OVERCOMING FIBROUS TISSUE GROWTH ON PLGA DISCS AND STUDY OF SCAVENGER RECEPTOR MEDIATED RESPONSES TO BIOMEDICAL MATERIALS

A DRUG DELIVERY APPROACH TO OVERCOMING FIBROUS TISSUE GROWTH ON POROUS POLY(LACTIC-CO-GLYCOLIC ACID) DISCS AND STUDY OF SCAVENGER RECEPTOR MEDIATED RESPONSES TO BIOMEDICAL MATERIALS

By

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TITLE: A Drug Delivery Approach to Overcoming Fibrous Tissue Growth on Porous Poly(Lactic-Co-Glycolic Acid) Discs and Study of Scavenger Receptor Mediated Responses to Biomedical Materials

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ABSTRACT

A compatible interface between a biomedical material and host tissue is paramount to the continual function and life-span of medical devices that reside in the body. However, the unfavourable host response that ensues when foreign materials inhabit the body must be overcome for sophisticated medical devices, such as artificial organs and real-time biosensors, to be used clinically. My thesis research commenced with a search to find a pharmaceutical compound that could be incorporated into a medical device to suppress the accumulation of fibrous tissue. A prolyl hydroxylase inhibitor, a drug developed to inhibit collagen synthesis, was found to be effective at inhibiting collagen deposition within and on the outer surface of a poly(lactic-glycolic acid) disc, and also limited connective tissue ingrowth. Furthermore, the drug suppressed Scavenger Receptor A (SRA) expression on a macrophage-like cell culture, a receptor known to contain a collagenous domain. The latter finding prompted a review of the literature, upon which it was discovered that SRA mediates leukocyte adhesion and binding to an assortment of materials, such as silica, modified polystyrene, titanium, and iron(III) oxide. As a result, a series of studies were initiated to investigate whether leukocytes use SRA to detect a range of different biomedical materials. Consequently, we found that SRA contributes very little to leukocyte binding of two common medical polymers, polystyrene and poly(lactic-co-glycolic acid), but may interact with the materials to affect the cytokine profile in the local environment. In a subsequent study, SRA was found to be crucial to the leukocyte binding of polyanionic hydrogels. In summary, we have identified a unique pharmaceutical strategy for suppressing the accumulation of fibrous tissue on medical devices in vivo, and uncovered a mechanism of leukocyte stimulation in response to incubation with biomedical materials that the material science research community was not previously aware of.

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On the topic of family, my Mother and Father were both absolutely critical to getting me to this point in my education. When presented with the fork in the road of life after the completion of my undergraduate degree, my father was certainly the prominent cheerleader for the path of more training. Without that encouragement, I might have made the mistake of following the route of instant gratification by joining the workforce. My mother, supportive of whatever I decided to do in life, was there for me as soon as the going got tough and even dedicated many long hours in the lab to assist me in taking blood and running the subsequent experiments. Though most of those experiments never made it into this thesis, the knowledge that we gathered will likely be the nucleus of many future projects.

I would be remiss to overlook the role that my brother has played in my success to this point in my life. We are vastly different, but I have always considered Thomas to be my number one ally and best friend. Furthermore, our competitiveness has pushed me to undertake ventures that I would have otherwise deemed impossible.

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can draw on when initiating future projects. Michel Paul was the first mentor that I ever had, and there is no question that I pursued a career in research primarily due to his guidance, and the enjoyment that I experienced working in his lab. Our research in fatigue endocrinology and circadian rhythms was both stimulating and fun, and I sincerely look forward to working with Michel again at DRDC for my post-doc.

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LIST OF ABBREVIATIONS

1,4-DPCA	1,4-dihydrophenonthrolin-4-one-3-carboxylic acid
α-SMA	α-Smooth Muscle Actin
AGE	Advanced glycation end products
ANG II	Angiotensin II
ANOVA	Analysis of variance
AP	Alternative pathway (of the Complement Cascade)
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
C3	Complement protein 3
C3R	Complement 3 receptor
CCL	Cysteine-cysteine (CC) chemokine ligand
CCR	Cysteine-cysteine (CC) chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMC	Carboxymethylcellulose
CP	Classical pathway (of the Complement Cascade)
CTGF	Connective tissue growth factor
DAMP	Damage-associated molecular patterns
DC-STAMP	Dendritic cell-specific transmembrane protein
DMC	Dimethyl Carbonate
DMEM	Dulbecco's Modified Eagle Medium
DxSO4	Dextran sulfate
ECM	Extracellular matrix
EndMT	Endothelial-to-mesenchymal transition
EMT	Epithelial-to-mesenchymal transition
ELISA	Enzyme-linked immunosorbent assay
FBGC	Foreign body giant cell
FBS	Fetal bovine serum
Fc	Constant fragment
FcR	Constant fragment receptor
FIH	Factor inhibiting hypoxia-inducible factor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HIF	Hypoxia-inducible factor
HMWK	High molecular weight kininogen
HSP	Heat Shock Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

KO	Knockout
LAP	Latency-associated protein
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich regions
Mac-1	Macrophage-1 antigen
MARCO	Macrophage receptor with a collagenous structure
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAAGA	N-acetyl aspartyl glutamic acid
NFκB	Nuclear factor κB
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PGA	Poly(glycolic acid)
PHI	Prolyl hydroxylase inhibitor
PiCMg	Phosphate buffered saline with calcium, magnesium, and dextrose
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PMN	Polymorphonuclear leukocyte
PNIPAm	Poly(N-isopropylacrylamide)
PolyIC	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptors
PVA	Poly(vinyl alcohol)
RGD	Arginylglycylaspartic acid (L-arginine–glycine–L-aspartic acid)
SCARA5	Scavenger Receptor A5
sCR1	Soluble complement receptor 1
SE	Serum-enriched
SF	Serum-free
SR	Scavenger Receptor
SRA	Class A Scavenger Receptor
SRCL	Scavenger Receptor with C-type Lectin
TCPS	Tissue culture polystyrene
TGF	Transforming growth factor
Th1/2	T-helper lymphocyte type 1/2
TIMP	Tissue inhibitor of metalloproteinases
TIPS	Thermally-induced phase separation
TLR	Toll-like receptor
Tr	Regulatory T- lymphocytes
WT	Wild-type (non-genetically modified mouse)

DECLARATION OF ACADEMIC ACHIEVEMENT

The research performed for this thesis was conceived, conducted, analyzed, written, and submitted for publication primarily by the author of this thesis with the following exceptions:

- In Chapter 4, the phlebotomy and some of the subsequent polymorphonuclear leukocyte separation steps were performed by Brenda Love.
- In Chapter 5, the synthesis and characterization of Poly(NIPAM-co-ADH), the synthesis and characterization of the aldehyde-functionalized polysaccharides, the quantification of aldehyde functionality, and the hydrogel preparation, characterization and rheology were performed by Mat Patenaude. Mr. Patenaude also wrote the sections in the chapter that describe the methods and results of the aforementioned techniques, and created Figures 5.1 and 5.S1.

INTRODUCTION

The compatibility between man-made materials and host tissues is critical to the ultimate performance of indwelling medical devices, affecting the function and life-span of biosensors, drug-delivery devices, engineered tissues, catheters, orthopaedic implants, heart valves, and other medical instruments that interface with living material. Recent technological advances have led to the development of sophisticated real-time biological sensors and artificial organs that will eventually benefit a large portion of our population. However, a significant challenge to still overcome is the unfavourable tissue response that ensues upon inserting foreign materials into the human body. A completely "bioinert" material, one that is resistant to inciting a host response, has not yet been developed, which may be due to the currently poor understanding of the interactions between host tissues and foreign materials. 'Hiding' a material from the host can be achieved through the use of immunosuppressing drugs, namely glucocorticoids. Conveniently, these drugs can often be incorporated into the material itself, and allowed to slowly leach out, providing resistance against the instigation of a host response. The major drawback of delivering glucocorticoids from the material is that they make the local environment susceptible to infection and suppress proliferation of functional tissue in the local environment.

Therefore, the cells of the organ that are near the end of their lifespan are not able to be replaced by new cells, which causes weakening or damage to the tissue. Furthermore, glucocorticoids are only effective while the drug elutes from the material and do not confer long-term suppression of inflammation. As a result, the development of other countermeasures is necessary to defend future medical devices from the onslaught of the host response. Thus, my graduate research at McMaster began as a quest to find other pharmaceutical inhibitors to 'hide' a foreign material, specifically a synthetic tissue engineering scaffold, from the host defence. Chapter 1 provides an introduction to this quest with an overview of the host responses to biomedical materials and pharmaceutical strategies that can be used to inhibit such responses. In Chapter 2, research into a selection of the pharmaceutical strategies from Chapter 1 is discussed.

While investigating the specific mechanism of action of the pharmaceutical used in Chapter 2, a prolyl 4-hydroxylase inhibitor, a drug developed to inhibit collagen synthesis, we discovered that the expression of a surface receptor with a collagen domain, Scavenger Receptor A (SR-A), was inhibited. Upon reviewing the literature, SR-A was found to be implicated in leukocyte adhesion and binding to an assortment of materials, such as silica, modified polystyrene, titanium, and iron(III) oxide. This led to a series of studies investigating if, or how, our cells use SRs to detect a range of different

materials. Of course, a very strong understanding of cellular detection of foreign materials has already been developed from past research. Therefore Chapter 3 provides a review of the research on cellular recognition of biomedical materials, which is summarized briefly below.

When implanted into a living body, the surface of a polymeric material adsorbs a layer of protein. It is generally accepted that local cells become 'activated' by binding to the foreign material via the adsorbed protein layer. However, as material surface engineering has advanced, the resistance to such protein adsorption has improved, yet we still face the same challenges with blood coagulation, inflammation, and foreign body responses as before. Classical medical polymers that readily adsorb proteins have been shown to ligate integrins on the surface of interrogating leukocytes. In our research on SR-dependent recognition of biomedical materials, we were expecting to implicate a role for class A SRs in the binding of classical medical polymers but, as Chapter 4 shows, we found that these receptors contribute very little to the binding of two common medical polymers, polystyrene and poly(lactic-co-glycolic acid). Nevertheless, in the absence of an adsorbed protein layer, host cells seem to be capable of responding to a foreign material through the use of other surface receptors. For example, hydrogels and protein resistant surfaces both incite inflammation and foreign body responses despite adsorbing relatively little protein. Scavenger receptors are the most likely

candidates for such material detection as they have broad ligand specificity, and are expressed by a significant number of different cell types in the human body. The prototypic member of the SR family, SR-A, was first identified in macrophages for binding modified low density lipoproteins, such as acetylated LDL and oxidized LDL. Many SR-A ligands have since been found, with the similarity among them being a net-negative charge. Consequently, we hypothesized that polyanionic medical polymers carry the risk of being detected by SR-A through direct pattern recognition of the surface. The testing of this hypothesis is described in Chapter 5, in which we found that SR-A is indeed a receptor for polyanionic hydrogels. Furthermore, leukocytes appear to rely exclusively on SR-A to bind a model polyanionic hydrogel that we tested *in vivo*.

In summary, we have found that inhibiting proline hydroxylation through controlled drug-release is an effective strategy for modulating the host response to biomedical materials. Though the specific mechanisms of action were not elucidated, we found that the expression of SR-A on a macrophage-like cell line was reduced dose-dependently with a prolyl-4 hydroxylase inhibitor. Subsequently, the role of SR-A in host responses to biomedical materials was evaluated. This led to the finding that intraperitoneal injections of polystyrene or poly(lactic-co-glycolic acid) particles stimulate a similar inflammatory response in SR-A knockout mice as

in wild-type mice, though the cytokine response varied between mouse types. Since SR-A is known to preferentially ligate polyanionic substrates, we also tested the host response to intraperitoneal injections of polyanionic and uncharged hydrogels in SR-A knockout and wild-type mice. From this, we found that SR-A is critical to the leukocyte binding of polyanionic hydrogels. In conclusion, this research shines light on the intricate leukocyte pattern recognition system that permits the identification of almost any foreign body in the host. Much information is already known about integrins, the major pattern recognition receptors responsible for biomedical material identification; however, less is known about the role that scavenger receptors play in host responses to such materials. Our findings show that scavenger receptors play a major role in leukocyte binding to polyanionic hydrogels, but also contribute to cellular activation/signaling in response to two biomedical materials, polystyrene and poly(lactic-co-glycolic acid).

CHAPTER 1: BIOMATERIALS, FIBROSIS, AND THE USE OF DRUG DELIVERY SYSTEMS IN FUTURE ANTI-FIBROTIC STRATEGIES

This chapter is a review of the literature that pertains to the fibrosis of biomaterial medical devices. When a biomaterial is implanted into the body, the presence of the material elicits an inflammatory response that evolves into fibrovascular tissue formation on and around the material. The fibrotic response is similar to pathological fibrosis of the liver, lung, kidney, and peritoneum in many ways: (1) the presence of mononuclear leukocytes are common in the local environment of both pathological fibrosis and biomaterial-induced fibrosis even though cells of mesenchymal origin are responsible for laying the majority of the extracellular matrix; (2) paracrine signaling molecules, such as TGFβ1, are essential mediators of fibrosis, whether it is pathological or biomaterial-induced; and (3) injury and/or the presence of foreign materials (including bacterial components, toxins, or man-made objects) are essential initiators for the development of the fibrotic response. As a result, this chapter discusses mechanisms and research methodology related to pathological fibrosis that is relevant to biomaterial fibrosis research. Potential research models for the study of fibrosis from the fields of biomaterials and drug delivery are also discussed. This chapter provides the background on which Chapter 2 is based.

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I. INTRODUCTION

Fibrosis occurs when extracellular matrix (ECM) protein synthesis exceeds degradation. Generally, this trend occurs in response to injury whereby a regenerative or wound healing response becomes over active, with matrix metalloproteinases (MMPs) unable to degrade ECM components at a rate equal to ECM protein synthesis. As a result, the ECM builds and overtakes essential components, such as the tubules and capillaries in the kidney, or the parenchyma in the liver. Ultimately, the overgrowth of fibrous tissue adversely affects organ function, and depending on the affected tissue, can lead to organ failure and death. In response to a biomaterial, the problem of fibrosis is exacerbated by the existence of the foreign polymer that cannot be taken up by infiltrating immune cells, namely macrophages. In such cases, a foreign body response will inevitably ensue, a process that has been well documented by Anderson and colleagues.[1, 2] Biomaterials are herein considered as inanimate materials, natural or synthetic, that are used in medical devices and interact with components of the human body. For the purpose of this review, we exclude fixed or decellularized autografts from our definition of biomaterials as these constructs are derived from the host and are therefore compatible with his/her immune system.

Similarities between the fibrotic response in the kidney, liver, pancreas, lung, peritoneal cavity, etc., have led us to believe that the key to

curing fibrotic disease and end-stage organ failure is understanding and modifying the pro-fibrotic microenvironment. A common feature of fibrosis is the presence of mononuclear leukocytes. Unfortunately, to this point we do not fully understand or appreciate the role of mononuclear leukocytes in the pro-fibrotic environment. Work involving molecular mechanisms in such environments have focused on the presence or absence of various signaling molecules, especially TGF-β. Tissue damage, leading to a pro-inflammatory environment, initiates cell migration into the tissue. In the case of pathogen presence, which occurs in superficial wounding, such cellular migration is advantageous. Unfortunately, with a lack of a pathogen, migrated mononuclear cells take up a pro-fibrotic phenotype, which leads to the production of pro-fibrotic mediators and production of ECM proteins, namely Collagen-1.

The fibrotic response that occurs in the organs of the human body is not unlike the fibrotic response to foreign materials. In both cases, responsive mononuclear cells produce cytokines, chemokines and growth factors associated with ECM production. Biomaterials are used in many non-blood contacting applications in the human body, such as for sutures, pacemakers, real-time glucose monitors, peritoneal dialysis, artificial joints, and cosmetic augmentation. Currently, each device has a limited amount of time for which it can be left in the body due to the fibrotic host response to

foreign materials. Furthermore, the fibrotic response to stents, glucose monitors, artificial joints, etc. significantly affect the function of the device. If the fibrotic host response were manageable, materials could be left in the body for significantly longer amounts of time and many devices would not need to be replaced as frequently. Unfortunately, a solution to the fibrotic response has yet to be determined, which limits the use of biomaterials in other applications such as for tissue engineering scaffolds, or non-cellular tissue replacement.

In the following sections, a brief overview of tissue engineering and scaffold materials is provided for understanding of the subsequent discussion on fibrotic responses to these constructs. Anderson has written thorough reviews on the biological responses to foreign materials in 2001 and 2008,[1, 2] and several good reviews on various fibrotic diseases have recently been written.[3-7] Therefore, the focus of this paper is to highlight similarities and differences between the foreign body response and fibrotic diseases as both are characterized by excessive deposition of ECM proteins. Due to similarities of these conditions, we suggest several implantable constructs for future fibrosis research in animal models.

II. GENERAL MECHANISMS OF FIBROSIS

The general accepted model of fibrosis is as follows: In response to injury, epithelial and/or endothelial cells release mediators of the

antifibrinolytic coagulation cascade.[8] Platelets, when exposed to fibrin and other ECM components, aggregate and form a blood clot, which results in hemostasis.[9, 10] Platelets then degranulate, which promotes vasodilation, MMP production by epithelial/endothelial cells, and growth factor, cytokine and chemokine release by epithelial/endothelial cells. Affected tissues at the wound site also produce prostaglandins, which enhances vasodilation, vascular permeability and leukocyte migration. As a result of an increased permeability of the local vasculature, leukocytes migrate through vascular walls and to the site of damage.[11] Thus, acute inflammation ensues with its severity closely related to the following factors: (1) the extent of damage and therefore the magnitude of release of prostaglandins, other signaling factors (cytokines and chemokines), and MMPs; (2) the site of damage and tissue(s) affected; and (3) the extent of provisional matrix formation.[2] Neutrophils and mast cells make up the majority of the initial extravasating cells and are therefore characteristic of acute inflammation. Mast cells release histamine from their granules, which exacerbates the initial inflammatory response by recruiting phagocytes to the implantation site.[12, 13] Neutrophils far outnumber mast cells, but both cells are responsible for providing recognition of the material and guiding further cell migration with the release of cytokines and chemokines. Monocytes and macrophages are slower to appear in the wound site, but neutrophils and mast cells soon

degranulate and apoptose, leading to the predominance of monocytes/macrophages in the local environment. In the wounded tissue, both the neutrophils and macrophages phagocytize tissue debris, dead cells and foreign organisms/materials. Both cells also produce cytokines and chemokines dependent on the phagocytized materials, which amplify the wound-healing response. Several of the signaling factors produced are also chemotactic and mitogenic for endothelial/epithelial cells, which initiate the formation of new blood vessels. Lymphocyte migration to the wound site results in T cell activation and further production of profibrotic cytokines (IL-4, IL-13 and TGFβ). Fibroblasts in the local environment are stimulated by the abundance of profibrotic mediators and become α-SMA-expressing myofibroblasts, which are primarily responsible for the local ECM protein production. At this point in time, if macrophages and the other immune cells are not able to clear the initial stimulus (such as damaged tissue, foreign organisms, or foreign materials), activation of the myofibroblasts persists and collagen-1-rich fibrous tissue is formed. As long as the fibrous tissue remains cellular, fibrosis is reversible. However, as ECM production continues, the immune cells undergo apoptosis and subsequently die, leaving an irreparable scar that likely impairs function of the organ/tissue.

II.A. The initiation of the fibrotic pathology and inflammation – disruption of tissue homeostasis

Fibrotic conditions can result from very minor perturbations in tissue homeostasis. Therefore, severe damage to tissue is not a necessary initiator for fibrosis. For example, in the liver, stellate cells have been shown to express toll-like receptor (TLR) 4. As a result, lipopolysaccharides or endogenous ligands of TLR4 are capable of activating these cells, which respond by down-regulating bone morphogenetic protein (BMP) and activin membrane-bound inhibitor, ultimately increasing the production of Transforming Growth Factor (TGF) β1.[14, 15] At this point, it is important to note that though the presence of inflammatory cells is common in the pro-fibrotic environment, inflammatory cells may not be necessary for the development of fibrotic conditions. For example, hemochromatosis frequently presents itself, without the presence of inflammatory cells, but still results in liver cirrhosis.[16] Furthermore, Gauldie and colleagues have also shown that lung fibrosis progresses due to over-expression of TGFβ1 without apparent inflammation,[17] and Gauldie has since argued that inflammation is a minor component of idiopathic pulmonary fibrosis.[18] Inflammatory cells are often the producers of profibrotic signaling molecules, and may accelerate the development of fibrosis; however, inflammatory cells (leukocytes) may not be essential to fibrogenesis as pro-fibrotic signaling factors can be

produced by other cell types. Regardless of the initiating event, fibrogenesis does not occur in the absence of signaling factors, cytokines and growth factors that polarize the present cells to a regenerative/fibrotic phenotype.

II.B. Cytokines and Signaling Molecules

1. TGF-β and BMP1

Several cytokines and growth factors are critical to the development and regulation of wound healing. Among the most important mediators of the fibrotic response is TGFβ. This is partly due to its effect on fibroblast differentiation, extracellular matrix formation and epithelial-to-mesenchymal transition,[19] and also partly because of its effects on downstream mediators, such as CTGF.[20] Until recently, roles of different isoforms were thought to be identical. However, it is now understood that the balance between TGFβ1 and TGFβ3 is critical to proper wound healing as TGFβ1 promotes fibroproliferation by inducing production of TIMP-1, and TGFβ3 promotes non-fibrotic tissue repair.[21] The primary cell sources of TGFβ are monocytes and tissue macrophages, but other cells may also contribute to TGFβ signaling. Unlike other cytokines, TGFβ is stored intracellularly non-covalently bound to a latency-associated protein (LAP), which deactivates the cytokine. Dissociation of LAP is catalyzed by cathepsins, plasmin, calpain, thrombospondin, integrin-αvβ6 and MMPs.[22-24]

Once LAP is cleaved and TGFβ is active, it is capable of interacting with its transmembrane receptors, which use the Smad proteins as signaling intermediates.[25] The Smad proteins then modulate transcription of target genes, such as procollagen I and III.[25] Bone Morphogenetic Protein (BMP) 1 and BMP7 have been shown to oppose the production and activation of TGBβ1.[26]

2. IL-4 and IL-13

Other cytokines that directly mediate the development of fibrosis are IL-4, and IL-13, which are both capable of initiating fibrosis in the absence of TGFβ.[27-30] IL-4 is a well researched cytokine in both the pathology and biomaterials literature, and has been shown to stimulate production of collagen types I and III and fibronectin. IL-13 has the same signaling pathways as IL-4, IL-4Rα/Stat6, and therefore has many similar functions as IL-4.[31] Nevertheless, IL-13 is a critical mediator of fibrosis. In schistosomiasis blocking IL-13 leads to a decrease of over 85% of collagen deposition with undiminished production of IL-4.[32-34] Over-expression of IL-13 triggered significant subepithelial airway fibrosis,[35] whereas IL-4 over-expression induced little subepithelial airway fibrosis despite an intense inflammatory response.[36] Furthermore, anti-IL-13 antibody treatment is capable of reducing collagen deposition in the lungs of animals with fibrosis induced by

either *A. fumigatus* conidia[37] or bleomycin.[38] IL-13 has also been found to upregulate TGFβ signaling by inducing TGFβ production in macrophages and stimulating LAP cleavage through MMP and cathepsin pathways.[39, 40] Due to the important role of IL-13, the IL-13 decoy receptor (IL-13Rα2-Fc) is capable of blocking the progression of fibrosis by binding IL-13, leaving it unable to bind to the IL-4R complex.[41-43] This has been confirmed in both IL-13Rα2 deficient mice and IL-13Rα3$^{-/-}$ mice, in which the enhanced IL-13 activity led to more rapid and advanced liver fibrosis.[44, 45] However, there is now evidence that IL-13 may actually be capable of promoting fibrosis through the IL-13Rα2 chain.[46]

3. IL-5 and IL-21

IL-5 and IL-21 act similarly, in that, they do not contribute directly to the development of fibrosis, but promote the production of the aforementioned cytokines. Eosinophils, which are a source of TGFβ1 and IL-13, are dependent on IL-5 for differentiation, activation and recruitment. Several studies have found benefit of neutralizing IL-5 activity,[47-50] while others have found no benefit.[51, 52] Therefore, IL-5 and eosinophils might not directly mediate fibrosis, but might amplify the production of other fibrotic mediators. Like IL-5, IL-21 is thought to enhance fibrosis signaling by promoting Th2 cell migration and survival.[53] Also, IL-21 has been shown to

increase IL-4 and IL-13 receptor expression on macrophages, which promotes the development of a pro-fibrotic macrophage phenotype.[54]

4. IL-10

IL-10 is an immunosuppressive cytokine that is known to inhibit fibrosis and contribute to the resolution of chronic inflammation.[55-57] The main sources of IL-10 are regulatory T- lymphocytes (Tr1), T-helper type 2 (Th2), Th1, and Th17 cells.[58, 59] Regulatory macrophages and monocytes are also important producers of IL-10 and may be responsible for immune regulation in neoplastic tissue.[60] In the liver, since IL-10 deficiency alone is not sufficient to affect the progression of fibrosis,[61] there is evidence that Th1 cytokines (IFN-γ and IL-12) and the IL-13 decoy receptor (IL-13Rα2), act together with IL-10 to reduce collagen production.[62, 63]

5. Angiotensin II

Other critical mediators of fibrosis include the components of the renin-angiotensin-aldosterone system. The most critical of these hormones is angiotensin II (ANG II), because of its ability to directly upregulate TGFβ1 and stimulate fibroblast proliferation and differentiation into myofibroblasts.[64-67] ANG II is produced by activated macrophages and fibroblasts, and by inducing NADPH oxidase activity, TGFβ1 production is

stimulated.[64] ANG II also stimulates TGFβ1 signaling by increasing SMAD2 levels. The renin-angiotensin-aldosterone system is generally overlooked with respect to material-induced fibrosis, but may be an important component capable of being blocked.

6. Chemokines

The presence of chemokines in the pro-fibrotic environment are poorly understood; however, several studies have indicated that chemokines play a critical role in the promotion of fibrogenesis. In the biomaterials literature, both Rhodes *et al.* and Jones *et al.* have shown that production of chemokines, such as CCL2, CCL4, CCL13 and CCL22, are produced by macrophages responding/adhered to biomaterials and might also be important for further macrophage recruitment.[68, 69] CXC chemokines might also be critical in promoting fibrosis, especially macrophage inflammatory protein (MIP)-2, as neutralization of MIP-2 has been shown to effectively suppress bleomycin-induced pulmonary fibrosis.[70]

II.C. Myofibroblasts

The next important step in the development of fibrosis is the development of α-SMA-expressing myofibroblasts from epithelial cells, endothelial cells and/or fibroblasts. This step is well researched in the

pathological fibrosis literature, but not often mentioned in the biomaterials literature. Myofibroblasts are responsible for the majority of ECM protein production in all fibrotic conditions and therefore their presence in the fibrogenic environment is critical. There are three known origins of myofibroblasts in pathological fibrosis: (1) mesenchymal cells, such as fibroblasts or hepatic stellate cells, resident in the tissue may become activated by a stimulus;[71] (2) Epithelial cells or endothelial cells may undergo a transition to become myofibroblasts, a process well known by many as the epithelial-to-mesenchymal transition (EMT), or endothelial-to-mesenchymal transition (EndMT);[4, 72, 73] (3) fibrocytes, circulating in the blood, derived from bone marrow stem cells, can extravasate through the nearby vascular walls and become activated in the tissue.[74, 75] Once activated, myofibroblasts migrate toward the wound or foreign material and produce the majority of new collagen, as well as other ECM proteins. The exact origin of resident myofibroblasts in the fibrogenic environment of most fibrotic conditions, including the foreign body response to biomaterial implantation, is unknown.

II.D. Normal Wound Repair

In normal wound repair, myofibroblasts are responsible for wound contracture, whereby edges of the wound migrate towards the center.[32] Wound repair is achieved by endothelial cells and/or epithelial cells migrating and dividing over the basal layer of the wound. The damaged tissue is regenerated from this cell division, and the wound healing process is concluded. However, with the presence and activation of immune cells and/or myofibroblasts, the synthesis of ECM components is not always reduced. As a result, if the rate of ECM protein synthesis by myofibroblasts exceeds the rate of degredation by MMPs, fibrotic tissue develops and a permanent scar forms, which hampers function in local organ structures. In later stages of wound healing, macrophage cells are indespensible as they are responsible for the majority of MMP production and therefore ECM degradation. Since additional monocytes/macrophages migrate to the wound site during inflammation, there may in fact be a benefit of inflammation for normal wound repair.

III. BIOMATERIALS AND TISSUE ENGINEERING

Each year, thousands of eligible organ recipients die due to the shortage of donor tissues.[76, 77] The field of tissue engineering was born as a result of the worldwide shortage of donor tissues and aims to develop

biological tissue substitutes for patients with end-stage organ failure.[78-80] Various conceptual designs exist for each organ, but most models include both cellular and acellular components.[81, 82] The acellular aspect of the first generation engineered organs, likely a microporous polymer, would be responsible for providing structure to the construct. Either differentiated cells of particular types or undifferentiated stem cells will then be seeded into the polymer with conditions suitable for cell proliferation.

Major challenges facing tissue engineering are the immune response to tissue-engineered products.[83, 84] When foreign materials, such as synthetic polymers, are implanted into the body, proteins adhere to the surface; immune cells recognize these proteins and try to phagocytize the material.[85] With respect to polymers, immune cells cannot successfully phagocytize the material and thus initiate a foreign body response whereby the material is essentially walled off from the rest of the body, a response that would significantly impact the function of an engineered tissue.[2]

Aside from use in tissue engineering applications, biomaterials are used extensively in medicine. Catheters, stents, real-time glucose monitors, pacemakers, bone plates, joint replacements, artificial ligaments/tendons, etc., are all examples of the wide use of biomaterials in medicine. Currently, the host response to implanted biomaterials affects the lifespan and function of many of the aforementioned medical products. Significant research efforts

have focused on coagulation and hemostasis in response to biomaterials in vascular applications with a goal of engineering an antithrombogenic material. However, the fibrotic response to materials in non-vascular applications has been underappreciated. There are some slight differences between the fibrotic response to materials and fibrosis in response to tissue injury or infection, but the different responses have mostly similar characteristics.

Many different materials, both synthetic and natural polymers are used in both tissue engineering and medicine. Natural polymers, such as alginate, collagen, laminin, fibronectin, hyaluronic acid and chitosan, allow for better cell adhesion,[86, 87] and therefore may be more appropriate in certain applications. These materials are also considered to be more biocompatible and are similar to the ECM of normal tissue. As a result, natural polymers are increasingly being used in tissue engineering.[88] Also increasingly used are acellular tissue matrices, which are prepared by removing the cellular components of tissues, leaving the ECM to provide structural support for new tissue ingrowth.[88] The biocompatibility and minimal host responses to natural and acellular matrices make them a frequently desirable alternative to synthetic materials. However, synthetic polymers, such as polylactic acid (PLA), polyglycolic acid (PGA) and poly (lactic-co-glycolic acid) (PLGA), are generally easier to work with as

structure, porosity, degradation rate and mechanical properties are modifiable.[87]

Synthetic and natural biomaterials elicit a host immune response. The characteristics of the host immune response vary with different polymers and surface chemistries due to the differences in protein adsorption between different biomaterials and surfaces.[2, 85, 89] Much effort has gone into modifying surfaces of polymers in the hopes of creating a material that does not elicit a host response, but that goal has yet to be realized. It should be noted that characteristics of biomaterials, such as hydrophobicity/hydrophilicity, surface charge, porosity, and degradation rate, affect protein adsorption and subsequent immune response.[85, 89] Porosity of a biomaterial scaffold has also been shown to affect vascular ingrowth.[90] With respect to synthetic polymers especially, hydrophobicity/hydrophilicity and surface conformation can be modified by treating the fabricated scaffold with acids, bases, or solvents.[91, 92] Different materials can also be blended together to modify hydrophobicity of the scaffold. For example, polyvinyl alcohol can be added to PLGA to increase its hydrophilicity.[93] The biocompatibility of synthetic polymers can be altered by adding RGD groups, and other cell recognition groups to the surface of the material. If the host response and biocompatibility issues can be resolved for synthetic polymers their use in tissue engineering applications will increase,

since processing of these materials is much simpler than the processing of natural polymers.

III.A. The Foreign Body Response

The initial stages of the foreign body response are very similar those mentioned previously in the 'general mechanisms of fibrosis' section. It has been noted in the biomaterials literature that once macrophages are recruited to the implantation site, they produce tumor necrosis factor (TNF-α), interleukin-6 (IL-6), platelet-derived growth factor (PDGF), granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF) to recruit additional macrophages to the area.[94, 95] As mentioned above, chemokines, such as CCL2, CCL4, CCL13 and CCL22, are produced by macrophages responding/adhered to biomaterials and may also be important for further macrophage recruitment.[68, 69]

1. Cell Adhesion to Material Surfaces

Identified as one of the most important parts of the foreign body response, is the adhesion of cells to the provisional matrix around the biomaterials and the subsequent response of these cells. Integrins are the receptors responsible for this phenomenon and mediate intracellular responses to environmental stimuli.[96-98] Integrin receptors are heterodimers

comprised of α and β subunits. The partnering of different subunit chains provides the variability in terms of ligand binding and receptor function.[98] Multiple protein ligands likely participate in the receptor-ligand binding and monocyte adhesion.[99-101]

2. Macrophages and Foreign Body Giant Cells

Another integral part of the foreign body response is the fusion of macrophages to form foreign body giant cells (FBGCs). The exact mechanism of macrophage fusion is not yet known [2]. Mannose receptors appear to be important, since inhibitors of mannose receptor activity prevents/reduces macrophage fusion.[102, 103] Other important receptors for macrophage fusion include β1-integrin receptors, CD44, and dendritic cell-specific transmembrane protein (DC-STAMP).[104-106] In terms of signaling molecules, most important appear to be IL-4, IL-13 and CCL2.[102, 107-110] These molecules, and potentially others, upregulate the expression of binding molecules that is absolutely necessary for fusion to occur. That is, phenotypic alteration with one or more of these signaling molecules is required for macrophage fusion. Consequently, the surface upon which the macrophages are adhered to must also have an appropriate protein layer, otherwise fusion will not occur. This is indicated by the fact that macrophage fusion is material dependent, and therefore ultimately dependent on specific protein adsorption.[111] For

example, vitronectin adsorption has been shown to increase FBGC formation, whereas fibronectin reduces FBGC formation.[112, 113] As for intracellular signaling, rac1 and diacylglycerol kinase have been suggested as important transducers of macrophage fusion; however, the intracellular mechanics of macrophage fusion have not yet been well researched.[114, 115]

III.B. Fibrotic response to engineered-tissue

As discussed previously, when tissue injury occurs the normal wound healing response involves cells from edges of the wound migrating and proliferating towards the center to replace the damaged tissue. However, if the wound is larger than a few millimeters, this process is accompanied by fibrosis and scar tissue formation. Implantation of a biomaterial matrix actually lengthens the distance that cells can traverse without the initiation of a fibrotic response, up to one centimeter.[116] If a wound greater than one centimeter is treated with a biomaterial matrix alone, fibrosis occurs. However, if the matrix is cellularized with autologous cells from the wounded tissue, damage up to 30 centimeters in distance or depth can be treated without an adverse fibrotic response.[88]

The fibrotic response to engineered-tissue becomes a concern when the cellular source is allogeneic or xenogeneic to the host and tissue rejection takes place. However, engineered tissue can be made with cytokine

producing components, which might be advantageous in preventing tissue rejection. There is currently much literature on the immune response to allogeneic tissue, which point to regulatory leukocytes as importance mediators of tolerance induction and downregulators of graft versus host disease.

IV. SIMILARITIES AND DIFFERENCES BETWEEN PATHOLOGICAL FIBROSIS AND THE FOREIGN BODY RESPONSE

A unique characteristic of the foreign body response is the presence of multinucleated giant cells, formed by the fusion of macrophages on the surface of a material. In the foreign body response, these cells are viewed as an indispensible component, whereby fibrotic tissue does not form on the surface of the material if monocytes/macrophages do not adhere to the surface and FBGCs do not form.[2] However, the existence of giant cells is not characteristic of fibroproliferative conditions; in fact, even the role of mononuclear leukocytes is unclear. As previously mentioned, some fibrotic conditions develop in the absence of immune cells, but these conditions generally develop over extended periods of time (months to years). The inflammatory response to materials generally lasts a couple months, with a mostly acellular capsule developed around the material by the end of two months. Therefore, the role of immune cells in fibrotic conditions may be to

speed the process of successful regeneration or fibrous tissue formation, which is capable of slowly developing in their absence. This is done primarily by producing cytokines and growth factors that mediate fibrous tissue formation. On the other hand, since macrophages are the primary producers of MMPs, they may be essential mediators of matrix degradation in the later stages of wound healing. Macrophage depletion, which naturally occurs in the late stages of the foreign body response, likely results in the irreversibility of the acellular fibrotic capsule that forms around the material. Therefore, though macrophages may exacerbate the fibrotic response in initial stages, they likely play a major role in the promotion of wound healing as opposed to fibrosis.

In the foreign body response to biomaterials, the role of MMPs and TIMPs is not well understood. It is known that ECM synthesis exceeds the rate of catabolism, but the rate of either process has never been determined. Furthermore, MMPs have been shown to modulate macrophage adhesion and fusion on biomaterial surfaces.[117] Jones *et al.* showed that MMP-1, -8, -13 and -18 increase macrophage fusion, but by unknown mechanisms.[117] This demonstrates the multifunctional roles of MMPs whereby cell migration, adhesion, fusion, but also ECM catabolism are modulated by this family of enzymes. A potentially valuable future study would involve the quantification of MMP concentration and determination of ECM protein synthesis and

catabolism rates at different time points in the fibrotic cascade by *ex vivo* elucidation.

Finally, the phenotype of mesenchymal cells that accumulate in the local environment of implanted materials is unknown. In fibrotic diseases these cells are referred to as myofibroblasts because of their expression of α-SMA. Myofibroblasts are known to arise from three sources: (1) local tissue fibroblasts become activated and express α-SMA; (2) other local cells of mesenchymal origin, such as epithelial cells, endothelial cells, or hepatic stellate cells, transform into fibroblasts and then myofibroblasts; and (3) bone marrow derived fibrocytes migrate from the blood. Researchers of the foreign body response to biomaterials have yet to study the origin of the ECM-producing mesenchymal cells, and refer to these cells as fibroblasts, not myofibroblasts as the pathology literature does. Identifying the origin and phenotype of the ECM-producing mesenchymal cells is essential for the understanding of the foreign body response to biomaterials and doing so would be relatively straight forward, as biomaterials can be explanted and subjected to immunohistochemistry to determine adhered cell types. Currently, EMT-derived myofibroblasts are known to be responsible for the majority of capsular opacification in response to intraocular lens implantation.[118]

Unlike fibrotic diseases, the response to a material implant is known prior to its implantation, and an approximate timeline of fibrogenesis is known.[1, 2] Therefore, anti-fibrotic countermeasures and the timing of their application following material implantation can be optimized to circumvent the synthesis of fibrovascular tissue. Also, incorporating anti-fibrotic treatment compounds into medical devices may be a viable method of dramatically increasing the lifespan of such devices. As a result, several anti-fibrotic treatment strategies are reviewed below.

V. BIOMATERIALS AS ANTI-FIBROTIC DRUG CARRIERS

Tissue engineering and biomaterials offer research models that allow researchers some control of the local fibrotic environment *in vivo*. Biomaterials can be made to act as drug carriers that slowly release their contents, and engineered tissues allow researchers to implant simplified tissues to study the effects of one or a few cell types. The advantage of using biomaterials as drug carriers is that they can be made to release a drug at a relatively steady rate. As a result, enzymes or cytokines, or other proteins can be inhibited constantly. In situations where knockout animal models are not possible or appropriate, such as the use of $TGF\beta^{-/-}$ or $MMP\text{-}2^{-/-}$ mice, inhibiting the pathways through the use of enzyme inhibitors or monoclonal antibodies, released from a biomaterial, may be an effective alternative. As

fibroproliferative diseases affect a significant number of people, several inhibitors of related pathways have already been developed. Many of these inhibitors are capable of being loaded into polymeric drug delivery devices that can be utilized prior to, or following induction of a fibrotic condition. Known inhibitors of fibrosis include inhibitors to TGFβ, Angiotensin II, Endothelin-1, connective tissue growth factor (CTGF), Rho kinases (which prevent epithelial-mesenchymal transdifferentiation), collagen synthesis, MMP 2 and 9 and Wnt-4. A list of these agents is provided in Table 1.1.

Since the response to biomaterial implantation is well known and characterized, biomaterial researchers have the ability to manipulate the fibrotic response to their constructs at different time points through the use of treatment agents. In fact, as discussed, an anti-fibrotic strategy can be worked right into a material that is to be implanted into the body such that the response remains regenerative, and not fibrotic in nature. Such a strategy may significantly elongate the lifespan of medical devices and preclude their loss of function. The following are a few notable methods by which material scientists could intervene in the wound healing/fibrotic cascade.

Table 1.1. Fibrotic pathways and inhibiting compounds

Fibrotic Pathway/ Target	Inhibiting compound(s)
Inflammation/immunosuppression	Glucocorticoids, retinoids, colchicine, azathioprine, cyclophosphamide, thalidomide, pentoxifylline, theophylline
Collagen synthesis	Prolyl-4-hydroxylase inhibitors (eg. HOE0 077 or phenanthrolinones)
TGFβ	Decorin, pirfenidone, relaxin, BMP-7, hepatocyte growth factor, SMAD7
CTGF	Antisense oligonuceotides, cAMP, TNFα
Endothelin-1	Bosentan
Angiotensin II	ARBs, ACE inhibitors
Rho GTPases	Y-27362, fasudil
MMP2 and 9	Bay 12-9566
TIMP-1	Monoclonal antibodies specific for TIMP-1
B cell antagonists	Rituximab

V.A. Inflammation and glucocorticoids

Interfering with the inflammatory response could theoretically be successful at suppressing fibrous tissue development by reducing the sources of pro-fibrotic molecules that mediate the development of fibrosis. Glucocorticoids are probably the best treatment agents to use, for this purpose because they are generally non-irritating and suppress cell proliferation. In addition, glucocorticoids modulate the phenotype of infiltrating macrophages and lymphocytes.[60, 119] However, several other anti-inflammatory/immunosuppressive drugs have been proposed as suitable treatment agents for fibrotic conditions, which may also be of value. Other

immunosuppressive drugs include: colchicine, azathioprine, cyclophospamide, prednisone, thalidomide, pentoxifylline, and theophylline.

V.B. Artificially modulating the signaling cascade

Several profibrotic signaling molecules have been implicated in the development of fibrosis. TGFβ signaling modifiers, cytokines (e.g. IFNγ, IL-12, IL-10, etc.), chemokine antagonists (e.g. inhibitors of CXCL1, CXCL2, CXCL12, CCL2, CCL 3, etc.), and cytokine/chemokine receptor antagonists (inhibitors of IL-1β, IL-5, IL-6, IL-13, CCR2, CCR3, CCR5, etc.) have each been shown to be successful at suppressing the fibrotic response in various tissues. Incorporation of these compounds into an implantable biomaterial is likely to result in reduced fibrous tissue formation.

V.C. Regulating ECM production and catabolism

Since fibrosis results directly due to an imbalance between ECM production and catabolism, regulating these two processes could be an appropriate method of inhibiting fibrotic tissue formation. Furthermore, it is conceivable that manipulation of these processes could successfully reverse fibrous tissue formation. The most practical way of regulating ECM production is by inhibiting enzymes involved in collagen synthesis. An alternative method is by taking a cellular approach and target myofibroblasts with pro-apoptotic drugs. An enzyme that has been of interest to fibrosis

researchers is prolyl 4-hydroxylase, because of the importance of hydroxyproline to the triple helix structure of collagen. ECM catabolism is regulated by MMPs, namely MMP1. Therefore, up-regulating the production of MMP-1 and down regulating TIMP-1 could be a strategy to reduce the quantity of fibrous tissue and may lead to a regeneration of fibrosed tissue. Other MMPs (specifically MMP2, MMP9 and MMP12) have also been shown to have a significant impact on the deposition of fibrous tissue, due to their influence on cells in the local pro-fibrotic environment. That is, MMP2, MMP9 and MMP12 each play a role in the promotion of fibrotic tissue formation by supporting the EMT and increasing permeability of vascular tissue to immune cells. As a result, some researchers have successfully utilized inhibitors of MMP2, MMP9 and MMP12, which have an impact on immune cell infiltration and myofibroblast formation. Due to the actions of these MMPs, there is a time window for which the benefit of inhibiting the MMPs is realized and therefore the inhibitors must be delivered early in the host response.

VI. CONCLUSIONS

Fibrogenesis in response to tissue injury/infection is not unlike the foreign body response to implanted materials and biomaterial scientists should be attentive to research on pathological fibrosis. Since the

characteristics and approximate timing of the foreign body response are known, material scientists have the opportunity to alter the response by incorporating biologically active compounds into the materials. Current strategies aimed at alleviating damaging scar tissue formation need to be evaluated for their efficacy and ability to be incorporated in materials. In addition to relieving the fibotic response to the material itself, antifibrotic compounds are likely to have a significant impact on further development of fibrotic pathology in organ systems. The primary advantage of polymeric drug delivery of anti-fibrotic compounds is the local application of the therapy and avoidance of systemic adversities. Furthermore, the local delivery of these compounds allows greater doses to be applied to the tissue/cells of interest. Obviously, a major disadvantage of this technique is the requirement to surgically place the treatment patch in the affected area. However, if avoidance of end-stage organ failure can be achieved, such a procedure could be a welcome alternative to organ transplantation, which would otherwise be required.

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CHAPTER 2: TRANSIENT INHIBITION OF CONNECTIVE TISSUE INFILTRATION AND COLLAGEN DEPOSITION INTO POROUS POLY(LACTIC-CO-GLYCOLIC ACID) DISCS

Connective tissue rapidly proliferates on and around biomaterials implanted *in vivo*, which impairs the function of the engineered tissues, biosensors, and devices. Glucocorticoids can be utilized to suppress tissue ingrowth but can only be used for a limited time because they non-selectively arrest cell proliferation in the local environment. The present study examined the hypothesis that a prolyl-4-hydroxylase inhibitor,1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA), would suppress connective tissue ingrowth in porous PLGA discs implanted in the peritoneal cavity for 28 days. Our findings show that the prolyl-4-hydroxylase inhibitor was effective at inhibiting collagen deposition within and on the outer surface of the disc, and also limited connective tissue ingrowth, but not to the extent of glucocorticoid inhibition. Finally, it was discovered that 1,4-DPCA suppressed Scavenger Receptor A expression on a macrophage-like cell culture, which may account for the drug's ability to limit connective tissue ingrowth *in vivo*.

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INTRODUCTION

The meaning of 'biocompatibility' for biomaterials is largely dependent on the intended purpose of the material. For most applications, biomaterials are simply required to be tolerated by the host environment, which means that the material itself incites a low degree of inflammation and thus is able to reside in the body for a long period of time.[1] However, the development of next-generation real-time biosensors, vascular catheters and engineered tissues would benefit from the development of bioinert materials that are ignored by the surrounding tissues and the host immune system. A well-recognized method of thwarting the host response to such biomaterials is to incorporate pharmaceutical inhibitors into the construct to suppress the responses of host cells to the material. The research described in this manuscript compares a new pharmaceutical inhibitor, a prolyl-4-hydroxylase inhibitor, to glucocorticoids for the purpose of inhibiting inflammation, connective tissue infiltration, and collagen deposition on a standard porous poly(lactic-co-glycolic acid) (PLGA) tissue scaffold following intraperitoneal implantation.

Glucocorticoid release from a biomaterial construct with subsequent evaluation of inflammation and connective tissue infiltration has been performed before. Bhardwaj and colleagues found that Dexamethasone from PLGA/PVA composite materials did not provide protection against the

foreign body response once the drug was exhausted from the construct.[2] Nevertheless, long-term drug release of glucocorticoids still remains an option for 'hiding' a biomaterial from host cells, be they hematopoietic, mesenchymal or endothelial, though the unavoidable effect of arresting cellular proliferation in local functional tissue makes long-term glucocorticoid delivery unappealing for most applications. Hence our objective was to evaluate other potential pharmacological inhibitors to modulate inflammatory and fibrotic responses, so as to create an environment that supports long-term functioning of a sensor, catheter, or organ scaffold.

Prolyl-4-hydroxylase inhibition is an appealing technique to modulate the inflammatory and fibrotic responses to biomaterial implantation because of its capacity to impair collagen fibril synthesis and secretion and its concomitant inhibition of proteins that contain collagenous domains, such as complement protein C1q and Scavenger Receptor A.[3] Furthermore, proline hydroxylation is required for hypoxia-inducible factor (HIF) inhibition.[4-6] Similarly, β-oxocarboxylic acids, such as 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA), inhibit factor inhibiting HIF (FIH), an asparaginyl-hydroxylase, with an IC_{50} value of 60 μM.[7] As a result, prolyl 4-hydroxylase inhibition results in elevated intracellular HIF-1α, which promotes cell migration,[8-10] angiogenesis,[11-13] and regulation of glucose

metabolism,[14] which leads to protection against hypoxia-induced cell death. We opted to use 1,4-DPCA due to the existing literature on its ability to inhibit collagen hydroxylation[15] and FIH.[7] Specifically, the half-maximal inhibitory concentration (IC_{50}) of 1,4-DPCA for proline hydroxylation of collagen in human foreskin fibroblasts is 2.4 μM.[15]

The hypothesis for this research was that 1,4-DPCA would reduce tissue infiltration and collagen deposition. To test this hypothesis, porous PLGA discs were impregnated with 1,4-DPCA or one of two glucocorticoids, corticosterone or hydrocortisone, which were utilized as negative controls. Drug release kinetics were determined to ascertain the rate at which the drugs eluted from the discs. The discs were then implanted in the peritoneal cavity of C3H/HeJ mice and incubated for 28 days. Upon explantation, a peritoneal lavage was performed to determine the concentrations of interleukin (IL)-1β, IL-4, IL-6 and IL-10. The cytokines chosen are established biomarkers of acute and chronic inflammation (IL-1β and IL-6, respectively), the resolution of inflammation (IL-10) and foreign body responses (IL-4).

In summary, the tissue response to all the drug loaded discs was found to be favourable compared to control (unloaded) discs, and there was a reduction of inflammatory cytokine concentration compared to the control discs. However, the suppression of the tissue infiltration to the 1,4-DPCA discs was not as complete as we had hoped, as the discs accumulated an

appreciable quantity of connective tissue after only 28 days. Collagen deposition however was considerably reduced.

MATERIALS AND METHODS

Disc Fabrication and Drug Loading

The drug-loaded polymer discs were fabricated using a thermally-induced phase separation (TIPS) technique [16]. Cao et al systematically studied solvents for the TIPS technique and found that Dimethyl Carbonate (DMC) was superior to other solvents due to its high vapour pressure, high melting point, and absence of toxic effects.[17] As a result, DMC was selected as the solvent for scaffold fabrication. To cast the drug-loaded scaffolds, 0.5 g PLGA was dissolved into 10 ml of DMC in a 15 ml glass beaker and stirred at 45°C for 2 hours. The containers for the PLGA and DMC were only opened in the laminar flow biohood. All glassware and instruments, including magnetic stirring rods, were autoclaved prior to use. Drug loading was determined on the basis of solubility in organic solvents such that drugs were able to fully dissolve in the PLGA/DMC mixture. Each of the drugs was added to an autoclaved glass tube that was 0.7 mm in diameter. The glass tubes were then sealed and vigorously stirred at 45°C for another 2 hours. The stirring rods were removed from the glass tubes in the laminar flow biohood and the solution was immediately placed in the freezer at -80°C. The solution was left

overnight to freeze. In the morning, the glass tubes were unsealed and immediately placed in a freeze drier. The mixture was lyophilized for 72 hours resulting in the creation of porous, drug-loaded PLGA discs. The thickness of the discs were approximately 4.0 mm.

Drug Release Kinetics

To determine drug release *in vitro*, discs were placed in 3 ml of phosphate-buffered saline (PBS) and incubated at 37°C. Samples were 1 ml in volume and replaced with 1 ml of fresh PBS when taken. Samples were taken every 5 minutes in the first hour, every 15 minutes in the next hour, every 30 minutes in the third and fourth hour, and once an hour for the remainder of the first day. Absorbance measurements were made on a Beckman Coulter DU 800 Spectrophotometer and compared to a standard curve to determine the quantity of drug within the sample.

Disc Implantation and Explantation Procedures

Ethics approval for all animal work was obtained from the Animal Research Ethics Board at McMaster University prior to the commencement of the research (Animal Utilization Protocol 08-12-61). Sixteen female C3H/HeJ mice, three to six weeks old, were ordered from The Jackson Laboratory (Bar Harbor, Maine) and housed in the Central Animal facility at McMaster

University. The mice were allowed to acclimate to their environment for at least 3 days prior to surgery. Following acclimatization, drug-loaded PLGA discs, or a PLGA-only control disc, were implanted into the peritoneal cavity of the mice (one disc per mouse, four mice per condition). The mice recovered well from surgery and the discs were left in the peritoneal cavity for 28 days prior to explantation. After 28 days, the mice were euthanized with carbon dioxide and then immediately injected with 3 ml of saline and 3 ml of air in the peritoneal cavity. A small incision in the skin was then made, followed by a small incision in the muscle layer. The hole created was held open with forceps and a 5 ml transfer pipette was inserted into the peritoneal cavity to remove fluid. The fluid was then transferred into a 15 ml centrifuge tube. Fluid in the peritoneum was removed in this manner until the majority was extracted. Generally, 2ml of fluid was recovered from each mouse. Following explantation of all the discs, each tube was spun at 900 rpm for 5 minutes to separate cells from the supernatant. The supernatant was stored at -80°C for future use. After collecting peritoneal fluid from a mouse, the PLGA disc was explanted. In most cases, this involved slight expansion of the skin and muscle layer incisions. Once the disc was retrieved, it was placed in a plastic protective covering and inserted into a container with 10% formalin for fixation.

Histological Analysis

After 72 hours of formalin fixation, the discs were placed in 70% ethanol for sterilization. The discs and associated tissue were then processed such that they were dehydrated with ethanol, cleared with xylene, and infiltrated and embedded with paraffin wax. The discs were then cross-sectionally cut and stained with either hematoxylin and eosin (H&E) or Masson's Trichrome.

Enzyme-Linked Immunosorbent Assays

The peritoneal lavage fluid was analyzed by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits from Biolegend (San Diego, CA, USA). Samples were run in duplicate and 100µl of lavage fluid was used for each well. The assay was performed exactly according to the manufacturer's instructions and a standard curve was performed on every plate.

Effect of 1,4-DPCA on Scavenger Receptor-A Expression

RAW 264.7 macrophage-like cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 10nM lipopolysaccharides (LPS) from *Escherichia coli*

055:B5 to stimulate expression of SR-A. Increasing amounts of 1,4-DPCA were added to the cell cultures, from 3μM to 125μM.

After 72 hours of LPS and 1,4-DPCA treatment, adherent cells were removed from the plate with a cell scraper. The medium was then pipetted from the dish, placed in a centrifuge tube, and spun at 200 x g for 5 minutes. The supernatant was then discarded, and the cells were resuspended in PBS containing 10% FBS to a concentration of 10^6 cells in 100μl. Fluorescein-conjugated anti-mouse CD204 monoclonal antibody (10μl) was then added to the cell suspension, and the cell suspension was incubated for 30 minutes at 4°C. The cells were then washed twice and resuspended in PBS containing 10% FBS. Flow cytometry was then performed, with 5×10^4 events collected for each sample. Two completely independent experiments were run with similar results collected from each.

Cellular Proliferation Assay

To determine if the use of 1,4-DPCA addresses the limitation of glucocorticoids indiscriminately arresting cellular proliferation, a three-day study of cell proliferation was performed on a NIH/3T3 cell line. Prior to the study, the cells were cultured in DMEM supplemented with 10% FBS, and subcultured with trypsin treatment. At the onset of the experiment, the cells were lifted from the tissue culture plate with a 0.05% trypsin-EDTA solution

and cultured on a 6-well plate at a density of $8x10^5$ cells per well with 10μM of drug. The cells were then incubated for 72 hours at 37°C. Upon retrieval, the cells were treated with typsin and enumerated using Accucheck Counting Beads (Life Technologies, Burlington, ON, Canada), exactly according to the manufacturer's instructions , with a Flow-Cytometer (Beckman Coulter FC500, Mississauga, ON). The mean number of proliferation cycles was then calculated from the final cell count. A total of 6 repeats was performed for each condition.

Statistical Analysis

Cytokine concentrations from the peritoneal lavage fluid and the cellular proliferation data were subjected to a one-way analysis of variance using Microsoft SPSS software. Main effects were considered significant when $p>0.05$. Significant main effects were subjected to a Tukey's Honestly Significant Difference (HSD) test to determine significant differences between independent means.

RESULTS

Drug Release Kinetics

Porous PLGA discs, created using a thermally-induced phase separation technique,[16,17] were loaded with 1,4-DPCA, hydrocortisone, or

corticosterone. The drugs generally displayed second-order release from the scaffold (Figure 2.1), but as expected, the relative elution rate of the individual drugs related to their solubility in water.

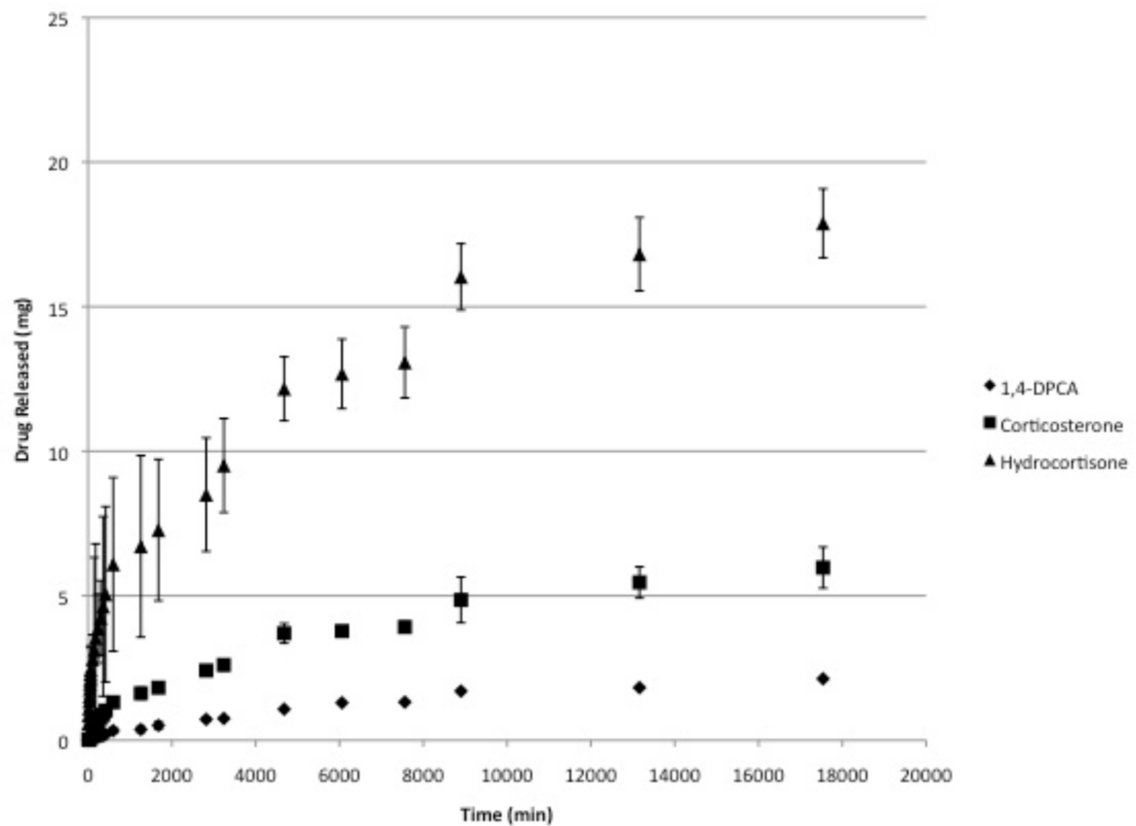

Figure 2.1. Drug release from the porous PLGA discs. Values represent mean ± SD.

Histological Analysis

The constructs were implanted into the peritoneal cavity of C3H mice and left for 28 days. Post-explantation histological analysis of the PLGA discs showed complete connective tissue infiltration in the discs that did not

contain any pharmacological inhibitors (Figure 2.2A). Observation of the histological sections revealed that some foreign body giant cells resided in and around the control (non-loaded) PLGA constructs, but no polymorphonuclear leukocytes were apparent on the slides. The glucocorticoid-containing discs (Figure 2.2B, 2.2C) had a significantly suppressed tissue infiltration, yet cell migration into the disc was evident on the outer edge of all the glucocorticoid-containing discs. The 1,4-DPCA discs were found to contain dramatically less connective tissue on the interior of the discs compared to the control discs, but a thicker layer of cell infiltration on the outer edge of the discs relative to the glucocorticoid discs (Figure 2.2D). There was a striking difference in the level of collagen deposition within and around the control discs and those containing 1,4-DPCA, which was revealed by staining the sections with Masson's Trichrome (Figure 2.3). Very little collagen was visible in the sections of the discs containing 1,4-DPCA, whereas the control discs all contained significant collagen both within and on the outer surface of the discs.

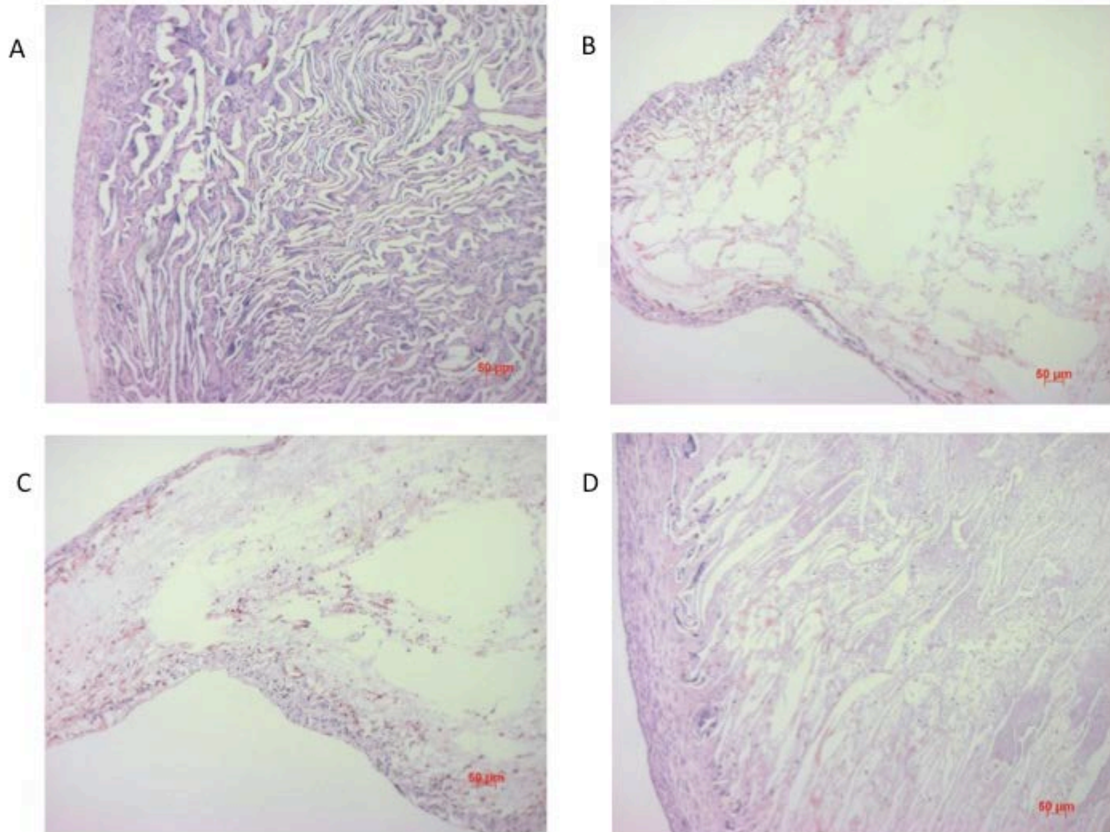

Figure 2.2. Histological sections of the PLGA discs following a 28-day incubation in the peritoneal cavity in C3H mice. Tissues were stained with hematoxylin and eosin and viewed at 100x magnification. (**a**) Control (unloaded) disc; (**b**) Disc containing corticosterone; (**c**) Disc containing hydrocortisone; (**c**) Disc containing 1,4-DPCA. Substantially reduced tissue infiltration is evident in all the drug-loaded discs, but to a greater degree in the discs containing a glucocorticoid (hydrocortisone or corticosterone). The histological images of the corticosterone-containing disc, and the hydrocortisone-containing disc show that incorporating these drugs into the PLGA discs compromised the integrity of the construct.

Cytokine Concentration in the Peritoneal Lavage Fluid

The cytokine concentration in the peritoneal cavity was assessed by lavaging the peritoneum and subsequently performing ELISAs for the various cytokines. The glucocorticoids and 1,4-DPCA each suppressed the IL-1β, IL-4,

IL-6, and IL-10 concentration in the peritoneal cavity relative to the unloaded control discs; however, due to the low sample size, the reduced cytokine levels did not reach statistical significance for IL-1β, IL-4, and IL-10 (Figure 2.4). A significant main effect of the drug impregnated in the discs was found for IL-6 [$F(3,11) = 3.979$, $p = 0.38$). Tukey's HSD post-hoc analysis revealed that the only significant difference between the independent means was between the control and hydrocortisone conditions ($p = 0.026$).

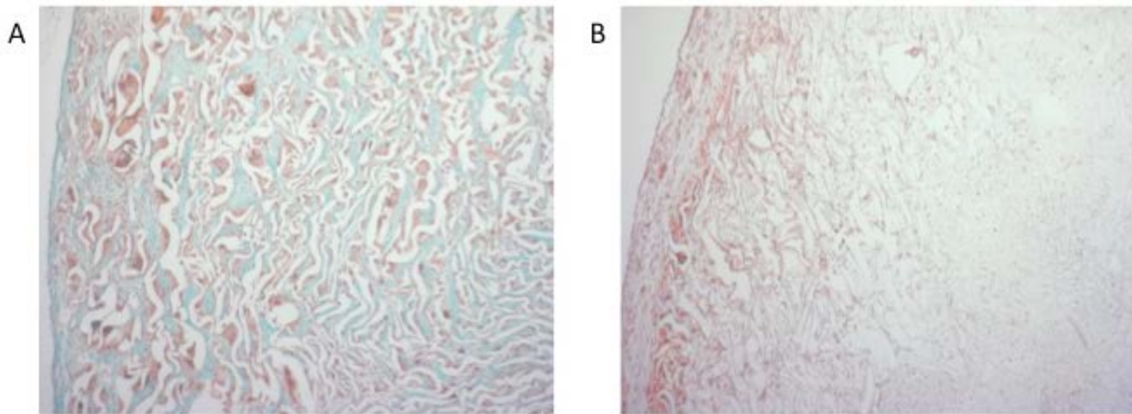

Figure 2.3. Histological sections of the control (**a**) and 1,4-DPCA-containing (**b**) discs. Tissues were stained with Masson's Trichrome to highlight collagen deposition (green). Images were taken at 100x magnification.

Effect of 1,4-DPCA on Scavenger Receptor-A Expression

The capacity of 1,4-DPCA to inhibit cell infiltration of the PLGA discs and reduce cytokine concentrations in the peritoneal cavity might be due to a number of reported effects of prolyl-4-hydroxylase inhibitors: (1) The expression prolyl-hydroxylase domain protein, factor inhibiting hypoxia-

inducible factor, is reduced by prolyl-4-hydroxylase inhibition. As a result, HIF-1α signaling is amplified, which regulates factors associated with cell proliferation, cell adhesion, cell migration and angiogenesis.[7,18] (2) The triple-helical super-secondary structure of collagen is stabilized by hydroxylation of the proline component in the continuously repeated Pro–Hyp–Gly sequences of collagen.[3,19,20] Therefore, prolyl-4-hydroxylase inhibition substantially impairs collagen expression by preventing formation of its triple-helical structure. (3) Consequently, expression of proteins with collagenous domains, such as complement protein C1q and scavenger receptors, should theoretically be impaired by the inhibition of prolyl-4-hydroxylase.

Since complement activity on a biomaterial is known to be dominated by the alternative pathway,[21,22] meaning that complement activity should not be greatly affected by inactivity of C1q, we looked at an intriguing possibility that prolyl 4-hydroxylase inhibition suppresses the expression of Scavenger Receptor A (SRA) on macrophages, as suggested by Gorres and Raines.[3] This was specifically tested by culturing RAW 264.7 macrophage-like cells for 72 hours in increasing amounts of 1,4-DPCA in parallel with cell stimulation using lipopolysaccharides from *Escherichia coli* 055:B5. SR-A expression was subsequently tested by flow cytometry with a fluorescein-conjugated anti-mouse CD204 monoclonal antibody.[23] The purpose of this experiment was

simply to determine whether Scavenger Receptor A (SR-A) expression on RAW 264.7 cells was effected by 1,4-DPCA. From this experiment we found that SR-A expression in the RAW264.7 cells was inhibited in a dose-dependent fashion, with a corresponding reduction in cell adhesion (Figure 5).

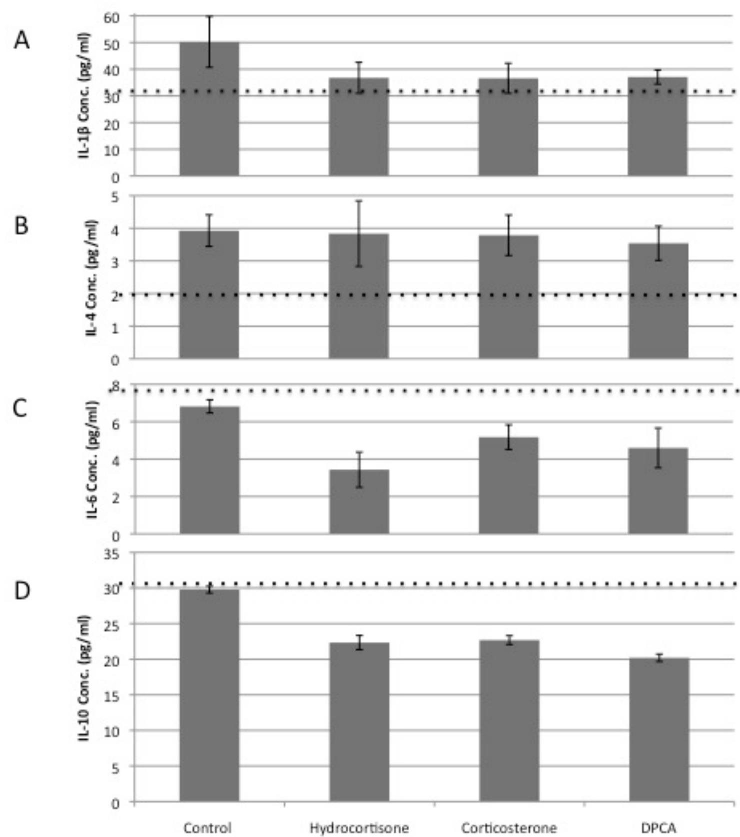

Figure 2.4. Cytokine concentrations in the peritoneal lavage fluid. Testing was performed with sandwich ELISA kits purchased from Biolegend, inc. (San Diego, CA, USA). Dotted lines represent the lowest concentration on the standard curve. Therefore, values below the dotted line were obtained by extrapolating the standard curve downwards. Values represent mean ± SD (n=4). (**a**) IL-1beta concentrations; (**b**) IL-4 concentrations; (**c**) IL-6 concentrations; (**d**) IL-10 concentrations. Statistical analysis by one-way ANOVA indicated that hydrocortisone significantly reduced the IL-6 concentration in the peritoneal cavity compared to control (unloaded) discs (p = 0.026).

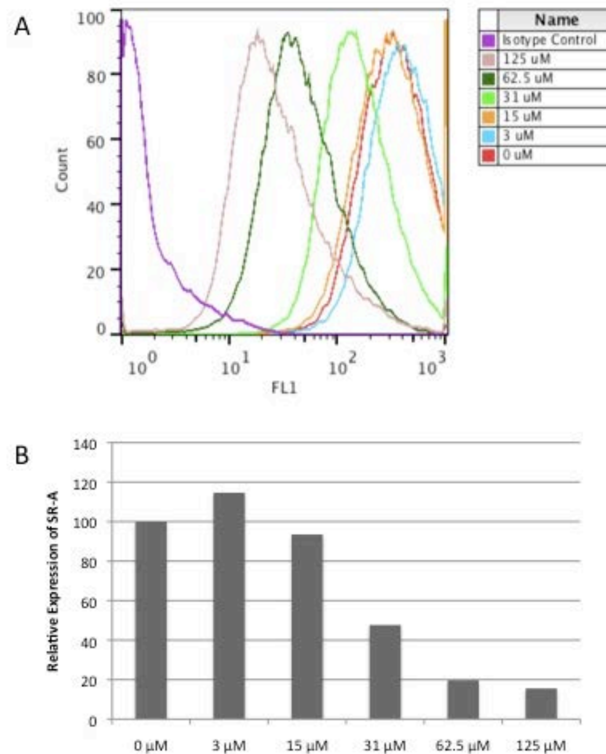

Figure 2.5. 1,4- DPCA suppression of SR-A expression in a RAW 264.7 cell-line. SR-A expression was stimulated for 72 hours with 10nM lipopolysaccharides (LPS) from *Escherichia coli* 055:B5. The cultures were concurrently treated with 1,4-DPCA, with concentrations ranging from 3µM to 125µM. SR-A was detected with a fluorescein-conjugated rat anti-mouse CD204 monoclonal antibody (Accurate Chemical, Westbury, NY, USA). A rat IgG2b isotype control antibody was used to ensure that there was no non-specific binding. (**a**) An overlay of the flow cytometry histograms. (**b**) A graphical representation of the relative expression of SR-A on the surface of RAW 264.7 cells following treatment with 1,4-DPCA. Sizes of the bars are representative of the x-axis means from the flow cytometry histograms as a percentage of the untreated control.

Cellular Proliferation Assay

A significant main effect of drug was observed from the results of the cellular proliferation assay [$F(3,20) = 4.060$, $p=0.021$]. Tukey's HSD post-hoc analysis revealed that only corticosterone (10µM) significantly suppressed

the cellular proliferation of the NIH/3T3 cell line compared to the control (untreated) condition ($p = 0.012$). However, it was apparent that 1,4-DPCA (10μM) and hydrocortisone (10μM) also suppressed the cellular proliferation of the NIH/3T3 cell line, such that less cells were present in the treated culture wells after 72 hours compared to the untreated control well (Figure 2.6).

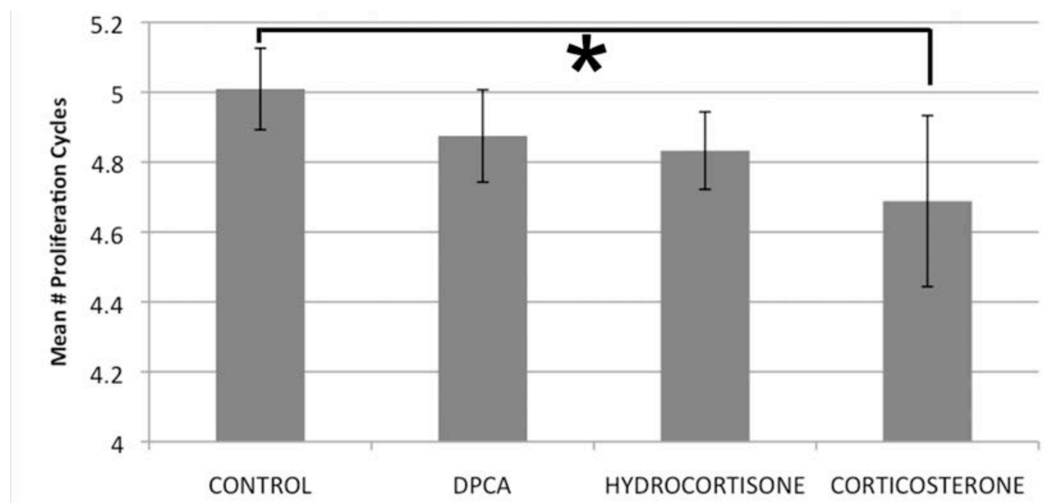

Figure 2.6. Inhibition of cellular proliferation of a NIH/3T3 cell line. Cells were cultured on a 6-well plate at a density of $8x10^5$ cells/well with 10μM of drug and incubated for 72 hours at 37°C. After 72 hours, the cells were lifted from the wells with trypsin and enumerated on a Flow Cytometer using Accucheck Counting Beads according to the manufacturer's instructions. The mean number of proliferation cycles was calculated from the final cell count. A total of 6 repeats was performed for each condition. Corticosterone significantly suppressed the cellular proliferation of the NIH/3T3 cell line compared to the control (untreated) condition ($p = 0.012$), as indicated in the figure.

DISCUSSION

Fibrosis occurs in response to the implantation of solid biomedical materials in the body and is generally comprised of two elements: (1) extracellular matrix protein synthesis that exceeds degredation; and (2) fibrovascular (connective) tissue proliferation around the material.[24] By using an inhibitor of proline hydroxylation, we intentionally targeted the former element by interrupting the synthesis of collagen, the primary component of dense irregular connective tissue. Though we suspected that fibrovascular tissue proliferation might be affected, it was an unintended consequence; the etiology of which is beyond the current understanding of the role of proline hydroxylation in fibrotic pathologies. As mentioned above, number of known consequences of inhibiting proline hydroxylation might be responsible: HIF-1α degradation is regulated by proline hydroxylation and affects cell adhesion, migration, and proliferation; other intracellular signaling molecules are also indirectly regulated by proline hydroxylation, including nuclear factor κB (NFκB) and activating transcription factor 4 (ATF-4); and finally any protein with a collagenous domain, such as C1q and SR-A, is likely to contain hydroxylated proline (see ref. 3 for a review). In the existing literature on SR ligation to biomaterials, there is significant evidence of opsonization-independent class A SR binding to various materials, such as silica,[25-27] tissue culture polystyrene(TCPS),[23] sulfated polystyrene,[28]

titanium,[29] titanium dioxide (TiO_2),[28] and iron(III) oxide (Fe_2O_3).[28] Several endogenous biomolecules, such as modified low-density lipoproteins and proteins with advanced glycation end products, that might adsorb to biomaterials are well known endogenous class A Scavenger Receptor ligands. Therefore, the ability of 1,4-DPCA to inhibit SR-A expression is a plausible mechanism by which it suppresses connective tissue proliferation into porous PLGA discs.

To our knowledge, the propensity of the aforementioned endogenous SR-A ligands to adsorb to biomaterial surfaces is not yet known, though it is probable that some degree of the proteins that adsorb to biomaterial surfaces *in vivo* contain advanced glycation end products. Furthermore, high-density lipoproteins have been shown to adsorb readily to polystyrene, poly(vinyl chloride), and n-Butyl-methacrylate: N-vinyl-pyrolidinone copolymers.[30,31] Therefore, it is probable that endogenous modified low-density lipoproteins also adsorb to biomaterial surfaces to some degree. Future investigation is required to evaluate potential endogenous SR ligands for their ability to compete for space in the biomolecular layer that encompasses biomaterials when implanted *in vivo*. Since a number of SRs are known to bind a wide variety of polymeric ligands, there is also a remote possibility that direct cell interaction with biomaterial surfaces occurs through SRs. To date the role of SRs in anti-biomaterial responses is

generally unknown, and is thus a subject requiring extensive study in the future.

A notable deficit of this study was that the ideal drug loading and release kinetics were not investigated here. The quantity of drug used for each disc was determined on the basis of solubility in organic solvents, such that the drugs were able to fully dissolve in the PLGA/DMC mixture. Therefore, a greater percentage of the final weight of the sterone discs was drug. Likewise, the final percentage (by weight) of PLGA in those discs was lower, though only marginally. This permitted us to observe a maximal effect of each drug, which resulted in almost complete inhibition of cellular infiltration into the discs in the case of the glucocorticoids. However, an apparent consequence of maximally impregnating the discs with drug was the compromised integrity of the glucocorticoid-PLGA discs.

The drug release kinetics that we observed are comparable to the findings of Leelarasamee and colleagues, who report kinetics associated with polylactic acid impregnated with hydrocortisone.[32] Notably the polymer [i.e. polylactic acid vs. poly(lactic-glycolic acid)] and the geometry of their construct (i.e. microcapsules vs porous discs) is different from what we used; however the drug release curves appear quite similar nevertheless. No other comparable studies on the kinetics of hydrocortisone or corticosterone release from PLGA were found. Seigel and colleagues studied the release of

corticosterone from solid PLGA discs, but report vastly different drug release kinetics.[33] The differences between the release kinetics reported here and those from Seigel et al., who observed a very delayed burst release of corticosterone from their PLGA discs, are presumably due the porosity, and therefore much greater surface area, of our constructs.

We believe that biomaterials impregnated with antifibrotic compounds, such as 1,4-DPCA, could be useful clinically as a means of locally regulating extracellular matrix production and catabolism (see ref. 14 for a review). Such a strategy could be particularly effective for fibroproliferative pathologies of organ systems. Regarding 1,4-DPCA specifically, local administration with a medium that facilitates prolonged drug release would impair collagen expression and deposition and may slow the progression of fibrosis and the permanent damage of an organ system.

CONCLUSIONS

The prolyl-4-hydroxylase inhibitor, 1,4-DPCA, was found to be effective at inhibiting collagen deposition in and around porous PLGA discs implanted in the peritoneal cavity and left for 28 days. Furthermore, the drug limited connective tissue ingrowth in the discs, but not to the extent achieved by corticosterone or hydrocortisone. A corresponding reduction in inflammatory cytokine concentration was observed, but only IL-6

concentration differences reached statistical significance. The efficacy of 1,4-DPCA to limit tissue ingrowth may be associated with its previously documented effect on HIF-1 α, but it may also be associated to its ability to impair SR-A expression, which was shown in the RAW264.7 cell line.

ACKNOWLEDGMENTS

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CHAPTER 3: THE RECOGNITION OF BIOMATERIALS: PATTERN RECOGNITION OF MEDICAL POLYMERS AND THEIR ADSORBED BIOMOLECULES

This chapter is a review of the activation and adhesion of leukocytes on the surface of biomaterials. Immune cells (leukocytes) respond to biomaterials in very similar ways regardless of the nature of the biomaterial. Hydrophobic biomaterials, hydrogels, biomaterials with low protein binding surfaces, and those that readily adsorb a protein layer all seem to incite similar leukocyte responses that may differ in magnitude, but ultimately result in encapsulation by fibrotic tissue. This occurs despite the vastly different surface and bulk properties of many biomedical materials. However, leukocytes have a very intricate pattern recognition system made up of integrins, toll-like receptors, scavenger receptors, and other surface proteins that enable them to perceive almost any foreign body in the host. This chapter provides the background on which Chapters 4 and 5 are based.

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I. INTRODUCTION

The implantation of a synthetic polymeric material triggers an inflammatory response, which leads to fibrous tissue accumulation on and around the material. The response is from the presence of the material and is independent of the wounding due to implantation: materials implanted in the peritoneal cavity, distant from the implantation site, evoke inflammation, as do materials implanted through minimally invasive procedures.[1,2] The nontoxic, inert, and non-immunogenic nature of synthetic polymers makes the inflammation and subsequent fibrosis a somewhat peculiar phenomenon. However, it is well known that a protein layer is adsorbed onto the polymer almost immediately upon interaction with a biological fluid.[3,4] Therefore, it is accepted that elements of this initial protein coat mediate the response.[5,6] Fibrinogen (Fg), albumin, and immunoglobulin (Ig) are the most abundant proteins in plasma, and typically dominate on the material surface upon implantation.[7,8] Since pre-adsorption of albumin has been shown to passivate polymers to the inflammatory, and thrombogenic responses, it likely has little role.[9-11] On the other hand, pre-adsorption of Fg and other serum proteins leads to near normal inflammatory responses, which supports the possibility that these proteins initiate the host response to polymeric materials.[11-13] Complement protein 3 (C3), high molecular weight kininogen (HMWK), fibronectin (Fn), vitronectin, the factor proteins of the coagulation

cascade, and other plasma proteins that associate with hydrophobic polymer surfaces all contribute to the host response to these materials and there is substantial overlap with respect to the cell receptors used to detect many of these proteins. For example, the αMβ2 integrin (also known as Mac-1, CD11b/CD18, and CR3) has been shown to ligate C3,[14,15] HMWK,[16] Fg,[17,18] and Factor X.[19-21] Therefore, though the proteins that adsorb to hydrophobic surfaces are structurally and functionally unique in soluble form, they each contribute to the stimulation of interrogating leukocytes via an overlapping mechanism. We have therefore classified such integrin-mediated leukocyte activation and adhesion to the adsorbed biomolecule layer on biomaterials as a process of pattern-recognition.

There is substantial evidence that the aforementioned protein adsorption mediates the activation and adhesion of monocytes, macrophages, and neutrophils to many of the existing solid biomedical materials that are clinically used today. However, hydrogels, and materials with a low-binding interface adsorb far less protein to their surfaces, yet these materials seem to incite similar leukocyte responses as the classical hydrophobic biomaterials and too become encapsulated by fibrotic tissue eventually.[22] Moreover, when neutrophils are incubated with hydrophobic polymer microspheres in protein-free conditions, the neutrophils produce a significant respiratory burst and dump granule contents.[23] Therefore, we can conclude that there

must be other mechanisms that leukocytes use to detect foreign materials. Furthermore, these other mechanisms must not solely be dependent on an adsorbed protein layer. In the case of hydrogels, and materials with a slight charge, the most likely candidate for these responses is scavenger receptors (SRs), which are pattern-recognition receptors (PRRs) known to be expressed by macrophages and, to a more limited extent, monocytes.[24-27] Upon review of the literature, we find that these receptors have been shown to bind a number of different biomedical materials including titanium dioxide, iron oxide, and silica.[28-30] In the case of leukocyte recognition of hydrophobic materials in protein-free conditions, the leading theory is likely Seong and Matzinger's 'Hyppos-hypothesis', an evidence-based theory suggesting that the identification of molecular-patterns is based on the exposure of hydrophobic portions of molecules.[31]

In summary, neutrophils and monocytes have multiple PRRs that enable them to perceive and respond to foreign materials. The following review is an in-depth look at the current knowledge of leukocyte ligation and responses to foreign materials, with a particular focus on biomaterials. A small glossary of the acronyms that are used in this chapter has been provided (see Table 3.1). A summary of the pattern recognition receptors and their ligands that are discussed in this review has also been provided (see Table 3.2).

Table 3.1. List of Abbreviations for Chapter 3

AGE	Advanced glycation end products
AP	Alternative pathway (of the Complement Cascade)
BSA	Bovine serum albumin
C3	Complement protein 3
C3R	Complement 3 receptor
CP	Classical pathway (of the Complement Cascade)
Fc	Constant fragment
FcR	Constant fragment receptor
Fg	Fibrinogen
Fn	Fibronectin
HMWK	High molecular weight kininogen
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LRR	Leucine-rich regions
MARCO	Macrophage receptor with a collagenous structure
PAMP	Pathogen-associated molecular patterns
PRR	Pattern-recognition receptors
RGD	Arginylglycylaspartic acid (L-arginine–glycine–L-aspartic acid)
SCARA5	Scavenger Receptor A5
sCR1	Soluble complement receptor 1
SR	Scavenger Receptor
SRA	Class A Scavenger Receptor
SRCL	Scavenger Receptor with C-type Lectin
TLR	Toll-like receptor

II. PATTERN RECOGNITION RECEPTORS – A GENERAL OVERVIEW

Professional phagocytes of the innate immune system rely on both opsonization and pattern-recognition receptors to identify foreign, and potentially harmful, organisms and inanimate materials.[32-34] Pattern-recognition receptors (PRRs) have broad ligand specificity and bind molecules with similar molecular patterns.[35] Unlike lymphocyte receptors, PRRs are germline-encoded and nonclonal, meaning that they do not change from one generation to the next, or between individuals of the same species.

In their original conception of pattern recognition, Janeway and Medzhitov focused on host identification of microbial structures, pathogen-associated molecular patterns (PAMPs), by germ-line encoded PRRs.[33] Since then, the concept of pattern recognition has been broadened to include PRRs that recognize endogenous ligands of the host that are associated to tissue damage, the so-called damage-associated molecular patterns.[36] Remarkably, some PRRs are able to bind a range of molecular ligands, which may include proteins, lipids, carbohydrates and nucleic acids of either exogeneous or endogenous origin. Similarly, some ligands, such as bacterial lipopolysaccharide (LPS), are able to bind several distinct PRRs.[37-41]

Pattern-recognition receptors can be loosely divided into two categories: phagocytic PRRs, and signaling PRRs. Phagocytic PRRs promote the attachment, engulfment and destruction of microorganisms or foreign materials by phagocytes and generally require anchoring to the intracellular cytoskeleton to do so. Mannose receptors and β-glucan receptors both fit in this category. On the other hand, signaling PRRs identify pathogens in the local environment and subsequently trigger intracellular signaling to orchestrate an inflammatory response. Examples of signaling PRRs include Toll-like receptors and NOD-LRR proteins. Some PRRs are members of both categories. For example, two of the PRRs that we discuss here, integrins and SRs, seem to function as both phagocytic receptors and signaling receptors.

Table 3.2. Overview of Pattern Recognition Receptors and Their Ligands

Pattern Recognition Receptor	Ligands
Integrins	Complement Component C3 and Derivatives Various Adsorbed Plasma Proteins
Toll-Like Receptors	Adsorbed Endogenous Alarmins (see Table 3) Cationic Polymers (e.g. polyethyleneimine, polylysine, cationic dextran, cationic gelatin) Alginate (Mannuronic acid component) Poly(Anhydride) Nanoparticles Hydroxyapatite Modified Alkane Polymers
Scavenger Receptors	Latex Microspheres Charged Environmental Particles (e.g. TiO_2, Fe_2O_3, SiO_2) Anionic Polymers Unknown Adsorbed Serum Component(s) (may include proteins with advanced glycation end products or modified low density lipoproteins)

III. PATTERN RECOGNITION OF ADSORBED BIOMOLECULES BY INTEGRINS

Integrins have historically been excluded from identification as PRRs; however, integrins are polygamous receptors that bind conserved regions (patterns) on endogenous proteins, and therefore fit perfectly in the PRR category. A good example of this is integrin αMβ2 (CD11b:CD18), also known as complement 3 receptor (CR3) and macrophage-1 antigen (Mac-1), because of its broad ligand repertoire that includes binding sites on Fg, HMWK, complement protein 3b (C3b), and prothrombinase (Factor X). As these endogenous plasma proteins denature on a biomaterial surface, their

integrin binding sites become exposed, leaving them prone to ligation by interrogating leukocytes. Due to the magnitude in which this occurs, the process of protein denaturation followed by integrin ligation explains the vast majority of biomedical material recognition events by leukocytes. Opsonization of biomaterials only exacerbates the dominance of integrin-mediated leukocyte responses as Ig adsorption to a surface initiates the classical pathway of the complement cascade, which results in more C3b deposition on the material. These processes are described in further detail below.

III.A. Leukocyte Binding of Opsonins

Once an insoluble biomaterial is implanted *in vivo*, the material quickly adsorbs IgG and complement protein 3 (C3).[42-44] In immunology, this is known as opsonization, as the process readily occurs on the surface of bacterial pathogens to target them for phagocytosis and destruction by neutrophils and macrophages. On biomaterials, the process occurs in a non-specific fashion: when Ig adsorbs to the surface, the constant fragment (Fc) is available for binding to the Fc receptor (FcR) on the aforementioned phagocytes. Similarly, when C3 adsorbs to the surface it quickly cleaves into C3a and C3b via the alternative pathway (AP) of the complement cascade.[43]

Immunoglobulin G adsoption to a biomaterial surface usually occurs through its hinge and C(H)2 domain of Fc,[45] which suggests that the terminal Fc region is available for binding to the Fc receptors (FcR) of interrogating phagocytes. However, since adjacent FcRs on the cell membrane of the phagocytes must be crosslinked for an intracellular signal to be initiated, and since the number of crosslinking events must reach a minimum threshold for activation of the cell to occur,[46] Ig adsoption to a material *in vivo* is unlikely to directly contribute much to the observable cellular activation of phagocytes on material surfaces. Rather, Ig adsorption on a biomaterial indirectly contributes to cellular activation by initiating the classical pathway (CP) of the complement cascade (see Figure 3.1).[47] Specifically, complement component C1q binds to the Fc domain of either IgG or IgM on a biomaterial (or pathogen) surface (Figure 3.1B). The C1q molecule is composed of a collagenous domain with six globular heads, resembling a bouquet of six flowers.[48] Two molecules of each C1r and C1s are bound to each C1q.[49] When C1q binds two or more IgG molecules within a 30-40nm area, or one IgM molecule, the enzymatic activity in C1r becomes activated.[50] The activated C1r then cleaves the inhibiting fragment of C1s, which permits its activity as a potent serine protease for C2 and C4 (Figure 3.1C). Upon cleavage, the large subcomponents of C2 and C4, C2a and C4b respectively, associate with the surface of the material and function together as a serine protease for C3

(Figure 3.1D). The large fragment of C3, C3b, then deposits on the biomaterial surface (Figure 3.1E). As a result, surfaces pre-coated with IgG eventually become covered in C3, masking the expression of IgG and C1q.[51] While IgG and IgM are activators of the complement cascade, C3 deposition also occurs readily on non-coated surfaces by the AP.[52,53]

Complement activation generally starts immediately during the initial adsorption of the protein layer on the surface of a biomaterial (Figure 3.1A). This generally occurs via the CP, either from direct C1q binding to the surface, or through C1q bound to Ig.[44] However, following the adsorption of the initial biomolecule layer, AP complement activation provides the bulk of the C3b deposition on the surface.[44] As Andersson and colleagues reported in 2002, the C3 adsorbed to a polymer surface can bind Factor B in the presence of properdin to form a functional AP convertase (C3b,Bb).[43] This convertase then cleaves C3 into C3a and C3b (see Figure 3.1E, top right). Component C3a is a small soluble (extremely hydrophilic) anaphlotoxin, which diffuses out and away from the material, stimulates smooth muscle contraction and vascular permeability, and acts as a chemoattractant for phagocytes. Furthermore, C3a stimulates degranulation of mast cells and neutrophils, resulting in histamine release and generation of cytotoxic oxygen radicals, respectively. Component C3b is far more bulky and hydrophobic, and rapidly adsorbs to the material surface (Figure 3.1E). Therefore, C3b is available for

binding to Factor B resulting in more C3b,Bb Convertase complexes being available on the biomaterials surface. In fact C3b does not even need available space directly on the biomaterial surface, as it will bind atop albumin and IgG that is already bound to the surface.[44] Consequently, the main C3b binding on a biomaterial surface is mediated by the AP atop the adsorbed protein film.[44,54] This is important because C3b subsequently acts as a ligand for leukocyte adhesion to the biomaterial surface through integrin receptor αMβ2 (Figure 3.2A). As complement activation is rapid, most of the C3b is already deposited on the surface once leukocytes reach the local environment of the medical device.

The complement cascade is generally highly regulated as spontaneous hydrolysis of the thioester bond in C3, referred to as 'tickover', occurs at a significant rate in plasma. The result, C3(H_2O), is able to bind factor B, which can then be cleaved by the plasma protease factor D to liberate Ba and ultimately form C3(H_2O)Bb, a fluid phase C3 convertase. Host cells are generally protected from such spontaneous complement activation through regulatory proteins that are present on their membranes. Since biomaterials are devoid of such regulation, the complement amplification loop is uncontrolled. Thus, efforts have been made to enrich biomaterial surfaces with such regulatory proteins to prevent continuous amplification of complement. Andersson and colleagues successfully limited complement

activation on a polystyrene surface by binding factor H.[55] Engberg and colleagues used peptides derived from streptococcal M protein that specifically binds C4 binding protein in plasma to inhibit complement activation via the CP.[56] Inhibiting complement can also be achieved with soluble inhibitors. Compstatin, soluble complement receptor 1 (sCR1), pentamidine, pyridoxal-5-phosphate, and N-acetyl aspartyl glutamic acid have all been used successfully to inhibit complement activation on biomaterial surfaces.[57-59] Blocking the various pathways of the complement cascade results in a suppression of the leukocyte activation that otherwise occurs in response to biomaterial microspheres.[57] The importance of complement on biomaterial surfaces was well demonstrated by McNally and Anderson who depleted serum of complement component 3 (C3), and found substantially reduced monocyte adhesion to fluorinated, siliconized, anionic, and cationic surfaces.[60] When C3 was added back into the C3-depleted serum monocyte adhesion was restored to near normal levels.[60] However, regardless of whether complement is inhibited, many of the adsorbed proteins in the biomolecule layer that encapsulates a biomaterial upon implantation in the body contain integrin binding sites for leukocyte adhesion and activation (see Figure 3.2B); therefore, with or without the complement cascade, the innate immune system is capable of recognizing common biomedical materials and launching an attack.

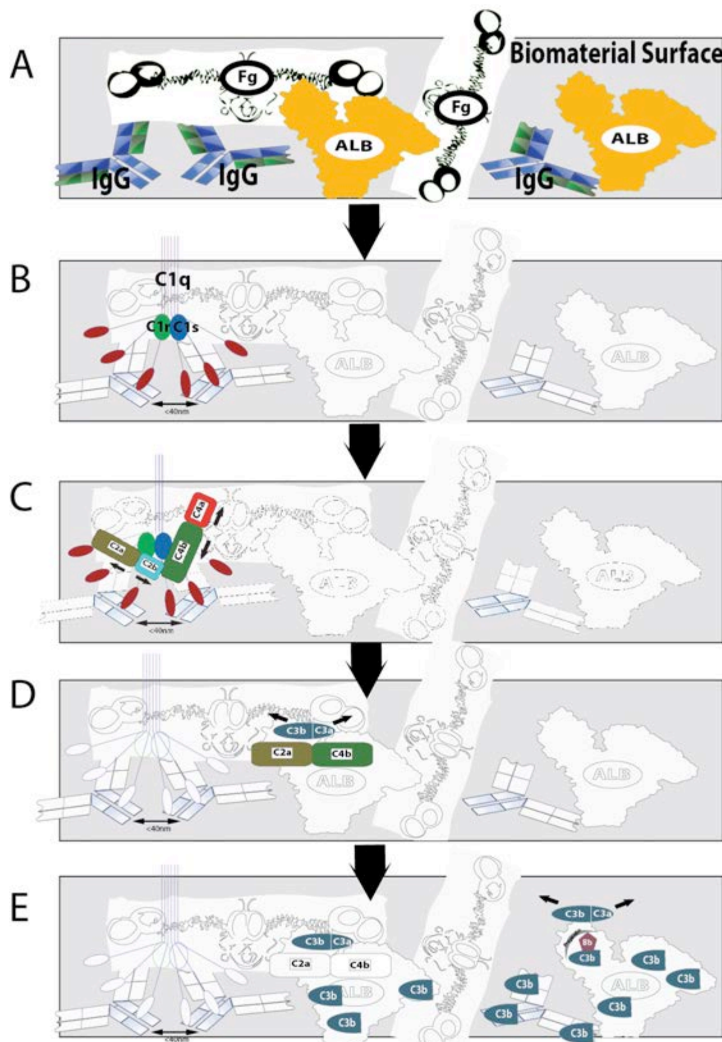

Figure 3.1. ***Complement activation on a biomaterial surface by the Classical Complement Cascade.*** (A) A layer of adsorbed proteins accumulates immediately upon contact of a biomedical material with a biological fluid. (B) The classical pathway of the complement cascade is initiated by the C1 complex binding to one adsorbed IgM molecule or two adsorbed IgG molecules within 40nm of each other. The C1 complex is composed of two molecules of each C1r and C1s bound to C1q, a collagenous hexamer with six heads (red). (C) Binding of C1q activates the autocatalytic enzymatic activity of C1r, which cleaves C1s to form an active serine protease. C1s then cleaves C2 and C4 into their respective components. (D) C4a is a hydrophilic anaphlotoxin that assists in the initiation of inflammation and chemotaxis of leukocytes. C4b is a larger hydrophobic molecule that generally associates with the surface of the material. C4b binds to C2, which in turn, is cleaved by C1s. The C4bC2a complex is a serine protease for C3, referred to as C3 convertase. (E) The C3 convertase continually cleaves C3 into C3a and C3b. C3a is another small, hydrophilic anaphlotoxin that assists with leukocyte migration. C3b is a larger hydrophobic protein that readily deposits directly on the biomaterial surface, or on top of pre-adsorbed proteins such as albumin and fibrinogen. Adhesion of C3b to a surface permits its binding to Factor B, which is cleaved into Bb and Ba by Factor D (not shown). The resulting C3b and Bb complex is another C3 convertase, and is stabilized by Properdin. Ultimately the biomaterial surface becomes coated in C3b, permitting ligation of neutrophils and monocytes/macrophages with integrin αM β2, also known as Mac-1 and Complement Receptor 3 (see Figure 3.2).

III.B. Leukocyte Binding of Plasma Proteins

It has long been known that proteins adsorb to the surface of hydrophobic polymers almost immediately upon incubation of the material in a protein-containing fluid.[61] The protein adsorption is random and non-specific, with abundant proteins dominating the surface initially, which are gradually replaced with proteins that have a greater affinity for the surface.[3] Following adsorption, proteins generally change in conformation, or denature.[4,62-64] Cells attach to the protein-coated biomaterial surface, with β2 integrin-mediated adhesion dominating this process at early timepoints.[65-67] As a result, β2 integrins, especially αMβ2, are able to bind to commonly adsorbed proteins, with some proteins having multiple adhesion sites: Fg is an abundant plasma protein and contains two αMβ2 binding sites that become exposed when Fg is adsorbed to hydrophobic surfaces;[17,18] HMWK has at least one αMβ2 adhesion sites that appears to have a higher affinity for αMβ2 than the Fg αMβ2 binding sites;[16] C3b is another ligand for αMβ2, as discussed in the previous section.

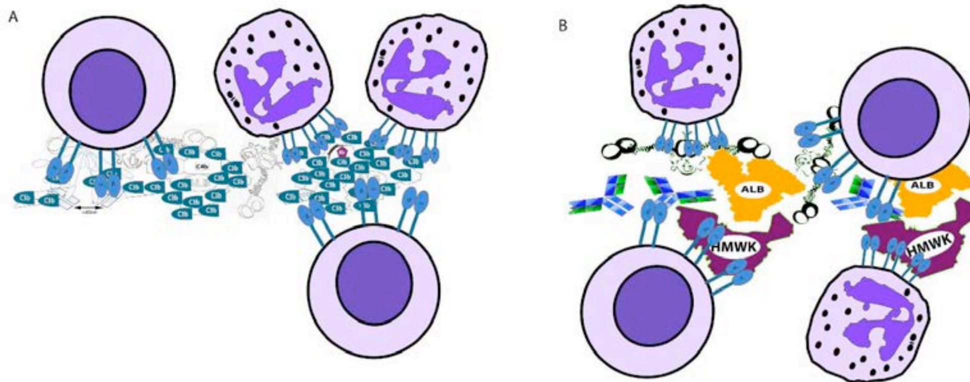

Figure 3.2. Cellular recognition of the adsorbed protein layer. Regardless of whether the complement cascade is permitted to coat a biomedical material surface with C3b (A), integrin receptors on neutrophils and monocytes remain capable of detecting the surface from other adsorbed proteins such as HMWK, fibrinogen, factor X, and vitronectin. (B).

Fibronectin (Fn) and vitronectin are two of the most important cell adhesion proteins found in plasma. This designation has been bestowed upon Fn and vitronectin because they both contain Arg-Gly-Asp (RGD) sequences that strongly adhere alpha V, alpha 1, alpha 3, and alpha 5 integrins. Laminins and collagens also contain RGD peptide adhesion sequences that bind multiple integrin dimers, but are not present in high concentration in plasma, and therefore are not generally found in abundance within the adsorbed biomolecule layer on biomaterials.

The consequence of integrin binding to the protein film is dependent on the cell type. For example, neutrophils dominate biomaterial implantation sites at early time points, but generally do not produce cytokine responses. Instead, neutrophil β2 integrin ligation to the adsorbed protein layer causes

degranulation, resulting in the release of various enzymes involved in the production of cytotoxic oxygen radicals, and the promotion of histamine release from mast cells and/or basophils.[11,68] Mast cells, though not found in abundance at material implantation sites, will arrive in low numbers with neutrophils and similarly degranulate upon integrin ligation resulting in an amplification of the acute inflammatory response.[69-74] By the time most of the monocytes reach the implantation site, the inflammatory response is fully underway.[75] As a result, monocytes are mostly activated by the inflammatory mediators in the local milieu. For example, the IL-4 and/or IL-13 released by mast cells during the acute inflammatory response will consequently direct monocytes to differentiate into macrophages and, subsequently, foreign body giant cells.[69,76] However, monocytes are capable of responding directly to the protein film on a biomaterial surface, and generally use the adsorbed protein layer to guide their differentiation and fusion into foreign body giant cells using β1 and β2 integrins.[60,77-80]

Integrin binding of the adsorbed protein layer that coats a material upon implantation to the body explains the vast majority of the cellular adhesion and activation that occurs on biomaterial surfaces. When McNally and Anderson blocked CD18 (integrin β2) with a monoclonal anti-CD18 antibody, monocyte adhesion was again substantially reduced on fluorinated, siliconized, anionic, and cationic surfaces.[60] However, in the absence of

integrin binding, some leukocytes, especially neutrophils and macrophages, remain capable of recognizing and adhering to common biomaterial surfaces.[23,81] The receptors responsible for such interactions are well-known pattern recognition receptors that may have the ability to recognize materials in the absence of a biomolecular layer. This may explain why hydrogels and low-binding surfaces suffer from foreign body responses that are similar to the responses to other biomaterials.

IV. TOLL-LIKE RECEPTORS AND THE IMMUNE-STIMULATING EFFECTS OF DAMAGE-ASSOCIATED MOLECULAR PATTERNS ON BIOMATERIALS

Toll-like receptors (TLRs), named after the *Drosophila* Toll receptor, are hallmarks of the innate immune system because of their ability to bind several distinct pathogen-associated molecular patterns (PAMPs), and thus strongly initiate immune responses upon infection without the need for priming. So far, 12 members of this family have been identified in mammals and all are type I transmembrane proteins that contain hydrophobic LRRs in the extracellular domain that mediate ligand binding.[34,82]

IV.A. Toll-Like Receptor Responses to the Associated Biomolecule layer

Most classical biomaterials do not themselves ligate to TLRs, though exceptions occur. Generally this is a good thing for the biomedical device as

TLR activation can cause very significant cellular activation and subsequent host responses. However, TLR ligands are released upon tissue injury, inflammation, and cell necrosis (see Table 3.3 for list of endogenous TLR ligands). As tissue injury and inflammation are an inevitable consequence of material implantation, there is the potential for materials to sequester such endogenous damage signals.[83] These endogenous damage signals are more formally referred to as alarmins, and together with PAMPs, make up the damage-associated molecular pattern family.[36] As Babensee hypothesized, since the implantation of biomedical materials generally causes some tissue damage, and subsequent cellular necrosis, the biomedical material may trap such alarmins at its surface for subsequent detection by interrogating leukocytes.[83] For example, tissue damage stimulates production of cellular Fn with extra domain A (EDA), which activates TLR4.[84] Fibronectin is known to readily deposit on biomaterials implanted *in vivo*, and therefore it is not unreasonable to suspect that some of the Fn found on biomaterial surfaces *in vivo* contains EDA.

The adsorption of alarmins on biomaterial surfaces may, alternatively, have an anti-inflammatory effect. Lee and colleagues recently showed that various cationic biomaterials can be used to reduce the inflammation and macrophage activation associated with incubating the cells with single stranded RNA (ssRNA), double stranded RNA (dsRNA), or hypomethylated

DNA.[85] Presumably, the anti-inflammatory effect is of these materials is due to sequestering of the alarmins at the biomaterial surface, reducing their concentration and making them less available for binding to the professional phagocytes.

Table 3.3 Endogenous Ligands of the Toll-Like Receptor Activation System

Toll-Like Receptor	Endogenous Ligands
TLR2	Heat-Shock Proteins (HSP60[136], HSP70[137,138]), Necrotic cells[139,140], High-mobility group protein B1 (HMGB1)[141-144], Biglycan[145,146], Hyaluronan[147,148], Serum Amyloid A[149]
TLR3 (expressed intracellularly)	Messenger RNA (mRNA)[150]
TLR4	Heat-Shock Proteins (HSP60[136,151], HSP70[137,138]), Necrotic cells[152], HMGB1[142-144], Extra Domain A of Fibronectin[84] (Fn-EDA), Biglycan[145,146], Hyaluronan[148,153-155], Heparin Sulfate[156], Tenacin-C[157], AGE-low density lipoproteins[158], Surfactant Protein A[159], β-defensin[160], Serum Amyloid A[161], Angiotensin II[162], Fibrinogen[163]
TLR9 (expressed intracellularly)	Nuclear Histone Proteins[164]

IV.B. Direct Toll-Like Receptor Responses to Biomaterials

Contrary to the findings of Lee and colleagues described above,[85] cationic polymers appear to be capable of directly stimulating TLR responses in the absence of alarmins. Chen and colleagues found that several cationic polymers including polyethyleneimine, polylysine, cationic dextran and cationic gelatin were independently capable of stimulating TLR4, resulting in the production of IL-12 and type 1 T helper cell responses to the polymers.[86]

Both Chen and Lee tested and confirmed the respective effectiveness of polylysine; therefore polylysine (and potentially other cationic polymers) appears to be capable of both stimulating TLR responses independently, and inhibiting TLR responses by sequestering TLR ligands.

Cellular activation from TLR ligation to biomaterial surfaces is not limited to cationic materials. Alginate, for example, has long been known to activate monocytes and macrophages[87-89] and it has since been shown that the mannuronic acid component activates both TLR2 and TLR4.[90] Poly(anhydride) nanoparticles have been shown to act as Th1 vaccine adjuvants by interacting with TLRs.[91] The immunogenicity of hydroxyapatite and modified alkane polymers has also been shown to be mediated by TLR4 and dimerized TLR1/TLR2, respectively.[92,93] Furthermore, Shokouhi and colleagues have quite elaborately shown that dendritic cells can sense a wide array of biomedical materials through a general mechanism that involves multiple TLR/MyD88-dependent pathways, specifically TLR2, TLR4, and TLR6.[94] The results of the latter study suggest that the cellular activation was caused by the general hydrophobicity of the polymers, as described by the Seong-Matzinger 'Hyppos Hypothesis,' and therefore we discuss these results in more detail in Section IV.C.

V. SCAVENGER RECEPTOR RECOGNITION OF ADSORBED ENDOGENOUS BIOMOLECULES AND UNOPSONIZED BIOMATERIAL SURFACES

Scavenger receptors were first identified for their ability to bind modified lipoproteins, which contributed to cholesterol deposition in macrophages.[95] Since their discovery, SRs have been found on macrophages, dendritic cells (DCs),[96,97] and some endothelial cell populations.[98-102] A protein receptor is included in the SR family if it has the ability to bind modified low-density lipoprotein, and other polyanionic ligands.[24] There are 8 classes of SRs, denoted as class A through to class H, with each class containing receptors with similarities in their molecular structures.[26] Many of the single receptors in the SR superfamily recognize multiple ligands, and though the chemical nature between ligands varies, each generally has a polyanionic nature . Many of the SRs are vital to host defense against fungal, parasitic, bacterial, or viral infections, as they recognize common microbial components; however, modified host molecules, such as advanced glycation end products (AGE),[103] β-amyloid protein,[104] and oxidized low density lipoprotein,[105] are also recognized by SRs . Therefore, SRs are important to the pathophysiology of diabetes, Alzheimer's disease, and atherosclerosis, respectively. In these conditions, SR binding of the respective ligand at an accumulation site induces macrophage recruitment. The macrophages then use the SRs to take up the materials, but if the deposits are too large, or if the products are

unable to be digested and used by the cells, 'frustrated' phagocytosis occurs, and the macrophages stimulate inflammation.[106,107]

V.A. Class A Scavenger Receptors

The class A SR subfamily is comprised of SR-AI/II, Macrophage receptor with a collagenous structure (MARCO), SR with C-type Lectin (SRCL), and SR-A5 (SCARA5).[108] Since SR-A and SRCL undergo alternative splicing, there are, in total, seven structurally similar members. Each member is a homotrimeric type II transmembrane glycoprotein. While most macrophage populations express SR-A,[109] MARCO is only expressed by macrophages in the spleen marginal zone, the lymph nodes, and the peritoneal cavity.[110] However, MARCO expression can be induced with pathogen stimulation in a TLR-dependent mechanism.[111-114] Like macrophages, dendritic cells also express SR-A, and can be stimulated to express MARCO.[96,97,115,116] Scavenger Receptor with C-type Lectin (SRCL) is not expressed by macrophages at all, and is predominantly found on endothelial cells.[117] Scavenger Receptor A5 (SCARA5) is also not expressed on macrophages, but is found on epithelia.[118]

V.B. Evidence of Class A Scavenger Receptor Ligation to Biomaterials

In 1993, Fraser and colleagues isolated a rat antibody, famously known as 2F8, that blocks cation independent adhesion of macrophage like RAW264 cells to tissue culture plastic when incubated with serum.[81] Subsequent

analysis revealed that the antibody recognized SR-AI/II. In a similar macrophage adhesion assay to frozen tissue sections, the antibody was found to block EDTA-resistant adhesion.[119] Since then, Suzuki and colleagues found that SR-A$^{-/-}$ macrophages take almost twice as long as WT macrophages to adhere to a 'tissue-culture treated' plastic vessel or glass surface and spread their cytoplasm to the same degree.[120] Robbins and Horlick have shown that transfecting HEK 293 cells with SR-A enhances their ability to adhere to polystyrene.[121] Finally, Santiago-Garcia and colleagues demonstrated that SR-A binds to several proteoglycans, which may be important for macrophage binding to engineered materials that mimic the endogenous extracellular matrix. The MARCO receptor may also contribute to the adhesion and spreading of cells, as transient transfection of various cell lines (CHO, HeLa, NIH3T3, and 293) induces dramatic changes to the morphology of the cells on tissue culture dishes, such as the formation of long dendritic processes and large lamellipodia-like structures.[122]

Latex beads and charged environmental particles, such as titanium dioxide, iron oxide, and silica, are taken up in the lung by alveolar macrophages. Kobzik found that this uptake can be blocked by incubating the cells with polyanionic ligands of scavenger receptors, such as polyinosinic acid.[28] Subsequently, the major receptor on lung alveolar macrophages (AM) for these particles was found to be MARCO in both mice,[29] and humans.[30]

On whether or not SR-A contributes to intercellular signaling, the scientific literature is conflicted. On the one hand, SR-A was shown to be exclusively a phagocytic receptor for *Neisseria meningitides*, with the induction of proinflammaotry cytokines depending on TLR4 but not SR-A;[123] SR-A binds DNA and CpG oligonucleotides, but is not necessary for uptake or cytokine induction;[124] and Fucoidan and LTA, known SR-A ligands, are able to activate the signaling pathways in SR-A$^{-/-}$ macrophages through a CD14 mechanism.[125] On the other hand, in mice challenged intraperiteoneally with thioglycollate, lack of SR-A leads to an increase in the secretion of MIP-2 and keratinocyte-derived chemokine, and greater neutrophil recruitment. Peritoneal macrophages from the SR-A$^{-/-}$ mice were also found to secrete more chemokine *in vitro* after exposure to thioglycollate, dependent on particle internalization. This was also seen when the cells were incubated with latex beads coated with AGE-BSA, but not when incubated with soluble AGE-BSA.[126] When alveolar macrophages and Kupffer cells are cultured with *M. tuberculosis* cord factor, both cell types from SRA$^{-/-}$ mice respond with an increased production of TNF-α, and the alveolar macrophages from the knockout animals also secrete more MIP-1α.[127] Lastly, when peritoneal macrophages are stimulated with LPS, or LPS and interferon-γ together, SR-A$^{-/-}$ cells produce more IL-12 than WT cells.[128,129] Furthermore, the anti-

CD204 antibody, 2F8, inhibits IL-12 production in the stimulated WT cells, but stimulates hydrogen peroxide production in unstimulated alveolar and peritoneal macrophages when immobilized to a surface.[129] Taken together, the ligation of SR-A does seem to cause fluctuations cytokine secretion, though the mechanism by which it does this is unknown. Moreover, SR-A ligation seems to draw cells away from Th1 cytokine secretion and towards the production of regulatory/Th2 cytokines.

VI. PATTERN RECOGNITION RECEPTOR RESPONSES TO HYDROPHOBIC PORTIONS OF BIOMATERIALS AND ADSORBED PROTEINS: THE SEONG-MATZINGER HYPPOS HYPOTHESIS

In 2004, Seong and Matzinger described evidence from the literature to support their hypothesis, that pattern-recognition is little more than an ancient detection mechanism for identifying hydrophobic portions (hyppos) of biomolecules.[31] Their theory explains the considerably promiscuous binding properties of the various PRRs, especially TLR2 and TLR4. Like many other PRRs, TLR2 and TLR4 contain leucine-rich regions (LRRs) that bind predominantly through hydrophobic interactions. As a result, they are able to bind a very wide array of different molecules with vastly different structures, including endogenous proteins with exposed hydrophobic domains. For example, TLR4 recognizes β-defensin, surfactant protein A, EDA+Fn, Heat

Shock Protein (HSP) 60, and HSP70, Hyaluronan break-down products, and atherosclerotic plaques in addition to various bacterial and viral products. Though these particular endogenous ligands may be present on a biomaterial in small concentrations, a biomaterial surface is a unique environment in which adsorbed proteins spontaneously unfold. According to the Hyppos-hypothesis, this spontaneous denaturation of endogenous proteins has the potential to at least transiently expose their hyppos, which then become available ligate a cell-surface PRR.

In addition to binding to hyppos of endogenous molecules, there is limited evidence that hydrophobic materials themselves may be detectable by PRRs. Seong and Matzinger, in support of their theory, referred to work by Hunter and colleagues, who showed that multi-block copolymer surfactants with large hydrophobic regions (polypropylene oxide) and relatively small hydrophilic regions (polyethylene oxide) could be used as adjuvants to stimulate antibody production to an antigen.[130-132]

Perhaps the most intriguing consequence of the Hyppos Hypothesis is that it provides an explanation of how leukocytes bind and respond to biomedical polymers in protein-free conditions. For example, Volle and colleagues demonstrated that neutrophils produce a robust respiratory burst in response to polystyrene microparticles in the absence of added protein in the

culture media.[23] Several other groups have shown abundant cellular attachment to various materials in protein-free media or saline with an assortment of cell types. For example, Zhou and colleagues found that L929 fibroblasts adhere equal well to gold surfaces modified with cysteine in the presence or absence of serum.[133] Dewez and colleagues reported that epithelial cells attached and spread on tissue culture polystyrene and primaria polystyrene in protein-free media after only 1.5 hours; though treatment with cycloheximide inhibited such adhesion.[134] Oji and colleagues reported that osteoblasts adhered to commercially pure titanium (cpTi) and Ti6Al4V alloy substrates better in phosphate-buffered saline and protein-free media than in media supplemented with serum.[135] Finally, Fraser and colleagues demonstrated strong macrophage adhesion to tissue culture polystyrene in the protein-free media that was not inhibited with a functional anti-CD204 (Scavenger Receptor A) antibody.[81] Therefore, even in the absence of adsorbed proteins on a surface, many different cellular populations appear to be capable of binding and responding to material surfaces. Given the diversity of the cell types capable of such adhesion, the receptor is possibly different for each cell population; however, each cell type seems to express a PRR that binds non-specifically to material surfaces, permitting cellular attachment.

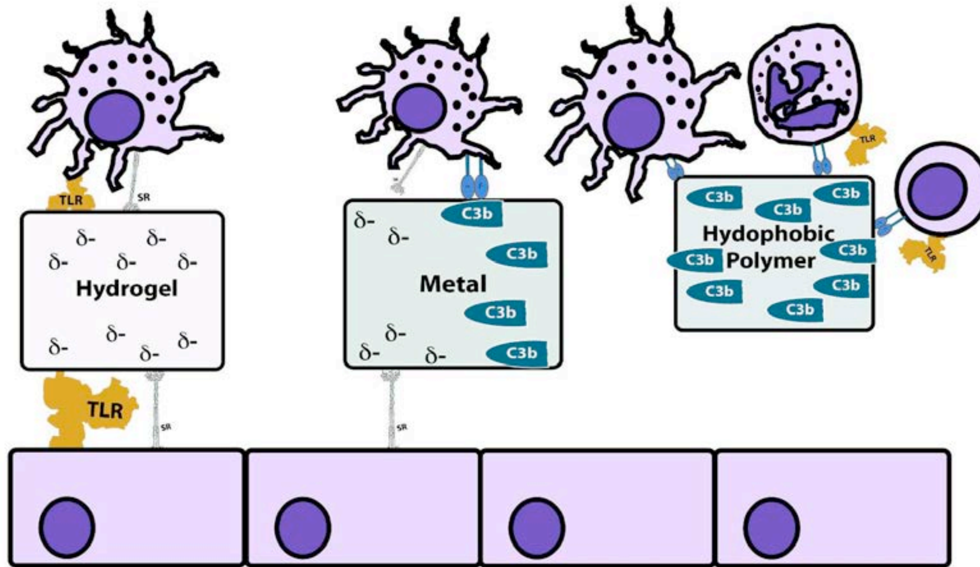

Figure 3.3. A summary of the leukocyte pattern recognition system for detecting foreign materials. Scavenger receptors (SR) and toll-like receptors (TLR) on endothelial cells (bottom) and macrophages (top left) are often capable of identifying polar hydrogels that may not adsorb the proteins with the same propensity as other, more hydrophobic, materials. Such is the case with alginate and other highly charged hydrogels. Similarly, several metal oxides have been found to be recognized by scavenger receptors on endothelial cells and macrophages. Alternatively, metals may adsorb a protein layer and initiate activation of the complement cascade on their surface. Hydrophobic materials readily adsorb a protein layer and initiate the complement cascade when implanted in the body, and are therefore most often recognized by integrin receptors on neutrophils, monocytes, and macrophages.

VII. SUMMARY

In summary, there are three main ways that a biomaterial may be recognized by the leukocyte pattern recognition system (see Figure 3.3). Protein adsorption and integrin ligation of the resulting biomolecule layer is by far the most common and explains the majority of leukocyte responses to biomaterials. TLR responses to components of the adsorbed biomolecule

layer seem to be less common and explain only a small percentage of immune responses to biomaterials. To date, the only cell type that has been specifically tested to produce TLR/MyD88-dependent responses is the dendritic cell; however work involving dendritic cells suggests that TLR-mediated cellular activation of these cells may occur on a far larger number of materials than we originally thought. This may or may not be due to the exposure of 'Hyppos', hydrophobic portions of biomolecules, in the adsorbed biomolecule layer. Some biomaterials, such as polylysine and a number of other cationic polymers, appear to stimulate TLRs directly. Conversely, these polymers appear to be capable of sequestering damage-associated molecular patterns, resulting in the materials exhibiting an anti-inflammatory effect in the presence of TLR-stimulating ligands. Finally, scavenger receptors appear to play a back-up role within the body. For example, when biomaterials are introduced to the airways, where they cannot be coated with plasma proteins, these receptors are critical to the phagocytosis and disposal of various biomaterial particulates. More work is required in the investigation of these receptors in the body; however, they may also play a role in detection of materials that are not coated with an adsorbed biomolecule layer, such as extremely hydrophilic hydrogels.

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CHAPTER 4: CLASS A SCAVENGER RECEPTORS DO NOT MEDIATE THE ACUTE INFLAMMATORY RESPONSE TO POLYSTYRENE OR POLY(LACTIC-GLYCOLIC ACID) PARTICLES IN THE PERITONEAL CAVITY

As I explained in the previous chapter, the binding and uptake of various biomaterials has been shown to be mediated by opsonization-independent, integrin-independent leukocyte binding mechanisms. However, the role of such mechanisms in the detection of common hydrophobic polymers at tissue sites that readily permit plasma protein adsorption has not been ascertained. Therefore, in this study we tested whether or not class A scavenger receptors mediate recognition and subsequent inflammatory responses to polystyrene or poly(lactic-co-glycolic acid) particles in the peritoneal cavity. Since previous research has shown that plasma proteins readily adsorb to the surface of the aforementioned materials, we hypothesized that class A scavenger receptors would not mediate the acute inflammatory response to the particles in the peritoneal cavity. To test this hypothesis, mice deficient for scavenger receptor class A type I/II or macrophage-associated receptor with collagenous domain (MARCO), and wild-type control mice were given an intraperitoneal injection containing one type of particle. The particles were allowed to incubate in the peritoneal cavity for 12, 24 or 48 hours, following which the particles were explanted and a peritoneal lavage was performed. Enzyme-linked immunosorbent assays were used to quantify the concentration of interleukins 1β, 4, 6 or 10. These tests revealed that class A scavenger receptors do not mediate the acute inflammatory response to polystyrene or poly(lactic-glycolic acid). Subsequently, scavenger receptors were targeted for material recognition by pre-adsorbing the polystyrene particles with maleated bovine serum albumin. Polymorphonuclear leukocyte (PMN) binding to uncoated particles was also tested in the presence of competitive SR ligands. These experiments confirmed that class A scavenger receptors do not mediate the acute inflammatory response or PMN binding/phagocytosis to polystyrene microspheres in the peritoneal cavity.

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INTRODUCTION

Pattern recognition mechanisms mediate foreign body identification. Material opsonization is known as the dominant intermediary step in such recognition as it occurs immediately following contact of the material with a biological fluid. Following opsonization, integrin-dependent leukocyte binding often occurs; however, opsonization-independent, integrin-independent leukocyte binding mechanisms to various biomedical materials have been discovered in the past 20 years. Class A scavenger receptors (SR-A) have specifically been implicated in the binding and uptake of several particulate materials such as silica,[1-3] tissue culture polystyrene (TCPS), [4] sulfated polystyrene,[5] titanium,[6] titanium dioxide (TiO_2),[5] and iron(III) oxide (Fe_2O_3). [5] Such material recognition is particularly prevalent in the lung, as polystyrene beads and charged environmental particles have been shown to be taken up by alveolar macrophages utilizing SR-A.[5,7] Beamer and Holian demonstrated that material recognition through SR-AI/II (CD204) specifically leads to chronic host responses, as SR-AI/II null mice do not produce excessive connective tissue following inhalation of silica.[1] Therefore, the binding of SR-A to medical polymers might contribute to the tissue responses to and dictate the lifespan of biomedical materials depending on the properties of the material and the region of the body.

The expression of SR-A is not universal throughout the body. Scavenger

receptor-AI/II is expressed by a wide array of cell types and by virtually all macrophage populations;[8] however, a macrophage receptor with a collagenous structure (MARCO) is only expressed by macrophages, and only in the lung, peritoneal cavity, lymph nodes, and marginal zone of the spleen.[9] *In vitro* experiments have demonstrated that SR-A mediates binding of macrophage-like RAW264 cells to polystyrene tissue culture plates when incubated with serum in the absence of divalent cations,[4] and isolated murine macrophages from SR-AI/II null mice take almost twice as long as WT macrophages to adhere to polystyrene tissue culture plates in complete media.[10] Similarly, HEK 293 cells transfected with CD204 have an enhanced adherence to polystyrene.[11]

In this article, we discuss a series of studies that test whether or not Class A Scavenger Receptors mediate acute inflammatory responses to polystyrene mono-sized microparticles and a poly(lactic-co-glycolic acid) particle emulsion in the peritoneal cavity. The use of particles is critical to this research as it enables delivery to the body through a minimally-invasive injection, so as to not induce local wounding. We opted to deliver these materials to the peritoneal cavity for several reasons. First, injection of an aqueous solution containing particles in the peritoneal cavity will not displace tissue, which might otherwise lead to tissue damage and inflammation. Second, the peritoneal cavity can be lavaged upon material

extraction to evaluate the concentrations of cytokines. Finally, several medical devices (e.g. peritoneal dialysis catheters and pacemaker leads) are necessarily implanted in the peritoneal cavity, and are therefore subject to serous protein adsorption and complement cascade activation. In the lung, these pathways are deficient as the airway epithelia are coated by a surfactant-rich non-serous fluid. Therefore, though airway macrophage cells are known to rely on SR-A to identify particulate materials, elsewhere in the body the role of SR-A in material recognition and cellular activation is not known. The *in vivo* research was followed up with a flow cytometry assay to assess whether PMN binding and uptake of polystyrene microspheres could be reduced with competitive ligands for SR-A. The ligands used for these assays (polyinosinic acid, fucoidan and dextran sulfate) are known ligands for SR-A and have been previously shown to competitively inhibit macrophage binding to polystyrene microspheres.[12]

MATERIALS AND METHODS

Processing/synthesis of PLGA and PS particles

Poly(lactic-co-glycolic acid) particles were synthesized by dissolving 10 mg PLGA (Sigma-Aldrich, Oakville, Canada), in 10 ml ethyl acetate under constant stirring. After the PLGA was completely dissolved, 10 ml of 0.1% poly(vinyl alcohol) (PVA) solution was added to the solvent mixture under constant stirring. Thereafter, 100 ml of distilled water was slowly added and

the solution was subsequently homogenized with an ultrasonicator (Misonix Qsonica S-4000, Newtown, CT, USA). To remove the solvent, the particle solution was left to stir vigorously in a desiccator with continuous vacuum suction. Particle size was determined by dynamic light scattering using a Lexel 95 argon ion laser with a detector angle of 90°, operating at a wavelength of 633 nm and a power of 200 mW. The data was analyzed with a BI-9000AT digital autocorrelator (version 6.1; Brookhaven Instruments, Holtsville, NY). The apparent particle size was found to be 381.3 nm using the CONTIN algorithm. Prior to injection, the particles were autoclaved, centrifuged, and then re-suspended in sterile phosphate buffered saline (PBS).

Polystyrene (PS) microspheres (3 μm) were purchased from Polysciences, Inc. (Warrington, PA, USA). Prior to injection, the microspheres were washed twice with a filter sterilized 0.1 M borate buffer (pH=8.5) and once with sterile PBS (pH=7.4) before being suspended at a final concentration of 10^8 particles/ml in PBS.

Bovine Serum Albumin Maleylation and Adsorption to PS Particles

Bovine Serum Albumin (BSA) was maleylated by slowly adding maleic anhydride (0.1 M) to a BSA (10 mg/ml) solution while maintaining alkalinity with 5 M sodium hydroxide. Polystyrene particles were washed three times

with 0.1 M borate buffer (pH=8.5), and then resuspended in the maleylated BSA solution and left at room temperature overnight with gentle end-to-end mixing.

Mice

Ethics approval for all animal work was obtained from the Animal Research Ethics Board at McMaster University prior to the commencement of the research. Wild-type C57BL/6 mice, three to six weeks old, were ordered from Charles River (Montreal, Quebec) and housed in the Central Animal facility at McMaster University. The mice were allowed to acclimatize to their environment for at least 3 days prior to use. The MARCO$^{-/-}$ and SRA $^{-/-}$ mice were also from the C57BL/6 genetic background, and were provided by the laboratory of Dr. Dawn Bowdish, and bred at the McMaster University Central Animal Facility.

Intraperitoneal Particle Injection and Explantation Procedures

The particle emulsions were delivered to the peritoneal cavity by injection with a 25 gauge needle so as to induce as little wounding as possible. The total injected volume of the emulsions was 0.5ml, which contained approximately 2 x 10^8 particles of PLGA, or 5 x 10^7 particles of PS. The mice were then left for 12, 24 or 48 hours, and then individually sacrificed with carbon dioxide gas. A post-mortem peritoneal lavage and

tissue extraction was immediately performed on each mouse. For the peritoneal lavage, 3 ml of PBS was injected into the peritoneal cavity along with an equivolume of room air. The fluid inside the peritoneal cavity was agitated slightly and then extracted with a plastic transfer pipette by making a small cut in the skin and peritoneum. For the tissue extraction, the peritoneal cavity was opened and the particles were visually identified, and then extracted along with any adhered tissues (usually adipose tissue or peritoneum). The tissues were then fixed in formalin (10%) and sent to the histology lab for processing. Unfortunately, due to the lack of colouring of the PLGA nanoparticles, and potentially the small size, the PLGA particles were not visible in the peritoneal cavity. Therefore, histological analysis was not done on the PLGA injections.

Enzyme-Linked Immunosorbent Assays.

The peritoneal lavage fluid was analyzed by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits from Biolegend (San Diego, CA, USA). Samples were run in duplicate and 100 μl of lavage fluid was used for each well. The assay was performed exactly according to the manufacturer's instructions and a standard curve was performed on every plate.

Blood Collection and Polymorphonuclear (PMN) Leukocyte Separation

Blood was collected using standard clinical laboratory methods. Blood that was to be used for PMN isolation was withdrawn in BD Vacutainer tubes containing heparin; blood that was collected for serum was taken in BD Vacutainer tubes coated with silica. Following blood collection, the serum tubes were stored at room temperature for 1 hour, and then spun at 1300 rcf. The isolation of PMNs was performed as described by Clark and Nauseef.[13] Briefly, the heparinized whole blood was subjected to dextran sedimentation by mixing with cold 3% dextran in saline. After storage for 30 minutes at 4°C, the top leukocyte-plasma layer was removed by pipetting. The PMN-RBC pellet at the bottom of the tube was then cleared of RBCs by hypotonic lysis using ice-cold 0.2% NaCl for 30 seconds followed by resolution of isotonicity by adding an equal volume of ice-cold 1.6% NaCl. The cell suspension was then centrifuged and the RBC lysis was repeated. Subsequently, the PMNs were separately cultured in RPMI media with no supplements.

Flow Cytometry Assessment of PMN binding to Polystyrene Particles

The PMN suspension ($1x10^6$ cells/100 µl) was cultured in 1.5 ml eppendorf tubes containing nanoparticles such that the final nanoparticle to cell ratio was 10:1. Immediately following culture, the tubes were placed in

an incubator with a rotating platform. After 15 hours, the cells were agitated by pipetting and flow cytometry was immediately performed.

Statistics

The intraperitoneal cytokine concentrations were submitted to a two-way ANOVA using IBM SPSS software. The independent variables were defined as the presence/absence of the functional SR-AI/II gene (mouse type), and the time of explantation/lavage (time). Statistically significant main effects were assessed by the Tukey HSD (Honestly Significant Difference) method of post-hoc analysis.

RESULTS

Histological Analysis of Uncoated Particles

The histology slides from the PS injections showed no difference between WT and KO mice in the acute inflammation that followed the injection of PS microspheres (Figure 4.1). Significant inflammation was evident by 12 hours and peaked by 24 hours post-injection in all conditions.

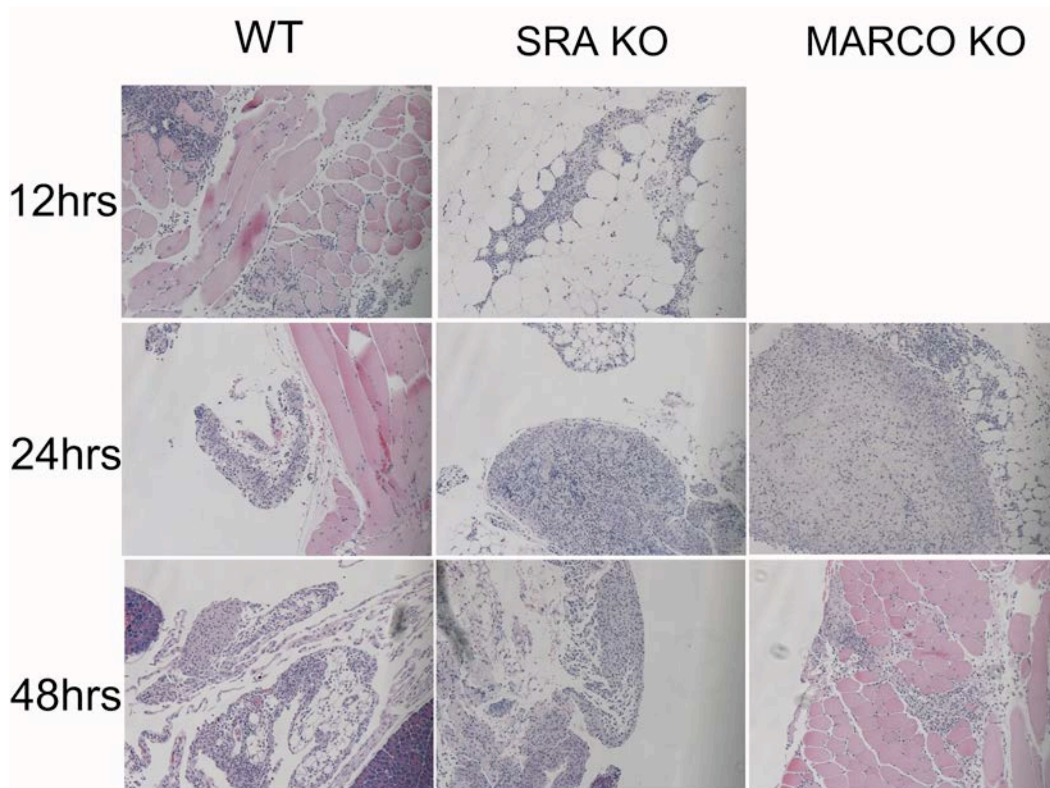

Figure 4.1. Leukocyte inflammation 12 hours, 24 hours, and 48 hours after PS particle injection in WT, SR-AI/II knock-out, and MARCO knock-out mice (H&E staining, 100x magnification).

Following injection of the malBSA-coated beads, histological assessment of the extracted tissue showed that the acute inflammatory responses to the malBSA-coated beads (Figure 4.2), and to the uncoated beads, were similar in all mice (SR-AI/II$^{-/-}$ and WT).

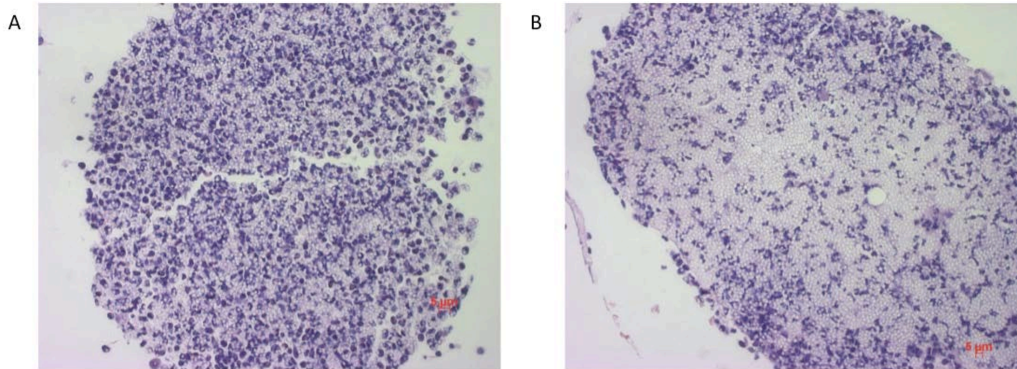

Figure 4.2. Leukocyte inflammation 12 hours after injection of malBSA-coated PS microspheres in SR-A$^{-/-}$ (A) and WT (B) mice (H&E Stain; 100x magnification).

Cytokine Concentration in the Peritoneal Lavage Fluid

Generally, the cytokine data for the PS injections showed elevated IL-1β, IL-4 and IL-10 in the WT mice compared to all KO mice, and the SRA KO mice showed elevated IL-6 compared to the WT and other KO models (Figure 4.3). A significant main effect of time (time of explantation) was found for IL-1β [$F(2,24) = 4.235$, $p = 0.033$], and post-hoc analysis showed that there was significantly more IL-1β in the peritoneal cavity at 12 hours post-injection compared to 48 hours post-injection ($p=0.037$). For IL-6, significant main effects of time [$F(2,24) = 9.872$, $p = 0.002$], animal type [$F(2,24) = 12.607$, $p = 0.001$], and an interaction effect between the independent variables was found [$F(3,24) = 7.032$, $p = 0.003$]. Post-hoc analysis showed that there was significantly more IL-6 recovered from the lavage of the SR-A null mice, than that from the WT ($p=0.001$) or MARCO null mice ($p=0.003$). Furthermore, there was significantly less IL-6 in the peritoneal lavage at 48 hours post-

injection than there was at 12 hours ($p=0.009$) or 24 hours ($p=0.001$) post-injection.

The cytokine data from the mice that received PLGA injections showed more similarity between the WT and SR-A KO mice, especially for IL-1β, IL-4, and IL-10 (Figure 4.3). Again, significant main effects of time [$F(2,24) = 59.056$, $p < 0.001$], animal type [$F(2,24) = 31.944$, $p < 0.001$], and a significant interaction between independent variables [$F(3,24) = 30.872$, $p < 0.001$] was found for IL-6. Generally, the SR-A KO mice that were sacrificed at 12-hours post-injection responded to the PLGA nanospheres with strong IL-6 production, though the mice sacrificed at 24 and 48 hours post-injection did not have as great a concentration of IL-6 in the peritoneal lavage. Indeed, post-hoc analysis revealed that there was significantly more IL-6 in the peritoneal cavity at 12 hours post-injection than there was at 24 hours ($p<0.001$) or 48 hours ($p<0.001$) post-injection, and significantly more IL-6 found in the peritoneal lavage of SR-A null mice than in WT ($p<0.001$) or MARCO null mice ($p<0.001$).

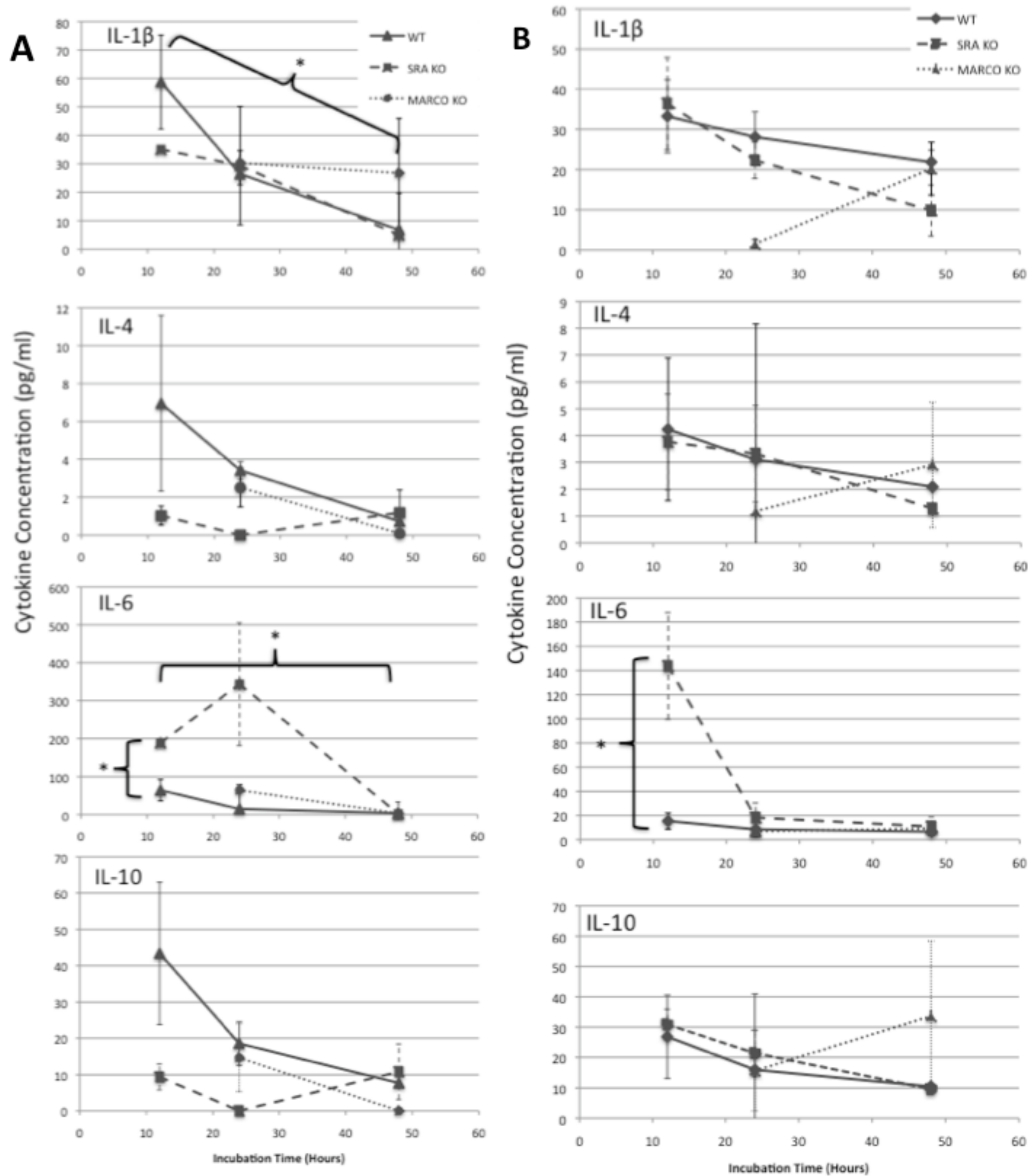

Figure 4.3. Cytokine concentrations in peritoneal lavage fluid at each explantation time-point. (A) Cytokine responses following injection of PS microspheres. (B) Cytokine responses following injection of PLGA nanoparticles. Explantation time-points were 12, 24 and 48-hours. Data points indicate mean ± SD (n = 3 animals per condition for each explantation time-point).

Following injection of the malBSA-coated particles, the cytokine data from the peritoneal lavage showed that the WT mice had reduced production of IL-1β, IL-4, and IL-10, compared to the SR-A KO mice (Figure 4.4).

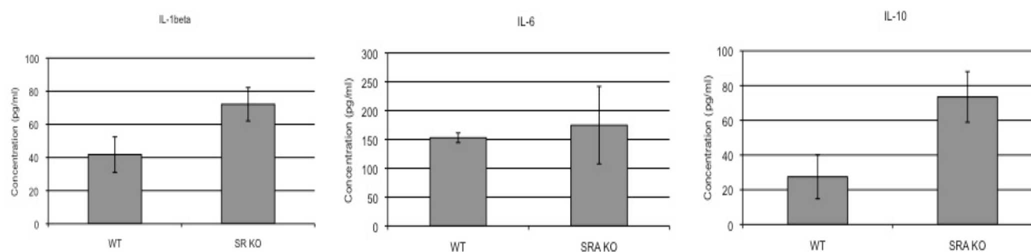

Figure 4.4. Cytokine concentrations in the peritoneal lavage fluid obtained 12 hours after injection of malBSA-coated PS microspheres in SR-A$^{-/-}$ and WT mice.

Flow Cytometry Assessment of PMN Binding to Polystyrene Particles

Polymorphonuclear (PMN) leukocyte binding to PS microparticles was not affected by SR-A ligands (Figure 4.5), which indicates that SR-A is not critically involved in the binding or uptake of PS by PMNs. This finding is not surprising considering the number of integrin ligands present in serum, which are known to readily adsorb to hydrophobic polymer surfaces such as PS.

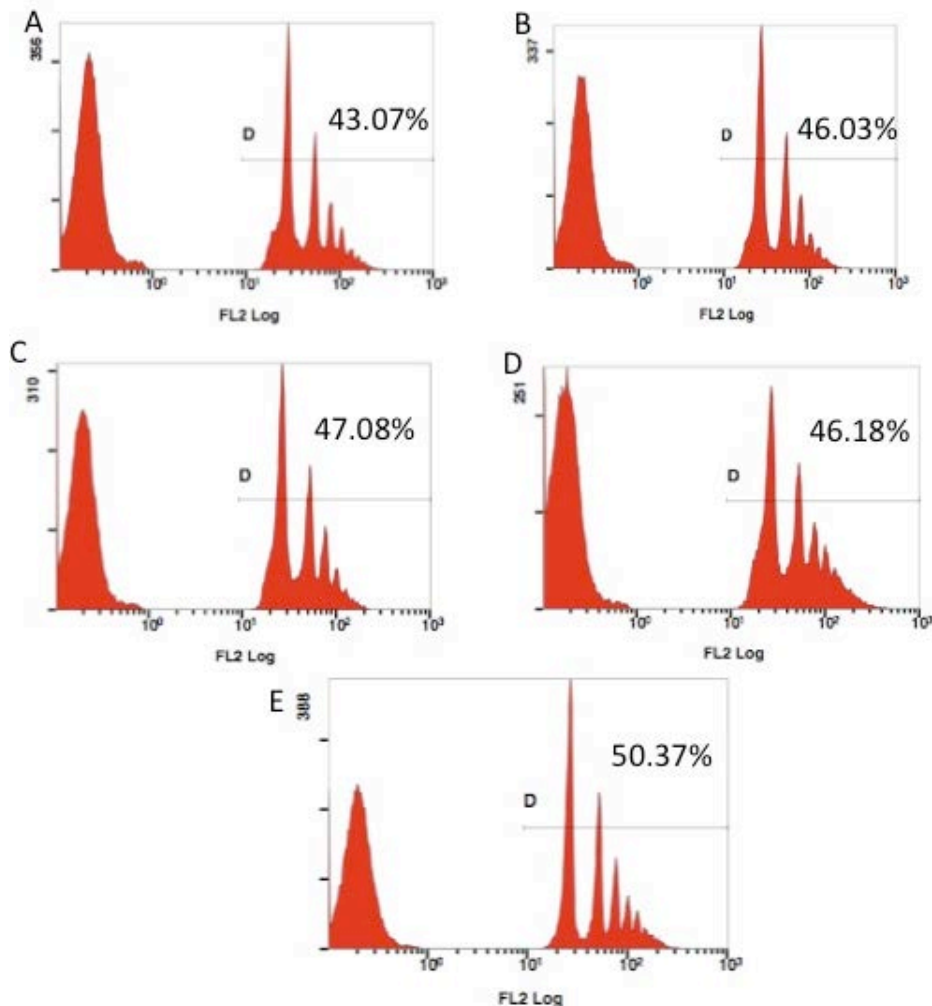

Figure 4.5. SR-A ligands do not inhibit PMN binding and phagocytosis of PS microspheres. (A) Control, (B) Polyinosinic-polycytidylic acid, (C) Polyinosinic acid, (D) Dextran sulfate, (E) Fucoidan.

DISCUSSION

In this study we observed equivalent inflammation between knockout and WT mice between mouse models for both types of particles; however, the cytokine response profiles were notably different between the conditions. The variability in cytokine concentrations between mouse models

indicates that though SR-A does not seem to participate in initiation and perpetuation of the inflammatory response, it may participate to some degree in material recognition and intra/intercellular signaling. There are, after all, a number of endogenous SR ligands that are present in the body and are likely found adsorbed on material surfaces in low concentrations such as modified low density lipoproteins and serum proteins with glycation end products. In the lung, the fibrotic response to silica exposure has been shown to be deficient in SR-A$^{-/-}$ mice even though the inflammatory response is normal or slightly elevated in those mice compared to WT control animals.[1] Long-term studies comparing SR KO mice models to WT animals for their ability to develop a fibrotic response to commonly used biomaterials in the peritoneal cavity is necessary. Injection of material emulsions is a particularly neat method of material delivery as wound induction is minimal.

Soluble polyanionic SR-A ligands were not capable of inhibiting PMN binding to PS beads, which indicates that SR-A is not a necessary mediator of PMN binding and phagocytosis of PS particles. Therefore, SR-A might be participating in the responses to PS beads, but likely only in a signaling transduction capacity.

The inability of malBSA to inhibit responses in SR-A$^{-/-}$ mice suggests that preadsorbing proteins to biomaterials, in an effort to target a specific leukocyte recognition pathway, is ineffective due to the protein turnover on a

biomaterial surface, and also due to the opsonization pathways that are present in the body. Deducing the meaning of these results is limited, however, as multiple SRs are capable of ligating malBSA. In other words, though SR-A is the primary receptor for malBSA, there are other receptors that may have contributed to the recognition of the beads.

CONCLUSIONS

Taken together, these results indicate that scavenger receptor A alone does not mediate the acute inflammatory response to PS or PLGA particles in the peritoneal cavity. However, our experiments were not long enough to evaluate the role of SR-A, if there is one, in the downstream foreign body response to the particles. Notably, pre-coating the PS microspheres in malBSA was not capable of inhibiting the inflammatory response to the PS microspheres.

ACKNOWLEDGMENTS

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CHAPTER 5: SCAVENGER RECEPTOR A MEDIATES LEUKOCYTE BINDING OF POLYANIONIC POLYMER HYDROGELS BUT NOT THEIR UNCHARGED COUNTERPARTS

There is an increasing body of evidence showing that cellular adhesion to and recognition of various biomaterials is mediated by opsonization-independent, integrin-independent pattern-recognition mechanisms. In this study, we investigated the role of scavenger receptor A (SR-A) in leukocyte binding to a series of novel polyanionic and uncharged hydrogels on a poly(N-isopropylacrylamide) backbone. Our hypothesis for this research was that SR-A would mediate the binding of polyanionic polymer hydrogels, but not their uncharged counterparts. To test this hypothesis, the hydrogels were injected in the peritoneal cavity of SR-A knockout (KO) and wild-type mice using a minimally invasive procedure and allowed to set *in situ*. After 24 hours, the hydrogels were recovered and analyzed, and the peritoneal cavity was lavaged for determination of cytokine concentrations. The polyanionic hydrogels retrieved from the knockout animals were found to be completely devoid of adhered leukocytes, which were present in other materials regardless of the mouse type in which they were injected. Subsequently, an i*n vitro* cellular adhesion study with a RAW264.7 cell line revealed that an unidentified serum component mediated the interaction between SR-A and the polyanionic hydrogel. Taken together, the results of this study show that SR-A is critical to leukocyte adhesion on a polyanionic hydrogel both *in vitro* and *in vivo*.

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INTRODUCTION

There has been evidence accumulating over the past several years that cellular adhesion to and recognition of various biomaterials is at least partially mediated by an opsonization-independent, integrin-independent pattern-recognition mechanism. Class A Scavenger Receptors (SR) have been implicated in this process for their ability to bind tissue culture polystyrene(TCPS) [1], sulfated polystyrene [2], silica [3-5], titanium [6], titanium dioxide (TiO_2) [2], and iron(III) oxide (Fe_2O_3) [2]. Since the aforementioned research was either performed *in vivo* or utilized heat-treated serum, it is reasonable to assume that the SRs were actually bound to an adsorbed biomolecular layer. Several known endogenous Class A SR ligands exist, including modified low-density lipoproteins [7, 8] and proteins with advanced glycation end products [9], which may adsorb to biomaterial surfaces and mediate cellular adhesion through ligation of SRs (see [10] for a review on Scavenger Receptor binding to host-derived ligands). However, since SRs bind a wide variety of biomolecules with diverse structures, there is an intriguing possibility that SRs have the ability to directly bind to a biomaterial surface.

Class A SRs are expressed by a number of different cell types including macrophages [11-13], dendritic cells [14-17] and endothelia [18]. The archetypal Class A SR, SR-AI/II, is known to preferentially bind polyanions

such as polyinosinic acid, fucoidan, dextran sulphate, and maleylated BSA, though charge density and molecular structure seem to contribute to binding as not all polyanions are SR-A ligands (e.g. chondroitan sulphate) [19, 20] (reviewed in [21]). As a result, we studied the inflammatory responses to novel polyanionic and neutrally-charged hydrogels in SR-A null (SR-A$^{-/-}$) and wild-type (WT) animals. The materials used for these experiments were poly(N-isopropylacrylamide) (PNIPAm) with hydrazone cross-linking provided by aldehyde-functionalized carboxymethylcellulose (CMC), and the neutrally charged hydrogels were similar, but with aldehyde-functionalized dextran cross-linking [22]. A major advantage of these materials is that they can be charge-modulated by varying the dextran content and CMC content. That is, the charge of the CMC-PNIPAm can be reduced by adding dextran and reducing the CMC content of the material prior to extrusion. This is critical to studying SR-A interactions since ligand binding is known to rely on ionic interactions [20], with charge distribution and ligand structure being important factors to binding specificity. Furthermore, the hydrogel is cross-linked *in situ* by extruding through a double barrel syringe upon injection, and holds its shape when extracted at room temperature unlike other PNIPAm-based hydrogels. This allows for *in vivo* testing with minimally invasive material delivery.

There is evidence in the research literature that CMC might ligate to SR-A, as Rice and colleagues reported that CMC partially inhibited binding of U937 cells with an immobilized acetylated low density lipoprotein (AcLDL) [23]. However, binding of SR-A directly to polyanionic biomaterial surfaces has not yet been shown, and is unlikely to play a major role in cell adhesion to classical hydrophobic polymers such as polyesters, polyurethanes, polystyrenes, and silicones, due to the rapidity of protein adsorption that encapsulates such materials. Since hydrogels are hydrophilic by nature, and adsorb relatively little protein as a result, much of the surface is exposed to leukocyte interrogation, which may permit direct cellular binding to the material surface. Therefore, we followed up the *in vivo* experiments with an investigation into the nature by which a macrophage-like cell line, RAW 264.7 cells, binds to the charged/uncharged hydrogel surfaces. Taken together, our results show that SR-A is critical to leukocyte binding onto a novel polyanionic hydrogel, CMC-PNIPAm, with an unknown serum component mediating the interaction between receptor and polymer.

MATERIALS AND METHODS

Materials

N-Isopropylacrylamide (NIPAM, 99%), acrylic acid (AA, 99%), thioglycolic acid (MAA; ≥98.0%), adipic acid dihydrazide (ADH, 98%), N′-

ethyl-N-(3-dimethylaminopropyl)-carbodiimide (EDC, commercial grade), carboxymethyl cellulose (CMC; MW 700000, DS = 0.9), dextran from *Leuconstroc* spp (Dex; Mr, 500000), sodium periodate (>99.8%), ethylene glycol (99.8%), bupivacaine hydrochloride (99%), silver (I) oxide (≥99.9%) were all purchased from Sigma-Aldrich (Oakville, Ontario). 2,2-Azobisisobutyric acid dimethyl ester (AIBME) was purchased from Wako. All water used in material preparation was of Milli-Q grade.

Synthesis and Characterization of Poly(NIPAM-co-ADH)

Copolymers of NIPAM and acrylic acid were prepared via free radical chain transfer polymerization. N-Isopropylacrylamide (4 g, 35 mmol), AA (0.952 g, 13 mmol), AIBME (0.056 g, 0.243 mmol), and thioglycolic acid (80 μL, 1.15 mmol) were dissolved in 20 mL of anhydrous ethanol at 56 °C. The reaction was allowed to proceed overnight at 56 °C under a nitrogen atmosphere. Solvent was then removed under reduced pressure at 50 °C. The resulting viscous product was redissolved in 200 mL of diH_2O and underwent exhaustive dialysis in a 12−14 kDa MWCO membrane. The purified polymer solution was lyophilized to dryness, yielding a white powder. The acid content of the polymer was determined using conductometric titration (ManTech Associates). Polymer molecular weight was determined with aqueous gel permeation chromatography (Agilent), using a pH 7.4 sodium acetate buffer as the eluent and polyethylene oxide standards for calibration

(Varian). Carboxylic acid groups from AA residues were then given hydrazide functionality by adding ADH (10.15 g, 58.26 mmol) to a 0.5 %(wt/vol) solution of 20 wt % poly(NIPAM-co-AA). The pH of this solution was then adjusted to 4.75 with HCl. EDC (5.58 g, 35.94 mmol) was then added to this solution and the pH was maintained at 4.75 by dropwise addition HCl for about 5 h until no further change in pH was observed. The reaction was left to stir overnight, at which point the pH of the solution was raised to 7.00 with NaOH. The resulting solution was exhaustively dialyzed using a 12−14 kDa MWCO membrane, after which the polymer solution was lyophilized to dryness, yielding a white powder as the final product. The degree of functionalization was determined by a conductometric titration by assaying the number of unreacted acid groups. 1H NMR in DMSO-D_6: 0.24H, δ =10−10.5 ppm; 0.12H, δ = 8.5−9.0 ppm; 1.12H, δ = 6.65−7.88 ppm; 1.6H, δ = 3.0−3.7; 9.72H, δ = 0.0−2.5 ppm.

Synthesis and Characterization of Aldehyde-Functionalized Polysaccharides.

The oxidation of polysaccharides, including CMC and dextran, to form aldehyde groups was carried out using sodium periodate as the oxidizing agent (Figure 5.1). Sodium periodate (0.8 g, 3.74 mmol) was added to 150 mL of diH_2O containing 1.5 g of polysaccharide and the reaction proceeded with stirring for 2 h at room temperature. To stop the reaction, 0.4 mL of ethylene

glycol was then added. The reaction mixture was then allowed to stir for an additional hour. The reaction solution was purified by exhaustive dialysis for 3 days using a 3500 MWCO membrane, and the purified oxidized polysaccharide was lyophilized, yielding a white final product.

Quantification of aldehyde functionality

The degree of aldehyde functionalization of oxidized carboxymethyl cellulose (oxCMC) and dextran (oxDex) was determined using an in-house silver oxide aldehyde detection assay. 0.1 g of oxDex were added to 10 mL of deionized water in a 20 mL glass scintillation vial along with excess sodium hydroxide. 0.4 g of silver (I) oxide were then added to the solution which was then left to stir overnight, leading to the quantitative precipitation of elemental silver. 5 mL of the reaction solution were then added to 45 mL of deionized water, and the resulting solution was titrated at room temperature with 0.1 N NaOH. Based on this assay, approximately 45 ± 3 % of dextran residues underwent oxidative cleavage of vicinal diol groups, yielding approximately 2400 aldehyde groups per 500 kDa chain of dextran. A similar assay was performed to quantify the number of oxidized groups on CMC; however, since oxCMC possesses carboxylic acid groups prior to silver oxide-mediated aldehyde oxidation, the titration results of oxCMC that did not undergo silver oxide oxidation were subtracted from the results of oxCMC that did undergo silver oxide oxidation, yielding 13 ± 3 % oxidation of

CMC residues at the vicinal diol moieties or approximately 324 aldehyde groups per 700 kDa chain.

Hydrogel Preparation

Hydrogels were fabricated by mixing solutions of oxidized polysaccharides with solutions of poly(NIPAM-co-ADH). The polymeric mass concentrations of each solution were held constant in each gel. Gels were made by dissolving 0.06 g of each reactive polymer in 1 mL of 0.15 mM NaCl solutions and subsequently coextruding each precursor solution through a double barrel syringe [in which poly(NIPAM-co-ADH) and oxidized polysaccharides were loaded into separate barrels (Figure 5.1)] into a 0.23 cm^3 cylindrical silicone rubber mold. In cases where mixtures of aldehyde-functionalized polymers were used, the overall mass concentration was maintained constant (6 wt% total polymer). The percentages indicated in the hydrogel codes in Table 5.1 (xCMC/yDex) in the results represent the mass percentages of each carbohydrate loaded into the syringe to make a 6 wt% total carbohydrate solution.

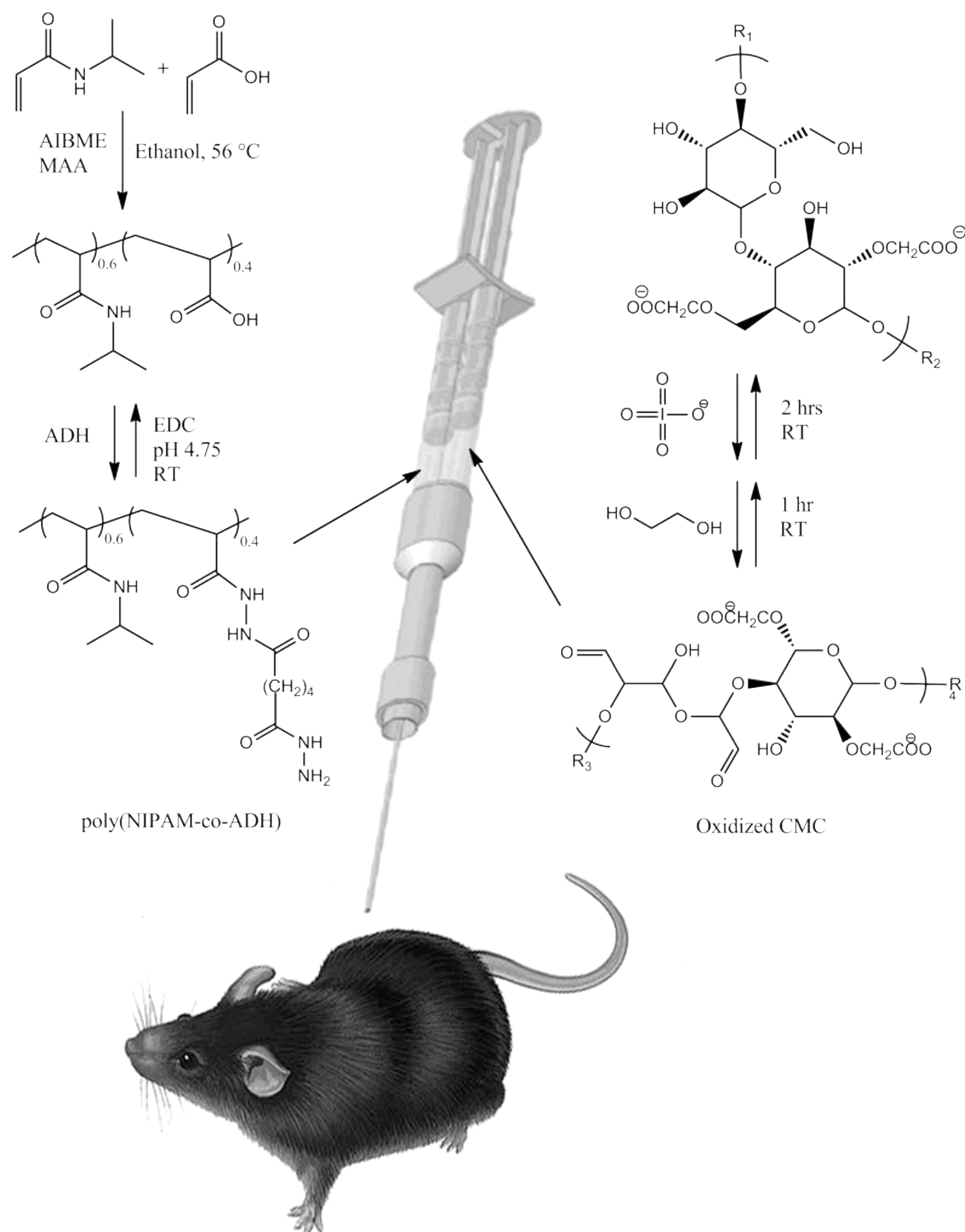

Figure 5.1. Poly(NIPAM-co-ADH) and oxidized CMC/Dextran were preloaded into separate barrels of a dual barrel syringe. Upon intraperitoneal injection of the material, the separate components were co-extruded as the mouse was anesthetized with general anesthetic (isofluorane). The material was given three minutes to cross-link in the peritoneal cavity until the anesthetic was discontinued. This procedure permitted implantation of the material without minimal wounding or tissue displacement.

Table 5.1. Weight percent content of polymeric precursor solutions composed of poly(NIPAM) and oxidized polysaccharides used to fabricate hydrogels.

Hydrogel	Poly(NIPAM-co-ADH) (g/mL)	oxCMC (g/mL)	oxDex (g/mL)
100CMC	0.06	0.06	0
50CMC/50Dex	0.06	0.03	0.03
100Dex	0.06	0	0.06

Hydrogel characterization experiments were prepared by introducing aqueous solutions of complimentary polymers into different barrels of a double-barrel syringe. The mass content of these polymer solutions, containing either poly(NIPAM-co-ADH) or oxidized polysaccharide, was held constant in each gel. Specifically, gels were made by dissolving 0.06 g of the reactive precursors in 1 mL of 0.15 mM NaCl, and complimentary polymer solutions were coextruded through a double-barrel syringe into a 0.23 cm^3 cylindrical silicon rubber mold. When the oxidized polysaccharide solutions were composed of a mixture of both oxDex and oxCMC, the total mass concentration was maintained constant (6 wt% polymer in total) (Table 5.2).

Table 5.2. Plateau modulus and cross-link density of poly(NIPAM)/CMC/Dex hydrogels.

Hydrogel	Plateau modulus (Pa)	Cross-link Density (10^{-24} m^{-3})
100CMC	167 ± 20	0.17 ± 0.02
50CMC/50Dex	1485 ± 27	1.57 ± 0.03
100Dex	2351 ± 30	2.48 ± 0.03

Hydrogel Rheology

The mechanical properties of these hydrogels were measured using oscillatory rheology. The determination of gel storage moduli (G') were made with an ARES rheometer (Texas Instruments) under parallel-plate geometry (7 mm diameter, 1 mm spacing). Gels were first made using a cylindrical mold and were subsequently incubated at 37 °C until reaching an equilibrium swelling/de-swelling state. Gels were then taken from their incubation chamber and immediately underwent oscillatory rheology. The hydrogel linear visco-elastic ranges were determined by performing a strain sweep between 1-100% at 1 Hz. A frequency sweep was then performed from 1-100 rad/s using a strain within the linear visco-elastic range of the hydrogels. A strain was selected from within this linear range and set as a constant to perform a frequency sweep from 1 to 100 rad/s to measure the complex viscosity, elastic modulus, and loss modulus. The cross-link density of the

hydrogel networks tested was estimated based on rubber elasticity theory (see equation below).

$$\rho = \frac{G'_p N_A}{RT} \Big/ V_G$$

where G'_p is the plateau modulus (taken as the maximum gel storage modulus), R is the gas constant, T is temperature in Kelvin, N_A is the Avogadro number, and V_G is the gel volume, which was taken to be 0.23 cm^3 in all cases.

Mice

Ethics approval for all animal work was obtained from the Animal Research Ethics Board at McMaster University prior to the commencement of the research. Wild-type C57BL/6 mice (six per condition), three to six weeks old, were ordered from Charles River (Montréal, Québec) and housed in the Central Animal facility at McMaster University. The mice were allowed to acclimatize to their environment for at least 3 days prior to use. The SRA $^{-/-}$ mice were also from the C57BL/6 genetic background, and were provided by the laboratory of Dr. Dawn Bowdish, and bred at the McMaster University Central Animal Facility.

Intraperitoneal Hydrogel Injection and Explantation Procedures

Prior to injection of the polymer hydrogels, the mice were immobilized with a general anesthetic (isoflurane) so that the hydrogels would have time to set once injected. The injection site was then sterilized with 70% alcohol, and the injection was given. Following injection, the mice were kept under anesthetic for 3 minutes, and awakened promptly thereafter. After 24 hours, the mice were euthanized with carbon dioxide gas and immediately injected with 3 ml of saline in the peritoneal cavity. A small incision in the skin was then made, followed by a small incision in the muscle layer. The hole created was held open with forceps and the fluid was collected and immediately refrigerated. After collecting the peritoneal fluid, the incision in the peritoneum was extended, and the hydrogel was removed and fixed with 10% neutrally-buffered formalin.

Enzyme-Linked Immunosorbent Assays

The peritoneal lavage fluid was analyzed by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits from Biolegend (San Diego, CA, USA). Samples were run in duplicate and 100μl of lavage fluid was used for each well. The assay was performed exactly according to the manufacturer's instructions and a standard curve was performed on every plate.

Cellular Adhesion Studies

Investigation of cellular adhesion to the polymeric hydrogels was performed using the methods of Fraser and colleagues with the exception that cells were pre-stained with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) instead of post-experimental staining with Giemsa [1]. A 48-well plate was coated with the Dextran-PNIPAm and CMC-PNIPAm polymers and given up to an hour to set. The wells were then filled with sterile phosphate buffered saline (PBS) so that the hydrogels could swell to their maximal volume. The macrophage-like cell line, RAW264.7, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (Life Technologies, Burlington, Ontario, Canada). The cells were collected from culture flasks by cell scraping and stained with CFSE immediately prior to use by the methods outlined by Quah and Parish [24]. After the final wash step, the cells were suspended in PiCMg buffer (PBS supplemented with 5mM dextrose, 0.6mM calcium chloride, 1.0 mM magnesium chloride). The cells were added to the individual wells at a concentration of $1x10^6$ cells/well. The wells were then supplemented with serum and/or PiCMg buffer until a total volume of 200µl was reached. Anti-CD204 inhibition of adhesion was evaluated in both serum-free (SF) and serum-enriched (SE) culture environments. Polyanionic inhibition of adhesion was evaluated only in SE culture environments

(inhibitor concentration = 100 µg/ml). The plate was then covered and placed in a refrigerator (4°C) for 30 minutes to permit binding of anti-CD204 antibody to SR-AI/II, and then subsequently moved to an incubator (37°C) for 1.5 hours to permit cellular adhesion to the material surfaces. Following incubation, the wells were washed three times with PBS and each time the supernatant was removed by vacuum suctioning. Cellular adhesion to the dextran and CMC hydrogels was evaluated by measuring the fluorescence emission of the cells in the wells following the three wash steps.

Statistics

The intraperitoneal cytokine concentrations were submitted to a two-way ANOVA using IBM SPSS software. The independent variables were defined as the presence/absence of the functional SR-AI/II gene (mouse type), and the type of material injected into the peritoneal cavity.

RESULTS

Hydrogel Rheology

The elastic moduli of the three hydrogels, determined from a 1-100 rad/s frequency under constant strain, are shown in the supporting information (Figure S1). The plateau moduli of 100 Dextran gels are shown to be an order of magnitude larger than 100 CMC gels, with the 50/50

CMC/Dextran hydrogel showing an intermediate plateau elastic modulus. These results are consistent with previous findings where higher charge content within a gel affords a greater degree of water within the hydrogel matrix, making it less elastic. Calculations of gel cross-link density are shown in Table 2. These calculations demonstrate that 100 Dextran hydrogels possess an order of magnitude greater cross-link density compared with 100 CMC hydrogels. Previous work has demonstrated that there exists a strong correlation between a gel's mechanical properties and its influence in directing cell behavior [25]. In order to account for this, the current work was extended to include a 100 Dextran hydrogel possessing similar mechanical properties to those of the reported 100 CMC hydrogels (See Supporting Information, Figures 5.S1 and 5.S2).

Histological Analysis

An assessment of the histology slides revealed that the dextran and hybrid hydrogels contained a cell layer on their surface following implantation in both WT and SR-A$^{-/-}$ mice. The encapsulating cell layer on the hydrogels appeared to be mostly composed of polymorphonuclear (PMN) leukocytes. Migration of PMNs into the interior of the dextran and hybrid hydrogels was also evident. Similarly, the CMC hydrogels implanted in the WT mice had a distinctive cell layer on the surface of the material with some PMN migration into the interior; however, the CMC hydrogels recovered from

the SR-A$^{-/-}$ animals contained no cells adhered to the outer surface or migrated into the interior of the disc (Figure 5.2).

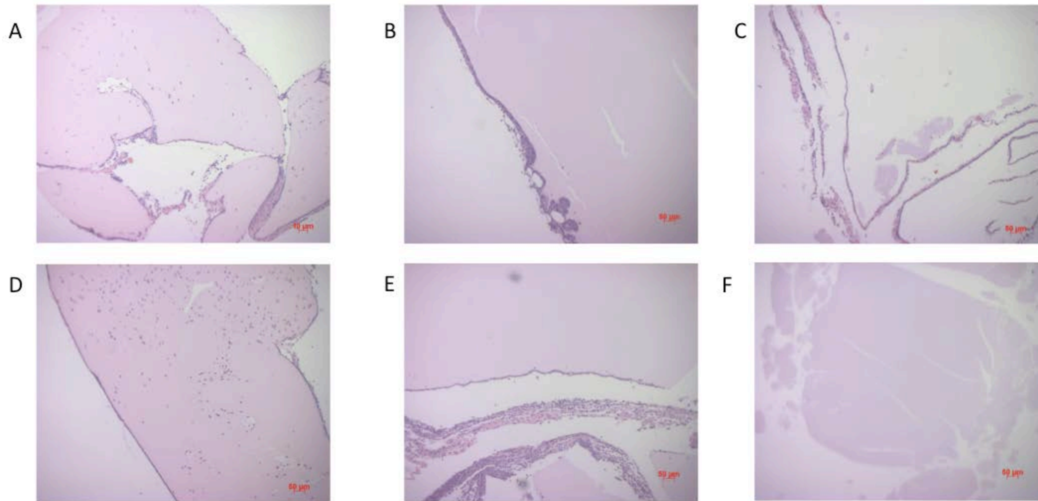

Figure 5.2. Histological analysis of the materials following explantation from the WT mice revealed the existence of a cell layer on the dextran (A), hybrid (B) and CMC (C) hydrogels with some cellular migration towards the interior of the gel. The materials recovered from the SR-A$^{-/-}$ mice showed a similar cell layer on the dextran (D) and hybrid (E) hydrogels; however, the CMC hydrogel (F) did not contain a cell layer (n = 6/condition, figures are representative images of the histology slides created from each sample).

Cytokine Concentration in the Peritoneal Lavage Fluid

Enzyme-linked immunosorbent assays were performed for IL-1β, IL-4, IL-6, and IL-10 (Figure 5.3). Overall, the cytokine data was found to be highly variable with only modest levels of IL-1β, IL-4, and IL-10. Statistical analysis revealed no main effects between conditions (material type or mouse type) and no interaction between independent variables with respect

to the IL-1β, IL-4 and IL-10 concentrations detected in the peritoneal lavage. In contrast, the IL-6 concentration in the peritoneal cavity of all the mice was high, though a main effect of mouse type (SR-$A^{-/-}$ vs. WT) was observed, which indicates that the SR-$A^{-/-}$ mice produced significantly more IL-6 when responding to the hydrogel injections [$F(1,36) = 5.860$, $p = 0.021$]. To confirm that the high IL-6 concentrations were not caused by free (non-cross-linked) PNIPAm that may have partially migrated away from the injection site prior to cross-linking, we injected the non-cross-linked PNIPAm hydrogel backbone alone into the peritoneal cavity of 6 mice (3 SR-AI/$II^{-/-}$, 3 WT). After 24 hours, the mice were sacrificed and a peritoneal lavage was performed and subsequently assayed for IL-6. Only low/moderate levels of IL-6 were found from this experiment, which indicates that the PNIPAm was not responsible for the elevated IL-6 concentrations observed following injection of the hydrogels (See Supporting Information, Figure 5.S3).

Cellular Adhesion Studies

The CMC gels were generally more cell adhesive than the dextran hydrogels. Furthermore, the presence of serum was found to improve the adhesion of cells to the CMC hydrogels. The anti-CD204 mAb reduced the cell adhesion to the CMC hydrogels in the SE condition, but not in the SF condition. Similarly, polyI, polyI:C, $DxSO_4$, and fucoidan were found to inhibit

cellular adhesion to the CMC hydrogels, whereas only $DxSO_4$ inhibited adhesion to the dextran hydrogels (Figure 5.4).

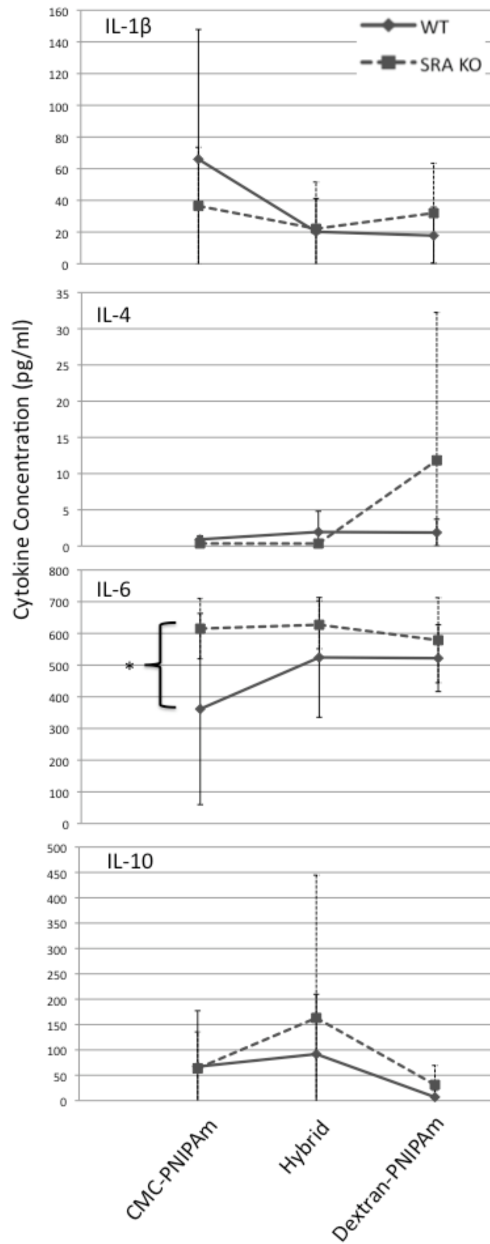

Figure 5.3. Cytokine concentrations in the intraperitoneal lavage fluid. The intraperitoneal lavage fluid recovered from the mice at the time of hydrogel explantation was found to contain significant amounts of interleukin 6 in all conditions (n = 6, mean ± SD). This was also true for the $SR\text{-}A^{-/-}$ mice injected with the CMC hydrogel, though the WT mice injected with the CMC hydrogel had significantly less IL-6 in the peritoneal cavity.

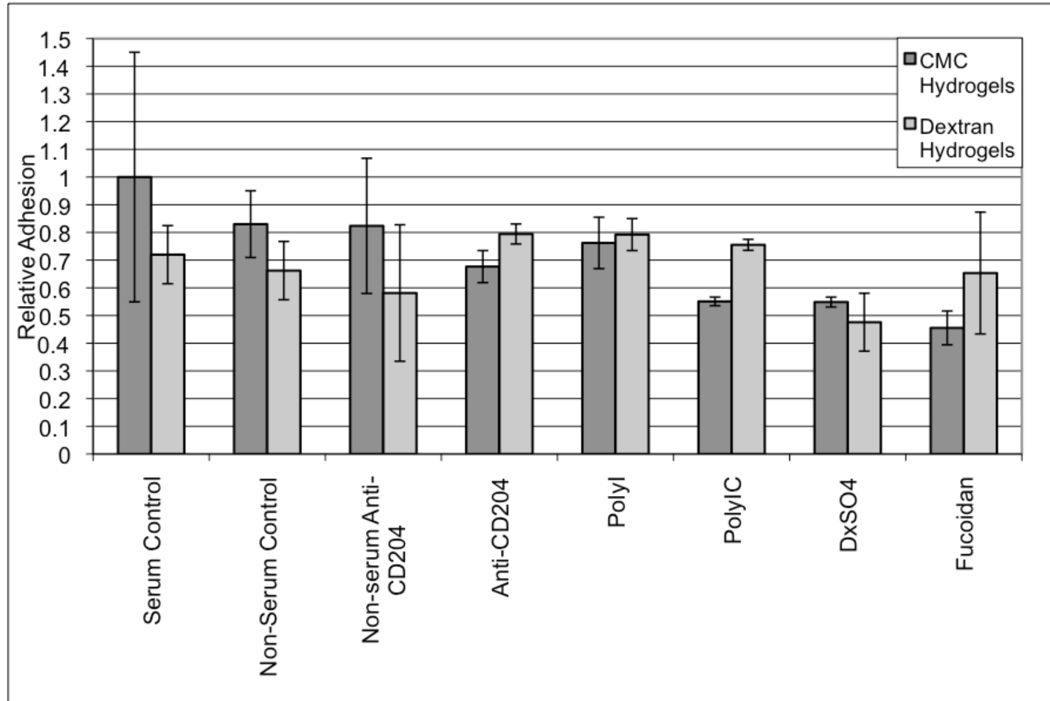

Figure 5.4. RAW264.7 Cellular Adhesion to Dextran:PNIPAm and CMC:PNIPAm Hydrogels. Adhesion was measured by cellular fluorescence using an internal stain, carboxyfluorescein diacetate succinimidyl ester (CFSE). Cellular adhesion was calculated relative to the control condition containing 5% serum (n = 2, mean ± SD). A non-serum control was also performed. Inhibition of cellular adhesion was demonstrated using an anti-CD204 antibody, polyinosinic acid (PolyI), polyinosinic:polycytidylic acid (PolyIC), dextran sulfate (DxSO4), and Fucoidan. Except where indicated, the inhibition of cellular adhesion was performed in culture conditions containing 5% serum.

DISCUSSION

The tunable charge density of the CMC/Dextran:PNIPAm co-polymers provided us the opportunity to study the effect of charge on SR-A mediated cell adhesion, which consequently permitted the evaluation of intraperitoneal cytokine responses to materials that exclusively bind SRA in both WT and knock-out mouse models. Though the CMC hydrogels were clearly devoid of an adhesive cell layer following 24 hours of incubation in

the peritoneal cavity of SR-A$^{-/-}$ mice, the intraperitoneal IL-6 concentration was still significantly elevated (as observed in the other conditions) indicating that the polymers initiated a significant biological response. The statistical main effect of mouse type (SR-A$^{-/-}$ vs. WT) for IL-6 suggests that SR-AI/II was activated/bound to each of the hydrogels and suppressed cellular IL-6 production in the WT mice. Since the soluble PNIPAm polymer backbone did not stimulate high levels of IL-6 when injected independently in the periteonal cavity, we attribute the significant biological responses incited by the hydrogels to the glucan linkages within dextran and cellulose. Beta-glucans, such as those found in cellulose, have long been classified as immunomodulatory, especially when in an insoluble form (see [26] for a review). Though alpha-glucan polysaccharides (e.g. dextran) are not generally regarded as being immune stimulators, modified dextrans, such as sulphated dextran and acetylated dextran, are known to cause very significant responses [27, 28]. Therefore, we suspect that the aldehyde functionalization of dextran significantly enhanced its immunostimulatory property.

The cell adhesion studies with RAW264.7 macrophage-like cells confirmed that the CMC-PNIPAm hydrogels were primarily recognized and bound by cell-surface SR-A, as partial inhibition of cell adhesion was possible with an anti-SRAI/II antibody (anti-CD204) and various polyanionic SR-A

ligands. As far as we know, no other polymeric material has been shown to be exclusively recognized/bound through an SRAI/II mechanism in the peritoneal cavity. Furthermore, it is notable that the PNIPAm backbone of the CMC:PNIPAm polymers did not seem to lead to non-SRA mediated cellular adhesion. However, the suppression of RAW264.7 cell binding to the gels in SF conditions indicates that SRA binding was mediated by an unknown protein found in FBS. This finding was similarly reported by Fraser and colleagues when studying integrin-independent RAW264 cell binding to tissue culture polystyrene [1].

CONCLUSIONS

Scavenger receptor A-I/II was found to be the primary receptor for leukocyte binding and recognition of a PNIPAm hydrogel crosslinked with CMC. The interactions between SR-AI/II and the CMC/Dextran-PNIPAm hydrogels seem to be mediated by an unknown protein found in fetal bovine serum and present in murine peritoneal fluid.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

A series of experiments were carried out using 100Dex possessing similar mechanical properties and cross-link density to 100CMC. The synthesis of oxDex required to achieve these properties required the use of 0.03 g of sodium periodate in the oxidative cleavage of 500 kDa dextran. All other steps in this oxidation remained consistent with all other oxidations.

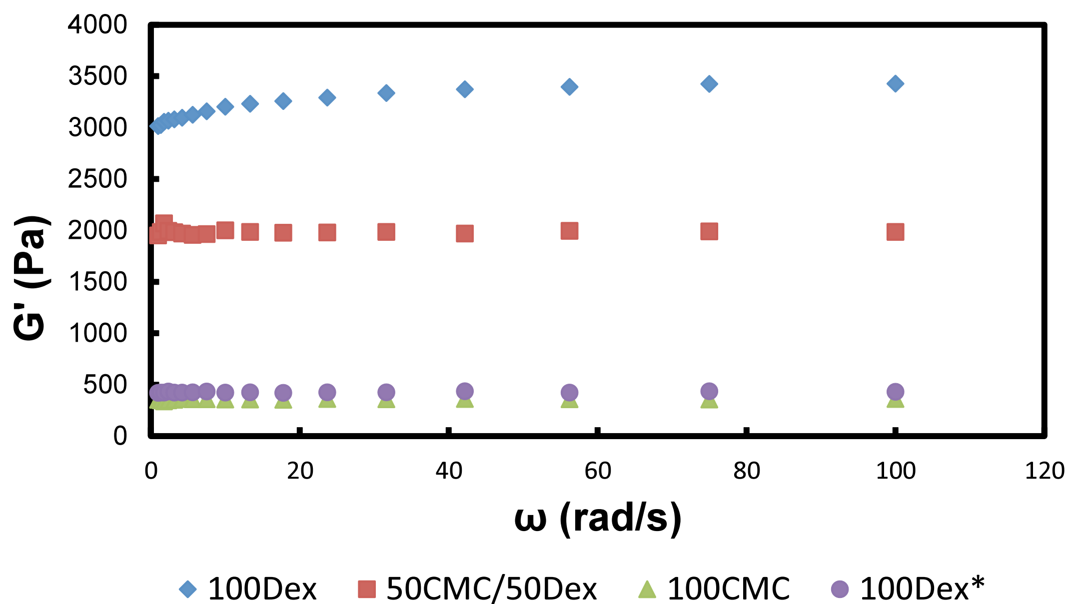

Figure 5.S1. Rheology of 100Dex, 50CMC/50Dex, and 100CMC hydrogels formed at 37 °C showing elastic modulus (G') as a function of frequency (ω). The elastic modulus vs. frequency is also shown for modified 100Dex possessing similar mechanical properties to 100CMC and is denoted with *.

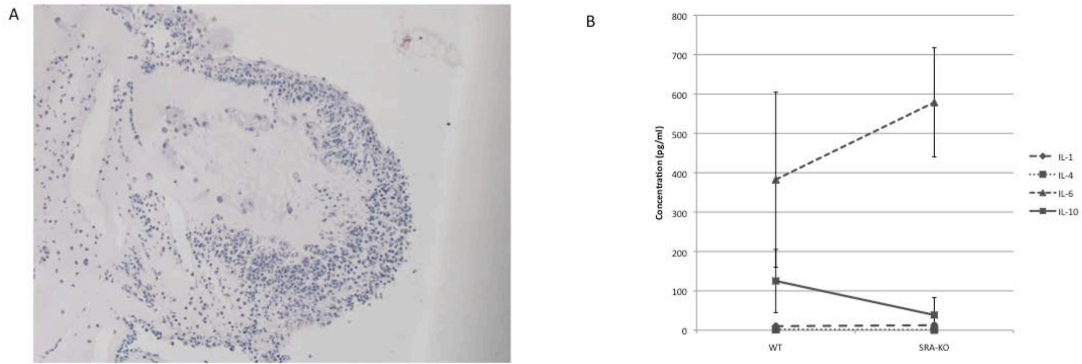

Figure 5.S2. Histological assessment (A) and cytokine concentrations (B) from the 100Dex material that possessed similar mechanical properties and cross-link density to 100CMC. The histology image is taken from a 100Dex gel recovered from the peritoneal cavity of an SR-AI/II null animal and is a representative image of the 100Dex gels recovered from both types of mice (WT and KO).

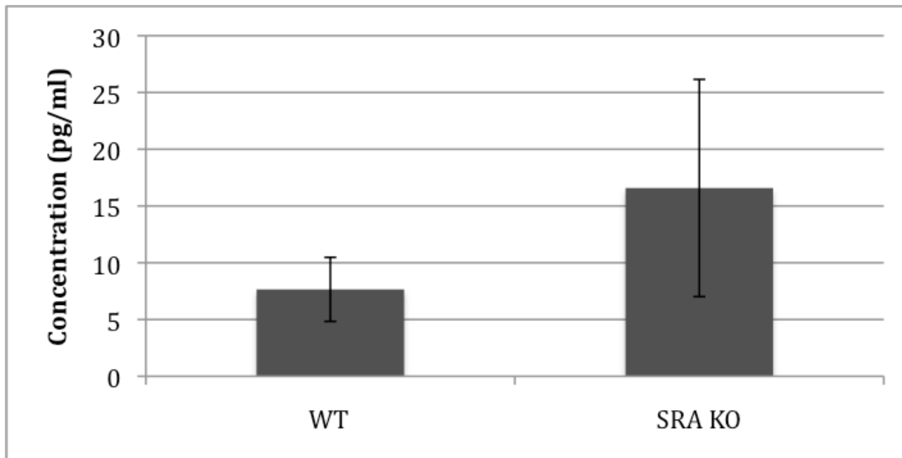

Figure 5.S3. Interleukin-6 concentration in the intraperitoneal lavage fluid 24 hours following injection of free (non-cross-linked) PNIPAm (0.3ml of a 3 wt% solution; n= 3/condition).

CHAPTER 6: SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

Summary of Thesis

In Chapter 1 we reviewed the literature to provide an overview of the chronic host response to biomedical material introduction to the body, with a particular focus on fibrosis and the pharmaceutical strategies that can be used to inhibit such fibrous tissue growth. In Chapter 2 we investigated the efficacy of proline hydroxylase inhibition in modulating the host response to porous poly(lactic-co-glycolic acid) (PLGA) discs. From this research, we found that inhibiting proline hydroxylation through controlled drug-release can be used to reduce collagen deposition and retard fibrous tissue growth into the disc. Furthermore, the inhibition of prolyl-4-hydroxylase appears to be effective at suppressing the expression of a cellular receptor with a collagenous domain, Scavenger Receptor AI/II (SR-AI/II), on a macrophage-like cell line. In Chapter 3 we again reviewed the literature, this time to provide an overview of biomedical material recognition and the role of pattern recognition in cellular responses to biomaterials. In Chapter 4, we studied two class A SRs, SR-AI/II and MARCO to determine whether they contribute in cellular activation/signaling in response to injection of

polystyrene or PLGA particles in the peritoneal cavity. We found that though both particles stimulate a similar inflammatory response in SR-A knockout mice as in wild-type mice, the cytokine responses vary between mouse types, indicating that SR-A and MARCO affect cellular signaling and downstream responses. In Chapter 5, we tested the hypothesis that SR-A may be an essential cell adhesion receptor to polyanionic hydrogels, which are among the most hydrophilic biomaterials. From this research, we found that SR-A is critical to the leukocyte binding of the most polyanionic hydrogel tested. Taken together, this research brings attention to the repertoire of pattern recognition receptors, which enables the identification of any foreign biomedical material by the host immune system.

Recommendations for Future Work

Three distinct projects have emerged from my thesis work, all of which require future study. The first project to emerge is the role of proline hydroxylation in host responses against biomaterials. We have found that proline hydroxylation is not only necessary for collagen synthesis, but also for expression of a cellular surface receptor, Scavenger Receptor AI/II (SR-AI/II), that ligates to a novel polyanionic hydrogel (see Chapter 5). It has been suggested in the literature that proline hydroxylation may play a role in the post-translational modification of opsonization proteins, such as

complement protein 1q (C1q), which contains a collagenous domain, and other scavenger receptors with collagenous domains. Actual experimental evidence is required to confirm that proline hydroxylation is a requirement for the expression/secretion of these proteins. Since these proteins may be critical for initiating the host response to various biomedical materials, it would be meaningful to subsequently look at the effect of inhibiting proline hydroxylation in responses to various materials, especially polyanionic hydrogels. As the inhibition of proline hydroxylation is known to modulate intracellular signaling molecules such as hypoxia-inducible factor 1α, it will be important for future researchers to keep the potential of such side effects in mind when deducing meaning from experimental results. Finally, the use of proline hydroxylase inhibitors should be evaluated in various medical device constructs (e.g. engineered-tissues) which may benefit from the favourable side effects (e.g. hypoxia-resistance) that these drugs confer.

The second project to emerge from my thesis work is the evaluation of scavenger receptors in host responses to biomedical materials. My research indicates that scavenger receptors are critical for leukocyte adhesion to a novel polyanionic hydrogel, but they also appear to play a role in cellular activation/signaling in response to more hydrophobic materials. Studies with longer incubation times still need to be performed to determine the role of SR-A in the foreign body response to various biomedical materials.

Furthermore, various sites of the body should be studied. Evidence in the research literature shows that SR-A mediates fibrosis in the lung following inhalation of various materials; however, the role of SR-A in foreign body responses elsewhere in the body is not known. With respect to the apparent cellular attachment function of SR-A on a polyanionic hydrogel, other materials need to be tested to establish a trend of SR-A ligation to negatively charged hydrogels. A good starting point would be alginate as it is commonly utilized in several biomedical engineering applications. Another interesting set of materials to test would be those that have been developed for blood-contacting applications, especially heparin-coated and polyethylene glycol surfaces. There is some evidence in the literature that class B SRs, such as CD36, which is found on platelets, erythrocytes and various leukocytes, binds to heparin. Therefore, heparin surfaces cause binding/activation of platelets, erythrocytes, and leukocytes, which may lead to coagulation/inflammation downstream. Polyethylene glycol, though not charged, is used to repel proteins at a surface due to its highly polar, and therefore hydrophilic, nature. As discussed in chapter 3, we know that SRs preferentially bind polyanions; therefore, it is logical that one or more classes of SRs may also ligate highly polar polymers, such as polyethylene glycol. Though assays for detection of new SR ligands have been described in the literature, I believe that the best way to test these polymers for SR ligation would be through the

use of transgenic cell lines expressing one or more of the receptors of interest.

The third project to emerge from my thesis work is the evaluation of neutrophil responses to materials and the mechanisms responsible for such responses. Neutrophils are known to be the first cells to respond to a biomaterial upon its introduction to the body. Upon arrival to the site of the biomedical material, neutrophils produce and release a burst of reactive oxygen species that damage local tissue and may harm the implanted device, especially if it is cellularized (e.g. engineered tissue). As discussed in Chapter 3, neutrophils respond to such materials even in the absence of protein in the culture media indicating that such a response can be initiated by a mechanism apart from the protein adsorption, integrin ligation mechanism of cellular activation that dominates on most materials. The receptors responsible for neutrophil activation in the absence of environmental proteins is unknown. Scavenger receptors (SR) were originally thought to mediate these responses, but neutrophils do not express the class A SRs; though, it is possible that other SR classes are expressed on neutrophils and mediate biomaterial recognition. Determining the mechanism responsible for these neutrophil responses in the absence of environmental protein would permit a more thorough understanding of acute inflammatory responses to biomaterials. In order to protect the device and local tissue, the reactive

oxygen species released from neutrophils can be quenched with antioxidants; however, the sustained release of antioxidants from the device does not protect the construct from the progression of the foreign body response. Like every biomedical material in the body, the device will eventually become encapsulated by fibrotic tissue.

Aside from the aforementioned three projects, several avenues of future research have emerged from my work. As I discussed in Chapter 1, several anti-fibrotic pharmaceutical agents have been developed for the purpose of treating fibrotic pathologies. However, the pharmaceutical companies that have developed such drugs need to keep in mind that a drug is only useful if it is delivered in an appropriate manner. In the case of treatment agents for fibrotic pathologies, the only suitable delivery method, in my opinion, is local elution from a polymeric drug carrier. Poly(lactic-co-glycolic acid) microparticles, which could be introduced to the tissue non-invasively through fine-needle injection, would be perfect drug carriers for such applications. For example, one of the more promising pharmaceutical strategies for the treatment of fibrotic pathologies is proline hydroxylase inhibition; however, as far as I know, the only method of drug delivery currently being investigated is oral delivery even though local delivery of these drugs would be far more suitable. Investigation of these compounds, using local delivery methods, must be done *in vivo* using mouse or rat models

that have been developed for fibrosis research. Following the induction of a fibrotic pathology, a locally-focused treatment should only require occasional re-administration. Again, a slow-eluting drug-delivery technique that can be applied with a minimally-invasive method would be ideal and should be strived for.

Another pathology-focused area of future research is the development of a drug-delivery system for bronchodilators that could be used for individuals that suffer from asthma. Such a drug-delivery system could be useful for asthma-sufferers that are about to enter an environment in which an asthmatic attack is highly likely. Clinical-utility would be particularly apparent for asthma-sufferers that are preparing for a long-distance exertion or about to enter an extremely cold environment. Slow delivery of a bronchodilator is achievable by impregnating such drug in a quickly-degradable particulate material that could be aspirated. The main challenge is engineering a particulate material that can be inhaled safely, without inciting a host response. Class A SRs have been shown to be critical identification receptors of environmental particles in the lung, and to frequently initiate the host response following inhalation of a particulate material. Therefore, polyanionic polymers are likely not appropriate for such application. A novel fast-degrading polymer based on monomeric components of pulmonary surfactant would be ideal.

A final area of future research is the identification of host proteins that passivate biomedical materials to host responses. Albumin has long been known as a passivating protein, but others likely exist. Parasite immunology would be useful to look to for guidance as parasites frequently coat themselves in host-proteins to avoid detection. Determining a protein surface-coat that effectively hides a polymer from recognition by interrogating leukocytes would be useful in the quest to prevent host responses to biomedical materials. Such research is particularly called-for now that we have uncovered a new way in which leukocytes identify a biomedical material (specifically, a charged hydrophilic polymer) that does not readily adsorb protein.