THE *IN VITRO* AND *IN VIVO* EFFECTS OF ALGINATE ON IMMUNE RESPONSE IN MODEL SYSTEMS

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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 (2007) McMaster University
Hamilton, Ontario

TITLE: The *in vitro* and *in vivo* effects of alginate on immune response in model systems

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NUMBER OF PAGES: x, 123

ABSTRACT

The use of polymeric biomaterials in regenerative medicine and drug delivery is a continually growing practice. Alginic acid (alginate) is widely used in these fields because of its beneficial properties from an engineering and mechanical perspective. Still, alginate has not yet been fully investigated from a biological perspective. For disciplines that anticipate *in vivo* use of their devices, it is crucial to understand the biological interactions between the device and the host.

In this project, the *in vitro* and *in vivo* immunological effects of alginate are examined in two model systems: one with a protein antigen and one with a xenogeneic cell antigen. The former system is used as a proof of principle study for alginate's immunological effect on simple protein-based systems, similar to those found in protein/drug delivery applications and certain types of vaccines. This model uses bovine serum albumin (BSA) as the protein antigen. The latter system is used to demonstrate alginate's effect on more complex antigens, such as whole cells. Thus, Chinese hamster ovary (CHO) cells are used as the as the cell antigen. This model represents a system that may be found in tissue engineering applications, where whole cells are delivered with a biomaterial scaffold.

Antibody production from blood serum indicated that alginate solution has adjuvant abilities while alginate microspheres do not. Thus, alginate solution possesses great potential in the field of vaccines. In addition, *in vivo* alginate challenges were found to have effects on second-set responses of splenocytes to *in vitro* alginate and antigen

challenges. Splenocytes from alginate-injected mice were overall equally or less responsive to *in vitro* challenges than splenocytes without previous alginate immunization. Therefore, alginate solution may also have immunosuppressive effects, although the results from this project merely speculate on this possibility. Still, this ability would be helpful in overcoming current transplantation problems as well as certain tissue engineering hurdles.

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank God for giving me the opportunity to have been a part of McMaster University as a graduate student. I thank You whole-heartedly for providing me with guidance, love, knowledge, wisdom, and faith, and delivering these gifts through wonderful people.

One of these amazing people is my supervisor, Dr. Kim. S. Jones. I thank you indefinitely for all your support and guidance. This year has been quite overwhelming, but I have learned so much. You are an inspiration to me, and I thank you sincerely. I would also like to thank Dr. Heather Sheardown, and everyone from the Jones and Sheardown labs for their friendship, insight, and encouragement. You've all taught and inspired me in various ways (academic and non-academic), and I am grateful to have worked with you. Thank you to Dr. Robert Pelton for chairing my defense and to Dan Mclean for putting up with me all year (but really, for all your help)! To Dr. Jonathan Bramson, Tony Yang, Jen Bassett, and Jamie Millar, thank you for your words of wisdom and kindness. To the wonderful staff of Chemical Engineering, thank you for all your hard work!

Thank you to my wonderful family, who has been an outstanding supporter of my adventures throughout the years. Mom and Pat, thank you for supporting me through another one. I love you both very much. Last but not least, I could not have done this without my other half, Kalvin. Without you, none of this would have been possible. Thanks for your continuous support and optimism. I love you very much.

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1. INTRODUCTION

Polymeric materials have contributed to various disciplines of modern science. Many of these polymers are considered biomaterials, which are materials that are compatible with biological systems. In tissue engineering, biomaterials are often used as scaffolds to repair or regenerate damaged tissues. The ideal material is inexpensive, non-toxic, and conducive to cell growth. Moreover, its structural integrity, porosity, and degradability should be controllable. Scaffolds are usually used in conjunction with various cells, growth factors, and other biomaterials.

A similar range of polymers can also used in drug delivery. In this application, one of the most important roles of the material is to protect the drug without altering the function of the molecule. The material can also target the drug's delivery to a particular site while withstanding the physiological challenges en route. Moreover, the rate of decay of the material usually correlates to the release rate of the drug. Therefore, it is important that the degradability of the material be controllable. Many studies examine these few topics of drug delivery. However, few studies investigate the biological impact of these systems. Immunological studies are more commonly seen in vaccine-specific applications.

In vaccines, the antigen alone is usually too weak to stimulate a sufficient antibody response from the host. Therefore, an adjuvant is used to amplify the host response against the antigen. Certain polymers, such as poly(lactic-co-glycolic acid) (PLGA), have been shown to have adjuvant effects [1] while other materials, like poly(ethylene glycol) (PEG) do not [2]. Moreover, polymeric adjuvants can also be used to protect the antigen (like in drug delivery systems) while triggering a stronger host response.

Degradable polymers are used very often in the applications named above. Some of the most common degradable natural polymers are alginic acid (also called alginate), chitosan, collagen, hyaluronan, and cellulose. Synthetic polymers such as PLGA, polycaprolactone (PCL), and poly(vinyl alcohol) (PVA) are also commonly used in these fields. Certainly, natural and synthetic polymers have also been used together for optimal

properties of scaffolds and delivery systems. For example, studies have examined release kinetics, effects of particle size, effects of porosity, and optimization of polymer blend ratios. Again, very few studies have examined the immunological effect of such devices, even though many of the applications are intended for *in vivo* use.

Thus, this project investigates the effects of a biomaterial on the immune response in model systems. The material of choice is sodium alginate, a polysaccharide that commercially comes from brown algae such as kelp. It is also a component of some bacterial biofilms [3], thus it exists in many different types. Alginate has been used alone or in combination with other polymers such as chitosan [4, 5], poly(oxyethylene) and poly(oxypropylene) copolymer [6, 7], and PLGA [8]. This material was chosen because of its common use in tissue engineering and drug/protein delivery. For example, it has been used as a drug and cell encapsulant [9-11], tissue scaffold for cell growth [4], as well as a wound healing dressing [12]. Since these applications all include interactions with the host, the immune response to the devices is an important aspect to investigate. Moreover, the immunological effect of alginate may spark new potential uses of this polymer, such as in vaccines.

Alginate solution (non-crosslinked) as well as alginate microspheres (crosslinked) are examined in two model systems: one with a protein antigen and one with a xenogeneic cell antigen. The former system uses bovine serum albumin (BSA) as the model protein. This system is used as a proof of principle study for alginate's immunological effect on simple protein-based systems, similar to those found in protein/drug delivery applications and certain types of vaccines. The latter system uses Chinese hamster ovary (CHO) cells as the cell antigen. This model demonstrates alginate's effect on more complex antigens, such as whole cells. This cell-based model system represents a system that may be found in tissue engineering applications, where whole cells are delivered with a biomaterial scaffold. Although both systems are proof of principle type studies, they both represent current research strategies that target *in vivo* purposes. Thus, it is crucial to evaluate the immunological effect of these systems.

The overall goal of this project is to explore the immunological effect of alginate on commonly used model systems. Specifically, this project examines:

1) The adjuvancy of alginate solution and microspheres.
2) The effects of immunization with alginate on *in vitro* challenge assays.
3) The physical effects of alginate on cells *in vitro*.

These objectives will be examined with two types of antigens: BSA (protein) and CHO cells (whole cells), both of which are xenogeneic in nature. From the literature and from past research done in the Jones lab, I hypothesize that:

1) Alginate solution and alginate microspheres will have different effects on cells *in vitro*. Still, both forms of alginate will stress cells, leading to varied rates of proliferation, cell death, cell behavior, and morphology. This stress may be triggered from impurities (i.e. proteins) in the polymer, which can be recognized by cells.
2) Alginate solution possesses adjuvant effects with both model antigens. This is due to its ability to trigger an innate immune response.
3) Alginate will cause cells to alter their behavior and shape due to the interactions between the two components. This is also likely due to the stresses caused by the biomaterials.

This project is broken down into two main methodologies: the *in vitro* and the *in vivo* immunological effects of alginate. For the *in vitro* testing, naïve splenocytes will be challenged with alginate and/or model antigens (proteins and cells) *in vitro*. In the *in vivo* portion of the study, mice will be immunized with alginate and/or protein/cells. Their splenocytes will be challenged with the same *in vitro* stimuli as previously mentioned. The responses from naïve and immunized mouse splenocytes will be compared and analyzed. Antibody production will also be measured and examined.

The results from this project indeed demonstrate that alginate solution possesses adjuvant effects while alginate microspheres do not. Cells did behave differently when

challenged with alginate solution compared to microspheres, as expected. However, it was unexpected that mice immunized with alginate responded differently to *in vitro* challenges. Specifically, these mice were not as responsive to *in vitro* challenges as mice without alginate injections. This was true for both model systems.

2. LITERATURE REVIEW

2.1 Immune Response

Biomaterials are used in various scientific disciplines including tissue engineering and vaccine development. Both of these fields involve foreign materials in contact with or entering the body, and therefore, host response is a major issue that must be addressed. The goal of tissue engineering and regenerative medicine is to repair and/or replace damaged tissue with functional alternatives, thereby restoring the tissue. These fields aim to minimize the immune response against the implant thus limiting inflammation, fibrosis, and implant rejection. On the other hand, the basic principle behind vaccines is to benefit from the body's defense mechanism (immune system) to protect the body from infections. Vaccines stimulate the adaptive immune system to generate antibodies and memory cells against specific pathogens (infectious agents), such as viruses and parasites. With these antibodies and memory cells, the body can react faster when faced with the same pathogen again. So, even though the same materials can be used in both fields, the desired immune response for each application is quite different.

An immune response is orchestrated by various types of cells, cytokines, and proteins. Cytokines are low molecular weight proteins released by cells that facilitate communication between cells and trigger cellular processes. The immune system can be divided into two categories: the innate (natural) and adaptive (acquired) immune systems. Although each system serves a distinct and specific purpose, both systems work together to optimize host defense against harmful antigens. An antigen is generally defined as any substance that causes *anti*body *gen*eration. This includes exogenous and endogenous, infectious and non-infectious, and both living and non-living agents. Not all foreign materials generate antibodies, therefore, foreign materials cannot automatically be classified as antigens.

Complement is a major component of the innate immune system that actively participates in host defense. This system contains over 20 unique plasma proteins that

help recognize antigens (non-specifically) and mediate the inflammatory response [13]. The complement system can be activated by the presence of the antigen-antibody complex (classical pathway) as well as the recognition of a foreign surface (alternative pathway). With biomaterials, the latter pathway is the more common activation pathway. In both cases, the pathways lead to the recruitment of inflammatory cells, opsonization of the antigen (i.e. coating the antigen with identifying tags), and destruction of the foreign materials.

2.1.1 *Immune Cells*

Leukocytes, also known as white blood cells, are the key cells of host defense. These cells are derived from hemopoietic stem cells and make up less than 1% of the cells found in blood. White blood cells help control inflammation, dispose of dead tissues, and control cell signaling. Leukocytes can be categorized as phagocytes, granulocytes, and lymphocytes (see Table 1). The main purpose of phagocytes is to engulf and destroy antigens and necrotic tissues. These cells are the main immune cells of the innate immune system, although many of them continue to be activated in an adaptive immune response. Granulocytes predominately participate in innate immune defense by releasing their granules within, which also aim to destroy the antigen. Lymphocytes are more involved with the adaptive immune response.

Phagocytes	Granulocytes	Lymphocytes
Neutrophils		T-lymphocytes
Monocytes	Eosinophils	B-lymphocytes
Macrophages	Basophils	Natural killer cells
Dendritic cells	Mast cells	

Table 1: List and categories of white blood cells.

The innate immune system is composed of many specific proteins and cell types, including neutrophils, monocytes, and macrophages. Neutrophils are small (1-2μm), short-lived, and terminally-differentiated cells that play an important role in the

inflammatory response [14]. These cells are the first to flood the site of infection and reside at the site for 1-2 days before apoptosing [14, 15]. Their main role involves cell signaling (i.e. release of proteins, peptides, amino acids), although they also help remove debris at the site via phagocytosis. Monocytes are free-flowing cells in the blood that are chemically attracted to injury sites (chemotaxis). Depending on the signals monocytes receive, they can: i) continue phagocytosing debris and antigens at the site, ii) begin the wound-healing process, or iii) prepare for the arrival of adaptive immune cells [14]. The phagocytic abilities of monocytes are enhanced when they differentiate into macrophages.

Macrophages and dendritic cells are the key phagocytic cells of the immune system and are discussed in more detail below (Sections 2.1.2.2.1 and 2.1.2.2.2). Eosinophils are key cells for fighting against parasitic infections and allergic reactions (i.e. asthma) [14]. They are relatively small in size (10-12 μm) and make up 1% - 5% of white blood cells. Basophils are found even less frequently, making up less than 0.3% of circulating white blood cells. These cells contribute to host defense against allergy and anaphylaxis. Mast cells have similar functions to basophils, but are found in loose connective tissues rather than in blood. T- and B-lymphocytes are key players in adaptive immunity while natural killer (NK) cells contribute significantly to both the innate and adaptive immunity. These cells are discussed in more detail in later sections (Sections 2.1.2.3 and 2.1.3.1).

2.1.2 *Innate Immunity*

The innate immune system is the first line of defense against exogenous and endogenous antigens. In many cases, pathogens are recognized and destroyed by this system. The purpose of the innate system has been defined numerous times over the past few decades. One of the most general descriptions and accepted definition of innate immunity (until the 1950s) was: a mechanism that differentiates between self and non-self [16]. However, this definition fails if one considers the case of pregnancies, where immune rejection of the fetus is bypassed. The innate immune system has also been described as: a method to distinguish between infectious (i.e. bacteria) and non-infectious

agents [17]. Like the previous definition, however, there are cases where this definition fails. For example, certain microflora found in the gut have probiotic effects. The same bacteria in the lungs, however, would cause serious damage. Thus, the classification of infectious versus non-infectious is an insufficient description about the role of this system. Currently, the most widely accepted definition of the innate immune system is titled the Danger Model, introduced by Matzinger [16]. She suggests that the innate immune system is more responsive to damage than foreignness. In other words, the system is activated by signals due to injury rather than by the recognition of foreign materials. By this definition, the dichotomy of self and non-self, and even infectious or non-infectious is rejected. When danger signals are recognized, the innate immune system becomes activated, causing two major reactions:

1) enhancing/inducing an inflammatory response;
2) triggering an adaptive immune response for a specialized attack against the antigen.

These responses are discussed in more detail in the following sections.

The innate immune system contains cells whose role is to recognize and eliminate antigens. Innate immune cells, which includes phagocytes and granulocytes, have pattern recognition receptors (PRRs) that recognize molecular sequences from pathogens, also known as pathogen-associated molecular patterns (PAMPs) [18]. These PAMPs are segments from the antigen, usually bacterial carbohydrates and nucleic acids [14]. The recognition of PAMPs is non-specific, and therefore immune cells do not generate memory to the antigens. Such receptors can be found on the cell surface (membrane-bound PRRs), inside the cell (cytoplasmic PRRs), and outside of the cell (secreted PRRs). Babensee *et al.* hypothesized that biomaterials are recognized by the same PRRs that recognize pathogens [19]. This hypothesis sparked from her previous study linking biomaterials to dendritic cell maturation [20]. A follow-up study suggested that PLGA was recognized through a specific PRR called toll-like receptor-4 (TLR4) [21]. Still, additional studies are needed to fully exploit the recognition mechanism of innate immune cells on biomaterials.

2.1.2.1 Inflammatory Response

The main purpose of the inflammatory response is to repair damaged tissue and regain homeostasis. This response can be local to the site of injury and/or systemic. An inflammatory response can be acute (short term) or chronic (long term). Figure 1 shows a typical timeline of response after injury.

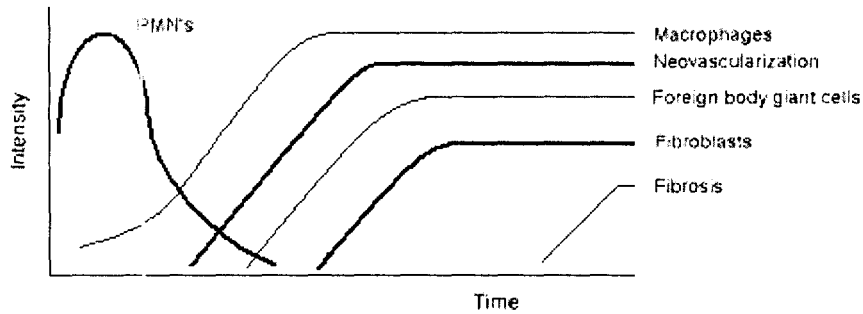


Figure 1: Timeline of events upon activation of the immune system in response to injury.
PMN = polymorphonuclear cells. Adapted from [15].

Janeway *et al.* explain the inflammatory response in a simplistic manner in [14]. Briefly, inflammation can be physically identified by redness, swelling, pain, and heat. These observations are caused by cellular-level changes within the body. Cytokines are released by immune cells to communicate with surrounding cells. For example, interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α) are pro-inflammatory cytokines. These signals stimulate increased blood flow to the site, localizing many types of immune cells. Endothelial cells from blood vessels are also stimulated to up-regulate adhesion molecules helping cells localize in the region of the damaged tissues. In addition, endothelial cells become 'leaky' allowing the influx of leukocytes to traverse the vessel wall into the tissue. At the same time, debris and waste fluids are excreted to the bile for disposal. These changes in cell conformation and movements in fluids cause the four signs of inflammation listed above. When the site has been repaired, anti-inflammatory cytokines such as IL-10 are released to help restore homeostasis. Specific cytokines also exist for directing the adaptive immune response, which will be discussed in the corresponding section.

2.1.2.2 Antigen Presenting Cells

Macrophages and dendritic cells are the main phagocytic components of the innate immune system and the main antigen presenting cells (APCs) in the body. Their main role is to engulf, process, and present peptides of surrounding agents on their major histocompatibility complexes (MHC) to T-cells (see Figure 2). Almost all cells of the body contain MHC class I receptors. MHC class II receptors, however, are predominantly only found on macrophages, dendritic cells and B-cells [22]. B-cells are sometimes classified as APCs, although they have been found to present antigen far less efficiently than dendritic cells and macrophages [22-24]. As an adaptive immune cell, B-cells have many other important roles to play in host defense (to be discussed in Section 2.1.3.1).

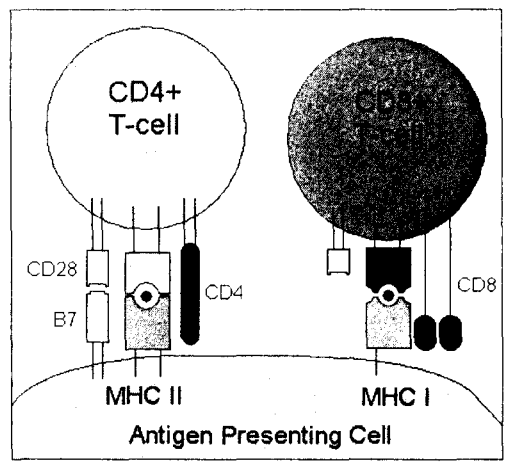


Figure 2: A typical schematic of an antigen presenting cell presenting antigen peptides on MHC receptors to CD4+ T-helper cells and CD8+ cytotoxic T-cells.
(Adapted from [14].)

Extracellular antigens (i.e. bacteria) are normally presented on MHC class II, whereas intracellular antigens (i.e. virus) are normally presented on MHC class I receptors [25]. Cross-presentation (also called cross-priming) can also occur, where protein peptides (extracellular) are presented on MHC class I receptors [25, 26]. This type of antigen presentation is usually seen only with dendritic cells. These receptors are monitored by cells of the adaptive immune system. Peptides presented on MHC class II

are examined by naïve CD4+ T-cells, while peptides displayed on MHC class I are recognized by CD8+ cytotoxic T-cells [25]. Therefore, APCs presenting antigenic peptides will trigger an adaptive immune response.

2.1.2.2.1 Macrophages

Macrophages exist ubiquitously in connective tissues and are usually the first to identify pathogens [14]. During an inflammatory response, monocytes that migrate into connective tissue differentiate to macrophages for increased phagocytic capabilities. Blood-derived monocytes can be stimulated *in vitro* with growth factors granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) to become macrophages [27]. Compared to neutrophils, these cells are much larger (30-40 μm), although their roles are similar (to recognize and destroy invaders). Macrophages use various mechanisms to destroy antigens, including acidification (endosomal pH ~4), oxygen reactive intermediates (superoxides, hydrogen peroxide), enzymes (lysozyme, acid hydrolase), and antimicrobial peptides (defensin, cationic proteins) [14].

Upon recognition of dangerous invaders, macrophages induce an inflammatory response by releasing cytokines, such as IL-1, IL-6, and TNF-α [17]. When the response has expanded to an adaptive response, adaptive immune cells take over most of the cell signaling, although macrophages continue to release cytokines to aid the process [14]. Macrophages can be activated by CD40 ligands from T-helper cells, as well as by the cytokine interferon-gamma (IFN-γ). Upon activation, macrophages recognize and destroy opsonized antigens more efficiently [14].

Innate immune cells have a variety of PRRs, many of which are found on macrophages. Scavenger receptors recognize microbes and lipoproteins [28], while mannose-binding lectin receptors are specific to mannose patterns found in bacteria [29]. These surface-bound receptors can also be found on dendritic cells

[14]. In the last fifteen years, a new class of receptors has been identified: the toll-like receptor (TLR) [17]. These receptors have been shown to cause synthesis and secretion of cytokines leading to host (innate and adaptive) activation. There are at least 11 TLRs identified to-date in mammals, the most well-known being TLR4 [30]. Lipopolysaccharide (LPS) is recognized by TLR4 and stimulates macrophage activation [31]. Antigens bound to this receptor trigger signaling via the NF-κB pathways leading to increased production of cytokines and co-stimulatory molecules [32]. Antigens binding to different receptors will inherently lead to different cellular responses.

2.1.2.2.2 Dendritic Cells

Although dendritic cells are classified as innate immune cells, their function as antigen presenters plays a key role in the activation of the adaptive immune system [18]. In a normal physiological environment, dendritic cells make up < 1% of blood mononuclear cells [33]. *In vitro*, stimulation with GM-CSF and IL-4 causes blood-derived monocytes to differentiate into immature dendritic cells [34]. Cells are deemed 'mature' upon cell activation.

Dendritic cells have a large role in the presentation of antigenic peptides to adaptive immune cells, as they are much more powerful and efficient at T-cell activation than macrophages [14]. Like macrophages, dendritic cells are derived from the blood and travel to the peripheral tissues [14, 18]. However, these cells are very mobile, traveling from the tissue to the lymphoid organs and presenting the antigen peptides to naïve T-cells [14, 18, 35]. During this process, dendritic cells mature and up-regulate other surface stimulatory molecules such as CD80, CD86, and CD40 to enhance communication with and activation of T-cells. This identification process activates the adaptive immune system, allowing T-cells to generate a specific attack system towards the antigen. Once the system has been activated, dendritic cells return to the site of infection to help macrophages destroy unwanted materials.

2.1.2.3 Natural Killer Cells

Natural killer (NK) cells are special lymphocytes that belong to the innate immune system. Like monocytes and dendritic cells, these cells are derived from the blood and migrate to connective tissues [36]. One of the main roles of NK cells is to monitor MHC class I receptors [37]. To prevent the spread of infections, these cells kill any cell that has a reduced expression of MHC class I markers (an indication that the cell has been infected). Therefore, from a tissue engineering perspective, removing these identifiers cannot resolve the problem of foreign tissue rejection. Cells that physiologically do not possess these surface receptors (i.e. red blood cells, neurons) are resistant to NK cell lysis [37]. After the adaptive immune response has been activated, NK cells also help destroy opsonized antigens.

2.1.3 *Adaptive Immunity*

Unlike the innate immune system, the adaptive immune system is an antigen-specific defense mechanism. It usually takes the adaptive immune system several days to become fully activated. Lymphocytes (T-cells and B-cells) are the key cell types of this system. They contribute to the overall immune response by enhancing innate immune cell functions as well as eliciting their own effector functions. An adaptive immune response is also called an acquired immune response because the host develops memory to the antigen after the first encounter. As such, vaccines are designed to expose the body to the antigen (as a first encounter) so that the following encounter with the pathogen can be fought off quickly.

2.1.3.1 B-cells and T-cells

B-cells are derived from bone marrow and mature in lymphoid organs. Each cell has membrane-bound immunoglobulins (Ig) that are specific for a particular antigen. When the antigen and its corresponding Ig interact, the antigen-Ig complex is internalized, processed, and presented to T-cells via the B-cell's MHC molecules. In this method, the

adaptive immune system can be activated directly. B-cells are more likely to recognize antigens than T-cells; B-cells recognize peptides, proteins, polysaccharides, and lipids in their linear or conformational forms, whereas T-cells can only recognize linear peptides. B-cells have another important role of producing antibodies, which is discussed below (Section 2.1.3.2).

T-cells are derived from bone marrow but mature in the thymus. In their naïve state, T-cells are uncommitted. During an adaptive immune response, T-cells help stimulate the proliferation and differentiation of B-cells, dendritic cells, and macrophages. The stimulated cells continue a positive feedback loop by activating T-cells. Naïve T-cells can differentiate into many types of lymphocytes, including three important classes: CD8+ cytotoxic T-cells, CD4+ T-helper1 (Th1) cells, and CD4+ T-helper2 (Th2) cells. These cells are more generally known as effector T-cells. Cytotoxic CD8+ T-cells target cells that have been infected with cytosolic antigens. By the surveillance of their MHC class I receptors, infected cells are quickly eliminated. Naïve T-helper cells examine MHC class II receptors and differentiate to Th1 and Th2 cells when an antigenic peptide is displayed. Th1 cells communicate with macrophages via their MHC class I receptors and help activate macrophages to phagocytose the antigen more efficiently. The same cells can also interact with B-cells, stimulating an increase in antibody production to opsonize the antigens. This response is also known as a cell-mediated response (see Section 2.1.3.3). On the other hand, Th2 cells interact with and stimulate B-cells to differentiate into plasma cells, triggering an antibody-mediated response (see Section 2.1.3.2).

Indirect T-cell activation follows a three-step process (see Figure 3). First, T-cells interact with MHC molecules on APCs (class II for naïve CD4+ cells and class I for CD8+ T-cells). Next, these interactions are enhanced by surface co-stimulatory molecules, such as CD80 or CD86 on APCs (bind with CD28 on T-cells). Another well-known co-stimulatory pair is between CD40 ligands on T-cells and CD40 receptors on APCs. This type of communication enhances T-cell proliferation and activation. Lastly, cytokine signaling (from self or surrounding cells) is required to completely activate the cell. T-

cells secrete cytokines (i.e. IL-2, IL-4, IFN-γ) either to sustain their own productivity (autocrine effect), or to activate their surrounding immune cells (paracrine effect) [18]. Once activated, T-cells stimulate both innate and adaptive immune cells to perform more efficiently.

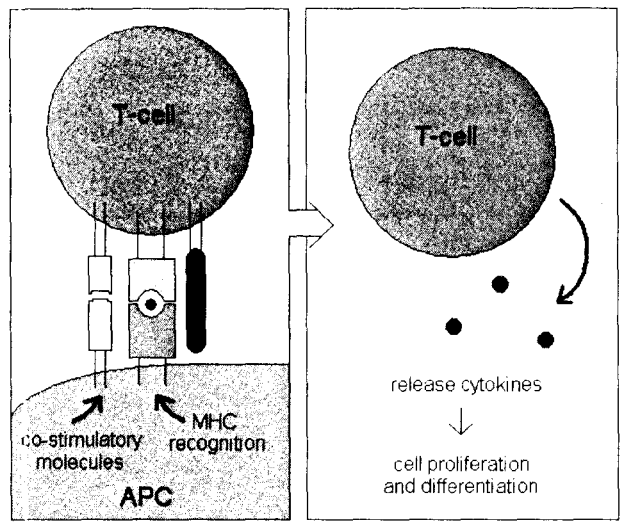


Figure 3: Three basic steps for T-cell activation.
1) recognition of antigen on MHC molecules, 2) up-regulated co-stimulatory molecules, and 3) the release of cytokines. Adapted from [14].

This indirect pathway for adaptive immune activation can occur from both CD4+ and CD8+ T-cells. However, activation facilitated by Th cells can bias Th1- (cell-mediated) or Th2- (antibody-mediated) type responses (see Sections 2.1.3.2 and 2.1.3.3 for details). The response defaults to a Th2 polarization if insufficient signals are given for a Th1 bias [38]. Activation via the MHC class I receptor defaults to a cell-mediated response. The adaptive immune system can also be activated directly from CD8+ T-cells that inspect infected cells directly.

2.1.3.2 Antibody-Mediated Immunity

Antibody-mediated immunity, also known as humoral immunity, is geared to combat against extracellular pathogens, such as parasites. One method of pathogen recognition is through phagocytosis by APCs and presentation to Th cells. These cells secrete specific cytokines (i.e. IL-4, IL-5, IL-6) that stimulate B-cell activation, differentiation, and proliferation. B-cells can differentiate fully to plasma cells, releasing immense quantities of Ig specific to an antigen for opsonization. The five major classes (in humans) of these glycoproteins are IgA, IgD, IgG, IgM, and IgE [14]. The first antibody to be produced during a humoral response is IgM. This antibody has a pentameric structure in serum, thus is efficient in activating the complement system [14]. The function of IgD remains unclear, although it is known that this antibody is co-expressed with IgM on many mature B cells [14]. The least common immunoglobulin is IgE, which has a role in allergic and parasitic infections. In the gut, IgA is secreted as a monomer or dimer and has a role in hypersensitivity, as well as in the neutralization of bacterial and inhibition of viral infections. The most abundant antibody, IgG, performs similar tasks to IgA, although this antibody circulates in the blood. This antibody exists as IgG1, IgG2, IgG3, and IgG4 in humans, while mice have distinguished IgG2a and IgG2b antibodies (homologues of IgG1 and IgG3 in humans) [14]. The former (IgG2a) is an indicator and enhancer of Th1-type responses, whereas the latter (IgG2b) can be induced by transforming growth factor-beta (TGF-β), a marker of fibrosis. A summary of antibody functions can be found in Table 2. The activation of Th2 cells (Th2-type response) also stimulates the production of IgG and IgE (IgG, IgE, and IgA production in mice) [39].

Some B-cells do not fully differentiate to plasma cells, but instead, become memory B cells. These cells allow the body to respond quickly against a re-encounter with the same antigen by quickly mass-producing antibodies for a specific antigen. Over time, however, these cells may die, and memory against a specific antigen may be lost. This is one reason certain viral vaccines require re-vaccination over time.

	Neutralization	Opsonization	Complement Activation	Sensitization for immune cells
IgA	++	+	+	-
IgD	-	-	-	-
IgG				
- IgG1	++	+++	++	NK cells, mast cells
- IgG2	++	+*	+	-
- IgG3	++	++	+++	NK cells, mast cells
- IgG4	++	+	-	-
IgM	+	-	+++	-
IgE	-	-	-	Mast cells

Table 2: List of antibody functions from immunoglobulins in humans.
Legend: +++frequent, ++average, +sometimes, -rarely, *if specific Fc receptor present. Adapted from [14].

2.1.3.3 Cell-Mediated Immunity

Cell-mediated immunity (Th1-type response) targets intracellular pathogens, such as microbacteria and viral infections. Antigen peptides presented on MHC class I will automatically trigger this type of a response. This method of adaptive immune activation can be direct (recognition of foreign peptide on infected cells) or indirect (recognition of foreign peptides as presented by APCs). Cytotoxic CD8+ T-cells kill all cells that present foreign peptides. The body uses this method to prevent the spread of infections. Antigen peptides presented on MHC class II can also stimulate cell-mediated immunity indirectly by Th cells. Specifically, Th cells secrete IL-2 and IFN-γ to stimulate a cell-mediated response, the activation of CD8+ T-cells, and the generation of antigen-specific antibodies [39]. Infected cells and invading antigens are destroyed more quickly with the help of B-cells. As previously mentioned, B-cells release IgG1 antibodies to opsonize antigens and infected cells. These opsonized cells and antigens are then destroyed by CD8+ T-cells, NK cells, and macrophages.

2.2 Types of Biomaterials

A biomaterial can be defined as a natural or synthetic material suitable for use within a living body. Both types of materials have their respective benefits and drawbacks, depending on the application. For example, most synthetic materials have a larger range in mechanical properties that can be adjusted and controlled by composition. Many natural materials do not have this versatility. Still, synthetic polymers usually have much greater strength than natural polymers, which is more beneficial in the structural design of certain devices. Different types of materials will also have different compatibility challenges with the host. For example, polymers are susceptible to many forms of degradation in comparison to metals. At the same time, metallic elements can corrode *in vivo*, leading to other challenges. Thus, it is crucial to evaluate the chemical stability, mechanical/physical strength, and electrical/magnetic properties of the device prior to implantation. It is equally as important to examine the biological interactions of the device to the host.

The charge of each biomaterial can also be used to improve the release rates of the device. Table 3 gives an example of different polymers and their charges that have all previously been used in drug delivery applications. Thus, even though the polymers have different properties, they can all be used for the same purpose, depending on the design of the device.

Polymer Charge	Examples
Anionic	Hyaluronic acid, alginic acid, carboxymethyl cellulose
Cationic	Chitosan, poly-L-lysine, polyethylenimine
Amphipathic	Collagen
Neutral	Dextran, agarose, polyethylene oxide, polystyrene

Table 3: Natural and synthetic polymers of different charge used in biomedical applications.

2.2.1 Natural Biomaterials

Natural biomaterials can be described as materials extracted from the natural environment. Proteins and polysaccharides are the most widely used natural biomaterials in regenerative medicine. Polynucleotides (i.e. deoxyribonucleic acid (DNA), ribonucleic acid (RNA)) are also considered natural polymers, but target a limited scope of applications. Proteins are composed of different amino acids and are the basis of all living organisms. Albumin, for example, is a globular protein found in blood and is commonly used in drug delivery. Other commonly used proteins include collagen, elastin, gelatin, casein, and fibrinogen [40]. Polysaccharides are complex carbohydrates easily found in plants/vegetables, crustaceans, and synthesized by bacteria [41]. Most polysaccharides have the structural form $C_n(H_2O)_n$, where n can range from 200 to 2500. Cellulose, alginate, hyaluronic acid, chitin, and starch are examples of polysaccharides commonly used as biomaterials. Physiologically, these natural polymers are needed to provide mechanical support to tissues.

Components of connective tissue are likely choices for biomaterials, such as the proteins collagen and elastin. Collagen is the most abundant component of connective tissue and provides mechanical support for the tissue [41]. There are at least 13 types of collagen, many derived from different tissues of the body with different characteristics. Type I (skin, bone), II (cartilage), and III (skin, blood vessels) collagen are the most commonly examined types of collagen in tissue engineering. These proteins can be formed into fibrils, increasing the strength of the scaffold. Still, the collagen network produced by fibroblasts in the native environment is much stronger than most of the matrices produced *in vitro*. For example, collagen (type II) scaffolds have been used to seed chondrocytes for cartilage regeneration [42]. Lee *et al.* were able to show that the implant did indeed improve the regeneration of damaged tissue, however, the tissue was 20-fold weaker than native tissue.

Elastin is another biomaterial used in regenerative medicine that is found in connective tissue. In comparison to collagen, elastin lacks in strength and complexity. However, elastin has an elastic ability, that is, it can recoil after the removal of stresses.

This characteristic can not be found with collagen. This property of elastin is due to its highly crosslinked chains in the fiber. Because elastin lacks mechanical strength, it is more commonly used for its elasticity property, such as artificial skin [43] and aortic scaffolds [44].

2.2.1.1 Bovine Serum Albumin and Ovalbumin

Bovine serum albumin (BSA) and ovalbumin (OVA) are two commonly used proteins in drug delivery studies. Both proteins have been well characterized [45-47]. Although they differ slightly in molecular weight (MW_{BSA} ~ 67 kDa, MW_{OVA} ~ 43 kDa), these proteins have a pI ~ 4.7 and are both commercially available at an affordable cost (i.e. from Sigma Aldrich). These proteins are sometimes called carrier proteins as they can induce an immune response on their own. When employed with less immunogenic materials (i.e. potential adjuvants), these proteins help initiate an adaptive immune response. The extent of immune response induced becomes an indicator of biomaterial adjuvancy.

The globular protein BSA is a key component in bovine blood. It is a transporter of fatty acids and denatures at ~55°C [48]. On the other hand, OVA is found in chicken egg white and denatures at ~78°C [49]. Its biological function remains unclear, although many believe it is a storage protein [50]. SIINFEKL is a peptide sequence of OVA (257-264) that can bind to MHC class I receptors ($H\text{-}2K^b$) recognized by cytotoxic T-cells [51]. Therefore, this epitope is commonly used in host response research, including therapeutic anti-virus and anti-cancer vaccine studies [51].

2.2.1.2 Sodium Alginate

Sodium alginate, pI ~ 5.4, is a non-toxic, affordable, and mucoadhesive natural polymer with many uses in regenerative medicine [52]. In its physiological environment, alginates provide structural support to their organism. Commercially-available alginates come from brown algae and are composed of β-(1,4)-D-mannuronic acid and α-(1,4)-L-

guluronic acid residues (see Figure 4). Each type of alginate has a different ratio of each residue (mannuronic acid, M; guluronic acid, G). Alginates rich in mannuronic acid are usually less viscous, which may be beneficial as a gel capsule for the delivery of xenogeneic materials [53]. In comparison, alginates with high G content are more viscous, and thus more brittle as matrices. A review by Wee and Gombotz showed that many studies have indicated an immune response could be generated by alginate [54]. More specifically, these studies found that the mannuronic acid residue induces more of an innate immune response than guluronic acid segments. However, Klock *et al.* demonstrated that the immune response induced by alginate was due to impurities in the alginate [53]. Alginates extracted from nature contain many impurities, including endotoxin, metals, and proteins [52]. After purification of the alginate (high M content), Klock *et al.* implanted alginate capsules into rats and did not see a significant inflammatory response [53]. Thus, it is clear that alginate on its own has low chemical reactivity. However, when interacting with proteins and other polymers, alginate can cause significant side effects.

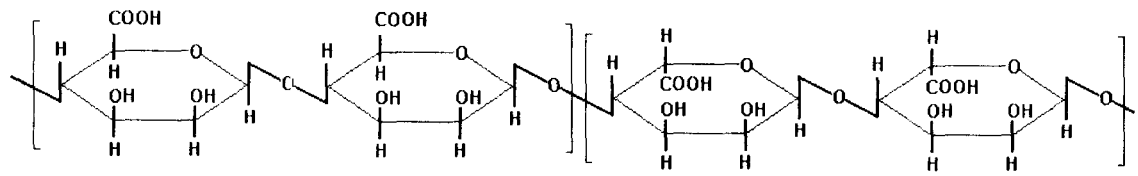


Figure 4: Mannuronic acid and guluronic acid residues of alginate.
(Image adapted from [54])

Babensee and Paranjpe found that alginate and hyaluronic acid films induced a lower expression of CD86, CD40, and HLA-DQ molecules on dendritic cells, whereas PLGA and chitosan films induced an increase in these molecules [19]. The upregulation of these receptors is an indication of dendritic cell maturation. Thus, it was found that alginate films did not support dendritic cell maturation. At the same time, alginate

oligomers have been found to stimulate cytokine secretion from the macrophage cell line RAW264.7, thereby activating the innate immune system [55]. When used as a wound healing dressing, some types of alginate were found to activate macrophages, causing an undesirable pro-inflammatory response [12]. In our own lab, alginate has been found to activate the NF-κB pathway, a pathway that can be activated via TLR4, leading to innate immune activation [56]. This coincides with previous research that showed certain biomaterials cause dendritic cell maturation via TLR4 [21]. These exciting discoveries present an opportunity to use alginate in an immunomodulatory manner.

Current research uses alginate in both its crosslinked and non-crosslinked forms. A summary of aqueous alginate's properties is found in Table 4. Alginate can be crosslinked using divalent cations, such as calcium, strontium, or barium [54]. Sometimes, a combination of cations can be used. For example, a previous study showed that alginate crosslinks more efficiently with calcium and zinc together than with calcium alone [57]. Ion exchange between divalent and monovalent cations leads to destabilization of the gel [52]. However, the destabilization and degradation of the gel has advantageous uses, for example, in drug delivery. Drug delivery has also profited by crosslinked alginate's stability (or instability) as a function of its surrounding pH [52]. Particularly in this field, alginate is used as a drug delivery vehicle in the form of particles.

Properties of Alginate	Strength
Solubility	Low solubility in water; Insoluble in most organic solvents
Gelation	Divalent cations (i.e. Ca^{2+}, Ba^{2+}, Sr^{2+}) to stabilize gel; Monovalent cations (i.e. Na^{+}) or complex anions (i.e. PO_4^{3-}) to destabilize gel
Stability	Solution is stable between pH 4-10
Effects of pH	Stable in acidic conditions; Swell in basic conditions (followed by degradation)

Table 4: Summary of alginate solution properties. Adapted from [52].

Alginate particles have been created and characterized using many methods [58, 59]. Protein-loaded alginate particles have also been developed following similar techniques for a variety of purposes [48, 60, 61]. Many studies have been done to optimize encapsulation efficiency [62] and controlled release of the antigen [5, 63]. These delivery systems have also been used specifically for vaccine development. Antigen-loaded alginate particles have been delivered orally to animals including cattle [64, 65], rabbits [64], and rodents [64, 66, 67]. These studies showed that oral delivery of encapsulated antigen increased its bioavailability and antibody production in comparison to non-encapsulated antigen delivery. Even though encapsulated particles have shown great potential as oral delivery systems, they have yet to show their effectiveness as injectable delivery systems. It is unlikely that intramuscular administration of these particles will provoke the same response than from its oral route since each route faces different biological barriers. Practically, these particles may have similar (possibly better) effects when delivered intramuscularly. In such a case, these systems can be used to improve currently available vaccines, as well as the development of new vaccines. Thus, studies are needed to examine the effects of injected encapsulated antigens.

In addition, there have been very few studies that examine the effect of aqueous solutions as delivery vehicles. Some polymers, like alginate, can easily de-crosslink (partially or fully) in physiological settings (by pH, temperature, etc.), resulting in the aqueous alginate solution. The immunological effect of these solutions is also unclear. Thus, it is also necessary to pursue further studies in the immunological effect of polymeric solutions as delivery vehicles. In summary, the methodology used in related previous studies may be of great benefit in the improvement of current vaccines, and thus merits further investigation.

2.2.2 Synthetic Biomaterials

Synthetic materials are also commonly used in biomedical research and cover a wide range of materials, including polymers, metals, and ceramics. Synthetic and natural polymers have both been used in the same applications, sometimes even combined

together. The benefit of blending the two types of materials is that the device now has a greater range in properties and applications. As Table 5 shows, synthetic polymers have been used to make products (i.e. lab ware) as well as components of implants (i.e. grafts).

Polymer	Applications
Polyvinyl chloride (PVC)	Tubing, blood bags, IV shunts
Polystyrene (PS)	Lab ware
Polyethylene (PE) - LDPE, LLDPE, HDPE, UHMWPE	Health care products (containers, packaging, tubes), orthopedic implants, hip replacements
Polypropylene (PP)	Lab ware, membranes, sutures
Polymethylmethacrylate (PMMA)	Dental implants, ocular lens, blood pumps, bone cement
e-Polytetrafluoroethylene (e-PTFE)	Sealing tape, vascular grafts, coatings
Poly(lactic-co-glycolic) acid (PLGA)	Scaffolds, encapsulating agents, sutures
Polyurethane (PU)	Scaffolds, membranes (many cardiovascular applications)
Polyethylene terephthalate (PET)	Meshes, sutures, wound dressings, vascular grafts

Table 5: Examples of synthetic polymers used in tissue engineering.

Some materials are rarely implanted into the body, but used frequently to hold bodily fluids. Polyvinyl chloride (PVC), for example, is used in many hospital products but is non-ideal for *in vivo* use. This material can be rigid and firm, while remaining flexible. Moreover, it has low protein binding, instigating fewer infections. Therefore, its properties make PVC useful in handling sensitive materials like blood. Some polymers like PLGA, polymethylmethacrylate (PMMA), e-polytetrafluoroethylene (e-PTFE), and polyurethane (PU) have been used both internally and externally as biomaterials. Polystyrene, however, is very rarely implanted. Instead, it is most commonly used in tissue culture. Some materials, like PLGA, are quite hydrophobic compared to proteins, leading to incompatibility and instability of the system [40]. Thus, it is clear that natural and synthetic materials both have their advantages and shortcomings.

The list of polymers in Table 5 is a non-exhaustive list of synthetic polymers currently in use. The table indicates only examples of uses for these polymers, as there are many more possible applications. Still, it can be seen that synthetic polymers have a far greater span of applications than natural polymers. Another benefit of synthetic polymers over natural polymers lies in the sterilization process. Natural polymers are difficult to sterilize, as many of them will degrade or denature with heat and/or moisture. Sterilization will be discussed in further detail in Section 2.3.1.

Metals are another class of synthetic materials commonly used in tissue engineering. In general, metals have higher mechanical strength than other types of materials. Moreover, their mechanical properties are also quite easily adjustable and fabricated when using metallic alloys. Stainless steel, titanium alloys, and cobalt-chromium-molybdenum (CoCrMo) alloys have been used to regenerate and remodel damaged bone. In these applications, degradation of the implant is usually undesirable. Instead, the material must maintain wear resistance, mechanical strength, and sometimes electrical conduction. Although metals can withstand great loads, there are two major disadvantages of using metals *in vivo*. The first hurdle to overcome is the possibility of stress shielding, where the metal withstands the load, preventing the proper formation of bone. This can be overcome by adjusting the conformation and amount of metal used in the implant. The second disadvantage of using metals in vivo is the possibility of corrosion of the implant, leading to further problems. Therefore, a second surgery is required to remove or replace the implant to overcome this problem. In summary, even though metallic implants may possess optimal properties in development, its effects in the physiological environment raise many concerns. The invasive nature of this solution is non-ideal in tissue engineering applications.

Ceramics are another type of synthetic materials that are commonly used in tissue engineering applications. These materials are relatively inert and can be degradable. Ceramics are widely used in implants alongside with metals. Aluminum trioxide (Al_2O_3), calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), and stabilized zirconium dioxide (ZrO_2) are commonly used for dental and orthopedic implants. Generally, these materials are

chemically stable and have good wear resistance. On the other hand, these materials tend to be very fragile, thus the brittleness of the material is another factor that influences the choice of material to be used.

2.2.3 Hydrogels

Hydrogels are a special category of biomaterials that are used in a wide variety of applications, including contact lenses [68], wound dressings [69], and breast implants [70]. These materials begin as soluble polymer chains and form insoluble networks when crosslinked. These networks can be made from physical entanglements, covalent crosslinks, or strong hydrogel bonds. Hydrogels are extremely hydrophilic, absorbing water and swelling in aqueous solutions. This property causes the material to also have very low mechanical abilities. Most hydrogels are degradable and can be a mix of natural and synthetic materials. Alginate, poly (N-isopropylacrylamide) (pNIPAAm), and various acrylates are common materials used to form hydrogels. They can also be made to be responsive to pH [71, 72] and temperature [72], making hydrogels an excellent vehicle for drug delivery. They are advantageous as wound dressings as they can crosslink and/or polymerize in situ to fit wounds perfectly. For example, Balakrishnan *et al.* applied oxidized alginate, gelatin, and borax to a wound in rats [69]. With this hydrogel, new epithelium was able to cover the entire wound without causing significant side effects.

2.3 Uses of Polymers

A biomaterial is ideally non-toxic, inexpensive, and versatile to manipulate. It can be used either to help regenerate damaged tissue, or to maintain/repair native tissues. Polymers particularly have a wide range of use. Polymers have contributed to the development of medical devices such as stents, heart valves, implants, intraocular lenses, hip replacements, and intervertebral disc replacement. With each application, the desired properties of the polymer are different. Polymers placed in biological environments are

susceptible to physical and chemical degradation. Table 6 lists some of the most common mechanisms leading to polymer degradation *in vivo*. From an immunological perspective, hydrolysis and oxidation are the two mechanisms of biggest concern. Hydrocarbons (i.e. polyethylene), halocarbons (i.e. PTFE), dimethylsiloxane, and sulphones do not undergo hydrolysis. However, anhydrides, esters (PLGA), amides, and urethanes can hydrolyze in the presence of water. Therefore, most hydrogels are vulnerable to hydrolysis. Blood also contains many hydrolytic enzymes, catalyzing the hydrolysis of ester bonds.

Physical	Chemical
Swelling	Oxidation
Mineralization	Solvolysis (i.e. hydrolysis)
Fractures	Radiolysis (i.e. gamma rays)
Crystallization	Depolymerization
Decrystallization	Photolysis

Table 6: Physical and chemical mechanisms that lead to polymer degradation.

Degradation of the implanted material is desirable in certain instances. For example, it may be desirable for a tissue substrate to degrade at a comparable rate of proliferation from seeded cells. The biomaterial begins as a structure for cell growth. Over time, the degradation of the material would allow the tissue to survive independently of the material. Still, the degradation of these materials can cause unwanted side effects, such as the release of acidic by-products from PLGA [73] and chitosan [74] scaffold degradation. On the other hand, it may also be desirable for an implant to remain intact without degradation. For example, stents (usually metal) are used to uphold the walls of the blood vessel, allowing the passage of blood. Some stents are coated with polymers (and sometimes drugs, too) to minimize cell adhesion and occlusion to the structure. Therefore, it is crucial that the biomaterial resist degradation, corrosion, and wear. It is clear that the ideal behavior of the material depends on its use. Here, the focus will remain on two common uses of polymers: scaffolds and encapsulation agents in delivery systems. First, a brief summary of sterilization techniques will be discussed.

2.3.1 Sterilization of Polymers

One of the most important steps while working with tissues and biomaterials is sterilization. This procedure kills bacteria that can infect the device and harm the host. Steam from autoclaves is hot enough to destroy cellular structures and denature proteins. This method of sterilization is often used on glass and metals, as well as certain types of synthetic polymers. However, it cannot be used with certain materials such as PVC, low density PE, polyamides, and polyacetals due to hydrolysis. Ethylene oxide sterilization is another method of sterilize biomaterials. At room temperature, ethylene oxide is a gas, thus is able to penetrate biomaterials very easily. This gas alkylates amine groups on nucleic acids, thereby destroying cells. The main disadvantage of this technique is the carcinogenic/toxic and flammable nature of the gas. Radiation sterilization (i.e. gamma irradiation) can also penetrate biomaterials easily without leaving behind toxic residues. However, this method is expensive to maintain and takes longer periods of time as it is used (due to its half-life). In addition, some materials degrade when exposed to gamma rays, such as PTFE and PE. Klock *et al.* developed a special method of purifying alginate using dialysis [53], although ethylene oxide has also been used to sterilize alginate [75]. Certain materials can also be sterile-filtered using 0.22 μm-sized filters. Table 7 gives a summary of sterilization methods for commonly used polymers.

Polymer	Sterilization Method
Chitosan	ethylene oxide
Alginate	ethylene oxide
Polypropylene	ethylene oxide
Polyethylene	ethylene oxide, gamma irradiation
Poly(tetrafluoroethylene)	ethylene oxide, autoclaving
Poly(ethylene terephthalate)	ethylene oxide, gamma irradiation

Table 7: Sterilization methods and applications of natural and synthetic polymers
Adapted from [75].

2.3.2 *Scaffolds*

Polymers are commonly used as tissue scaffolds in regenerative medicine for tissue reconstruction. They are used not only as structural supports, but also as substrates supportive of cell growth. The goal of using a biomaterial is to simulate the native tissue, creating an environment conducive for natural cell growth. Scaffolds also offer different configurations for structure and organization, and add strength to the tissue. Ideally, the development of live tissue is proportional to the decay of the biomaterial scaffold without compromising the structural integrity of the construct. Some degradable materials such as PLGA [76], poly(caprolactone) [77], alginate [4, 78], collagen [42], and chitosan [4] are used for tissue scaffolding. Whole cells are used with the scaffold and grown either *in vitro* or *in vivo* [78]. Previously in Section 2.2.1, a recent study by Lee *et al.* showed how collagen scaffolds were conductive to chondrocyte growth [42]. Several years later, Li and Zhang showed how alginate-chitosan scaffolds stimulated even more chondrocyte proliferation and the production of type II collagen, an important component of cartilage [4]. Many studies have also examined the effects of degradation on tissue growth [4, 73, 74]. Thus, much research is still being done in this field to understand the effects of different biomaterials on tissue development.

2.3.3 *Delivery Systems*

Polymers are also commonly used as drug and protein delivery vehicles in the form of particles or films (crosslinked). In some cases, direct drug administration to the target site can be difficult because of the physiological defense mechanisms of the body. For example, an orally administered drug must endure enzymatic degradation and acidic pHs before reaching the intestines. The delicate nature of most drugs would not survive these barriers unless with protection, such as an encapsulant. The polymer properties can not only help protect the drug/protein within, but can also help target the delivery of the antigen to the proper site of absorption. For example, chitosan has mucoadhesive properties, and thus is frequently used in nasal and oral delivery systems [79]. This

system was found to not only increase the bioavailability of the drug, but also to help localize the drug to the absorption site.

For degradable polymers, controlled delivery of the antigens is greatly dependent on the properties of its encapsulating material. Most commonly, the antigens are encapsulated (or loaded) within the biomaterial. This delivery method has been used for the delivery of model proteins (i.e. OVA, BSA) [8, 61, 64, 80, 81], dextran [62], rotavirus proteins [64, 82], tetanus toxoid [9], and various cells [83]. Bhumkar *et al.* used adsorption to delivery insulin to rats via chitosan-reduced gold nanoparticles [84]. Thus, the agents being delivered can also be adsorbed or absorbed to the biomaterial, protecting them from premature release. The slow degradation of the material is important for implantable devices such as stents. Grassi *et al.* coated metal stents with drug-loaded alginate gels, allowing slow release of the anti-proliferative agents at the target site [6]. Drachmann *et al.* did a similar study, coating a stent with poly(caprolactone) and poly(lactide) co-polymer with Paclitaxel to prevent restenosis [85, 86].

The field of drug delivery uses many methods of delivery, including passive diffusion, injections (subcutaneous, intraperitoneal, intramuscular), and oral and nasal delivery. In all cases, the devices aim to release the drug at a constant rate within the therapeutic range. The challenge arises when the rupture of the polymer is inconsistent since many polymers are prone to degradation, ultimately leading to inconsistent drug release. Thus, controlled release of the antigen is a necessity and can be managed by manipulating its encapsulating polymer.

2.3.3.1 Immune Response to Vaccines

Vaccines are a special type of delivery system. Vaccines deliver attenuated antigens to the body to protect the host from infection (i.e. deliver influenza peptides for protection against the influenza virus). Ideally, the antigens have sufficient epitopes for the host to recognize, but at the same time, are weak enough so that the response to the vaccine itself has few side-effects. These antigens are usually co-delivered with adjuvants,

which are used to amplify the immune response to the antigen (details on adjuvants are given in Section 2.5).

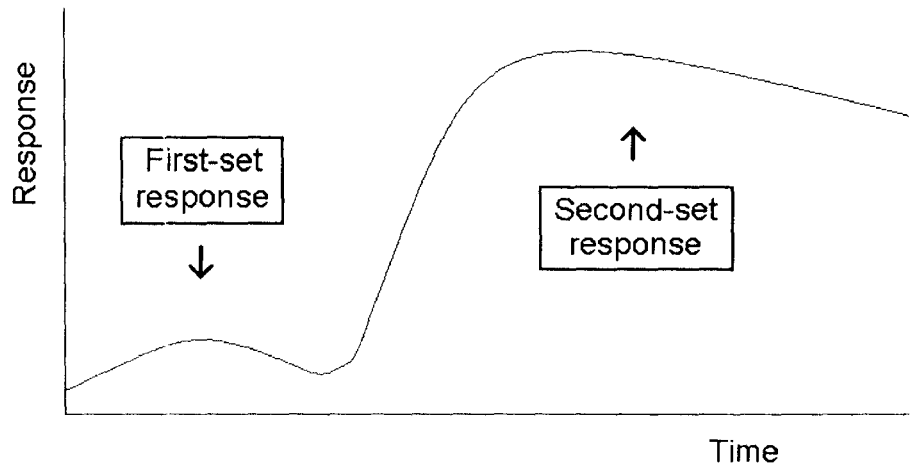


Figure 5: Typical immune response from the first and second encounters with an antigen.

When a vaccine is received, the body encounters the antigen for the first time and a small immune response is triggered (see Figure 5). This response, also called a first-set response, is usually relatively small and slow because the body requires time to formulate the proper adaptive response against the antigen. The outcome of the first-set response is the generation of memory B-cells (and/or T-cells), which are able to produce antigen-specific antibodies if the antigen is encountered again (the second-set response). The second encounter usually comes in the form of the virus itself. However, because the body has developed memory B-cells for the virus, the body is able to respond quickly and efficiently to eliminate the antigen and infected cells. Immune cells release signals for cell apoptosis to restore homeostasis once the antigen and infected cells have been destroyed.

2.3.3.2 Biomaterials and Model Proteins

Model proteins are often used in proof-of-principle studies. The effects of temperature, pH, formulation method, and crosslinking materials have been shown to vary the properties of the system [87]. Many studies aim to optimize the release kinetics of the device, controlled degradation of the material, modify the functional properties of the material, and also investigate the interactions between the material and the protein [5, 61, 62, 87]. These studies give us much information about the biomaterial itself as a medical tool.

Previous studies have also investigated blends of biomaterials to improve protein loading and controlled release. Some common blends currently used in drug delivery include PEG and PLGA [80, 81], 1,2,3-tridecanoyl glycerol (tricaprin) and PLGA [81], and a mix of alginate and chitosan, with and without PLGA [5, 8]. These studies show how these blends provided more stability of the system and slower degradation of the materials than of a single material alone. Therefore, the rate of release of the protein is also diminished. One method of loading these polymeric particles is to pre-mix the proteins with the aqueous polymer solutions before crosslinking [52]. In this method, the proteins are evenly distributed throughout the particle. For hydrogels, the particles can be loaded by swelling the particles and allowing the proteins to diffuse into the particles [52]. This method requires that the hydrogel contains pore sizes large enough for proteins to pass.

Although the final purpose of these systems is to be applied into living beings, few studies have investigated the immunological effects of the biomaterial and cells/proteins. The insufficiency of this information creates another hurdle when moving from *in vitro* to *in vivo* models.

2.3.3.3 Immune Response to Xenogeneic Materials

For many years, doctors and scientists have searched for new methods of replacing damaged organs with functional tissues. The idea of transplantation appeared to be a plausible alternative as organs serve the same purpose for every individual.

Unfortunately, immune rejection of such organs has greatly hindered the success of organ transplantation. Transplantations can be autogenic (from self), allogeneic (different individuals from the same species) or xenogeneic (individuals from different species). Currently, these types of transplantations are used for cardiac failures (i.e. heart valve replacement), diabetes, kidney replacements, burn victims, and bone replacement, amongst many others.

Another alternative to whole organ transplantation is to replace only partial (yet specific) portions to regenerate organ function. For example, xenogeneic islet transplantation is a popular, though not clinically accepted, therapy for type I diabetes. Unfortunately, this treatment has demonstrated only bittersweet success. Although the porcine islets have been shown to effectively reduce diabetic symptoms by producing insulin, immunosuppressants are needed to treat the chronic rejection of the transplanted cells [88]. Therefore, some researchers have been looking at alternatives to xenogeneic transplantation that remove the dependency of immunosuppressants.

2.3.3.4 Biomaterials and Model Cells

Biomaterials have been used to avoid immune rejection of delivered antigenic components. For example, one tactic is to mask the presence of xenogeneic cells using a biomaterial encapsulating agent, such as alginate. Unfortunately, molecules released by immune cells were still able to pass through the encapsulating layer, identifying the antigen within [89]. Still, alginate has shown success in the encapsulation and delivery of bacterial cells as a probiotic remedy [10].

Some biomaterial encapsulants have been found to maintain or improve the metabolic activity of the cells within. Methyl methacrylate (MMA) and HEMA copolymer was used to encapsulate CHO cells, which were found to be stable and non-toxic throughout the 14 day observation period [90]. Although this study did not use animal models, the preliminary *in vitro* results suggest that the biomaterial has at least a short-term ability to prevent immune rejection of the xenogeneic cells. In a related study, mice were given a hamster skin graft and challenged intraperitoneally with different

biomaterials, including HEMA-MMA [91]. Mouse splenocytes were challenged *in vitro* with hamster splenocytes and measured for cell proliferation. The results indicated that splenocytes from mice that received biomaterial implants were less responsive to the *in vitro* challenges than the splenocytes retrieved from mice with the skin graft alone. Specifically, mouse splenocyte response to the *in vitro* xenogeneic challenge was suppressed due to the addition of biomaterials. The result of this study raises a number of questions. First, is the physical barrier (by the biomaterial) between the xenogeneic challenge and host required to suppress immune rejection? The former study uses HEMA-MMA as an encapsulating agent, which physically separates the CHO cells from the host. In the latter study, however, the xenogeneic challenge and biomaterial interact with the host in two separate locations. Still, the results show that the addition of the biomaterial suppressed splenocyte proliferation. This dichotomy leads one to question the effect of HEMA-MMA that caused the host to reduce immune rejection of the xenogeneic challenge. More generally, what are the effects of biomaterials on host response?

In this project, I have investigated whether or not alginate has immunosuppressive effects like HEMA-MMA. It has been shown that alginate cannot shield xenogeneic islets from immune recognition and rejection [89]. However, can the effect of alginate reduce the host response to a xenogeneic challenge? The immunological effect of alginate with xenogeneic cells is still unclear. This relationship between the device and host is an important aspect of regenerative medicine, and thus merits further examination.

2.4 Immune Response to Biomaterials

The study of the immunological effects of biomaterials has grown drastically over the past few decades because of its increasing use in medical applications. This research area is quite broad and can be broken down into specific effects such as inflammatory response, fibrotic response, and adjuvancy. In the case of implants, a fibrotic response is usually not wanted around the device. The fibrotic capsule (essentially collagen) is laid

down by fibroblasts and is usually non-vascular. Thus, surface modifications can be done to try to minimize fibrosis.

Some researchers, like Babensee, believe that biomaterials only induce an innate response but not an adaptive response [19]. Indeed, some biomaterials have been shown to have the ability to induce an innate immune response, being recognized by the same receptors as other antigens. Furthermore, some of these biomaterials have shown success as an adjuvant [39]. Still, much research is needed to fully understand the interactions between the biomaterial and the host.

Preliminary studies usually begin with *in vitro* studies using either immortalized cells (i.e. macrophage-like cell line RAW264.7, dendritic cell line DC2.4) or cells isolated from peripheral blood. Using these cells, researchers have been able to identify specific immune cell responses to biomaterials. Raghuvanshi *et al.* showed that APCs were more attracted to PLA particles than PLGA particles, causing a greater immune response from PLA than PLGA [9]. This greater immune response can be explained by the hydrophobic nature of PLA. Therefore, it can be hypothesized that the greater the hydrophobicity of the material, the greater an immune response it can induce. PLGA particles have also been used to demonstrate immune cell functionality and performance. Lutsiak *et al.* showed that macrophages phagocytosed PLGA nanoparticles equally as efficiently as dendritic cells [92].

The physical form of the material has been found to influence the response type. For example, alginate solution was found to activate the inflammatory response [56] while alginate film did not activate innate immune cells [19]. Also, alginate as an encapsulating agent was found to induce an antibody-mediated response in animals [66]. Similarly, chitosan and PLGA films did not induce dendritic cell maturation [19, 20] whereas PLGA particles do stimulate dendritic cells, as explained earlier [9]. PLGA microparticles have been shown to effectively present OVA to dendritic cells 100-fold better than soluble antigen [93]. Hydrophobically modified poly(γ-glutamic acid) was also found to deliver OVA peptide to dendritic cells leading to their maturation [94]. The use of biomaterials to increase peptide uptake and induce maturation in dendritic cells is

an important research step while investigating materials with potential vaccine applications. The diverse effect of biomaterials on immune response continues to be explored today. The effect of particle size difference on antibody response was investigated between PLGA nanospheres and microspheres. It was found that the difference in particle size had no effect on the elicited IgG1 or IgG2a-type responses. This was found to be true for particles administered subcutaneously, orally, and intranasally [95]. Just as films and scaffolds are investigated for compatibility of cell growth, encapsulating materials are usually examined for adjuvancy.

2.5 Adjuvancy of Biomaterials

The word 'adjuvant' comes from Latin term *adjuvare*, which means "to help" [39]. Although adjuvants are commonly used in research and clinical practice, the mechanisms of its functionality is still unclear. It is known, however, that adjuvants used in vaccines help prolong protection against infections. According to Guy, adjuvants are "compounds that can increase and/or modulate the intrinsic immunogenicity of an antigen" [39]. When administered with a purified antigen, the adjuvant facilitates and amplifies an antibody-mediated and/or cell-mediated immune response to the antigen. The use of an adjuvant allows a lesser amount of antigen to be administered for equal immunogenicity of just the antigen alone.

Innate activators like cholera toxin (CT) and LPS have the ability to generate a non-specific innate immune response. If an activator can be directed to generate a specific response to a co-delivered antigen, then the activator is also an adjuvant. With more recent molecular technologies, researchers were able to determine specific sequences of these components that activate the response. For example, N-acetylmuramyl-L-alanyl-D-isoglutamine (also named muramyl dipeptide, MDP) was found to be an adjuvant peptide able to stimulate both humoral and cell-mediated immune responses [96]. These peptides, derived from bacterial cell wall, were shown to enhance immune responses similarly to its original whole structure.

Although an adjuvant can stimulate an innate immune response, the adaptive immune system is unaffected. Many studies utilize carrier proteins like BSA to stimulate the adaptive immune system. Therefore, the combination of biomaterial and carrier protein demonstrates the biomaterial's adjuvancy. Most adjuvants can enhance both antibody-mediated and cell-mediated immunity, depending on the antigen. Cytokines [97], heat-shock proteins [98], polymers [99, 100], bacterial DNA [101], mineral salts [1], and emulsions [1] are all common adjuvants. Since there are many types of adjuvants, these items are groups into general categories, such as bacterial adjuvants, oil-based adjuvants, and mineral-based adjuvants

One of the first adjuvants discovered was an oil-based adjuvant discovered by Le Moignic and Pinoy (referenced in [102]). These men showed that killed *S. typhimurium* mixed in mineral oil increased immune response. One of the most potent adjuvants to date was a as an oil-based adjuvant developed by Jules Freund (referenced in [102]). This emulsion contained water-soluble antigen with killed mycobacteria in mineral oil and was named Freund's Complete Adjuvant (FCA). Its potency can lead to highly severe reactions, including organ failure, inflammation, fever, induced autoimmunity, arthritis, hypersensitivity, even death. Therefore, this adjuvant is not used in humans, but has been approved for use in rodents. Freund's Incomplete Adjuvant (FIA) contains the same formulation without the mycobacteria. Unlike FCA, this adjuvant predominantly stimulates antibody production over cell-mediated responses. Previously, FIA had been used in human vaccines. Even though this adjuvant is slightly less potent than FCA, it can still lead to severe side effects such as inflammation, granulomas, abscesses, and cysts. Therefore, this adjuvant is no longer permitted for human use. Freund's adjuvants have been found to be very effective with water-soluble antigens.

Other oil-based adjuvants have also been developed as safer alternatives to Freund's adjuvants. Non-Ulcerative Freund's Adjuvant (NUFA) triggers an antibody-mediated response without the undesirable formation of lesions obtained with FIA. Gerbu Adjuvants (GA) utilize water-soluble, aliphatic amines instead of oil to minimize the side effects of oil. Bacterial components are also replaced by GMDP, a synthetic glycopeptide

from mycobacterial cell wall. The Ribi Adjuvant System (RAS) is another oil-in-water emulsion adjuvant that uses components of bacterial products instead of whole bacterial cells to lower undesirable toxic side effects. This system also uses a metabolizable oil called squalene, diminishing the side effects from non-metabolizable oils (i.e. paraffin oil). Freund's complete adjuvant is also known as a bacterial adjuvant because of the use of mycobacteria. Adjuvants using whole bacterial cells, cell wall skeletons, bacterial toxins (i.e. CT), and endotoxin (i.e. LPS and its derivatives) all fall under this classification.

For humans, mineral-based adjuvants such as aluminum hydroxide (i.e. Alhydrogel®) and aluminum potassium (Adju-Phos®) are regarded as safe and effective. Aluminum salts have been found to increase IL-1 and IL-4 production, an indication of innate and antibody-mediated immunity. They have also been found to be the best enhancer of immune response to weak antigens. Furthermore, mineral-based adjuvants can be combined with bacterial components to further enhance immunological responses. However, aluminum-based adjuvants have been found to have several considerable complications. It has been found that some proteins adsorb to the aluminum salts, rendering them less thermally stable [103]. Moreover, allergic reactions to aluminum occur sporadically and can be very dangerous [1]. Furthermore, aluminum salts can only induce an antibody (or Th2-type) response [39]. Therefore, biomaterials with adjuvant effects offer a safer alternative to current aluminum-based adjuvants. Alhydrogel is a commercially-available aluminum hydroxide gel (Cedar Lane Laboratories; Hornby, ON, Canada). Alhydrogel 2.0% is used internationally as a standard for comparison when identifying potential adjuvants. This formulation contains 2% aluminum oxide (equivalent to 3% aluminum hydroxide).

In many cases, adjuvants also act as a carrier, protecting the antigen before reaching its target site. Polymers such as polystyrene [99, 100] and PLGA [99, 100] have been used as encapsulating agents and investigated for adjuvancy. Specifically, PLGA has demonstrated adjuvancy with immunoreactive tetanus toxoid, but was not as powerful as traditional aluminum-based adjuvants [9]. Interestingly, a recent study found

that PEG 400 showed no adjuvant effects. Instead, it was found to induce an immunosuppressive effect [2]. Thus, it has been shown that biomaterials can potentially amplify or mask an immune response.

4. MATERIALS AND METHODS

4.1 Animals

Male Balb/c mice (Charles Rivers Laboratory, Wilmington, MA) were used throughout this project. The mice were housed at the McMaster University Central Animal Facility (CAF) in level B care. Animals were acclimated for at least one week prior to research use. On average, mice were 2-3 months old when experiments were carried out. All treatments have been approved by McMaster's Animal Research Ethics Board (AREB) committee under Animal Usage Protocol # 04-11-52.

4.1.1 Immunization

Mice were immunized following the standard operating procedures of the CAF. The mice were anesthetized using Isoflurane in a level B operating room. Intramuscular injections were given in the thigh, at a volume of 50 µl using a 30-gauge needle (CAF455.sop). For single injections, only the left thigh was used. For double injections, both thighs were used. The injection site was first shaved, and then cleaned with ethanol. Animals were monitored closely post-injection until they had regained normal movement, usually within minutes.

Two experiments were carried out *in vivo*: protein-challenged and cell-challenged experiments. The protein-challenged experiment contained 5 mouse groups whereas the cell-challenged experiment contained only 4 (see Table 8). Alginate solution was injected at 1% w/v concentration (see Section 4.2 for details). Inoculations with BSA contained 0.02 mg BSA per injection. Alhydrogel® is a known adjuvant (aluminum hydroxide) and was used as a positive control in the protein-challenged experiment. No positive control existed for the cell-challenged experiment. CHO cells were injected in media at approximately 10^8-10^9 cells/ml. The alginate/CHO-injected mouse group was the only alginate/CHO-injected group of both experiments. The alginate and CHO cells were injected intramuscularly into opposite legs.

Protein-challenged	Cell-challenged
Naïve (non-treated)	Naïve (non-treated)
Alginate-injected	Alginate-injected
BSA-injected	CHO-injected
Alginate/BSA-injected	Alginate/CHO-injected
Alhydrogel/BSA-injected	

Table 8: Mouse groups for protein-challenged and cell-challenged *in vivo* experiments.

4.1.2 Tissue retrieval

Animals were sacrificed 10 days post injection. Several tissues were taken for further analysis: the blood, spleen, and draining lymph nodes (inguinal, popliteal). After anesthetization with Isoflurane, mice underwent cardiac puncture (GEN754.sop). Blood volume obtained by this method ranged on average from 0.5 – 0.8 ml per mice. The animals were cervically dislocated to ensure that mice were fully euthanized (CAF460.sop). Spleen and lymph node tissues were removed using aseptic technique (Bramson lab, McMaster University) and stored in cold Dulbecco's phosphate buffered saline (PBS). The animals were discarded following CAF standard procedures.

4.2 Alginate solution and microspheres

Alginic acid sodium salt from brown algae (low viscosity, MW: 12 – 80 kDa, 61% mannuronic acid [104]) was purchased from Sigma Aldrich (A2158, St. Louis, MO). Aqueous alginate was made at a 2% w/v concentration in PBS. The solution was sterile-filtered using 0.45 μm and 0.22 μm low protein-binding Acrodisc® syringe filters (PALL Life Sciences, East Hills, NY). Detailed procedures for preparing alginate solution and microspheres are found in Appendix A.

Microspheres were created following a previously described method [48]. Briefly, 1% alginate solution was emulsified in canola oil and crosslinked using a 0.5% $CaCl_2$/0.05%$ZnCl_2$ solution. Poly-L-lysine had been used in the past to enhance crosslinking, however, it has been shown to lead to macrophage activation [105]. Thus,

poly-L-lysine was not used in these batches. The alginate microspheres were washed once with PBS, resuspended in PBS, and sonicated for 1 h in the Branson 1200 sonicator (Ultrasonic Corp., Danbury, Connecticut). In preliminary batches, particles were sized using the Mastersizer (Malvern, UK). Alginate solution and microspheres were stored at 4°C in 50 ml Falcon centrifuge tubes. Both forms of alginate were used as stimuli in challenge reaction experiments (see Section 3.5.1). Particles were counted using a hemocytometer prior to its addition as a challenge.

Protein-loaded particles were also used to examine particle uptake by dendritic cells. Two different types of labelled particles were used:

1) Alginate particles fluorescently labeled with Rhodamine B Ethylenediamine (Molecular Probes, Eugene, Oregon). Rhodamine B Ethylenediamine was added to freshly prepared microspheres (see Appendix B). The fluorescent solution and microspheres were incubated for 4 h in the dark at room temperature (RT), and then rinsed 4 times with PBS. Fluorescently-labeled alginate particles were added immediately to dendritic cell cultures and examined using confocal microscopy (see Section 3.6 for details).
2) Alginate particles loaded with fluorescent-OVA (Biosearch Technologies, Novato, CA). This experiment highlights how a model protein such as ovalbumin is processed by dendritic cells when loaded into microspheres. The protocol to create these particles is identical to that of plain alginate particles, except the protein solution was mixed with alginate solution prior to emulsification. Fluorescent-OVA was of cell culture grade and used immediately upon reconstitution. Details of these experiments are found below (Section 3.6). Non-fluorescent OVA microspheres were also created to examine peptide presentation on dendritic cells. Ovalbumin-loaded alginate particles (non-fluorescent) were created and examined for protein release using the Bradford Assay (see Appendix C). At set time points (0h, 1h, 2h, 3h, 5h, 12h), microspheres were centrifuged at 1500 rpm for 5 min. Samples of the supernatant were taken and stored for protein quantification. After the collection for all time points was complete, samples were plated in duplicates onto a flat bottom

96-wellplate (Falcon 35-3072, Becton Dickinson and Co., Franklin Lakes, NJ). Half dilutions of ovalbumin solution (2 mg/ml) were used to generate a standard curve. Bio-Rad Reagent (B6916, Sigma-Aldrich, St. Louis, MO) was added to each well and incubated at room temperature for 5 minutes. The plate was read on the Victor^{3}V (Wallac 1420 multilabel counter, PerkinElmer, Waltham, MA) at 595 nm.

4.3 Proteins and peptides

Albumin from chicken egg white (A5253, Grade II) and albumin from bovine serum (B4287, for molecular biology) were purchased from Sigma Aldrich (St. Louis, MO). Both BSA and OVA solutions (in PBS, 2 mg/ml) were sterile-filtered in the same manner as the alginate solution. Protein solutions were stored at 4°C in polystyrene centrifuge tubes for up to 2 weeks.

Ovalbumin (fluorescent and non-fluorescent) was used to produce protein-loaded alginate microspheres, as mentioned in Section 3.2. BSA was used to examine protein release from microspheres and was also used in the *in vivo* portion of this project. Specifically, 0.02 mg BSA was injected with alginate, with Alhydrogel, or alone into mice, as mentioned in Section 3.1.1.

4.4 Cell lines and reagents

Three different cell lines were used in this project: CHO cells, dendritic cells, and T-cell hybridomas (dubbed 'DKL' cells). All cells were handled in NAPCO NapFLOW™ Class II Type A/B3 Biosafety Cabinet (Krackeler Scientific Inc., Albany, NY). Cells were cultured using plug seal cap tissue culture treated polystyrene flasks (Corning Inc., Corning, NY) and grown at 37°C with 5% CO_2 incubators (Sanyo Electric Co., Tokyo, Japan). Cells were cultured following standard procedures using sterile techniques. The protocol and reagent list can be found in Appendix C.

4.4.1 Chinese hamster ovary cells

CHO fibroblasts (CHO-K1 cell line, ATCC, Manassas, VA) were cultured in Minimal Essential Medium (MEM, 1X), with Earle's salts and L-glutamine + 10% fetal bovine serum (FBS) + 1% penicillin-streptomycin (P/S, all items from Invitrogen, Burlington, ON) and were used in cell-challenged mice experiments as a xenogeneic challenge. Cultured cells were pelleted in a 50 ml centrifuge tube, and supernatant media was aspirated. Cells were loaded into a 1 ml syringe, with minimal amounts of media present. Mice receiving CHO cell injections received 50 µl of cells (~10^9 cells) intramuscularly.

These cells were also used as a stimulus for *in vitro* challenge reactions (described in Section 3.5.2). Cells were suspended in CHO-S-SFM supplemented with 10% FBS and 1% P/S at 4 x 10^3 cells/ml and 4 x 10^4 cells/ml. Cells were gamma irradiated for 8 minute at 2500 rad using the Gammacell 1000 irradiator (Atomic Energy of Canada, Ottawa, ON) to slow proliferation. Finally, cells were added into challenge plates (50 µl), each sample having a CHO cell density of 10^3 cell/ml (low density challenge) or 10^4 cell/ml (high density challenge).

4.4.2 Dendritic cells

Dendritic cells (DC2.4 cell line) were cultured in cRPMI (RPMI-1640 medium (1X) + 10% FBS + 1% P/S + 1% HEPES + 1% non-essential amino acids + 1% L-glutamine + 0.1% β-mercaptoethanol; all from Invitrogen, Burlington, ON) and had several functions in this project. First, dendritic cells were combined with alginate particles (with and without protein) to investigate particle uptake and processing (see Section 3.6). Cells were also verified for MHC class I expression using flow cytometry (Section 3.8), since these cells were immortalized. This was an essential step since these receptors must be present for protein cross-priming to be possible.

4.4.3 'DKL' cells

These T-cell hybridomas were created in Dr. Jonathan Bramson's lab (McMaster University) by Deb K. Leung (hence, 'DKL'), following a set procedure [106]. Briefly, mouse (C57BL/6 OT-1) T-cells specific for the OVA epitope SIINFEKL (OVA 257-264) were fused together with immortal myeloma (BWZ.36) cells to create immortal T-cell hybridomas that are specific for SIINFEKL. This peptide binds to H-2K^b MHC class I receptors and activates CD8+ T-cells. DKL cells were used to observe cell behavior in response to alginate solution and microspheres under light microscopy (Section 3.6), mimicking naïve T-cells. Cells were cultured in cRPMI + 1% sodium pyruvate following standard sub-culturing procedures.

4.5 Challenge reactions

Animal tissues were harvested and examined *in vitro* for response against xenogeneic protein and cell challenges. Although the spleen contains mostly T- and B-lymphocytes, the cells may be at a different stage of maturation than other circulating lymphocytes. Thus, preliminary tests of T-cell response from the draining lymph nodes were also examined for comparison.

Spleen and lymph node tissues were processed in a similar manner, but challenged with different stimuli (due to limited number of cells). Both tissues were dissociated and resuspended in RPMI 1640 medium containing 10% FBS, 1% P/S, 0.1% β-mercaptoethanol. Originally, splenocytes were isolated using Lympholyte-M (Cedar Lane Laboratories; Hornby, ON, Canada). However, this method incurred large variability in the number of cells retrieved. Therefore, the protocol was modified to lyse red blood cells from the culture of splenocytes using ACK buffer (see Appendix D). Cells were enumerated using trypan blue and a hemocytometer, and resuspended at 2 x 10^6 cell/ml.

The DELFIA® cell proliferation assay (PerkinElmer, Waltham, MA) was used following the manufacturer's instructions to test mouse splenocyte and lymph node cells responses after a 3-day challenge incubation at 37°C and 5% CO_2. After processing the

tissues, challenges (100µl) were added to the wells of the 96-wellplate. Splenocytes ($2x10^5$ cell/well) and lymph node cells ($\sim10^5$ cell/well) were added last to ensure consistency between plates and sub-samples. After 3 days, BrdU was added to the plates and incubated for another 24 h. At that time, plates were centrifuged for 10 minutes at 300g and then fixed for 30 mins at RT with the DELFIA fixing reagent. Samples were washed with PBS-Tween (0.5%) and then anti-BrdU was added and incubated for 1h at RT. Samples were then rinsed and the inducer was added. The inducing reaction was stopped at 15 mins with sulfuric acid and analyzed immediately using Microsoft Excel. In all tables below, 'X' indicates the combinations of materials that were tested. Statistics were also calculated using Excel's ANOVA (one- and two-way). Significance was measured using either the Student t-test or the Dunnett test.

4.5.1 Protein challenge reactions

Alginate's effect on protein-challenged mice was tested using four mouse treatments, plus one naïve group. Immunized groups received one of the following (*in vivo* challenges):

i) Alginate solution (1%)

ii) BSA solution (2 mg/ml)

iii) Alginate/BSA solution (1%, 2 mg/ml)

iv) Alhydrogel/BSA solution (3% Al_2O_3, 2 mg/ml)

Some of the *in vitro* treatments performed on splenocytes were also repeated for lymph node cells. All test samples contained 100 µl of the challenge with 100 µl cells at concentrations listed above. Mouse splenocytes were challenged *in vitro* with different combinations of BSA solution and alginate (see Table 9). The lymph nodes produced fewer cells, therefore, only a select number of combinations were tested (see Table 10).

Alginate challenge	BSA challenge			
	0 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
No Alginate	X	X	X	X
1% Alginate solution	X	X	X	X
1% Alginate microspheres	X	X	X	

Table 9: *In vitro* stimuli on spleen cells in protein challenge reactions.

Alginate challenge	BSA challenge			
	0 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
No alginate	X			X
1% alginate solution	X			X
1% alginate microspheres	X			

Table 10: *In vitro* stimuli on lymph node cells in protein challenge reactions.

4.5.2 Cell challenge reactions

Alginate's effect on xenogeneically-challenged mice was investigated using sets of mice immunized with one of the following (*in vivo* challenges):

i) Alginate solution (1%)

ii) CHO cells (~10^8 – 10^9 cells)

iii) CHO cells with alginate solution (~10^8 – 10^9 cells, 1%)

A naïve group was also used as a negative control. The procedures to analyze splenocyte proliferation were identical to that of the protein challenge reactions. *In vitro* challenges of CHO cells were plated at specific final densities, namely 10^3 cell/ml (low density) and 10^4 cell/ml (high density). Mouse splenocytes from each set of mice were challenged *in vitro* with different combinations of alginate (solution and microspheres) and CHO cells (see Table 11). Lymph node cell challenges were limited to alginate solution and CHO cells only (see Table 12).

Alginate Challenge	CHO challenge		
	No CHO cells	Low density (10^3 cell/ml)	High density (10^4 cell/ml)
No alginate	X	X	X
1% alginate solution	X	X	X
1% alginate microspheres	X	X	X

Table 11: *In vitro* stimuli on spleen cells in xenogeneic challenge reactions

Alginate Challenge	CHO challenge		
	No CHO cells	Low density (10^3 cell/ml)	High density (10^4 cell/ml)
No alginate	X	X	
1% alginate solution	X		
1% alginate microspheres			

Table 12: *In vitro* stimuli on lymph node cells in xenogeneic challenge reactions

4.6 Microscopy

The light microscope Wilovert 30 (Hund Wetzlar, Germany) and the light/fluorescence microscope Axiovert 200 (Carl Zeiss MicroImaging, Inc., Germany) were used to assess cell morphology, cell viability, microsphere morphology and cell/microsphere enumeration. Photos were taken using AxioVision 3.1 (Carl Zeiss Vision, USA). Initial viability was measured using trypan blue and a hemocytometer. Cells were added to a 96-wellplate along with challenges. After a 24h of incubation, samples were photographed and measured for cell viability. Viability was also observed using the Live/Dead assay (Invitrogen, Burlington, ON) following manufacturer's instructions. In short, the combined live/dead reagents were added to cells and incubated for 30 minutes at RT. The cells were then viewed under the Axiovert 200 for analysis. This assay labels live fluorescent green and dead cells fluorescent red.

The Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Germany) was used to observe the uptake and processing of alginate microspheres (with and without fluorescent-OVA) by dendritic cells. Images were analyzed using the

corresponding LSM software. Dendritic cells were seeded onto pre-autoclaved glass slides and wells (200,000 cells/well) and incubated for 2 h at 37°C. Labelled alginate particles (without OVA) and alginate-encapsulated fluorescent-OVA particles were added (100 ul) to wells at approximately 1:100 ratio (cells:particles) and incubated at 37°C for specific time periods up to 24h. After incubation, cells were washed and fixed prior to observation under the confocal microscope. Detailed procedures can be found in Appendix E.

4.7 Antibody Titering

Antibody titering was performed to investigate alginate's adjuvancy. Blood samples were taken from mice and allowed to clot. Samples were centrifuged at 14,500 rpm for 30 minutes. Serum was removed and immediately frozen at -80°C until analysis. All data was analyzed using Microsoft Excel. Statistics were also calculated using Excel's ANOVA and significance was measured using the Dunnett test.

For protein-challenged mice, antibody response was assayed using a sandwich ELISA-directed method [107]. In summary, BSA (10μg/ml) and OVA (10μg/ml) were used as the specific and control antigen, respectively, in this experiment. Protein solutions were added in triplicates to coat ELISA plates and stored overnight at 4°C. The next day, plates were rinsed and blocked with 5% skim milk powder in PBS overnight at 4°C. Serum was added in serial dilutions (1:1 to $1:10^8$) the following day and incubated for 1h at 37°C. HRP-conjugated monoclonal rat anti-mouse IgG1, IgG2a, or IgM antibody diluted 1:5000 (Beckman Coulter, Mississauga, ON) was added to each sample and incubated for 1h at RT. Next, tetramethylbenzidine (TMB; Zymed (Invitrogen), Burlington, ON) was added to each well and incubated for 30 min in the dark. Finally, sulfuric acid was used as the stop solution, and plates were read at 450 nm within 30min with λ correction 620nm. Thorough washes were done in between each step (see Appendix F for full details).

A similar method for antibody testing was applied for cell-challenged mice, following the protocol described in [91] (see Appendix G). In brief, CHO cells and PBS

were used as the specific and control antigens. Cells were grown to confluence for the experiment. After washing with PBS, cells were fixed with 1% glutaraldehyde (Sigma-Aldrich, PN# G6257) in PBS and blocked with 5% skim milk in PBS. The subsequent steps are identical to the method mentioned above.

5. RESULTS AND DISCUSSION

The overall goal of this project was to examine the immunological effect of alginate co-delivered with either protein or cells. These combinations of biomaterial and antigen are model (yet typical) combinations that are used for tissue engineering and drug delivery purposes. Alginate solution and alginate microspheres have both been used as drug delivery vehicles in the past, thus, both forms of alginate were tested in this study. This study also investigated whether the physical state of alginate affected cellular response. While drug delivery models use protein antigens, tissue engineering models frequently combines biomaterials with cells. Therefore, this project examines both whole cell and protein antigens.

From this study, alginate solution was found to have significant potential as a vaccine adjuvant. The results indicate that alginate can trigger both Th1- and Th2-type responses. On the other hand, preliminary work also reveals that alginate microspheres have low adjuvancy. Moreover, the microspheres provoked splenocyte cell death *in vitro*. Still, microspheres were found to help deliver protein antigens to dendritic cells, enabling their activation.

5.1 *In vitro* effect of alginate on immune response

The effect of alginate on immune response was first tested *in vitro*. This assay measured the first-set response of splenocytes to alginate (solution and microspheres), as well as with model antigens: BSA and CHO cells.

5.1.1 Adjuvant effect of alginate in protein model systems

Naïve (non-treated) mouse splenocytes were challenged with BSA, alginate solution, and alginate microspheres. Alginate solution alone triggered a significant response from splenocytes over the blank (PBS challenge) sample (see Figure 6). Interestingly, the incorporation of BSA (1 μg/ml, 10 μg/ml, 100 μg/ml) to the alginate

challenges induced further cell proliferation. The response is evidently due to the complex of alginate-BSA, as BSA alone did not significantly affect the mouse splenocytes (Figure 7). The combination of alginate microspheres and BSA did not induce such a response (Figure 8). In fact, microsphere challenges appear to have lessened the proliferative response of cells.

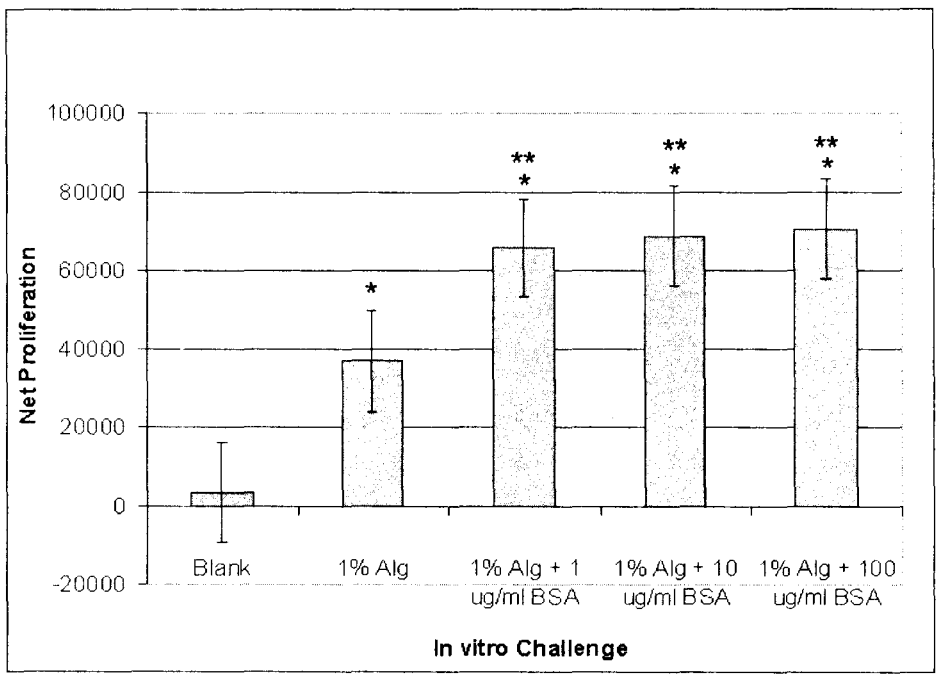


Figure 6: Alginate and BSA solutions combined stimulated significantly greater naïve splenocyte proliferation than either solution alone.

Splenocytes were incubated for 72h in each treatment, and then measured for proliferation using the DELFIA BrdU assay. Legend: Alg=alginate solution, Blank = PBS. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from blank treatment, ** $p \leq 0.05$ from 1% alginate solution treatment; $n = 4$)

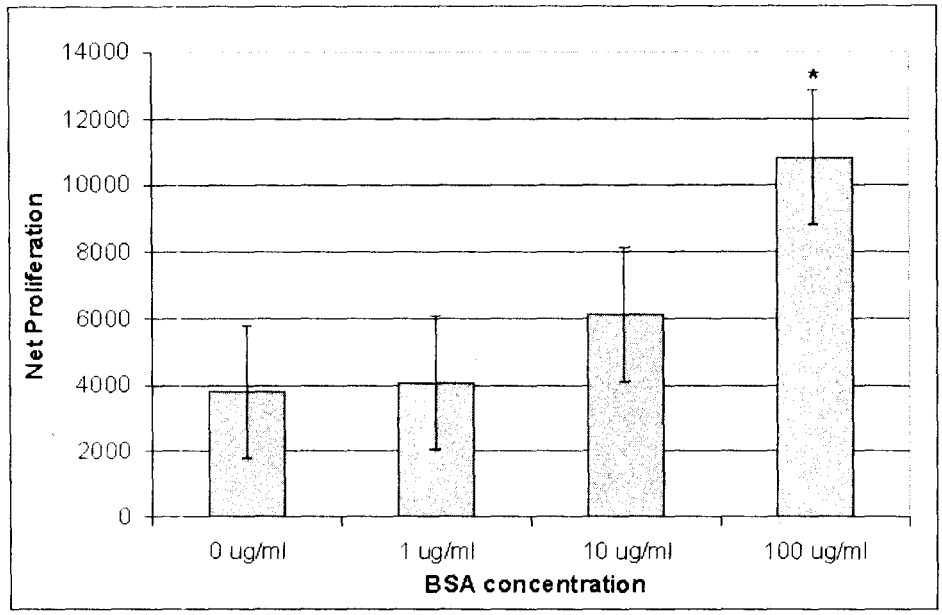


Figure 7: Naïve splenocytes were challenged with different concentrations of BSA solution (in PBS). Splenocytes became slightly more reactive with every 10-fold increase in BSA concentration. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from blank (0 µg/ml BSA) and 1 µg/ml BSA treatments; n = 4)

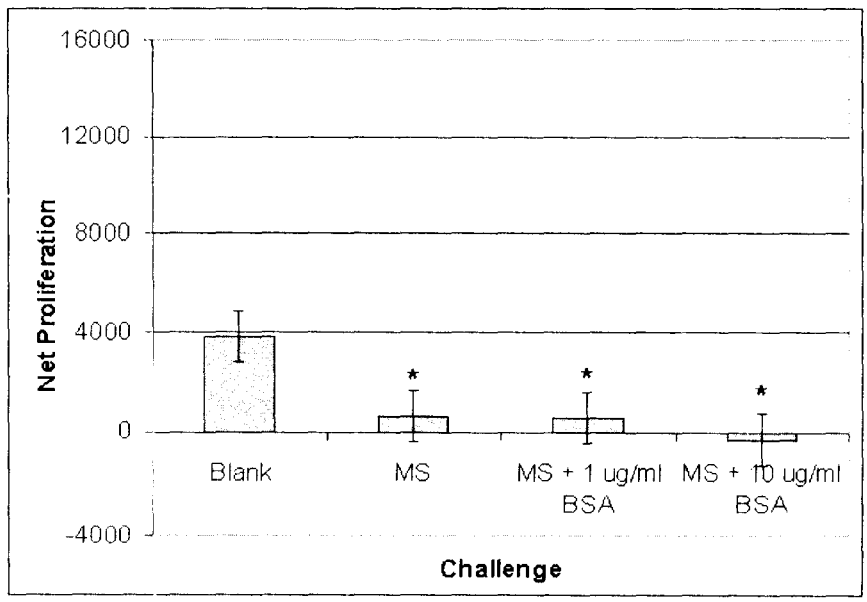


Figure 8: Naïve splenocytes were challenged with 1% alginate microspheres with and without BSA solution (in PBS).
These challenges did not induce splenocyte proliferation. Rather, a significantly lowered response was observed. Legend: MS = 1% alginate microspheres, Blank = PBS. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from blank treatment; n = 4)

The graphs (Figure 6-8) depict the first-set response of naïve splenocytes to alginate and BSA challenges. Of all the stimuli, the alginate/BSA combination induced the greatest response on naïve splenocytes. This result suggests that alginate possesses adjuvant abilities, which is further supported by the cell responses due to other challenges. The BSA challenges induced low responses, as was expected from a xenogeneic protein. Alginate solution induced a slightly greater response, which may be related to its characteristic as an innate activator [56]. Another likely interpretation of the response from alginate solution is that the response to 'alginate solution alone' may in fact be one with slight adjuvant effects. These experiments all contain background levels of BSA in cell media (<0.3μg/ml). Therefore, it is possible that this minute amount of BSA may have been sufficient to combine with alginate solution "alone" to induce the final response. In this case, this challenge would be demonstrating alginate's adjuvancy as well as the sensitivity to the antigen concentration present in the sample. This speculation is supported by the response from the combined alginate and BSA challenges. Even though the alginate/BSA challenges were serially diluted, their responses were nearly identical. The lowest dilution, 1 ug/ml BSA, may therefore have already reached the maximum concentration to interact with alginate solution for a maximum response level. Still, at this concentration, the splenocyte response is significantly greater than BSA alone and alginate alone. The literature tells us that polysaccharide-protein interactions can alter polymer and protein structures [87]. It is equally possible that the complex inducing the great response is between alginate and another protein (or non-specifically with any foreign protein) in the cell medium. Overall, the complex formed may have induced the exposure of stimulatory epitopes, influencing the apparent adjuvant effect.

5.1.2 Adjuvant effect of alginate in model cell systems

In tissue engineering applications, the delivery of whole cells into the body is a common practice. Thus, it is important to understand the immunological effects of delivering alginate with whole cells. In this project, CHO cells were used as the model cell. The addition of CHO cells induced a slight increase in cell proliferation over the

blank sample. This effect was comparable to that from alginate solution alone (Figure 9). Still, the combination of CHO cells and alginate stimulated a significantly greater response from mouse splenocytes. However, the stimulatory effects of combined alginate and CHO cells were not seen with alginate microspheres (data not shown).

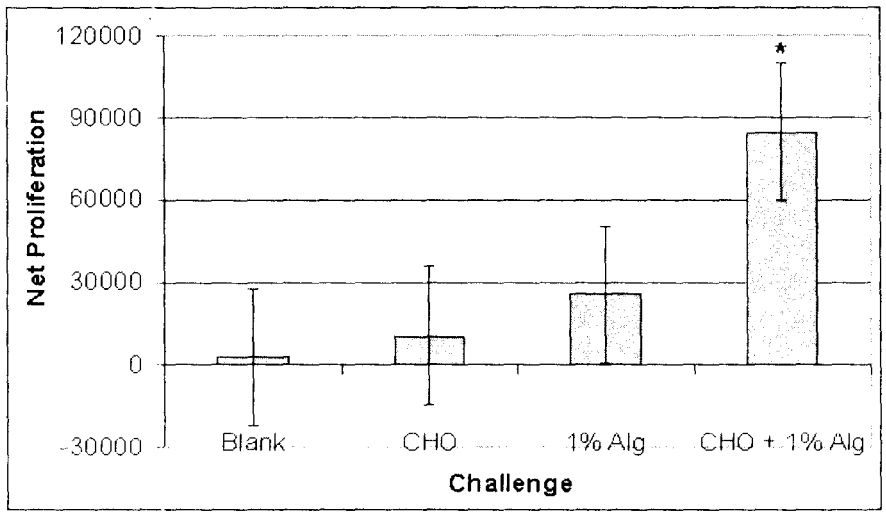


Figure 9: The in vitro challenge of alginate solution and CHO cells stimulated the greatest response from naïve mouse splenocytes.
This combination stimulated more cell proliferation than from either challenge alone. Legend: Blank = PBS, CHO = 10^4 CHO cells, Alg = alginate solution. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from blank and CHO cell treatments; n = 4)

Thus, the combination of CHO cells and alginate solution induced the greatest first-set response on naïve mouse splenocytes in this model system. This response was statistically greater than the cumulative responses from alginate solution alone and CHO cells alone. These results do not clearly depict alginate's adjuvancy, however, they do not rule out the possibility. All challenges other than those with microspheres induced a greater response than the blank. Thus, it is likely that the microspheres are causing splenocytes (and possibly CHO cells as well) to suppress proliferation. Further experiments were done (described below) to investigate this question. Cells contain many surface receptors that alginate can interact with, causing the cells to react. These reactions

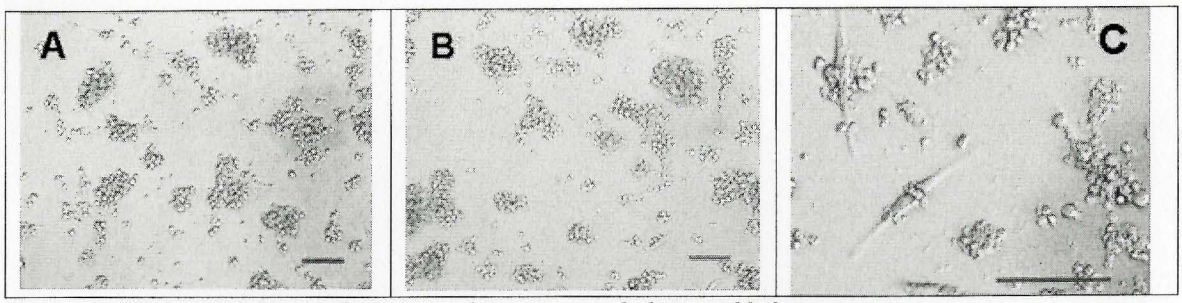


Figure 1: Effects of alginate solution on splenocyte morphology and behavior.
Cells were challenged with: A) 1% alginate solution; or B) 1% alginate solution + 100 μg/ml BSA; Figure C shows a zoomed in version of Figure B, depicting the elongated and clusters of cells. (bar = 50 um).

Examination of splenocytes with alginate microspheres was also done using the light microscope (Figure 11). Unlike the alginate solution, microspheres were found to have no effect on cell morphology after 24 h. Splenocytes challenged with microspheres without BSA (Figure 11B) and with BSA (Figure 11C) had a murky/cloudy aqueous phase, likely due to degraded microspheres. Still, challenged samples showed no physical difference in cell morphology compared to non-treated cells (Figure 11A).

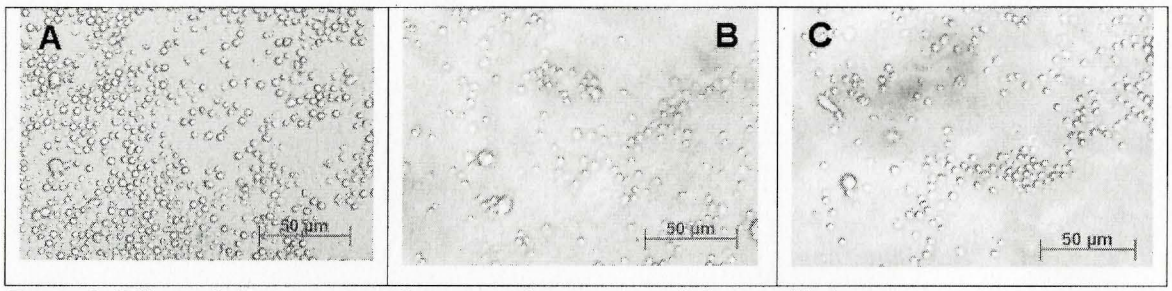


Figure 2: Effects of alginate microspheres on splenocyte morphology and behavior.
Cells were challenged with: A) PBS; B) 1% alginate microspheres; or C) 1% alginate microspheres + 100 μg/ml BSA. No significant changes in cell morphology or behavior were observed. (bar = 50 um).

With both the splenocytes and CHO cells, background levels of BSA exist in the cell media. It is unclear whether this background level had an effect on either model system when combined with alginate, however, it is a possibility that cannot be ruled out. Serum is used to facilitate cell development, although alternatives (i.e. mouse serum) are

available. Still, using serum-free media is not an option since it is required for T-cell activation [108].

Alginate's impurities may have also influenced the stimulation on splenocytes. The effects of such impurities may have an augmented effect when used in combination with xenogeneic antigens. This query can be investigated easily by using ultra pure alginate, as available from NovaMatrix (Sandvika, Norway). The impurities in the alginate solution may have also caused variability in splenocyte proliferation between different repetitions of the experiments (new lot of alginate solution each time). Although the solutions were produced by identical techniques and stored in the same conditions, *in vitro* re-challenge of alginate solution caused varying levels of proliferation from mouse splenocytes. Since solutions are derived from the same source of alginate powder, the non-specific proliferation could have been caused by small impurities that passed through the syringe filters, and/or the results are simply due to mouse-to-mouse variability. Still, the trend of responses from the challenges was quite consistent between repetitions of the experiment.

5.2 *In vivo* effect of alginate on immune response

In the previous section, the effect of alginate as an *in vitro* challenge was examined. Here, the *in vivo* effects of alginate (injected) and its effects on *in vitro* challenges are investigated. Injections of aqueous alginate were found to lower the general splenocyte response to *in vitro* challenges, although the general trend of responses was maintained. Sections 5.2.1 and 5.2.2 explain more details of the second-set responses from the protein and cell model systems, respectively.

5.2.1 Alginate with model protein

The effect of various *in vitro* challenges on naïve splenocytes was examined in the previous section. In this subsection, the same *in vitro* challenges were used to stimulate splenocytes from immunized mice. Mice were immunized with alginate

solution, BSA solution, alginate/BSA solution, or Alhydrogel/BSA solution. Splenocytes from alginate-injected and BSA-injected mice were less responsive to 100 μg/ml BSA than naïve splenocytes (Figure 12), as with alginate/BSA-injected mouse splenocytes (Figure 13). Splenocytes from Alhydrogel/BSA-injected mice, however, had significantly greater cell proliferation from the same BSA challenge. This was expected since Alhydrogel (aluminum hydroxide) is a known adjuvant. Splenocytes from these four mouse groups were also challenged with alginate solution (with and without BSA). While naïve splenocytes had an increased response to alginate/BSA together, the response from immunized mouse splenocytes remained unchanged (Figure 14 and Figure 15). Still, all mouse groups gave consistent responses when challenged with microspheres (with and without BSA). That is, alginate microspheres did not stimulate the proliferation of mouse splenocytes (Figure 16 and Figure 17).

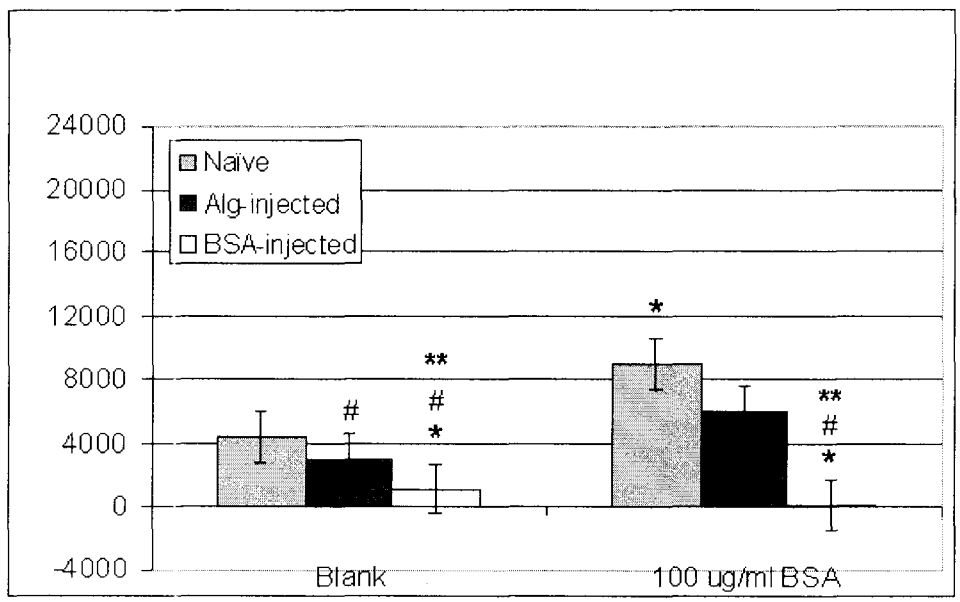


Figure 12: Alginate- and BSA-injected mouse splenocytes were challenged in vitro with 100 μg/ml BSA. Their response was compared to that of naïve splenocytes. The response from BSA-injected mice was significantly less than from naïve splenocytes challenged with alginate. In addition, BSA-injected mouse splenocytes produced a significantly lower response to 100 μg/ml BSA challenge than the naïve splenocytes. Error bars show standard errors of the mean. (Statistics: *$p \leq 0.05$ from naïve splenocytes with blank treatment, # $p \leq 0.05$ from naïve mouse splenocytes challenged with 100 μg/ml BSA, **$p \leq 0.05$ from alginate-injected mouse splenocytes with 100 μg/ml BSA treatment; $n = 5$)

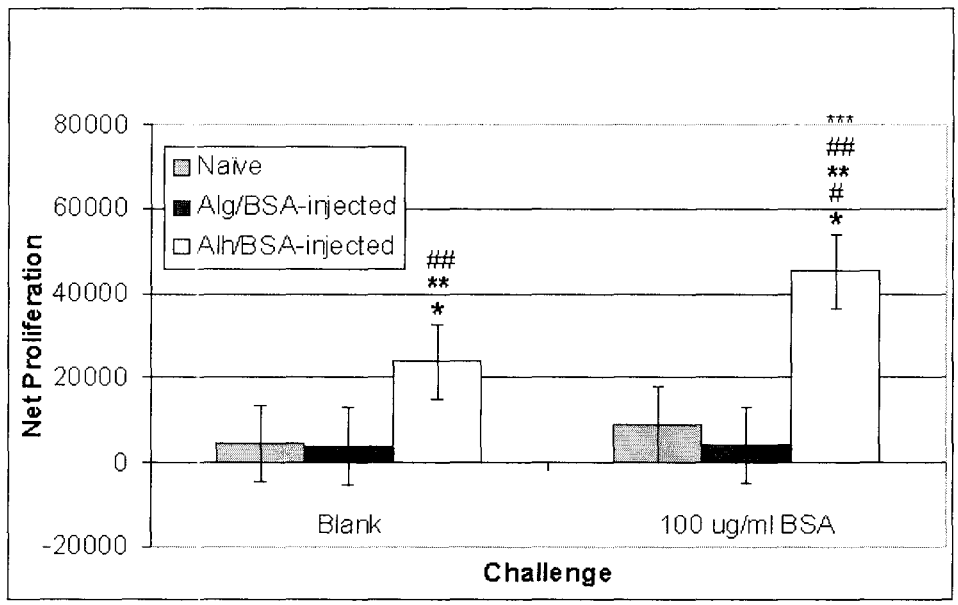


Figure 13: Alginate/BSA-injected and Alhydrogel/BSA-injected mouse splenocytes were challenged in vitro with 100 µg/ml BSA.

Alhydrogel is a known adjuvant, thus a significantly increased response was expected from *in vitro* BSA challenge, as is shown here. Alginate/BSA-injected mice, however, were not affected by the BSA challenge, in comparison to naïve splenocytes. Error bars show standard errors of the mean. (Statistics: *$p \leq 0.05$ from naïve splenocytes with blank treatment, # $p \leq 0.05$ from naïve mouse splenocytes challenged with 100 µg/ml BSA, **$p \leq 0.05$ from alginate/BSA-injected mouse splenocytes with blank treatment, ## $p \leq 0.05$ from alginate/BSA-injected mouse splenocytes challenged with 100 µg/ml BSA, ***$p \leq 0.05$ from Alhydrogel/BSA-injected mouse splenocytes with blank treatment; $n = 5$)

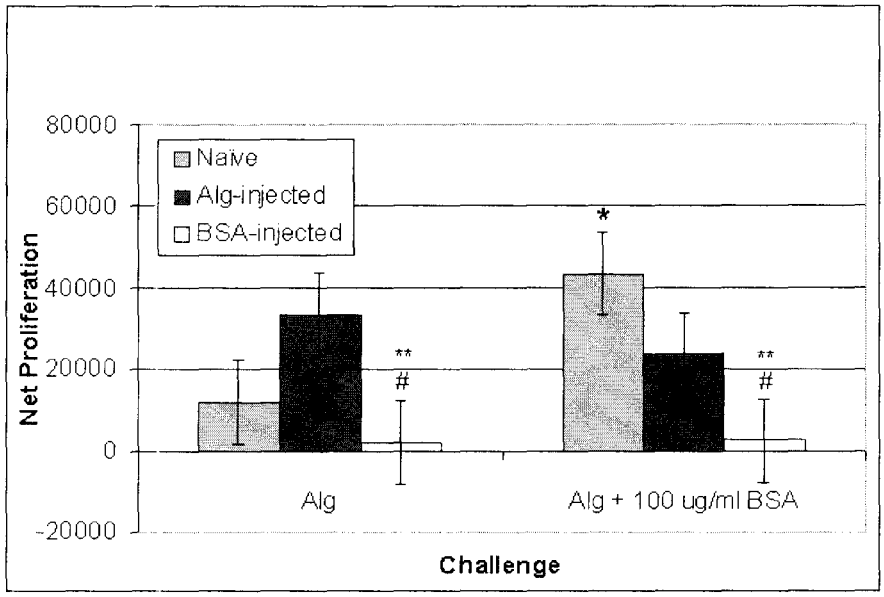


Figure 14: Alginate- and BSA-injected mouse splenocytes were challenged in vitro with alginate solution, with and without 100 µg/ml BSA.

The response from BSA-injected mice was significantly less than from naïve splenocytes. Legend: Alg = 1% alginate solution. Error bars show standard errors of the mean. (Statistics: *$p \leq 0.05$ from naïve splenocytes with 1% alginate solution treatment, # $p \leq 0.05$ from naïve mouse splenocytes challenged with alginate solution + 100 µg/ml BSA, **$p \leq 0.05$ from alginate-injected mouse splenocytes challenged with 1% alginate solution; n = 4)

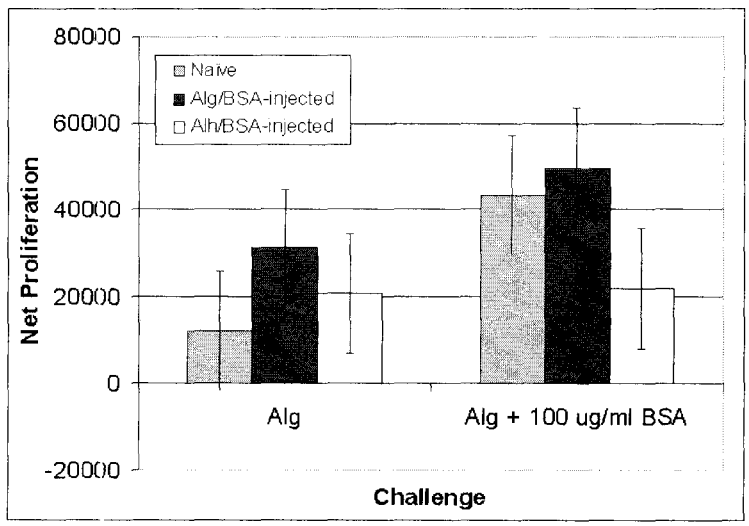


Figure 15: Alginate/BSA- and Alhydrogel/BSA-injected mouse splenocytes were challenged in vitro with alginate solution, with and without 100 µg/ml BSA.

Alginate/BSA-injected mice were slightly more responsive to challenges than naïve mice, but not statistically significant. In addition, Alhydrogel/BSA-injected mice responded identically with or without BSA. Legend: Alg = 1% alginate solution. Error bars show standard errors of the mean. (n = 4).

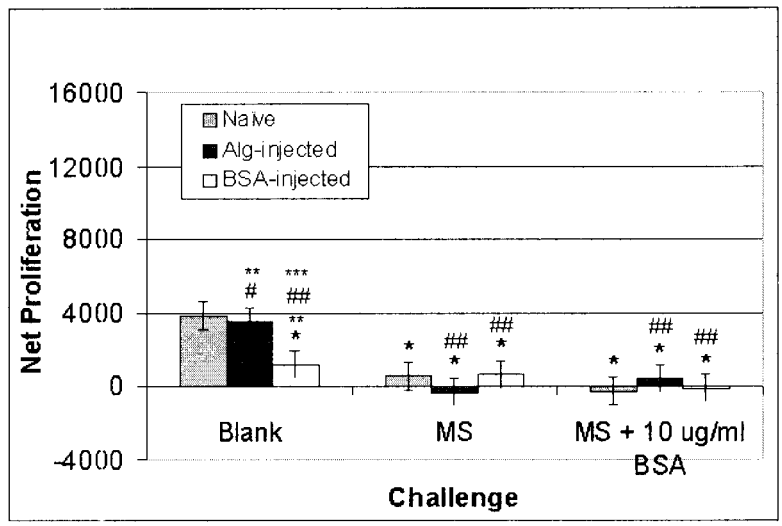


Figure 16: Alginate- and BSA-injected mouse splenocytes were challenged in vitro with alginate microspheres, with and without 10 µg/ml BSA.

In comparison to the naïve mouse group, these immunized mouse groups were not stimulated by microspheres, with or without BSA. Legend: MS = 1% alginate microspheres. Error bars show standard errors of the mean. (Statistics: *$p \leq 0.05$ from naïve splenocytes with blank treatment, #$p \leq 0.05$ from naïve mouse splenocytes challenged with 1% alginate microspheres, **$p \leq 0.05$ from naive mouse splenocytes challenged with 1% alginate microspheres + 10 µg/ml BSA, ##$p \leq 0.05$ from alginate-injected mouse splenocytes with blank treatment, ***$p \leq 0.05$ from alginate-injected mouse splenocytes challenged with 1% alginate microspheres, ###$p \leq 0.05$ from alginate-injected mouse splenocytes challenged with 1% alginate microspheres + 10 µg/ml BSA; n = 4)

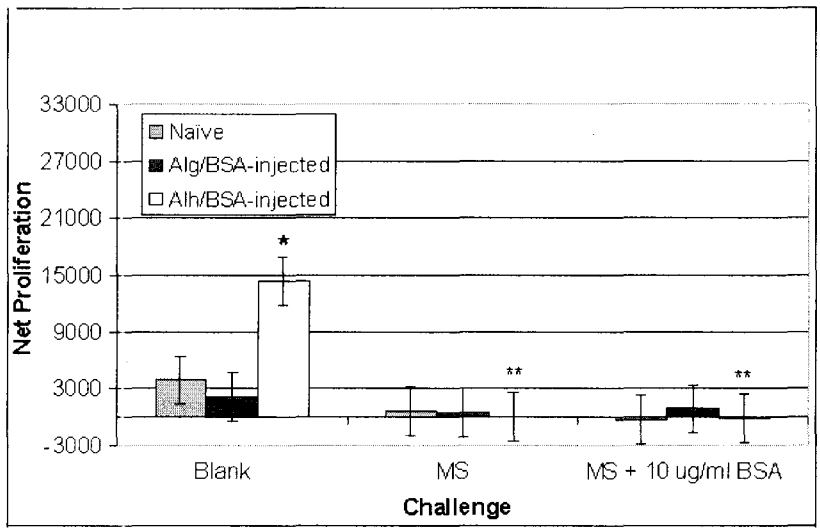


Figure 17: Alginate/BSA- and Alhydrogel/BSA-injected mouse splenocytes were challenged in vitro with alginate microspheres, with and without 10 μg/ml BSA.
No mouse group gave a significant response to microspheres, with or without BSA, except for the positive control group (Alhydrogel/BSA-injected mice). Legend: MS = 1% alginate microspheres. Error bars show standard errors of the mean. (Statistics: *$p \leq 0.05$ from naïve splenocytes challenged with blank and alginate microsphere (with and without 10 μg/ml BSA) treatments as well as from alginate-injected mouse splenocytes challenged with the same treatments, **$p \leq 0.05$ from Alhydrogel/BSA-injected mouse splenocytes with blank treatment; n = 4)

In the previous section, the first-set response to challenges was examined. Here, the second-set responses to the same challenges are explored. The previous section presented evidence of alginate solution's adjuvancy. Here, however, alginate does not display such effects. As an adjuvant, a response should be triggered upon re-encountering the antigen (i.e. the second-set response). Alginate/BSA-injected mouse splenocytes were unaffected by the re-challenge of BSA (Figure 13). In fact, their response was nearly identical to that of naïve splenocytes. On the other hand, the splenocytes from Alhydrogel/BSA-injected mice had an immense response to the BSA challenge, as was expected. This mouse group was also quite responsive to the blank sample of PBS, which is likely due to the BSA within the cell media itself, as previously discussed. No adjuvant effect was seen from the BSA-injected and alginate-injected mice, as expected.

Alginate/BSA-injected mouse splenocytes were most responsive when re-challenged with alginate solution, with and without BSA (Figure 15). The addition of alginate solution to the samples may have increased the number of alginate/BSA

complexes, which have been shown (in the previous section) to stimulate splenocyte proliferation. Thus again, the BSA in each sample is present in abundance, thus the addition of alginate solution determines the amount of increased response. This hypothesis can easily be tested by challenging splenocytes with a range of alginate solution concentrations (0.1% - 5%) with a constant (yet abundant) amount of BSA. Still, this hypothesis is not consistent with the response from alginate-injected mouse splenocytes when re-challenged with the combination of alginate and BSA. In fact, splenocytes from this mouse group appeared to have a dampened response similar to the trend of naïve mouse splenocytes in response to the various challenges. The reason for and significance of this trend is still unclear. Finally, alginate microspheres had no proliferative effect on splenocytes from immunized mice, as was seen with the naïve group.

Although the results from this assay provide important information about the effects of alginate *in vivo*, the inspection of the tissues at harvesting also illuminate some interesting observations. The appearance of the spleen and lymph nodes for all mouse groups were comparable in size, color, and luster. Still, there were variations in cell count between mice. Table 13 shows the highest, lowest, and average splenocyte cell count for each mouse group. Naturally, a range in cell counts is expected due to mouse-to-mouse variations. However, a large range of splenocyte cell count is due to the method of cell isolation that was used at the beginning of the project (splenocyte isolation using Lympholyte-M). The current method of cell isolation (using ACK lysing buffer) has shown to produce more consistent cell counts.

In general, all immunized mice had a greater splenocyte count than naive splenocytes, which is expected. The average cell count for immunized mouse groups is comparable, suggesting the host to each injection triggered a similar immune response. It is also interesting that BSA-injected mice had the greatest average splenocyte count, yet the splenocytes were least responsive to *in vitro* challenges.

Mouse Group	Lowest count (x 10^6 cells/ml)	Highest count (x 10^6 cells/ml)	Average spleen cell count (x 10^6 cells/ml)
Naïve (n=4)	1.82	7.6	4.41
BSA-injected (n=4)	6.5	10	8.25
Alginate-injected (n=4)	3.13	9.4	7.73
Alginate/BSA-injected (n=5)	3.15	11	6.69
Alhydrogel/BSA-injected (n=6)	2.03	12.9	6.65

Table 13: Splenocyte cell counts from different mouse groups.

Mouse Group	Lowest count (x 10^6 cells/ml)	Highest count (x 10^6 cells/ml)	Average LN cell count (x 10^6 cells/ml)
Naïve (n=3)	0.11	1.42	0.93
BSA-injected (n=4)	2.32	4.5	3.46
Alginate-injected (n=4)	1.11	3.94	2.51
Alginate/BSA-injected (n=3)	1.12	2.9	1.91
Alhydrogel/BSA-injected (n=5)	1.2	3.48	2.34

Table 14: Lymph node cell counts from different mouse groups.

Lymph nodes contain mostly T-cells which may be at a different activation stage than splenocytes. Therefore, lymph node tissues were examined after preliminary splenocyte experiments to verify the consistency of the splenocyte responses. Cell counts from lymph nodes were more consistent within groups than from splenocytes (see Table 14). Still, immunized-mouse lymph nodes increased in size two- to three-fold. Again, BSA-injected mice had the greatest lymph node cell count in the experiment, consistent with the splenocyte cell counts. This large response was not expected, and may have been due to impurities. Further studies with purified alginate and BSA are required to better assess these numbers.

5.2.2 Alginate with model cells

Like the protein model system, mice were immunized with antigen (CHO cells), alginate solution, or alginate and CHO cells (in different legs), and then re-challenged *in vitro*. Mice injected with alginate were less responsive to *in vitro* challenges. Specifically, alginate-injected mouse splenocytes were equally or less responsive to the *in vitro* challenges than naïve splenocytes. In addition, alginate/CHO-injected mouse splenocytes were equally or less responsive to the *in vitro* challenges than CHO-injected mouse splenocytes.

Alginate-injected mouse splenocytes responded similarly to naïve splenocytes when challenged with CHO cells (Figure 18). CHO-injected and alginate/CHO-injected mouse splenocytes, on the other hand, were more proliferative to the *in vitro* challenge (Figure 19). When challenged with alginate solution (with and without CHO cells), the response from alginate-injected mouse splenocytes was statistically the same as from naïve mouse splenocytes, although a slightly dampened response trend is visible (Figure 20). With alginate-injected and alginate/CHO-injected mouse splenocytes, a slight increase in response is seen. Finally, all splenocytes challenged with microspheres (with and without CHO cells) did not show significant changes in cell response (Figure 21 and Figure 22, respectively).

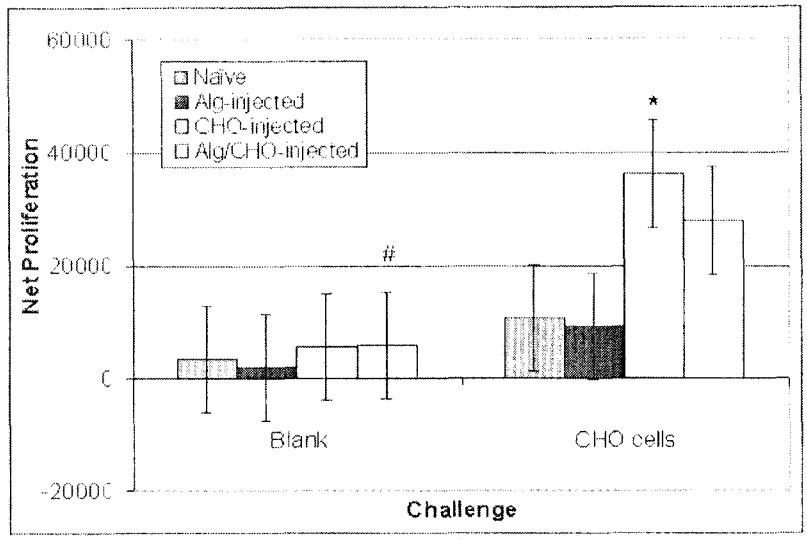


Figure 18: Alginate-injected mouse splenocytes responded comparably to naïve splenocytes when re-challenged in vitro with CHO cells (10^4 cells).
However, CHO- and Alginate/CHO-injected mice were produced a significantly larger responses to CHO cells than naive mice. Legend: CHO = CHO cells at 10^4 cells. Error bars show the standard error of the mean. (Statistics: *p ≤ 0.05 from naïve, alginate- and CHO-injected mouse splenocytes with blank treatment, **p ≤ 0.05 from CHO-injected mouse splenocytes challenged with CHO cells; n = 4)

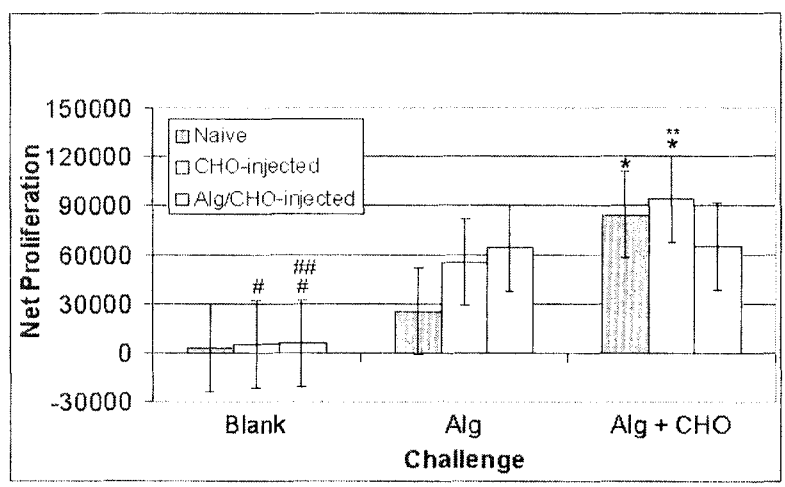


Figure 19: In vitro challenges of alginate (with and without CHO cells) were given to mouse splenocytes that were immunized with either CHO cells alone or CHO cells with alginate solution.
Both immunized mouse groups were affected by *in vitro* challenges, significantly more than naïve mouse splenocytes to the blank. Interestingly, alginate/CHO-injected mice responded identically to both challenges. Legend: Alg = 1% alginate solution, CHO = CHO cells at 10^4 cells. Error bars show the standard error of the mean. (Statistics: *p ≤ 0.05 from naïve mouse splenocytes with blank treatment, #p ≤ 0.05 from naïve mouse splenocytes challenged with CHO cells + alginate solution, **p ≤ 0.05 from CHO-injected mouse splenocytes with blank treatment, ## p ≤ 0.05 from CHO-injected mouse splenocytes challenged with CHO cells + alginate solution; n = 4)

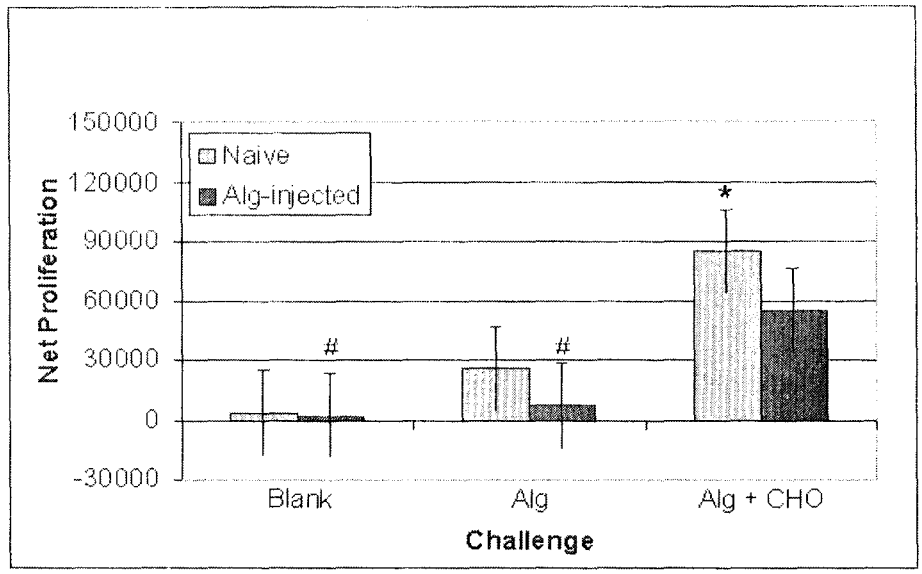


Figure 20: Alginate-injected mice challenged in vitro with alginate solution (with and without CHO cells) produced responses slightly lower than those of naïve mice.
Still, the response to alginate + CHO cells from alginate-injected mouse splenocytes was still significantly greater than the response from naïve splenocytes on the blank. Legend: Alg = 1% alginate solution, CHO = CHO cells at 10^4 cells. Error bars show the standard error of the mean. (Statistics: *$p \leq 0.05$ from naïve mouse splenocytes with blank treatment, #$p \leq 0.05$ from naïve mouse splenocytes challenged with CHO cells + alginate solution; n = 4)

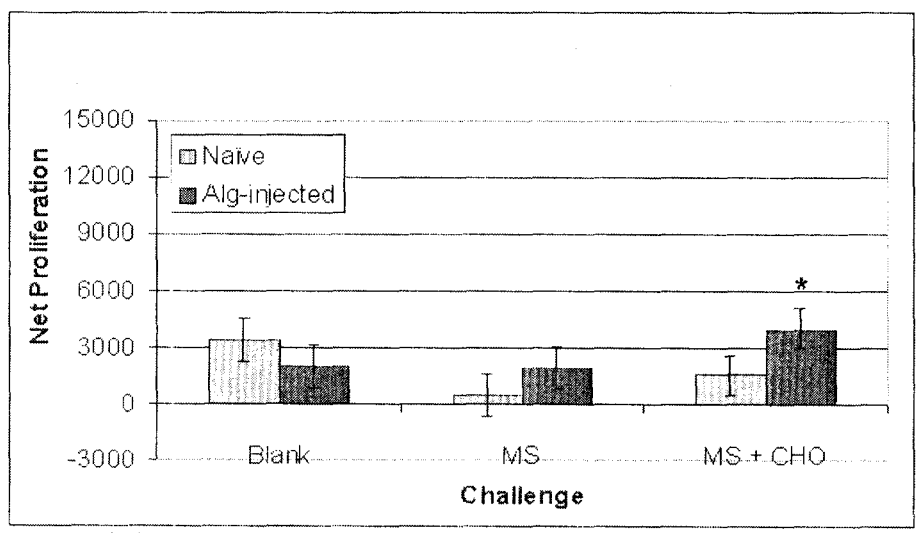


Figure 21: In vitro challenges of 1% alginate microspheres, with and without CHO cells
Alginate microspheres, with or without CHO cells, did not significantly affect alginate-injected mouse splenocytes in comparison to naïve splenocytes. Legend: MS = 1% alginate microspheres, CHO = CHO cells at 10^4 cells, *shows statistical differences in response compared to naïve splenocytes response to blank treatment. Error bars show the standard error of the mean. (Statistics: *$p \leq 0.05$ from naïve mouse splenocytes challenged with 1% alginate microspheres; n = 4)

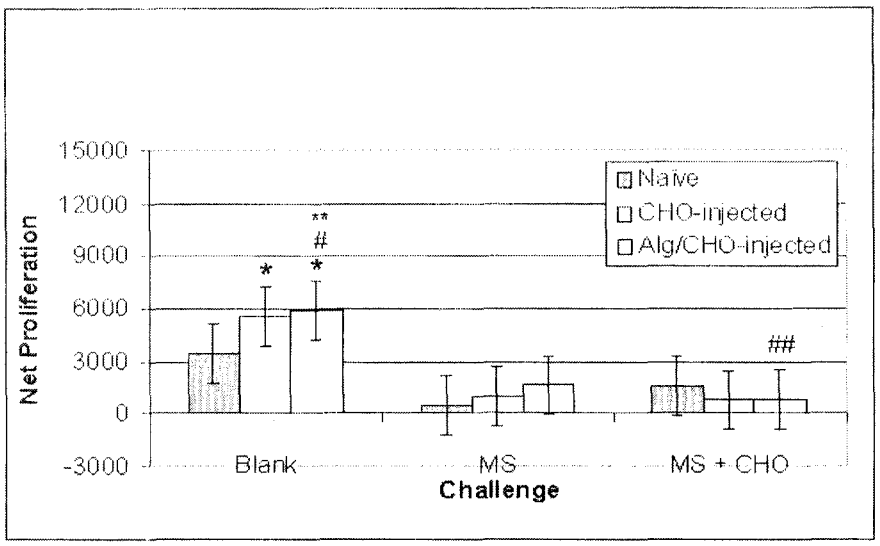


Figure 22: Response to in vitro challenges of 1% alginate microspheres, with and without CHO cells. Alginate microspheres appear to lower the splenocyte response from CHO-injected and alginate/CHO-injected mice, in comparison to naïve splenocytes. However, this trend was not significant. Legend: MS = 1% alginate microspheres, CHO = CHO cells at 10^4 cells. (Statistics: *$p \leq 0.05$ from naïve mouse splenocytes challenged with 1% alginate microspheres, #$p \leq 0.05$ from CHO-injected mouse splenocytes challenged with 1% alginate microspheres, **$p \leq 0.05$ from CHO-injected mouse splenocytes challenged with 1% alginate microspheres + CHO cells, ##$p \leq 0.05$ from alginate/CHO-injected mouse splenocytes with blank treatment; n = 4)

The injected alginate appeared to diminish the responsiveness of splenocytes to some of the *in vitro* challenges. At first glance, the results seem to indicate alginate-injected splenocyte proliferation was suppressed. However, there is insufficient evidence to make this claim. Still, this trend is particularly interesting in regenerative medicine applications. This experiment indicates that immunization with alginate lowers the immune response to the biomaterial itself (second exposure), with or without xenogeneic cells (Figure 20). The ability for alginate to lower the T-cell response to a xenogeneic antigen can translate to fewer problems of immune rejection and less side effects caused by immunosuppressants. The reason for this trend is unknown, although several basic tests can be done to better understand this response.

In general, CHO-injected and alginate/CHO-injected splenocytes gave similar responses to the *in vitro* challenges. However, several challenges induced a lowered (but

not significant) response from the doubly-injected mouse splenocytes than from CHO-injected splenocytes (Figure 18 and Figure 19). Moreover, splenocytes from the CHO-injected mice induced the greatest immune response of the experiment when re-challenged with alginate and CHO cells. The lesser response from alginate/CHO-injected mouse splenocytes is comparable to the trend from alginate-injected mice, as previously mentioned.

As with the protein model system, challenges with microspheres did not induce cell proliferation for any mouse groups (Figure 21 and Figure 22). Because this trend is repeated for all mouse groups in this model system as well as in the protein model system, further work with these microspheres were completed. This trend is quite interesting and will be discussed in the following section.

Also like the protein model system, the tissues were inspected during their harvesting. The spleen and lymph node sizes varied greatly depending on the injection. This was particularly true for mice injected with CHO cells. Splenocyte numbers of these mice were about double that of naïve mice (see Table 15). Lymph nodes from CHO-injected and alginate/CHO-injected mice were drastically larger than those from naïve and alginate-injected mice (see Table 16). The luster and color of the organs, however, did not change. Although CHO- and alginate/CHO-injected mice had larger lymph nodes, the nodes closest to the site of CHO cell injections were significantly (~10- to 20-fold) larger than the other naïve lymph nodes. This increase in size is indicated by the great increase in lymph node cell count. These cell counts, however, were greatly dependent on the retrieval of the tissues. Poor surgical technique led to low counts for the CHO- and Alginate/CHO-injected mouse groups (lowest counts on Table 16), both of which stem from the first test trial. Thus, these values are a poor reflection of the actual lymph node cell numbers from those mice. The subsequent trials gave more accurate cell counts due to improved surgical performance. These values hover around the highest cell count range. The large lymph node cell counts of these mice are indicative of the complexity of CHO cells as the antigen.

Mouse Group	Lowest count (x 10^6 cells/ml)	Highest count (x 10^6 cells/ml)	Average spleen cell count (x 10^6 cells/ml)
Naïve (n=4)	2.23	12	7.71
Alginate-injected (n=3)	2.89	10.8	7.04
CHO-injected (n=4)	4.7	11.7	9.25
Alginate/CHO-injected (n=3)	3.65	18	12.55

Table 15: Splenocyte cell counts from different mouse groups.

Mouse Group	Lowest count (x 10^6 cells/ml)	Highest count (x 10^6 cells/ml)	Average LN cell count (x 10^6 cells/ml)
Naïve (n=3)	0.60	2.85	2.08
Alginate-injected (n=3)	1.11	13	5.55
CHO-injected (n=3)	2.71	34	19.35
Alginate/CHO-injected (n=2)	27.7	36	31.85

Table 16: Lymph node cell counts from different mouse groups.

5.2.3 Comparison of model systems

The responses from protein and cell model systems both had interesting peaks and flats, which are examined in more detail in this section. The two major peculiarities from these experiments are the lowered proliferative abilities of alginate-injected splenocytes, and the non-proliferative effect from alginate microspheres. The lowered cell response from alginate-injected mice is unexpected, especially since alginate has been shown to stimulate innate activation. The lack of responsiveness from alginate microspheres is also strange, when comparing the stimulatory effects of alginate solution and from Alhydrogel (also microspheres).

Injections with alginate lowered some of the responses from splenocytes when re-challenged *in vitro*. With these results, it is unclear whether the response was suppressed, delayed, or other. However, since this trend is also partially seen in alginate/CHO-injected mice, it is logical to speculate that the lowered immune response is due to the interaction of the alginate with the host, and not a physical (i.e. barrier) or chemical (i.e. toxicity) effect of alginate. A feasible explanation is that immunization with alginate

induced (partial) tolerance against further challenges. If this were true, this could explain the responses seen above. Whether this tolerance is temporary (i.e. in longer term studies, alginate-injected mouse splenocytes would respond similarly to other mouse groups) or permanent (i.e. alginate-injected mouse splenocytes will always have a lowered response against *in vitro* challenges) is currently unknown. A similar study examining the effects of implanted HEMA-MMA and agarose on xenogeneically-challenged mice found that the HEMA-MMA/agarose implants lower splenocyte proliferation initially, but not after two months [91]. Longer-term studies and cytokine analysis can help obtain a clearer picture of whether alginate is mimicking the effect of HEMA-MMA, or if alginate has a completely different effect on host response. It is also possible that the initial encounter with alginate (the inoculation) may have intercepted a signaling pathway causing lowered response. The body as a system works in balance with itself. Therefore, the alginate challenge may have led to the effect of a disrupted immune response. This cause-and-effect relationship would be difficult to prove due to the complexity of the immune system working in conjunction with other systems of the body.

Another interesting result that is common for all mouse groups in both model systems is the lack of responsiveness from splenocytes that are challenged with alginate microspheres. It's possible that the microspheres (crosslinked) have more difficulty interacting with cells, and therefore are not stimulating their proliferation. The assay used is too broad to justify that the microspheres were simply not stimulating cell proliferation. It is equally possible that the microspheres were killing the cells, thus generating a 'no response' result. It is possible that the stress imposed on cells by microspheres is hindering cell proliferation. After 72h, it is likely that too few cells remain to produce a significant response. To verify cell viability, microspheres were added to cultured DKL cells and examined with trypan blue and the fluorescent Live/Dead Assay 24 hours later. These cells were chosen because they are immortalized T-cells, mimicking most closely mouse splenocytes used in this project. Since BSA and alginate solution were both PBS based, a PBS challenge was used as a control. Viability was measured as a percentage based on standard culture conditions (media only; set as 100% viability). Figure 23 shows

the adjusted viability of cells to each *in vitro* challenge. Preliminary results show that alginate microspheres greatly affected the viability of the cells. This can also be seen in images taken using a light microscopy (Figure 24).

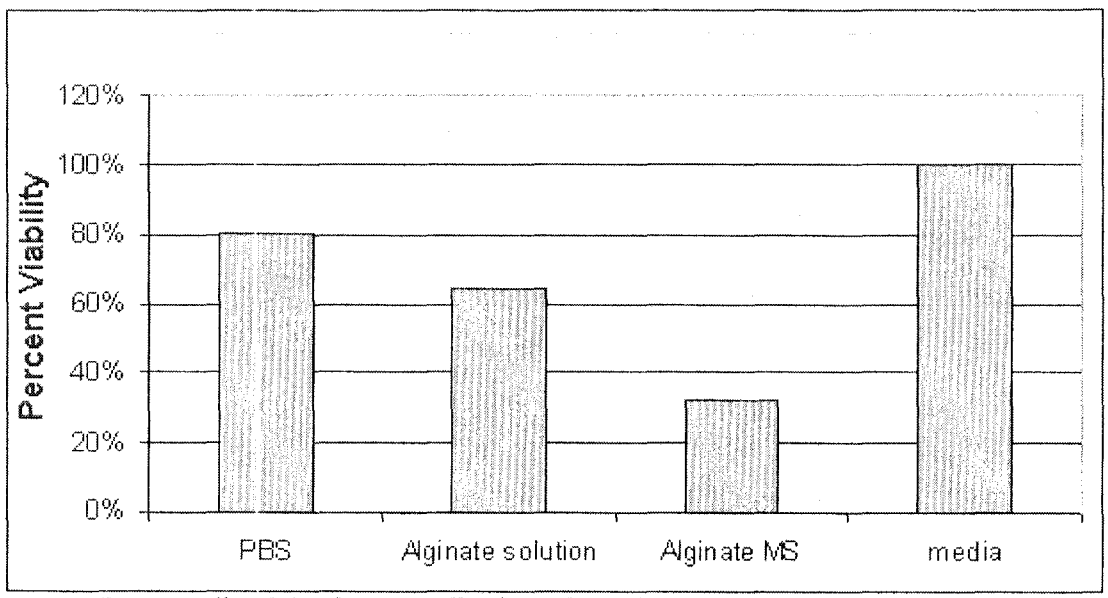


Figure 23: Percent cell viability for DKL cells after 24h-incubation of 1% alginate challenges.
Legend: MS = microspheres; media = RPMI1640 + supplements.

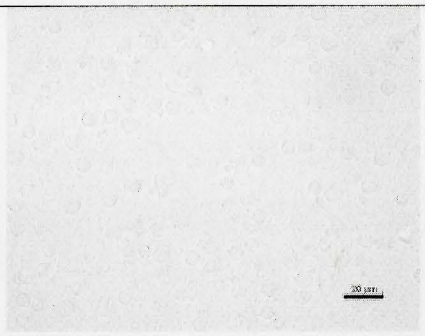
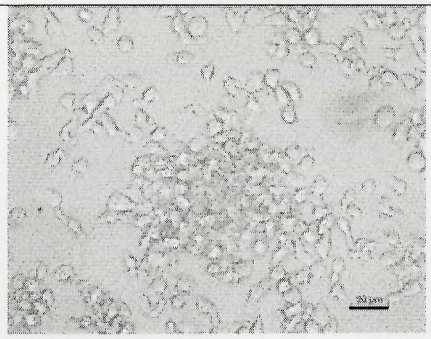
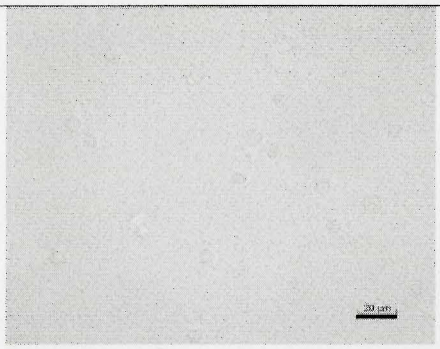
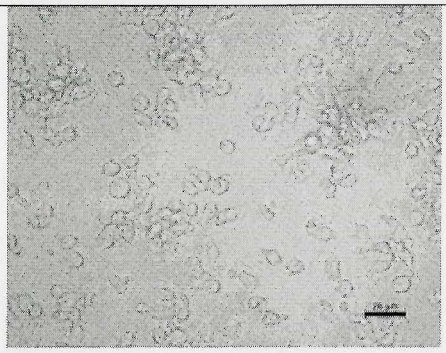
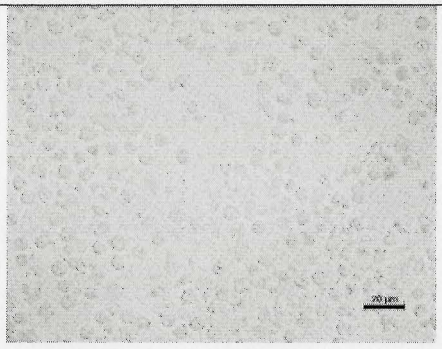
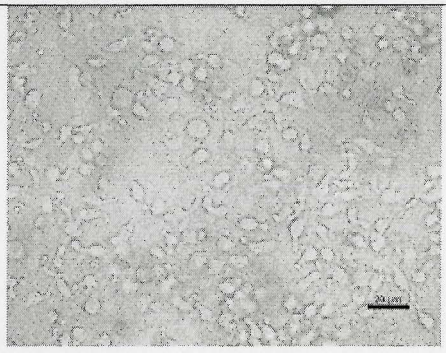
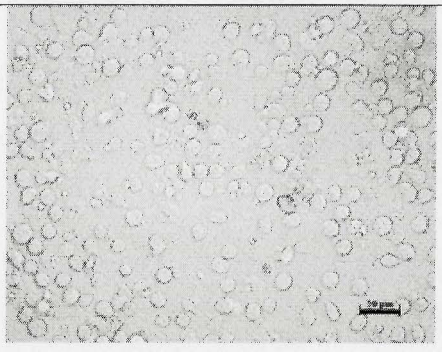
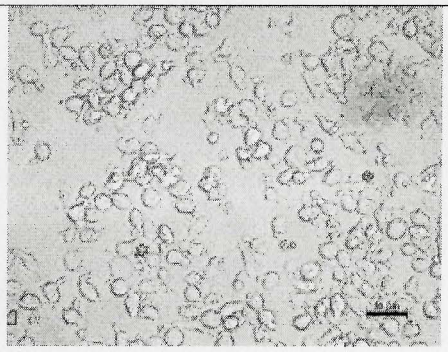
Challenge	Before incubation	After 24h incubation
PBS		
Alginate solution		
Alginate microspheres		
Media		

Figure 24: Light microscopy images of DKL cells before and after 24h challenge incubation.
Cells were challenged with PBS, 1% alginate solution in PBS, 1% alginate microspheres in PBS, and RPMI1640 medium. (40X magnification, bar = 20 μm).

Figure 24 shows a comparison of before and after photos of the cells for each challenge. The alginate solution challenge "before incubation" photo appears to have less cells than the others, however, the cells were merely suspended in solution. The actual number of cells present was in fact comparable to other challenges (data not shown). After 24h, the number of cells is visibly less in the microsphere-challenged sample. In fact, a closer inspection of this sample (Figure 25) shows much debris in between cells, not found in the other samples. Likely, this debris came from dead cells, accounting for the low viability of the sample. The number of cells and viability of cells present in each sample is best seen in Figure 26, where live cells fluoresce green and dead (whole) cells fluoresce red. As expected, cells incubated with media (Figure 26D) contained the least number of dead cells. Cells challenged with PBS and alginate solution (Figure 26A and 26B, respectively) contain similar cell numbers with slightly more dead cells. As previously found, samples challenged with alginate microspheres contained the least number of viable cells, as confirmed by Figure 26C. This sample also contains the greater number of dead cells, as shown by the red fluorescence.

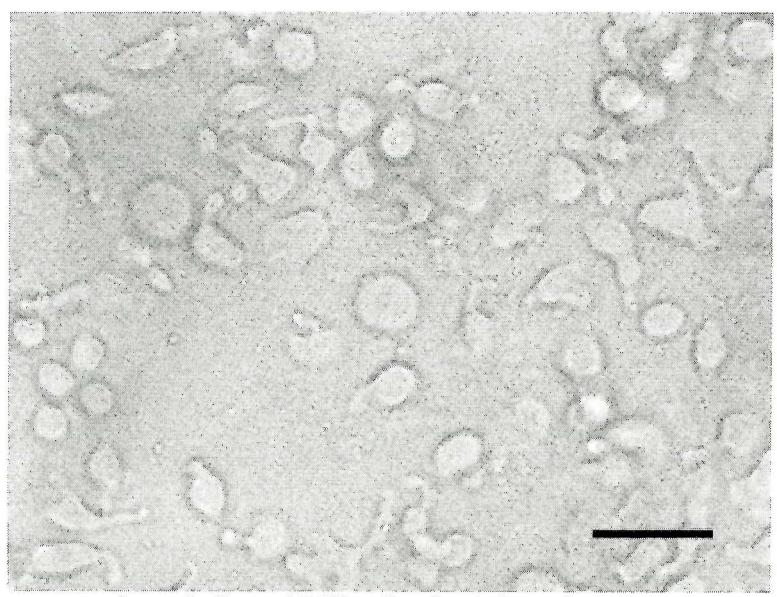


Figure 25: Alginate microsphere-challenged DKL cells after 24h incubation.

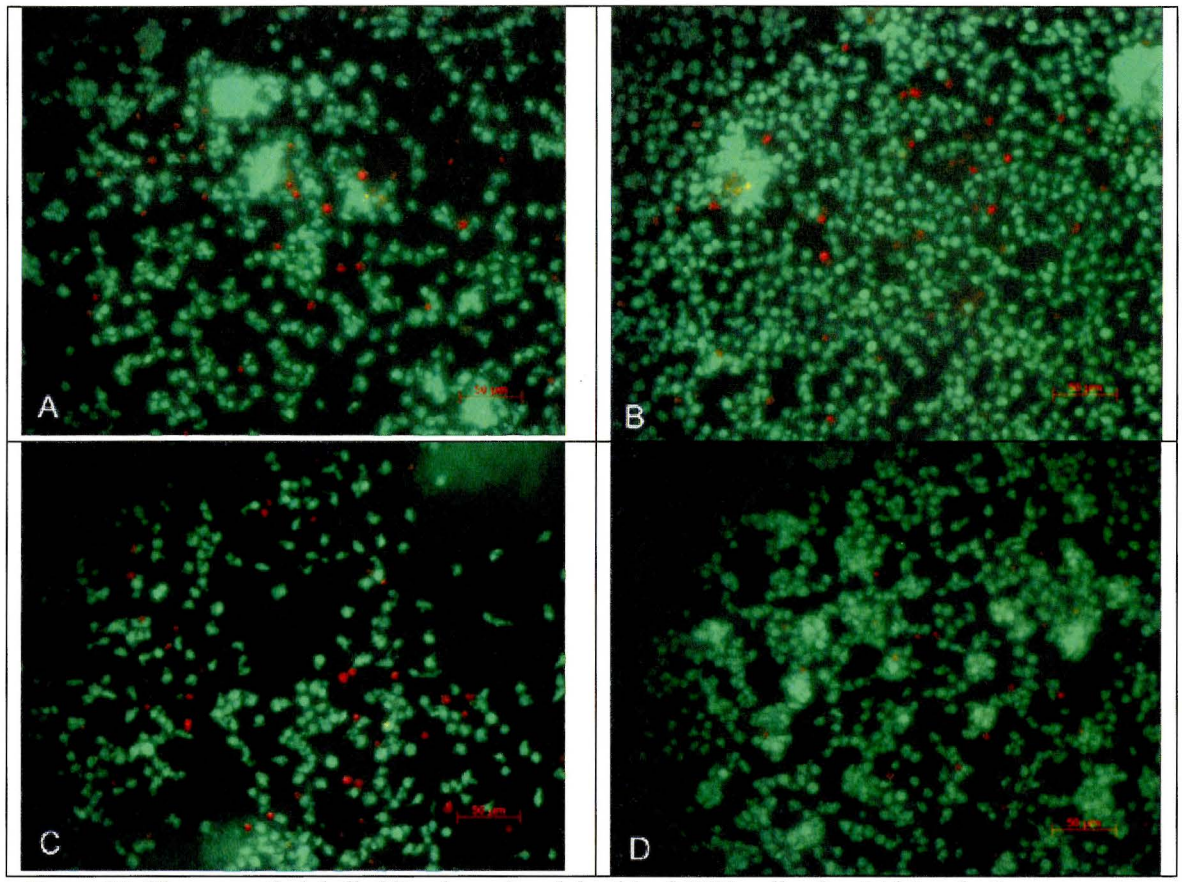


Figure 26: Fluorescently labelled live (green) and dead (red) DKL cells after 24 h incubation.
Cells were challenged with: A) PBS; B) 1% alginate solution in PBS; C) 1% alginate microspheres in PBS; and D) RPMI1640 medium. (20X magnification).

The images show that the microsphere challenges cause cell death. A simple experiment can be done to narrow down more specifically the cause of cell death. For example, a supernatant test can help determine whether the microspheres are releasing impurities leading to cell death. If in fact the alginate microspheres contained impurities that are causing cells to apoptose, the same response should be seen from the supernatant test. Subsequently, if toxicity was found from the supernatant, the question shifts to investigate why alginate solution does not have the same effect. Another possible explanation for the cell death seen here is due to the size of the particles. It's possible that the bulk of the particles were of a size that stimulates cell apoptosis. Therefore, differently-sized particles can be used to examine whether the size is indeed influencing

differently-sized particles can be used to examine whether the size is indeed influencing cell viability. The particles used in these experiments were sized using the Malvern Mastersizer and found to be ~1μm in diameter (normal distribution).

Because the microspheres caused cell death *in vitro*, no adjuvancy was seen from microspheres. Still, this experiment is insufficient to define the adjuvancy of alginate microspheres. Therefore, a preliminary *in vivo* study was done to evaluate the adjuvant effect of alginate microspheres. Following the previously mentioned methods, mice were injected with alginate microspheres in BSA solution (MS/BSA-injected mice). Splenocytes were then challenged *in vitro* with 100 μg/ml BSA.

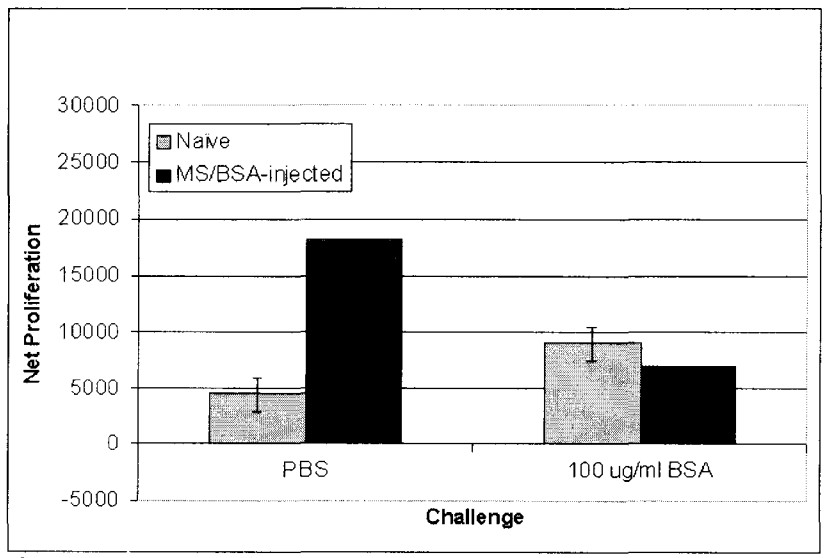


Figure 27: A preliminary test was done to test the adjuvancy of 1% alginate microspheres.
Mice were immunized with alginate microspheres and BSA, and then re-challenged *in vitro* with 100 μg/ml BSA. The response from mouse splenocytes was comparable to that of naïve mice, thus did not show adjuvant effects.

Figure 27 indicates that alginate microspheres do not possess adjuvant effects. Alginate solution did not show adjuvancy when challenged with BSA solution either. However, the combined alginate and BSA solution did induce significant cell proliferation from naïve splenocytes (Section 5.1.1). Further tests will be done to confirm the adjuvancy of alginate solution and microspheres.

Another preliminary test done was to check if splenocyte responses were reproducible by lymph node cells. Figure 28 indicates the responses from lymph node cells to the same BSA and alginate challenges given to splenocytes previously. Similarly, Figure 29 indicates the responses from lymph node cells to the same CHO cell and alginate challenges as previously done.

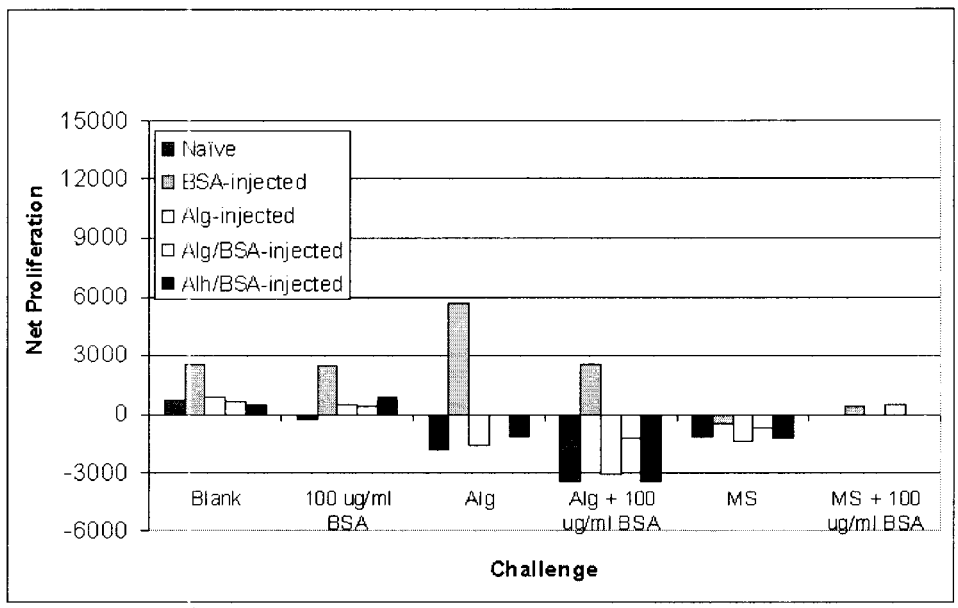


Figure 28: Lymph node cell responses to alginate and BSA challenges.
Lymph node cells were examined after several repetitions of splenocyte experiments to verify consistency of results between lymphoid organs. The responses from lymph node cells to alginate and BSA challenges were inconsistent animal-to-animal, as well as in comparison to splenocyte responses. Legend: Alg = 1% alginate solution, MS = 1% alginate microspheres.

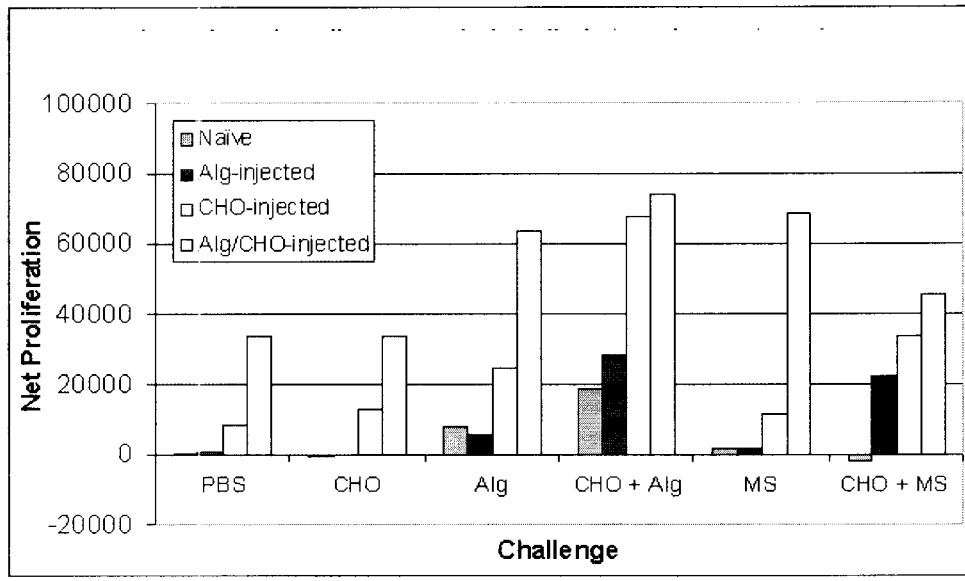


Figure 29: Lymph nodes cells were also challenged in vitro with CHO cells and alginate.
The responses from these cells were somewhat similar to splenocyte responses to the same challenges. Legend: CHO = 10^4 CHO cells, Alg = 1% alginate solution, MS = 1% alginate microspheres.

Figure 28 shows very little response from mouse lymph node cells, which is inconsistent with the results seen by splenocytes. On the other hand, Figure 29 shows responses that somewhat resemble the response from splenocyte in the cell model system. The main difference in response is seen with the microsphere challenges, where splenocytes had no proliferative effect due to the microspheres at all. A later study showed that microspheres could be causing the cells to apoptose, thus masking any proliferative effect they may be having on cells. Here, it can be seen that microspheres do have an effect on alginate/CHO-injected mouse splenocytes, and somewhat on CHO-injected and alginate-injected mice as well. Still, conclusions cannot be pulled from these findings as they represent only preliminary results. However, both graphs do indicate that T-cells from different lymphoid tissues can react very different to antigen challenge. Taken in a broader sense, the body can react differently to the same challenge depending how, when, and where the antigen is recognized.

5.2.4 Antibody response from immunized mice

In addition to splenocyte challenges, antibody production was also measured as an indicator of adjuvancy. The tests show that alginate has adjuvant potential, inducing antigen-specific IgG1 and IgG2a production. Thus, antibody titers were measured and quantified from blood serum. From the protein model system, Alhydrogel/BSA-injected mice produced the most IgG1 (Figure 30) and IgG2a (Figure 31) antibodies. Still, alginate/BSA-injected mice had the second highest antibody titer for both immunoglobulins, indicating that alginate is less potent than Alhydrogel as an adjuvant. Naïve and alginate-injected mice showed background levels of antibodies, while BSA-injected mice produced more antibodies than expected.

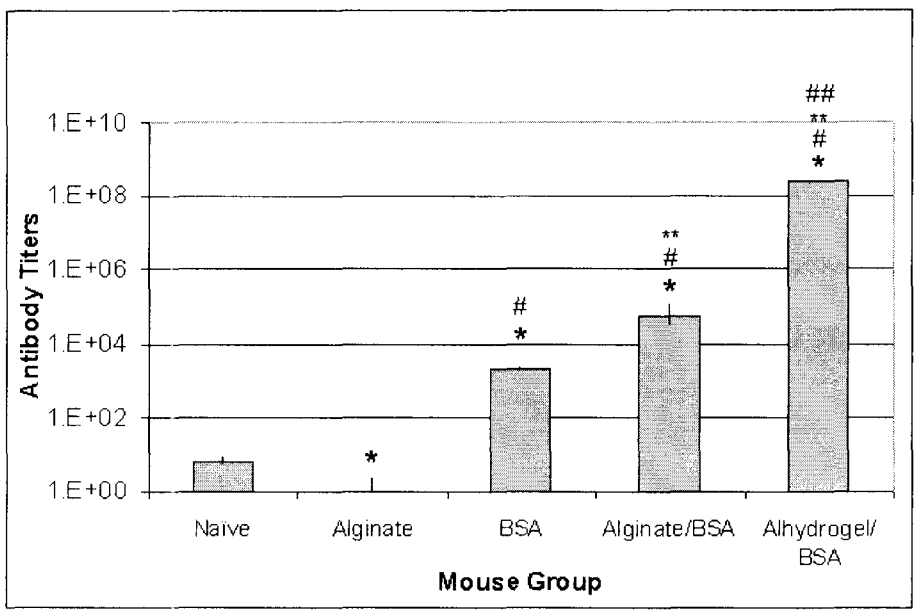


Figure 30: BSA-specific IgG1 antibody produced from mice.
Alhydrogel/BSA-injected mice had the greatest antibody production, as expected. Alginate/BSA-injected mice also produced significant amount of antibodies over naïve mice. Error bars show standard error of the mean. (Statistics: *p≤0.05 from naïve mouse group, #p ≤ 0.05 from alginate-injected mouse group, **p≤0.05 from BSA-injected mouse group, ##p ≤ 0.05 from alginate/BSA-injected mouse group; n = 4)

BSA-specific IgG2a antibody was also produced by Alhydrogel/BSA- and alginate/BSA-injected mice, although to a lesser extent than to IgG1 antibody. This slight response may be due to the xenogeneic nature of the protein. Again, only background

levels of this antibody are produced from the naïve and alginate-injected mouse groups, with higher than expected readings from BSA-injected mice. Preliminary experiments for BSA-specific IgM production was also examined, although no measurements above background were found for all mouse groups (data not shown). In summary, the IgG1 response was significantly greater than the IgG2a response, suggesting that alginate can induce a Th2-type response.

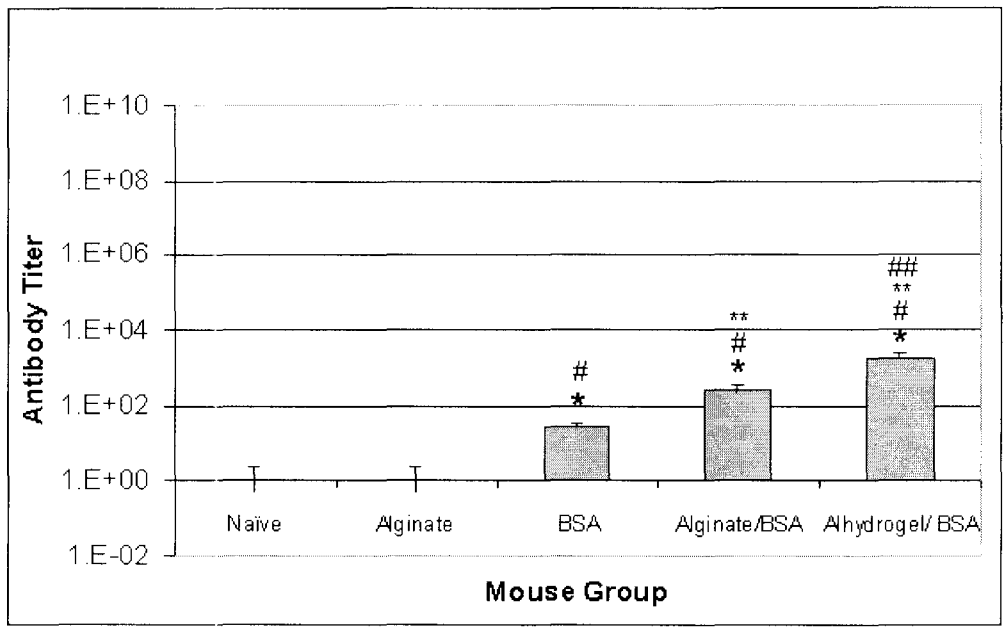


Figure 31: BSA-specific IgG2a antibody produced from mice.
Alhydrogel/BSA-injected mice still had the greatest antibody production, although alginate/BSA-injected mice produced a nearly comparable amount of antibodies to the positive control. Mouse groups marked with * produced significantly greater amounts of BSA-specific IgG2a than from the naïve mouse group. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from naïve mouse group, #$p \leq 0.05$ from alginate-injected mouse group, **$p \leq 0.05$ from BSA-injected mouse group, ##$p \leq 0.05$ from alginate/BSA-injected mouse group; $n = 4$)

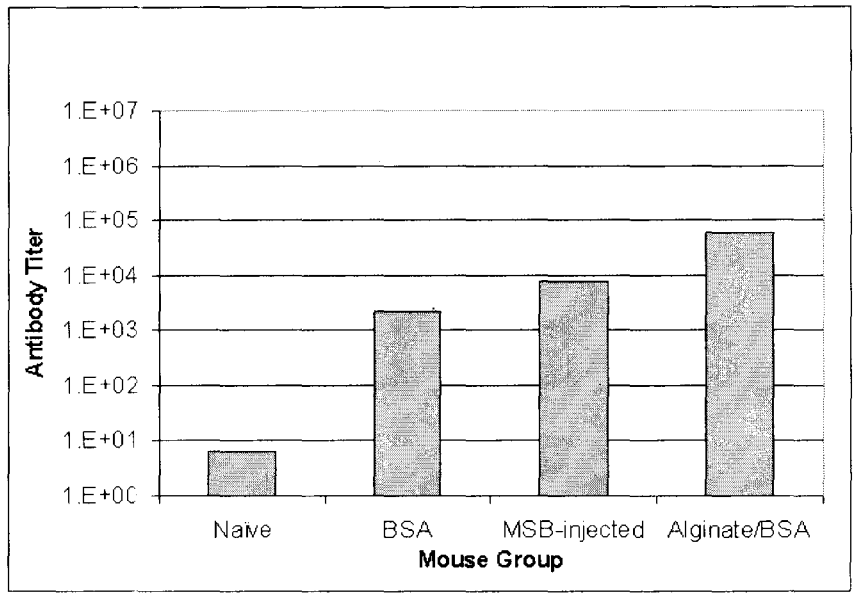


Figure 32: Antibody production of BSA-specific IgG1 was measured for MS/BSA-injected mice. The results were most comparable to BSA-injected mice.

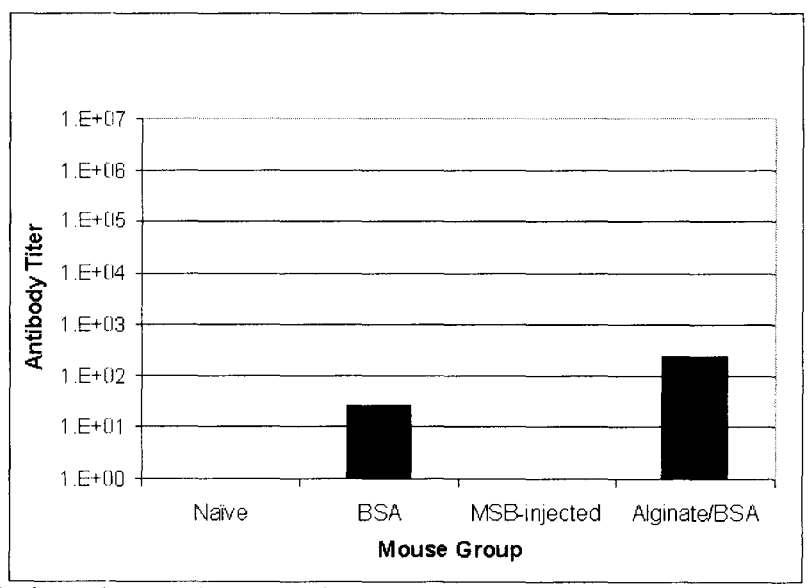


Figure 33: Antibody production of BSA-specific IgG2a was measured for MS/BSA-injected mice. This figure shows that only background levels of this antibody were detected in this mouse group, similar to the naïve mouse group.

The preliminary test results from MS/BSA-injected mice indicate that alginate microspheres triggered slight IgG1 production (Figure 32), but did not support IgG2a production (Figure 33). As Figure 34 shows, the antibody response from MS/BSA-injected mice is similar to that of BSA-injected mice. I believe that alginate microspheres do not possess adjuvant effects. After 10 days in the body, it is likely that the microspheres have degraded/de-crosslinked at least partially or fully. What is seen in Figure 34 is merely an effect of degraded/de-crosslinked microspheres interacting with BSA, leading to an immune response. Antibody titering of alginate/BSA-injected mice clearly shows alginate's adjuvancy. The microspheres merely mimicked this ability when they degraded and/or de-crosslinked *in vivo*.

Alginate's adjuvancy in the protein model system showed a slight bias to a Th2-type response (greater production of IgG1 than IgG2a). The adjuvant effect of alginate is re-examined following the same method with the cell model system. Again, preliminary results showed that only background levels of IgM were produced for all mouse groups (data not shown). Figure 32 and Figure 33 shows CHO cell-specific IgG1 and IgG2a production, respectively. In both graphs, CHO-injected and alginate/CHO-injected mouse groups produced more antibody than naïve and alginate-injected mice. Statistically, CHO-injected and alginate/CHO-injected mouse groups have identical antibody production, indicating no adjuvant effect from alginate. An antibody response was expected from this mouse group since the spleen and lymph node sizes were so greatly affected by the injection of CHO cells.

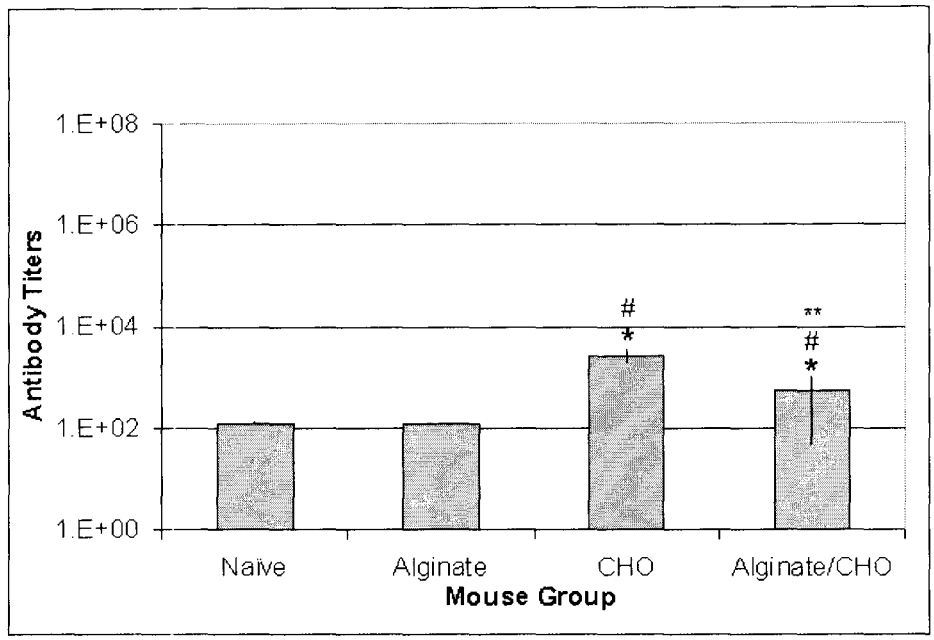


Figure 34: Antibody production of CHO cell-specific IgG1 was measured for naïve, alginate-, CHO-, and alginate/CHO-injected mice.

This figure suggests that alginate does not possess adjuvant effects since CHO- and alginate/CHO-injected mice produced similar amounts of antibody, both of which were significantly greater than from naïve mice. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from naïve mouse group, #$p \leq 0.05$ from alginate-injected mouse group, **$p \leq 0.05$ from CHO-injected mouse group; $n = 4$)

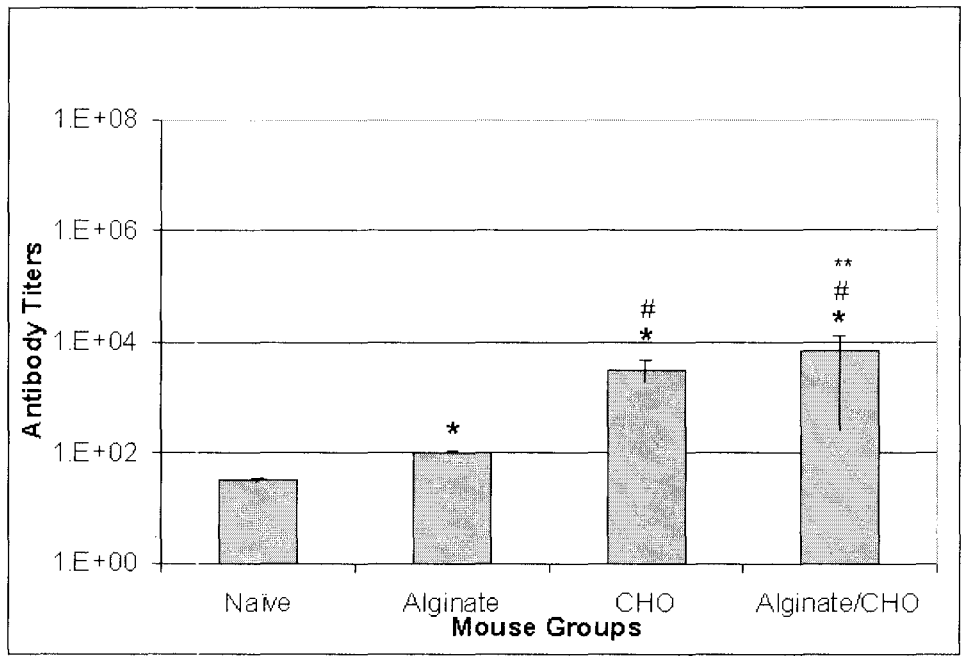


Figure 35: Antibody production of CHO cell-specific IgG2a was measured for naïve, alginate-, CHO-, and alginate/CHO-injected mice.
This figure suggests that alginate does not possess adjuvant effects since alginate-, CHO- and alginate/CHO-injected mice all produced significantly greater amounts of IgG2a than the naïve mouse group. Error bars show standard error of the mean. (Statistics: *$p \leq 0.05$ from naïve mouse group, #$p \leq 0.05$ from alginate-injected mouse group, **$p \leq 0.05$ from CHO-injected mouse group; $n = 4$)

Between the protein-challenged and cell-challenged experiments, it is interesting to see the difference of alginate's adjuvancy. With BSA, alginate tended towards an IgG1 response (Figure 30) while with CHO cells, no adjuvant effect was seen (Figure 34 and Figure 35). However, the adjuvant effect (and hence antibody production) is probably not seen in these figures because the injections of alginate and CHO cells were made in different legs (different site). Hence, the response that is seen in the two figures is likely predominantly triggered by xenogeneic effects of CHO cells. Still, this difference is useful for regenerative medicine and vaccine applications.

5.3 Applications of Alginate Solution and Microspheres

Alginate was delivered with BSA and CHO cells in two different xenogeneic model systems (protein and cell). In the protein model system, the results demonstrated alginate solution's adjuvancy supporting Th2-type responses. This useful result can be applied to vaccines, where an immunological response is desired. Still, the current model lacks potency in comparison to the positive control Alhydrogel, which can be a disadvantage. Booster shots are unappealing to most people, but preliminary tests were still conducted to see its effects. The results suggest that booster shots of BSA (with or without alginate solution) did not significantly alter splenocyte responses (data not shown). Thus, the animals used in this thesis did not receive booster shots.

Since alginate solution has shown potential as an adjuvant, future work should consider its use in vaccines. Alginate is a natural polysaccharide with non-toxic effects to the host. In comparison, traditional aluminum adjuvants can cause hypersensitivity reactions to certain people [109]. Moreover, many studied have found that aluminum-based adjuvants can only induce antibody-mediate immune responses, which greatly limit the versatility of their use [109]. Alginate solution's ability to induce a cell-mediated response has not been fully explored (i.e. different types of antigens), but will certainly be another advantage over aluminum-based adjuvants if the ability exists. DNA vaccines have gained popularity over the past few years as a preferred method of vaccination, although their effectiveness has been debated. Studies have shown that their efficiency in antibody production has varied depending on the antigen. For example, in a cancer-related study, vaccination with HER-2 DNA induced only partial protection, whereas the HER-2 protein vaccine induced almost full protection against a tumor challenge [110]. Others have shown that DNA vaccination worked equally as efficiently as protein vaccines [111]. In this case, DNA vaccines becomes a preferred method of vaccination since they do not require protein purification, which can be time consuming and costly. The combination of protein- and DNA-based vaccines have also been used and found to trigger both antibody- and cell-mediated responses [112]. Still, the debate between which vaccine type is better is unique to the antigen.

Alginate solution has been used with Pluronic® (block copolymers of ethylene oxide and propylene oxide) [113] as a gelling matrix for drug delivery. These matrices have been used for arterial and ocular drug delivery [6, 7]. The adjuvant effect of alginate solution in these studies was not considered but may have had an effect on *in vivo* applications. The immune-privileged status of the eye may have been a reason that the immunological effect of the gel was not investigated. The application of the gel on arterial stents has also yet to reach *in vivo* studies, thus no immunological studies have been done to-date. In this project, alginate solution has been shown to have adjuvant effects when delivered with proteins. It is unclear how this finding will affect delivery systems, such as those mentioned above, that utilize alginate solution. It is likely that its effects will vary depending on the other materials used in the system, the device's purpose, and the affected site. Still, *in vivo* studies should consider the immunological effects of alginate prior to implantation. Models using crosslinked alginate should also consider the immunological effect of the device, as the physiological environment can alter the crosslinking and degradability of polymer.

Extensive research has examined the use of alginate microspheres as drug delivery and vaccine vehicles. Microspheres have often been used for oral and nasal vaccination. These studies proved their models as successful delivery systems, using mostly protein antigens. Many of the studies found secretion of antigen-specific IgG1 [65, 66, 114], however, the induction of IgA has been inconsistent between studies [64, 66, 115]. These studies indicate that microspheres do improve antigen delivery and are suggestive of the adjuvant effects of alginate microspheres. Still, the preliminary studies of alginate microspheres in this thesis show that intramuscularly-delivered microspheres do not possess adjuvant effects. Previous studies have also found that alginate films do not cause the maturation of dendritic cells, an indication of a poor adjuvant [19]. Taken together, these results suggest that crosslinked alginate does not stimulate the immune system. However, preliminary *in vitro* tests completed in this thesis show that alginate microspheres may activate dendritic cells. This may be due to the round (and phagocytosable) morphology of the microspheres versus the flat surface of films. Cells (2

x 10^6 cell/ml) were stimulated with microspheres (~1:100 ratio of cells to particles) and observed for uptake and processing. In the initial experiments, Rhodamine B Ethylenediamine-labeled alginate particles were used. Confocal pictures were taken 2h, 15h, and 21 h (Figure 38, A-C respectively) after its addition. Separated fluorescent and light microscope pictures can be found in Appendix H.

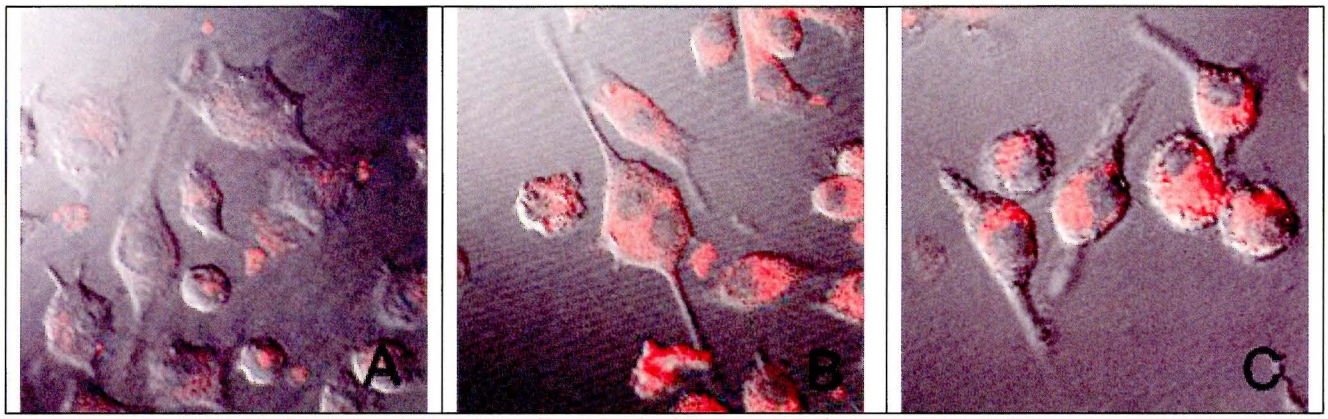


Figure 36: Confocal images of dendritic cells with Rhodamine B Ethylenediamine-labeled alginate particles. Images were taken after: A) 2h incubation; b) 15h incubation; and C) 21h incubation. The red fluorescence indicates that the alginate has been taken up by dendritic cells. (63X magnification).

The shortest incubation time of alginate microspheres with dendritic cells was 2h (Figure 36A). As expected, alginate particles were beginning to be taken up by the cells. After 15h of incubation (Figure 36B), it is clear that the alginate had been taken up and has filled the cytosol. This can be seen by the red fluorescence throughout the cell leaving the nucleus untouched. By 21h (Figure 36C), the alginate has dispersed throughout the cytosol of the cells without entering the nucleus. Cells have also developed a rounder morphology and larger cell size, a possible indication of cell activation. The images do not show the structure of the alginate, thus it is not possible to conclude that the fluorescing color comes from intact microspheres; it is possible that the microspheres have de-crosslinked or degraded before its ingestion. If indeed the microspheres de-crosslinked, activation of dendritic cells would be expected since alginate solution has adjuvant abilities. However, if the microspheres are intact, these images suggest that alginate microspheres can activate dendritic cells.

A similar test with added ovalbumin was conducted to examine protein release and uptake from alginate microspheres to dendritic cells. Because the OVA protein is dispersed throughout the alginate particle, observing a doubly-fluorescent OVA/alginate particle would have been very difficult. Most likely, the fluorescence of one material would overpower the other, providing very little beneficial information. Thus, fluorescent-ovalbumin was delivered to dendritic cells with (non-florescent) alginate microspheres. PBS was delivered to negative control samples while positive control samples received fluorescent-ovalbumin solution only. The delivery of straight protein solution *in vivo* is impractical because of the rapid degradation of the unprotected protein. Thus, alginate is used to protect the protein for delivery to the cells.

Figure 37 below shows confocal pictures of dendritic cells with no challenge (negative control), alginate-encapsulated fluorescent-ovalbumin, and fluorescent-ovalbumin solution (positive control). Separate fluorescent and light microscope pictures can be found in Appendix H. At all time points, the negative controls display no fluorescence, although cell numbers do appear to decrease with time (Figure 37A, D, G). Because these cells are grown on glass slides, the lowered number of adherent cells is likely due to the glass surface; cell death due to the challenge is unlikely the main cause of decreased cell numbers.

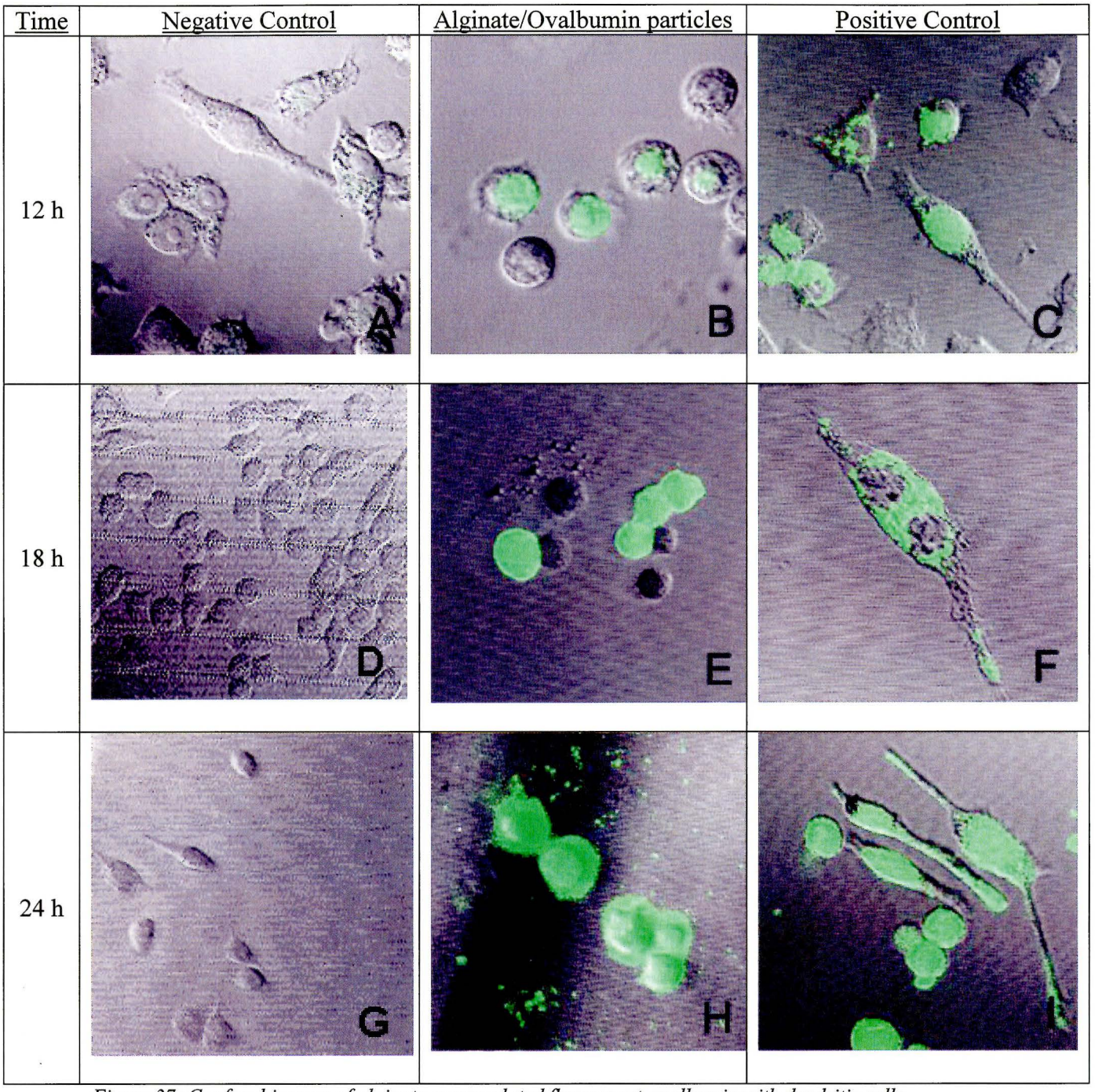


Figure 37: Confocal images of alginate-encapsulated fluorescent-ovalbumin with dendritic cells (B, E, H). Cells from negative control samples (A, D, G) received PBS only, while positive control samples (C, F, I) were given fluorescent-ovalbumin solution. (40X and 63X magnification).

Cells incubated with alginate/ovalbumin particles had significantly less cells adherent to the surface than the negative controls (data not shown). Moreover, as they were much larger in size and had a spherical conformation, possibly a sign of activation. After 12h of incubation (Figure 37B), the ovalbumin from alginate/ovalbumin particles has clearly been released into the cytosol. By 18h and 24h (Figure 37E, H), the ovalbumin has penetrated throughout the cell, including the nucleus. In the positive control, the ovalbumin is seen to have partially invaded the cell space after 12h (Figure 37C). At this point, the cells still maintain their elongated shape. Six hours later (Figure 37F), the ovalbumin has almost completely filled the cell, including the nucleus. Some cells have also begun to change morphology into a sphere like the test samples. Finally, at 24h (Figure 37J), the ovalbumin has completely penetrated through some cells. The different cell morphologies are equally divided between round and elongated shapes.

Again with these images, it is not clearly defined how the ovalbumin entered the cells. It is possible that the loaded microspheres were taken up. The acidic pH of the endosomes would have caused microspheres to shrink, releasing some of the ovalbumin. The ovalbumin could then spread throughout the cell. Because of the size of ovalbumin, some say it is passively diffused into the nucleus [116], while others believe there is a transport mechanism allowing it to enter the nucleus [117]. Others have found that ovalbumin contains sequences with nuclear targeting characteristics (also called Nuclear Localization Sequence, NLS) [118]. The explanation of how the ovalbumin reached the nucleus is not as important as whether anything was transported into the nucleus along with the ovalbumin. It is unlikely that alginate was transported into the nucleus, as it is too large in size. However, many more specialized tests will need to be done to verify this case.

Another possibility is that the protein was released by microspheres (possibly due to degradation/de-crosslinking) before being taken up. Therefore, cell would be taking up both microspheres and free ovalbumin. The alginate would still stimulate the activation of dendritic cells, causing a conformational change. This would explain the round cell

shapes from the alginate/ovalbumin particles as early as 12 h (Figure 37B), while the same shapes are seen in the positive control after 24 h (Figure 37I).

The second system examined in the project was the cell model system, using CHO cells as the model antigen. This experiment showed two key results of interest. Firstly, the splenocyte responses from alginate/CHO-injected mice would suggest that alginate does not possess adjuvant effects. However, the results may have been due to separate injection sites of the CHO cells and alginate solution. In comparison, naïve splenocytes were very responsive to *in vitro* challenges of CHO cells in alginate solution (Figure 9), suggesting that this combination has a great stimulatory effect. The immunological effect of cells with biomaterials is most crucial in the field of tissue engineering. In many cases, a large immune response is not desirable since a prolonged inflammatory response may be detrimental to the patient and/or the device. Further studies on the stimulatory effect of alginate/CHO cells would greatly enhance the study of alginate as an adjuvant. In comparison with the protein model, the response seen from splenocytes to alginate/CHO cells is likely due to alginate solution's adjuvancy.

Alginate-encapsulated cells have been shown to provoke less of an immune response *in vivo* than cells alone. Here, this thesis shows that mice injected with alginate solution were less responsive to *in vitro* challenges of CHO cells than mice without alginate treatment. A longer-term study is needed to examine whether the responses are delayed, suppressed, or other. Cells should also be examined to see if the injections of alginate altered their ability to withstand challenges; an increased tolerance for challenges would show less response, as would a lowered tolerance causing cell death. If pre-injection of alginate solution has similar abilities to lower immune rejection to CHO cells as encapsulation, then the former method would be faster and less time consuming. It's possible that the alginate from the surface of encapsulated cells de-crosslink, interacting with the environment as alginate solution does. If this were true, however, an augmented response would be expected since alginate has innate immunity activating abilities. What is shown in this project is the opposite effect. Thus, it is curious and well-deserved to

investigate further the interactions between alginate and xenogeneic cells, as well as their interaction and effect on immune response.

6. CONCLUSION AND RECOMMENDATIONS

This project examined the adjuvant potential of alginate solution and microspheres, the effects of injected alginate, as well as the physical effects of alginate on cells. From the results I have gathered, these are the overall conclusions of this thesis:

- Alginate solution acts as an adjuvant with the model protein BSA, triggering greater IgG1 production than IgG2a. Therefore, there is great potential for alginate in the development of vaccines.
- Alginate solution may have adjuvant potential with CHO cells. The comparison of naïve splenocyte responses between both systems suggests alginate has the same potential on cellular antigens.
- Alginate microspheres do not possess adjuvant abilities. Microsphere/BSA-injected mice responded very similarly to BSA-injected mice to *in vitro* challenges, displaying no adjuvant characteristics.
- Alginate microspheres (with or without BSA) used as *in vitro* challenges provoked cell death. This was discussed upon further examination when all splenocytes (from both model systems) were not responsive to these particles.
- The uptake of alginate microspheres, with and without protein, has also shown interesting results. The preliminary study done here shows the effect of this polymer and protein with dendritic cells. Still, many tests are needed to establish the form of the alginate, mechanisms of delivery and uptake, and interactions between alginate and cells.
- Immunization with alginate solution was found to have an effect on both protein and cell model systems. In general, mice injected with alginate were equally or less responsive to *in vitro* challenges than naïve splenocytes.

From these results, further work is needed to support these findings. In addition, these results are suggestive to other characteristics of alginate which require much more examination, including:

- Cytokine analysis from splenocytes. Cytokine production is a good indicator of the type of immune response being activated.
- Injections of CHO cells and alginate solution together. CHO cells and alginate solution were injected in different legs for the purpose of examining the effect of the alginate on the xenogeneically-challenged mice. To examine the adjuvancy of alginate, the solution can be mixed together with CHO cells prior to injection, as like the protein model's injection of alginate/BSA.
- The use of alginate-encapsulated CHO cells. Injections of alginate-encapsulated CHO cells can also be used as a control to compare the immune response to alginate/CHO injections.
- Investigation of the reason behind cell death due to alginate microspheres (*in vitro* challenge). The reason for such behavior is unclear, but is speculated to be due to impurities of the alginate. Purified alginate can be used to re-do the experiment. Supernatant tests can also be done to see if the cell response is due to the microspheres or the supernatant. Endotoxin tests can also be performed to verify its level within each sample.
- Examination for the cause of lowered immune response from alginate-injected mice to *in vitro* challenges. If indeed the lowered response is due to the host response to biomaterial, then such interactions would be greatly useful in regenerative medicine applications where an immune response is ideally minimized. Experiments of different duration (short and long term) may help distinguish whether the lower immune response from alginate-injected mice is delayed, suppressed, or other (cell death, tolerance, etc).

Examination of possible cross-presentation effects of protein-loaded alginate microspheres had begun, but the results were inconclusive given the duration of this

project. This project was able to show that alginate microspheres efficiently delivery ovalbumin antigens to dendritic cells. I also wanted to show how the antigen peptides can be presented on MHC class I instead of its classical presentation on MHC class II. This demonstration would verify how protein vaccines can be used to trigger Th1-type responses. The SIINFEKL-specific DKL cells would have been used in an ELISPOT to examine IFN-γ secretion from dendritic cells. Before this assay could be done, tests were completed verifying that MHC class I receptors were present on DC2.4 cells. It was also found that MHC class I receptors are found on DKL cells but not on L929 fibroblast cells (protocol in Appendix I). This may be due to its derivation from a cell line and not native tissue. The results of these preliminary studies can be found in Appendix J and may be useful to an individual pursuing a similar objective.

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8. APPENDICES

Appendix A: Preparing alginate solution and microspheres

Prepared by: Pearline Lung	**Date**: May 12, 2007
Revision: 2	**Supercedes**: June 15, 2006

MATERIALS

Preparation	Preparation + Procedure	Procedure
• Beaker + stir bar • 0.45 μm filter • 0.20 μm filter • stir plate • Biological safety cabinet (BSC)	• sterile distilled water (DW) • 50 ml centrifuge tubes • 10 ml pipette	• Flask + stir bar • Phosphate-buffered saline (PBS) • Canola Oil • 5 ml pipette • 25 ml pipette • Aspiration pipette tips • $CaCl_2/ZnCl_2$ solution • Poly-L-lysine

Preparation

1. Rinse beaker and magnetic stir bar with ethanol. Autoclave.
2. Suspend 0.4 g alginate (1%w/v) in 40 ml DW (in BSC) in beaker with magnetic stir bar. Stir at low-medium speed (~5500 rpm) for 2 h.
3. Filter with 0.45 μm filter in BSC.
4. Filter with 0.20 μm filter in BSC.
5. Label tube and cap (name, date, substance, #)

Procedure

1. In BSC, add 14 ml of 1% alginate solution into glass autoclaved flask.
2. If microspheres are to be loaded, add in protein solution.
3. Add 70 ml canola oil.
4. In hood, mix for ~1 minute at 5500 rpm (low-medium setting) until an emulsion is obtained.
5. Add 17.5 ml 0.5% $CaCl_2$/0.05% $ZnCl_2$ (in PBS) **drop wise** while mixing.
6. Mix for 5 minutes.
7. In BSC, transfer solution to centrifuge tube. Centrifuge 10 min at 1500 rpm.
8. Remove oil supernatant with pipette and aspirate (suction) the remainder of the supernatant.
9. Resuspend microspheres in 10 ml PBS. Vortex at medium-high.
10. Centrifuge for 10 min at 1500 rpm.
11. Aspirate supernatant, Resuspend in 30 ml PBS.
12. Sonicate 1 h. Store in fridge.

Reference: Lemoine D, Wauters F, Bouchend'homme S, Preat V "Preparation and Characterization of Alginate Microspheres Containing a Model Antigen" Int J Pharm 176, 9-19, 1998

Appendix B: Fluorescently labeling alginate microspheres

Prepared by: Daniel McLean/Pearline Lung	**Date**: January, 2007
Revision:	**Supercedes**:

Purpose

To label alginate microspheres with a fluorescent tag through coupling to carboxylic acid functional groups

Protocol

Preparation
-Prepare the following solutions
-500μL of 2mg/mL Rhodamine B Ethylenediamine (suspended in 100mM EDC, 25mM NHS)
-2.5mL 1% or 2% alginate microspheres

Experiment
1. Add 100mL of Rhodamine B Ethylenediamine solution to 2.5mL microsphere solution
2. Let reaction proceed for 4 hours in dark at room temperature
3. Centrifuge for 10 mins at 1500rpm in centrifuge and discard supernatant
4. Wash with PBS
5. Repeat centrifugation four more times
6. Resuspend in 100mM PBS

Explanation

The fluorescent molecule is coupled to ethylenediamine. Alginate contains carboxylic acid functional groups (mannuronic and guluronic acid) dispersed throughout the particle. Under the reaction conditions, 100mM EDC and 25mM NHS, the ethylenediamine and carboxylic acid functional groups incorporated into the particle covalently react. The resultant particles can be detected using a fluorescent microscope.

Source: Shunxing Su, sus5@mcmaster.ca

Appendix C: Standard Sub-culturing Protocol

Prepared by: Pearline Lung	**Date**: Jan. 22, 2007
Revision: 1	**Supercedes**:

*All reagents from Invitrogen (Burlington, ON, Canada)

Cell Line	Medium*
CHO-K1 (ATCC, Manassas, VA)	Minimal Essential Medium (1X), with Earle's salts and L-glutamine + 10% fetal bovine serum + 1% penicillin-streptomycin
DC2.4 (ATCC, Manassas, VA)	RPMI-1640 Medium (1X) + 10% fetal bovine serum + 1% penicillin-streptomycin + 1% HEPES + 1% non-essential amino acids + 1% L-glutamine + 0.1% β-mercaptoethanol
'DKL' (gifted from Dr. J. Bramson, McMaster University)	RPMI-1640 Medium (1X) + 10% fetal bovine serum + 1% penicillin-streptomycin + 1% HEPES + 1% sodium pyruvate + 1% L-glutamine + 0.1% β-mercaptoethanol

Other Reagents*:

- Dulbecco's Phosphate Buffered Saline (PBS)
- 0.05% Trypsin, with EDTA

1. Observe cell culture under a light microscope. For DKL cells, proceed to step 6.
2. Aspirate supernatant.
3. Wash cell surface with 1 ml PBS.
4. Add 1mL of trypsin to the flask. Incubate for ~ 5 mins, or until cells detach from the surface.
5. Deactivate trypsin by adding 9mL of medium to the flask.
6. Mix media/cells and wash adherent surface (~ 10 times). Avoid forming bubbles.
7. Centrifuge cell suspension for 4 mins at 900rpm.
8. Aspirate supernatant. Resuspend the pellet of cells with 10mL of medium.
9. Add 1mL of cell suspension to a new flask containing 9mL of medium.
10. Place flask in incubator at 37°C, 5% CO_2.

Appendix D: ACK lysing buffer recipe

Prepared by: Pearline Lung	**Date**: March 1, 2007
Revision: 1	**Supercedes**:

Materials

8.29 g	NH_4Cl (0.15M)
1g	$KHCO_3$ (10.0 mM)
37.2 mg	Na_2EDTA (0.1 mM)

Procedure

- Add materials into a 1L container.
- Add 800 ml H_2O and adjust pH to 7.2-7.4 with 1N HCl.
- Fill bottle with H_2O to 1 L mark.
- Filter sterilized solution through a 0.2 μm filter and store at room temperature.

Source: Dr. Bramson's Lab, McMaster University

Appendix E: Tracking fluorescent-OVA from alginate microspheres

Prepared by: Daniel McLean/Pearline Lung	**Date**: January, 2007
Revision: 1	**Supercedes**:

Purpose

To verify the delivery of ovalbumin to the cytosol of dendritic cells through an alginate particle

Protocol

Preparation

- Load alginate microspheres with fluorescently-labeled ovalbumin protein (see *Appendix B*: Fluorescently labeling alginate microspheres).
- Subculture DC2.4 cells and resuspend to 200,000 cells/mL.
- Prepare ten confocal wells with autoclaved glass slides, silicone gel, and glass wells.

Experiment

1. Add 1mL of cell suspension to each confocal well.
2. Incubate cells at 37°C for 2 hours to allow cells to attach.
3. Add 100μL of alginate-encapsulated ovalbumin particles to six wells at specific time points (i.e. 24 hours, 16 hours, and 8 hours)
4. Add 100μL of particles to two wells (test sample).
5. Add 100μL of ovalbumin solution to two wells (positive control).
6. Incubate at 37°C for specified period of time.
7. Aspirate media and wash twice with 500μL of PBS, gently!
8. Add 500μL of 4% paraformaldehyde and incubate at room temperature for 20 minutes.
9. Aspirate paraformaldehyde and add 500μL of PBS.
10. View under Zeiss LSM Confocal Microscope.
11. Take Z-stack pictures and "field of view" pictures for each time point.

Confocal microscope location and contact information

- 3rd Floor Health Sciences Building "Electron Microscope" room
- Extension 22496
- Assistance: Marnie Timlec

Appendix F: ELISA-based antibody titering for BSA-specific antibodies

Prepared by: Pearline Lung	**Date**: May 15, 2007
Revision: 3	**Supercedes**: March 27, 2007

Protocol is suitable for IgG1, IgG2a, or IgM! Recipe suitable for one full plate (96 wells).

MATERIALS

Capture Abs: 10μg/ml BSA and OVA → *sterile-filtered both!*

- 15 μl of BSA (2 mg/ml) + 3 ml Coating Buffer (specific Ag)
- 15 μl of OVA (2 mg/ml) + 3 ml Coating Buffer (control Ag)

Coating Buffer: 0.1M Sodium Carbonate, pH 9.5

Working Detector: HRP-conjugated monoclonal rat anti-mouse IgG1, IgG2a, or IgM antibody

- Dilute to 1:4000 – 1:8000
  - volume needed: 200 μl x 48 wells = 9.6 ml → 12 ml
  - to make 1:5000 → 2.4 μl Ab_1 + 12 ml PBS-T
    → 2.4 μl Ab_2 + 12 ml PBS-T

Samples:

Std	A 1:10	B 1:100	C $1:10^3$	D $1:10^4$	E $1:10^5$	F $1:10^6$	G $1:10^7$	H $1:10^8$
Serum	70 μl of Original	70 μl of 1:10	70 μl of 1:100	70 μl of $1:10^3$	70 μl of $1:10^4$	70 μl of $1:10^5$	70 μl of $1:10^6$	70 μl of $1:10^7$
PBS-T	630 μl	630 μl	630 μl	630 μl	630 μl	630 μl	630 μl	630 μl

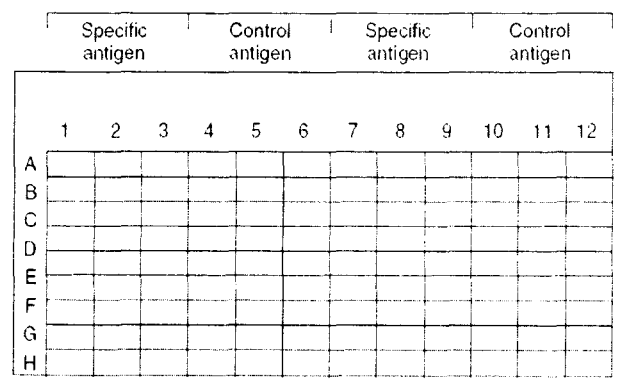
**volumes can be halved if serum is low*

Wash Buffer: PBS + 0.05%Tween-20 (PBS-T)

- 100 ml (PBS 10X) + 900 ml Millipore water + 500 μl Tween-20

Substrate Solution (TMB)
100ul/well ×96 = 9.6ml
→ 11 ml (5.5 ml A + 5.5 ml B)

Stop Solution (1M H_2SO_4)
50ul/well ×96 = 4.8ml → 5.5 ml



PROCEDURE

1. Add 50 µl of **Capture Ab** to each well (see diagram above - Specific = BSA solution; Control = OVA solution). Incubate **overnight at 4°C.**
2. Aspirate by inverting plate and wash 2 times with PBS (using squirt bottle).
3. Add 100 µl of 5% skim milk powder in PBS. Block **overnight at RT**.
4. Aspirate by inverting plate and wash 2 times with PBS (using squirt bottle).
5. Add 50 µl **sample** (Std. A-H) to each well. Incubate **1 hr at 37°C.**
6. Aspirate and wash 4 times. Pat dry on paper towel.
7. Add 200 µl **Working Detector** to each well. Remember there are 2 tests per plate! Incubate **1hr at RT.**
8. Aspirate and wash 4 times. Pat dry on paper towel.
9. Add 100 µl **Substrate Solution** to each well. Incubate **30 min at RT in dark**.
10. Add 50 µl **Stop Solution** to each well. Read at 450 nm within 30min with λ correction 620nm.

REFERENCES

1. Current Protocols in Molecular Biology (2000), 11.17.1 – 11.17.3
2. The Journal of Immunology (1998), 160: 4114–4123
3. [Online] at: http://www.komabiotech.com/technical/protocol/ELISA.htm

Appendix G: ELISA-based antibody titering for CHO-specific antibodies

Prepared by: Pearline Lung	**Date**: May 15, 2007
Revision: 1	**Supercedes**:

Protocol is suitable for IgG1, IgG2a, or IgM! Recipe suitable for one full plate (96 wells).

MATERIALS

Cells: CHO cells – *specific antigen*; L929 cells or no cells – *control antigen*

Working Detector: HRP-conjugated monoclonal rat anti-mouse IgG1, IgG2a, or IgM antibody

- Dilute to 1:4000 – 1:8000
  - volume needed: 200 µl x 48 wells = 9.6 ml → 12 ml
  - to make 1:5000 → 2.4 µl Ab_1 + 12 ml PBS-T (columns 1-6)
    → 2.4 µl Ab_2 + 12 ml PBS-T (columns 7-12)

Samples:

Sample	A 1:10	B 1:100	C $1:10^3$	D $1:10^4$	E $1:10^5$	F $1:10^6$	G $1:10^7$	H $1:10^8$
Serum	70 µl of Original	70 µl of 1:10	70 µl of 1:100	70 µl of $1:10^3$	70 µl of $1:10^4$	70 µl of $1:10^5$	70 µl of $1:10^6$	70 µl of $1:10^7$
PBS-T	630 µl	630 µl	630 µl	630 µl	630 µl	630 µl	630 µl	630 µl

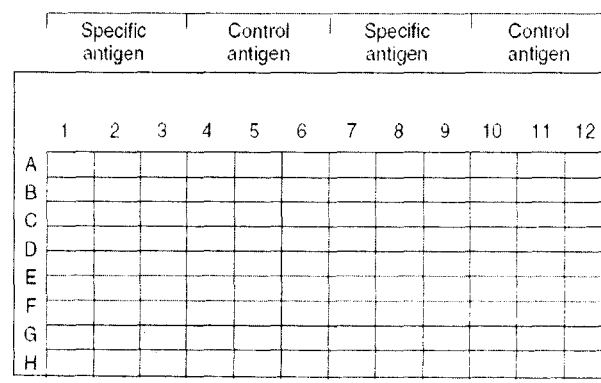
**volumes can be halved if serum is low*

Wash Buffer: PBS + 0.05%Tween-20 (PBS-T)

- 100 ml (PBS 10X) + 900 ml Millipore water + 500 µl Tween-20

Substrate Solution (TMB)
100ul/well ×96 = 9.6ml
→ 11 ml (5.5 ml A + 5.5 ml B)

Stop Solution (1M H_2SO_4)
50ul/well ×96 = 4.8ml → 5.5 ml



PROCEDURE

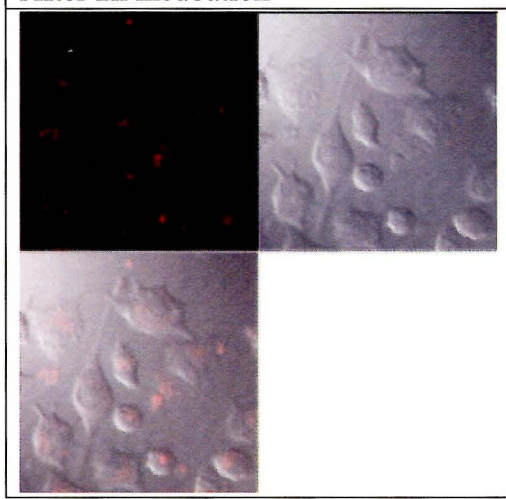
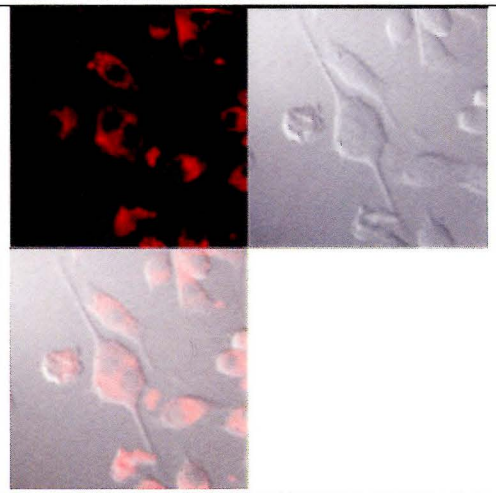
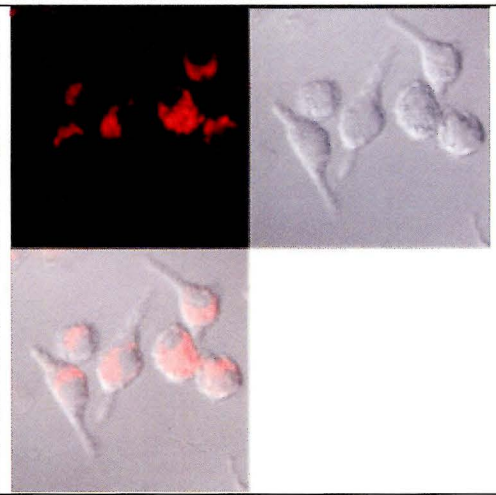
11. Add 50 µl of **Cells** to each well (see diagram above - Specific = CHO cells; Control = L929 cells OR no cells). Incubate **overnight at 37°C** (cells should be confluent).
12. Wash once with 200 µl PBS (using squirt bottle).
13. Fix using 1% glutaraldehyde in PBS for **1 hr at RT**.
14. Wash 2 times with PBS (using squirt bottle).
15. Add 100 µl of 5% skim milk powder in PBS. Block **overnight at RT**.
16. Wash 2 times with PBS (using squirt bottle).
17. Add 50 µl **sample** (Std. A-H) to each well. Incubate **1 hr at 37°C.**
18. Aspirate and wash 4 times. Pat dry on paper towel.
19. Add 200 µl **Working Detector** to each well. Remember there are 2 tests per plate! Incubate **1hr at RT.**
20. Aspirate and wash 4 times. Pat dry on paper towel.
21. Add 100 µl **Substrate Solution** to each well. Incubate **30min at RT in dark**.
22. Add 50 µl **Stop Solution** to each well. Read at 450 nm within 30min with λ correction 620nm.

REFERENCES

1. Current Protocols in Molecular Biology (2000), 11.17.1 – 11.17.3
2. The Journal of Immunology (1998), 160: 4114–4123
3. Jones KS, Sefton MV, Gorczynski RM. Suppressed Splenocyte Proliferation Following a Xenogeneic Skin Graft due to Implanted Biomaterials. Transplantation 2006;82:415-21.

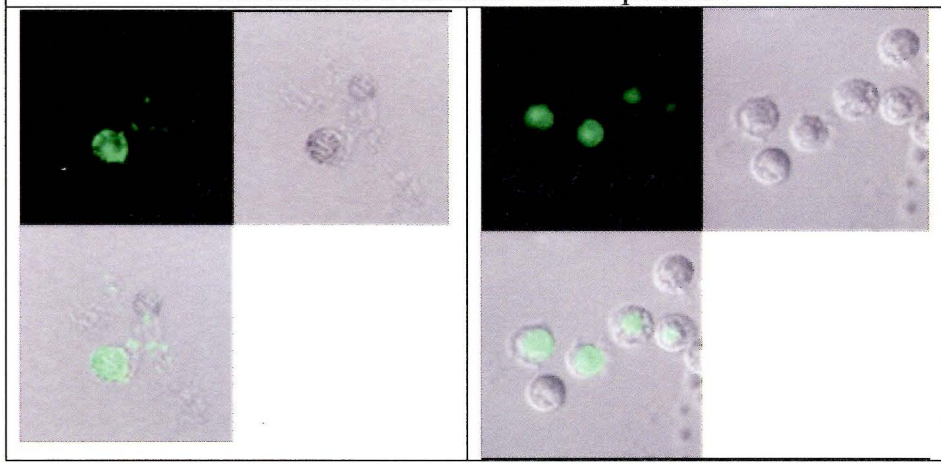
Appendix H: Confocal images of dendritic cells with alginate microspheres (with/without ovalbumin)

Dendritic cells with Rhodamine B Ethylenediamine-labeled alginate microspheres (no protein)

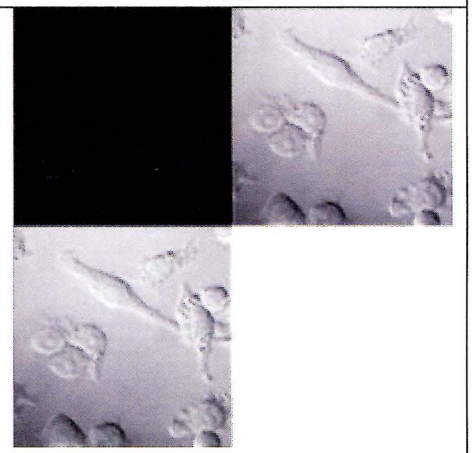
After 2h incubation	After 15h incubation	After 21h incubation
		

A 12h incubation of dendritic cells with:

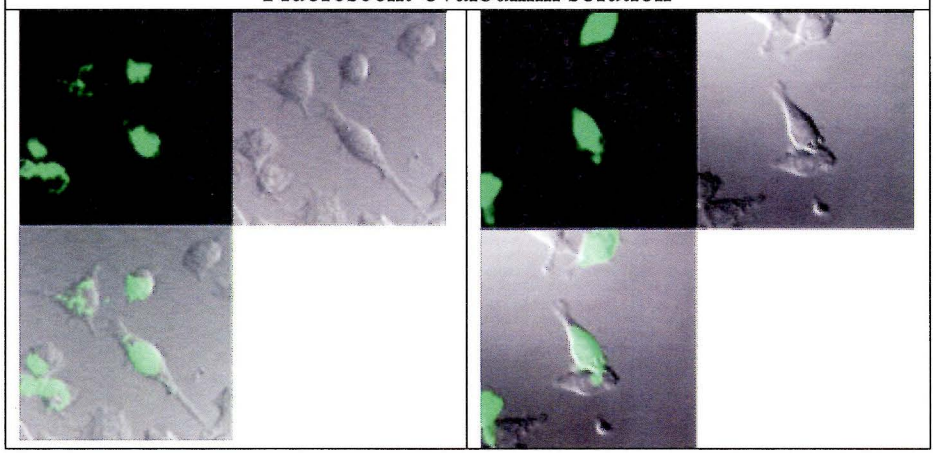
Fluorescent-ovalbumin-loaded particles



PBS

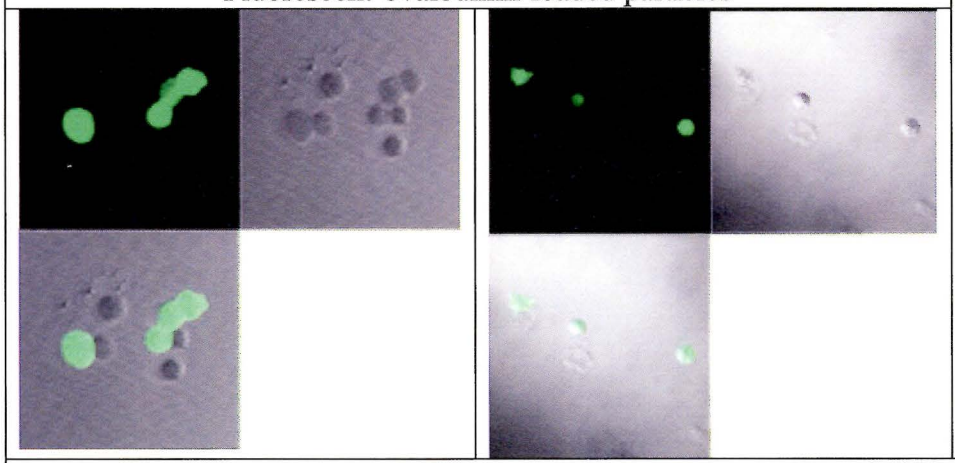


Fluorescent-ovalbumin solution

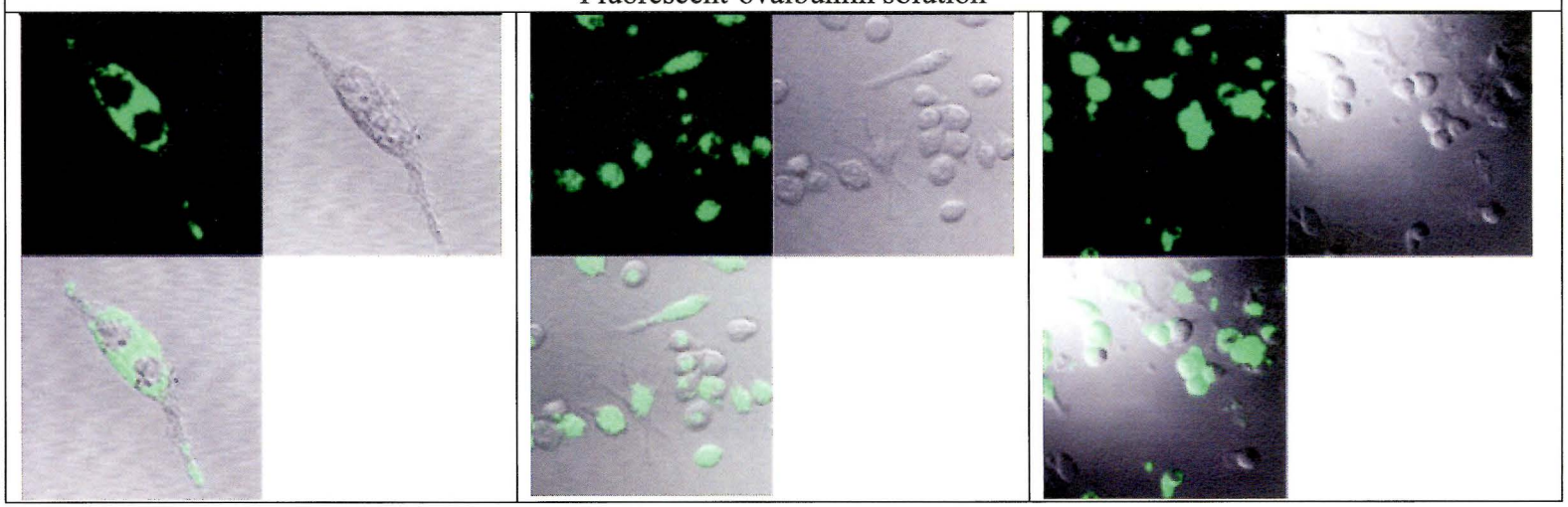


A 18h incubation of dendritic cells with:

Fluorescent-ovalbumin-loaded particles

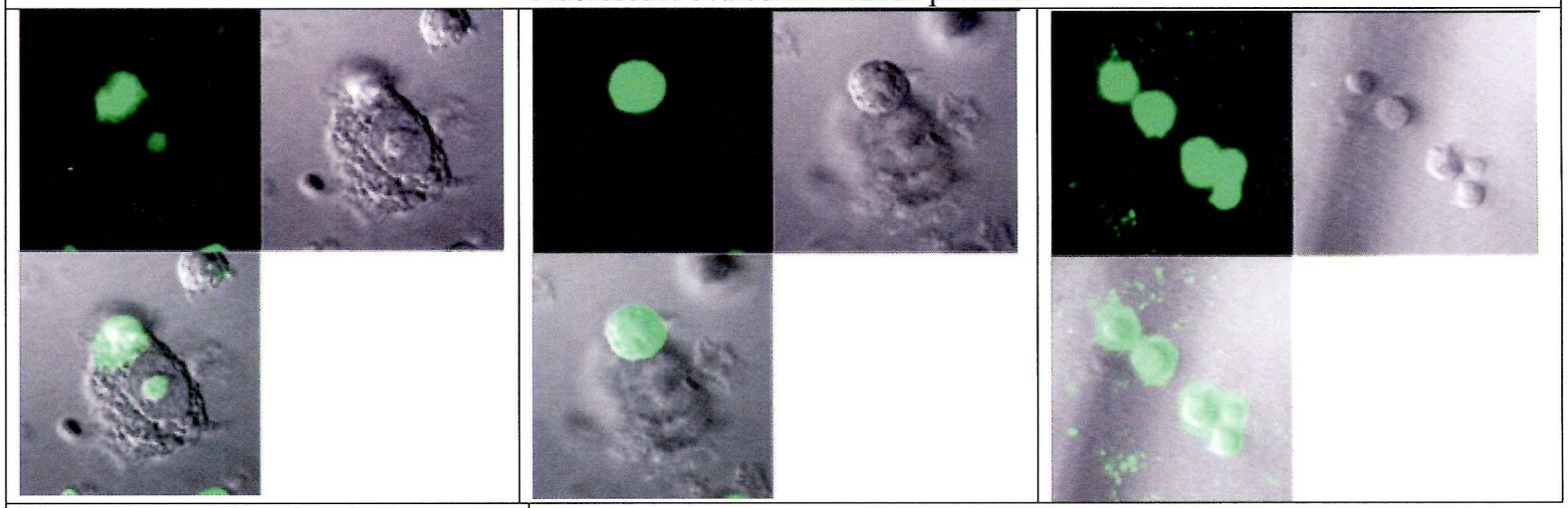


Fluorescent-ovalbumin solution

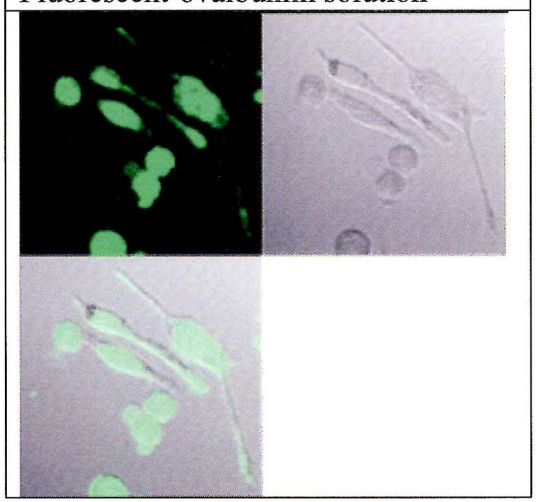


A 24h incubation of dendritic cells with:

Fluorescent-ovalbumin-loaded particles



Fluorescent-ovalbumin solution



Appendix I: FITC-H-2K^b surface staining for flow cytometry

Prepared by: Pearline Lung	**Date**: March 23, 2007
Revision: 1	**Supercedes**:

Cells to compare:
- DC 2.4 dendritic cells
- L929 mouse fibroblast cells
- DKL T-cells

cRPMI:

RPMI	500 ml
FBS	50 ml
Penn/strep	5 ml
L-glut	5 ml
Hepes	5 ml
Na pyruvate	5 ml
B-mercaptoethanol	0.5 ml

Procedures:
1. Resuspend cells at 2 x 10^7 cells per ml in cRPMI.
2. Add 100 ul of cell suspension to 1.5 ml Eppindorf tube.
3. Spin tubes for 3 min, 1500 rpm. Aspirate supernatant. Resuspend cells by tapping.
4. Prepare Fc Block at 1:100 in FACS buffer (0.5% BSA in PBS ie. add 2.5g of BSA to 500 ml of PBS). Make 25 ul per tube (plus a bit extra).
5. Add 25 ul of Fc Block to each tube and incubate on **ice for 15 min**.
6. Spin tubes for 3 min, 1500 rpm. Aspirate supernatant. Resuspend cells by tapping.
7. Add 50 ul of FITC-H-2K^b diluted to 1:50 to each **STAINED** tube. Incubate on ice for **30 min (in dark)**.
8. Add 150 ul of FACS buffer and pellet cells.
9. Add 200 ul of FACS buffer and pellet again.
10. Add 200 ul FACS buffer and transfer to 5 ml flow tube. Add 800 ul FACS to flow tube.

Source: Bramson Lab (McMaster University)

Appendix J: Flow cytometry results of MHC class I staining on dendritic cells, DKL cells, and L929 cells

The dendritic cells used in this experiment were derived from a cell line, thus, may have lacked receptors found on blood-derived dendritic cells. Therefore, flow cytometry was used to verify the presence of MHC class I receptors on these cells (see Figure 38). Mouse fibroblast L929 cells and DKL cells were used as controls. All flow cytometry analysis was done using the Beckman Coulter FC500 and the FC500 analysis software. Cells were blocked with Fc block (PN# 553142, BD Biosciences, Mississauga, ON) and then stained with FITC-H-2K^{b} antibody (Dr. Bramson's lab, McMaster University). FACS buffer (0.5% BSA in PBS) was used to wash cells in between steps.

These results confirm the presence of MHC class I receptors on the dendritic cells. The DKL cells also showed the presence of MHC class I receptors. Interestingly, no MHC class I receptors were found on L929 cells.

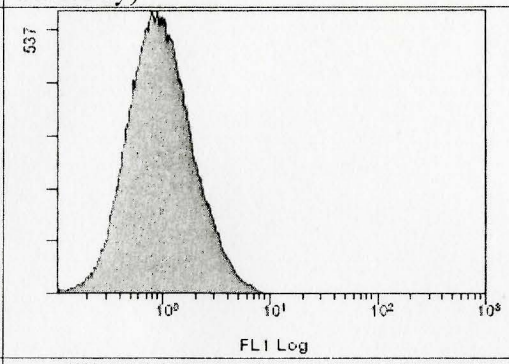
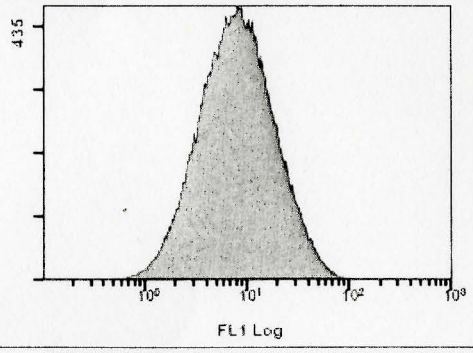
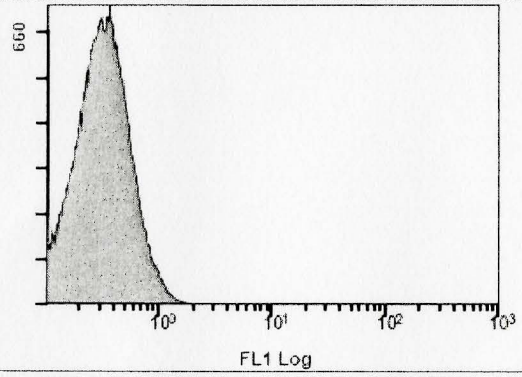
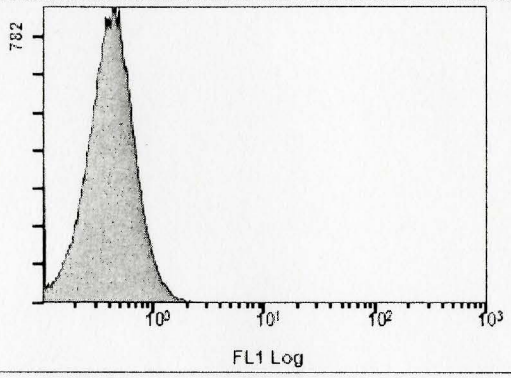
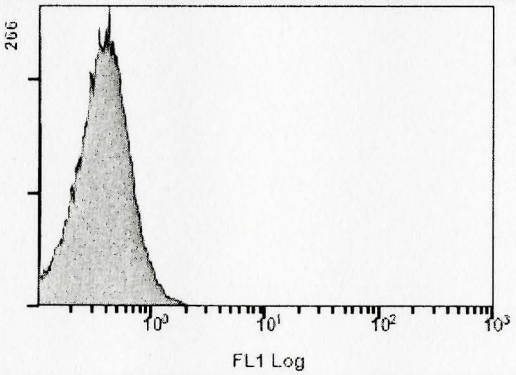
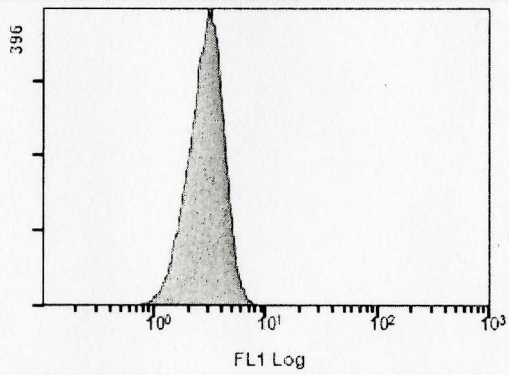
Cell Type	Unstained cells (no FITC-H-2K^b antibody)	Stained cells (added FITC-H-2K^b antibody)
Dendritic cells		
L929 cells		
DKL cells		

Figure 38: Presence of MHC class I receptors in dendritic cells, DKL cells, and L929 cells.