Biomaterial-based Ligand Presentation to Induce Notch Signaling *in Vitro*

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1. Abstract

T cell adoptive transfer is an important procedure in immunotherapy. In immuno-compromised patients, T cells may need to be developed from stem cells, requiring the induction of Notch signaling events responsible for native T cell differentiation in the thymus. Current methods to develop T cells *in vitro* fail to mimic the 3D thymic niche and are not mechanically tunable. In this study, we improve on these approaches with a 2D polymer system with controllable mechanical properties, and with a 3D system that more accurately mimics the thymic niche. 3D inverse opal poly(ethylene glycol) (PEGDA) scaffolds were fabricated with defined pores using poly(methyl methacrylate) microspheres that are soluble in acetic acid, leaving negative space for cell growth. The PEGDA surface was made bioactive via biotinylation, followed by a streptavidin linker attaching biotinylated Notch ligand Delta-Like Ligand 4 (bDLL4). 2D polyacrylamide gels were prepared by sandwiching a pre-polymer droplet between two glass surfaces during redox initiation. Mechanical properties were modified by adjusting the concentrations of acrylamide and bis-acrylamide. Gels were functionalized by immobilizing streptavidin via a SANPAH linker and binding bDLL4. After fabricating the systems and demonstrating their chemical and mechanical tunability, RT-PCR was used to detect *Hes-1* expression, a downstream target of Notch, and flow cytometry was used to detect T cell differentiation levels following incubation of T cell progenitors with functionalized polymer systems. We show that a) the thymic niche can be effectively mimicked by synthetic systems, b) these systems effectively induce Notch signaling, and c) Notch signaling results in early T cell differentiation. These data are promising and suggest the potential to develop T cell banks from stem cells for the purposes of immunotherapy.

2. Background

2.1 Adoptive Transfer and Medical Motivation

Cancer immunotherapy is a promising approach in cancer therapeutics. With this strategy, the body's immune cells are trained to attack malignant cells as they would any other invader, by educating the effector cells of the immune system to recognize cancer cells are foreign and pathogenic. Immunotherapy is an attractive alternative or complement to the current paradigm of surgery, radiation, and chemotherapy, as these traditional approaches are very non-specific and can often be cripplingly painful or uncomfortable for patients. The immune system, however, is effective due to its precision and highly amplified response.

The presence of tumor-infiltrating lymphocytes (TILs) in malignant cancer sites underscore the potential for immunotherapy's efficacy[1]. High numbers of effector T cells in the tumor microenvironment are indicative of a favorable prognosis, demonstrating the role that immune cells can play in the reduction of tumor burden[1]. While the potential therapeutic benefits of a strong immune response are clear, however, the tumor microenvironment secretes immunosuppressive signals that can inhibit expansion and proliferation of immunological effector cells, essentially silencing the immune system[1]. As a result, medical engineers are attempting to strengthen the immune response. One technique already in clinical practice is called adoptive transfer. In this technique, T cells from cancer patients are extracted from peripheral blood, pulsed with antigen from the tumor microenvironment, and the antigen-specific T cells are isolated, expanded, and infused back into the patient[2]. This method has been especially effective in the treatment of melanomas[3].

However, there are several limitations to adoptive transfer[4]: The procedure requires the isolation of peripheral blood, which can be invasive to the patient. Furthermore, expanding T

cells *ex vivo* can be time-consuming, a non-trivial barrier in the case of time-sensitive treatment requirements. Perhaps most significantly, however, a patient's T cells may not be available due to severe immunodeficiency, and obtaining T cells from an alternative source poses the possibility of host rejection and forces patients to become dependent on long donor waitlists.

The limitations in the current practice of adoptive transfer therapy combined with immunotherapy's enormous potential creates an urgent need to develop therapeutic banks of antigen-specific T cells against various diseases, including cancer. This would allow clinicians to reliably access effector cells for the purpose of immunotherapy. Furthermore, to reduce the dependence on cell donors, these disease-specific T cells may be derived from stem cells. A critical first step in the realization of this vision is the engineering of early-stage immunological cells from stem cells. Biomaterials functionalized with biological signals show enormous potential in the direction of stem cells down the T cell lineage. The efficacy of this strategy will be explored in the studies that follow.

2.2 T Cell Development In Vivo

To accurately induce T cell development *in vitro* for therapeutic purposes, native T cell differentiation *in vivo* must be explored. Hematopoietic stem cells (HSCs) are multipotent stem cells that can repopulate all cells of the blood. HSCs initially develop in the bone marrow, but for HSCs to progress down the T cell lineage, they must leave the bone marrow and migrate to the thymus. There hematopoietic progenitor cells are directed to differentiate into mature T cells[5]. Microenvironments that direct stem cell differentiation down a particular pathway are called stem cell niches and can be comprised of several complicated signals that synergize to determine a stem cell's eventual fate[6].

Mature T cells are positive for one of two surface protein markers: either CD4 or CD8. CD4 single-positive cells are helper T cells that support the immune system via the release of cytokines: soluble signals in the immune system that regulate immune responses[7]. CD8 single-positive cells are cytotoxic T cells that kill invading pathogens. As HSCs from the bone marrow migrate to the thymus, however, they are $CD4^{-}CD8^{-}$, or double negative (DN) cells[5]. Upon entering the thymus, two main phases comprise the T cell developmental pathway. HSCs arrive and congregate in the cortical region of the thymus, where the first phase begins, before moving to the medullary segments of the thymus for the second phase[5]. The two phases are characterized by different signaling events and different levels of T cell development. A schematic of these steps can be seen in *Figure 1*.

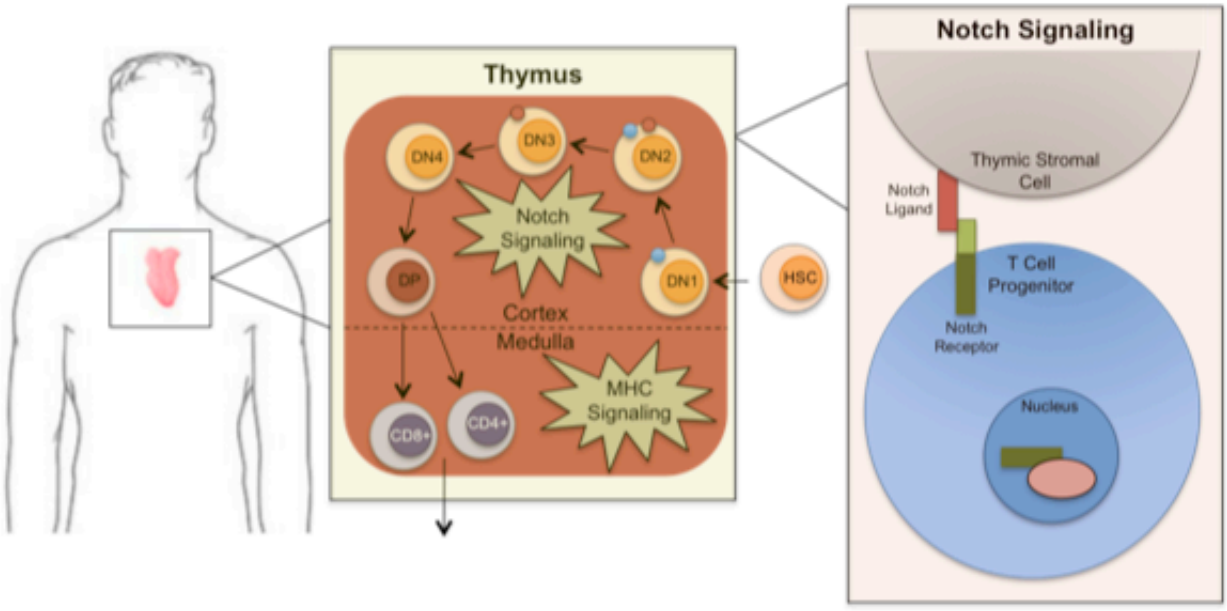

Figure 1. Simplified schematic of T cell differentiation steps.

In the first phase, HSCs undergo Notch signaling and several forms of cytokine signaling via stromal cells in the cortex to differentiate into naïve, non-antigen-specific early T cells[5]. Infiltrating DN cells are characterized by two other surface markers: CD25 and CD44[5]. DN

developmental stages are traced by measuring the levels of expression of these two markers: the first stage of DN development (DN1) is demarcated by a $CD25^{-}CD44^{+}$ fingerprint, DN2 by $CD25^{+}CD44^{+}$, DN3 by $CD25^{+}CD44^{-}$, and DN4 by $CD25^{-}CD44^{-}$ [5]. At this point, cells begin to express CD4 and CD8, or double positive (DP) cells[5]. Cells throughout this phase also express Thy1.2, a T cell marker that is constitutively expressed on all DN cells. Because CD25 is transiently expressed in the DN2 and DN3 stages of development, double expression of Thy1.2 and CD25 is indicative of T cell maturation past the DN1 stage but before the DN4 stage.

In the second phase of T cell development, DP cells bifurcate into either $CD4^{+}$ helper T cells or $CD8^{+}$ cytotoxic T cells, also known as single positive (SP) cells[5]. The fate difference is determined by interactions with major histocompatibility complexes (MHCs) on professional antigen-presenting cells (APCs) in the thymus[5]. Early T cells with strong affinity for MHC Type 1 molecules express CD8 while cells with strong affinity for MHC Type 2 molecules express CD4[5].

2.3 Notch Signaling

The goal of this study is to direct the differentiation of HSCs towards a naïve T cell state, or through the first phase of the developmental pathway described above. Because of this, the key signaling event in the experiments that follow is Notch signaling. Notch signaling is an insoluble signaling system. *In vivo*, the Notch ligand is immobilized to the surface of a cortical thymic stromal cell[8]. Ligands are physically presented on the plasma membrane of the signaling cell and sensed by receptor molecules on the plasma membrane of the receiving cell, in this case a T cell progenitor[8]. Notch signaling is present in several embryonic differentiation pathways,

and has even been implicated in cancer, but this study focuses on the role of Notch signaling in T cell development[8,9].

In mammals, five Notch ligands have been found to signal via four receptor molecules[8]. The five ligands are Delta-like ligands 1, 2, and 3 (DLL1-3) and Jagged-1 and Jagged-2. All five signal through Notch receptors simply named Notch-1 through Notch-4[8]. The Notch receptor is composed of both an extracellular region and an intracellular region; the extracellular region interacts with Notch ligands while the intracellular region interacts with coactivators in the nucleus to influence gene expression, triggering downstream phenotypic effects[8]. When Notch receptors are activated, metalloproteases cleave residues in the extracellular region, and the lost fragment is commonly endocytosed by the signaling cell[8]. This extracellular cleavage event is followed by a second cleavage event in the intracellular portion of the receptor, freeing the intracellular domain and allowing it to migrate to the nucleus to form a transcription intitiation complex with the transcription factor CSL[8].

In the immune system, Notch signaling is critical to T cell lineage commitment. Notch-1 inhibits several potential non-T cell differentiation fates for HSCs, including myeloid cells, dendritic cells, and B cells[8]. By inhibiting these cell fate potentials, Notch signaling helps induce efficient T cell differentiation. The delta-like ligands, DLL1 and DLL4, have been implicated in this process to a greater extent than DLL3 or the Jagged ligands, and data suggest that DLL4 may be essential to T cell differentiation[8].

2.4 Prior Work Towards in vitro T Cell Differentiation

Several groups have worked on differentiating T cells *in vitro*. Two in particular are highly relevant to this study's attempt at artificial Notch ligand presentation. The Zúñiga-

Pflücker group at the University of Toronto obtained a bone marrow stromal cell line called OP9 cells and transfected them to induce constitutive DLL1 expression. The modified cell line was named OP9-DL1[10]. Studies have shown that growing HSCs in the presence of these Notch ligand-presenting stromal cells effectively directed the cells through the DN stages and formed DPs, even resulting in the development of $CD8^+$ SP cells, although not $CD4^+$ SP cells[10]. Without the artificial introduction of Notch ligand, however, non-T cell lineages develop, whereas when DLL1 is transduced, T cells develop at the expense of these other fates, demonstrating the expected role of the Notch ligand[10].

For clinical purposes, however, it is desirable to create a stromal cell-free differentiation system. Ideally, T cells that develop should not come in contact with any other cell source—especially not an immortalized cell line—to avoid the risk of contamination. For this reason, other groups have focused on developing stromal-free T cell differentiation systems. One notable example of this is the Varnum-Finney group. There, researchers identified a set of soluble cytokine signals that synergize with insoluble Notch ligand presentation on tissue culture-treated polystyrene to direct T cell differentiation[11]. Instead of relying on stromal cells for DLL1 presentation, tissue culture wells were treated with an antihuman IgG antibody, blocked with bovine serum albumin (BSA), and incubated with DLL1-IgG, a construct in which an IgG domain was attached to the ligand to allow for binding with the receiving antibody in the tissue culture dish[11]. In addition, the group identified a defined set of cytokines to induce differentiation: murine stem cell factor (SCF), human Flt-3 ligand (hFlt3-l), human IL-6 (hIL-6), and human IL-7 (hIL-7) at 100 ng/mL, and human IL-11 (hIL-11) at 10 ng/mL. The group saw a significant increase in Thy^+CD25^+ cells in conditions with Notch signaling.

2.5 This Study's Approaches and Potential Benefits Over Prior Work

From the standpoint of tissue engineering, there are several potential issues with the *in vitro* systems described above. First, the T cell differentiation methods outlined above are all in two-dimensional culture conditions, while the thymic niche is a complex three-dimensional environment where relative spatial orientation is critical, such as the relationship between the cortical and medullary regions of the thymus[5]. Furthermore, studies have indicated that even if components of the thymus are isolated, they must reaggregate into a three-dimensional structure to effectively induce T cell differentiation[5]. Even if three-dimensional environments are not essential, however, it is reasonable to hypothesize that three-dimensional ligand-presentation environments will more efficiently expose cells to Notch signals.

Another concern with previous attempts at *in vitro* T cell differentiation is that they are both carried out on hard tissue culture-treated plastic. These environments have significantly less compliance than the thymus. Recently, studies have identified the mechanical microenvironment to be critical to the detection of a stem cell niche. Pioneering work done by the Engler group at the University of California, San Diego has demonstrated that mesenchymal stem cells will differentiate into bone, muscle, or neuronal cells based exclusively on the stiffness of the substrate on which they are grown[12]. Thus, engineering the stem cell niche must take into account both the chemical microenvironment—by introducing the correct cocktail of cytokines and Notch signals—and the mechanical microenvironment—by striving to mimic the mechanical compliance of the thymus.

In this study, two novel independent methods of *in vitro* T cell differentiation are presented. One solves the problem of two-dimensionality while the other solves the problem of mechanical stiffness. Three-dimensional inverse opal (IO) scaffolds with highly ordered pores

for cell residence were developed from poly(ethylene glycol) diacrylate (PEGDA) and functionalized with Notch ligands to direct T cell differentiation in three dimensions. Two-dimensional polymer gels were made of polyacrylamide (PA) and similarly functionlized with Notch ligands to induce T cell differentiation, but the ratios of the acrylamide and bis-acrylamide monomers could be fine-tuned to fabricate gels with a specific elasticity. By analyzing these systems, the roles of three-dimensionality and substrate stiffness in T cell differentiation could be independently analyzed.

This project consisted of three main phases: 1) each polymer system was fabricated and characterized both chemically and mechanically; 2) mouse HSCs were incubated with these systems in the short term and analyzed for Notch signal induction; 3) mouse HSCs were incubated with these systems in the long term and analyzed for T cell differentiation efficiency.

3. Materials and Methods

3.1 Fabrication of PEGDA IO Scaffolds

PEGDA scaffolds were prepared based on a modified protocol developed by the Irvine group[13]. 100 μm poly(methyl methacrylate) (PMMA, Fluka) microparticles were suspended at 60% w/w. Microsphere suspension was slowly added at 20 μl increments to PDMS molds shaking at 280 rpm to allow the microspheres to settle into a crystalline lattice shape while the ethanol evaporated. 40% PEGDA solutions were prepared with PEG-diacrylate and biotin-PEG acryl, added at 500:1 PEG:biotin molar ratios, using Irgacure 2959 (Ciba) at 0.05% w/w as the photoinitiator. 20 μl of the prepolymer solution were added to the microsphere structures and constructs spun down at 1500 rpm for three minutes to allow polymer solution to infiltrate between the packed microspheres. Gels were formed under UV radiation for eight minutes. The scaffolds were then incubated in 100% acetic acid for three days, to dissolve the PMMA microspheres, replacing with fresh acid every two to four hours initially and then every day. Scaffolds were then transferred to sterile PBS and washed several times before use. These steps are outlined in *Figure 2* below.

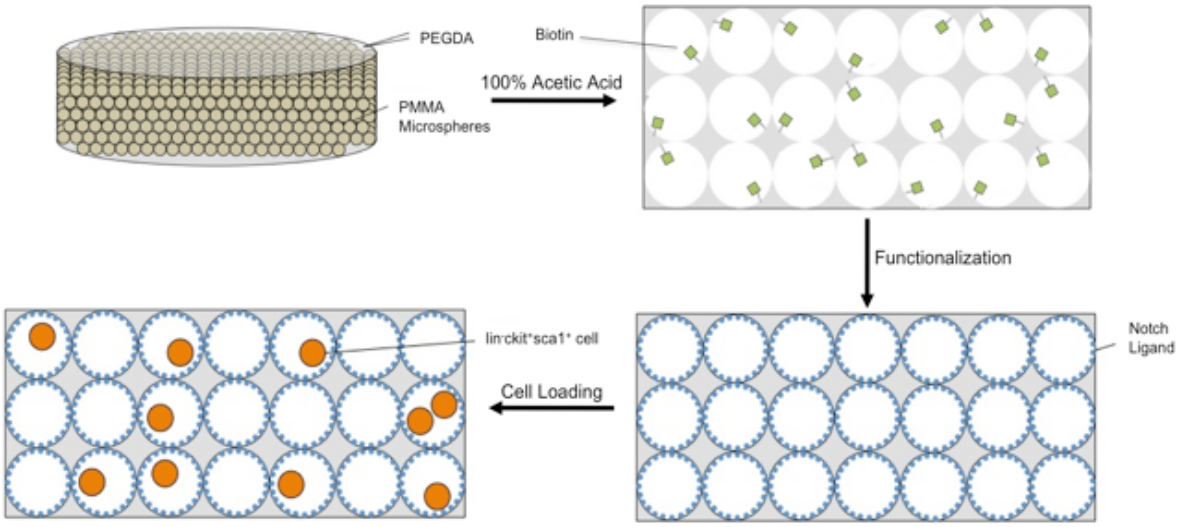

Figure 2. Steps in PEGDA IO scaffold fabrication

3.2 Fabrication of PA Gels

2D PA gels were fabricated based on the protocol developed by Tse, et al[14]. Thin, hydrophilic gel disks were polymerized between hydrophilic glass coverslips and hydrophobic glass slides, allowing the disks to adhere to the coverslips and to be easily removed from the slides, as seen in *Figure 3*. Circular glass coverslips were made hydrophilic via amino-silanation. Coverslips were treated with 0.1 M sodium hydroxide (NaOH) in water. Solvent was evaporated with heat, leaving a uniform residue of NaOH. Free ions were treated with aminopropyltriethoxysilane (APES, Acros Organics). Frosted glass slides were made hydrophobic via treatment with dichlorodimethylsilane (DCDMS, Acros Organics). Acrylamide and *n,n'* methylene-bis-acrylamide (Sigma-Aldrich) were polymerized using redox chemistry initiated by 1/1000 volume of *n,n,n',n'*-tetramethylethylenediamine (TEMED, Sigma-Aldrich) and 1/100 volume of 10% ammonium persulfate (APS, Sigma-Aldrich). Acrylamide and bis-acrylamide were added at 100:1 ratios. During polymerization, 25 µl droplets were sandwiched between the coverslip and slides and allowed to crosslink for thirty minutes at room temperature before removal. Gels were subsequently washed twice with PBS to remove unpolymerized acrylamide, which can be toxic to cells.

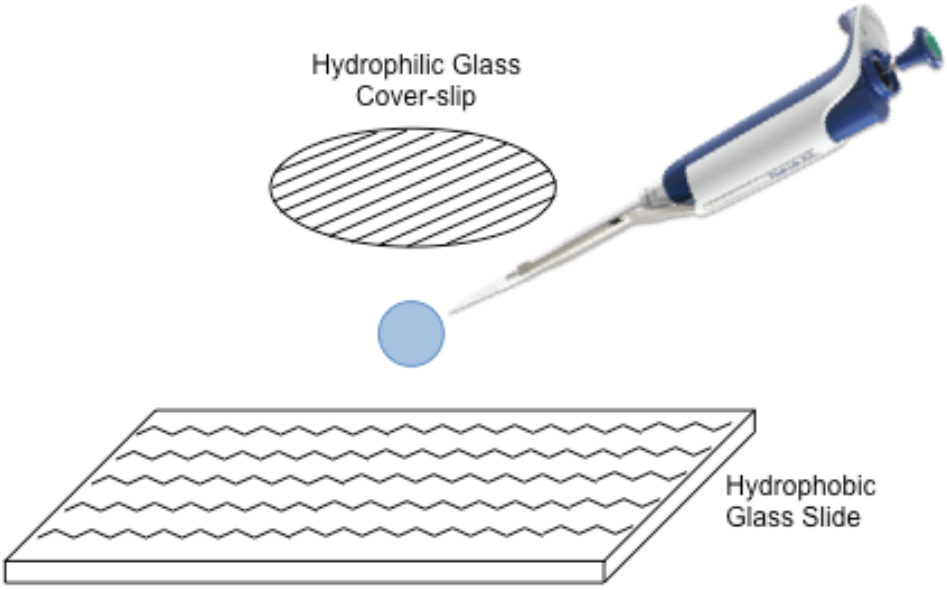

Figure 3. Polymerizing PA between two modified glass surfaces

3.3 Ligand Biotinylation

Mouse DLL4 obtained from R&D Systems was conjugated to biotin using the Biotin-XX Microscale Protein Labeling Kit (Life Technologies). Biotin-XX-succinimidyl ester was incubated in a sodium bicarbonate buffer solution for fifteen minutes, and unbound biotin was removed via a resin in a centrifugal filter. This was done to prevent free biotin from out-competing biotinylated DLL4 (bDLL4) and rapidly binding streptavidin sites on polymer surfaces during functionalization. The yield of the reaction was determined using absorbance on a plate reader (BioTek).

3.4 Functionalization of Tissue Culture Plates

Hard tissue culture plates were used as controls against which soft polymer substrates could be compared. Tissue culture plates were bound with ligand using methods similar to those described by Varnum-Finney[11]. 48-well plates were incubated in 150 µl of 10 µg/mL streptavidin (Promega) for thirty minutes at 37ºC. Wells were then washed twice with PBS and blocked with

Hank's Balanced Salt Solution (HBSS, Gibco) + 2% BSA for thirty minutes at room temperature, and washed twice upon completion of the incubation period. Finally, wells were incubated with 150 µl of bDLL4 at the desired concentration, followed by two more PBS washes.

3.5 Functionalization of PEGDA IO Scaffolds

Biotinylated scaffold constructs were placed in 1.5 mL low-adhesion tubes and incubated with 500 µl of 5 µg/mL streptavidin solution overnight at 4ºC. Unbound streptavidin was washed twice with PBS for twenty-four hours at 4ºC and then incubated with 500 µl of the desired bDLL4 concentration in low-adhesion tubes overnight at 4ºC. Two more PBS washes followed to remove unbound bDLL4. A schematic of the final chemical linkages in this functionalization scheme can be seen in *Figure 4*.

3.6 Conjugation of Streptavidin and SANPAH

N-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, Pierce) is a common reagent to bind free amine groups on polyacrylamide with a photoactivatable nitrophenyl azide, and was thus used to functionalize the surface of 2D PA gels. An N-hydroxysuccinimide (NHS) ester that reacts with amino groups on proteins was used to conjugate streptavidin to sulfo-SANPAH to form streptavidin-SANPAH. Streptavidin was incubated with sulfo-SANPAH at room temperature for three hours, and unconjugated SANPAH removed via centrifugal filtration.

3.7 Functionalization of 2D PA Gels

Sulfo-SANPAH was used to attach streptavidin to gels by photo-crosslinking SANPAH to amines on the acrylamide backbone. 150 µl of streptavidin-SANPAH solution at 5 µg/mL were exposed to UV radiation for ten minutes. Modified gels remained on their glass coverslips, and fit into 24-well plates that were outfitted with PDMS molds to fit the dimensions of the coverslip. Following two PBS washing steps, streptavidin-modified gels were incubated with 200 µl of solution at the desired concentration of bDLL4 at 4ºC overnight, and again washed twice in PBS before cell seeding to remove unbound ligand. The chemical presentation scheme is outlined in *Figure 4* below.

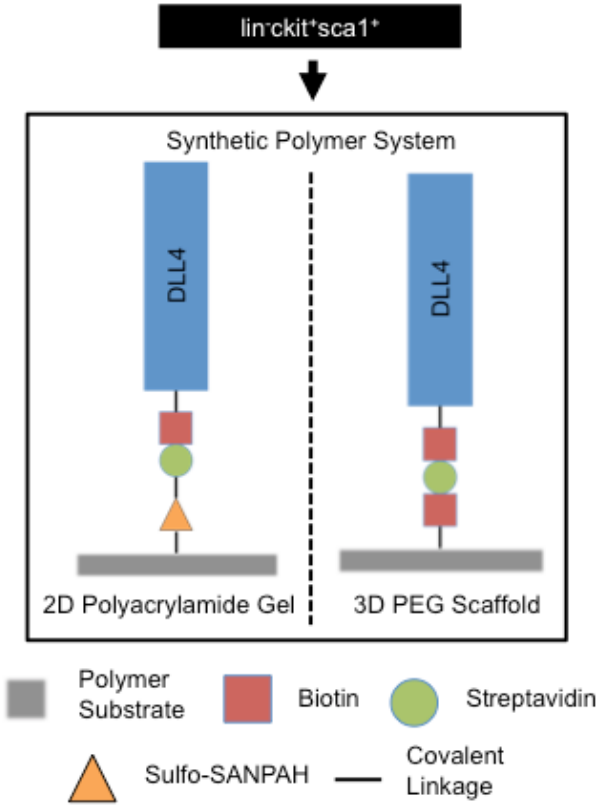

Figure 4. Ligand presentation schemes for both 2D and 3D systems

3.8 Ligand Density Studies

Ligand density was measured indirectly. BSA tagged with biotin and fluorescein (biotin-BSA-FITC, Nanocs) was used as a model for bDLL4 due their to similar molecular weights. Biotin-BSA-FITC was bound in an identical manner and concentration as bDLL4 to all streptavidin-modified *in vitro* systems. Three PBS washes were collected and analyzed on a fluorescent plate reader (Biotek) against a biotin-BSA-FITC standard curve to determine the mass of biotin-BSA-FITC remaining unbound. This was subtracted from the total probe added to determine the density of bound model protein.

3.9 Rheology

The mechanical properties of hydrogels and tissues were determined using a rheometer (Anton Paar Physica MCR 101) utilizing a 7.974 mm diameter oscillatory measuring system. Measurements were taken at room temperature with a 1 Hz frequency and 5% strain, as these conditions were in the linear regions of representative frequency and amplitude sweeps for all material types. Gap sizes were chosen to approximately equalize initial compression across all samples. Thymus samples were obtained from BALB/c mice (Jackson Laboratory), PEGDA gel samples were photopolymerized in PDMS templates, and PA gel samples were polymerized in 8 mm silicone isolators. The storage moduli were compared between materials and tissue samples to determine relative mechanical properties.

3.10 HSC Isolation

Primary HSCs were isolated from four to six week old BALB/c mice (Jackson Laboratory). Mice were euthanized and legs were dissected and de-fleshed to collect the femur

and tibia. Bone tips were cut and the bone marrow flushed with PBS into a conical tube using a 21-gauge needle. Cells were suspended via repeated aspirations through a 16-gauge needle. Any remaining flesh or bone debris was removed by straining through 40 μm cell strainers, and red blood cells were lysed using a red blood cell lysis buffer (eBioscience). Hematopoietic cells already committed to a mature lineage were removed using magnetic-activated cell sorting (MACS). Cells were stained with a cocktail of antibodies bound to magnetic beads that identify mature cell markers using a mouse lineage depletion kit (Miltenyi Biotec) and these cells were captured on a magnetic column. Lineage negative cells were stained with anti-cKit APC and anti-sca1 PE antibodies (eBioscience) and sorted for double positive cells on a BD FACS Aria II (Beckton Dickinson) to select for $lin^{-}ckit^{+}sca1^{+}$ cells, markers characteristic of HSCs.

3.11 Notch Signaling Studies

Cells were seeded onto 48-well plates, 2D PA gels, or 3D PEGDA gels and cultured with IMDM (HyClone) with 20% FBS (Stem Cell Technologies) and the cytokine cocktail previously described by the Varnum-Finney group[11]: SCF, hFlt3-l, hIL-6, and hIL-7 at 100 ng/mL and hIL-11 at 10 ng/mL. Cells were loaded onto PEGDA gels by adding 15 μl of cell suspension onto the hydrogel surface and spinning at 800 rpm for one minute at 4ºC. After three hours in culture, cells were removed with two rounds of vigorous PBS washes. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen), and the mass of isolated RNA was quantified using the Quant-iT RiboGreen RNA Kit (Life Technologies). Reverse transcription was performed using Superscript II First Strand Synthesis System for RT-PCR (Life Technogies). Levels of *Hes-1*, a downstream target of Notch signaling, were used to determine whether the functionalized substrates had induced Notch signaling. *Hes-1* expression was measured using quantitative real

time PCR (RT-PCR). An ABI Prism 7900HT Real Time PCR System measured the fluorescence emitted by dyes in the RT^2 SYBR Green/ROX qPCR Mastermix (SA Biosciences). *Hes-1* levels were normalized against *β-actin* levels, a gene that is constitutively expressed. The $2^{-\Delta\Delta Ct}$ method was used to determine fold differences in *Hes-1* levels between samples.

3.12 Differentiation Studies

Differentiation studies were performed only in 48-well plates and in 2D PA gels. 1,000 $lin^-ckit^+sca1^+$ cells were seeded onto the substrates in the same media used for the Notch signaling studies. Media was replaced along with fresh cytokines every three to four days by gently aspirating out media without disturbing cells resting on the substrate floor. Fresh cytokines were added such that their total concentration matched the levels described above. If culture became too confluent, 4/5 of the cells were removed and discarded.

On day nine of culture, the differentiation state of the cells was measured using flow cytometry. Cells were suspended in FACS buffer (PBS + 2% FBS + 0.1% NaN_3 + 2 mM EDTA). Non-specific binding was avoided by incubating with Fc Block (BD Pharmingen) at 4ºC for five minutes. Cells were then stained with three antibodies: anti-CD25 Alexafluor 488, anti-CD44 PE, and anti-Thy1.2 APC (eBioscience). In addition, a live/dead stain (7-AAD, eBioscience) was added to differentiate viable from dead cells during the analysis. Antibody incubation proceeded for thirty minutes at 4ºC wrapped in foil. Cells were washed once in FACS buffer and flow cytometry was performed on an Accuri machine (Beckton Dickinson). Cells were isotype-stained as negative controls, and single-stained samples were used to optimize compensation values. The presence of $Thy1.2^+CD25^+$ double positive cells indicated either the

DN2 or DN3 stage of development and their abundance was used to quantify T cell differentiation.

4. Results

4.1 High surface area scaffolds can be fabricated

One concern while developing 3D porous materials is maintaining pore integrity. If pore sizes are too large or if the material is too soft and compressible, pores tend to collapse, disturbing the controlled cellular microenvironment. The stiffness obtained with 40% PEGDA gels, however, was sufficient to prevent the collapsing of the pores. Surface area calculations indicate a highly spatially efficient substrate. The entire scaffold has a volume on the order of millimeters cubed, but has a surface area of 80.2 cm^2. This is greater than the area of T75 tissue culture flasks that are commonly used in culture.

4.2 Ligand density can be effectively controlled on well plates and 2D PA gels

Ligand density was measured using the indirect techniques described in the methods section. Biotin-BSA-FITC was incubated on either 48-well plates or 2D PA gels at a low concentration (2 μg/mL), moderate concentration (5 μg/mL), or high concentration (10 μg/mL). These were compared against a negative control with no fluorophore. A dose-dependence was observed on both hard tissue culture well plates and on 2D PA gels. The surface areas of the well plates and PA gel surfaces were calculated and used to determine ligand density in units of molar density of probe per area (pmol/cm^2). The data are displayed in *Figure 5*. Wells in 48-well plates had molar densities of 0.96 ± 0.31 pmol/cm^2, 2.0 ± 0.6 pmol/cm^2, and 2.9 ± 0.6 pmol/cm^2 at low, moderate, and high concentrations, respectively. Gels had molar densities of 4.4 ± 0.3 pmol/cm^2, 7.5 ± 0.5 pmol/cm^2, and 13.3 ± 0.4 pmol/cm^2 at low, moderate, and high concentrations, respectively. Gels were able to bind more biotin-BSA-FITC than hard tissue culture wells, but a dose-dependency was observed on both substrates.

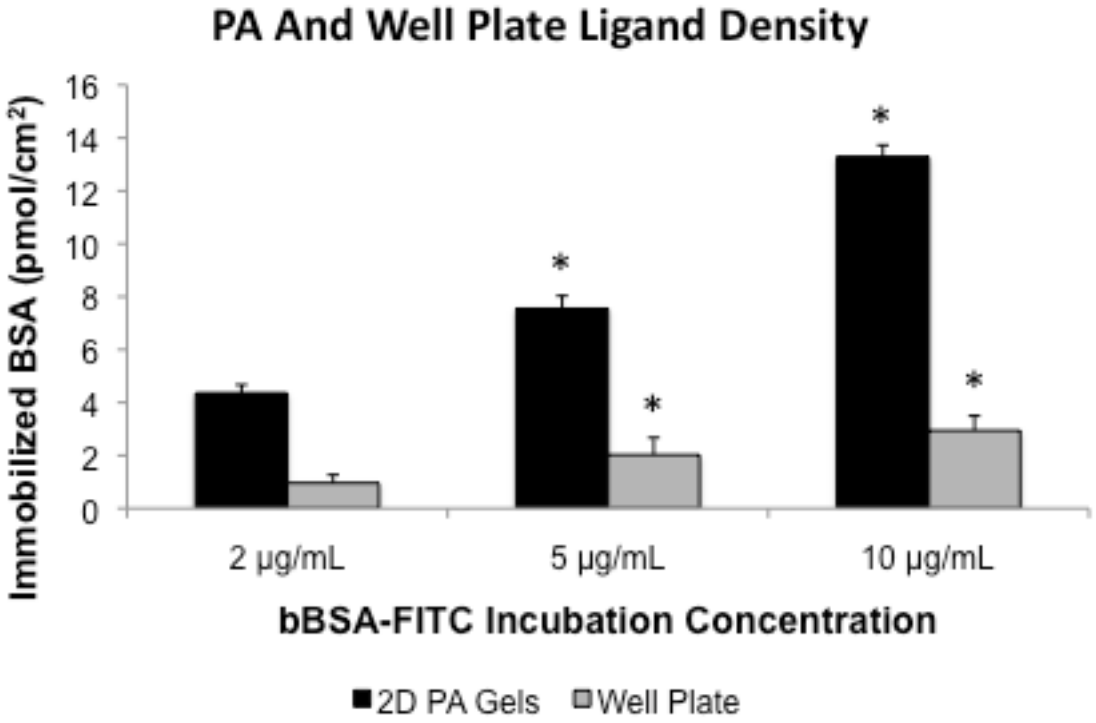

Figure 5. PA ligand density. Increasing ligand model protein incubation concentration increases final immobilized density. * indicates $p<0.05$

4.3 Ligand density is independent of acrylamide concentration

One concern was that ligand density and mechanical properties could not be tuned independently. Because acrylamide concentrations were found to correlate with mechanical properties, gels with different levels of acrylamide concentration were incubated with low and high concentrations of biotin-BSA-FITC to determine whether there were significant differences in density that resulted from different acrylamide concentrations. Gels with low concentration (3% acrylamide, 0.03% bis-acrylamide), moderate concentration (4% acrylamide, 0.04% bis-acrylamide), and high concentration (10% acrylamide, 0.1% bis-acrylamide) of polymer were functionalized with biotin-BSA-FITC at low concentrations (2 µg/mL) and high concentrations (10 µg/mL) of ligand model protein. No statistical differences were found between the different stiffnesses, as seen in *Figure 6*.

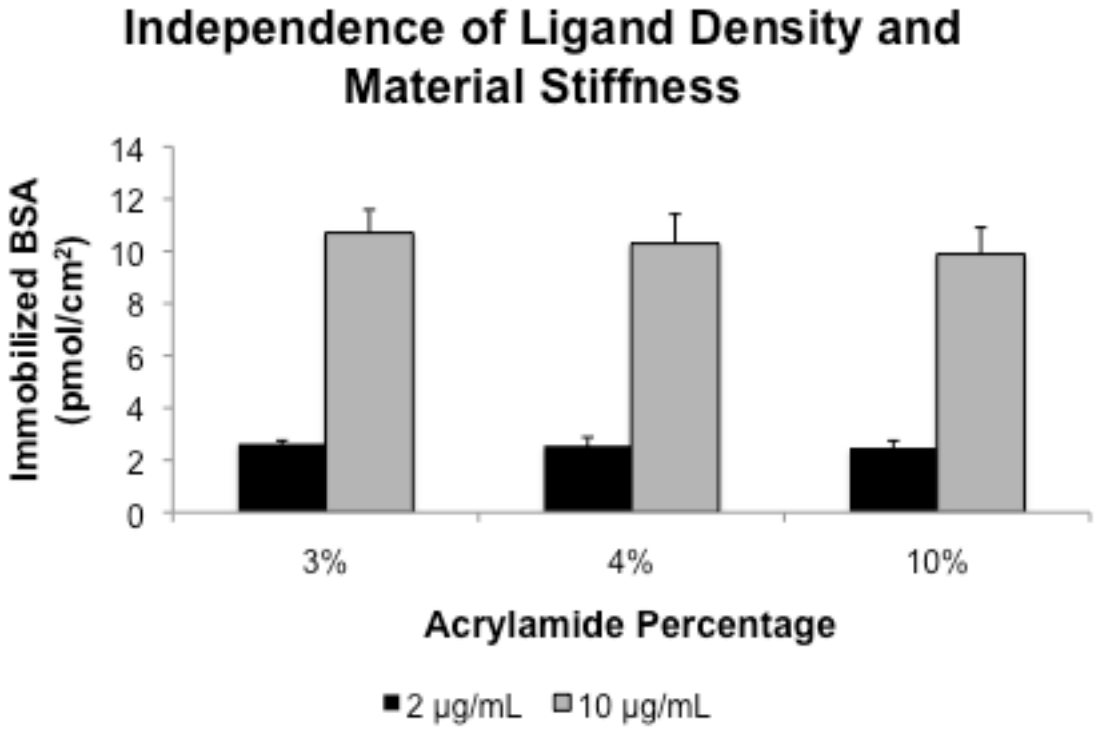

Figure 6. Independence of ligand density and material stiffness. The final immobilized ligand density is independent of acrylamide percentage.
* indicates $p<0.05$

4.4 Streptavidin density can be effectively controlled on PEGDA IO scaffolds

Ligand density was again measured using the indirect method. Problems in the sensitivity of the assay prevented accurate measurements for the final biotinylated probe. Instead, biotinylated PEGDA scaffolds were incubated in 1 mL of solution with incrementally increasing streptavidin-PE concentration. The data are shown in *Figure 7*. Scaffolds incubated with 1 µg/mL streptavidin-PE bound 0.0045 ± 0.0056 pmol/cm^2. Gels incubated with the next lowest concentration (2 µg/mL) bound 0.027 ± 0.006 pmol/cm^2. Scaffolds incubated with 3 µg/mL streptavidin-PE bound 0.034 ± 0.007 pmol/cm^2, while the highest concentration (4 µg/mL) yielded the highest density (0.053 ± 0.008 pmol/cm^2). All increasing concentrations resulted in significantly higher densities than that of scaffolds incubated with only 1 µg/mL streptavidin-PE.

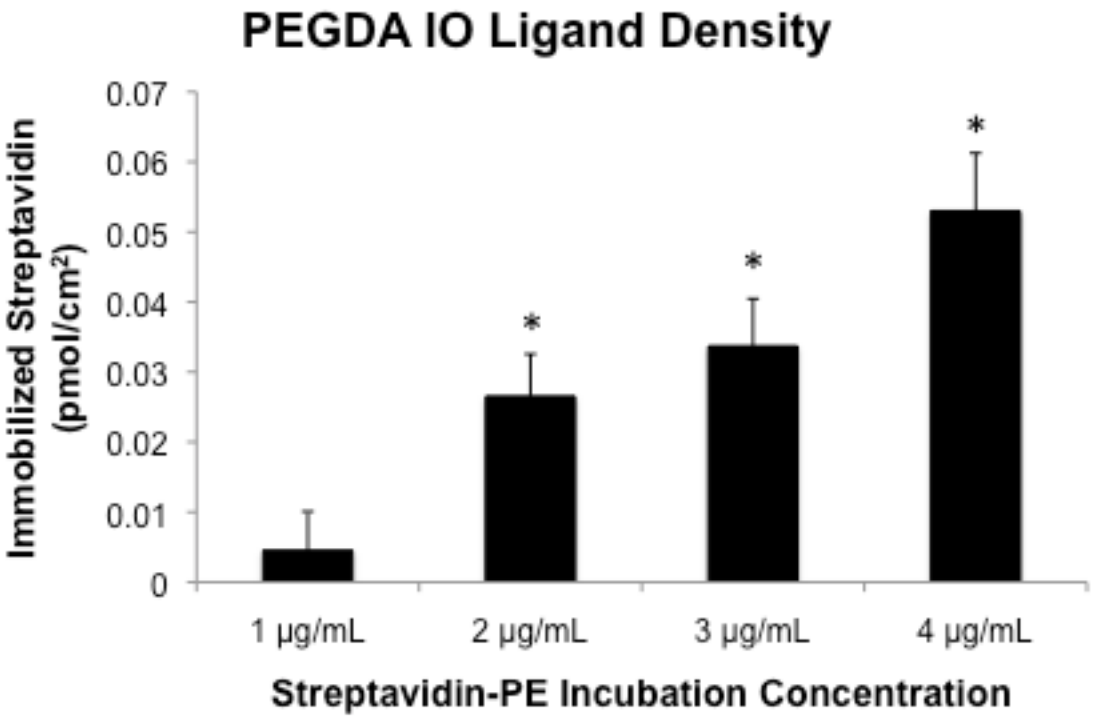

Figure 7. PEGDA IO ligand density. Increasing cross-linker model protein incubation concentration increases final immobilized density. * indicates $p<0.05$

4.4 Rheology is an effective measure of mechanical properties for tissues and soft substrates

Rheology optimization requires determining a strain (%) and frequency (Hz) of oscillation that provide linear storage modulus curves for all substrates examined. In this study, five substrates were examined: mouse thymuses, 3% PA gels, 4% PA gels, 5% PA gels, 10% PA gels, and 40% PEGDA gels. A sweep from 0.1% to 10% strain at 1 Hz and a sweep from 0.1 to 10 Hz at 5% strain were performed. The strain sweep was linear at 5% strain and the frequency sweep was linear at 1 Hz for all materials, both synthetic and native, as seen in *Figure 8*. Thus, all rheological studies performed were executed with 5% strain and 1 Hz.

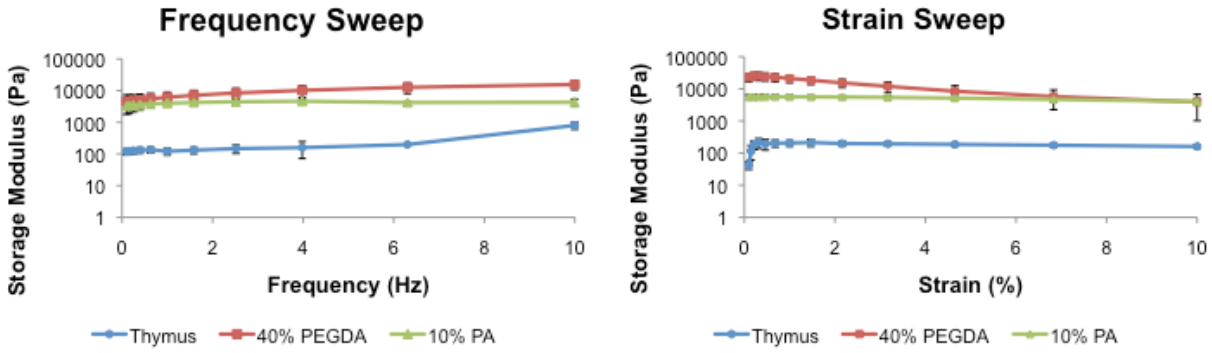

Figure 8. Frequency and strain sweeps for rheology optimization. A 1 Hz, 5% strain protocol is linear for all samples.

4.6 4% acrylamide, 0.04% bis-acrylamide PA gels mimic the thymus

Rheological data is visualized in *Figure 9*. The thymus was found to have a storage modulus of 110 ± 30 Pa. A 40% bulk PEGDA gel with no pores had a storage modulus of 7300 ± 3500 Pa. PA gels decreased with decreasing concentrations of acrylamide. 10% PA gels had a storage modulus of 5100 ± 4400 Pa; 5% PA gels had a storage modulus of 220 ± 66 Pa; 4% PA gels had a storage modulus of 120 ± 35 Pa; 3% PA gels had a storage modulus of 3.3 ± 3.4 Pa. T-tests were performed comparing the storage modulus of the synthetic materials to that of the thymus. 40% PEGDA and 10% PA were both significantly stiffer than the thymus while 3% PA was significantly softer. Both 5% and 4% PA gels were not statistically different from the thymus, but 4% gels had an average storage modulus closer to that of the thymus. For all subsequent experiments, 4% gels were used to mimic the mechanical microenvironment of the thymus.

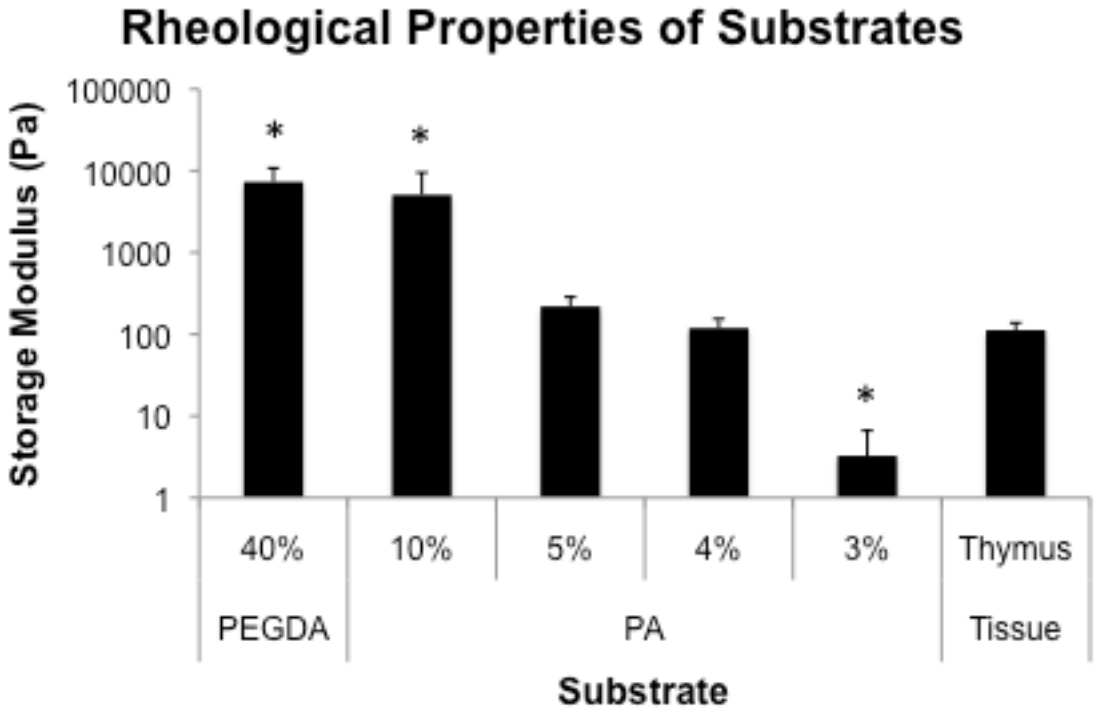

Figure 9. Rheological properties of substrates. 4% PA gels mimic the thymus.

4.7 PEGDA IO scaffolds induce Notch signaling with dose dependency

Hes-1 gene expression was measured using RT-PCR. PEGDA IO gels were fabricated and functionalized with low and high concentrations of Notch ligand (2 μg/mL and 10 μg/mL, respectively). Following three hours of culture, levels of *Hes-1* expression were found to be dose-dependent on the density of ligand bound, as seen in *Figure 10*. Levels of expression were compared against plain scaffold unmodified with DLL4, and normalized to calculate the fold change in *Hes-1* expression. Cells seeded in scaffolds with low ligand density had a 1.9 ± 0.8 fold increase in *Hes-1* expression while cells in scaffolds with high ligand density experienced a 2.9 ± 0.4 fold increase. The large standard deviation in the low-density scaffold data made the fold increase insignificant. However, results at 10 μg/mL demonstrated a significant increase in Notch signaling in the presence of a high concentration of immobilized bDLL4.

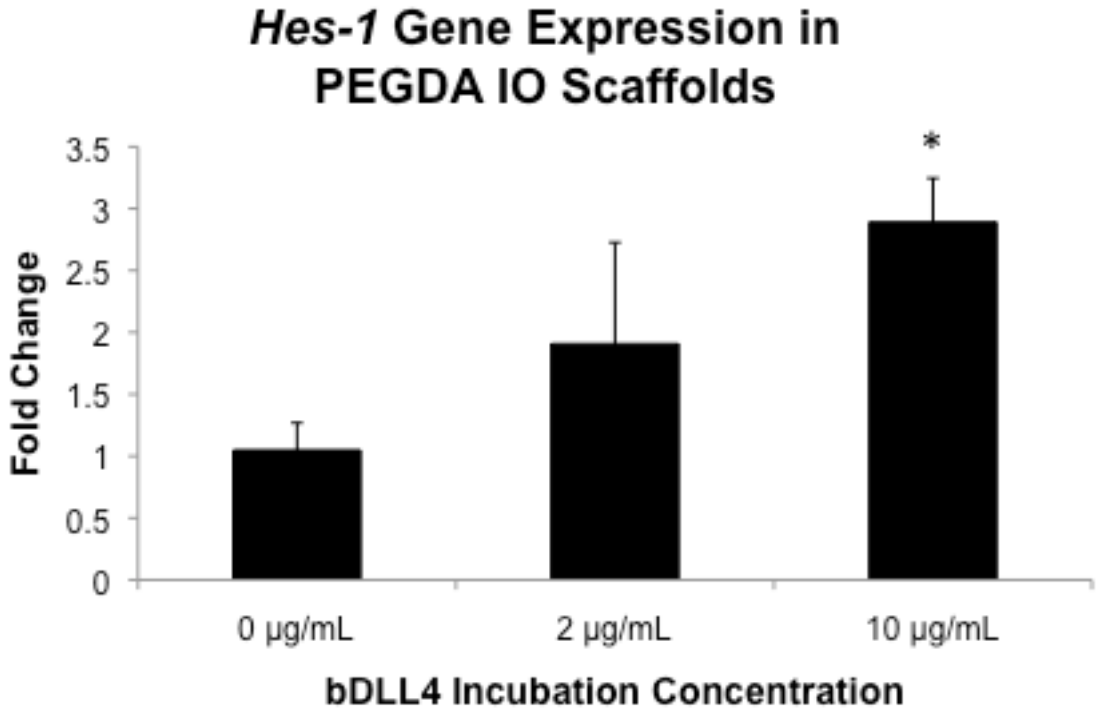

Figure 10. *Hes-1* gene expression in PEGDA IO scaffolds. Increasing ligand density results in increasing levels of Notch signaling.
* indicates $p<0.05$.

4.8 Soft PA gels induce latent Notch signaling

RT-PCR with cells seeded on 2D PA gels revealed interesting patterns in Notch signaling, as seen in *Figure 11*. *Hes-1* expression data was normalized against untreated well plates. Well plates treated with low and high concentrations of bDLL4 (2 µg/mL and 10 µg/mL, respectively) to demonstrate the effectiveness of immobilized Notch signaling in 2D. Wells modified with low concentrations of bDLL4 showed a significant increase in gene expression with a 2.8 ± 0.8 fold increase in *Hes-1* expression. Furthermore, a significant increase in *Hes-1* expression was detected in 10% gels treated with a high concentration of bDLL4, with a 1.8 ± 0.2 fold increase in *Hes-1* expression. Interestingly, non-functionalized soft 4% and 10% surfaces also showed an increase in gene expression, although they were not bound with signaling ligand. 4% gels exhibited a fold increase of 2.3 ± 0.5 in levels of *Hes-1* and 10% gels

displayed a fold increase of 1.9 ± 0.6 in levels of *Hes-1*. Only the 4% gels, however, had an increase in *Hes-1* gene expression that was statistically significant.

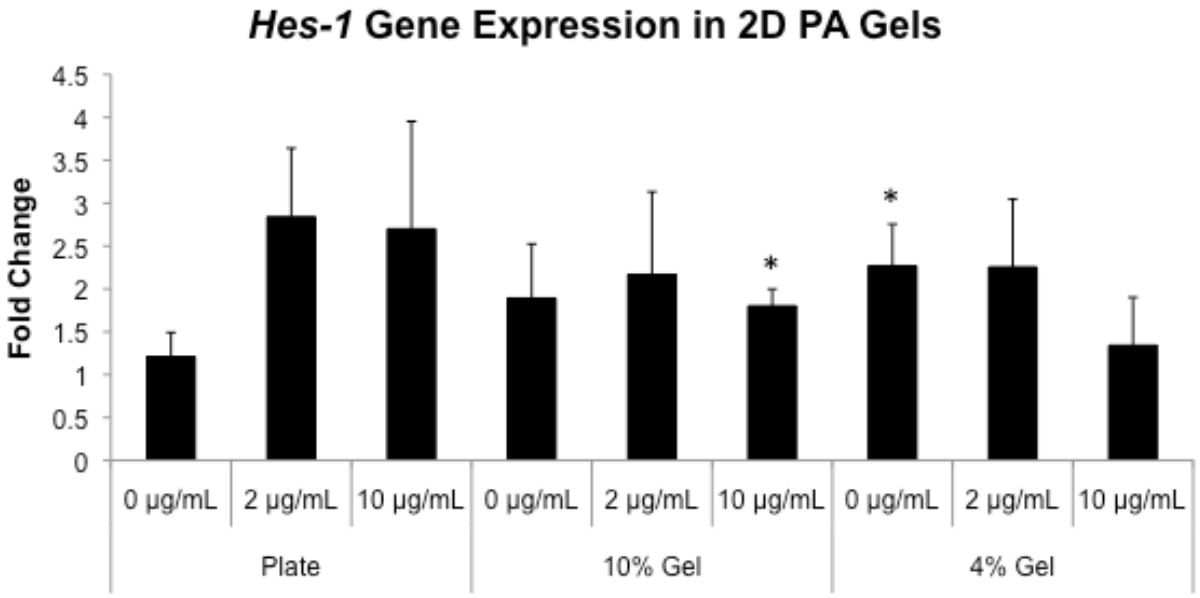

Figure 11. *Hes-1* gene expression in 2D PA gels. No clear link between binding density and gene expression can be seen. However, there is some latent *Hes-1* expression in 4% gels. * indicates $p<0.05$.

4.9 PA gels induce early T cell differentiation after nine days of culture

The fraction of cells with a Thy1.2$^+$CD25$^+$ double-positive phenotype was measured after nine days in culture. The percentage of cells was relatively low, less than 5%, and due to large standard deviations, means and statistical analysis did not yield statistically significant results. Thus, a representative flow cytometry plot is shown below in *Figure 12* that demonstrates the common trends. In well plates, cells cultured on substrates with no ligand or with low concentration of ligand (2 µg/mL) exhibited low levels of differentiation, and less than 1% of the cells were Thy1.2$^+$CD25$^+$. Plates with high density (10 µg/mL), however, showed 3.83% differentiation efficiency. 4% and 10% PA gels both induced a higher degree of differentiation in low DLL4 density conditions, with 1.11% and 2.94% fractions of double-positive cells. Gels

with high DLL4 densities exhibited 3.03% differentiation in 4% gels and 3.76% differentiation in 10% gels.

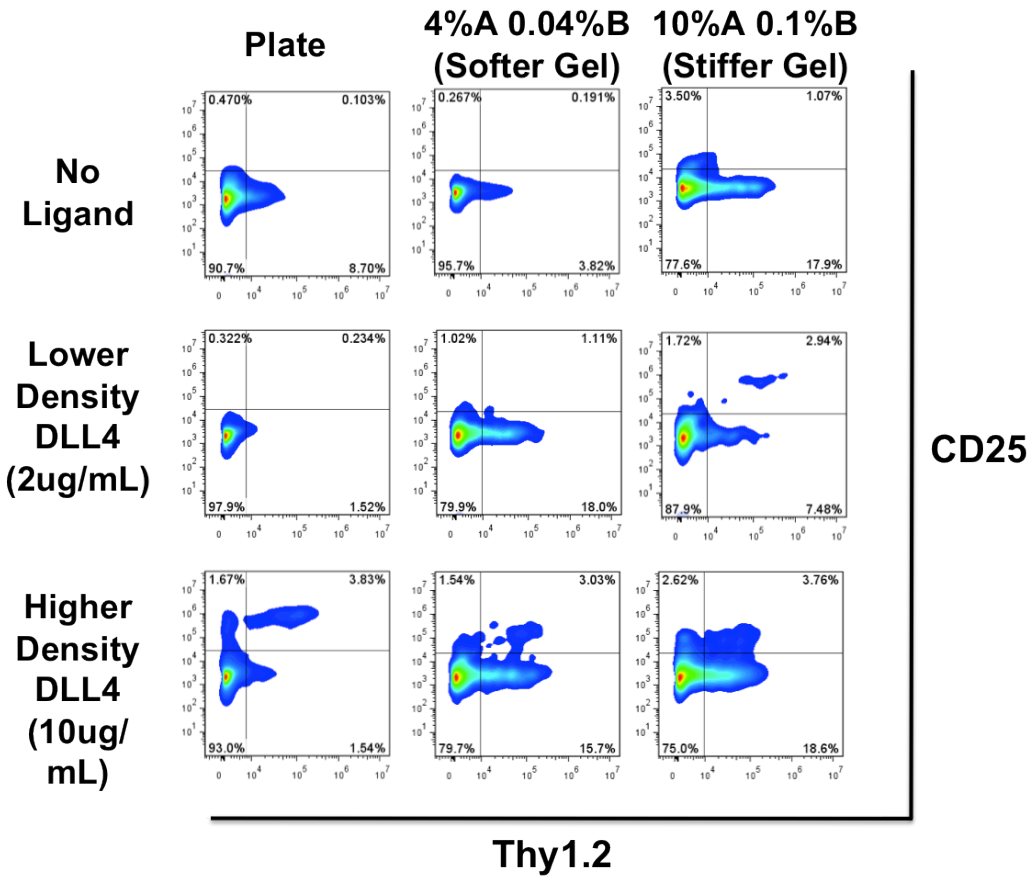

Figure 12. Induced differentiation on PA gels. Thy1.2^{+}CD25^{+} cells are considered early T cells.

5. Discussion

In adoptive transfer therapy, autologous T cells are expanded *ex vivo*, pulsed with antigen, and re-infused back into the patient in the hopes that these T cells will help the patient defend against an invasive pathogen or malignant tumor[2–4]. However, if the patient is severely immunocompromised, finding sufficient T cells to culture *ex vivo* may be challenging if not impossible. Perhaps even more significantly, expanding and maturing T cells in the laboratory can be extremely time-consuming, and waiting for T cell growth in imperfect laboratory conditions is a luxury that patients suffering from life-threatening diseases may not have. Because of this, there is an urgent need to have T cell banks on store for patients in case they become severely immunocompromised or need immediate immunotherapy to defend against an infiltrating pathogen.

Stem cells are an attractive source from which to develop these banks because of their differentiating potential and tendency to self-replicate, providing a potentially steady and constant source of T cells. The field of tissue engineering has made great strides in the past decades regarding optimal conditions to induce stem cell differentiation. Engineers have utilized the idea of bio-mimicry to as accurately as possible replicate the conditions of the native tissue in synthetic system, with the hypothesis that stem cells sense their local niche to determine towards which lineage they should differentiate. To this end, tissue engineers have fabricated elaborate three-dimensional systems that present growth factors and signals to cells occupying carefully designed pores, because cells grow in three-dimensions in the body, and not on flat surfaces. Similarly, engineers have concluded that the mechanical properties of a stem cells' microenvironment also contribute to the stem cell niche, and cells will differentiate into different types of cells depending on the stiffness and compliance of the substrate on which they reside[12].

The development of a precisely-designed spatial, chemical, and mechanical microenvironment for differentiation appears particularly critical in T cell differentiation. T cells natively develop in the thymus, an organ with an elaborate three-dimensional architecture. There are distinct cortical and medullar regions, each of which are responsible for different stages in the T cell developmental pathway. Thus, it is reasonable to believe that a three-dimensional environment could be substantially more efficient at inducing T cell differentiation than a flat surface more traditionally used in cell culture techniques.

In addition, the chemical microenvironment of the T cell differentiation scheme must be finely tunable. Immunologists have elucidated several factors in the differentiation pathway, but one of the most critical is an immobilized Notch ligand, such as DLL4, that blocks B cell differentiation and thus promotes T cell differentiation instead. Any substrate capable of directing stem cells toward the T cell lineage must thus be capable of immobilizing these ligands.

Furthermore, the mechanical environment must be finely tuned. One of the prevailing hypotheses describing the mechanism for a cell's sensing of its mechanical microenvironment is that mechanosensors in the cytoskeleton of a cell pull on the extracullular substrate[12]. If the substrate is non-compliant and tugs back with a force that is too great, the mechanotransducing structure can unfold, making it non-functional. On the other hand, if the substrate is too soft, the mechanosensor remains in a conformation that obscures its active site, also making it non-functional. Only if the substrate is ideally compliant will the mechanosensor remain natively folded but with an exposed active site, facilitating down-stream signaling events. While HSCs are non-adherent cells and thus do not exchange forces with a permanent substrate with defined mechanical properties, they do come into contact with a physical substrate during the process of Notch signaling, because the Notch ligand must be immobilized. During the first step in Notch

ligand sensing, the receptor binds to the ligand and the force results in a cleavage event in the extracellular portion of the receptor. Because of this, it is reasonable to believe that mechanotransduction could be a part of the Notch transduction pathway. Thus, mimicking thymic mechanical properties could significantly increase T cell differentiation potential.

Unfortunately, current methods of *in vitro* T cell differentiation are neither three-dimensional nor are they of suitable material stiffness. One current method of *in vitro* T cell differentiation involves growing stem cells on a modified layer of stromal cells derived from the bone marrow that has been transfected with DLL4 to induce T cell differentiation. Another isolates DLL4 and independently binds the ligand to tissue culture treated plates to promote Notch signaling and T cell lineage commitment. Neither are adequate simulations of the thymic microenvironment.

To this end, this study presents two differentiation protocols that improve synthetic conditions using biomaterials, or materials that can interface with biological systems. One substrate is an ordered, porous scaffold called an inverse opal structure made from PEGDA. The other is a two-dimensional gel with finely tunable mechanical properties composed of PA. Because the pores in the PEGDA IO scaffolds collapse if the gel is too soft, this study does not present a system that solves both the three-dimensional problem and the problem of the tunable mechanical environment. Instead, model systems explore each variable independently.

Data presented above demonstrate that both the PEGDA IO and 2D PA systems provide intricate control over the chemical microenvironment. Chemical connection systems have been developed to present streptavidin on the surfaces of functionalized polymer gels that can then be bound to biotin-modified DLL4. Incubating with higher concentrations of streptavidin and/or biotin result in higher final spatial densities of DLL4 on the polymer surface. Thus, the chemical

microenvironment can be finely tuned to develop any density of DLL4 between zero and the saturation point on the gel, a point that was never reached in the experiments described here.

Similarly, the mechanical properties of the 2D PA gel system can be tightly controlled by modifying the amount of acrylamide versus bis-acrylamide, the two monomer types in the PA chain. Rheological studies demonstrated that 4% acrylamide, 0.04% bis-acrylamide gels accurately mimic the mechanical properties of the thymus, which has a storage modulus of approximately 110 Pa. These gels can be made more or less stiff by increasing or decreasing the concentration of acrylamide and bis-acrylamide, respectively. Importantly, modifying the stiffness of the gel does not affect the density of ligand that can be bound. Thus, for the 2D PA system, mechanical stiffness and ligand density are orthogonal systems each independently tunable for optimization.

Although PEGDA scaffolds could not be mechanically modified to the same extent as 2D PA gels, rheological studies were also performed to elucidate their mechanical strength. The concentration of PEGDA used in this study (40%) yielded a storage modulus several orders of magnitude higher than that of the thymus or 4% PA gels, averaging at approximately 7000 Pa. However, this elasticity is still a significant improvement over traditional polystyrene surfaces, which have elastic moduli on the order of GPa. Thus, even though the 3D PEGDA system suffers from a lack of mechanical tunability, it still offers compliance far closer to the thymus than customary tissue culture practice.

None of the attempts to mimic the thymus are meaningful, however, unless they 1) successfully induce Notch signaling and 2) result in T cell differentiation. The most promising Notch signaling data collected thus far is with the 3D PEGDA scaffolds, which show a clear dose dependency in levels of *Hes-1* expression following incubation of cells in functionalized

scaffolds for three hours. The clear relationship between ligand density and Notch signaling was absent when cells were instead seeded on 2D softer surfaces. On one hand, this indicates that the tunability of the PA system is somewhat lacking, because although ligand density can be finely regulated, this does not translate into effective control over the level of Notch signaling. On the other hand, however, the basal level of Notch signaling on soft 4% PA surfaces that mimic the mechanics of the thymus (~2.5 fold above the control) is comparable to the high levels of Notch signaling induced on the slightly harder 3D surfaces with the highest density of Notch ligand (~2.75 fold above the control).

The idea that soft surfaces can induce basal levels of Notch signaling is an interesting one that has not yet been explored in the literature. Further mechanical studies on the molecular interactions between ligand-bearing stromal cells and receptor-bearing progenitor cells *in vivo* must be conducted to determine whether there is a causal relationship between mechanical properties and Notch induction or whether the observed apparent relationship is instead due to some other variable that has not been controlled in this study.

Finally, differentiation data was collected for $lin^{-}ckit^{+}sca1^{+}$ cells cultured on 2D PA gels with a cytokine cocktail designed for expansion and differentiation. After nine days of culture, about 3% of the cells had a $Thy1.2^{+}CD25^{+}$ double-positive phenotype indicative of early T cells. This efficiency is relatively low compared to other *in vitro* T cell differentiation systems that have been developed. In their studies using OP9-DL1 co-culture, the Zúñiga-Pflücker group reports nearly 95% CD25 expression, whereas the experiments described above produced roughly 4% CD25. Differentiation on 2D PA gels also yields high numbers of $CD44^{+}CD25^{-}$ cells (data not shown), indicative of the DN1 stage in development, whereas CD25 expression is indicative of DN2 and DN2 expression. Thus, compared to the Zúñiga-Pflücker method, the soft-

substrate differentiation strategy appears to arrest the cells at an earlier stage in development. The Varnum-Finney group has also reported highly efficient T cell differentiation using immobilized ligands on tissue culture treated polystyrene surfaces. They report plots where >50% of the cells are Thy1.2^{+}CD25^{+} double positive. However, it should be noted that these results were measured after three weeks of culture, versus culture just over one week on soft surfaces.

These data indicate that further optimization may be required to induce differentiation beyond the DN1 stage in development. Nonetheless, the systems presented in this thesis have clear advantages. First, the creation of highly porous 3D systems potentially allows for much more robust scale-up in the future. These scaffolds have significantly higher spatial efficiency than conventional tissue culture flasks. If the dream of creating a bank of T cells is to be realized, using porous *ex vivo* scaffold technology would be a far more space-efficient means of growing, cultivating, and differentiating cells prior to re-implantation into the immunocompromised host. Second, although the 2D PA system seems less effective at inducing efficient T cell differentiation, it provides a novel means to study the effects of mechanical properties on differentiation, which may be critical to further research in the field. Results hint at a non-canonical form of Notch signaling that should be further explored.

6. Conclusions

The data described in this thesis indicate that soft polymer systems can be developed to mimic the thymic microenvironment both chemically and mechanically. These include a polyacrylamide two-dimensional system and a porous three-dimensional poly(ethylene glycol) system. Furthermore, these systems are suitable as substrates for cell growth and differentiation. Studies presented above show that the polymers can be chemically modified to be bioactive. Ligands, or biological signals, can be attached in a reliable fashion, and used to direct stem cells down a particular differentiation pathway. Gene expression studies indicate that when ligands responsible for Notch signaling—the key signaling event in early T cell differentiation—are chemically bound to these polymer surfaces, they increase the expression of genes downstream in the Notch signaling pathway. These short-term studies have also been translated into long-term differentiation studies, and functionalized polyacrylamide has been shown to produce early-stage T cells. Together, these results demonstrate the potential that biomaterials have in the field of immunotherapy, in particular in the differentiation of T cells from stem cells.

7. Acknowledgments

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