

The Effect Of The Physical Form Of Biodegradable Polymer Carriers On The  
Humoral Immune Response To Co-Delivered Antigen

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The Effect Of The Physical Form Of Biodegradable Polymer Carriers On  
The Humoral Immune Response To Co-Delivered Antigen

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## TABLE OF CONTENTS

Acknowledgements	iii
List of Tables	vi
List of Figures	vii
List of Abbreviations	viii
Summary	x
Chapter I Introduction	1
Chapter II Literature Review	4
2.1 Motivation for Tissue Engineering	4
2.2 Biomaterials used in Tissue Engineering	5
2.3 Natural Biomaterials for Tissue Engineering	8
2.4 Synthetic Biomaterials for Tissue Engineering	10
2.5 Biocompatibility	13
2.6 Inflammatory Response to Implanted Biomaterials	15
2.7 Innate and adaptive immunity	20
2.8 The ‘Danger’ Model of Adaptive Immunity	31
2.9 Immune Responses in Tissue Engineering	32
Chapter III Methodology	34
3.1 Animals	34
3.2 Polymeric Carrier Vehicles Preparation and Characterization	35
3.3 Humoral Immune Response Assay	40
3.4 <i>In Vitro</i> Protein Release Kinetics	43

3.5 Data Analysis	44
Chapter IV Results	45
4.1 Polymeric Carrier Vehicle Preparation and Characterization	45
4.2 <i>In Vitro</i> Protein Release Kinetics	48
4.3 Humoral Immune Response Assay	51
Chapter V Discussion	61
Chapter VI Conclusions and Future Recommendations	69
References	71

## LIST OF TABLES

Table 3-1	Treatment Groups and Physical and Chemical Properties of PLGA MP and PLGA SC	37
Table 4-1	Cumulative Release of OVA from PLGA SC and PLGA MP during <i>in vitro</i> Protein Release Kinetics	50

## LIST OF FIGURES

Figure 2-1	Antigen Shedding and Immune Stimulation	24
Figure 2-2	Danger Signals Induce Dendritic Cell Maturation	28
Figure 2-3	Adjuvanticity of Biomaterials	30
Figure 4-1	Representative size distribution of 50/50 PLGA MP	47
Figure 4-2	Protein release kinetics of OVA from PLGA SC and PLGA MP over 28 days.	49
Figure 4-3	PLGA SC and PLGA MP without OVA do not produce IgG levels significantly different than that of PBS.	53
Figure 4-4	Anti-OVA IgG (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA.	54
Figure 4-5	PLGA SC and PLGA MP without OVA do not produce IgG1 levels significantly different than that of PBS	57
Figure 4-6	PLGA SC and PLGA MP without OVA do not produce IgG2a levels significantly different than that of PBS	58
Figure 4-7	Anti-OVA IgG1 (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA	59
Figure 4-8	Anti-OVA IgG2a (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA	60

## LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
BCA	Bicinchoninic Acid
bFGF	basic Fibroblast Growth Factor
CFA	Complete Freund's Adjuvant
EGF	Epidermal growth factor
FBGC	Foreign body giant cells
FGF	Fibroblast growth factor
FN	Fibronectin
HA	Hyaluronic acid
HAC	Hydroxyapatite/Collagen
HSP	Heat-shock protein
IgG	Immunoglobulin G
IL	Interleukin
IFN- $\gamma$	Interferon- $\gamma$
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MP	Microparticles
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCL	Poly(epsilon-caprolactone)

PDGF	Platelet-derived growth factor
PE	Polyethylene
PEG	Polyethylene Glycol
PGA	Poly(glycolic acid)
PLLA	Poly(L-lactic acid)
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PMMA	Polymethylmethacrylate
PPRs	Pattern recognition receptors
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl alcohol
SC	Scaffold
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UNOS	United Network for Organ Sharing
VEGF	Vascular endothelial growth factor

## SUMMARY

The biomaterial component of a tissue engineered device has been shown to enhance the immune response to a co-delivered model shed antigen. The purpose of this research was to investigate *in vivo* the differential level of the immune response toward different forms of the biomaterial. A model shed antigen, ovalbumin (OVA), was incorporated into polymeric biomaterial carriers made of 50:50 poly(lactic-*co*-glycolic acid) (PLGA) in the form of microparticles (MP) or scaffolds (SC). These MP and SC biomaterial carrier vehicles with incorporated antigen were then injected or implanted, respectively, into C57BL6 mice to investigate the differential level of the immune response towards OVA controlled release from PLGA MP and PLGA SC. For each polymeric carrier, the resulting time-dependent systemic humoral immune response towards the incorporated OVA, the OVA-specific IgG concentration and isotypes (IgG2a or IgG1, indicating a predominant Th1 or Th2 response, respectively) were determined using ELISA. To assess the differential level of the immune response depending on the form of PLGA, the total amounts of polymer and OVA delivered were kept constant as well as the release rate of OVA. The *in vitro* protein release kinetics were studied for both PLGA microparticles and PLGA scaffolds to examine the release rate of OVA from the polymeric carriers.

The level of the humoral immune response was higher and sustained for OVA released from PLGA SC which were implanted with associated tissue damage, and lower and transient when the same amount of polymer and OVA

were delivered from PLGA MP, which were minimally invasively delivered by injection. This immune response was primarily Th2 helper T cell-dependent as exemplified by the predominance of IgG1 isotype, although for the strong adjuvant, Complete Freund's adjuvant (CFA), and PLGA SC carriers the anti-OVA IgG2a isotype levels were also significant, potentially indicating both a Th2 and Th1 response.

The PLGA SC and PLGA MP exhibited similar protein release kinetics, releasing similar amounts of OVA at each time point. Each carrier incubated contained the same ratio of OVA to polymer. *In vitro* protein release kinetics experiments suggest that the rate of release of OVA from PLGA SC and PLGA MP was similar, and therefore the enhanced immune response induced by PLGA SC is most likely due to 'danger signals' from implantation which primed the system for an enhanced immune response and not from a difference in concentration of OVA released from the carriers.

# CHAPTER I

## INTRODUCTION

Tissue engineering is an emerging multidisciplinary field involving biology, medicine, and engineering to revolutionize the way patients are treated by restoring, maintaining, or enhancing tissue and organ function. Tissue engineering devices usually consist of a polymeric biomaterial component as well as associated cells or biomolecules, such as proteins. These transplanted cells or biomolecules can be recognized as foreign by the host's immune system depending on the cell source (allo- or xenogeneic), which stimulates an antigen-specific immune response. The biomaterial may trigger a non-specific inflammatory response including the recruitment and activation of antigen presenting cells such as dendritic cells and macrophages. The biomaterial component has been previously shown to act as an adjuvant in the enhancement of the immune response towards to the cellular component of a tissue engineered construct. The hypothesis of this research is that biomaterials in the form of scaffolds, which are implanted with associated tissue damage, induce a higher level of immune response to associated co-delivered antigen as compared to biomaterials in the form of microparticles, which are minimally invasively delivered by injection.

## Objectives

Specific Aim 1: *Determine the formulation necessary to maintain a common ratio of OVA: polymer dose for both PLGA MP and PLGA SC carrier vehicles. A ratio of 1.075 mg OVA/mg polymer was formulated to keep the same ratio of OVA: polymer for both the PLGA MP and PLGA SC carrier vehicles. A double-emulsion solvent-extraction technique with a polyvinyl alcohol coat allowed for the controlled encapsulation of OVA into the PLGA MP. Ovalbumin was directly incorporated into PLGA SC in the commonly used solvent casting particulate leaching technique.*

Specific Aim 2: *Study the in vitro protein release kinetics of OVA from both PLGA MP and PLGA SC to insure a constant release rate for both carrier vehicles. The in vitro release kinetics of OVA into PBS from OVA-loaded PLGA MP and PLGA SC were evaluated over 28 days.*

Specific Aim 3: *Assess the differential level of the enhancement in the immune response towards OVA when it is controlled released from PLGA MP or PLGA SC. To investigate the differential level of the enhancement of the humoral immune response towards OVA controlled release from PLGA MP or PLGA SC, the total amount of polymer and OVA delivered (1.075 mg OVA/mg PLGA) was kept constant as well as the release rate of OVA from these two vehicles. The controlled release of OVA from the carriers then allowed testing of the hypothesis that a differential level of biomaterial adjuvant effect will be observed in the immune response towards associated antigen*

depending on the form of the carrier vehicle, which is proportional to the extent of tissue damage associated with its insertion in agreement with the 'danger' model of immunity. In the serum of C57BL/6 mice receiving these OVA carrier vehicles, the anti-OVA total IgG concentrations and isotypes were determined in order to study the differential level of the immune response towards the different forms of PLGA.

## CHAPTER II

### LITERATURE REVIEW

#### Motivation for Tissue Engineering

Across the United States, an average of seventeen men, women, and children die every day due to lack of sufficient numbers of organ donors. According to the United Network for Organ Sharing (UNOS), currently there are over 85,000 patients waiting for organs in the United States alone, with 58,000 of these patients needing kidneys [1]. Every twelve minutes another name is added to the growing organ transplant wait list due to injury, disease, or autoimmune disorders irreversibly damaging organs.

Tissue engineering introduced an alternative to waiting for appropriate organ transplants in the 1970's by the concept of applying a combination of polymers (natural or synthetic), cells, and biomolecules for the regeneration, repair, or modification of damaged tissues or organs. Tissue engineering seeks to regenerate tissues with methods other than conventional medicines and drug therapies. Included in tissue engineering is the development of cell lines and tissues for the intent of restoring function and physiology lost due to damaged tissue or organs. A large amount of research focuses on restoring function through the use of cell therapies derived from autologous cells, cells from an established cell line, stem cells, or genetically modified cells [2,3]. To expand the possible sources of cells, cells from human donors (allografts) [4] or from other animals (xenografts) [5] are being explored, however there is still the concern of immunological rejection as in organ transplantation. These modified cells can then be

seeded into a three-dimensional device component such as a scaffold, matrix, or membrane, in order to regenerate damaged tissue. For the tissue engineered construct to be successful in regenerating tissue, the cells must be viable and able to carry out their metabolic functions within the matrix. The tissue engineered device allows stimulation, directly or indirectly, of cells into a specific activity [6]. Also, the biomaterial component, typically a polymer, must sustain long-term structural and functional properties in order to endure the harsh physiological environment. Along with this harsh physiological environment is the potential immune response towards the tissue engineered construct *in vivo* due to the cellular component which may be of allogeneic or xenogeneic origin. An inflammatory response will typically be directed against the biomaterial component of the construct while a specific immune response may be directed against incorporated foreign cells of either allogeneic or xenogenic origin. In order to be successful and fully integrate into neighboring tissue, a tissue engineered construct must also be able to evade the host inflammatory and immune response.

### Biomaterials Used in Tissue Engineering

Biomaterials used in tissue engineering may be of either natural or synthetic origin. Synthetic biomaterials [e.g., poly( $\alpha$ -hydroxy acid) family of polymers [e.g., poly(lactic-*co*-glycolic acid) (PLGA)]] are typically biodegradable, temporary three-dimensional scaffolds which allow cells to attach and eventually grow and differentiate in a preferred manner while depositing their own natural extracellular matrix as the biomaterial degrades. This property of biodegradation makes PLGA scaffolds attractive

for gradually regenerating tissues, yet the PLGA scaffold may only be used as a temporary implant as a result [7]. Natural biomaterials include protein and polysaccharide polymers such as collagen, fibrin, elastin, alginate, chitosan, and hyaluronic acid. These biomaterials are similar in nature to the host tissue and have less toxicity. However, they are also quite immunogenic and structurally complex, making them difficult to manufacture [7].

Scaffolds may also be used as a method of gradually delivering growth factors to encourage new cell growth and direction [8]. The biomaterial scaffold will eventually degrade and be replaced by a cell-derived extracellular matrix, resulting in the formation of natural tissue. Biomaterials may also be fabricated in other forms besides scaffolds such as injectable systems for cell printing (assembling cells in a pre-defined pattern onto a biomedical construct) and injectable polymeric microparticles. These injectable biomaterials allow for great ease in implantation and less complications as observed with invasive surgeries. Overall, a biomaterial will influence surrounding natural tissue as well as be influenced by the tissue to degrade over time and be replaced by the host natural tissue [8].

The advantage of using biomaterials in tissue engineering is the ability to restore function and physiology lost due to damaged tissue or organs and the ability to transplant altered cells into specific locations, including immunoprivileged sites. These cells can be manipulated, unlike with transplant organs, to enhance a specific function or to reduce immunogenicity so that a transplant is more successful in its ability to integrate into the host tissue. Altered cells for transplantation may also be cryopreserved for off the shelf availability and joined with a combination of other cells in a biomaterial scaffold to

enhance a specific function [9]. Since the cells may be manipulated in order to decrease immunogenicity, allogenic and xenogenic cells may be used for this form of tissue engineering, potentially offering unlimited cell sources. In reality, these transplanted cells will induce an immune response and may cause severe complications upon implantation. More research is needed to find a method to truly immunoisolate these foreign cells from the host defense system and thereby avoid implant rejection.

For each specific application, a biomaterial must be considered for its composition, mechanical properties, porosity, degradation rate, reproducibility, biocompatibility, sterilizability, and shelf-life. It is important to consider such properties as the topography for cell attachment and migration or differentiation. Most scaffolds are porous so that surrounding tissue may infiltrate the scaffold and begin to regrow [10]. Polymers such as nylon and polyester are commonly used as sutures and artificial blood vessels due to their resilient strength. Biodegradable polymers are resilient and easy to fabricate, yet they are also not very strong and will deform with time as they degrade. As a polymer degrades, it will release degradation products that will change the acidity of the local environment, resulting in changes in homeostasis [7]. Metals, such as titanium and stainless steels, are used as bone plates and joint replacements due to their strong and ductile mechanical properties. Stainless steels were used as early joint replacement prosthesis components and found to be inadequate in their fatigue strength, allowing bending and breaking of the device with stress over time. These metals may be chemically passivated in order to increase biocompatibility with the host, yet the properties of the metal may change with this passivation such as the hardness of the metal. A major concern with metals as biomedical implants is their ability to corrode

upon removal of this passivation coating. Ceramics, such as calcium phosphates and hydroxyapatite, are biocompatible and are used for orthopedic implant parts [7]. They are well tolerated by the body, yet they are brittle and difficult to manufacture.

### Natural Biomaterials For Tissue Engineering

Natural biomaterials include such materials as protein polymers, polysaccharide polymers, and lipids. Of these materials, collagen, chitin, and hyaluronic acid (HA) are the most commonly used for tissue engineering devices. Advantages of natural materials include biofunctionality, biodegradability, and less of an inflammatory response upon implantation into the host. However the disadvantages include low mechanical properties, low stability, and complex processing [7, 11].

Natural biomaterials are biomimetic in that they are able to imitate the extracellular matrix of natural tissues. Chitosan, manufactured from polysaccharide chitin found in crab shell, is often used for absorbable sutures as well as for skin and cartilage tissue engineering [12,13]. Chitosan fiber scaffolds with seeded chondrocytes are able to produce new extracellular matrix in a way similar to natural cartilage. The randomly arranged chitosan fibers are able to distribute the strain and allow for the integration of new cartilage [13]. These scaffolds offer promise in using natural biomaterials for the repair of defects in articular cartilage as they have the appropriate structure and the desired composition, mechanical properties, and durability similar to articular cartilage *in vivo*. Chitosan fiber scaffolds have proven to be more successful for this area of tissue engineering versus foams and hydrogels which do not meet these

physical and mechanical needs [13]. However, chitosan is highly immunogenic and there are some concerns about its toxicity.

Collagen, a family of fibrous insoluble proteins, is another promising natural biomaterial which is used for skin and cartilage tissue engineering, heart valves and vascular grafts, orthopedic applications, and drug delivery. Collagen is commonly used for such medical purposes as replacing ligaments due to its mechanical strength [14] and for facial dermal repair [14]. Studies have suggested that collagen with immobilized heparin prevents surface thrombus formation, making collagen safe and efficient for vascular grafts [16]. However, it is still significantly immunogenic. Collagen with incorporated human fibroblasts exhibits high mechanical strength, which makes it a useful seeded scaffold for potential use in an artificial heart [17]. One of the most popular uses of collagen is for cartilage tissue engineering. As collagen scaffolds gradually degrade, they form homogenous cartilage much like the natural host cartilage. Cartilage tissue engineering efforts have focused on combining collagen with synthetic polymers in order to increase the mechanical properties of a scaffold as well as to increase cell-seeding of chondrocytes [18].

Hyaluronic acid (HA) is a component of the extracellular matrix of embryonic mesenchymal tissue which is often used for dermal wound healing applications as well as bone tissue engineering. When fibronectin (FN) is combined with HA in collagen scaffolds, fibroblasts are encouraged to migrate into the scaffolds and stimulate wound healing. The HA component of the scaffold will enhance wound healing and tissue regeneration, even in areas where scar tissue may block natural wound healing [19]. Hyaluronic acid in the form of hydrogels is unique in its wound healing and angiogenesis

ability. As a natural polymer, it is easily manufactured and modified, as well as hydrophilic and biodegradable. Hyaluronic acid is also nonadhesive, a required property for vascular grafts [20]. There is some question about the long-term mechanical properties of HA. Research is currently underway to strengthen HA by the addition of other materials or chemicals.

Two types of biomaterials may also be combined in a scaffold in order to form a device with multiple properties of the individual components. For example, polysaccharide hybrid materials for cartilage tissue engineering scaffolds can be composed of alginate and chitosan fibers to allow for enhanced chondrocyte adhesion [21]. The combination of these two polymer fibers allows for enhanced adhesion of chondrocytes as well as the maintenance of cellular structure. In the area of bone tissue engineering, hydroxyapatite/collagen (HAC) scaffolds mimic the extracellular matrix of bone. The collagen component allows for natural-like apatite formation [22]. Agarose combined with fibronectin has been used as conduits to repair short gaps in peripheral nerves, and recently for large gaps [23]. These conduits allow growth of Schwann cells and fibroblasts by contact guidance. In deciding what material to use it is important to consider the intended application of the device and whether the materials used are appropriate.

### Synthetic Biomaterials For Tissue Engineering

Of the synthetic biomaterials (including polymers, metals, ceramics, and glass) polymers are most commonly used in tissue engineering. Much research focuses on the

use of polymers from the poly( $\alpha$ -hydroxy acid) family, which are thermoplastic aliphatic polymers with many desirable properties for tissue engineering constructs such as their ability to biodegrade, their biocompatibility, and their relatively good processability. Disadvantages include immunogenicity, poor mechanical properties, and acidic degradation products which may effect homeostasis. Common homopolymers in this family are poly(L-lactic acid) (PLLA) and poly(glycolic acid) (PGA) which may be joined in various molar ratios to form the copolymer poly(DL-lactic-*co*-glycolic acid) (PLGA).

The copolymer PLGA is a biodegradable polymer which undergoes non-enzymatic bulk degradation by hydrolysis of the ester linkages in the backbone of the polymer [24]. The degradation rate of PLGA is dependent to an extent on the molar ratio of lactic to glycolic acid. The degradation time of PLGA can be controlled from weeks to over a year by varying the ratio of monomers. For example, increasing glycolic acid, the hydrophilic component of the copolymer, increases the biodegradation rate [25]. As the PLGA biodegrades, lactic acid is naturally metabolized in the tricarboxylic acid cycle and then eliminated from the body in the form of water and carbon dioxide removed through respiration [26]. Glycolic acid may also follow this cycle or be excreted unmodified from the body as water and carbon dioxide. During biodegradation of PLGA, it is important that these acidic degradation products are removed from the body to maintain homeostasis [27].

The strong interest in biodegradable polymers such as PLGA is that they do not elicit permanent chronic foreign-body reactions, as they will be gradually absorbed by the host. They also are able to regenerate tissues through the interaction of their

biodegradation with immune cells. However, even with this appeal, the biodegradable polymer implants release acidic degradation products which cannot be eliminated quickly enough from the body, resulting in changes in homeostasis. A solution to this problem would be to allow for a slower release of degradation products versus a burst release so that the products could be timely metabolized. The development of new biodegradable polymers is a research challenge as they must be biocompatible and release low-toxicity degradation products.

The poly (alpha-hydroxy acid) family is commonly used for a variety of biomedical purposes [28] including sutures, drug delivery devices, orthopedic fixation devices, as well as tissue engineered blood vessels, and nerve regeneration channels. The biodegradable polymer poly(L-lactic acid) (PLLA) has a high tensile strength and low elongation which allows it to have a high modulus suitable for load-bearing applications such as bone fracture fixation. Poly(glycolic acid) (PGA) was first used as resorbable sutures, and then became material for meshes and surgical products [29]. A limitation of these sutures is that they lose their mechanical strength at 2 to 4 weeks after implantation. Sponge tubes made of PGA-collagen for nerve regeneration show more promise than the composite tubes in maintaining structural integrity for neuronal support [30]. The copolymer PLGA is commonly used for cartilage tissue engineering due to its ease in incorporating chondrocytes and supporting their differentiation into an appropriate phenotype [31]. Many studies focus on using PLGA in the form of microparticles for drug or growth factor delivery. An attractive use of PLGA in tissue engineering is the potential to transplant pluripotent stem cells inside PLGA microparticle beads to stimulate tissue regeneration [32].

There is also a motivation to manufacture biomaterial surfaces to which proteins will not adhere. Polyethylene oxide (PEO) aids in making a biomaterial more biocompatible by reducing non-specific protein adherence to biomaterials [33,34]. These biomaterials are able to resist leukocyte adhesion, therefore inducing less of an inflammatory response. However, fibrous encapsulation will still occur due to macrophages at the implant site. Hydrophilic and anionic substrates allow for decreased rates of macrophage adhesion on biomaterial surfaces [35].

The decision of which biodegradable material to use for an application should consider the kinetics of degradation as well as the mechanical properties. For a material to be more susceptible for degradation so that the natural tissue will replace the scaffold, bonds need to be susceptible to cleavage so that enzymes may gradually digest the biomaterial *in vivo*. It is also important that the degradation products be nontoxic [36]. Another advantage of biodegradable polymers is the ability to manufacture the polymers in different geometries and different sizes for tissue regeneration as well as the incorporation of growth factors and other bioactive molecules [27].

### Biocompatibility

In 2003 it was suggested that the term biocompatibility should be based on the application of the biomaterial versus the composition of the material [6]. Previously the definition had been based on the ability of a material to perform with the appropriate response in a specific application, and considered mainly only the biological safety of the material. How the biomaterial performs should be included in the definition, not just that

it is of a certain material. It is important to recognize that the host response to the material will vary and some materials are not as applicable and efficient in certain situations as others. Biomaterials today are varied in their applications and responses so that a new definition of biocompatibility must be considered [6].

Long-term implanted biomaterials, such as titanium, cobalt-chromium alloys, platinum, carbon, alumina, silicone, polytetrafluoroethylene (PTFE), polyester, polymethylmethacrylate (PMMA), and polyethylene (PE), are used as such due to their mechanical properties as well as ability to resist corrosion and degradation. For these long-term implants, the importance is the outlasting mechanics and relatively low reactivity [6,7]. Although some of these long-term implants have been coated with surface coatings, these coats can be subjected to degradation (i.e. by the changing pH of the local environment due to metabolic products), rendering them more likely to induce an inflammatory response. Also, the corrosion products may elicit an adverse biological reaction in the host. To control this corrosion, researchers have used noble metals (i.e. gold, silver, and platinum) which exhibit good corrosion resistance. However, these are costly and have poor mechanical properties. Therefore most research focuses on passivation of mechanically strong biomaterials. Biocompatibility should be thought of as a two way street with the material affecting the host either systemically or locally and the host affecting the degradation of the material as well. The biocompatibility of a tissue engineered device is described as the ability of a scaffold, matrix, or membrane to support cellular activity in order to optimize tissue regeneration without any damaging effects to these cells or the host either locally or systemically [6].

It would be ideal to make a biomedical polymer that is relatively unreactive and non-toxic. However, no biomaterial is inert and unreactive when in contact with the host environment. Most biomaterials will exhibit some thrombogenic and inflammatory properties.

### Inflammatory Response to Implanted Biomaterials

Tissue damage and changes in homeostasis occur with the implantation, insertion, or injection of a biomaterial, resulting in damage to connective and adipose tissue and the release of proteins from these damaged tissues into the surrounding area. Injury to tissue caused by the insertion of a biomaterial leads to a tissue response in the form of inflammation, wound healing, and foreign body response [37]. The extent and duration of the host tissue response will depend on the implant procedure as well as the physical and chemical properties of the biomaterial implanted. Many events occur during the host response of inflammation and wound healing after an injury, and may follow the sequence of acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrosis [37].

After a biomaterial is implanted, inflammation occurs in order to contain any harmful agent at the site of injury as well as to begin regenerating natural parenchymal cells at the site. Blood proteins, cells, and plasma fluid may leave damaged blood vessels through exudation, stimulating cellular responses and chemical factors for mediating inflammation [37]. At this time thrombosis may occur due to an accumulation of activated platelets, forming a blood clot which aids in immune cell migration. Platelets

adhere to collagen exposed during blood vessel injury and form a thrombus by the binding of plasma fibrinogen and von Willebrand Factor to the integrin  $\alpha$ (IIb) $\beta$ (3) [38]. Activated platelets express P-selectin which will later mediate leukocyte rolling to the injury site [39]. They also express platelet-derived growth factor (PDGF) and transforming growth factors (TGF B1 and B2) from their granules, activating macrophages and fibroblasts to migrate to the injury site and initiate wound healing [37]. Platelet adhesion to biomaterial surfaces may be mediated by platelet glycoprotein IIb/IIIa as well as through the GP IIb-vWF interaction. Platelet activity is studied to produce a non-thrombogenic tissue engineered construct. If a biomaterial induces thrombosis platelets will be removed from circulation at a rapid rate, resulting in platelet consumption. In a study of the importance of PDGF in regulating wound healing, transgenic mice with PDGF-D showed increased macrophage recruitment and angiogenesis as compared to control mice [40].

Acute inflammation occurs over minutes to days and is important for the emigration of leukocytes, mainly neutrophils, from the blood vessels to the implantation site [37]. Leukocytes migrate by “rolling” along the endothelium, a process which is assisted by adhesion molecules and chemotactic stimuli. Selectins, such as L-selectin expressed on neutrophils, initiate primary interaction between the endothelium and leukocytes, resulting in “rolling” of the leukocytes along the venous wall [41]. This rolling induces leukocyte activation due to contact with activating molecules (e.g., interleukin 8) resulting in an upregulation of the functional activation of beta 2-integrins (CD11/CD18) which interact with the counter receptor ICAM-1 on the endothelium, leading to a secondary, tighter adhesion between leukocytes and the endothelium [42].

After migration to the implantation site, the leukocytes initiate phagocytosis by releasing cytokines, proteolytic enzymes, and reactive oxygen intermediates. At the same time, monocytes are stimulated to migrate in response to TGF-beta and monocyte chemoattractant protein-1, and will differentiate into macrophages at the injury site [43]. Macrophages work alongside leukocytes in the phagocytosis of bacteria, debris, or foreign particles as well as initiate wound healing by releasing a variety of growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to stimulate angiogenesis [40].

During phagocytosis, neutrophils and macrophages must first recognize and bind to harmful agents which have been coated with opsonins (serum factors such as immunoglobulin G (IgG), complement-activated fragment C3b, and fibrinogen), engulf the agent, and finally degrade it [37, 44, 45, 46]. Sometimes, however, the biomaterial is too large for this engulfment and “frustrated” phagocytosis occurs so that phagocytes resort to releasing enzymatic products in an attempt to degrade the foreign material [37]. Macrophages will secrete different cytokines and growth factors depending on the particular opsonins coating a biomaterial. This also controls the ability of the fibroblasts to proliferate as different cytokines will have different influences on fibroblast growth [46]. Protein adsorption on a biomaterial surface produces different levels of macrophage cytokines IL-1 beta, IL-6, and TNF-alpha, suggesting that macrophage activation is indeed dependent on the type of polymer as well as the type of adsorbed proteins on the polymer [47].

Chronic inflammation develops with the prolonged presence of inflammatory stimuli, macrophages, and lymphocytes as well as the regeneration of blood vessels and

connective tissue [37]. Macrophages are able to process foreign antigens and present them to other immune cells to stimulate an adaptive immune response towards the specific antigen. They also are able to secrete important growth factors for wound healing such as fibroblast growth factor (FGF) for the proliferation of fibroblasts, tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) for the regeneration of blood vessels. Growth factors from macrophages will encourage cells to proliferate, migrate, and differentiate as well as stimulate tissue remodeling [37, 40].

As little as four days after biomaterial implantation, fibroblasts and endothelial cells may begin to proliferate and form granulation tissue, a sign that wound healing is initiated. Angiogenesis occurs when the endothelial cells mature and form an organized layer within capillary tubes [40] and new tissue is formed by the migrating fibroblasts [48,49]. Macrophages at the site continue to release growth factors and cytokines, for example PDGF, VEGF, and FGF [48]. The new tissue is a matrix for macrophages and fibroblasts to migrate and initiate tissue regeneration and remodeling. Eventually the tissue will be remodeled and cross-linked collagen with a high tensile strength, mainly collagen III, will be formed on this matrix [48, 49]. Fibroblasts will continue to proliferate and differentiate into myofibroblasts as they manufacture new collagen. This fibroblast proliferation is controlled by a complex signaling pathway involving TNF-beta as well as epidermal growth factor (EGF) [49]. Once the injury site is cleared of harmful agents, fibroblasts will proliferate at the injury site, depositing new tissue, and initiate wound closure [48].

The foreign body response, the last stage of the wound healing process, is dependent upon the topography of the biomaterial [37]. A smooth surface may attract minimal macrophages while a rough surface will also result in the formation of foreign body giant cells (fused monocytes and macrophages) (FBGC). The macrophages are activated by both chemical and physical cues from the biomaterial [50] and foreign body giant cells may stay on the surface of an implant throughout its *in vivo* use. The final phase of wound healing is the fibrous encapsulation of the biomaterial, isolating it from the rest of the host. There is an interest of the effect of this fibrous barrier on the diffusion of nutrients and drugs. It is important to keep any implanted cells within a biomaterial alive and able to receive nutrition as well as discard metabolic waste [51]. The amount of encapsulation also depends on the form of the biomaterial and the intensity of the implantation (for example injection versus surgical implantation). Small-diameter microfiber implants do not become encapsulated after subcutaneous implantation, although large-diameter fibers (5.9 micron) are encapsulated [52]. It is believed that the larger diameter fibers have a more pronounced dead space area between collagen fibers which attracts the infiltration of inflammatory cells into the space, stimulating fibrous encapsulation. The overall wound healing is highly dependant on adequate blood supply, the ability to receive nutrition, and the ability of the cells at the site to proliferate and differentiate.

Many studies have focused on reducing the host inflammatory response towards a biomaterial and thus increasing its biocompatibility. There is a motivation to decrease the amount of macrophages and foreign body giant cells which release harmful degradative enzymes towards the biomaterial. For example, polyurethanes with modified

surfaces containing silicone can alter and decrease macrophage adhesion, improving the biostability of polyurethane [53]. To inhibit FBGC, diacylglycerol kinase inhibitor R59022 has been discovered as a potent inhibitor of macrophage and monocyte fusion to form FBGCs. On the other hand, alpha-tocopherol stimulates FBGC formation [54]. As mentioned before, different surface properties of a biomaterial may alter the degree of macrophage and FBGC formation at the surface. For example, an interpenetrating polymer network composed of polyacrylamide and poly(ethylene glycol) restricts cell adsorption while N-(2 aminoethyl)-3-aminopropyltrimethoxysilane promotes cell adhesion. This difference in surface chemistry controlled FBGC formation and macrophage fusion so that the interpenetrating polymer network did not form FBGCs [55]. Specifically, the copolymer PLGA has shown good biocompatibility with little FBGC formation at its surface. The two components of PLGA, PGA and PLLA, have shown to cause little inflammatory response (a scarce number of monocytes and leukocytes at the site) as biodegradable pins and screws for the fixation of bone fractures [56], and in fact the copolymer PLGA shows little inflammatory response *in vivo*, making it a biocompatible polymer [57].

### Innate and Adaptive Immunity

In order to create a tissue engineered device with incorporated cells (such as allogeneic or xenogenic cells) which will fully integrate into host tissue without rejection, it is necessary to understand both the innate immunity towards the implant (typically

considered the inflammatory response) as well as the adaptive immunity towards the transplanted cells. Innate and adaptive immunity are not independent processes and it is vital to understand their interconnections in order to learn how to engineer the immune response and allow a device to evade this response, thereby integrating into the host tissue successfully.

Within hours of infection due to a pathogen or injury due to an implantation, innate immunity takes effect and stimulated phagocytes and natural killer cells arrive at the site of inflammation. Innate immunity is the first line of defense against foreign materials (e.g., pathogens or biomaterials) and has physical barriers such as the epithelium as well as blood proteins such as complement, inflammatory molecules, and cytokines [58]. The innate immune response is largely mediated by phagocytes (such as neutrophils, macrophages, and natural killer cells) which engulf the foreign materials and release mediators such as reactive oxygen intermediates in an attempt to destroy them. Innate immunity is responsible for recognizing microbes or other foreign materials such as biomaterials whereas adaptive immunity recognizes both microbes and non-microbial agents such as transplanted cells which are allo- or xenogeneic. Unlike innate immunity, there is more diversity with adaptive immunity as well as B memory cells which can retain memory of an antigen. The innate response to a foreign body, either a pathogen or an implanted biomaterial, is the same as the inflammatory response to biomaterials.

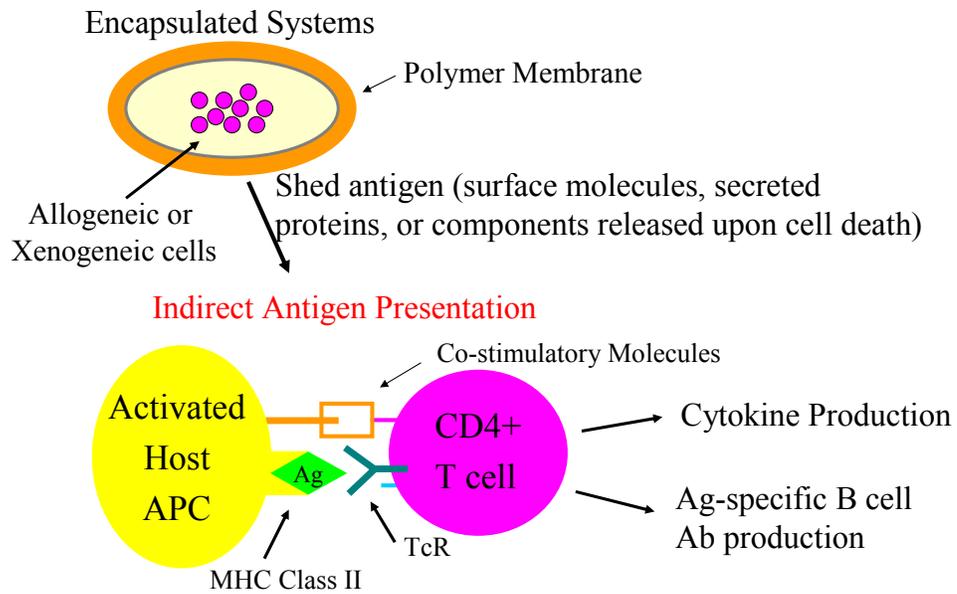
The innate immune system may recognize foreign agents through either evolutionary conserved structures belonging to pathogens or through opsonin proteins coating a foreign object. Once a foreign antigen is recognized as such by an antigen-presenting cell (APC), the antigen is engulfed, processed, and then presented on the APC

to lymphocytes, a process which initiates adaptive immunity. Adaptive immunity is responsible for clearance of antigens, cells, or pathogens and is composed of cellular immunity and humoral immunity. Cellular immunity involves the clonal expansion of antigen specific T helper cells which will secrete cytokines for the activation of T and B cells to proliferate and differentiate. During cellular immunity, cytotoxic T cells or natural killer cells are involved in direct killing of cells containing foreign antigen. In humoral immunity B cells produce antigen specific antibody for the clearance of a pathogen [59].

There are two different pathways for antigen presentation depending on where the antigen was derived. Antigens in the extracellular environment (exogenous antigens) will be engulfed and processed by an APC and then presented in the direct pathway of antigen presentation, major histocompatibility complex (MHC) class II pathway. The host APCs in the MHC class II pathway present antigen to T helper cells via the CD4 molecule on the T cells. MHC class II is expressed on mononuclear phagocytes, B cells, dendritic cells, endothelial cells, as well as thymic endothelium. These complexes are responsible for binding antigen and presenting it to T cells. They define self and each individual's MHC molecules differ from the next. Different MHC molecules also present different antigens. During the second, indirect pathway of antigen presentation, or MHC class I pathway, antigens are processed from the cytosol (intracellular) and then presented by APCs to the CD8 molecule on cytotoxic T cells. MHC I is expressed on nucleated cells as well as dendritic cells [58]. Cross-presentation of MHC molecules is also possible, providing immunity to viruses and tolerance to self. There is new evidence that dendritic cells have an ability to process exogenous antigens into the MHC class I pathway [59].

The bridge between innate and adaptive immunity occurs when the innate immune system's pattern recognition receptors (PRRs) located on phagocytotic cells recognize and bind pathogen associated molecular patterns (PAMPs). These PRRs, such as C-type lectins and Toll-like receptors, recognize pathogens as well as stimulate intracellular signaling which leads to the activation of the antigen presenting cells such that they are efficient at antigen presentation and T cell stimulation. For example, the mannose receptor on macrophages binds certain sugar molecules on pathogens and toll-like receptor 4 (TLR4) stimulates intracellular signaling upon contact with lipopolysaccharide (LPS) by associating with CD14 on a macrophage [58, 60, 61].

During the MHC class II pathway of antigen presentation, a foreign antigen from the extracellular region is engulfed by an APC and degraded in the endocytotic vesicles within the cell which contain proteolytic enzymes. After the antigen is degraded into peptide, MHC class II molecules, which are originally manufactured in the cell's endoplasmic reticulum, fuse with the endocytotic vesicles to associate with the processed antigens inside the vesicles. After fusion, they form an antigen-MHC class II complex which is transported to the cell surface to be presented by the APC to CD4+ T helper cells (Figure 2-1). During MHC class I antigen presentation, either pathogenic proteins or self-proteins which have mutated within the cell cytosol are processed in the cell proteasome and then transported to the endoplasmic reticulum where they fuse with MHC class I molecules. After fusion, the antigen-MHC class I complex is transported to the surface of the APC for presentation to CD8+ T lymphocytes [58, 62].



**Figure 2-1 Antigen Shedding and Immune Stimulation**

Antigen-presenting cells, such as macrophages displaying the antigen-MHC complex, which have been activated during the innate immune response are the link between innate and adaptive immunity as they become efficient at stimulating the activation of T and B lymphocytes and the eventual production of antigen-specific antibodies (Figure 2-1). In particular, dendritic cells are unique from all other APCs, as they are the only cells with the ability to stimulate naïve T lymphocytes [63]. Once an APC has processed an antigen, it enters the lymphatic capillaries to travel to the lymph nodes where many naïve T lymphocytes are situated. These T cells are formed in the thymus and travel to the lymph nodes to come into contact with the APC antigen-MHC complexes via the T cell receptor (TCR). Each TCR is specific for a particular antigen-MHC complex [58]. On a T cell, the co-receptor molecule CD4 will bind to specific parts of the MHC class II molecule. During MHC class I antigen presentation, CD8 binds the MHC class I molecule. This T cell activation is also regulated by co-stimulatory molecules such as CD80 and CD68 on APCs which bind to CD28 on T cells [58]. Once T cell activation has occurred, naïve T cells synthesize the autocrine growth factor interleukin-2 (IL-2) which stimulates the growth and differentiation of both CD4+ and CD8+ T cells. Clonal expansion of the T cells occurs when the expression of the alpha chain of the IL-2 receptor is upregulated. Eventually large numbers of antigen-specific T cells are manufactured. These T cells, either CD4+ or CD8+, will differentiate and mature. The T helper cells will secrete IL-4 to stimulate B cell growth and IL-5 to stimulate B cell differentiation. The cytokine INF- $\gamma$  is responsible for activating macrophages and therefore inducing delayed hypersensitivity towards an antigen (Figure 2-1). The CD4+ T cells may be further divided into two classes of T helper cells: Th1,

which secrete cytokines that support cell-mediated immunity, and Th2, which secrete cytokines during humoral immunity [58, 59].

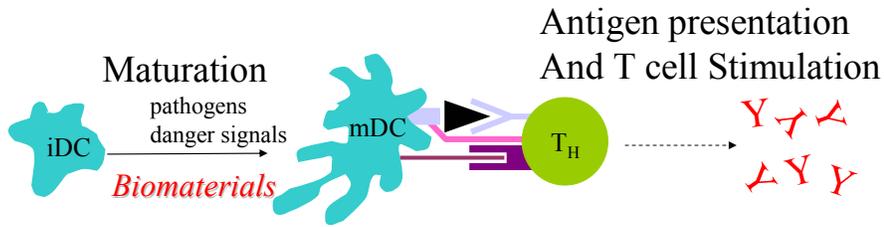
Naïve T cells are stimulated via IL-12 from macrophages to become Th1 cells. Th1 helper T cells activate macrophages to increase expression of MHC class I and MHC class II molecules by secreting interferon- $\gamma$  (IFN- $\gamma$ ). Also as a result of secretion of IFN- $\gamma$ , B cells undergo isotype switching to produce IgG2a. IL-2 and IFN- $\gamma$  encourage CD8+ T cells to grow and become cytotoxic T cells. IFN- $\gamma$  will also stimulate B cells to initiate complement and the opsonization of a foreign antigen to be phagocytosed. Th1 cells also activate macrophages to internalize antigen, undergo cell-mediated toxicity, and delayed-type hypersensitivity [58].

Activation and differentiation of B cells and eosinophils is stimulated by Th2 T cells by the secretion of IL-5, as well as B cell isotype switching. These Th2 cells secrete IL-4 which stimulates B cells to produce IgG1 and promote the growth and survival of T cells. Once the B cells have matured, IL-10 increases expansion of MHC class II molecules on the B cells as well as inhibits cytokine release from macrophages. The Th2 cytokines IL-4 and IL-13 are responsible for the alternative activation of macrophages [64].

The CD8+ family of T cells differentiate into cytotoxic T cells in response to IL-2 for the destruction of antigen-MHC class I complexes. These T cells secrete IFN- $\gamma$ , TNF- $\beta$ , and TNF- $\alpha$  to activate macrophages. They are also responsible for inhibiting pathogens by increasing expression of MHC class I molecules on macrophages by the secretion of IFN- $\gamma$ . The CD8+ T cells may trigger cell apoptosis by expressing Fas ligand or by releasing lytic granules, such as perforin to form pores in the target cell's membrane [58].

It is now thought that dendritic cells and complement are the bridge between innate and adaptive immunity [65,66]. Dendritic cells are special macrophages, unique in their ability to stimulate naïve T cells. Originally hematopoietic stem cells in the bone marrow, immature dendritic cells migrate through blood to non-lymphoid tissues when guided by chemokines. While immature, they reside in peripheral tissues and capture and process any antigen they receive. When stimulated by inflammatory signals produced by pathogens or danger signals, they mature and migrate to the lymph nodes where they present antigen for T cell priming (Figure 2-1, Figure 2-2).

Direct contact with a biomaterial is also a maturation stimulus for dendritic cells. Dendritic cells treated with PLGA films or PLGA MP show moderate levels of dendritic cell maturation as compared to the potent dendritic cell maturation activator, LPS. Films and microparticles made of PLGA were also found to moderately increase expression of co-stimulatory and MHC class II molecules [67]. Much research focuses on DC maturation in order to find a method to suppress their maturation with a view towards not enhancing any potential immune responses such as allogeneic cell rejection [64].

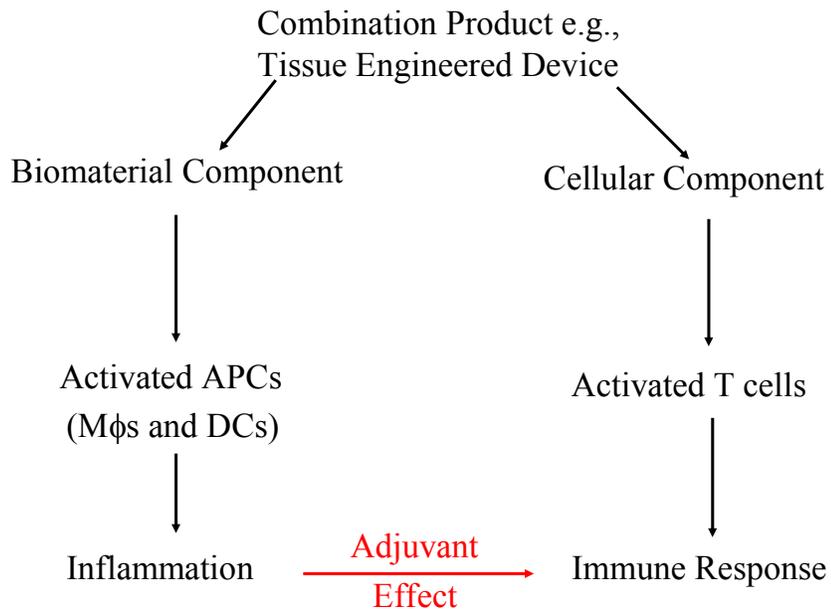


- Danger Signals Include:
- Heat shock proteins
  - Necrotic Cells
  - Small hyaluronan fragments
  - Heparin sulfate proteoglycans
  - Fibrinogen
  - Fibronectin
- Bind to Toll-like receptors on DCs → DC maturation  
 resulting in NF-κB activation regulating many proinflammatory and immune regulatory genes

**Figure 2-2 Danger Signals Induce Dendritic Cell Maturation**

Antibody-mediated adaptive immunity is responsible for rejection of implants containing foreign antigen such as xenogenic or allogeneic tissues. Current interest lies in methods to induce tolerance such as by deletion of T cells, as in mixed bone chimeras, or by costimulatory blockade or APC depletion so that T cells are not able to respond to an antigen. The adaptive immune response towards xenogenic tissue is mediated largely by CD4<sup>+</sup> T cells via the indirect pathway of antigen presentation (Figure 2-1). Dendritic cells may also be manipulated to induce tolerance. For example, blocking the second signal pathway of antigen presentation by anti-CD28, anti-B7, anti-CD40, anti-CD40L, and CTLA4-Ig may reduce the activity of antigen presenting cells [65].

A biomaterial, as an adjuvant, may boost the immune response, stimulating the humoral and cell-mediated immune responses (Figure 2-3). A strong adjuvant, Complete Freund's adjuvant (CFA) induces a high immune response to co-delivered foreign antigen. There is a great advantage in using a biomaterial with an antigen in a vaccine to boost the immunogenicity of protein or DNA vaccines which induce low T and B cell mediated adaptive immune response. Adjuvants also may contain heat inactivated microbacterial particles which activate and mature dendritic cells to produce cytokines and a phenotype with stable expression of MHC and costimulatory molecules to support effective antigen presentation and T cell stimulation [37].



**Figure 2-3 Adjuvanticity of Biomaterials**

## The 'Danger' Model of Adaptive Immunity

Dendritic cells are not only stimulated to act as antigen-presenting cells after exposure to exogenous pathogens as described above, but may in fact become mature upon ligating endogenous proteins called 'danger signals' which are released from stressed, damaged, or dying cells (Figure 2-2). Such endogenous proteins are cell and tissue derived endogenous ligands which include heat-shock proteins, necrotic cells and extracellular-matrix breakdown products [68]. Specifically, hyaluronan fragments and heparin sulfate proteoglycan [69] will bind to TLRs on DCs to result in NF- $\kappa$ B activation, which regulates many proinflammatory and immune regulatory genes resulting in dendritic cell maturation [70,71,72]. Also, proteins which adsorb to biomaterials such as fibrinogen [73] and fibronectin [74] bind to TLRs on DCs, mediating an adaptive immune response. Stressed and necrotic epithelial cells will activate human eosinophils [75]. The 'danger' model of immunity, which was proposed in 1994, suggests that only an association between an antigen and a 'danger signal' will trigger adaptive immunity after the recognition of these endogenous proteins by TLRs [68, 69].

The human 60-kDa heat-shock protein (HSP60) is a danger signal to the innate immune system, stimulating APCs. Macrophages will respond to HSP60 via the release of TNF- $\alpha$  depending on the dosage of HSP60 present [76]. Heat-shock protein 60 is also responsible for inducing gene expression of Th1 cytokines IL-12 and IL-15, suggesting it plays a role in expression of Th1 tissue inflammation [76]. Different HSPs may have different capacities to activate APCs. For example, HSP60 induces DC maturation and

activation to produce proinflammatory cytokines while HSP71 comparatively is a weak inducer of DC maturation [77].

Uric acid is a well known endogenous danger signal which cells may release during injury. It can stimulate dendritic cells to mature and initiates the activation of cytotoxic T helper cells when co-injected with antigen. If uric acid is removed, the immune response towards antigens associated with injured cells is inhibited. This suggests a link between cellular injury and the immune response towards injured and dying cells. Uric acid is a product of the degradation of RNA and DNA into purines in injured cells [78]. Understanding the mechanisms of activating dendritic cells by such endogenous 'danger signals' may lead to a better understanding of transplant rejection and immune responses to tissue engineered constructs which are often implanted into a tissue site, resulting in cell stress, inflammation and tissue damage [79,80,81].

### Immune Responses in Tissue Engineering

During biomaterial implantation, a non-specific inflammatory response is initiated including aspects of protein adsorption, complement activation, coagulation, and neutrophil and macrophage adhesion and activation [82]. In addition to this inflammatory response, transplanted cells (particularly if they are allogenic or xenogenic) seeded into a biomaterial will be recognized as foreign by the host, inducing an antigen-specific immune response [82]. Encapsulated cells may shed antigens, which are able to pass through the biomaterial capsule and are then internalized, processed and presented in association with the host MHC class II molecules by APCs to host CD4+ helper T cells

in the indirect pathway of antigen recognition [82] (see Figure 2-1). Although polymer membranes are to provide immunoisolation of transplanted cells, these shed antigens are able to stimulate the immune system which can lead to the destruction of the encapsulated cells [82]. The biomaterial component of the tissue engineering device also acts as an adjuvant, enhancing the humoral immune response towards a specific antigen. For example, the model shed antigen, ovalbumin (OVA), adsorbed to different biomaterial carriers in the form of microparticles or scaffolds formed a moderate immune response as compared to the polymer or protein alone [83].

Xenografts pose the greatest risk of an immune response. However, reconstituted extracellular matrix proteins have been used for vascular tissue, abdominal wall tissue, and ligament tissue after modifications. Porcine small intestinal submucosa (SIS) is an acellular extracellular matrix which is used for tissue repair in many species. The SIS does cause an acute inflammatory response with IL-4 and IL-10 expressed at the graft site and eventual tissue remodeling. Anti-SIS antibodies were also produced, which were restricted to the IgG1 isotype [84]. This immune response suggests that SIS is immunogenic, but Th2 restricted which suggests that the biomaterial will be accepted.

All biomaterials with time become surrounded by fibrous capsules, impeding interaction of the biomaterial with the surrounding host tissue [85]. Long-term tissue engineered constructs in the future will attempt to achieve reduced fibrous capsule formation, making tissue engineering devices more biocompatible and able to incorporate into host tissue efficiently.

## CHAPTER III

### METHODOLOGY

#### Animals

Male C57BL/6 mice (six to eight weeks old) (Charles Rivers Laboratory, Wilmington, MA) were allowed to acclimate to their new environment for one week prior to receiving the antigen-polymer vehicle combinations. Mice were housed (six per cage with two cages per treatment type) in the Whitehead Center animal facility at Emory University. Animal care and treatment were in compliance with the Institution Animal Care and Use Committee (IACUC) of Emory University according to protocol #040-2000.

## Polymeric Carrier Vehicles Preparation and Characterization

### Preparation of Polymeric Biomaterial Microparticles

Poly(lactic-*co*-glycolic acid) microparticles (PLGA MP), coated with polyvinyl alcohol (PVA), were prepared using a double-emulsion solvent-extraction technique as previously described [86]. Briefly, 200 mg of 50:50 PLGA (MW = 125,000; Birmingham Polymers, Birmingham, AL) was dissolved in 1.8 ml dichloromethane (DCM) (Sigma, St. Louis, MO) on a shaker overnight. An OVA (chicken egg, Grade VI, Sigma) solution with concentration of 100 µg/µl was prepared in 1 % (w/v) PVA (87-89 % hydrolyzed, Avg MW=13,000-23,000; Sigma) in distilled/deionized water. Under high-speed vortexing, 100 µl of the protein solution containing 10 or 0 mg (negative control) OVA was mixed with the PLGA/DCM solution for 1 minute to form the first emulsion. This emulsion was then rapidly added to 50 ml of 1 % (w/v) PVA in distilled/deionized water with stirring at 1400 rpm. After 5 minutes, 100 ml of 2 % (v/v) isopropanol (Sigma) in distilled/deionized water was added. The mixture was then allowed to stir for an additional two hours during which time the DCM solvent evaporated. The microparticles were collected by centrifugation in preweighed centrifuge tubes and then washed 5 times with 20 ml of sterile filtered distilled/deionized water, collecting between washes by centrifugation and resuspension in 2% (v/v) isopropanol solution. The MPs were then washed with 2 % (v/v) isopropanol twice and sterile filtered distilled/deionized water three times. After suspending the MP in sterile filtered distilled/deionized water they were placed under ultraviolet light in a laminar

flow hood for an hour for sterilization before injection. A Limulus Amebocyte Lysate endotoxin assay was performed on the PLGA MPs, prepared as for injection, according to the manufacturer's instructions (BioWhittaker, Walkersville, MD). For 3.88 mg of PLGA MPs without incorporated OVA (as used for injection) the effective endotoxin content was  $0.204 \pm 0.2$  EU/mL. For 8.05 mg of PLGA MPs with incorporated OVA (as used for injection) the effective endotoxin content was  $0.267 \pm 0.1$  EU/mL. For MPs which would be injected into mice, air-drying was used instead of freeze-drying to decrease the amount of MP clumping, whereas, for protein quantification, MPs were freeze-dried. Once dry, the total batch of PLGA MPs weighed 80 mg. The mean size and standard deviation of the PLGA MP were obtained using a Coulter Multisizer II (Coulter Corporation, Miami, FL). For the PLGA MP, sizes ranged from 3 to 20  $\mu\text{m}$  with a mean diameter of 3.5  $\mu\text{m}$  (Table 3-1).

#### Preparation of PLGA Scaffolds

The PLGA scaffolds (PLGA SC) were prepared by a salt-polymer casting particulate-leaching technique with NaCl as the leachable component [87,88]. Sodium chloride (Sigma) was sieved into particle size range using a sieve shaker (W.S. Tyler, Mentor, OH) and 355-425  $\mu\text{m}$  NaCl particles were used as the leachable component. Briefly, 0.5 g of 50:50 PLGA (molecular weight of 125,000 Daltons) (Birmingham Polymers) was dissolved in 5 ml DCM and the polymer was allowed to completely dissolve. Solid OVA [600 or 0 mg (negative control)] was added and this PLGA-

**Table 3-1 Treatment Groups and Physical and Chemical Properties of PLGA MP and PLGA SC**

Treatment Group	Mean size (MP) or dimension (SC)	Percent OVA Incorporation	OVA/Polymer Ratio	Polymer Mass Delivered (mg)	OVA Mass Delivered (mg)
PLGA MP With OVA	3.5 $\mu$ m diameter	20%	1.075 mg OVA/ mg PLGA	3.88	4.17
PLGA SC With OVA	0.7 cm diameter, 0.2 cm thick	36.50%	1.075 mg OVA/ mg PLGA	3.88	4.17
PBS With OVA	NA	NA	NA	0	4.17
CFA With OVA	NA	NA	NA	0	4.17
PLGA MP Without OVA	3.5 $\mu$ m diameter	0	0	3.88	0
PLGA SC Without OVA	0.7 cm diameter, 0.2 cm thick	0	0	3.88	0
PBS Without OVA	NA	NA	NA	0	0
CFA Without OVA	NA	NA	NA	0	0

NA=not applicable

DCM/OVA mixture was homogenized at 1,000 rpm for 1 minute using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). The PLGA-DCM/OVA mixture was poured over the 4.5 g NaCl (355-425 nm) and pre-spread evenly in a 50 mm-diameter Teflon Petri dish (VWR, Bristol, CT) in a 9:1 salt ratio. The mixture was stirred completely, covered, and placed in a fume hood overnight. The cover was removed the next day and the dish left uncovered in the fume hood overnight to allow the DCM to evaporate. The disks were then removed and placed in distilled/deionized water on a shaker to dissolve out the leachable component (NaCl), while refreshing the water every couple of hours. The disks were kept in water overnight and then allowed to air dry on sterile pads in a laminar flow hood. Once dry, 0.7 cm diameter and 0.2 cm thick SC were punched out of the disk using an ethanol-sterilized punch. These SC (for either implantation or protein quantification) were then freeze-dried overnight using a freeze dryer (Labconco Incorporation, Kansas City, MO). The SCs were washed with 70 % ethanol three times and then sterile-filtered distilled/deionized water three times for 10 minutes each on a shaker 24 hours before implantation. The SC were placed on sterile pads under UV light in a laminar flow hood for 30 minutes per side before implantation. For PLGA SC (0.7 cm diameter, 0.2 cm thick) as used for implantation, the effective endotoxin contents were determined, using Limulus Amebocyte Lysate endotoxin assay (BioWhittaker), to be  $0.015 \pm 0.07$  EU/mL and  $0.055 \pm 0.2$  EU/mL for PLGA SC without and with incorporated OVA, respectively (Table 3-1).

### Polymer Quantification

Dry weights were determined for both PLGA SC and PLGA MP in pre-weighed cryovials (VWR). The cryovials containing the polymers were flash frozen with liquid nitrogen and then lyophilized overnight using a freeze dryer (Freeze Dry System/Freezone 4.5, Labconco Incorporation, Kansas City, MO). Polymer weights were determined in triplicate and the mean weight and standard deviation were determined.

### Quantification of OVA Associated with PLGA SC and PLGA MP

In the same manner as described above, PLGA disks were fabricated and freeze-dried and then digested overnight, rotating at 37 °C in 44 ml 0.1 M NaOH with 5 % (w/v) sodium dodecyl sulfate (SDS) (Sigma). The PLGA SC punched out of a whole disk were treated the same manner, but with a digest volume of 4 ml 0.1 M NaOH with 5 % (w/v) SDS. Two PLGA MP batches prepared simultaneously as described above were combined and then split in two halves. Then one half was freeze dried (24 hours) and digested for determination of OVA incorporation. The other half was used ‘fresh’ for injection into mice, the next day. In this way, the actual amount of OVA associated with the polymer was determined for the sample which was then injected into mice. This was necessary because of variability in the amount of OVA incorporation (even with the same starting amount of OVA) and given the constraint of keeping the OVA/polymer ratio the same for both the microparticle and scaffold forms. Lower variability in the incorporation

of OVA into scaffolds (as compared to microparticles) permitted such an approach. Freeze-dried MPs (80 mg) were digested with 15 ml 0.1 M NaOH and 5% SDS overnight at 37 °C. The total protein contents of the digested PLGA SC and PLGA MP were then determined using the Bicinchoninic Acid (BCA) protein assay (Sigma) and used to determine the amount of OVA incorporated into each form considering the initial amount of OVA used during preparation. The BCA assay was performed according to the manufacturer's instructions. The samples were transferred to a cuvette and the absorbance values were read at 562 nm using an Ultraspec 4300 UV/Visible Spectrophotometer (Amersham Pharmacia, Cambridge, England). Samples were read in triplicate, the concentrations determined using the standard curve and the means calculated. Using these concentrations, the actual OVA amount incorporated into the PLGA MPs or SC was determined. A constant ratio of amount of OVA to amount of polymer (OVA/polymer ratio) was determined to be 1.075 mg OVA/mg PLGA for both PLGA MP and PLGA SC (Table 3-1). The percent OVA incorporation was 36.50% for PLGA SC and 20% for PLGA MP (Table 3-1).

### Humoral Immune Response Assay

#### Co-Delivery of OVA with PLGA MP and PLGA SC in Mice

The treatment groups for mice are summarized in Table 3-1 with the amounts of OVA and polymer delivered indicated for each. Prior to murine implantation, SC with OVA and SC without OVA were placed under UV light in a laminar flow hood for 30 minutes per side and then added to sterile PBS. Scaffolds with incorporated OVA

weighed approximately 8.75 mg, whereas PLGA SC without incorporated OVA weighed 4 mg. Prior to murine injection, MP were centrifuged (260 g, 5 minutes) and 8.05 mg of air-dried PLGA MP with incorporated OVA or 3.88 mg PLGA MPs without OVA were resuspended in 100  $\mu$ l PBS. These weights corresponded to approximately  $2.69 \times 10^5$  microparticles. For either the MP or SC form, the OVA/polymer ratio was the same at a value of 1.075 mg OVA/mg polymer (determined and set as described above) and the amount of polymer delivered for either form was constant at 3.88 mg and the amount of OVA was constant at 4.17 mg (or 0 mg as a control).

C57BL/6 mice were given 100  $\mu$ l dorsal subcutaneous injections of MP with or without OVA. Other C57BL/6 mice were given a dorsal subcutaneous implantation of SC with or without OVA under sterile surgical conditions. Immunization with PBS with (4.17 mg OVA in 100  $\mu$ l PBS) and without OVA served as negative controls and immunization with (8.34 mg OVA in 50  $\mu$ l PBS) or without OVA in PBS in a 1:1 dilution with Complete Freund's Adjuvant (CFA) (Sigma) served as the positive controls. Three weeks after primary immunization, all mice except for those in the CFA group, received a booster of an OVA solution in PBS at the same concentration of 4.17 mg OVA in 100  $\mu$ l PBS into the injection/implantation site (no additional polymer was delivered). For the positive control, CFA group, Incomplete Freund's Adjuvant (IFA) (Sigma) was injected instead of CFA to prevent a potential lethal dosage of CFA. Six mice were placed in each treatment or control group.

Blood samples were collected from the retro-orbital plexus of the mice at 2, 3 (prior to boosting), 4, 8, 12, and 18 weeks after the primary immunization. The bleeds clotted overnight at 4  $^{\circ}$ C and the serum was removed after centrifugation at 2300 g for 10

minutes. Serum was stored at  $-20\text{ }^{\circ}\text{C}$  until analysis for anti-OVA IgG antibodies and isotypes (IgG1, IgG2a) by ELISA.

#### Quantification of Anti-OVA IgG and Isotypes by ELISA

The production of anti-OVA total IgG and the isotypes, IgG1 and IgG2a, in mouse serum samples was measured by ELISA as previously described [83]. Standard wells of Nunc Immuno<sup>TM</sup> MaxiSorp ELISA plates (Life Technologies, Paisley, UK) were coated with a  $1\text{ }\mu\text{g/ml}$  solution of goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL) in  $0.1\text{ M}$  sodium bicarbonate ( $\text{NaHCO}_3$ ) ( $\text{pH } 9.5$ ). Sample wells of the plate were coated with a  $40\text{ }\mu\text{g/ml}$  solution of OVA in  $0.1\text{ M}$   $\text{NaHCO}_3$ . After incubation overnight at  $4\text{ }^{\circ}\text{C}$ , the plates were washed four times with  $0.5\text{ }\%$  (v/v) Tween 20 in PBS (PBT) and blocked with  $5\text{ }\%$  (w/v) condensed milk in PBT (PBT-CM) for 2 hours at  $37\text{ }^{\circ}\text{C}$ . After washing the plates 4 times with PBT, triplicates of each serum sample were diluted 1/100 or 1/1000 in PBT-CM and added to the sample wells. The standard wells received mouse IgG, IgG1, or IgG2a standard diluted 1:1 in PBT-CM starting at  $1\text{ }\mu\text{g/ml}$  to a final concentration of  $0.488\text{ ng/ml}$ , each concentration in duplicate. The plates were allowed to incubate at  $37\text{ }^{\circ}\text{C}$  for 2 hours and then washed four times with PBT and incubated at  $37\text{ }^{\circ}\text{C}$  for 2 more hours with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates) in PBT-CM. After four washes with PBT, the reaction was developed using a *p*-nitrophenyl phosphate solution prepared from tablets dissolved in 1 X diethanolamine buffer prepared from an alkaline phosphate substrate kit (Bio-Rad, Hercules, CA). The reaction was stopped after 2 minutes by adding  $0.4\text{ M}$  sodium

hydroxide (NaOH) and the absorbance values were read at 405 nm using a Powerwave<sub>x</sub>340 ELISA plate reader. The mean absorbance was calculated for each set of triplicate serum samples and the mean concentration and standard deviation for each sample was determined using a standard curve.

#### *In Vitro* OVA Protein Release Kinetics From PLGA SC and PLGA MP

The *in vitro* OVA protein release kinetics from PLGA SC and PLGA MP were determined as previously described [86,89]. To keep the polymer amount constant between the two different carriers, 40 mg PLGA MP (with and without OVA) were suspended in 15 ml sterile PBS in a glass vial (VWR) and 40 mg PLGA SC (5 small SC, with and without OVA) were suspended in 15 ml sterile PBS; each sample in duplicate vials. These glass vials were then incubated at 37 °C on a rotating shaker. At various time intervals, day 1, 4, 7, 14, 21, and 28, the vials were centrifuged and the supernatant was removed and stored at -20 °C until total protein analysis using a BCA assay. Fresh 15 ml sterile PBS was added to each vial and the release experiment continued. In preparation for the BCA protein assay, the protein samples were concentrated using Centricon Plus-20 centrifugal filter devices (Millipore Incorporation, Billerica, MA), per manufacturer's instructions. Briefly, the 15 ml protein solution was added to the sample filter cup and centrifuged for 10 minutes at 2,000 g. Flow collected in the filtrate collection tube was discarded and the filter unit was inverted to sit on a retentate cup and centrifuged at 1,000 g for 2 minutes to collect approximately 3 mL of concentrated OVA solution in PBS. All concentrated samples were analyzed on the same day using the BCA protein assay as described above and using the same standards.

## Data Analysis

Statistical analysis was performed using an ANOVA general linear model with Minitab software (Version 13, Minitab Inc., State College, PA).  $p$ -values of  $\leq 0.05$  were denoted as significant.

## CHAPTER IV

### RESULTS

#### Polymeric Carrier Vehicle Preparation and Characterization

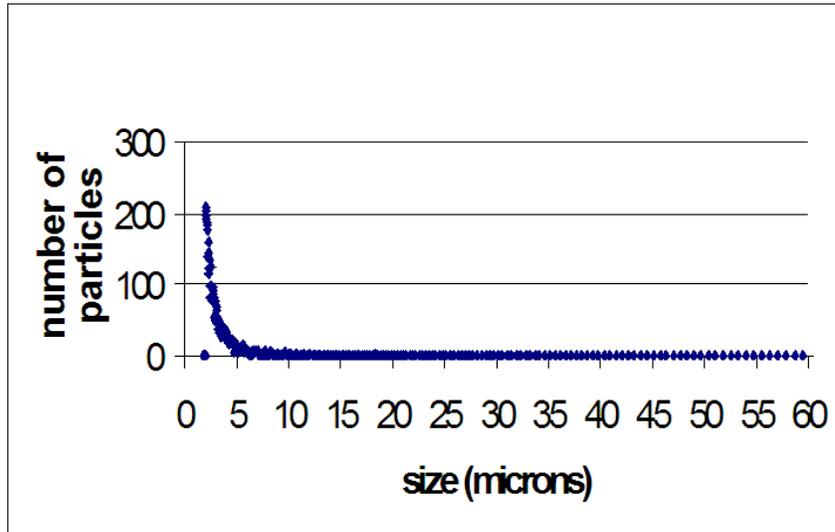
##### Preparation of Polymeric Biomaterial Microparticles

The 50/50 PLGA MP were fabricated using a double-emulsion solvent-extraction technique. The resulting microparticles had a size range from 3-20  $\mu\text{m}$  and a mean diameter of 3.5  $\mu\text{m}$  (Table 3-1). Figure 4-1 shows a representative size distribution of the 50/50 PLGA MP.

##### Polymer and Protein Quantification

For both the PLGA MP and the PLGA SC the same OVA/polymer ratio of 1.075 mg OVA/mg polymer was delivered *in vivo*. To keep the same amount of polymer and protein between the two types of polymeric carriers, MP were centrifuged and 8.05 mg of air-dried PLGA MP with incorporated OVA or 3.88 mg PLGA MPs without OVA were resuspended in 100  $\mu\text{l}$  PBS. These weights corresponded to approximately  $2.69 \times 10^5$  microparticles. The PLGA MP had a percent incorporation of OVA of approximately 20% while the PLGA SC had a percent incorporation of OVA of approximately 36.50%.

Each PLGA SC with incorporated OVA weighed approximately 8.75 mg (PLGA SC without OVA weighed 4 mg) and was 0.7 cm in diameter and 0.2 cm thick (Table 3-1).

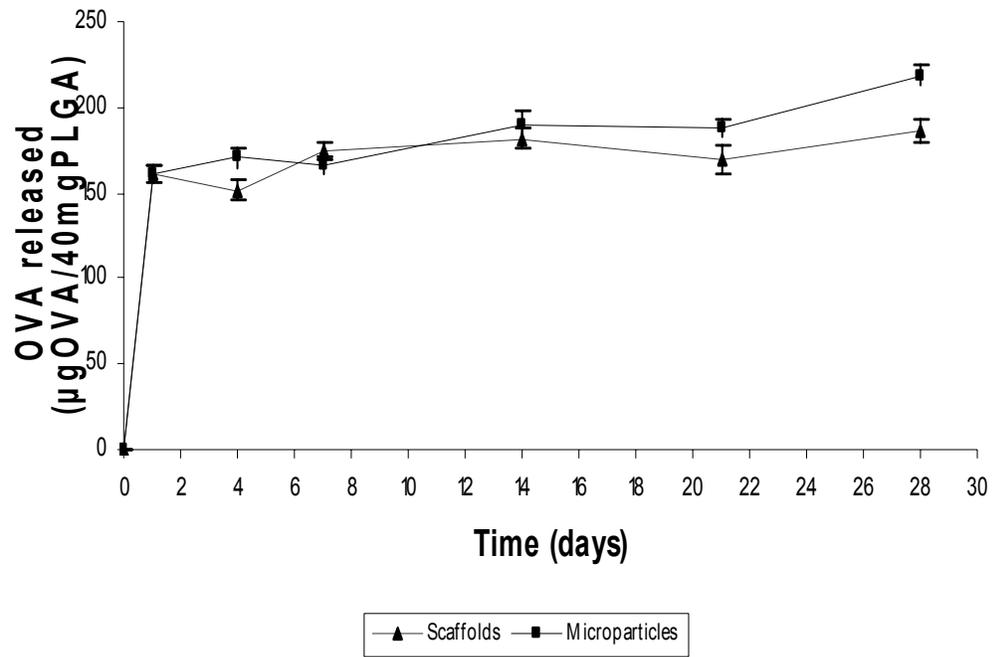


**Figure 4-1 Representative size distribution of 50/50 PLGA MP**

### *In Vitro* Protein Release Kinetics

*In vitro* release kinetics of OVA into PBS from OVA-loaded PLGA MP and PLGA SC were evaluated over 28 days. For both carriers, 40 mg of polymer was incubated in PBS to keep the polymer amount consistent and each carrier incubated contained the same OVA/polymer ratio (1.075 mg OVA/mg polymer). The PLGA MPs and PLGA SCs remained intact over the 28 day protein release experiment although there was some loss of mechanical properties for the PLGA SC over this time period. The overall release rates of OVA from the polymer carriers were similar for both PLGA SC and PLGA MP (Figure 4-2). The release rate was determined by the ratio of amount of OVA released at each time point to the amount of polymer incubated over the 28 days of the experiment. Each data point indicates the amount of OVA released per 40 mg polymer during the period concerned between consecutive time points (Figure 4-2, Mean  $\pm$  S.D.; n=2 independent samples; m= 3 determinations).

The release rates of OVA from PLGA SC and PLGA MP at day one were the same with a release of 6.44 mg OVA/24 hours. Thereafter, the release rates for the rest of the first week were similar at 2 mg/day for both carriers. For the remainder of the experiment, the release rates were also similar at 1 mg/day. The total amount of OVA released from the PLGA MP over the 28 days of the experiment was 43 mg (100%). Forty milligrams (93%) of OVA was released from the PLGA SC over the 28 days of the experiment (Table 4-1).



**Figure 4-2 Protein Release Kinetics from PLGA SC and PLGA MP over 28 days**

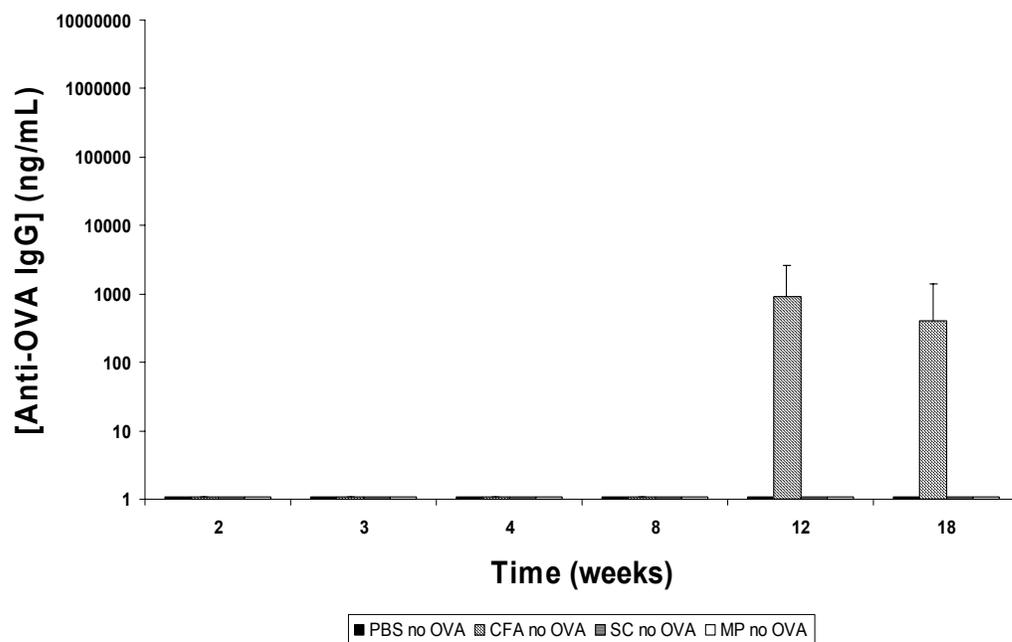
**Table 4-1 Cumulative Release of OVA from PLGA SC and PLGA MP during *in vitro* Protein Release Kinetics**

Polymeric Carriers	OVA to Polymer Ratio	Amount of OVA lost over 28 days total	% OVA lost
PLGA SC with OVA	1.075 mg OVA/mg Polymer	40 mg	93%
PLGA MP with OVA	1.075 mg OVA/mg Polymer	43 mg	100%

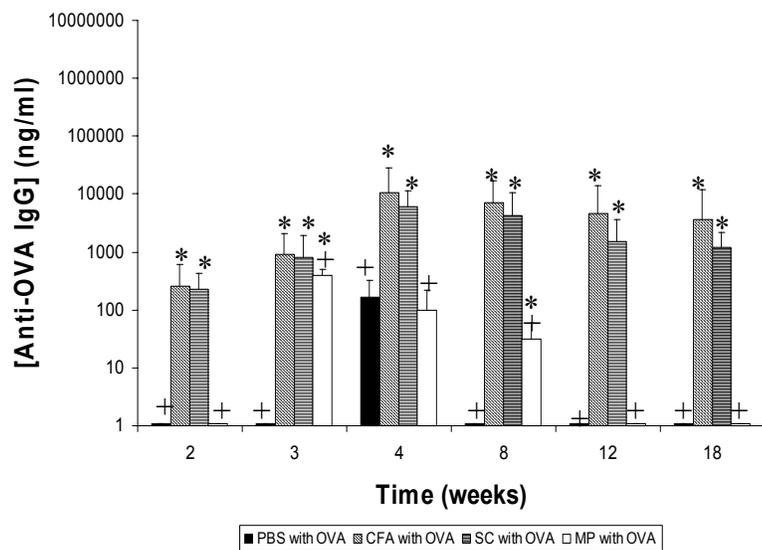
## Humoral Immune Response to Antigen Co-delivered with Polymeric Vehicles

The production of anti-OVA total IgG and the isotypes, IgG1 and IgG2a, in mouse serum samples was measured by ELISAs to assess the humoral immune response to OVA released from PLGA MP and PLGA SC as compared to appropriate controls. Polymeric scaffolds and polymeric microparticles delivered without OVA did not result in anti-OVA IgG antibody production being statistically different from the antibody levels observed for the negative control, PBS without OVA (Figure 4-3, Mean  $\pm$  S.D.; n=4-6 mice; note the ordinate is in log scale). At a few time points CFA without OVA did produce moderate levels of anti-OVA IgG, in contrast with the PLGA SC and PLGA MP (Figure 4-3). Ovalbumin delivered with PBS (negative control) into C57BL6 mice resulted in low baseline levels of anti-OVA IgG antibody production while OVA co-delivered with CFA (positive control) elicited an intense humoral immune response characterized by high levels of anti-OVA IgG (Figure 4-4, Mean  $\pm$  S.D.; n=4-6 mice; note the ordinate is in log scale. \* represents significant different from PBS, + represents significant difference from CFA). Controlled release of OVA from PLGA SC resulted in high, sustained levels of OVA-specific IgG for the 18 week duration of the experiment (Figure 4-4). For this polymeric vehicle, these levels of OVA-specific IgG were not statistically different from the positive control of OVA delivered in CFA, while being statistically different from the low baseline levels observed for the negative control of OVA delivered in PBS alone. In contrast, OVA released from PLGA MPs induced transient, moderate levels of anti-OVA IgG which peaked at 3 weeks and tapered off thereafter to be at background levels by 12 weeks. These levels of anti-OVA IgG were

statistically different from those observed for OVA delivered in PBS at 3 and 8 weeks (boost given at 3 weeks) but at all time points, the levels were statistically different from those of the positive control of OVA delivered in CFA.



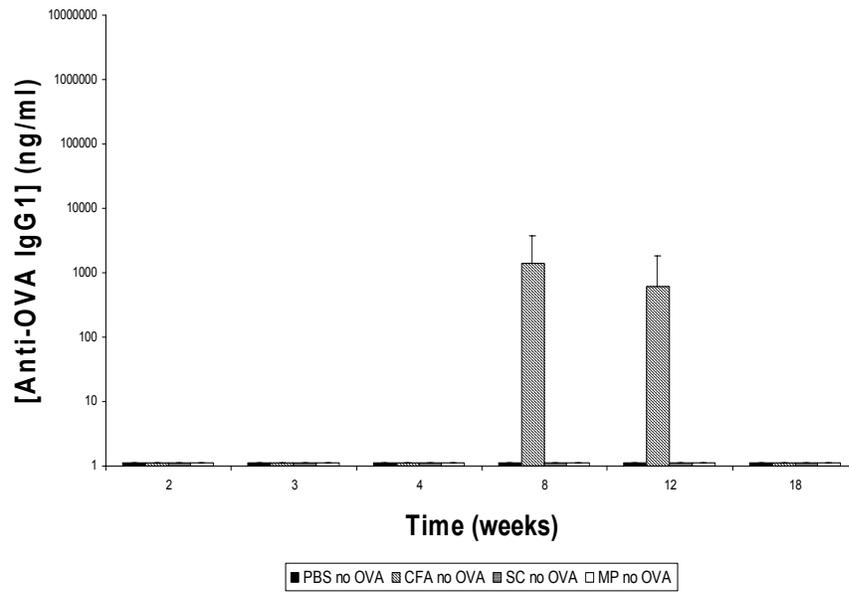
**Figure 4-3 PLGA SC and PLGA MP without OVA do not produce IgG levels significantly different than that of PBS**



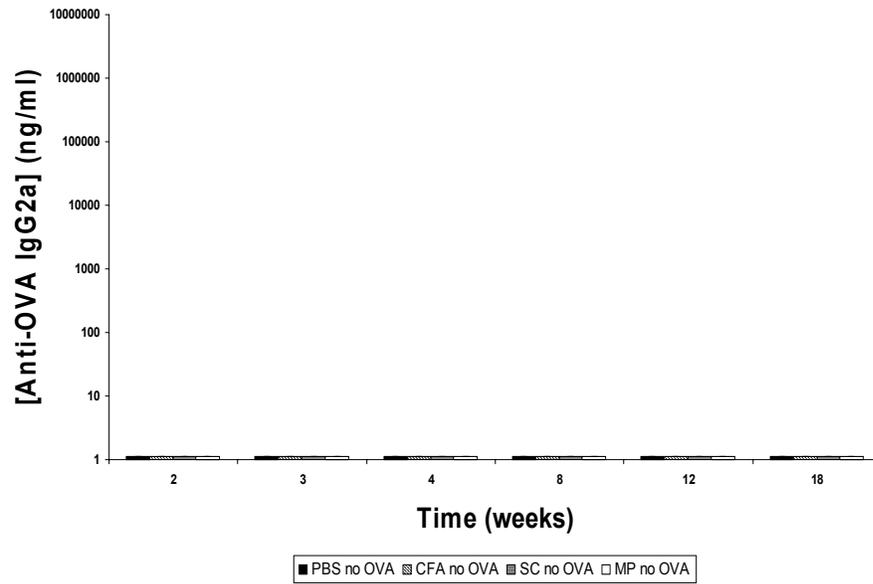
**Figure 4-4 Anti-OVA IgG (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA.**

To assess the predominance of a Th1 versus a Th2 helper T cell response in the humoral immune response to OVA delivered with the two forms of polymeric carrier vehicles, the IgG isotypes were determined. The IgG2a isotype is characteristic of a Th1 response, whereas the IgG1 isotype is characteristic of a Th2 response. Again, the polymeric carriers without OVA did not produce IgG1 (Figure 4-5, Mean  $\pm$  S.D.; n=4-6 mice; note the ordinate is in log scale) or IgG2a (Figure 4-6, Mean  $\pm$  S.D.; n=4-6 mice; note the ordinate is in log scale) antibody levels significantly different from that of PBS. The generation of anti-OVA IgG1 antibodies in response to OVA co-delivered with each polymeric vehicle and controls (Figure 4-7, Mean  $\pm$  S.D.; n=4-6 mice; note the ordinate is in log scale. \* represents significant different from PBS, + represents significant difference from CFA) showed similar trends to those observed for total anti-OVA IgG (Figure 4-4). The levels of OVA-specific IgG1 for OVA released from PLGA SC were not statistically different from the levels observed for the positive control of OVA delivered with CFA (Figure 4-7). Furthermore, the levels of OVA-specific IgG1 for OVA released from PLGA MP were significantly different from PBS only just after the 3 week boost at 4 weeks (Figure 4-7). There were some differences in the times at which the levels of IgG or IgG1 antibody levels peaked for the responses to OVA delivered with PLGA MPs. Specifically, the anti-OVA IgG levels peaked earlier at week 3 while the anti-OVA IgG1 levels lagged with the peak at a later time point, week 4 (Figure 4-4 and Figure 4-7). Significant levels of anti-OVA IgG2a were observed for OVA released from PLGA SC at the time points between 4 and 12 weeks or delivered with the positive control, CFA, at the time points between 3 and 12 weeks, as compared to the low baseline levels observed for OVA delivered with PBS alone (Figure 4-8, Mean  $\pm$  S.D.;

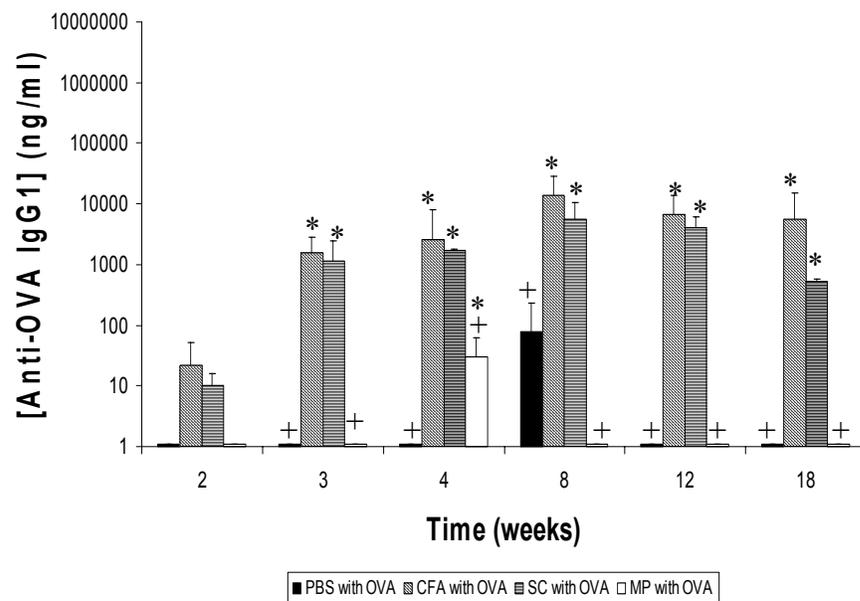
n=4-6 mice; note the ordinate is in log scale. \* represents significant different from PBS, + represents significant difference from CFA). Controlled release of OVA from PLGA MPs did not induce anti-OVA IgG2a at any time point (Figure 4-8). Anti-OVA IgG2a levels were not detected at the 2 and 18 week time points for any of the carriers tested. The IgG1 levels (Figure 4-7) were slightly higher for CFA and PLGA SC than the IgG2a levels (Figure 4-8). These results suggest the predominance of IgG1 isotype, although for CFA and PLGA SC carriers, anti-OVA IgG2a isotype levels were also significant.



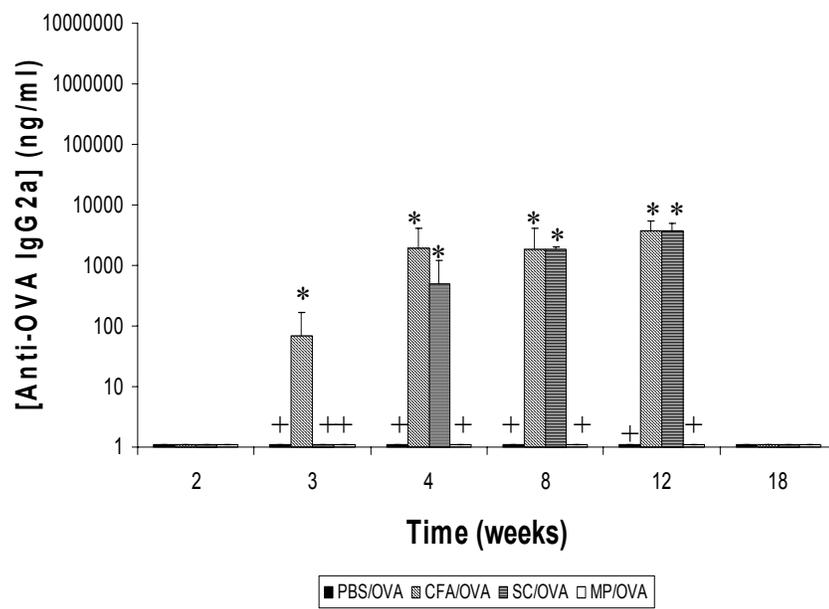
**Figure 4-5 PLGA SC and PLGA MP without OVA do not produce IgG1 levels significantly different than that of PBS**



**Figure 4-6 PLGA SC and PLGA MP without OVA do not produce IgG2a levels significantly different than that of PBS**



**Figure 4-7 Anti-OVA IgG1 (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA**



**Figure 4-8 Anti-OVA IgG2a (ng/ml) production for PLGA SC and PLGA MP with incorporated OVA**

## CHAPTER V

### DISCUSSION

The biomaterial component of a tissue engineered device acts as an adjuvant in mediating an immune response to co-delivered antigen. Polymeric vehicles without incorporated antigen in the form of scaffold or microparticles did not induce an immune response, indicating that the biomaterial alone does not induce an antigen-specific antibody response. In addition, the biomaterial chemistry does not seem to have a significant influence on the immune response, at least with the limited materials tested in a previous experiment [83]. In this research, the extent of the enhancement of the antigen-specific antibody response towards incorporated OVA depended on the form of the polymeric vehicle carrier, either as a scaffold or a microparticle.

The humoral immune response towards OVA controlled released from PLGA SC was sustained for the duration of the experiment at a high level, and not significantly different than that of OVA delivered with the positive control of CFA. When the same amount of polymer and antigen was delivered and released at the same rate from PLGA MPs, the level of the humoral immune response was transient and at a moderate level as compared to SC and CFA. These results indicate that PLGA in the form of a SC acted as a stronger adjuvant in the enhancement of the immune response toward incorporated antigen than PLGA in the form of MPs. It should be noted that PLGA MPs did produce some moderate adjuvanticity.

The immune response induced by the PLGA MP and PLGA SC with incorporated OVA was dominated by a Th2-type immune response as indicated by the production of

IgG1 antigen-specific antibodies. This suggests that adjuvanticity of the biomaterial results in the activation of CD4<sup>+</sup> T cells and the proliferation of isotype switching of B cells. However, for CFA and PLGA SC carriers, anti-OVA IgG2a isotype levels were also significant, potentially indicating both a Th2 and Th1 response. In previous research it was discovered that the immune response elicited by both MP and SC carriers with adsorbed OVA versus incorporated OVA were dominated by a Th2-type immune response as indicated by the production of OVA-specific IgG1 [83]. However, it has also been shown that PLGA MPs with adsorbed OVA were able to elicit a delayed type hypersensitivity (DTH) reaction in mice, which is a Th1-dependent response [67]. The mechanism by which PLGA contributes to the activation of T cells is through its adjuvant effect with the maturation of DCs upon biomaterial contact into efficient antigen presenting cells *in vivo* for the presentation of co-delivered OVA to prime T lymphocytes [67]. Other studies have confirmed an adjuvant effect of biomaterials to polarize Th response with varying results. Allergen-loaded PLGA MP can stimulate a Th2 response [90], *Brucella ovis* bacteria antigens encapsulated in poly-epsilon-caprolactone (PEC) MP further enhanced the already Th1 response to the unencapsulated antigen whereas PLGA MP encapsulation induced a Th2 response [91], and OVA encapsulated in PLGA MPs elicited a Th1 response [92]. The disparity between these results may be due to the differences in the type and form of the antigen used (peptide vs protein vs allergen), the mode of antigen co-delivery (encapsulated vs adsorbed), and the method of determining the nature of the Th response (serum IgG isotypes levels vs cytokines secreted by *in vivo* primed lymphocytes following *in vitro* second challenge with antigen vs DTH response).

To facilitate a distinction of the enhancement of the humoral immune response based on the polymeric vehicle form, OVA was incorporated into PLGA in the form of SCs or MPs for controlled release at the same rate with a constant amount of polymer and antigen delivered at a constant OVA/polymer ratio for these two carrier forms. In this way, the key difference between the two carrier vehicle forms was the means by which the polymeric vehicle was introduced into the host. The scaffolds, because of their size, required invasive surgical implantation, with associated tissue damage whereas the microparticles were minimally invasively delivered by injection.

In a previous experiment [83], polymeric carriers of both microparticle and scaffold form were soaked in OVA concentrations of 1 mg OVA/ 1 ml PBS. The OVA adsorbed to the surface of the polymeric carriers and its release was not controlled. In this current study, a greater OVA concentration was used with 1.075 mg OVA/mg polymer and this OVA was incorporated into the microparticles via a double emulsion technique with a PVA coat and into scaffolds via homogenizing. Keeping the same amount of OVA:polymer delivered constant as well as the amounts of OVA and polymer delivered allowed for determinations of the differences in the immune response between the two forms of carriers.

In the innate response, DCs may be activated in the absence of foreign pathogens through endogenous ‘danger signals’ from necrotic or stressed cells, or released from damaged or inflamed tissue. In the context of biomaterials and tissue engineering, ‘danger signals’ could result from surgical implantation of a biomaterial or construct or other stress or necrosis of cells or inflammation caused by the presence of a foreign material. The TLRs recognize endogenous ligands which are relevant in the innate

response to biomaterials and have a significant role in the maturation of DCs. Therefore, maturation of DCs in the context of implanted biomaterials would support their maturation to become efficient antigen presenting cells with an enhancement of the immune response towards associated antigen.

The implantation of the PLGA SCs undoubtedly caused tissue damage with associated necrosis and inflammation. In contrast, injection of PLGA MPs was a minimally invasive means of delivery. In the innate response, DCs can be activated in the absence of foreign pathogens through endogenous ‘danger signals’ from necrotic or stressed cells, or released from damage or inflamed tissue [68, 69, 70]. These molecular danger signals are cell and tissue derived endogenous ligands such as heat shock proteins, small hyaluronan fragments, and heparin sulfate proteoglycan [71], or even proteins which adsorb to biomaterials such as fibrinogen [73], fibronectin [74] which bind to TLRs on DCs mediating their maturation to be efficient at stimulating T cells for an adaptive immune response. The danger hypothesis states that an immune response only occurs if the antigen is presented along with ‘danger signals’ from stressed or necrotic cells or with associated tissue damage [70]. In the context of biomaterials and tissue engineering, ‘danger signals’ could result from surgical implantation of a biomaterial or construct or other stress or necrosis of cells or inflammation caused by the presence of a foreign material. The TLRs recognize endogenous ligands which are relevant in the innate response to biomaterials and have a significant role in the maturation of DCs. Therefore, maturation of DCs in the context of implanted biomaterials would support their maturation to become efficient antigen presenting cells with an enhancement of the immune response towards associated antigen, with the biomaterial acting as an adjuvant.

The results presented herein indicate the significant consideration of delivering a tissue engineered construct as non-invasively as possible to minimize any potential immune responses. Furthermore, there is the potential to ‘hide’ an immunological tissue engineered construct if it is delivered as non-invasively as possible to avoid the generation of ‘danger signals’. Experimental evaluation of the implantation environment for the molecular basis for biomaterial and tissue engineered construct associated ‘danger signals’ remains to be elucidated.

To further strengthen the biomaterial implantation associated ‘danger signals’ explanation for the results herein, other possible explanations and contributions can be ruled out. The *in vitro* protein release experiment demonstrated similar OVA release kinetics for both the PLGA SC and PLGA MP over the 28-day duration of this study. This was evident even though the method used to prepare the PLGA MPs, coating with PVA, has been shown to result in an initial burst of release and a faster release rate of protein due to its hydrophilic nature as compared to uncoated PLGA MPs with no effect on the bioactivity of the released protein [86]. A possible difficulty with the incorporation of OVA into PLGA SCs was the salt leaching step during which protein may be released. However, this was taken into account by determining protein incorporation into the PLGA SCs after the salt leaching step and keeping the OVA/polymer ratio constant for both carrier vehicles. Therefore, the stronger humoral immune response towards OVA released from PLGA SCs as compared to PLGA MPs was not due to a higher rate of OVA release from the PLGA SC carrier. While there was a change in the mechanical properties of the PLGA SC over the 28 days of the experiment, becoming slightly more brittle, consistent with the observations of others

[93,94], this did not lead to changes in protein release kinetics *in vitro*. Presumably, on a smaller scale, changes in the mechanical properties of the PLGA MPs were also occurring, but were not as readily observable. Furthermore, these changes in mechanical properties are likely to also occur *in vivo* as the polymer degrades, but the PLGA MPs and PLGA SCs being of the same polymer, do not likely explain the differences in the humoral immune response to OVA released from these two carrier forms.

The hydrophilic nature of the PVA coating on the MP will render it more biocompatible than that of PLGA presumably due to lower protein adsorption. However, PVA is also highly water soluble and has been found to dissolve after 24 hours in water and is thought to dissolve at a similar rate *in vivo* [83,95]. It is possible that this PVA coat minimized the PLGA adjuvant effect associated with PVA-coated PLGA MP than that associated with the PLGA SC. Possible dissolution of the PVA coat may have contributed to a more significant immune response at later time points for the PLGA MP. However, the MP did induce only a transient response which was moderate and significantly less than that of PLGA SC and CFA, tapering off during the final weeks of the experiment. After the expected time required for the PVA coat to dissolve, the PLGA MP still did not produce significantly high levels of anti-OVA IgG, IgG1, or IgG2a.

Another difference between the two polymer carriers is their endotoxin levels. The OVA-loaded PLGA MP had an endotoxin level of  $0.267 \pm 0.1$  EU/mL, much higher than that of the OVA-loaded PLGA SC at  $0.055 \pm 0.2$  EU/mL. The PLGA SC and PLGA MP had the same ratio of OVA:polymer. Therefore this difference may be due to the higher surface area of the spherical PLGA MP, which is available for endotoxin exposure for detection. It is possible that the PVA coat on the PLGA MP, which is exposed to the

environment, contains additional endotoxin levels, which therefore increases the endotoxin levels of the PVA-coated PLGA MP as compared to the PLGA SC.

The protein release kinetics experiment demonstrated similar release kinetics for the PLGA SC and PLGA MP *in vitro*. There is a concern of how these results will translate to the *in vivo* situation of antigen delivery from PLGA MP and PLGA SC as described herein for the resultant humoral immune response and the adjuvant effect of the biomaterials. Presumably, host proteins will adsorb to the PLGA MPs and PLGA SCs *in vivo* which may alter the protein release profile from that measured *in vitro*. If the effect of adsorbed host proteins was the same for both PLGA MPs and PLGA SCs, then the release profiles would be altered to the same extent for both carrier vehicles. Performing the *in vitro* protein release experiments in PBS containing serum may more accurately approximate the *in vivo* situation. The increased protein concentration in serum changes the dynamics of the experiment and may actually slow the release of OVA from polymeric carriers. *In vivo* experiments for protein release kinetics are limited. One of the complications is that proteins released from polymeric carriers, such as growth factors, may be cleared due to filtration, bind to receptors, or be enzymatically degraded. This may affect the biological efficacy of using such a protein-release system [96]. One possible complication with the *in vitro* release kinetics experiment presented herein is the compatibility of the digest reagents with the BCA assay. Some detergents may interfere with absorbance readings, resulting in incorrect analysis. This was avoided by using 5 % SDS as suggested by the manufacturer (Sigma) to be a compatible concentration of the detergent.

Another consideration which may have affected the enhancement of the immune response depending on the form of the polymeric carrier form is the actual surface area available for interaction with the host and for release of OVA. For PLGA SC (as a non-porous cylinder) the surface area was calculated to be 1.21cm<sup>2</sup>. The total surface area for the PLGA MPs (2.69 x 10<sup>5</sup> microparticles of 3.5 μm diameter) which were injected (based on non-porous spheres) was calculated to be 1,035.11cm<sup>2</sup>. Therefore, if any effect of surface area of the polymeric carriers was important, it did not translate into a higher release rate of incorporated antigen nor a greater adjuvant effect on the humoral immune response. In fact, the results were quite to the contrary.

## CHAPTER VI

### CONCLUSIONS AND FUTURE RECOMMENDATIONS

The higher level of humoral immune response observed for OVA delivered with PLGA SC implicates implantation associated 'danger signals' due to tissue damage in the enhancement of this immune response. Evaluation of the implantation environment for the molecular basis for biomaterial and tissue engineered construct associated 'danger signals' are necessary to confirm this hypothesis. This might be done either by western blots of exudates for known danger signals or treatment of dendritic cells *in vitro* with biomaterial implant site exudates to assess the extent of DC maturation. For example, PLGA MP with incorporated OVA may be delivered into a surgical cut, much like that of the PLGA SC with implantation, to determine if 'danger signals' released from the surgical cut are indeed enhancing the immune response towards PLGA SC.

An issue to consider with the *in vitro* protein release kinetics is how it will translate to *in vivo* experiments where there are more dynamics, such as serum proteins and a constantly changing environment. It would also be best to find what is happening between time 0 and 24 hours during the protein release kinetics and consider how this would translate to release kinetics *in vivo*.

The humoral immune response towards PLGA SC and PLGA MP was primarily Th2 helper T cell-dependent as exemplified by the predominance of IgG1 isotype, although for CFA and PLGA SC carriers, anti-OVA IgG2a isotype levels were also significant, potentially indicating both a Th2 and Th1 response respectively. The results

presented herein indicate the significant consideration of delivering a tissue engineered construct as non-invasively as possible to minimize any potential immune responses.

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