Investigation of the Cytokine and Chemokine Response of Dendritic Cells Following Gold Nanoparticle Treatments and Variations in Hydrogels

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Abstract

Immunotherapies have significant potential for implementation towards personalized medicine through avenues such as vaccine, gene, and cancer applications. Dendritic cells are a major contributor in the immune system, functioning as antigen-presenting cells that aid in orchestrating the immune response towards pathogenic activators. Manipulation of these cells can allow for the optimization of immune responses, which can be achieved through tissue engineering and modulation via gold nanoparticles. This study seeks to provide a basis for elucidating the secretory responses of immature dendritic cells as they progress through maturation following treatment with modified gold nanoparticles. Additionally, findings are presented on the manipulation of dendritic cells with a variety of treatments such as PEGylation and thiolation to understand resulting effects on IL-10 expression. Biomaterial-based matrices are additionally investigated for their roles in dendritic cell viability and functionality – results of variation in weight percentage of hydrogels, used for dendritic cell encapsulation, will be shown. With some notable exceptions, such as IL-4, the majority of cytokine and chemokine expressions in experimental cell groups compared to baseline immature dendritic cell expression levels decreased. Future studies should continue to characterize and solidify this response, both with regard to modulation in hydrogels and gold nanoparticles. These studies can aid in paving the way for individualized treatment for autoimmune disorders.

Introduction

Within the field of immunotherapeutic techniques, an increasingly investigated method for augmenting the immune response is the manipulation of dendritic cells with biomaterials. One such biomaterial that can be used is gold nanoparticles, which have the ability to target dendritic cell surface receptors in order to induce a phenotypic, observable response that orchestrates greater immune activity. Researchers are currently aiming to understand how characteristics of gold nanoparticles can assist in determining the phenotypic response of the cells. If understood, this knowledge could be used to engineer a specific and personalized immune response. Furthermore, researchers are investigating how these cellular responses can change due to variation in the nanoparticles' parameters. Some research has shown that as gold nanoparticles interact with dendritic cells, they can initiate the maturation process of immature dendritic cells to have them express activated or tolerogenic phenotypes.[1]

This maturation process is an integral component to the immune response of the body. Maturation of a dendritic cell occurs upon exposure and subsequent phagocytosis of antigens, followed by the production of corresponding peptides that are expressed on the cell surface. Dendritic cells additionally maintain an immune tolerance towards self-antigens. Studies with gold nanoparticle methodology have shown promising results thus far in inducing maturation, with researchers confirming the efficacy of the nanoparticles as an adjuvant to vaccine delivery and for eliciting an anti-tumor response in cancer patients.[2,3] However, as this treatment gains more potential for clinical application, the potential cytotoxicity of the nanoparticles and their safety in patients must also be considered.

The immune responses by dendritic cells in the body are mediated through varying conditions in the treatment groups involving gold nanoparticle application. This response by the

dendritic cells is shown through the release of corresponding cytokines and chemokines into the cell environment.[4] Currently, the cytokine and chemokine expression of dendritic cells upon treatment with various coatings is not fully established. By identifying what the supernatant is comprised of as a result of nanoparticle application with varying coatings and concentrations, researchers can better understand what specific response is being triggered and what phenotype is being expressed within the dendritic cells, thus giving greater ability to assess their potential risks.

In addition to this, biomaterials have been shown to modulate dendritic cell viability and functionality.[5] Research has been done to explore the potential of various materials to activate dendritic cells, as well as their mechanisms of maturation.[6,7] Further work, however, still needs to be done in the identification of the structural components of biomaterial-based matrices that support non-activated or tolerogenic dendritic cells. By investigating the structure and function relationships that govern hydrogel matrices and dendritic cell interactions, this knowledge can direct the design of hydrogels to support dendritic cells. Further biochemical modifications can be introduced to the matrices that can be used in the improvement of the scaffold's ability to maintain encapsulated cell viability and tolerogenic functionality.[8]

In this study, assays will be conducted to identify the cytokine and chemokine profile that is released from treated dendritic cells. The goal of this experiment is to more clearly understand the cellular immune response elicited by different treatment groups of coatings and concentrations. During the preliminary treatment steps, dendritic cells are treated with three different conditions of nanoparticle groups: bare, serum-coated, and polyethylene glycol (PEG) coated. The PEG groups contain PEG-2K (short chain PEG) and PEG-5K (long chain PEG) variations. Each of these treatments are administered at concentrations of 0.1 pM, 1.0 pM, and 10

pM, for a total of twelve treatment groups. Following treatment by these groups, the cytokine and chemokine release response can be characterized via Luminex analysis (Millipore). This is done in order to study the levels of the pro- and anti-inflammatory cytokines IL-1β, IL-1Ra, MIP-1α, TNF-α, and IL-10 present in the dendritic cell response. These cytokines are detectable upon cell contact with biomaterials and indicate the dendritic cell phenotype that is being expressed. Ultimately, these assays will help determine how the immune system responds to gold nanoparticles and will inform nanoparticle design criteria for immunotherapeutic application in patients. Additionally, secretory profiles of dendritic cells treated with various modifications, such as thiolation and PEGylation, as well as within various hydrogel weight percentages, will be presented.

Literature Review

The immune system response to a pathogen is twofold, including both the innate and adaptive immune responses. The two aspects of response work in conjunction with one another but differ in their approach towards establishing immunity for the body. The innate response provides an immediate immune response, but it does not provide a tailored mechanism to the antigens being defended against. Examples of this include inflammation and non-specific cellular responses. In contrast, the adaptive immune response does not act immediately; instead, it takes several weeks to develop, but provides a tailored response to the antigens present. This response functions through T and B lymphocytes and retains memory of the response towards a specific antigen in order to elicit a quicker reaction upon subsequent exposure. Both of these responses are mediated in the body through the dendritic cells of the immune system.[9]

Dendritic cells work to conduct both the innate and adaptive immune response in the body by activating T-cells.[10] The dendritic cells function first as antigen-capturing cells, then as antigen-presenting cells, meaning they capture antigens through phagocytosis, perform internal processing, and then present the resulting peptide of the antigen on their cell surface. Dendritic cells are a product of monocytes, a type of differentiated cell from bone marrow.[11] When an innate immune response is initiated, monocytes congregate at the site of inflammation and further develop into immature dendritic cells. Upon producing antigenic peptides after phagocytizing antigens and expressing them on their surface, the phenotype of the immature dendritic cell is changed to a mature dendritic cell.[12]

Nanoparticles are defined as particles ranging in size from 1-100nm; their characteristics vary broadly in size, shape, material, and coating. They are used in medicine to carry and deliver drugs to specific targets with a controlled release and specified targeting due to their low cell

toxicity. Within immunotherapy specifically, gold nanoparticles have shown to be promising for dendritic cell manipulation in order to augment the immune response. Gold nanoparticles are favorable in this application due to their inertness, solubility in water, and low toxicity in cells.[13] These nanoparticles can be used to heighten the activity of dendritic cells in stimulating specific T-cell responses, which has shown potential in developing strategies for vaccine, gene, and cancer therapies.[14]

However, some questions remain in regard to the potential cytotoxicity of the nanoparticles. As the characteristics of the gold nanoparticles are varied and administered to dendritic cells, the cells release corresponding cytokines and chemokines into the cell environment.[15] Studies have shown that as particle size increases, efficiency of particle uptake by dendritic cells decreases. The nanoparticles must be optimized to balance the ability to carry what they are loaded with but cannot be too large to be eliminated before they have a chance to interact with dendritic cells.[16] Small gold nanoparticles (diameter of 3 nm) have been shown to be noncytotoxic and nonimmunogenic, as the secretion of proinflammatory cytokines TNF-α and IL-1β were not observed.[17] Another study showed results where smaller gold nanoparticles sizes, such as 0.8 nm, were associated with increased expression of proinflammatory cytokines IL-1, IL-6 and TNF-α, as well as higher cytotoxicity.[18] Noncytotoxicity has been shown for dendritic cells with the use of gold nanoparticles of larger size (10 nm and 60 nm in diameter) even at high concentrations.[18,19]

Additionally, the concentration of gold nanoparticles present also plays a role in uptake and cytotoxicity. Higher gold nanoparticle concentrations have shown to increase rates of toxicity to cells, so in addition to size and coating, the amount per volume of nanoparticle present must also be investigated and optimized for greatest uptake and minimal cell toxicity.[20] This

occurs because with excess nanoparticles beyond what is effective, there is an increased chance of the nanoparticles creating nanopores within the cell that ultimately can engender interference with normal cellular function and damage the cell. The threshold at which gold nanoparticle concentrations with particular specifications in size or coating result in cytotoxicity is not established and needs further investigation.

Finally, the coatings of nanoparticles can affect their uptake. Serum-coating the gold nanoparticles or attaching a long chain polymer, such as polyethylene glycol (PEG), has been shown to decrease phagocytic uptake of nanoparticles in order to increase their circulation time. This increases the efficacy of the nanoparticles in situations where their phagocytosis can cause insufficient exposure to the site being targeted.[21,22] Conversely, coatings can be used to enhance phagocytosis, where the uptake of the gold nanoparticles increases their target efficiency. Coatings can also be used to induce a specific phenotype in the dendritic cell, resulting in either immature, mature, or tolerogenic cells, and consequently engineering a specific immune response. However, strong correlations between uptake and maturation as a result of surface chemistries have not yet been established. Variations in nanoparticle coating not only affords different chemical properties to nanoparticles – different densities of the same coating can additionally effect change in nanoparticle modulation. For example, one study used increasing PEG-coating densities with gold nanoparticles and found that macrophage uptake efficiency was high and serum-dependent at low PEG densities, while less efficient and serum-independent at high PEG densities.[23] Additionally, selecting nanoparticle coatings has important implications in the formation of a gold nanoparticle-protein corona. This corona is a dynamic biopolymer layer serving as the first nano-bio interface, thus determining the interactions of gold nanoparticles with living cells.[24] As many as sixty-nine plasma proteins are able to bind to the surface of a gold

nanoparticle,[25,26] and these proteins largely pertain to the fate of a gold nanoparticle in the body through biodistribution, cellular uptake and clearance efficiency, as well as immunological properties. As shown by the literature, the cytokine and chemokine response from dendritic cells is not fully established, thus demonstrating the need for more studies to inform how particle coating, in addition to concentration, modulates cellular interactions.

In this experiment, the supernatant will be analyzed once obtained from the filtration of cells from the initial culture using a filter plate. By understanding what comprises the supernatant resulting from nanoparticle application, researchers can better understand what exact response is being triggered within the dendritic cells and assess their potential risks. This study uses Luminex analysis to analyze the supernatant and characterize dendritic cell response by investigating the levels of the pro- and anti-inflammatory cytokines IL-1β, IL-1Ra, MIP-1α, TNF-α, IL-10, IFN-γ, IL-12p70, MCP-1, and RANTES. The goal is to more clearly elucidate the effect of gold nanoparticle coating and concentration on dendritic cell response, paving the way for gold nanoparticles' optimization in immunotherapy.

Biomaterials have additionally been shown to modulate dendritic cell viability and functionality.[5] The potential of various materials to activate dendritic cells, as well as their mechanisms of maturation, is currently being researched.[6,7] By elucidating the qualities and benefits of a biomaterial-based scaffold that can support the functionality and induced outcomes by transferred, antigen-specific, tolerogenic dendritic cells, this knowledge can be applied to dendritic cell therapies in humans. The relationships that govern hydrogel matrices and dendritic cell interactions can be used in directing the design of hydrogels to support dendritic cells. Using a murine model provides for an anatomically relevant and scalable design. The project uses a PEG-4MAL hydrogel which is functionalized with bioactive ligands as a delivery scaffold for

tolerized dendritic cells. This can be used to ameliorate experimental autoimmune encephalomyelitis (EAE), as well as diseases such as multiple sclerosis (MS). Dendritic cells which are treated with tolerizing agents and antigen result in antigen-specific immunosuppressive dendritic cells. This function by conditioning T cells towards tolerance. Cells delivered subcutaneously or intravenously incur unwanted effects and distribution, so in developing an injectable hydrogel scaffold, viability and localization of delivered dendritic cells can be improved.[27,28] Secretory profiles of immature dendritic cells will be analyzed following a variety of treatment methods, as well as encapsulation within modified hydrogels. These serve to illustrate the effects on IL-10 expression following modification, as well as the impact caused on dendritic cells as a result of varying hydrogel stiffness.

Methods and Materials

This experiment is conducted through a Luminex assay in order to study the levels of the pro- and anti-inflammatory cytokines IL-1β, IL-1Ra, MIP-1α, TNF-α, and IL-10 present in the dendritic cell response. Initially, an assay must be conducted in order to obtain the linear range for the cytokines and chemokines to be investigated, which relates amount loaded to instrument fluorescent readout. This allows for the determination of the optimal concentrations that the supernatant should be diluted to for testing, so that ranges can be observed prior to saturation. After the linear range is identified, each treatment group (bare, serum-coated, PEG-2K coated, PEG-5K coated) can have an assay conducted at each concentration (0.1 pM, 1.0 pM, 10 pM). The cytokines specified are detectable upon cell contact with biomaterials and indicate the dendritic cell phenotype that is being expressed.

Materials

Necessary materials are included in Milliplex kit HCYTOMAG-60K. Materials that are required but not provided in Milliplex kit include reagents including Luminex sheath fluid or Luminex drive fluid as used for machine protocol.

Reagents Supplied	Volume	Quantity
Human Cytokine / Chemokine Standard	lyophilized	1 vial
Human Cytokine Quality Controls 1 and 2	lyophilized	2 vials
Serum Matrix (Contains 0.08% Sodium Azide)	lyophilized	1 vial (serum and plasma sample only)
Set of one 96-Well Plates with 2 Sealers		1 plates 2 sealers
Assay Buffer	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	60mL	1 bottle

Cytokine Detection Antibodies	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	3.2 mL	1 bottle
Bead Diluent (not provided with premixed panel)	3.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)		1 bottle

For instrumentation, materials needed include the following: adjustable pipettes with tips capable of delivering 25 µL to 1000 µL, multichannel pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL, reagent reservoirs, polypropylene microfuge tubes, absorbent pads, laboratory vortex mixer, sonicator, and titer plate shaker. Luminex 200, HTS (High Throughput Screening), FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex Corporation will also be used, as well as an automatic plate washer for magnetic beads or handheld magnetic separation block.

Methods

Results are read on MAGPIX software. Following sample collection of cells from initial murine or human cell culture done in lab, the cells are filtered out using a filter plate. 25 uL of supernatant from each of the below wells is then transferred. to each new corresponding sample well for cytokine assay (pictured below for reference). Mix supernatant well by vortexing and centrifuging prior to use in the assay to remove particulates. Following this, add a maximum of 25 µL per well of neat or diluted serum. All samples must be stored in polypropylene tubes. Do not store samples in glass.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Antibody 150 ul/well iDC			Isotyp 150 ul/well iDC			Antibody 50nm - 150 ul 0.1pM Au(PEG5K)			Cells-Back-ground	Cells-Back-ground	Cells-Back-ground
B	Antibody 150 ul/well mDC			Isotyp 150 ul mDC			Isotyp 50nm - 150 ul 0.1pM Au(PEG5K)			Media	Media	Media
C	Antibody 150 ul/well tDC			Isotyp 150 ul tDC			Antibody 50nm - 150 ul 1pM Au(PEG5K)			Isotyp 50nm - 150 ul 1pM Au(PEG5K)		
D	Antibody 50nm - 150 ul 0.1pM Au			Isotyp 50nm - 150 ul 0.1pM Au			Antibody 50nm - 150 ul 10pM Au(PEG5K)			Isotyp 50nm - 150 ul 10pM Au(PEG5K)		
E	Antibody 50nm - 150 ul 1pM Au			Isotyp 50nm - 150 ul 1pM Au			Antibody 50nm - 150 ul 0.1pM Au(PEG2K)			Isotyp 50nm - 150 ul 0.1pM Au(PEG2K)		
F	Antibody 50nm - 150 ul 10pM Au			Isotyp 50nm - 150 ul 10pM Au			Antibody 50nm - 150 ul 1pM Au(PEG2K)			Isotyp 50nm - 150 ul 1pM Au(PEG2K)		
G	Antibody 50nm - 150 ul 0.1pM Au(Serum)			Isotyp 50nm - 150 ul 0.1pM Au(Serum)			Antibody 50nm - 150 ul 10pM Au(PEG2K)			Isotyp 50nm - 150 ul 10pM Au(PEG2K)		
H	Antibody 50nm - 150 ul 1pM Au(Serum)			Isotyp 50nm - 150 ul 1pM Au(Serum)			Antibody 50nm - 150 ul 10pM Au(Serum)			Isotyp 50nm - 150 ul 10pM Au(Serum)		

Figure 1. Plate layout of initial gold nanoparticle and dendritic cell treatments in 96-well plate. Gold nanoparticle concentrations of 0.1 pM, 1.0 pM, and 10 pM were added to dendritic cells in individual wells for all gold nanoparticle treatment groups (bare, serum-coated, PEG-2K coated, and PEG-5K coated). Antibody staining was added to three wells of six wells per treatment, and isotype staining was added to the others. Cells-background indicates untreated cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard Background)	400 pg/mL Standard	QC-2 Control	Antibody mDC sample	Antibody tDC sample	Antibody 0.1pm Au	Antibody 10pm Au	Antibody 0.1pm Au(serum)	Antibody 1pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)
B	0 pg/mL Standard Background)	400 pg/mL Standard	QC-2 Control	Antibody mDC sample	Antibody tDC sample	Antibody 0.1pm Au	Antibody 10pm Au	Antibody 0.1pm Au(serum)	Antibody 1pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)
C	3.2 pg/mL Standard	2,000 pg/mL Standard	Antibody iDC sample	Antibody mDC sample	Antibody tDC sample	Antibody 1pm Au	Antibody 10pm Au	Antibody 0.1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 1pm Au(PEG5K)	
D	3.2 pg/mL Standard	2,000 pg/mL Standard	Antibody iDC sample	Antibody mDC sample	Antibody tDC sample	Antibody 1pm Au	Antibody 10pm Au	Antibody 0.1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 1pm Au(PEG5K)	
E	16 pg/mL Standard	10,000 pg/mL Standard	Antibody iDC sample	Antibody mDC sample	Antibody 0.1pm Au	Antibody 1pm Au	Antibody 10pm Au	Antibody 1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)	
F	16 pg/mL Standard	10,000 pg/mL Standard	Antibody iDC sample	Antibody mDC sample	Antibody 0.1pm Au	Antibody 1pm Au	Antibody 10pm Au	Antibody 1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 0.1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)	
G	80 pg/mL Standard	QC-1 Control	Antibody iDC sample	Antibody tDC sample	Antibody 0.1pm Au	Antibody 1pm Au	Antibody 0.1pm Au(serum)	Antibody 1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)	
H	80 pg/mL Standard	QC-1 Control	Antibody iDC sample	Antibody tDC sample	Antibody 0.1pm Au	Antibody 1pm Au	Antibody 0.1pm Au(serum)	Antibody 1pm Au(serum)	Antibody 10pm Au(serum)	Antibody 1pm Au(PEG5K)	Antibody 10pm Au(PEG5K)	

Figure 2. Plate layout for use in Luminex cytokine assay of uncoated, serum coated, and PEG 5K coated nanoparticles.

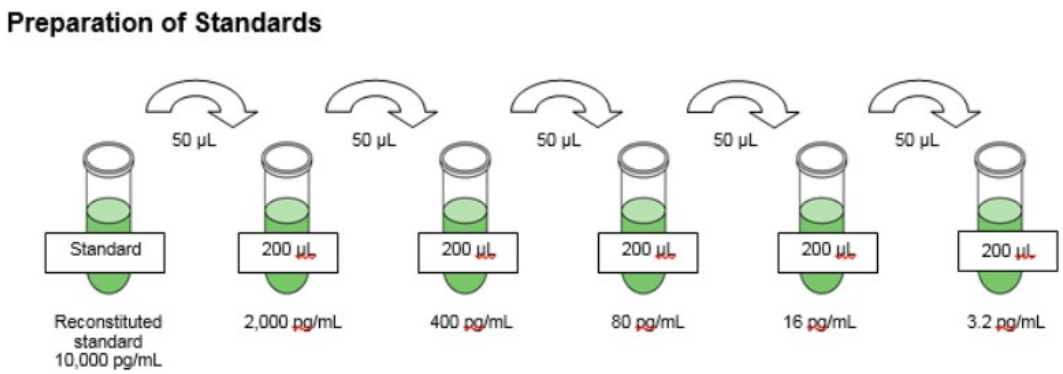
Preparation of Reagents for Immunoassay

In preparing the reagents for immune assay for individual bead vials, sonicate each antibody-bead vial for 30 seconds. Following this, vortex for 1 minute. Add 60 µL from each antibody bead vial (5 vials) to the mixing bottle and bring final volume to 3.0 mL with Bead Diluent by adding 2.7 mL. Vortex the mixed beads well using 5 cytokine antibody-immobilized beads – add 60 µL from each of the 5 bead sets to the mixing bottle. Then, add 2.7 mL Bead Diluent. To prepare quality controls, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times, vortex, and allow the vial to sit for 5-10 minutes. Following this, transfer the controls to appropriately labeled polypropylene microfuge tubes

To prepare wash buffers, bring the 10X Wash Buffer to room temperature. Mix to bring salts into solution, and dilute 60 mL to 10X Wash Buffer with 540 mL deionized water. Preparation of Human Cytokine Standard requires the following before use: reconstitution of the Human Cytokine Standard with 250 µL deionized water – this gives a 10,000 pg/mL concentration of standard for all analytes. Then invert the vial several times, vortex for 10 seconds, and let stand for 5-10 minutes. Then transfer the standard to an appropriately labeled polypropylene microfuge tube and use as the 10,000 pg/mL standard. To prepare the working standards, label five polypropylene microfuge tubes 2,000, 400, 80, 16, and 3.2 pg/mL. Then add 200 µL of Assay Buffer to each. Prepare serial dilutions by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2,000 pg/mL tube and mix well by vortexing. Transfer 50 µL of the 2,000 pg/mL standard to the 400 pg/mL tube and mix well by vortexing. Transfer 50 µL of the 400 pg/mL standard to the 80 pg/mL tube and mix well by vortexing. Transfer 50 µL of the 80 pg/mL standard to 16 pg/mL tube and mix well by vortexing. Finally, transfer 50 µL of the 16

pg/mL standard to the 3.2 pg/mL tube and mix well by vortexing. The 0 pg/mL standard (Background) will be Assay Buffer.

Serial Dilutions Chart – adapted from Valeková et al.[29]:



Standard Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 µL	0
2,000	200 µL	50 µL of 10,000 pg/mL
400	200 µL	50 µL of 2000 pg/mL
80	200 µL	50 µL of 400 pg/mL
16	200 µL	50 µL of 80 pg/mL
3.2	200 µL	50 µL of 60 pg/mL

Immunoassay Procedure

Before beginning the assay, allow all reagents to warm to room temperature (20-25°C) before use in the assay. If using a filter plate, set the filter plate on a plate holder. To begin the assay, add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature. Then, decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Following this, add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer

should be used for 0 pg/mL standard (Background). Then add 25 µL of Assay Buffer to the sample wells. Add 25 µL of appropriate matrix solution (from initial cell culture) to the background, standards, and control wells. Use proper control culture medium as the matrix solution. Then add 25 µL cell culture sample into the appropriate sample wells, vortex mixing bottle, and add 25µL beads to each well. Following this, seal the plate with a plate sealer and cover with foil. Incubate with agitation on a plate shaker overnight at 4°C or 2 hours at room temperature, preferably overnight. Gently remove well contents and wash plate two times by following directions in the plate washing section below. Allow to warm to room temperate and add 25 µL of Detection Antibodies into each well. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Do not aspirate after incubation. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Gently remove well contents and wash plate two times by following directions in the plate washing section below. Add 150 µL of Sheath Fluid to all wells and resuspend the beads on a plate shaker for 5 minutes. Run plate on Luminex 200, HTS (High Throughput Screening), FLEXMAP 3D® or MAGPIX® with xPONENT® software and save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. For diluted samples, multiply the calculated concentration by the dilution factor.

Plate Washing

To wash the plates, use a solid plate with a handheld magnet. Rest the plate on the magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent

pads to remove residual liquid. Wash plate with 200 μL of Wash Buffer by removing plate from magnet. Add wash buffer, shake for 30 seconds, reattach to magnet, allow beads to settle for 60 seconds, remove well contents as previously described after each wash twice, and repeat accordingly to assay procedure.

Data Analysis

Results of Luminex assays were analyzed and visually represented through the creation of heatmaps in MATLAB. This heatmaps were coded to provide a gradient scale of fold change in order to indicate lower expression, higher expression, or no change in expression of a given cytokine or chemokine. Values for controls (immature dendritic cells) were compared to treatment groups, such as mature, tolerogenic, PEG-4MAL-IL-10-treated, or hydrogel-modified dendritic cells. The ratio between the two was plotted on the heatmap. Sample MATLAB code is provided in the Addendum.

Results

The following contains results of cytokine and chemokine analysis assays conducted on murine dendritic cell supernatant samples following encapsulation in hydrogels. The supernatant was obtained from the initial cell culture conducted by other members of the lab after removing cells using a filter plate. Testing was then conducted on the remnants of the sample. Data was obtained from Luminex machine quantification and visual heatmap representations were coded using MATLAB.

In this study, cytokine and chemokine expression in treated dendritic cells will be compared to that of immature dendritic cells, which serve as a baseline. Levels seen in immature dendritic cells will be used to determine whether a certain cytokine and chemokine has increased or decreased with a given treatment. The linear range is first obtained in order to determine the optimal concentration of supernatant to diluent across all cytokines being tested. Figures 3, 4, and 5 depicts how results are displayed in heatmaps using fold changes. "Fold change" is defined as the ratio of each individual cytokine or chemokine between immature dendritic cells and treated cells.

Pro-inflammatory cytokines of interest included TNF-alpha, IL-1alpha, IL-1beta, IL-6, IL-12beta, IL-8, and IFN-gamma, and anti-inflammatory cytokines of interest included IL-4, IL-5, and IL-10. Pro-inflammatory chemokines of interest included MCP-1. Additional cytokines of interest include: IL-1R α, MIP-1 α, IL-12p70, and RANTES.

iDC = immature dendritic cells; tDC = tolerogenic dendritic cells; mDC = mature dendritic cell.

Figure 3 presents a controls fold change between immature dendritic cell cytokine and chemokine expression levels and those of experimental murine cells. These cells are compared to tolerogenic and mature dendritic cells in order to establish a baseline expression level for the cytokines and chemokines being observed. Mature dendritic cells expressed noticeably higher levels of inflammatory cytokines as compared to tolerogenic ones – exceptions to this include IL-4 and IL-10, both anti-inflammatory cytokines, as well as GM-CSF, a modulator of stem cells to differentiate cells involved in innate (non-specific) immune response.[30] All fold changes for previously noted cytokines of interest were significantly different between mature dendritic cells and immature dendritic cells, with the exception of IL-4.

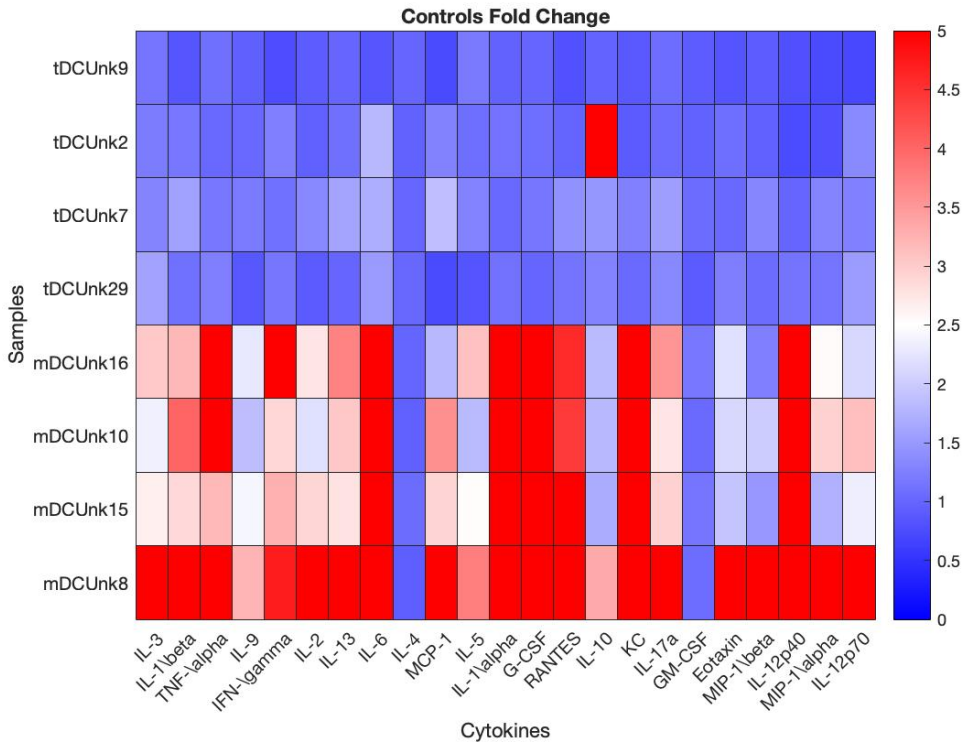


Figure 3. Controls fold change between iDC levels and those of experimental murine cells: tDCs and mDCs. Untreated tDCs and mDCs are compared to iDC expression levels as a baseline. The scale of colors is indicated as per the following: blue (low change) to white (moderate change) to red (high change). The baseline for comparison is immature dendritic cell cytokine and chemokine expression. For all cytokines of interest except IL-4, all fold changes between mature dendritic cells and immature dendritic cells were significant with $P < 0.05$.

Figure 4 depicts the PEG-4MAL-IL-10 fold change between untreated iDC levels and those of experimental mouse cells. Immature dendritic cells are treated with one of the following: IL-10, thiolated IL-10 (S-IL-10), PEGylated and thiolated IL-10 (PEG IL-10), or PEG (polymer without IL-10).

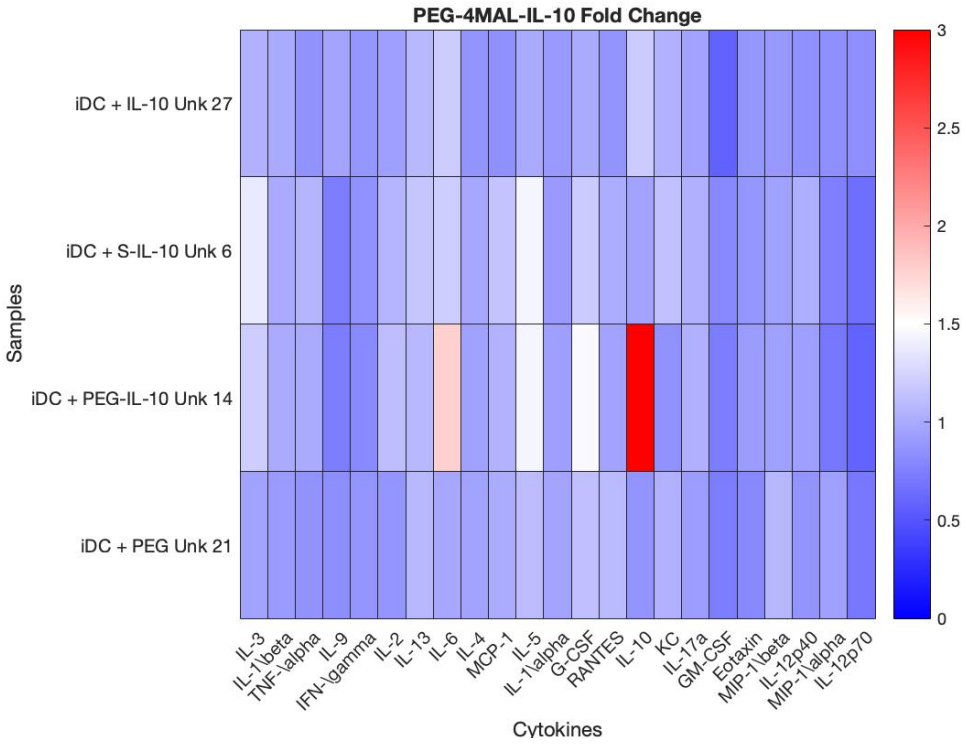


Figure 4. PEG-4MAL-IL-10 fold change between untreated iDC levels and those of experimental mouse cells: iDCs treated with PEG and/or IL-10. The expression of IL-10 in the DCs were observed after being modified in different ways (S-IL10: IL-10 with thiolation, PEG IL-10: PEGylated and thiolated, PEG: polymer without IL-10).

Finally, Figure 5 presents results of observing dendritic cells encapsulated in hydrogels with varying polymer densities. This sought to understand the gel stiffness within the scaffold and its corresponding effect on dendritic cells. Phenotype, secreted molecules, and morphology of the cells were further pursued, but only for 3.5 weight percent and 4.5 weight percent only. This was due to the fact that dendritic cells in scaffolds of higher weight percentages (6.0% and 10.0%) died due to gel being too stiff.

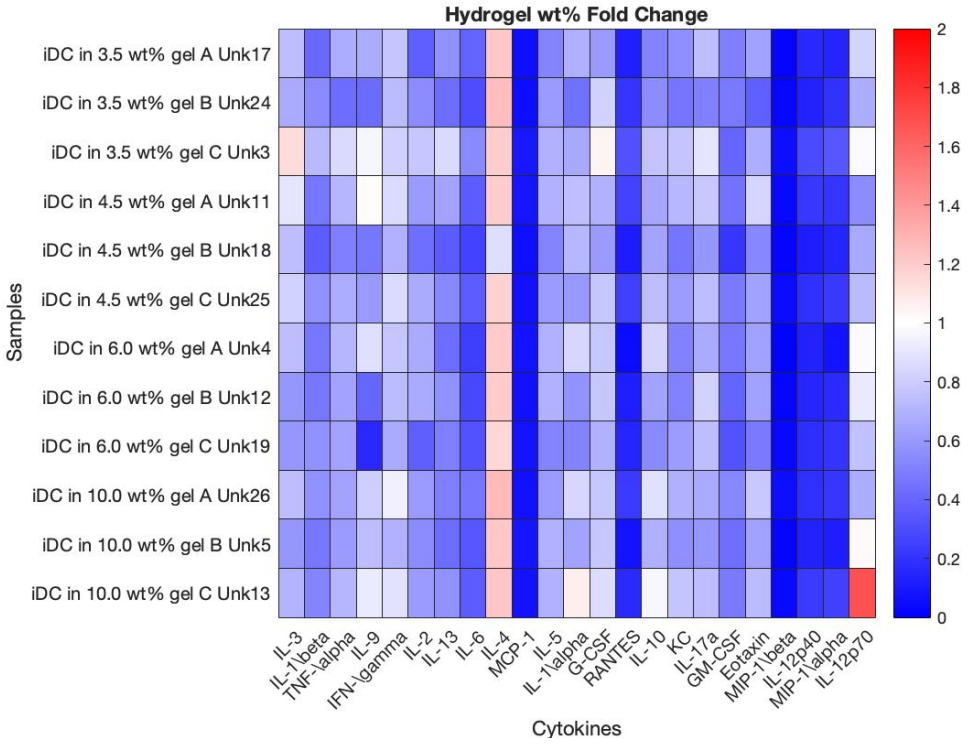


Figure 5. Hydrogel wt% fold change between untreated iDC levels and those of experimental murine cells: iDCs in 3.5, 4.5, 6.0, or 10.0 wt% hydrogel. DCs encapsulated in hydrogels with varying polymer densities were observed to see the effect of gel stiffness in scaffold on DCs. Phenotype, secreted molecules, and morphology of the cells were then investigated. Only 3.5 wt% and 4.5 wt% were viable as cells in higher wt% (6.0% and 10.0%) died due to gel being too stiff.

In investigating the fold change between controls, many of the cytokine and chemokine expressions associated with inflammation were highly expressed in mature dendritic cells, while those involved with anti-inflammatory responses were not. Tolerogenic dendritic cells, conversely, did not present high inflammatory cytokine levels. A particularly high expression of IL-10, an anti-inflammatory cytokine, was observed in tDCUnk2, which is representative of the tolerogenic phenotype. In regard to the PEG-4MAL-IL-10 results, a notable increase in expression occurred in in IL-10 for the iDC + PEG-IL-10 sample. The expression of IL-10, an anti-inflammatory cytokine, was increased across all four modifications tested, but particularly

so with PEGylation and thiolation, which suggests this may be the most potent modification to elicit IL-10 expression.

In the hydrogel weight percent analysis, IL-4 expression was consistently elevated, across all weight percent samples with the exception of iDC in 4.5 weight percent gel B, which exhibited a slightly less extreme increase. IL-4 is a critical cytokine involved in anti-inflammatory responses as well as the promotion of the alternative activation of macrophages into M2 cells. Additionally, there was a significant increase in IL-12p70 expression within the iDC in 10.0 wt% gel C sample, and a slight increase in IL-3 within the iDC in 3.5 wt% gel C sample. IL-12p70 is involved in the regulation of T cell responses, while IL-3 stimulates multipotential hematopoietic progenitors, as well as lineage-committed cells.[31]

While the assay gives a comprehensive visualization of cytokine expression via quantification of their secretory profiles, specific pro-inflammatory cytokines of interest included TNF-alpha, IL-1alpha, IL-1beta, IL-6, IL-12beta, IL-8, and IFN-gamma, and anti-inflammatory cytokines of interest included IL-4, IL-5, and IL-10. Pro-inflammatory chemokines of interest included MCP-1. Additional cytokines of interest include: IL-1R α, MIP-1 α, IL-12p70, and RANTES. Subsequent runs of this experiment should continue to focus on the secretion of these biomolecules.

Discussion

With some notable exceptions, the majority of cytokine and chemokine expressions in experimental cell groups compared to baseline immature dendritic cell expression levels were highly indicative of whether the dendritic cell was of a mature or tolerogenic phenotype. A less extreme response for the tolerogenic dendritic cells may likely be due to the fact that there is no antigen present to truly induce the immune response, so the mature dendritic cells are not secreting significant amounts of cytokines and chemokines that would require an anti-inflammatory response to counterbalance it.

With regard to the PEG-4MAL-IL-10 fold change between untreated immature dendritic cell levels and those of experimental mouse cells, it seems possible that PEGylation and thiolation confer a synergistic effect on IL-10 expression, or simply that PEGylation is more efficacious than thiolation. Further experimentation would need to be conducted with PEGylated IL-10 alone in order to distinguish the effects of each modification.

The remaining samples generally exhibited slight increases in cytokine and chemokine expression, including those specified earlier as cytokines and chemokines of interest, with greater degrees of increase observed with higher weight percent hydrogel. The significant and consistent increase of IL-4 in the hydrogel weight percent analysis across all samples raises an interesting avenue of whether the hydrogel qualities potentially contributes towards anti-inflammatory activity. This may be a result of the material comprising the hydrogel. M2 macrophages, promoted by IL-4, encourage vessel maturation and tissue regeneration, suggesting that the hydrogel does encourage dendritic cell viability, functionality, and compatibility. This is additionally supported due to being coupled with low expression seen in pro-inflammatory cytokines. That being said however, cells in scaffolds of higher weight

percentages (6.0% and 10%) died, indicating these scaffolds' weight were not compatible with the growth of dendritic cells.

Despite the trends these data show, these results are preliminary – replications of these experiments and assays will need to be completed in order to confirm the cytokine and chemokine response. Additional experimental runs with murine samples can aid in establishing statistical significance for the results presented. Solidifying the dendritic cell response can aid to inform development and design of hydrogels for maximal dendritic cell compatibility, viability, and functionalization. Due to unforeseen difficulties with human dendritic cell culture, gold nanoparticle analysis was unable to be completed for inclusion in this thesis. These assays will need to be completed again upon achievement of AuNP treatment on human dendritic cells. The continuation of this work is paramount in order to identify how AuNPs can be developed to elicit an optimal or desired response from dendritic cells.

Conclusion and Future Work

These results indicate that generally, IL-10 expression increased with the four tested IL-10 modification. Similar results of increased cytokine and chemokine secretion overall were observed with a corresponding increase in the weight percent of hydrogel, with limits of 6.0%, as cell death was observed at 6.0% and 10.0%. However, further research should be done to see whether the observed cytokine and chemokine response is consistent upon treatment of varying hydrogel modifications. By optimizing the hydrogel to function with dendritic cells, this technology can be leveraged to improve the viability, cellular response, and localization of dendritic cells that are delivered to patients for the use of amelioration of autoimmune disease such as EAE or MS.

Determining the specific cytokine and chemokine response of dendritic cells, as well as what resulting cell phenotype is expressed, are critical to nanoparticle design and application within immunotherapy. Additionally, a wider range of nanoparticle concentrations can be investigated to fully establish cytotoxicity thresholds. Finally, the methods and data analysis used in this study will need to be applied to the AuNP treated-dendritic cells in order to ascertain their optimal design. By optimizing AuNP characteristics and conducting studies to continue assessing cytotoxicity and internalization of AuNPs in dendritic cells, greater clarity of the effect of AuNPs on the body can be obtained. This information can then subsequently be used in engineering and optimizing AuNP treatments for immunotherapeutic application and the creation of personalized immune responses. In doing so, the ultimate application of these technologies towards personalized medicine can work towards being achieved.

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Addendum

```
[n, t, r] = xlsread('iDCcontrols.xlsx');
[n2, t2, r2] = xlsread('comparecontrols.xlsx');

ratiotDC = n2(1:4, :) ./ n(:, :);
ratiomDC = n2(5:8, :) ./ n(:, :);
ratios = [ratiotDC;ratiomDC];
ylabels = r2(2:end, 1);
xlabels = t(1, 2:end);

length = 1000;
blue = [0, 0, 1];
white = [1, 1, 1];
red = [1, 0, 0];
colors1 = [linspace(blue(1),white(1),length)', linspace(blue(2),white(2),length)',↙
linspace(blue(3),white(3),length)'];
colors2 = [linspace(white(1),red(1),length)', linspace(white(2),red(2),length)',↙
linspace(white(3),red(3),length)'];
colors = [colors1;colors2];
h = heatmap(xlabels, ylabels, ratios,'Colormap',colors); % Create heat map
h.Title = 'Controls Fold Change';
h.XLabel = 'Cytokines';
h.YLabel = 'Samples';
h.ColorLimits = [0 5];
```

Figure A1. Sample MATLAB code used in data analysis.