Evaluating the Use of Monocytes with a Degradable Polyurethane for Vascular Tissue Regeneration

by

Kyle Giovanni Battiston

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Monocytes are one of the first cell types present following the implantation of a biomaterial or tissue engineered construct. Depending on the monocyte activation state supported by the biomaterial, monocytes and their derived macrophages (MDMs) can act as positive contributors to tissue regeneration and wound healing, or conversely promote a chronic inflammatory response that leads to fibrous encapsulation and implant rejection. A degradable polar hydrophobic ionic polyurethane (D-PHI) has been shown to reduce pro-inflammatory monocyte/macrophage response compared to tissue culture polystyrene (TCPS), a substrate routinely used for in vitro culture of cells, as well as poly(lactide-co-glycolide) (PLGA), a standard synthetic biodegradable biomaterial in the tissue engineering field. D-PHI has also shown properties suitable for use in a vascular tissue engineering context. In order to understand the mechanism through which D-PHI attenuates pro-inflammatory monocyte response, this thesis investigated the ability of D-PHI to modulate interactions with adsorbed serum proteins and the properties of D-PHI that were important for this activity. D-PHI was shown to regulate protein adsorption in a manner that produced divergent monocyte responses compared to TCPS and PLGA when coated with the serum proteins α2-macroglobulin or immunoglobulin G (IgG). In the case of IgG, D-PHI was shown to reduce pro-inflammatory binding site exposure as a function of the material’s polar, hydrophobic, and ionic character. Due to the favourable monocyte activation state supported by D-PHI, and the importance of monocytes/macrophages in regulating the response of tissue-specific cell types in vivo, the ability of a D-PHI-stimulated monocyte/macrophage activation state to contribute to modulating the response of vascular smooth muscle cells (VSMCs) in a vascular tissue engineering context was investigated. D-PHI-
stimulated monocytes promoted VSMC growth and migration through biomolecule release. Coupling monocyte-VSMC co-culture with biomechanical strain further enhanced these effects, while also promoting extracellular matrix deposition (collagen I, collagen III, and elastin) and enhancing the mechanical properties of VSMC-monocyte seeded tissue constructs. This thesis identifies the use of biomaterials with immunomodulatory capacity to harness the stimulatory potential of MDMs and contribute to tissue engineering strategies in vitro. This latter work in turn has contributed to identifying aspects of biomaterial design that can contribute to supporting desirable monocyte-biomaterial interactions that can facilitate this process.
Acknowledgments

I would like to thank my supervisors, Dr. J Paul Santerre and Dr. Craig Simmons, for being excellent mentors. This thesis would not have been possible without their support. I would also like to thank my committee members, Drs. Alison McGuigan and Christopher Yip, whose insights and comments helped improve the quality of this PhD project. I would also like to thank Dr. Rosalind Labow for always taking the time to critically analyse all my documents and provide support for this project.

I would like to thank all of the undergraduate students I supervised who helped this thesis progress in one way or another, namely Maxfield Bradshaw, Samantha Chow, Devika Jain, Eilyad Honarpavar, Sina Makaremi, Bradley Menezes, Hanmin Miao, Arezou Ossareh, Ben Ouyang, Jenny Qian, and Charlie Wu.

I have been lucky to work with great people in the Santerre laboratory, who have helped create a great work environment: Patrick Blit, Maher Bourbia, Kate Brockman, Jane Cheung, Yasaman Delaviz, Marisa Lopez Donaire, Zac Grodzinski, Yi Guo, Mike Laschuk, Joanne McBane, Mitchell Nascimento, Maneesha Rajora, Soroosh Sharifpoor, Ken Shiguetomi, Hans Shih, and Meghan Wright. I would also like to thank members of the Simmons laboratory, including Oleg Chebotarev, Zahra Mirzaei, and Agnes Soos.

I would also like to thank the following people for their valuable contributions to this thesis through their suggestions and expertise and their willingness to give their time to answer my questions: Gurbaksh Basi, Dr. Peter Brodersen, Kuihua Cai, Dr. Siew Ging-Gong, Dr. Aleksander Hinek, Dr. Jonathan Rocheleau, Dr. Fred Keeley, Feryal Sarraf, Pamuditha Silva, Nancy Valiquette, Dr. Jian Wang, Dr. Eric Yang, Dr. Meilin Yang, and the staff of the MC-78 machine shop (Tai Do, Fred Gebeshuber, Ryan Mendell, Jeff Sansome).

I am also very grateful to my family for their support throughout this process, in particular my parents and my wife Alejandra.
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**Supplemental Figure 6.4** 20 μm thick cross-sections of D-PHI scaffolds seeded with VSMCs, monocytes, or VSMCs and monocytes in co-culture after 4 weeks of static or dynamic (10% strain, 1 Hz) culture. Samples were stained with H&E, picrosirius red, or alcian blue (pH=2.5) to assess cellularity, collagen deposition, and GAG deposition, respectively, throughout the entire thickness of the wall of D-PHI scaffolds. Scale bar represents 250 μm.
Supplemental Figure 6.5 40X magnification image of monocytes after 4 weeks of culture on D-PHI that is representative of morphology under both static and dynamic culture for monocytes cultured alone and in co-culture with VSMCs. Arrow indicates a FBGC. While the majority of cells remain mononucleated, isolated cases of FGBCs can be found. The image shown is from the dynamic monocyte culture at 4 weeks, but is representative of monocyte/macrophage morphology for all of the different conditions.

Supplemental Figure 6.6 Densitometric quantification of western blot data α-SMA and calponin expression for D-PHI scaffolds seeded with VSMCs (white bars) or VSMCs and monocytes in co-culture (grey bars). Data are the mean ± S.E. n=6. Data are presented as band intensity normalized to each samples respective GAPDH loading control, as well as to the W0 sample to give a measure of the loss or gain of expression after initial cell seeding. *p<0.05 vs. respective VSMC control.

Supplemental Figure 6.7 Quantification of apoptosis expressed as the percent of caspase 3 positive cells from immunofluorescence image analysis after 4 weeks of culture for (A) VSMCs and (B) monocytes/macrophages. Data are the mean ± S.E. n=9. *p<0.05 vs. static control.

Supplemental Figure 6.8 Quantification of sulphated GAG content from samples subjected to papain digestion. Data are the mean ± S.E. n=5. *p<0.05.

Supplemental Figure 6.9 Quantification of immunofluorescence images staining for the M1 markers CD80 and CD86, as well as the M2a marker CD206 for monocytes alone on D-PHI scaffolds. Dashed line indicates levels for each marker at W0 (24 hr post-seeding). Data are the mean ± S.E. n=9. *p<0.05 vs. week 2 timepoint for the same culture condition.

Supplemental Figure 6.10 Quantification of immunofluorescence images staining for the M1 markers CD80 and CD86, as well as the M2a marker CD206 for co-culture samples. Dashed line indicates levels for each marker at W0 (24 hr post-seeding). Data are the mean ± S.E. n=9. †p<0.05 vs. static condition at the same timepoint.
Supplemental Figure 6.11 Cytokine and growth factor release quantification for (A) TNF-α and (B) IL-10. Data are the mean ± S.E. n=4-5. *p<0.05 vs. co-culture at same time point under same culture conditions. °p<0.05 vs. W0 for same culture condition. †p<0.05. S- static. D- dynamic. Dashed line indicates level at W0. Where no dashed line is indicated, W0 levels were below sensitivity of the assay.

Appendix B: Seeding VSMCs on tubular D-PHI scaffolds

Figure B1 (a) CAD drawing of the perforated stainless steel tube used for vacuum seeding (OD 3.175 mm, ID 2.175 mm, 1.5 cm section filled with 1 mm diameter holes, 6 rows and 7 holes per row). (b) Schematic showing the set-up of the vacuum seeding apparatus. (c) Photograph of a partially dehydrated D-PHI scaffold prior to seeding. (d) Photograph of a D-PHI scaffold after vacuum cell seeding.

Figure B2 Comparison of modified static and vacuum seeding techniques using (a) A-10 rat aortic SMCs and (b) human coronary artery SMCs. *p<0.05. Data are the mean ± S.E.

Figure B3 H&E staining of tubular D-PHI scaffolds seeded with (a,b) rat aortic SMCs using the static (a) or vacuum (b) technique, or human coronary artery SMCs (c,d) using the static (c) or vacuum (d) technique. Scale bar represents 100 μm (a-d). (e) Representative H&E image showing human coronary artery SMCs seeded on D-PHI scaffolds using the previous static technique (McBane et al., 2012; Sharifpoor et al., 2011).

Appendix C: Drawings for the Bioreactor

Figure C1 Actuator to syringe coupler male ¼-28 UNF to male #6-32. Type 304 stainless steel. Dimensions are in mm.

Figure C2 Syringe termination to manifold adapter. Female ¼-28 UNF to female #10-32. Type 304 stainless steel. Dimension are in mm.

Figure C3 Front and top views of 50 ml syringe holder. Type 304 stainless steel.
Dimensions are in mm……………………………………………………………………………….. 288

**Figure C4** Bioreactor chamber lid. Polycarbonate. Dimensions are in mm………………. 288

**Figure C5** Bioreactor chamber. Polycarbonate. Dimensions are in mm…………………. 289

**Appendix D: Stress-strain curves**

**Figure D1** Representative stress-strain curves for the different sample types from the bioreactor study reported in Chapter 6. a) Co-culture dynamic, b) co-culture static, c) VSMC dynamic, d) VSMC static, e) monocyte dynamic, f) monocyte static, g) negative control (scaffold incubated in medium)………………………………………………………….. 290
### List of Abbreviations

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<tr>
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<td>Poly(lactic-co-glycolic acid)</td>
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<td>Self-assembled monolayer</td>
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Chapter 1
Introduction

1.1 Introduction

Following the implantation of a biomaterial, a cascade of events occurs in a material-specific manner that will ultimately determine the success or failure of the implanted biomedical device. Nanoseconds (Utesch et al., 2011) following exposure to protein-rich bodily fluids (such as blood and interstitial fluid), proteins adsorb to the material surface, forming the biological substrate with which cells will interact (Wilson et al., 2005). This is followed by the arrival of white blood cells such as neutrophils and monocytes (Anderson et al., 2008). Of particular importance for the success of the implanted device is the response of the monocyte. Following adhesion to the biomaterial surface, monocytes will differentiate to macrophages. Macrophage phenotype is plastic and covers a wide range of cellular states, ranging from the classically activated M1 (pro-inflammatory) state to alternatively activated M2 states that can be wound healing (tissue repair, M2a) or regulatory (anti-inflammatory, M2c) (Mosser and Edwards, 2010). The polarization of macrophages is determined in large part by the microenvironment provided by the biomaterial, including the nature of the tissue if the construct has been cultured with cells in vitro prior to implantation, as well as the adsorbed protein layer (Wilson et al., 2005; Anderson et al., 2008; Collier and Anderson, 2002). Initial (acute) stages of positive wound healing are characterized by a pro-inflammatory macrophage phenotype that is accompanied by the release of pro-inflammatory cytokines and growth factors that act to recruit inflammatory cells and act as mitogens for fibroblasts and smooth muscle cells (SMCs) (Barrientos et al., 2008). Over time, however, macrophage phenotype should shift towards a wound healing M2 state, as chronic inflammation can ultimately lead to implant failure (Brown et al., 2012b). Biomaterials that have been implanted with positive outcomes have been shown to promote this phenotypic macrophage transition over time (Brown et al., 2012a).

Prior to the arrival of monocytes to the surface of the implanted biomaterial, proteins will adsorb to the surface resulting in a new biological substrate that is specific to the biomaterial (Wilson et al., 2005). The type (Collier and Anderson, 2002), amount (Jenney and Anderson, 2000), and conformation (Jenney and Anderson, 2000; Lan et al., 2005) of specific serum proteins has
important implications for regulating the monocyte response to a biomaterial. Several biomaterial properties play crucial roles regulating protein-surface interactions, such as surface chemistry, surface energy, roughness, and wettability (Barrias et al., 2009; Arima and Iwata, 2007; Xu and Siedlecki, 2007; Rechendorff et al., 2006). Contradictory results regarding the effects of different types of surface chemistry effects using different base materials, however, suggests that some of these effects may be biomaterial specific, demonstrating the need to understand the factors resulting in specific protein effects for new biomaterials that are developed. Hydrophilic and anionic surfaces, for example, have been shown to be less inflammatory, supporting reduced monocyte adhesion and increased anti-inflammatory cytokine release (MacEwan et al., 2005; Brodbeck et al., 2002a), while hydrophobic and cationic surfaces have been shown to support foreign body giant cell (FBGC) formation, an indication of a more pro-inflammatory phenotype-inducing substrate. However, in other cases the hydrophobic substrate from a set of test biomaterials has been shown to support the least amount of monocyte/macrophage adhesion (Young et al., 2000).

A degradable polar hydrophobic ionic polyurethane (D-PHI) was developed in the Santerre laboratory for use in tissue engineering applications (Sharifpoor et al., 2009). Preliminary studies on the interaction of this polymer with monocytes indicated that D-PHI supported a heightened anti-inflammatory and reduced pro-inflammatory response from adherent monocytes (McBane et al., 2009). The different aspects of D-PHI that may be responsible for these effects, however, remain to be elucidated. Dissecting how D-PHI regulates adherent monocyte activation may provide insight into aspects of biomaterial design and biomaterial-cell interactions that can have important implications for the development of new biomaterials, and in particular materials with significant relevance to tissue engineering applications.

In this thesis, the properties of D-PHI responsible for regulating acute interactions with monocytes were investigated. While aspects of surface chemistry, topography, and wettability can directly interact with cells, previous work with D-PHI has been performed in conditions with high concentrations of proteins (either serum proteins in medium (McBane et al., 2009) or in vivo studies (McBane et al., 2011)), suggesting that the regulation of protein interactions by different surface properties of D-PHI may be important in regulating monocyte response. Differences in the amount (Jenney and Anderson, 2000) or conformation (Jenney and Anderson,
2000; McNally and Anderson, 2002) of specific serum proteins have been implicated in the monocyte/macrophage response to biomaterial surfaces. The presence of proteins such as immunoglobulin G (IgG), vitronectin, and fibronectin, amongst others, is known to support various responses from monocytes/macrophages, including adhesion, fusion to FBGCs, and cytokine release (Brodbeck et al., 2002a; McNally et al., 2008). Many serum proteins also possess distinct domains that have been identified as critical for interacting with monocytes, which can become exposed when the protein denatures upon binding to the biomaterial (Jenney and Anderson, 2000). The amount of PEG in tyrosine/PEG-derived polycarbonates, for example, has been shown to regulate the conformation and bioactivity of adsorbed fibronectin (Tziampazis et al., 2000). In this thesis the effect of protein adsorption on regulating monocyte response to D-PHI was evaluated by assessing differences in the type and amount of serum proteins that adsorbed to D-PHI from a complex protein solution, namely serum supplemented medium, as well as by assessing differences in binding site exposure of specific serum proteins known to be important in monocyte-biomaterial interactions. Properties of D-PHI, in particular its surface chemistry, were also explored as a means to determine aspects of D-PHI important for specific protein-surface, and also cell-surface, interactions.

D-PHI has been evaluated for its suitability for vascular tissue engineering applications (Sharifpoor et al., 2009), as there is a significant need for new biomaterial strategies to develop small diameter vascular grafts due to the limited availability of donor vessels and low patency rates associated with currently available synthetic materials (McBane et al., 2012). Desirable properties for a synthetic material to be used for vascular tissue engineering include the ability to support cell adhesion and growth, to promote tissue production and allow for tissue infiltration, to have sufficient mechanical properties relevant to the physiological structure being engineered, and to biodegrade with non-toxic degradation by-products (McBane et al., 2012). D-PHI has been shown to have mechanical properties comparable to those measured for human coronary arteries (Sharifpoor et al., 2011); to support the growth, viability, and appropriate phenotypic state of relevant vascular cell types, including endothelial cells (ECs) (McDonald et al., 2011), fibroblasts (Cheung et al., 2013), and vascular SMCs (VSMCs) (Sharifpoor et al., 2010); and to degrade in vitro and in vivo at an appropriate rate with non-toxic degradation by-products, while also maintaining its structural integrity and supporting tissue in-growth (McBane et al., 2011).
While it is known that monocytes and their derived macrophages (monocyte-derived macrophages, or MDMs) will interact with implanted tissue such as an engineered vascular graft, there is little understanding of how these cells respond to environments involving new biomaterials and the VSMCs and their derived tissue products. While there has been significant research into factors regulating monocyte response to biomaterial surfaces, the factors regulating the response of monocytes will be unique to each material and these monocyte-biomaterial interactions will determine in part how MDMs interact with pre-seeded vascular cell types. Furthermore, monocytes/macrophages that have been activated by appropriate stimuli can be positive contributors to tissue production, remodeling, and ultimately regeneration (Brown et al., 2012b; Brown et al., 2012a).

Given that D-PHI has been shown to support a reduced pro-inflammatory monocyte response, the cytokines and growth factors released by monocytes adherent to D-PHI may be used to support a desired response from VSMCs seeded on D-PHI scaffolds. In contrast to dosing specific growth factors and cytokines into culture medium, monocytes are an autologous source of biomolecules that avoid several disadvantages inherent to growth factors, such as high cost and endotoxin contamination of recombinant proteins (Wakelin et al., 2006; Daly et al., 2012). M1 and M2 macrophages have divergent responses with respect to their influence on cell migration and proliferation, two important parameters for the in vitro development of a tissue engineered vascular graft. The conditioned medium from M2, but not M1, macrophages has been shown to support migration of myoblasts (Brown et al., 2012a), while M2 but not M1 stimulated macrophages have been shown to support the proliferation of VSMCs (Khallou-Laschet et al., 2010). The bioactivity of polarized macrophages has been taken advantage of in other areas of tissue engineering, such as in bone tissue engineering where macrophages have been used as a means to guide the differentiation and growth of mesenchymal stem cells (MSCs), with effects dependent on the activation state of the macrophage (Dong and Wang, 2013). In this thesis, the effect of co-culturing monocytes with primary VSMCs derived from human coronary artery was explored for its influence on VSMC growth, migration, and phenotype. VSMCs are the primary cellular component of the tunica media of an artery, and in combination with their derived ECM products are responsible for the strength and contractility of this tissue (Beamish et al., 2010). In this thesis, the mechanism through which monocytes adherent to D-PHI influenced VSMC
response was explored to provide an understanding of the bioactive agents responsible for monocyte-induced effects on VSMCs.

A common method for achieving the maturation of tissue engineered constructs is the use of biomechanical stimulation applied with bioreactor systems (Sharifpoor et al., 2011; Syedain et al., 2011; Wang et al., 2010; Stegemann and Nerem, 2003). In native vasculature, VSMCs are exposed to biomechanical stimulation in the form of cyclic circumferential strain due to the pulsatile nature of blood flow (Lehoux et al., 2006). Furthermore, previous studies that have looked at the source of new VSMCs following the implantation of vascular grafts note that they derive from migrating cells at the anastomosis sites (Hibino et al., 2011). As a result, the in vivo development of new tissue requires the interaction of native VSMCs (e.g., coronary artery SMCs in the case of a coronary artery bypass procedure) with host monocytes under physiological biomechanical stimulation. Therefore, for both the purpose of understanding the role of monocytes in contributing to tissue regeneration with VSMCs from an in vitro perspective, as well as for understanding the contribution of monocytes to VSMC function under physiological conditions, it is important to understand how monocytes influence VSMCs under biomechanical stimulation, and specifically under exposure to cyclic mechanical strain. In this thesis, a custom-designed bioreactor was conceived, fabricated, and used to study the effect of physiological cyclic mechanical strain levels on VSMC growth, infiltration, phenotype, and ECM deposition when in co-culture with primary human monocytes. Furthermore, the effect of culture conditions on monocyte/macrophage activation was explored to understand the role of biomaterial-mediated macrophage activation in supporting desirable outcomes for vascular tissue engineering applications.

1.2 Hypothesis

Central Hypothesis: D-PHI will support a reduced pro-inflammatory monocyte activation state in part directed by the make-up of the adsorbed serum protein layer. The influence of D-PHI-stimulated monocytes and mechanical stimulation will together support VSMC growth, infiltration into porous scaffolds, and extracellular matrix production, and enhance the mechanical properties of cell-seeded D-PHI constructs.
1.3 Objectives

The central objective for this thesis was to investigate the role of protein adsorption in regulating monocyte response to D-PHI, while exploring the potential role of D-PHI-stimulated monocytes in contributing to strategies for vascular tissue regeneration.

1.3.1 Objective 1

To evaluate the role of protein adsorption in regulating the differential monocyte response to D-PHI vs. TCPS, while understanding the chemical and physical make up of D-PHI’s surface, and the latter’s influence on mediating protein interactions.

**Approach:**

- Characterized D-PHI, TCPS, and PLGA surface properties by performing water contact angle measurements (surface wettability), scanning electron microscopy (SEM [surface morphology]), and low and high resolution x-ray photoelectron spectroscopy (XPS [surface chemistry]).
- Investigated protein interactions with D-PHI, TCPS, and PLGA. Quantified total adsorbed protein from serum containing medium as well as from single protein solutions (bicinchoninic acid [BCA] assay). Identified protein adsorption profiles from serum containing medium for D-PHI, TCPS, and PLGA by removing adsorbed protein and identifying the presence of different serum proteins on these materials (2-D electrophoresis and liquid chromatography tandem mass spectrometry [LC-MS/MS]). Evaluated the exposure of established protein domains important for monocyte interactions on D-PHI vs. TCPS (enzyme linked immunosorbent assay [ELISA]).
- Evaluated monocyte response to protein pre-adsorbed surfaces. Proteins evaluated included those identified to be present in the adsorbed protein layer for D-PHI, TCPS, or PLGA from 2-DE and LC-MS/MS studies, as well as serum proteins with distinct binding sites identified in the literature to be important for monocyte-biomaterial interactions. Monocyte response was evaluated by assessing the release of characteristic pro- and anti-inflammatory cytokines (ELISA), cell adhesion (DNA mass quantification), and cell morphology (SEM).
• Determined the mechanism of monocyte-protein interactions for several proteins identified to produce biomaterial-specific effects by performing inhibition studies. Monocyte response was assessed by evaluating cell adhesion (DNA mass quantification), cell morphology (SEM), and/or cytokine release quantification (ELISA).

• Synthesized a family of D-PHI materials by varying monomer ratios to assess the role of D-PHI chemistry in regulating protein-surface interactions. D-PHI surfaces were characterized by water contact angle measurements (surface wettability), XPS (surface chemistry), surface morphology (SEM), swelling (gravimetric analysis), and/or percent monomer conversion (gel content). D-PHI surfaces were characterized for their interactions with a serum protein (IgG) previously identified to be important for understanding D-PHI’s interactions with adherent monocytes (BCA for total protein content and ELISA for binding site exposure), and select formulations were subsequently evaluated for monocyte interactions in the presence or absence of this serum protein by assessing cell adhesion (DNA mass quantification) and cell morphology (SEM).


In the latter paper, Ben Ouyang performed initial experiments investigating monocyte interactions with fibronectin and IgG-coated surfaces and investigated IgG interactions (total protein adsorption, Fab exposure) with the different materials. Eilyad Honarparvar performed some of the antibody inhibition experiments. Jenny Qian performed experiments investigating differences in D-PHI chemistry on IgG adsorption and monocyte response. My contribution was performing some of the experiments (protein adsorption, monocyte interactions), conceiving the idea for the study, designing the experiments, manuscript writing, and supervising the studies performed by Ben Ouyang, Eilyad Honarparvar, and Jenny Qian.
1.3.2 Objective 2

Evaluate the influence and possible mechanisms for monocyte-mediated changes in VSMC growth, infiltration, and/or contractile marker expression in monocyte-VSMC co-culture on D-PHI scaffolds.

**Approach:**

- Co-cultured primary human monocytes with VSMCs on D-PHI scaffolds and evaluated VSMC response by assessing cell number (DNA mass quantification), contractile marker expression (western blotting), and cell infiltration (histology – H&E staining). Compared the effects of direct monocyte co-culture to those induced by monocyte-conditioned medium derived from monocyte-only cultures on D-PHI scaffolds.
- Identified cytokines released by monocytes on D-PHI scaffolds (cytokine antibody array), and screened select cytokines for their ability to influence VSMC response (cell number, phenotype, migration) using doses produced by monocytes on D-PHI.
- Confirmed the importance of cytokines shown to regulate VSMC response in the co-culture system with VSMCs on D-PHI scaffolds by performing antibody inhibition experiments. Neutralizing antibodies for specific cytokines were dosed into medium for D-PHI scaffolds seeded with VSMCs and monocytes in co-culture, and the effect on contractile marker expression (western blotting) and cell number (DNA mass quantification) was assessed.


In this study, Ben Ouyang performed experiments investigating effects of MCP-1, IL-6, and GM-CSF on VSMC response in monoculture (DNA mass quantification). My contribution was conceiving the experiments, designing the studies, supervising Ben Ouyang’s work, writing the manuscript, and performing most of the experiments.
1.3.3 Objective 3

Evaluate the effect of combined monocyte co-culture and biomechanical stimulation on VSMC growth, infiltration, phenotype, and ECM deposition.

Approach:

- Designed a custom bioreactor to apply physiological strain levels (10% strain, 1 Hz) to cell-seeded tube-shaped D-PHI scaffolds.
- Studied the effects of dynamic mechanical strain (10% circumferential strain, 1 Hz) relative to static culture on D-PHI scaffolds seeded with VSMCs, monocytes, and VSMCs and monocytes in co-culture for 4 weeks. VSMC response was assessed by evaluating cell number (DNA mass quantification, immunofluorescence [IF]), phenotypic marker expression (western blotting, IF), and distribution (histology, IF).
- Evaluated the effects of dynamic (10%, 1 Hz) vs. static culture for VSMC, monocyte, and co-culture samples over 4 weeks for their effect on ECM production, including collagen (western blotting, IF, histology), glycosaminoglycan (dimethylmethylene blue dye binding, histology), and elastin (Fastin elastin assay, western blotting, IF) production.
- Evaluated the response of monocytes/macrophages in dynamic (10% circumferential strain, 1 Hz) vs. static culture on D-PHI scaffolds, both in monoculture and in co-culture with VSMCs, to understand the role of MDMs in regulating VSMC response under dynamic co-culture. Monocyte/macrophage response was evaluated by assessing growth factor and pro- and anti-inflammatory cytokine release (ELISA) and through the expression of characteristic M1 and M2 markers (IF).
- Assessed the effect of dynamic (10% circumferential strain, 1 Hz) vs. static culture on the mechanical properties of D-PHI scaffolds seeded with VSMCs, monocytes, or VSMCs and monocytes in co-culture for 4 weeks. Elongation-at-yield, elastic modulus, and tensile strength were measured using an Instron testing machine.
Relevant Papers: Battiston KG, Labow RS, Simmons CS, Santerre JP. Monocyte co-culture with arterial smooth muscle cells in an immunomodulatory scaffold enhances vascular tissue production under dynamic biomechanical stimulation. Submitted.

1.4 Additional contributions

Outside of the core objectives of my thesis, I have provided additional contributions to the literature through several studies on topics closely related to my thesis objectives. The first study sought to investigate differences in commercially sourced TCPS by assessing surface properties, protein adsorption, and monocyte response. Surface properties were assessed by water contact angle measurements (surface wettability), surface chemistry (low and high resolution XPS), and surface morphology (SEM). Protein adsorption was assessed by measuring total adsorbed protein from serum containing medium (BCA assay), as well as by identifying specific proteins present in the adsorbed protein layer (2-DE and LC-MS/MS). Finally, monocyte response was evaluated by assessing cell adhesion (DNA mass quantification), cytokine release (ELISA), and enzymatic activity (acid phosphatase activity). The abstract of this published paper is in Appendix E. (Battiston KG, McBane JE, Labow RS and Santerre JP. Differences in protein binding and cytokine release from monocytes on commercially sourced tissue culture polystyrene. Acta Biomater 2012;8(1):89-98).

Initial studies on the feasibility of co-culturing VSMCs with primary human monocytes was conducted on 2D D-PHI films, rather than the 3D porous scaffolds used in the work discussed in Objectives 2 and 3. This work was conducted by a postdoctoral fellow in our research group, Dr. Joanne McBane, as well as other researchers at the University of Ottawa Heart Institute. Monocytes were co-cultured with VSMCs on D-PHI, TCPS, and PLGA surfaces at different ratios. Monocyte response was assessed by evaluating cytokine release (ELISA), cell adhesion (DNA mass quantification), enzymatic activity (esterase and acid phosphatase activity), and cell morphology (SEM). VSMC response was evaluated by investigating cell adhesion (DNA mass quantification), cell morphology (SEM), and contractile marker expression (western blotting). Surfaces properties of the three polymers were compared by evaluating surface wettability (water contact angle measurements), surface chemistry (low and high resolution XPS), and surface morphology (SEM). My contribution to this paper was the performance of surface

In vascular tissue engineering strategies, while it is initially desirable to promote the synthetic VSMC phenotype *in vitro* since it is associated with increased proliferation and matrix deposition, ultimately a phenotypic switch to the contractile state is desired since this is the VSMC phenotype predominant in healthy vasculature (Chan-Park et al., 2009). Work presented in this thesis as part of Objectives 2 and 3 demonstrated that monocyte co-culture with VSMCs supported increased VSMC numbers and matrix deposition, but also suppressed the expression of the contractile marker proteins α-SMA and calponin, which suggests a shift to a more synthetic phenotype. It was thus desirable to explore a strategy to promote a phenotypic switch to a contractile phenotype without mitigating the ability of co-culture to promote VSMC growth. TGF-β1 is a growth factor that has been employed by others in vascular tissue engineering strategies for this purpose (Stegemann and Nerem, 2003; Chan-Park et al., 2009; Syedain and Tranquillo, 2011; Risinger et al., 2010). A strategy was evaluated where TGF-β1 was supplemented into medium for VSMC-monocyte co-cultures on D-PHI scaffolds, and contractile marker expression (western blotting) and DNA mass quantification were assessed. While TGF-β1 successfully upregulated α-SMA and calponin expression after 4 weeks of culture, it also significantly reduced VSMC number. The ability of TGF-β1 to upregulate contractile marker expression using shorter exposure periods was subsequently evaluated, where TGF-β1 was supplemented into medium for the final 4 weeks, 3 weeks, 2 weeks, 1 week, or 1 day of culture. This study demonstrated that supplementation of TGF-β1 into culture medium for only the final 2 weeks of culture was able to successfully upregulate α-SMA and calponin expression, with no detrimental effects on VSMC number observed. This study was published in a paper highlighting the tissue engineering work being performed in the Santerre laboratory. This paper also included a preliminary experiment that demonstrated that dynamic monocyte-VSMC co-culture upregulated collagen deposition relative to static co-culture using a custom-designed bioreactor system developed by Sharifpoor et al. (Sharifpoor et al., 2010). My contributions to this paper were the design and performance of experiments relating to studies involving VSMCs, as well as
While VSMCs are a significant component of any vascular tissue engineering strategy, the endothelial cell (EC) layer, covering the lumen of the blood vessel, is also of critical importance (Melchiorri et al., 2013). The source of ECs for tissue engineering strategies, however, can be problematic. Circulating angiogenic cells (CACs) are a promising cell source derived from the mononuclear cell fraction of whole blood that possess angiogenic potential that may be able to provide a microenvironment suitable for developing a confluent EC layer on a vascular construct (Favre et al., 2013). The biocompatibility of D-PHI with regards to its interactions with CACs was assessed, with the study conducted by Dr. Eva Mathieu and other researchers at the University of Ottawa Heart Institute. Monocytes, CACs, or monocytes and CACs in co-culture were maintained on D-PHI and TCPS surfaces, either coated with fibronectin or non-coated, and the cellular response was investigated. Cell response was assessed by evaluating cell adhesion (DNA mass quantification), pro- and anti-inflammatory cytokine release (ELISA), nitric oxide production, metabolic activity (WST-1), expression of monocyte and endothelial markers (western blotting), and cell morphology (SEM). My contribution to this study was to synthesize D-PHI films and perform SEM imaging and analysis. (Mathieu E, Battiston KG, McBane JE, Davidson L, Suuronen EJ, Santerre JP and Labow RS. Characterization of a degradable polar hydrophobic ionic polyurethane with circulating angiogenic cells in vitro. J Biomater Sci Polym Ed 2014;25(11):1159-73).

1.5 References


Chapter 2
Literature Review

2.1 Biomaterials

Biomaterials are materials that are used in contact with biological systems, and can range from biomedical devices to artificial organs to drug delivery systems, amongst many others. More specifically, Williams has recently sought to redefine a biomaterial as “… a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine.” (Williams, 2009) This broad definition includes biomaterials outside the classical categories of metals, polymers, and ceramics, with quantum dots and even viruses now found within the scope of the biomaterials field (Williams, 2009). Their utility is evident by their widespread use in many applications, from commonly used sutures to advanced procedures such as heart valve replacement.

Tissue engineering, often viewed as a sub-field within the study of biomaterials, aims to restore the loss of function of whole tissues or parts thereof, through a combination of biomaterials, cells, and appropriate stimulation methods, which can take the form of biochemical, biomechanical, and electrical stimulation, amongst others. While tissue engineering strategies do not necessarily require the use of materials, the combination of biomaterials and cells to produce new tissue holds several advantages. Compared to tissue engineering strategies that use cells and their derived matrix proteins to produce a mechanically robust product, biomaterials can be designed to possess the initial mechanical integrity required for specific applications, thus limiting the in vitro culture time needed prior to implantation. Furthermore, cell instructive cues, whether through surface chemistry or cell-adhesive peptides, can be incorporated into the material to direct particular cellular activity, such as proliferation, migration, or tissue-specific phenotypes. Biomaterials can also be designed to be biostable or with desired degradation rates, as well to release bioactive agents in a highly tailored manner for certain applications. Synthetic biomaterials in particular can be made with high reproducibility, whereas cell-derived products suffer from source-to-source variability. With the advent of tissue engineering in the 1990s (Langer and Vacanti, 1993), the initial choice of biomaterials often involved those that were
FDA-approved, such as PLGA, due to the thought that clinical translation would be easier and that their biological interactions were generally well understood. However, prior to use in tissue engineering these biomaterials were designed primarily for structural support (e.g. sutures), rather than to interact with host tissue and direct cellular responses in a deliberate manner.

A concept of critical importance to implanted biomaterials and tissue engineered constructs is biocompatibility. The definition of biocompatibility has shifted over the years (Williams, 2008), with the goal originally for new materials to be bioinert, or not interacting with their biological environment. This notion came from the observation that optimal performances for several implanted materials were obtained with biomaterial options that were mechanically robust but chemically and generally biologically non-reactive, such as oxide ceramics for bone implant applications and polytetrafluoroethylene (PTFE) for vascular grafts. No biomaterial, however, can be completely inert as there is always some degree of interaction between the material and the surrounding cells and tissue. Given that the material must interact with the biological system, biocompatibility then refers to the ability of a material to elicit an appropriate biological response for the stimulation of cells in a specific tissue microenvironment (Williams, 2008). A material that may be appropriate for cardiovascular applications, for example, may demonstrate poor biocompatibility in the context of neural applications. Given the prominent role of biomaterials in tissue engineering in particular, the concept of inertness is counterproductive to the ultimate goals of tissue engineering, where the aim is to regenerate new tissue, a process that will necessitate directed guidance of cellular activity, and thus require interaction with the biological environment (Williams, 2014). An understanding of the different biological components interacting with biomaterials, as well as the biomaterial properties influencing these interactions, is thus necessary for the development of new biomaterials for tissue engineering applications.

2.2 Protein adsorption

Protein adsorption refers to the partitioning of protein between a bulk phase and an interface (Vogler, 2012), namely the biomaterial surface, and is an important factor governing the biological response to any biomaterial. Within nanoseconds (Utesch et al., 2011) of exposure to biological fluids (e.g. blood, serum containing medium, interstitial fluid etc.) proteins begin to adsorb to a biomaterial’s surface, resulting in a new biological substrate containing numerous
biomolecules that will ultimately determine the subsequent cellular response. In a process termed the Vroman effect (Figure 2.1), proteins in high abundance initially adsorb to the surface, but over time these proteins are displaced by proteins with stronger binding affinities for that particular substrate (Vroman and Adams, 1969). The adsorbed protein layer is thus biomaterial-specific, making it an important factor in contributing to differential biological responses to different materials.

**Figure 2.1** Graphical representation of the Vroman effect. Proteins present in high abundance (e.g. albumin, IgG) will initially adsorb to the biomaterial surface, but will subsequently be displaced by proteins with higher affinity for the particular substrate, leading to a biomaterial-specific adsorbed protein layer that will then direct the cellular response.

### 2.2.1 Protein-surface interactions

Protein interactions with a given substrate are governed both by the properties of that substrate, as well as the type of protein(s) being tested. The amount of a protein that adsorbs to a surface is dependent on different properties of the protein, including source (e.g. bovine vs. human) (Lu et al., 1999b), molecular weight (Parhi et al., 2009; Green et al., 1999), charge (Mathes and Friess, 2011), and rigidity (Ouberai et al., 2014; Mathe et al., 2013). Adsorption of different serum albumins to Teflon has been reported to differ in terms of the thickness of the adsorbed protein layer for bovine vs. human albumin (Lu et al., 1999b). Despite having similar sequence homology, isoelectric point, and molecular weight, differences in protein glycosylation between species can render human vs. xenoproteins with distinct properties that will result in varying interactions with biomaterials (Vogler, 2012). Depending on the intermolecular interactions primarily responsible for protein interactions with a material (electrostatic, van der Waals, etc.),
protein rigidity can also play an important role in the affinity of a protein for a particular substrate. Adsorption of proteins from serum to silica nanoparticles has been reported to favour flexible over rigid proteins, as flexible proteins upon interacting with the silica through electrostatic interactions are able to spread and experience further electrostatic interactions that results in a more strongly bound protein to the material surface (Mathe et al., 2013).

The interaction of a particular protein with a biomaterial will also be dependent on the conditions under which the experiment is performed, as factors including solution pH (Mathes and Friess, 2011; Lu et al., 1999a; Holmberg et al., 2008), solution composition (the presence of other proteins (Zhuo et al., 2007; Lassen and Malmsten, 1997), buffer properties (Mathes and Friess, 2011; Vutukuru et al., 2006)), and even the container the experiment is performed in (Mathe et al., 2013) influence protein-material interactions. In order to perform certain functions, some proteins must be converted to an active form or be presented in a conformation that exposes specific domains. While experiments using single protein solutions can provide important insights into biomaterial-mediated changes in protein activation or conformation, biological environments contain a complex mixture of proteins that can effect protein-surface interactions. Factor XII (FXII) is a protein that, when activated to FXIIa, initiates the intrinsic coagulation pathway and is reported to be activated by hydrophilic and anionic surfaces. This specificity for hydrophilic surfaces that has been reported in previous studies has been shown to be due to interactions of FXII with proteins not involved in the coagulation cascade, such that FXII activation does not show a preference for hydrophilic surfaces in the absence of other serum proteins (Zhuo et al., 2007). Adsorption from single protein solutions can also suggest a high affinity of a particular protein for a surface, but this same protein may be excluded in the presence of other proteins in a competitive environment. Poly(hexamethyl disiloxane), for example, will adsorb more fibrinogen than IgG or human serum albumin (HSA) from equal concentrations of single protein solutions. However, adsorption from a ternary protein mixture of fibrinogen, IgG, and HSA results in the exclusion of fibrinogen and a preference for HSA and IgG (Lassen and Malmsten, 1997). Likewise, adsorption kinetics of single protein solutions vs. complex mixtures can differ drastically. For the adsorption of albumin and IgG to poly(ethylene terephthalate), for example, from single protein solutions, adsorbed protein mass increases from 1 hr to 24 hr, but when adsorption is performed from a 25% serum solution, the quantity of
albumin or IgG adsorbed after 24 hr is less than half of that initially adsorbed after 1 hr due to the Vroman effect (Holmberg et al., 2008).

Following the implantation of a biomaterial, the nature of the protein-rich environment encountered cannot be controlled. Protein-material interactions must therefore be understood in terms of how aspects of a biomaterial’s surface interact with complex protein solutions in order to inform the design of new biomaterials and to support desirable protein-surface interactions for different applications. Parameters that define a biomaterial surface include surface chemistry, surface wettability (hydrophobicity), and roughness. Depending on the application, biomaterials can be designed to prevent or limit protein adsorption (non-fouling) or to adsorb certain proteins as a means to promote cell attachment and growth. Non-fouling strategies include surface modifications with grafted polymer chains, such as 500-2000 MW PEG, which repel proteins due to steric interactions (Price et al., 2001); conjugation of anti-thrombotic agents, such as the anticoagulant hirudin (Alibeik et al., 2010); as well as many other techniques (Hoffman, 1999). For tissue engineering applications, however, it is not desirable to resist protein adsorption, as adsorbed proteins present cell-binding domains that support desirable cellular activity. Rather it is desired to modulate the amount, type, and conformation of the adsorbed proteins.

Hydrophobic surfaces are generally considered to have greater capacity for protein adsorption. Advancing water contact angle measurements of θ<65° are typically defined as hydrophilic, and those with θ>65° as hydrophobic. This definition is based on the observation that many biological processes, such as protein adsorption and coagulation, tend to change drastically at this threshold (Vogler, 2012). In addition to the amount of adsorbed protein, the strength of the interaction between hydrophobic surfaces with adsorbed proteins is generally reported to be stronger. Low density polyethylene (LDPE) surfaces treated with glow discharge plasma to generate a range of surface hydrophilicity showed that stronger adhesion forces were observed for FXII, fibrinogen, and BSA for θ>60-65°, and lower adhesion forces observed for θ<60° (Xu and Siedlecki, 2007). In a study by Luan et al., self-assembled monolayers (SAMs) of 1-undecanethiol on gold were used to prepare surfaces with θ ranging from 30-100°, with more hydrophobic surfaces generated due to an increased percentage of 1-undecanethiol. Fibrinogen adsorption on these surfaces was shown to be positively correlated with increasing hydrophobicity, as evidenced by a number of analytical methods including radiolabeling, surface
plasmon resonance (SPR), and quartz crystal microbalance with dissipation (QCM-D) (Luan et al., 2014). Surface modifications applied to biomaterials in order to change the hydrophobicity have yielded similar results, such as decreased adsorption of human fibrinogen with an increase in OH content of hydroxyl/methyl terminated SAMs (Rodrigues et al., 2006); decreased BSA adsorption to hydrophobic polycaprolactone by increasing surface hydrophilicity with hyaluronan (Li et al., 2012); and decreased protein adsorption to PLGA films blended with PEG-containing Pluronic molecules with increasing hydrophilicity, where hydrophilicity increased as a function of film thickness (20-200 nm) (Gyulai et al., 2011).

While hydrophobic surfaces may typically adsorb more protein, the type of proteins that adsorb and their conformation is also important. Co-polymer blend films of N-isopropylacrylamide (NiPAAm) and N-butylacrylamide (NBAAm) showed different adsorption trends for albumin and fibronectin, with increasing hydrophilicity supporting greater albumin adsorption, while the opposite trend was apparent for fibronectin (Allen et al., 2006). However, hexamethyldisiloxane (HMDSO) surfaces treated with oxygen plasma suggested an affinity of fibronectin for hydrophilic and albumin for hydrophobic surfaces under competitive protein adsorption conditions (Wei et al., 2009). The affinity of fibronectin for hydrophobic surfaces may be due to increased denaturation, leading to more protein-surface interactions, as fibronectin has been shown to adopt more extended conformations on hydrophobic vs. hydrophilic Ti6Al4V, resulting in differential exposure of biologically active domains on surfaces with differing hydrophobicity (Vadillo-Rodriguez et al., 2013). Surfaces prepared with varying hydrophobicity through CH3 and OH-terminated SAMs also support the ability of hydrophobic surfaces to promote greater protein denaturation, as BSA and fibrinogen were both shown to have less ordered secondary structure on hydrophobic surfaces (Roach et al., 2005). Likewise, silicon wafers with increased hydrophilicity following treatment with 5% HF showed differences in the sheet/turn ratio of adsorbed fibrinogen compared to the unmodified hydrophobic surface, suggesting differences in conformation as a function of surface wettability (Steiner et al., 2007). In cases where hydrophobic surfaces do bind large quantities of protein, the bioactivity of the adsorbed protein is not necessarily increased on these surfaces. Hydrophilic polyurethanes (PUs) have been shown to bind less fibrinogen than hydrophobic PUs; however, both of these surfaces supported
minimal platelet interactions because the bioactive domains of the adsorbed fibrinogen were not exposed on the hydrophobic PU surface, despite more protein being present (Wu et al., 2005).

Modifying a base polymer to regulate hydrophobicity enables one to study the role of wettability with minimal influences of changes in surface chemistry. With significant changes in surface chemistry, however, an increase in surface hydrophobicity does not necessarily lead to an increase in protein adsorption. Self-assembled monolayers (SAMs) prepared with NH$_2$ (θ=42°), CH$_3$ (θ=80°), and CF$_3$ (θ=107°) terminal groups showed the lowest amount of BSA adsorption to the most hydrophobic surface, CF$_3$-terminated SAMs (Kim et al., 2011). Changes in surface chemistry thus play an equally important role in regulating the adsorption of proteins to biomaterial surfaces. Different functional groups can also introduce charge to a surface, which has the potential to impact protein adsorption as different proteins can possess more or less charged amino acid side groups (Lubarsky et al., 2005; Kreke et al., 2005). IgG and BSA, for example, have shown higher affinity for alginate-chitosan-alginate microcapsules possessing increased positive surface charge (Xie et al., 2010a). Poly(l-lysine) surfaces have also been shown to support a greater amount and faster adsorption of fibronectin relative to dextran sulfate surfaces, a phenomenon believed to be due to greater complexation between the negative protein and positive poly(l-lysine) surface (Wittmer et al., 2007).

SAMs with varying percentages of different terminal function groups demonstrate how surface chemistry can change protein adsorption characteristics despite similar changes in surface wettability. NH$_2$, COOH, and OH terminated SAMs show different trends in protein adsorption when their hydrophobicity is varied by changing the percent NH$_2$, COOH, or OH terminal groups relative to CH$_3$ terminal groups on the surface. On OH terminated SAMs protein adsorption remained constant with changes in wettability, while COOH and NH$_2$ terminated SAMs showed an increase in protein adsorption with increasing hydrophobicity (Arima and Iwata, 2007). Other types of surface chemistry that have been shown to regulate protein interactions include stearyl groups as a means to promote albumin adsorption (Ji et al., 2001), lysine groups for interactions with plasminogen (Woodhouse and Brash, 1992), as well as sulfonate-modified polyurethanes, which have been shown to strongly bind fibrinogen (Chen et al., 2002). Within certain biomaterials, regions may vary in surface chemistry that can lead to heterogeneous protein-surface interactions. This is particularly true for polyurethanes, which can
experience phase separation due to incompatibility between the polar hard segments and nonpolar soft segments. In a study by Xu and Siedlecki, BSA was shown to preferentially adsorb to the apolar hydrophobic soft segments of a poly(urethane urea) (PUU) (Xu and Siedlecki, 2010). IgG, an important serum protein involved in the immune system, has also shown surface dependent adsorption, with increased IgG adsorption observed for hydrogels of dodecyl methacrylate and 2,3-dihydroxypropyl methacrylate with increasing proportion of the latter component in the hydrogel structure (Haigh et al., 2000). Metal surface chemical composition has also shown important implications for protein adsorption, with immunofluorescence staining showing reduced albumin and fibronectin adsorption, but not IgG, to aluminum regions of metal-oxide surfaces relative to niobium, vanadium, and titanium regions (Scotchford et al., 2003).

Another surface property that can influence protein adsorption is roughness, both in terms of the amount of adsorbed protein as well as specificity for certain proteins over others from complex solutions. Introducing surface features to a biomaterial has the potential to increase adsorbent capacity, as molecular simulations have shown that concave nanostructures support greater protein density due to their ability to maximize short-range dispersion forces (Elter et al., 2011). Nanostructured gold, produced by depositing gold nanoparticles on gold film as a means to prevent changes in chemistry, has been shown to reduce complement activation, even when such surfaces are pre-adsorbed with complement activation-inducing IgG (Hulander et al., 2011). Ti-6Al-4V surfaces of varying roughness have demonstrated protein-dependent differences in adsorption, with albumin showing preference for smooth surfaces, while total protein and fibronectin showed a preference for rough surfaces when adsorbed from serum-containing medium (Deligianni et al., 2001). In other cases, however, such as titanium surfaces with root mean square surface roughness ranging from 2-21 nm, no differences in protein adsorption have been observed (Cai et al., 2006). The ability of these different surface properties to regulate protein interactions will have important implications for the cellular response to implanted biomaterials as well as cell-biomaterial interactions in vitro.

2.2.2 Adsorbed protein-cell interactions

Understanding mechanisms of protein-surface interactions is critical since protein adsorption, rather than surface properties directly, will determine the cellular response to an implanted
biomaterial (Chandler-Temple et al., 2013). Whether a biomaterial is being used as a non-thrombogenic surface, tissue engineering scaffold, or other application, as long as it is interfacing with biological tissue the appropriateness of the cellular response for a given application will determine the success or failure of the implanted biomaterial (Williams, 2014). Three distinct areas that demonstrate the importance of cell-protein mediated interactions to an implanted biomaterial are coagulation (platelet response), immune response (white blood cell interactions), and cell attachment and growth (tissue-specific cells for different tissue engineering applications).

Several biomaterial applications require favourable blood-material interactions to ensure proper function, such as catheters, blood pumps, and vascular grafts. Research in the field of blood-material interactions has demonstrated the importance of several different plasma proteins that play a role regulating the response of platelets, the activation of which can lead to thrombosis and fouling of the biomedical device, resulting in occlusion, which can have life-threatening implications for the patient (Raad et al., 1994). Specific proteins that have been identified as being pro-thrombotic include fibrinogen, von Willebrand factor (vWF), fibronectin, immunoglobulins, Factor XII, and complement factors, amongst others (Jung et al., 2013). Fibrinogen in particular has been widely studied for its interactions with biomaterial surfaces as it is widely regarded as a pro-thrombotic protein. Locations on a fluorinated surface-modified polyetherurethane that show fibrinogen aggregation have also been shown to correlate to increases in platelet activation (Massa et al., 2005). However, as with many proteins, the conformation of the adsorbed fibrinogen will influence its bioactivity. A series of PUs of varying wettability, for example, have shown decreased fibrinogen adsorption to hydrophilic vs. hydrophobic formulations; however, due to lack of fibrinogen binding site exposure on the hydrophobic formulations, no differences in platelet adhesion or activation were associated with the increased fibrinogen content (Wu et al., 2005).

Binding of platelets to vWF occurs through the A1 domain of the protein to platelet receptor GPIbα. This interaction requires a conformational change in the protein, as loss of alpha-helical secondary structure increases binding affinity between A1-GPIbα 20-fold relative to the native conformation (Auton et al., 2010). Biomaterials that support protein denaturation will thus be more likely to support greater platelet activation, even if similar quantities of vWF are adsorbed.
Other plasma proteins, such as albumin, are typically considered to have a passivating effect with limited bioactivity. This quality of albumin, however, has recently been shown to be biomaterial-specific, as albumin denaturation leading to less than an approximate 30% loss in alpha-helical structure does not activate platelets, but above this threshold albumin adopts a conformation that produces RGD-like domains that subsequently cause increased platelet adhesion and activation with increased albumin adsorption (Sivaraman and Latour, 2012).

For tissue engineering applications, cellular activity has to be directed in a manner that is desirable for the specific physiology of different tissues. This can involve promoting cell migration, proliferation, and certain phenotypes of tissue-specific cells (Williams, 2014). Adsorption of fibronectin to biomaterial surfaces has often been correlated with the ability of surfaces to promote the adhesion and growth of several cell types, with surface chemistry (Lan et al., 2005; Keselowsky et al., 2004) and hydrophobicity (Vadillo-Rodriguez et al., 2013) shown to be important in regulating the exposure of cell-binding domains that render the adsorbed fibronectin more or less bioactive as a result of adsorption-induced changes in conformation. Adhesive peptide sequences of fibronectin are so critical for cell adhesion that they are often chemically incorporated into biomaterial surfaces to render a non-adhesive surface capable of supporting cell growth or desirable interactions with stem cells. This allows for the presentation of cell-binding domains without the need for materials that specifically promote the appropriate conformation of the adsorbed proteins (Grafahrend et al., 2011; Smith et al., 2013). Protein adsorption also has potential to control guidance of stem cell differentiation, as differences in the adsorbed protein layer have been implicated in the difference in the adipogenic and osteogenic differentiation capacity of mesenchymal stem cells (MSCs) adherent to SAMs of L- or D-cysteine (Yao et al., 2013). The ability of phosphate-functionalized PEG hydrogels to support osteogenic difference of MSCs has also been reported to be due to their ability to support the adsorption of ECM proteins, such as collagen and fibronectin, from serum (Gandavarapu et al., 2013). The ability of adsorbed vitronectin to support embryonic stem cell (ESC) adhesion, proliferation, and differentiation requires a threshold density of 250 ng/cm² (Yap et al., 2011); however, given the presence of bioactive domains on vitronectin, this concentration may be dependent on the conformation of the adsorbed protein. This is also the case for adsorbed fibronectin, which when adsorbed on a vinyl backbone chain with the side groups -
COO(CH\(_2\))\(_x\)H, with x = 1, 2, or 4, showed similar amounts of total adsorbed protein on all surfaces, but surface-dependent changes in fibronectin conformation as a result of surface mobility that resulted in changes in the ability of surfaces to promote osteogenic differentiation of MSCs (Gonzalez-Garcia et al., 2012).

All implanted biomedical devices will be subjected to a foreign body response (discussed in detail in section 2.3.3) involving white blood cells, particularly monocytes and their derived macrophages. These latter cells play a critical role directing this inflammatory process, with their cellular activity regulated in part by the biomaterial’s adsorbed protein layer. Different serum proteins have been implicated in different aspects of macrophage activity, including adhesion, apoptosis, cytokine release, fusion to foreign body giant cells (FBGCs), and release of degradative reaction oxygen species (ROS) (Collier and Anderson, 2002; Schmidt and Kao, 2007). Adsorbed fibrinogen has been shown to support the release of bone- and angiogenic-related factors from adherent monocytes/macrophages (Maciel et al., 2014). Adsorbed vitronectin, but not other common proteins known for cell-interactions such as complement C3bi, collagen types I or IV, fibrinogen, plasma fibronectin, fibroblast fibronectin, laminin, thrombospondin, and vWF, has been shown to support long-term macrophage adhesion and fusion to FBGCs (McNally et al., 2008). IgG is another important serum protein found in the adsorbed protein layer for numerous biomaterials that has been shown to mediate increases in long-term macrophage adhesion, with specific regions of the protein implicated in this response (Jenney and Anderson, 2000b), as well as the maturation of dendritic cells (Carrillo-Conde et al., 2012). Albumin has also been reported to facilitate nanoparticle uptake by mononuclear cells, where this response is due to the exposure of a cryptic epitope on albumin upon binding to layered silicate nanoparticles that results in their uptake via macrophage class A scavenger receptors (Mortimer et al., 2014). These changes in monocyte/macrophage response as a function of the adsorbed protein layer will determine the extent of the inflammatory response, and whether constructive wound healing occurs or a chronic inflammatory response results in the failure of the implanted device.
2.3 Wound healing and the foreign body response

Wound healing is a complex process involving multiple cell types and biochemical factors that is initiated with an inflammatory response before ultimately being resolved with new tissue formation and remodeling, or chronic inflammation and fibrous tissue formation (Barrientos et al., 2008). The implantation of a biomaterial typically requires the formation of an injury site, which is the initial step in the foreign body reaction. In general, the response involves four phases: hemostasis, which results from the activation of platelets by tissue factor following tissue damage, promoting the release of clotting factors that initiate the formation of a provisional matrix, consisting of fibrin, aggregated platelets, and erythrocytes; inflammation, initially involving neutrophils (phagocytosis of foreign material) and mast cells (release of histamine and other inflammatory mediators), but primarily dominated by macrophages 48-72 hr post-injury (Brown et al., 2012b); proliferation, involving cell growth and the deposition of ECM proteins due to the release of growth factors and cytokines (ex. TGF-β, EGF, FGF, and others (Barrientos et al., 2008)) by cells at the wound site; and remodeling, where new tissue deposited in the proliferative phase is reorganized due to the release of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which can ultimately result in the formation of a scar (Brown et al., 2012b).

While a number of different cellular and protein components play important roles in these processes, including platelets, complement activation, neutrophils, lymphocytes, and fibroblasts, amongst others, perhaps the most important is the monocyte/macrophage as these cells have been shown to play a role in all of the phases to some extent (Anderson et al., 2008). Monocytes are important contributors of tissue factor, which can activate platelets, as well as membrane-derived microparticles, which provide a surface for the assembly of the components of the coagulation cascade (Owens, A. Phillip., III and Mackman, 2011). Monocytes extravasate from circulating blood and enter tissue due to the presence of chemoattractants (cytokines and growth factors), which are released by platelets and mast cells, amongst other cell types, at the wound site (Anderson et al., 2008). Recruited monocytes differentiate to macrophages after entering tissue, and the further release of chemoattractants by recruited macrophages leads to the recruitment of more leukocytes to the wound site. Depending on the activation of macrophages by the
microenvironment supported by the biomaterial, macrophages can polarize towards a pro-inflammatory (M1) or pro-wound healing (M2) state (Brown et al., 2012b) (Figure 2.2).

**Figure 2.2** Description of different activation states of monocyte-derived macrophages in the presence of different stimuli. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Gordon S. and P.R. Taylor *Nature Reviews Immunology* 2005;5:953-964, copyright 2005.

Depending on the activation state supported, implantation of a biomaterial can lead to chronic inflammation, fibrous capsule formation, and device failure, or alternatively, wound healing and tissue regeneration that results in integration of the implanted device with the host tissue. The following sections will highlight the importance of monocytes/macrophages in tissue repair and homeostasis in healthy tissue and disease, and subsequently discuss the difference in macrophage activation leading to positive vs. negative outcomes in response to an implanted biomaterial.

### 2.3.1 Monocytes/macrophages in homeostasis and tissue repair

Macrophages are derived following the differentiation of peripheral-blood mononuclear cells (PBMCs) that migrate into tissue to replenish resident macrophage populations in homeostasis, or in response to an inflammatory response (Mosser and Edwards, 2010). These PBMCs develop
from a myeloid progenitor cell in the bone marrow, which differentiate sequentially to monoblasts, pro-monocytes, and then monocytes in response to stimulation from specific cytokines, including macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF). Similar to macrophages, monocytes in the blood are not a homogeneous population, and in humans are classified according to the relative expression of characteristic cluster of differentiation (CD) cell surface markers. The two general populations of monocytes include the inflammatory (or classical) subset (CD14^{hi}CD16^{-}, 90% of human monocytes) and a resident (or non-classical) subset (CD14^{+}CD16^{+}). While the specific role of each subset is not fully known, it is believed that the inflammatory (CD14^{hi}CD16^{-}) population rapidly migrates to sites of inflammation in response to tissue injury or pathogen presence, while the resident population (CD14^{+}CD16^{+}) play a more important role in maintaining tissue homeostasis through replenishing resident tissue macrophage populations and acting as regulators of the inflammatory response (Woollard and Geissmann, 2010).

Classical characterization of macrophage populations consisted of two subsets inspired by nomenclature in the T-cell literature. Classically activated pro-inflammatory macrophages (M1) were considered responsible for enhanced microbicidal or tumoricidal activity and pro-inflammatory cytokine release, and the alternatively activated state (M2) was assigned to all other macrophage-related activities (Mosser and Edwards, 2010; Sica and Mantovani, 2012). However, recent advances in the field of macrophage biology have led to the conceptual framework wherein macrophage activation is viewed as consisting along a spectrum with three predominant phenotypes described, namely classically activated (M1 or pro-inflammatory), wound healing (M2a), and regulatory (M2b,c), though macrophages can possess facets of different activation states at the same time and are no longer considered to be discrete populations (Mosser and Edwards, 2010). This view is supported by in vivo work, where isolated macrophage populations either consist of M1 and M2 polarized cells within the same microenvironment, or cells that express markers characteristic of both previously-thought discrete macrophage populations simultaneously (Sica and Mantovani, 2012).

Macrophage polarization can occur due to innate or adaptive immune responses. Classically activated macrophages require stimulus from interferon-γ (IFN-γ) with or without co-stimulation
with tumour-necrosis factor (TNF) or lipopolysaccharide (LPS), which can derive from immune cells such as natural killer (NK) and T-cells (IFN-γ or TNF) or microbes directly (LPS) (Mosser and Edwards, 2010; Martinez et al., 2008). The M1 state is associated with the release of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-23, and TNF; release of superoxide anions and oxygen and nitrogen radicals, which increase macrophage pathogen killing capacity; and the expression of characteristic cell surface markers, including CD80, CD86, CCR7, and IL-1R, amongst others (Mosser and Edwards, 2010). The wound healing phenotype, on the other hand, is induced by the cytokines IL-4 or IL-13, which can be produced by granulocytes such as basophils, as well as T-cells (Van Dyken and Locksley, 2013). Wound healing macrophages display markedly different characteristics from classically activated macrophages, such as the inability to present antigens to T-cells in vitro and a greater susceptibility to infection due to reduced microbicidal activity (Mosser and Edwards, 2010). This activation state is also associated with decreased pro-inflammatory cytokine release, including many of the markers positively associated with M1 macrophages, such as IL-1, IL-6, TNF, and IL-12; reduced production of oxygen and nitrogen radicals, a factor that makes them less effective at pathogen killing and thus more susceptible to some infections; secretion of ECM components, including collagens, polyamines, and fibronectin; release of cytokines such as IL-1ra and IL-10; as well as an increase in expression of the mannose receptor (CD206). The third activation state, namely the regulatory macrophage, has been further subdivided by some into two subsets, M2b and M2c (Martinez et al., 2008). M2b macrophages are derived following stimulation with IgG-immune complexes in combination with toll-like receptors (TLRs), while M2c macrophages develop in response to stimulation with IL-10. The M2c phenotype has also been termed the anti-inflammatory phenotype due to its role in immunoregulation, including the release of cytokines such as IL-10 and TGF-β. Like wound healing macrophages, the regulatory population is also associated with reduced pro-inflammatory cytokine release (IL-1, IL-12, TNF), and thus may be characterised by the changing ratio of IL-10 (increasing) to IL-12 (decreasing). Regulatory macrophages are thought to still possess antigen-presenting capacity, and are thus found to express co-stimulatory molecules also associated with M1 macrophages, such as CD80 and CD86. The main differentiating cell surface marker for this population, however, is CD163, a haptoglobin-hemoglobin scavenger receptor responsible for clearing pro-inflammatory debris from erythrocytes, thus lending this cell population an anti-inflammatory property (Kowal et al.,
While some suggest that regulatory macrophages, unlike wound healing macrophages, do not contribute to ECM deposition (Mosser and Edwards, 2010), others suggest a potential role for matrix deposition and remodeling with a role in proteoglycan production (Sica and Mantovani, 2012).

A hallmark feature of macrophages is their plasticity. Unlike some other cell types, where differentiation results in a relatively fixed phenotypic state, macrophages are capable of progressing from an M1 state to an M2 state or vice versa in response to appropriate stimuli in their microenvironment. While macrophage phenotypic switching is commonly observed during the progression of wound healing in vivo, it is not always clear that this shift in activation state is due to phenotypic switching of the previously recruited macrophage population, or the recruitment of a new population of macrophages. In an in vitro model of atherosclerosis, however, macrophage plasticity has been demonstrated using polarizing stimulants over short exposure periods. Bone marrow-derived macrophages from the femur of C56BL/6 and ApoE knock-out mice exposed to pro-M1 conditions (exposure to IFN-γ and LPS) for 10 hr were able to revert to an M2 state following exposure to pro-M2 conditions (IL-4) for 10 hr. Similar results were observed when first polarizing to an M2 state followed by M1-polarizing conditions, as demonstrated by the release of characteristic markers such as IL-6 and iNOS (M1 markers) as well as Arg I and Arg II (M2 markers) (Khallou-Laschet et al., 2010).

Resident macrophages are found in many tissue types, including bone (osteoclasts), the central nervous system (microglial cells), lung (alveolar macrophages), liver (Kupffer cells), and connective tissue (histiocytes), amongst others (Mosser and Edwards, 2010). The presence of these cells in healthy tissue suggests an important role in maintaining tissue homeostasis, which can range from pathogen killing and phagocytosis to ECM turnover and clearance of apoptotic cells. Because different tissue types have different requirements for maintaining proper function, the activation state of resident macrophage populations in healthy tissue is tissue-type dependent. Macrophage populations in the lung and liver, for example, must have greater pathogen killing capacity, a feature of M1 polarized macrophages, due to their exposure to airborne pathogens and microbes from the gut barrier, respectively (Weiclenbusch and Anders, 2012). In the central nervous system, however, microglia are responsible for promoting neuronal survival, removing dead neurons, and synaptic remodeling, processes more commonly associated with M2 activated
macrophages (Davies et al., 2013). In the case of adipose tissue macrophages, IL-4 stimulated (M2a) phenotypes are present in healthy tissue and are required for maintaining insulin sensitivity and lipolysis of stored fat. In the case of splenic macrophages, phenotypic heterogeneity can be seen within the organ itself depending on discrete microanatomical niches. Macrophages present in the red pulp, white pulp, inner layer of the marginal zone, and outer layer of the marginal zone express distinct sets of surface receptors that are particular to their specific functions (Davies et al., 2013).

There are three main phases to the activity of monocytes following injury: inflammation, proliferation, and remodeling (Novak and Koh, 2013). Each of these phases is characterized by different processes that are necessary for successful wound repair, with macrophages playing an important but different role in each stage of the process. Monocytes are recruited to the injury site within hours, and over the first few days their differentiation is mainly directed towards the M1 state and is characterized by pro-inflammatory cytokine release (IL-1, TNF, IL-6, IL-12) (Novak and Koh, 2013). These early-stage macrophages are responsible for the phagocytosis of debris and apoptotic neutrophils (Mantovani et al., 2013). The pro-inflammatory mediators promote the recruitment of matrix-producing cells, such as fibroblasts, and initiate the process of neovascularization (Lucas et al., 2010), while also promoting the activation of stem cells. These early macrophages also release a number of chemokines that recruit PBMCs to the tissue. Clearance of apoptotic debris is thought to promote a shift towards an M2-like state, and this population of macrophages secrete TGF-β and IL-10, which dampen the inflammatory response (Mosser and Edwards, 2010). This transition from an M1 to M2 activation state has been observed in several different tissue types and is associated with beneficial tissue remodeling; however, the persistence of either activation state can lead to impaired wound healing. Classically activated macrophages can cause excessive tissue damage, while M2 macrophages typically associated with normal wound healing can promote fibrosis.

2.3.2 Monocyte/macrophage function in disease

The importance of macrophage function has been demonstrated for many different disease states, including obesity, cancer, and periodontal disease (Brown et al., 2012b). Macrophage
contributions to atherosclerosis, however, will be highlighted due to the relevance of the subject to this thesis.

Atherosclerosis is a chronic inflammatory disease that results from endothelial dysfunction, and is most commonly associated with regions of the vasculature associated with disturbed flow (curvatures, bifurcations) that results in abnormal shear stress and alteration of endothelial gene expression (Dunn et al., 2014; Malek et al., 1999). This results in enhanced permeability of the endothelium, leading to the accumulation of lipoproteins characteristic of this disease state. Once in the arterial wall, lipoproteins become modified (aggregation, oxidation, enzymatic modification) that renders them pro-inflammatory, leading to further activation of the endothelium (Moore et al., 2013). This leads to the recruitment of leukocytes to the subendothelial space, where they ingest the modified lipoproteins and become foam cells. Foam cells release a number of pro-inflammatory mediators, including cytokines and reactive oxygen species that can promote intimal migration of VSMCs and their subsequent proliferation, leading to a narrowing of the blood vessel lumen. Furthermore, these macrophages release matrix-degrading proteases that can destabilize the plaque, as well as undergo apoptosis at a rate that prevents proper clearance of cell debris, resulting in the formation of a thrombotic necrotic core, which can ultimately lead to plaque rupture and cause a stroke or myocardial infarction (Moore et al., 2013). The process of atherogenesis and atherosclerotic plaque maturation is depicted in Figure 2.3.
Figure 2.3 a) Atherogenesis and the formation of fatty streaks. Subendothelial recruitment of monocytes that differentiate into macrophages results in accumulation of lipids and foam cell formation. b) Maturation into atherosclerotic plaque and rupture. Plaque progression is characterized by smooth muscle cell recruitment and proliferation. Macrophage apoptosis within the lesion results in release of proteases that thin the fibrous plaque leading to plaque rupture and thrombosis. Gr1^+/Ly6C^{high} and Gr1^-/Ly6C^{low} are monocyte subsets in the blood circulation of mice with human homologs being CD14^{hi}CD16^- and CD14^-CD16^+ monocytes, respectively. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cardiology, Wollard K.J. and F. Geissmann Nature Reviews Cardiology 2010;7:77-86, copyright 2010.

Monocyte recruitment to atherosclerotic lesions is mediated by the upregulation of cell surface receptors on activated endothelium that mediate interactions with circulating blood monocytes through the leukocyte adhesion cascade, which involves four steps: capture, rolling, arrest, and extravasation. The first two phases, capture and rolling, are dependent on EC-bound
glycosaminoglycans (GAGs) binding chemokines (CXCL1, CXCL5), as well as P-selectin, that interact with monocyte cell surface receptors (CCR1, CCR5) (Moore et al., 2013). High affinity adhesion between monocytes and activated endothelium that occurs during the arrest phase is due to the interaction of EC receptors (vascular cell adhesion molecule 1 [VCAM1], intercellular cell adhesion molecule 1 [ICAM1], plus others) with monocyte integrins. Transmigration to the subendothelial space subsequently occurs due to the release of chemokines, such as MCP-1, from intimal ECs, macrophages, and SMCs. Modified lipoproteins in the plaque are then phagocytosed by macrophages through select scavenger receptors, promoting foam cell formation. Due to excess lipoprotein and cholesterol uptake, macrophage metabolic pathways become overwhelmed, resulting in cholesterol enrichment in macrophage cell membranes, which promote inflammatory signalling. Furthermore, because apoptotic cell clearance by macrophages requires lipid metabolism, this process is impeded, resulting in necrosis, a feature of advanced atherosclerotic plaques (Moore et al., 2013).

Macrophages expressing different markers have been associated with regressing vs. progressing atherosclerotic plaques. Upon entering a plaque, macrophage activation by TLRs promotes polarization to an M1 state, resulting in the release of pro-atherosclerotic cytokines, such as IL-12, and oxygen and nitrogen radicals that can destabilize the plaque (Moore et al., 2013). Lipid-enriched macrophages have also been shown to express typical M1 markers of activation. Despite this fact, the role of oxidized lipoproteins on macrophage polarization is unclear, as they have been shown to induce expression of some markers commonly associated with the M2 state. In plaques where there is a progression towards a more inflammatory state, M1 macrophages become more predominant, while in regressing plaques M2 macrophages become more frequent. Indeed, treatment of mice with the M2a-polarizing cytokine IL-13 has been shown to inhibit the progression of atherosclerosis, suggesting a beneficial role of M2-activated macrophages (Moore et al., 2013). It should also be noted that macrophages found within a plaque often do not correspond directly to M1 or M2 activation states, and for this reason efforts have been made to categorize them separately. Macrophage activation states identified as contributing to or being predominant in plaque development include the M4 and Mox states, respectively. M4 macrophages are differentiated in the presence of CXCL4, and are considered to promote atherosclerosis. They are characterized by a lack of CD163 expression and characteristic gene
expression that differs from other macrophage activation states (Gleissner et al., 2010). Mox macrophages are also found in progressing plaques, are induced by oxidized phospholipids and nitrosylated fatty acids, and express high levels of ROS (Moore et al., 2013).

2.3.3 The foreign body response and the importance of macrophages

The host response to implanted biomaterials follows similar stages to that observed for tissue repair following injury with the exception that the material itself has the potential to perturb steps within this process (Anderson et al., 2008). Protein adsorption transforms the biomaterial surface into the substrate with which newly recruited cells will interact. Initially, this includes platelets, which upon activation release stimulatory cytokines and growth factors, such as platelet-derived growth factors (PDGFs) and interleukins (ILs) that facilitate the recruitment of inflammatory cells within hours. Neutrophils and mast cells predominate during acute inflammation, while the cellular make-up of the wound site over time begins to transition to monocyte-derived macrophages (MDMs) following neutrophil apoptosis in the more chronic stages of inflammation (days to weeks). It is primarily at this stage of the host response that the immune response begins to diverge for biomaterials that will support a chronic, pro-inflammatory foreign body reaction, and those that will experience constructive remodeling and tissue regeneration (Figure 2.4).
Figure 2.4 Stages in the foreign body response to an implanted biomaterial. Following exposure to blood, interstitial fluid or other protein-rich media, biomaterials are coated with an adsorbed protein layer that is biomaterial-specific (Step 1, seconds to minutes). White blood cells subsequently adhere to the biomaterial substrate interacting with the adsorbed protein layer (Step 2, hours to days), and adherent monocytes subsequently differentiate to macrophages (Step 3, days to weeks), with different activation states supported by features of the biomaterial-supported microenvironment. Activated macrophages will stimulate tissue cells (fibroblasts, smooth muscle cells) through release of growth factors and cytokines (Step 4). Depending on the activation state supported, wound healing or chronic inflammation (Step 5) will occur, characterized by tissue-specific cell proliferation, matrix deposition, and vascularization, or fibrous tissue encapsulation, respectively.

In the case of a foreign body reaction, the formation of granulation tissue due to fibroblast proliferation and migration follows chronic inflammation. This is followed by fibrous capsule formation, which can ultimately lead to failure of the implanted biomaterial (Anderson et al., 2008). In these phases of the host response, macrophages fuse to form foreign body giant cells (FGBCs), multinucleated phagocytes that release pro-inflammatory mediators, degradative
enzymes, and reactive oxygen species (ROS) that induce tissue damage and contribute to biomaterial degradation and ultimately failure. Because this process is facilitated by macrophages in a biomaterial-dependent manner, it is important to understand the processes involved in the different stages of the host response that lead to a macrophage phenotype supportive of this adverse outcome.

Monocyte recruitment to implanted biomaterials is an initial event required for tissue repair to occur. Until relatively recently, biomaterials were designed with the goal of inertness, which in the context of inflammation meant minimizing the inflammatory response as this was thought to be primarily associated with fibrosis and device failure. However, studies that sought to associate inflammation with the thickness of the fibrous capsule revealed no correlation between the number of macrophages at a wound site and the extent of fibrosis (Jones, 2008). Furthermore, in a number of disease states, during tissue regeneration following injury, as well as in response to implanted biomaterials, a lack of macrophages at the site of injury or implantation is associated with negative outcomes (Zhang et al., 2012). The process of inflammation is thus not detrimental in and of itself, but must be regulated in a tissue- and context-dependent manner to facilitate wound healing and regeneration. In most cases monocyte recruitment is mediated by chemokines released by activated platelets or neutrophils (Anderson et al., 2008). In the case of vascular grafts, however, monocyte pre-seeding has also been shown to support positive tissue remodeling (Mirensky et al., 2010), with this effect demonstrated to be due to release of MCP-1 from adherent monocytes that recruit host monocytes to the implanted material. This effect could be mimicked by a biomaterial modified with MCP-1-releasing alginate particles (Roh et al., 2010). Without pre-seeding, the initial migration-inducing cytokine burst depends on initial cell interactions with the biomaterial, such as the release of granules from activated neutrophils that mediate monocyte recruitment. PEG-containing hydrogels have been shown to support greater granule release than TCPS or PDMS-adherent neutrophils, suggesting biomaterial-dependent differences that can result in reduced or enhanced recruitment of monocytes (Cohen et al., 2014). While studies such as these suggest initial PMN-biomaterial interactions may be important in regulating differential inflammatory responses to biomaterials, PMN-biomaterial and PMN-monocyte interactions regulating monocyte response to biomaterials, including initial monocyte
recruitment, are not as well studied as direct monocyte/macrophage-biomaterial interactions, but could provide important insights into the inflammatory response to different surfaces.

Following the recruitment of monocytes, the next phase involves monocyte adhesion to the biomaterial surface, which, as discussed previously (section 2.2.2), is mediated in large part by the composition of the adsorbed protein layer. Specific proteins possess amino acid sequences recognized by monocytes that can promote adhesion, fusion, and cytokine release. The presence of these proteins alone, however, does not guarantee their bioactivity as in some cases conformational changes are required to expose cryptic cell binding motifs, which can occur in a biomaterial-dependent manner. Proteins that have been reported to have an effect on monocyte adhesion include IgG, vitronectin, fibronectin, von Willebrand factor, serum amyloid P component, fibrinogen, albumin, and laminin, amongst others (Collier and Anderson, 2002; Maciel et al., 2014; Jenney and Anderson, 2000a; Kim et al., 2005; Anderson et al., 1999). Several serum proteins (vitronectin, fibrinogen, fibronectin, albumin, high molecular weight kininogen [HMWK], C3, factor X) have sites recognized by integrins, which in addition to mediating cell adhesion also allow for cell migration and can activate signal transduction pathways leading to cellular activation (Love and Jones, 2013). Monocytes/macrophages express several different types of integrins, including α4β1 and α5β1 (fibronectin); α6β1 (laminin); αLβ2, αMβ2, and αDβ2 (ICAMs on ECs); αXβ2 (C3bi, fibrinogen); and αVβ3 (recognizes RGD, present in vitronectin and fibronectin, amongst others) (Anderson et al., 2008). With regard to monocyte/macrophage-biomaterial interactions, integrins are the most studied components with regards to regulating initial adhesion, but scavenger receptors (SRs) and toll-like receptors (TLRs) have also been shown to play a role in this process (Love and Jones, 2013). SRs can recognize β-amyloid protein and low-density lipoproteins (LDLs), which have been shown to adsorb to biomaterial surfaces (Cornelius et al., 2002). Macrophages lacking SRs have been shown to take twice as long to adhere to TCPS in vitro (Love and Jones, 2013). SRs and TLRs both possess leucine-rich regions that bind predominantly through hydrophobic interactions, which may mediate adhesion to the hydrophobic portions of biomaterials as well as exposed hydrophobic regions of proteins. Immunogenicity of several different biomaterials, including alginate, hydroxyapatite, and poly(anhydride) particles has also been shown to be mediated by interactions with different TLRs (Love and Jones, 2013).
The nature of the adsorbed protein layer in combination with the cytokine milieu present at the wound site prior to monocyte arrival will result in monocyte-to-macrophage differentiation and subsequent macrophage activation. Primary contributors of cytokines to this microenvironment include platelets, neutrophils, and mast cells (Anderson et al., 2008). Neutrophils have been shown to regulate monocyte/macrophage adhesion, viability, and pro-inflammatory cytokine release (TNF-α, IL-1β) in a biomaterial-dependent manner (Cohen et al., 2013), while mast cells are a source of IL-4 and IL-13, cytokines that support M2 macrophage polarization (Love and Jones, 2013). Whether adherent macrophages become polarized towards an M1 or M2 state or fuse to form FBGCs is also regulated by the adsorbed protein layer. Fusion to FBGCs is supported by surfaces with adsorbed vitronectin and fibronectin, proteins containing RGD-domains, suggesting a role for integrins in this process (McNally et al., 2008). IgG has been shown to mediate long-term macrophage adhesion, potentially through preventing apoptosis that commonly occurs over time when monocytes/macrophages are adherent to a biomaterial surface (Jenney and Anderson, 2000b). Biomaterial surface properties, such as hydrophobicity and ionicity, have been implicated in regulating adherent macrophage apoptosis in a material-dependent manner (Brodbeck et al., 2002; Waldeck et al., 2012). Regulation of apoptosis can have an effect on macrophage polarization, as phagocytosis of apoptotic cells provides a stimulus that can promote polarization towards an M2 state (Mosser and Edwards, 2010).

Pro-inflammatory macrophages and FBGCs are thought to be the primary mediators of an adverse foreign body response. FBGCs derived from arthroplasties have been shown to express receptors for pro-inflammatory mediators, such as IL-1R, IL-6R, and TNFR, but not those associated with pro-wound healing macrophages, such as the receptor for IL-4 (Anderson et al., 2008). FBGCs also release ROS, degradative enzymes, and acid that can cause damage to tissue and the biomedical device. This combination of pro-inflammatory stimuli and mediators of degradation ultimately leads to fibrous tissue encapsulation and failure of the material. For applications such as tissue engineering scaffolds, drug release devices, and sensors, the presence of a fibrous capsule directly impedes the function of the biomaterial by preventing cell-material interactions (tissue engineering) or shielding the functions of the device (drug delivery, sensors), while in other cases mechanical failure results from degradation of the device (Santerre et al., 2000).
Generally, biomaterials that have been shown to promote constructive remodeling support a shift in macrophage activation from M1 to M2 over time. This has been demonstrated recently with a number of biologically derived surgical mesh materials, where a positive correlation was observed between positive remodeling outcomes and the ratio of M2:M1 macrophages over time (Brown et al., 2012a). Factors that have been shown to support such a transition include scaffold porosity and pore size (Sussman et al., 2014), surface roughness (microstructures vs. nanostructures) (Paula et al., 2008), substrate stiffness (Blakney et al., 2012), incorporation of anti-inflammatory peptides (Kim et al., 2014), electrospun fiber thickness (Wang et al., 2014b), and surface chemistry (Brodbeck et al., 2002). While an increase in M2:M1 ratio over time is associated with positive outcomes, it should be noted that both activation states are required for proper wound healing, as both phenotypes play important roles in different stages of the wound healing process. In some cases having a mix of both populations is beneficial, such as in promoting neovascularization (Spiller et al., 2014). While some studies have demonstrated the mechanisms supporting an M1 to M2 transition, such as the release of ECM degradation by-products from ECM-derived scaffolds (Sicari et al., 2014), in most cases even though an M2 state is shown to be supported by the material, the mechanism through which the different parameters influence macrophage polarization are not well understood (Sussman et al., 2014; Blakney et al., 2012; Franz et al., 2013; Vasconcelos et al., 2013). Further evaluation of how certain parameters (porosity, fibre thickness, roughness etc.) regulate molecular interactions and protein adsorption may provide insight to allow for intelligent design of immunomodulatory materials capable of supporting an increase in M2:M1 macrophages over time, and thus positive remodeling outcomes.

Regulation of macrophage polarization leading to an adverse foreign body reaction or constructive wound healing involves many different factors. While clues have been provided with regards to surface properties, protein adsorption, and the ability of materials to support adhesion and specific cytokine release, further work needs to be done to fully elucidate the mechanisms involved in these processes.
2.4 Vascular tissue engineering

Cardiovascular disease, and coronary artery disease in particular, is one of the leading causes of death worldwide (Seifu et al., 2013). While less advanced arterial disease can be treated by medication, angioplasty, or stents, when there is significant vessel occlusion due to plaque deposition or intimal hyperplasia surgical intervention is required in the form of a bypass graft. The preferred source of grafts for these procedures is from autologous sources, such as the long saphenous vein, internal mammary artery, or radial artery. However, due to progressive vascular disease in these vessels or use in previous procedures, autologous vessels are unavailable in approximately 1/3 of cases. Dacron® and Goretex® are two synthetic non-degradable materials that have performed well in large diameter vascular graft applications; however, they perform relatively poorly to autologous vessels when used to bypass small diameter blood vessels such as the coronary artery, due to complications such as thrombosis and intimal hyperplasia at the anastomoses, which is thought to derive in part from compliance mismatch between the synthetic graft and native vasculature in combination with reduced blood flow velocity in the smaller vessels (Seifu et al., 2013). Furthermore, while monocytes and macrophages are thought to be critical to supporting healing in the initial stages after implantation, the use of non-degradable materials is also thought to promote a chronic inflammatory response by macrophages and FBGCs that can compromise graft performance and inhibit long-term tissue remodelling and healing (Zilla et al., 2007; Hagerty et al., 2000).

Vascular tissue engineering has been explored as a means to develop a vascular graft with similar mechanical, cellular, and physiological properties as native vasculature. Arteries and veins have a hierarchical structure consisting of three distinct layers: the tunica intima, tunica media, and tunica adventitia. The tunica intima consists of a monolayer of ECs that resides on a basal lamina consisting primarily of collagen IV and laminin, which is supported by an internal elastic lamina containing microfibrils and collagen fibers (Wagenseil and Mecham, 2009). The tunica media is rich in SMCs found within an extracellular matrix consisting primarily of elastin and collagens I and III. The outermost layer of the arterial wall, the tunica adventitia, contains myofibroblasts in a collagen-rich ECM that is separated from the tunica media by an external elastic lamina (Wagenseil and Mecham, 2009). The tunica intima provides the vessel with its
non-thrombogenic properties, while the *tunica media* is associated with vessel contractility and mechanical strength. Some efforts to tissue engineer a blood vessel attempt to recreate this hierarchical structure using purely cell-based approaches that have demonstrated some success (Laflamme et al., 2006; McAllister et al., 2009). Because one of the main challenges associated with the *in vitro* development of a tissue engineered vascular graft is demonstrating sufficient mechanical properties, others have focused on the use of biomaterials in combination with SMCs to develop a highly cellularized, ECM-rich tissue engineered media equivalent. The general pathway for the development of a tissue-engineered media using a scaffold support is shown below in Figure 2.5.

**Figure 2.5** General pathway for generation of a tissue engineered vascular graft using a scaffold-guided approach. A biopsy is taken from a patient (1) and used to isolate specific cell types (2). Cells are expanded *in vitro* (3) before being seeded on an ECM-based (4i) or polymer-based (4ii) scaffold. Cells are allowed to adhere to the biomaterial surface (5) before being subjected to stimulation (biomechanical or other) using a bioreactor (6) to promote tissue maturation prior to being implanted in the patient. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cardiology*, Seifu D.G. et al. *Nature Reviews Cardiology* 2013;10:410-421, copyright 2013.
This section will focus on the methods that have been used to stimulate the production of vascular tissue in a laboratory setting. A detailed review of the different materials that have been investigated as vascular grafts and their performance in vivo is provided in Appendix A.

2.4.1 Cell types and sources

While vascular grafts can be implanted without pre-seeding, several studies have demonstrated the benefit of pre-seeding vascular grafts as a means to improve graft performance (Syedain et al., 2011; Niklason et al., 1999). The source, type, and phenotype of these cells, however, vary widely depending on the nature of the study and the approach towards clinical application. This section will highlight the different sources of cells used to study and/or implement vascular tissue engineering, focusing specifically on cells used to generate the medial layer of a vascular substitute. Primarily, these cells fall into two main categories: differentiated cells harvested from vascular tissue and stem cells with varying degrees of stemness from a variety of sources (Krawiec and Vorp, 2012).

2.4.1.1 Differentiated cells

The medial layer of vascular tissue is composed primarily of VSMCs embedded in an ECM network of collagen I and elastin. The most straight-forward strategy for vascular tissue engineering, and the first that was explored, involves isolating tissue-specific cells from the graft recipient and culturing them on a biomaterial substrate for a period of time prior to implantation (Niklason et al., 1999; Shin'oka et al., 2001). This strategy eliminates immunological complications associated with graft-versus-host disease, wherein an allogeneic cell source results in the graft recipient’s immune cells recognizing the implanted graft as foreign. While this approach has demonstrated clinical success for vascular tissue engineering by Shin’oka et al. (Shin'oka et al., 2001), it required 10 weeks of ex vivo cell expansion, a time period that eliminates this approach from being used in situations where a graft is needed on short notice. Acquisition of VSMCs from vascular tissue requires an invasive procedure which may not be possible for all patients. Furthermore, the case study reported by Shin’oka et al. involved a pediatric patient. It is well known that cells from adult tissue, the primary population requiring vascular grafts due to advanced peripheral arterial disease or coronary artery bypass procedures,
possess limited proliferative capacity and produce little to no elastin in vitro. Efforts to improve adult VSMC proliferative capacity have included gene therapy to induce telomerase expression, with positive results reported (Fields et al., 2003). However, the long-term effect of genetic manipulation needs to be further explored to eliminate the possibility of unwanted mutagenesis.

An alternative to VSMCs for producing a media equivalent is fibroblasts (Syedain et al., 2011). While fibroblasts do not possess the same phenotype as VSMCs, they are robust producers of ECM and thus can contribute to the deposition of collagen in a timely manner. Furthermore, fibroblasts can be isolated from non-critical organs (ex. dermal fibroblasts using skin biopsies), making their acquisition less invasive than VSMCs. The use of dermal fibroblasts to generate a tissue engineered media-equivalent has been demonstrated by Konig et al. for clinical use in humans, requiring around half a year for tissue maturation in the absence of a biomaterial support, as well as by Syedain et al. in an ovine model, requiring 5 weeks for construct maturation using a fibrin scaffold and bioreactor conditioning (Syedain et al., 2014). The approach by Syedain et al. further involved decellularization of the graft following maturation, which removes the fibroblasts prior to implantation which, though present in the adventitia of a blood vessel, are not found in the media. While this approach is promising due to the non-invasiveness of the cell source, it is unclear if the non-VSMC specific ECM produced by fibroblasts will adequately support a desirable VSMC phenotype. Furthermore, because cells are patient-derived, off-the-shelf potential is limited. For these reasons, other cell sources, particularly stem cells, have also been explored.

2.4.1.2 Stem cells

Stem cells are defined by their ability to self-renew and differentiate into multiple cell types. They possess significantly greater proliferative capacity and can be isolated from non-critical organs (ex. adipose-derived stem cells [ASCs], induced pluripotent stem cells [iPSCs]) in relatively non-invasive procedures. For vascular tissue engineering, sources of stem cells that have been explored include embryonic stem cells (ESCs), iPSCs, mesenchymal stem cells (MSCs), ASCs, and pericytes (Krawiec and Vorp, 2012).
In a study by Sundaram et al., human ESC-derived MSCs were differentiated to SMCs in the presence of TGF-β1 for 14 days, demonstrating positive staining for VSMC contractile proteins SMA, SM22α, and calponin. ESC-MSC-derived SMCs seeded on PGA scaffolds and cultured in a pulsatile perfusion bioreactor for 8 weeks demonstrated similar cellularity and phenotypic marker expression to native vessels (Sundaram et al., 2014). However, detection of bone and cartilage markers in differentiated cell populations raises concerns about stable lineage commitment. Furthermore, ethical concerns associated with the use of ESCs have prompted most research into stem cell strategies to focus on adult stem cells. The use of iPSCs, however, has the potential to overcome this ethical hurdle. Wang et al. differentiated human iPSCs into proliferative SMCs, seeded them onto a nanofibrous PLLA scaffold, and demonstrated the maintenance of mature contractile phenotype following subcutaneous implantation in a nude mouse model (Wang et al., 2014a). iPSC cell sheets produced by Hibino et al. have also been seeded onto a biodegradable scaffold of polyglycolic acid–poly-l-lactide and poly(l-lactide-co-ε-caprolactone) and implanted in the inferior vena cava of mice (Hibino et al., 2012). While around 10% of seeded iPSC cells were found to remain in grafts after 10 weeks, they were not the main source of ECs or SMCs within the graft. These preliminary findings make the use of iPSC cells an attractive area for future research.

ASCs differentiated to SMCs through a combination of BMP4 and TGF-β1 were used by Wang et al. to seed a PGA mesh that was subsequently cultured for 8 weeks under dynamic stimulation, producing an organized ECM, high cellularity, and mechanical properties suitable for implantation (Wang et al., 2010). Human bone marrow-derived MSCs have also been shown to be capable of differentiation to SMCs that could be seeded on PGA mesh scaffolds, cultured in a pulsatile perfusion bioreactor for 8 weeks, and generate a vascular substitute substantially equivalent to a native vessel (Gong and Niklason, 2008). These studies, as well as many others (Krawiec and Vorp, 2012), have demonstrated the great potential that lies in the use of stem cell strategies for vascular tissue engineering.

2.4.1.3 Other approaches

The aforementioned strategies, regardless of the source of the cells, require prolonged in vitro culture periods as they aim to generate a mature vascular construct (cellularity, ECM
composition, cell phenotype) prior to implantation. Other groups, in particular studies by the group of Christopher Breuer, have performed pre-seeding with bone marrow mononuclear cells for 2 hr prior to implantation with clinical success (Shin’oka et al., 2005). Studies have demonstrated that monocytes within the heterogeneous bone marrow mononuclear cell population recruit host monocytes following implantation, which help direct the tissue regeneration process (Roh et al., 2010). In this case, new vascular cells are recruited from the anastomoses to repopulate the graft (Hibino et al., 2011a). This strategy relies on the use of materials that modulate monocyte activation in a manner that promotes wound healing, as well as grafts that derive their mechanical strength from the material rather than cell-derived products. In such cases, it is also imperative that degradation occurs at a controlled rate such as not to outpace new tissue deposition. Studies by other groups have also demonstrated that the source of cells that repopulate the graft following implantation are not from pre-seeded cells, but of bone marrow origin (Enomoto et al., 2010), further demonstrating the importance of supporting cell recruitment.

2.5 Strategies for culturing VSMCs

In order to tissue engineer the medial layer of a vascular graft, there are several factors that must be promoted by the in vitro culture system. To enable the production of adequate cellularity (native vessels possess on the order of $10^8$ cells/ml (Niklason et al., 1999)), culture systems should promote VSMC proliferation. In addition, with the use of 3D tissue constructs and porous biomaterials, it is imperative that heterogeneous cell distribution is obtained in order to have uniform cellularity, ECM deposition, and ultimately mechanical properties. With porous biomaterials in particular, the ability to support cellular infiltration and avoid monolayer formation on the seeding surface has proved to be a significant challenge (Villalona et al., 2010). Ideally, VSMCs will also be induced to lay down ECM proteins, such as elastin and collagen I, that will contribute to the graft’s mechanical strength. VSMC phenotype is also an important characteristic to consider. While mature arteries typically possess VSMCs in a mature contractile state, as opposed to a synthetic, proliferative state, in vitro strategies must both initially promote a proliferative response followed by a transition to a more contractile phenotype. The following
sections will highlight the main strategies that have been employed in the field of tissue engineering towards achieving these goals.

2.5.1 Exogenous factor supplementation

Biochemical stimulation is a potent strategy for regulating VSMC phenotype. Depending on the type of factor used for stimulation, dosing, and co-delivery strategies, it is possible to obtain tight control over VSMC response (Chan-Park et al., 2009). These stimulants, in the form of growth factors, cytokines, and vitamins, typically fall under two categories: those promoting the synthetic, proliferative phenotype, and those promoting a contractile phenotype, with effects on ECM production varying depending on the individual stimulant used.

Growth factors such as PDGF (Stegemann and Nerem, 2003), FGF (Segev et al., 2002), insulin (Liu et al., 2011a), and EGF (Yu et al., 1997) have been shown to be potent stimulators of VSMC proliferation. For this reason, vascular tissue engineering strategies often involve the use of culture medium that has these growth factors added to promote faster cell and tissue growth (Syedain et al., 2014; Gui et al., 2014). However, they also possess the side-effect of downregulating VSMC contractile marker expression (Stegemann and Nerem, 2003). In contrast, TGF-β1 and BMP4 are growth factors known to increase VSMC contractile marker expression, while having the side effect of inhibiting VSMC proliferation (Stegemann and Nerem, 2003; King et al., 2003). Combination strategies involving sequential addition of different growth factors can provide further control over VSMC phenotype (Risinger et al., 2010). With regards to ECM production, insulin and TGF-β1 have both been shown to be potent inducers of elastin synthesis in vitro (Syedain and Tranquillo, 2011; Shi et al., 2012), while TGF-β1 also has the effect of diminishing collagen production when used in combination with long-term mechanical strain (Syedain and Tranquillo, 2011). In order to maximize both collagen and elastin production, some studies have involved time-dependent strategies that optimize production of both elastin and collagen depending on the temporal addition pattern of TGF-β1 (Syedain and Tranquillo, 2011). Another common strategy for promoting tissue production in vitro involves addition of sodium ascorbate in culture medium, which can induce elastic fiber production and has been used successfully by several groups to enhance tissue maturation. However, sodium ascorbate has a short half-life under typical culture conditions (37°C, pH 7.0) and so must be replenished
frequently, which increases risk of contamination during long culture periods (Laflamme et al., 2006; Gui et al., 2014). Disadvantages of these approaches include the significant costs associated with growth factor use on a large scale, as well as issues regarding endotoxin contamination in cases where recombinant growth factors are used (Wakelin et al., 2006; Daly et al., 2012).

### 2.5.2 Co-culture systems

Natural tissues, such as arteries and veins, are multicellular systems where cellular crosstalk plays a critical role in regulating and maintaining cell phenotype. Arteries possess an adventitial outer layer rich in fibroblasts, an SMC-rich medial layer, and an EC monolayer on the luminal surface. Most co-culture strategies in vascular tissue engineering thus attempt to recreate this hierarchical structure more to support proper tissue function (contractile medial layer, non-thrombogenic intimal layer) than as a means of controlling cell phenotype through cell-cell interactions (Laflamme et al., 2006). Primarily, this involves prolonged culture of VSMCs on a biomaterial scaffold using a bioreactor system in order to generate robust tissue and high cellularity, followed by a seeding of the lumen with ECs and either immediate implantation, or a short period of pre-conditioning using a flow bioreactor system to expose ECs to shear stress (Syedain et al., 2014; Zhang et al., 2009; Niklason et al., 1999).

While EC-SMC co-culture systems in vascular tissue engineering are often used to mimic cellular organization in a healthy blood vessel, numerous studies have also demonstrated the importance of these cells in regulating the phenotype of the other (Hergenreider et al., 2012). ECs have been shown to regulate SMC proliferation (Kader et al., 2000), adhesion, and spreading (Wang et al., 2007). Using a hydrogel system (methacrylated dextran-graft-lysine and methacrylamide-modified gelatin), Liu et al. demonstrated the ability of ECs adherent to TCPS to upregulate contractile marker expression, matrix deposition (collagen, elastin), and induce stimulatory growth factor release (TGF-β1, PDGF) of hydrogel-encapsulated SMCs exposed to EC conditioned medium (Liu et al., 2012), a finding that has also been demonstrated by others using other model systems (Williams and Wick, 2005; Wallace et al., 2007b). The importance of performing EC-SMC co-cultures under physiological conditions has also been demonstrated by studies showing that EC-regulated VSMC migration and VSMC-induced induction of
inflammatory gene expression in ECs is protected by the exposure of ECs to shear stress (Wang et al., 2006; Sakamoto et al., 2006; Chiu et al., 2005; Zhou et al., 2013). VSMCs have also been demonstrated to affect EC phenotype. EC-seeded collagen cylinders embedded with VSMCs were shown by Leung et al. to regulate EC morphology, proliferation, and NO release (Leung and Sefton, 2007). Other effects promoted by VSMCs include reduced cell spreading and focal adhesion formation and increased expression of inflammatory receptors (ex. E-selectin) (Wallace et al., 2007a; Chiu et al., 2007).

Monocyte/macrophage interactions with VSMCs are also a relatively well-studied phenomenon. While much of the knowledge garnered on VSMC-monocyte interactions have been performed with an interest in atherosclerosis, where macrophages are in intimate contact with VSMCs, it should also be noted that following implantation monocytes have been shown to be recruited to grafts and contribute to remodelling (Roh et al., 2010; Hibino et al., 2011b). In this context, VSMC-monocyte interactions can be studied with regards to the ability of monocytes to govern VSMC phenotype towards promoting tissue regeneration. Monocytes and MDMs are potent producers of stimulatory growth factors and cytokines that have been shown to promote VSMC proliferation and migration, an effect desirable in vascular tissue engineering strategies. These stimulatory factors include pro-inflammatory mediators such as PDGF, IL-6, MCP-1, and MCP-3 that promote proliferation (Stegemann and Nerem, 2003; Maddaluno et al., 2011; Schepers et al., 2006; Nam et al., 2011), while anti-inflammatory cytokines such as IL-10 have the opposite effect (Mazighi et al., 2004). Adhesion of monocytes to biomaterial surfaces typically results in the upregulation of pro-inflammatory cytokine release (such as MCP-1 (Roh et al., 2010)), which can have the benefit of supporting an initial proliferative response from adherent VSMCs, as well as their migration into 3D tissue constructs or porous biomaterials. Biomaterials that support macrophage polarization towards an M2 activation state will subsequently release cytokines that inhibit this proliferative activity and promote a more contractile state (Mazighi et al., 2004), a VSMC phenotype that is desirable once sufficient cellularity and tissue deposition is achieved in the construct. Furthermore, monocytes can relatively non-invasively be isolated from a patient’s peripheral blood, making them an attractive autologous cell source for tissue engineering strategies, without the drawbacks associated with growth factor supplementation (Wakelin et al., 2006; Daly et al., 2012).
2.5.3 Biomechanical stimulation

Another method commonly used in the vascular tissue engineering field for regulating VSMC response is biomechanical stimulation. In the context of regulating VSMC response this is predominantly explored with regards to the use of circumferential strain (Huang and Niklason, 2014). Depending on location in the vasculature, blood vessels are exposed to longitudinal strain ranging from 10-70% (Wagenseil and Mecham, 2009). Due to the pulsatile nature of blood flow, changes in the diameter of blood vessels exposes VSMCs in the media to circumferential strain ranging from 5-15%. This exposure to strain has an effect on VSMC phenotype, including regulation of cell signalling pathways, proliferation, ECM production, and migration (Lehoux et al., 2006). The following sections will provide a review of mechanical stresses in vascular tissue engineering, including factors contributing to vascular mechanics, as well as the roles of substrate stiffness and externally applied strain on VSMC phenotype.

2.5.3.1 The extracellular matrix and vascular mechanics

The major ECM components in the medial layer of an arterial wall are collagen and elastin deposited by VSMCs, which constitute approximately 50% of the dry weight of the vessel (Wagenseil and Mecham, 2009). Elastin is arranged in fenestrated sheets (lamellae), between which are collagen fibers, proteoglycans, and VSMCs. VSMCs themselves are not a major contributor to the strength of an artery, as the elastic modulus of smooth muscle is on the order of 10 kPa in the relaxed state, while that of elastin and collagen are 600 kPa and $10^6$ kPa, respectively (Matsumoto and Nagayama, 2012). Furthermore, other studies have shown that elimination of VSMC function does not significantly change the mechanical properties of mouse aorta, implicating collagen and elastin as the primary components responsible for these properties (Wagenseil and Mecham, 2009). However, other studies have indicated that VSMC phenotype can result in changes in stiffness on the order of tens of kPa (Sehgel et al., 2013).

Elastin, which provides the property of elasticity to blood vessels, is incorporated in elastic fibres that also contain microfibrils, which are 10-15 nm diameter filaments that facilitate elastin assembly. Microfibrils also have the potential to provide elasticity. In invertebrates, which lack elastin and possess open circulatory systems, they are the ECM component that provides vessel
wall elasticity. In the high pressure closed circulatory system of vertebrates, however, the presence of microfibrils alone is not sufficient to provide the necessary elastic recoil, and elastin-null mice die shortly after birth from aortic obstruction (Wagenseil and Mecham, 2009), for which elastic recoil is necessary in order to transform pulsatile flow from the heart into nearly steady flow for peripheral vasculature (Kassab, 2006). Elastin is formed from the cross-linking of tropoelastin, which contains hydrophobic sequences alternating with lysine-rich cross-linking motifs. Per unit, elastin contains 15-20 cross-links, which helps provide elastin its elastic properties. The mechanical properties of arteries at low strains are thought to be derived from the deformation of the elastin component.

The main collagens responsible for imparting strength to the vessel wall are collagen I and III, though up to 17 different collagen types have been identified in the mouse aorta (Wagenseil and Mecham, 2009). Collagen I and III form fibrils that contain 1-4 cross-links per collagen unit. Collagen I is found predominantly in the media and collagen III in the adventitia, though their distribution can vary depending on location in the vasculature. Mice deficient in collagen I or collagen III have been shown to die at various stages of development due to vessel rupture, highlighting the importance of these proteins to vascular mechanics (Wagenseil and Mecham, 2009). Under high pressure, collagen fibers become circumferentially aligned, with previous studies showing that only 10% of collagen fibers are required to be engaged at physiological pressures in order to provide mechanical support (Wagenseil and Mecham, 2009). Collagen is thought to be responsible for mechanical properties observed at high strain levels.

Proteoglycans are proteins that have covalently attached glycosaminoglycan (GAG) chains through O-glycosidic linkages to serine residues in the core protein of the proteoglycan. Proteoglycans that are found in the vessel wall include hyaluronan, versican, aggrecan, and small leucine-rich proteoglycans (decorin, biglycan, lumican), and constitute approximately 5% of the dry weight of an artery (Roccabianca et al., 2014). These molecules play importance roles in sequestering growth factors, VSMC phenotype regulation, mechanotransduction, and collagen and elastic fiber formation. Mice lacking proteoglycans such as decorin and biglycan, for example, demonstrate dysregulated collagen fiber formation, including differences in diameter and organization that can result in aortic rupture between the media and adventitia (Wagenseil and Mecham, 2009). Recent computational studies have demonstrated that while collagen and
elastin are the most significant contributors to vascular mechanics, proteoglycans and glycosaminoglycans can also play a role in overall structural integrity of the vessel, as well as in VSMC mechanosensing (Roccabianca et al., 2014; Shi et al., 2010).

2.5.3.2 Biomaterial stiffness and cellular response

Substrate stiffness has been shown to play an important role governing cell response, including stem cell differentiation (ex. MSC differentiation to EC vs. SMC phenotypes (Wingate et al., 2012)), cell phenotype, and migration, amongst other responses. In healthy arteries VSMCs are exposed to substrate stiffness ranging from 2-10 kPa, while in atherosclerotic plaques stiffness increases significantly to up to hundreds of kPa (Lee et al., 2008). VSMCs have been shown to be sensitive to substrates of stiffness varying from 0.3-500 kPa, which covers the range of healthy to pathological tissue.

In a study by Peyton et al., PEG hydrogels with varying degrees of crosslinking were prepared to generate surfaces with tensile moduli ranging from 13.7-423.9 kPa. Increased stiffness was associated with VSMC proliferation, suggesting a shift towards a synthetic phenotype that is further supported by a decrease in VSMC differentiation markers, such as reduced association of calponin and caldesmon with α-actin fibrils as a function of increased stiffness (Peyton et al., 2006). This response mirrors the phenotypic shift of VSMCs in atherosclerosis, which is also associated with a stiffer ECM. This response has also been demonstrated with polyacrylamide gels having modulus values varying from 19-84 kDa, suggesting that this response is not an artifact of the material chosen, but is reflective of the response to substrate modulus (Brown et al., 2010). VSMCs can also respond to gradients in modulus, where durotaxis was shown to increase on substrates with gradients of 0-4 kPa/100 μm. In the latter studies, the magnitude of the gradient but not the absolute modulus was shown to increase the rate of durotaxis from softer to harder areas (Isenberg et al., 2009). The response of VSMCs to stiffness, however, is sensitive to other aspects of the culture system. While polyacrylamide gels of 135 vs. 25 kPa modulus values demonstrated greater cell area and focal adhesion protein expression, this effect was attenuated when VSMCs were cultured at higher density and allowed to form cell-cell contacts (Sazonova et al., 2011). Furthermore, when comparing PDMS substrates of 1.79 and 0.05 MPa, cells were sensitive to differences in modulus with respect to attachment and spreading only in
the absence of serum (Brown et al., 2005). ECM coating can also regulate VSMC response to substrates of different stiffness. When cultured on acrylamide/bisacrylamide hydrogels of 1-308 kPa, migration speed of cells on fibronectin coated surfaces of 0.8 μg/cm² was greatest for 51.9 kPa, while when the surface was coated with 8.0 μg/cm² the maximum migration speed was observed at 21.6 kPa (Peyton and Putnam, 2005). The aforementioned data, however, may be specific to VSMCs only, as other cell types have been shown to have different responses to changes in substrate modulus. For example, with PEG hydrogels of 0.3-13.7 kPa, while fibroblasts showed increased proliferation on stiffer substrates, ECs showed the opposite trend (Robinson et al., 2012). Collectively, these data illustrate the importance of the underlying substrate on governing VSMC response in combination with other parameters of the culture system, including serum type, cell density, the presence of other cell types (co-cultures), and matrix composition.

2.5.3.3 Mechanical strain and vascular tissue engineering strategies

Bioreactor systems are often used in tissue engineering strategies to impart mechanical stimuli to cells, with the goal of supporting growth, specific phenotypes, oxygenation, and other critical factors for promoting new tissue production. These systems have successfully been transferred into practice, such as for the use of a bioreactor to provide hydrodynamic shear stress, nutrient supply, and waste removal through rotation to a decellularized trachea re-seeded with donor epithelial cells and MSC-derived chondrocytes, for use as a tissue engineered airway (Asnaghi et al., 2009; Gonfiotti et al., 2014). For vascular tissue engineering strategies targeting the medial (VSMC-rich) layer of a graft, bioreactors are typically designed to apply mechanical strain to cell-seeded constructs. These bioreactor systems typically take two general approaches: those that isolate the effect of mechanical strain and those that use pulsatile flow, such that cells experience both mechanical strain and shear stress.

Pulsatile perfusion bioreactors have been successfully used to accelerate the development of vascular tissue in vitro. Parameters that have been varied using this approach to gauge the effect on VSMCs include flow rate, shear stress, pressure, distension, and pulse frequency. In a study by Niklason and Langer, an implantable tissue engineered vascular graft was generated using a
pulsatile perfusion bioreactor for a period of 8 weeks, exposing VSMCs seeded on a PGA mesh to 5% radial strain at 2.75 Hz with exposure to shear stress of 0.3 dynes/cm² (0.1 ml/s) (Niklason et al., 1999). This dynamic culture system promoted increased collagen production and mechanical strength of the tissue engineered construct, but no increase in VSMC number. Other systems have shown that pulsatile loading with pressures ranging from 60/40 mmHg to 120/70 mmHg, radial distension from 3-10%, and flow rate from 5-3000 ml/min in various combinations can increase cell number, promote contractile marker expression, and increase ECM deposition (Zhang et al., 2009; Opitz et al., 2004; Engbers-Buijtenhuijs et al., 2006; Xu et al., 2005; Jeong et al., 2005; Hahn et al., 2007; Song et al., 2011). Fluid flow in these systems, when reported, is described as laminar with Reynolds numbers ranging from 96 to 249 (Zhang et al., 2009; Engbers-Buijtenhuijs et al., 2006). In studies with rat aortic SMCs, turbulent flow has been shown to be a greater inducer of cell proliferation (Rosati and Garay, 1991; Shigematsu et al., 2000), though no studies have involved the study of turbulent vs. laminar flow in the context of vascular tissue engineering. Furthermore, the aforementioned studies using pulsatile perfusion bioreactors are limited in their ability to identify how individual factors are regulating VSMC response due to a wide range of factors (shear stress, flow rate, pressure, radial distension) being varied simultaneously. One factor that has been identified as a possible explanation for increased cell number is an increase in aerobic respiration under pulsatile perfusion as demonstrated by glucose consumption, medium pH, and pCO₂ values (Engbers-Buijtenhuijs et al., 2006), suggesting improved mass transport (nutrient delivery, waste removal).

Controlled studies in which individual factors have been varied provide some insight into how pulsatile perfusion bioreactors regulate VSMC response. In a study by Crapo et al., PGS scaffolds were seeded with baboon arterial SMCs in a modified bioreactor such that pressure could be controlled independently of flow rate. Constructs were exposed to 10 ± 5 mmHg for the entire 21 day culture period, or to 60 ± 10 mmHg that was gradually increased to 120 ± 20 mmHg. Constructs exposed to higher hydrostatic pressure experienced increased production of soluble and insoluble elastin as well as burst pressure, suggesting that exposure to hydrostatic pressure is one mechanism through which pulsatile perfusion supports a synthetic VSMC phenotype (Crapo and Wang, 2011). In another study by Wayman et al., shear stress was controlled independently of mechanical strain for porcine carotid arteries cultured in a custom-
designed bioreactor. Circumferential stress was varied from 50 to 150 kPa while keeping shear stress constant at physiological levels of 15 dynes/cm², or alternatively shear stress was changed from 7.5 to 22.5 dynes/cm² while circumferential stress was maintained at 100 kPa. High circumferential strain, but not shear stress, resulted in medial and adventitial layer proliferation as well as enhanced ³H-Proline incorporation (increased matrix synthesis) (Wayman et al., 2008). While this study suggests mechanical strain can increase VSMC number and matrix production, it should also be noted that in this study VSMCs were not exposed directly to shear stress due to the presence of a complete endothelium. Furthermore, the presence of ECs can modify VSMC response to shear stress (Redmond et al., 2001). Specifically, the effects observed in the presence of ECs are not transferrable to pulsatile perfusion bioreactor systems where VSMCs in monoculture are exposed directly to flow. Studies on the effects of VSMCs in monoculture to shear stress suggest that this stimulus can result in increases in proliferation-inducing cytokines and growth factors such as FGF-2 and PDGF (3-25 dynes/cm²), VSMC alignment, either inhibit (laminar, steady flow) (Fitzgerald et al., 2008) or promote (pulsatile, oscillatory flow) VSMC proliferation, as well as reduce contractile marker expression, suggesting that shear stress can promote a synthetic VSMC phenotype (Shi and Tarbell, 2011).

Unlike pulsatile perfusion systems, cyclic stretch bioreactors are able to isolate the effects of flow from mechanical strain, and allow for a more fundamental understanding of the factors regulating VSMC response. Furthermore, these systems represent a more physiologically relevant biomechanical stimulation for VSMCs, since VSMCs in vivo do not directly experience the effects of shear stress due to the presence of the endothelium. Isolating effects of strain from flow is achieved with most bioreactor systems by using a distensible mandrel (ex. silicone, latex) as a support for the tissue engineered construct, such that distension of the mandrel transfers the strain to the cell-seeded material without exposure to the pressurizing medium (typically air or water). In addition to percent strain, other factors that can be varied with these systems include frequency and duty cycle. In a study by Wang et al, a pulsatile perfusion bioreactor was used to apply strain (5% radial strain, 1.25 Hz, 100% duty cycle) for 8 weeks to ASC-derived SMCs seeded on a PGA mesh that was mounted on silicone tubing, resulting in increased mechanical properties (ultimate tensile strength, elastic modulus, suture retention strength, burst pressure), cell number, and collagen content (Wang et al., 2010). Similar results have been achieved with
human aortic SMCs embedded in a collagen gel and exposed to 10% radial strain (1 Hz) for 4-8 days (Seliktar et al., 2000). In a detailed study by Isenberg and Tranquillo using rat aortic SMCs embedded in a collagen gel, the various factors involved in the application of cyclic strain (percent strain, duty cycle, frequency) were varied in order to determine the optimal conditions for enhancing construct mechanical properties and promoting tissue growth (Isenberg and Tranquillo, 2003). Circumferential strain had significant effects on elastic modulus (EM) (10% > 5% = 2.5% > static) and ultimate tensile strength (UTS) (2.5% = 5% > 10% > static), as did duty cycle with strain held constant at 5% and relaxation time at 1.75 sec while varying the stretch time (UTS: 6.7% = 12.5% > 18% = 22% = static; EM: 6.7% > 12.5% > 18% > 22% > static). The effect of frequency was also investigated by maintaining stretch time constant at 0.25 sec and strain at 5%, while varying relaxation time, demonstrating effects on UTS (0.25 Hz > 0.5 Hz = 1 Hz > static) and EM (0.25 Hz = 1 Hz > 0.5 Hz > static). In a further study by Syedain et al. using human dermal fibroblasts embedded in a tubular fibrin gel, constructs were either subjected to a single strain rate over 3 weeks (2.5%, 5%, 10%, or 15%), or had their strain rate increased in either 2 (ICD2: 5% → 10% → 15%) or 4 (ICD4: 5% → 7.5% → 12.5% → 15%) equal time steps from 5% to 15%. In all cases, a 12.5% duty cycle and 0.5 Hz frequency were used. Results indicated that incrementally changing the strain level maximized UTS and EM, with 4 equal time intervals promoting optimal results. Within the constant cyclic strain conditions, trends were also observed for UTS and EM (15% = 10% > 5% = 2.5% = static). While increasing cell number and collagen content were observed with increasing cyclic strain level, ICD2 and ICD4 conditions were the only ones to show increases in collagen produced when normalized to cell number (Syedain et al., 2008). Such studies provide support for the use of mechanical strain as a means to enhance vascular tissue formation in vitro.

Combining the effects of biomechanical stimulation with biochemical stimulation has also been used to further optimize the production of vascular tissue. Rat aortic SMCs exposed to mechanical strain (10% radial strain, 1 Hz) showed an increase in cell number. Exposure to PDGF resulted in an increase in cell number in general, but when exposed to PDGF, mechanical stimulation had a negative effect on cell number, while exposure to TGF-β1 had a drastic negative effect on cell number and also eliminated the benefits of the observed mechanical effects (Stegemann and Nerem, 2003). TGF-β1 is a promising growth factor for use in vascular
tissue engineering as it has the ability to promote elastin synthesis, the lack of which is one of the main limitations of many tissue engineered vascular constructs. However, Syedain et al. demonstrated that stimulation of fibrin constructs embedded with human dermal fibroblasts with TGF-β1 (1 ng/ml) in conjunction with mechanical strain (0.5 Hz, 12.5% duty cycle, radial strain increased from 5% → 7.5% → 12.5% → 15% over 3 weeks), while similar to non-TGF-β1 stimulated constructs after 5 weeks, prevented the upregulation of collagen production and mechanical properties observed without biochemical stimulation over 7 weeks (Syedain and Tranquillo, 2011). However, TGF-β1-stimulated cultures did demonstrate the desirable property of increasing elastin production. By limiting TGF-β1 exposure to the final 2 weeks of the 7 week culture period, Syedain et al. were able to utilize the initial 5 weeks of mechanical conditioning to increase the mechanical properties (via increased collagen deposition) while the final 2 weeks involving strain in combination with TGF-β1 stimulation were sufficient to enhance elastin production without a negative impact on the construct’s mechanical properties. Combining mechanical strain with growth factor supplementation, or other means of introducing stimulatory factors, thus has the potential to further optimize the production of tissue engineered vascular grafts.

Independent of mechanical strain variables, the underlying substrate on which VSMCs are cultured can play a role in the ability of VSMCs to sense this stimulus. Neonatal rat VSMCs cultured on 2D silicone elastomers and exposed to a strain rate of 1 Hz demonstrated effects dependent on the type of protein that substrates were coated with. VSMC proliferation was enhanced with the application of strain on substrates coated with fibronectin and collagen (fibronectin > collagen), but not on substrates coated with elastin, laminin, or polylysine. Furthermore, when substrates were coated with a mixture of laminin and vitronectin, proliferation increased with a corresponding increase in the concentration of adsorbed vitronectin on the surface. Promotion of proliferation by fibronectin-coated surfaces under strain could be blocked through soluble RGD peptides as well as blocking specific integrins (β3 and α3β5), suggesting a critical role of these integrins in mechanotransduction (Wilson et al., 1995). Such studies have further applicability to the application of strain with different biomaterials, as differences in the adsorbed protein layer in addition to the surface chemistry itself can potentially result in changes to the ability of adherent VSMCs to respond to strain.
While not as widely studied as VSMCs, monocytes/macrophages and other immune cells are also affected by mechanical strain. Studies demonstrating that macrophages contribute to neovessel formation following the implantation of tissue engineered vascular grafts (Hibino et al., 2011b) demonstrate that these cells will be exposed to mechanical strain in such situations, and how their phenotype is affected may play a role in their subsequent polarization and ability to contribute to vascular remodelling. U937 cells, a macrophage-like cell line, have been shown to be responsive to both uniaxial and biaxial strain. Uniaxial strain was shown to decrease DNA levels on polyRGD, but not col I, coated surfaces, with cells aligning in the direction of the applied strain. Both biaxial and uniaxial strain were shown to upregulate intracellular esterase activity, IL-6 but not IL-8 production, as well as total protein production by U937 cells (Matheson et al., 2007; Matheson et al., 2006). Exposure of peritoneal macrophages to 5% strain (1 Hz) suppressed their ability to phagocytose latex particles after 24 and 48 hr (Miyazaki and Hayashi, 2001), while other studies have shown that biaxial strain at 1, 2, and 3% (1 Hz) promotes macrophage scavenger receptor (CD36) expression, which may suggest increased phagocytic capacity (Sakamoto et al., 2001). These studies highlight the importance of considering the magnitude of the applied strain, rather than solely the presence of mechanical stimulation itself. Using human peripheral blood mononuclear cells seeded on PCL bisurea nanofibrous strips, Ballota et al. further demonstrated the importance of strain magnitude on macrophage phenotype. Following 1 day of exposure to strain, 7% strain upregulated several pro- and anti-inflammatory cytokines, including MCP-1, IL-6, and IL-10 in addition to MMP9, while 12% strain upregulated SDF1α expression. These effects appear to be evident only initially following the application of strain, as no effects were observed after 2 days of culture. With regards to phenotype polarization, exposure to no strain as well as 7% strain resulted in an increase in immunoregulatory macrophages over time (increase in the ratio of CD206/CCR7 expression), while 12% strain promoted a decrease in this ratio. Increasing strain magnitude was also associated with decreased cell numbers (0% > 7% > 12%) (Ballotta et al., 2014). Studies with dendritic cells, which like macrophages can be derived from monocytes, further demonstrate the strain-responsiveness of immune cells, as well as the dependence of these effects on adsorbed proteins. Exposure of cells to 3% or 10% strain resulted in increased apoptotic and necrotic cells. CD86 expression was also shown to increase on cells exposed to 3% strain on
collagen and fibrinogen, but not laminin, coated surfaces after 1 hr, while IL-12 release was only increased in the presence of fibrinogen coating (Lewis et al., 2013).

2.6 Summary

The immune response plays an integral role in determining the fate of implanted biomaterials, whether they are part of a medical device used to obtain physiological measurements, provide a stimulus to biological tissue, or as part of a tissue engineered product. Monocytes and their derived macrophages are a significant cellular component of this immune response, and their biomaterial-dependent activation has been shown to be critical to the success or failure of many implanted devices. When chronic pro-inflammatory macrophage polarization is attenuated, and an immunoregulatory, wound healing state is supported, macrophages have the ability to support constructive tissue remodeling and regeneration. A significant factor regulating biomaterial-mediated monocyte/macrophage response is the adsorbed protein layer. This thesis investigated the role of protein adsorption in regulating the inflammatory response of monocytes to a degradable polyurethane developed in the Santerre laboratory (i.e. D-PHI). Furthermore, different aspects of D-PHI’s chemistry were evaluated for their role in supporting specific outcomes with regards to protein adsorption and monocyte/macrophage activation. Due to the ability of D-PHI to reduce pro-inflammatory monocyte activation and previous work demonstrating the importance of monocytes/macrophages in contributing to improved healing outcomes, monocytes/macrophages were evaluated for their potential use in a tissue engineering strategy. In particular, due to the suitability of D-PHI for vascular tissue engineering applications, monocyte/macrophage use in vascular tissue engineering was investigated as a case study to evaluate the use of monocytes/macrophages in tissue engineering. Studies were also performed to demonstrate the mechanisms through which monocytes regulate VSMC response. Furthermore, a custom-designed bioreactor was developed in order to test the ability of monocyte/macrophage-VSMC co-cultures to be combined with biomechanical strain, a stimulation method commonly used in vascular tissue engineering strategies, to enhance the desirable effects on VSMCs cultured within D-PHI scaffolds, such as cell growth, infiltration, phenotype, and vascular tissue production, while also investigating the macrophage activation state supported by D-PHI under both static and dynamic culture conditions.
2.7 References


Chapter 3
Protein binding mediation of biomaterial-dependent monocyte activation on a degradable polar hydrophobic ionic polyurethane

3.1 Foreword

Protein adsorption is an important event that transforms biomaterial substrates into biological surfaces that subsequently guide cellular interactions. Following biomaterial implantation, the ability of monocytes to adhere to a surface and differentiate to pro- or anti-inflammatory macrophages is governed in large part by the nature of the adsorbed protein layer. To understand the role of protein adsorption in regulating monocyte activation on D-PHI vs. TCPS and PLGA, this study sought to identify differences in the adsorbed protein layer between these biomaterials and to screen proteins differentiated between these materials for their effect on monocyte response. This study identified differences in the amount and type of protein adsorbed to D-PHI vs. TCPS and PLGA, and also investigated surfaces properties known to regulate protein and monocyte interactions, including surface wettability, surface morphology, and surface chemistry. PLGA was shown to support heightened pro-inflammatory cytokine release regardless of the type of protein present, while TCPS supported sustained TNF-α release when compared to D-PHI, which supported a reduction in pro-inflammatory cytokine release over time. One protein in particular that was identified as being present in the adsorbed protein layer, α2-macroglobulin (A2M), was shown to differentially regulate pro-inflammatory cytokine release (TNF-α) on D-PHI vs. TCPS and PLGA. The mechanism of A2M-monocyte interactions was demonstrated. When cultured in protein-rich medium (10% FBS-containing medium) the pro-inflammatory effects of A2M on D-PHI were masked and anti-inflammatory cytokine release increased in general. This study identified the potential for protein adsorption to contribute to differential monocyte activation on D-PHI vs. TCPS and PLGA, while also highlighting the importance of complex protein solutions in masking pro-inflammatory protein interactions with monocytes on D-PHI. Furthermore, D-PHI was demonstrated to be a degradable material with reduced pro-inflammatory monocyte interactions as an alternative to the widespread use of the degradable polyester PLGA.
This chapter is published in ‘Biomaterials’ as: Battiston KG\textsuperscript{1}, Labow RS\textsuperscript{2,3}, Santerre JP\textsuperscript{3,4}. Protein binding mediation of biomaterial-dependent monocyte activation on a degradable polar hydrophobic ionic polyurethane. *Biomaterials* 2012;33(33):8316-28.

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### 3.2 Introduction

Following the implantation of a foreign body, the first event that occurs is protein adsorption to the surface of the material (Anderson et al., 2008; Horbett, 1993; Wilson et al., 2005). The adsorbed biomolecular layer contains a number of different bioactive proteins, growth factors, cytokines, and other agents that will impart the biomaterial with specific cell-responsive characteristics. The particular nature of the cell response resulting from the cell-protein interactions will depend on the defined composition of the adsorbed protein layer, as well as differences in protein conformation that occur upon adsorption to the biomaterial (Campillo-Fernandez et al., 2009).

A critical cellular component of the foreign body response to implanted biomaterials is the monocyte/macrophage (Anderson et al., 2008). Monocytes are one of the first cell types present following implantation and play an important role in directing the inflammatory and wound healing processes (Anderson et al., 2008). As a result, prospective biomaterials are often
Monocyte-biomaterial interactions have also been evaluated to better understand the use of monocyte pre-seeded biomaterials in improving wound healing in vivo following implantation (Roh et al., 2010). There are several examples in the scientific literature wherein monocyte pre-seeding, either directly (McBane et al., 2011) or indirectly (Roh et al., 2010; Mirensky et al., 2010; Cho et al., 2009), has been used with the intent of eliciting a desired biological response. As an example, vascular grafts pre-seeded with bone marrow mononuclear cells have previously been shown to remain patent and exhibited evidence of growth and venous development in a lamb model (Brennan et al., 2008). While bone marrow derived mononuclear cells consist of varied cell types, including monocytes, endothelial progenitor cells, natural killer cells, and mesenchymal stem cells, amongst others (Udelsman et al., 2011), depletion of the monocyte fraction alone prior to pre-seeding the vascular grafts eliminated the beneficial response in terms of maintenance of graft diameter, demonstrating the importance of monocytes in maintaining graft patency (Mirensky et al., 2010). This beneficial effect of monocytes has also been linked with the release of MCP-1 from biomaterial-adherent monocytes upon pre-seeding, which has been shown to be critical for the recruitment of host monocytes to the site of implantation (Roh et al., 2010).

Previous work has assessed the inflammatory response of peripheral blood monocytes to a degradable polar hydrophobic ionic polyurethane (D-PHI) that is being evaluated for potential applications as a small diameter tissue engineered vascular graft (McBane et al., 2009; Sharifpoor et al., 2009). It has been reported that monocytes adherent to D-PHI secrete less of the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) up to 72 hr relative to tissue culture polystyrene (TCPS), while also supporting greater release of the anti-inflammatory marker interleukin-10 (IL-10) at 72 hr (McBane et al., 2009). Furthermore, monocytes on D-PHI supported less pro-inflammatory cytokine release in terms of TNF-α levels evaluated in vitro for possible biocompatibility concerns by assessing how they interact with monocytes/macrophages (Schutte et al., 2009; Matheson et al., 2007; McBane et al., 2009; Anderson et al., 1999; Gretzer et al., 2006; Greulich et al., 2011; Hsu et al., 2010; Chin-Quee et al., 2010). The extent of the inflammatory response elicited by the biomaterial may be evaluated in terms of the release of specific pro-inflammatory cytokines from adherent monocytes, such as TNF-α, IL-1β, IL-6, and IL-12, amongst others (McBane et al., 2009; Bonfield et al., 1989).
at later time points (>14 days) relative to the biodegradable polyester poly(lactide-co-glycolide) (PLGA) (McBane et al., 2011). However, the role of protein binding in this differential response of monocytes to D-PHI, PLGA, and TCPS has yet to be investigated.

While protein adsorption has an important influence on cell-biomaterial interactions (Horbett, 1993), protein adsorption, itself, is influenced by the chemical composition of the biomaterial surface, such as the presence of specific surface functional groups (hydroxyl, carboxyl, and amine, to mention a few) (Barrias et al., 2009; Arima and Iwata, 2007). Biomaterial properties, such as surface wettability and surface chemistry, have also been correlated directly with cell response (Arima and Iwata, 2007). It is not always clear whether the specific chemistry of the biomaterial is directly imparting the material with cell-responsive characteristics, or rather if it is a mediation of the adsorbed protein layer by the biomaterial surface properties that elicits the observed differential cellular response. Another parameter known to influence protein adsorption is surface topography. It has recently been demonstrated that gold nanoparticle morphology results in differences related to the retention of an enzyme’s secondary structure, and thus activity, upon adsorption to the nanoparticles (Gagner et al., 2011). In other work, fibrinogen adsorption has been shown to increase with a corresponding increase in surface roughness (Rechendorff et al., 2006).

The effect of surface chemistry on monocyte cytokine expression in vitro has previously been demonstrated, with IL-10 (anti-inflammatory) expression increased in monocytes/macrophages adherent to hydrophilic and anionic surfaces, and decreased on cationic surfaces, while the opposite effect was apparent for the expression of IL-8 (pro-inflammatory) (Brodbeck et al., 2002). Foreign body giant cell (FBGC) and monocyte adhesion have been shown to be inhibited on hydrophilic and anionic surfaces, further suggesting that surfaces with this chemical nature promote an anti-inflammatory response (Brodbeck et al., 2002). Anionic and cationic surface chemistry have also been shown to influence monocyte adhesion to biomaterials (MacEwan et al., 2005). Monocyte/macrophage interactions have also been shown to be dependent on a number of different plasma proteins, including IgG (Shen et al., 2004), vitronectin (McNally et al., 2008), and fibronectin (Sudhakaran et al., 2007), amongst others. These examples illustrate the importance of studying protein adsorption in order to provide a deeper fundamental understanding of monocyte activation to biomaterial surfaces.
Previous studies that have highlighted the role of specific proteins, such as vitronectin (McNally et al., 2008), with respect to cell-biomaterial interactions have investigated the monocyte/macrophage activation to the surfaces following pre-adsorption with these proteins. This approach requires previous knowledge of the proteins that may influence monocyte/macrophage activation. Hence, there is a need to identify such proteins that may be playing an important role in monocyte/macrophage activation when studying new biomaterials. Studies, such as those performed by Kim et al. (Kim et al., 2005) and others (Battiston et al., 2012), have compared the composition of the bound protein layer of different biomaterials in order to identify proteins that can then be examined in isolation for their effect on cell response (Kim et al., 2005). Due to the large number of proteins that have been implicated in the potential activation of monocyte-biomaterial interactions, the latter approach was used in the present study. The composition of the bound protein layer on three different biomaterials, D-PHI, PLGA, and TCPS, was compared in order to identify bound proteins that were more specific to one surface over another. Hence, the objective of the current study was to determine the effect of binding of the specific proteins identified and which may have the potential to mediate the differential activation of monocytes to D-PHI relative to well-established biomaterial substrates, namely PLGA and TCPS.

### 3.3 Materials and methods

All materials were purchased from Sigma-Aldrich unless stated otherwise. TCPS materials used in this study were 96-well plates manufactured by Becton Dickinson (BD).

#### 3.3.1 Biomaterial preparation

#### 3.3.1.1 D-PHI films

D-PHI films were prepared as in previously established methods (Sharifpoor et al., 2009). Briefly, methyl methacrylate (MMA), methacrylic acid (MAA), and a divinyl oligomer (DVO) were mixed in a 15:5:1 molar ratio in the presence of the initiator benzoyl peroxide (BPO, 0.032 mol/mol vinyl group) for 24 hr in a light-shielded bottle. 50 µl of the resulting mixture was
pipetted into a 96-well polypropylene plate (BD) and cured at 110°C for 24 hr in a dry nitrogen-filled environment.

### 3.3.1.2 PLGA films

A PLGA (lactide:glycolide 75:25, MW 66kDa-107kDa) solution was prepared by dissolving PLGA pellets in chloroform at 5 wt/wt%. After the PLGA had dissolved, the solution was cast onto 5 mm diameter round glass cover slips (Electron Microscopy Sciences). Prior to casting the PLGA solution onto the cover slips, these substrates were cleaned by incubation in a 5% chromic acid solution, followed by sonication in ddH2O. The PLGA chloroform solution was cast onto the coverslips and the solvent was allowed to evaporate for 24 hr in a fumehood. Residual chloroform was removed by placing the PLGA-coated surfaces in a vacuum oven for 3 hr at room temperature, followed by 3 hr at 50°C.

### 3.3.2 Biomaterial characterization

#### 3.3.2.1 X-ray photoelectron spectroscopy (XPS)

XPS analysis was performed as previously reported (Battiston et al., 2012). Briefly, a Thermo Scientific K-Alpha XPS system (East Grinstead, UK) located at Surface Interface Ontario, University of Toronto, was used to obtain XPS spectra at a take-off angle of 90° (three samples per experimental group) with data collected from one spot per sample (n=3). High and low resolution analysis of all data was performed with Avantage 1.68 software (Thermo Scientific Fisher, Waltham, MA, USA). Percent atomic composition was determined by low resolution analysis of carbon, oxygen, and nitrogen, while functional group data for the C 1s region were collected in high resolution mode. Background subtraction was performed using Shirley’s method (Repoux, 1992). All peaks were referenced relative to the main carbon peak at 285.0 eV.

#### 3.3.2.2 Contact angle measurements

Advancing water contact angle measurements were obtained as outlined previously (Battiston et al., 2012) using a goniometer (NRL C.A. goniometer, Ramé-Hart, Inc., Mountain Lakes, NJ). Briefly, a microsyringe was used to place a 20 µL droplet of distilled/deionized water on the
biomaterial surfaces (n=12-18). For each droplet, the contact angle on either side was measured and the average ± standard deviation was reported as a single measurement.

3.3.3 Protein analysis

3.3.3.1 Total protein binding

A protein solution consisting of 10% fetal bovine serum (FBS) in RPMI-1640 medium was incubated on each of the biomaterial surfaces for 24 hr at 37°C and 5% CO₂ to simulate standard \textit{in vitro} cell culture conditions. Subsequently, surfaces were rinsed three times with Dulbecco’s phosphate buffered saline (DPBS, Gibco), similar to rinsing protocols well-established in the literature (Cornelius et al., 2002; Gessner et al., 2003; Higuchi et al., 2003) and the bound protein layer was then immediately eluted by treatment with a 2% sodium dodecyl sulphate (SDS, Bio-Rad) solution for 24 hr, as per standard protocols (Cornelius et al., 2002; Gessner et al., 2003; Magnani et al., 2004; Higuchi et al., 2003). Rinsing steps were performed immediately following removal of the protein solution and the elution solution was added immediately after removal of the rinsing buffer to minimize exposure of the surface to the vapor interface. To quantify the mass of protein present, a micro bicinehinonic acid (BCA) protein assay was used (Thermo Scientific).

3.3.3.2 2-D electrophoresis (2-DE)

Initial preparation of samples for 2-DE containing proteins eluted from the different biomaterial surfaces was the same as described above. Eluates were concentrated using a 3 kDa Amicon Ultra-15 centrifugal device (Millipore). Residual SDS was removed from samples using a chloroform-methanol precipitation protocol (Candiano et al., 2009) since ionic detergents can result in excess streaking and are incompatible with the 2-DE process (Battiston et al., 2012). The resulting protein pellet was resolubilized in a ReadyPrep Rehydration/Sample buffer (Bio-Rad, 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 0.001% bromophenol blue). Protein concentration was assessed with a CB-X protein assay (G-Biosciences). Protein samples were then incubated with 1x Bio-Lyte 3/10 ampholyte (Bio-Rad) and reduced with 5 mM tributylphosphine. Immobilized pH gradient (IPG) strips (Bio-Rad, pH 3-10, 17 cm, non-linear gradient) were rehydrated with sample protein solutions containing 65 µg protein, as determined
by the CB-X assay. First dimension separation consisted of four steps and was performed with a Protean IEF cell (Bio-Rad): 1) 250 V, 1 hr; 2) 8000 V, 2.5 hr; 3) 80,000 V-hrs at 8000 V; and 4) 500 V. The samples were then either stored at -80°C or underwent second dimension separation by SDS-PAGE.

Following first dimension separation, IPG strips were reduced and alkylated for 10 min each with 2% dithiothreitol (Bio-Rad) and 2.5% iodoacetamide (Bio-Rad), respectively, in order to break disulphide bonds (Galvani et al., 2001). IPG strips were then cut in half, loaded onto Criterion™ Precast Gels (Bio-Rad, 10.5-14% Tris-HCl gels), overlayed with 1 ml of agarose containing trace amounts of bromophenol blue, and separated for 1 hr at 200 V. Gels were stained using a Silver Stain Plus staining kit (Bio-Rad) and subsequently imaged with a ChemiDoc™/Gel Doc™ XRS imager (Bio-Rad). While protocols were optimized to minimize protein streaking, including removing residual SDS, some streaking was still apparent in the silver stained 2-D gels.

### 3.3.3.3 In gel digestion

Proteins of interest were first excised from gels. Protein extraction was performed by incubation with 100 mM ammonium bicarbonate, followed by 60 mM ammonium bicarbonate/40% acetonitrile. Gel pieces were then dried with a SpeedVac vacuum concentrator (Thermo Scientific), swelled with a trypsin solution (Promega, 1 part 200 µg/ml trypsin solution with 3 parts 100 mM ammonium bicarbonate), and then incubated with 100 mM ammonium bicarbonate overnight at 37°C. Gel pieces were then incubated with a 5% formic acid solution, followed by a 5% formic acid/30% acetonitrile solution, after which the supernatant was collected and concentrated using the SpeedVac.

### 3.3.3.4 Liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was carried out by an Agilent 1100 HPLC-chip and 6340 ion trap system with MS scan range from 300 to 1,300 m/z and back-to-back CID/ETD (collision-induced-dissociation/electron transfer dissociation) at Sunnybrook Hospital’s proteomics facility. Raw data files from LC-MS/MS were searched against a custom protein sequence representing GST-Fra2 using Spectrum Mill MS Proteomics Workbench (v03.03.084, Agilent Technologies). Peak
lists were searched by the following criteria: two missed trypsin cleavages, fixed modification (carbamidomethylation on cysteine), variable modifications (oxidized methionine, pyro-glutamic acid modification at N-terminal glutamines, phosphorylated-serine, -threonine, and -tyrosine), precursor mass tolerance +/- 2.5 Da, product mass tolerance +/- 0.7 Da. The spectra identified by Spectrum Mill to be phosphorylated were manually verified and reported. In instances where multiple candidate proteins were identified in a single spot, the protein most likely to be present was determined by spectral intensity (high), the number of distinct peptides identified (≥2), and the MS score (≥10 when divided by number of distinct peptides).

3.3.4 Cell culture and analysis

3.3.4.1 Monocyte isolation and culture

Monocytes were isolated from whole blood obtained from healthy volunteers at Mount Sinai Hospital, Toronto, Ontario (University of Toronto ethics approval protocol #22203) by layering approximately 27 ml of blood on 20 ml of histopaque-1077 followed by centrifugation at 2000 rpm for 30 min. The platelet-rich plasma top layer was discarded and the buffy coat and histopaque layer were collected and transferred to a separate tube. Multiple washes with RPMI containing EDTA and FBS were performed as described previously (McBane et al., 2011). Monocytes were seeded on the different biomaterials at a concentration of 400,000 per well in 96-well plates with media exchange occurring every 48 hr. Experiments were repeated for three distinct donors.

To assess the effect of specific proteins (identified from the above LC-MS/MS studies described in section 2.3) bound to the biomaterial substrates on monocyte response, biomaterials were first pre-coated with 100 µl of α1-antitrypsin (Calbiochem-EMD), α2-macroglobulin (Calbiochem-EMD), antithrombin III (Calbiochem-EMD), apolipoprotein E (Calbiochem-EMD), tetranectin (R&D Systems), or vitronectin (Invitrogen) at a concentration of 25 µg/ml (McNally et al., 2008) in RPMI-1640 medium for 24 hr. Monocytes were seeded on the protein pre-coated surfaces and cultured for 72 hr and analysed for DNA mass and TNF-α and IL-10 release.

In a separate experiment the role of the low density lipoprotein receptor related protein-1 (LRP1) in monocyte interactions with α2-macroglobulin (a key protein that was identified to modulate
the monocyte response on D-PHI in the above studies) pre-adsorbed biomaterials was assessed by supplementing medium with 200 nM of receptor-associated protein (RAP, Molecular Innovations), a naturally occurring antagonist of LRP1. Monocyte response (DNA mass and TNF-α and IL-10 release) at 24 hr post-seeding was assessed in the presence or absence of 200 nM RAP, and in the presence or absence of 10% fetal bovine serum (FBS), for α₂-macroglobulin pre-adsorbed and non-coated D-PHI, PLGA, and TCPS surfaces.

3.3.4.2 DNA mass quantification

DNA mass was quantified as described previously (McBane et al., 2011). Cell culture media were removed from wells and biomaterials were incubated with lysis buffer containing 0.05% Triton X-100/EDTA for 1 hr. Samples were mechanically disrupted by vigorous pipetting. For analysis, 10 µl of lysates were loaded into black flat-bottom 96-well plates containing 1x TNE (Tris/NaCl/EDTA) with Hoechst 33258 DNA stain. Plates were read with an FL600 Microplate Fluorescence Reader with excitation and emission wavelengths of 360 and 460 nm, respectively. Sample values were compared to a standard curve prepared from DNA standards of calf thymus DNA.

3.3.4.3 Cytokine release

Enzyme-linked immunosorbent assays (ELISAs) for human TNF-α and IL-10 (eBioscience) were performed as per the manufacturer’s instructions on cell supernatants. Supernatants were collected from samples where media had been changed 24 hr prior to the selected time-point. Depending on the experiment, media were collected at 1- and/or 3-day time-points for analysis with n=3 for each donor.

3.3.5 Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) by analysis of variance (ANOVA) or by an independent samples t-test where appropriate, with statistical significance reported for \( p < 0.05 \).
3.4 Results and discussion

3.4.1 Total protein binding to D-PHI, PLGA, and TCPS surfaces

A degradable polyurethane material has demonstrated unique anti-inflammatory properties relative to established biomaterials, such as PLGA and TCPS (McBane et al., 2009; McBane et al., 2011), and it has been hypothesized that this difference is in part reflected by the unique nature of the bound proteins interacting with the biomaterial. Hence, the objective of this work was to further probe the influence of biomaterials on serum proteins and their subsequent interactions with an isolated population of human white blood cells. This was considered particularly important considering the relevance of these cells to the ultimate processes of wound repair and tissue regeneration. Figure 3.1 shows the total surface protein content (µg/cm²) bound to D-PHI, PLGA, and TCPS from a 10% FBS solution, indicating that D-PHI (5.84 ± 0.23 µg/cm²) has more than three times the amount of bound protein when compared to both PLGA (1.65 ± 0.16 µg/cm²) and TCPS (1.58 ± 0.07 µg/cm²), both of which had comparable levels of total bound protein.

Figure 3.1 Total protein binding to biomaterial surfaces (n=9). RPMI-1640 medium with 10% FBS was incubated on surfaces for 24 hr at 37°C and subsequently eluted with 2% SDS for 24 hr. Values shown are the mean ± S.E. * Significant difference compared to PLGA and TCPS (p<0.05).
Protein adsorption is known to be influenced by different surface properties, including surface wettability and surface chemistry (Barrias et al., 2009; Arima and Iwata, 2007). These properties are highlighted for D-PHI, PLGA, and TCPS in Table 3.1 (reported previously in (McBane et al., 2011) and (Battiston et al., 2011)) and contains advancing water contact angle measurements, and both low and high resolution XPS data.

Table 3.1 Biomaterial surface properties

<table>
<thead>
<tr>
<th>Material</th>
<th>Contact Angle (n=12-21)</th>
<th>Low Resolution XPS (n=3)</th>
<th>High Resolution XPS (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbon</td>
<td>Oxygen</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>D-PHI</td>
<td>80.5 ± 2.2</td>
<td>71.05 ± 0.48</td>
<td>26.63 ± 0.53</td>
</tr>
<tr>
<td>PLGA</td>
<td>89.8 ± 8.3</td>
<td>60.71 ± 0.09</td>
<td>39.29 ± 0.08</td>
</tr>
<tr>
<td>TCPS</td>
<td>48.2 ± 2.7</td>
<td>91.14 ± 0.87</td>
<td>8.36 ± 0.10</td>
</tr>
</tbody>
</table>

All values are reported as mean ± S.D. N/A – not applicable. Data have previously been reported in (McBane et al., 2011) and (Battiston et al., 2011).

The most hydrophilic material in this study, TCPS, had similar levels of bound serum proteins as PLGA and lower levels relative to D-PHI, which has a significantly higher contact angle than TCPS, but a lower value than PLGA (refer to Table 3.1). Hence, surface wettability alone does not provide a clear correlation with total bound protein to the surfaces. Furthermore, differences in surface chemistry do not completely explain the increase in protein binding to D-PHI. TCPS is the only biomaterial with hydroxyl chemistry presented at its surface (Table 3.1) and also possesses some polar carboxyl chemistry, while PLGA contains no proton donating functional group in its final processed form, but a large number of carbonyl groups and hydrophobic C-H groups for protein interactions. However, it should be noted that PLGA will rapidly begin to hydrolyze in aqueous medium, generating carboxyl and hydroxyl functionality (Houchin and
Topp, 2008). D-PHI surface chemistry is very diverse, presenting secondary amine (urethane bond), hydrophobic (-CH), and polar (carbonyl, carbonate) chemistry. This diverse combination of chemistry may be an important mediator in the increase in protein binding seen in the present study. Another factor known to influence protein adsorption is surface roughness (Scopelliti et al., 2010). However, no significant differences in surface morphology or roughness could be detected at 500x magnification using scanning electron microscopy (SEM) (Supplemental Figure 3.1).

Measurements of total bound protein to D-PHI, PLGA, and TCPS provided preliminary insight into how protein binding differs between the biomaterials. This is consistent with the findings of others (Woodhouse et al., 1992), which showed no correlation to simple aspects of surface character. However, Jozefowicz had previously discussed the importance of chemical diversity on interactions with biological systems (Jozefowicz and Jozefowicz, 1997). Hence, given the poor specific correlation of protein binding to the above parameters of the biomaterial surfaces, a more in depth study to define the nature of the bound protein layer in terms of the presence of specific serum proteins was desired in order to gain further insight into the differential monocyte activation that has previously been reported for the aforementioned biomaterials (McBane et al., 2009; McBane et al., 2011).

3.4.2 Composition of the bound protein layer

Differences in the composition of the bound protein layer between D-PHI, PLGA, and TCPS were determined using 2-DE and LC-MS/MS using established methods in the literature (Kim et al., 2005). Identical masses of eluted protein (65 µg) from the three biomaterials were loaded and run for all gels (Supplemental Figure 3.2). Following silver staining for protein visualization, gels were imaged and qualitatively analyzed to determine relative differences (high, medium, low) in staining intensity for the isolated proteins (Table 3.2) between the 2-D gels for D-PHI, PLGA, and TCPS (n=3 for each surface type).
Table 3.2 Staining intensity of proteins identified on biomaterial surfaces (\(\text{H}\) = high, \(\text{M}\) = medium, \(\text{L}\) = low, \(\text{□}\) = not detected).

<table>
<thead>
<tr>
<th>Identified Protein</th>
<th>D-PHI</th>
<th>PLGA</th>
<th>TCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1)-antitrypsin (AAT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha_2)-macroglobulin (A2M)</td>
<td>M</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>(\alpha_2)-antiplasmin (AAP)</td>
<td>L</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Antithrombin III (ATIII)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein E (ApoE)</td>
<td>H</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>Flavin reductase (FR)</td>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(GAPDH)</td>
<td>L</td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>Hemoglobin (HG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase 1 (PGK)</td>
<td>M</td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>Tetranectin (TN)</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>H</td>
<td>M</td>
<td>H</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>M</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Vitronectin (VN)</td>
<td>L</td>
<td>L</td>
<td></td>
</tr>
</tbody>
</table>
While many of the proteins identified are implicated in the scientific literature with respect to their importance in influencing monocyte activation, as either adsorbed proteins or soluble factors, six of the proteins identified (which were readily available in commercially pure form) were selected for further evaluation of their effect on monocyte activation when pre-adsorbed onto D-PHI, PLGA, or TCPS, namely α1-antitrypsin (AAT), α2-macroglobulin (A2M), antithrombin III (ATIII), apolipoprotein E (ApoE), tetranectin (TN), and vitronectin (VN). AAT, present in solution, has been shown to regulate CD14 expression, an important phenotypic marker for monocytes (Ziegler-Heitbrock, 2007), and has been suggested to be involved in the reduction of pro-inflammatory monocyte activation in vivo (Nita et al., 2007). Biomaterials pre-adsorbed with A2M (Anderson et al., 1999) and VN (McNally et al., 2008) have previously been shown to facilitate macrophage fusion and foreign body giant cell (FBGC) formation, suggesting that they may be linked to a more pro-inflammatory phenomenon. ATIII is known to inhibit NF-κB activation induced by lipopolysaccharides (LPS) (Mansell et al., 2001), where NF-κB is an important transcription factor involved in pro-inflammatory cytokine production in monocytes (Sprague and Khalil, 2009). ApoE, as a soluble factor, has previously been shown to have an anti-inflammatory effect by inhibiting macrophage inflammatory responses to toll-like receptor (TLR-3 and TLR-4) agonists (Zhu et al., 2010), while TN is a protein secreted by monocytes that has been shown to facilitate spontaneous monocyte migration (Nielsen et al., 1993), and has also been implicated in playing an important role in cutaneous wound healing in mice (Iba et al., 2009). While some proteins, such as vitronectin, were detected on some biomaterials but not the others, this may reflect the sensitivity of the method used in the present study rather than the complete absence of a specific protein from the bound protein layer for a biomaterial.

The differential presence of the above proteins in the bound protein layer on D-PHI, PLGA, and TCPS surfaces may be important in understanding the nature of monocyte activation that has been observed previously for D-PHI. However, in addition to differences in protein quantity, cell response may also be influenced by differential protein conformation by exposing specific binding sites when pre-adsorbed onto D-PHI vs. PLGA and TCPS (Garcia et al., 1999). As a result, it was necessary to evaluate how each of these proteins interacts with monocytes when they are pre-adsorbed to each of these biomaterials.
3.4.3 Monocyte response to protein pre-adsorbed biomaterials

The aforementioned proteins (AAT, A2M, ATIII, ApoE, TN, VN) were pre-adsorbed to D-PHI, PLGA, or TCPS from serum-free RPMI-1640 medium at a concentration of 25 µg/ml (McNally et al., 2008). The effect of these pre-adsorbed surfaces on monocytes was evaluated at 24 and 72 hr by DNA (Figure 3.2) and cytokine analysis (Figure 3.3). As a control, monocytes were also seeded on non-coated (NC) biomaterials to determine if differences in material chemistry influenced monocyte response. DNA mass quantification for monocytes on D-PHI indicated that AAT, ATIII, ApoE, and VN supported greater initial day 1 monocyte attachment as compared to the relative NC D-PHI control. However, none of the proteins promoted a significant increase in monocyte attachment above the NC baseline TCPS and PLGA controls at day 1 when pre-adsorbed to these two materials (Figure 3.2). Over the 72 hr culture period monocyte retention was greater on protein-coated and non-coated D-PHI relative to TCPS, but significantly less relative to PLGA (Figure 3.2), which showed increased cell adhesion for AAT and A2M pre-coated PLGA (p<0.05).
Figure 3.2 DNA mass quantification for monocytes seeded on protein pre-adsorbed biomaterial surfaces \((n=9)\) at day 1 (black bars) and day 3 (white bars) post-seeding. DNA measurements for protein-coated D-PHI, PLGA, and TCPS were normalized to their respective non-coated (NC)
D-PHI, PLGA, and TCPS DNA levels at day 1, indicated by the dashed line. Values shown are mean ± S.E. and are the combination of 3 donors, with n=3 per donor. (A) AAT – α₁-antitrypsin, (B) ATIII – antithrombin III, (C) A2M – α₂-macroglobulin, (D) ApoE – apolipoprotein E, (E) TN – tetranection, (F) VN – vitronectin, (G) NC – non-coated control. DNA density for non-coated biomaterials at day 1 are: D-PHI: 20,978.23 ± 908.47 ng/cm², PLGA: 5795.27 ± 1036.00 ng/cm², TCPS: 20,370.82 ± 1546.06 ng/cm². * p<0.05 compared to NC control for the same material at the same time-point. † p<0.05 compared to day 3 for the same material with the same protein coating. p<0.05 compared to ● PLGA or ○ TCPS for the same protein coating at the same time-point.

Adherent monocytes to D-PHI have previously been imaged using SEM and have been shown to have a round, non-spread morphology (McBane et al., 2011), suggesting that while D-PHI protein-coated surfaces supported greater monocyte retention, the above increased cell attachment may not be associated with an increase in monocyte activation for D-PHI-adherent monocytes. Enhanced monocyte recruitment has been shown to be promoted by monocyte pre-seeding to biomaterials prior to implantation, which has previously been shown to contribute to aiding in the wound healing process in vivo (Roh et al., 2010; Mirensky et al., 2010). However, if monocytes are recruited to the biomaterial surface and then induced into a pro-inflammatory state by the material chemistry or adsorbed proteins, greater monocyte attachment may not be beneficial. In this regard, D-PHI has been shown to be a relatively non-activating surface for monocytes relative to the levels of the release of the pro-inflammatory markers TNF-α and IL-1β (McBane et al., 2009; McBane et al., 2011). While DNA mass decreased over time on D-PHI and TCPS for the different proteins, several protein-coated PLGA surfaces (AAT and A2M) showed an increase in DNA mass from 24 to 72 hr. Although an increasing monocyte presence over time was an unexpected finding because they are generally considered to be a non-proliferating cell type, sub-populations (such as progenitor cells) in the mononuclear fraction have been shown to proliferate in vitro (Udelsman et al., 2011). Alternatively, a higher activation, supported by PLGA, may have supported a greater adhesion by 72 hr for monocytes on PLGA since samples processed for 72 hr measurements had a media change at 48 hr, allowing for an accumulation of cytokines that could influence cell adhesion between 24-48 hr (Vigetti et al., 2010).
The effect of bound proteins and material chemistry on monocyte activation was evaluated by assaying for the release of a representative pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine from adherent monocytes (Figure 3.3).
Figure 3.3 TNF-α (black bars) and IL-10 (white bars) release from monocytes adherent to protein pre-adsorbed D-PHI, TCPS, and PLGA. Cytokine levels were measured with an ELISA using the conditioned media from cultured monocytes, with cytokines allowed to accumulate in media for 24 hr prior to collection. Values shown are mean ± S.E. and are the combination of 3 donors, with n=3 per donor. (A) AAT – α1-antitrypsin, (B) ATIII – antithrombin III, (C) A2M – α2-macroglobulin, (D) ApoE – apolipoprotein E, (E) TN – tetranectin, (F) VN – vitronectin, (G) NC – non-coated control. * p<0.05 for TNF-α compared to NC control for the same material at the same time-point. p≤0.05 for ● TNF-α or ○ IL-10 relative to D-PHI for the same protein coating at the same time-point. p≤0.05 for ◊ TNF-α or▲ IL-10 compared to day 3 for the same protein coating on the same material.

For monocytes seeded on the NC controls, a significant increase in TNF-α release was apparent for PLGA-adherent monocytes relative to those adherent to D-PHI. Furthermore, while no differences in pro-inflammatory cytokine release were apparent between D-PHI and TCPS, a significant decrease in pro-inflammatory TNF-α levels was evident for D-PHI and PLGA over the 72 hr culture period (Figure 3.3g), whereas TNF-α release remained steady over the 72 hr culture period on TCPS, which may indicate a more chronic inflammatory response of monocytes on TCPS relative to D-PHI.

In addition to the differences in monocyte activation caused by surface functionality, it was hypothesized that monocyte activation would also be mediated by protein binding in a biomaterial-dependant manner, meaning that the same protein pre-adsorbed to D-PHI vs. PLGA or TCPS would differentially activate monocyte cytokine release. Individual proteins were first assessed to determine if their presence on the biomaterial surfaces could alter monocyte activation relative to their respective NC control. In this regard, the only protein to have a significant effect on monocyte activation on D-PHI was A2M, which elicited an increase in TNF-α release when pre-adsorbed to D-PHI for 24 hr, but not when pre-adsorbed to TCPS or PLGA. Note that D-PHI also showed the highest level of protein binding for A2M in a non-competitive single protein binding study (Figure 3.4), suggesting that D-PHI may have specific affinity for this protein.
Figure 3.4 Total A2M bound to biomaterial surfaces. Biomaterials were incubated with 100 μL of a 25 μg/ml solution of A2M in serum-free RPMI-1640 medium. Proteins were eluted with 2% SDS for 24 hr. Values are mean ± S.E. (n=13-15). * p<0.05 compared to D-PHI and PLGA.

Further differences between the biomaterials were noted when comparing conditions where individual proteins were coated on D-PHI, PLGA, or TCPS. In general, protein-coated PLGA surfaces generated significantly greater TNF-α release relative to protein-coated D-PHI at both 24 and 72 hr time-points (Figure 3.3a-f). PLGA has previously been shown to support the activation of macrophages, as demonstrated by the release of TNF-α and IL-1β from PLGA-adherent macrophages (Ding et al., 2009). Furthermore, treatment with PLGA microparticles has been shown to support the maturation of human monocyte-derived dendritic cells (DCs) in terms of pro-inflammatory cytokine secretion and the expression of costimulatory and MHC class II molecules, independent of microparticle phagocytosis (Yoshida and Babensee, 2006). This effect of PLGA on DC maturation was also apparent when monocyte-derived DCs were treated with 75:25 PLGA films, confirming the effect seen with PLGA microparticle treatment (Babensee and Paranjpe, 2005). When compared to other biomaterials, such as a poly(oxalate-co-oxamide), PLGA has also been shown to induce greater inflammatory TNF-α release from murine macrophage RAW 264.7 cells (Song et al., 2011). The pro-inflammatory nature of PLGA is thus well documented, and has even been taken advantage of in the case of Ali et al. (Ali and
Mooney, 2008), who used PLGA along with GM-CSF as a means to elicit an immune response by the conditioning of DCs for the purpose of vaccination or the treatment of autoimmunity.

Differences in the temporal trend in monocyte activation supported by protein-coated biomaterials were also evident. Over the 72 hr culture period, both D-PHI and PLGA surfaces, in general, displayed a decrease in pro-inflammatory TNF-α levels over time, indicative of an initial burst in pro-inflammatory cytokine levels characteristic of an acute response to the biomaterial that subsides over time. Several TCPS surfaces, however, including the NC control and AAT, ATIII, A2M, and VN pre-adsorbed surfaces do not support this same decrease in pro-inflammatory markers over time. Such findings may provide insight into the response that has been observed for TCPS relative to D-PHI (McBane et al., 2009). Previously, TCPS was shown to be the most activating surface for monocytes from a subset of candidate biomaterials (Matheson et al., 2007), and also supported a lower ratio of IL-10/TNF-α over time relative to D-PHI (McBane et al., 2009), where an increase in this ratio over time has previously been associated with a transition to a wound healing phenotype (Gretzer et al., 2006).

### 3.4.4 Mechanism of monocyte activation on α2-macroglobulin pre-adsorbed biomaterials

Among the six proteins that were investigated in the current study, A2M was considered of particular interest (Figure 3.3) given its reported role in the activation of monocytes/macrophages and its implication in the modulation of macrophage interactions on biomaterial surfaces (Anderson et al., 1999). The changes in monocyte activation between D-PHI, PLGA, and TCPS pre-adsorbed with A2M, in particular regarding differences in TNF-α release from adherent monocytes/macrophages, were investigated further by assessing how protein-receptor interactions could potentially be contributing to the observed cell response on the biomaterials. The A2M receptor, low density lipoprotein receptor-related protein 1 (LRP1), also known as α2-macroglobulin receptor (A2MR), is the principal known receptor for A2M and is primarily present during macrophage development (Williams et al., 1994). Furthermore, A2M will only interact with LRP1 when it has been protease- or methylamine-activated (denoted as A2M*) (Birkenmeier and Stigbrand, 1993). In this case the binding of proteases to A2M causes conformational changes that expose binding sites, resulting in increased binding capacity and
allowing for receptor-mediated endocytosis, necessary for the clearance of A2M-proteinase complexes.

A natural antagonist of LRP1 is receptor-associated protein (RAP), which is not present extracellularly; rather, it is an intracellular chaperone protein that associates with newly synthesized proteins, such as LRP1, preventing premature ligand interactions during transport to the cell surface (Willnow, 1998).

While RAP has not been used previously with primary human monocytes, it has been shown to block the upregulation of matrix metalloproteinase 9 (MMP-9) in macrophage-derived cell lines caused by A2M* (activated A2M) (Caceres et al., 2010). RAP is hypothesized to inhibit A2M*-LRP1 interactions through steric or competitive inhibition, or by inducing a conformational change that prevents ligand binding (Bu and Marzolo, 2000). Thus, RAP was used in the current studies to investigate the potential role of LRP1 in monocyte activation when the cells are interacting with A2M that has been pre-adsorbed to D-PHI, PLGA, and TCPS. Specifically, RAP was used to determine if the increase in TNF-α seen when A2M is pre-adsorbed to D-PHI is due to interactions of A2M with the receptor LRP1. To verify that RAP was not having an adverse effect on cell attachment, which may have confounded the interpretation of cytokine release data, DNA mass was quantified in the presence and absence of RAP on A2M pre-adsorbed and NC surfaces, as well as in the presence and absence of FBS (Figure 3.5). The inclusion of FBS was necessary in order to determine if the differential effects of A2M and the effect of the receptor LRP1 were also evident when competitive protein binding was occurring. The latter condition modelled aspects of in vivo conditions, as well as standard in vitro cell culture conditions.
Figure 3.5 DNA mass quantification at day 1 with (RAP+, black bars) or without (RAP-, white bars) the inhibitor RAP for monocytes cultured on biomaterial surfaces with (A) no FBS, A2M pre-adsorbed, (B) no FBS, no A2M pre-adsorbed, (C) FBS, A2M pre-adsorbed, or (D) FBS, no A2M pre-adsorbed. Values shown are mean ± S.E. and collected from 3 donors (FBS+ samples) or 4 donors (FBS- samples) with n=3 for each donor. For (A) and (B) DNA values are normalized to non-coated controls without FBS present. For (C) and (D), DNA values are normalized to non-coated controls with 10% FBS supplemented medium. DNA density for non-coated biomaterials without FBS are: D-PHI: 24,672.57 ± 1229.47 ng/cm², PLGA: 12,300.68 ± 1594.35 ng/cm², TCPS: 16,640.62 ± 801.93 ng/cm². DNA density for non-coated biomaterials with FBS are: D-PHI – 23,350.54 ± 540.60 ng/cm², PLGA – 3828.87 ± 746.80 ng/cm², TCPS – 20,480.50 ± 939.27 ng/cm². * p<0.05 compared to the same condition with RAP.

RAP was found to have no significant effect on cell attachment at 24 hr on D-PHI or TCPS, both in the presence (FBS+) or absence (FBS-) of serum proteins, and in the presence (A2M+) or absence (A2M-) of pre-adsorbed A2M. However, a reduction in cell attachment was observed for PLGA in both FBS+/- and A2M+/- conditions, although results were not significant for
samples with A2M pre-adsorbed to PLGA and incubated in FBS-containing medium (Figure 3.5). Based on these data it was concluded that the negative effect of RAP on cell attachment to PLGA is A2M-independent and was most likely biomaterial specific.

Previous experimental evidence exists for discriminating the effects of RAP on cell adhesion onto substrates. It has been shown that RAP inhibits connective tissue growth factor (CTGF) interactions with hepatic stellate cells (HSCs), where HSC adhesion to different forms of CTGF was inhibited in the presence of RAP (Gao and Brigstock, 2003). These reports suggest that LRPI mediates HSC adhesion to CTGF. RAP has also been shown to inhibit platelet adhesion to immobilized protein C (White et al., 2008), thus further demonstrating that LRPI mediates cell adhesion to a number of different proteins in addition to A2M. As a result, since the effect of RAP on cell adhesion was observed for PLGA both in the presence and absence of A2M, it is hypothesized that monocyte adhesion to PLGA is mediated by interactions of LRPI with another possible ligand, rather than by A2M.

To assess the potential implication of LRPI in A2M-induced TNF-α release on D-PHI relative to PLGA and TCPS, monocytes were incubated in the presence of RAP, for surfaces coated or non-coated with A2M, and the release of TNF-α (Figure 3.6) and IL-10 (Figure 3.7) from adherent human monocytes was assessed at 24 hr post-seeding.
Figure 3.6 TNF-α release from monocytes adherent to A2M pre-adsorbed (A) D-PHI, (B) PLGA, or (C) TCPS, with (black bars) or without (white bars) the inhibitor RAP. TNF-α levels were determined by measuring the TNF-α concentration in conditioned medium from cultured monocytes with an ELISA, with TNF-α allowed to accumulate for a 24 hr period prior to collection. Values shown are the mean ± S.E. and are from 3 donors with n=3 per donor. * p<0.05 compared to same condition with RAP. † p<0.05 compared to the same condition with FBS.

The hypothesized role of LRP1 in the A2M-mediated process of TNF-α release on D-PHI was confirmed, as it was demonstrated that the presence of RAP decreased TNF-α release on A2M pre-adsorbed D-PHI to the levels measured for the D-PHI NC control (Figure 3.6). Since LRP1 is known to only interact with A2M* (Birkenmeier and Stigbrand, 1993), this result suggests that A2M* is present in the bound protein layer when A2M is pre-adsorbed to D-PHI.

While previous studies have demonstrated that D-PHI reduces pro-inflammatory monocyte activation, as determined through TNF-α and IL-1β release from monocytes adherent to D-PHI vs. PLGA (>14 days) (McBane et al., 2011) and TCPS (24-72 hr) (McBane et al., 2009), results
with A2M (Figure 3.3c, Figure 3.6) suggested that D-PHI may be the more activating surface in the presence of bound A2M alone; however, in the presence of other serum proteins (Figure 3.6), D-PHI no longer supported the enhanced activation of monocytes in the presence of A2M, suggesting that D-PHI is a relatively non-activating surface for monocytes in the presence of bound serum proteins as compared to PLGA and TCPS.

While the current results support the concept that protein binding, in addition to surface chemistry, appear to contribute to the differences in monocyte activation observed previously between D-PHI and PLGA and TCPS, it was found that A2M alone does not appear to play a key role in these differentiated cell responses for substrates incubated in serum. It is hypothesized, rather, that a more complex group of the bound proteins and their interactions with one another that, along with the substrate surface chemistry of the biomaterial, resulted in the low-activating character of D-PHI when interacting with human monocytes. This hypothesis is supported by the data presented in Figures 3.6 and 3.7, where the presence of FBS (which is made up of multiple proteins), both in the presence and absence of pre-adsorbed A2M, resulted in a decrease in pro-inflammatory TNF-α for D-PHI and TCPS. While not statistically significant, TNF-α and IL-10 release for PLGA dropped to levels comparable to that for D-PHI and TCPS in the presence of serum proteins. An increase in anti-inflammatory IL-10 release from adherent monocytes in the presence of FBS was only observed for D-PHI and TCPS, but was not seen for PLGA. In general, there was greater donor to donor variability associated with the PLGA substrates despite it being a commercially pure material with surface topography that was similar to that of the other materials (Figure 3.6 and Figure 3.7), though PLGA displayed a consistent trend towards increased pro-inflammatory cytokine release in the absence of serum proteins.
Figure 3.7 IL-10 release from monocytes adherent to A2M pre-adsorbed (A) D-PHI, (B) PLGA, or (C) TCPS, with (black bars) or without (white bars) the inhibitor RAP. IL-10 levels were determined by measuring the IL-10 concentration in conditioned medium from cultured monocytes with an ELISA, with IL-10 allowed to accumulate for a 24 hr period prior to collection. Values shown are the mean ± S.E. and are from 3 donors with n=3 per donor. † p<0.05 compared to same condition with FBS.

A2M* interactions with LRP1 have previously been shown to induce proliferation (Bonacci et al., 2007) as well as MMP-9 expression (Caceres et al., 2010) in a macrophage-derived cell line. These A2M*-LRP1 interactions have previously been shown to be mediated by MAPK-ERK1/2 activation (Caceres et al., 2010; Bonacci et al., 2007). The levels of phosphorylated-ERK1/2 (p-ERK1/2, also reported as p-p44/p42) normalized to total intracellular ERK1/2 (p44/p42) were measured both in the presence and absence of RAP, as well as in the presence and absence of FBS and A2M, at 2 hr post-seeding to determine if the induction of TNF-α release, supported by A2M pre-adsorbed D-PHI, was also mediated by this intracellular signaling pathway. p-ERK1/2 was not significantly affected by the presence of A2M pre-adsorbed to any of the biomaterial surfaces (data not shown).
While the present study identified a number of candidate proteins potentially related to the differential activation of monocytes to D-PHI vs. PLGA and TCPS, other proteins not identified in the current study may have an important role in influencing monocyte response to these surfaces. Proteins such as immunoglobulin G (Jenney and Anderson, 2000), fibronectin (Sudhakaran et al., 2007), and serum amyloid P component (Kim et al., 2005) are known to influence monocyte activation. While these proteins may not differ in amount in the bound protein layer between D-PHI, PLGA, and TCPS, changes in conformation upon adsorption could occur that may elicit different biological responses (Garcia et al., 1999). Such proteins remain to be further investigated.

Differences in protein conformation supported by biomaterials have been observed between hydrophobic and hydrophilic surfaces resulting in differences in enzyme activity (Huang et al., 2011). In a separate study, it was demonstrated that polymer chain chemistry, rather than end-group chemistry, had a greater influence on adsorbed protein conformation (Abraham et al., 2011). There are significant experimental challenges in studying protein conformation on two-dimensional substrates (Huang et al., 2011). Furthermore, studies that are able to successfully probe protein conformation are limited to studying single-protein systems (Huang et al., 2011; Abraham et al., 2011). A possible future direction to pursue in order to better understand the differential monocyte activation observed between D-PHI, PLGA, and TCPS surfaces in the present study as well as in previous experiments (McBane et al., 2009; McBane et al., 2011) would be to probe the conformation of specific adsorbed proteins, such as A2M, on the aforementioned biomaterials both alone and during competitive protein adsorption in the presence of serum proteins. The previously observed differences in surface chemistry and surface wettability (Table 3.1) between D-PHI, TCPS, and PLGA would be anticipated to support differences in protein conformation that may be important in influencing monocyte activation to these surfaces.

3.5 Conclusions

The present study highlights the importance of protein binding in the context of mediating differential monocyte activation in a biomaterial-dependent manner. *In vitro* cell response to biomaterials relies primarily on the surface characteristics of the biomaterial and the surface-
specific composition of the bound protein layer in cases where the medium contains a complex mixture of proteins. Results indicated that D-PHI bound more protein than TCPS, a standard cell culture biomaterial, and PLGA, a non-cytotoxic and biodegradable polyester. The increase in bound protein on D-PHI is suspected to be associated with the diverse chemical functionality displayed at the D-PHI surface. Proteomic analysis identified several proteins differing in the composition of the bound protein layer for D-PHI, PLGA, and TCPS, which were then screened for their effect on monocyte activation. D-PHI was shown to be a relatively non-activating surface (low TNF-α) when compared to both non-coated and protein pre-adsorbed PLGA, while also supporting a different trend in activation when compared to TCPS. One serum protein in particular, A2M, was shown to support more TNF-α release when bound to D-PHI, with no effect when pre-adsorbed to TCPS and PLGA. This biomaterial-specific effect was shown to be due to the A2M receptor LRP1 being active on D-PHI, but not for the two other substrates. The effect of A2M, however, was not present under competitive protein binding conditions, demonstrating that A2M does not appear to be a critical protein implicated uniquely in the reduced monocyte activation seen for D-PHI vs. TCPS and PLGA. This study further demonstrates the importance of protein binding to implanted materials. It also illustrates the challenge of assigning a single factor, such as material surface chemistry or relative hydrophobicity, to explain complex phenomena such as protein binding and the subsequent cell response.

3.6 Acknowledgements

Special thanks to Dr. Eric Yang from Sunnybrook Hospital’s proteomics facility for discussions and help with the 2-DE, in gel digestion, LC-MS/MS, and protein identification. This study was funded by an NSERC/CIHR collaborative grant #337246/83459 (J.P. Santerre, R.S. Labow) and CIHR operating grant #230762 (J.P. Santerre, R.S. Labow). K.G. Battiston was funded by a CIHR Strategic Training Fellowship (STP-53877) and by an Ontario Graduate Scholarship (OGS).
3.7 Supplemental figures

Supplemental Figure 3.1 SEM images depicting (A) D-PHI, (B) PLGA, and (C) TCPS surfaces at 500x magnification.
Supplemental Figure 3.2 Silver-stained 2-D gels of bound proteins eluted from (A) D-PHI, (B) PLGA, and (C) TCPS biomaterial surfaces. Protein solutions obtained from incubating biomaterial surfaces in RPMI-1640 medium with 10% FBS and eluting the bound proteins with 2% SDS were concentrated with a 3 kDa MWCO membrane and excess SDS was removed with a chloroform-methanol precipitation protocol. 65 µg of protein was then loaded onto each gel. Arrows indicate spots that were excised from gels and identified by LC-MS/MS.
3.8 References


Chapter 4
Synthetic polymeric biomaterials that attenuate the interaction of immunoglobulin G with human monocytes: insights into immunomodulatory biomaterial design

4.1. Foreword

Differences in protein conformation upon adsorption to different biomaterials can alter the biological activity of serum proteins. This study investigated the hypothesis that D-PHI could attenuate pro-inflammatory monocyte response by limiting the exposure of pro-inflammatory binding sites on serum proteins. In particular, interactions of D-PHI with IgG were of interest due to the prominence of this protein in the adsorbed protein layer for many different biomaterials. IgG coating on TCPS supported monocyte retention and activation, while IgG coating on D-PHI had minimal effects relative to a non-coated control surface. This reduction in IgG-induced monocyte activation was shown to be due to D-PHI’s ability to limit exposure of the Fab region of the IgG molecule. It was further shown that D-PHI chemistry could be changed to modulate IgG Fab exposure, and in particular that D-PHI’s chemical heterogeneity plays a role in this process. This study identified the ability of D-PHI to render pro-inflammatory proteins non-activating by reducing key binding site exposure, and indicated aspects of material design important in this process that can inform the design of new immunomodulatory biomaterials.

This chapter has been submitted as: Battiston KG1, Ouyang B1, Honarpav Var E2, Qian J1, Labow RS3, Simmons CA1,2, Santerre JP1,2. Synthetic polymeric biomaterials that attenuate the interaction of immunoglobulin G with human monocytes: insights into immunomodulatory biomaterial design.

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4.2. Introduction

The implantation of a biomaterial initiates a series of events as part of the inflammatory response to a foreign body. This process significantly limits the effectiveness of many traditional and novel polymeric biomaterials, which have not considered the inflammatory process as an inherent feature of the material design. Within nanoseconds of exposure to blood and interstitial fluid following injury, proteins begin adsorbing to the material surface, transforming the surface character of the biomaterial into a biological substrate that will in turn direct the cellular response (Anderson et al., 2008). A significant component of the cellular response that occurs regardless of the site of implantation involves inflammatory cells, primarily neutrophils and monocytes (Anderson et al., 2008). Of particular importance for the long-term viability of the implanted biomaterial is the monocyte, as these cells will subsequently differentiate to macrophages (monocyte-derived macrophages, MDMs), which play a significant role in determining whether tissue regeneration and favourable wound healing occurs, or if the implanted object will be encapsulated and rejected via a classical foreign body response by the immune system (Brown et al., 2012a; Brown et al., 2012b).

There has recently been a focus on biomaterial based immune-stimulated therapies, wherein the use of specific biomaterials results in a desired immune response that has therapeutic value (Brown et al., 2012b). While in some cases these biomaterials have been used with success, their immunomodulatory capability has typically been revealed as a result of experimentation to assess the impact of the immune system’s response rather than as an initial design criterion. Examples include the use of methacrylic acid (MAA) beads, which have been shown to regulate macrophage gene expression (Fitzpatrick et al., 2011) and to promote a shift towards an alternatively activated macrophage phenotype (Patel and Sefton, 2012), as well as the pre-seeding of polyglycolic acid (PGA) mesh with a copolymer sealant solution of poly-l-lactide and -e-caprolactone [P(CL/LA)] with bone marrow mononuclear cells, where biomaterial-stimulated release of MCP-1 by adherent monocytes was determined to be responsible for the recruitment of host monocytes that would in turn support new tissue regeneration (Mirensky et al., 2010; Roh et al., 2010; Hibino et al., 2011). Other therapies involve biomaterial design with specific immune-targeting strategies, such as lentiviral delivery of IL-10 to maintain an anti-inflammatory
macrophage phenotype, where biomaterials are used as the delivery vehicle (Boehler et al., 2011; Boehler et al., 2014).

Without specific biochemical or molecular targeting features, however, there are few examples of biomaterials designed with the main goal being the modulation of interactions with immune cells which could lead to tissue regeneration and wound healing. The biomaterials field has aimed to understand the role of different biomaterial properties with respect to activating monocytes/macrophages. This provided insight into the importance of features such as surface chemistry and topography, amongst others (McWhorter et al., 2013; Ghrebi et al., 2013; Almeida et al., 2014; Vaine et al., 2013; Bota et al., 2010). Surface features are known to play an important role in regulating macrophage polarization, in particular with recent studies demonstrating that topographical features promote elongation of macrophages that subsequently favour polarization towards the M2 state (McWhorter et al., 2013; Ghrebi et al., 2013). Large pores and wide angles in 3D chitosan scaffolds have also been associated with increased pro-inflammatory cytokine release (Almeida et al., 2014), while textured microparticles (Vaine et al., 2013) and PTFE surfaces with different intranodal distances (Bota et al., 2010) have also provided an understanding of how surface topography regulates monocyte/macrophage activity. Pore size and fiber diameter of electrospun polydioxanone scaffolds have also been shown to regulate macrophage response, where pore size in particular has been shown to be an important regulator of macrophage polarization (Garg et al., 2013).

Surface chemistry is another material feature that has been shown to play an important role in regulating monocyte cytokine release. Hydrophilic and anionic surfaces have been shown to inhibit long-term monocyte adhesion and to support anti-inflammatory (increased IL-10, decreased IL-8) monocyte response (Brodbeck et al., 2002a; Brodbeck et al., 2002b; MacEwan et al., 2005), while hydrophobic and cationic surfaces have been shown to support foreign body giant cell (FBGC) formation (Brodbeck et al., 2002a; Brodbeck et al., 2002b; Shen et al., 2004). The findings of these and future studies will ultimately determine the success of immune-based strategies for improved healing and tissue regeneration as the field develops a better understanding of how different materials can be used to favour particular interactions with immune cells.
Effects of surface chemistry and other biomaterial properties on monocyte response, however, cannot be viewed in isolation from their effects on protein adsorption, as it is the material’s modulation of the adsorbed protein layer that will determine the nature of the surface which cells will adhere to and interact with. While there are studies that have looked at the effect of surface chemistry on protein adsorption (Barrias et al., 2009; Arima and Iwata, 2007), surface chemistry on monocyte activation (Brodbeck et al., 2002a; Brodbeck et al., 2002b; MacEwan et al., 2005), and adsorbed proteins on monocyte response (McNally et al., 2008; Kim et al., 2005; Collier and Anderson, 2002; Jenney and Anderson, 2000a; Jenney and Anderson, 2000b), there are relatively few studies that have explored the topic of monocyte activation as a function of surface chemistry by understanding how different surface chemistries regulate the bio-activity of the adsorbed protein layer. The work that has been undertaken in this area has been largely focused on coagulation proteins (Horbett, 1993). There is thus a significant need to understand how aspects of material chemistry can regulate protein interactions as a means to support favourable interactions with immune cells.

Studies have linked specific serum proteins as being important regulators of monocyte/macrophage response, such as immunoglobulin G (IgG) (Jenney and Anderson, 2000b), serum amyloid P component (Kim et al., 2005), fibrinogen (Gretzer et al., 2002; Kreuzer et al., 1996), fibronectin (Bajaj et al., 2007; Marom et al., 2007; Sudhakaran et al., 2007), and vitronectin (McNally et al., 2008), amongst others. In particular, IgG has been reported to be a strong supporter of long-term macrophage adhesion (Collier and Anderson, 2002; Jenney and Anderson, 2000a; Anderson et al., 1999). A degradable polar hydrophobic ionic polyurethane (D-PHI) has recently been shown to reduce the pro-inflammatory activation of adherent monocytes relative to tissue culture polystyrene (TCPS) (McBane et al., 2009). It was hypothesized that the adsorbed protein layer was an important factor in the differential response of monocytes to D-PHI vs. TCPS, and initial studies sought to identify the adsorbed proteins that were different between the two materials, of which α2-macroglobulin (A2M) was identified as a candidate protein. While A2M showed differential effects on D-PHI vs. TCPS, these differences were only observed in medium without serum, but not apparent in high serum containing medium (10% FBS) (Battiston et al., 2012b). In the present study, it was hypothesized that D-PHI may attenuate monocyte activation by supporting adsorption of serum proteins, such
as IgG, in a manner that is passivating. This is in contrast to the conventional knowledge about these proteins being pro-inflammatory in nature in their interactions with biomaterials, by supporting a conformation that triggers inflammatory cells via select binding sites (Jenney and Anderson, 2000b). The objective of the present study was therefore to assess IgG-induced monocyte activation on D-PHI and TCPS surfaces, and to determine any differences in the manner by which D-PHI interacts with IgG. D-PHI was then used as a case study in order to facilitate our understanding of how aspects of this material’s chemistry elicit favourable protein-monocyte interactions. The ultimate aim is to provide insight into features that can be useful for the design of new immunomodulatory biomaterials.

4.3. Materials and methods

4.3.1. D-PHI film preparation

D-PHI films were prepared by previously established methods (Sharifpoor et al., 2009). A divinyl oligomer (DVO), methacrylic acid (MAA), and methyl methacrylate (MMA) were mixed with the initiator benzoyl peroxide (BPO, 0.032 mol/mol vinyl group) and cured at 110°C in a nitrogen-filled oven. D-PHI formulations with varying hydrophobic and ionic character were also prepared by varying the DVO:MAA:MMA molar ratio, including 1:20:40 (↑ hydrophobic, ↑ ionic [HHHI]), 1:2:24 (↑ hydrophobic, ↓ ionic [HHLI]), 1:10:10 (↓ hydrophobic, ↑ ionic [LHHI]), and 1:1:7 (↓ hydrophobic, ↓ ionic [LHLI]).

4.3.2. Monocyte isolation and culture

Monocytes were isolated from the peripheral blood of healthy volunteers at Mount Sinai Hospital, Toronto, Ontario (University of Toronto ethics approval protocol #22203). Blood was layered onto Histopaque-1077 and separated by density gradient centrifugation. The buffy coat, containing mononuclear cells, was collected and subjected to a series of washes as described in detail elsewhere (Battiston et al., 2012b). Monocytes were seeded at a concentration of 200,000 per well in 96-well plates on non-coated (NC) or IgG-coated (human IgG, Jackson ImmunoResearch) D-PHI and TCPS surfaces. Protein pre-coating was performed by first incubating surfaces with PBS supplemented with 1% Penicillin/Streptomycin for 24 hr, followed
by a 24 hr incubation period with IgG in serum-free RPMI-1640 medium (200 µg/ml, 100 µl per well). Monocytes were cultured for 1 or 3 days on IgG and non-coated (NC) surfaces in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 10% autologous human serum (AHS), or no serum supplementation (serum free).

To assess the role of the Fab domain for IgG exposed to monocytes and its impact on monocyte activation, prior to cell seeding IgG-coated surfaces were incubated with a Fab-specific IgG (20 µg/ml) for 1 hr. Blocking antibody was also supplemented into medium with each medium change (20 µg/ml).

4.3.3. Scanning electron microscopy (SEM)

Samples were fixed with a 3% glutaraldehyde solution, subjected to ethanol dehydration, and critically point dried prior to sputter coating (8 nm platinum). Samples were analyzed using a Hitachi S 2500 Scanning Electron Microscope (Hitachi, Mito City, Japan) at an operating voltage of 10 kV (Faculty of Dentistry, University of Toronto).

4.3.4. DNA mass quantification

DNA mass was quantified following incubation of samples with a lysis buffer (0.05% Triton-X/EDTA). DNA mass was quantified with Hoechst 33258 DNA stain and compared to DNA standards prepared from calf thymus DNA (McBane et al., 2009).

4.3.5. Enzyme linked immunosorbent assays (ELISAs)

ELISAs for human TNF-α and IL-10 (eBioscience) were performed as per the manufacturer’s instructions on supernatants. Supernatants were collected 24 hr after a medium change, such that any cytokines detected would have been released over this time period.

4.3.6. Total protein quantification

Samples were coated with IgG as described in section 4.3.2, and were then rinsed 3x with PBS, taking care to minimize sample exposure to the air interface, after which surfaces were incubated
for 24 hr with a 2% SDS solution (200 µl per well). Protein content of the resulting solution was quantified using a micro bicinchoninic acid (BCA) assay (Thermo Scientific).

4.3.7. Fab exposure quantification

Following protein coating as described previously, Fab exposure was quantified using an HRP-conjugated Fab-specific IgG (Sigma) and a tetramethylbenzidine (TMB) substrate solution (eBioscience), with absorbance read at a wavelength of 450 nm.

4.3.8. Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) by analysis of variance (ANOVA) or by an independent samples t-test, where appropriate, with statistical significance reported for p<0.05. All experiments were repeated in triplicate with three technical replicates for each experimental value. Experiments performed with monocytes were repeated with three distinct donors.

4.4. Results

4.4.1. Surface bound IgG-induced monocyte activation – influence of serum and biomaterial type

The role of substrate dependent IgG-induced monocyte response was evaluated by coating IgG on D-PHI and TCPS surfaces and then culturing freshly isolated human monocytes from peripheral blood for up to 3 days on these surfaces. Cells were cultured in medium supplemented with either 10% FBS, 10% AHS, or no serum supplementation as previous studies have indicated a serum-dependence for monocyte response to biomaterials (Schmidt et al., 2011). IgG coating had minimal effect on monocyte adhesion, both initially and after 3 days of culture, when cells were cultured in serum free medium (Figure 4.1). However, when monocytes cultured on IgG-coated TCPS were maintained in serum-containing medium, there was a 3-4 fold increase in cell number at day 3 relative to a non-coated TCPS control (p<0.05). The same coating condition for
D-Phi, however, yielded marginal differences in DNA values between IgG-coated and non-coated conditions for any medium type (Figure 4.1).

**Figure 4.1** Effects of IgG pre-adsorption in different growth media on monocyte attachment to TCPS and D-Phi substrates. Data are the mean ± S.E. N=8 from 3 donors. All DNA values are normalized to the non-coated condition for their respective growth medium (no serum, 10% AHS, or 10% FBS). *p<0.05 vs. respective non-coated control.

Cytokine release studies were performed to determine the release of TNF-α (pro-inflammatory) and IL-10 (anti-inflammatory) due to differences in protein coating (IgG, no coating) as well as serum type (no serum, AHS, FBS). Cytokine levels were plotted as the ratio of TNF-α/IL-10, such that a higher number indicates a more pro-inflammatory state. IgG-coated TCPS supported an initial burst in TNF-α/IL-10 at day 1, whereas there was no protein dependence in cytokine release for monocytes adherent to D-Phi. No differences in protein coating were seen for either material at day 3 (Figure 4.2). Trends in cytokine serum-dependence were the same for both TCPS and D-Phi, however overall ratio values were generally lower when cells were cultured
on D-PHI. Initially at day 1, AHS supported an increase in TNF-α/IL-10, regardless of protein coating, whereas by day 3 AHS had the lowest level of TNF-α/IL-10, while no serum and FBS both had higher levels of TNF-α/IL-10. In terms of absolute cytokine levels, however, FBS supported the greatest amount of both TNF-α and IL-10 release (approximately 10-fold greater), regardless of protein coating or material type (data not shown, p<0.05).
**Figure 4.2** Cytokine release presented as the ratio of TNF-α to IL-10 and expressed as a function of protein coating (white bars) or growth medium (black bars). When expressed as a function of protein coating or growth medium data reflect the aggregate of all medium conditions or all protein coatings, respectively, in order to emphasize the effect of protein coating or growth medium. Data represent the mean ± S.E. n=24 from 3 donors. *p<0.05.

SEMs of adherent monocytes, similar to DNA and cytokine data, showed a protein and serum dependence for TCPS, and only a serum-type dependence for D-PHI. Monocytes found on IgG-coated TCPS appeared much larger with greater cell spreading when compared to the non-coated
control, while no difference in cell morphology is observed for monocytes adherent to IgG-coated vs. non-coated D-PHI. Monocytes on both materials, however, exhibited a serum dependence. Monocytes appear the largest with the greatest amount of cell spreading in AHS, followed by FBS and the no serum conditions, respectively (Figure 4.3).

![Figure 4.3](image)

**Figure 4.3** Representative SEM micrographs demonstrating monocyte morphology on TCPS and D-PHI surfaces 3 days post-seeding for the different protein pre-adsorption conditions (IgG, non-coated) in different growth media (no serum, 10% AHS, 10% FBS). Scale bars represent 30 µm.

**4.4.2. D-PHI and TCPS differ in adsorbed IgG conformation**

No difference was observed in total bound IgG between D-PHI and TCPS (Figure 4.4).
Figure 4.4 Quantification of IgG pre-adsorption to TCPS and D-PHI substrates. (A) Total IgG bound to D-PHI and TCPS surfaces following pre-adsorption. n=6. (B) IgG Fab exposure on TCPS vs. D-PHI surfaces in the presence of 10% AHS (black bars), 10% FBS (gray bars) or no serum (white bars). n=24 for 3 donors for AHS samples, while n=8 for FBS and no serum samples. Data represent the mean ± S.E. (C) DNA mass quantification and (D) SEM images for monocytes seeded on IgG pre-adsorbed and non-coated D-PHI and TCPS surfaces, and exposed or not exposed to an Fab-specific blocking antibody. n=9 from 3 donors. Data represent the mean ± S.E. Scale bar represents 30 μm. *p<0.05 vs. NC condition.

However, it was found that TCPS supported a significant increase in Fab exposure, which was apparent both immediately after coating the surfaces with IgG, as well as following a 24 hr
incubation in either AHS or FBS supplemented medium (Figure 4.4). Fab exposure was also shown to decrease following incubation in serum containing medium for both TCPS and D-PHI.

4.4.3. Limiting adsorbed Fab-site exposure reversed pro-adhesion effects of IgG

Because Fab exposure was shown to be the highest on TCPS, the surface on which IgG coating resulted in increased monocyte survival and cell spreading, experiments were performed to confirm the specific association of Fab exposure in the observed IgG-monocyte interactions. Monocytes were cultured for 3 days on non-coated TCPS and D-PHI, as well as on IgG-coated surfaces, both in regular medium or medium supplemented with a Fab-specific blocking antibody. Experiments were performed only for AHS supplemented medium since under this medium condition IgG produced the greatest effects (Figures 4.1, 4.3). Blocking the exposed IgG Fab domain prevented IgG-induced increases in monocyte adhesion and spreading, while blocking exposed Fab on D-PHI had no effect on monocyte response (Figure 4.4).

4.4.4. Polyurethane chemistry influences adsorbed IgG Fab site exposure and monocyte activation

Given the unique attribute of low IgG-monocyte interactions for D-PHI, it was of interest to further examine the inherent chemistry of D-PHI and to determine which aspects of the chemical features of the material are relevant to its innate interaction with IgG and human monocytes. D-PHI is fabricated using the monomeric components DVO (polar), MAA (anionic), and MMA (hydrophobic) combined in a 1:5:15 (DVO:MAA:MMA) molar ratio (Sharifpoor et al., 2009). To explore the role of D-PHI chemistry in modulating IgG Fab site exposure, a family of polyurethanes with increased/decreased anionic and/or hydrophobic content relative to the polar DVO were fabricated (refer section 4.3.1 in Materials and Methods). None of the formulations indicated that they had a significantly reduced Fab site exposure relative to the original D-PHI formulation, but one formulation (LHLI) supported a significant increase in Fab site exposure relative to D-PHI (Figure 4.5A). No differences in total bound IgG were observed between the different formulations (Figure 4.5B).
Figure 4.5 (A) Quantification of Fab exposure and (B) total IgG adsorbed following incubation of different surfaces with 200 µg/ml IgG solution. Ratios on the x-axis indicate the molar ratio of DVO:MAA:MMA used in the different D-PHI formulations. * p<0.05 vs. the original D-PHI formulation (1:5:15). Data represent the mean ± S.E. n=9.

IgG-coated LHII D-PHI was subsequently shown to support an increase in monocyte adhesion and cell spreading, effects which were similar to that observed with IgG-coated TCPS, but not IgG-coated D-PHI (Figure 4.6).
Figure 4.6 (A) SEM images and (B) SEM image quantification for monocytes adherent to IgG-coated and non-coated D-PHI (1:5:15) vs. LHLI D-PHI. n=9 from 3 donors. Data represent the mean ± S.E. Scale bar represents 30 µm. (C) DNA mass quantification for original TCPS, the original D-PHI formulation (1:5:15 DVO:MAA:MMA), and the new formulation with decreased hydrophobic and ionic content (1.4:2:10). n=9 from 3 donors. Data represent the mean ± S.E. *p<0.05 vs. non-IgG-coated control (dashed line).

Characterization of the original D-PHI formulation vs. LHLI D-PHI indicated differences in contact angle were marginally different, with LHLI D-PHI possessing a slightly lower contact angle than D-PHI. Despite this finding, LHLI D-PHI showed less swelling than D-PHI when incubated in culture medium for 5 days (Table 4.1). No differences were observed in gel content. XPS analysis indicated reduced surface nitrogen and carbonate content for LHLI D-PHI vs. the original formulation (Table 4.1).
Table 4.1 Summary of physical and chemical properties of D- PHI vs. LHLI D-PHI *p<0.05 vs. LHLI D-PHI

<table>
<thead>
<tr>
<th>Property</th>
<th>D-PHI</th>
<th>LHLI D-PHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Resolution XPS (atomic %) (n=3, ± S.E.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon</td>
<td>71.05 ± 0.28</td>
<td>70.93 ± 1.77</td>
</tr>
<tr>
<td>Oxygen</td>
<td>26.63 ± 0.30</td>
<td>25.39 ± 1.37</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>2.32 ± 0.03*</td>
<td>2.08 ± 0.05</td>
</tr>
<tr>
<td>High Resolution XPS (atomic %) (n=3, ± S.E.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CH (285 eV)</td>
<td>57.39 ± 2.08</td>
<td>62.50 ± 5.63</td>
</tr>
<tr>
<td>-COOH or CO(O-) (287 eV)</td>
<td>30.22 ± 1.60</td>
<td>28.50 ± 3.35</td>
</tr>
<tr>
<td>O=C(O-)₂ (289 eV)</td>
<td>6.37 ± 0.98*</td>
<td>2.51 ± 0.45</td>
</tr>
<tr>
<td>O=C(O-)(N-) (289 eV)</td>
<td>6.02 ± 0.26</td>
<td>6.49 ± 1.89</td>
</tr>
<tr>
<td>Contact angle (°) (n=10, ± S.E.)</td>
<td>81.4 ± 1.4*</td>
<td>77.8 ± 2.9</td>
</tr>
<tr>
<td>Gel content (n=5, ± S.E.)</td>
<td>99.23 ± 0.94</td>
<td>99.55 ± 0.47</td>
</tr>
<tr>
<td>Swelling (%) (n=5, ± S.E.)</td>
<td>11.7 ± 0.6*</td>
<td>4.1 ± 0.3</td>
</tr>
</tbody>
</table>

4.5. Discussion

Protein adsorption has long been viewed as a critical component regulating the cellular response to biomaterial surfaces. Of particular importance to implanted biomaterials is the response of the monocyte, as these cells will subsequently differentiate to macrophages and contribute to a chronic inflammatory response, leading to implant failure, or constructive remodeling as a function of the nature of their interaction with the biomaterial and its adsorbed protein layer.
While many studies have linked specific serum proteins with different monocyte responses on biomaterials, there has been little work reported on with respect to introducing material chemistry changes based on how the conformational expression of these serum proteins may be involved in regulating aspects of the monocyte response to biomaterial surfaces.

IgG comprises 75% of serum immunoglobulins and is consistently found to be a significant component of the adsorbed protein layer for many different biomaterial surfaces (Shen et al., 2004; Walkey et al., 2014). While interactions of IgG through Fc receptors on monocytes is well recognized, the ability of the Fab domain within the IgG molecule to interact with monocytes is significantly less studied and well understood (Jenney and Anderson, 2000a; Polat et al., 1993). Work in the biomaterials field, however, has indicated that the ability of IgG to support macrophage adhesion is mediated by the Fab region of the molecule (Jenney and Anderson, 2000b). In the present study, we have used a degradable polyurethane, which has previously been shown to reduce pro-inflammatory monocyte response (McBane et al., 2009), as a case study to illustrate how material chemistry can be used to regulate protein-material interactions and thus subsequently protein-cell interactions, with particular focus on IgG due to its known importance in biomaterial-monocyte interactions (Shen et al., 2004; Jenney and Anderson, 2000b; Kalltorp et al., 1999). TCPS was used as a reference material as it has been well characterized with respect to its ability to support a pro-inflammatory monocyte state, as well as for its wide relevance to in vitro cell culture studies (McBane et al., 2009; Schmidt et al., 2011).

The initial response of monocytes to a biomaterial surface can be evaluated in terms of how well the substrate supports monocyte adhesion. The spreading of the cell on the surface subsequent to adhesion can provide a measure of cell activation (McWhorter et al., 2013), as well as the release of characteristic pro- and anti-inflammatory cytokines (Anderson et al., 2008). IgG was coated onto biomaterial surfaces specifically designed to assess the relevance of the biomaterial substrate in determining subsequent inflammatory cell interactions. IgG coating on D-PHI and TCPS resulted in significant differences in monocyte attachment. IgG coating on TCPS produced a 3–4 fold increase in adherent monocyte number (dependent on serum type), whereas there was no effect of IgG coating with D-PHI (Figure 4.1). Increases in monocyte attachment to biomaterial surfaces has long been viewed as being positively correlated with an adverse acute immune response to the biomaterial surface (Schutte et al., 2009), and IgG is typically viewed as
a protein that can induce pro-inflammatory monocyte interactions (Jenney and Anderson, 2000b). However, an increase in monocyte attachment does not always indicate an enhanced pro-inflammatory response, as these cells, while present on any material surface, although increased in number, may or may not be affected by that substrate to be activated in a pro-inflammatory manner (Brown et al., 2012a; Mosser and Edwards, 2010). SEM and cytokine analysis, however, both confirmed the ability of IgG-coated TCPS, but not IgG-coated D-PHI, to support activation of adherent monocytes to a pro-inflammatory state through both cell spreading/enlargement, indicative of monocyte-to-macrophage differentiation (Figure 4.3), as well as an increase in the release of TNF-α/IL-10 (Figure 4.2). Monocyte activation on a biomaterial surface can be evaluated in part by the morphology of these cells, where increased cell size, cytoplasmic spreading, and pseudopodia are markers of activation (Boccafoschi et al., 2013). Together these data support IgG’s role in propagating an acute pro-inflammatory monocyte response on TCPS, while an attenuation of the pro-inflammatory monocyte response in the presence of IgG is observed on D-PHI.

Many studies using primary human monocytes maintain their cultures in xenogeneic serum-containing medium, mostly commonly fetal bovine serum (FBS) (McBane et al., 2009; Schmidt et al., 2011). Given the large disconnect that already exists between in vitro culture platforms for testing new biomaterials and the in vivo response to these materials, the use of autologous human serum, which can readily and non-invasively be isolated from the same peripheral blood used to collect the patient’s monocytes, can provide a more relevant culture environment for testing monocyte-biomaterial interactions. Recent studies have indicated differences in cytokine release and monocyte adherence in the presence of FBS vs. AHS containing medium that displays a biomaterial dependence (Schmidt et al., 2011). For example, while differences in adherent cell density displayed an order of magnitude difference between FBS and AHS for PEG hydrogels, no difference was observed between serum conditions for PDMS substrates (Schmidt et al., 2011). Differences in monocyte response to serum sources can be associated with a number of factors. First, there is potential for a difference in both the type and amount of proteins found in bovine vs. human sera. Second, while many bovine and human proteins contain significant sequence homology, differences exist that can make human cells more responsive to the human version of the same protein. Indeed, it has been shown that human cells can be more responsive
to the human form of certain proteins, which show higher bioactivity at lower concentrations vs. xeno-versions of the same protein (Meszaros et al., 1995). In the present study, monocyte spreading was significantly enhanced by the presence of AHS vs. FBS or no serum (Figure 4.3). Cytokine release profiles also showed a serum dependence, with AHS producing the highest ratio of TNF-α/IL-10 at day 1, but the lowest at day 3 (Figure 4.2). This trend was apparent for both TCPS and D-PHI, and was independent of protein coating. The lack of monocyte response to IgG-coated TCPS in no serum medium, and the reduced cell spreading and adhesion in FBS containing medium vs. AHS, highlight the importance of using AHS for such studies and the possibility of missing important effects when xenoproteins are used instead.

Because the principal material-dependent observation for differentiated monocyte interactions with TCPS and D-PHI occurred in the presence of AHS containing medium, subsequent experiments exploring the monocyte response to the IgG coated surfaces focused solely on this medium condition. Differences in monocyte response to protein coated surfaces may be due to differences in protein conformation due to the biomolecule’s potential denaturation upon interaction with the biomaterial, which can lead to differential exposure of key ligands important for protein-cell interactions (Thyparambil et al., 2014). Alternatively, differences in protein-substrate affinity can result in differences in the total amount of bound protein, leaving one surface with more protein available for cellular interactions (Jenney and Anderson, 2000b; Battiston et al., 2012a). No differences were observed in total bound IgG between TCPS and D-PHI, while TCPS was shown to support a greater increase in the exposure of the Fab domain (Figure 4.4). While Fc interactions with monocytes have been well characterized, in terms of supporting enhanced cell spreading and long-term attachment, previous studies have implicated the Fab domain as being critical for monocyte interactions (Jenney and Anderson, 2000b). In a study by Jenney et al., adsorbed IgG was shown to support enhanced long-term macrophage adhesion, and this effect was also shown to be induced by adsorbed Fab and Fab₂, but no effect was seen for the adsorbed Fc fragment (Jenney and Anderson, 2000b). Surfaces that support IgG upon adsorption in a manner that preferentially exposes the Fab region might then be expected to support adhesion and spreading of monocytes/macrophages, as is the case with the quantified Fab exposure for D-PHI and TCPS. Here, TCPS, with a higher Fab exposure than D-PHI,
supported enhanced adhesion and spreading of monocytes when coated with IgG, whereas D-PHI did not.

While data showing that higher Fab exposure correlates to specific effects with monocytes may implicate Fab exposure as being critical to monocyte response, it is not definitive. Further experiments were performed to confirm the critical role of exposed Fab in adsorbed IgG-monocyte interactions by comparing the monocyte response to adsorbed IgG relative to Fab-blocked. Since Fab site blocking prevented IgG-mediated increases in cell spreading and adhesion for TCPS and not D-PHI, it can be concluded that IgG-Fab exposure is a critical factor necessary for IgG to support increases in monocyte adhesion and spreading. Thus, it is a parameter that should be considered in all biocompatibility assessments, just as important as clotting time and platelet aggregation.

The ability of D-PHI to limit Fab exposure upon binding of IgG from solution thus prevents IgG from imparting its pro-inflammatory effects on adherent monocytes. Furthermore, the aspect of D-PHI responsible for these surface-protein interactions can help provide insight that would be useful in the design of new immunomodulatory biomaterials. A significant factor differentiating D-PHI from TCPS is the surface chemistry of the two materials (Battiston et al., 2012a). Of note, D-PHI possesses polar, hydrophobic, and ionic character, not dissimilar to the diversity of chemistry on display in the different amino acid residues that are typically found in proteins. The chemical diversity present in the structure of D-PHI may be a critical factor in supporting favourable biomaterial-protein interactions. To explore this hypothesis the chemistry of D-PHI was manipulated by varying the hydrophobic (MMA) and anionic (MAA) monomer content relative to the polar (DVO) content. These formulations were screened for their effect on Fab exposure. Of the formulations tested, one formulation (LHLI – low hydrophobic, low ionic) supported a significant increase in Fab exposure similar to TCPS (Figure 4.5), suggesting that the content and possibly distribution of ionic and hydrophobic moieties was critical in the design of the biomaterial. To confirm the role of Fab exposure in supporting monocyte adhesion and spreading, IgG-coated LHLI D-PHI was compared to IgG-coated D-PHI and TCPS. If Fab exposure is important for supporting monocyte interactions, it would be expected that IgG-coated LHLI D-PHI would support increased monocyte adhesion and spreading due to its
support of increased Fab exposure upon IgG binding. Indeed, IgG-coated LHLI D-PHI, similar to TCPS, supported increases in monocyte attachment and spreading (Figure 4.6).

By decreasing the hydrophobic and ionic monomer content of D-PHI, the final polymer resembles more of a homopolymer (DVO) structure with reduced hydrophobic and ionic character. This reduction in chemical diversity may limit the ability of proteins to maintain their native conformation upon interaction with the biomaterial surface, resulting in changes in conformation and unfolding that are necessary to expose distinct binding sites that are important for supporting protein-cell interactions (Thyparambil et al., 2014; Lan et al., 2005). The degree of denaturation a protein experiences can have a critical role in its ability to interact with different cell types. Adsorbed albumin, for example, is considered to be a passivating surface that does not support specific interactions with cells. However, it has recently been shown that surfaces that induce a minimum change in albumin unfolding upon interacting with the biomaterial surface can result in similar levels of platelet activation as that reported for adsorbed fibrinogen. Likewise, the ability of fibrinogen to induce platelet activation is positively correlated with the degree to which the protein is denatured by the surface (Sivaraman and Latour, 2012).

While changing D-PHI chemistry produced changes in Fab exposure, other material properties in addition to surface chemistry can also be affected by this change. In particular, surface wettability and morphology are important factors with known effects on monocyte response to biomaterial surfaces (Bota et al., 2010; Brodbeck et al., 2002a; Arima and Iwata, 2007) that can be impacted by the changes in chemistry introduced with LHLI D-PHI. Hydrophobic polymer surfaces have been associated with increased monocyte adhesion and spreading, while hydrophilic surfaces have been associated with decreased monocyte adhesion (Patel et al., 2003). In the current study, there were very little differences in the contact angle values (Table 4.1). In other studies, hydrophobic surfaces have also been shown to promote the release of pro-inflammatory cytokines (TNF-α, IL-1β, MCP-1), while monocytes adherent to some hydrophilic surfaces secrete fewer pro-inflammatory cytokines and more anti-inflammatory mediators, such as IL-10 (Patel et al., 2003). Ionic surface character, as well as the nature of that ionicity (cationic vs. anionic), is another chemical feature with important implications for monocyte-biomaterial interactions. Anionic surfaces have been shown to promote decreased adhesion and
macrophage fusion to foreign body giant cells, with the opposite effect observed for cationic surfaces (Brodbeck et al., 2002b; Brodbeck et al., 2002a).

Material properties that were compared for the two D-PHI formulations included gel content (monomer conversion), surface wettability (advancing water contact angle), and surface chemistry (low and high resolution XPS). No difference was observed in gel content between the two D-PHI formulations, with both demonstrating gel content values of >99%, indicating similar monomer conversion (Table 4.1). While a statistical difference was observed in contact angle measurements for LHLI D-PHI (77.8 ± 2.9) vs. original D-PHI (81.4 ± 1.4), these differences were minimal and such differences would not be expected to influence protein or cellular activity (Arima and Iwata, 2007). No differences were observed for low resolution XPS of the two surfaces. High resolution XPS analysis revealed a 2-fold decrease in carbonate bond presence on the surface of LHLI D-PHI (p=0.05) despite DVO comprising a significantly greater proportion of the monomer mixture. None of the properties measured to compare LHLI D-PHI vs. the original D-PHI formulation (contact angle, XPS) explain the differences in protein (IgG) or cellular (monocyte) interactions observed with the change in chemistry introduced with LHLI D-PHI, suggesting that the specific change in chemistry is likely the primary mediator resulting in the different protein and cell response observed with this material.

4.6. Conclusions

The results presented here demonstrate that a degradable polyurethane, D-PHI, is able to reduce monocyte retention and spreading through limiting exposure of the Fab region of the IgG molecule. We further show that Fab exposure can be modulated by producing a D-PHI formulation that moves towards a homopolymer composition (DVO), i.e. by minimizing the contribution of MMA and MAA to the material chemistry, demonstrating the importance of biomaterial chemistry in regulating biomaterial-protein and ultimately biomaterial-cell interactions. While the present study used D-PHI to illustrate the importance of biomaterials in regulating cellular interactions through the biomaterial’s mediation of the adsorbed protein layer, there are important insights that have applicability beyond D-PHI, to be applied to other biomaterials, degradable or non-degradable. An understanding of how biomaterial chemistry interacts with proteins important for governing cellular interactions can lead to the design of new
biomaterials that enable the promotion of specific biological effects through their mediation of the adsorbed protein layer.

4.7. Acknowledgements

This study was funded by CIHR operating grant #230762 (J.P. Santerre, R.S. Labow, C.A. Simmons). K.G. Battiston was funded by a CIHR Strategic Training Fellowship (STP-53877) and by an Ontario Graduate Scholarship (OGS).

4.8. References


Chapter 5
Monocyte/macrophage cytokine activity regulates vascular smooth muscle cell function within a degradable polyurethane scaffold

5.1. Foreword

Biochemical strategies to promote the growth and infiltration of VSMCs in 3D biomaterial scaffolds require the use of recombinant growth factors that are expensive and have the potential for endotoxin contamination that can result in an adverse foreign body response following implantation. Monocytes and their derived macrophages are a readily available autologous source of stimulatory biomolecules provided they are used in combination with biomaterials that elicit a favourable monocyte/macrophage activation state. To investigate the use of monocytes to contribute to a vascular tissue engineering strategy, monocytes were co-cultured with VSMCs on D-PHI scaffolds for 28 days, and the results were compared to monocultures of VSMCs and monocytes run in parallel. Monocyte-VSMC co-culture was shown to support a shift to a synthetic VSMC phenotype (increased growth and infiltration into porous D-PHI scaffolds, decreased contractile marker expression) through the release of stimulatory biomolecules, such as IL-6. This study supports the use of D-PHI to modulate the activation of monocytes in a manner that allows them to positively regulate the response of VSMCs for a vascular tissue engineering strategy.

This chapter has been published in ‘Acta Biomaterialia’ as: Battiston KG\(^1\), Ouyang B\(^1\), Labow RS\(^2,3\), Simmons CA\(^{1,4}\), Santerre JP\(^{1,4}\). Monocyte/macrophage cytokine activity regulates vascular smooth muscle cell function within a degradable polyurethane scaffold. *Acta Biomaterialia* 2014;10(3):1146-55.

5.2. Introduction

Small diameter vascular grafts (<6 mm internal diameter) are prone to failure due to complications arising from thrombogenesis and neointimal hyperplasia (associated with vascular smooth muscle cell (VSMC) migration and proliferation). VSMCs can shift between contractile and synthetic phenotypes depending on the stimuli to which the VSMCs are exposed (Chan-Park et al., 2009). For strategies related to the in vitro development of tissue-engineered vascular grafts, the synthetic phenotype is initially desirable as it is associated with VSMC proliferation and matrix synthesis, which leads to a faster maturation of the vascular tissue in vitro (Chan-Park et al., 2009; Laflamme et al., 2006). Accelerating the rate of cell and tissue growth into biomaterials intended for use as tissue engineered constructs is important because, typically, in vitro strategies to develop a vascular graft with sufficient cellular and mechanical properties have required prolonged culture times (≥ 4 weeks) (Laflamme et al., 2006; Syedain et al., 2011). These extended culture times increase the risk of bacterial contamination, introduce labor-intensive costs for the culture practises, and ultimately increase the wait time for a patient to receive treatment if the graft is being prepared using an autologous cell source.

A commonly used strategy to promote a desired VSMC phenotypic state involves the use of growth factor stimulation (Chan-Park et al., 2009; Stegemann and Nerem, 2003; Ross et al., 2006; Risinger et al., 2010; Risinger et al., 2010). Transforming growth factor-β1 (TGF-β1) is one example of a biological factor that is used for such purposes and has been able to promote the contractile VSMC phenotype, which is characterized by the expression of α-smooth muscle
actin (α-SMA), calponin, and smooth muscle myosin heavy chain (SMMHC) (Ross et al., 2006; Gong et al., 2009; Wang et al., 2010). Moreover, TGF-β1 has been shown to support an increase in matrix production, such as collagen and elastin (Syedain and Tranquillo, 2011). Despite these effects, TGF-β1 exposure is also associated with the inhibition of proliferation (Stegemann and Nerem, 2003; Risinger et al., 2010), which thus hinders the necessary expansion of VSMCs during the in vitro development of tissue engineered vessels. In contrast to TGF-β1, growth factors that can be used to promote the synthetic VSMC phenotype include platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF), and fibroblast growth factor (FGF), all of which promote the growth of VSMCs (Chan-Park et al., 2009). Despite their promise, the use of exogenous growth factors can add significant cost and time to the production of a tissue engineered graft, particularly if its supplementation is required over long culture times. Furthermore, the issue of endotoxin contamination associated with the use of recombinant proteins (Wakelin et al., 2006) can ultimately lead to impaired in vivo wound healing responses (Daly et al., 2012) in addition to affecting the phenotype of cells cultured in vitro (Bajaj et al., 2007; Sandbo et al., 2007).

An alternative strategy for modulating VSMC phenotype involves the use of monocytes and monocyte-derived macrophages (MDM). These are early cell types present following biomaterial implantation (Anderson et al., 2008), and depending on their phenotypic state they can be positive contributors to tissue regeneration and remodelling (Brown et al., 2012a; Brown et al., 2012b). Monocytes can easily and relatively non-invasively be isolated from the mononuclear fraction of peripheral blood and can act as an endogenous source of stimulatory cytokines and growth factors, which can be used in place of growth factor medium supplementation. The ability of monocytes and MDM to provide stimuli to induce a desired phenotype has also been used in several stem cell differentiation strategies, such as promoting osteoblast formation from mesenchymal stem cells (MSCs) (Nicolaidou et al., 2012), as well as regulating their growth (Freytes et al., 2013). These studies highlight the successful use of monocytes in modulating the phenotypic state of other cells. Such strategies require the use of a biomaterial that supports a particular MDM state, which in the case of vascular tissue engineering is one that is supportive of VSMC growth and function, whether it is through direct cell-cell interactions or the release of
particular cytokines and growth factors (Kunigal et al., 2003; Boyle et al., 2001; Proudfoot et al., 1999; Ikeda et al., 1998).

The phenotype of monocytes and MDM is heterogeneous and can vary depending on different environmental stimuli, both in vitro and in vivo (Mosser and Edwards, 2010). Biomaterial surface properties (Brodbeck et al., 2002; Bota et al., 2010) and the adsorbed protein layer (McNally et al., 2008; Sudhakaran et al., 2007) are two important factors influencing the monocyte’s activation state. Biomaterials that have been designed to elicit a desirable activation state of the monocyte/MDM can harness the potential of these cells to act as stimulatory agents for other cell types through the release of appropriate cytokines and growth factors. It has been shown that the M2 (anti-inflammatory) macrophage state is associated with the promotion of VSMC proliferation (Khallou-Laschet et al., 2010) and that supernatants derived from M2 vs. M1 (or classically activated, pro-inflammatory) macrophages support enhanced migration of C2C12 myoblasts (Brown et al., 2012a).

Monocyte/MDM dysfunction with respect to their activation in healthy tissue has been associated with adverse outcomes. Co-culture systems with monocytes/MDM that are induced into a pro-wound healing, anti-inflammatory state by a biomaterial thus hold promising potential in tissue engineering (Brown et al., 2012a; Brown et al., 2012b). With particular regard to vascular tissue engineering, monocytes have successfully been shown to increase graft patency when pre-seeded on grafts composed of a non-woven poly-glycolic acid mesh with a 50:50 copolymer sealant of poly-ε-caprolactone-L-lactide (Mirensky et al., 2010). A critical aspect of the monocyte’s role in this process is thought to occur through the release of secreted factors, of which MCP-1 was shown to be of particular importance. MCP-1 aids in the recruitment of host monocytes following implantation, which are then able to contribute to the regeneration of vascular tissue (Roh et al., 2010).

Previous work with a degradable polar hydrophobic ionic polyurethane (D-PHI) has illustrated the ability of this material to support a 2D VSMC-monocyte co-culture system for prolonged culture times with limited inflammatory cytokine production (McBane et al., 2011a) as well as being able to support tissue infiltration with minimal inflammation relative to PLGA when implanted subcutaneously in rats (McBane et al., 2011b). The objective of the present study was
to investigate the effect of monocyte/MDM-released factors on VSMC response within 3D, porous D-PHI scaffolds with regards to growth, 3D migration, and contractile marker expression, and to identify key proteins that may contribute to specific effects on VSMC phenotype observed in a VSMC-monocyte co-culture system on D-PHI scaffolds. These factors were compared to the observations seen within the co-culture in order to determine if monocyte/MDM-released factors vs. cell-cell interactions are primarily responsible for the observed VSMC activity in monocyte-VSMC co-culture on 3D D-PHI scaffolds.

5.3. Materials and methods

5.3.1. D-PHI scaffold preparation

D-PHI scaffolds were prepared using previously established methods (Sharifpoor et al., 2010; Sharifpoor et al., 2009). Briefly, a divinyl oligomer (DVO), methacrylic acid (MAA), and methyl methacrylate (MMA) were mixed in a 1:5:15 molar ratio along with the initiator benzoyl peroxide (0.032 mol/mol vinyl group) and 10 wt% polyethylene glycol and 65 wt% sodium bicarbonate as porogens. The resulting mixture was packed into Teflon molds and allowed to cure for 24 hr at 110°C. Porogen leaching was performed by 14 x 2 hr periods of sonication with ddH₂O, with water changed between each sonication period. This process resulted in a porous scaffold with 79 ± 3 % porosity (Sharifpoor et al., 2010). Scaffolds used for cell culture were in the form of 6.5 mm diameter discs with a thickness of approximately 1 mm, such that they fit within the wells of a 96-well plate.

5.3.2. Culture of vascular smooth muscle cells

SMGM®-2 coronary artery smooth muscle cells (Lonza, CC-2583) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were obtained from Lonza at passage 3 and were further passaged and used between passage 7-9 with at least one passage occurring between thawing and seeding. For experiments where VSMCs were cultured alone on D-PHI scaffolds, 100,000 VSMCs (100 µl of a 1,000,000 VSMCs/ml suspension) were seeded per scaffold in 50:50 RPMI-1640:DMEM medium, containing 10% FBS, 1% penicillin/streptomycin, and 0.35 mM L-glutamine.
5.3.3. Isolation and culture of monocytes

Monocytes were isolated from whole human peripheral blood obtained from healthy volunteers (University of Toronto ethics approval #22203). Approximately 100 ml of blood was collected into EDTA-containing Vacutainers (Becton Dickenson, Toronto, Canada). The blood was layered onto Histopaque 1077 (Sigma) and the blood components separated by centrifugation to isolate the mononuclear cell fraction. A series of washes were performed, after which cells were re-suspended in 50:50 RPMI-1640:DMEM medium, containing 10% FBS, 1% penicillin/streptomycin, and 0.35 mM L-glutamine and counted. For monocyte-only cultures on D-PHI scaffolds, an estimated 200,000 monocytes were seeded per scaffold (100 µl of a 10,000,000 cell/ml solution, of which 20% were previously shown to be monocytes, with mostly monocytes remaining after performing a medium change 2 hr post-seeding, and with 95% of the cells previously shown to be CD68+ [monocytes and MDM] after 72 hr, with only 3% staining positive for CD3 [T-cells]) (Boynton et al., 2000; Dinnes et al., 2008).

5.3.4. Comparison of monocyte/MDM-conditioned medium (MCM) to direct co-culture

VSMCs were co-cultured with monocytes on D-PHI scaffolds in a 1:2 ratio (100,000 VSMCs and 200,000 monocytes per scaffold) by combining VSMC and monocyte suspensions such that 100 µl of 50:50 RPMI-1640:DMEM medium (with 10% FBS, 1% penicillin/streptomycin, and 0.35 mM L-glutamine) contained 100,000 VSMCs and 200,000 monocytes, which was then seeded onto D-PHI scaffolds. Co-cultures were maintained in the 50:50 RPMI-1640:DMEM medium for the duration of the experiment. To generate MCM, supernatants from monocyte/MDM-only cultures were collected during each medium change and centrifuged for 5 min at 1400 rpm to remove any non-soluble components. The resulting supernatant was used to culture VSMC-seeded D-PHI scaffolds (100,000 VSMCs per scaffold). This supernatant was retrieved from monocyte-only cultures on D-PHI scaffolds that were run in parallel with co-culture vs. VSMC+MCM experiments, such that VSMCs cultured in MCM on a given day were exposed to the monocyte/MDM released factors obtained from monocyte-only cultures on the very same day of culture.
5.3.5. Effects of cytokines on VSMC response in monoculture and in co-culture with monocytes

To evaluate the effect of specific cytokines on VSMCs, VSMCs were first seeded on D-PHI scaffolds (100,000 VSMCs per scaffold) and left for 24 hr to allow initial cell attachment. After 24 hr, monocyte chemotactic protein-1 (MCP-1, 5 ng/ml, Peprotech), interleukin-6 (IL-6, 1 ng/ml, Peprotech), or granulocyte macrophage-colony stimulating factor (GM-CSF, 0.25 ng/ml, Peprotech) were supplemented into culture medium and replenished with medium changes every 48 hr. To assess the role of MCP-1 and IL-6 in VSMC-monocyte co-culture on D-PHI scaffolds, antibodies for MCP-1 (Human CCL2/JE/MCP-1 antibody monoclonal mouse IgG1, 4.5 µg/ml, R&D systems) or IL-6 (Human IL-6 antibody monoclonal mouse IgG1, 1 µg/ml, R&D systems) were supplemented into culture medium after allowing an initial 24 hr period for cell attachment post-seeding (100,000 VSMCs and 200,000 monocytes per scaffold). Antibodies were supplemented with each medium change every 48 hr.

5.3.6. DNA mass quantification

Scaffolds were removed from 96-well plates, placed in microcentrifuge tubes, and rinsed twice with PBS. The scaffolds were then minced using a needle, and 300 µl of lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA (TNE), 0.1% SDS, pH 8.0) was subsequently added. Samples were vortexed briefly and incubated at 65°C for 60 min with intermittent vortexing. To quantify the DNA mass extracted from the scaffolds, 10 µl of the lysates were loaded into black flat-bottom 96-well plates containing a 1X TNE solution with Hoechst 33258 DNA stain. Fluorescence was read using an FL600 Microplate Fluorescence Reader with excitation and emission wavelengths of 360 and 460 nm, respectively. To determine the amount of DNA associated with a given fluorescence intensity, sample values were compared to a standard curve prepared from DNA standards of calf thymus DNA (Sigma).

5.3.7. Immunoblotting

Whole cell lysates (as obtained in section 5.3.6) were quantified for protein concentration using a modified Bradford assay (Bio-Rad Protein Assay Dye Reagent) using a bovine serum albumin
(BSA) standard (Bio-Rad). Volumes equivalent to 2 µg of total protein were separated by SDS-PAGE using a Protean II Cell System (Bio-Rad) by loading onto a 5% stacking and 12% separating gel. Proteins were then transferred to nitrocellulose membranes for 90 min at 130 V, washed twice for 10 min in Tris-buffered saline-Tween 20 (TBST; 10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.5), and blocked for 1 hr with 5% non-fat dry skim milk in TBST. Membranes were subsequently incubated with primary antibody solutions for α-SMA (mouse monoclonal to α-SMA, Abcam, 1:2000 in 5% BSA in TBST) or calponin (rabbit monoclonal [EP798Y] to calponin, Abcam, 1:10000 in 5% BSA in TBST) overnight at 4°C. Primary antibodies were also used for GAPDH (mouse monoclonal to GAPDH clone 6C5, Millipore, 1:2000 in 5% milk in TBST) or β-actin (beta-actin (13E5) rabbit mAb, Cell Signaling Technology, 1:1000 in 5% BSA in TBST) to serve as loading controls. Membranes were subsequently washed 8 times for 5 min in TBST, incubated with horseradish peroxidase conjugated secondary antibodies (1:2000 goat anti-rabbit IgG in 5% milk in TBST, Bioshop or 1:2000 goat anti-mouse IgG in 5% milk in TBST, Thermo Scientific) for 1 hr, followed by 8 more 5 min washes. Blots were visualized with a chemiluminescence detection kit (Pierce) with images obtained using a ChemiDoc™ XRS Imager and densitometric analysis performed using Quantity One software (Bio-Rad).

5.3.8. Histology

At 1, 7, 14, and 28 day time-points, scaffolds were washed twice with PBS (pH 7.4) and subsequently fixed with a 4% paraformaldehyde solution on ice for 20 min. Samples were then stored in 15% sucrose at 4°C overnight, followed by 30% sucrose until scaffolds were paraffin-embedded. 20 µm sections of paraffin-embedded samples were stained with hematoxylin and eosin (H&E). For each condition, 18 sections were taken and analysed to ensure images shown were representative of each condition.

5.3.9. Boyden chamber assay

Cell migration was assessed using a 96-well BME Cell Invasion Assay (Trevigen). 24 hr prior to starting the migration assay, VSMCs were serum-starved (DMEM supplemented with 0.5% FBS) to allow for receptor expression. Cells were subsequently harvested and resuspended in
0.5% FBS supplemented DMEM. 10,000 VSMCs were added to the top chamber of each well, with 150 µl of medium supplemented with MCP-1 (5 ng/ml), IL-6 (1 ng/ml), or GM-CSF (0.25 ng/ml) added to the bottom chamber. Cells were allowed to migrate for 18 hr, with 0.5% FBS and 10% FBS containing medium used as negative and positive controls, respectively. Migrated cells were then dissociated from the bottom of the membrane, stained with calcein-AM, and fluorescence intensity was read using a Microplate Fluorescence Reader, with excitation and emission wavelengths of 485 and 520 nm, respectively.

5.3.10. Cytokine antibody array

An antibody array (RayBio Human Cytokine Antibody Array G Series 3) was purchased from RayBiotech Inc. and performed according to the manufacturer’s instructions. Sample releasates were obtained from monocyte-only seeded D-PHI scaffolds at 1- and 7-days post-seeding, with 24 hr given for cytokine accumulation in the medium prior to collection. As a negative control, medium supplemented with 10% FBS was used to determine background intensity contributed from cytokines in non-monocyte/MDM conditioned medium. The intensity readings obtained for each marker were normalized to the positive control contained within the array, such that each value is a fraction of the maximum possible signal intensity.

5.3.11. ELISAs

Enzyme-linked immunosorbent assays (ELISAs) were performed for human MCP-1, IL-6, GM-CSF, and IL-13 as per protocols provided with the kits purchased from the manufacturer (eBioscience). Supernatants were collected from samples 24 hr post-medium change, such that cytokines were allowed to accumulate in culture medium for 24 hr prior to collection.

5.3.12. Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) by analysis of variance (ANOVA) or an independent samples t-test where appropriate, with statistical significance reported for $p<0.05$. A minimum of three blood donors were used for all experiments involving monocytes.
5.4. Results and discussion

The objective of the present study was to investigate the interplay between monocyte/MDM-released factors on VSMC-monocyte co-cultures when grown in D-PHI scaffolds in order to assess their potential role in regulating VSMC growth, 3D migration, and contractile marker expression, and to identify some of the key proteins released by monocytes/MDM influencing the observed VSMC response on D-PHI scaffolds.

5.4.1. Monocyte/MDM-released factors regulate VSMC response similar to direct co-culture on D-PHI scaffolds

In order to isolate the effects of monocyte/MDM-released factors from the effects due to cell-cell contact between VSMCs and monocytes as they undergo a phenotypic change to macrophages, VSMCs were cultured in the presence of MCM. MCM was generated from monocyte-only seeded D-PHI scaffolds that were run in parallel with all experiments. This allowed for the isolation of monocyte/MDM-released factors produced at the same time point as a given VSMC culture condition. This protocol is important since monocyte phenotype will change over time on a biomaterial (Brown et al., 2012a) and this will alter the make-up of the conditioned medium, and therefore is anticipated to affect VSMC response differently. VSMC growth (DNA mass quantification) and contractile marker expression (α-SMA, calponin) were compared between VSMCs cultured in MCM to those cultured in regular medium and VSMCs in co-culture with monocytes/MDM.

Co-culturing monocytes/MDM with VSMCs was shown to increase DNA from day 7 onwards when compared to the sum of DNA from individual VSMC and monocyte/MDM cultures (Figure 5.1).
Figure 5.1 DNA mass quantification for (A) VSMC-monocyte co-culture (C) samples compared to the sum of individual VSMC (V) and monocyte (M) monocultures on D-PHI scaffolds. The total DNA in co-culture is compared to both the sum of the two monocultures when VSMCs are cultured in regular medium (V) and when they are cultured in the presence of MCM (V+MCM). Data represent the mean ± S.E. n=9, with three distinct donors used for samples containing monocytes. (B) Comparison of DNA mass for VSMCs cultured on D-PHI scaffolds in the presence of MCM or in regular medium. Data represent the mean ± S.E. n=9, with three distinct donors used for samples containing monocytes. * p<0.05.

However, there was no difference in total DNA between the co-culture and monoculture conditions when VSMCs were cultured in the presence of MCM. As shown in Figure 5.1B, this was due directly to the increase in DNA obtained from VSMCs when cultured in MCM. This
increase in DNA was not the result of residual monocytes remaining in the MCM, as the MCM was acellular (Figure 5.1B), but was hypothesized to be due to an increase in VSMC growth supported by the presence of predominantly monocyte/MDM-released factors. Monocyte co-culture with VSMCs has previously been shown to support VSMC proliferation (Bonin et al., 1989; Nam et al., 2011). However, since other studies have contrastingly shown that monocyte co-culture can also inhibit VSMC proliferation (Kunigal et al., 2003; Proudfoot et al., 1999), this effect could be dependent on the differential activation state of the macrophage within the M1 to M2 spectrum when cultured on different biomaterial substrates or in different microenvironments (Mosser and Edwards, 2010). It should be noted that over the 28 days of culture, MDM number was shown to decrease while VSMC number increased in monoculture (Figure 5.1), suggesting that by day 28 the 1:2 VSMC:monocyte had changed to contain more VSMCs relative to monocytes.

A suppression of the contractile phenotypic markers α-SMA and calponin was observed in VSMC-monocyte co-culture over time and in particular between day 1 and day 28 of culture, in contrast to the similar expression of these markers at day 1 and day 28 when VSMCs were in monoculture on D-PHI scaffolds (Figure 5.2).
Figure 5.2 Western blot data showing (A-C) the expression (representative samples) and (D-I) densitometric quantification of α-SMA and calponin expression for VSMCs alone (A,D,E), in co-culture (B,F,G), and for VSMCs cultured in MCM (C,H,I) for up to 28 days. Data represent the mean ± S.E. n=6, with three distinct donors used for samples containing monocytes. Values are normalized to their respective β-actin loading control as well as to the level of the marker expression at day 1 under the same condition. * p<0.05 compared to expression of the marker under the same culture conditions at day 1.
MCM had a similar effect on VSMC calponin expression as was observed for direct co-culture, suppressing levels below those measured after initial seeding, while there was no suppressive effect observed between day 1 and day 28 for the expression of α-SMA. This may suggest that the biological factors regulating calponin and α-SMA expression are not the same for the cells cultured on D-PHI, and that while the former is potentially mediated by the proteins released from monocytes/MDM, the latter may be more affected by direct co-culture of the two cell types on D-PHI scaffolds.

Since the loss of contractile phenotypic marker expression and an enhanced proliferative phenotype is also associated with cell migration (Chan-Park et al., 2009), sections of D-PHI scaffolds were stained with H&E in order to visualize the extent of VSMC infiltration into the porous D-PHI scaffolds (Figure 5.3).
Figure 5.3 Representative images of histological analysis (H&E) at (A) day 1, (B) day 14, and (C) day 28 for D-PHI scaffolds seeded with (i) VSMCs cultured in regular medium, (ii) VSMCs cultured in MCM, (iii) VSMC-monocyte co-culture, and (iv) monocytes alone. Arrows indicate cells. Scaffold pores appear as white voids and cells as deep purple, while the scaffold itself stains pink, particularly at later time-points. Scale bar represents 500 µm.
By later time points (> 14 days), there was a notable increase in the presence of VSMCs within the middle portion of the D-PHI scaffolds, which was an effect also observed with MCM. In contrast, VSMCs cultured in regular medium formed a monolayer on the surface with little to no cellular penetration into the scaffold observed by day 14 and beyond.

5.4.2. Factors released by monocytes/MDMs during culture on D-PHI scaffolds

To provide initial insight into the proteins present within MCM that may be contributing to the regulation of VSMC phenotype and cell migration, a cytokine antibody array was used to screen 42 potential markers (Figure 5.4). As a control, 10% FBS containing medium was also screened to determine background contributions from regular medium.

Figure 5.4 Relative expression of various cytokines and growth factors identified with a cytokine antibody array (RayBiotech) in releasates obtained from monocytes cultured on D-PHI scaffolds for 1 or 7 days. Data are normalized relative to the internal positive control in the array (i.e. maximum signal intensity). Data represent the mean ± S.E. n=3.
Of the identified cytokines and growth factors, several stood out for their role in regulating VSMC growth and migration, while others provided important insight into the shifting activation state of the monocytes when cultured on D-PHI scaffolds. IL-1β, a pro-inflammatory cytokine involved in the acute immune response (Barrientos et al., 2008), was detected at day 1 but was no longer present by day 7. Furthermore, IL-13, a cytokine that is used to polarize macrophages towards the M2, or anti-inflammatory, state (Martinez et al., 2008) was not detected at day 1 but was upregulated at day 7. These findings provide further support for the ability of D-PHI to promote a monocyte/MDM activation state that is anti-inflammatory.

The ability of a biomaterial to support a phenotypic transition from a pro-inflammatory to pro-wound healing monocyte/MDM over time in vivo is important since during normal wound healing processes there are dynamic changes in the monocyte/MDM phenotype that are characteristic of different wound healing stages (Mosser and Edwards, 2010). Pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α initiate the wound healing process by promoting recruitment of inflammatory cells to the wound area and acting as mitogens for fibroblasts and SMCs (Werner and Grose, 2003). The absence or presence of low levels of these cytokines has been associated with impaired wound healing, while excessive levels have been associated with enhanced fibrotic scarring (Thomay et al., 2009; Lin et al., 2003; Shinozaki et al., 2009). Furthermore, persistence of pro-inflammatory cytokine levels at the wound site promotes a chronic inflammatory phase that is detrimental to the processes of wound repair and tissue regeneration (Sindrilaru et al., 2011). There is thus a desired shift towards pro-wound healing and anti-inflammatory cytokine production to facilitate the wound healing process. The presence of different cytokines will in part be dependent on the activation state of the macrophage, with M1 macrophages associated with the release of pro-inflammatory mediators (i.e. TNF, IL-12, IL-1, IL-6), while alternatively activated macrophages are associated with a different secretory profile (i.e. IL-10, IL-1ra) (Mosser, 2003).

Cytokines detected by the array screen that have been shown to promote VSMC migration and proliferation included MCP-1 and IL-6 (Ma et al., 2007; Schepers et al., 2006; Spinetti et al., 2004; Nilsson et al., 2007; Wang and Newman, 2003). In addition, GM-CSF was of interest for its known role in promoting extracellular matrix production (Postiglione et al., 2005). ELISAs were performed to determine the concentration of these cytokines in MCM and their temporal
dependence. MCP-1, IL-6, GM-CSF, and IL-13 all showed different temporal trends in their concentrations (Figure 5.5).

Figure 5.5 Quantification of IL-6 (black diamonds), MCP-1 (white circles), GM-CSF (white triangles), and IL-13 (black exes) in conditioned medium from monocytes cultured on D-PHI scaffolds for 1, 7, and 14 days. n=9 from three donors. mean ± S.E.

The concentration ranges measured for MCP-1 (5-25 ng/ml) and IL-6 (1-10 ng/ml) include values that have been shown in the literature to influence both VSMC proliferation and migration (Ma et al., 2007; Nilsson et al., 2007; Wang and Newman, 2003), making these two cytokines attractive candidates for investigating the regulation of VSMC phenotype observed in VSMC-monocyte co-culture on D-PHI scaffolds, and reflected in part by the presence of monocyte/MDM-released factors in MCM (Figures 5.1-5.3).

As shown in Figure 5.4, IL-1β levels decreased over 7 days of culture for monocyte-seeded D-PHI scaffolds. Similarly, the pro-inflammatory cytokine IL-6 showed its highest concentration of 10 ng/ml at day 1, but dropped by an order of magnitude to 1 ng/ml over two weeks of culture (Figure 5.5). MCP-1 is also important as its release from bone marrow mononuclear cell pre-seeded vascular grafts has been shown to be critical in the recruitment of host monocytes that initiate remodelling within the region of the implanted biomaterial (Roh et al., 2010). It has also been suggested that macrophages and MCP-1 play a key role in tissue reparative processes with respect to skeletal muscle regeneration (Shirernan et al., 2007; Shireman et al., 2006). The ability
of bone marrow-derived cells (BMDCs) to mediate vascular growth and tissue healing has also been reported to require the production of MCP-1 (Schatteman et al., 2010).

5.4.3. Response of VSMCs to cytokine stimulation

In order to test the ability of MCP-1, IL-6, and GM-CSF to affect VSMC growth and contractile marker expression, VSMCs were cultured on D-PHI scaffolds and exposed to MCP-1 (5 ng/ml), IL-6 (1 ng/ml), or GM-CSF (0.25 ng/ml) for 7 days since the stimulatory effects of co-culture and MCM were apparent in this time frame (Figure 5.1 and Figure 5.2). These particular concentrations were chosen since they were concentrations assayed to be present in VSMC+MCM experiments (Figure 5.5). After 7 days of culture on D-PHI scaffolds, MCP-1 had a modest but significant positive effect on DNA mass (22 ± 7 % increase vs. regular medium), while IL-6 (+6.3 ± 5.1 %) and GM-CSF (+12 ± 9 %) did not have a significant effect on growth over this time period (Figure 5.6).

![Figure 5.6](image)

**Figure 5.6** Effect of GM-CSF, MCP-1, and IL-6 on VSMC growth over 7 days on D-PHI scaffolds for VSMCs cultured alone. n=7-9. Data represent the mean ± S.E. The dashed line indicates the level of DNA supported by VSMCs in 10% FBS containing medium without any exogenous stimulus added. * p<0.05 compared to regular medium.

With regards to contractile marker expression, IL-6 had a suppressive effect on both α-SMA (74 ± 9 % of regular medium) and calponin (69 ± 6 % of regular medium), whereas only calponin
was significantly affected by MCP-1 (67 ± 10% of regular medium) (Figure 5.7). GM-CSF did not have any effect on either α-SMA or calponin expression.

Figure 5.7 Western blot data showing the expression (A) and densitometric quantification (B,C) of α-SMA (B) and calponin (C) when exposed to GM-CSF, MCP-1, or IL-6. Data represent the mean ± S.E. The dashed line indicates the expression level supported by VSMCs in 10% FBS containing medium without any exogenous stimulus added. n=6. * p<0.05 compared to regular medium.

Because MCM and co-culture also promoted VSMC migration, the impact of MCP-1, IL-6, and GM-CSF on migration was tested in a Boyden chamber assay. However, none of the cytokines had a significant effect on migration (data not shown). MCP-1 has previously been shown to have a stimulatory effect on rabbit VSMC migration in doses ranging from 5-20 ng/ml (Ma et al., 2007; Spinetti et al., 2004). MCP-1 has also been studied with respect to VSMC proliferation, where inhibition of MCP-1 reduced the proliferation of human SMCs explanted from saphenous veins (Schepers et al., 2006), whereas the proliferation of rat VSMCs has been shown to not be affected by MCP-1 (Spinetti et al., 2004). This difference in response to MCP-1 between human and rat VSMCs may explain the lack of migration seen in response to MCP-1 in the present study, while an increase in proliferation in response to MCP-1 was observed. This is similar to other studies with human VSMCs (Schepers et al., 2006), but contrasted to studies with rat
VSMCs (Spinetti et al., 2004). Similar to MCP-1, IL-6 has been shown to support the migration of VSMCs isolated from rat (Wang and Newman, 2003) or mouse aortas (Yu et al., 2011). IL-6 has also been shown to support proliferation of both mouse (Yu et al., 2011) and human VSMCs (Nilsson et al., 2007). While none of these studies have looked specifically at VSMC contractile marker expression, it would be expected based on the enhanced proliferative and migratory state of VSMCs exposed to MCP-1 and IL-6, indicative of the synthetic phenotype, that these proteins would also suppress contractile marker expression (Beamish et al., 2010).

5.4.4. Inhibition of IL-6 and MCP-1 in VSMC-monocyte co-culture

Since MCP-1 and/or IL-6, but not GM-CSF, were shown to be capable of influencing VSMC proliferation (Figure 5.6) and contractile marker expression (Figure 5.7) in monoculture on D-PHI scaffolds, the specific effect of MCP-1 and IL-6 in VSMC-monocyte co-culture on D-PHI was investigated. VSMCs and monocytes were co-cultured on D-PHI scaffolds for 7 days with or without the presence of antibodies for MCP-1 or IL-6. Neutralization of IL-6, but not MCP-1, was shown to have a suppressive effect on DNA mass (Figure 5.8) as well as a stimulatory effect on calponin, but not α-SMA, expression (Figure 5.9). This suggests that IL-6, but not MCP-1, regulates VSMC phenotype with respect to the aforementioned outcomes when in co-culture with monocytes/MDM on D-PHI scaffolds. Furthermore, while calponin expression was increased following neutralization of IL-6, there was no effect on α-SMA, which was similarly not affected by MCM (Figure 5.2).
Figure 5.8 DNA mass quantification for VSMC-monocyte co-cultures on D-PHI scaffolds for 7 days in regular medium or in the presence of antibodies for MCP-1 or IL-6. Data represent the mean ± S.E. n=7-9 from three distinct donors. * p<0.05 compared to regular medium.

Figure 5.9 Western blot data showing the expression (A) and densitometric quantification (B,C) of calponin (B) and α-SMA (C) when VSMC-monocyte co-cultures are exposed to antibodies for MCP-1 or IL-6. Data represent the mean ± S.E. n=6 from three distinct donors. * p<0.05 compared to regular medium.
The relationship of calponin and IL-6 vs. the absence of IL-6 association with α-SMA expression suggests different regulation mechanisms. One possible explanation for this difference is that α-SMA expression requires direct cell-cell contact interactions between VSMCs and monocytes/MDM, as occurs in direct co-culture. Direct VSMC-monoocyte contact has been reported to occur through interactions with β1 and β2 integrins, and that these interactions are increased in the presence of growth factors such as PDGF-BB (Cai et al., 2004). Furthermore, the regulation of other effects by monocytes/macrophages on VSMCs, such as MMP production, have been shown to have dependence on both released factors as well as cell-cell contact (Ikeda et al., 1998; Zhu et al., 2000). Another possibility is that while MCM was used to approximate the presence of cytokines and growth factors being released by monocytes in co-culture, it is also anticipated that the presence of VSMCs will alter the products being released by monocytes/MDM over time. The factors that are more important in the regulation of α-SMA may only be present under direct co-culture conditions. Finally, calponin is more specifically associated with smooth muscle tissues, while α-SMA can be expressed by a number of other cells and is less specific to the contractile VSMC phenotype (Miano and Olson, 1996; Ratajska et al., 2001). Calponin is also typically detected later in the maturation of SMCs during development, while α-SMA is detected earlier on, suggesting that calponin is a marker of a higher SMC differentiation stage (Frid et al., 1992). For this reason, it would be expected that calponin expression may be affected more significantly by IL-6 alone, while α-SMA down regulation may occur in the presence of multiple stimulatory cytokines and growth factors, rather than only exposure to IL-6.

IL-6 promoted a shift in VSMCs to a synthetic phenotype, characterized by enhanced VSMC proliferation and migration, when VSMCs were co-cultured with monocytes. Depending on the cytokine profile being released from the monocytes/MDM, which is known to be biomaterial dependent (Brodbeck et al., 2002; McBane et al., 2011a; MacEwan et al., 2005; Jenney and Anderson, 2000), different effects will be expected in monocyte co-culture systems. Furthermore, it should also be noted that while the current study indicated IL-6 as being key to regulating VSMC response in the present culture system, VSMCs sourced from different locations in the vasculature are known to respond differently to cytokine and growth factor stimulation (Li et al., 2011). Therefore, the specific cytokines predominantly responsible for
inducing effects on VSMCs may differ depending on the source of VSMCs and the specifics of the culture system used. Several studies have looked at the effect of polarizing macrophages towards the M1 vs. M2 activation state and using the conditioned medium from these cells to determine their differential effects (Brown et al., 2012a; Khallou-Laschet et al., 2010). In particular, it has been shown that the M2, or the “wound healing macrophage”, produces cytokines and growth factors that promote enhanced proliferation of VSMCs (Khallou-Laschet et al., 2010) and greater migration of myoblasts (Brown et al., 2012a) when compared to M1 polarized macrophages. Therefore, depending on the particular goals of the co-culture system, a biomaterial that supports different macrophage polarization will be required. In the present study, both the cytokine profile (decreasing IL-6 and IL-1β, increasing IL-13) and the enhanced proliferation and migration of VSMCs (associated with an M2 secretory profile (Khallou-Laschet et al., 2010)) are indicative of a hypothesized shift towards a more M2, or anti-inflammatory state for monocytes cultured on D-PHI scaffolds in vitro.

5.5. Conclusions

The present study investigated the contribution of monocyte/MDM-released factors after exposure of the cells to D-PHI on the stimulatory effects of monocyte co-culture on VSMC migration and proliferation, as well as the suppressive effects on contractile marker expression. Similar to direct co-culture, monocyte/MDM-released factors were observed to increase VSMC penetration into porous D-PHI scaffolds, enhance growth, and suppress calponin expression. IL-6 and MCP-1 were identified as two potential markers contributing to this response, and were confirmed to affect proliferation and/or contractile marker expression of VSMCs cultured alone on D-PHI scaffolds and exposed to concentrations of these proteins relevant to the culture system. In co-culture, however, only IL-6 and not MCP-1 was shown to be linked to enhanced proliferation of VSMCs and suppression of calponin, but not α-SMA, expression. This study highlights the use of monocytes/MDM as stimulatory agents in combination with biomaterial strategies for directing VSMC response, and that in the present system involving the degradable polyurethane D-PHI, IL-6 is an important contributor to this process.
5.6. Acknowledgements

This study was funded by an NSERC/CIHR collaborative grant #337246/83459 (J.P. Santerre, R.S. Labow) and CIHR operating grant #230762 (J.P. Santerre, R.S. Labow, C.A. Simmons). B. Ouyang was funded by an NSERC USRA. K.G. Battiston was funded by a CIHR Strategic Training Fellowship (STP-53877) and by an Ontario Graduate Scholarship (OGS). Thanks to Feryal Sarraf for help with preparation of histology samples and staining and to Gurbaksh Basi for collection of the microarray data.

5.7. References


present functional properties similar to those of their native blood vessels. *Tissue Eng.* 12:2275-2281.


Chapter 6

Monocyte co-culture with arterial smooth muscle cells in an immunomodulatory scaffold enhances vascular tissue production under dynamic biomechanical stimulation

6.1. Foreword

The use of biomechanical stimuli to improve the cellular and mechanical properties of tissue engineered constructs is a commonly used strategy in vascular tissue engineering. Efforts to combine the use of biomechanical and biochemical stimulation, however, can result in a dominant response of the VSMC to the biochemical stimulus, mitigating the desirable effects observed with biomechanical stimulation. In this study, the use of monocyte co-culture as a biochemical stimulus in combination with biomechanical stimulation through the application of strain (10% circumferential strain, 1 Hz) was investigated. A custom bioreactor was designed to apply uniform circumferential strain to tubular D-PHI scaffolds (3 mm internal diameter) seeded with VSMCs and monocytes in co-culture, as well as with control VSMC and monocyte monoculture conditions. Dynamic monocyte-VSMC co-culture supported the greatest VSMC presence, elastin content, and sulphated GAG content for cell-seeded D-PHI scaffolds, while dynamic culture increased collagen content and tissue construct mechanical properties for both co-culture and VSMC monoculture samples. Monocytes/macrophages were also shown to contribute significantly to vascular ECM deposition, while their polarization state shifted towards a more wound healing phenotype on D-PHI over time. This study highlights the ability to combine the biochemical stimulation provided by D-PHI-stimulated monocytes with biomechanical stimulation to enhance tissue regeneration in an in vitro vascular tissue engineering strategy. Furthermore, monocytes/macrophages are identified as a novel source of vascular ECM proteins.

This chapter has been submitted as: Battiston KG\textsuperscript{1}, Labow RS\textsuperscript{3}, Simmons CS\textsuperscript{1,2}, Santerre JP\textsuperscript{1,2}. Monocyte co-culture with arterial smooth muscle cells in an immunomodulatory scaffold enhances vascular tissue production under dynamic biomechanical stimulation.
6.2. Introduction

Developing a tissue engineered construct requires the ability to promote cell growth, obtain a homogeneous cell distribution, promote extracellular matrix (ECM) production, and support specific cellular phenotypes (Huang and Niklason, 2014). In the field of vascular tissue engineering (VTE), it is desirable to promote the growth of vascular smooth muscle cells (VSMCs), which should be stimulated to produce ECM proteins characteristic of the tunica media, including collagen I, collagen III, and elastin, while also maintaining the expression of specific contractile marker proteins (e.g., α-smooth muscle actin [SMA], calponin) (Villalona et al., 2010). For three dimensional constructs it is also important that the distribution of these cells is uniform throughout the scaffold architecture in order to achieve uniform ECM deposition and mechanical properties (Villalona et al., 2010). Methods to achieve these goals have traditionally included the use of bioreactors (to apply perfusion, mechanical distension, or other stimuli), co-culturing VSMCs with other cell types, dosing specific growth factors and cytokines into culture medium (Chan-Park et al., 2009), and using extracellular matrix proteins scaffolds (Seifu et al., 2013) or modifying pro-inflammatory synthetic scaffolds, such as lactic acid based materials (Kim et al., 2011; Park and Babensee, 2012; Yoon et al., 2008), to mask their inflammatory character and enable effective cell interactions with the scaffold.

VSMCs in their natural environment are exposed to biomechanical strain, which for arteries is on the order of 5-15% (circumferential strain) (Larsson et al., 2011; Yuda et al., 2011; Teixeira et al., 2013). This biomechanical stimulation has important implications for the regulation of VSMC phenotype, proliferation, migration, and ECM synthesis, and as such has been used as a method for directing VSMC response for tissue engineering purposes (Huang and Niklason, 2014). Studies have also indicated that the effect of strain is substrate-dependent, which suggests
that these effects may differ in a biomaterial- or culture-environment-specific manner (Wilson et al., 1995). The use of biochemical stimulation in combination with biomechanical stimulation has also been explored in the context of VTE, and has been shown to alter the manner in which VSMCs are able to respond to mechanical stimuli (Stegemann and Nerem, 2003). In addition to growth factors and cytokines, co-culturing VSMCs with other cell types, such as endothelial cells (ECs), can also modify their response to biomechanical stimuli. For EC-VSMC co-cultures, this has been demonstrated in the context of shear stress, where the presence of ECs has been shown to mitigate the effect of shear stress on promoting VSMC migration (Sakamoto et al., 2006; Wang et al., 2006).

Following the implantation of a tissue engineered vascular graft, the initial recruitment of monocytes has been shown to be critical for supporting long-term tissue remodelling (Hibino et al., 2011b). Monocyte recruitment to the site of implantation suggests that these cells are exposed to the same biomechanical stimulation as VSMCs, which can regulate their response to the biomaterial (Mirensky et al., 2010; Roh et al., 2010). Monocytes and their derived macrophages (MDMs) release a number of pro- and anti-inflammatory biomolecules that can regulate VSMC response. Pro-inflammatory cytokines such as IL-6 and MCP-1 promote a proliferative, migratory VSMC phenotype (Nilsson et al., 2007; Schepers et al., 2006), while anti-inflammatory cytokines such as IL-10 inhibit proliferation (Mazighi et al., 2004). The initial burst in inflammatory cytokines that is released by monocytes upon adherence to a biomaterial substrate can therefore be beneficial to promote VSMC proliferation, so long as the biomaterial ultimately supports a phenotypic transition towards a wound healing and regulatory macrophage phenotype that enables neotissue formation and not chronic inflammation (Hibino et al., 2011b; Brown et al., 2012a; Brown et al., 2012b). Monocytes are thus a promising source of stimulatory cytokines with inherent temporal-dependant pathways to control (i.e., negative and positive feedback controls) and contribute to VTE, while also being inherent to the host since they are isolated from peripheral blood as a readily available autologous cell source. Despite immune cells being widely regarded as critical in supporting tissue regeneration, there are few examples of synthetic biomaterials designed with the intention of harnessing the capacity of pro-regeneration immune cell phenotypes towards promoting tissue repair, and instead widely
regarded pro-inflammatory synthetic biomaterials such as PLGA predominate (Yoon et al.,
2008).

A degradable polar hydrophobic ionic polyurethane (D-PHI) has previously been demonstrated
as being suitable for VTE applications (Sharifpour et al., 2009). Specifically, this material has
been shown to inhibit pro-inflammatory responses with monocytes/macrophages in vitro
(McBane et al., 2009) as well as in vivo compared to established cell culture substrates such as
tissue culture polystyrene (TCPS) and poly(lactide-co-glycolide) (PLGA) (McBane et al., 2011).
Dynamic mechanical strain, commonly used in VTE strategies to promote cell proliferation and
matrix deposition, has recently been shown to regulate monocyte/macrophage phenotype
(Ballotta et al., 2014). However, the use of mechanical strain to sustain a macrophage activation
state that can positively support the growth and tissue deposition of other cell types (e.g.
VSMCs) has not previously been investigated, despite the fact that monocytes/macrophages are
present in remodelling tissues that are subjected to mechanical loading (Hibino et al., 2011b). It
was thus important to investigate the effect of combining dynamic mechanical strain with
monocyte co-culture on an immunomodulatory material, such as D-PHI, that has been shown to
reduce pro-inflammatory and heighten anti-inflammatory cytokine release (McBane et al., 2009),
and determine whether this could lead to enhanced cellularity and matrix deposition in a tissue
engineering strategy. In addition to the widely acknowledged role of monocytes/macrophages in
regulating tissue production from tissue-cell types (e.g. VSMCs, fibroblasts), this study sought to
determine the direct contribution of macrophages themselves to the deposition of new ECM
components. This study seeks to determine the ability of monocytes in co-culture with VSMCs
to regulate the biomolecular phenotype of cells and the ECM of engineered vascular tissue in
vitro, the ability of dynamic mechanical strain to enhance these effects, and the contribution of
monocytes/macrophages to new tissue deposition.

6.3. Materials and methods

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.
6.3.1. D-PHI scaffold fabrication

Tubular D-PHI scaffolds were prepared according to previously established protocols (Sharifpoor et al., 2009). A divinyl oligomer (DVO) was mixed with methacrylic acid and methyl methacrylate in a 1:5:15 molar ratio with the initiator benzoyl peroxide and polyethylene glycol and NaHCO$_3$ as porogens (Sharifpoor et al., 2010). The resulting mixture was packed into cylindrical Teflon molds with a 3.5 mm inner diameter, with a stainless steel rod (2.6 mm diameter) used to create the luminal diameter. Following a 24 hr curing period at 110°C, porogens were leached from scaffolds by immersion in water and 14 x 2 hr periods of sonication, resulting in a scaffold with a porosity of 79 ± 3% (Sharifpoor et al., 2010) (Supplemental Figure 6.1A).

6.3.2. Bioreactor design

A custom bioreactor was designed to provide 10% circumferential strain to tubular scaffolds (3 mm ID) at a frequency of 1 Hz. 10% strain was defined as an increase in internal diameter of the scaffold, which was measured as a function of the increase in the diameter of the silicone tubing that the scaffold was mounted on. This is consistent with the method of characterizing circumferential strain in vivo (Larsson et al., 2011; Yuda et al., 2011; Teixeira et al., 2013). It should be noted that based on this characterization and the fact that the D-PHI wall thickness (approximately 1 mm) does not change during the application of cyclic strain, the strain experienced by cells on the interior of the scaffold was 10% while those on the exterior experienced closer to 6% strain, both of which are in the strain range to which VSMCs are sensitive (Isenberg and Tranquillo, 2003). The set up and components of the bioreactor are depicted in Supplemental Figure 6.1. The bioreactor consists of a stepper motor actuator (Digit-HT17, Ultramotion), the speed and displacement of which is controlled by a programmable stepper driver/indexer (Si2035, Applied Motion Products). The actuator is used to control the displacement of a water-filled 50 ml gas-tight syringe (Hamilton), which is mounted on a custom made stainless steel syringe holder. The water from the syringe is distributed to six custom made bioreactor chambers through a stainless steel manifold with six outlets. Soft silicone tubing is mounted within each bioreactor chamber, around which are mounted tubular D-PHI scaffolds that are in intimate contact with the silicone tubing, such that the strain from the silicone tubing...
is translated to the mounted scaffold (Isenberg and Tranquillo, 2003). The silicone tubing is then plugged at one end, such that pushing water into the system with the syringe results in uniform distension of the silicone tubing, and thus the D-PHI scaffolds, without exposing the inner portion of the scaffolds to fluid flow.

6.3.2.1. Scaffold preparation for cell culture

D-PHI scaffolds were immersed in 70% ethanol for 24 hr. Scaffolds were then incubated in complete medium (50:50 RPMI-1640:DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.35 mM L-glutamine) for a minimum of 24 hr prior to seeding. 30 min prior to seeding cells, scaffolds were air dried in a biosafety cabinet to yield a partially hydrated D-PHI scaffold.

6.3.2.2. VSMC culture, monocyte isolation, and co-culture conditions

VSMCs (human coronary artery SMCs, Lonza, CC-2583) were obtained at passage 3 and used for all experiments at passage 6. Prior to co-culture studies, VSMCs were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS.

Monocytes were isolated from whole peripheral blood donated by healthy volunteers according to previously established protocols using Histopaque 1077 and density centrifugation (University of Toronto ethics approval #22203) (McBane et al., 2009). Of the isolated mononuclear cells, 20% were previously shown to be monocytes, with mostly monocytes remaining after performing a medium change 2 hr post-seeding, and with 95% of the cells shown to be CD68+ (monocytes and MDM) after 72 hr, with only 3% staining positive for CD3 (T-cells) (Boynton et al., 2000; Dinnes et al., 2008).

For seeding scaffolds, cell suspensions were prepared containing 250,000 monocytes, 125,000 VSMCs, or a co-culture mixture of 250,000 monocytes and 125,000 VSMCs in complete 50:50 RPMI:DMEM medium for monocyte-only, VSMC-only, and co-culture samples, respectively. Scaffolds were rehydrated with 25 µl of the appropriate cell suspension and left for 30 min in an incubator (37°C, 5% CO₂) to allow for initial cell adhesion, after which 40 ml of medium was
added to each chamber for overnight incubation. Each bioreactor chamber contained seven 5 mm x 3 mm (length x ID) D-PHI scaffolds seeded with cells. An equal number of static chambers, identical in every aspect to the dynamic bioreactor system except not connected to a pressurized fluid source, were maintained in parallel (static condition). All experiments were repeated with a minimum of 3 different monocyte donors.

6.3.2.3. Bioreactor operation

24 hr after seeding to allow for cells to further adhere to the D-PHI scaffolds, the dynamic bioreactor system was started. Prior to starting the bioreactor, the actuation distance required to provide 10% strain to D-PHI scaffolds was determined by using a Canon Rebel SL1 camera to take pictures of D-PHI scaffolds at rest and under strain. Images were analyzed in ImageJ and the actuation distance was modified to impart 10% circumferential strain to the cell-seeded scaffolds. Strain was maintained between 7-10%, which is within the physiological range for a human coronary artery (Larsson et al., 2011), for up to 4 weeks.

6.3.3. DNA mass quantification

DNA mass was quantified from tissue constructs according to previously published protocols using a lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 25 Mm EDTA (TNE), 0.1% SDS, pH 8.0) and incubation in a 65°C water bath for 1 hr with intermittent vortexing. DNA mass was quantified with Hoechst 33258 DNA stain and using a standard curve prepared from DNA standards of calf thymus DNA (Sharifpoor et al., 2010).

6.3.4. Histological analysis

Samples were rinsed once with pH 7.4 PBS and then incubated on ice for 20 min in a 4% paraformaldehyde solution (in pH 7.4 PBS). Samples were then embedded in paraffin and 20 µm thick sections of samples were used for H&E, alcian blue (pH 2.5), and picrosirius red staining, as well as for all immunofluorescence analysis. For histological staining, a minimum of 3 sections were stained for each stain type from each experiment to ensure images shown were representative.
6.3.5. **Sulphated GAG quantification**

Samples were rinsed twice with PBS and subsequently minced with a needle before being placed in a papain digestion buffer (35 mM ammonium acetate, 1 mM EDTA, 2 mM DTT, 50 µg/ml papain, pH 6.2). Samples were incubated in a 65°C water bath for 48 hr with intermittent vortexing. Samples were then stored at -80°C for further analysis. Aliquots of papain digestion solutions were added into wells of a 96-well plate, along with standards prepared from chondroitin sulphate. To wells containing standards and samples, a dimethylylthylene blue (DMMB) solution was added and absorbance was subsequently read at 525 nm.

6.3.6. **Total elastin quantification**

Samples were rinsed once with PBS and then minced with a needle. Samples were then incubated with 0.25 mM oxalic acid at 95-100°C using a heat block for 1 hr. The resulting solution was collected and the oxalic acid treatment was repeated a further 2 times to ensure that all elastin was solubilized. The oxalic acid digest was then stored at -80°C for further analysis. Elastin from the oxalic acid digests was quantified using a Fastin elastin assay according to the manufacturer’s protocol (Naito et al., 2012).

6.3.7. **Western blotting**

Total protein content in the cell lysates (obtained as detailed in section 2.3) was determined using a modified Bradford assay. For quantification of VSMC contractile markers (α-SMA, calponin) and the loading control (GAPDH), sample volumes equivalent to 5 µg of total protein were loaded per sample. Western blotting for contractile marker proteins was thus performed in a manner to assess the amount of marker expression on a per cell basis to determine if on average cells are losing or gaining contractile marker expression over time. For ECM proteins (collagen I, collagen III, and elastin), equal volumes (20 µl) of lysate were loaded per well. Proteins were separated by SDS-PAGE according to previously established protocols (McBane et al., 2009). Blots were stained with primary antibody solutions for α-SMA (mouse monoclonal to α-SMA, abcam, 1:2000 in 5% BSA in TBST), calponin (rabbit monoclonal [EP798Y] to calponin, abcam, 1:10000 in 5% BSA in TBST), GAPDH (mouse monoclonal to GAPDH clone 6C5, Millipore,
1:2000 in 5% milk in TBST), collagen I (rabbit polyclonal to collagen I, abcam, 1:4000 in 1% BSA in TBST), collagen III (rabbit polyclonal to collagen III, abcam, 1:4000 in 1% BSA in TBST), or elastin (rabbit polyclonal to elastin, 1:2000 in 5% milk in TBST), followed by incubation with horseradish peroxidase conjugated secondary antibodies (1:2000 goat anti-rabbit IgG in 5% milk in TBST, Bioshop or 1:2000 goat anti-mouse IgG in 5% milk in TBST, Thermo Scientific). Blots were visualized with a chemiluminescence detection kit (Pierce). Images were obtained using a ChemiDoc™ XRS Imager. Densitometric analysis was performed using Quantity One software (Bio-Rad). Quantified data for VSMC marker expression were presented as normalized to week 0. This normalization was performed by dividing intensity readings at each time point to the intensity obtained for week 0 samples, such that a value of 1 indicates no difference in marker expression, a value of less than 1 indicates a decrease in marker expression relative to week 0, and a value of greater than 1 indicates an increase in marker expression relative to week 0.

6.3.8. Immunofluorescence

20 µm thick sections of paraffin-embedded cell-seeded scaffolds were processed according to previously published protocols (Sharifpoor et al., 2011). Immunostaining for VSMC contractile marker proteins α-SMA and calponin were performed in order to distinguish between VSMCs from monocytes in the co-culture system in order to determine the number of VSMCs in co-culture conditions relative to monoculture. Rather than being an assessment of contractile marker expression normalized to all cell presence, immunostaining for calponin and α-SMA was thus used as a tool to assess VSMC presence in the scaffolds after 4 weeks of culture. Immunostaining was performed by incubating in primary antibody solutions for α-SMA (mouse monoclonal to α-SMA, abcam, 1:50), calponin (rabbit monoclonal [EP798Y] to calponin, abcam, 1:50), collagen I (rabbit polyclonal to collagen I, abcam, 1:50), collagen III (rabbit polyclonal to collagen III, abcam, 1:50), elastin (rabbit polyclonal to collagen III, 1:50), CD163 (mouse monoclonal to CD163, abcam, 1:25), CD206 (mouse monoclonal to CD206, abcam, 1:100), CD80 (rabbit monoclonal to CD80, abcam, 1:100), or CD86 (mouse monoclonal to CD86, abcam, 1:100). Samples were then incubated in secondary antibody solutions in 10% goat serum at 1:200 (AlexaFluor® goat anti-mouse IgG or AlexaFluor® 568 goat anti-rabbit IgG). Nuclear
counter-staining was performed with Hoechst 33342 (1:1000). Slides were then mounted with PermaFluor (Thermo Scientific) and subsequently imaged with a fluorescence microscope (DM-IRE2, Leica Microsystems, Germany) at the Faculty of Dentistry, University of Toronto.

To quantify VSMC scaffold coverage from immunofluorescence images, total positive calponin and α-SMA area were normalized to total scaffold area using ImageJ. M1 and M2 marker quantification (10x magnification), as well as caspase 3 staining (20x magnification), was performed by manually counting nuclei and positively stained cells for the different markers, and normalizing total number of positively stained cells to the number of nuclei counted. Quantification of col I, col III, and elastin from IF images was performed by quantifying the total area staining positive for these proteins relative to total scaffold area. For each sample, three regions of interest were chosen for quantification. Regions of interest were chosen such that they were approximately equally spaced from one another so as to obtain a representative distribution of the entire scaffold cross-section, and were chosen under white light illumination such that the experimenter would not be biased by knowing the cellularity or marker expression of the region chosen. Furthermore, in order to minimize bias from oversampling one region of the scaffold during sectioning, sections were never taken adjacent to one another and were chosen to represent the middle and both ends of the tissue construct. While this may help minimize bias, it should be noted that any quantification of tissue sections in this manner will be semi-quantitative as the entire specimen is not being analyzed. While both nuclei and scaffold material stained with the Hoechst 33342 stain (Supplemental Figure 6.2), cells only appear in the scaffold pores and so are easily distinguished against the black background in the scaffold pores.

6.3.9. ELISA

Enzyme-linked immunosorbent assays (ELISAs) from eBioscience (TNF-α, IL-10) or R&D Systems (FGF-2) were performed for human TNF-α, IL-10, and FGF-2 from concentrated supernatants from bioreactor chambers at 0, 2, and 4 week timepoints 24 hr after a medium change, such that quantified biomolecules were the result of 24 hr of cellular activity. Medium that was incubated with non-seeded D-PHI scaffolds was used as a control. 40 ml of culture supernatant was concentrated to approximately 0.6 ml using Amicon Ultra centrifugal filter devices with a 10 kDa molecular weight cut off membrane. ELISAs were performed according
to the manufacturers’ protocols. Values are reported in pg and represent cytokines present in each bioreactor chamber, such that they are the combined released products from 7 individual scaffolds. It should be noted that measured cytokine values reflect soluble cytokines and do not include those that have been sequestered in the ECM by sulphated GAGs.

6.3.10. Mechanical testing

Following 4 weeks of static or dynamic culture, D-PHI scaffolds were dismounted from the distension tubing by using a surgical blade to slice along the length of the tube. Tensile mechanical testing was performed using an Instron uniaxial servo-hydraulic testing machine (Instron model 8501) with a 10 N load cell and a strain rate of 1 mm/min. Scaffolds (7-9 per condition) were mounted such that they were tested in the direction of the applied strain (circumferentially). All testing was performed in a wet condition within 5 min of removing samples from culture medium. Control samples were also included for week 0 and week 4 time points, which consisted of non-seeded D-PHI scaffolds that had been incubated in medium for 24 hr (week 0) or 4 weeks (week 4).

6.3.11. Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) by analysis of variance (ANOVA) using Bonferroni for pair-wise comparisons or an independent samples t-test where appropriate, with statistical significance reported for \( p<0.05 \). Tests for homogeneity of variance (Leven’s test) and if the data were normally distributed were performed in SPSS to ensure the assumptions inherent to the statistical tests were valid. A minimum of three blood donors were used for all experiments involving monocytes.

6.4. Results

To evaluate the effect of monocyte co-culture in combination with mechanical stimulation on VSMC response, monocytes isolated from the peripheral blood of human volunteers were co-cultured with VSMCs on D-PHI scaffolds alongside control monoculture conditions of both VSMCs or monocytes alone. Scaffolds were either cultured under dynamic (10% circumferential strain, 1 Hz) or static conditions for up to 4 weeks.
6.4.1. Mechanical stimulation and monocyte co-culture synergistically increase VSMC number

Dynamic stimulation resulted in an increase in DNA mass for both VSMCs alone (392 ± 38 [static] vs. 715 ± 94 [dynamic] ng/scaffold) as well as the co-culture condition (2482 ± 286 [static] vs. 3125 ± 263 [dynamic] ng/scaffold) at 4 weeks, with no significant effect observed for the monocyte monoculture (Supplemental Figure 6.3). VSMCs readily penetrated into D-PHI scaffolds under co-culture or dynamic monoculture conditions, but not when in static monoculture (Supplemental Figure 6.4). Histological analysis detected primarily mononucleated macrophages after 4 weeks of culture, with few foreign body giant cells (FBGCs) present (Supplemental Figure 6.5).

α-SMA and calponin expression experienced an initial decrease (relative to week 0) for all culture types and did not show a dependence on mechanical stimulation. Following this initial decrease in contractile marker expression, α-SMA and calponin expression were maintained with no change in expression levels for up to 4 weeks (Supplemental Figure 6.6).

After 4 weeks of culture, quantification of α-SMA+ and calponin+ cell coverage of tissue constructs showed an increase in VSMC number in co-culture under dynamic stimulation. Calponin+ area quantification further confirmed that static co-culture had greater VSMC coverage than static VSMC monoculture, that dynamic culture increased VSMC scaffold coverage in monoculture, and that VSMC scaffold coverage was greatest when both monocyte co-culture and dynamic stimulation were employed simultaneously (Figure 6.1).
Figure 6.1 (A) Immunofluorescent staining of 20 µm cross-sections of D-PHI scaffolds after 4 weeks of static or dynamic (10% strain, 1 Hz) for the contractile marker proteins α-SMA (green) and calponin (red). Nuclei are stained blue. Scaffold architecture is also stained blue. Scale bar represents 60 µm. Quantification of IF images for VSMC area relative to total scaffold area for (B) calponin and (C) α-SMA.

No differences in VSMC apoptosis were observed under co-culture or mechanical stimulation conditions, though monocytes did experience greater apoptosis (30 ± 7 [static] vs. 53 ± 9 % [dynamic] caspase3 positive cells) following the application of mechanical strain at 4 weeks (Supplemental Figure 6.7).
6.4.2. Vascular ECM components are produced by MDM and are regulated by co-culture and mechanical stimulation

Western blotting results showed a trend towards increased collagen I production in dynamic vs. static co-culture, as well as increased collagen III under dynamic stimulation for both co-culture and VSMC monoculture conditions (Figure 6.2).
Figure 6.2 Collagen analysis of cell-seeded D-PHI scaffolds. Densitometric quantification of western blot data for (A) collagen I and (B) collagen III after 4 weeks of culture. Data are the mean ± S.E. n=5. C – co-culture. V – VSMC. M – monocyte. S – static. D – dynamic. Immunofluorescence images of 20 µm cross-sections of D-PHI scaffolds after 4 weeks of culture showing staining for (C) collagen I and (D) collagen III (red), as well as quantification of immunofluorescence images (E,F). D-PHI scaffold architecture also appears blue. Scale bar represents 60 µm. *p<0.05. †p<0.05 vs. monocytes under same culture conditions.
Western blotting also demonstrated production of both collagen I and collagen III by monocytes/macrophages. Immunofluorescence image quantification demonstrated significantly (p<0.05) more scaffold coverage for both collagen I and III under dynamic culture for both co-culture and VSMC monoculture conditions, but not for monocytes alone (Figure 6.2).

Total elastin quantification (Figure 6.3A,D,E) demonstrated greater elastin production in the co-culture regardless of mechanical stimulation, with both VSMCs and monocytes producing similar levels of elastin. In general, western blotting, which is a measure of intracellular tropoelastin levels since cross-linked elastin cannot be solubilized by the lysis buffer, showed that dynamic culture increased tropoelastin production regardless of culture type (Figure 6.3B,C), suggesting a potential for increased elastin production that has not been translated yet into deposited extracellular elastin. Immunofluorescence imaging demonstrated that elastin deposition was present throughout the thickness of the scaffold constructs for all culture conditions, with slightly elevated scaffold coverage observed for co-culture vs. monoculture conditions (Figure 6.3).
Figure 6.3 Analysis of elastin content of cell-seeded D-PHI scaffolds. (A) Total elastin content of D-PHI scaffolds after 4 weeks of culture. Data are the mean ± S.E. n=6-8. *p<0.05 vs. dynamic co-culture. (B) Densitometric quantification of western blot data for soluble (non-cross-linked) and intracellular tropoelastin after 4 weeks of culture. n=5. (C) Aggregating western blot results for static vs. dynamic irrespective of cell type to emphasize the importance of dynamic culture. Data are the mean ± S.E. n=15. *p<0.05. (D) Immunofluorescent staining and (E) quantification of immunofluorescence images for elastin for cell-seeded D-PHI scaffolds after 4 weeks of culture. Elastin is depicted in red and scaffold architecture in blue. †P<0.05 vs. monocytes under same conditions. Scale bar represents 60 µm.

At the initial week 2 time point, co-culture supported greater GAG deposition relative to the monoculture controls. However, by 4 weeks of culture, co-culture only showed an increase in GAG deposition relative to monoculture under static culture conditions (Supplemental Figure 6.8).
6.4.3. Monocyte/macrophage activation shows temporal but not mechanical stimulation dependence

The M2c macrophage marker CD163 was not detected for any conditions at any of the time points. For D-PHI scaffolds seeded with monocytes alone, the expression of the M1 marker CD80 increased from week 0 to week 2, and further increased at week 4, with no difference between static and dynamic conditions (Supplemental Figure 6.9).

![Figure 6.4](image)

**Figure 6.4** Temporal changes in the ratio of the M2a marker CD206 to the M1 marker CD86 for (A) monocytes seeded alone on D-PHI scaffolds or (B) in co-culture with VSMCs. Dashed line indicates levels for each marker at W0 (24 hr post-seeding). Data are the mean ± S.E. n=9. *p<0.05 vs. week 2 time point for the same culture condition.

The M1 marker CD86, however, remained at similar levels throughout the culture period. The M2a marker CD206 remained the same from week 2 to week 4 under static culture, but increased from week 2 to week 4 in dynamic culture (Supplemental Figure 6.9). The ratio of M2/M1 marker expression was also presented as a means to gauge whether there was a population shift between a primarily M2 vs M1-like state as a function of time. Due to the lack of expression of CD163, this was specifically performed for CD206 only, an M2a marker, such that the M2/M1 ratio calculated specifically refers to M2a and not M2c macrophages. CD206+ macrophages were normalized to CD86+ macrophages since these two markers were co-stained on the same sections, though results did not differ significantly when CD206+ staining was normalized to CD80+ staining (data not shown). Under dynamic culture, the M2/M1 ratio increased from week 2 to week 4, but remained the same under static culture. Both static and dynamic culture
experienced an increase in the M2/M1 ratio from week 0 at week 4, suggesting an overall shift to a more M2-like state.

For monocytes seeded in co-culture with VSMCs (Supplemental Figure 6.10), expression of the M1 markers CD80 and CD86 both increased from week 0 to week 4. For CD80, static culture showed a greater expression level at week 2 relative to dynamic culture, though no differences were apparent as a function of mechanical stimulation by week 4. For the M2a marker CD206, no differences were observed between static and dynamic conditions, with a general trend towards increased marker expression over time. The ratio of M2/M1 marker expression in co-culture dropped at week 2, but regained week 0 expression levels by week 4. One difference apparent between monoculture and co-culture conditions was the initial higher M2/M1 ratio in co-culture vs. monoculture at week 0 (p<0.05). However, by week 4 the M2/M1 ratio showed no culture-type dependence.

Cytokine analysis revealed a decrease in both TNF-α and IL-10 over the 4-week culture period (Supplemental Figure 6.11). As anticipated, negligible contributions to TNF-α and IL-10 release were observed for VSMC monoculture samples. At week 2, greater TNF-α and IL-10 levels were produced in dynamic co-culture vs. dynamic monocyte culture, though no differences were apparent between the two static conditions. For monocytes alone, static culture supported a greater release of IL-10 relative to dynamic culture. The ratio of TNF-α to IL-10 can be an indicator of whether a shift towards a more pro-inflammatory (increasing ratio) or anti-inflammatory (decreasing ratio) state is occurring over time. This ratio decreased significantly for monocytes under static and dynamic culture, as well as co-culture samples under dynamic culture (Figure 6.5).

FGF-2 was detected in both co-culture and VSMC only samples, but not in significant levels from monocyte-only seeded scaffolds. FGF-2 levels increased significantly from week 0 to week 2 for the co-culture condition, at which time co-culture produced more FGF-2 than VSMCs cultured alone. By week 4, however, no differences were observed between static and dynamic or monoculture vs. co-culture conditions (Figure 6.5).
Figure 6.5 Cytokine and growth factor release quantification for (A) FGF-2 and (B) TNF-α/IL-10. Data are the mean ± S.E. n=4-5. *p<0.05 vs. co-culture at same time point under same culture conditions. °p<0.05 vs. W0 for same culture condition. S- static. D- dynamic. Dashed line indicates level at W0. Where no dashed line is indicated, W0 levels were below sensitivity of the assay.

6.4.4. Mechanical stimulation enhances contract mechanical properties for VSMC-containing scaffolds

Dynamic mechanical strain resulted in an increase in elastic modulus and tensile strength for VSMC and co-culture conditions, with no changes observed for monocytes under static vs. dynamic culture (Figure 6.6). Dynamic co-culture, but not dynamic VSMC culture, also resulted in greater elastic modulus than dynamic monocyte culture. Elongation at yield remained the same regardless of culture condition.
Figure 6.6 Mechanical testing analysis of cell-seeded D-PHI scaffolds for (A) elongation-at-yield, (B) yield strength, and (C) elastic modulus after 4 weeks of culture. Data are the mean ± S.E. n=9-10. *p<0.05. † p<0.05 vs. dynamic monocyte.

6.5. Discussion

Biomaterials with the ability to attenuate a pro-inflammatory monocyte/macrophage response have been shown to promote cell growth and migration in vitro as well as positive tissue remodeling outcomes in vivo (Brown et al., 2012a). In contrast to the ability of synthetic ECM-derived scaffolds to achieve this response (Sicari et al., 2014), there are few examples of synthetic biomaterials designed to support these interactions with immune cells, and instead pro-inflammatory materials such as PLGA are commonly employed, requiring coating with bioactive components to mask their inflammatory character (Kim et al., 2011; Yoon et al., 2008). It was thus hypothesized that a critical strategy for new synthetic biomaterials must consider the ability to harness monocyte/macrophage cytokine activity by promoting a desirable activation state as these cells will ultimately always be present in the remodelling of implanted tissue engineering constructs. If such cells can be included in the early phase of building the tissue engineered...
construct then the potential for yielding a desirable cellular environment prior to implantation would be a favorable sign for the ultimate successful integration of the construct post-implantation. Furthermore, since these cells are present early on in tissue remodelling, and many tissue types (e.g. circumferential strain for arteries and veins) are exposed to biomechanical stress during the remodelling process, it is important to better comprehend the behaviour of monocytes/macrophages under relevant mechanical loading conditions to understand how this may affect their capacity to regulate other cell types, as well as generate new tissue themselves.

D-PHI, a degradable elastomeric polyurethane, was shown in previous studies to attenuate pro-inflammatory cytokine release (TNF-α, IL-1β), promote anti-inflammatory cytokine release (IL-10) (McBane et al., 2009), and support tissue integration when implanted subcutaneously in a rabbit model (McBane et al., 2011), results which suggest an immunomodulatory capability. This study demonstrates that monocytes cultured on an immunomodulatory biomaterial, such as D-PHI, can be used in co-culture strategies for tissue engineering to promote cell growth and tissue deposition. Furthermore, combining co-culture strategies that provide biochemical stimulation to VSMCs in combination with biomechanical stimulation through dynamic circumferential strain can have a complementary effect on promoting ECM deposition and increased cellularity of tissue engineered constructs. Macrophages are also revealed as potential contributors to significant levels of vascular tissue production, including collagen I, collagen III, sulphated GAGs and in particular elastin, a protein that is often referenced as lacking in many reported articles on vascular tissue engineering (Venkataraman et al., 2014; Mecham, 2008).

Previous studies have demonstrated that the use of dynamic stimulation in the form of circumferential strain can promote heightened matrix deposition and circumferential organization of collagen fibrils due to VSMC proliferation or greater collagen production per cell, which in turn results in enhanced mechanical properties (Stegemann and Nerem, 2003; Syedain and Tranquillo, 2011; Syedain et al., 2008). Some efforts that have attempted to explore the possibility of coupling biomechanical stimulation with biochemical stimulation (e.g., PDGF, TGF-β1), however, have demonstrated that the biochemical stimulus dominates and attenuates VSMC sensitivity to mechanical strain (Stegemann and Nerem, 2003; Syedain and Tranquillo, 2011). The use of growth factors as a source for biochemical stimulation also poses challenges due to potential endotoxin contamination (Wakelin et al., 2006) as well as considerable cost,
which can lead to impaired tissue remodeling following implantation (Daly et al., 2012) and a failure to enable the therapy to enter the clinical market (Prestwich et al., 2012). Since human monocytes would be associated with the host, this latter problem is minimized, and in the former challenge, monocyte cytokine release can be regulated by environmental factors such that they are primed to release appropriate stimulatory biomolecules without the need for exogenous stimulation.

In the present study, DNA mass increased in the presence of mechanical stimulation for VSMC and co-culture samples (Supplemental Figure 6.3). While quantification of DNA mass is limited in terms of understanding what is happening with individual populations within the co-culture system, immunostaining for VSMC contractile marker proteins showed that the increase in DNA mass in co-culture is attributed in a significant manner to an increase in the VSMC population (Figure 6.1) despite an increase in monocyte apoptosis under mechanical strain (Supplemental Figure 6.7). The effects of mechanical strain and co-culture simultaneously increase VSMC scaffold coverage (Figure 6.1), an effect that previous studies have not consistently demonstrated with combined biochemical and biomechanical stimulation (Stegemann and Nerem, 2003). The ability of mechanical strain to augment the effect of monocytes/macrophages with regard to increasing VSMC number demonstrates the importance of using an appropriate biomechanical environment in order to understand the activity of VSMCs during tissue remodelling in vivo and in vitro, when both the cellular (monocyte/macrophage) and biomechanical (circumferential strain) stimuli will be present.

Quantification of released FGF-2 demonstrated the presence of this growth factor in co-culture and VSMC monoculture samples at all time-points, with co-culture significantly increasing FGF-2 levels relative to VSMC monoculture at week 2 (Figure 6.5). This increase in FGF-2 levels is most likely due to monocyte-induced stimulation of VSMC-FGF-2 production, since only marginal levels of FGF-2 were observed in monocyte monoculture. FGF-2 is a potent inducer of VSMC proliferation (Nugent et al., 1993), suggesting that the upregulation of FGF-2 production in co-culture may be responsible in part for the increase in VSMC number observed in co-culture vs. VSMC monoculture under both static and dynamic conditions (Figure 6.1). FGF-2 is also known to bind to vascular ECM components such as sulphated GAGs, including heparin sulphate proteoglycans (Govindraj et al., 2006). Sulphated GAGs were present in greater
amounts in co-culture at week 2, indicating the possibility that FGF-2 sequestration in the co-culture ECM may further enhance FGF-2 availability to VSMCs leading to greater cell proliferation.

Increased cellularity without the deposition of defined extracellular matrix proteins will not lead to the generation of a more mechanically robust tissue engineered construct (Syedain et al., 2008). For VTE, this refers to the production of collagens (primarily I and III) and elastin, the latter of which is particularly difficult to induce in cultured adult cells (Bashur et al., 2012). Collagen and elastin combined make up approximately 50 wt% of a dried, defatted artery (Govindraj et al., 2006). Western blotting for collagens I and III suggested an increase in the production of collagens by mechanical strain for VSMCs and co-culture at 4 weeks, and for all culture types under dynamic stimulation for elastin (Figures 6.2, 6.3).

When comparing plots for the effects of culture condition on collagen production (Figure 6.2) to those for construct mechanical properties (Figure 6.6), it can be seen that increases in elastic modulus and tensile strength were only observed for those conditions that also supported an increase in collagen. In contrast, while static co-culture demonstrated similar cellularity to dynamic VSMC culture (Figure 6.1), the increased presence of VSMCs in static co-culture did not result in the same increase in mechanical strength observed for dynamic VSMC culture (Figure 6.6). This would be expected since the stiffness of VSMCs (10 kPa) is significantly less than that of elastin (600 kPa) and collagen (10^6 kPa) (Matsumoto and Nagayama, 2012). While VSMCs can contribute to increases in tissue stiffness, with studies showing that VSMCs can contribute to increases in the stiffness of vascular tissue on the order of approximately 10 kPa (Sehgel et al., 2013), it would be expected that increased collagen content would have a more significant impact due to the greater inherent stiffness of the ECM vs the cellular component (Matsumoto and Nagayama, 2012; Wagenseil and Mecham, 2009). In contrast to the ECM dependence of elastic modulus and tensile strength, elongation at yield appears to be dominated by the biomaterial itself, with no changes observed for any of the culture conditions tested (Figure 6.6). This demonstrates the ability of the material to withstand certain strain levels during the dynamic state of tissue regeneration or remodelling.
Interestingly, scaffolds seeded with monocytes alone also demonstrated substantial deposition of collagen I, collagen III, elastin, and GAGs (Figures 6.2, 6.3 and Supplemental Figure 6.8). While the immunoregulatory role of macrophages in tissue regeneration is well appreciated in the tissue engineering field (Brown et al., 2012b), the ability of these cells to contribute to ECM deposition in the context of tissue engineering has not yet been extensively explored. Recent studies in the field of cell biology, however, have demonstrated that macrophages are capable of producing both collagens and elastin in vitro and in vivo (Schnoor et al., 2008; Krettek et al., 2003). In a study by Schnoor et al., monocytes and macrophages were shown to express virtually all known collagen and collagen-related mRNAs (Schnoor et al., 2008). In contrast to VSMCs, collagen production appears to be used by macrophages to modulate cell-cell and cell-matrix interactions (e.g., adhesion), rather than contribute to the native ECM. For example, collagen VI was shown to be present on the cell surface, and macrophages were shown to express receptors that interact with collagen VI. However, with the co-culture system, the presence of VSMCs may lead to the incorporation of macrophage-derived ECM products into the fibrillar collagen network that provides mechanical integrity to tissues. The macrophage activation state has been shown to influence collagen VI production. Stimulation with pro-inflammatory mediators (LPS, IFN-γ, TNF-α) suppressed collagen VI production whereas stimulation with cytokines known to induce M2 polarization (IL-10, IL-4, IL-13, TGF-β1) supported enhanced collagen VI production (Schnoor et al., 2008). Collagen VI production has also been shown to be supported by apoptosis, another factor known to promote M2 polarization (Johann et al., 2007; Weigert et al., 2007). While there are currently no studies demonstrating polarization dependent changes in collagen I or collagen III production, the ability of pro-inflammatory cytokines to down-regulate collagen VIII (Weitkamp et al., 1999) and collagen VI (Schnoor et al., 2008), while increasing collagen VI production is supported by M2-polarizing cytokines, suggests that this may be a general effect of M2-polarized macrophages in increasing all types of collagen. The ability of monocytes/macrophages to produce elastin in particular is a promising result due to the limited ability of cultured VSMCs to produce this matrix protein in vitro (Mecham, 2008) on established synthetic biomaterials. However, the extent of tissue analysis performed in this study does not provide full information on the maturity and organization of the deposited tissue. In order to determine if collagen and elastin produced by monocytes/macrophages is capable of contributing to construct mechanical properties, as well as if it is being incorporated into the ECM or if it
solely associated with the cell surface, further analysis including collagen fibril thickness, collagen and elastin organization (dominated by scaffold architecture vs. aligned as a function of mechanical strain), as well as elastin cross-link density would be necessary. More comprehensive analysis of collagen fibril and fiber dimensions as well as orientation can be achieved, for example, using the highly sensitive ultrastructural approach demonstrated by Dahl et al. (Dahl et al., 2007). Such data would further be informative with regards to the increase in collagen produced as a function of mechanical stimulation shown in the present study through IF analysis.

The presence of monocytes and their derived macrophages has been demonstrated to be critical for proper healing following injury (Novak and Koh, 2013), as well as for supporting appropriate tissue integration and regeneration for implanted tissue engineered constructs (Hibino et al., 2011b). Of equal importance to the presence of monocytes/macrophages, however, is their activation state. While no correlations have been observed between fibrous capsule formation and macrophage number for long-term implants (Jones, 2008), a positive correlation has been observed between the proportion of M2-polarized macrophages and positive remodeling outcomes, such as vascularity, tissue organization, cellular infiltration, and degree of encapsulation (Brown et al., 2012a). Rather than the number of monocyte/macrophages present, it is thus their relative activation state that will determine whether their presence will support tissue remodeling or chronic inflammation. *In vitro*, monocyte/macrophage co-culture with VSMCs and other cell types (ex. myoblasts (Brown et al., 2012a)) has also demonstrated polarization-dependent effects on proliferation and migration (Khallou-Laschet et al., 2010; Freytes et al., 2013). Conditioned medium derived from M2-polarized macrophages has been shown to induce greater proliferation and migration, suggestive of a synthetic VSMC phenotype that is supported by macrophage-derived cytokines and growth factors (Khallou-Laschet et al., 2010).

For both monocytes alone and in co-culture with VSMCs on D-PHI scaffolds, there was a general trend towards an increase in M2a-polarization from week 0 to week 4, suggesting a shift towards a more wound healing phenotype macrophage. Furthermore, the lack of positive staining for CD163 suggests a wound healing rather than a predominantly anti-inflammatory M2c state, which would be desirable in terms of promoting cell growth and tissue deposition, both of which were supported by the co-culture system in this study. The expression of both M1 and M2c
markers were shown to increase from week 0 to week 4, and this was anticipated as monocytes at week 0 either do not express or express at low levels characteristic M1 and M2 markers, and begin to demonstrate greater expression only as they begin to differentiate to macrophages on the material. Furthermore, the presence of both M1 and M2 macrophages has been shown to be associated with positive outcomes in the context of tissue engineering (Spiller et al., 2014), since both overactive M1 and M2 macrophage activation can result in poor outcomes due to chronic inflammation or fibrosis, respectively (Mosser and Edwards, 2010). For monocytes alone on D-PHI, a significant increase in M2/M1 ratio was only observed for dynamic culture, while this ratio remained constant under static culture during this time period. While the present study quantified M1 and M2 marker expression based on the number of positively stained cells, it should also be noted that M1 and M2 macrophages can express small amounts of each other’s polarization state. The IF analysis in this study thus is not able to detect the absolute level of expression, which could be accomplished by flow cytometry or PCR analysis.

In addition to immunofluorescence staining, cytokine analysis further supports a shift towards a more M2-like state. While both TNF-α and IL-10 levels decreased over time, the ratio of TNFα/IL-10 can provide further insight into the macrophages’ activation state, with a decrease in this ratio suggesting a shift away from a pro-inflammatory phenotype. For all culture conditions (except static co-culture), a decrease in TNF-α/IL-10 occurred from week 0 to week 4. This mirrored the increase in the M2/M1 ratio (Figure 6.4). Furthermore, the initial reduction in pro-inflammatory macrophage activation demonstrated by the increase in the M2/M1 ratio for co-culture is also seen with cytokine analysis, where TNF-α/IL-10 is greater for monocytes alone vs. co-culture at week 0. In the present study, cytokine levels were measured to determine the effect of the specific culture conditions (monoculture vs. co-culture, static vs. dynamic culture) on monocyte/macrophage activation. Assessing the monocyte activation state by cytokine expression levels can also be performed by determining how monocytes react under different culture conditions and respond to inflammatory triggers, such as lipopolysaccharide (LPS) or IFN-γ, which is especially important since these types of triggers may be present at an implantation site as a result of bacterial presence (LPS) or activation of other white blood cells types (IFN-γ). A more complete characterization of the activation state of the monocyte could
include an assessment of cytokine expression levels under the different culture conditions tested following exposure to an inflammatory trigger such as LPS.

While some benefits of combining monocyte co-culture with biomechanical stimulation have been demonstrated by the present study, some limitations of the study parameters are also apparent if one wished to subsequently optimize the effects observed in the current study for ultimate translation into clinical applications. In order to illustrate the feasibility of coupling biomechanical strain with monocyte co-culture, this study utilized a strain level (10% circumferential strain) and rate (1 Hz) that have been shown in other studies to promote VSMC proliferation and promote tissue production (Sharifpoor et al., 2011; Seliktar et al., 2000). However, VSMCs are also sensitive to differences in strain amplitude, strain frequency, and duty cycle (Isenberg and Tranquillo, 2003), as well as the use of incremental changes in strain amplitude as culture period progresses (Syedain et al., 2008). Furthermore, monocytes have also recently been shown to be strain-sensitive in terms of their polarization and cytokine producing capabilities (Ballotta et al., 2014). In order to optimize the ability of a biomechanically stimulated monocyte-VSMC co-culture system to support vascular tissue production, a thorough study of the different factors involved in the definition of the strain profile is required.

Furthermore, while previous studies with the monocyte-VSMC co-culture system on D-PHI evaluated the effects of different monocyte-VSMC ratios (McBane et al., 2011), cell response to some mechanical stimuli (e.g. substrate modulus) has been shown in some studies to be cell density dependent (Sazonova et al., 2011), and thus the optimal cell density should be further evaluated in the context of the biomechanical loading environment in order to optimize the ability of this culture system to promote new tissue production.

The results presented here are in accordance with recent findings in the area of implanted biomaterials and tissue engineered constructs that implicate biomaterial-mediated control of macrophage phenotype as an important factor that can support tissue regeneration (Brown et al., 2012b; Hibino et al., 2011a). In the development of vascular tissue and particularly following the implantation of degradable vascular grafts, the initial presence of monocytes on the graft has been shown to be critical for the release of cytokines, such as MCP-1, that support the recruitment of host cells that subsequently contribute to new tissue formation (Roh et al., 2010). Furthermore, treatments that prevent macrophage infiltration into the implanted material
demonstrate significantly reduced neovessel formation, as evidenced by a lack of recruited VSMCs and ECs, as well as a lack of collagen deposition (Hibino et al., 2011b). Positive macrophage interactions are thus important for supporting integration with host tissue, which is especially critical for tissue engineered constructs as their function relies on the ability to communicate with host tissue, whereas an adverse foreign body response can mitigate this process (Anderson et al., 2008).

While there are several benefits to harnessing monocytes/macrophages and their cytokine activity to promote desirable biological outcomes, including ease of isolation, low cost vs. recombinant growth factors and reduced concern of endotoxin contamination, some potential drawbacks should be noted. When using autologous cell sources, there is significant potential for donor-to-donor variability. While in the present work multiple donors were used for all experiments, this study is a relatively small-scale one and did not include any unhealthy individuals, who may be more likely to be the recipients of grafts and may have monocytes with different cytokine profiles. It remains to be seen if across larger population scales the use of this cell source would produce consistent enough effects to be universally applicable. However, work by others (Mirensky et al., 2010; Shin'oka et al., 2005) has demonstrated the clinical use of bone marrow mononuclear cells (BMMNCs) despite this donor-to-donor variability, which is of particular relevance to the present study since the monocyte fraction was shown to be the cellular component linked to the benefits of BMMNC use in the latter studies. This suggests that despite significant variability in cytokine activity from different patients, strategies employing monocytes may potentially be of clinical benefit.

Work that has demonstrated the positive contribution of macrophages to tissue remodelling and regeneration has predominantly been explored in in vivo settings (Brown et al., 2012a) or with scaffolds that are seeded with macrophages in typical cell culture dishes (Almeida et al., 2014). While the former approach is most relevant to the activity of these cells following the implantation of a tissue engineered product, the complex in vivo environment prevents a detailed investigation of the different factors that could play a role in regulating the activation and polarization of macrophages. In the case of mechanically loaded tissues such as arteries and veins (e.g. vascular grafts), this makes it impossible to sort out the contributions of the biomaterial, the biomechanical environment, and the surrounding tissue environment (i.e. site of
implantation) on the state of the cells and their ability to regulate new tissue production. In contrast, while *in vitro* testing of biomaterials can specifically determine the ability of different biomaterials to regulate the activation state independent of implantation site, simple static culture environments may not be representative of the stresses imposed on both the biomaterial and its associated cells *in vivo*. The results presented here identify the importance of investigating the ability of monocytes/macrophages to regulate the activity of tissue-specific cell types using *in vitro* platforms that mimic *in vivo* biomechanical loading conditions, as they have the ability to perturb the activity of both cell populations. Furthermore, the ability to include appropriate controls, such as the culture of monocytes/macrophages alone under biomechanical strain, has allowed for the identification of ECM production by monocytes/macrophages as a novel source of new tissue production. This factor would not have been able to be identified using a more complex *in vivo* study, where new tissue deposition would have likely been attributed only to classical cell types such as VSMCs and fibroblasts. The present study illustrates the ability to harness the natural cross-talk that exists between macrophages and tissue cells in the context of vascular tissue engineering within a physiologically relevant biomechanical environment, applied to a unique degradable polyurethane (D-PHI). These finding may also be applied in the context of other tissue engineering disciplines.

### 6.6. Conclusions

This study used a monocyte co-culture on a degradable polyurethane (D-PHI) to regulate the response of VSMCs in combination with biomechanical strain in a vascular tissue engineering context. The use of circumferential strain was shown to influence the ability monocyte-VSMC co-cultures to regulate VSMC growth and new tissue production, highlighting the importance of understanding the ability of macrophages to regulate the response of VSMCs using physiologically relevant biomechanical loading environments. Results demonstrated that monocyte co-culture and biomechanical strain have a complementary and non-mitigating effect on increasing VSMC number. The application of biomechanical strain was shown to be required to increase the collagen content of VSMC-containing scaffolds (in monoculture or in co-culture with monocytes), with a corresponding increase observed in construct mechanical properties. Monocytes/macrophages were shown to shift away from a pro-inflammatory state over time, as
demonstrated through both cell surface marker expression and cytokine analysis. Furthermore, in addition to regulating the response of VSMCs, monocytes/macrophages were shown to produce significant amounts of vascular ECM components, including collagen I collagen III, elastin, and GAGs. This study highlights that immunomodulatory biomaterials, such as D-PHI, that support a desirable macrophage activation state can be combined with biomechanical strain to augment vascular tissue production in vitro, in part due to the unexpected contribution of monocytes/macrophages themselves producing vascular ECM proteins.

6.7. Acknowledgements

This study was funded by CIHR operating grant #230762 (J.P. Santerre, R.S. Labow, C.A. Simmons). K.G. Battiston was funded by a CIHR Strategic Training Fellowship (STP-53877) and by an Ontario Graduate Scholarship (OGS). Special thanks to the staff at the MC-78 machine shop (Mechanical and Industrial Engineering, University of Toronto); Maxfield Bradshaw, Sina Makaremi, and Bradley Menezes for assistance with bioreactor design; Dr. Jian Wang for help with mechanical testing; and Feryal Sarraf for preparation of histology samples and staining.
Supplemental Figure 6.1 Bioreactor set-up. (A) Tubular D-PHI scaffolds. (C) Close-up of D-PHI chamber with (1) D-PHI scaffolds mounted on (2) distensible silicone tubing. (B) Picture of bioreactor set-up, showing (i) syringe holder, (ii) 50 cc syringe, (iii) bioreactor tray, (iv) manifold, (v) actuator, (vi) bioreactor chamber with syringe filter for gas exchange, and (vii) controller.
Supplemental Figure 6.2 Immunofluorescence image showing stained nuclei within the pores of D-PHI scaffolds, which also stains with the Hoechst 33342 stain. Scale bar indicates 60 μm.

![Immunofluorescence image](image)

Supplemental Figure 6.3 DNA mass quantification for tubular D-PHI scaffolds seeded with (A) VSMCs and monocytes in co-culture (grey bars), (B) VSMCs (black bars), or (C) monocytes (white bars). (D) Comparison of DNA in co-culture samples to the sum of DNA from individual VSMC and monocyte monocultures (V+M). Constructs were cultured for up to 4 weeks under dynamic (10% circumferential strain, 1 Hz) or static conditions. n=8-10. Data represent the mean ± S.E. *p<0.05.
Supplemental Figure 6.4 20 µm thick cross-sections of D-PHI scaffolds seeded with VSMCs, monocytes, or VSMCs and monocytes in co-culture after 4 weeks of static or dynamic (10% strain, 1 Hz) culture. Samples were stained with H&E, picrosirius red, or alcian blue (pH=2.5) to assess cellularity, collagen deposition, and GAG deposition, respectively, throughout the entire thickness of the wall of D-PHI scaffolds. Scale bar represents 250 µm.
Supplemental Figure 6.5 40X magnification image of monocytes after 4 weeks of culture on D-PHI that is representative of morphology under both static and dynamic culture for monocytes cultured alone and in co-culture with VSMCs. Arrow indicates a FBGC. While the majority of cells remain mononucleated, isolated cases of FGBCs can be found. The image shown is from the dynamic monocyte culture at 4 weeks, but is representative of monocyte/macrophage morphology for all of the different conditions.
**Supplemental Figure 6.6** Densitometric quantification of western blot data α-SMA and calponin expression for D-PHI scaffolds seeded with VSMCs (white bars) or VSMCs and monocytes in co-culture (grey bars). Data are the mean ± S.E. n=6. Data are presented as band intensity normalized to each samples respective GAPDH loading control, as well as to the W0 sample to give a measure of the loss or gain of expression after initial cell seeding. *p<0.05 vs. respective VSMC control.

**Supplemental Figure 6.7** Quantification of apoptosis expressed as the percent of caspase 3 positive cells from immunofluorescence image analysis after 4 weeks of culture for (A) VSMCs and (B) monocytes/macrophages. Data are the mean ± S.E. n=9. *p<0.05 vs. static control.
**Supplemental Figure 6.8** Quantification of sulphated GAG content from samples subjected to papain digestion. Data are the mean ± S.E. n=5. *p<0.05.

**Supplemental Figure 6.9** Quantification of immunofluorescence images staining for the M1 markers CD80 and CD86, as well as the M2a marker CD206 for monocytes alone on D-PHI scaffolds. Dashed line indicates levels for each marker at W0 (24 hr post-seeding). Data are the mean ± S.E. n=9. *p<0.05 vs. week 2 timepoint for the same culture condition.
Supplemental Figure 6.10 Quantification of immunofluorescence images staining for the M1 markers CD80 and CD86, as well as the M2a marker CD206 for co-culture samples. Dashed line indicates levels for each marker at W0 (24 hr post-seeding). Data are the mean ± S.E. n=9. †p<0.05 vs. static condition at the same timepoint.

Supplemental Figure 6.11 Cytokine and growth factor release quantification for (A) TNF-α and (B) IL-10. Data are the mean ± S.E. n=4-5. *p<0.05 vs. co-culture at same time point under same culture conditions. °p<0.05 vs. W0 for same culture condition. †p<0.05. S- static. D- dynamic. Dashed line indicates level at W0. Where no dashed line is indicated, W0 levels were below sensitivity of the assay.
6.9. References


Chapter 7
Conclusions

The studies presented in this thesis generated an understanding of the importance of adsorbed serum proteins for influencing the response of monocytes upon adhering to a biomaterial substrate. A biomaterial, D-PHI, that was shown to reduce pro-inflammatory monocyte activation through its modulation of the adsorbed protein layer was subsequently shown to harness the stimulatory potential of these white blood cells to direct the response of VSMCs in the context of a vascular tissue engineering strategy. This monocyte-induced stimulation could further be augmented when used in combination with biomechanical stimulation. In the following section a summary of the main findings and their importance to the field is given. In addition, recommendations for future studies based on the insights provided by this thesis are included that can further enhance the field’s understanding of the contribution of adsorbed protein-monocyte interactions in governing the successful remodeling of implanted biomaterials, as well as realizing the potential of such materials to guide the immune system to regenerate new tissues in both *in vitro* and *in vivo* settings through the stimulation of tissue-specific cell types.

7.1. Summary and scientific contributions

Understanding the mechanisms of adsorbed-protein interactions is an important aspect that is often neglected in protein adsorption studies (Shen et al., 2004; Maciel et al., 2014; Schmidt et al., 2011), but which can provide further insight into the biological implications of the observed results. Studies in Chapters 3 and 4 investigated the role of protein adsorption in regulating monocyte activation to a degradable polyurethane, D-PHI, and compared the response observed with D-PHI to conventional biomaterial substrates, namely TCPS and/or PLGA. The study in Chapter 3 sought to detect proteins that were differentiated in terms of their presence in the adsorbed protein layer for D-PHI, TCPS and PLGA, and identified a set of candidate proteins for further studies regarding their effect on monocyte activation, some of which would not otherwise have been identified from a survey of the biomaterials literature regarding protein-monocyte interactions (Collier and Anderson, 2002; Jenney and Anderson, 2000a). An approach such as this has the potential to identify new proteins not thoroughly studied in the biomaterials literature that are important for governing monocyte interactions, as opposed to simply choosing from a
list of proteins which have already been studied comprehensively. In this study, A2M was identified as a protein that induced a pro-inflammatory effect on D-PHI (increased TNF-α release), and the mechanism of the interaction of A2M with monocytes on D-PHI was confirmed through inhibition studies. Another aspect of this study that contributes to the understanding of biomaterial-monocyte interactions to the field is the ability of D-PHI to attenuate the pro-inflammatory effects of A2M in the presence of other serum proteins. In many instances, the effects of single protein solutions on cell response are observed (Collier and Anderson, 2002; Jenney and Anderson, 2000a; Anderson et al., 1999); however, without conducting such studies in complex biological environments (in this case, complex protein solutions such as serum-containing medium), other aspects such as protein-protein interactions and phenomena such as the Vroman effect are not taken into account (Vroman and Adams, 1969; Vogler, 2012). In addition, the degradable polyester PLGA was shown to induce a heightened pro-inflammatory response regardless of protein coating. PLGA is a commonly used degradable biomaterial in many different tissue engineering applications (Sicchieri et al., 2012; Yoshida and Babensee, 2006; Niklason et al., 1999). However, recent studies have indicated the propensity for this material to act in a pro-inflammatory manner (Yoshida and Babensee, 2006; McBane et al., 2011; Babensee and Paranjpe, 2005; Song et al., 2011; Ali and Mooney, 2008), possibly due to a local increase in acidic degradation by-products that are harmful to cells. In other cases, degradation by-products of PGA have been shown to support dedifferentiation of VSMCs (Higgins et al., 2003). This study demonstrates that in terms of interactions with monocytes, a key cell type in the foreign body reaction to implanted biomaterials, that alternatives to PLGA exist that may result in improved performance of implanted materials and tissue engineered products.

In Chapter 4, a different approach to studying the phenomenon of protein adsorption was pursued. It was hypothesized that a difference in protein conformation, rather than protein amount, was the critical factor regulating monocyte response to D-PHI. As such, key proteins that have been identified in the biomaterials literature were screened for their potential to induce divergent monocyte responses to D-PHI and TCPS. In particular, IgG was shown to support an initial increase in TNF-α/IL-10, greater monocyte retention over time, and greater cell spreading when pre-adsorbed to TCPS, but not when pre-adsorbed to D-PHI. This was subsequently shown
to be due to an increase in the Fab binding site exposure after pre-adsorption of IgG to TCPS. This was demonstrated through both ELISA quantification for Fab exposure as well as inhibition experiments. Much of the literature which has reported on protein-biomaterial interactions simply identifies a certain protein as being pro-inflammatory or not for monocytes (Collier and Anderson, 2002; Jenney and Anderson, 2000a; Jenney and Anderson, 2000b). This is one of the first studies to identify the ability of a biomaterial to inhibit a pro-inflammatory monocyte response by supporting a different conformation of a protein, despite adsorbing the same amount if not more of that protein when compared to TCPS. Furthermore, the role of D-PHI chemistry in regulating IgG Fab exposure was investigated by systematically increasing or decreasing the ionic (MAA) and hydrophobic (MMA) content relative to the polar (DVO) component of D-PHI. Through these studies, it was demonstrated that IgG Fab exposure was decreased by increasing both MMA and MAA content and, conversely, increased by decreasing MMA and MAA content relative to DVO. A formulation that resulted in increased Fab exposure had similar effects to TCPS in supporting increased monocyte adhesion and spreading when coated with IgG, further confirming the role of IgG Fab exposure in these processes. Supporting a reduced monocyte/macrophage activation is desirable in many different applications, and has been shown to be supported by several different kinds of biomaterials (Sicari et al., 2014; Sussman et al., 2014; Vasconcelos et al., 2013). In some cases, such as ECM-derived surgical meshes, soluble ECM components have been implicated in supporting a favourable macrophage polarization (Sicari et al., 2014). For synthetic biomaterials, however, there is little understanding of the underlying mechanisms supporting the desirable monocyte activation state (Sussman et al., 2014). As a result, while some insights can be provided by these studies, ultimately they are not as instructive for the design of new immunomodulatory materials. In this thesis, the data presented suggest that an increase in chemical heterogeneity (ionic from MAA, hydrophobic from MMA, non-ionic polar from DVO) is desirable, and that moving towards a more homopolymer structure leads to greater changes in protein presentation that can expose pro-inflammatory binding sites, such as the Fab site on IgG. A significant contribution of this thesis is thus to identify aspects of D-PHI chemistry that contribute to its favourable interactions with proteins and monocytes that can be used to guide the design of new immunomodulatory biomaterials. This specific observation also resulted in the submission of a provisional patent application in August 2014.
The work presented in Chapter 5 demonstrated the potential of monocytes to be used in tissue engineering strategies by demonstrating their use in vascular tissue engineering as a case study. Co-culturing monocytes with VSMCs was shown to promote increased cellular infiltration into porous D-PHI scaffolds, support greater VSMC numbers, and also down-regulate contractile marker expression, all of which are indicative of a synthetic VSMC phenotype. Experiments using MCM implicated monocyte-released factors as important in regulating these processes. Using a cytokine antibody array and ELISAs identified IL-6 as a potential cytokine involved in regulating VSMC response, and the role of IL-6 in supporting VSMC growth and suppressing contractile marker expression was confirmed through inhibition experiments in the monocyte-VSMC co-culture system. Biochemical stimulation is typically achieved in the form of recombinant growth factors when used in vascular tissue engineering strategies (Gui et al., 2014; Stegemann and Nerem, 2003; Chan-Park et al., 2009; Syedain and Tranquillo, 2011). This comes with great costs in materials, especially when prolonged culture periods are involved, and the risk of endotoxin contamination that can promote an adverse foreign body response following implantation (Wakelin et al., 2006; Daly et al., 2012). This thesis thus demonstrates that monocytes/macrophages and their cytokine activity can be harnessed in co-culture strategies to promote desired cellular responses when coupled with biomaterials that induce an appropriate monocyte/macrophage activation state. Furthermore, the identification of the cytokines in part responsible for monocyte effects on VSMCs can be used as a potential screening tool to determine if other biomaterials are promoting sufficient cytokine release to be used in a similar strategy. While this thesis used vascular tissue engineering to evaluate the potential of monocytes to act as a stimulus for another cell type, this strategy has further applicability beyond vascular tissue engineering, including tissue engineering disciplines where monocytes contribute to the natural development of the tissue (e.g. bone (Dong and Wang, 2013)), during healing (e.g. skeletal muscle (Novak and Koh, 2013; Novak et al., 2014)), or are present as a resident cell type during homeostasis (e.g. Kupffer cells in the liver (Zinchenko et al., 2006) and microglial cells in the central nervous system (Olah et al., 2012)).

Monocyte-VSMC co-culture was coupled with the use of biomechanical stimulation in Chapter 6 to determine if these two strategies could have a complementary effect on promoting increased VSMC number and tissue deposition in a vascular tissue engineering strategy. Previous work in
the literature has suggested that combining mechanical stimulation with growth factor delivery can attenuate the positive effects induced by mechanical stimulation, such as cell proliferation, collagen production, and increased mechanical properties (Stegemann and Nerem, 2003; Syedain and Tranquillo, 2011). In this thesis, however, biomechanical stimulation and monocyte co-culture were shown to have a complementary and non-mitigating effect on increasing VSMC number. Furthermore, co-culture was shown to support increased elastin and sulphated GAG deposition, while not having a detrimental effect on the ability of dynamic culture conditions to upregulate collagen production and construct mechanical properties. An important contribution of this thesis to the scientific literature is thus to identify monocytes/macrophages as a source of biochemical stimulus that can be coupled with biomechanical stimulation to increase VSMC number and tissue deposition, replacing the use of more expensive specific molecular biology strategies. This work supports the notion of a macrophage polarization shift towards an M2 activation state as being an important factor necessary for macrophages to support these outcomes. Furthermore, macrophages are identified in this thesis as potent producers of vascular tissue components, including collagens, elastin, and GAGs, which is a topic that has not received much attention in the tissue engineering literature and warrants further investigation.

7.2. Recommendations

1. This thesis identified protein adsorption as being critical to understanding D-PHI-mediated monocyte activation, and specifically identified exposure of the Fab region of IgG as playing a role in this process. Exposure of the Fab region has been correlated with increases in monocyte spreading and adhesion to biomaterials during acute periods (3 days). While this study has aided in the understanding of D-PHI-monocyte interactions in vitro, in vivo studies have also implicated D-PHI as supporting reduced inflammation and enhanced tissue integration when compared to materials such as PLGA (McBane et al., 2011). Protein adsorption is an important regulator of monocyte response in vivo with a strong biomaterial dependence (Anderson et al., 2008). It is important to establish whether the relationship between Fab exposure and monocyte response in vitro can be correlated to the inflammatory response observed following biomaterial implantation. Such a study could be implemented by evaluating Fab exposure to a set of comparable biomaterials (same shape, size, porosity,
etc.) in vitro and subsequently correlating Fab exposure with the immune response following implantation. Parameters that could be used to assess the immune response include number of monocytes/macrophages at different time points post-implantation (CD68+ cells), M1 (CCR7, CD80) and M2 (CD163, CD206) marker expression (as well as M2/M1 ratio), cytokine release in tissue exudates (TNF-α, IL-10, IL-12, IL-1, IL-1ra, MCP-1, IL-6 etc.), and scoring of histological images similar to that performed by Brown et al., which includes factors such as fibrous encapsulation, number of blood vessels, tissue infiltration, number of immune cells, and extent of biodegradation (Brown et al., 2012). If a correlation were observed between Fab exposure and in vivo foreign body response endpoint markers, Fab exposure would be a valuable tool in initial in vitro biocompatibility testing. Furthermore, D-PHI chemistry has the potential to allow coupling of peptides. It would thus be possible to artificially introduce IgG Fab exposure into D-PHI in a dose dependent manner by coupling Fab peptides to the material, which can be accomplished by acidifying the methyl ester of LDI and coupling via an amine group on the peptide using diisopropylcarbodiimide chemistry. A series of D-PHI formulations with increased Fab exposure through Fab-peptide coupling could be implanted and the immune response as a function of Fab exposure evaluated. This strategy has the advantage maintaining material mechanical properties, chemistry, and other factors constant, such that any differences observed can be more confidently attributed to differences in Fab exposure.

2. In a study by Jenney et al., the Fab region of IgG was implied to be important for long-term macrophage adhesion by comparing macrophage response to surfaces adsorbed with total IgG to those adsorbed with Fc or Fab fragment (Jenney and Anderson, 2000b). This thesis provided further evidence for the importance of Fab interactions supporting monocyte adhesion and spreading through antibody inhibition experiments. However, a fundamental understanding of the mechanism of Fab-monocyte/macrophage interactions is still lacking. Unlike the Fc fragment, which has many different macrophage receptors to which it can bind (Ishiguro et al., 1991; Polat et al., 1993), there are no known Fab receptors. Proposed theories for Fab-monocyte interactions include lectin binding with Fab-associated carbohydrates and Fab-mediated complement activation, with complement products supporting enhanced macrophage adhesion (Jenney and Anderson, 2000b). However, no experimental support for
these theories has been demonstrated. Unlike the Fc region, which is most commonly associated with binding to Fc receptors leading to phagocytosis, the Fab region is predominantly studied for its ability to bind ligands. One possible way in which Fab exposure could promote adhesion is through the binding of growth factors and cytokines from the culture medium that are subsequently presented to cells and regulate their attachment to the surface (Heidenhain et al., 2011). This latter hypothesis is supported by data presented in Chapter 4 that show a lack of IgG-induced monocyte adhesion when cells are cultured in serum-free medium. Because IgG plays a critical role in so many processes, the identification of the mechanism for Fab-monocyte interactions would be a significant finding not only for the field of biomaterials, but also immunology in general.

3. Studies in Chapter 4 indicated that reducing DVO content relative to the combined MAA and MMA content of D-PHI resulted in an increase in Fab exposure. It is hypothesized that this is due to a transition to a more homopolymer structure that gives the protein fewer options in terms of interacting with different chemical groups on the material surface, resulting in conformational changes upon adsorption that expose key binding sites. In addition to the relative amount of DVO, another variable that could be studied is the MMA:MAA ratio. Furthermore, for D-PHI the hydrophobic character is provided by the methyl group in MMA and the ionic character by the carboxylic acid group in MAA. Future work should explore different chemical groups as a means to provide this functionality to determine the importance of chemistry vs. general hydrophobicity and ionic character in regulating Fab exposure. Initial synthesis of D-PHI was also performed using a 3:1 MMA:MAA ratio as previous studies had indicated that this was favourable for supporting cell adhesion (Van Wachem et al., 1985). Dramatic differences in D-PHI’s mechanical properties and swelling were also observed with the different formulations tested in Chapter 4. In addition to testing Fab exposure, experiments should also assess how these new formulations perform in swelling and mechanical testing experiments, as well as if they are able to support adhesion and proliferation of different tissue cell types.

4. The phenotypic state of macrophages is highly plastic, existing on a spectrum between classically activated (M1) and alternatively activated (M2) (Mosser and Edwards, 2010),
with monocyte subsets also existing in circulation prior to tissue infiltration and subsequent polarization to macrophages (Gordon and Taylor, 2005; Randolph et al., 2002). The initial adhesion of different monocyte subsets to a foreign surface has yet to be investigated. In particular, the recruitment of different monocyte subsets to biomaterials used in vascular grafts under flow conditions has not been studied. To investigate this phenomenon, the presence of phenotypic markers expressed on monocytes recruited to different biomaterials and their means of adhesion during the initial monocyte-biomaterial interaction could be achieved via live-cell imaging using monocytes circulated through tubular biomaterials with a flow bioreactor system (e.g. BioDynamic test chamber from Bose) using various synthetic (e.g. PLGA and polyurethane) and natural (e.g. collagen) materials. The expression of markers characteristic of different monocyte subpopulations could be observed by taking time-lapse images using GFP and CFP-labelled phenotypic markers (Sahai et al., 2005) with two-photon microscopy (Sahai et al., 2005; Devi et al., 2013; Li et al., 2012; Laperchia et al., 2013; Haka et al., 2012). Two-photon microscopy allows for thicker samples (e.g. scaffold thickness of 2 mm) to be imaged with greater penetration of excitation light, which would be necessary due to the thickness of samples being imaged. Also, the use of excitation wavelengths in the near-infrared range minimizes tissue damage due to phototoxicity. It is expected that different monocyte subpopulations will be recruited preferentially to different biomaterial substrates, and that the engagement of different integrins will be involved in monocyte adherence to different biomaterials (Anderson et al., 2008). This study could provide important insights into the mechanisms and processes underlying monocyte recruitment and activation to implanted biomaterials.

5. In Chapters 5 and 6, the role of monocytes/macrophages in tissue engineering strategies was explored exclusively in the context of vascular tissue engineering. However, there are many tissue types that contain resident macrophage populations (e.g. Kupffer cells in the liver, microglia in the central nervous system) as well as many biomaterial applications where monocytes/macrophages will interact with the implanted product and contribute to the foreign body reaction (Mosser and Edwards, 2010). Macrophages thus have the potential to be used in different tissue engineering strategies, and have been explored to some extent with regard to this for regulating hepatocyte function (Zinchenko et al., 2006), remyelination in
the central nervous system (Olah et al., 2012), and to regulate MSC function for bone tissue engineering (Dong and Wang, 2013). Each of these areas has specific design criteria for materials in terms of mechanical properties and promoting specific cell phenotypes, but all have the requirement of supporting a macrophage activation state that is supportive of tissue-specific cellular activity. In order to evaluate the potential for macrophages to contribute to different tissue engineering strategies, polarized macrophages (using IFN-γ and LPS for M1 polarization and IL-10, IL-13, or IL-4 for M2 polarization) should be co-cultured with different cell types to determine (1) if macrophages can contribute to different tissue engineering disciplines and (2) the appropriate phenotypic state required for supporting desirable cellular activity. In addition to populations of pure M1 or M2 polarized populations, mixed populations should also be explored since in some contexts, such as promoting vascularization, the presence of both subsets is required (Spiller et al., 2014). Knowledge gained from such studies could inform the design of biomaterials to promote certain macrophage activation states for different tissue engineering applications.

6. In the present thesis, bioreactor studies involved exposing cells to dynamic mechanical stimulation with 10% circumferential strain with a 1 Hz frequency. Previous studies in the Santerre laboratory have demonstrated that this mode of stimulation can increase VSMC number (Sharifpoor et al., 2011), and it is also within the physiological range for human coronary artery (Larsson et al., 2011). However, VSMCs have been shown to be sensitive to different strain levels (2.5-15%), as well as the frequency and duty cycle of the applied strain (Isenberg and Tranquillo, 2003). Likewise, monocytes and dendritic cells have also been shown to be sensitive to the magnitude of the applied strain (Ballotta et al., 2014; Lewis et al., 2013). In order to optimize the dynamic co-culture system, studies should be undertaken to explore the effect of the magnitude of strain, frequency, and duty cycle on D-PHI scaffolds seeded with monocytes and VSMCs. Furthermore, there are few studies to date studying the response of immune cells to mechanical strain (Ballotta et al., 2014; Lewis et al., 2013; Matheson et al., 2007), and none in the context of 3D porous biomaterial scaffolds. To provide a more complete understanding of the impact of mechanical stimulation on monocytes/macrophages, experiments wherein strain rate, magnitude, and duty cycle are controlled should be performed for monocytes alone on 3D D-PHI scaffolds. Furthermore,
Syedain et al. have also demonstrated that incrementally increasing strain up to 15% with 4 equal time-steps over 3 weeks results in enhanced mechanical properties and collagen production when compared to consistently applying the same magnitude of strain (Syedain et al., 2008). Parameters that should be assessed include cell number (DNA mass quantification), apoptosis/necrosis (immunofluorescence), M1/M2 marker expression (immunofluorescence, PCR), cytokine release (ELISA), and ECM production.

7. One of the unexpected findings of this thesis was the extent to which monocytes/macrophages are capable of producing ECM proteins, including collagen I, collagen III, elastin, and sulphated GAGs. A limited number of studies have been performed on this subject, but those that have been published indicate that macrophages express mRNA for nearly all collagens (Schnoor et al., 2008) as well as elastin (Krettek et al., 2003). In the case of collagen VI, production is supported by polarizing macrophages with biomolecules that induce an M2 state, and inhibited by polarizing biomolecules that support an M1 state (Schnoor et al., 2008). However, with respect to vascular ECM, there is no understanding of how collagen I, collagen III, and elastin production are influenced by macrophage polarization. In order to provide insight into this phenomenon, studies should be undertaken where monocyte populations are polarized to favour M1 (IFN-γ, LPS, TNF-α) vs. M2 macrophages (IL-10, IL-13, IL-4, TGF-β1) and detailed analysis of ECM production is performed. For elastin, this would include a Fastin elastin assay (total elastin), western blotting (intracellular elastin production), immunostaining (extracellular vs. intracellular elastin), and PCR (gene expression). The same assays could be employed for collagens I and III as well as sulphated GAGs, with the Fastin elastin assay replaced by OH-Pro quantification for total collagen and a dimethylmethylene blue dye binding assay for total sulphated GAGs. In addition to total protein production, the organization of the ECM proteins is equally important for their function. For collagen VI, secreted protein from macrophages was shown to associate with the cell surface and was not organized into filaments, possibly due to the lack of proteins required for filament assembly, aggregation, and network formation (such as fibrillin, decorin, and lumican, amongst others) being expressed at low levels by macrophages (Schnoor et al., 2008). The formation of collagen fibrils can be assessed by polarized light microscopy, whereas elastin cross-linking can be
assessed by quantification of desmosine content by ELISA or radioimmunoassay. Formation of collagen fibrils from collagen secreted by macrophages could also be analyzed in the co-culture system, as VSMCs express proteins required for filament organization that may allow macrophage-secreted collagen to be incorporated into the vascular ECM (Schnoor et al., 2008).

8. This thesis demonstrated the ability of monocyte co-culture to increase GAG and elastin production, but did not have an effect on total collagen production. Furthermore, while detectable levels of elastin were produced in the culture system used, these levels are still below that observed in healthy arteries (15 % (coronary) to 40 % (aorta) by mass of the dry weight of arterial walls (Fischer and Llaurado, 1966), compared to approximately 1% in the present in vitro system reported in this thesis). Due to the importance of collagen and elastin in providing mechanical integrity of blood vessels, efforts should be undertaken to further support the production of these ECM proteins. A commonly used method to enhance cell proliferation and collagen production in tissue engineering is by dosing ascorbic acid into culture medium (Gui et al., 2014; Laflamme et al., 2006; Syedain et al., 2011). This is a strategy that can be achieved with minimal cost using synthetically derived molecules. With regard to elastin production, a number of strategies could be explored, including growth factor delivery (TGF-β1, insulin, sodium ascorbate) and a different cell source (Syedain and Tranquillo, 2011; Shi et al., 2012). In this thesis (Appendix G), the use of TGF-β1 to promote a contractile VSMC phenotype was demonstrated, and a time-dependent supplementation strategy was employed to mitigate the negative effects of this growth on VSMC number. Similarly, Syedain et al. have demonstrated that supplementing TGF-β1 into culture medium for only the final two weeks of culture results in enhanced elastin production, without the limitations in terms of decreased collagen production and cell growth attributed to prolonged exposure to TGF-β1 under mechanical strain (Syedain and Tranquillo, 2011). The use of different growth factors for promoting increased elastin production should thus be evaluated in the context of the dynamic co-culture system. In addition to evaluating elastin production, experiments should also confirm that increases in collagen production, cell proliferation, and mechanical properties promoted by dynamic co-culture are not attenuated by growth factor supplementation. Furthermore, since these biomolecules can also affect
monocyte/macrophage activation, the effect of their doses used in the dynamic co-culture system should also be screened with respect to their effect on monocyte response (polarization [immunofluorescence and PCR for M1 and M2 markers], cytokine release [ELISA]). TGF-β1, for example, has been shown to support macrophage production of type VI collagen (Schnoor et al., 2008). The effect of these biomolecules on elastin, collagen, and GAG deposition should thus also be taken into consideration.

9. In this thesis mechanical testing was performed by assessing construct mechanical properties such as elastic modulus, tensile strength, and elongation at yield. Another type of mechanical test that can provide insight into the organization of the ECM is hysteresis testing. Hysteresis refers to the loss of energy when a material is cyclically loaded and unloaded. In vascular tissue engineering, this refers to the ability of a vascular construct to act as an efficient elastic reservoir, and is a property primarily provided by elastic fibres, but to which collagen fibres can also contribute (Wagenseil and Mecham, 2009). Hysteresis testing can thus provide insight into the organization of the ECM that is being quantified with other assays, and would be useful in determining differences in ECM produced by VSMCs and monocytes/macrophages. Hysteresis testing is performed by repeatedly loading and unloading a vascular construct between two strain values and analyzing the subsequent force-displacement curves. Hysteresis can be calculated from the area under the inflation and deflation curves, the difference between which is the energy lost in the cycle (Gauvin et al., 2011).

10. Many tissue engineering strategies attempt to promote a certain phenotypic state of cells in vitro (Chan-Park et al., 2009). In vascular tissue engineering, this means that while synthetic VSMCs are desirable for promoting tissue production and increased cell proliferation, a phenotypic switch should ultimately be supported that promotes a contractile VSMC phenotype, which is associated with healthy vasculature (Chan-Park et al., 2009). In this thesis, such a phenotypic shift has been demonstrated in the co-culture system on D-PHI scaffolds with supplementation of TGF-β1 during the final 2 weeks of culture, resulting in upregulation of the contractile marker proteins α-SMA and calponin (Appendix G). Such a strategy, however, is based on the assumption that the VSMC phenotype supported in vitro
will be maintained in the complex in vivo environment. In a study by Bochaton-Piallat et al., SMCs derived from newborn rats were 96% α-SMA+ and 50% SM-MHC+ (mainly contractile) and those from old rats were 48% α-SMA+ and 1% SM-MHC+ (mainly synthetic) in vitro. Following seeding into an injured rat carotid artery, similar to in vitro culture, newborn rat SMCs expressed higher levels of α-SMA and SM-MHC relative to SMCs from old rats (Bochaton-Piallat et al., 2001). Two important notes should be made about this study. (1) The authors concluded that genetics, rather than the in vivo environment, was the principal factor governing VSMC phenotype. In the case of VSMCs induced to adopt a phenotype with biochemical stimulation, will this stimulation be maintained in vivo in the absence of the growth factors, and will they revert to a phenotype dictated by their source (i.e. determined by genetics) following implantation? Furthermore, will the presence of the biomaterial (absent in the study by Bochaton-Piallat et al.) help maintain the in vitro supported phenotype? (2) While SMCs from newborn vs. old rats maintained higher contractile marker expression in vivo, it should also be noted that the proportion of positively stained cells changed from in vitro to in vivo, suggesting some dependence on the in vivo environment. In order to evaluate whether VSMC phenotype in vivo is dominated by the in vivo environment or the in vitro-induced phenotypic state, vascular grafts with similar cellularity, ECM composition etc. could be prepared, followed by a short period of growth factor stimulation to induce a synthetic (e.g. PDGF-BB (Chan-Park et al., 2009)) or contractile (e.g. TGF-β1 (Chan-Park et al., 2009)) state without otherwise altering graft properties significantly. Following implantation, VSMC phenotype would be tracked at different time-points, with donor VSMCs distinguished from host VSMCs by using GFP-labelled VSMCs in vitro. This study would have significance beyond vascular tissue engineering, with applicability to the field of tissue engineering in general.

11. While the use of the dynamic co-culture system in this thesis resulted in enhanced construct cellularity, collagen deposition, and mechanical properties, one significant limitation of the final construct is the inability to be sutured using traditional techniques. Several strategies could be taken in order to improve this property. (1) Using longer culture times (e.g. 12 weeks) has been shown by other research groups to be required for supporting sufficient collagen production to generate a vascular graft with mechanical properties suitable for
implantation (Niklason et al., 1999; Laflamme et al., 2006; Syedain et al., 2011). A similar strategy with D-PHI could be used to generate greater construct cellularity and collagen deposition, possible in combination with medium supplementation of factors known to induce matrix synthesis (e.g. ascorbic acid) (Niklason et al., 1999; Syedain et al., 2011). (2) In Chapter 2, different D-PHI formulations were identified with reduced Fab exposure similar to the original D-PHI formulation, suggesting that these materials may also promote the same reduced pro-inflammatory macrophage activation state. Furthermore, mechanical testing showed that increasing the relative proportion of DVO to combined MMA and MAA resulted in decreased mechanical strength. Formulations with increased MMA and MAA content, which showed reduced Fab exposure, may thus have greater mechanical strength than the original D-PHI formulation while demonstrating the ability to support a reduced pro-inflammatory macrophage activation state. This would require testing different D-PHI formulations with increased MMA and MAA content for their compatibility with VSMCs as well as their mechanical properties and biodegradability. (3) Preliminary experiments with D-PHI have been performed wherein the D-PHI scaffold is reinforced with an electrospun polyurethane membrane, which has the mechanical strength required to permit suturability, allowing the graft to be implanted in a rabbit model without cell seeding. Use of such a graft allows for implantation without an in vitro culture period, permitting off-the-shelf capability, where the body will act as a bioreactor to support tissue regeneration that will provide sufficient mechanical strength so that after the electrospun coating has degraded, sufficient mechanical strength is provided by the D-PHI biomaterial in combination with the in vivo developed neotissue. Ultimately, if a material could be achieved with sufficient mechanical strength a strategy could be employed where a short period of pre-seeding with monocytes is performed prior to implantation without any prolonged culture period. The feasibility of such a strategy has been demonstrated by Shin’oka et al (Shin’oka et al., 2005). This strategy could take advantage of the immunoregulatory property of D-PHI to allow for macrophage-guided tissue regeneration in vivo, allowing for an off-the-shelf capability that is not possible with prolonged in vitro culture.

12. In this thesis primary human coronary artery SMCs from elderly donors were used as the VSMC source. In terms of clinical translation of a vascular tissue engineering strategy,
coronary artery SMCs have limited availability. Furthermore, adult cell sources typically have limited proliferative capacity as well as reduced matrix deposition, particularly for elastin (Mecham, 2008; Petersen et al., 2010). Stem cells, however, have significantly greater proliferative capacity than adult cells and can be induced to differentiate to VSMCs (Huang and Niklason, 2014; Krawiec and Vorp, 2012). ASCs, in particular, can be relatively non-invasively isolated as an autologous cell source from a patient’s adipose tissue and differentiated to VSMCs in vitro with appropriate growth factor stimulation (e.g. BMP4, TGF-β1) (Wang et al., 2010). The use of ASC-derived VSMCs would allow for greater cell proliferation and matrix deposition, and would thus potentially not require culture times as long as those needed with human coronary artery SMCs to generate a tissue engineered construct with sufficient mechanical properties for implantation.

13. VSMCs are important contributors to vascular ECM production that is required for a mechanically robust tissue engineered vascular graft. The presence of a non-thrombogenic intimal layer, however, has also been shown to be of critical importance for the long-term success of tissue engineered vascular grafts (Zhang et al., 2009; Zilla et al., 2007; Quint et al., 2012). While for VSMCs the primary biomechanical stimulus is dynamic strain, for ECs stimulation with shear stress is important for proper function and survival (Dunn et al., 2014; Hergenreider et al., 2012; Chiu et al., 2005). Following the generation of the medial layer using a bioreactor to apply circumferential strain, it is important to endothelialize the constructs and expose them to shear stress using a pulsatile perfusion bioreactor. In addition to regulating EC response, shear stress has also been shown to regulate the ability of ECs to influence VSMC activity (Chiu et al., 2005; Wang et al., 2006; Sakamoto et al., 2006). In order to determine the optimal conditions for such a co-culture system, different seeding strategies to achieve an EC monolayer on the luminal surface of the D-PHI scaffolds, with limited EC infiltration into the medial layer, should be explored. In addition, in order to optimize EC response and EC-mediated effects on VSMCs, the application of different shear stresses should be investigated. EC response could be evaluated by assessing expression of desired phenotypic markers (von Willebrand factor, CD31, VE-cadherin), pro-inflammatory markers (ICAM-1, VCAM-1, E-selectin), growth factor release (VEGF, FGF), cell proliferation, and cell morphology.
7.3. References


Appendix A
Vascular graft literature review

Several different strategies in the biomaterials field have been explored for developing a tissue engineered vascular graft, including modifications to Goretex® and Dacron® to improve their performance, decellularized xenografts, cell-based strategies, biopolymers, and synthetic polymers (primarily polyurethanes and polyesters). The following section will review the major advances made in each of these areas and highlight the benefits and drawbacks of these approaches.

A.1. Modifications to conventional materials

The main limitations with currently used synthetic biomaterials, primarily Goretex® and Dacron®, for small diameter vascular grafts are the promotion of intimal hyperplasia and thrombus formation, resulting in vessel occlusion, as well as poor tissue integration and endothelialisation (Guidoin et al., 1993; Pasquinelli et al., 1990). Because these materials have a long history for use in vascular graft applications, an attractive strategy has been to perform modifications to Goretex® or Dacron® that specifically address limitations of these materials. This maintains the primary benefits associated with these materials, namely mechanical strength and in vivo stability, while also avoiding the risks that can be associated with the development of a completely new material.

Studies examining Dacron® and Goretex® grafts following explantation report the appearance of organized fibrin and exposed polymer with a lack of endothelialisation (Guidoin et al., 1993; Pasquinelli et al., 1990). One of the main strategies to modify these materials thus aims to promote the formation of a complete endothelial monolayer. Typically this is achieved by surface grafting of specific bioactive compounds. The use of EC-specific peptides such as REDV (Lei et al., 2012) as well as the combination of RGD and WQPPRARI (Gauvreau and Laroche, 2005) in vitro can support the adhesion and spreading of ECs, and have been used with other materials as a means to promote the recruitment of endothelial progenitor cells (EPCs) in vivo (Seeto et al., 2013). Immobilized growth factors, such as VEGF, have also been shown to be potent supporters of EC adhesion (Crombez et al., 2005).
In addition to enhancing endothelialisation, other strategies also aim to reduce thrombus formation and inhibit the SMC proliferation that can lead to intimal hyperplasia. To address the latter issue, dip-coated electrospun PET grafts for PEI-siRNA complex release have been developed that have the potential to alter VSMC phenotype to attenuate a proliferative response (Nabzdyk et al., 2014). Grafting of molecules such as dextran and heparin to PTFE have also been shown to reduce SMC adhesion and proliferation, as well as demonstrate greater EC adhesion and proliferation and enhanced non-thrombogenic properties (Hoshi et al., 2013). The combination of heparin with immobilized antibodies, such as CD133, targeting ECs has also been shown to promote faster endothelialisation in a porcine carotid artery transplantation model (Lu et al., 2013). While promising results have been observed with some of these strategies in vitro as well as in animal models, those that have reached clinical testing have resulted in much less dramatic results, such as the case of heparin-bonded PTFE, which showed no difference in patency rates compared to unmodified PTFE grafts and reduced patency rates relative to autologous saphenous vein (Dorigo et al., 2012). Furthermore, the non-degradable nature of these materials means that the synthetic polymer will always be present, in some cases requiring prolonged anticoagulant therapy, and will never result in full tissue integration or neovessel formation.

A.2. Natural biomaterials

Biological sources for vascular grafts have also been studied extensively, including decellularized tissue, ECM-derived biopolymers, non-ECM derived biopolymers, and purely cell-based approaches that do not rely on a biomaterial for growth and structural support. The following sections will highlight the significant advancements that have been made in each area, as well as highlighting the challenges associated with each approach.

A.2.1. Decellularized tissue

Decellularized tissues are produced by removing antigenic material, such as cells, in order to mitigate immune-mediated implant rejection. Typically, strategies involve the use of detergents, enzymes (DNases, RNases, proteases), or some combination thereof to eliminate components that will be recognized by the graft recipient as foreign, leaving behind the extracellular matrix.
framework. Removal of antigenic material allows the use of xenogeneic and allogeneic sources of grafts and has the advantage of potentially being an off-the-shelf strategy since decellularized tissue can be cryopreserved for long-term storage. This strategy has the further advantage of producing a construct that contains many of the intrinsic cues present in the ECM, both in terms of cell recognition sequences important for promoting phenotypic states, adhesion, and proliferation, as well as the 3D architecture that is critical for many cellular processes. Decellularized tissues have also been shown to work clinically in other settings, such as for heart valve replacements.

The most common types of decellularized vessels that have been evaluated include animal arteries and veins (xenografts), predominately of porcine origin due to similarities in physiology to humans, as well as human umbilical arteries (allografts). Decellularized grafts are often used in conjunction with cell-seeding strategies, typically ECs on the luminal surface, prior to implantation (Dahan et al., 2012). Decellularized porcine vascular tissue implanted as an interposition graft in the carotid artery of a rat was shown to maintain patency for 6 months without evidence of stenosis or dilation when pre-seeded with EPCs (Zhu et al., 2008). In some cases, pre-seeding strategies have been critical for the success of the implanted construct. Kaushal et al. demonstrated that decellularized ovine iliac vessels occluded following 15 days when implanted in a carotid interposition model in sheep, but remained patent for up to 130 days when pre-seeded with EPCs. Explanted grafts at this time point also showed natural physiological responses, including contractile activity and NO-mediated vascular relaxation similar to native carotid arteries (Kaushal et al., 2001). Decellularized canine jugular vein has also demonstrated good results in a bilateral carotid interposition model, with cellular repopulation of the graft and desirable mechanical properties observed after 8 weeks (Martin et al., 2005). Human umbilical arteries used as abdominal aorta interposition grafts in a rat model have demonstrated patency and endothelialisation after 8 weeks (Gui et al., 2009). In addition to decellularized vasculature, intestinal submucosa, rich in collagen I, has demonstrated patency and cellularization when implanted in a rabbit arterial bypass model (Huynh et al., 1999).

Despite these promising results, a lack of long-term studies has yet to prove the success of this approach. In addition, there are several issues associated with the use of decellularized vessels. Incomplete endothelialisation prior to implantation has been associated with adverse blood
interactions for decellularized arteries, most likely due to the thrombogenic nature of the underlying ECM. Furthermore, despite removal of most antigenic material, enzymatic degradation of decellularized tissue has been reported *in vivo*, particularly of the elastic component, which can result in impaired mechanical properties (Walles et al., 2003; Pennel et al., 2014). Decellularized constructs also suffer from poor re-cellularization upon implantation. While some have reported enhanced cellularization using a filler material, such as agarose, containing growth factors such as FGF or VEGF, others have demonstrated that the incorporation of such factors results in massive stimulation of FBGCs and increased intimal hyperplasia, thus ultimately compromising graft performance (Kurane et al., 2007; Heidenhain et al., 2011). Lastly, as has been shown with heart valves, decellularized tissues are often subject to calcification that can impede their performance.

### A.2.2. ECM-based approaches

Similar to decellularized tissue, the use of ECM components has also been explored for vascular tissue engineering strategies due to the fact that they innately express cell signalling domains that can support differentiated cell phenotypes, attachment, and proliferation. With regards to vascular tissue, the primary components that have been investigated are collagen I, elastin, and hyaluronan. The first demonstration of a tissue engineered vascular graft by Weinberg and Bell involved the use of a collagen scaffold reinforced with a Dacron® mesh that was seeded with ECs on the luminal surface and VSMCs in the vessel wall (Weinberg and Bell, 1985; Weinberg and Bell, 1986). Despite demonstrating promising biological properties, these grafts ultimately exhibited insufficient strength to be used in the high pressure arterial circulation. This poor mechanical strength is thought to be due to a lack of circumferential alignment of collagen fibrils and VSMCs found in healthy arteries. Furthermore, while collagen is a significant component of vascular ECM, elastin is also a critical factor in terms of providing compliance and elastic properties, and is notoriously difficult to generate from cultured cells (Mecham, 2008). To address these issues a number of strategies have been employed, including mechanical conditioning to promote collagen fibril alignment and incorporating other ECM proteins, such as elastin, or synthetic polymers into the graft structure (Syedain et al., 2011; Stitzel et al., 2006). Rat aortic SMCs seeded in a collagen I gel and cultured under dynamic mechanical strain
produce a graft with increased modulus and tensile strength, though not as strong as a typical blood vessel (Cummings et al., 2004). An important aspect of the increased strength associated with these constructs is that they demonstrated circumferential alignment of collagen following mechanical stimulation (Syedain et al., 2011). Mechanical properties similar to bovine iliac artery have also been observed by controlled electrospinning of collagen I, elastin, and PLGA, illustrating the potential combination of the bioactivity of biopolymers such as collagen with the mechanical strength of synthetic polymers as a potential vascular tissue engineering strategy (Stitzel et al., 2006).

Elastin makes up 15% (coronary) to 40% (aorta) by mass of the dry weight of arterial walls (Fischer and Llaurado, 1966), making it an attractive option for vascular tissue engineering. In addition to its role in contributing to vessel mechanics, there has recently been a greater understanding of elastin’s bioactivity. Elastin-mimetic peptides have been shown to have good hemocompatibility properties, supporting limited platelet adhesion and activation, EC adhesion, and greater eNOS expression from ECs when covalently immobilized on a polyurethane surface (Blit et al., 2011). While scaffolds made solely of elastin do not possess sufficient mechanical strength, strategies have been employed wherein electrospun human recombinant tropoelastin cross-linked with disuccinimidyl suberate demonstrated similar elastic modulus and tensile strength as elastin extracted from porcine carotid artery, though still significantly lower than native carotid artery (McKenna et al., 2012). In order to increase the strength of elastin-based grafts, recombinant tropoleastin has been electrospun in a bilayered graft with PCL and cross-linked with glutaraldehyde vapors, such that the luminal surface is pure synthetic elastin. These grafts were shown to possess permeability, elastic modulus, compliance, and burst pressure similar to human internal mammary artery and saphenous vein, while also remaining patent with no change in mechanical properties following a pilot in vivo study as a carotid artery interposition graft following one month in a rabbit model (Wise et al., 2011). Due to limited sources of elastin, tropoelastin is typically produced recombinantly with bacterial production systems, which may lead to concerns associated with unwanted endotoxin contamination that can lead to a detrimental inflammatory response (Wakelin et al., 2006; Daly et al., 2012).

Hyaluronan has also been shown to be a promising alternative for ECM-based vascular tissue engineering. HYAFF-11™ is a degradable material produced by total esterification of
hyaluronan, which is synthesized from 80-200 kDa sodium hyaluronate (Lepidi et al., 2005). When implanted in the abdominal aorta of rats (2 mm ID), complete endothelialisation was observed in less than a week, as well as complete vascular wall regeneration after only 2 weeks. Of particular importance was the robust production of elastin, which is one of the most common issues associated with vascular tissue engineering strategies (Lepidi et al., 2005). When the same material was investigated as a carotid artery interposition graft in a porcine model (4 mm ID), 3/10 grafts experienced complete or partial occlusion after 5 months, with the remaining grafts demonstrating complete degradation of the graft material, which was replaced by a neovessel with a medial layer composed of VSMCs, collagen, and elastin, and a luminal endothelial lining (Zavan et al., 2008).

While not a typical vascular ECM protein, fibrin has also been explored for vascular tissue engineering purposes as it contains many of the integrin-binding domains that make collagen an attractive candidate. Used on its own, fibrin is not sufficiently mechanically robust to withstand the high pressure arterial circulation. For this reason, strategies involving fibrin typically involve a cell-seeding approach followed by dynamic culture in a bioreactor system, or the use of fibrin as a sealant for a porous synthetic material (Koch et al., 2010). Syedain et al. demonstrated that culturing fibroblasts in a fibrin gel for 7-9 weeks under mechanical conditioning resulted in a graft that had sufficiently high burst pressure (1400-1600 mmHg) and compliance similar to native vasculature that it could be implanted with PLA sewing rings in an ovine model (Syedain et al., 2011). Using a similar strategy with a 5-week maturation time, these fibrin-based grafts could be decellularized and stored in PBS at 4°C prior to implantation as an interposition graft in the femoral artery of an ovine model. These conduits had burst pressures exceeding 4000 mmHg and compliance similar to native ovine vasculature. In addition, these grafts remained patent for 24 weeks with endothelialisation complete by this time point, with cells in the medial area primarily expressing α-SMA and elastin deposition evident (Syedain et al., 2014). Using a similar approach, Niklason’s group has shown that fibrin gels seeded with a 50:50 mixture of bovine SMCs and neonatal human dermal fibroblasts and cultured for 30 days with pulsatile stretching contained circumferentially aligned collagen fibrils and elastic fibers, which contributed to mechanical properties sufficient for implantation in terms of burst pressure (913
mmHg), suture retention strength (53.3 g), and compliance (3.1% per 100 mmHg) (Gui et al., 2014).

Non-defined ECM grafts have also been used by implanting Silastic tubing in the peritoneal cavity of the graft recipient, allowing a fibrous capsule to form a construct around the implanted tubing after 2 weeks, consisting of non-thrombotic mesothelial cells (lumen); myofibroblasts, collagen, and elastin in the media; and a collagenous adventitia. This construct could be grafted by end-to-end anastomosis and remain patent for 4 weeks in both rats and rabbits (Campbell et al., 1999). While such an approach provides an interesting opportunity for so-called designer grafts, their long-term viability has not yet been reported upon. As with all biopolymer approaches, a further issue relates to batch-to-batch variability. Due to the biological source of biopolymers, differences in properties between batches make it difficult to predict how a particular batch will perform.

A.2.3. Other biopolymers

In addition to ECM-based biopolymers, other biopolymers not associated with native vasculature have been considered, primarily due to desirable mechanical properties. Example of such biopolymers include cellulose (Fink et al., 2011) and silk (Lovett et al., 2010), the latter of which has been explored the most extensively with regards to applications in vascular tissue engineering. Zhang et al. seeded human VSMCs and ECs on electrospun silk scaffolds and cultured them under dynamic flow conditions, demonstrating appropriate phenotypic marker expression, well-distributed cell populations, and suitable mechanical properties (Zhang et al., 2009). Silk tubes have been produced by aqueous gel spinning and implanted into the abdominal aorta of rats. Over 4 weeks, these constructs demonstrated graft patency and supported an endothelial lining on the luminal surface (Lovett et al., 2010). In terms of bioactivity, immobilized silk fibroin on PLGA scaffolds has also been used as a means to improve hemocompatibility and endothelialisation (Liu et al., 2011b). However, despite such promising results, silk alone has been shown to have the potential to promote a pro-inflammatory foreign body response (Liu et al., 2014).
A.3. Synthetic polymers

Synthetic polymers are attractive candidates for vascular tissue engineering strategies. A significant advantage of synthetic polymers is the ability to tailor different properties, such as degradation rate, chemistry, and the introduction of chemical cues for desired protein and cellular interactions. In contrast, while there is some flexibility with biopolymers in terms of cross-linking and other strategies, they do not demonstrate the same versatility as their synthetic counterparts. Furthermore, synthetic polymers possess the added advantage of reproducibility. For vascular tissue engineering applications, the broad classes of polyesters and polyurethanes have been explored in the greatest detail and thus will be the focus of this review.

A.3.1. Polyesters

The primary polyesters that have been explored for vascular tissue engineering applications are poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), and poly(glycerol sebacate) (PGS). These materials have been used in strategies where the material is implanted without pre-seeding with cells, as well as in cases where long in vitro culture periods are required to generate a vascular graft with sufficient mechanical strength to be implanted.

The first significant achievement using polyester-based vascular grafts was reported by Niklason et al. in 1999. In this seminal paper, SMCs from bovine aorta were cultured on tubular PGA scaffolds treated with sodium hydroxide to increase surface hydrophilicity. These constructs were cultured for 8 weeks in a pulsatile perfusion bioreactor, after which bovine aortic ECs were seeded on the luminal surface. This study demonstrated the importance of mechanical stimulation in promoting cell growth and collagen deposition, while producing a graft that had similar collagen levels (50% dry weight) to native vessels, as well as significant cellularity and suturability. When implanted in the saphenous artery in a porcine model, grafts remained patent for 30 days, while non-dynamically stimulated constructs developed thrombosis after 3 weeks (Niklason et al., 1999). A significant limitation of techniques such as the aforementioned that rely on cells to derive mechanical strength is that they typically require long culture periods, limiting off-the-shelf potential. Furthermore, degradation by-products from PGA have been
shown to de-differentiate SMCs from their mature contractile phenotype (Higgins et al., 2003). However, Dahl et al. demonstrated that grafts generated from PGA mesh seeded with canine SMCs or human aortic SMCs and cultured for 7-10 weeks in a distension bioreactor (2.5% radial strain at 2.75 Hz) in the presence of several growth factors could be decellularized, leaving behind cell-derived ECM architecture with no remaining polymer. Decellularized grafts were stored at 4°C long-term without loss of mechanical properties, which were similar to native human blood vessels (Dahl et al., 2011). Prior to implantation, grafts were coated with fibronectin, pre-seeded with ECs (3 weeks required for expansion prior to pre-seeding), and exposed to a shear pre-conditioning protocol for less than 2 days. Grafts derived from human cells were implanted in a baboon model of arteriovenous access for hemodialysis, while canine-derived grafts were tested in a canine model of peripheral and coronary artery bypass. In both cases, grafts demonstrated excellent patency and remodelling after 1 month (Dahl et al., 2011). Longer-term studies in rats in an abdominal aortic interposition graft also demonstrated a high patency rate, with recellularization of SMCs and a confluent endothelium (Quint et al., 2012). Such strategies can significantly reduce the generation of a tissue engineered vascular graft from 10 weeks to less than 30 days.

One of the more promising strategies developed involves the use of a PGA mesh with a PCL-PLLA copolymer sealant (80% porosity, 20-100 µm pores). In 2001 it was reported that a 4-year old girl received such a graft to reconstruct an occluded pulmonary artery. The graft was pre-seeded for 10 days with cells derived from autologous saphenous vein following 8 weeks of expansion *in vitro* (Shin'oka et al., 2001). The group subsequently transitioned to pre-seeding with autologous bone marrow derived cells (obtained from bone marrow aspirate, 5 ml/kg) with only 2-4 hr of pre-seeding and successfully transplanted grafts as extracardiac cavopulmonary connections in 42 patients. After a mean follow-up time of 490 days, no complications related to thrombosis, stenosis, or obstruction were observed with all grafts remaining patent (Shin'oka et al., 2005). However, it is unclear if success in the relatively low pressure pulmonary circulation will translate to high pressure environments such as the coronary artery. Furthermore, polymers such as PLLA and PGA can release acidic degradation by-products that, when they build up in a microenvironment, can reduce local pH and contribute to an inflammatory response (Higgins et al., 2003).
PCL-based constructs have also demonstrated some positive results. Electrospun PCL scaffolds (5-6 µm diameter fibres, 30 µm pores) implanted for up to 100 days in a rat abdominal aorta model demonstrated a regenerated SMC layer, vascularization, and physiological responsiveness to adrenaline (contraction) as well as acetycholine-induced relaxation (Wang et al., 2014b). However, longer-term studies with PCL micro and nanofibrous grafts in rat abdominal aorta demonstrate that while cellular infiltration, endothelialisation, and neovascularization are observed 6 months post-implantation, these effects regress by 12 and 18 months, with calcification observed (de Valence et al., 2012). This demonstrates the importance of longer-term studies to validate promising in vitro and in vivo studies performed over short time periods. PCL nanofibers have also been used to reinforce microporous PGS scaffolds, which when implanted as an interposition graft in a rat abdominal aorta demonstrated 80% patency after 1 year, with tissue architecture similar to native arteries, innervation, a confluent endothelium, and the same amount of elastin as observed in mature arteries (Wu et al., 2012; Allen et al., 2014).

A.3.2. Polyurethanes

Polyurethanes (PUs) are block co-polymers with alternating hard and soft segments that possess a carbamate, or urethane, link (\(\text{NH}-(\text{C}=\text{O})-\text{O}-\)), and are typically produced by reacting a diisocyanate (or other polyisocyanate), polyol, and chain extender (low molecular weight hydroxyl- or amine-terminated compounds). The isocyanate and chain extender comprise the hard segment, which are rigid at room temperature due to the adhesiveness of the significant polar region generated from the high density of urethane groups. The soft segment, composed of the polyol, has a low density of urethane groups and is thus flexible at room temperature. Due to the different polarity of the hard and soft segments, the hard segments can aggregate in a process called phase separation, such that pseudo-crystalline areas are located within a flexible matrix (Zdrahala, 1996). Flexibility in the choice of hard and soft segment components means that PUs can be made with tailored properties for different applications, including degradation rate (substantially non-degradable or biostable to rapidly resorbing), swelling, and mechanical properties. In particular, PUs are noted for their processability, high elasticity, excellent mechanical properties, and in the context of many biomedical applications, good biocompatibility (Santerre et al., 2005).
Biostable PUs have for a long time been considered as potential alternatives to standard Goretex® and Dacron® vascular grafts, in particular due to their optimal mechanical properties (such as compliance) (Zdrahala, 1996). More recently, however, with the advent of tissue engineering strategies, biodegradable PUs have been developed such that long-term remodeling following implantation will result in the elimination of the polymeric component, and replacement with native host tissue. Choice of PU, in particular of the soft segment, has been shown to be critical in order to control degradation rate while maintaining optimal mechanical properties and cellular compatibility. Dependence of graft performance on polyurethane chemistry was demonstrated by Xie et al. who evaluated five types of PU vascular grafts in a canine model as an infrarenal aortic interposition graft (Xie et al., 2010b). Corvita® (polycarbonate urethane [PCU]) grafts were the least thrombogenic, demonstrated low inflammation, good tissue ingrowth, and minimal degradation on their external surface with respect to both hard and soft segments after 6 months. Thoratec® (polyether urethane urea) grafts showed moderate levels of inflammation with marginal tissue ingrowth, and degradation evidenced by loss of material around the sutures resulting from degradation of soft segment and more ordered hard segments. Finally, Pulse-Tec® (polyether urethane) grafts were covered with a thrombogenic inner capsule, had high inflammation and good tissue ingrowth, and experienced severe degradation, particularly of the soft segment, with complete breakdown of portions of the graft wall observed (Xie et al., 2010b).

Polyester urethanes have demonstrated positive results in preliminary animal models. He et al. seeded a polyester urethane urea graft (porous inner layer with a fibrous external coating) with muscle-derived stem cells using a rotational vacuum seeding technique and cultured grafts in a spinner flask for 2 days prior to implantation as an aortic interposition graft in rats. Pre-seeding increased patency from 38% to 100% after 8 weeks, and resulted in extensive remodeling with collagen and elastin, an EC monolayer, and a medial layer expressing VSMC contractile marker proteins (Nieponice et al., 2008; He et al., 2010). A graft composed of a polyesterurethane-PDMS semi-interpenetrating polymer network with two different porous layers was able to be implanted in the common carotid artery of adult sheep, and demonstrated superior handling and patency relative to ePTFE, while gradually being replaced by host tissue (Soldani et al., 2010). In other cases, however, polyester urethane urea grafts have required the incorporation of non-
thrombogenic additives in order to increase graft patency (67% with coating vs. 40% without in a rat abdominal aorta model) (Hong et al., 2009).

Significant results have also been reported with polyether urethane grafts. When used as hemoaccess bridge fistulas in humans, polyether urethane urea grafts (15 μm pore size) have been shown to be better at enabling early access to the graft and good hemostasis, and were equivalent to ePTFE with regards to long-term (1-2 years) patency (Kiyama et al., 2003). Electrospun Pellethane 2663-80A grafts (71% porosity, 0.88 μm fiber diameter) have demonstrated 95% patency following 6 months of implantation in a rat abdominal aorta model, with cellular infiltration observed (Bergmeister et al., 2012). Polyether urethane grafts (PTMO soft segment) have also been modified to possess a NO-producing peptide, which has demonstrated the ability to promote EC growth while inhibiting SMC proliferation (Jun et al., 2005).

PCU grafts have also been recently investigated with varying success. POSS-PCU grafts (polyhedral oligomeric silsesquioxane poly(carbonate-urea)urethane) implanted in the carotid artery of sheep demonstrated 65% patency after 9 months, while all ePTFE control grafts occluded. No differences were observed in terms of compliance and blood flow relative to native artery (Ahmed et al., 2014). PCU grafts have also been shown to promote faster endothelialisation with a thinner neointima when implanted in the abdominal aorta of rats relative to ePTFE (Jeschke et al., 1999). A poly(carbonate-urea) urethane (MyoLink™) implanted in the aorta-iliac position of beagle dogs for 3 years demonstrated biostability over the implantation period with minor hydrolysis of the amorphous segment (Seifalian et al., 2003). Incorporation of heparin and sirolimus in microporous polycarbonate-siloxane PU grafts, however, was required to produce patency rates and EC coverage similar to ePTFE after 6 months in the abdominal aorta of rabbits (Ishii et al., 2008).

While the results reported above demonstrate several promising results using PU grafts, several disadvantages should be noted. In order for the implanted polymer to be replaced by natural tissue, degradation rates must be tailored such that they balance the rate of new tissue deposition and matrix organization. In many cases, grafts are either significantly non-degradable (Seifalian et al., 2003) or resorb at a rate that outpaces tissue growth (Hong et al., 2010). Furthermore,
degradation by-products from conventional polyurethanes include toxic diamines from the use of aliphatic and aromatic isocyanate monomers (Santerre et al., 2005). The Santerre laboratory has developed a degradable polyurethane using a pre-polymer synthesized with a 2:2:1 molar ratio of 2-hydroxyethylmethacrylate, lysine diisocyanate, and poly(hexamethylene carbonate)diol that is subsequently cross-linked in the presence of monomers with single vinyl functionality (MMA and MAA) to introduce hydrophobic and ionic character (DVO:MAA:MMA in a 1:5:15 molar ratio) (Sharifpoor et al., 2009). Degradation by-products from this novel polyurethane are non-toxic (CO₂, H₂O, short-chain diols, lysine, and oligomeric ionomers, all of which can be metabolized by the body), with degradation occurring at a controlled rate that does not outpace tissue deposition (10% in vitro after 4 months and 20% in vivo after 100 days), while supporting integration with host tissue (McBane et al., 2011). Preliminary results also demonstrate a limited pro-inflammatory and enhanced anti-inflammatory response from human monocytes (McBane et al., 2009), mechanical properties similar to native vasculature (Sharifpoor et al., 2010), and compatibility with vascular cell types, such as VSMCs (Sharifpoor et al., 2011) and ECs (McDonald et al., 2011), suggesting potential applicability in vascular tissue engineering.

A.4. References


Appendix B
Seeding VSMCs on tubular D-PHI scaffolds

B.1. Introduction

A significant challenge inherent to tissue engineering strategies that use three-dimensional scaffolds is the ability to achieve high seeding efficiency and homogeneity with respect to the distribution of cells seeded within porous scaffolds (Villalona et al., 2010). In particular, vascular tissue engineering strategies involve seeding tubular scaffolds, a geometry that is not easily seeded using traditional techniques which involve pipetting a cell suspension onto a scaffold in a tissue culture plate. Using this approach, cells are not typically constrained to adhering on the scaffold and often adhere instead to the underlying tissue culture vessels (Villalona et al., 2010). Obtaining a uniform cell distribution should contribute to enabling uniformity of extracellular matrix (ECM) deposition and ultimately mechanical properties of the tissue engineered construct. Achieving a high initial seeding density reduces the number of donor cells that are required should a tissue engineering strategy be translated clinically. The current study compared the use of a vacuum seeding technique to a modified static seeding technique with two different cell types: A-10 rat aortic smooth muscle cells (SMCs) and human coronary artery SMCs. Using A-10 cells, the modified static seeding technique demonstrated increased seeding efficiency compared to the vacuum technique, with both methods achieving a uniform cell distribution. However, using human coronary SMCs, there was no difference in efficiency between the static and vacuum techniques, though both techniques did demonstrate an improvement compared to conventional static cell seeding and demonstrated a uniform cell distribution throughout the porous D-PHI scaffolds.

B.2. Materials and methods

B.2.1. D-PHI scaffold fabrication

D-PHI scaffolds were synthesized by mixing a divinyl oligomer (DVO), methacrylic acid (MAA), and methyl methacrylate (MMA) in a 1:5:15 molar ratio in the presence of the initiator
benzoyl peroxide (BPO, 0.0032 mol/mol vinyl group) (Sharifpoor et al., 2009). Sodium bicarbonate and polyethylene glycol were added at 65 and 10 wt%, respectively. The resulting mixture was packed into a cylindrical Teflon mold with a 5 mm inner diameter. A 3 mm diameter mandrel used to make the luminal diameter of the D-PHI tube. Scaffolds were cured for 24 hr at 110°C and then subjected to 14 x 2 hr periods of sonication in ddH₂O. Cured scaffolds had dimensions of 5 mm (OD) x 3 mm (ID) when dry and 6 mm (OD) x 3.5 mm (ID) when hydrated.

B.2.2. Cell seeding

Two different forms of cell seeding were explored in the present study: vacuum and a modified static seeding technique. The vacuum seeding apparatus consisted of an Eppendorf tube as the cell suspension reservoir, a perforated stainless steel vacuum cylinder (Figure B1a), PTFE medical tubing (3.175 mm ID), a specimen trap (Erlenmeyer flask), and a pump with a regulator (Figure B1b). D-PHI tubes that had been hydrated in culture medium (DMEM supplemented with 10% FBS and 1% PenStrep) were mounted on the perforated end of the stainless steel vacuum cylinder with sutures and Tegaderm as a sealant (Figure B1c) and left to partially dry for 90 min. Scaffolds were then placed in a cell suspension (500 μl at 668,000 cells/ml) and vacuum pressure (25 mmHg) was applied to draw cells through the scaffold.
Figure B1 (a) CAD drawing of the perforated stainless steel tube used for vacuum seeding (OD 3.175 mm, ID 2.175 mm, 1.5 cm section filled with 1 mm diameter holes, 6 rows and 7 holes per row). (b) Schematic showing the set-up of the vacuum seeding apparatus. (c) Photograph of a partially dehydrated D-PHI scaffold prior to seeding. (d) Photograph of a D-PHI scaffold after vacuum cell seeding.

Static seeding was performed by partially drying fully hydrated D-PHI tubes in a laminar flowhood for 90 min. Scaffolds were then seeded with a concentrated cell suspension containing 334,000 cells. Following the initial cell seeding, samples were maintained in DMEM supplemented with 10% FBS and 1% PenStrep for 24 hr prior to analysis. For both static and vacuum seeding, cell seeding density was kept constant at approximately 3500 cells/mm². Furthermore, both static and vacuum seeding techniques were performed with both A-10 rat aortic SMCs (ATCC, CRL-1476) and human coronary artery SMCs (Lonza, CC-2583) at the same seeding density.
B.2.3. DNA mass quantification

Samples seeded with cells were washed twice with PBS, minced with a needle, and incubated with lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.1% SDS at pH 8.0). To determine the amount of DNA associated with the number of seeded cells, a cell suspension containing 334,000 cells was centrifuged at 1400g for 5 min, the supernatant was discarded, and the cell pellet was resuspended with lysis buffer. All samples were incubated for 60 min at 65°C with intermittent vortexing. DNA mass was quantified by mixing aliquots of cell lysates with a DNA dye containing Hoechst 33258 DNA stain in a TNE (Tris/NaCl/EDTA) buffer and obtaining fluorescence measurements with an FL600 Microplate Fluorescence Reader using excitation and emission wavelengths of 360 and 460 nm, respectively. Fluorescence readings from samples were compared to a standard curve prepared from DNA standards of calf thymus DNA.

B.2.4. Histological staining

Cell-seeded scaffolds were washed with PBS, fixed in 4% paraformaldehyde in PBS for 20 min on ice, infiltrated with 15% sucrose overnight, and then stored in 30% sucrose prior to paraffin embedding and sectioning. 20 μm sections of scaffolds were stained using H&E. Images were obtained using a Spot RT camera (Diagnostic Instruments Inc.) mounted on an Olympus BX51 light microscope (Olympus Canada Inc.).

B.2.5. Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0 (SPSS Inc.) with an independent samples t-test. Data are presented as the mean ± S.E., with statistical significance reported for p<0.05.

B.3. Results and discussion

Using A-10 rat aortic SMCs, the modified static seeding technique (58 ± 7%) was shown to improve seeding efficiency relative to vacuum seeding (18 ± 3%) (Figure B2a). However, when
experiments were repeated using human coronary SMCs, there was no difference observed between static (29 ± 5%) and vacuum (23 ± 8%) techniques (Figure B2b).

![Graph showing comparison of static and vacuum seeding efficiencies](image)

**Figure B2** Comparison of modified static and vacuum seeding techniques using (a) A-10 rat aortic SMCs and (b) human coronary artery SMCs. *p<0.05. Data are the mean ± S.E.

H&E staining of seeded tubes demonstrated that regardless of seeding technique, and with both A-10 rat aortic SMCs and human coronary artery SMCs, a uniform cell distribution was achieved throughout the thickness of the porous scaffolds, in contrast to previous studies that have shown monolayer formation and a lack of cellular infiltration following seeding (Figure B3).

Previous seeding techniques employed with D-PHI scaffolds using irregular geometries (e.g. dumbbells) obtained seeding efficiencies <10%, with cells typically forming a monolayer on the seeding surface, with little cellular infiltration (Sharifpoor et al., 2011; McBane et al., 2012) (Figure B3e). Initial experiments with A-10 rat aortic SMCs suggested that hydrating D-PHI scaffolds with a saturating volume of cell suspension could improve seeding efficiency and cell distribution (Figures B2 and B3), even compared to a vacuum technique that has been reported in the literature (Udelsman et al., 2011). However, while employing this static technique with human coronary artery SMCs improved seeding efficiency compared to previous studies (Sharifpoor et al., 2011), there was no difference compared to vacuum seeding (Figure B2).

While rat aortic SMCs have a diameter on the order of 5-10 μm in suspension and 10-20 μm when adhered to a surface, human coronary artery SMCs are larger, with diameters of 10-20 μm
and 100-200 μm when in suspension and adhered, respectively. Furthermore, A-10 cells were observed to adhere quicker to TCPS following passaging than human coronary artery SMCs. While the static technique may provide similar initial seeding efficiency with human coronary artery SMCs vs. A-10 cells, it is hypothesized that the addition of medium after 45 min results in a significant loss of poorly adhered cells, as evidenced by a larger number of cells observed on the underlying TCPS well following 24 hr of incubation for tubes seeded with human coronary artery SMCs vs. A-10 cells (data not shown). Furthermore, efforts to prolong the static incubation time with human coronary artery SMCs from 45 to 90 min did not manage to improve efficiency, which may be due to a lack of healthy cells as the scaffolds begin to dehydrate during longer incubation times without culture medium. For A-10 cells, however, their ability to rapidly adhere to a substrate results in a lower loss of cells following the addition of cell culture medium after 45 min, while a lower seeding efficiency with vacuum seeding is likely observed with these cells due to cells passing directly through the pores and not adhering to the scaffold due to their smaller size.
Figure B3 H&E staining of tubular D-PHI scaffolds seeded with (a,b) rat aortic SMCs using the static (a) or vacuum (b) technique, or human coronary artery SMCs (c,d) using the static (c) or vacuum (d) technique. Scale bar represents 100 μm (a-d). (e) Representative H&E image showing human coronary artery SMCs seeded on D-PHI scaffolds using the previous static technique (McBane et al., 2012; Sharifpoor et al., 2011).
B.4. Conclusions

The current study investigated the use of a modified static seeding technique and a vacuum seeding technique for improving seeding efficiency and cell distribution in a 3D porous D-PHI scaffold. A modified static seeding technique was developed where D-PHI scaffolds were rehydrated with a cell suspension using a volume of medium that would saturate the scaffolds. While the static technique improved seeding efficiency relative to the vacuum technique for rat aortic SMCs, there was no difference observed when using human coronary artery SMCs. However, compared to techniques previously employed, the static technique managed to triple seeding efficiency and obtain a homogeneous cell distribution with minimal handling. This study demonstrates the importance of considering both cell type and biomaterial properties (e.g. swellability) when evaluating cell seeding techniques.

B.5. References


Appendix C
Drawings for the Bioreactor

Figure C1 Actuator to syringe coupler male ¼-28 UNF to male #6-32. Type 304 stainless steel. Dimensions are in mm.

Figure C2 Syringe termination to manifold adapter. Female ¼-28 UNF to female #10-32. Type 304 stainless steel. Dimension are in mm.
Figure C3 Front and top views of 50 ml syringe holder. Type 304 stainless steel. Dimensions are in mm.

Figure C4 Bioreactor chamber lid. Polycarbonate. Dimensions are in mm.
Figure C5 Bioreactor chamber. Polycarbonate. Dimensions are in mm.
Figure D1 Representative stress-strain curves for the different sample types from the bioreactor study reported in Chapter 6. a) Co-culture dynamic, b) co-culture static, c) VSMC dynamic, d) VSMC static, e) monocyte dynamic, f) monocyte static, g) negative control (scaffold incubated in medium).
Appendix E

Differences in protein binding and cytokine release from monocytes on commercially sourced tissue culture polystyrene

**Abstract:** Tissue culture polystyrene (TCPS) is a ubiquitous substrate used by many researchers in the biomedical and biological sciences. Different parameters involved in the production of TCPS, including the treatment time and the use of reactive gases and chemical agents, can have a significant influence on the ultimate surface properties achieved. The assumption that they will all yield a consistent and controlled product has not proven to be true. To provide a better insight into the bioactivity differences in TCPS supplied by different manufacturers, TCPS from three different companies (Sarstedt, Wisent Corp., and Becton Dickinson (BD)) were analyzed for their surface properties, protein adsorption characteristics, and interactions with human monocytes. Marked differences were observed in terms of surface wettability and surface chemistry. Furthermore, Wisent TCPS adsorbed more than twice the amount of serum proteins compared with BD and Sarstedt TCPS. Sarstedt showed significantly more cell retention (more DNA) compared with both BD and Wisent TCPS brands over a 7 day culture period. Cytokine release from monocytes adherent on the three different TCPS also differed significantly, suggesting that the differences in the surface properties were sufficient to differentially mediate monocyte activation. These results have important implications for TCPS research use, in terms of appreciating the interpretation of the data when TCPS is used as a control substrate as well as when it is used where a pre-conditioned state would influence the outcome of the study.

Appendix F  
The effect of degradable polymer surfaces on co-cultures of monocytes and smooth muscle cells

Abstract: Strategies to optimize biomaterial chemistry for applications in vascular tissue engineering attempt to promote endothelial and smooth muscle cell recruitment into porous material constructs. The primary objective is to facilitate relevant tissue formation in a wound healing versus pro-inflammatory manner. The present work investigated the interactive co-cytocellular response of human monocytes and human vascular smooth muscle cells (VSMCs) with a novel degradable, polar/hydrophobic/ionic (D-PHI) polyurethane and compared it to a commercially available biomaterial, poly-lactic-glycolic acid (PLGA) as well as tissue culture polystyrene (TCPS). D-PHI triggered a smaller pro-inflammatory response (acid phosphatase, esterase, tumor necrosis factor-α) at later time points (>14 d) than PLGA suggesting that monocytes may be transitioning to a more wound-healing phenotype on the D-PHI surface. When D-PHI was coated with collagen, monocyte cell attachment did not differ with the native D-PHI; however, PLGA showed significant differences between collagen coated versus uncoated surfaces. There were more VSMCs and monocytes attached in co-culture to D-PHI when compared to PLGA. Co-cultures on D-PHI released more IL-10 (anti-inflammatory) than monocytes cultured alone, while the VSMCs retained the expression of its marker protein calponin. Together the above data suggest that co-culturing monocytes with VSMCs may aid in stimulating the attachment of VSMCs to D-PHI while eliciting the desired functional phenotypes for both monocytes (i.e. low inflammation based on IL-10 values) and VSMCs (expressing calponin, a marker of contractility). Moreover, the results of this study demonstrated that D-PHI performed equally or better to PLGA in terms of the assayed biological parameters.

Appendix G

New degradable polyurethanes for use in tissue engineering: inspired by mechanisms of biodegradation and wound healing

Abstract: The Biomedical Polymers Laboratory at the Institute of Biomaterials and Biomedical Engineering in Toronto in the Faculties of Applied Science and Engineering, Dentistry and Medicine is coordinated by Professor J Paul Santerre in the Institute’s Biomaterials, Tissue engineering and Regenerative Medicine theme area. Research in the Santerre group is focused on new polymer synthesis and specifically polyurethane chemistry for biomedical implants and their bio-stability, conceiving new biomaterials for tissue engineering applications of vascular and connective tissues, and utilizing co-culture techniques as a means of circumventing complicated biochemical modifications of materials. In this reported work, D-PHI interactions in both two and three dimensional architectures with human gingival fibroblast (HGFs) and endothelial cells (ECs) show good cell growth, viability and collagen production relative to established degradable biomaterials such as polylactic-glycolic acid (PLGA) and tissue culture materials such as tissue culture polystyrene (TCPS). In addition, HGFs cultured on D-PHI show VEGF production under optimal perfusion flow conditions, thereby enabling the potential to contribute to micro-vessel formation when the materials are applied towards the development of gingival tissues. The work further shows the potential use of D-PHI as an elastomer under cyclic mechanical loading for vascular tissue engineering applications, as indicated by the material’s ability to support an anti-inflammatory monocyte state, which combine to also support the growth and modulated contractile phenotype of vascular smooth muscle cells (VSMC).

Appendix H
Characterization of a degradable polar hydrophobic ionic polyurethane with circulating angiogenic cells in vitro

Abstract: This study investigated the interaction of human circulating angiogenic cells (CACs) with a degradable polar hydrophobic ionic polyurethane (D-PHI) which has been previously shown to exhibit anti-inflammatory character and favorable interactions with human endothelial cells (ECs). Given the implication of the CACs in microvessel development it was of intrinsic interest to expand our knowledge of D-PHI biocompatibility with this relevant primary cell involved in angiogenesis. The findings will be compared to a well-established benchmark substrate for CACs, fibronectin-coated tissue culture polystyrene (TCPS). Immunoblotting analysis showed that CACs were a heterogeneous population of cells composed mostly of monocytic cells expressing the CD14 marker. Assessment of the cytokine release profile, using ELISA, showed that D-PHI supported a higher concentration of interleukin-10 (IL-10) when compared to the concentration of tumor necrosis factor alpha, which is indicative of an anti-inflammatory phenotype, and was different from the response with TCPS. It was found that the CACs were attached to D-PHI and remained viable and functional (nitric oxide production) during the seven days of culture. However, there did not appear to be any significant proliferation on D-PHI, contrary to the CAC growth on fibronectin-coated TCPS. It was concluded that D-PHI displayed some of the qualities suitable to enable the retention of CACs onto this substrate, as well as maintaining an anti-inflammatory phenotype, characteristics which have been reported to be important for angiogenesis in vivo.