THE IN VITRO EFFECTS OF BIOMATERIALS ON LYMPHOCYTE RESPONSES TO AN ALLOGENEIC CHALLENGE

THE IN VITRO EFFECTS OF BIOMATERIALS ON LYMPHOCYTE RESPONSES TO AN ALLOGENEIC CHALLENGE

By

Nadira Farooqui, B.A.Sc

A Thesis Submitted to the School of

Graduate Studies in Partial Fulfillment

of the Requirements for the Degree

Master of Applied Science

McMaster University

MASTER OF APPLIED SCIENCE
(Chemical Engineering)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE:	The *in vitro* Effects of Biomaterials on Lymphocyte Responses to an Allogeneic Challenge
AUTHOR:	Nadira Z. Farooqui B.A.Sc (University of Waterloo)
SUPERVISOR:	Dr. Kim Jones (Chemical Engineering)
NUMBER OF PAGES:	xiii, 108

ABSTRACT

It has been shown that when implanted individually, both cells and biomaterials elicit biological responses. Implanted cells are often destroyed by the host's immune system, while biomaterials activate foreign body reactions which can result in inflammation and fibrotic encapsulation. However, when implanted simultaneously, the inflammatory responses to the biomaterial component can alter the immune responses to the cellular component.

The experiments described in this thesis were designed to characterize the effect of different biomaterials on adaptive immune responses towards an allogeneic challenge. Balb/c splenocytes were challenged with irradiated allogeneic L929 cells, and treated with different biomaterials. Alterations in adaptive immune responses were quantified by T cell proliferation and cytokine release (i.e. IL-1β, IL-4, IL-12, and IFN-γ). The roles various cell types played in first set responses were investigated.

Experimental results indicated that biomaterials had a significant influence on non-specific proliferation of splenocytes. In particular, analysis of the degree to which biomaterials affected specific proliferation indicated that the soluble alginate treatment significantly increased proliferation differences when compared to the control. However, biomaterials neither significantly affected specific splenocyte proliferation to an allogeneic challenge, nor the profile of secreted cytokines. To elucidate this response, alginate-treated splenocytes were depleted of adherent macrophages, CD4+ cells or

CD8+ cells. Within non-challenged mixtures, CD4+ depletion had the most obvious effect. These results were supported by the non-depleted challenges, and indicated the direct influence biomaterials on CD4+ T cell proliferation.

ACKNOWLEDGEMENTS

During the course of my thesis work, there were many people who were instrumental in helping me. It is a pleasure for me to take this opportunity to thank them.

I would like to begin by expressing my sincere gratitude to my supervisor, Dr. Kim Jones. With her enthusiasm, encouragement, and ability to explain things clearly and simply, she helped me through many moments in which nothing seemed to work.

I would also like to thank all the summer students who provided their assistance on this project, particularly Lauren Davies. With her ability to devise and carry out a viable experimental plan, Lauren has proven herself to be a vital asset to any team.

To the faculty, staff, and fellow graduate students in the Department of Chemical Engineering, I would like to thank you for providing me with a stimulating and fun environment in which to grow and learn. To everyone at McMaster's Central Animal Facility, thank you for making my transition in to animal research one of ease.

Finally, I would like to thank my friends and family for their constant support and encouragement during my graduate studies. Most importantly, I wish to thank my parents and my brother. Together they raised me, supported me, taught me, and loved me. To them I dedicate this thesis.

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ANOVA – Analysis of variance

APC – Antigen presenting cell

Balb/c – "Bagg albino" mouse

BCR – B cell receptor

CTL – Cytotoxic T lymphocyte

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal bovine serum

HLA – Human leukocyte antigen

HUVEC – Human umbilical vascular endothelial cell

IFN – Interferon

Ig - Immunoglobin

IL – Interleukin

LPS - Lipopolysaccharide

LVAD – Left ventricular assist device

KIR – Killer immunoglobulin-like receptors

mAbs – Monoclonal antibodies

MAP-kinase – Mitogen activated protein kinase

MBL – Mannan-binding lectin

MHC – Major histocompatibility complex

MLR – Mixed lymphocyte reaction

MS - Microsphere

NF-κB – Nuclear factor kappa B

NK cell – Natural killer cell

NO – Nitrous oxide

OVA – Ovalbumin

PAMP – Pathogen-associated molecular pattern

PBS – Phosphate buffered saline

PDMS – Peroxide-catalyzed polydimethylsiloxane

PET – Polyester terephthalate

PGA – Poly(glycolic acid)

PLA – Poly-L-lactic acid

PLL – poly(L-lysine)

PMMA – Poly(methyl methacrylate)

PRR – Pattern recognition receptor

PS – Polystyrene

PVA – Polyvinyl alcohol

SR – Scavenger receptor

TCR – T-cell receptor

T_h cells – T helper cells

TLR – Toll-like receptor

Treg cells – Regulatory T cells

CHAPTER 1

INTRODUCTION

Organ failure due to damage or disease poses a major problem to today's population. Organ availability is seriously limited, and even those who receive transplants are not necessarily out of harm's way. In the mid-nineteen hundreds, early experimental skin transplantation between different strains of mice predicted that the immune system governed the process of transplant rejection and, hence, presented a great challenge to the success of organ transplantation. The required anti-rejection drugs that go hand-in-hand with an organ transplant have been shown to impose many risks to the organ recipient – risks that include hypertension, diabetes, high cholesterol levels, opportunistic infection, anaphylactic reactions, and abnormal wound healing (1). Even when all precautions are taken, graft survival rates are far from perfect.

One way by which researchers sought to address the problem of supply and demand was by engineering organs. Tissue engineers have since made much progress - from the synthesis of skin and blood vessels to advances with more complex structures such as livers. As in the case of cadaveric organs, tissue-engineered organs have to face the recipient's immune system upon transplantation.

The two main components of tissue-engineered devices are transplanted cells (likely to be allogeneic) and the biomaterial in which these cells are seeded. Both of these

components have been shown to elicit biological responses when implanted individually. However, when implanted concurrently, the inflammatory responses to the biomaterial component and immune responses to the cellular component are interconnected, and determine the ultimate function of the device.

The experiments described in this thesis were designed to characterize the effect of different biomaterials on adaptive immune responses towards an allogeneic challenge. I hypothesized that biomaterial treatments (possible components of tissue-engineered constructs) altered the first set response to an allogeneic challenge, and that this alteration differed between biomaterials. Specifically, it was hypothesized that the adaptive immune responses (quantified by increased T cell proliferation and cytokine release) will be greater for non-mammalian polysaccharides than synthetic polymers. Measured cytokines would also help determine if T cells were polarized to elicit a Th1 or Th2 response.

Further investigation was made into the roles various cell types play in the first set response. A 0.1% alginate treatment was used as the basis for this comparison. Prior to the allogeneic challenge, naïve splenocyte suspensions were depleted of: (1) adherent macrophage cells (antigen presenting cells or APCs), (2) CD4+ T cells, and (3) CD8+ T cells. Previous studies have indicated that direct recognition plays a vital role in allogeneic transplant rejection. Mediated primarily by CD8+ T cells and not requiring host-APCs, direct recognition has been associated with the acute and more immediate

phase of rejection. Therefore, I hypothesized that splenocyte suspensions depleted of CD8+ T cells would show a dramatic reduction in proliferation. Also playing a role in CD8+ T cell mediated recognition is the indirect pathway known as cross-priming. Facilitated through donor antigens that bind to host-MHC class I molecules on host-APCs, the effect of cross- priming would be seen in adherent macrophage and CD8+ depleted suspensions.

Although I set out to investigate the hypotheses explained above, the most interesting finding within this experimental work lay outside these hypotheses, and dealt with direct biomaterial influences over adaptive immune responses.

The following literature review is provided as an overview of the topics specific to the work presented in this thesis. I begin with a review of the function and composition of tissue-engineered constructs. This is followed by current data on immune responses to the specific components of tissue-engineered constructs – including inflammation, immune recognition and mechanisms of rejection. Minimal research has been done to understand the interactions between the host responses elicited by the biomaterial and cellular components of tissue-engineered constructs, but the available literature will be discussed.

CHAPTER 2

LITERATURE REVIEW

2.1 Tissue-Engineered Devices

In the hope to restore proper biological function to tissues and organs, tissue engineers have combined cells and biomaterials to create engineered implants. The first definition of tissue engineering was given by Langer and Vacanti. They stated that tissue engineering was "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." (2)

It has been shown that both cells and biomaterials elicit biological responses when implanted into a recipient (i.e. host). Implanted cells can remodel and differentiate *in vivo* or be destroyed by the host's immune system. Research suggests that it may be possible to direct biological responses by modifying implanted devices. For example, some polymer scaffolds not only function to transport cells to diseased tissues, but control and guide wound healing and tissue regeneration (3). Generally, upon implantation, biomaterials activate foreign body reactions, resulting in inflammation, altered wound healing, and fibrotic encapsulation (4,5).

Though many advances have been made in tissue engineering, researchers still look to optimize the biocompatibility of tissue-engineered devices by modifying implants to induce a desirable host response.

Before diving into the specific immune responses elicited by both cells and biomaterials (components of tissue-engineered constructs), I will first discuss the applications of such devices and the evolution of its components.

2.1.1 Applications of tissue-engineered devices

The applications of tissue-engineered constructs may one day reach every biological ailment. Depending on the problem, different principles of tissue engineering can be employed.

When naturally released proteins (e.g. insulin, dopamine, Factor VIII or Factor IX) are not secreted properly or unable to reach the tissues which require them, cell transplants can be utilized to deliver these required proteins (4,6). In cases where purified pharmaceuticals are preferred, tissue engineering principles have also been applied. For example, polymer based biomaterials have been shown to effectively deliver drugs orally, transdermally, and to other sites within the body (7,8).

Regeneration of the body's proper biological function is one goal of tissue engineering, but not always realistic. Organ transplantation is required in cases where organ function

has been dramatically reduced and regeneration is not possible. Cadaveric organ transplantation is not always an option for patients since the supply of organs is relatively low. In cases involving organ failure, tissue-engineered organs are an attractive solution. With both regeneration and tissue-engineered organs, researchers have focused on the implantation of cells, scaffolds, and cell-seeded scaffolds.

2.1.1.1 Cell transplants

Many diseases affect the biological production of proteins. The lack of these required proteins, due to the absence or improper function of the cells that produce them, can make simple biological functions impossible. These diseases include, but are not limited to, diabetes (4,5), hemophilia (9), and CNS diseases (10). For such diseases, the tissue engineering solution points to cell transplantation.

One advantage to cell transplantation is the need for lower levels of immunosuppression. This can be partially attributed to lack of MHC class II expression on implanted cells, as MHC class II molecules are required for direct recognition via CD4+ T cells. Some researchers are striving for a further reduction in direct rejection through the isolation of cells with a decreased expression of MHC class I molecules (required for CD8+ T cell recognition) (11). Immune rejection is thought to be further decreased by the implantation of encapsulated cells (4,10). However, there has been evidence showing that encapsulation does not actually decrease host immune responses, and that the

inflammatory response to the implanted biomaterial contributes to encapsulated cell death (5).

When comparing protein delivery via pharmaceutical methods to cell transplantation, another advantage to cell transplantation is highlighted. By replacing damaged or diseased cells, protein production would become continuous and/or environmentally-responsive – requiring no prescriptions to be refilled or invasive drug administration to be repeated. Researchers have found that transplanted islet cells not only continually produced insulin, but also produced the protein as needed – by taking cues from the host's biological system (12).

2.1.1.2 Regeneration

Regeneration in terms of tissue engineering is the ability to re-develop the function of diseased or damaged tissues and organs. A few human organs, such as the liver, can regenerate unaided. However, for the body to restore function to nerves, tissues, and the majority of other organs, it must be assisted in the process of regeneration.

Unlike human organs, embryos have the ability to regenerate. Embryos contain undifferentiated stem cells, a cell population capable of self-renewal and differentiation into different cell lines. With this vast potential, stem cells have been used to guide the process of regeneration (13,14). Olfactory mucosa is a stem-like progenitor cell for neural repair. One study showed that autologous olfactory mucosa transplantation into a

injured human spinal cord is feasible, relatively safe, and beneficial in the regeneration neural function (15).

Biomaterials have also been used to guide the regeneration process. Scaffolds can be designed to promote cell invasion and migration. One study suggested that migration and long-term survival of myoblasts could be greatly increased by optimizing scaffold composition, architecture, and growth factor delivery. This indicates the possibility for the clinical use of biomaterials in muscle tissue regeneration (16).

Studies have also shown that functionalized polymers can be used to guide regeneration. Lee, J. et al, showed that surface modification of scaffold materials with biological moieties enhanced the biomaterial-tissue interface and promoted desired tissue responses (17). The modifications allowed human umbilical vascular endothelial cells (HUVECs) cultured on these functionalized-surface modified materials to have improved attachment and spreading.

2.1.1.3 Tissue transplants

To address the lack of cadaveric organs, researchers are working on engineering organs. The two main components being utilizing in this process are transplanted cells and biomaterials in which these cells are seeded. Although much of the research on tissue-engineered organs has been plagued with problems, progress has been made.

One of the early applications of tissue-engineered transplants was for use in wound healing. Today, skin substitutes are used to treat burns and chronic non-healing wounds when conservative treatments fail and skin grafting is not possible. Grown from human foreskin cells, Apligraf is composed of keratinocytes and fibroblasts (18). Such skin substitutes are nearly impossible to tell apart from physiologically normal skin, and have the same two-dimensional structure (19,20).

Researchers have also seen some progress in the engineering of 3-dimernsional structures such as bone, cartilage, and heart valves. Cartilage and bone have low cell densities and, therefore, do not require blood vessels, eliminating the persistent problem of proper nutrient distribution. Currently, allogeneic bone replacements are done without cells, because the inclusion of cells has been shown to cause immune reactions that compromised function (21).

Research suggests that tissue-engineered heart valves are associated with limited durability and calcification as a result of foreign material implantation (22). However, the emergence of the biodegradable polymer scaffold has been shown to provide a structural profile until seeded cells produce their own extracellular matrix (23). There are still many hurdles that must be overcome before tissue-engineered heart valves are introduced into clinical use, but the potential is there.

As previously mentioned, properly supplying nutrients to implanted cells is a major problem, yet essential when referring to the production of larger organs. Another problem is associated with cellular interactions. In biologically-produced organs, pathways for cellular interactions naturally develop. In the case of tissue-engineered organs, these pathways have to be facilitated. Researchers are striving for success with larger organs, varying from livers (22,24,25) to hearts (26-28).

2.1.2 Tissue Construct Components

Tissue-engineered constructs can be composed of biomaterials, cells, or a combination of the two. With continuing advancements in cellular biology and materials science, the source and composition of these components are evolving. The following sections discuss the evolution of these sources and current focus. Within the biomaterials section, I will also discuss the possible biological responses to various material types, as this information is important to understand the hypotheses made within this thesis.

2.1.2.1 Biomaterials

Biomaterials are materials that are intended to interact with biological systems. As such they have evolved over the last 2000 or more years, from simple sutures and wound dressings to joint replacements and breast implants.

Biomaterials can be natural or synthetic, unmodified or functionalized, permanent or degradable. They range in shapes and sizes from thin films to complex three-dimensional structures. Porous structures have been developed to allow oxygen permeation, cell migration and angiogenesis. Re-absorbable (or biodegradable) polymers have been developed to provide a temporary structural silhouette for seeded cells to produce their own extracellular matrix and grow into their own biological entity (23). One early *in vivo* application of biomaterials was the use of metals in bone repair.

Several mammalian proteins have been studied for use as biomaterials (e.g. fibrin (29) and collagen (19)). Often devised from extracellular matrix, such materials retain many natural mechanical and biological properties of tissues. For example, natural acellularized extracellular matrix proteins, such as collagen, incorporate structures and proteins to which cells recognize and respond (30). These structures have been shown to facilitate host cell migration and integration (31). However, one major problem associated with the use of mammalian proteins as biomaterials is unwanted biodegradation (32).

Naturally occurring materials, other than mammalian proteins, have also been studied for tissue engineering applications. Many non-mammalian polysaccharides have been used as biomaterials, including alginate (33), agarose (34), and chitosan (35,36). Such materials have the necessary structural integrity (in some applications) and the ability to provide the body with many of the appropriate cues needed for proper function. For

example, chitosan sponges have been studied for cartilage regeneration. These studies have shown that the pores within these sponges can promote chondrocyte proliferation and metabolic activity (35). One problem with the use of non-mammalian polysaccharide biomaterials is that they can be composed of carbohydrates that are also present on microorganisms. Several of these common non-mammalian carbohydrates have been shown to be perceived by immune cells as pathogen-associated molecular patterns in the context of microorganisms (37,38). For example, mannose (a component of alginate) has been shown to activate pathogen recognition receptors on immune cells (i.e. the mannose receptor), resulting in receptor-mediated endocytosis and phagocytosis. It has been shown that when the mannose receptor binds to microorganisms it triggers a variety of intracellular responses, including cytokine secretion, lysosomal enzyme secretion, and modulation of other cell surface receptors (37). Ligation of the mannose receptor has, thus, been associated with innate immunity and inflammation. Therefore, the possibility exists that the carbohydrates composing non-mammalian polysaccharide biomaterials can activate innate responses in a similar manner. Another problem with the use of non-mammalian polysaccharides, is the lack of compatible sterilization techniques – as current techniques often result in reducing polymerization (39,40). The implantation of non-sterile biomaterials results in the activation of host responses associated with pathogen elimination (e.g. responses to lipopolysaccharide (LPS) of gram-negative bacteria).

Synthetic polymers have also been used as scaffolds for tissue-engineered constructs, and the family of poly(α-hydroxy acid) polymers are one major focus. For examples, the use of poly(glycolic acid) (PGA) has been suggested for many tissues, including cartilage (35), intestine (41), and livers (42). Many synthetic polymers have been developed to have different structural (from gels to solids) and chemical properties (charge, hydrophobicity, and reactivity). Surface-modified synthetic polymers have also been developed, and shown to guide nerve regeneration (43) and prevent protein adhesion (44). With the wide range of polymerization techniques available, synthetic polymers can be fabricated to allow for appropriate sterilization, avoiding host responses elicited by foreign pathogens.

2.1.2.2 Cells

Organs are comprised of cells supported by extracellular matrices. This may sound simple to imitate, but, in fact, has posed an enormous problem for tissue engineers. Efficiently packing large numbers of cells together, while providing the right biological cues and nutrients (by nearby capillaries) – has yet to be achieved.

Cell sources used by tissue engineers have come from autologous (host and donor are the same), allogeneic (within species), and xenogeneic sources (between species). The advantage of using autologous cells is their obvious lack of immunogenicity, but few autologous cell types can be cultured in significant numbers for transplantation.

Allogeneic and xenogeneic cells have also been studied, and although potential has been seen, a range of immunological events have shown to occur (4,12).

Adult stem cells from the host or allogeneic embryonic stem cells are hoped to eliminate the problem of culturing autologous cell types. With the ability to differentiate in to a large number of cell types the potential of such cells may be endless. While this strategy is still in its developmental stages, the therapeutic potential of injecting stem cells systemically or locally has been seen (45-47). Stem cells are predicted to affect a range of disorders, including neurodegenerative diseases, spinal cord injuries, and cardiac diseases.

Although stem cells hold a lot of promise, it is unlikely that they will be widely used in tissue engineering applications. Once implanted into the body, it would be very difficult to provide biomaterial embedded-stem cells with the appropriate cues they needed to differentiate into the cell type required. Autologous cells would be an ideal cell source, but the lack of proper cell culturing techniques steers tissue engineers toward the use of foreign cells (i.e. allogeneic and xenogeneic cells).

2.1.2.3 Cells and materials together

The incorporation of cells into biomaterial scaffolds is achieved using various techniques. Biomaterials can be seeded with cells *in vitro* prior to implantation. This technique is frequently employed when fabricating blood vessels (48) and cartilage (21). For many

more complex organs, a vascular system is required. A study conducted by Mooney and Park showed that sponges fabricated from poly-L-lactic acid (PLA) and polyvinyl alcohol (PVA), on which hepatocytes adhered, would allow fibrovascular tissue invasion. The pores permitted the formation of tissue consisting of the polymer sponge, hepatocytes, blood vessels, and fibrous tissue (49).

Many researchers are investigating the ability of certain materials to allow for the direct migration of autologous cells *in vivo*. For example, recovery after spinal cord injury depends on the directed re-growth of transected axons. Alginate-based hydrogel beads have been studied for this potential application. After implantation into cervical spinal cord lesions in adult rats, beads integrate into the spinal cord parenchyma and *in vitro* results showed directed axon regeneration across the alginate scaffold (50).

As we move towards developing tissue-engineered constructs that incorporate both biomaterials and cells, the interconnections between the host responses elicited by both components become increasingly important. The following sections discuss the damaging effects elicited by these components and the potential interactions.

2.2 A Brief Overview of Host Responses

To be able to understand or predict host responses to tissue-engineered constructs, we must first review general host responses and, where applicable, host responses to transplants.

It was recognized in the mid-nineteen hundreds that the immune system governed the process of transplant rejection. Genetically identical animals accepted grafts from one another, but allografts generated a rejection response (first set rejection). Re-grafting using the same donor was shown to generate an even faster rejection process known as second set rejection. The major genes responsible for allogeneic rejection are inheritable and highly polymorphic. Before performing transplants from cadavers, clinical laboratories perform tissue matching and sequence the DNA on the major histocompatibility locus, which contains the genes responsible for rejection processes.

2.2.1 The Major Histocompatibility Complex

Governing the process of transplant rejection, the major histocompatibility complex (MHC) is a large gene-dense region, which plays an important role in immunological processes. The MHC region is best known for the human leukocyte antigen (HLA) genes that encode cell surface antigen presenting proteins.

MHC proteins act in cell surveillance and display self and non-self peptides on cell surfaces. MHC class II molecules display non-self peptides, while MHC class I molecules display both self and non-self peptides (Figure 2.1).

Non-self antigens displayed on MHC molecules initiate specific host immune responses. Class II molecules display antigens originating outside cells, while MHC class I

molecules display intracellular antigens that are produced within the cell (this process is explained further in the next section).

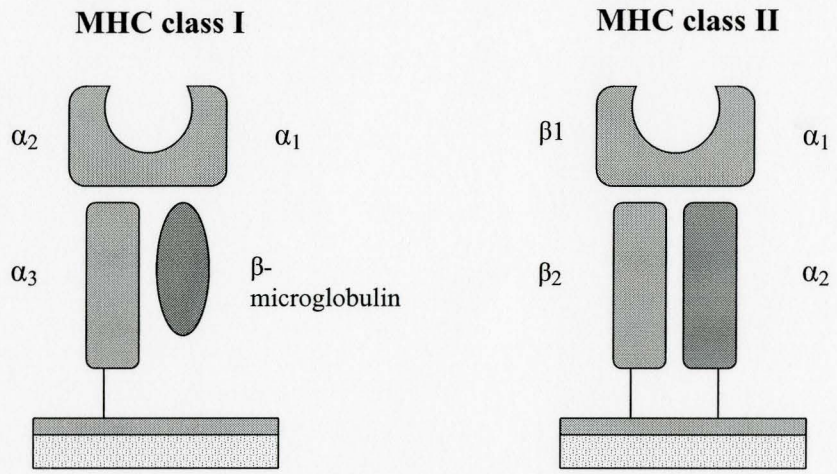

Figure 2.1: MHC class I and MHC class II molecules. Shown is the heterodimer formation and single transmembrane polypetide of MHC class I molecules. Similarly, MHC class II molecules are also heterodimers, but consist of two homologous transmembrane polypeptide chains.

2.2.2 Adaptive Responses: Mechanisms of Antigen Recognition

MHC molecules play a vital role in antigen recognition and maintain constant dialog with surrounding T cells. Anchored into cell membranes, MHC molecules display polypeptides that can be recognized by T cell receptors (TCR). In the case of non-self polypeptides, MHC-TCR interactions are designed to initiate an appropriate response.

Figure 2.2 illustrates the predominant cells and molecules involved in T cell mediated antigen recognition.

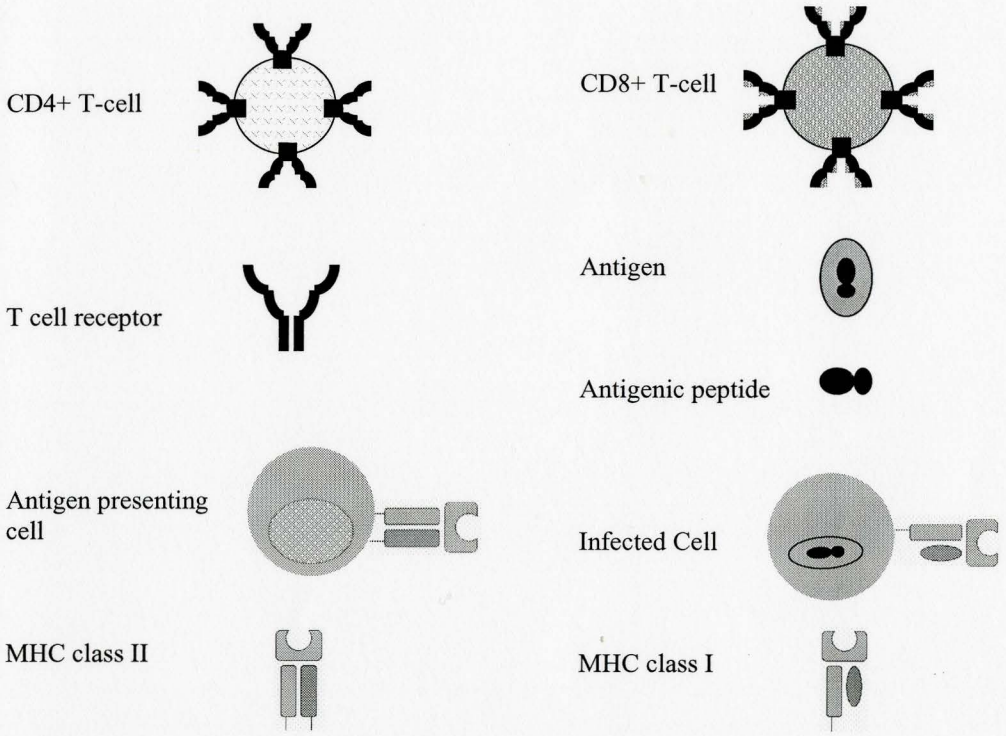

Figure 2.2: Representation of the cells and molecules involved in T-cell mediated antigen recognition.

As previously mentioned, MHC class I molecules are associated with the presentation of intracellular antigens. An excellent example of such an antigen would be a virus. Viruses infect a cell by entering its cytoplasm. The MHC class I pathway of antigen presentation allows virus-infected cells to signal T cells (Figure 2.3). CD8+ T cells, also know as cytotoxic T lymphocytes (CTL), interact exclusively with MHC class I

molecules and almost always induce apoptosis of the infected cell. Self-destruction of virus-infected cells reduces the risk of contaminating neighboring cells.

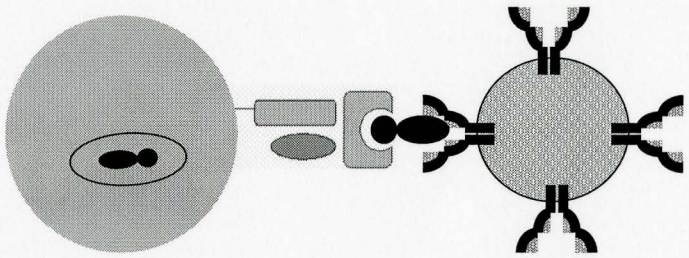

Figure 2.3: MHC class I stimulation of T cells. Intracellularly infected cells present antigen associated peptides to CD8+ T cells via their MHC I molecule.

Cells, including macrophages, dendritic cells, and B cells, display MHC class II molecules and are known as antigen presenting cells (APCs). Class II molecules present peptides derived from extracellular proteins or microbes and are thus associated with extracellular pathogens (e.g. bacteria). Antigens engulfed by APCs are digested within lysosomes and bound by MHC class II molecules prior to surface presentation (Figure 2.4). Class II molecules interact exclusively with CD4+ T cells, also known as helper T cells (T_h cells). Helper T cells eliminate the infection by triggering the appropriate immune response. Downstream responses can include localized inflammation, further phagocyte recruitment, or B cell activation followed by a strong antibody response.

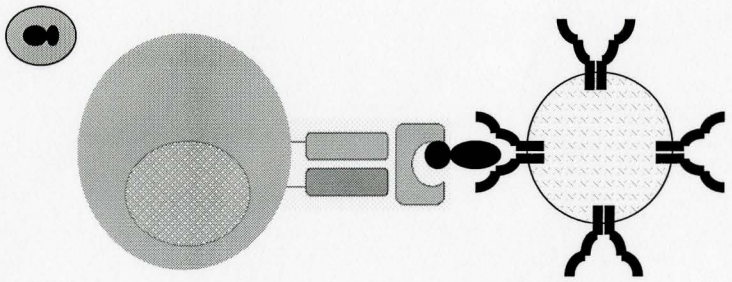

Figure 2.4: MHC class II stimulation of T cells. Antigen presenting cells pick up extracellular antigens, phagocytose them, process them into peptides and present them to CD4+ T cells via their MHC class II molecules.

Another important method for antigen identification is through membrane bound antibodies on B cells, known as B cell receptors (BCRs). In contrast to T cell recognition, B cells bind antigens without a great deal of assistance. This triggers antibody production.

Antigen recognition through adaptive immune processes, specifically through MHC-TCR interactions, has proven to be a major obstacle in transplantation (discussed in more detail later). Innate immunity has also been shown to affect the outcome of transplantation by the release of immediate indicators that warn of abnormalities (51-53).

2.2.3 Innate Responses

The adaptive immune system has evolved thorough means of handling antigens that are specific and involve immune memory, but can take several days to be effective. The role of the innate immune system is to take immediate action. The innate immune system is comprised of many processes, some of which have been observed in tissue-engineered implantations, specifically in response to biomaterial implantation.

Physical barriers are the first line of defense against antigen invasion, the most visible of all being skin. Made up of dead cells and rich in keratin, acid lipidic secretions from sebaceous and sweat glands impede bacteria growth. Mucus is another major defense mechanism, and coats all openings to the body. With the ability to catch and immobilize invading organisms, its composition is often deadly (54). Other physical barriers include saliva, tears, and gastric acid.

Phagocytes such as macrophages, dendritic cells, and neutrophils are involved in innate immunity. Phagocytes act as security guards, patrolling the body without stimulus, but can also react to cytokines produced by lymphocytes. Table 2.1 gives a brief overview the function of the various innate immune cells.

Phagocyte:	**Function:**
Monocytes	• Protect against blood-borne pathogens. • Move quickly into tissues and sites of infection, where they mature into macrophages.
Macrophages	• Act in nonspecific and specific defense against pathogens, by phaocytosis. • Large cells directly related to the mature form of monocytes.
Neutrophils	• Quickest reacting and most abundant of the immune cells. • Serve to destroy pathogens with lysosomal enzymes and hydrogen peroxide.
Eosinophils	• Important in the defense against invading parasites.
Basophils	• Important in parasitic and allergic reactions. • Release histamines upon activation.
Mast Cells	• Important in allergic reactions. Release histamine upon activation.

Table 2.1: The function of various innate immune cells.

Natural killer (NK) cells are specialized lymphocytes that play an important role in innate immunity. The Fc region on antigen bound antibodies can activate NK cells through their Fc receptor, leading to antibody-dependent cell-mediated cytotoxicity. NK activation can also occur through killer immunoglobulin-like receptors (KIRs), located on the surface of NK cells. KIRs recognize MHC class I molecules. The affinity of this interaction is related to NK cell reactivity (55).

The complement system is a chemical cascade derived from small plasma proteins that help clear pathogens without a need for previous exposure. Directly activated by surface structures, complement by-products serve as opsonins and chemokines. Formation of the membrane attack complex (the final product of the cascade) results in cell lysis. Humoral aspects can also activate the complement cascade. Naturally circulating antibodies can bind to structures on pathogen surfaces. Bound antibodies act to opsonize by activating complement, or facilitating phagocytosis through Fc receptors located on phagocytes.

Pattern recognition receptors (PRRs) located on APCs recognize pathogen-associated molecular patterns (PAMPs) (52). One such PRR is the mannose receptor, which binds a range of microorganisms by recognizing non-mammalian patterns such as glycoproteins terminated in mannose (38).

The family of Toll-like receptors (TLRs) is another important group of PRRs responsible for recognition of PAMPs (53,56). There have been ten TLRs identified in humans (TLR1-TLR10) and 11 in mice. TLR ligation leads to immediate responses, which include production of cytokines, reactive oxygen and nitrogen intermediates, and upregulation of chemokine receptors and costimulatory molecules - responses that eventually lead to NF-κB and MAP-kinase activation. An example of a TLR is TLR4, which not only recognizes exogenous ligands (such as lipopolysaccharide), but endogenous ligands as well (57).

Chemotactants such as chemokines and cytokines secreted in innate reactions have been shown to magnify the later responses of adaptive immunity. As seen in Table 2.2, IL-1β is a cytokine commonly secreted by several phagocytes. Lattman et al. found chronic rejection brought on by adaptive immune responses is more prevalent in patients with increased levels of IL-1β (58). This research illustrates the bridge between innate and adaptive immune responses.

Cytokines:	**Produced by:**	**Target:**	**Effect:**
IL-1β	Monocytes Macrophages B cells Dendritic cells	Th cells	Co-stimulation
		B cells	Maturation and proliferation
		NK cells	Activation
		Other	Inflammation, acute phase response and fever
IL-4	Th2 cells	T cells	Proliferation
		Activated B cells	Proliferation and differentiation IgG_1 & IgE synthesis
		Macrophages	MHC expression
IL-12	Macrophages B cells	Naïve T cells	Th1 differentiation
		Th1	IFN-γ production
		NK cells	Activation
IFN-γ	Th1 cells NK cells	Naïve T cells	Th1 differentiation
		Activated B cells	Ig class switching to IgG
		Macrophages	MHC expression Pathogen elimination

Table 2.2: An overview of several cytokines relevant to the scope of this thesis.

Graft survival is affected by many factors. The following sections will discuss the factors that directly affect the field of tissue engineering – including biocompatibity, inflammation, and rejection responses.

2.3 Investigating Host Responses to Tissue-Engineered Devices

Tissue-engineered constructs are devices for the restoration or modification of tissue function. Constructs can consist of either transplanted cells or biomaterials, or a cell-biomaterial combination. Although this thesis focuses on the third category of devices, it is important to review the literature on the first two categories. Understanding host responses to biomaterials and cells in isolation of each other will help us understand and narrow the possible interactions between the two. Although minimal research has been done to understand these interactions, the available literature is discussed.

2.3.1 Biocompatibility of the Biomaterial Component

Biomaterial implantation initiates a sequence of events similar to a foreign body reaction. Starting with acute inflammation, the foreign body reaction may lead to one or more of the following:

1. immune cell recruitment and activation
2. chronic inflammatory responses
3. granulation tissue development
4. fibrous capsule development

The intensity and duration of a foreign body response is dependant on several factors, which include the extent of injury created upon implantation and biomaterial chemical composition, surface charge, roughness, and porosity.

For biomaterials contacting blood, aspects such as protein adsorption must be considered. Typically, when a biomaterial contacts blood it initiates the coagulation cascade, platelet activation, and complement activation. Such events interfere with the function of the device through clot formation and can even promote leukocyte adhesion to the biomaterial surface and leukocyte activation (59,60).

If biomaterial particles are much smaller than cell size, leukocytes are able to phagocytose them. On the other hand, if they are much larger than cell size they incite a foreign body reaction, as foreign body giant cells are attracted/produced (61). Downstream immune responses are amplified by cytokine secretion and often end in fibrosis, resulting in total biomaterial encapsulation. Clinically, PGA and PLA have been used as fixation devices in internal fractures, but tissue responses to these materials often result in the presence of macrophages, foreign body giant cells, and fibrous encapsulation (62).

Biodegradability and porosity also affect material biocompatibility. For biodegradable materials, the intensity of host responses are determined by degradation processes which can lead to structural changes, and more importantly particulate formation (63,64). The

porosity of a biomaterial also affects biocompatibility. Porous materials with pore sizes ranging from 100-200 microns promote cell invasion and angiogenesis (61). Specific materials, such as PLLA, have shown that the presence of pores evokes a strong inflammatory response and results in the presence of more foreign body giant cells surrounding the implant (64).

2.3.2 Inflammatory Responses

Inflammatory responses include a cascade of events the body uses to stop additional tissue damage, engulf and destroy foreign entities, and trigger the initiation of the repair process. Straightforward surgeries, such as gallbladder removal, result in inflammation followed by wound healing. However, biomaterials have been shown to stimulate fibrosis, and divert wound healing to scarring.

The first interactions between implanted devices with serum and tissue proteins result in non-specific protein adsorption to the biomaterial surface. Blood proteins, like albumin, fibrinogen, and fibronectin, interact with biomaterial surfaces to form a heterogeneous protein layer. Many of the downstream responses to biomaterials have been attributed to this adsorbed protein layer. Biomaterial surface modification has been suggested to control protein and cell adhesion (65).

Acute inflammation begins as cells migrate to the site of injury. Migration occurs in response to cell-derived cytokines and chemokines, the adsorbed protein layer, and

factors associated with both the coagulation and complement cascade. Proteins adsorbed onto biomaterial surfaces, such as fibronectin, activate macrophages through integrin receptors. Conformational changes in fibronectin, which can occur after adsorption, stimulate TLR4 (66) which, in turn, activates the innate immune system. Macrophage activation can also occur via complement. Biomaterial surfaces adsorb C3 inducing conformational changes which lead to complement activation and expression of surface bound C3b, C5b, and soluble C5a (67). Receptors on macrophage surfaces recognize and bind to adsorbed complement, as well as biomaterial-surface bound IgG and IgM (68). Summoned by these events, macrophages (as well as neutrophils, which arrive first) bind to implanted biomaterial surface-adsorbed opsonins (C3, IgG, and fibronectin). This results in a number of possible outcomes, including cell attachment and activation, secretion of inflammatory mediators (which include IL-1β, TNF-α, and IL-6 (69)), reactive oxygen intermediates, release of proteolytic enzymes, and phagocytosis of the implant (63).

The chronic phase of inflammation is less uniform than the acute phase. This healing response is initiated by macrophages and results in fibroblast proliferation, collagen and proteogylcan synthesis, and angiogenesis. In the case of biomaterial implants, macrophage phagocytosis of degradation products leads to the formation of foreign body giant cells, which has shown to be IL-4 dependent (70,71).

Typically in the late stages of the wound-healing process, fibroblasts respond to repair damaged tissue. With biomaterial implantation, this response results in fibrosis and often total encapsulation by collagen and proteoglycans (72).

As this section illustrates, inflammatory and wound healing responses to biomaterial implantation often results in fibrotic encapsulation, which effectively reduces the function of tissue-engineered devices.

2.3.3 Immune Responses to the Cell Component

To restore proper biological function, it is important that tissue-engineered devices contain appropriate cell numbers (varying with application) that express the desired phenotype, and that these aspects are maintained for the length of time the device is to be used. Host responses to the cellular components of engineered implants can effectively reduce cell numbers, alter phenotypes, and reduce the functionality of tissue-engineered devices.

As mentioned previously, immunological responses can be diminished and potentially avoided by using autologous cells. It has been shown through bone grafting that autografts are more successfully incorporated into patients when compared to allografts. Allografts evoke both local and systemic immune responses that diminish or destroy assimilation processes (73). Since the extracellular matrices of the autografted and allografted bone were essentially the same, the elicited immune responses can be directly

attributed to the cells contained within the grafts and, thus, illustrate the immunological differences between autologous and allogeneic cells. Although autologous cells have shown to be essentially non-immmunogenic, other studies have suggested that the cellular components released upon cell death can cause undesired immune responses (63).

Currently, the possibility of using autologous cells for most applications does not exist – due to the lack of proper culturing techniques and availability of required cells. Hence, allogeneic or xenogeneic cells become important cell sources. When incorporated into devices, preventative measures must be taken to avoid immune rejection; such measures include immune suppression, induction of tolerance, and immunomodulation. A large fraction of the immunogenicity towards a graft can be attributed to the presence of cells. In the case of allografts and xenografts, the resulting responses often end in immune rejection. The following sections focus on allogeneic cell transplants and incorporation of such cells in tissue-engineered constructs, as that is the focus of this thesis.

2.3.3.1 Allogeneic Responses

When an allograft is transplanted, most molecules associated with the transplant are compatible. However, MHC molecules, due to their polymorphic nature, are usually not. Polymorphic differences between donor and host-MHC molecules have shown to activate 1-7% of naïve T cells (74). Early experimental skin grafts between different mice strains

(including T cell deficient nude mice) illustrated T cell mediation of rejection processes. T cell mediated rejection is classified into two categories: acute and chronic rejection.

Early rejection processes are referred to as acute rejection, and occur within days or weeks. Clinically, acute rejection is regarded as the most serious threat to allograft survival for it poses a very immediate danger. Anti-rejection drugs have been employed as preventatives in acute rejection, and have been shown to work effectively by interfering with lymphocyte proliferation (1). Since they leave patients immunosuppressed, anti-rejection drugs have been shown to impose many risks to the organ recipient – risks that include opportunistic infection, anaphylactic reactions, and abnormal wound healing.

Typically, chronic rejection occurs over years. Characterized by a fibrotic response, chronic rejection is associated with cumulative damage involving a series of time-dependent immune (and non-immune) processes that result in graft rejection (75). This latter stage of rejection does not seem to be affected by immunosuppression, and is thus a serious problem.

2.3.3.2 Mechanisms of Immune Rejection

Within rejection processes, there are two pathways in which T cells can be activated. The direct pathway plays a dominant role in acute or early allograft rejection, while the indirect pathway is central to the understanding of chronic rejection.

Direct recognition involves host T cells directly recognizing allo-MHC. T cell receptors have been designed to react with self-MHC plus foreign peptide, but through direct pathways can cross-react with donor-MHC (with or without peptide). Foreign-MHC molecules presenting host-self peptides can react with both host CD8+ and CD4+ T cells (Figure 2.5). Allo-MHC molecules have shown to cross-react 1-7% of naïve T cells (74).

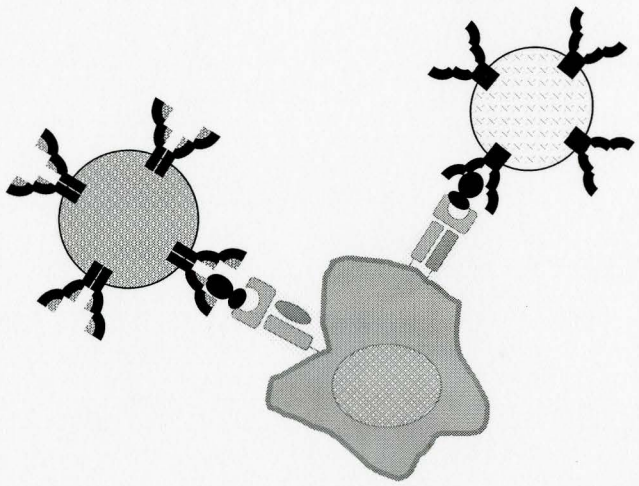

Figure 2.5: Direct recognition. Foreign cells (egg-like cells) are recognized directly by CD8+ and CD4+ T cells through self-peptide in foreign-MHC I and II complexes (respectively).

In addition to MHC-TCR interactions, co-stimulatory signals are required for T cell recognition and activation. The presence of donor APCs is a large factor in direct CD4+ T cell mediated recognition, because APCs possess the proper surface costimulatory and MHC class II molecules (51,76).

The role of costimulatory molecules in T cell activation has been shown with the effect of anti-B7 antibodies (mAbs). The family of B7 surface molecules has been identified on APCs, and implicated in APC costimulation and T cell activation. Administration of anti-B7 mAbs has been shown to delay CD4+ T lymphocyte infiltration into a graft and effectively prolong allograft survival, thus illustrating the immunosuppressive effects *in vivo* of such mAb treatment (77-79) and the need for costimulation in T cell activation.

Indirect recognition is comparable to extracellular pathogen recognition. Antigens shed from the allograft are phagocytosed by host-APCs and presented in the host-MHC II binding cleft. Host T cells recognize the peptide as non-self, and with proper costimulation (provided by host-APCs) activate CD4+ T-helper cells (Figure 2.6).

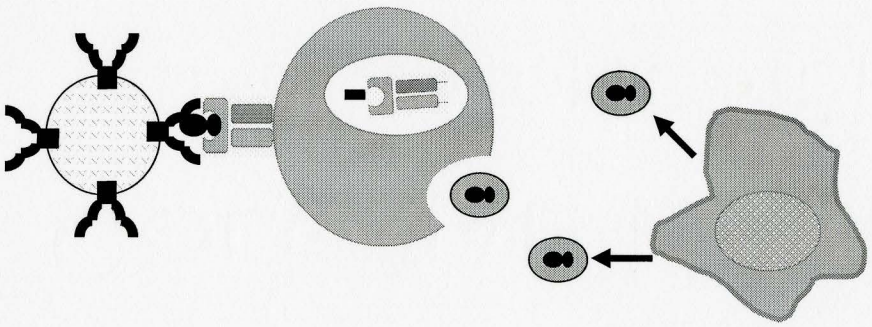

Figure 2.6: Indirect recognition. Recognition occurring through antigens shed from foreign cells (egg-like cells). CD4+ cells recognize foreign peptides in host-MHC class II molecules.

Another indirect pathway of allograft recognition is known as cross-priming (80). Once again, this indirect process is facilitated through donor antigens shed from the graft and phagocytosed by host-APCs. In cross-priming, antigens shed from the allograft bind to host-MHC class I molecules where they can be recognized by CD8+ T cells. With the proper APC costimulation, CD8+ cells can be primed to respond directly to the foreign parenchymal cells (Figure 2.7).

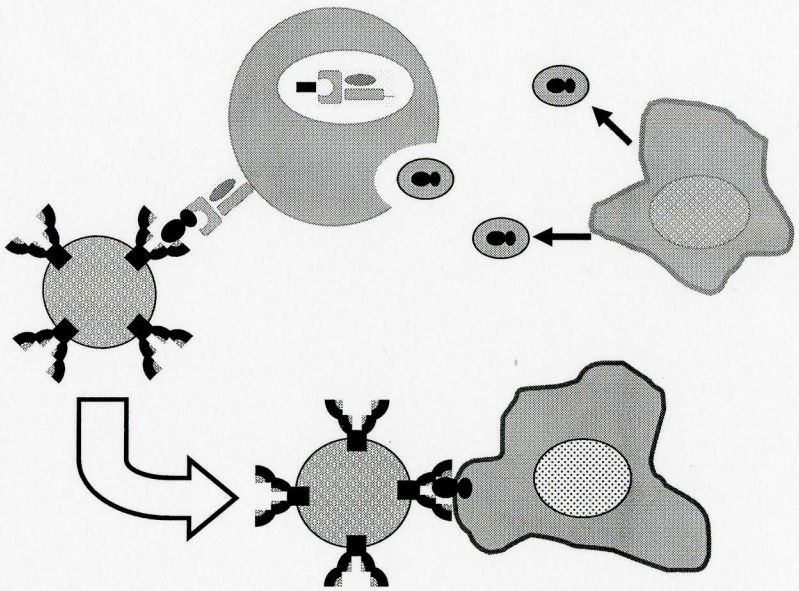

Figure 2.7: Cross-priming. Recognition occurring through antigens shed from foreign cells (egg-like cells). CD8+ cells recognize foreign peptides in host-MHC class I molecules, and are primed to respond directly to foreign cells.

The exact mechanism for CD8+ T cell cross-priming has yet to be fully defined, but SCID mice have been used to demonstrate the direct participation of CD8+ T cell cross-

priming in allograft rejection. Allogeneic SCID recipients receiving MHC class I-deficient knockout allografts and purified CD8+ T cells were shown to reject grafts. Thus, rejection can be strictly associated with CD8+ T cells and host-APC/MHC recognition. Further analysis detected CD8+ T cells in the recipient lymphoid organs and skin graft (81).

2.3.3.3 Th1/2 deviation

As a consequence of foreign antigen recognition, CD4+ T cells are stimulated to secrete cytokines. Depending on their cytokine secretion profile, helper T cells can be classified as Th1 or Th2 cells. The degree of Th1/Th2 polarization is dependent on the nature of the immune responses, and the actions of the cytokines secreted after naïve lymphocyte activation. Th1/Th2 biases have been shown to play a role in transplant rejection; the cytokine profiles associated with transplanted allografts have helped identify some key mediators of graft rejection.

Th1 cells promote cell-mediated immunity and produce the characteristic cytokines IL-2, IFN-γ, and TNF-β. Th1 cells stimulate CTLs, delayed-type hypersensitivity, and IgG2a, often resulting in cytotoxicity. Th2 cytokines lead to eosinophilic and allergic–type responses, as well as T cell dependent B cell responses. Cytokines associated with Th2 responses include IL-4, IL-5, IL-6, IL-13, and IL-10. Th2 cytokines are also associated with anti-inflammatory actions (e.g. IL-4, IL-13, and IL-10) and immune regulation (82).

Experimental evidence associates Th1 responses with allograft rejection and Th2 responses with allograft acceptance (83,84). *In utero* transplantation (IUT) studies have explored the role of CD4+ T cells and cytokines in developing tolerance versus rejection. Results showed that tolerant Balb/c mice secreted higher levels of IL-4, and non-tolerant animals produced higher levels of IFN-γ (85). Although controversial, this illustrates that shifting from a Th1 to Th2 response may prevent allograft rejection.

Allografts are occasionally accepted naturally. This phenomenon has been attributed to CD4+ CD25+ T cells, also known as regulatory T cells (Treg cells) (86,87). Treg cells have been shown to produce an array of cytokines, many of which are associated with Th2-type responses - further supporting the notion of Th2 allograft acceptance. Many researchers have seen the utility of Treg cells, and strive to induce allograft tolerance by priming naïve T cells to become Treg cells (88). Such research focuses on blocking costimulatory pathways in the production of Treg cells.

As discussed, immune responses to tissue-engineered constructs can be associated with both biomaterial and cell components. The biomaterial may initiate a host response upon implantation, but the response to the cellular components often results in rejection processes that can be mediated by cytokines. If researchers can characterize the effect of the biomaterial component and host responses to implanted cells, they can optimize graft design and possibly combat rejection.

2.3.4 Interconnections: Material & Cell Responses

When implanted together, the host response to biomaterial-cell constructs is not a simple sum of the inflammatory response to the biomaterial and T cell mediated response to the transplanted cells. The possible interconnections between the responses elicited by the biomaterial and cell components of tissue-engineered devices shall be discussed in this section.

The current literature on encapsulated cells provides some insight on the possible interactions between the host response elicited by biomaterial-cell transplants. Early research suggested that polymer capsules provided a physical barrier between the host environment and encapsulated cells, and thus prevented immune contact with immunoglobulin, complement components, leukocytes, and lymphocytes (89-91). However, recent research suggests cell encapsulation does not effectively prevent strong recipient responses (4,5,10,92). This indicates that antigens shed from biomaterial-capsule surfaces or encapsulated cells (that diffuse through the capsule wall) are responsible for encapsulated cell death. These findings from tissue-engineered encapsulation devices can be potentially applied to the understanding of how inflammatory responses elicited by biomaterial scaffolds can influence the immune responses towards the cell components, and vice versa.

In response to biomaterial-dependant activation, leukocytes can secrete highly reactive intermediates, including lysosomal enzymes, oxygen (e.g. H_2O_2), and nitrogen (e.g. NO).

These free radicals usually function directly at the site of secretion, but have been implicated in non-specific lysis of embedded foreign cells. *In vitro* studies demonstrate that NO secreted by activated macrophages are responsible for the destruction of islets despite encapsulation in alginate (93). Therefore, lysis independent of cell contact poses another potential problem for tissue-engineered constructs.

The possibility exists for antigens to be shed from the cell components of tissue-engineered constructs. Foreign secreted antigens could range from a variety of cellular components to the therapeutic agent intended for delivery. Once shed, antigens diffuse through the biomaterial scaffold into the host environment where they can be recognized by host immune cells (94). Innate-type immune responses can then be elicited directly towards a static biomaterial scaffold, including phagocytosis by foreign body giant cells and fibrosis (95).

Providing further insight on the possible interactions between the host responses elicited by biomaterial and cell transplants is the current research on adjuvants. These studies have shown that biomaterials can act as adjuvants. Not having any specific antigenic effect themselves, biomaterials can stimulate the immune system and increase the response to simultaneously implanted cellular components (96-98). This increase in antigenicity towards cellular components can result in the sensitization of host immune cells, as well as rejection processes. Providing a path for CD4+ T cell activation, several

studies have shown that biomaterials induce dendritic cell maturation and up-regulation of MHC class II and costimulatory molecules (99,100). Involved in APC and lymphocyte recruitment, biomaterial implantation has been associated with pro-inflammatory cytokine secretion and translocation of NF-κB (a transcription factor which regulates various genes involved in inflammatory responses) (101). Studies have also shown that implantation of ovalbumin (OVA), a model antigen, incorporated into a polymeric biomaterial resulted in a time-dependant systemic humoral immune response towards the released OVA (102,103). This humoral immune response towards the incorporated antigen was found to be primarily Th2-dependent, as indicated by the predominance of the IgG1 isotype. On the other hand, several studies have associated the resultant adjuvant dependant response with a Th1-type response and the production of IFN-γ (104,105). Although researchers have shown the specific responses to vary, it appears that biomaterials can act as adjuvants in the development of an adaptive immune response to co-delivered antigens.

The current research on encapsulation and biomaterial adjuvants has shed some light on the interactions between foreign body responses elicited by biomaterial scaffolds and immune responses towards cells. It is my hope that the experimental work in this thesis will bring tissue engineers one step closer to understanding and characterizing these interactions.

CHAPTER 3

PROJECT SCOPE

In this study, Balb/c splenocytes were exposed to irradiated L929 cells (allogeneic cells from C3H mice) in the presence of different biomaterials. Biomaterials were either in a soluble or solid microsphere form (Table 4.1). Adaptive immune responses to the L929 cells produced by Balb/c splenocytes were then quantified by T cell proliferation and cytokine release. I hypothesized that the biomaterial treatment altered the first set response to the allogeneic challenge, and that this alteration differed between biomaterials. Specifically, it was hypothesized that the adaptive immune responses would be greater for non-mammalian polysaccharides than synthetic polymers. Measured cytokines would also help determine if T cells were polarized to elicit a Th1 or Th2 response.

By depleting splenocyte suspensions the roles that various cell types play in the first set response was also studied. A 0.1% alginate treatment was used as the basis of this comparison. Naïve splenocyte suspensions were depleted of: (1) adherent macrophage cells (antigen presenting cells), (2) CD4+ T cells, and (3) CD8+ T cells. Previous studies have indicated that direct recognition plays a vital role in allogeneic transplant rejection. Mediated by CD4+ and CD8+ T cells, not requiring host-APCs, direct recognition has been associated with the acute and more immediate phase of rejection. Due to the lack of MHC class II expression on implanted allogeneic cells, it was hypothesized that cell

recognition would occur directly via CD8+ T cells. Therefore, splenocyte suspensions depleted of CD8+ T cells would show a dramatic reduction in proliferation. Also playing a role in CD8+ T cell mediated recognition is the indirect pathway known as cross-priming. Facilitated through donor antigens that bind to host-MHC class I molecules, the effect of cross-priming would be seen in adherent macrophage and CD8+ depleted suspensions. Similarly, the effect of indirect activation of CD4+ T cells by host-APCs would be seen by CD4+ depletion.

By characterizing the interactions between inflammatory responses to the biomaterial component and immune responses to the cellular component, we can optimize the design of tissue-engineered constructs. Guides to material selection will allow for safer biomedical devices, more effective drug delivery, and enduring graft survival.

CHAPTER 4

MATERIALS AND METHODS

4.1 Animals

Male Balb/c mice (~4 weeks old, Charles Rivers Laboratory, Wilmington, MA) were allowed to acclimate to their new environment for 1-3 weeks prior to sacrifice. Mice were housed (5 per cage) in the Central Animal Facility at McMaster University. Animal care and treatment were in compliance the Animal Usage Protocol # 05-01-02.

4.2 Tested Materials

Seven different materials were studied in either a soluble or solid microsphere form (Table 4.1). Material selection was based on potential application in tissue engineering. Endotoxin levels were determined in duplicated by Limulus Amebocyte Lysate Test (Cape Cod Incorporated, East Falmouth, MA), as per the manufacturer's instructions.

Biomaterials	**Soluble Form**	**Microsphere Form**
Chitosan	x	x
Alginate	x	x
Agarose	x	-
Poly(methyl methacrylate)	-	x
Polystyrene	-	x

Table 4.1: Materials investigated.

4.2.1 Soluble Materials

Soluble materials were made at a 1% concentration. Alginic acid sodium salt (Sigma, St. Louis, MO) was added to endotoxin free water, and stirred until dissolved. Then the solution was filtered with a 0.44 μm, followed by 0.20 μm syringe filter (PALL Life Sciences, East Hills, NY). Chitosan (Aldrich, St. Louis, MO) was added to acetic acid, and stirred until dissolved. Agarose (Sigma, St. Louis, MO) was dissolved in distilled water and autoclaved, facilitating the dissolution process. The final concentration used within splenocyte challenges for all soluble materials was 0.1% (w/v).

4.2.2 Microspheres

Alginate and chitosan microspheres were fabricated using an emulsification process (106,107), refer to Appendix A for the procedure. Particle size was determined using a mastersizer (Appendix B), and found to be approximately 1μm for both alginate and chitosan microspheres. The initial concentrations of the microsphere solutions were determined using the hemocytometer, and found to be approximately 10^8 microspheres/mL. To ensure cell-bead contact, the number of microspheres used within splenocyte challenges for these 1 μm diameter beads were 10 particles per splenocyte.

Polystyrene and Poly(methyl methacrylate) microspheres (Polysciences, Inc., Warrington, PA) had particle sizes of 0.35 μm and 0.30 μm, respectively. Both synthetic microspheres were sterilized by irradiation (2 Mrad over 24 hours, with the help of

Robert Pasuta from the McMaster Nuclear Reactor Facility), as per manufacturer's instructions. As these particles had a diameter one third the size of the emulsified microspheres, three times as many particles were used per splenocyte.

4.3 Splenocyte Challenge Reactions

Balb/c mice were sacrificed by cervical dislocation under anaesthesia. Their spleens were removed and placed in 1 mL of ice cold phosphate buffered saline (PBS). Cells in the spleens were dissociated into a single cell suspension using a tissue grinder. Erythrocytes were separated from the cell suspension using Lympholyte-M gradient (Cedarlane Laboratories), as per manufacturer's instructions. The buffy coat, containing mononuclear cells, was carefully pipetted into a new centrifuge tube, suspend in 10 mL of RPMI 1640 (10% FBS, 1% penicillin-streptomycin, 50 μM β-mercaptoethanol), and centrifuged at 800 g for 20 min. Splenocytes were washed three times and resuspended in RPMI 1640 at 1.666 x 10^6 cells/mL (cell counting was done using Trypan Blue and a hemocytometer). These cells (60 μL) were added to a flat-bottom 96-well plate in quadruplicate and incubated with either:

- medium (120 μL) and endotoxin free water (20 μL),
- medium (120 μL) and material solution (20 μL),
- medium (20 μL), L929 cells (100 μL), and endotoxin free water (20 μL), or
- medium (20 μL), L929 cells (100 μL), and material solution (20 μL).

L929 cells (ATCC, Manassas, VA) were irradiated (2500 rad), and suspended to 1 X 10^5 cells/mL. The splenocytes were incubated for 72 hours at which a time resolved fluorescence-BrdU assay (DELFIA® Assay, PerkinElmer, Wellesley, MA) was used to assess splenocyte proliferation, as per the manufacturer's instructions with the following modification: 1 hr incubation at 60°C after initial centrifugation and medium removal.

In some cases, Balb/c splenocytes were depleted of either: (1) adherent macrophage cells (antigen presenting cells), (2) CD4+ T cells, or (3) CD8+ T cells. Adherent macrophages were depleted by incubating splenocyte suspensions for 3hrs on a tissue culture dish to remove adherent cells. CD4+ and CD8+ T cells were depleted using purified Anti-Mouse CD4 and CD8a Monoclonal Antibody (respectively) which bound CD8 and CD4 surface molecules. Low-Tox Rabbit Complement was then used for the cytotoxic destruction of antibody bound T cells. The products used for T cell depletions were purchased from Cedarlane Laboratories (Hornby, Ontario), and the protocol used was as per the manufacturer's instructions.

4.3 Cytokine ELISAs

Duplicate plates were used to measure for cytokines elicited during splenocyte challenges. After 48 hours, plates were centrifuged at 300 g for 10 min. Then 120 μL of the supernatant from each well was transferred to a new plate and frozen for later analysis. Levels of IL-1β, IL-4, IL-12, and IFN-γ were determined in duplicate by

sandwich ELISA (BD OptEIA™ Mouse IL-1β, IL-2, IFN-γ, and IL-4 sets) with dilutions where necessary, as per manufacturer's instructions. IL-1β was chosen due to its pro-inflammatory effects and role in rejection processes. IFN-γ, IL-4, and IL-12 were chosen as signature cytokines of the Th1/2 subsets.

4.4 Optimizing Responses

In order to determine the optimum times for pre-BrdU and BrdU incubations, two preliminary studies were performed. The highest fluorescence was shown at 72 hr pre-BrdU incubation, and 24 hr BrdU incubation (data not shown). In addition, preliminary splenocyte challenges showed highest cell responses at a seeding density of 1×10^5 responder cells and 1×10^4 stimulator cells per well (data not shown).

4.5 Data Analysis

Statistical analysis was performed using t-tests and ANOVA general linear modeling with Microsoft Excel's Data Analysis Tool-Pack. P-values of ≤ 0.05 were designated as significant.

CHAPTER 5

RESULTS

5.1 Splenocyte Challenges

As researchers move towards developing tissue-engineered constructs that incorporate both biomaterials and cells, the interconnections between the host responses elicited by both components become increasingly important. This experimental work was designed to characterize the effect of different biomaterials on adaptive immune responses towards an allogeneic challenge. It was hypothesized that biomaterial treatment altered the first set splenocyte response to a L929 cell challenge, and that this alteration differed between biomaterials. Adaptive responses were quantified by increased splenocyte proliferation and cytokine release.

Analysis of proliferation from challenged splenocytes in the presence of various material treatments highlighted several trends (Figure 5.1). The proliferation from challenged splenocytes for all material treatments was not significantly different from the challenged control (consisting of no material treatment), with the exception of the soluble alginate treatment. Within the soluble alginate treatment group, challenged and unchallenged splenocytes proliferation were approximately the same. To determine non-specific biomaterial effects, proliferation of unchallenged splenocytes from all material treatments were compared to the unchallenged control (consisting of no material treatment). Significant differences in proliferation were seen among several material treatments,

suggesting that material treatments elicited non-specific splenocyte proliferation. To further investigate the degree to which biomaterials affected specific proliferation, a graph representing the differences between unchallenged and challenged proliferation was created (i.e. challenged proliferation results were subtracted by unchallenged results). The soluble alginate treatment showed a significant decrease in proliferation differences when compared to the control (Figure 5.2), indicating that the soluble alginate treatment had a strong effect on specific splenocyte proliferation.

To examine the effect that material structure had on splenocyte proliferation towards an allogeneic challenge, the results obtained from soluble versus solid (microsphere) treatments were compared. Initially, chitosan and alginate treatments were used as the basis for this comparison (i.e. the chitosan microsphere treatment was compared to the chitosan soluble treatment). This produced conflicting results, as the chitosan comparison appeared to support the notion that microspherical treatments increased splenocyte proliferation, while the alginate comparison did not (Figure 5.3). With the exception of the soluble alginate treatment, which showed relatively high splenocyte proliferation (challenged and unchallenged), analysis of proliferation suggests that solid microsphere treatments increased splenocyte proliferation toward the L929 cell challenge in comparison to soluble treatments.

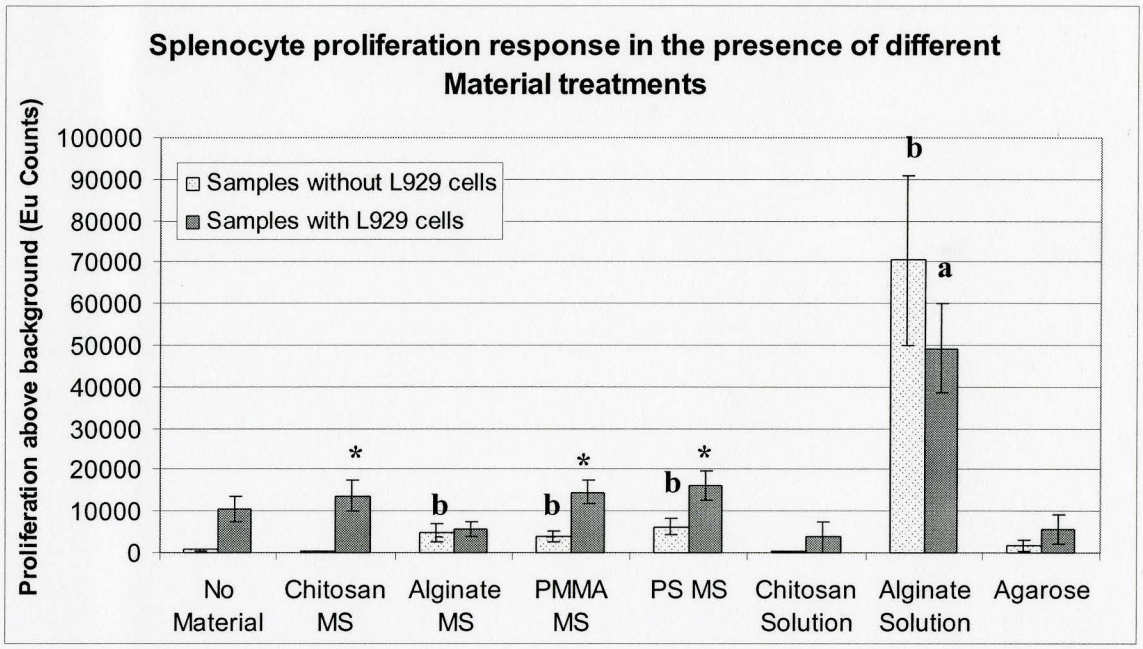

Figure 5.1: Splenocyte proliferation response to L929 cells in the presence of different material treatments. The experimental work represented in this graph was performed to investigate the effects different biomaterials had on splenocyte proliferation towards an allogeneic challenge (first set proliferation). Error bars are standard error of the mean; n=5 full material trials, 4 replications of each treatment within each plate; "" corresponds to significant differences between challenged and unchallenged treatments; "a" corresponds to significant differences between challenged material treatments and the challenged control; "b" corresponds to significant differences between unchallenged material treatments and the unchallenged control; significant differences are denoted as* $p \leq 0.05$.

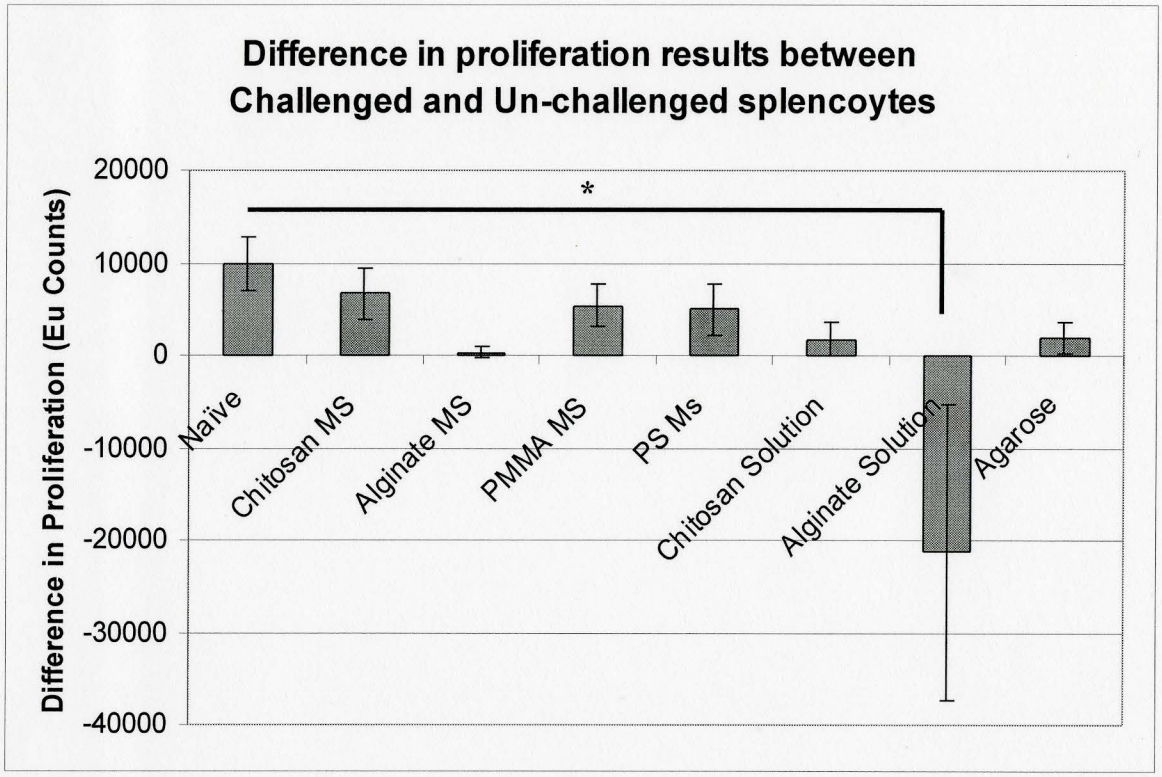

Figure 5.2: Difference in proliferation results between challenged and unchallenged splenocytes for all material treatments. The results represented in this graph were obtained by subtracting the unchallenged proliferation results from the challenged results for each material treatment. Error bars are standard error of the mean; n=5; "" corresponds to significant differences denoted as* $p \leq 0.1$.

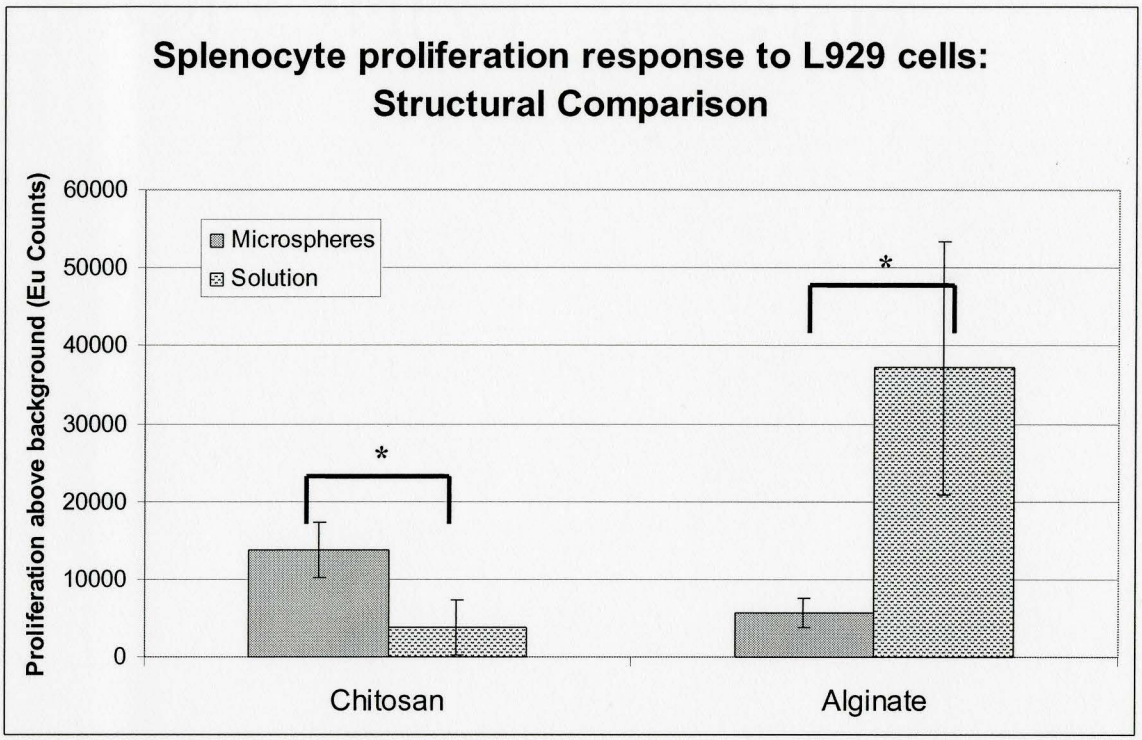

Figure 5.3: The influence of material structure on splenocyte proliferation in response to L929 cells. Chitosan and alginate were used to compare the effect material structure had on splenocyte proliferation towards an allogeneic challenge. The result obtained from the soluble form of each material was compared to the microsphere form. Error bars are standard error of the mean; n=5 material treatments, 4 replications of each treatment within each plate; "" corresponds to significant differences denoted as $p \leq 0.1$.*

To determine the effect that material composition had on splenocyte proliferation towards an allogeneic challenge, the results from non-mammalian polysaccharide and synthetic polymer treatments were pooled (Figure 5.4). To eliminate structural effects, only solid microsphere treatments were pooled. It was initially hypothesized that non-mammalian polysaccharide treatments would show increased splenocyte proliferation toward the allogeneic challenge. However, analysis of proliferation from pooled results was not significantly different; the trend indicated that the synthetic polymer group increased splenocyte proliferation in response to L929 cells.

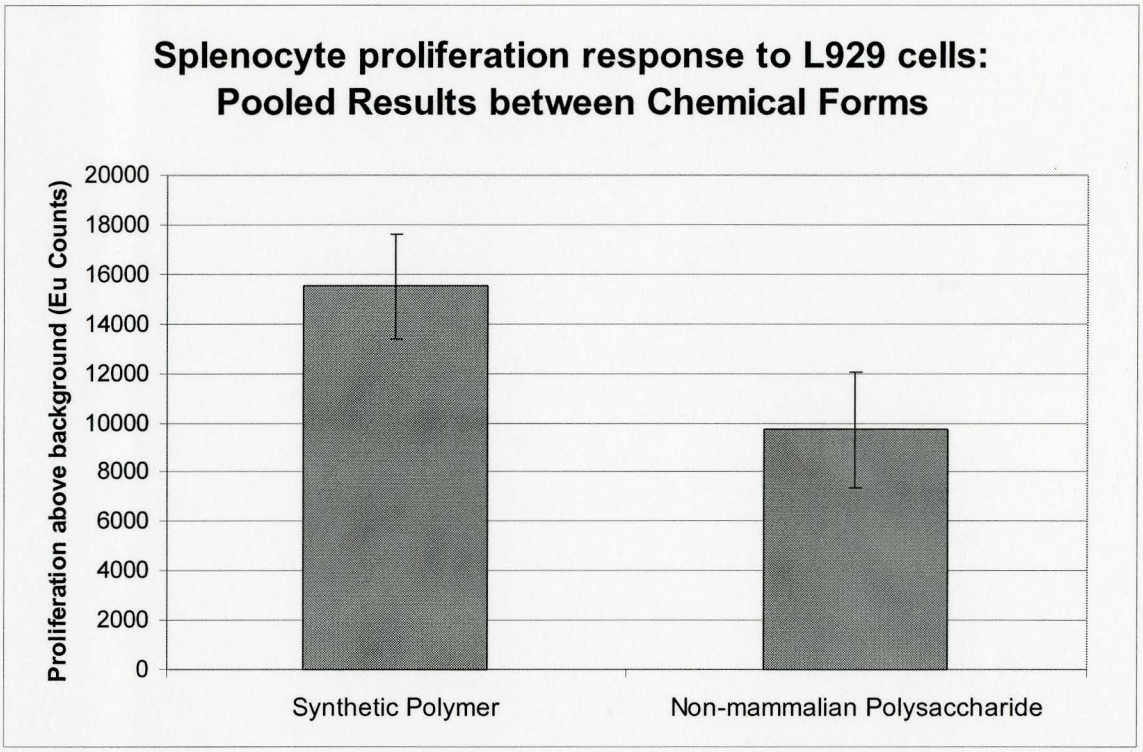

Figure 5.4: Pooled splenocyte proliferation in response to L929 cells for microspheres of non-mammalian polysaccharide and synthetic polymer treatment groups. The results represented in this graph were obtained by the pooling of the various groups of materials. To eliminate structural effects, only the proliferation results obtained from the solid microsphere treatments were pooled. Error bars are standard error of the mean; n=10, 4 replications of each treatment within each plate.

5.2 Depleted Splenocyte Challenges

Previous studies have indicated that direct recognition plays a vital role in allogeneic transplant rejection. Mediated by CD4+ and CD8+ T cells, not requiring host-APCs, direct recognition has been associated with the acute phase of rejection. Due to the lack of MHC class II expression on implanted allogeneic cells, it was hypothesized that cell recognition would occur directly via CD8+ T cells. To investigate the roles various cell types played in the first set response naïve splenocyte suspensions were depleted of adherent macrophage cells, CD4+ T cells, and CD8+ T cells prior to challenges. Therefore, it was hypothesized that splenocyte suspensions depleted of CD8+ T cells would show a dramatic reduction in proliferation.

Analysis of proliferation from depleted splenocyte challenges in the presence of a 0.1% soluble alginate treatment illustrated several effects biomaterials had on proliferation responses (Figure 5.5). Proliferation from depleted splenocyte challenges were all significantly different from the non-depleted challenge consisting of no material treatment. This indicates that biomaterial treatment can stimulate the immune system and increase the responses elicited toward foreign cellular components.

Comparisons between depleted challenges were not significantly different from the non-depleted control (challenged by L929 cells and consisting of the alginate treatment). Within the alginate treated groups, no major differences were seen among the various challenges, including challenges depleted of CD8+ T cells.

Within the non-challenged groups, splenocytes depleted of CD4+ T cells proliferated less than non-depleted and CD8+ T cell depleted splenocytes, and significantly less than adherent macrophage depleted splenocytes. Since there were no proliferation differences seen among depleted material and L929 challenged splenocytes, this data implicates CD4+ T cells in non-challenged splenocyte proliferation induced by the presence of a biomaterial (i.e. non-specific proliferation).

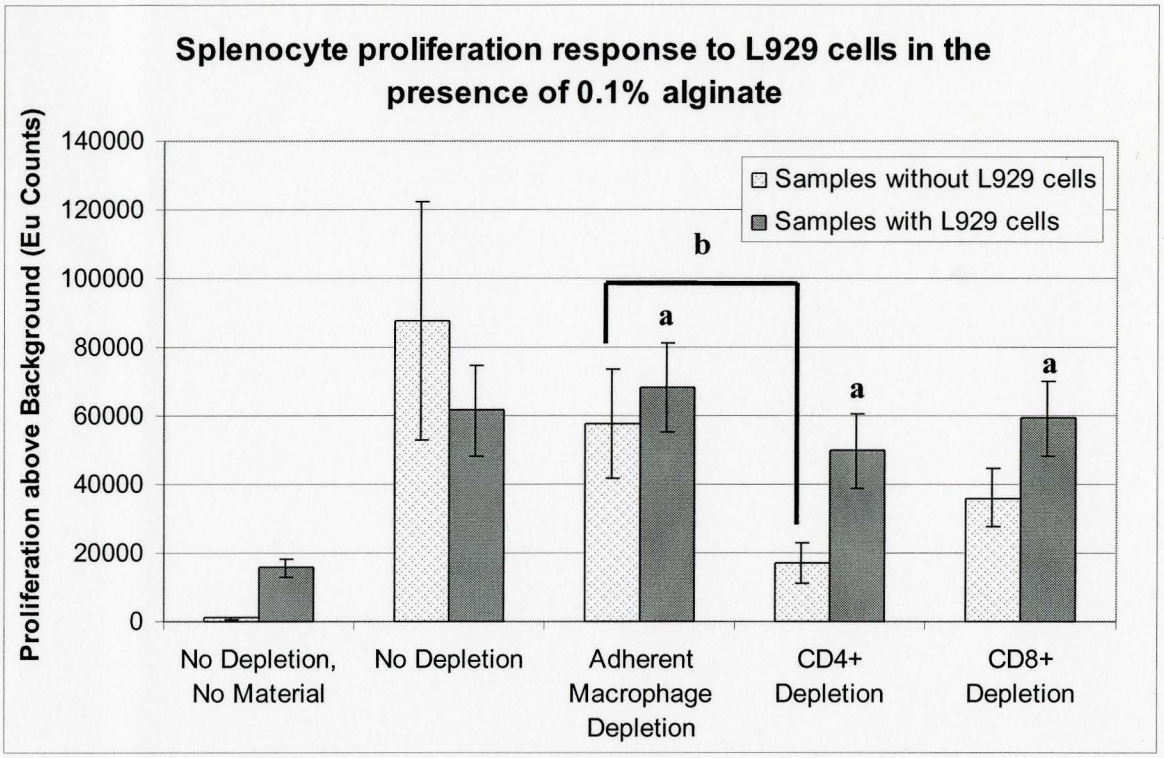

Figure 5.5: Proliferation response to L929 cells in the presence of 0.1% alginate treatment by depleted splenocyte suspensions. The experimental work represented in this graph was performed to investigate the roles various cell types play in the first set response towards an allogeneic challenge. This was done by depleting naïve splenocyte suspensions of adherent macrophage cells, CD4+ T cells, and CD8+ T cells prior to challenges. Error bars are standard error of the mean; n=5 full material trials, 4 replications of each treatment within each plate; "a" corresponds to significant differences between depleted challenges and the non-depleted, non-material treated challenge; significant differences are denoted as $p \leq 0.05$.

5.3 Th1/2 Bias

To help determine biomaterial effects on adaptive immune responses towards an allogeneic challenge, splenocytes treated with various materials were quantified for secretion of IL-4, IL-12, and IFN-γ. These measured cytokines would help determine if T cells were polarized to elicit a Th1 or Th2 response.

IFN-γ, a signature cytokine for the Th1 subset, was found to be produced at undetectable levels (data not shown) for all material treatments.

No significant differences in cytokine production from splenocytes were seen between non-mammalian polysaccharide and synthetic polymer treatments, or solid microsphere and soluble treatments (Figure 5-6-5.7).

High IL-12 levels with low IL-4 levels is consistent with a Th1-type response, whereas the opposite (low IL-12 levels with high IL-4 levels) is consistent with a Th2-type response. However, for this experimental work only slightly higher IL-12 levels were detected for all material treatments (Figure 5.8).

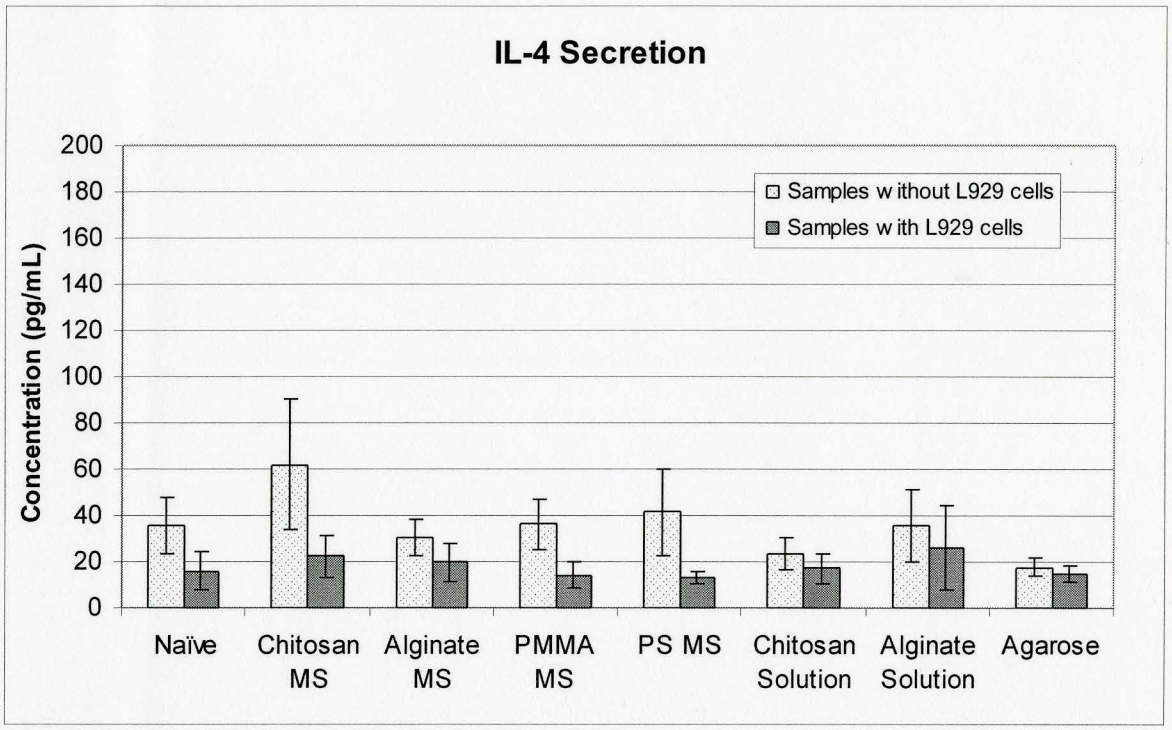

Figure 5.6: IL-4 secretion from splenocytes in the presence of various material treatments. The experimental work represented in this graph was performed to investigate the effects different biomaterials had on IL-4 secretion from splenocyte in the presence of an allogeneic challenge (first set response). IL-4 has been shown to be a signature cytokine of the Th2 subset. No significant differences were seen among material treatments. Error bars are standard error of the mean; n=4-5 full material trials, 2 replications of each treatment within each plate.

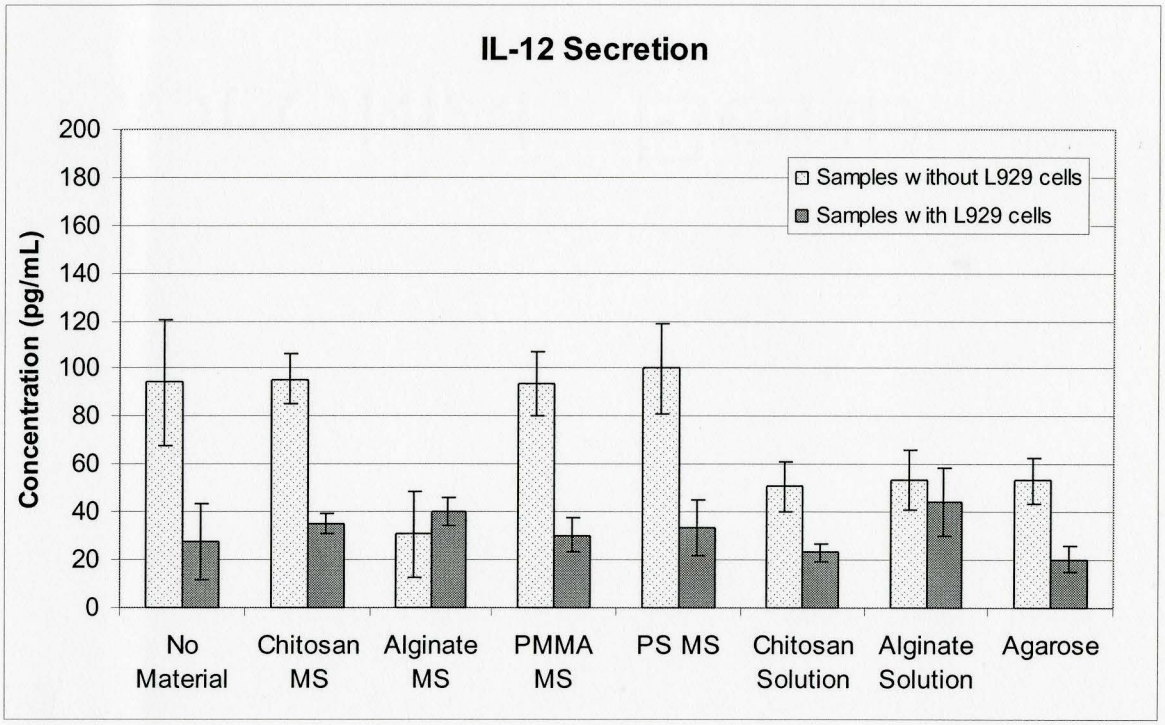

Figure 5.7: IL-12 secretion from splenocytes in the presence of various material treatments. The experimental work represented in this graph was performed to investigate the effects different biomaterials had on IL-12 secretion from splenocytes in the presence of an allogeneic challenge (first set response). IL-12 has been shown to be involved in Th1-type responses. Error bars are standard error of the mean; n=5 full material trials, 2 replications of each treatment within each plate.

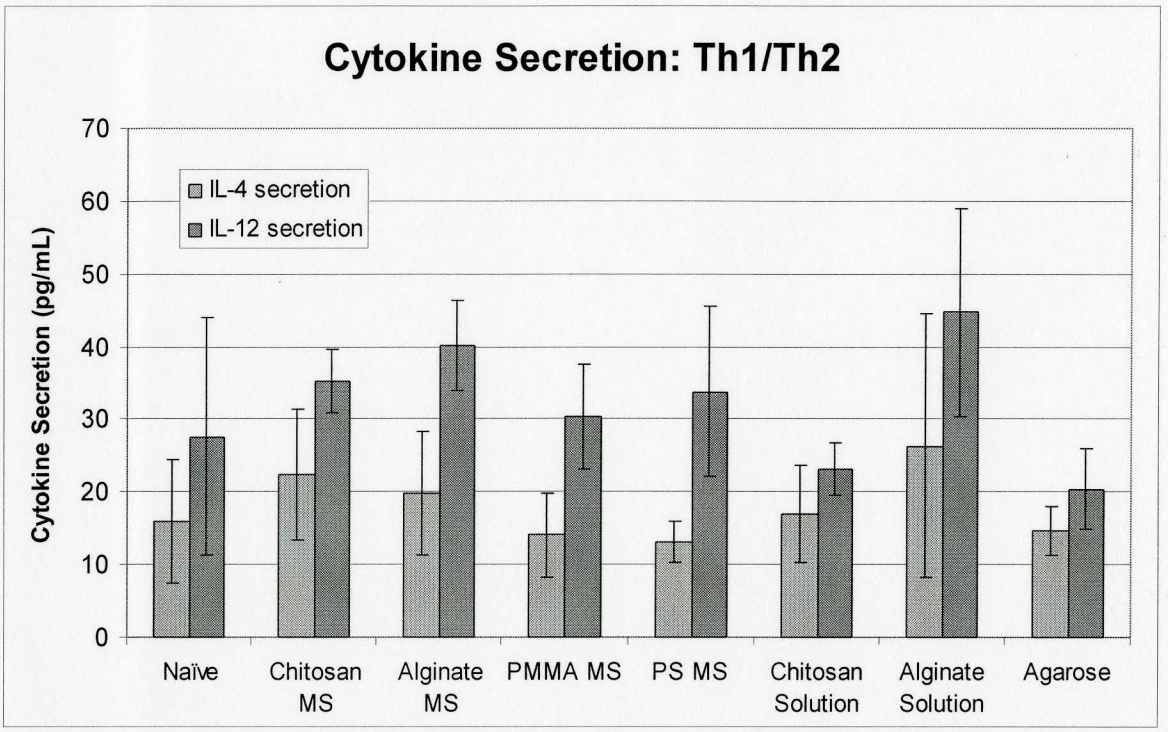

Figure 5.8: Comparing IL-4 and IL-12 secretion from challenged splenocytes in the presence of various material treatments. IL-12 and IL-4 have shown to be involved in Th1 and Th2-type responses, respectively. This graph indicates slightly higher IL-12 secretion levels. Error bars are standard error of the mean; n=4-5 full material trials, 2 replications of each treatment within each plate.

5.4 Pro-Inflammatory Cytokine Secretion

To evaluate the effects of biomaterial treatments on inflammatory responses, splenocyte production of IL-1β (a pro-inflammatory cytokine) was examined. IL-1β has been shown to be a strong mediator of inflammation, NK activation, and T cell co-stimulation, and therefore, has also been implicated in enhancing adaptive immune responses.

When compared to the challenged control (consisting of no material treatment), the production of IL-1β from challenged splenocytes was not significantly different (Figure 5.9). In fact, most materials showed the same subsequent levels of IL-1β production as the challenged control, with a few treatments showing slight higher levels of IL-1β.

IL-1β production was significantly higher for challenged splenocytes treated with chitosan microspheres then those treated with soluble chitosan.

No differences in IL-1β production was seen between challenged splenocytes treated with non-mammalian polysaccharides and synthetic polymers.

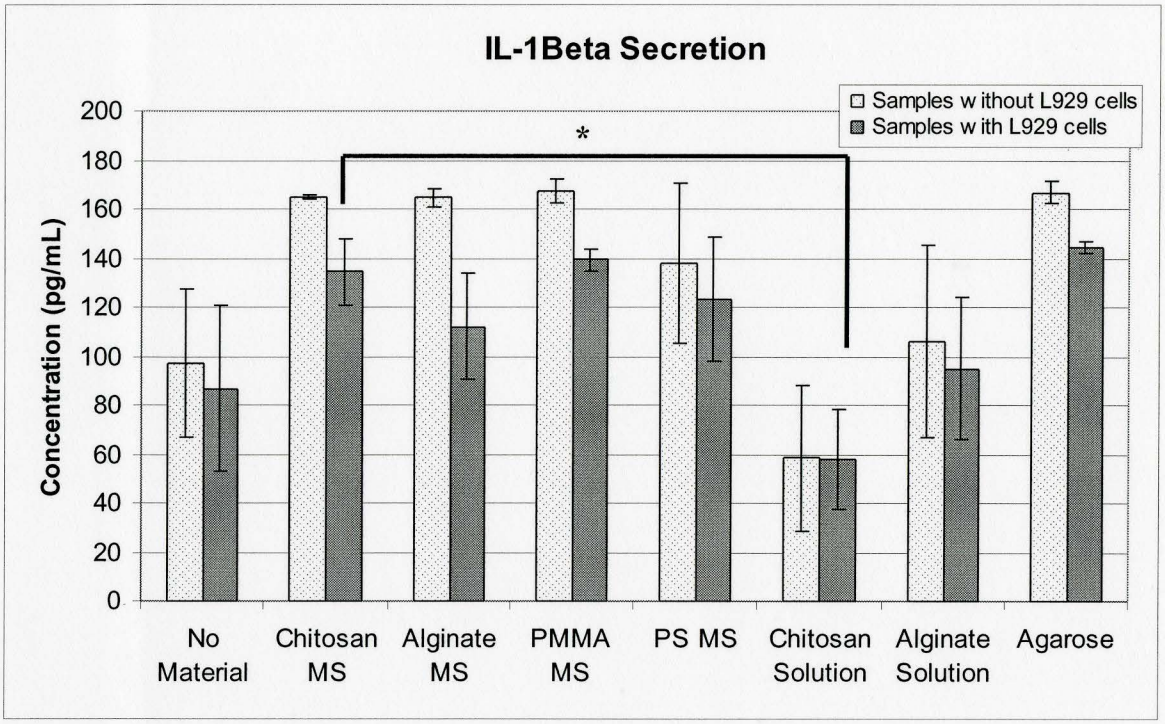

Figure 5.9: IL-1β secretion from splenocytes in the presence of various material treatments. The experimental work represented in this graph was performed to investigate the effects that different biomaterials had on IL-1β secretion from splenocytes in the presence of an allogeneic challenge (first set response). Error bars are standard error of the mean; n=4-5 full material trials, 2 replications of each treatment within each plate; "" corresponds to significant difference between bars at $p \leq 0.05$.*

5.5 Endotoxin Tests

Endotoxin contamination, also know as LPS contamination, is associated with the release of lipopolysaccharide (LPS) from gram-negative bacteria. To examine the effects of endotoxin contamination, material endotoxin levels were quantified (Figure 5.10). Significant differences in endotoxin levels were seen between alginate and chitosan treatments compared between the same structural forms (i.e. the soluble chitosan compared to the soluble alginate treatment), as well as between the same chemical composition in different structural forms. To determine if endotoxin levels directly correlated to an increase in splenocyte proliferation, proliferation was plotted against endotoxin levels (Figure 5.11). As this graph shows, an increase in endotoxin levels did not directly institute an increase in splenocyte proliferation.

The endotoxin results for both polystyrene and poly(methyl methacrylate) microspheres were inconclusive due to high backgrounds resulting from the beads and suspension solution (as the beads and solution were not colourless). They were sterilized as per manufacturer's instruction for the elimination of bacterial and mold contamination.

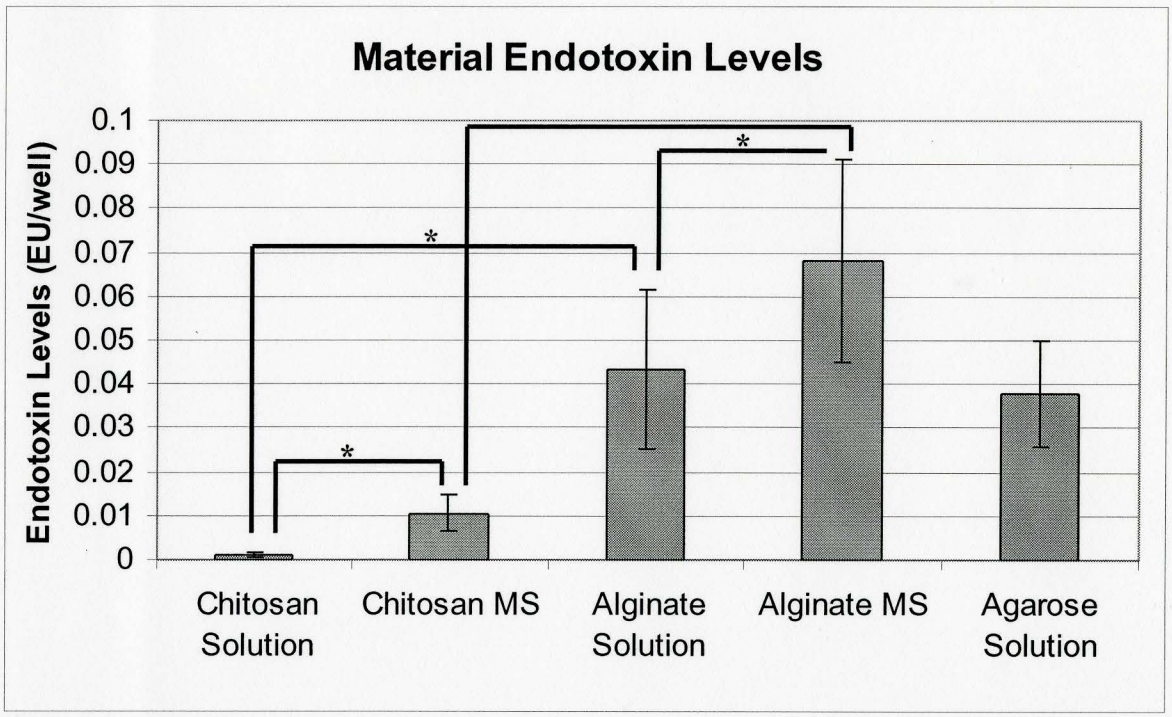

Figure 5.10: Endotoxin results for fabricated materials. The endotoxin levels were quantified to determine if splenocyte proliferation and cytokine profiles could be attributed to the presence of endotoxin. Significant differences were seen among material endotoxin levels. Error bars are standard error of the mean; n=5 full material trials, 2 replications of each treatment within each plate; "" corresponds to significant difference between bars at $p \leq 0.05$.*

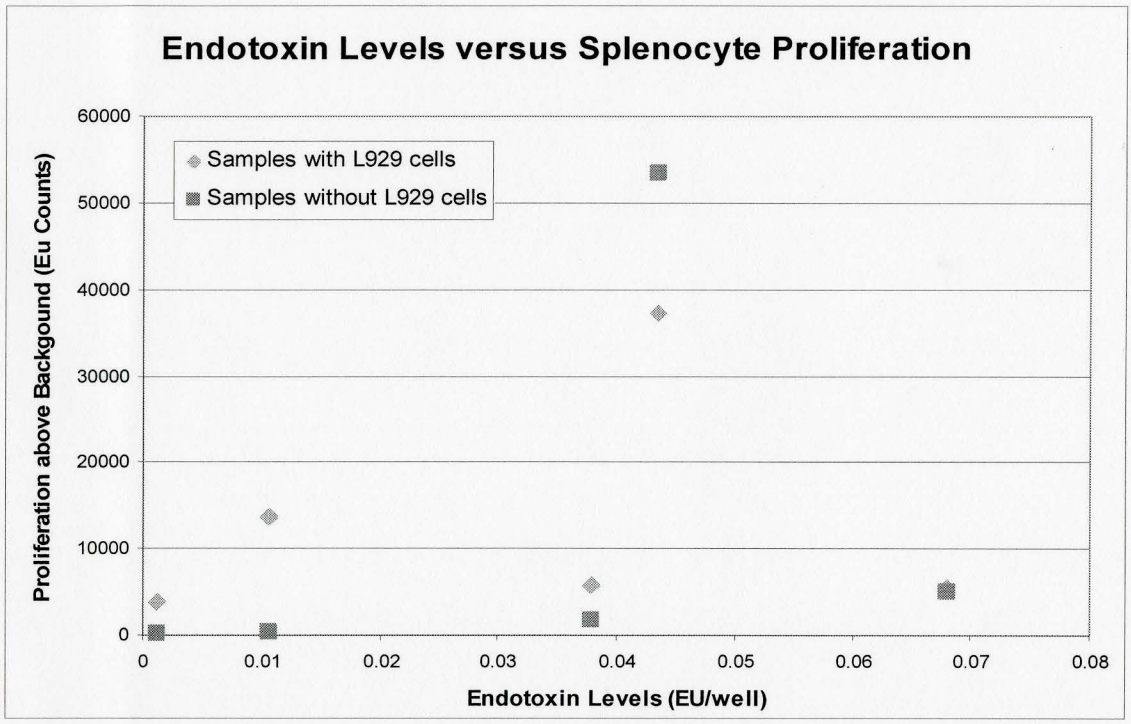

Figure 5.11: Endotoxin results plotted against splenocyte proliferation. This graph was created to determine if splenocyte proliferation increased with elevated endotoxin levels, as significant differences were seen among material endotoxin levels.

CHAPTER 6

DISCUSSION

Biomaterial scaffolds and cell transplants, the two main components of tissue-engineered devices, have both been shown to elicit biological responses when implanted in isolation. Biomaterial implantation has been associated with foreign body responses, and cell transplantation with adaptive responses. When implanted at the same time, the host responses elicited by these components have been shown to influence the other, as seen in cellular encapsulation and biomaterial adjuvant research. The experimental work described in this thesis was designed to characterize the effect of different biomaterials on adaptive immune responses towards an allogeneic challenge. In this study, Balb/c splenocytes were exposed to L929 cells in the presence of different biomaterials (Table 4.1). The adaptive immune responses produced by Balb/c splenocytes were then quantified by splenocyte proliferation and cytokine release. In the following sections the important findings from this experimental work shall be discussed.

6.1 Structural Influences of Materials in Host Responses

To examine if the structure of a biomaterial affects host responses, the results from splenocyte challenges obtained from solid (microsphere) and soluble treatments were compared. Initially, chitosan and alginate were used as the basis for this comparison, because they were present in both soluble and solid microsphere forms. Hence, the chitosan microsphere treatment was compared to the soluble chitosan treatment, while the

alginate microsphere treatment was compared to the soluble alginate treatment (Figure 5.3). This produced conflicting results, as the chitosan comparison appeared to support the notion that microspherical treatments increased splenocyte proliferation, while the alginate comparison did not. With the exception of the soluble alginate treatment which showed relatively high splenocyte proliferation (challenged and unchallenged), analysis of proliferation suggests that solid microsphere treatments increased splenocyte proliferation toward the L929 cell challenge in comparison to soluble treatments. This increase in splenocyte proliferation can be attributed to the relative size of the microspheres.

The microspheres used within these experiments ranged from 0.3 to 1 micron. Previous studies have shown that particles much smaller than cells will be phagocytosed by macrophages, leading to inflammation and further immune responses (61). One studied indicated that titania and polystyrene particles (< 2 microns in size) stimulated macrophages in a size dependant manner (108). Therefore, we can attribute the increase in proliferation to the relatively small size of the microspheres used.

To evaluate the effects of biomaterial structure on inflammatory responses, the production of a pro-inflammatory cytokine was examined. IL-1β was selected since it has been shown to be a strong mediator of inflammatory responses (58,109-111). Significant differences in IL-1β production were seen between challenged splenocytes treated with chitosan microspheres and soluble chitosan (Figure 5.9). This increase in IL-

1β production in the presence of microspheres supports the notion that small biomaterial particles can enhance inflammation and acute phase responses, as seen with biomaterial degradation particulates.

The effect of small biomaterial particles on inflammatory responses has been thoroughly studied in the context of joint replacements. The failure of joint replacements is characterized by macrophage phagocytosis of biomaterial debris that occurs as a result of wear. Biomaterial particulates induce macrophages to release a variety of inflammatory mediators and signaling proteins that lead to bone resorption, which results in loosening of the prosthetic joint (112,113). Analysis of gene expression found that exposure to biomaterial implants resulted in the expression of several genes responsible for pro-inflammatory cytokine production, including the genes for the assembly of TNF-α and IL-1β (110). Biomaterial degradation products associated with joint replacements have also been shown to provoke fibrotic responses (114), probably as a result of increased inflammation and cytokine secretion from invading macrophages.

The increase in proliferation and IL-1β secretion from challenged splenocytes treated with solid microspherical materials can be attributed to the size of the microspheres. Small biomaterial particles increase inflammatory and acute phase responses, and can possibly alter the later responses of the adaptive immune system.

6.2 The Effect of Biomaterials with different Chemical Compositions: Non-mammalian Polysaccharides versus Synthetic Polymers

As with biomaterial structure, it is thought that the chemical composition of a biomaterial influences host responses. In this experimental work, it was hypothesized that treatment with materials of various compositions would alter the adaptive immune responses towards an allogeneic challenge *in vitro*. Specifically, it was hypothesized that the first set response elicited towards the L929 challenge, would be greater for non-mammalian polysaccharides than synthetic polymers.

This hypothesis was made because non-mammalian polysaccharide biomaterials can be composed of carbohydrates that are also present on microorganisms. Several of these common non-mammalian carbohydrates have shown to be perceived by immune cells as pathogen associated molecular patterns in the context of microorganisms (37,38). For example, mannose (a component of alginate) has been shown to activate pathogen recognition receptors on immune cells, resulting in receptor-mediated endocytosis and phagocytosis. It has been shown that when the mannose receptor binds to microorganisms it triggers a variety of intracellular responses, including cytokine secretion, lysosomal enzyme secretion, and modulation of other cell surface receptors (37). Ligation of the mannose receptor has, thus, been associated with innate immunity and inflammation. Therefore, the possibility exists that the carbohydrates composing non-mammalian polysaccharide biomaterials can activate innate responses in a similar

manner. However, innate and adaptive immune activation have been shown to be interlinked. As such the mannose receptor plays an important role in adaptive immune responses. Involved in APC mediated endocytosis and phagocytosis, the mannose receptor in an important pathway to antigen uptake and delivery to MHC class II molecules (37), and has also been implicated in antigen presentation via MHC class I molecules (115,116). Similarly, other natural non-mammalian polysaccharide based biomaterials are likely to interact with various lectins, resulting in similar downstream immune responses. Thus, immune activation by non-mammalian polysaccharides was hypothesized to elicit an increased first set proliferation response towards the L929 cell challenge.

To determine the effect that different materials had on splenocyte proliferation towards an allogeneic challenge, the results from non-mammalian polysaccharide and synthetic polymer treatments were pooled (Figure 5.4). To eliminate structural effects, only solid microsphere treatments were pooled. The polystyrene and poly(methyl methacrylate) microspheres were pooled representing the synthetic polymer group, and the alginate and chitosan microspheres were pooled representing the non-mammalian polysaccharide treatments. It was initially hypothesized that non-mammalian polysaccharide treatments would show increased splenocyte proliferation toward the allogeneic challenge. Although the analysis from pooled results was not significantly different, the trend indicated that the synthetic polymer group increased splenocyte proliferation in response to L929 cells. In terms of cytokine secretion, no differences were seen from challenged

splenocytes treated with either non-mammalian polysaccharides or synthetic polymers (Figure 5.6–5.7, 5.9). These results suggest the first set response elicited towards the L929 challenge was greater for synthetic polymers than non-mammalian polysaccharides.

Although, many synthetic polymers have been developed to have a range of chemical properties (charge, hydrophobicity, and reactivity), they are often immediately recognized as foreign upon implantation (117). Material recognition occurs as plasma proteins adsorb to form a heterogeneous protein layer. Some plasma proteins that have been implicated in this process include fibrinogen, fibronectin, and vitronectin. One study highlighted the importance of fibrinogen in mediating biomaterial induced inflammation to polyester terephthalate (PET) films (118). In the splenocyte challenges described, plasma proteins would be provided by the serum-contained medium used within the challenges.

The increase in splenocyte proliferation towards the L929 cell challenge in the presence of a synthetic polymer treatment, suggests the possibility that protein adsorption to synthetic polymer surfaces plays a strong role in immune activation. In order to overcome protein adsorption associated with synthetic polymer biomaterials, attempts have been made to modify biomaterial surface chemistry (44,65,119). One potential strategy is to 'mask' the synthetic polymer surface with a layer of albumin (120).

6.3 Th1/Th2 Bias

Experimental evidence associates Th1 responses with allograft rejection and Th2 responses with allograft acceptance (83,84). To determine if material treatments elicited either a Th1 or Th2-type response levels of IFN-γ, IL-4, and IL-12 were examined (Figure 5.6 and 5.7).

High IL-12 and IFN-γ levels with a low IL-4 level is consistent with a Th1-type response, whereas the opposite is consistent with a Th2-type response. Analysis of cytokines from challenged splenocytes produced inconclusive data. All material treatments produced undetectable levels of IFN-γ (data not shown), and slightly higher levels of IL-12 than IL-4 (Figure 5.8). These results indicate a possible Th1 bias even though IFN-γ, an important cytokine within the Th1 subset, was undetectable among material treatments.

IL-12 is a cytokine produced by monocytes, and has been shown to initiate Th1 responses by up-regulating CD4+ molecules on T cells and signaling the production of IFN-γ (121). Co-stimulation of APCs, mediated by B7 molecules, has been shown to work with IL-12 for the optimal production of IFN-γ (122). Therefore, IL-12 is a potent inducer of IFN-γ, and suggests the possibility that IL-12 is produced prior to IFN-γ. Thus within a Th1 response, undetectable levels of IFN-γ in the presence of IL-12 can be explained by the fact that IL-12 is secreted prior to the production of IFN-γ, and the production of IFN-γ has yet to occur.

The reason Th1 responses are associated with allograft rejection can be clearly illustrated by the actions of Th1-cytokines. Linked to both innate and adaptive mechanisms, Th1-cytokines mediate a range of responses. For example, IFN-γ contributes to phagocytic cell activation and inflammation (123), but has also been shown to induce cytokine production from NK and T cells, act as a growth factor for activated NK and T cells, enhance the cytotoxic activity of NK cells, and favor cytotoxic T lymphocyte generation. Th1 responses have also been shown to result in Th1-mediated humoral immunity (124).

Some studies have suggested that IFN-γ production occurs independently from IL-12 (125). This suggests that the results obtained from this experimental work might indicate neither a Th1 nor Th2 bias. Although the cytokine profile of the Th2-subset has been associated allograft acceptance, there is evidence suggesting that transplant rejection can occur via Th2-type responses (5,126). For example, the complications associated with the left ventricular assist device (LVAD) have been shown to be associated with the Th2-subset. The implantation of a LVAD has been shown to activate CD4+ T cells producing Th2-cytokines, resulting in B-cell hyperreactivity and immunoglobulin synthesis. Th2-cytokines have also been shown to increase costimulation through CD40 ligand-CD40 interactions (127). A rat allograft model has also associated Th2-type responses with chronic rejection. This study showed the presence of Th2-cytokines and Th2-regulated alloantibodies in long surviving grafts with chronic rejection (128).

Due to the lack of IFN-γ production and the subsequent levels of IL-4 and IL-12, it is questionable whether material treatments produced either a Th1 or Th2-type cytokine profile. The possibility exists for a slight Th1 bias to have been produced, although, if IFN-γ production occurs independently from IL-12 these results could suggest otherwise.

6.4 The Mechanism of Allogeneic Recognition

Previous studies have indicated that direct recognition plays a vital role in allogeneic transplant rejection (128,129). Mediated primarily by CD8+ T cells, but also CD4+, direct recognition does not require host-APCs and has been associated with the acute and more immediate phase of rejection.

Due to the lack of MHC class II expression on the L929 cell challenge, it was hypothesized that cell recognition would occur directly via CD8+ T cells. Therefore, splenocyte suspensions depleted of CD8+ T cells would show a dramatic reduction in proliferation.

Comparisons in proliferation from depleted challenges were not significantly different from the non-depleted challenged control (consisting of the alginate treatment). No major differences were seen between the challenges depleted of CD8+ T cells and all other splenocyte challenges (Figure 5.5). These results implicate indirect pathways via CD4+ T cells in L929 cell recognition.

Indirect recognition via CD4+ T cells would occur as CD4+ T cells are activated by host-APCs. Indirect recognition mediated by CD4+ T cell has been implicated in transplant rejection processes (130-132). Most studies suggest that CD4+ T cells responding to indirectly presented alloantigens act in a Th2-type manner, thus, providing further evidence for the role of Th2 responses in acute graft rejection (133). The Th1/Th2 cytokine results obtained by this experimental work can possibly support a Th2 bias, as IFN-γ was not produced. Although indirect recognition via CD4+ T cells would be an alternative to direct recognition via CD8+ T cells (as was hypothesized to occur), no obvious reduction in proliferation was seen in challenged splenocytes depleted of either CD4+ T cells or adherent macrophage cells (Figure 5.5).

Possibly also playing a role in recognition processes is the indirect pathway known as cross-priming. Facilitated through donor antigens that bind to host-MHC class I molecules, the effect of cross-priming would be seen in adherent macrophage and CD8+ depleted splenocyte challenges. Yet no obvious reduction in proliferation was seen in adherent macrophage or CD8+ T cell depleted challenges. This suggests that several mechanisms of recognition are being utilized within these experiments.

Examination of depleted splenocyte challenges suggests that indirect and direct recognition via CD8+ and CD4+ T cells are being utilized by challenged splenocytes within these experiments.

6.5 Biomaterial Induced Inflammation

Biomaterial implantation has been shown to initiate a sequence of events similar to a foreign body reaction, starting with inflammation (134-136). The intensity and duration of the inflammatory response is dependant on several factors, which include the extent of the injury created upon implantation and biomaterial chemical composition, surface charge, roughness, and porosity. One study investigated the influences of peroxide-catalyzed polydimethylsiloxane (PDMS) implants on foreign body responses in Balb/c mice. After subcutaneous implantation in the intercapsular region, mice were sacrificed at various time points ranging form 2-105 days. Tissue from the surgical site was then fixed, processed, and histologically examined. At 2 and 14 day time points, a mild to moderate inflammatory reaction was observed. The response peaked at 14 days, as granulation tissue (composed of fibroblasts, macrophages, and neutrophils) was first observed at this time point. After 105 days, the implantation site was surrounded by mature connective tissue (137).

To investigate the effect of biomaterial induced inflammation, I investigated the production of IL-1β. IL-1β has been associated with inflammatory responses leading to macrophage mediated pathogen elimination (138,139), as well as allograft rejection (58). A direct connection between IL-1β production and biomaterial-induced inflammation has also been seen (140,141). For example, the involvement of IL-1β in osteolysis of total joint replacements has been demonstrated by the increased expression of IL-1β mRNA in the presence of polyethylene particles (142).

Although I hypothesized that all material treatments would considerably increase IL-1β production from challenged splenocytes, this was not the case. When compared to the challenged control (consisting of no material treatment), the production of IL-1β from challenged splenocytes was not significantly different (Figure 5.9). In fact, most materials showed the same subsequent levels of IL-1β production as the control. Significant differences in IL-1β production was seen between challenged splenocytes treated with chitosan microspheres and soluble chitosan. The increase in IL-1β indicates the effect of small solid particles in inflammation and acute phase responses.

6.6 Biomaterial influences over CD4+ T cells

Host responses toward the biomaterial component of a tissue-engineered construct are often associated with a foreign body reaction, leading to inflammation. As such, biomaterials are often studied in terms of acute phase, inflammatory, or fibrotic mediators. However, the results obtained from this experimental work suggest the possibility that biomaterials directly influence adaptive immune responses.

Analysis of proliferation from depleted splenocytes (non-challenged) in the presence of a 0.1% soluble alginate treatment produced some very interesting data (Figure 5.5). Within non-challenged depletions, proliferation from splenocytes depleted of CD4+ T cells were lower than the depleted CD8+ T cell group, and significantly lower than the adherent macrophage and challenged CD4+ T cell depleted groups. These results possibly

indicate direct biomaterial influences over CD4+ T cell proliferation in a macrophage-independent manner.

The notion of direct biomaterial influences over CD4+ T cell proliferation is also supported by the non-depleted proliferation results (Figure 5.1). To determine non-specific biomaterial effects, proliferation of unchallenged splenocytes from all material treatments were compared to the unchallenged control (consisting of no material treatment). Significant differences in proliferation were seen among several material treatments, suggesting that material treatments elicited non-specific splenocyte proliferation.

To further investigate the degree to which biomaterials affected specific proliferation, a graph representing the differences between unchallenged and challenged proliferation was created (i.e. challenged proliferation results were subtracted by unchallenged results). The soluble alginate treatment showed a significant decrease in proliferation differences when compared to the control (Figure 5.2), indicating that the soluble alginate treatment had a strong effect on specific splenocyte proliferation.

Biomaterial influences on CD4+ T cell proliferation can be attributed to several hypothesized macrophage-independent mechanisms. Firstly, the presentation of antigens can occur independently from macrophages in the presence of B cells. Having the ability to phagocytose antigens, B cells present antigens within MHC class II molecules

(143,144). Through CD154-CD40 interactions, B cells are also able to provide CD4+ T cells with the appropriate co-stimulation required (145). Once B cell-dependant stimulation has occurred, B cells can then be primed to produce antibodies against the non-mammalian polysaccharide components. Antibody production towards foreign polysaccharides has been shown to occur towards capsular polysaccharides located on microorganisms (146). Some of these antibodies have also shown to cross-react with non-mammalian polysaccharide materials (147). Possibly playing a role in B cell mediated CD4+ T cell activation is a soluble pattern recognition protein. For example, mannan-binding lectin (MBL) has been shown to interact with B cells in the recognition of particular foreign carbohydrates (148).

Another possible mechanism for macrophage-independent biomaterial activation of CD4+ T cells can be attributed to the existence of a specialized surface receptor on CD4+ T cells. Ligation of such a receptor could possibly initiate CD4+ T cell activation and proliferation. Much like the mannose receptor on macrophages, which has evolved to detect microorganisms by recognizing non-mammalian patterns, a CD4+ T cell receptor could be shown to recognize many non-mammalian polysaccharides (such as the mannuronic component of alginate, which was used in this set of experiments). Thus, it would be feasible for biomaterial-derived particulates to cross-react with such a receptor and directly initiate CD4+ T cell activation and proliferation.

Whether mediated by B cells or an unknown surface receptor, the data obtained from this experiment work suggests biomaterials have an influence over CD4+ T cell proliferation. These results directly associate the presence of a biomaterial, in the absence of cells, with adaptive immune responses (i.e. non-specific proliferation).

6.7 Possible Endotoxin Effects

Endotoxin is one of the most widely studied pathogen associated molecular patterns. Biomaterial contamination with LPS has been shown to increase immune responses by activating macrophages and dendritic cells. It has been shown that LPS activation of APCs involves at least three cell-surface receptors: CD14, MD-2, and TLR4. CD14 initiates the process by binding to LPS and transferring it to MD-2, which leads to TLR4 aggregation and signal transduction (66,149-151). In addition to TL4 dependant LPS recognition, scavenger receptors (SR) have been implicated in endotoxin associated host responses (152).

A study of calcium-phosphate based biomaterials illustrated that LPS-activated monocytes released IL-1β and IL-6, and were responsible for biomaterial degradation (153). LPS contamination of biomaterials has not only been shown to affect cytokine release and biomaterial degradation, but has also been associated with the production of reactive intermediates, and upregulation of chemokine receptors and costimulatory molecules (154-156). As such, LPS contamination not only affects innate immune response, but adaptive responses as well.

To examine the effects of endotoxin contamination, material endotoxin levels were quantified (Figure 5.10). The results obtained for the polystyrene and poly(methyl methacrylate) microspheres were inconclusive due high backgrounds resulting from the beads and suspension solution (as the beads and solution were not colourless). Both types of microspheres were sterilized as per manufacturer's instruction for the elimination of bacterial and mold contamination. Among the remaining material treatments, significant differences in endotoxin levels were seen between alginate and chitosan treatments compared between the same structural forms (i.e. the soluble chitosan compared to the soluble alginate treatment), as well as between the same chemical compositions in different structural forms. To determine if endotoxin levels directly correlated to an increase in splenocyte proliferation, proliferation was plotted against endotoxin levels (Figure 5.11). It was found that an increase in endotoxin levels did not directly institute an increase in splenocyte proliferation. Therefore, endotoxin contamination did not play a primary role in the results seen within the splenocyte challenges (i.e. proliferation and cytokine release).

Although the results obtained from this experimental work did not directly correlate endotoxin levels to an increase in splenocyte proliferation, several studies have suggested that LPS contamination directly affects adaptive responses. One study indicated that different sources of LPS (e.g. Escherichia coli and Porphyromonas gingivalis) appeared to vary in their ability to signal through TLR4. These variations were shown to alter splenocyte proliferation and cytokine secretion. This suggests that LPS from different

bacterial sources induce APC production of different cytokines and alter adaptive immunity in various ways (157). Another study determined LPS selectively activated Th1 and Th2 subsets (158).

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Innate and adaptive immunity are interlinked. As such innate responses can magnify or reduce adaptive responses by employing several mechanisms. For example, cytokine secretion can result in the up-regulation of costimulatory molecules on antigen presenting cells and lymphocyte activation. On the other hand, innate responses can regulate T lymphocytes through cytokines, reactive intermediates, and activated complement. Therefore, to optimize the success of tissue-engineered constructs it is important to characterize the interactions between biomaterial-induced foreign body responses and adaptive immunity. In the following paragraphs I shall review some of the interactions between biomaterial-induced foreign body responses and adaptive immune responses that were seen within the scope of this experimental work.

The most interesting finding within these experiments was the influence of biomaterials on CD4+ T cells. Within non-challenged depletions, proliferation from splenocytes depleted of CD4+ T cells was lower than the depleted CD8+ T cell group, and significantly lower than the adherent macrophage and challenged CD4+ T cell depleted groups. These results indicated the direct influence that biomaterials had in macrophage-independent CD4+ T cell proliferation. This was supported by the results obtained from

the non-depleted challenges, as significant differences in proliferation were seen among several unchallenged material treatments when compared to the unchallenged control

To investigate the degree to which biomaterials affected specific proliferation, the differences between unchallenged and challenged proliferation was compared. This comparison indicated that the soluble alginate treatment had the capacity to significantly alter proliferation differences when compared to the control.

It was initially hypothesized that non-mammalian polysaccharide treatments would show increased splenocyte proliferation toward the allogeneic challenge. Yet analysis of proliferation indicated that the synthetic polymer group increased splenocyte proliferation in response to L929 cells, with the exception of the soluble alginate treatment. It was also hypothesized that cell recognition would occur directly via CD8+ T cells. However, examination of depleted splenocyte challenges suggested that indirect and direct recognition via CD8+ and CD4+ T cells were being utilized by challenged splenocytes within these experiments, as all depleted challenges showed the same subsequent levels of proliferation.

By analyzing splenocyte challenges in terms of proliferation and cytokine release, the experimental work represented in this thesis has highlighted some important factors in biomaterial effects over adaptive immune responses. Although biomaterial structure (or size) and chemical composition were shown to influence adaptive immune responses,

there were also clear non-specific biomaterial interactions with CD4+ T cells. Therefore, there remains a need to characterize specific biomaterial interactions with adaptive responses as this study has shown that biomaterials can have a large capacity for non-specific interactions.

7.2 Recommendations for Future Work

Although this experimental work has highlighted some important interactions between biomaterial-induced inflammation and adaptive immune responses, there is still a lot of work to be done. In the following paragraphs I shall discuss further experimental work that I believe will contribute to the understanding of host responses elicited by tissue-engineered constructs.

This experimental work has shown that broad conclusions can not be made about any one particular material group. Therefore, splenocyte proliferation (challenged and unchallenged) should be quantified for a larger set of materials. Non-mammalian polysaccharides are one particularly interesting group of materials, as they have already shown a large capacity for non-specific proliferation (as seen with the soluble alginate treatment). It would be interesting to examine other non-mammalian polysaccharides to see if they elicit non-specific proliferation to the same degree. Depletion studies would also be helpful in understanding the various cell types that play a predominant role in this non-specific interaction.

As CD4+ T cells have shown to be involved in biomaterial induced non-specific proliferation, the mechanism to which this interaction occurs should be studied. Elimination of B cells from splenocyte suspensions would help to determine if T cell activation occurred via B cells. Antibody titers and isotyping from non-depleted B cell challenges would establish if B cells were primed to produce antibodies directly against polysaccharide biomaterial components. The investigation into a possible CD4+ T cell surface receptor would be interesting, as this has been implicated as a possible mechanism for the non-specific biomaterial interactions.

Examining material size in terms of splenocyte proliferation and cytokine release would also be useful in the search for biomaterial adjuvants. This study has shown that small solid microspheres elicited increased adaptive responses towards an allogeneic challenge compared to their soluble counterparts. This increase in antigenicity towards cellular components may result in the sensitization of host immune cells, thus, showing biomaterial-associated adjuvant effects.

Ultimately, biomaterials must be quantified for the responses they elicit *in vivo*. APC infiltration and fibrotic encapsulation can be characterized by histological examination of the tissues surrounding the implant site. Second set responses can be examined by re-exposing splenocytes to biomaterial (and/or cell) transplants, once re-exposed, quantifications of splenocyte proliferation, cytokine release, antibody titers, and antibody isotypes can be made.

The experimental work represented in this thesis has brought tissue engineers one step closer to understanding and characterizing the interactions between biomaterials and adaptive immune responses. However, there is there still is a lot of work to be done in characterizing the specific effects of biomaterials over host responses.

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APPENDIX A: Chitosan and Alginate Microsphere Emulsification Procedure

Chitosan Microsphere Emulsification Procedure:

Materials and Equipment:
-Chitosan
-Distilled water
-Tripolyphosphate (TPP)
-Magnetic mixer
-Vortex
-Stir bar

Methods:

Preparations
- Dissolve 60.2mg TPP in 40mL distilled water.
- Dissolve 156.8mg chitosan in 0.261mLacetic acid and 40mL distilled water (vortex vigorously and mix with stir bar).

Procedure:
1. Add 2mL of TPP solution to 5mL chitosan solution dropwise.
2. Mix with magnetic stir bar until solution turns opalescent.
3. Centrifuge at 1500rpm for 10 minutes.
4. Remove supernatant using suction and resuspend in 10mL distilled sterile water.
5. Repeat once.

References:

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Alginate Microsphere Emulsification Procedure:

Materials and Equipment:
-Alginate
-Zinc Chloride
-Calcium Chloride
-Canola Oil
-PBS

Methods:

Preparations:
- Suspend 0.4g alginate (1%w/v) in 40 mL distilled sterile water using magnetic stir bar at setting 5 for 2 hours.
- Filter with 0.45μm filter.
- Filter with 0.20μm filter.
- Autoclave all solutions.

Procedure:
1. Add 3.5mL PBS, pH7.2 to glass autoclaved flask.
2. Add 14mL 1% Alginate solution.
3. Add 70mL canola oil.
4. Mix for 1 minute @ 5500rpm.
5. Add 17.5mL 0.5%$CaCl_2$, 0.05%$ZnCl_2$ (in PBS) dropwise.
6. Mix for 2 minutes.
7. Centrifuge 10 minutes @1500rpm.
8. Remove supernatant using suction, resuspend (vortex) in 10mL distilled sterile water and 5mL poly-L-Lysine.
9. Centrifuge 10 minutes @ 1500rpm.
10. Remove supernatant using suction, resuspend (vortex) in 30mL PBS.
11. Sonicate for one hour.

References
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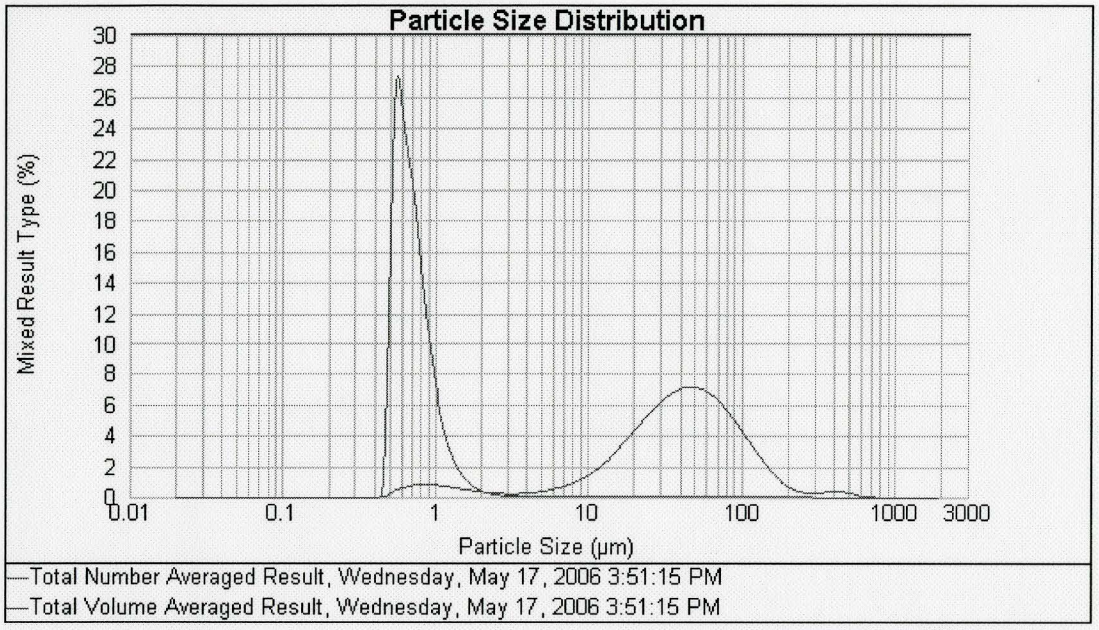
APPENDIX B: Mastersizer Results

Chitosan Microsphere Size Distribution:



Alginate Microsphere Size Distribution: