Viability of encapsulated adherent and non-adherent dendritic cells through the manipulation of PEG-4MAL hydrogel weight percent

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Viability of encapsulated adherent and non-adherent dendritic cells through the manipulation of PEG-4MAL hydrogel weight percent

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List of Symbols and Abbreviations

MS- multiple sclerosis

CNS-central nervous system

PNS-peripheral nervous system

BBB-blood brain barrier

DC-dendritic cell

MRI- magnetic resonance imaging

CSF-cerebrospinal fluid

APC-antigen presenting cell

EAE- experimental autoimmune encephalomyelitis

iDC- immature dendritic cell

PEG- polyethylene glycol

mDC-mature dendritic cell

tDC-tolerogenic dendritic cell

MHC-II- major histocompatibility complex II

Summary

In the United States alone, 400,000 people suffer from Multiple Sclerosis(MS), a disease in which the immune system attacks neurons in the central and peripheral nervous systems (CNS, PNS). Currently, no cure exists, and treatment methods often only mildly alleviate symptoms. Therefore, current researchers are finding new methods to treat the disease, specifically the role that tolerized dendritic cells (DCs) combined with varying biomaterials and cytokine delivery mechanisms play when exposed to an *in vitro* murine model. The research performed in this paper examined the morphology and the viability of adherent vs non-adherent DCs encapsulated in PEG4MAL hydrogel with varying weight percentages of the polymer. Confocal microscopy and flow cytometry were used to analyze cell viability. Moreover, tolerization of DCs with interleukin-10 (IL-10) was examined and the most promising method for cytokine delivery to DCs in a PEG hydrogel was investigated. It was found that adherent DC's have highest viability in lower weight percent PEG4MAL hydrogels compared to non-adherent DC's and that PEGylated IL-10 is the most efficient mechanism to introduce the cytokine to the DCs. Analyzing the thiolation of IL-10 was then the first step of this process, and results are underway. Future studies will optimize the characteristics of the hydrogel to allow for the greatest DC phenotype and viability. In turn, this can lead to an *in vivo* model and offer a more successful form of treatment for the disease.

Introduction

Multiple Sclerosis

It is estimated that 400,000 people suffer from MS in the United States while 2.5 million patients endure the disease worldwide.[1] MS is defined as an autoimmune disease in which the immune system attacks the CNS, specifically the myelin sheath, a fatty compound that protects the axon of nerve cells.[1] Not only does this decrease the speed in which signals travel through nerve endings, but it causes inflammation and scarring of the nerve tissue, a process which creates plaques.[1] Neurons are found throughout the entire body, so the entirety of the pathophysiology and the mechanism for the deterioration of myelin in different locations (CNS and PNS) is unknown. However, experts in immunology and neurology have suggested that one of the first locations of the lesions occurs in the BBB, which prevents certain substances from entering the

brain and spinal tissue of the CNS.[1] A mechanism for this demyelination process begins with the activation of myelin-reactive T-cells by antigen presenting cells, microglia, macrophages, or B cells (Figure 1). The T-cells produce adhesion molecules that allow for entry through the BBB. Once in the CNS, T-cells can produce a variety of pro-inflammatory molecules that promotes increase in both inflammation and the recruitment of various other inflammatory promoting cells. Moreover, free radicals and toxins, usually released to protect the body in an immune response, can cause further damage to the myelin sheath.[2] In light of this information, MS is considered to be a T-cell mediated disease; the activation of these destructive T-cells, however, are still widely studied.[3] One of the most commonly investigated antigen-presenting cells that dictates a majority of the scope of T-cell activation and participation in MS is the tolerized DC, which can suppress an immunological response by activating T-regulatory cells (see *Dendritic cells and MS)*.

Demyelination Mechanisms
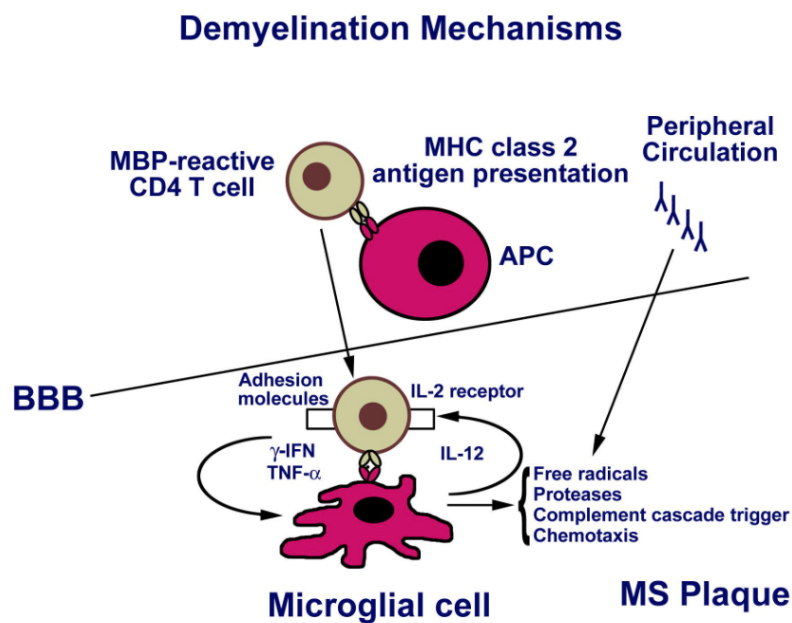
Figure 1: Image representing the potential mechanism of demyelination. There are various antigen-presenting cells (APCs); however, dendritic cells are widely studied because of their common involvement with T-cells[2].

Symptoms and Diagnosis

Because neurons and nerve tissues have varying functions, different symptoms can be experienced. For example, affected neurons associated with the vestibular system can cause dizziness while inflamed sensory or spinal neurons can cause chronic pain and tingling in 60% of patients (Figure 2). Lesions, scarring, and inflammation can occur in various areas of the body. Other common symptoms include double vision, fatigue, slurred speech, difficulty walking, and even depression.[4]
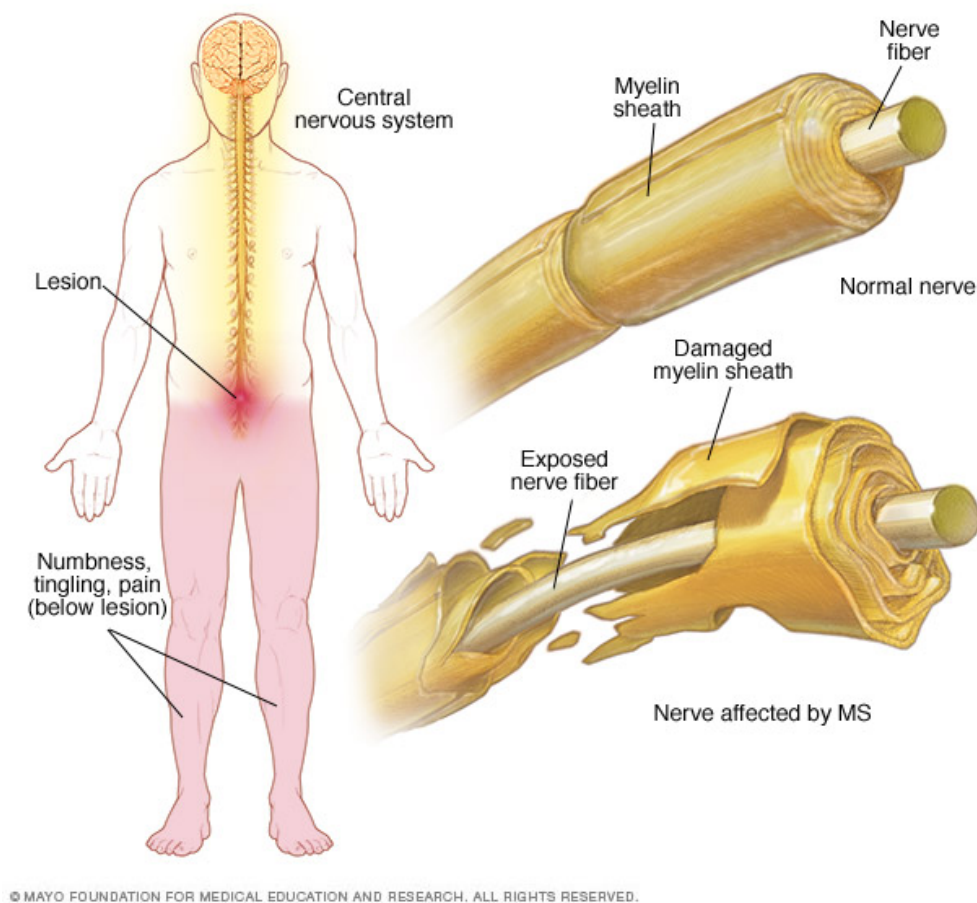
Figure 2: Representation of a symptom of pain and tingling experienced by a patient suffering from lesions in the lumbar and sacral nerves of the spinal cord.[3]

For some, symptoms are mild and manageable; however, for many, loss of bladder function and muscle tone cause serious complications and significantly decrease quality of life.

Therefore, proper diagnosis is often vital. Early diagnosis is rare due to the fact that the disease is not hereditary, as it is developed in patients over time. In order to properly diagnose MS, clinicians must use MRI technology and CSF analysis to find neuronal damage in two different areas of the body and provide evidence that the damage occurred different occasions.[5] After diagnosis, a treatment regimen is discussed, as there is no cure for the disease. Various treatments include therapies and drugs that modify and delay disease progression. Patients often alter medications due to ineffectiveness; therefore, novel forms of treatment and therapies are being widely studied to improve upon what exists on the market today.[6]

Dendritic Cells and MS

Common APCs found in the body and affect T-cell activation and function are known as DC's. DC's are known to boost primary immune function and induce immunological tolerance.[7] They link the innate and adaptive immune system by ingesting antigens and presenting potential harmful bacteria to T-cells via the cell surface.[8] Normal functioning DC's are vital to the proper functioning of the immune system, for they can activate, shape, and even prevent the harmful immune responses found in diseases such as MS.[7] However, studies have shown that patients with MS have abnormally functional DC's in their CSF.[9] These abnormalities are caused by miniscule changes in genetic information which can significantly compromise the body's ability to carry out an immune response and, according to recent findings, cause an immunoinflammatory response as found in MS.[10] A study by the International Multiple Sclerosis Genetic Consortium showed that genetic mutations can predispose patients to MS due to polymorphism while a study by Tailer et al. identified genetic mutations as a cause in the absence of certain subsets of DC's that are responsible for immunoregulation and producing interferons and cytokines that are used in current MS therapies.[11,12] Though aberrant DC's have shown to positively correlate with the progression of the disease, normal functioning DC's have shown to embody therapeutic properties as they can be tolerized to effect T-regulator cells to halt an immune response. Yogev et al. found that the extinction of DC's in the murine model of MS, known as EAE, was related to the advancement of the disease. Therefore, in order to find a more efficient therapy for the treatment of MS, we have turned to studying various methods of maintaining various DC viability (adherent vs. non-adherent) on various platforms.

Biomaterials and Dendritic Cells

A specific example of a platform utilized to investigate the viability and function of DC's is the *in vitro* manipulation and engineering of biomaterials as a platform for cell maturation and cytokine delivery (Figure 3).
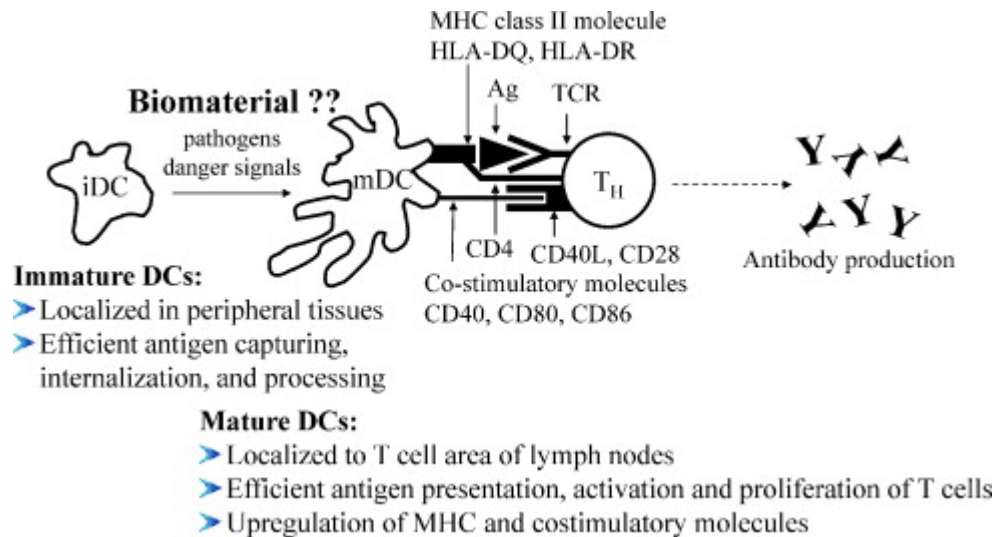
Figure 3: Descriptive image highlighting the use of biomaterials to allow for immature DC (iDC) maturation or tolerization. Eventually, allowing for antibody production and potential MS treatment.[14]

This is due to the fact that biomaterials, when designed with a compatibility specific to the cell, have shown to be agonists of maturation and an increased life span of the cell.[14] Cell compatibility of the material can be engineered by the manipulation of mechanical properties, weight percentage (wt%) of components, and varying cytokine and drug delivery mechanisms (see *Direct Conjugation* and *Microgel Delivery*). A common and extremely important cytokine delivered to DC's is interleukin-10 (IL-10).[15] IL-10 transforms the iDC's into tDC's which can then differentiate into T helper cells 1 and 2.[16,17,18] These cells allow for the secretion of antibodies from B cells as well as activate T regulatory cells to kill infected cells as seen with MS. Therefore, the successful delivery of IL-10 to iDC's on a properly chosen biomaterial platform is an extremely promising approach to a novel treatment for MS.

Depending on the strategy, biomaterials can induce maturation of cells or control it. In order to allow for the incorporation of cytokines such as IL-10, it is important to choose a biomaterial that suppresses the maturation of the iDCs so that it can be controlled. Materials such as PLGA and chitosan have induced maturation prematurely.[19] Experts at the Georgia Institute of

Technology have carried out experiments involving poly-ethylene glycol (PEG) hydrogels as a biomaterial suitable for seeding cells and incorporating various proteins.[19,20] For example, Phelps et al. found that a PEG hydrogel seeded with human umbilical vein endothelial cells and angiogenic factors (quantified by ELISA) and delivered in situ in the small bowel mesentery of diabetic mice significantly improved the islet-islet engraftment and vascularization as well as increased weight gain in the mice.[20] Moreover, Hume et al. found increased bioactivity and tolerized signals when seeding DC's in a PEG hydrogel and incorporating cytokines and secondary signals.[19] Therefore the biomaterial investigated in this study is a PEG-4MAL hydrogel with varying weight percent to analyze DC viability and activation.

Direct Conjugation vs. Microgel Delivery

Concurrent with hydrogel analysis is the analysis of incorporating IL-10 to DCs, as they induce tolerization. There are two main approaches to deliver IL-10 to DCs. Direct conjugation of IL-10 to the hydrogel (PEGylated IL-10) has shown that IL-10 has the ability to be thiolated as well as has an increased clinically relevant bioactivity. Meanwhile, loaded microgel delivery has a highly tunable release of IL-10. Direct conjugation can affect a functional network of DC's seeded onto a PEG hydrogel while the microgels have the potential to have issues with reproducibility of synthesis. We have analyzed both approaches in the Babensee lab. This research determined which delivery method of IL-10 allows for highest bioactivity through various experiments implemented in parallel (see *Conjugation vs Microgel Decision Matrix Generation*) and fine-tuning the hydrogel properties in order to allow for optimized dendritic cell tolerization and maturation. Both methods were analyzed using a flow chart and researching feasibility; however, the decision to move forward with PEGylated IL-10 instead of completing both experiments in parallel was made. The first experiment to confirm that direct conjugation is the correct method to deliver IL10 was the thiolation of IL-10 using Traut's Reagent. Verification of this thiolation was performed using a BCA Assay and Ellman's reagent almost immediately after. Results are currently still in progress.

Contributions

The research highlighted in this document was led by Nicholas Beskid and assisted by Naomi Wildschut. It examined the effect of varying weight percentages of PEG on adherent vs. non-adherent cells with iDC, mDC, and tDC controls. This is to determine which type of cell to utilize in further studies as well as analyze weight percentage of PEG. After cell culture and passaging, the iDC's were encapsulated in 20kDa PEG-4Mal hydrogels that were VPM crosslinked with 1mM RGD functionalization. The various weight percentages tested were 3.5%, 4.5%, 6%, and 10 %. Confocal microscopy and flow cytometry were utilized in order to analyze cell morphology and viability. It was deemed that lower weight percentage of PEG and utilization of adherent cells provided the highest viability as seen through the confocal microscope. Moreover, the testing of two IL-10 delivery approaches to iDCs were discussed in the Babensee lab. Analysis of DC activity when tolerized, formation of experimental plan, and thiolation of the IL-10 for the direct conjugation were completed and are detailed below. The direction of direct conjugation of IL-10 was chosen, and the results are underway.

Methodology

Dendritic Cell Culture

On Day 0, tubes were prepared with 100mL HBSS and 5% FBS. Four mice were sacrificed using proper sealed CO_2 containers, two at a time, for ten minutes each (proper and humane IACUC protocols were followed). Using forceps and tweezers, the skin was removed, the leg was clipped above the hip, and the muscles were disposed before placing the leg bone in a 50mL tube with HBSS and 5% FBS (goal is an intact femur and tibia to preserve cell viability). New tubes were used for each mouse to avoid cross contamination.

In aseptic environment, the ends of the bone were cut and flushed with HBS and 10% FBS. Cell clumps were broken up using the back of a plunger and washed with HBS; they were centrifuged for 10 minutes and 5 minutes respectively. Cells were counted using a multisizer (size>5um), suspended in media, and 3 ml of solution was added to each well in a labeled 24 well plate (mDC, tDC, iDC). Cells were supplemented with cytokines and swirled manually

clockwise and anti-clockwise. After this process, the overall expected outcome must yield more than 50e6 cells from the bone marrow isolation.

Media components : 429.5mL DMEM (Cellgro: 10-017-CV), 5mL pen/strep (Cellgro 30.002-Cl),5mL NE Amino Acids (Cellgro, 25-025-Cl),5mL Na Pyruvate (Cellgro, 25-000-Cl), 5mL HEPES (Cellgro, 25-060-Cl),0.5 2-mercaptoethanol (Gibco, 21985-023),50mL FBS heat inactivated (Gibco: 16000-044)

General Dendritic Cell Passaging

On days 2,4, and 6, well plates were swirled manually 10 times clockwise and counterclockwise. Half of the cell culture media was removed (18ml per mouse) and placed in 50ml centrifuge tube/centrifuged for 5 minutes (300XG/1200rpm). Cell media was removed from the centrifuge tubes and new media was added to the tubes. A pellet of cells on the bottom of the tube could be observed. The DC's were re-added to the wells. On day 6, various cytokines were added (20ng IL-4, 20ng GM-CSF per 1,000,000 cells) and swirled. To obtain mDC's, place 1 microgram (per 1,000,000 cells) of Lipopolysaccharide to the well. To obtain tDCs place 50ng of interleukin-10 to the well. Lastly, iDC's received no treatment. Moreover, non-adherent and adherent iDC's were encapsulated in VPM crosslinked gels of weight percents 3.5%, 4.5%, 6% and 10%.
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Phenotype and Viability Analysis

On day 7, brightfield microscopy, confocal microscopy and flow cytometry (FORTESSA) were used to analyze the physical and chemical characteristics of the adherent vs non-adherent cells with varying weight percentages.

Conjugation vs Microgel Decision Matrix Generation

An experimental plan was created in order to compare approaches of direct conjugation vs. loaded microgel delivery of IL-10 (Flow Chart 1).

Flow Chart 1. Experimental if/then statements detailing informed decision making process for IL10 delivery approach

Direct Conjugation vs. Microgel

Direct Conjugation - - - - - - - - tested in parallel - - - - - - - - **Loaded Microgel**

Step 1: Thiolation of IL-10 using Traut's

If thiolation is possible, proceed:

Verification with Ellman's

Step 2: *in vitro* bioactivity of PEGylated IL-10, in culture

If conjugating PEG to IL-10 does not alter
its bioactivity, or its ability to tolerize DCs, proceed:

CHECKPOINT 1:
Compare results from *in vitro* bioactivity
of PEGylated IL-10
to microgel-delivered IL-10
assessed in Transwell study.

Step 3: Rheological studies of networks with and without IL-10

If mechanical properties of networks with IL-10 are comparable
to mechanical properties of networks without IL-10, proceed

Step 4: Bioactivity of PEGylated IL-10 networks with cells encapsulated

Step 1: Troubleshoot the extent of loading capacity of microgels

If we can load enough without compromising
the structural integrity, proceed:

Step 2: *in vitro* bioactivity of microgel-delivered IL-10 using Transwell assay

If results show that loading microgels with IL-10 does not alter its bioactivity,
or its ability to tolerize DCs, proceed:

Step 3: Release Characterization

If release can be sufficiently controlled & tuned, proceed:

Step 4: Incorporation of IL-10 microgels in bulk network

CHECKPOINT 2:
Compare effectiveness of treatment of IL-10
directly conjugated to bulk hydrogel
networks to treatment of
microgel-delivered IL-10 within bulk hydrogels.

**If the bioactivity of loaded microgel is observed to be greater
than the bioactivity of PEGylated IL-10,
proceed with loaded microgel approach. However,
if the bioactivity of loaded microgel is observed to be less
than the bioactivity of PEGylated IL-10, proceed with PEGylated IL-10 approach.**

Thiolation of IL-10

Thiolation of IL-10 was performed using Traut's Reagent and verified via Ellmann's reagent. IL-10 at a concentration of no lower than 0.1mg/ml was to be thiolated in PBS with an adjusted pH of 8. 5mM EDTA was added to the buffer and protein solution to prevent oxidation. Traut's stock was made at 2mg/ml and initially diluted in H_2O. 30-fold excess of Traut's solution was added to protein in solution and incubated with gentle shaking. Spin columns then separated the thiolated protein.

The verification of thiolation using Ellman's reagent began by preparing a set of cysteine standards by dissolving Cysteine Hydrochloride Monohydrate at the following concentrations in Reaction Buffer (Table 1).

Table 1. Amounts of Cysteine needed to create standard solutions with reaction buffer.

Standard	Volume of Reaction Buffer	Amount of Cysteine (M.W. = 175.6)	Final Concentration
A	100mL	26.34mg	1.5mM
B	5mL	25mL of Standard A	1.25mM
C	10mL	20mL of Standard A	1.0mM
D	15mL	15mL of Standard A	0.75mM
E	20mL	10mL of Standard A	0.5mM
F	25mL	5mL of Standard A	0.25mM
G	30mL	0ml	0.0mM (Blank)

50 uL of Ellman's reagent was added to a mixture of 2.5mg/ml reaction buffer and 250uL of each standard solution (Table 1) to different test tubes. They were incubated for 15 minutes, placed in a plate reader, and varying absorbance were read at 412 nm.

Results

Phenotype and Viability

On Day 7, brightfield microscopic images were taken of the adherent and non-adherent cells to establish variance in morphology with each PEG weight percent. Figures 4 and 5 shows the sparse and elongated morphology of adherent and non-adherent cells with higher weight percent of PEG.

| 3.5% | 4.5% | 6% | 10% |
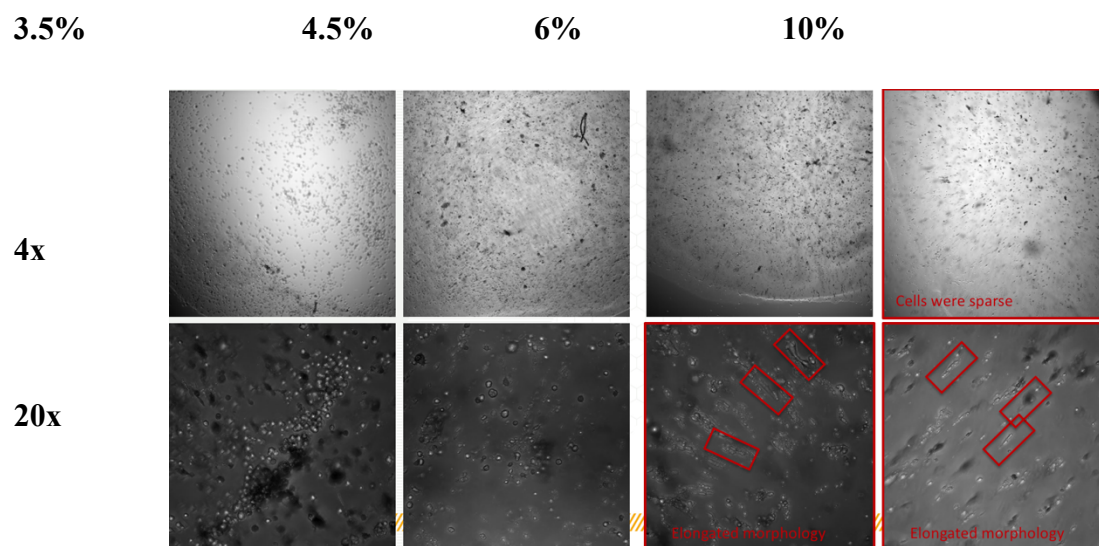
Figure 4. Brightfield microscopy images of the adherent cells encapsulated in PEG-4MAL hydrogel with varying weight percentages. It is shown that higher weight percent has sparse cells and elongated morphology when compared to lower weight percent of PEG
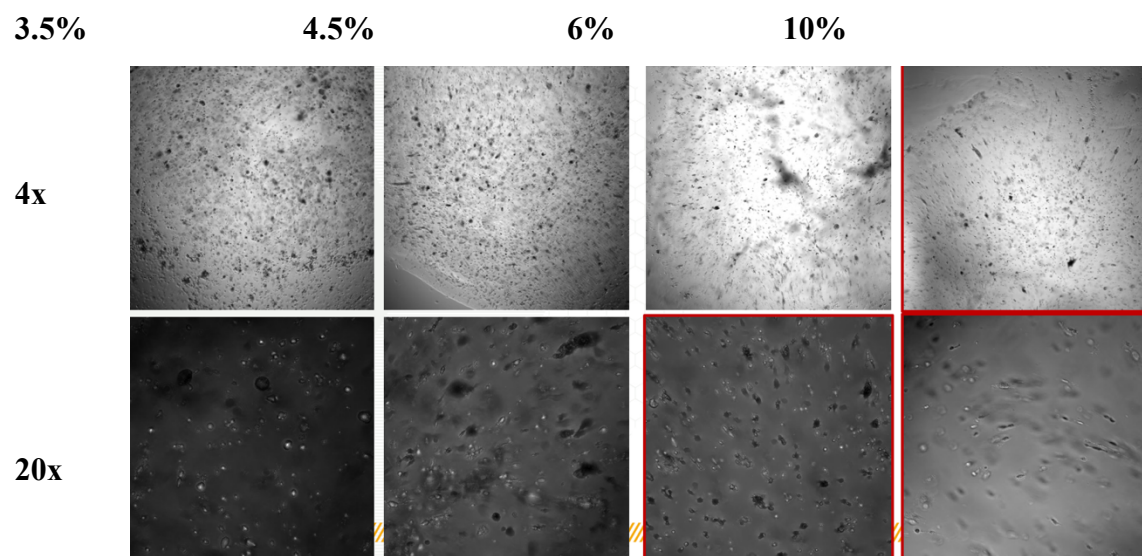
Figure 5. Brightfield microscopy images of the non-adherent cells encapsulated in PEG-4MAL hydrogel with varying weight percentages. It is shown that higher weight percent has sparce cells and elongated morphology when compared to lower weight percent of PEG, similar to the adherent cells.

Confocal microscopy was used to analyze the viability of the adherent and non-adherent cells in using calcien AM (4uM) and EthD-1 (4uM) in Figures 6 and 7. Additional analysis was performed to compare the adherent cells to the non-adherent cells after the confocal microscopy was performed
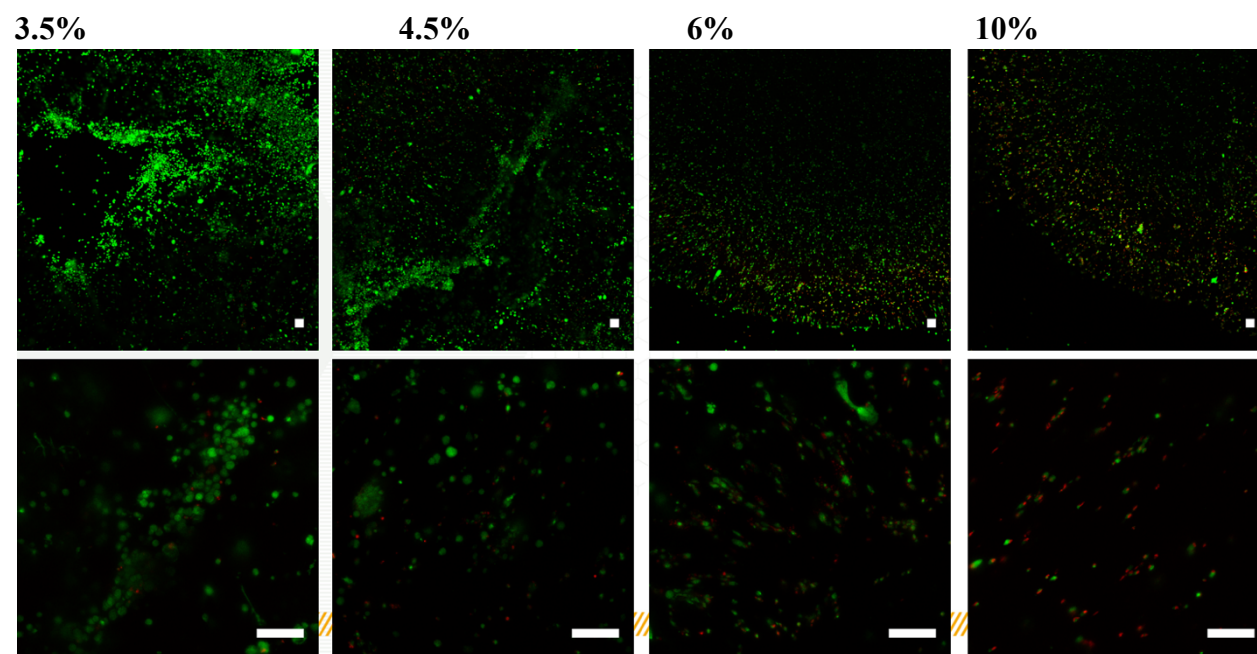

Figure 6. Confocal images of adherent cells at varying weight percentages of PEG-4MAL. The viability (green due to calcian AM, red due to EthD-1) of the cells in lower weight percentages is significantly higher than those in lower weight percentages. The top row is 4x and the bottom row is 20x zoom.

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3.5%   **4.5%**   **6%**   **10%**
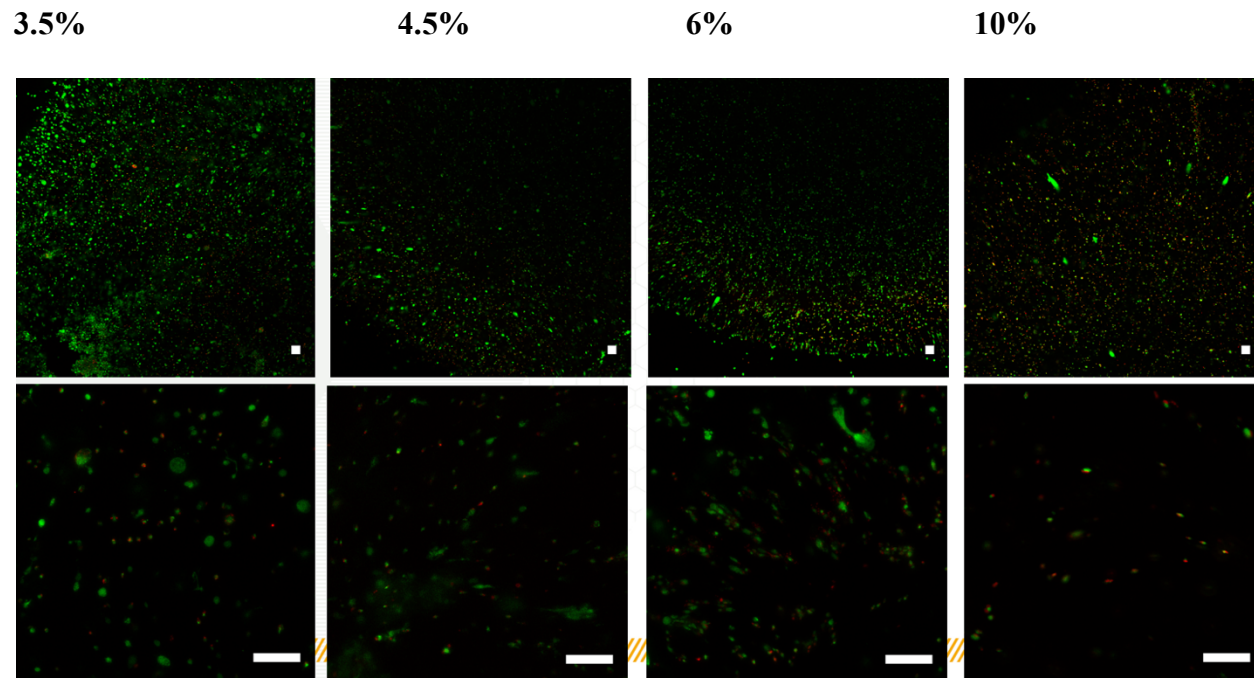
Figure 7. Confocal images of non-adherent cells at varying weight percentages of PEG-4MAL. The viability (green due to calcian AM, red due to EthD-1) of the cells in lower weight percentages is significantly higher than those in lower weight percentages. The top row is 4x and the bottom row is 20x zoom.

In order to confirm the results found using brightfield and confocal microscopy, flow cytometry was performed. Figure 8 shows higher percentage viability in adherent cells encapsulated in low weight percentage PEG. Specifically, 3.5% and 4.5%.
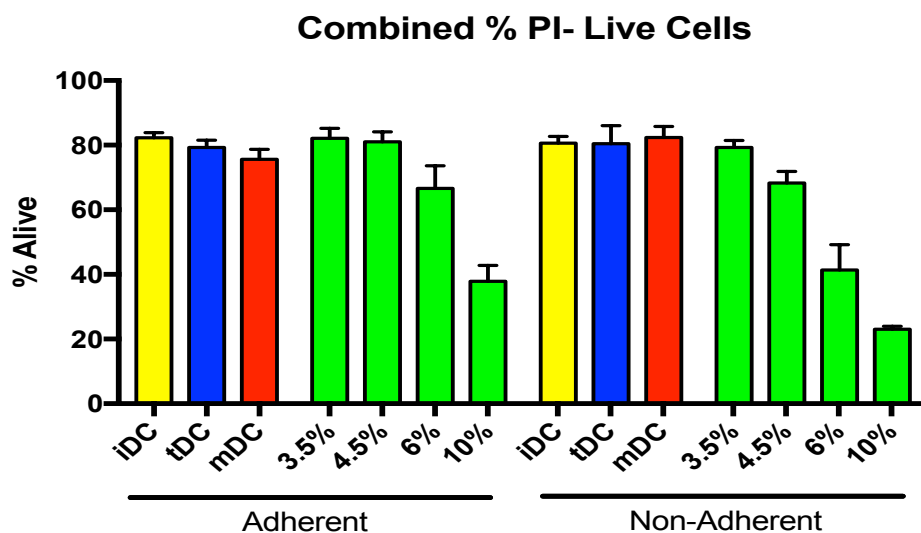
Figure 8 Graph of viability of adherent cells vs non-adherent cells from flow cytometry. The graph shows higher percentage viability in adherent cells encapsulated in low weight percentage PEG. Specifically, 3.5% and 4.5% and higher maturation in non-adherent cells.

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After analysis of the flow chart and discussion with the primary investigator, Dr. Julia Babensee, it was deemed that extensive experimentation to determine the best manner to introduce IL-10 to DC's was unnecessary. Due to the higher feasibility of PEGylated IL-10 as opposed to the microgel IL-10 delivery mechanism, the PEGylated IL-10 was chosen. The results for the first step in this process are set to be achieved on 01 May 2018.

Discussion and Conclusion

The differential effects PEG hydrogel on viability and functional changes of DC phenotypes were observed in the present study. Given the results of the study and extra analysis shown in Figure 9, it is shown that adherent DCs encapsulated in PEG-4MAL hydrogel with lower weight percent produce the higher viability and lower activation when compared to non-adherent DCs encapsulated in PEG-4MAL hydrogel.
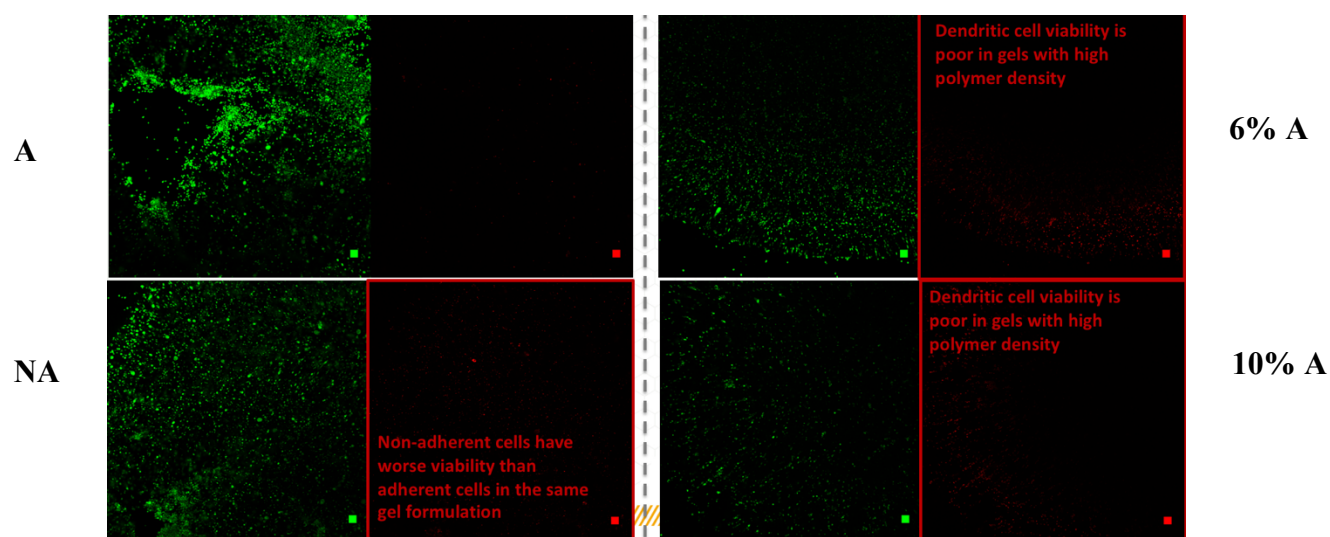
Figure 9. Images show the adherent cells had better viability than non-adherent cells in the same gel formulation. On the left, the images shown are taken from adherent and non-adherent at 3.5%. Moreover, the higher weight percentage for PEG was detrimental to cell viability. On the right, the images were taken from adherent cells at 6% and 10%

The higher viability in the adherent cell group is important to keep into consideration when moving forward with experimentations wherein other manipulations of the hydrogel can further optimize concentration and viability. The increased viability of adherent cells could be due to the shown increased proliferation that has been found to be stimulated by adherent cells. Moreover,

it has been shown that adherent cells are purer than non-adherent cells.[21] This data is significant because future work in the lab involving the manipulation of hydrogels (mechanical properties, cytokine delivery) will involve adherent dendritic cells to optimize viability, phenotype, and concentration. Moreover, we can now look at decreasing the weight percent to 2-3% in order to further examine the effect of the hydrogel on dendritic cell viability.

The overall goal is to have an optimized platform for tolerized dendritic cells. To do this, IL-10 needed to be incorporated in the model. The chosen direction was to directly conjugate the IL-10 and then encapsulate the dendritic cells with the PEGylated IL-10 because of the time and resources of the lab. A previous lab assistant successfully thiolated human IL-10 and a parallel was drawn to murine IL-10. Therefore, the decision to pursue direction conjugation was made and the ability to thiolate of murine IL-10 needed to be verified. Simply put, if sulfylhydrate groups are added on to the IL-10 structure, it can be crosslinked with the PEG to form a network that can then be encapsulated by dendritic cells and further analyzed. The results of this experiment are pending; however, if successful, the next steps include analysis of the IL-10 within the PEG network. Questions to be answered would include:

1. Does the PEG negatively affect the bio activity of the IL-10?

2. Does the IL-10 negatively affect the mechanical properties of the PEG hydrogel?

Future studies will continue the manipulation of the PEG-4MAL hydrogel in order to optimize dendritic cell phenotype and viability. This includes changes in mechanical properties to be tailored to conditions best suited for adherent dendritic cells. Similarly, if thiolation of IL-10 proves to be completely unsuccessful, it would be possible to pursue a microgel delivery mechanism in which IL-10 loaded microgels release the cytokine to the cells.

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