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Biomolecules that impact phenotypic transitions in fibroblasts and macrophages

Kiva Diane Forsmark

Iowa State University

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Biomolecules that impact phenotypic transitions in fibroblasts and macrophages

by

Kiva D. Forsmark

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Chemical Engineering

Program of Study Committee:
Kaitlin M. Bratlie, Major Professor
Balaji Narasimhan
Michael Wannemuehler

Iowa State University
Ames, Iowa
2016

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I would like to dedicate this thesis to those in my life who have provided me with unconditional support. This endeavor would not have been possible without each and every one of you.
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Macrophages and fibroblasts are intricately involved in many biological responses. The foreign body response (FBR) is orchestrated by macrophages, wherein the phenotypes of these macrophages exist plastically along a spectrum from M1, proinflammatory macrophages to M2a, anti-inflammatory, pro-wound healing macrophages, and M2c, regulatory macrophages. The timely progression of macrophages through these phenotypic changes is necessary for the resolution of this response. Interplay between M2a macrophages, myofibroblasts and M2c macrophages is responsible for the eventual resolution of this response. Following, transforming growth factor-β (TGF-β) activation, fibroblasts differentiate into myofibroblasts; these cells will secrete and contract extracellular matrix (ECM) components into a fibrous capsule. Herein, a library of modified poly-L-arginine (PLR) was used to influence the fibroblast to myofibroblast transition, and it was found that small variances in chemical modification allow for or impede this process. This excessive ECM deposition is also characteristic of some cancers that undergo what is known as a desmoplastic response. Desmoplasia is most often cancer promoting. Like the FBR, this response involves M1, M2a, and M2c macrophages and myofibroblasts. Increased levels and sulfation patterns of chondroitin sulfates (CS) and their associated proteoglycans are associated with this response. Increased arginase activity, increased iNOS activity, and morphological changes, indicating phenotypic changes in macrophages, were seen when CS-A, CS-B, CS-C, and CS-E were cultured with RAW 264.7 macrophages of five different phenotypes.
CHAPTER 1
FIBROBLASTS AND MACROPHAGES IN THE FOREIGN BODY RESPONSE AND CANCER

Section 1.1, Section 1.1.1 pages 1-5, and Section 1.2.1 are published in *Clinical and Translational Medicine*, 2014, volume 3, pages 1–13.

1.1 Foreign Body Response to Implanted Materials

Macrophages are involved in ECM remodeling, proliferation of epithelial cells, development of vasculature and the organization of tissues during development [1]. These functional capacities of macrophages extend into the wound healing response and the FBR to biomaterials. Macrophage phenotype is dynamic throughout the course of these processes, and the balance between phenotypes is instrumental in the timely progression of these responses from injury to successful healing. Macrophage phenotype affects the activation of fibroblasts during this healing process [2]. As with tumor associated macrophages (TAMs), macrophages involved in healing retain their plasticity and alter their phenotype in response to a variable cytokine microenvironment in the progression of these processes [3].

1.1.1 Overview of the foreign body response to implanted scaffolds

Surgical implantation or injection of a biomaterial-based construct injures the tissue, resulting in an influx of blood and cell death. Dying cells release danger signals (danger associated molecular patterns, DAMPs) that induce local inflammation [4] and activate resident macrophages [5,6]. These DAMPs include HMGB1, histones, and uric acid [4,5,7,8]. Blood proteins such as albumin, fibrinogen, fibronectin, immunoglobulin G (IgG), and various complement proteins adsorb to the surface of the biomaterial [9]. Activation of
the complement cascade results in opsonization of the biomaterial surface with C3b and induces inflammation through the anaphylatoxins C3a and C5a [10]. These anaphylatoxins recruit leukocytes to the site of inflammation, cause histamine release from mast cells, and induce oxidative bursts in neutrophils [11]. Release of histamine from mast cells attracts neutrophils and monocytes [12,13]. Neutrophils are the first immune cells to arrive at the implant site [14] and, along with mast cells, secrete Interleukin-4 (IL-4) and IL-13 early in innate immune responses [15].

Monocytes are the next immune cells to extravasate into the tissue, where they differentiate into tissue macrophages [16]. These macrophages are classically activated upon the adsorbed protein layer [17,18]. Proteins, such as fibrinogen, C3, and C3b on the surface of the biomaterial are bound by the integrin αMβ2 (CD11b:CD18), also known as complement receptor 3 (CR3), on the surface of macrophages [16,19–21]. Activated macrophages secrete tumor necrosis factor-α (TNF-α), IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, RANTES, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), IL-1β, and matrix metalloproteinases (MMPs) [22–24]. The chemokines macrophage inflammatory protein (MIP)-1α, IL-8, and MCP-1 attract additional monocytes [22]. These biomaterial-activated macrophages are also characterized by an increased phagocytic capacity [25]. Continued presence of pro-inflammatory macrophages causes acute inflammation to morph into chronic inflammation [26].

Attempted phagocytosis of biomaterials leads to the fusion of adherent classically activated macrophages into foreign body giant cells (FBGCs) [27]. IL-4 and IL-13 induce the fusion of adherent macrophages [27]. β1 and β2 integrins are involved in the fusion of these macrophages [28], and CCL2 guides the chemotaxis of adherent macrophages towards each
other [29]. FBGCs have a cytokine profile more characteristic of alternatively activated macrophages that includes transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), IL-1rα, and IL-10 [15,16,23,30]. FBGCs secrete protons, ROS, and MMPs in an attempt to eradicate the foreign body [31,32]. Like M1 macrophages, FBGCs secrete pro-inflammatory RANTES and the chemoattractant MCP-1 [23]. ECM breakdown by MMPs leads to increased DAMPs in the microenvironment and further macrophage activation [33].

The resolution stage of the FBR is dominated by alternatively activated macrophages. A profibrotic, alternatively activated, wound healing macrophage phenotype results from macrophage phagocytosis of dying cells, stimulation by IL-4 or by IL-13 [34,35]. These dying cells include epithelial and endothelial cells that are damaged by pro-inflammatory cytokines, such as TNF-α, and short-lived neutrophils [2,36]. Alternatively activated macrophages secrete profibrotic mediators such as TGF-β, IL-4, IL-13, IL-10, arginase, and ECM components [15,37]. These macrophages drive the wound healing response by activating mesenchymal cells that participate in the wound healing process [38,39]. TGF-β can also induce an M2-like phenotype in macrophages [40]. These M2 macrophages are profibrotic, but are still unable to reduce the pro-inflammatory response. Reduction of chronic inflammation requires IL-10-induced activation of regulatory M2-like macrophages [15,41]. These macrophages secrete high levels of the same protein that activates them [15]. IL-10 prevents the translation of pro-inflammatory cytokines by macrophages through STAT3 [42,43].

As in the immune response to parasitic infections, the early phase of wound healing and the FBR is characterized by M1-like macrophages and the late phases are controlled by M2-like macrophages [30,36,44–46]. In the healing of aseptic wounds regulatory M2 (IL-10
stimulated) macrophages rapidly downregulate the inflammatory response to promote tissue repair [15,47–50]. Conversely, in the FBR, further activation of macrophages will occur, resulting in continued chronic inflammation (pro-inflammatory macrophages and FBGCs) and continued wound healing (wound healing macrophages).

It has long been hypothesized that chronic inflammation is present until an extensive fibrous capsule surrounds the biomaterial [14]. Resident fibroblasts, fibrocytes, and macrophages are activated by TGF-β, and become myofibroblasts [51–55]. Myofibroblasts secrete high amounts of collagen I, collagen III, and fibronectin [50,56]. The expression of α-smooth muscle actin (α-SMA) permits myofibroblasts to contract collagen networks in a process known as contractile scarring [57,58]. Incessant activation of myofibroblasts results in continued secretion and contraction of ECM components. This eventually results in excessive scarring, and fibrous encapsulation. The fibrous capsule is a dense, hypocellular, avascular collagenous network that reduces the diffusion of all molecules, and results in the failure of scaffolds for applications in tissue engineering [59,60]. The entire process leading up to fibrous encapsulation is illustrated in Figure 1.1.
Figure 1.1. Macrophage phenotype in the wound healing and foreign body responses.
Most of what is known about myofibroblasts comes from research performed in relation to chronic fibrosis and wound healing. The contractile myofibroblast was first discovered in the granulation tissue of a skin wound [61]. Myofibroblasts are also present during renal fibrosis [62], hepatic fibrosis [63], scleroderma [64], and during fibrous encapsulation of implanted biomaterials [65]. Myofibroblast differentiation is a two-step process (Figure 1.2). First, in response to increasing rigidity in their microenvironment, fibroblasts will differentiate into an extended precursor cell known as the proto-myofibroblast [51]. Differentiation into an α-SMA-expressing myofibroblast can then occur in response to TGF-β. TGF-β, secreted by macrophages [66] during these responses, induces the myofibroblast phenotype [67]. This transition from fibroblast to myofibroblast is dependent on the presence of an alternative splice variant of fibronectin, known as EDA-FN, in the ECM [50,68].

TGF-β is secreted in an inactive form known as latent TGF-β (L-TGF-β) that is bound to the ECM [69–71]. MMPs -2 and -9, or myofibroblast contraction of the ECM can cleave the latent associated peptide (LAP) from L-TGF-β and release TGF-β into the microenvironment [70,72,73]. TGF-β is highly expressed in peri-implant tissue [16,65]. Though instrumental in traditional wound healing, excessive activation of the myofibroblast leads to fibrosis and fibrous encapsulation of implanted scaffolds.
1.1.2 Scaffolds to instruct phenotypic macrophage responses

Depending on biomaterial properties and the cytokines secreted by inflammatory cells in the biomaterial microenvironment, macrophages adopt either an M1- or M2-like state [74]. As macrophages are plastic, they can exist on a spectrum between these two states. This leads to the hypothesis that surface chemistry and physical properties of scaffolds can be used to polarize macrophages towards a specific phenotype, or away from another. In particular, some scaffolds have been engineered to reduce prolonged activation of M1-like macrophages, so that cell-laden scaffolds maintain cell viability [75,76]. Additional scaffolds have been engineered to reduce excessive fibrosis and decrease time to incorporation of the implant [77]. A balance in macrophage phenotype must be achieved for scaffold vascularization.

Varied scaffold chemistries suggest the ability to decrease the expression of M1 macrophages. Microgel conformational coatings formed from poly(N-isopropylacrylamide)
(pNIPAm) and poly(ethylene glycol) diacrylate (PEGDA) reduce fibrinogen adsorption, macrophage adhesion, macrophage spreading, and secretion of inflammatory cytokines [78]. Zwitterionic hydrogels are able to reduce protein adsorption and are characterized by anti-inflammatory, pro-healing macrophages that promote angiogenesis and show no evidence of a collagenous capsule for longer than three months [79]. The ability of macrophages to induce positive tissue remodeling on fourteen different biologically-derived surgical meshes was investigated, and suggested that a predominance of M2 macrophages could potentially lead to more constructive tissue remodeling after two weeks [74]. Sugisis and Matristem scaffolds – derived from porcine small intestinal submucosa, and urinary bladder, respectively – appeared to increase macrophage infiltration; whereas the other scaffolds, derived from human and porcine dermis, appeared to prolong the healing response and exhibited an increase in M1-like macrophages [74].

In addition to chemical properties, physical properties of scaffolds can significantly influence macrophage phenotype. Controlling the pore size of scaffolds is one technique that shows promise in decreasing pro-inflammatory macrophage presence and improving the healing outcome. A pore size of 30-40 µm within porous template scaffolds formed of five different synthetic polymers and one natural polymer appeared to increase infiltration of macrophages and vascular density, suggesting that these materials induce regenerative macrophages [80–82]. It is generally thought that geometric restriction of macrophages within these pores prevents them from spreading out into their phagocytic, inflammatory phenotype [83–85]. Vascular density is suggested to peak at pores size of 35 µm [80,86]. The degree of porosity in a material can also influence macrophage phenotypes, with more porous materials leading to decreased healing time of implants and, therefore, a reduced fibrous
capsule thickness. For example, even though porous polytetrafluoroethylene (PTFE) surfaces seemed to induce inflammatory cytokine secretion by macrophages, a thinner fibrous capsule was formed on porous versus nonporous PTFE [87]. BMDMs cultured on electrospun polydioxanone (PDO) of larger fiber length and pore size showed increased arginase, TGF-β, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor expression, characteristic of alternatively activated macrophages, than those cells cultured on scaffolds with smaller fiber length and pore size [77].

Substrate morphology and surface topography represent two other physical properties of scaffolds that are thought to influence macrophage phenotype and thus the foreign body response and material biocompatibility. 2D and 3D sP(EO-stat-PO) surface modified poly(D,L-lactide-co-glycolide) (PLGA) substrates were compared to find that the flat surfaces studied in this work lead to pro-inflammatory cytokine profiles while 3D nanofibers resulted in increased pro-angiogenic chemokines and angiogenesis [88,89]. Micro- and nanostructured surfaces have also been examined to determine the effect of surface topography on macrophage behavior [90–95]. Several studies have suggested macrophage responses are more greatly impacted by micro-patterned surfaces than corresponding nanostructures [90–92,95], however, few distinctive correlations have been revealed. Some trends indicate that larger posts or widely separated posts on material surfaces induce anti-inflammatory phenotypes in macrophages [90,91], while others suggest that nanostructured versus microstructured grooves decrease the pro-inflammatory response of macrophages [92,93,95]. Another surface property that has been examined with respect to macrophage phenotype is fiber diameter and orientation. Results from these studies indicate that aligned rather than randomly oriented nanofibers minimize inflammatory responses [75,96].
The processing of biologic scaffolds appears to alter macrophage phenotype. Processing of scaffolds such as subintestinal submucosa with a carbodiimide crosslinker can lead to a predominately M1 response resulting in chronic inflammation and prolonged healing; whereas the non-crosslinked scaffold appeared to induce a large M2-like response and constructive remodeling at sixteen weeks [85]. A low degree of acetylated chitosan scaffolds (5%) is suggested to induce a macrophage response characteristic of M2 macrophages and a reduced fibrous capsule. However, the 15% degree of acetylation resulted in adherent, activated pro-inflammatory macrophages [76,97], which again suggests that surface chemistry plays a role in macrophage response. Infiltration of blood vessels into a glutaraldehyde-crosslinked collagen scaffold was characterized by coordinated levels of M1- and M2-like macrophages [98].

It is suggested herein that a temporal balance between pro-inflammatory, wound healing, and regulatory (IL-10 stimulated) macrophages may be necessary for successful implantation of a scaffold for tissue engineering applications. Scaffold chemistry, pore size, and processing conditions appear to have the potential to regulate macrophage phenotype, and, therefore, the extent of inflammation, fibrous encapsulation, and angiogenesis of these materials. The effects of these biomaterial properties on macrophage phenotype and the fibrotic response are outlined in Table 1.1.
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1.1.3 Scaffold effects on fibrous encapsulation

Fibrous encapsulation of scaffolds and many other biomedical devices results in their failure. In the case of microencapsulated islets for diabetes therapeutics, the encapsulated cells become necrotic due to a lack of oxygen and other nutrients [99,100]. Biosensors are also rendered useless because analytes are unable to diffuse to and be detected by the sensor [59]. Additionally, long-term failure of implanted constructs, such as breast implants, often results from continued fibrous capsule formation and matrix remodeling [101,102]. Material properties such as chemistry, scaffold processing, pore size, and topography have effects on the thickness and character of the fibrous capsule that is the common end result to the FBR.

Variance in material chemistry, or surface chemistry can significantly affect capsule thickness and character. The degree of acetylation (DA) of chitosan scaffolds effects the thickness of the resulting fibrous capsule at four weeks: 15% DA caused a thick fibrous capsule (~70 μm), whereas 4% acetylation lead to a fibrous capsule of ~45 μm [97]. PEG-modification of nanoporous alumina membranes caused a reduction in granulation layer thickness at 4 weeks when compared to the unmodified control [103]. Ultra-high molecular weight polyethylene (UHMWPE), a common control biomaterial, only caused a 5 μm thick fibrous capsule at 90 days post implantation [101].

As aforementioned, toxins in the scaffold prior to processing, or how scaffolds are crosslinked, have an effect on the foreign body response. Biological scaffold processing by carbodiimide affects the end tissue result, contributing to an extended M1 response, FBGCs, and fibrotic tissue [85]. The absence of carbodiimide crosslinking of these porcine-derived small intestinal submucosa allowed for organized, but incorporated tissue [85]. Different curing agents of silicone breast implants can also cause differences in fibrous capsule
thickness. Treatment of silicone implants by a platinum curing system (PSE) or a free radical peroxide system (RSE) caused a 10 µm thick capsule at 30 days, but at 90 days the thickness around the PSE implant was 15 µm and the RSE implant had a 33 µm thick fibrous capsule [101,104].

One of the strongest effects on macrophage phenotype and the FBR is through changing pore size or topography of materials, thus leading to a reduction in fibrous encapsulation of these materials. Analysis of two PTFE scaffolds, both with a 2:1 fluorine to carbon ratio, showed that PTFE with a porous intranodal distance of 4.4 µm caused a capsule thickness of 36 µm, compared to the nonporous scaffold that induced a 50 µm thick capsule [87]. Cylindrical disks of porous collagen implanted subcutaneously allowed for fibrous encapsulation even at 10 days; however, if M1 macrophages were present, the implant character was characterized by cellular infiltrate [98]. Sphere-templated porous poly(hydroxyethylmethacrylate) implants did not cause fibrous capsulation even at four weeks [86]. The topography of polycaprolactone (PCL) scaffolds also had a significant effect on fibrous capsule thickness. Fiber scaffold surfaces had fibrous capsules that were thinner than 8 µm, whereas the film surface caused a 40 µm thick capsule [96]. Material properties affect macrophage phenotype, which has varied effects on the extent of fibrous encapsulation of these scaffolds.

1.2 Fibroblasts and Macrophages in Cancer

The excessive deposition of ECM components, akin to fibrous encapsulation, is also characteristic of the desmoplastic response associated with cancerous tumor progression
The timeline of tumor development and the desmoplastic response still needs to be determined. It is possible that the desmoplastic response is an attempt of the host to eradicate the cancerous tissue, in a manner similar to the FBR, or a cancer-cell induced response to promote their own growth [105,108]. Excessive fibrosis adjacent to cancerous or metastatic tissue defines desmoplasia, and is associated with a poor prognosis [109]. This desmoplastic reaction is present in colorectal [108], pancreatic [109], and breast cancers [110].

There are six hallmarks of cancer that are related to the malignant cells and their interactions with their tumor microenvironment. Cancer is characterized by the ability to sustain proliferative signaling, resist cell death, induce angiogenesis, enable replicative immortality, activate tissue invasion and metastasis, and evade growth suppressors [111]. TAMs, cancer-associated fibroblasts, and components of the ECM are important aspects of the tumor environment that contribute to the development of cancerous tissue and the desmoplastic response [111].

Macrophages recruited to the cancer site are involved in tumor-promoting inflammation, contributing to many of the hallmarks of cancer. TAMs include both M1 and M2 macrophages. Macrophages are recruited to the tumor site by CCL-2 (MCP-1), a molecule that also contributes to tumorigenesis and metastasis [112]. Growth factors such as VEGF, PDGF and macrophage colony-stimulating factor (M-CSF) are also known to recruit macrophages to cancerous tissue [113]. TAMs themselves also secrete many of these factors, contributing to a feed forward loop of macrophage recruitment. Initially, M1, pro-inflammatory macrophages are induced leading to anti-tumor effects due to TNF-α and iNOS activity [114]. There are recent reports that macrophage phenotype is stage dependent,
and as cancer progresses macrophages polarize from a more M1-like phenotype that suppresses tumor growth to a tumor-promoting M2-like state [115].

TAMs contribute to cancer progression in a multitude of ways. Macrophages in the tumor environment are educated to secrete a mitogen that promotes cancer cell replication [116]. Through interactions with cancer cells, macrophages can promote invasion and metastasis [117]. Aberrant, tumor-promoting angiogenesis that occurs adjacent to cancer tissues, is also promoted by macrophages [118]. TAMs secrete cathepsins that promote tumor growth, angiogenesis and invasion in vivo [119]. M2 macrophages also produce pro-fibrotic mediators, such as TGF-β, that orchestrate ECM remodeling [36], contributing to the likely cancer-promoting desmoplastic response.

Cancer-associated fibroblasts are also associated with the desmoplastic response, and are also known as activated fibroblasts or myofibroblasts. These cells promote cancer development by promoting cancer cell division, invasion, metastasis, and support cancerous tissues by promoting angiogenesis [106,111,120]. The desmoplastic response in pancreatic ductal adenocarcinoma (PDAC) is characterized by extensive fibroblast to myofibroblast differentiation, where the myofibroblasts are often more prevalent than cancerous cells [121]. These myofibroblasts secrete ECM components, such as collagens, in the tumor microenvironment as part of the desmoplastic response to tumors.

This loss of ECM homeostasis in cancerous environments is a result of macrophage and fibroblast activation. Increased collagen in this cancer-promoting ECM includes types I, III, IV, and V [108]. Increased collagen expression is associated with malignancy in pancreatic cancer [121], and upregulation of types I and IV collagen can enhance the metastasis of colorectal cancer cells to the liver [122]. Other aspects of the ECM that change
during the desmoplastic response include changed expression of proteoglycans and glycosaminoglycans – contributing to the regulation of growth factor activity and cancer progression [123–126].
CHAPTER 2
MYOFIBROBLAST TRANSITION IN RESPONSE TO MODIFIED POLY-L-ARGININES

2.0 Introduction

Synthetic materials are placed in contact with biological systems for the alleviation of disease symptoms, chronic wound healing, and pain. The biocompatibility of these materials is defined by how well they interface with the adjacent tissue. Due to the innate immune response, known as the FBR, that is mounted against these devices and scaffolds, excessive healing, also known as constrictive fibrosis, can occur [101]. Often, this excessive healing process results in fibrotic encapsulation and isolation of the implant [14].

There is evidence that myofibroblasts are responsible for the secretion and orientation of the collagen that forms this fibrous capsule [65,104]. Myofibroblasts differentiate from fibroblasts, the primary collagen-producing cell in the human body, predominately in response to TGF-β1 [127]. TGF-β1 induces α-smooth muscle actin (α-SMA) expression in fibroblasts, providing the resultant myofibroblasts with heightened contractile ability [58]. TGF-β1 also induces collagen I, III, and IV expression in myofibroblasts [128].

TGF-β1 is intricately involved in wound healing [129] and is an important regulator of tissue fibrosis [52]. TGF-β1 is involved in all of the stages of wound healing: inflammation, granulation tissue formation, reepithelialization, matrix formation and remodeling [129]. In mice that overexpress TGF-β1, there is excessive collagen deposition adjacent to subcutaneously implanted poly(vinyl alcohol) sponges [130]. TGF-β1 is highly expressed in the tissue surrounding implanted biomaterials [16,65] and is secreted by M2a
type wound healing macrophages, M2c type regulatory macrophages, platelets, and fibroblasts [129,131] as L-TGF-β that is localized to the ECM. Release of TGF-β1 from this latent complex occurs through proteolysis by MMPs and myofibroblast contraction of the ECM [70,72,73].

The chemical and physical properties of the implanted polymer affect protein adsorption [132,133], fibroblast morphology [133,134], and fibroblast adhesion [133,135]. The ability of a material to affect myofibroblast population in vivo [136] and inhibit fibroblast growth factor (FGF) stimulation of fibroblast to myofibroblast differentiation [137] has been demonstrated. Exploiting these properties could allow material-mediated control of the FBR and could improve the longevity and tissue incorporation of implanted artificial organs and other biomedical devices. Herein, we use a library of polymers composed of a poly(amino acid), poly-L-arginine (PLR), that is covalently-modified with small molecules that contain amidine-like functional groups to influence the TGF-β1 induced fibroblast to myofibroblast transition. Previously, the effects of these materials on fibroblast synthesis of collagen was demonstrated [138]. To determine if these materials had an effect on TGF-β1 induced myofibroblast population and final implant tissue character, myofibroblast population was assessed in vitro and in vivo. For all of the in vitro experiments three conditions were studied to elucidate the material-fibroblast interactions: 1) TGF-β1 stimulation was used as a positive control for the fibroblast to myofibroblast transition, 2) the downstream effects of materials surfaces on the release of TGF-β from L-TGF-β are examined herein through the addition of L-TGF-β to fibroblast culture, and 3) naïve fibroblasts, stimulated with PBS, serve as the negative control for fibroblast to myofibroblast differentiation for this study.
2.1 Materials and Methods

2.1.1 Materials

Unless otherwise indicated, all materials were purchased and used as received from Sigma (St. Louis, MO). Deionized (DI) water used in this study was obtained from a Milli-Q (Barnstead Nanopure, Thermo Scientific, Waltham, MA). The amidine derivatives (Figure 2.1) used to modify PLR were: L-2-amino-3-guanidinopropionic acid, 3-guanidinopropionic acid, N-nitro-L-arginine, creatine (Acros Organics, NJ), L-carnitine, L-citrulline, DL-5-hydroxylysine, Nα-acetyl-L-glutamine, N-carbamyl-α-amino-isobutyric acid, acetyl-DL-carnitine, D-L-2,4-diaminobutyric acid, Nα-acetyl-L-ornithine, albizziiin, and L-arginine (Amresco, Solon, OH).

2.1.2 PLR modification

Fourteen different molecules were used to modify poly-L-arginine (Almanda Polymers, Huntsville, AL) with an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, (Oakwood Chemical, West Columbia, SC)) coupling reaction, as previously described [138]. The amidine derivatives are numbered 2-15 to allow for comparison to results obtained by Bygd et al. [138]. Amidine derivative 1 is not studied herein due to its relative cytotoxicity in comparison to the other modifiers.
Figure 2.1. Amidine-like modifiers used to form the library of poly-L-arginines.

2.1.3 Surface modification with modified PLRs

Fluorescein (Acros Organics), 10 molar equivalents, was used to modify the modified PLRs, unmodified PLR, and poly-L-lysine (PLL) via an EDC coupling reaction as in Section 2.1.2. Prior to placement in 35x10 mm² Petri dishes (Corning, Corning, NY), glass coverslips (Corning) were cleaned for 2 h in coverslip cleaning solution (200 mL ethanol (DLI, King of Prussia, PA), 300 mL DI water, 50 g NaOH (Fisher) and rinsed with DI water for 10 min. Then, 400 µL of 0.05% solutions of fluorescein-labeled modified PLR, unmodified PLR, and PLL in phosphate buffered saline (PBS) were used to coat Petri dishes that contained glass
coverslips. Following 1 h incubation at 37°C, the samples were washed 2X with PBS. Prior to imaging, 1 mL of PBS was replaced with fresh PBS and images were acquired using a FLoid cell imaging station (ex 482/18nm, em 532/59nm, Life Technologies, Carlsbad, CA).

2.1.4 Cell culture

The NIH/3T3 Swiss mouse fibroblast cell line (American Type Cell Collection, ATCC, Manassas, VA) was cultured in complete medium (CM, Dulbecco’s modified Eagle’s medium, DMEM, Corning) containing with 10% heat-inactivated bovine calf serum (BCS, Mediatech, Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin, (MP Biomedical, Solon, Ohio) at 37°C in 5% CO₂. NIH/3T3 cells were passaged approximately every 3 days, to maintain proper cell growth and character through 0.025% trypsin-EDTA (Corning) detachment and subculturing at 4×10³ cells/cm².

2.1.5 Cell viability

Two different stimuli were used throughout this study: 5 ng/mL of TGF-β1 and 5 ng/mL of L-TGF-β (R&D Systems, Minneapolis, MN). Control, naïve cells were not stimulated with growth factor, but PBS was added as a volume control. During passaging, NIH/3T3 cells were seeded at 1.3×10⁵ cells/cm² in CM in 24-well plates (Griener bio-one, Monroe, NC). Prior to this, the wells were coated with 0.05% solutions of modified PLR, unmodified PLR, PLL, or PBS as described in Section 2.1.3. PBS coated wells (TCP, tissue culture plastic) served as controls. After allowing 2 h for the fibroblasts to adhere, TGF-β, L-TGF-β, or PBS was added to each non-PBS coated well. 48 h post-stimulation, an MTT assay was performed. First, the medium was replaced with fresh medium. Then, MTT (3-
(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (Acros Organics)) was
dissolved at 5 mg/mL in DI water and added in volumes of 50 µL to each well. The plate was
incubated for 2 h at 37°C. Then, 425 µL of supernatant was removed from each well, and
500 µL of dimethyl sulfoxide (DMSO, Fisher) was added to solubilize the purple formazan.
A BioTek Synergy HT Multidetection Microplate Reader (BioTek, Winooski, VT) was used
to read the absorbance at 540 nm with a reference at 690 nm. At least four replicates were
assayed for each experimental condition. Results are expressed as the mean value ± standard
error of the mean normalized to naïve cells cultured on uncoated TCP.

2.1.6 Immunocytochemistry-based determination of myofibroblast population

Coverslips were cleaned, placed in Petri dishes, and coated as in Section 2.1.3. Prior
to the addition of cells to each glass-coverslip containing Petri dish, the coated coverslips
were washed twice with sterile PBS. To each Petri dish, $2.5 \times 10^4$ cells/cm$^2$ were added in 2
mL of CM. The Petri dishes were incubated at 37°C and 5% CO$_2$ for 2 h; and then TGF-β, L-
TGF-β, or PBS was added to achieve final concentrations of 5 ng/mL of TGF-β, 5ng/mL L-
TGF-β, or 0 ng/mL growth factor. After a 48 h incubation, the samples were fixed with ice-
cold methanol (Fisher). Following fixation, the sample was blocked for 2 h. Consecutively at
4°C for 14-24 h the samples were placed in 150 µL each of 1:400 α-SMA antibody (ab5694,
Abcam, Cambridge, MA) solution, red-fluorescing secondary anti-rabbit antibody
(ab150080, Abcam) at 1:600, and 1:100 488-labeled α-tubulin antibody (Cell Signaling
Technologies, Boston, MA). Then, coverslips were stained with 100 µL of 0.1 µg/mL DAPI
for 2 min. Finally, the samples were mounted on microscope slides with 50 µL of glycerin
jelly and sealed with clear nail polish (Sally Hansen, Morris Plains, NJ). The samples were
stored at -20°C until imaging with the red (excitation/emission 586/646nm), blue (390/446) and green channels (482/532) of the FLoid ® Cell Imaging Station. At least three 20X 0.375 mm² images were taken and analyzed for each stimulation condition and material. The number of distinct nuclei, stained by DAPI and the number of red α-SMA expressing cells were counted manually for each image. Myofibroblast percentage (MF%) was determined manually as the number of red cells divided by the total number of blue nuclei in that full image.

2.1.7 Immunohistochemistry of tissue sections from SKH1-E mice implant sites

The methods used to prepare the tissue sections used herein were previously described in Bygd et al. [139]. Heat-mediated antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6.0 (Fisher), at 95-100°C for 20 min. Then the tissues were blocked for 2 h at room temperature (rt). Overnight at 4°C, 1:100 α-SMA antibody (ab5694, Abcam) was incubated with each slide. The slides were then incubated in 0.3% H₂O₂ (Fisher) for 15 min. A horseradish peroxidase conjugated 2° antibody (ab6721, Abcam, 1:1000) was applied for 1 h at rt, followed by development in 3,3’-diaminobenzidine (DAB) chromogen (Abcam) for 10 min at rt. The sections were counterstained with 0.1% w/v Mayer’s Hematoxylin (Electron Microscopy Sciences, Hatfield, PA) and incubated in bluing agent (Fisher) for 2 min. Dehydration of the samples was performed before mounting in CYTOSEAL™ XYL (Thermo Scientific). The mounted sections were stored at rt prior to imaging at 10X with an inverted microscope (Zeiss Axio Vert.A1, Jena, Germany) and digital camera (Canon EOS Rebel T3i).
2.2 Results

2.2.1 PLR polymer and coating characterization

To exert possible affects on fibroblast to myofibroblast differentiation PLR was modified by the small molecules containing amidine-like functional groups and must coat the glass or TCP substrate. Modification of PLR by each of the amidine-like molecules was previously confirmed by NMR analysis [138]. Glass and TCP electrostatically coated with fluorescently-labeled modified PLR 2-15, unmodified PLR, and PLL saturated the green fluorescent images taken with the FLoid ® Cell Imaging Station when focused on the coated plane (data not shown). Uncoated glass and TCP showed minimal green in the images.

2.2.2 Cell viability

For materials to interface successfully with cells in vitro or in vivo, they must be cytocompatible. The viability of NIH/3T3 cells cultured in the presence of 5 ng/mL TGF-β, 5 ng/mL L-TGF-β, or in the absence of growth factor stimulation is above 65% when compared to naïve cells cultured on TCP (Figure 2.2).
Figure 2.2 NIH/3T3 viability on modified PLR-coated surfaces in the presence of TGF-β, L-TGF-β, or no growth factor. Data represents the mean of at least four wells per treatment ± standard deviation.

2.2.3 *In vitro* myofibroblast population

Myofibroblasts were identified as the cells that expressed α-SMA, the most commonly used myofibroblast marker [139]. The positive control, performed on glass, for this differentiation process showed an equal MF% when stimulated by TGF-β and L-TGF-β, which both were higher than the MF% in the naïve cells (p<0.05, Figures 2.3 & 2.4). The NIH/3T3 response on modifications 2, 4, and 9 had the same trend as glass. The TGF-β MF% was higher than the L-TGF-β and naïve MF%, and the MF% was reduced to the level of naïve cells when cells were stimulated with L-TGF-β on modifications 3, 5, and 6.
(p<0.05). The MF% of cells of all stimulations on modifications 12, 15, and PLL were not statistically different (p<0.05). In response to glass coverslips coated with 8 and 14, the MF% in the presence of the growth factors are not statistically different, nor is the L-TGF-β MF% different from the naïve cell MF%; however, the TGF-β stimulated cells had a higher MF% than the naïve cells (p<0.05). A similarly inconclusive response occurs in response to modifications 7, 11, and 13: the MF% in the presence of growth factors is statistically similar, the MF% of TGF-β stimulated cells and naïve cells is not different; however, the L-TGF-β stimulated cells induced a MF% that is higher than the naïve cells (p<0.05). Unmodified PLR also causes an unclear MF% response. Though there is a higher MF% when stimulated by TGF-β than by L-TGF-β, there is no difference between the TGF-β and naïve MF%, nor the L-TGF-β and naïve MF% in response to unmodified PLR (p<0.05).
Figure 2.3 MF% determined from ICC-based myofibroblast detection. Statistical differences between MF% on each material, determined by Student’s t-test for p<0.05 are indicated by (*) for TGF-β compared to L-TGF-β, (#) for TGF-β compared to naïve, and (^) for L-TGF-β compared to naïve. Data represents the mean value of at least three replicates for each sample ± standard deviation.
Figure 2.4 Immunocytochemistry images depicting fibroblasts and myofibroblasts following incubation on poly(amino acid)-coated surfaces. Scale bar: 100 µm. Red is α-SMA, green is tubulin, and blue is DAPI. The numbers correspond to the PLR surface modifications, and G is glass. T is samples stimulated with TGF-β, L is samples stimulated with L-TGF-β and P is samples stimulated with PBS.

2.2.4 Histology

The in vivo myofibroblast presence was determined adjacent to these materials through IHC staining for α-SMA of tissue sections. The arrows in Figure 2.5 indicate myofibroblasts, which are stained in brown. Circular structures containing dense brown staining are indicative of smooth muscle cells, not myofibroblasts. There are myofibroblasts apparent in the tissue collected containing unmodified 105-150 µm diameter glass bead implants and in the tissue containing glass beads coated with modifications 2, 7, 10, 11, 12, 13, and 15. However, there are no myofibroblasts observed at this 28 day endpoint near glass bead implants coated with modifications 3, 4, 5, 6, 8, 9, 14, unmodified PLR, PLL, and skin. It is difficult to discern the location of the implant site in the tissue sections collected for 4, 9, and 10.
2.3 Discussion

A successfully healed wound is characterized by disorganized ECM, predominately consisting of collagen, containing resident fibroblasts [47]. Granulation tissue, the hallmark of the remodeling phase of wound healing, resolves into scar tissue following myofibroblast apoptosis [50]. Incessant activation and reduced apoptosis of myofibroblasts results in excessive secretion and contraction of collagen during chronic scarring [50,127]. Chronic pathological scarring is characterized by an extended presence of connective tissue bearing resemblance to granulation tissue, resulting in continued extracellular matrix deposition [127]. There are scaffolds that are designed to reduce the healing time of skin wounds [140], and materials that are designed for implantation [141]. Materials used to form the 3D scaffolds used to improve wound healing include: collagen-based matrices, electrospun PCL/gelatin scaffolds, PLGA and polyurethane [140]. An extensive discussion of implantable materials is also included in Section 1.1.

There is an understanding of how some of these materials affect macrophages and fibroblasts. In the case of fibroblast to myofibroblast differentiation, extensive studies have
been performed on the effects of material stiffness [142–144]. However, an incomplete understanding of how material chemistry can affect fibroblast to myofibroblast differentiation exists. As with the adhesion of cells, there is an effect of the water contact angle of materials on myofibroblast presence. This effect is seen as an increased α-SMA expression near hydrophobic silicone than near UHMWPE [104]. The following is an explanation of how materials within this PLR library have varied effects on the myofibroblast transition and the end result of the FBR to implanted materials.

The extent of fibroblast to myofibroblast differentiation was assessed on each of the modified PLR-coated surfaces using ICC detection of α-SMA. Some naïve samples have myofibroblasts; this is because fibroblasts may take on the myofibroblast phenotype spontaneously due to the mechanics of their substrates [145,146]; in valvular interstitial cells this value can range between 80% (on 32 kPa hydrogels) and <5% (on 7 kPa hydrogels) [142]. All samples studied here were compared to the glass control for typical fibroblast to myofibroblast differentiation of NIH/3T3 fibroblasts. On glass, the MF% was equal when stimulated by TGF-β and L-TGF-β and greater than the MF% without addition of growth factor. This is characteristic of the typical response when the material is not affecting the extent of the fibroblast to myofibroblast transition. When glass is coated with modifications 2, 4, and 9, the in vitro MF% trend of this typical response remains unchanged.

Coating of glass with modifications 3, 5, 6, or unmodified PLR causes a reduction in MF% in the presence of the L-TGF-β in comparison with TGF-β. Modifications 3, 5, and 6 caused the L-TGF-β stimulated cells to have the same MF% as naïve cells indicating that almost no TGF-β is released from the latent form on this surface. These are not the first materials to impede myofibroblast development; this has been shown by others in response to
lactose-conjugated silk fibroin [136]. It has also been demonstrated that p(NIPAm-co-AAc) particles functionalized with modifications 3-guanidinopropionic acid, creatine, carnitine, and unmodified PLR have an effect on the activity of MMP-13 [147]. In the presence of these particle modifications, MMP-13 activity was reduced to 8%, 27%, 21%, and 14% of the free enzyme activity, respectively [147]. It is possible that MMP-inhibition results in decreased release of TGF-β1 from L-TGF-β1 adjacent to these modifications, resulting in the decreased MF% seen when stimulated by the latent form with respect to the active form.

Glass is considered a poor biomaterial. Subcutaneously implanted 500 µm-diameter glass beads cause a deleterious FBR that results in an extensive fibrous capsule surrounding a collection of glass beads after only 14 days [148]. The characteristics of the tissue surrounding an implant, including collagen and neovasculature, are commonly visualized through the use of Masson’s trichrome stain. Masson’s trichrome was performed on these sections by our lab previously [138]. Generally, myofibroblasts are more prevalent in areas of thicker collagen. The dense, thick collagen evidenced in the Masson’s trichrome staining of glass implant tissue (Supplementary Figure 3r, [138]), is associated with brown staining of myofibroblasts in Figure 2.5G at the 28 day time-point, further alluding to an association of myofibroblasts with thick collagen. An excessive amount of collagen (Supplementary Figure 3b, [138]) is associated with the high myofibroblast presence next to the modification 2-coated glass bead implants (Figure 2.52).

The material coatings that caused minimal myofibroblast presence in the implant tissue were modifications 3, 4, 5, 6, 8, 9, 14, unmodified PLR, and PLL. All of these modifications had a myofibroblast presence similar to skin. Modifications 3, 5, and 6 also affected the myofibroblast transition in vitro. The tissue sections obtained adjacent to
modifications 4 and 9 do not contain obvious implant sites, so the lack of observed myofibroblasts could be explained by the fact that the collected tissue may not have interacted with these implants. As modifications 3, 5, and 6 exert a possible affect on the bioavailability of TGF-β, the *in vivo* reduction in myofibroblast population could also be due to this result. It is important to note that though PLL reduces the *in vivo* myofibroblast response at this 28 day endpoint; the collected tissue appears to still be characterized by immune cell infiltrate, a hallmark of chronic inflammation [85,149]. This apparent reduction of myofibroblasts may only be due to a delayed healing response due to the continued presence of M1 macrophages [98]. Modification 5 also showed no myofibroblasts present at this time, however, collagen and cellular infiltrate are observed. Though modification 5 is able to reduce MF% to the minimal amount possible on this surface *in vitro*, it appears that the response to this material is in the stage of chronic inflammation and has not yet reached the stage of myofibroblast ECM remodeling. The implant site near modification 3 is characterized by low collagen density, vascularization, and no apparent myofibroblasts; this is correlated with a reduction in MF% by this surface *in vitro*. Modification 6 created an ideal implant site that is vascularized, contains low collagen expression, and has no observed myofibroblasts at 28 days.

Though all of the small molecule modifiers used herein have chemical moieties that are similar to each other (Figure 2.1), small deviations in their chemical structure caused varied effects on myofibroblast population and implant character. For example, modifier 3 (3-guanidinopropionic acid) reduced myofibroblast differentiation *in vitro* and *in vivo*, but is only distinct from modification 2 by the lack of an amine group on carbon 2. Modification 2 caused the same deleterious foreign body capsule formation as in the typical response to
glass. This amine group seems to reduce the inhibitory effect of the guanidine group on the myofibroblast transition. In the case of L-arginine (modification 15), there is also an amine group on carbon 2, and though the alkyl chain is increased in length by two CH$_2$ groups with respect to modification 2, many myofibroblasts were present in vivo.

Modification 5, creatine, a naturally occurring biomolecule, has a methyl group in place of a hydrogen on the nitrogen that is part of the chain of the molecule, making it distinct from the guanidine group on modifications 2, 3, 4, and 15. This molecule exerts a stronger effect on the myofibroblast transition in vitro than the molecules containing this guanidine group. Carnitine, modification 6, is also a naturally occurring biomolecule. Carnitine impedes fibroblast to myofibroblast differentiation in vitro and in vivo; these effects appear to be exerted through the tri-methyl amine and the secondary alcohol, as these groups are distinct from all modifications except 11 and 8, respectively. Modification 11, a molecule containing tri-methyl amine groups and two acetyl groups does not reduce myofibroblast population in vitro or in vivo. Though no statistically significant effects of modification 8 on MF% were seen in vitro for the three different cell stimulations, this surface coating caused a reduction in myofibroblast population in vivo. The secondary alcohol exists on modification 6 and 8, which both have minimal myofibroblast population in vivo. This exemplifies how small deviations in chemical structure can cause varied effects on myofibroblast population in vivo and in vitro.

2.4 Conclusions

Material-based reduction of fibroblast to myofibroblast differentiation was demonstrated by a few of the materials within this library in vitro and in vivo when compared
to a glass control. Small deviations in the chemical structure of the molecules used to modify PLR effect the extent of fibroblast to myofibroblast differentiation. The materials that were able to reduce the extent of myofibroblast differentiation are PLR modified with 3-guanidinopropionic acid, creatine, and carnitine. Many other modifications, also with amidine-like functional groups, did not exert the same effect on this process and were unable to reduce the deleterious response that occurs to implanted glass beads. Further investigation into the intricacies of the factors, including macrophage phenotype, that lead to the transition from remodeling to resolution in the presence of these materials is required to more completely understand the relationships between TGF-β, myofibroblasts, and fibrous encapsulation.
CHAPTER 3
THE MACROPHAGE RESPONSE TO CHONDROITIN SULFATES

3.0 Introduction

The extracellular microenvironment is instrumental in controlling cellular behavior and responses [105]. Many components of the supportive ECM are responsible for providing the signals that maintain cellular homeostasis, and upon deregulation disease states, such as cancer, can result [150]. Cancerous cells develop due to an imbalance in proliferation and regulation signals in their microenvironment. The development of cancer is thought to be supported by TAMs [151]. All cells, including cancer cells and macrophages are affected by the components of the ECM including: proteins, proteoglycans, glycoproteins, collagens, and elastins [152]. Desmoplastic responses in cancer result in nonhomeostatic ECM [153]. There are extensive interactions between ECM molecules and growth factors, such as TGF-β [154]. Members of the glycosaminoglycan (GAG) families, such as chondroitin sulfate, heparan sulfate, keratan sulfate, and hyaluronan are commonly bound to glycoproteins to form proteoglycans (PGs). The chondroitin sulfate PGs include serglycin [155], decorin [156], and versican [157]. GAGs are negatively charged biopolymers of repeating N-acetylated hexosamine and uronic acid disaccharide units [158].

Chondroitin sulfates are covalently bound by a tetrasaccharide linkage to the serine residues in the protein core of the proteoglycan [158]. Chondroitin sulfates are formed of repeating N-acetylgalactosamine sugars (GalNAc) and glucuronic acid (GlcA) sugars through GalNAc transferase II (GalNAcT-II) and GlcA transferase II (GlcAT-II) [159]. In chondroitin sulfate-A (CS-A) or C4S, the 4th carbon of GalNAc is sulfated. For chondroitin
sulfate-C (CS-C), or C6S, carbon 6 of GalNAc is sulfated. In some cases, chondroitin sulfate can become highly sulfated into C4,6S, and this compound is known as chondroitin sulfate-E (CS-E). Chondroitin sulfate-B (CS-B), or dermatan sulfate, is similar to CS-A and CS-C, but has an iduronic acid (IdoA) group instead of a GlcA group. Cancer cell proliferation, cancer cell adhesion, and cancer cell invasion are affected by these different sulfation patterns [160].

These GAGs are modified by sulfotransferases including: chondroitin 4-O-sulfotransferase-1 (C4ST)-1, C4ST-2, C4ST-3, dermatan-4-O-sulfotransferase-1 (D4ST-1), chondroitin 6-O-sulfotransferase-1 (C6ST-1), and GalNAc 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST) [159].

The PG and GAG components of the ECM commonly change levels or sulfation patterns in cancer microenvironments. In some cancers, the expression of chondroitin sulfates can indicate a poor outcome [161]. Chondroitin sulfates, which normally only exist as the side chains of PGs, such as versican, have been shown to variously accumulate adjacent to breast and ovarian cancers [162,163]. Common PGs that have changed levels or sulfation patterns in cancer microenvironments related to these GAGs include: serglycin, decorin, and versican [156,164–169]. CS-A and CS-C have increased expression in colorectal cancer [170,171]. C4ST-1 is upregulated in breast cancer tissue and multiple myeloma, leading to increased expression of CS-A [124,172]. CS-C is increased in breast cancer tumors [173]. Highly-sulfated CS-E is expressed in ovarian cancer tissues [163,174].

Chondroitin sulfates are increased by a factor of five in esophageal squamous cell carcinoma; the enzymes that form IdoA and sulfate carbons 4 and 6 – leading to CS-A, CS-B, CS-C, and CS-E presence – also have increased expression in this carcinoma [175].
There is *in vitro* evidence that there is interplay between CS, stromal cells, cancer cells, and macrophages. Chondroitin sulfates, specifically CS-A and CS-B, have effects on the proliferation of osteosarcoma cells [176]. CS-B has concentration-dependent inhibitory or promoting effects on breast cancer cells, whereas CS-C has no effect [177]. Fibrosarcoma cell migration is promoted by CS-A [178]. Also, when stromal cells are co-cultured with cancer cells in 2D or 3D versican and chondroitin sulfates are produced by the stromal cells; and greater than 90% of these stromal cells differentiated into myofibroblasts in these co-cultures [179]. CS-E increases the metastatic ability of Lewis lung carcinoma, and CS-E is more highly expressed by cell lines with higher metastatic potential [180]. Chondroitin sulfate can have anti-inflammatory effects on primary cultures of macrophages [181], and the cancer cell response to macrophages is dependent on the macrophage phenotype [182].

Macrophages can account for more than 50% of the cells in breast cancer tumors [183,184]. This prevalence of macrophages in tumors, along with evidence that if macrophage migration and development at the tumor was delayed, the angiogenic switch necessary for malignant progression of the tumor does not occur [185], illustrates the importance of macrophages in cancer microenvironments. Macrophages exist on a spectrum of phenotypes with different roles in cancer ranging from anti-tumoral to pro-tumoral [186]. M1 macrophages, also known as classically activated macrophages, are thought to be involved in recognition and destruction of cancer cells through cytotoxicity, resulting from the release of TNF-α, reactive oxygen and nitrogen species [187,188]. On the other end of the spectrum lies M2 macrophages, also known as alternatively activated macrophages. M2 macrophages are anti-inflammatory cells that are thought to be the predominant phenotype found in TAMs. These macrophages are divided into M2a, M2b, and M2c macrophages.
M2a macrophages are also known as M(IL-4) or M(IL-13) cells, based on the biofactors that stimulate them into this phenotype [189]. M2c macrophages can be stimulated by IL-10 or TGF-β and are also known as M(IL-10 or TGF-β) macrophages; these cells are involved in tumor promotion, immune suppression, and tissue remodeling [182,187].

The metabolism of macrophages of different phenotypes is distinct and is reflective of their divergent functions [114]. L-arginine is a substrate for both inducible nitric oxide synthase (iNOS) and arginase, and can be used to assess phenotypic change [114]. M1 macrophages utilize arginine metabolism to produce cytotoxic nitric oxide (NO), whereas M2 macrophages metabolize arginine into tumor-promoting urea and L-ornithine [114]. An understanding of how different levels of CS-A, CS-B, CS-C, and CS-E effect macrophage phenotype, based on metabolic and morphological analyses, could assist in the elucidation of the role of macrophages in cancer development.

3.1 Materials and Methods

3.1.1 Preparation of chondroitin sulfate solutions

First, 5 mg/mL solutions of CS-A (Alfa Aesar), CS-B (Sigma), and CS-C (Spectrum, New Brunswick, New Jersey) were made in 1X PBS. These solutions were sterile filtered through 0.22 µm membranes (Corning). A 1:1 mixture of CS-A and CS-C were used to make a 5 mg/mL CS-E solution. This is used to approximate a similar environment to CS-E. These solutions were then diluted with sterile 1X PBS to make 25 µg/10µL, 5 µg/10µL, 2.5 µg/10 µL, and 0.5 µg/10µL solutions from each 5 mg/mL solution. These solutions were added in appropriate volumes to attain 100 µg/mL, 50 µg/mL, 10 µg/mL, 5 µg/mL, and 1 µg/mL of each chondroitin sulfate type in the following experiments.
3.1.2 RAW 264.7 cell culture

The RAW 264.7 macrophage cell line (ATCC) was cultured in complete medium (CM, Dulbecco’s modified Eagle’s medium (DMEM, (Corning)) supplemented with 10% fetal bovine serum (FBS, (Tissue Culture Biologics, Los Alamitos, CA)), 100 U/mL penicillin, and 100 µg/mL streptomycin, (MP Biomedical)) at 37°C in 5% CO₂.

3.1.3 RAW 264.7 viability in the presence of chondroitin sulfates

MTT assays were performed to determine the effects of different chondroitin sulfate types and concentrations on macrophages stimulated in five different ways. First, in 24-well plates (Griener Bio-One) 10 µL of a particular chondroitin sulfate type and concentration were added to four wells and an additional negative control well. After this - other than for naïve macrophages where no stimulus was added - cells were stimulated with 5 µg/mL lipopolysaccharide (LPS), 25 ng/mL IL-4 (eBioscience Inc., San Diego, CA), or 5 ng/mL TGF-β (R&D Systems, Minneapolis, MN). For M(LPS 24) macrophages, after 24 h the culture media was aspirated and replaced with chondroitin sulfate stimulated media. During passaging, RAW 264.7 macrophages were seeded at 1.3x10⁵ cells/cm² in CM in 24 well plates. 48 h later an MTT assay was performed as in Section 2.1.5. At least four replicates were assayed for each experimental condition. Results are expressed as the mean value ± standard deviation compared to cells of the same stimulation in absence of chondroitin sulfate.
3.1.4 Assessment of arginase activity

RAW 264.7 cells were seeded into 24 well plates as in Section 3.1.3. After 48 h the supernatant was collected for Section 3.1.5, and the wells were rinsed with sterile 1X PBS prior to addition of 100 µL of lysis buffer (DI water, 0.1% Triton X-100 (Acros Organics), and 1% protease inhibitor cocktail (Amresco)) on ice for 10 min. Samples were then thawed and 25 µL of lysis buffer were added to the wells of a 96 well plate. To activate arginase, 25 µL of 10 mM MnCl₂ (Fisher) and 50 mM Tris (pH 7.5, Fisher) were added to each well and the 96 well plate was incubated at 55°C for 10 min. The substrate of the enzyme, L-arginine (Amresco), was added to each well as 50 µL of a 1 M arginine solution (pH 9.7). The plate was then incubated at 37°C for 20 h. The urea concentration in 2-5µL aliquots of the post-20 h incubation samples were analyzed following the addition of 200 µL of a 1:2 mixture of solution 1 and solution 2 (Solution 1: 1.2 g o-phthaldialdehyde (Alfa Aesar), 1 L H₂O, and 500 µL HCl (Fisher); solution 2: 0.6 g N-(napthyhl)ethylenediamine dihydrochloride (Acros Organics), 5 g boric acid (Fisher), 800 mL H₂O, 111 mL H₂SO₄ (Fisher)) [190]. The plate was read on a BioTek Synergy HT Multidetection Microplate Reader at 520 nm with a reference at 630 nm.

3.1.5 Griess assay

Supernatant, 150 µL, from Section 3.1.4 was analyzed compared to a standard curve formed by serial dilutions of a 100 µM NaNO₂ solution. To each well of a 96 well plate, 130µL of DI water and 20 µL of Griess reagent (Acros Organics) were added to 150µL of sample or standard and incubated for 25 min. Due to high background absorbance of medium stimulated with 100 µg/mL of the chondroitin sulfates [181], an average background nitrite
level (for each chondroitin sulfate type) was subtracted from the collected nitrite levels. The plate was read at 448 nm with a reference of 690 nm on the same microplate reader as in Section 3.1.4.

3.1.6 Morphological Staining of Macrophages

Prior to placement in 35x10 mm² Petri dishes (Corning), glass coverslips (Corning) were cleaned for 2 h in coverslip cleaning solution (200 mL ethanol (DLI), 300 mL DI water, 50 g NaOH (Fisher) and rinsed with DI water for 10 min. To each Petri dish, 3.1x10⁴ cells/cm² were added in 2 mL of CM. CS-A solution, CS-B solution, CS-C solution, CS-E solution, or no solution was added to achieve final concentrations of 100 µg/mL, 50 µg/mL, 10 µg/mL, 5 µg/mL, 1 µg/mL and 0 µg/mL. After a 48 h incubation, the samples were fixed in paraformaldehyde fixation solution (3.7% para-formaldehyde (Fisher), in 100 mL of 1X PBS) at rt for 10 min. The cells were then permeabilized in 0.5% Triton X-100 in PBS for 5 min at rt. The samples were then placed in 200 µL of 100 nM phalloidin (Cytoskeleton, Inc., Denver, CO) for 1 h. The coverslips were then stained with 100 µL of 0.1 µg/mL DAPI for 2 min. Lastly, the samples were mounted on microscope slides with 50 µL of glycerin jelly and sealed with clear nail polish (Sally Hansen). The samples were stored at -20°C until they were imaged on the blue (390/446) and green channels (482/532) of the FLoid ® Cell Imaging Station.
3.2 Results

3.2.1 RAW 264.7 viability

MTT assays were performed to determine the effects of different chondroitin sulfate types and concentrations on macrophages stimulated into five different phenotypes. These five different phenotypes include M(LPS 48), M(LPS 24), M(IL-4), M(TGF), and naïve macrophages. When the viability of macrophages is examined after culture with chondroitin sulfates A, B, C, or E at concentrations of 100, 50, 10, 5, and 1 µg/mL compared to a positive control of the same stimulation in the absence of chondroitin sulfate, viabilities remain above 70% (Figure 3.1).
Macrophage viability in the presence of chondroitin sulfates of different types and concentrations. Macrophages are M(LPS 48), M(LPS 24), M(IL-4), M(TGF), and naïve. Viability percentage is determined with respect to a chondroitin sulfate negative positive control. Data represents the mean value of a minimum of four replicates for each sample ± standard deviation.

### 3.2.2 Arginase activity

Arginase activity was determined herein to express the extent of M2-like polarization. With respect to the positive controls, the maximal arginase activity was found in the M(IL-4) macrophages, followed by M(LPS 48), then M(LPS 24), M(TGF), and lastly the lowest activity was found in naïve macrophages (Figure 3.2). As M2 macrophages have higher arginase expression than M1 macrophages, it was expected that the M(TGF) macrophages would have higher arginase activity than the M(LPS 48) and the M(LPS 24) macrophages. However, this was not the trend seen in the absence of chondroitin sulfates. All associated
values for each stimulation, chondroitin sulfate type, and concentration are included within this figure to allow for comparison to the positive controls.

Figure 3.2 Urea levels for macrophages stimulated with chondroitin sulfates of different types and concentrations. Macrophages are M(LPS 48), M(LPS 24), M(IL-4), M(TGF), and naïve. Data are expressed as the mean value of at least four replicates ± the standard deviation. This data set is incomplete; values that appear as zero have not been completed.

There was a small increase in urea levels with respect to the M(LPS 48) control when cells are stimulated with 100 µg/mL of CS-A, CS-B, CS-C; or 10 µg/mL of CS-B and LPS for 48 h (p<0.05, Figure 3.3). Surprisingly, there was not an increase in urea levels with respect to stimulation with 50 µg/mL of CS-B. The only stimulation to cause a decrease in urea levels for M(LPS 48) cells was 5 µg/mL of CS-C (p<0.05). The analysis of urea levels after the addition of 1 µg/mL of each chondroitin sulfate type to M(LPS 48) macrophages has
not been completed and is not included in this analysis. For all other chondroitin sulfate types and concentrations there is not a difference with respect to the positive control. When stimulated with CS-A there was a higher urea level in response to 100 µg/mL and 50 µg/mL compared to the 10 µg/mL and 5 µg/mL stimulations (p<0.05). For CS-C: 100 µg/mL caused more measured urea than 10 µg/mL and 5 µg/mL, and 50 µg/mL induced higher urea levels than 5 µg/mL (p<0.05).

**Figure 3.3** Urea levels in M(LPS 48) macrophages. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

M(LPS 24) macrophages stimulated in the presence of 100 µg/mL of CS-A or CS-E caused an increase in urea concentration, whereas 1 µg/mL of CS-A, CS-B, CS-C; and 5
µg/mL of CS-B, CS-C, and CS-E decreased the urea concentration (p<0.05, Figure 3.4). CS-A stimulation at a high concentration, 100 µg/mL, caused a higher urea level when compared to the low CS-A stimulations (p<0.05). The urea levels following the addition of 50 µg/mL of each chondroitin sulfate type to M(LPS 24) macrophages has not been completed and is not included in this analysis. For most of the chondroitin sulfate types and concentrations there is no statistically significant difference in urea levels in M(LPS 24) cultures (p<0.05).

**Figure 3.4** Urea levels in M(LPS 24) macrophage culture. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.
All chondroitin sulfate types and concentrations added to M(IL-4) macrophages caused an increase in arginase activity relative to the positive control (p<0.05, Figure 3.5). This incomplete data set only includes analysis of urea levels following 50 and 5 µg/mL stimulations of CS-A, CS-B, CS-C, and CS-E to M(IL-4) macrophages. For CS-C and CS-E the 50 µg/mL stimulation caused an increase in urea levels when compared to the 5 µg/mL stimulation (p<0.05). This trend is not seen in M(IL-4) macrophages stimulated by CS-A and CS-B.

**Figure 3.5** Urea levels in IL-4 macrophage culture. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.
M(TGF) macrophages in the absence of chondroitin sulfate only caused a urea level of 24 ± 6 mg/dL (Figure 3.6). However, upon addition of chondroitin sulfates the urea level increased to nearly 10 times the level of chondroitin sulfate negative cells. For this incomplete data set, the increase in urea does not appear to be dependent on the chondroitin sulfate concentration. The addition of 5 and 1 µg/mL of each chondroitin sulfate type to M(LPS TGF) macrophages has not been completed and is not included in this analysis.

**Figure 3.6** Urea levels in M(TGF) macrophage culture. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

Naïve macrophages in the absence of chondroitin sulfate have almost no arginase activity. There was no observed increase in urea levels for naïve cells in the presence of 100
µg/mL of CS-A, CS-B, and CS-C (Figure 3.7). However, 100 µg/mL of CS-E and all other concentrations of the four chondroitin sulfates induced an increase in arginine metabolism by these cells (p<0.05). There was a dose dependence observed for naïve macrophages. For CS-A the highest urea production was in response to the 50 µg/mL concentration (p<0.05), showing minimal increases at the highest and lowest chondroitin sulfate concentrations. CS-B stimulation of these naïve macrophage urea levels peaks at 10 µg/mL with minimal urea at the 100 µg/mL and 1 µg/mL stimulations. For CS-C and CS-E there was an increase in urea levels by 50 and 5 µg/mL, but the urea levels were decreased by 100, 10, and 1 µg/mL concentrations.

**Figure 3.7** Urea levels in naïve macrophage culture. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.
3.2.3 iNOS activity

This assay determines the activity of the iNOS enzyme indirectly by measuring the cell culture supernatant level of nitrites. M(LPS 48) macrophages had the highest levels of iNOS activity (Figure 3.8). iNOS activity in M(LPS 24) macrophages was less than half of the M(LPS 48) macrophages. M(IL-4) iNOS activity was less than half of M(LPS 24) macrophages. Naïve macrophages and M(TGF) macrophages had almost no iNOS activity without the addition of chondroitin sulfate.

Figure 3.8 Nitrite levels for macrophages stimulated with chondroitin sulfates of different types and concentrations. Macrophages are M(LPS 48), M(LPS 24), M(IL-4), M(TGF), and naïve. Data are expressed as the mean value of at least four replicates ± the standard deviation. This data set is incomplete; values that appear as zero have not been completed.
M(LPS 48) nitrite production is only affected slightly by the addition of chondroitin sulfates (Figure 3.9). Decreased nitrite levels were observed for 50µg/mL and 5µg/mL of each type of chondroitin sulfate (p<0.05).

![Graph showing nitrite levels in M(LPS 48) macrophages](image)

**Figure 3.9** Nitrite levels in M(LPS 48) macrophages. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05).

When M(LPS 24) macrophages are stimulated with chondroitin sulfates of each type and concentration included herein there is a statistically significant reduction in iNOS activity compared to the positive control (p<0.05), except in the case of 50 µg/mL of CS-B (Figure 3.10). The addition of 100 µg/mL of each chondroitin sulfate type to M(LPS 24) macrophages has not been completed and is not included in this analysis.
Figure 3.10 Nitrite levels in M(LPS 24) macrophages. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

M(IL-4) macrophages induced a low, approximately 8 µM, nitrite response. Only the iNOS response to the 50 and 5 µg/mL stimulations of M(IL-4) macrophages has been completed. Stimulation by 50 µg/mL of CS-A, CS-B, CS-C, and CS-E caused an increase in nitrites to above 20 µM (Figure 3.11). In comparison to the positive control, M(IL-4) cells in the presence of 5 µg/mL of CS-A was not statistically different (p<0.05). Nitrite expression in M(IL-4) macrophages in the presence of 5 µg/mL of CS-B was reduced (p<0.05).
Figure 3.11 Nitrite levels in M(IL-4) macrophages. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

Except for 100 µg/mL of CS-A, there is an increase in nitrite expression by M(TGF) macrophages (Figure 3.12). All of the nitrite levels are distinct for 100 µg/mL of each of the chondroitin sulfates (p < 0.05). At the 10 µg/mL level the nitrite secretion is distinct for CS-B with respect to the other stimulations (p < 0.05). Here, 5 µg/mL of CS-B also decreases the extent of iNOS activity, at least with respect to the other CS-B concentrations (p < 0.05). For CS-A: 50 µg/mL, 10 µg/mL, and 1 µg/mL induced higher nitrite expression than 100 µg/mL and 5 µg/mL (p < 0.05).
Figure 3.12 Nitrite levels in M(TGF) macrophage culture. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

An increase in nitrite release when compared to naïve macrophages was seen upon stimulation with 10 µg/mL of CS-A, CS-B, CS-C, and CS-E; and 5 µg/mL of CS-C and CS-E (p<0.05, Figure 3.13). The addition of 50 and 1 µg/mL of each chondroitin sulfate type to naïve macrophages has not been completed and therefore the analysis of nitrite levels for these stimulations is not included in this analysis. Nitrite levels were increased to above the level for M(LPS 24) macrophages by 10 µg/mL of CS-A, 5 µg/mL of CS-C, and 5 µg/mL of CS-E.
Figure 3.13 Nitrite levels in naïve macrophages. These macrophages were stimulated with five different concentrations of four different chondroitin sulfate types. Data represents the mean value of at least four replicates for each sample ± standard deviation. (*) indicates a significant difference from the positive control (p<0.05). This data set is incomplete; values that appear as zero have not been completed.

3.2.4 Morphological staining

The positive controls for macrophage phenotype in response to the five stimulations studied herein are shown in figures 3.14, 3.15, 3.16, and 3.17. Naïve macrophages have a small circular morphology. M(TGF) macrophages are mostly circular but appear to be spread over a slightly larger area per cell than naïve macrophages. M(IL-4) macrophages are more elongated in the positive control than any other stimulation studied herein. M(LPS 24) and M(LPS 48) macrophages both have a flattened, extended morphology with diameters approximately equivalent to at least three naïve macrophages. The chondroitin sulfates seem
to have more of an effect on naïve, IL-4 and TGF stimulated macrophage morphology than on the LPS stimulated macrophage morphology.

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Figure 3.14 Macrophage morphology in the presence of CS-A. Macrophages are stained to show actin in green and nuclei in blue. CS-A concentrations (µg/mL) label the grid across the top, and stimulations are labeled along the left hand side. The last column is naïve, M(LPS 24), M(LPS 48), M(IL-4), and M(TGF) cells in the absence of CS-A. Scale bar is 50 µm.

Naïve macrophages stimulated with 1 µg/mL of CS-A have a similar morphology to naïve macrophages (Figure 3.14). Stimulation by CS-A at the concentrations 5 µg/mL and 10 µg/mL caused these cells to spread out marginally more. Further increase in CS-A concentration to 50 and 100 µg/mL caused the cells to look more dendritic in character. The same trend is seen for M(TGF) macrophages in the presence of this chondroitin sulfate. For
the M(IL-4) macrophages, stimulation with CS-A of any concentration seems to have stimulated some macrophages to have a morphology similar to that of M(LPS) macrophages and some to have a more dendritic character. M(LPS 24) and M(LPS 48) macrophages only have a minimally changed morphology in response to CS-A, wherein the edges of the cell are more contoured than in the absence of CS-A. CS-B, CS-C and CS-E caused more dendritic cell character than CS-A overall.

**Figure 3.15** Macrophage morphology in the presence of CS-B. Macrophages are stained to show actin in green and nuclei in blue. CS-B concentrations (µg/mL) label the grid across the top, and stimulations are labeled along the left hand side. The last column is naïve, M(LPS 24), M(LPS 48), M(IL-4), and M(TGF) cells in the absence of CS-B. Scale bar is 50 µm.

Macrophage morphology is affected by CS-B. Naïve macrophages look like M(TGF) macrophages (Figure 3.15) in the presence of 1 µg/mL of CS-B. An increase in CS-B
concentration to 5, 10, 50 and 100 µg/mL caused the naïve cells to spread out more and extend lamellipodia in many directions. There is no apparent difference in appearance between the four higher concentrations of CS-B. CS-B caused TGF macrophages to extend more at lower concentrations (1 and 10 µg/mL), but at higher concentrations (50 µg/mL and above) the cells also spread out extensively and appeared more dendritic. M(IL-4) macrophages are also affected by CS-B: CS-B caused these cells to appear like M1 macrophages in some cells and caused others to spread out into cells with distinct dendrites. Though CS-B does not change the morphology of M(LPS 48) cells, it does induce M(LPS 24) macrophages to adopt a more dendritic appearance.

As the concentration of CS-C used to affect naïve macrophages increases, the amount that these cells spread out into more dendritic-like cells increased (Figure 3.16). TGF and CS-C stimulated cells have an extended morphology that is characterized by extension of a few lamellipodia. The IL-4 stimulated macrophages in the presence of CS-C also have two different cellular morphologies: the first is the flattened, extended M1-like morphology, and the other is the spread out cells with small dendrites. M(LPS 24) macrophages also have two morphologies in the presence of CS-C: they have the normal M1 like morphology, but also have a less spread morphology that is dendritic in character. There is less of an effect on the M(LPS 48) macrophages by CS-C of any concentration: some of the cells have the M1 phenotype, but a few of them have adopted a more dendritic morphology.
Figure 3.16 Macrophage morphology in the presence of CS-C. Macrophages are stained to show actin in green and nuclei in blue. CS-C concentrations (μg/mL) label the grid across the top, and stimulations are labeled along the left hand side. The last column is naïve, M(LPS 24), M(LPS 48), M(IL-4), and M(TGF) cells in the absence of CS-C. Scale bar is 50 µm.

CS-E caused naïve macrophages to spread out into cells with long tendrils in a manner correspondent to the change in CS-E concentration (Figure 3.17). CS-E did not have as much of an effect on M(TGF) macrophages, these cells spread out slightly more and appeared more linear than when stimulated by any other chondroitin sulfate type or concentration. M(IL-4) macrophages stimulated by CS-E at lower concentrations (<5 μg/mL) have a dendritic character with the absence of M1-like morphologies (unlike in other M(IL-4) samples). M(LPS 24) macrophages do not seem to be extensively changed by this
chondroitin sulfate type, once the concentration is increased to at least 50 µg/mL the presence of dendrites appears on some cells. The addition of CS-E and LPS for 48 h causes dendritic-like cells at all concentrations. The penultimate concentration of CS-E, 100 µg/mL, caused M(LPS 48) macrophages to take on dendrites with a conifer-like appearance.

**Figure 3.17** Macrophage morphology in the presence of CS-E. Macrophages are stained to show actin in green and nuclei in blue. CS-E concentrations (µg/mL) label the grid across the top, and stimulations are labeled along the left hand side. The last column is naïve, M(LPS 24), M(LPS 48), M(IL-4), and M(TGF) cells in the absence of CS-E. Scale bar is 50 µm.
3.3 Discussion

Chondroitin sulfates are used as anti-inflammatory agents in the treatment of osteoarthritis [191]. Therefore, the anti-inflammatory effects of chondroitin sulfates on pro-inflammatory M1 macrophages have been extensively studied. Chondroitin sulfate had no significant effect on the release of NO by LPS stimulated RAW 264.7 macrophages [192]. However, murine M1 macrophages can be polarized towards an M2 phenotype in the presence of CS-C as evidenced by reduced NO production, reduced IL-6 secretion, reduced TGF-α secretion, and an increase in IL-10 levels [181]. The following elaborates on the effects of chondroitin sulfates on M1-like RAW 264.7 macrophages, and, for the first time, how chondroitin sulfates affect anti-inflammatory M2a and M2c macrophages.

In comparison to the anti-inflammatory and naïve macrophages, the M(LPS 48) macrophage iNOS and arginase activities are only marginally affected by the addition of chondroitin sulfates of different types and concentrations. This is tenuously similar to the response seen by primary macrophages by Habara et al., wherein there was no change in iNOS activity when CS-C was added to RAW 264.7 macrophages [192]. Though CS-E did not affect macrophage metabolism of arginine, CS-E induces extensive morphological changes to M(LPS 48) cells; they have processes that appear as miniature branches of pine trees. Though there were no extensive effects of chondroitin sulfates on arginase/iNOS, there could be effects on other inflammatory and anti-inflammatory cytokines, indicated by extensive changes in cellular morphology [193].

Chondroitin sulfates may be able to reduce the extent of M1 polarization – morphology and iNOS activity – by cells only inflamed for shorter periods of time. Lower concentrations of CS-A, CS-B, CS-C and CS-E caused a decrease in urea levels with respect
to the M(LPS 24) control, showing that lower concentrations of these molecules can reduce the M2 aspect of these cells. Nearly all chondroitin sulfate types and concentrations caused a slight reduction in iNOS activity, but did not increase arginase activity by M(LPS 24) cells. The morphology of these cells are also more dendritic upon the addition of CS-A, CS-B, CS-C, and CS-E. This loss of M1-like morphology is likely correlated with the reduction in iNOS activity.

The presence of chondroitin sulfates in M(IL-4) macrophage culture further increases arginine metabolism. This activation is concentration dependent, as 50 µg/mL of CS-C and CS-E induced higher urea levels than the 5 µg/mL stimulations. Further general activation, but not polarization of macrophages in the presence of IL-4 and chondroitin sulfates occurs because iNOS activity is also increased to the range bound by LPS stimulation for 24 and 48 h by chondroitin sulfate presence. It appears that only 5 µg/mL of CS-B promotes M2-like metabolism, while reducing M1-like metabolism, possibly indicating further M2 polarization. CS-A, CS-B, and CS-C of any concentration caused M(IL-4) macrophages to have a flattened and extended M1-like morphology. CS-B and CS-C also caused some cells to have more dendritic character, especially 5 µg/mL of CS-B, 5 µg/mL of CS-E, and 1 µg/mL of CS-E. It is likely that 1 µg/mL concentrations of CS-B, and CS-E would also induce a reduced iNOS activity and an increased arginase activity.

The other M2 type macrophage is also more extensively activated in the presence of chondroitin sulfates. It is possible that TGF and chondroitin sulfate synergize to promote M2 macrophage polarization. Urea levels are increased at least ten times above that for the M(TGF) culture in the absence of chondroitin sulfate, and at least two times above the M(IL-4) culture in the absence of chondroitin sulfate. There is also an increase in nitrite
concentration to the level of M(LPS 24) macrophages. Escalation in the activity of both enzymes indicates an overall increased activation of these cells in the presence of 50 µg/mL CS-A, 10 µg/mL CS-A, 100 µg/mL of CS-C, 10 µg/mL of CS-C, and 10 µg/mL of CS-E. Interestingly, CS-B added to M(TGF) macrophages raised the nitrite level only to that of M(IL-4) macrophages in the absence of chondroitin sulfate. CS-B is the only chondroitin sulfate studied herein that has an iduronic acid group; it is possible this repeat unit prevents activation of the M1-related iNOS activity. The flexible iduronic acid group – if present within the chondroitin sulfate chains attached to the proteoglycans versican, decorin, and CD44 – modulates tumor behavior such as cell growth, angiogenesis, and metastasis [194].

In general, the morphological changes of M(TGF) macrophages when stimulated by chondroitin sulfates elongate similar to M(IL-4) macrophages with more dendritic character, another possible indicator of phenotypic change.

Generally, naïve macrophages have increased arginase and iNOS activity in the presence of chondroitin sulfates. These cells had a more dendritic character as the concentrations of CS-A, CS-B, CS-C and CS-E increased. Again for 5 µg/mL of CS-B there is an increase in arginase, but no increase in iNOS activity; this response could also be due to the iduronic acid group. Chondroitin sulfates have an activating effect on macrophages in general. Though there appear to be transient interactions of CS-A, CS-B, and CS-C with RAGE (receptor for advanced glycation end products), only CS-E strongly binds to RAGE [195]. Additionally, advanced glycation end products (AGEs) promote iNOS expression, but only slightly increased arginase expression in bone marrow derived macrophages [196], similar to the majority of the responses seen in stimulated macrophages caused by the four chondroitin sulfate types.
3.4 Conclusion

Though this is an incomplete data set, some effects of different chondroitin types and concentrations on macrophage phenotype have been elucidated. Chondroitin sulfates had effects on the morphology of most types of activated macrophage. M(LPS) macrophages were more resistant to the extended, dendritic morphological change. In comparison to the anti-inflammatory and naïve macrophages, the iNOS and arginase activities for M(LPS 48) macrophages are only marginally affected by the addition of chondroitin sulfates of different types and concentrations. Chondroitin sulfates in M(IL-4) cultures caused increases in both iNOS and arginase metabolism, further activating these cells, but not polarizing them. It is possible that TGF and chondroitin sulfate synergize to promote M2 macrophage polarization. The activating effects of chondroitin sulfates on macrophages could be exerted through RAGE. Further studies are necessary to understand the effects of these chondroitin sulfates on macrophage polarization; in particular, analysis of the secretion of TNF-α, and IL-10 would be useful in elucidating macrophage phenotypic changes in response to these biomolecules.
REFERENCES


