0.0004 |
| VD3 vs. VD3/LPS | **** | <0.0001 |
| VD3 vs. IL-10 | * | 0.0281 |
| VD3 vs. IL-10/PGN | * | 0.0409 |
| VD3 vs. IL-10/LPS | *** | 0.0004 |
| VD3 vs. DEX | ns | 0.8587 |
| VD3 vs. DEX/PGN | **** | <0.0001 |
| VD3 vs. DEX/LPS | **** | <0.0001 |
| VD3 vs. mDCs | **** | <0.0001 |
| VD3 vs. iDCs | ** | 0.0063 |

**Table A.8 continued**

| | | |
|---|---|---|
| VD3/PGN vs. VD3/LPS | ns | 0.5794 |
| VD3/PGN vs. IL-10 | **** | <0.0001 |
| VD3/PGN vs. IL-10/PGN | ns | 0.6181 |
| VD3/PGN vs. IL-10/LPS | ns | >0.9999 |
| VD3/PGN vs. DEX | **** | <0.0001 |
| VD3/PGN vs. DEX/PGN | ns | 0.3612 |
| VD3/PGN vs. DEX/LPS | ** | 0.0012 |
| VD3/PGN vs. mDCs | **** | <0.0001 |
| VD3/PGN vs. iDCs | **** | <0.0001 |
| VD3/LPS vs. IL-10 | **** | <0.0001 |
| VD3/LPS vs. IL-10/PGN | * | 0.0138 |
| VD3/LPS vs. IL-10/LPS | ns | 0.5794 |
| VD3/LPS vs. DEX | **** | <0.0001 |
| VD3/LPS vs. DEX/PGN | ns | >0.9999 |

**Table A.8 continued**

| | | |
|---|---|---|
| VD3/LPS vs. DEX/LPS | ns | 0.1265 |
| VD3/LPS vs. mDCs | **** | <0.0001 |
| VD3/LPS vs. iDCs | **** | <0.0001 |
| IL-10 vs. IL-10/PGN | **** | <0.0001 |
| IL-10 vs. IL-10/LPS | **** | <0.0001 |
| IL-10 vs. DEX | ns | 0.5104 |
| IL-10 vs. DEX/PGN | **** | <0.0001 |
| IL-10 vs. DEX/LPS | **** | <0.0001 |
| IL-10 vs. mDCs | **** | <0.0001 |
| IL-10 vs. iDCs | ns | 0.9998 |
| IL-10/PGN vs. IL-10/LPS | ns | 0.6181 |
| IL-10/PGN vs. DEX | ** | 0.0011 |
| IL-10/PGN vs. DEX/PGN | ** | 0.0059 |
| IL-10/PGN vs. DEX/LPS | **** | <0.0001 |

**Table A.8 continued**

| | | |
|---|---|---|
| IL-10/PGN vs. mDCs | **** | <0.0001 |
| IL-10/PGN vs. iDCs | **** | <0.0001 |
| IL-10/LPS vs. DEX | **** | <0.0001 |
| IL-10/LPS vs. DEX/PGN | ns | 0.3612 |
| IL-10/LPS vs. DEX/LPS | ** | 0.0012 |
| IL-10/LPS vs. mDCs | **** | <0.0001 |
| IL-10/LPS vs. iDCs | **** | <0.0001 |
| DEX vs. DEX/PGN | **** | <0.0001 |
| DEX vs. DEX/LPS | **** | <0.0001 |
| DEX vs. mDCs | **** | <0.0001 |
| DEX vs. iDCs | ns | 0.1883 |
| DEX/PGN vs. DEX/LPS | ns | 0.2455 |
| DEX/PGN vs. mDCs | **** | <0.0001 |
| DEX/PGN vs. iDCs | **** | <0.0001 |

**Table A.8 continued**

| | | |
|---|---|---|
| DEX/LPS vs. mDCs | **** | <0.0001 |
| DEX/LPS vs. iDCs | **** | <0.0001 |
| mDCs vs. iDCs | **** | <0.0001 |

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