ABSTRACT

Title of Document: DEFINING CRITICAL PARAMETERS FOR PRODUCING AND MODULATING INFLAMMATION CAUSED BY CELL ENCAPSULATING ALGINATE MICROSPHERES

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Minimizing induced inflammation, particularly nitric oxide (NO) production, is critical to optimal function or failure of implanted encapsulated cells. The purpose of this study is to define critical factors that affect toxic NO production from the macrophage cell line RAW264.7 in response to alginate microcapsules. The effects of the following were determined: 1) concentration of endotoxin (LPS) contamination; 2) presence of interferon-gamma (IFN-γ); 3) bead diameter and alginate volume; and 4) anti-inflammatory drugs in the alginate. A higher concentration (5 X) of LPS was required in alginate to produce the effect seen by LPS free in medium, sensitivity was enhanced by IFN-γ, bead diameter was inversely proportional to NO_2 under low inflammatory conditions, and parthenolide in alginate significantly reduced inflammation. These results suggest that survival of implanted encapsulated cells may be improved by using highly purified alginate, avoiding ancillary inflammation, controlling surface area presentation, and incorporating anti-inflammatory drugs into the capsule matrix.

DEFINING CRITICAL PARAMETERS FOR PRODUCING AND MODULATING INFLAMMATION CAUSED BY CELL ENCAPSULATING ALGINATE MICROSPHERES

By

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Preface

The research presented in this thesis was done at the Food and Drug Administration, Center for Devices and Radiological Health, Office of Science and Engineering Laboratories (OSEL), Division of Biology, under the direction of Dr. Daniel B. Lyle. Financial support was provided by an Oak Ridge Institute for Science and Education (ORISE) Fellowship funded by OSEL.

Dedication

I dedicate this thesis to my parents, Alice and James Breger for all of their encouragement and support. I especially want to thank my mother for proof reading the entire thesis and trimming numerous run on sentences.

Acknowledgements

I would like thank to Dr. Dan Lyle and Jonathan Shallcross at the FDA for all of their help and support. I would also like to thank Dr. DeShong of the University of Maryland, Chemistry Department for generously allowing me to use his roto vap and Matthew Sandelier for his help.

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Chapter 1: Introduction

1.1 Introduction

This thesis is divided into six chapters. The first chapter provides a brief introduction to the thesis as well as a literature review. In the second chapter, background is given on each of the main components that are common themes in each of the preceding chapters. Chapters 3 through 5 each represent a study that addresses a goal of the thesis. At the beginning of each of these chapters is a more detailed introduction and background to the themes that are specific to that study. The third chapter determines the No Observable Effect Level (NOEL) of 2% w/v alginate in stimulating nitric oxide (NO) production by the cell line RAW264.7 by comparing free LPS to LPS incorporated into 2% w/v Pronova alginate. This was also done in the presence of IFN-γ to compare simple to more complex inflammatory responses. After establishing what levels of endotoxin could be present that did not stimulate a significant increase in NO_2 concentration compared to the control (NOEL), the effect of bead size on NO production of RAW264.7 was explored in Chapter 4. The last study, Chapter 5, explores the possibility of modulating NO production by RAW264.7 by incorporating inflammatory modulators directly into the alginate bead. Finally, Chapter 6 draws together all of the conclusions that were found in each chapter and extends the data to areas of further exploration.

1.2 Bioassays for Contamination

1.2.1 Alginate Purification Method

The development of the bioartifical pancreas is being hindered by the many things such as alginate contamination. Alginate is currently harvested from seaweed and as such the composition is highly variable, along with the amount of contamination present from lot to lot. The three main types of contamination are endotoxin, polyphenols, and proteins (Dusseault *et al.* 2005). Contamination is a very important consideration given the extreme difficulty and expense in removing it during processing. Trace amounts of contamination can lead to aggravated inflammatory responses by the host which can lead to graft failure. The main strategy for trying to minimize the problem is removing contamination to the lowest concentration possible, and then carefully defining what remains. Leinfelder *et al.* (2003) developed an assay for the validation of purification regimes of alginate. The assay is based on using the Jurkat cell line and measuring the induction of apoptosis. They also used a proliferation assay to study lymphocytes' response to different alginate lots. The two assays rely on determining the ratio of dead cells/debris to viable proliferating cells and the use of trypan blue along with flow cytometry respectively (Leinfelder *et al.* 2003).

The two cell lines did react in a dose dependent manner to the various types of contamination and were able to detect endotoxin concentrations of <2.5 EU/mL. These results were confirmed by doing histological analysis of implanted alginate microcapsules in diabetic BB rats and determining the level of fibrosis. Therefore immune cell preparation and cell lines can be used to study the effects of

contamination in biomaterials such as alginate, and be predictive of in vivo inflammation related adverse effects.

1.2.2 RAW264.7 Reacts to LPS and INF-γ

A study performed by Kim, Y.M. *et al.* (1996) developed a nitric oxide bioassay for the detection of IFN-γ. The assay was developed as a simple, inexpensive, and specific alternative to the ELISA and RIA assays. The colorimetric assay employed the RAW264.7 cell line to test for IFN-γ bioreactivity and was able to distinguish IFN-γ from other types of interferons. They also showed that RAW cells produced 2.5 to 3.5 times more NO in the presence of lipopolysaccharide (LPS) and IFN-γ than in the presence of either one alone. There was a linear correlation between NO production and low concentrations of IFN-γ (0-10 EU/mL) and logarithmic dependence at concentrations of 2-100 EU/mL. Other benefits to the assay were that it relatively rapidly yielded results after only 16 h, ease of adding colorimetric reagents (Griess reagent) to the medium for detection, inexpensive, nonradioactive, nonviral, and reproducible. (Kim *et al.* 1996)

Morphological and growth changes occur to macrophages when in the presence of certain stimuli. Zhuang *et al.* (1997) showed that the growth and viability of RAW was effected when continuously stimulated up to 23 days with LPS and/or IFN-γ. When exposed to either LPS or IFN-γ, the doubling times increased to 2-4 times the rate of unstimulated RAW cells. However, once either stimulant was removed, 24 h later the exponential growth rate returned. After LPS and IFN-γ were added together in culture with RAW, it took 4 days after stimuli were removed for the

exponential growth rate to return. RAW cells are capable of producing NO over extended periods of time when in the presence of stimuli, are resistant to its deadly effects, and are an excellent model system to study nitric oxide mediated inflammatory progress. (Zhuang *et al.* 1997)

1.3 Modulating Agents of RAW264.7

1.3.1 Introduction

Pancreatic islets are known to be sensitive to the toxic effects of macrophage-mediated nitric oxide due to low expression of antioxidant enzymes (Chae *et al.* 2004). There are very few studies where investigators have tried to incorporate modulating agents into alginate for the purpose of mitigating NO production. One such study performed by Chae *et al.* (2004) coencapsulated hemoglobin cross linked (Hb-C) with poly(ethylene glycol) with rat islets or insulinoma cells into alginate/poly(L-lysine)/alginate microcapsules. Hemoglobin acts as a NO scavenger and given NO's small molecular size, it can easily diffuse through alginate and react with hemoglobin forming nitrosylhemoglobin or methemglobin which is non-lethal to islets. A nitric oxide donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), was added to culture medium to generate varying concentrations of NO. The cellular viability along with insulin secretion was measured for two groups: the control which encapsulated islets contained no Hb-C or SNAP and encapsulated islets with Hb-C and SNAP. Indeed, islets coencapsulated with Hb-C had higher viabilities, insulin secretion abilities, and less evidence of apoptosis in the presence of SNAP compared to the

control. Encapsulated islets with no Hb-C exposed to SNAP showed concentration dependent cellular damage and apoptosis. Although NO scavengers such as Hb-C may be useful for acute NO scavenging in alginate capsules, chronic or high levels of production of NO by macrophages would probably saturate binding sites, leaving the islets defenseless.

1.3.2 Bisphosphonates

Other researchers have developed protocols that are used before encapsulated islets are transplanted into the body to try to reduce the numbers of reactive cells in advance of implantation. These preparation methods rely on removing the majority of macrophages already present to limit the production of NO before graft implantation. Bisphosphonates are most commonly used in the treatment of bone disease but have begun being studied in treatment of other inflammatory diseases. In one study performed by Monkkonen (1994), three types of bisphosphonates (clondronate, pamidronate, and etidronate) were studied using different preparations to inhibit growth of RAW264.7 and CV1-P. All three bisphosphonates were prepared separately in either liposomes, free in solution, or bound to calcium. Each of these preparations was then added to RAW264.7 or CV1-P and cultured for up to 72 h. Cellular growth was then evaluated using the MTT-assay. All three drugs that were encapsulated into liposomes significantly inhibited RAW264.7 and CV1-P growth; while the drugs free in solution were 20-1000 times less effective. The presence of high extracellular calcium increased the potency of all three bisphosphonates. Overall, pamidronate was the most potent bisphosphonate, regardless of preparation.

Bisphosphonates encapsulated into liposomes were used by Omer *et al.* (2003) as a preparatory treatment before transplanting encapsulated islets into the peritoneal of rats. The goal was to deplete the macrophage population present to extend the biocompatibility and function of encapsulated islets. Liposomes containing clondronate or saline was injected into the peritoneal cavity of normoglycemic Lewis rats two to five days before transplantation. Either empty PLL/alginate microcapsules or encapsulated neonatal porcine islets were then placed in the cavity. The insulin response and surrounding macrophage population was studied. When the cavity was just flushed with saline prior to transplantation, the empty and especially the islet containing microcapsules were overgrown with macrophages. However, when the cavity was flushed with the clondronate solution the empty capsules had no macrophage overgrowth while the encapsulated islets had much less macrophage overgrowth and a higher insulin response when compared to the control. These two studies show that bisphosphonate is effective in decreasing NO production by macrophages and can increase the lifespan of encapsulated islets. However, studies incorporating free or liposome-contained bisphosphonates directly into alginate have not been done yet.

1.3.3 Parthenolide

Parthenolide is a potent anti-inflammatory known to inhibit the NF-κB pathway, the main inflammatory pathway, by targeting IκB kinase. A study by Kang *et al.* (2001) showed that free parthenolide in the culture medium inhibited IL-12

production by RAW264.7 in a dose dependent manner through affecting the NF-κB binding site in the p40 promoter region.

Another study (Yip *et al.* 2004) explored the parthenolide mechanism further by inducing RAW264.7 to differentiate into osteoclasts and then stimulated them with LPS. Parthenolide was used as a possible inhibitor of LPS-induced osteolysis. The study showed when parthenolide was injected along with LPS into mice it blocked osteolysis. Other assays for NF-κB activation, p65 translocation, and IκB-α degradation were used to determine parthenolide's mechanism in osteoclasts. Parthenolide was shown to inhibit all three leading to the confirmation that parthenolide inhibits the NF-κB pathway. Overall, parthenolide has been shown to be a potent inhibitor of RAW264.7 production of NO in our lab and in others. Studies testing parthenolide's inflammation-inhibiting characteristics when directly incorporated into a biomaterial have not been done yet.

1.4 Summary

Though well studied for other endpoints, RAW264.7 has not been employed as an assay technique for determining LPS contamination levels in alginate capsules. Using RAW264.7 as a generator of inflammatory mediators such as nitric oxide determined by colorimetric assay could be a very powerful biological tool for detecting low levels of LPS contamination in alginate and other biomaterials. Since RAW264.7 is a model macrophage cell line, the data revealed by the assay could give insight into simple or complex inflammatory responses to alginate and the role of

concentration and presentation of LPS in biomaterials as to the final observed inflammatory response. Using IFN-γ should allow for even more sensitive detection of LPS contamination in alginate given IFN-γ's natural function in the body to help alert and amplify the body's response to initially low levels of pathogens.

The use of modulating agents incorporated into alginate has rarely been studied to date. Given the extreme sensitivity of islets to NO and the impossibility of completely removing all contamination from alginate, agents incorporated into alginate to mitigate NO production by impinging macrophages might be a key factor in the development of a successful artificial pancreas.

Chapter 2: Background

2.1 Introduction

The immune system is very complex, relying on a number of cell types and cellular signals to protect the host from possible infection that would lead to death. Therefore, creating treatments that rely on donor tissue and biomaterials (such as encapsulated islet cells) currently have very limited success due to a lack of understanding of the immune system. For the development of such a promising treatment that can be applied to a number of medical conditions, there needs to be a better grasp of the action of the immune system. The immune system is so sensitive that it will even react to something as infantile as a splinter in the thumb. Research should not only be focused on discovering ways to circumvent the immune system but to determine what modest level of immune reaction will not cause rapid graft failure. Also, a change of mindset should be considered. Instead of thinking, for example, that bioartifical organs will provide an ultimate cure they should be considered as one more tool for treatment in a chronic disease, thus providing a greater level of freedom for an extended period of time.

Below is an introduction to the basic players that effect the development of a bioartifical pancreas. These key concepts will enable the reader to better appreciate the complexity in developing new techniques in treating chronic diseases, such as type I diabetes with biomaterials and donor tissue. One piece of the puzzle is

determining what levels of nitric oxide (NO), an indicator of immune response that is produced by macrophages, is due to a biomaterial or to impurities such as bacterial endotoxin.

2.2 Role of Macrophages

2.2.1 Role of Macrophages in the Immune System

Macrophages are derived from monocytes/white blood cells and are part of the "innate" immune system. The innate immune system works in conjunction with the "adaptive" immune system. It is so highly evolutionary conserved that not only is the innate immune system found in vertebrates but invertebrates and even plants. As a first responder to potential infections, the innate immune system uses macrophages, along with other cell types, to phagocytize foreign bodies, produce NO, and an elaborate assortment of cytokines. Whereas the adaptive immune system is slow to respond to a new infection while antibodies from B and T cells are synthesized, the innate immune system responds quickly and imprecisely. The function of macrophages in the innate immune system is to stimulate "inflammatory responses" that phagocytize foreign cells and produce cytokines. An inflammatory response is characterized by pain, redness, heat, and swelling (Alberts *et al.* 2002). Macrophages are something like the police force of the body, quick to respond to attack as compared to the justice system which comes into play preventing future crime (adaptive immune system).

To recognize a pathogen and begin the phagocytizing process, macrophages rely on certain proteins that are only common to pathogens; which are located on the cellular surface. Once encountering a pathogen and recognizing it with cell surface receptors, macrophages will try to engulf the pathogen completely. Then a concerted attack begins with the production of highly acidic species and various highly toxic oxygen derived compounds. Important oxygen species include superoxide (O^-_2), hypochlorite (HOCl), hydrogen peroxide (H_2O_2), hydroxyl radicals, and nitric oxide (NO) (Swindle *et al.* 2007). These compounds, if prevalent in the body, would cause instantaneous death in susceptible tissue but has little effect on macrophages. If the pathogen is too big for a macrophage to phagocytize it, groups of macrophages and other phagocytic cell types surround the pathogen and exocytose their lethal compounds to obliterate the pathogen.

2.2.2 The Model Macrophage: RAW264.7

A cell line commonly used in studying macrophages is RAW264. It was developed by Rascke et al. in 1978 using the Abelson leukemia virus. This virus transformed macrophages into a cancerous cell line with no detectable virus activity. The RAW264 cell line is a specialized subset of peritoneal macrophages possessing the ability of anti-tumor activity. It was shown by Raschke et al. that the RAW264 cell line possessed all the same properties that native macrophages do; including taking up red dye, synthesizing and secreting lysozyme, phagocytizing latex beads, and mediating antibody-dependent lysis.

2.3 Role of NO

2.3.1 NO Is A Soluble Gas

Only recently discovered in the late 80's (Ignarro *et al.* 1987; Furchgott *et al.* 1989), NO has been a controversial substance in medicine and physiology. While not all of the myriad roles of NO have been discovered or fully understood, one thing is for sure: while NO may have seemingly contradictory effects in the body it is vitally important in the immune system, cellular signaling, neurotransmission, vasodilatation, inhibition of platelet aggregation, and inflammation (Ignarro 1990; Murad 1998; Coleman 2001). Some of the mechanisms of NO are fairly well understood, such as how NO causes endothelial cells to relax (Ignarro *et al.* 1987). Less well understood are how some cell types seem to be more resistant to NO compared to others. Within the innate immune system, the mechanism of action is still mysterious and sometimes contradictory. However, the overall effect of NO on foreign invading microbes or "innocent bystander" host cells is startlingly dramatic death.

2.3.2 Chemistry of NO

In the innate immune system, NO is structurally perfect for its role as stealthy annihilator. The small size of NO, a mere 30 Da, allows it to diffuse easily intracellularly and across cellular membranes due to having no specific cell surface receptor. Also, NO exhibits relatively stable free radical chemistry; it has no charge and one unpaired electron. The bioreactivity of NO can be extended from a few

seconds to a few hours by reversible reactions with cysteine and specialized proteins. These reversible reactions allow for the transportation of NO from macrophages to cells that might be relatively far away while keeping NO still highly reactive (Swindle *et al.* 2007).

The effectiveness of NO is extended by being able to react with other compounds to form other, equally harmful substances. Under aerobic conditions, NO is oxidized to form reactive nitrogen oxide species (RNOS). These RNOS, once excreted, can be hydrolyzed to form nitrites. The detection of nitrites in the solute is used as an indirect method for the detection of NO production. Another major product, under aerobic conditions, is N_2O_3. Under anaerobic conditions, NO can react with O_2^- to form the peroxynitrite anion ($ONOO^-$) which is highly toxic and mediates the most severe of toxic effects.

2.3.3 NO Is Involved In the Innate Immune System

In the innate immune system, NO is produced by a variety of immune cells such as macrophages, fibroblast, natural killer cells, etc (Alberts *et al.* 2002). Recent studies have shown that many autoimmune diseases that involve inflammation; such as asthma, rheumatoid arthritis, or type I diabetes, have been linked to the overproduction or poorly regulated production of NO. In healthy individuals, when NO is produced with regards to the innate immune system it is toxic to pathogens and regulates cellular death. In general, NO will cause apoptosis, necrosis, stimulate phagocytosis, cause death in erythrocytes, and used as a mediator in other immune cell types.

2.3.4 NO is produced by iNOS

There are three different enzyme isoforms that produce NO in the body for accomplishing a variety of purposes: NOS-1 (eNOS), NOS-2 (iNOS), and NOS-3 (nNOS). Originally named after the cell types where discovered or for function, it since has been determined that these isoforms are found in an assortment of cell types. In all three of the isoforms, NO is produced from the reaction of L-arginine with molecular oxygen utilizing NADPH (Weisz *et al.* 1994).

The isoforms, NOS-1 and NOS-3, are expressed constitutively. The expression is Ca^{+2} dependent, with a low level of expression, and produces NO is a pulse like nature; ideally suited for neurotransmission or quick cellular signaling. However, NOS-2 is only inducibly expressed (hence i-NOS) and is Ca^{+2} independent. The enzyme, NOS-2, is activated by LPS or cytokines. Once stimulated, the enzyme continuously produces sustained, high levels of NO production following a lag time of a few hours. The lag time occurs because necessary proteins for the enzyme must be synthesized from mRNA. Once there is a sufficient level of NOS-2, NO production can be sustained for a few hours to days. According to Weisz (1994), maximal mRNA concentration is achieved when macrophages have been exposed to a combination of LPS and interferon-γ (IFN-γ). The synergistic effect of the combination of LPS with IFN-γ is thought to be due to the stabilization of LPS on IFN-γ activated NOS-2 mRNA.

2.4 Role of LPS

Lipopolysaccharide (LPS), also known as endotoxin, makes up part of the cellular wall of gram negative bacteria. Bacterium can be generally separated into two distinct groups: gram-positive and gram-negative bacteria. Gram-positive bacteria are surrounded by a cellular wall composed of peptidoglycan; a substance completely foreign to the human body. Therefore, the body can efficiently attack and destroy gram-positive bacteria. However, gram-negative bacteria, in general, have a more robust cellular wall, a capsule, and are covered in a layer of slime that inhibits the immune system from recognizing antigens present on the cellular wall. These structural differences in cellular wall composition are the basis for distinction between gram-negative and gram-positive bacteria (Alberts *et al.* 2002). It is important to note that this classification system is completely empirical and there are many types of bacteria that fall in both or neither category.

For LPS to activate macrophages it must first bind to "LPS binding protein" found in blood, which then binds to the CD14 receptor on the macrophage cell surface which instigates a cascade of reactions ultimately concluding in the binding to Nuclear Factor-Kappa B (NF-κB) to DNA. The transcription factor NF-κB, specifically associated with the innate immune response and inflammation, causes the up regulation of genes leading to increased production of such chemicals as NO and cytokines. (Flo *et al.* 2000)

2.5 Role of a Cytokine

Cytokines play an important role in our body mainly as a means of amplifying an immune response. They are similar to hormones in that they are considered signaling compounds. However, they differ from hormones in that they are not produced by specific organs but by many different cell types. Some of the cytokines involved in immune response include interferons, interleukins, TGF-β, and TNF-α. Cytokines work by binding to specific cytokine receptors and setting off a cascade of events that lead to significant up regulation or down regulation of specific cellular products. Cytokines are used by macrophages to alert other immune cell types, stimulation activation, and increased phagocytic activity.

2.5.1 Interferon-γ

In this thesis, IFN-γ is used as a model cytokine to better understand complex immune reactions and the effect on NO production by RAW264.7 in the presence of LPS. It also increases phagocytic activity, which was not studied here. The cytokine IFN-γ can be produced by neighboring cell types such as T and/or B cells or by macrophages themselves. Cytokines, such as IFN-γ, act as chemoattractants, signaling other immune cell types that there is a pathogen and to respond. Besides altering the function of other immune cell types, IFN-γ, causes more NO production in macrophages and has a "synergistic" effect with LPS (Lowenstein *et al.* 1993).

2.6 Description of Alginate

Alginate is a very common biomaterial used in an assortment of industrial products and as medical treatments. As far back as the 18th century, there has been documentation of the health benefits and usefulness of alginate. Formally discovered in 1883 by E.E.C Stanford, a whole number of industries exploded on to the scene as the result of manufacturing alginate/alginc acid from brown seaweed. Besides food consumption, alginate was used in the manufacture of soda ash, glass, and for nutritional iodine. Alginate is still in the thick of it by currently being very popular in the pharmaceutical industry. Pharmaceutical alginate is used in sustained/controlled release drugs, wound healing dressings, blockage of esophageal /oesophageal reflux, and as a matrix in tissue engineering. (Myrvold *et al.* 2004)

The popularity of using alginate in a variety of medical applications is due in part to the fact that it is one of the few materials that can gel at physiological conditions. This property is currently very rare among biomaterials; as most are gelled using harsh chemicals and under non-physiological conditions. Currently, most alginate is still being produced from brown seaweed. In seaweed, alginate's role is to provide structural support and flexibility; two features of importance in many desired tissue engineered products. Other sources of alginate are the two types of soil bacteria, *Azotobacter vinelandil* and *Psesudomons*. The main disadvantage of alginate compared to other commercially available biomaterials is that the purity and chemical composition can vary considerably from lot to lot. Also, chemical homogeneity can differ greatly depending on species of brown seaweed, growing conditions, and reproductive cycle.

2.6.1 Chemical Composition of Alginate

Alginate is a polysaccharide made of β-D-mannuronate (M) and α-L-guluronate (G) units (Fig. 1) that can form common block patterns such as GG, MM, GM, MG through the formation of 1→4 glycosidic bonds. The amount or number of GG blocks is believed to be one of the leading factors in determining gel strength. This is due to the unique cavity structure that is formed when two G monomers common together causing divalent cations, such as Ca^{+2}, to preferentially bind over other block patterns (Fig. 2). When many GG blocks are strung together and are in the presence of a divalent cation, an "egg-box model" structure (Fig. 3), appears creating a mesh network that is suitable for immunoisolation or other tissue engineering projects.

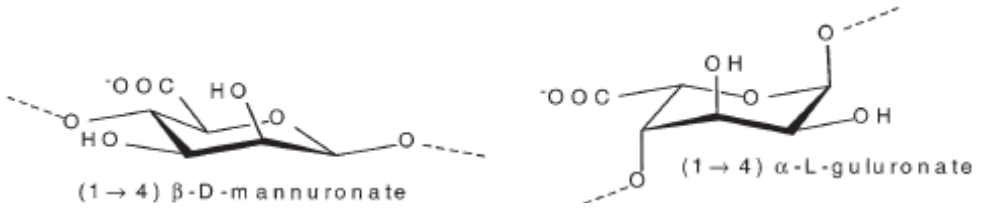

Figure 1. The Two Monomers, β-D-Mannuronate and α-L-Guluronate (G). These monomers can be randomly arranged to form linear block patterns such as MM, GG, MG, and GM. (Myrvold *et al.* 2004)

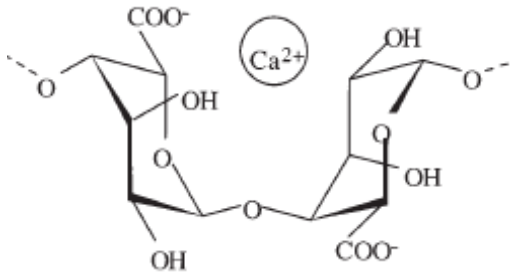

Figure 2. A Calcium Ion Binding In a Pocket Formed by Two Di-axially Linked G Monomers. (Myrvold *et al.* 2004)

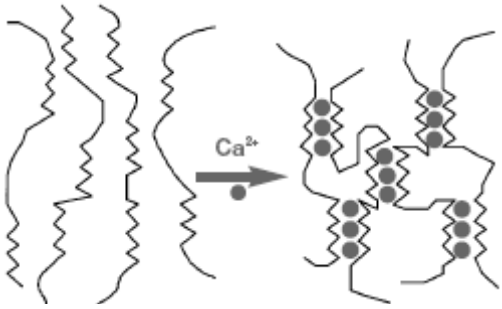

Figure 3. Schematic Showing Linearly Arranged M and G Units and the Effect of Adding a Divalent Cation, Such as Calcium. A mesh network is formed allowing for the flow of nutrients, waste removal, and products while providing an effective barrier to cells and molecules of the immune system.(Myrvold *et al.* 2004)

The ideal chemical composition of alginate is in contentious debate. It might be a mute point since most commercially available alginate is derived from the fickle brown seaweed. In regards to tissue engineering, the concern is the ratio of M/G blocks on rigidity and the effect on excluding immune components. Alginate containing a high amount of guluronate is more brittle and has a higher permeability to antibodies. Simply having grown in rough waters can cause brown seaweed to have a higher amount of guluronate. On the flipside, having a greater amount of mannuronate present makes the alginate more flexible and less permeable. Any divalent cation can bind preferentially to the GG blocks but depending on the cation there is a difference in gel strength. The gel strength of alginate increases as follows: $Mn^{+2} < Co^{+2} < Ni^{+2} < Zn^{+2} < Ca^{+2} < Sr^{+2} < Ba^{+2} < Cd^{+2} < Cu^{+2} < Pb^{+2}$ (Morch *et al.* 2006). However, calcium remains the popular choice for gelation since it is readily found in the body and is non-toxic. The other two most commonly used gelation ions are Barium and Strontium. These are not widely used for gelation of alginate due to Barium being a K^{+2} channel inhibitor and Strontium being a suspected cytotoxic agent.

There has been some debate about whether the ratio of M to G has an effect of the immune response (Otterlei *et al.* 1991; Klock *et al.* 1997; Orive *et al.* 2004), but this has since been put to rest due to better purification processes to get rid of contaminants. Types of contaminants that can themselves cause immune responses include: proteins, complex carbohydrates, fatty acids, phospholipids, toxins, polyphenols, and the ubiquitous endotoxin (LPS). Endotoxin is thought to be the culprit for most of the immune response seen to alginate.

2.7 Definition of Risk Assessment Parameters

In toxicology, risk assessment is used to determine acceptable levels of exposure to chemical agents in human health and ecology (Eason *et al.* 2002). In the case of human health, toxicological studies use animal and in vitro studies to determine acceptable concentration levels and lengths of exposure to suspected chemical agents. These data are then extrapolated to the human case and are used in regulatory decisions. In the case of ecology, this same type of paradigm is used in recognizing pollutants in the air, water, and the effects on wild life. The idea is that in a highly controlled experimental environment specific chemical agents can be more easily identified and assessed. However, one obvious disadvantage is that rarely do these experiments take into consideration complex interactions in the body or in the ecosystem.

2.7.1 Types of Risk Assessment Parameters

Traditionally, there have been a number of terms used to quantify the observable effects of chemical agents seen in toxicology such as LD_{50} (Lethal Dose, 50 %, the dose that kills 50% of the population), benchmark method, etc. To encapsulate either positive or negative observations, two other terms have been in vogue; the "No Observable Effect(s) Level" (NOEL) and the associated "Lowest Observable Effect(s) Level" (LOEL) (Haag-Gronlund *et al.* 1995). These terms are used in regulatory risk assessment of potential drugs, combination devices, or other materials/chemical that are suspected to cause an effect. Such regulatory terms are used in the assessment of experiments done with model animal specimens or in vitro models for the purposes of extrapolation to potential human side effects.

In the simplest of terms, if an experiment was conducted to elucidate the level of drug required to cause no significant change between it and a control that would be considered the NOEL, whether the chemical response was stimulatory or inhibitory. The very next higher concentration or dose of chemical would be considered the LOEL because it would be the lowest observable data point that was significantly different from the control. In layman terms, the NOEL would be "How Much Is Not Enough" to cause a response and the LOEL would be "How Much Is Just Enough" to cause a response.

For this thesis, the NOEL and LOEL were determined for a variety of conditions in the presence of RAW264.7 and LPS as a way to elucidate the effect of impurities in biomaterials on inflammatory immune responses. As is seen in vivo,

from patient to patient, and from experiment to experiment the NOEL and LOEL are not absolutely fixed quantities, but can change slightly due to systematic fluctuations.

2.8 NO and Islets of Langerhans

Islets of Langerhans are a unique cell type found in the pancreas. Their main job is to produce insulin in the regulation of glucose homeostasis. Insulin is also important in the regulation of a myriad of different bodily functions, making the loss of insulin production profoundly detrimental. As mentioned earlier, type I diabetes is an autoimmune disease that causes the destruction of β-cells and hence stops insulin production. The disease, unfortunately, is often only diagnosed once the majority of the islets have been irrevocably destroyed.

2.8.1 Current Treatments of Type I Diabetes

Current treatments rely on constant blood glucose monitoring and insulin injections. This situation is not ideal since insulin in the body is produced at a more continuous level and there may be severe side effects if insulin is incorrectly injected when the patient is hypoglycemic. In extreme cases, death or coma can occur. This has driven research aimed at coming up with novel solutions for the treatment of type I diabetes. Transplantation of panaceas' or islets has had limited success partially because islets are extremely sensitive and the necessary immunosuppressive drug regime may cause their premature death (Couzin 2004). This has encouraged investigators to explore creating a bioartificial pancreas. The bioartificial pancreas uses a biomaterial, such as alginate, to encapsulate donor islets before transplantation. The encapsulated islets are hence "shielded" or "hidden" from the immune system,

dramatically reducing or eliminating the need for immunosuppressive drugs, and consequently prolonging donor tissue survival.

2.8.2 NO Hinders Development of Bioartificial Pancreas

In this thesis, the production of NO is used as an indicator of inflammation and immune response which hinders the development of a bioartificial pancreas (Kroncke *et al.* 1993; Wiegand *et al.* 1993; Suarez-Pinzon *et al.* 1994; Kaneto *et al.* 1995) (Fig.4). The concern is that since NO is such a small compound it can easily diffuse through alginate and cause the cessation of insulin production by the islets of Langerhans. The biomaterial used in any encapsulation technique is required to allow the diffusion of oxygen, nutrients, chemical signals, etc. into the gel while also allowing diffusion of waste and cellular products out of the gel (De Vos *et al.* 2002). It would be highly improbable to create a biomaterial that will prevent the diffusion of NO without sacrificing the diffusion of these other requirements. Therefore, a concerted effort is underway to better understand the immune response to biomaterials and how to better manage the effect on any surgical grafts.

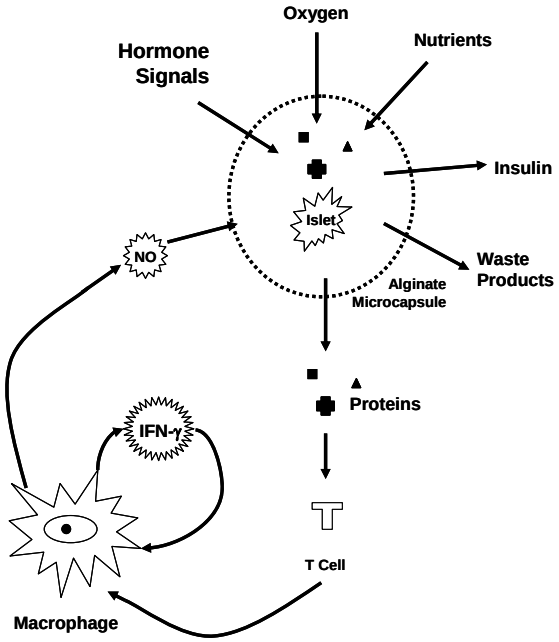

Figure 4. How Macrophages Can Effect the Development of the Bioartifical Pancreas. Macrophages produce a number of cytotoxic products including NO. NO can easily diffuse through alginate as a soluble gas. NO will kill islets limiting the effectiveness of the graft. Figure adapted from Thu, 1996.

2.9 Conclusion

Fortunately, the innate immune system possesses a variety of cell types and cellular products for the protection of the human body from deadly pathogens. Unfortunately, this complicates the discovery of preventing immune reactions to biomaterials used to encapsulate donor tissue. A defined strategy of using one major cell type (macrophages), cytokine (INF-γ), and material impurity (LPS) yields significant practical information about acceptable levels of NO in inflammatory immune responses to encapsulated cell grafts. This provides a better understanding of stringent regulations for the purity of pharmaceutical alginate and other biomaterials.

Chapter 3: Determination Of the "No Observable Effect Level" (NOEL) *in vitro* For Endotoxin Impurity in Manufactured Alginate Microcapsules

3.1 Introduction

The endotoxin LPS is a common contaminant in pharmaceutical grade alginate and is also known to cause an inflammatory response. If alginate is to be used to encapsulate islet cells for the treatment of type I diabetes, acceptable threshold levels of endotoxin need to be determined for providing insignificant immune responses that otherwise might be capable of damaging sensitive donor islets. It has been determined in pervious studies in this laboratory that islets are severely sensitive to NO produced by macrophages, effectively limiting their ability to respond to glucose and produce insulin. To help overcome this barrier to the development of encapsulation of donor tissue, the NOEL and LOEL of free LPS on RAW were determined. Those levels can then be compared to those seen when endotoxin contaminates in pharmaceutical alginate. These findings can then be applied in regulatory applications. Since macrophages produce significant, sustained amounts of NO when in the presence of a pathogen, and NO is readily converted to nitrite in media, measurement of nitrite concentration using the 2, 3-diaminonapthalene (DAN) assay directly correlates with the strength of the inflammatory response.

The T-cell mediator, IFN-γ, is produced in complex immune reactions and stimulates macrophages to produce even more NO. To simulate such a reaction, RAW264.7 was stimulated with an external dose of IFN-γ in the presence of free LPS. The NOEL and LOEL's of free LPS and LPS contaminated alginate with or with out IFN-γ were compared to determine acceptable levels of LPS contamination. The NOEL and LOEL was determined for LPS in the absence or presence of IFN-γ to simulate simple and complex immune responses and to show the full possible range of the inflammatory response.

3.2 Experimental Design

3.2.1 Reagents

Lipopolysaccharide (from *Escherichia coli* Serotype O26:B6) (LPS), mouse recombinant interferon gamma (mIFN-γ), and 2, 3-diaminonapthalene (DAN) were purchased from Sigma. LAL reagent water (endotoxin content < 0.005 EU/mL) was purchased from Cambrex. Sterile sodium alginate (Pronova SLG 20, viscosity >100 mPas, guluronate ≥ 60%, endotoxin ≤100 EU/g) was purchased from Nova Matrix (Drammen, Norway). Stock solutions were prepared as follows. LPS was diluted in dimethyl sulfoxide (DMSO) to a concentrate of 250 μg/mL and frozen at -70^{o}C until use. Mouse recombinant interferon gamma was initially diluted to 1 mg/mL in phosphate buffered saline pH 8.0, then to 1 μg/mL in complete medium (RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) (CM), and frozen at -20^{o}C until use. Stock solutions of DAN, at 10

mg/mL, were prepared by diluting 100 mg DAN in 5 mL of DMSO, and freezing at -20^{o}C.

3.2.2 Cell Culture

The murine macrophage cell line, RAW 264.7 (ATCC TIB71), was obtained from the American Type Culture Collection. The macrophage cell line was established through the inducement of a tumor in an adult male *Mus musculus* (mouse) with Abelson murine leukemia virus. The method is described in Raschke et al. (1978).

The RAW cells were cultured in CM, expanded in numbers, frozen down in 10% DMSO-containing CM and stored in liquid nitrogen. A vial containing up to a million cells of RAW 264.7 was taken from the standard stock passage maintained in liquid nitrogen, rapidly thawed, and seeded into a T75 flask containing 20 ml CM. The flask was cultured until the adhered monolayer of cells had reached 50% confluency (usually in one to two days). The monolayer was washed twice with calcium/magnesium-free phosphate buffered saline (Ca/Mg-free PBS), then allowed to sit in 10 ml of Ca/Mg-free PBS for five minutes. The flask was sharply rapped several times to dislodge the cells, poured into 10 ml of CM, cell density determined, and used to make sufficient volume in CM of a 1 x 10^5 cell/ml concentration.

3.2.3 96-well Plate Layout

The macrophage cell line, RAW was plated out into a 96-well plate as $1x10^4$ cells/well to a final volume of 0.2 ml (Fig. 5). After initial seeding, the cells were allowed to adhere to the bottom of the wells by further culturing for 24 hours. Following the adherence period, 10 µL of LPS standard or 40 µL of packed alginate beads spiked with LPS were added to the wells. The bottom half of the 96-well plate received an additional 10 µL dose of IFN-γ, maintaining the same volume in each well (Fig. 5). After the desired incubation period, the top 20 µl of supernatant was taken from each well to a duplicate plate, which was then stored at -70°C and later thawed to assay for nitrite concentration.

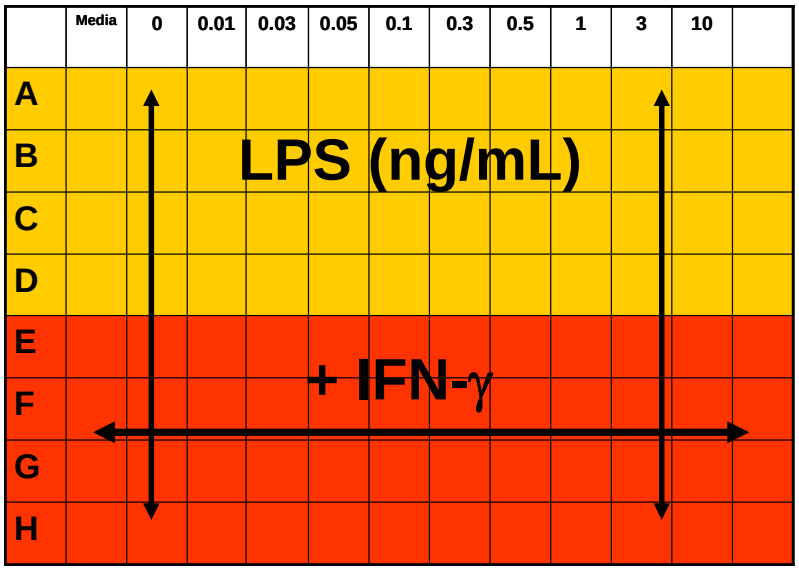

Figure 5. Layout of 96-well Plate Used for Free LPS Stimulation of RAW264.7 +/- IFN-γ. The volume of each well was maintained at 0.2 mL. Across the top of the "96-well plate" is the dose curve of LPS (ng/mL). Each well of the plate contains 10 µL of the appropriate concentration of LPS. The top half of the plate contains no IFN-γ. The bottom half of the plate contains an additional 10 µL of IFN-γ (50 ng/mL).

3.2.4 DAN Assay

A working DAN solution of 50 µg/ml was prepared by making in 0.62 M HCL a 1:400 dilution of thawed 20 mg/ml stock solution. All assays were conducted in 96-well plates, with each condition done in quadruplet. In each well, 20 µL of standard ($NaNO_2$), sample, or medium-only was added to 80 µL of d.i. water. Then, 10 µL of working DAN solution was added to each well. The plate was allowed to sit in the dark at room temperature for ten minutes. To stop the reaction, 20 µL of 2.8 M NaOH was added to each well in the same timing and sequence as for the additions of DAN. Then the plate was gently shaken for one minute in the dark; subsequently it was read in a fluorescence plate reader (Perkin Elmer HTS 7000 Plus Bio Assay Reader) with an excitation of 360 nm and an emission of 430 nm. Statistical analysis was performed using the t-Test assuming unequal variances method in Microsoft Excel.

3.2.5 Production of 2 % w/v "Spiked" Alginate Beads

Alginate was dissolved in LAL Reagent Water and left on the bench top over night to give a 2% w/v alginate solution. A dose curve was made by incorporating different concentrations of LPS into 2% "Super Pure" alginate (Pronova alginate ≤ 100 EU/g). The solution was then vortexed to ensure complete mixing. Beads of approximately 500-550 µm in diameter were made using a Nisco Engineering Encapsulator Unit (Norway) (Fig. 6) with the following settings: voltage 6.5 kV, flow rate 20 mL/hr, 0.5mm needle diameter, needle distance from gelation bath 1.3 cm.

The technology behind the encapsulator unit (Fig. 7) relies on creating an electrostatic field between the nozzle and the $CaCl_2$ bath (or hardening solution). The nozzle is electrically ground and the syringe pump provides steady flow through the nozzle causing it to vibrate. This causes the electrostatic field to "pull off" the beads from the outer part of the nozzle with uniform size (Fig. 8). However, there are deviations in bead size that can be due to mechanical and physical properties. The productivity and size distribution relies on nozzle diameter, flow rate, voltage, distance between the nozzle and surface of the $CaCl_2$ solution, and any pressure drops due to small nozzle diameters. Physical properties that affect size distribution are density, viscosity, and surface tension of the gel (Thu 1996).

Figure 6. Nisco Engineering Encapsulator. Used to produce 500 μm in diameter 2 % w/v alginate beads. Shown with syringe pump on top of unit.

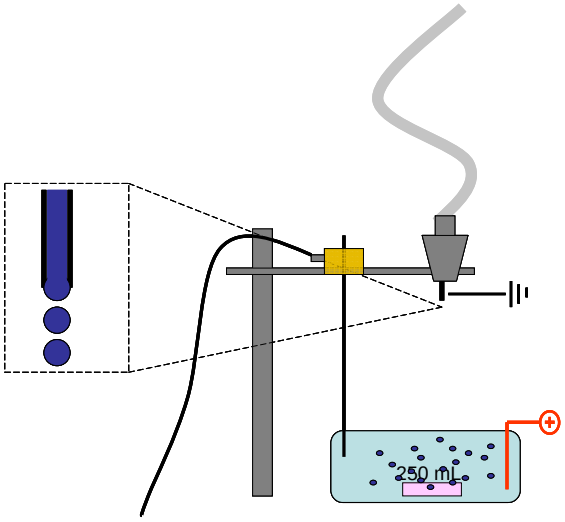

Figure 7. The Principle of the Encapsulator. Using an exact voltage setting and flow rate beads are pulled off the needle at a precisely controlled size. Beads form on the outer edges of the needle. Therefore, the given outer diameter of needle gives the approximate size of the beads. (Not drawn to scale)

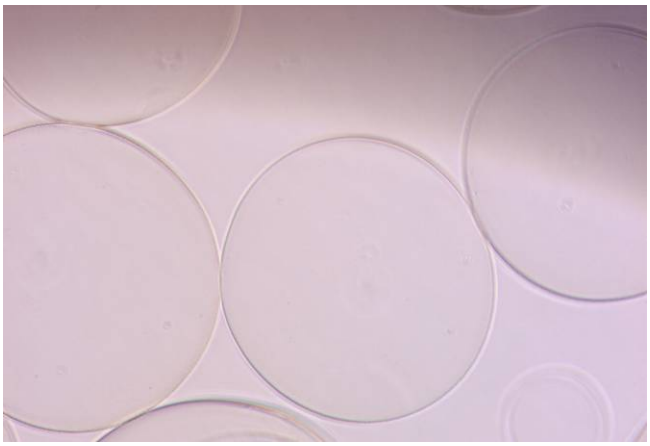

Figure 8. 2 % w/v Pronova- 20 Beads Made with Nisco Engineering Encapsulator. Settings: 6.5 kV, 0.5 mm diameter needle, 20 mL/hr pump speed, and 1.3 cm arm height. Beads are approximately 500 μm in diameter and are uniform in shape and size.

A crosslinking solution was prepared by dissolving $CaCl_2$ in d.i. H_2O to a concentration of 50 mM. To remove any contaminants that might be present, the solution was filtered using a Corning sterilizing, low protein binding membrane (0.22 μm). The beads were allowed to gel for 30 min in the $CaCl_2$ solution after the last of the alginate passed through the syringe under gentle agitation. The beads were then

collected, excess 50 mM $CaCl_2$ solution was removed, and the beads were washed three times with CM. The beads were incubated at 4^{o}C overnight in CM until use. "Packed Beads" were achieved by removing excess CM from the top of settled beads immediately before use (Fig.9). Using a clipped sterile pipet tip, 40 μL of beads were added to each well on top of RAW and incubated overnight.

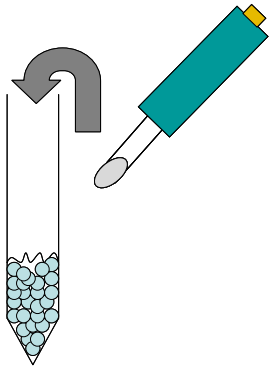

Figure 9. Schematic of Sterile "Packed Beads" with Pipet. Beads were incubated in a minimum of CM to ensure that a reproducible number of beads could be delivered to each well. The pipet was disinfected with a 70% EtOH solution prior to use and the end of a pipette tip was removed with a sterile scalpel at an angle.

3.3 Experimental Results

3.3.1 LPS Stimulation of RAW264.7

The supernatants of each well in the 96 well plate were assayed for nitrite concentration using the 2,3-diaminoaphthalene (DAN) fluorescence assay after exposures to a standard lot of LPS. The Endotoxin Unit (EU) activity was assayed previously in our lab and was found to be 0.6 EU/1.0 ng/mL. Micromolar concentrations of nitrite produced by RAW in response to LPS were observed at 20 h. The response to differing amounts of LPS followed in a dose dependent manner. This trend followed through the 24 and 48 h time points with amplification over time.

The RAW cells experience morphological and proliferative changes in response to varying doses of LPS. RAW exposed to no LPS were greater in number and increased in number over time (Fig. 10 A-B) and were more rounded in appearance covering less individual surface area. When exposed to LPS, RAW cells were fewer in number (denoting inhibited proliferation of the cell line), larger, and more spread out (denoting differentiation to mature, activated macrophages) over the surface of the well plate (Fig. 10 C-D). This trend was seen over increasing doses of LPS with even fewer cell numbers and increased cellular surface area up to 6.0 EU/mL LPS. After 48 h, the well surface of the control (Fig. 10 B) (RAW with no LPS) was completely covered in cells with crowding while at the highest dose of LPS (6.0 EU/mL) cells were spaced out with a dramatically different morphology (Fig. 10 D).

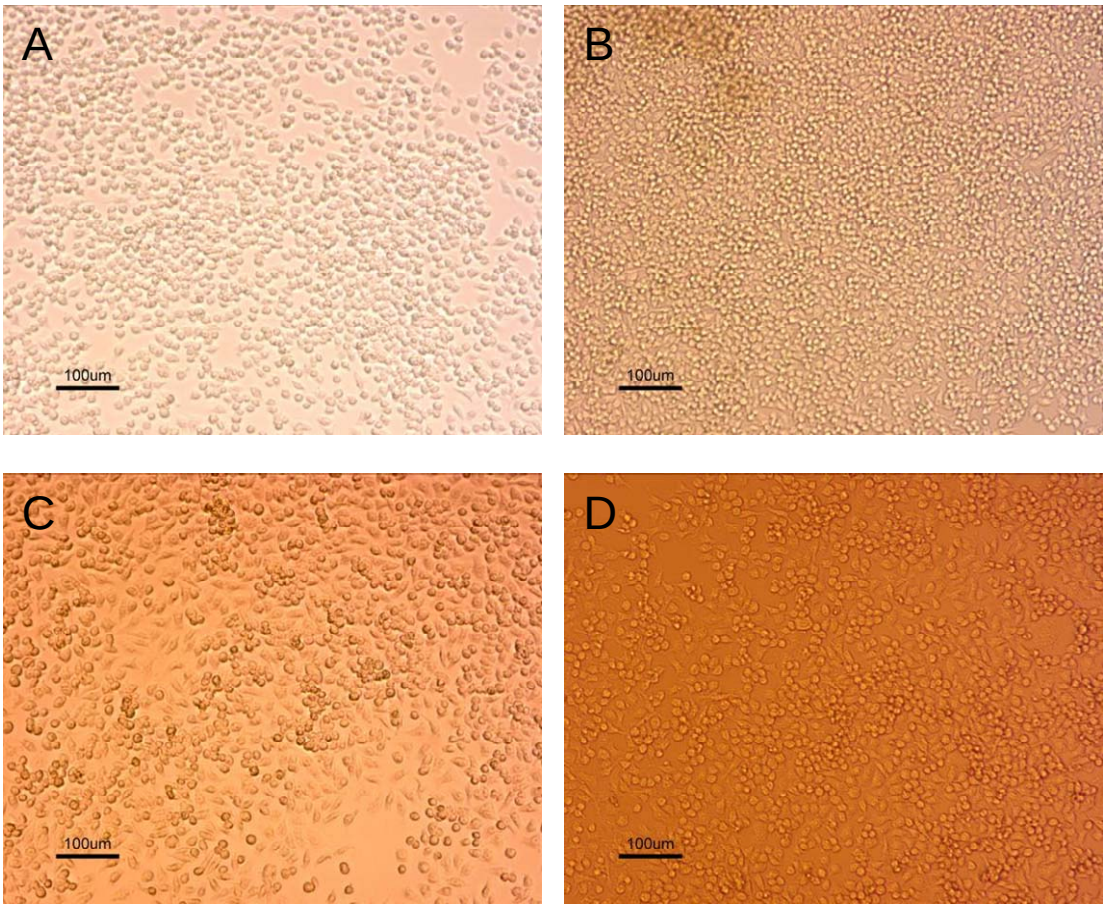

Figure 10. Comparison of RAW264.7 at 20 and 48 h In the Presence or Absence of LPS. A) RAW culture at 20 h in the absence of LPS and IFN-γ, cells are spaced out and round in appearance with very little crowding. B) Control RAW at 48 h without LPS or IFN-γ, cells completely covers the bottom of the well with significant crowding and overlap at the upper left of the picture. C) RAW exposed to LPS at 20 h. There are fewer cells than in control wells. Morphologically, cells are less round and many of the cells have adhered and spread out over the bottom of well in irregular shapes. These morphological changes indicate a slow down in proliferation with concomitant production of NO. RAW exposed to LPS at 48 h. There is much less cellular crowding even though are more cells compared to C. Many of the cells have flattened out indicating cell activation and NO production.

The NOEL and LOEL (Fig. 11) were determined at each time point and are summarized in Table 1. In general, the NOEL and LOEL for each time point is similar over the time course. Significance of LOEL was determined using the two-tailed Student's *t* test assuming unequal variances. The first significant difference between nitrite concentrations of LPS standard compared to the control (no LPS) was

taken as the LOEL with the NOEL being the previous concentration of LPS. For the 20 h time point, the LOEL was 0.03 EU/mL LPS which was the first concentration that elicited a significant increase in nitrite concentration compared to the control; therefore, the NOEL was 0.018 EU/mL of LPS. At 24 h, the NOEL and LOEL decreased to 0.006 EU/mL LPS and 0.018 EU/mL LPS respectively. While at 48 h, the NOEL and LOEL increased back to the original NOEL and LOEL. Overall, the NOEL was detected down to a level of 0.006 EU/mL LPS and the LOEL was detected from a range of 0.018 to 0.03 EU/mL LPS.

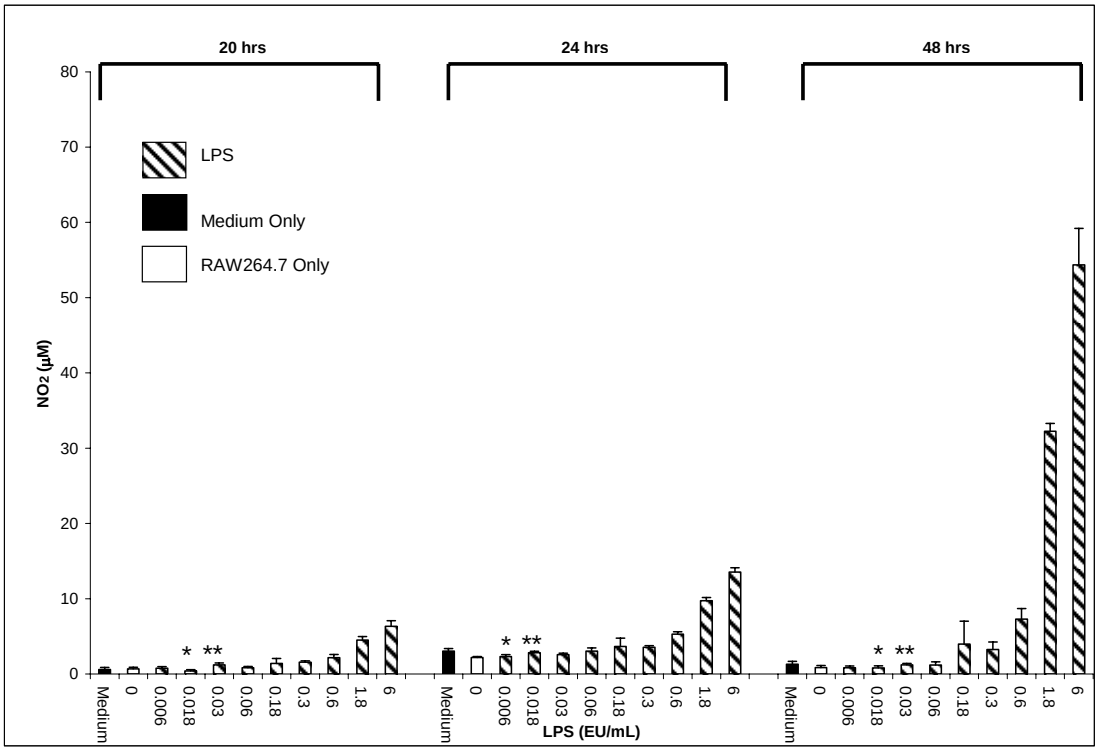

Figure 11. LPS Stimulation of RAW264.7 NO Production. LPS causes NO production in a dose dependent manner whose trend is amplified over time. Samples were taken over three time points: 20, 24, 48 hrs. The NOEL(*) and LOEL(**) values are indicated at each time point. The NOEL was detected down to the level of 0.006 EU/mL LPS with the LOEL ranging from 0.018 to 0.03 EU/mL.

Time		LPS (EU/mL)	NO_2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	0.018	0.40	0.06	0.27
	LOEL	0.03	1.2	7×10^{-3}	0.056
24 h	***NOEL***	0.006	2.3	0.2	0.66
	LOEL	0.018	2.8	6×10^{-4}	0.031
48 h	***NOEL***	0.018	0.80	0.4	0.79
	LOEL	0.03	1.2	0.04	0.029

Table 1. Summary of NOEL and LOEL Values for LPS Stimulation of RAW264.7 at 20, 24, 48 h. Significant NOEL and LOEL values were determined using the Student *t*-test and were presented with their corresponding NO_2 concentrations, P-values, and variance. Cut off for significance was P(T≤t) = 0.05.

3.3.2 Determination of NOEL and LOEL for LPS Contamination in 2 % w/v Alginate in the Absence of IFN-γ

A nitrite response was observed over the entire time course in a dose dependent manner compared to the control (Fig. 12). Even the 2 % w/v sterile alginate beads containing no added LPS elicited a significant nitrite response at 20 h. The sterile Pronova alginate contains less than 100 EU/g of endotoxin. This demonstrates the sensitivity of the assay, the reactivity (albeit very small) of even highly-purified alginate, and the applicability of using RAW264.7 as a way to predict acceptable levels of endotoxin contamination. Overall, the "spiked" alginate elicited a much lower nitrite response compared to its free LPS counterparts indicating that much higher amount of contamination can be present in alginate than free LPS.

Over the course of the experiment, the NOEL and LOEL increased. However, the NOEL's and LOEL's were much lower than expected so further investigation using a lower range of LPS contamination was conducted to determine if there were lower NOEL's and LOEL's that could be established (Fig. 13). Considering that 1

EU/mL “spiked” alginate beads produced a moderate NO response at each time point, 2 EU/mL “spiked” beads were used as the highest concentration so as to not miss any potential NOEL and LOEL values. Lower doses of “spiked” alginate beads were freshly made as described previously. Again, there was a significant nitrite response at all time points and at each dose of “spiked” beads. However, at 20 h and 24 h the LOEL was again 0 EU/mL. While at 48 h, the NOEL was 0.5 EU/mL LPS and the LOEL was 0.75 EU/mL LPS for RAW in the absence of IFN-γ (Table 3).

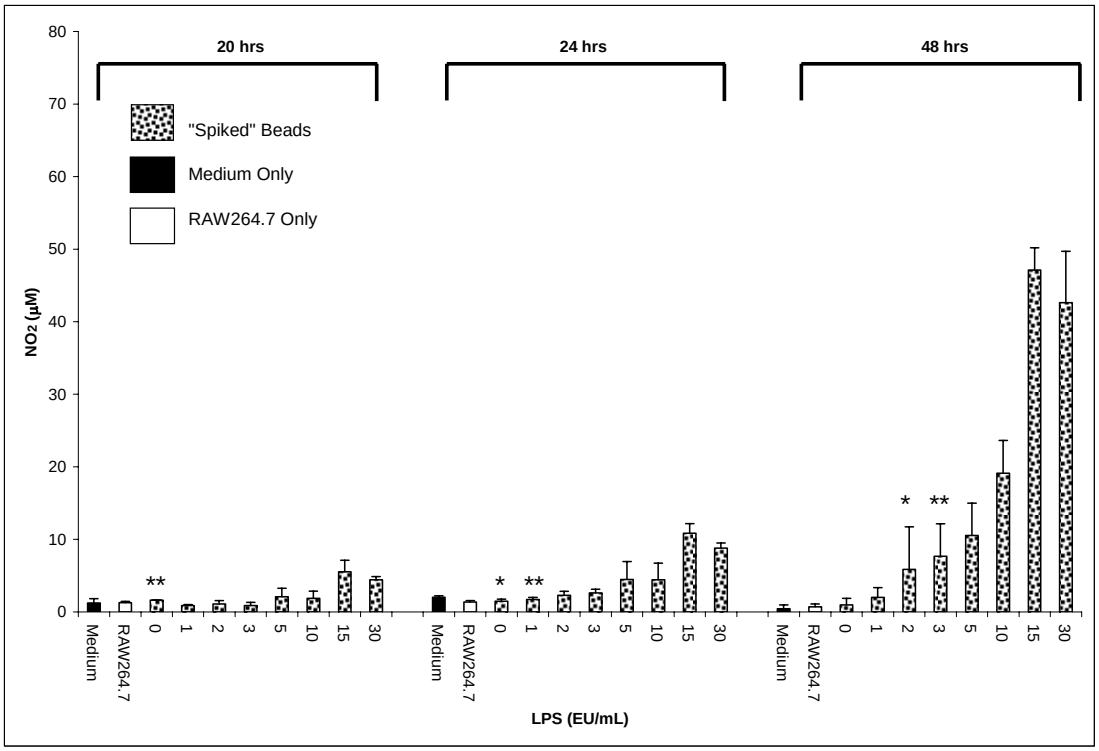

Figure 12. Stimulation of NO Production of RAW264.7 by LPS “Spiked” 2 % w/v Alginate Beads. A dose curve of nitrite concentration due to LPS “contamination” in alginate seen over each time point (20, 24, and 48 h). NOEL (*) and LOEL (**) values are indicated at each time point. Even the beads with no added exogenous LPS added or sterile alginate elicited a significant nitrite response.

Time		LPS (EU/mL)	NO2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	None			
	LOEL	0	1.6	0.01	3.4×10^{-4}
24 h	***NOEL***	0	1.5	0.3	0.085
	LOEL	1	1.7	0.05	0.078
48 h	***NOEL***	2	5.6	0.09	34
	LOEL	3	7.7	0.03	20

Table 2. Summary of NOEL and LOEL Values for "Spiked" Alginate Beads Stimulation of RAW264.7 at 20, 24, 48 h. The NOEL and LOEL values were determined using the Student *t*-test and were presented with their corresponding P values and variance. Cut off for significance was P(T≤t)=0.05. The NOEL and LOEL were both determined down to 0 EU/mL "spiked" 2 % w/v alginate beads.

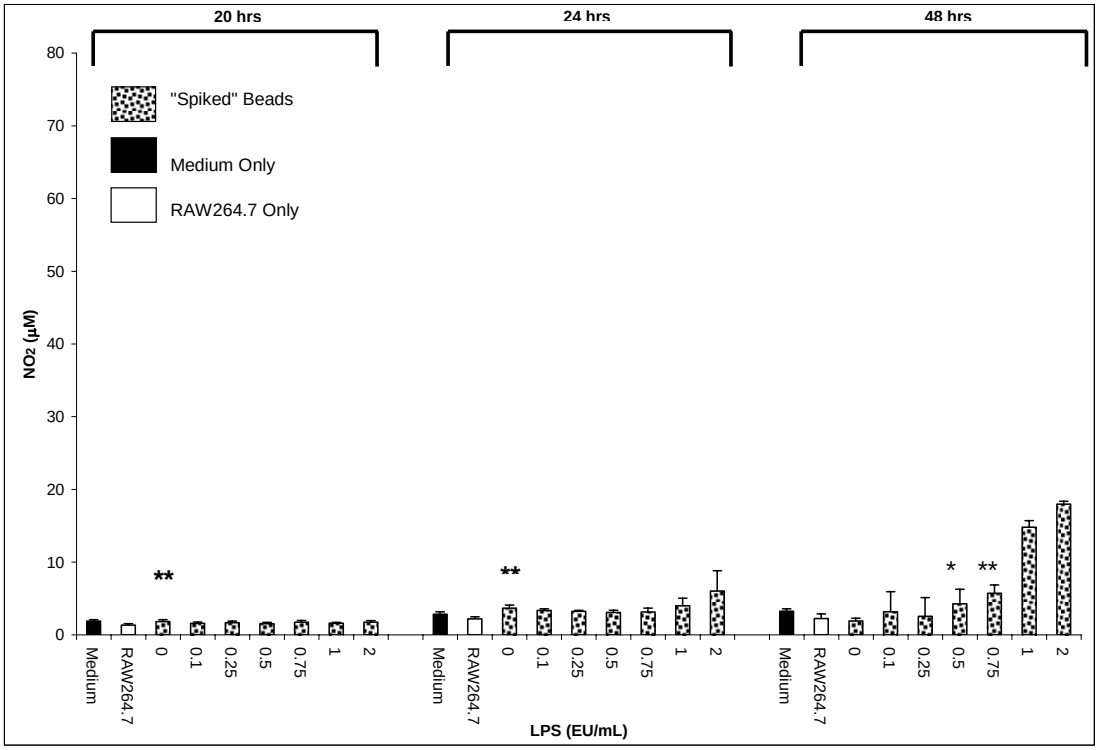

Figure 13. Narrower Range of LPS "Spiked" 2% w/v Alginate Beads for Stimulation of RAW264.7 Production of NO. NOEL(*) and LOEL(**) are indicated at each time point. There is a dose dependence of LPS contamination and NO_2 concentration. Only at 48 h could a NOEL be determined (0.5 EU/mL LPS).

Time		LPS (EU/mL)	NO_2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	None			
	LOEL	0	1.8	0.02	0.042
24 h	***NOEL***	None			
	LOEL	0	3.7	2×10^{-3}	0.083
48 h	***NOEL***	0.5	3.3	0.07	0.97
	LOEL	0.75	5.7	0.02	1.2

Table 3. Lower NOEL and LOEL Values for "Spiked" Alginate Beads Stimulation of RAW264.7 at 20, 24, 48 h. The NOEL and LOEL values were determined using the Student *t*-test and were presented with their corresponding, NO_2, P values, and variance.

Time (h)	Free LPS (EU/mL)	NO_2 (μM)	LPS (EU/mL) in 2% w/v Alginate Beads	NO_2 (μM)
20	0.6	0.821	3	0.894
24	0.6	3.05	3	2.62
48	0.6	1.18	3	7.67
20	6	6.35	30	4.44
24	6	13.5	30	8.78
48	6	54.4	30	42.6

Table 4. Comparison of Free LPS Versus Incorporated LPS Into Alginate NO_2 Concentrations. Overall, an amount five times the amount of free LPS can be incorporated into alginate to give similar a NO response by RAW264.7.

3.3.3 LPS Stimulation of RAW264.7 in the Presence of IFN-γ

To simulate a more complex immune reaction a T-cell mediator, IFN-γ, was used to further stimulate NO production by RAW. The same procedure was followed as above except that before the incubation period began an additional 10 μL of IFN-γ was added to the media along with a standard lot of LPS. The assay used to quantify the nitrite concentration was the same. As stated before, IFN-γ is produced by lymphocytes and other immune cell types. The cytokine is used as a cell mediator to

alert nearby cells to a pathogen and causes the amplification of cellular activity in immune cells, especially macrophages such as RAW264.7. Other studies have established the synergistic effect of IFN-γ and LPS on NO production (Lowenstein *et al.* 1993; Noda *et al.* 1997; Matsuura *et al.* 2003).

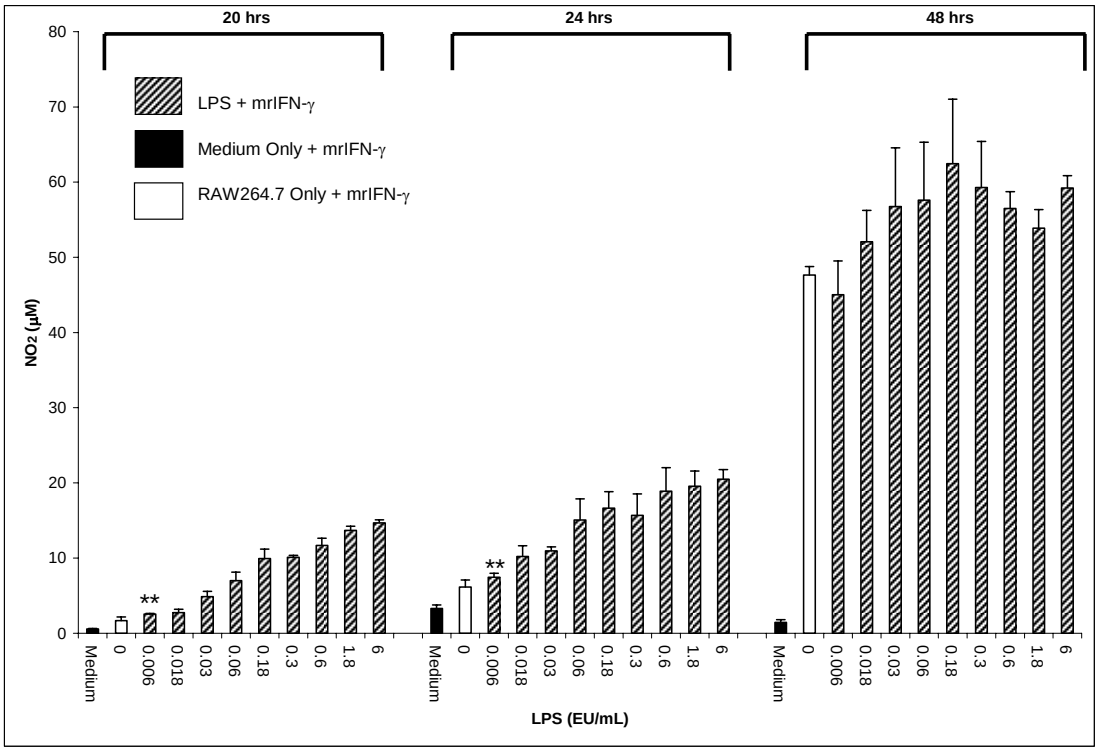

Figure 14. LPS Stimulation of RAW264.7 In the Presence of IFN-γ. The LOEL was determined to be 0.006 EU/mL for both 20 h and 24 h therefore the NOEL is less than 0.006 EU/mL. No NOEL and LOEL were reported for 48 h due to the large fluctuations in nitrite concentration. NOEL(*) and LOEL (**) values are indicated at each time point. There is an increase in nitrite concentration for each dose compared to LPS stimulation in the absence of IFN-γ.

Time		LPS (EU/mL)	NO_2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	≤ 0.006			
	LOEL	0.006	2.5	0.02	9.0×10^{-3}
24 h	***NOEL***	≤ 0.006			
	LOEL	0.006	7.4	0.03	0.27
48 h	***NOEL***	N/A		N/A	N/A
	LOEL	N/A		N/A	N/A

Table 5. Summary of NOEL and LOEL Values for LPS and IFN-γ Stimulation of RAW264.7. The significance of NOEL and LOEL values were determined using the Student *t*-test and were presented with their corresponding NO_2, P-values, and variance.

A strong nitrite response was seen over the range of LPS doses for 20 and 24 h (Fig. 14). The NO_2 concentrations for each LPS dose were dramatically higher than the previous corresponding graph (Fig. 11) where no IFN-γ was present. Also, there is still a correlation at these time points between NO_2 and LPS concentration. However, at 48 h no more correlation exists between LPS and NO_2. At 48 h, the nitrite concentrations seem to fluctuate or "max out" compared the control. Given the documented synergistic effect of IFN-γ on nitrite concentration, there were no detectable NOEL levels when compared to the control. The IFN-γ served as a tool to amplify the NO response by RAW giving much lower LOEL's compared to the absence of IFN-γ. The LOEL for 20 and 24 h was both 0.006 EU/mL LPS.

The control contained no LPS but 50ng/mL of IFN-γ (Fig. 15 A). The cellular morphologies and numbers were different then in the absence of IFN-γ (Fig. 10 A). The presence of a cytokine in solution causes the RAW cells to stop proliferating as rapidly as before. Therefore, the appearance of the interferon-only control was

different from that of the control containing no LPS or IFN-γ. The cellular morphology was more spread out and fewer in number corresponding to a greater nitrite concentration than the previous control. With the presence of IFN-γ, the appearance of RAW cells were more spread out at even lower doses of LPS compared to cells in the absence of IFN-γ.

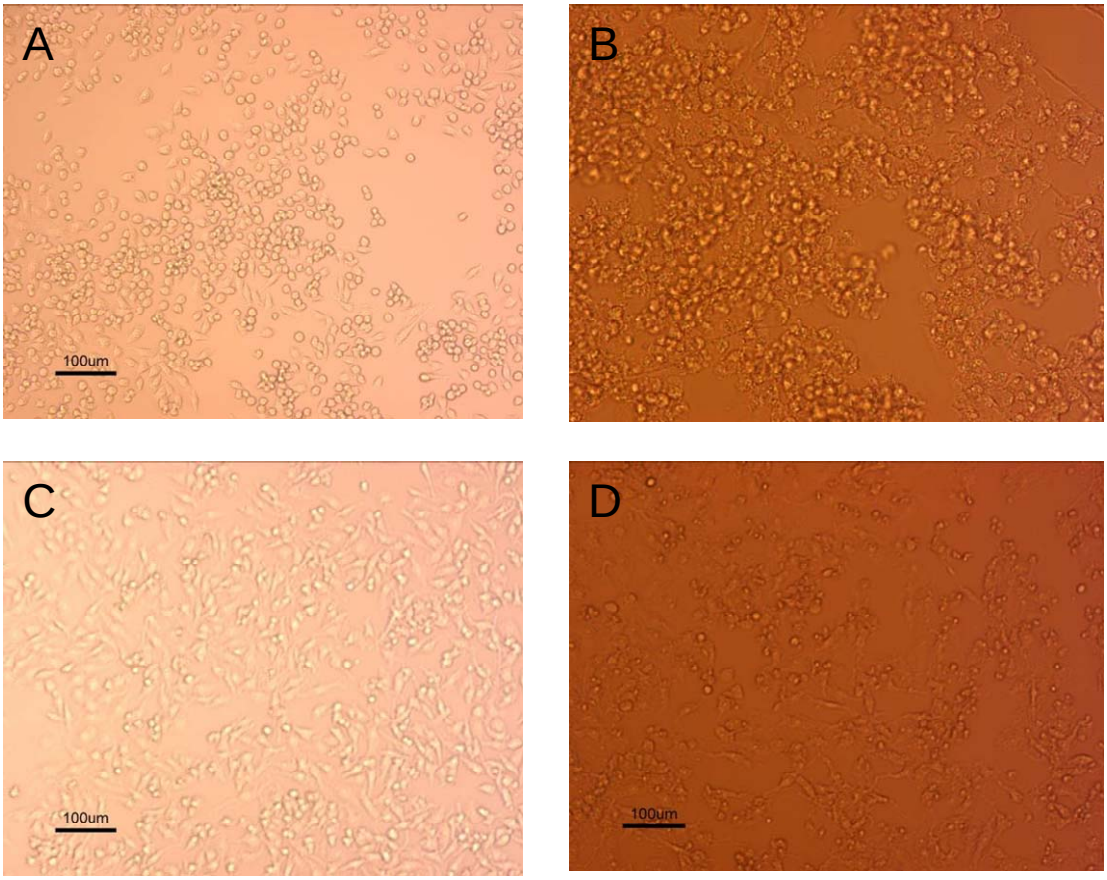

Figure 15. Comparison of RAW 264.7 at 20 and 48 h In the Presence of LPS and IFN-γ. A) Control at 20 h in the presence of IFN-γ. RAW cells are fewer in number than the previous corresponding medium only control and flatter in appearance. B) Cells at 48 h in the presence of only IFN-γ. RAW cells look dramatically different than from those in the medium only wells. There are fewer cells and a granular appearance to the cells indicating cellular death. C) RAW cells in the presence of LPS and IFN-γ. RAW cells are fewer in number, flatter, and more spread out. D) RAW cells in the presence of LPS and IFN-γ at 48 h. Much fewer cells are present and cellular debris appears.

3.3.4 Determination of NOEL and LOEL for LPS Contamination in 2 % w/v Alginate In The Presence of IFN-γ

In the presence of IFN-γ, similar trends of proliferation and morphology were seen for "spiked" alginate beads as in the free LPS case. A dose dependent nitrite response to entrapped LPS was observed in the presence of IFN-γ (Fig. 16). The response was amplified, however compared to the case when no IFN-γ was present, with much higher NO_2 concentration. The LOEL was determined to be 1 EU/mL for both the 20 h and 24 h, with the NOEL being 0 EU/mL (Table 6).

Considering the fact that the NOEL and LOEL were so low, a narrower range (0-2 EU/mL) of exogenous LPS contamination was examined in the presence of IFN-γ (Fig. 17). At 20 h, the LOEL was determine to be 0.25 EU/mL LPS and the NOEL of 0.1 EU/mL LPS. For 24 h, the LOEL was determined to be 0 EU/mL LPS (Table 7).

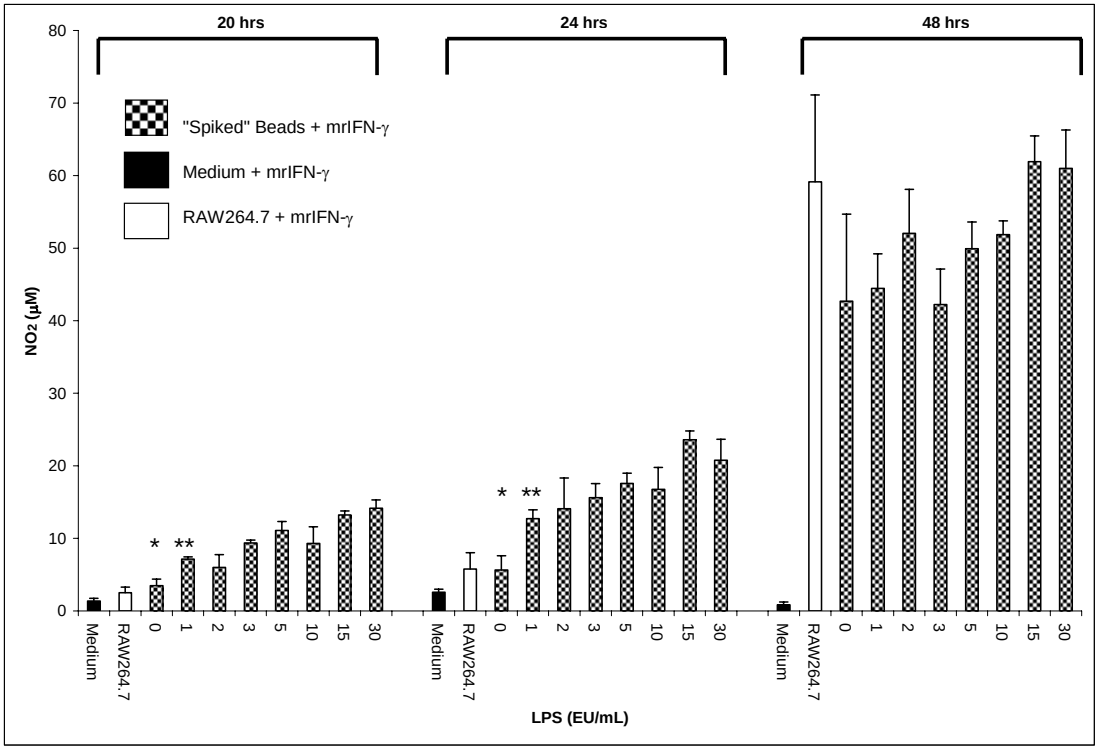

Figure 16 "Spiked" 2 % w/v Alginate Beads Stimulation of RAW264.7 In the Presence of IFN-γ. For 20 h and 24 h, a dose dependent nitrite curve is observed. At 48 h, RAW has "maxed-out" resulting in large fluctuations in nitrite concentration. The NOEL is indicated by (*) over the LPS dose and the LOEL (**).

Time		LPS (EU/mL)	NO_2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	0	3.5	0.2	0.86
	LOEL	1	7.1	7×10^{-4}	0.11
24 h	***NOEL***	0	5.6	0.2	3.8
	LOEL	1	12.7	3×10^{-3}	1.4
48 h	***NOEL***	N/A			
	LOEL	N/A			

Table 6. "Spiked" 2 % w/v Alginate Beads NOEL and LOEL Values In the Presence of IFN-γ. The NOEL and LOEL values were the same at 20 h and 24 h.

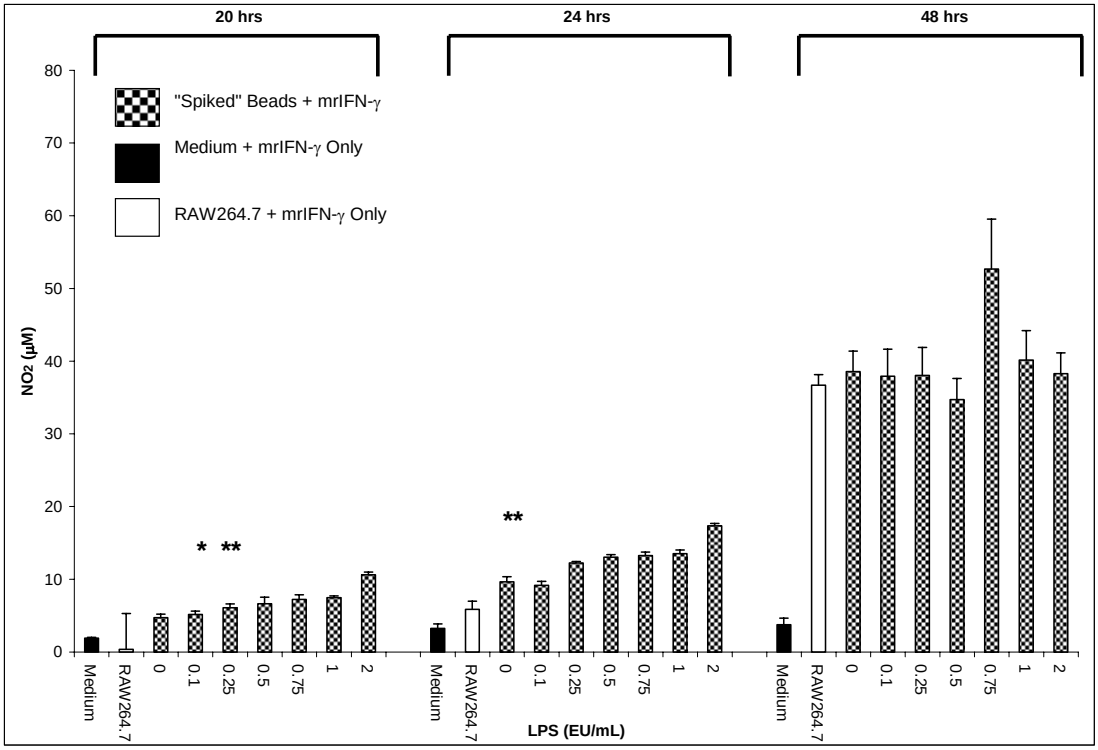

Figure 17. Stimulation of NO Production of RAW264.7 by LPS "Spiked" 2 % w/v Alginate Beads Over a Narrower Concentration of LPS In the Presence of IFN-γ. Lower values for the LOEL and NOEL were determined at 20 h and 24 h were determined. The LOEL was determined down to 0 EU/mL "spiked" beads. The NOEL(*) and LOEL (**) values are indicated at each time point with the exception of 48 h.

Time	20 h	LPS (EU)	NO_2 (μM)	P-value P(T≤t)=0.05	Variance
20 h	***NOEL***	0.1	5.2	0.3	0.21
	LOEL	0.25	6.1	7×10^{-3}	0.28
	24 h				
24 h	***NOEL***	None			
	LOEL	0	9.7	4×10^{-3}	0.48
	48 h				
48 h	***NOEL***	N/A			
	LOEL	N/A			

Table 7. Summary of NOEL and LOEL Values for LPS "Spiked" 2 % w/v Alginate Bead In the Presence of IFN-γ. Even lower LOEL and NOEL values were determined for exogenous LPS contamination of alginate for a narrower range of LPS concentrations.

Time (h)	Free LPS (EU/mL)	NO_2 (μM)	LPS (EU/mL) in 2% w/v Alginate Beads	NO_2 (μM)
20	0.6	6.98	3	9.35
24	0.6	15.1	3	15.6
48	0.6	N/A	3	N/A
20	6	14.7	30	14.1
24	6	20.5	30	20.8
48	6	N/A	30	N/A

Table 8. Comparison of Free LPS Versus Incorporated LPS In Alginate In the Presence of IFN-γ. Five times the amount of LPS incorporated in alginate needs to be present to give a similar NO response compared to free LPS.

3.4 Conclusions

The free LPS stimulation of RAW in the presence or absence of IFN-γ demonstrates the usefulness and sensitivity of using the DAN assay to quantify nitrite concentrations as a way to study NO production by RAW. The assay helps define the ability of the biomaterial alginate to sequester LPS. Two cases were studied: LPS stimulation and LPS stimulation with IFN-γ. For the case of the LPS dose curve in the absence of IFN-γ an overall increasing trend was observed, NOEL's and LOEL's were established, and the NOEL and LOEL levels were similar for each time point (Table 1).

When comparing the two sets of data together, there is a significant increase in nitrite concentration in the case of LPS + IFN-γ compared to LPS alone (Table 5). This is a demonstration of the synergistic effect of IFN-γ. A response to LPS by the

macrophage cell line was used in further studies as a way of quantifying contamination found in alginate. Using IFN-γ gives the assay a much greater sensitivity towards LPS concentration and demonstrates that LPS contamination of biomaterials needs to be viewed in both the simple and more complex immune states that individual recipients may exhibit. Even lower NOEL's and LOEL's can be established for alginate contamination in the presence of IFN-γ versus its absence. At the 48 h time point with LPS + INF-γ there was no more obvious trend in nitrite response (Figs. 16 & 17). This could be attributed to the death of RAW cells caused by high levels of metabolic activity.

Observationally, with time the media solution becomes increasingly more yellow due to an increase in acidity in the solution. By the 48 h time point, the media solution is yellow, indicating a highly acidic environment which is potentially unfavorable for cellular survival. The RAW cells eventually die, ceasing to produce more NO.

The seeming fluctuations in the NOEL and LOEL values between experiments and time points are not unexpected. The DAN assay gives benchmark values for the NOEL and LOEL to be used along with other similar cytokine assays in determining acceptable levels of contamination. The NOEL and LOEL values are expected to slightly fluctuate from experiment to experiment reflecting biological variability in the most carefully replicated experimental conditions. As demonstrated earlier, it is possible for the DAN assay to detect differences for very small LPS concentrations. The use of IFN-γ is used to determine even lower levels of significant

RAW response. Therefore, due to the biological variability it serves only as a tool for approximating a significant range for NOEL and LOEL values.

Exogenous LPS incorporated in 2 % w/v alginate beads made from pharmaceutical grade alginate produced similar trends for nitrite concentrations, as observed with free LPS +/- IFN-γ. However, a much higher concentration of LPS, at least five times more in both cases without or with IFN-γ (Tables 3 & 8), had to be incorporated into the alginate to give equivalent nitrite concentrations compared to free LPS. Given the structure of alginate mesh structure formed in the presence of Ca^{+2}, LPS could be trapped inside the gel and effectively removed from detection by RAW. Another possibility could be that one the main factors that contributes to RAW cells producing NO in response to LPS is a surface area exposure effect. As spheres, alginate has less surface area contact with RAW cells. LPS incorporated into alginate causes RAW to produce much less NO production than free LPS. Therefore, alginate seems to sequester LPS causing the RAW cells to be actually exposed to less direct LPS. Regardless of the mechanism, these results suggest the inflammatory potential of LPS within a biomaterial is less that that predicted on the basis of free LPS reactivity alone.

Chapter 4: Determining the Effects of Endotoxin and Surface Contact of Different Sized Microcapsules

4.1 Introduction

Given the small surface area of each well in the 96-well plate, having a uniform layer of alginate completely cover the bottom of the plate was impossible to achieve in our lab due to the rapid gelation effect of $CaCl_2$ solution. The alginate gel would end up all on one wall of the well or the surface would be highly congurated no matter the care in adding the $CaCl_2$ solution. Also, there was concern about the ability of the RAW cells adhering to the alginate surface, functioning properly, and proliferating. This led to using the encapsulator to make beads out of the alginate and putting the beads on top of the RAW cells that had already adhered to well surface.

Given increased NO production in response to LPS added to alginate, the effect of varying the macrophage contact with the alginate was important to investigate. There has been some debate in the literature about the benefits of small versus large beads in regards to diffusion of nutrients, waste, and soluble gases (De Vos *et al.* 2002). The effect of large versus smaller beads in regards to inflammation has not been previously determined.

To study the effect on inflammation, a variety of different total alginate volumes per well and different sizes of 5 EU/mL beads were used. A moderate amount of LPS (5 EU/mL) was chosen to test this effect so the assay would not be overpowered by a stronger dose and any subtle effects could be teased out. Only the 20 and 24 h time points were examined since at 48 h, especially in the presence of IFN-γ, the NO_2 concentrations become so high that dose curve responses would no longer be readily detectable.

4.2 Experimental Design

All reagents, cell culture methods, and assays were prepared in the same manner as described above with the inclusion of forming the different sized beads. Aliquots of packed beads were added to the 96-well plate in a similar manner as described in Chapter 3.

4.2.1 Production of Different Size Beads

Below is Table 9, gives the settings used on the encapsulator to achieve the appropriate bead diameters. All batches of beads were allowed to incubate for 30 mins. in $CaCl_2$ to ensure complete gelation. The beads were washed three times with CM and stored in CM at 4^oC until use.

Outer Needle Diameter (mm)	Flow Rate (mL/hr)	KV Setting	Approximate Bead Size (μm)
0.20	7.5	5.5	200
0.35	7.5	6.0	350-400
0.50	15	6.5	500
0.70	15	6.5	750
1.1	7.5	6.5	~1000

Table 9. Encapsulator Settings for the Production of Different Sized Beads. Bead sizes were confirmed with an inverted phase microscope using a calibrated eye piece.

4.3 Experimental Results Beads of a Different Size

4.3.1 Beads of a Different Size

Beads of different sizes were made by varying the voltage and the needle diameter. To study surface contact effects, it was thought that beads of small diameter would have more contact with the RAW cells which might exacerbate a NO response; while beads of a larger diameter would have less contact with the RAW cells (Fig. 18). In the previous study 40 μL aliquots of beads were used to ensure that the entire well was covered with alginate beads. In fact sometimes there would be multiple layers of beads. Therefore to negate any effect due to the pressure of beads pushing down on other beads a 10 μL aliquot of beads varying in diameter was first used to obtain a monolayer of beads over the bottom of the plate.

A study, shown below, consisted of using a range of differing delivery volumes (5-40 μL) for 500 μm diameter beads to determine if there was a difference in delivery volume of NO_2 concentration. The purpose was to determine the lowest volume of beads that could be delivered and give a detectable NO_2 level. This

information was used to determine what volume would deliver a monolayer of beads to the bottom of a well.

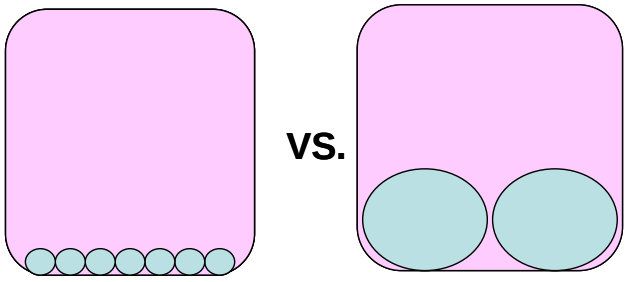

Figure 18. Schematic of Ideal Wells Containing Beads of Varying Diameters In Contact with RAW264.7 Adhered To the Bottom of the Plate. Beads of smaller diameter would have more direct contact with the bottom of the well while larger beads would not. (Not drawn to scale)

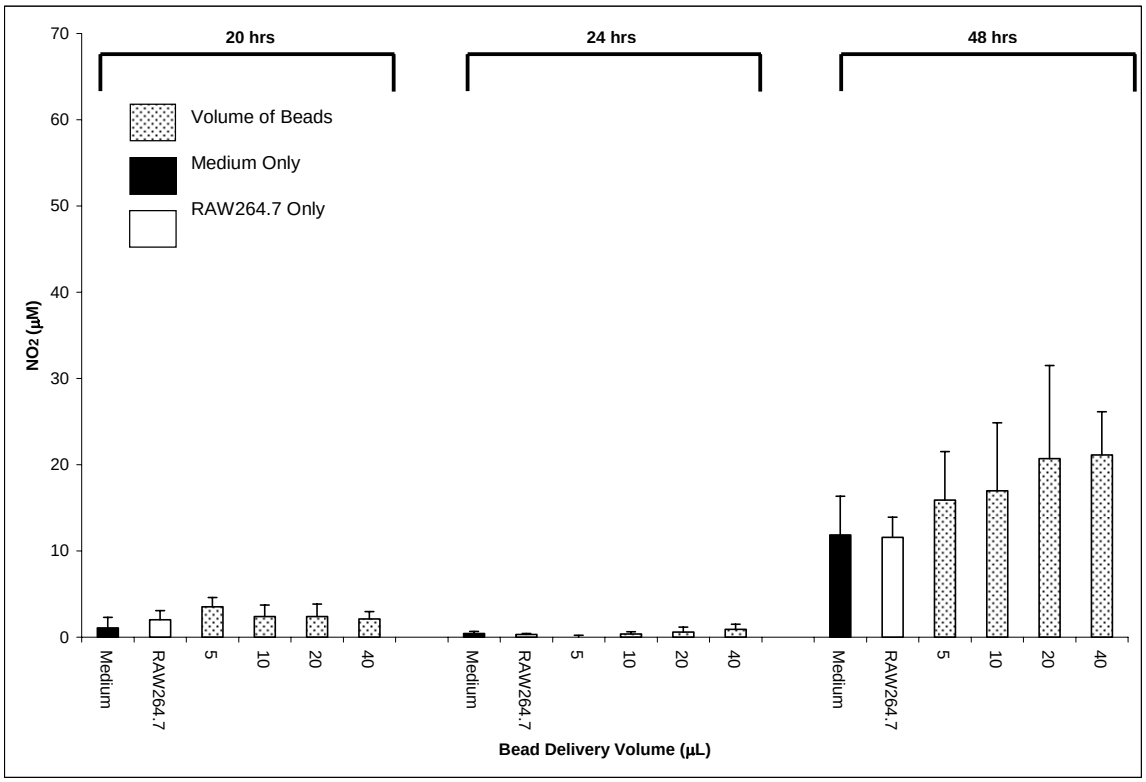

Figure 19. Different Delivery Volume for Beads of 500 μm In Diameter In the Absence of IFN-γ.

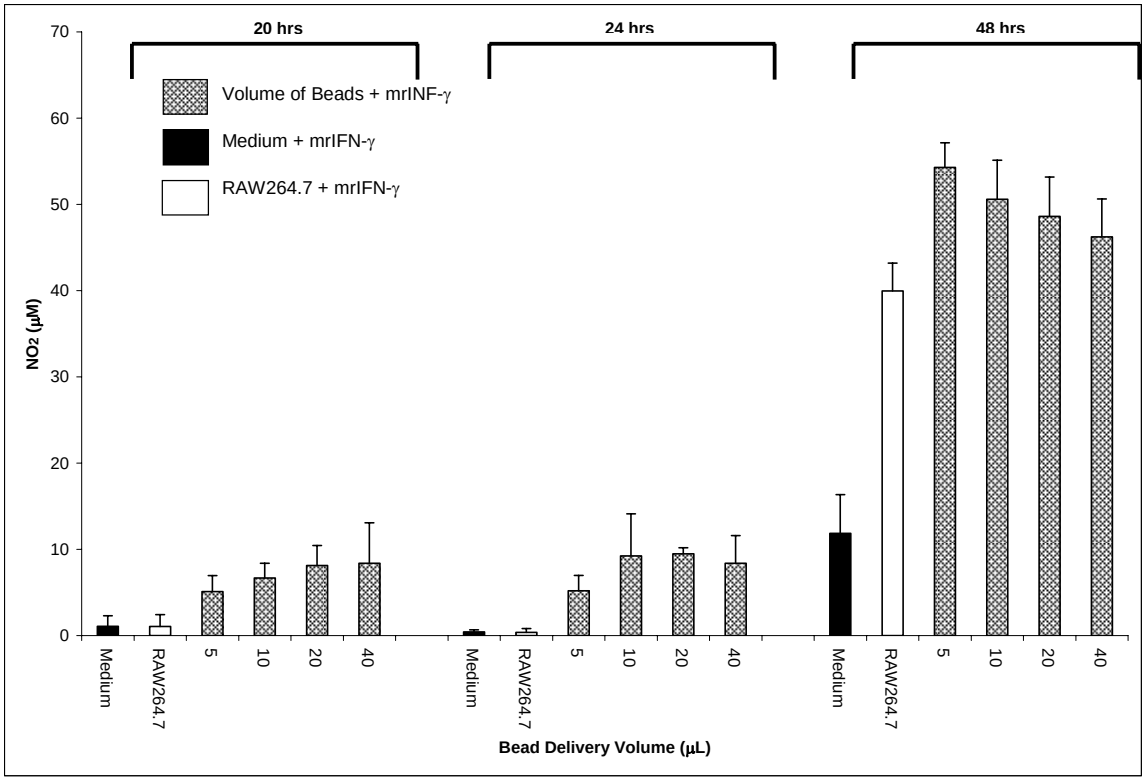

Figure 20. Effect of Delivery Volumes for Beads of 500 μm In Diameter On NO Production In the Presence of IFN-γ.

In the absence of IFN-γ, there seemed to be conflicting evidence whether increasing the volume causes an increase in NO_2 concentration (Fig. 19). At 48 h, there was some evidence that a dose curve based on delivery volume might be present. More evidence seemed to support this theory when IFN-γ was present at 20 and 24 h (Fig. 20). One possibility for this weak correlation was that the NO_2 concentrations are so low that the DAN assay cannot easily detect such minute differences in concentration. The beads contain 5 EU/mL of LPS (a moderate dose), however given that different volumes of beads are delivered to the well there are, theoretically, different amounts of LPS present due to the varying number of beads. Also, there were multiple layers of beads at the higher volumes. Given that 10 μL was the minimum delivery volume that reproducibly delivered beads and produced

significant NO_2 results, it was used as the aliquot volume in the subsequent experiment.

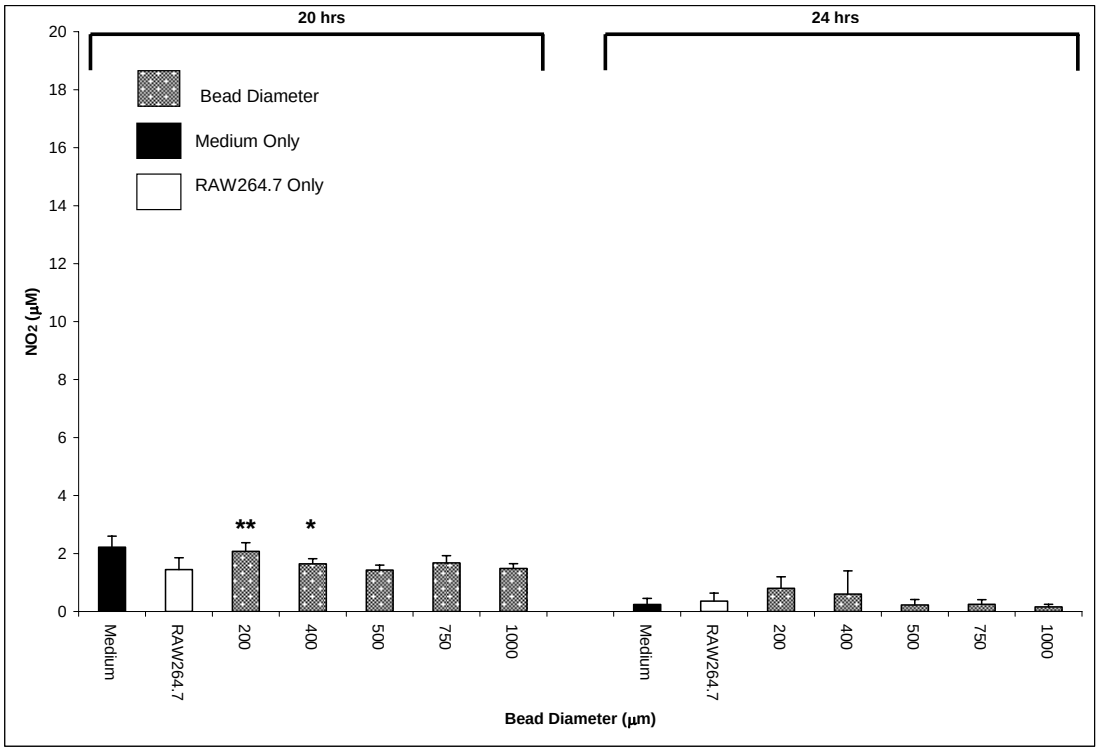

Figure 21. Using 10 µL Aliquots To Deliver Beads of Varying Diameter Containing 5 EU/mL of Exogenous LPS. A NO_2 dose response is seen at 20 h. The NOEL (*) occurs at beads of 400 µm and the LOEL is at beads of 200 µm. No obvious trend was seen at 24 h since none of the conditions were significantly different from the control RAW.

Time		Bead Diameter (µm)	NO_2 (µM)	P (t≤T) = 0.05
20 h	***NOEL***	400	1.644	0.3
	LOEL	200	2.073	0.05
24 h	***NOEL***	N/A		
	LOEL	N/A		

Table 10. Summary of NOEL and LOEL Values of Bead Stimulation of RAW264.7 at Two Time Points.

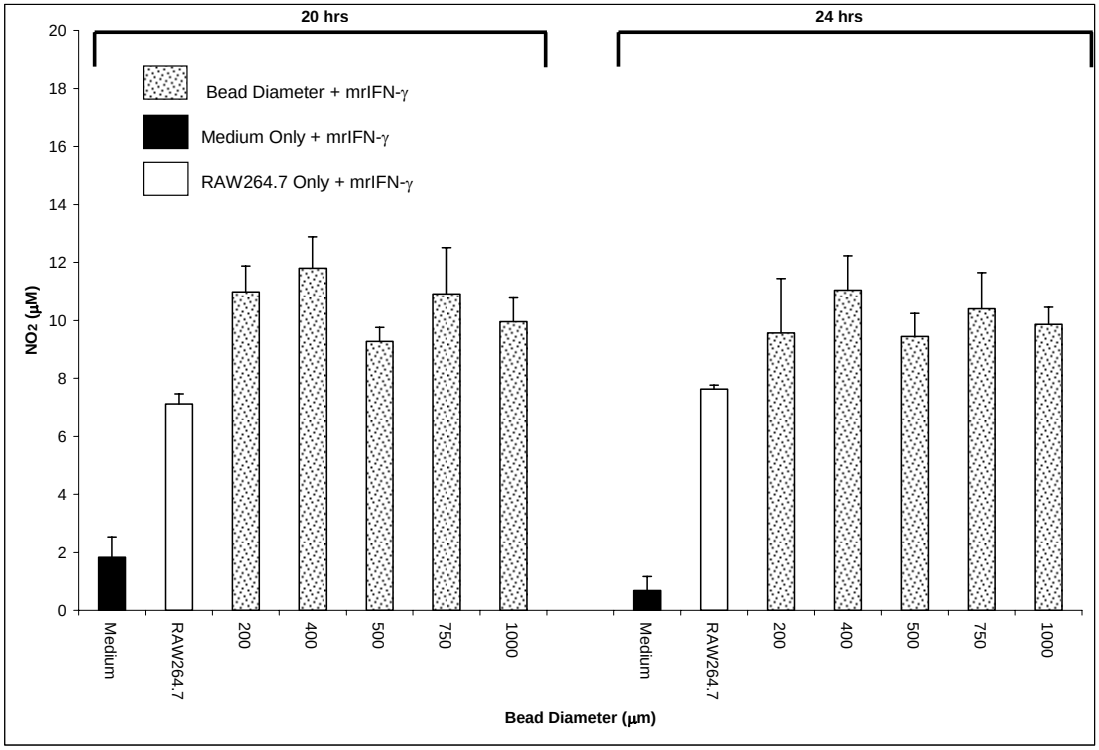

Figure 22. Graphical Results of 10 µL Aliquots of Bead Diameters Ranging From ~200-1000 µm In Diameter Containing 5 EU/mL LPS In the Presence of IFN-γ.
No obvious NO_2 trend or NOEL/LOEL values could be determined due to the increased sensitivity of RAW by IFN-γ.

All bead preparations contained a concentration of 5 EU/mL LPS to ensure a reasonable NO response that was measured by the DAN Assay. Bead diameters were varied from approximately 200 to 1000 µm. The increments and sizes were determined by varying the needle diameters. Bead diameters were confirmed using an inverted phase microscope. All measurements were done in quadruplet. In the absence of IFN-γ, a NO response was observed compared to the control (Fig. 21). The amount of NO_2 present decreased with increasing bead size. Upon inspection, the wells that had smaller bead diameters contained many more beads. In fact, for the 200 and 400 µm case, a simple monolayer was not achieved; instead there was overlap of beads indicating multiple bead layers. Wells that were suppose to contain a

monolayer of beads 750 or 1000 μm in diameter actually only had a few beads; insufficient for a monolayer (Fig. 23).

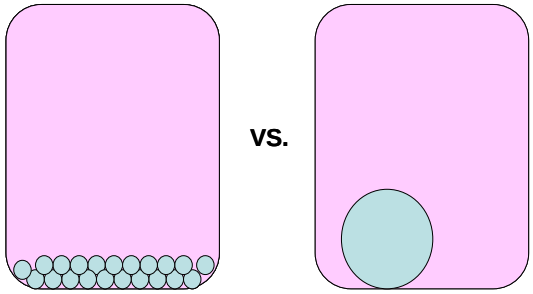

Figure 23. Schematic of Two Different Wells Containing Beads of Much Different Diameter. Beads of a smaller diameter formed multiple layers at the bottom of the well while beads of a larger diameter contained not enough beads to cover the bottom of the well. (Not drawn to scale)

In the presence of IFN-γ, the NO_2 concentration increased for every condition (Fig. 22). All of the bead diameters at both time points contained significantly more NO_2 compared to the control of RAW with no beads. However, the relationship between inverse bead diameters to NO_2 concentration was absent. Therefore, it was decided that different aliquots would be used for each bead diameter.

4.3.2 Beads of Varying Diameter and Delivery Volume

Since using a 10 μL aliquot of alginate beads did not ensure only a monolayer beads at the bottom of the well, the experiment was repeated with some modifications. Through trial and error different delivery volumes were determined for each bead diameter. For the case when 10 μL of 200 μm beads gave multiple layers of beads at the bottom of the well, the delivery volume was not changed due to decreased delivery accuracy of pipets below 10 μL.

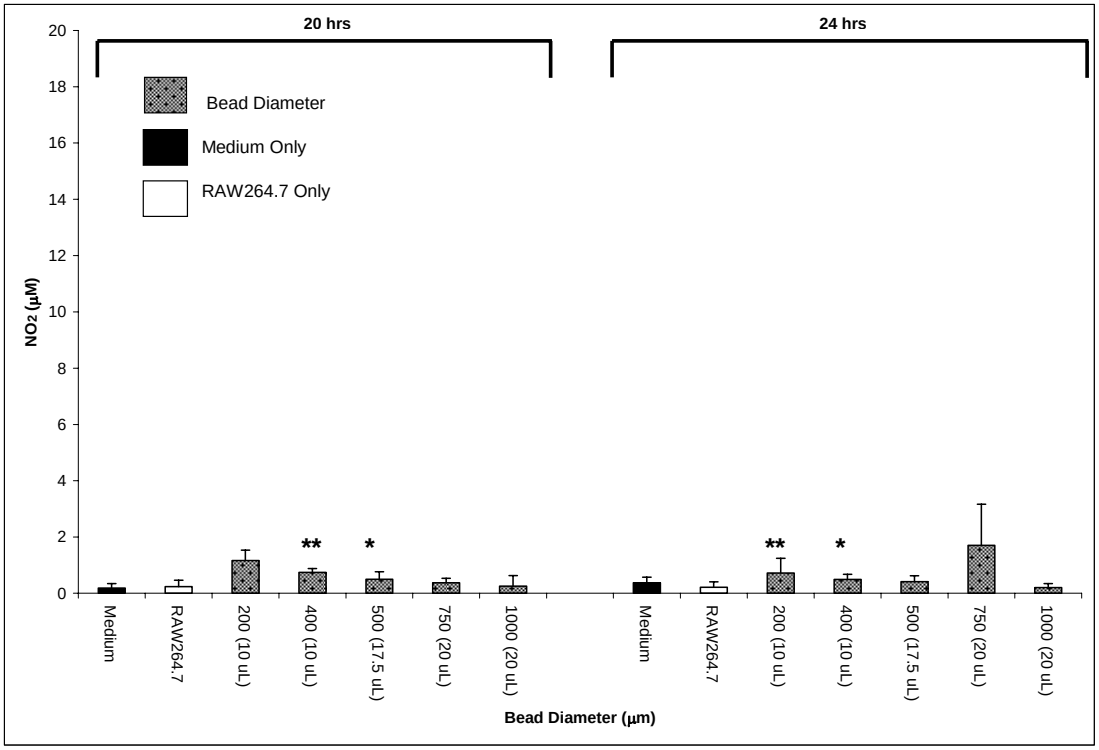

Figure 24. Modified Dose Curve with Bead Diameter and Delivery Volume In Parentheses In the Absence of IFN-γ. The NOEL(*) was for beads of 500 μm in diameter and the LOEL (**) was 400 μm for 20 h. For the 24 h time point, the NOEL (*) was 400 μm and the LOEL (**) was 200 μm. An inverse NO_2 response was seen in relation to the bead diameter.

Time		Bead Diameter (μm)	Delivery Volume (μL)	NO_2 (μM)	P (t≤T)= 0.05
20 h	***NOEL***	500	17.5	0.4976	0.06
	LOEL	400	20	0.7448	0.006
24 h	***NOEL***	400	10	0.4875	0.06
	LOEL	200	10	0.7161	0.04

Table 11. Tabular Results of Modified Dose Curve Experiment with NOEL and LOEL's Listed for Each Time Point.

Again, there was an inverse relationship between bead diameter and NO_2 concentration in the absence of IFN–γ (Fig. 24). Beads of 200 μm caused the highest increase in NO_2 compared to the control (RAW cells with no beads present) due to there being many more beads present then, say, 1000 μm. While the results from this

experiment are not as dramatic as in the previous study, the inverse relationship between bead diameter and NO_2 concentration lends support to the use of larger beads for encapsulation of islets. The larger beads might provide better protection from macrophages due to less actual contact per contained unit of islet cells. In the case when IFN-γ is present, similar results were obtained as before in the 10 μL case with no obvious dose trend except that there was a significant increase in NO_2 at each condition (Fig. 25).

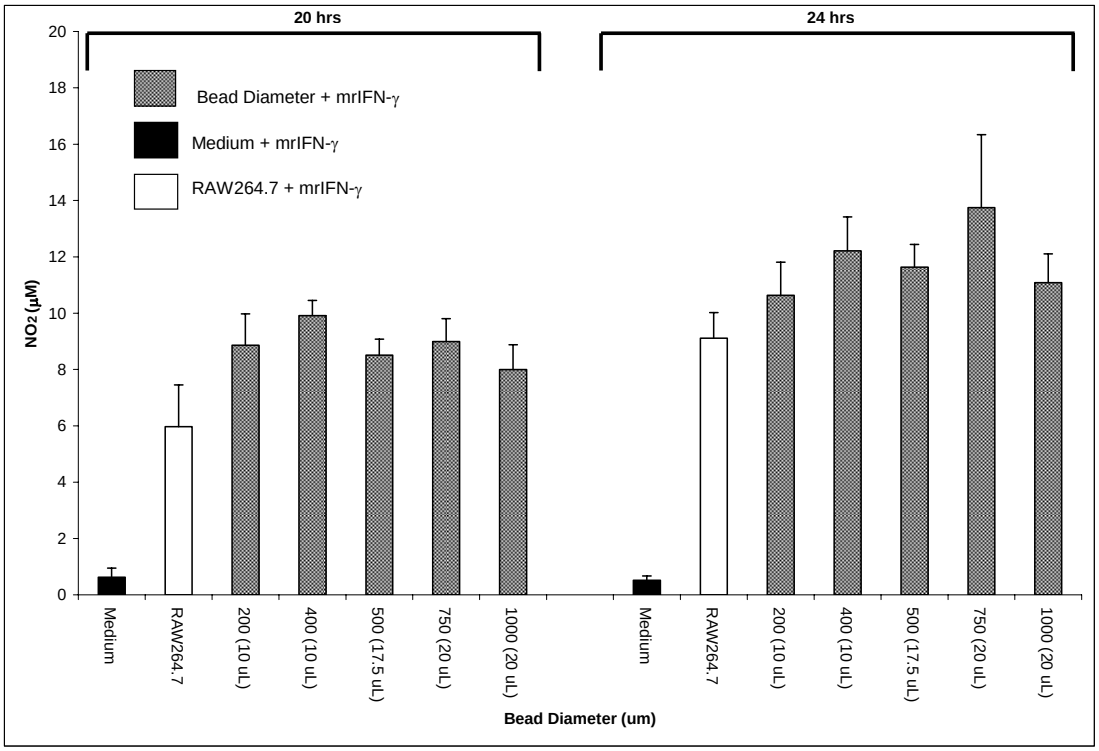

Figure 25. Modified Bead Delivery Experiment with Varying Delivery Volumes In the Presence of IFN-γ. An increase in NO production is seen at each condition which increases at the 24 h time point. No NOEL or LOEL could be determined.

4.4 Conclusions

4.4.1 Conclusions of Beads of a different size

The hypothesis for this set of experiments that increased direct contact between the beads and the RAW cells would cause an increase in NO production. The logic behind this was that smaller beads have a smaller surface area therefore many more beads would have a point of contact to the RAW cells (Fig. 18). This increase in contact with the RAW cells would increase NO production due, to the greater extent, of the documented phenomenon of macrophages becoming more active when they sense that they can not engulf foreign bodies and instead increase production of toxic immune mediators. Larger beads would have less actual contact with fewer RAW cells, decreasing RAW activation.

In the absence of IFN-γ, a dose response to beads of differing diameters was present (Table 10). The inverse relationship between bead diameter and NO_2 concentration was likely due to the wells with smaller sized beads contacting RAW cells more that the same volume large beads. The increased response to the LPS in alginate with beads of a smaller diameter was not evident in the presence of IFN-γ. This demonstrates the potency of IFN-γ to sensitize the RAW cells to produce maximal NO levels even in the presence of a minute amount of endotoxin. This would model a "HOT" immune response where macrophages have already been activated by a cytokine and produce a sustained level of NO. A minor variation on degree of direct contact between the encapsulated beads and the tissue macrophages may therefore be irrelevant.

4.4.2 Conclusions to Varying the Diameter and Volume of Beads

By maintaining a monolayer of beads between wells and while varying bead size, surface contact effects between beads and RAW could be studied. The observed inverse response to bead diameter and NO_2 concentration supports the idea that having smaller beads does result in more contact with the RAW cells, evoking a greater response (Table 11). An increase in the bead size, conversely, decreased the contact between the beads and the RAW cells, therefore causing a decrease in inflammatory response to endogenous LPS contaminated alginate. From the previous experiments it was seen that since much more LPS can be incorporated into the alginate compared to free LPS it must be sequestered by the alginate. However, in addition to direct contact effects, minute quantities of LPS may be leaching out over time adding to the degree of response.

With the addition of IFN-γ, there is a disappearance of any correlation between beads size and NO_2 concentration (Fig. 25). Increased macrophage sensitivity may cause a maximal response due to passing threshold levels of direct contact with the LPS-containing alginate or leached LPS. This information is important to graft implantation in that when the graft is first implanted there is an expected increase in inflammation solely due to tissue trauma at the site. Any IFN-γ being elaborated at the site could overwhelm mechanistic effects (such as varying bead size) to modulate subsequent inflammation.

Chapter 5: Comparison of Anti-Inflammatory Incorporated Drugs on NO Response

5.1 Introduction

Even small amounts of LPS contamination in alginate can cause a robust NO response from RAW. In this series of experiments, different anti-phagocytic drugs were incorporated into alginate capsules as a way to retard macrophage attack; specifically, NO production. The concept is that macrophages could be inhibited from reacting to the impurities in alginate, through drugs leaching out of the gel or through direct contact with drug-loaded alginate leading to macrophage death. The ideal drug candidate would have to be potent at low (nanomolar to low micromolar) concentration and have little to no effect on islet function. If shown to inhibit macrophage activation, such drugs might potentially increase the lifespan of implanted alginate encapsulated islets.

Two classes of drugs, bisphosphonates and parthenolide, were studied for their effectiveness on cessation of NO production by RAW; either free in the culture medium or incorporated into alginate beads. Both of these drugs have been previously studied with respect to macrophages or cell types similar in function to macrophages and are good candidates to provide NO inhibition as suggested above. The drugs

studied demonstrated strikingly different efficiencies for modulating NO production by RAW.

5.2 Bisphosphonates: Clondronate and Alendronate

5.2.1 Description of Bisphosphonates

Bisphosphonates were first used in the 1930's as a water-softener (Graham *et al.* 2007). Clinically, they are the drugs of choice when treating bone diseases involving hypercalcemia and bone resorption. Osteoporosis (brittle bones) is the result of overactive osteoclasts, the cellular type responsible for bone breakdown, which are derived from the macrophage lineage. Currently, bisphosphonates, though most commonly associated with the treatment of osteoporosis, are also used in treating Paget disease, myeloma, bone metastases, and pediatric bone diseases. Bisphosphonates are suspected to effect osteoclast recruitment, differentiation, resorptive activity, and may cause the cells to apoptose.

Bisphosphonates are analogs of inorganic pyrophosphate, which is a byproduct of hydrolyzed adenosine triphosphate (ATP), and is resistant to enzymatic breakdown. There are two main types of bisphosphonates (Fig 26): non-amino-bisphosphonates and amino-bisphosphonates which have different cellular mechanisms. Non-amino bisphosphonates, like clondronate (Fig. 27), may inhibit ATP-dependent cellular mechanisms possibly through the metabolite β,γ-dichlormethylene ATP ($AppCCl_2p$) (Makkonen *et al.* 1999). While amino-containing

bisphosphonates, such as alendronate (Fig. 27), inhibit enzymes of the cholesterol synthesis pathway, leading to apoptosis. The exact mechanism of action for either class of bisphosphonates is not fully understood but different bisphosphonates are more effective than others due to differences in R_2 groups. However, all bisphosphonates are capable of chelating calcium (Graham *et al.* 2007).

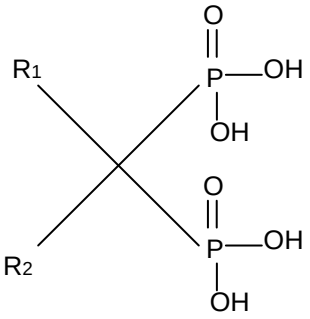

Figure 26. Basic Structure of Bisphosphonate.

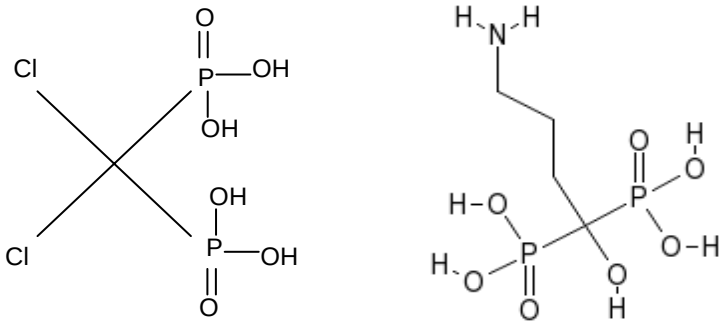

Figure 27. Chemical Structures of Clondronate (right) and Alendronate (left).

Osteoclasts and macrophages both originate from the monocyte/phagocyte system and have endocytic properties. Bisphosphonates are preferentially absorbed by osteoclasts over other cell types, adding to its success as an anti-resorptive drug. Due to the close relationship between macrophages and osteoclasts, researchers have

branched out to demonstrate the potential of bisphosphonates for treating inflammatory diseases such as rheumatoid arthritis.

New research has explored the opportunity of using bisphosphonates, specifically clondronate, to deplete peritoneal macrophages as a way to increase the survival of encapsulated porcine neonatal islets (Omer *et al.* 2003). Over time, fibroblast overgrowth and macrophages surround and adhere to alginate capsules instigating premature failure of islet grafts. A clondronate solution, flushed through the peritoneal cavity before graft transplantation, prevented the overgrowth of macrophages on the macrocapsules.

Macrophages become more susceptible to bisphosphonates when they are encapsulated in negatively charged liposomes. The Trojan Horse "liposome-mediated macrophage 'suicide' technique" was described by Van Rooijen et al (1989) as a way to deliver bisphosphonates to macrophages to prevent premature bisphosphonate hydrolysis in solution, dramatically increasing their potency. Once endocytosied by the macrophage, the liposome layer is degraded exposing the bisphosphonate.

In our present study, two types of bisphosphonates, clondronate and alendronate, were used to investigate a decrease in the NO production of RAW under a variety of conditions. Clondronate is a non-amino-containing bisphosphonate that inhibits DNA activation by NF-κB and AP-2, diminishing cytokines, NO release, and iNOS expression; a potentially ideal drug for an anti-inflammation study. Alendronate, on the other hand, is an amino-containing bisphosphonate that should have little effect on macrophages, and is used as a negative control.

5.2.2 Experimental Design of Alendronate and Clondronate Plates

The bisphosphonates were dissolved in CM and a dilution curve was made as indicated on the plate was prepared as indicated in Figure 28. RAW cells were cultured as described previously. All other reagents were made as described previously. Each plate contained a combination of LPS, drug, and IFN-γ, Samples were taken at 20, 24, and 44 h and analyzed using the DAN assay as described previously.

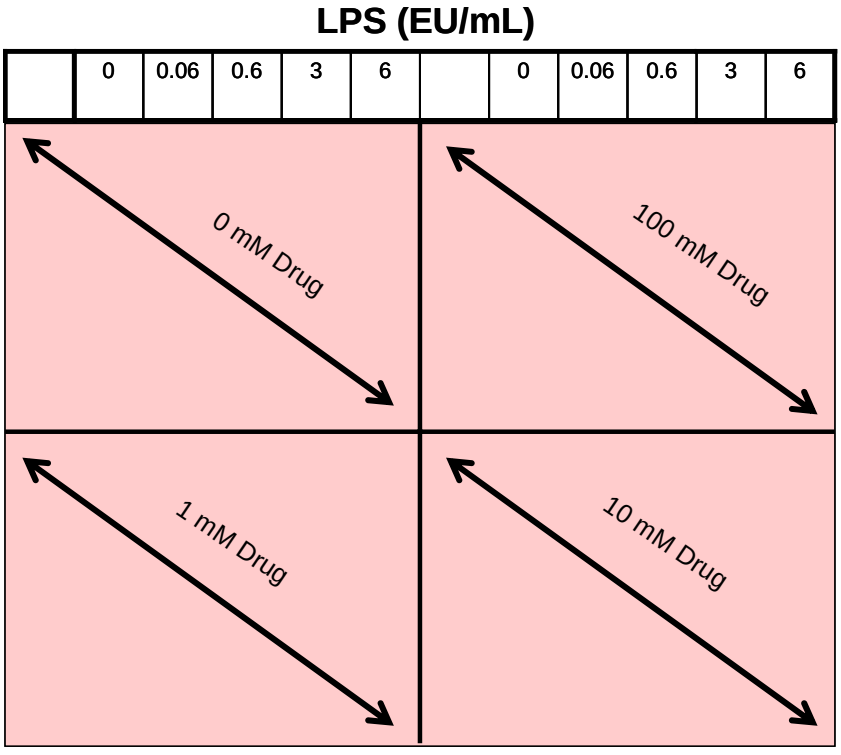

Figure 28. Example of Bisphosphonate Plate Setup.

5.2.3 Results of Alendronate

Alendronate was tested for the mitigation of NO production by macrophages. To simulate an inflammatory situation, low to moderate amounts of LPS (0-6 EU/mL) were added to the macrophages. Superimposed on the inflammatory stimuli was a dose curve (0-175 μM) of alendronate. Measurements were taken at three time points: 20, 24, 44 h. Each graph represents one time point in the absence or presence of IFN-γ.

As shown in previous experiments, increasing the amount of LPS present in the culture medium caused an increase in NO_2 concentration. However, at moderate amounts of LPS (3 or 6 EU LPS) there was no significant decrease in NO_2 concentration at 175 μM compared to 0 μM alendronate at 20 h (Fig. 29). In the presence of 0.6 EU/mL LPS there was an inverse dose response to alendronate at all the time points. RAW, in an unstimulated state, produced very little NO and was unreactive. With a low dose of LPS that is just enough to provide minimal stimulation, the RAW cells seemed more inclined to uptake alendronate.

At very low amounts of LPS stimulation, especially at 0.6 EU/mL LPS, there was an inverse dose response to alendronate. In the case were no LPS was present, there was a small inflammatory effect of alendronate seen by increased NO_2 levels. However, this trend did not progress over time (Figs. 29-31).

With a very low concentrations of LPS (0.6 EU/mL) there was a clear reduction in NO_2 with increasing alendronate. Over the course of the experiment, there was approximately a 40-55% reduction in NO_2 concentration at 175 μM

compared to 0 μM alendronate. Still, the NO_2 concentration at 175 μM in the presence of 0.6 EU/mL LPS was significantly higher than the control when no LPS or alendronate is present.

At moderate doses of LPS, 3 or 6 EU/mL, the RAW cells were unreactive to alendronate. The stronger LPS effect seemed to overtake any inhibitory effect of alendronate. Little decrease in NO_2 was observed at the highest concentrations of alendronate.

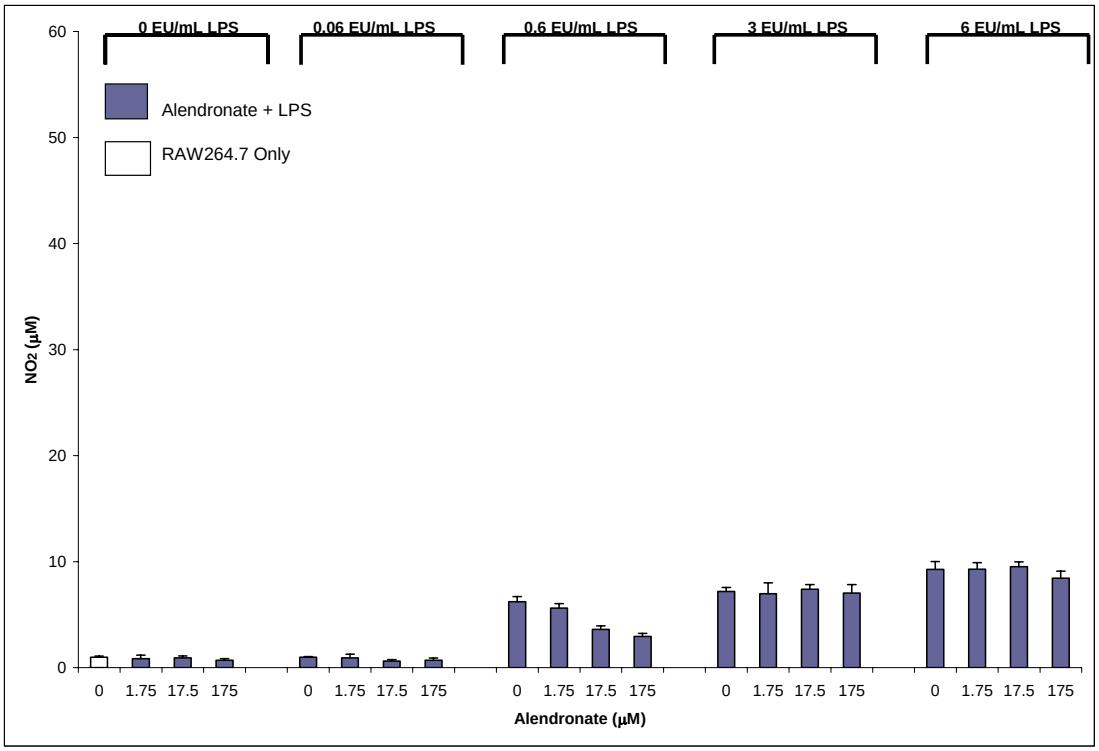

Figure 29: Alendronate and LPS Doses Curves at 20 hr. As LPS concentrations increase there is an increase in NO_2 at all alendronate concentrations. At 3 and 6 EU/mL LPS there is no significant difference between the control and the highest concentration of alendronate (175 μM). Under very mild stimulation of LPS (0.6 EU/mL) there is a 53% reduction in NO_2 at 175 μM alendronate compared to the control.

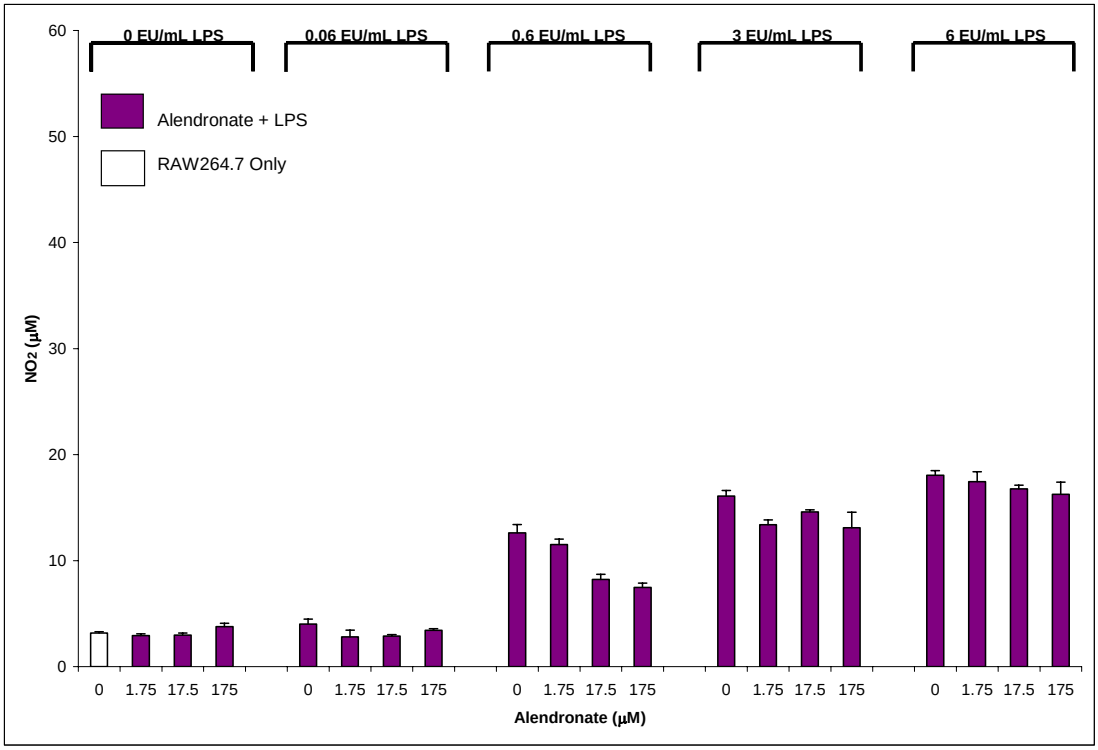

Figure 30. Alendronate and LPS Dose Curves at 24 h. As time and LPS concentration increase, NO_2 levels increase.

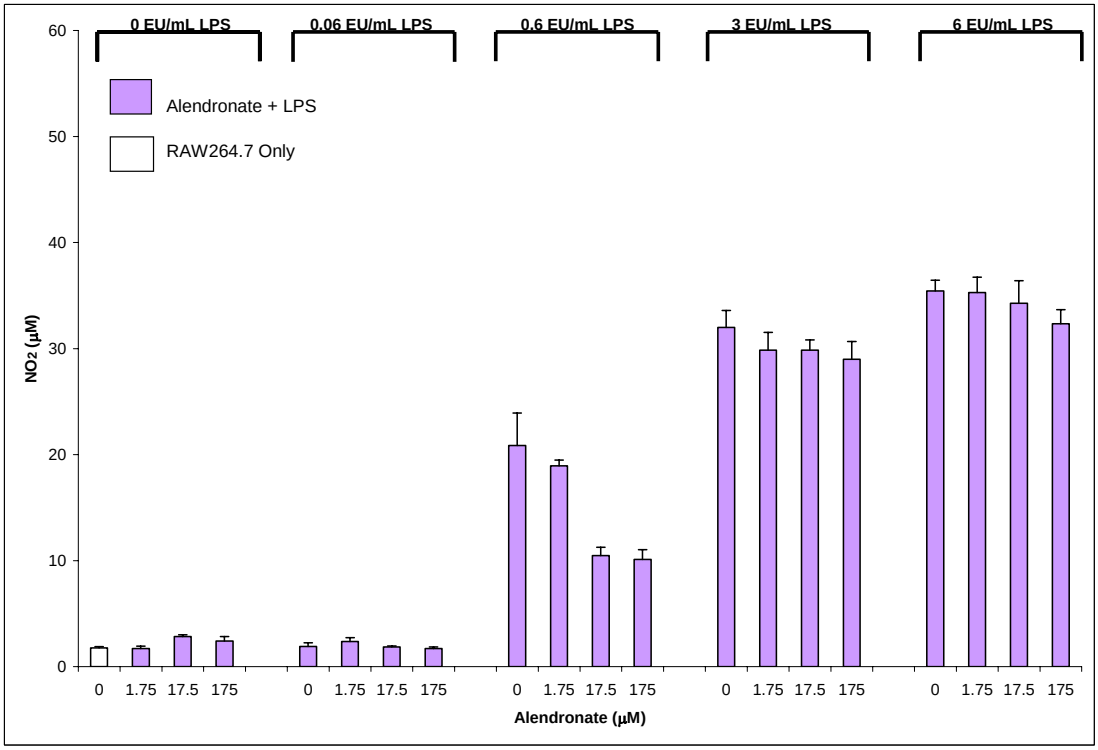

Figure 31. Alendronate and LPS Dose Curves at 44 h. The same trends continue with the largest decrease in NO_2 occurring when in the presence of 0.6 EU/mL LPS.

The addition of IFN-γ to LPS plus alendronate simulated a more complex immune reaction (Figs. 32-34). The addition of INF-γ caused a significant increase in NO production compared to the case where IFN-γ was absent. However, the LPS controls where no alendronate was present were questionable and the NO response was not as strong as expected. Adding alendronate seemed to cause no to very little effect on NO_2 concentration; neither stimulatory nor inhibitory. Overall, as time increased the NO_2 levels also increased to the 44 h mark were the RAW cells are normally seen to be unresponsive to additional stimuli.

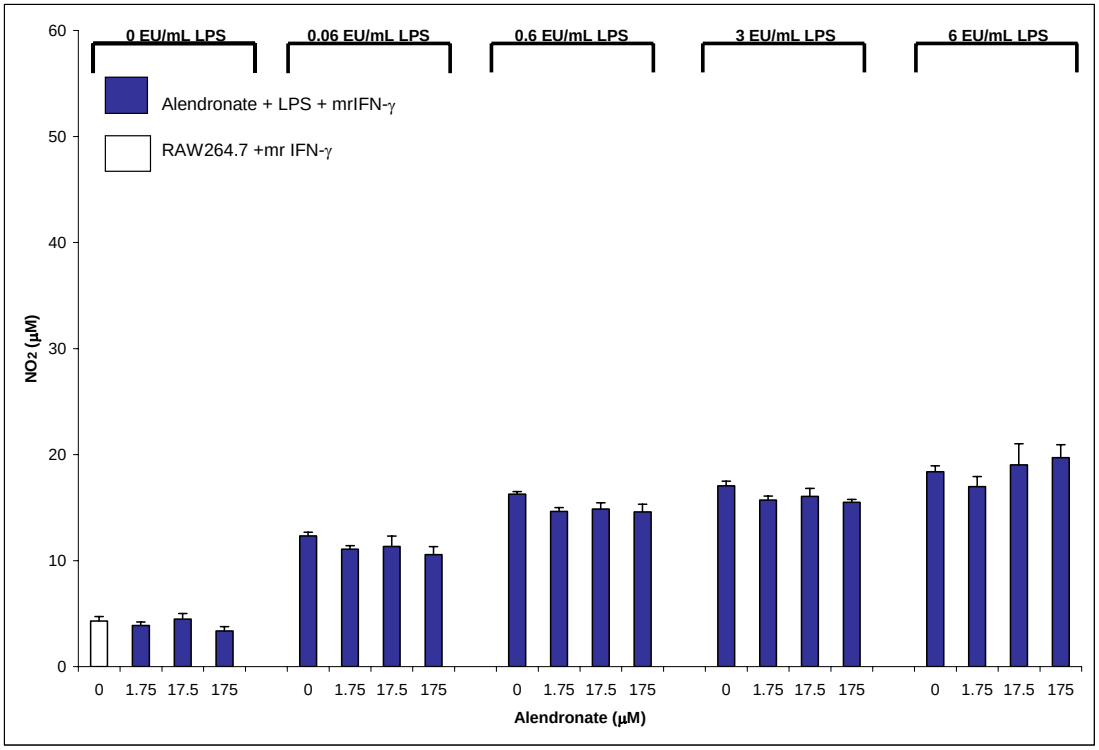

Figure 32. Alendronate and LPS Dose Curves In the Presence of IFN-γ at 20 hr. As LPS increases, NO_2 increases.

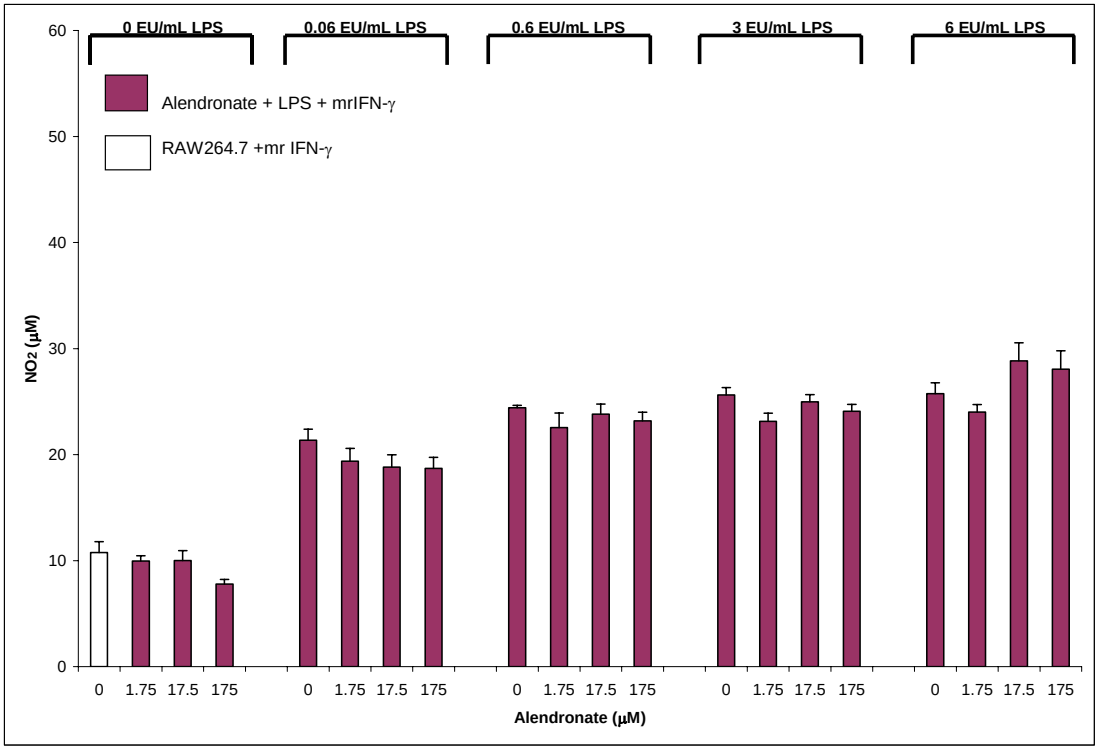

Figure 33. Alendronate and LPS Dose Curves In the Presence of IFN-γ at 24 h.
There is no significant decrease in NO_2 with increasing alendronate.

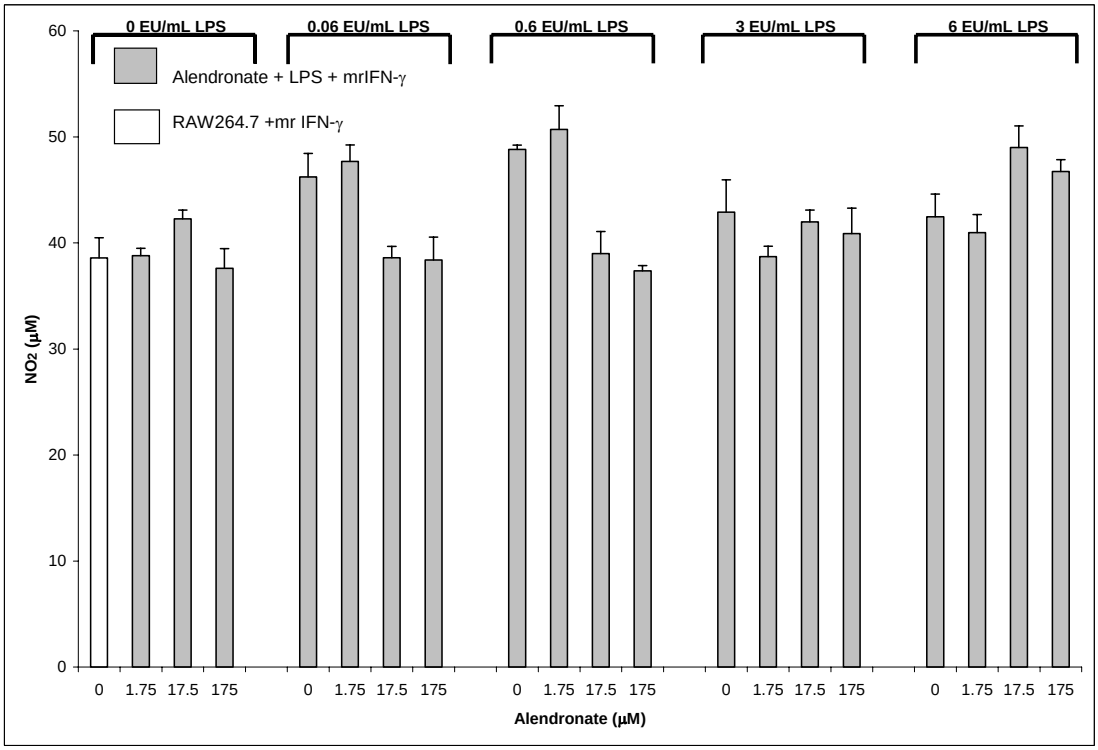

Figure 34. Alendronate and LPS Dose Curves In the Presence of IFN-γ at 44 hr.
No decrease in NO_2 due to alendronate is present.

Cytokines, such as IFN-γ, cause macrophages to produce more NO. In the previous set of experiments, there were two competing factors on NO production: alendronate and LPS. Adding IFN-γ caused less of a decrease in NO_2 in the presence of alendronate. As the reaction proceeds, the degree of decrease in NO_2 diminishes to the point where there is no more significant decrease at moderate amounts of LPS. At 44 h, there was no observable trend due to alendronate being present (Fig. 34).

5.2.4 Results of Clondronate

In the literature, clondronate has been seen in different in vivo systems to dramatically decrease the number of macrophages present. In this study, clondronate was tested over a range of concentrations, in the presence of various LPS concentrations, and the absence or presence of IFN-γ. Contrary to some published in vivo results, clondronate seemed to have little effect on NO production. Increasing LPS caused an increase in NO_2, but the inhibitor effects of clondronate are even less evident than was the case with alendronate. Only at 44 h (Fig.37), was a small decrease in NO_2 evident, and only at the highest concentration of clondronate.

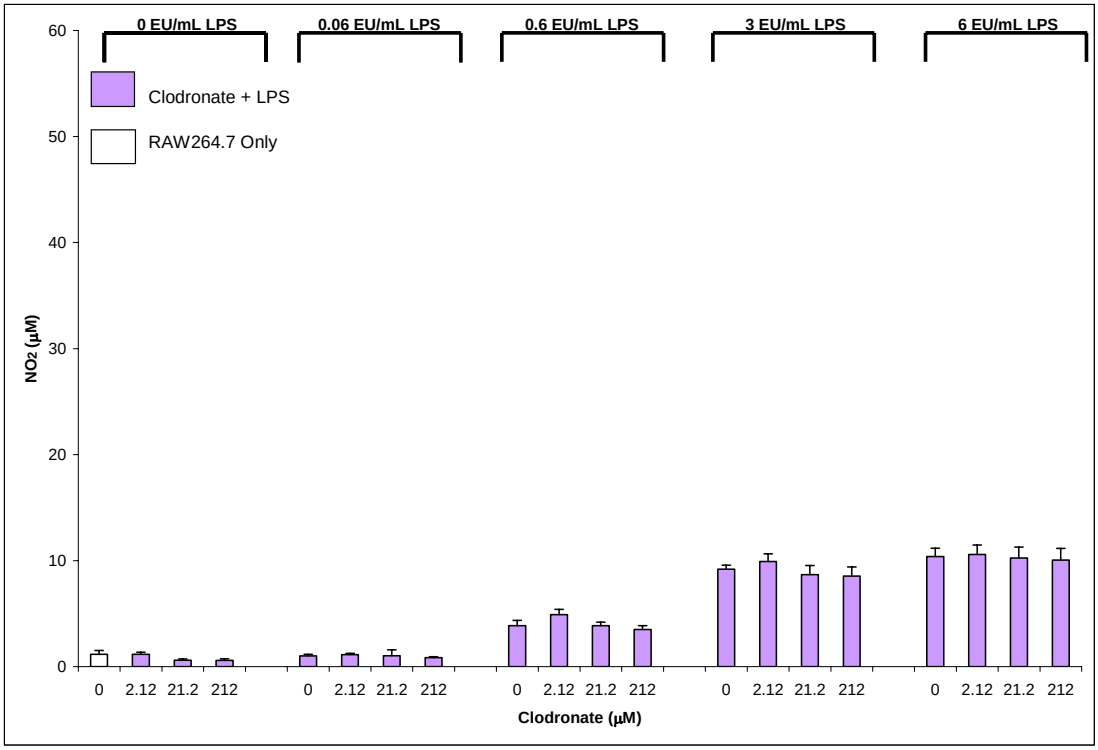

Figure 35. Clondronate and LPS Dilution Curve at 20 h. Overall there is no significant difference in NO_2 between the control and the highest clondronate concentration.

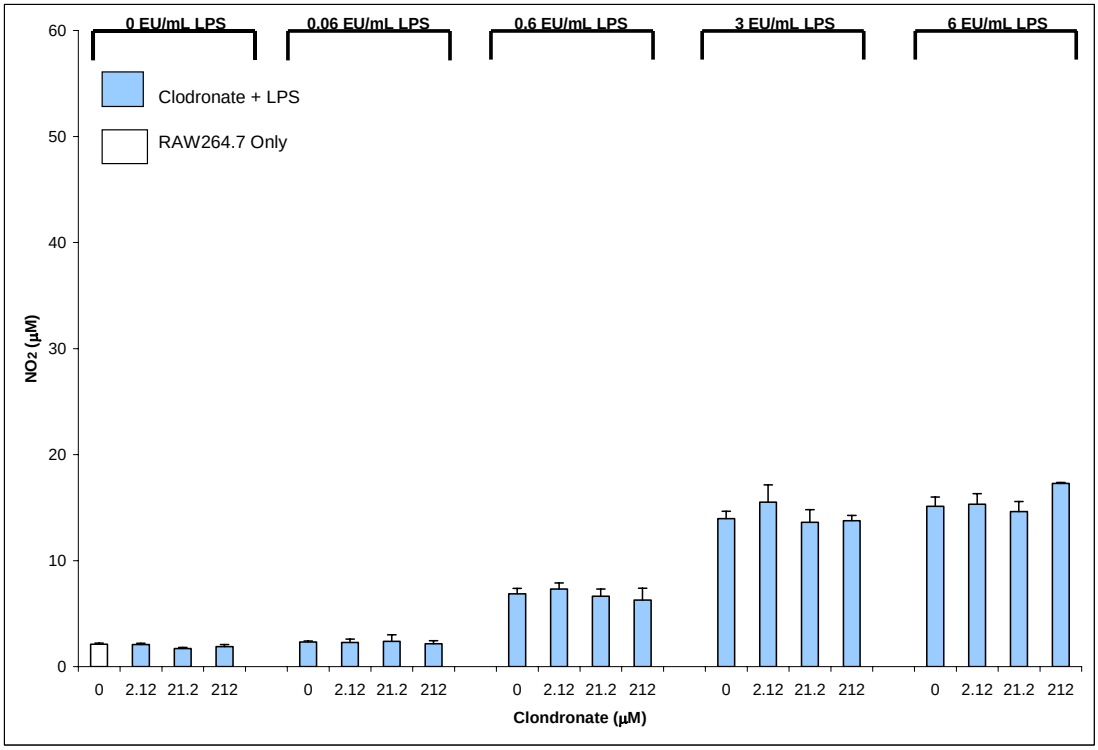

Figure 36. Clondronate and LPS at 24 h. No significant decrease in NO_2 due to clondronate in the presence of LPS. As LPS increases the NO_2 levels increase.

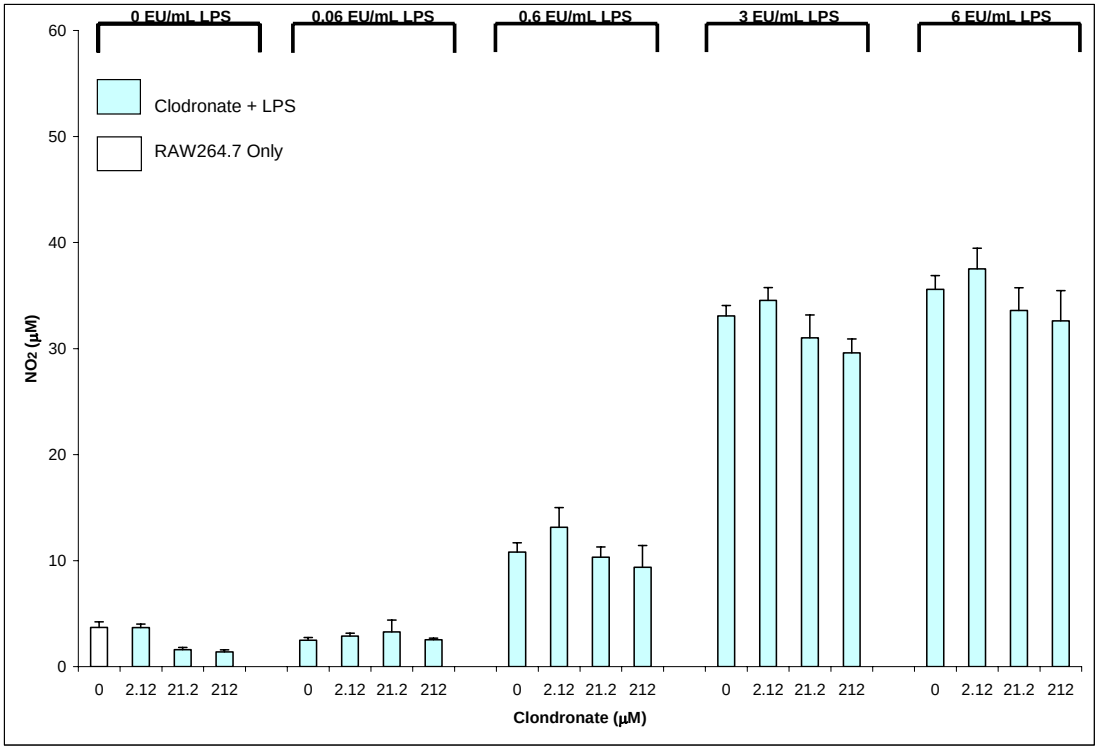

Figure 37. Clondronate and LPS Dose Curves at 44 h. Addition of LPS results in sporadic dose dependencies.

The addition of IFN-γ did not cause the clondronate to be inhibitory (Figs. 38-40). With the presence of IFN-γ, there was the expected increase in NO_2, however in this experiment the LPS controls (where no clondronate was present) did not respond as robustly as expected. Clondronate either has no effect on the interferon-mediated NO increase or only gave a sporadic, small decrease with no consistent (as was observed with LPS).

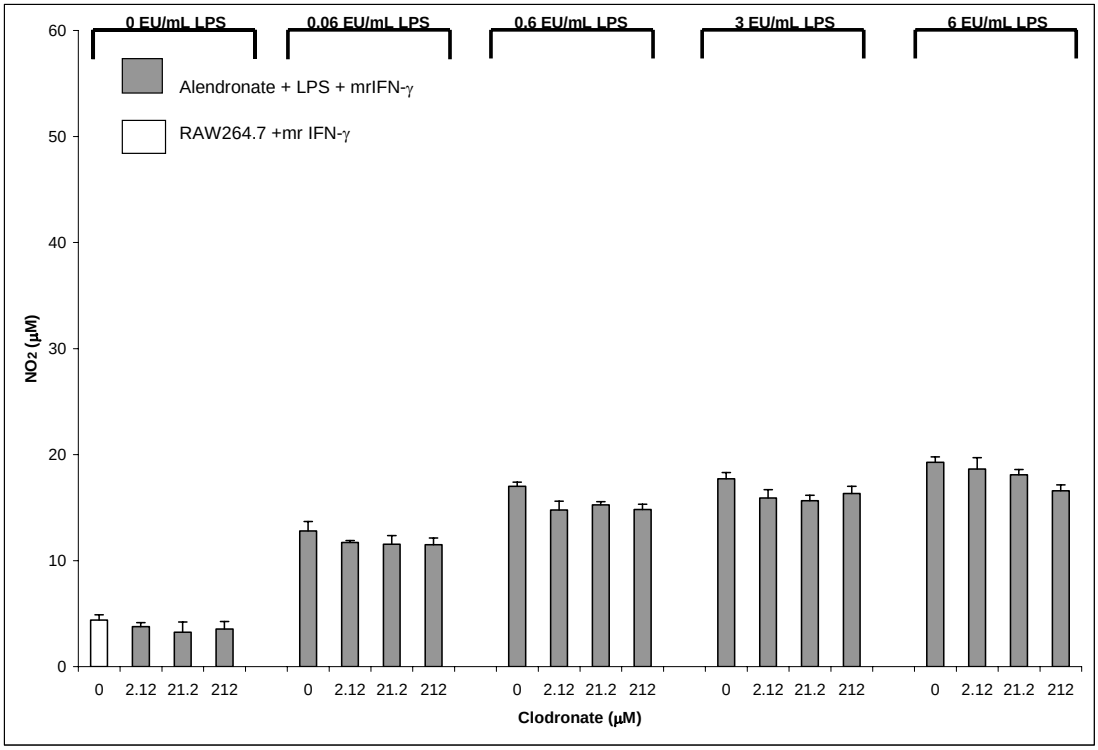

Figure 38. Clondronate and LPS In the Presence of IFN-γ at 20 h. No significant differences from the control by clondronate were observed.

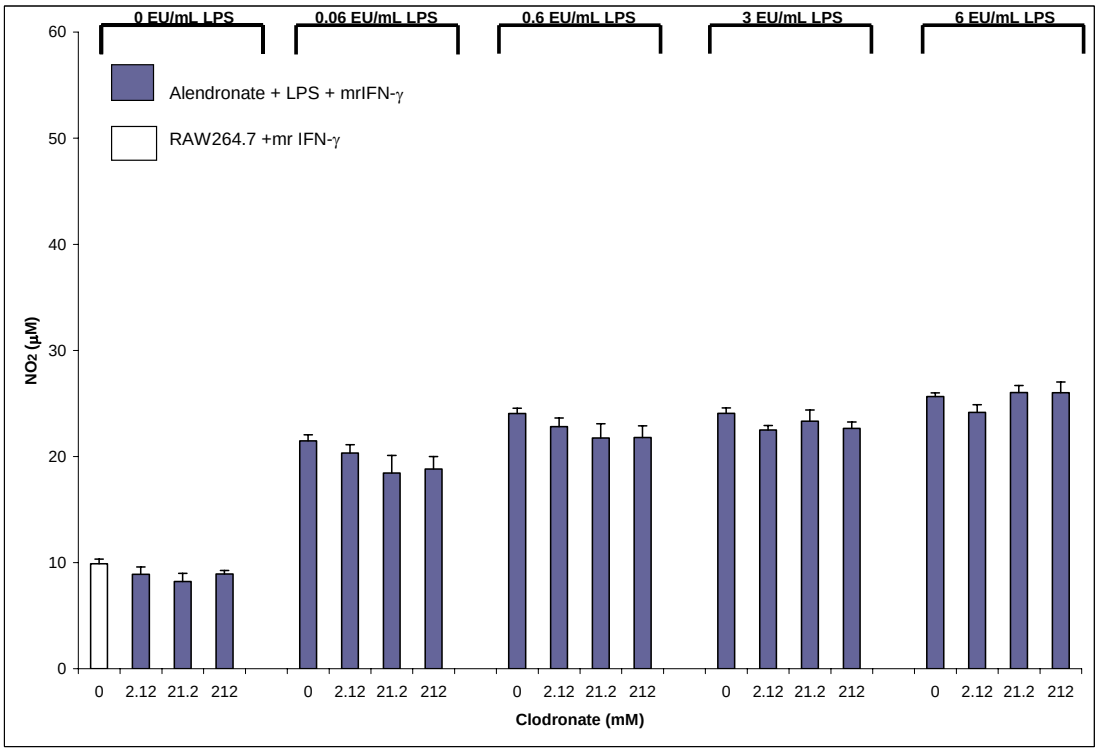

Figure 39. Clondronate and LPS In the Presence of IFN-γ at 24 h. There is an increase in NO_2 in response to LPS and IFN-γ. There is no consistent of significant clondronate effect.

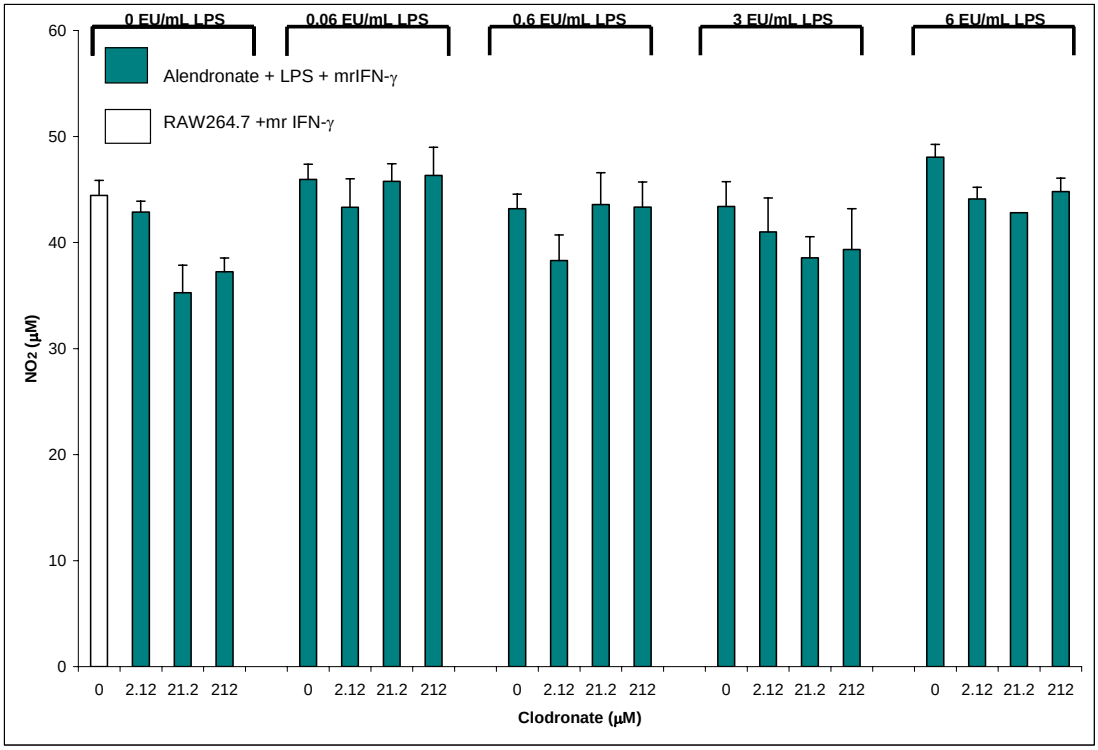

Figure 40. Clondronate and LPS In the Presence of IFN-γ at 44 h. There is no discernable dose dependence. Assay has "maxed" out.

5.3 Parthenolide

5.3.1 Description of Parthenolide

Parthenolide is a component of the herb, Feverfew (*Tanacetum parthenium*) (Fig. 41), which has been used for centuries in herbal medicine for the treatment of headaches, prevention of blood clots, and in treatment of arthritis (an inflammatory disease) (Heptinstal 1988). Sesquiterpene lactones, such as parthenolide, are known to be potent inhibitors of the NF-κB pathway (Hehner *et al.* 1999; Kang *et al.* 2001; Sheehan *et al.* 2002), the main intracellular signaling pathway for inflammatory cellular responses, which was the justification for using parthenolide as a possible anti-phagocytic drug. In the cell, NF-κB is a transcription factor located in the

cytoplasm. To keep NF-κB from entering into the nucleus and activating DNA, IκB is bound to NF-κB. When inflammatory signals stimulate cells, such as macrophages, IκB is phosphorylated by IκB kinase complex (IKC) ultimately causing the disassociation between NF-κB and IκB, allowing NF-κB to enter the nucleus. IKC is composed of three subunits: IKC-α, IKC-β, and IKC-γ. Parthenolide is believed to inhibit the IKC-β subunit (Kwok *et al.* 2001), leading to the inactivation of IKC thereby preventing NF-κB from entering the nucleus and activating DNA associated with and inflammatory response (Hehner *et al.* 1999).

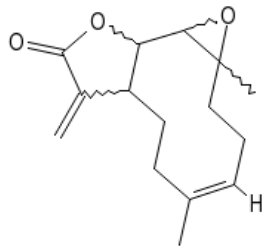

Figure 41. Parthenolide (pub med)

To determine if parthenolide would be a good candidate as a drug to incorporate into alginate for the mitigation of NO production by macrophages, free parthenolide in solution was first tested over a variety of conditions. Our previous experiments presented in this thesis have shown that even highly purified pharmaceutical grade alginate can cause a significant increase in NO_2 levels when in the presence of IFN-γ. Incorporating an anti-inflammatory drug into the alginate that has no effect on islet function could potentially extend the life span of an *in vivo* graft by retarding the initial inflammatory response upon implantation of the graft.

A dose curve of parthenolide was tested under a range of LPS stimuli. The LPS concentrations were chosen to model no contamination (0 EU/mL), moderate

contamination (0.6 EU/mL LPS), and high contamination (6 EU/mL LPS). The same experiment was then repeated in the presence of IFN-γ. Once the potency of parthenolide was confirmed it was then incorporated into 2% w/v alginate beads and then same experiments were repeated.

5.3.2 Experimental Design

The parthenolide dose curve was made by serial dilution of 20 mM parthenolide (dissolved in DMSO) into CM. All other reagents were prepared as described in Chapter 3. Figure 42 is a schematic of the 96-well plate set up with a dose curve of parthenolide for each concentration of LPS. The DAN assay procedure was used to assess NO_2 concentration over 20 and 24 h.

The production of "spiked" Pronova beads with parthenolide was performed by serial dilution using liquid alginate (2 % w/v). The solution was passed through the encapsulator with the following settings: 15 mL/hr, 0.35 mm OD needle, 6.5 KV. The beads were incubated in a 50 mM $CaCl_2$ and 200 mM mannitol solution for 30 mins. Mannitol serves as an osmalyte, regulating osmotic pressure. The beads were washed three times to remove excess $CaCl_2$ after incubation period. The beads were stored in an equimolar CM solution containing parthenolide to limit any potential parthenolide from leaching out. Before use, the beads were washed three times with CM to remove any excess drug. The excess solution was removed to give packed beads and 40 μL aliquots were delivered to the plate (Fig. 43). An aliquot of 6 EU/mL LPS was added where appropriate to the culture containing RAW to stimulate

a potential immune response. The NO_2 concentrations were measured at 20, 24, and 48 h with the DAN assay.

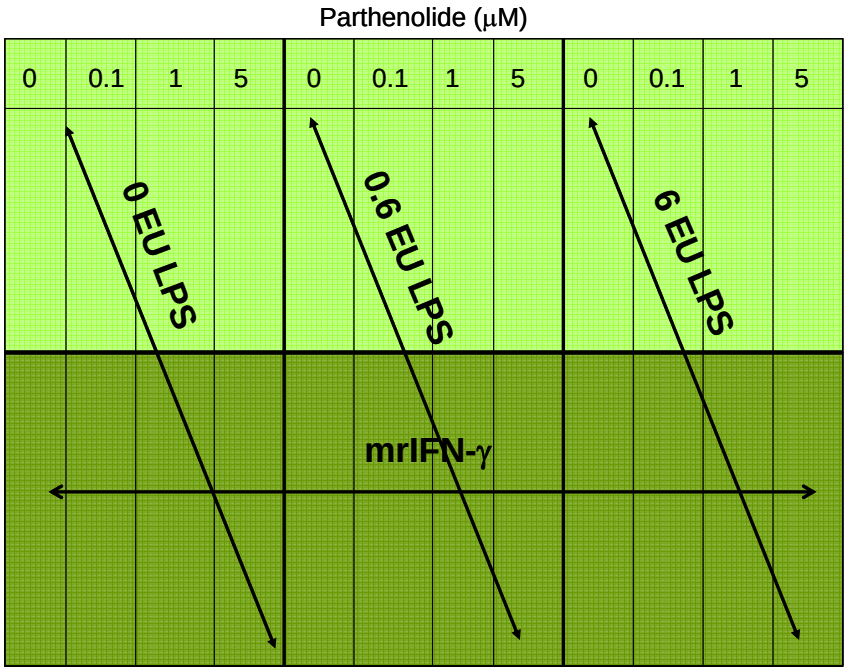

Figure 42. Schematic of 96-well Plate for Free Parthenolide Dose Curve with a Simultaneous LPS Dose Curve +/- IFN-γ (n=4 per condition).

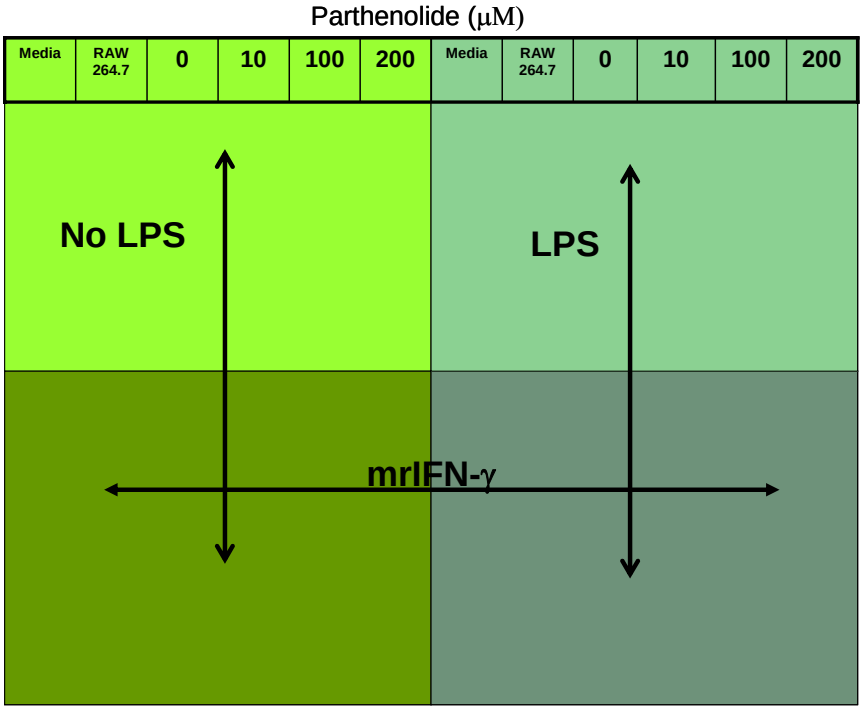

Figure 43. Schematic of 96-well Plate for Incorporated Parthenolide In 2% w/v Beads. Parthenolide concentration (μM) are listed across the top of the plate (n=4 per condition).

5.3.3 Free Parthenolide +/-LPS +/- IFN-γ Results

The use of parthenolide free in solution was used to test the inhibitory action of the drug on RAW. Therefore, only two time points, 20 and 24 h, and three doses (none to strong) were tested. Each graph represents only one time point. The media values were pooled from two previous experiments as a comparison tool since space on the plate was limited. LPS controls in the absence of parthenolide are present. As the concentration of LPS increases, in the absence of parthenolide, the NO_2 concentration increases in the same manner as seen in Chapter 3 confirming that RAW is reacting in the normal manner to LPS stimulation. In the absence of LPS, at 20 and 24 h (Figs. 44 & 45), parthenolide at all concentrations cause a significant decrease in NO_2 when compared to the control (RAW only). This confirms that parthenolide is an inhibitor of NO production by RAW264.7

Parthenolide was also tested in the presence of differing strengths of LPS to determine if parthenolide was a potent inhibitory of LPS-induced NO production. As LPS increased so did the NO_2 levels. At each dose of LPS, increasing the concentration of parthenolide caused a corresponding decrease in NO_2. These trends continued over time. At the highest concentration of parthenolide (5 μM), with the exception of 6 EU/mL LPS at 24 h (Fig. 45), the NO_2 level is either significantly lower then the RAW control or at the same level. This indicates that parthenolide, at 5 μM, can cause RAW cells, even in the presence of 6 EU/mL LPS, to not produce NO above the level of unstimulated RAW.

At 24 h in the absence of LPS and parthenolide, the typical morphology (rounded appearance) and cell number of RAW24.7 were present. As the amount of parthenolide increased, RAW cell numbers decreased with the most dramatic reduction at 5 μM parthenolide. With the addition of LPS, in the absence of parthenolide, the RAW cells flatten out indicating typical differentiation with concomitant production of NO. However, as the parthenolide concentration increases, the cell number decreases indicating the cessation of RAW proliferation. Regardless of the exact mechanism of inhibition (decrease in proliferation, differentiation, cytotoxcity, or signaling pathway inhibition), parthenolide free in solution caused a profound inhibition of RAW both morphologically and functionally (NO production).

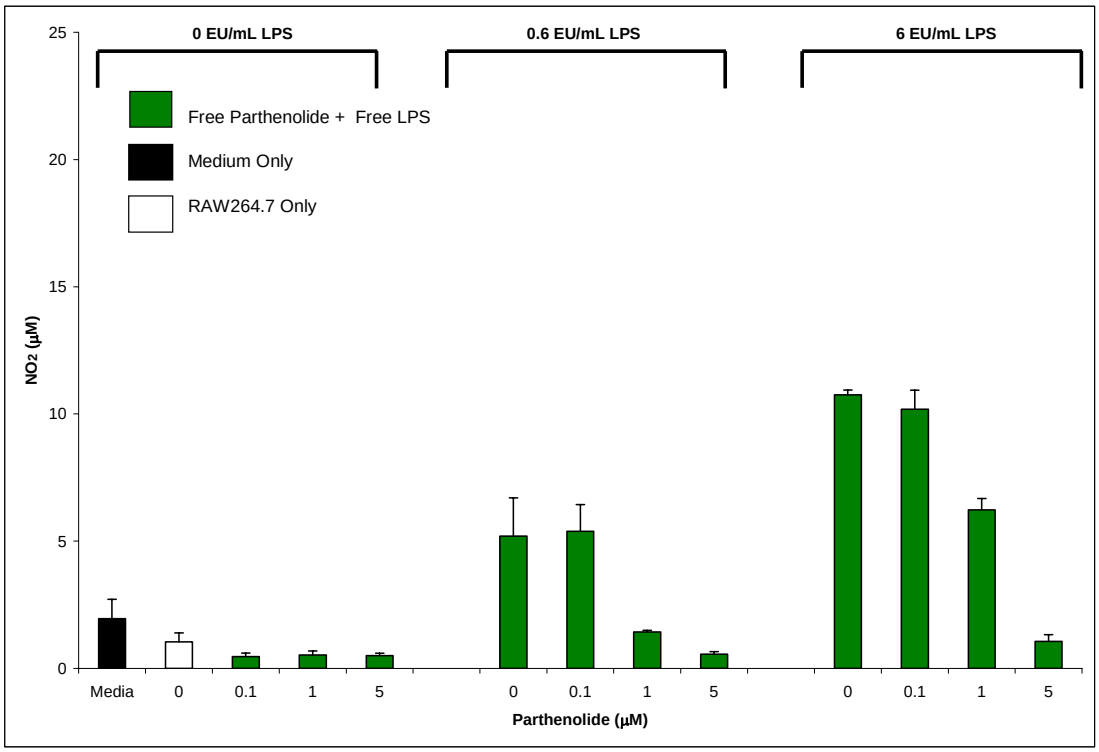

Figure 44. Parthenolide with LPS Dose Curves In the Absence of IFN-γ. Overall, as parthenolide concentration increases, NO_2 decreases even in the presence of 6 EU/mL LPS.

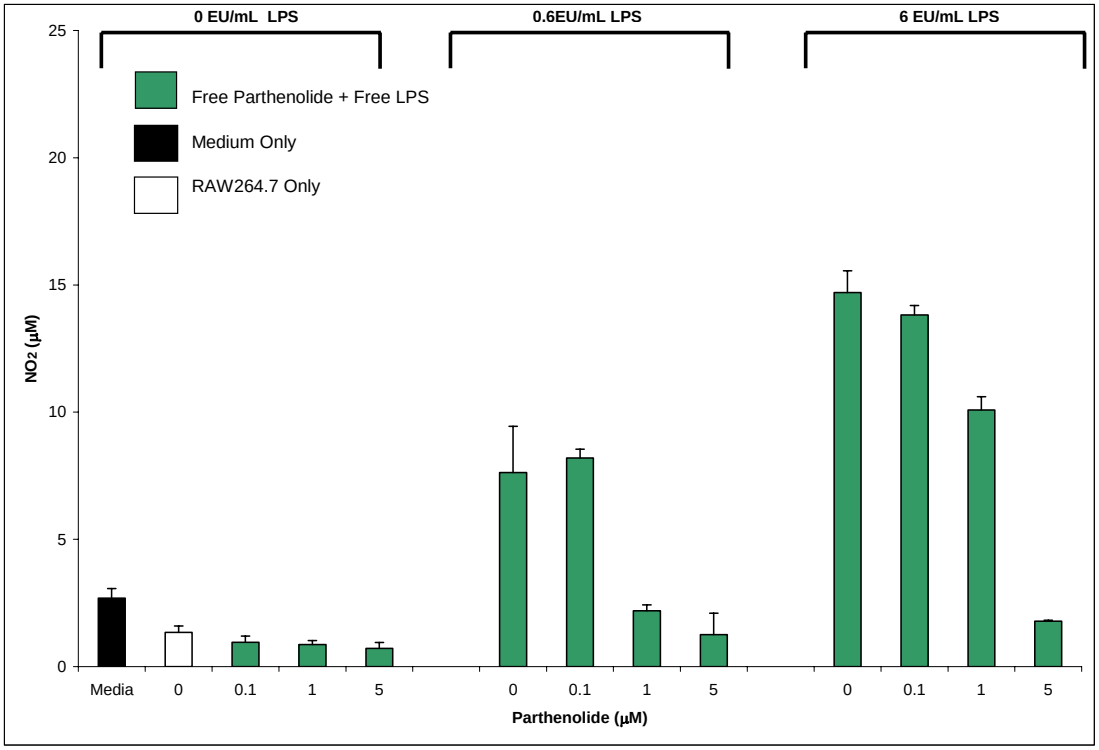

Figure 45. Parthenolide and LPS Super-imposed Dose Curves Free In Solution In the Absence of IFN-γ at 24 h. The same trends as at 20 h are seen at 24 h with the exception that overall NO_2 increases with increasing time.

With the addition of IFN–γ, there are now two stimuli present to increase NO production by RAW. Parthenolide was tested in the presence of these two stimuli to establish if parthenolide could cause the same dramatic decrease in NO_2 as above (Fig. 46 & 47). In the absence of parthenolide, the NO_2 concentration increased in an expected manner when LPS and IFN-γ were present. This indicated that the RAWcells were reacting in the normal, synergistic fashion to IFN-γ and increasing LPS. IFN-γ caused a greater increase in NO_2 and amplifies the effects of parthenolide. In the presence of IFN-γ in the absence of LPS, even a nanomolar concentration of parthenolide caused a significant decrease in NO_2 (Fig. 46). At each parthenolide dose of 5 µM, there was a dramatic decrease in NO_2 compared to the

control. With the exception to exposure to 6 EU/mL LPS, even 1 μM parthenolide caused a significant decrease in NO_2.

Morphologically, in the presence of IFN-γ and the absence of parthenolide and LPS, the cells become bigger and flatter. In the presence of parthenolide alone as the concentration increased, the RAW cells become more speckled in appearance and decreased in number. When LPS was added to the parthenolide and interferon, the RAW cells become bigger and flatter with more debris present.

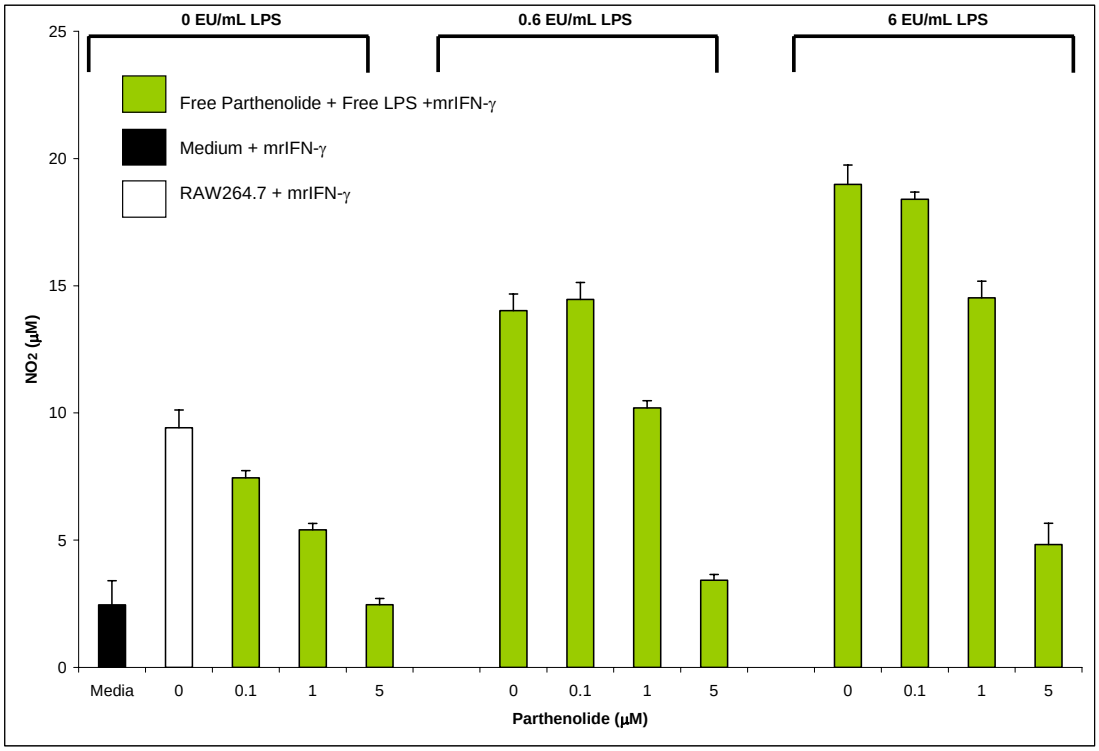

Figure 46. Parthenolide and LPS Curves Free In Solution Super-imposed Dose In the Presence of IFN-γ at 20 h.

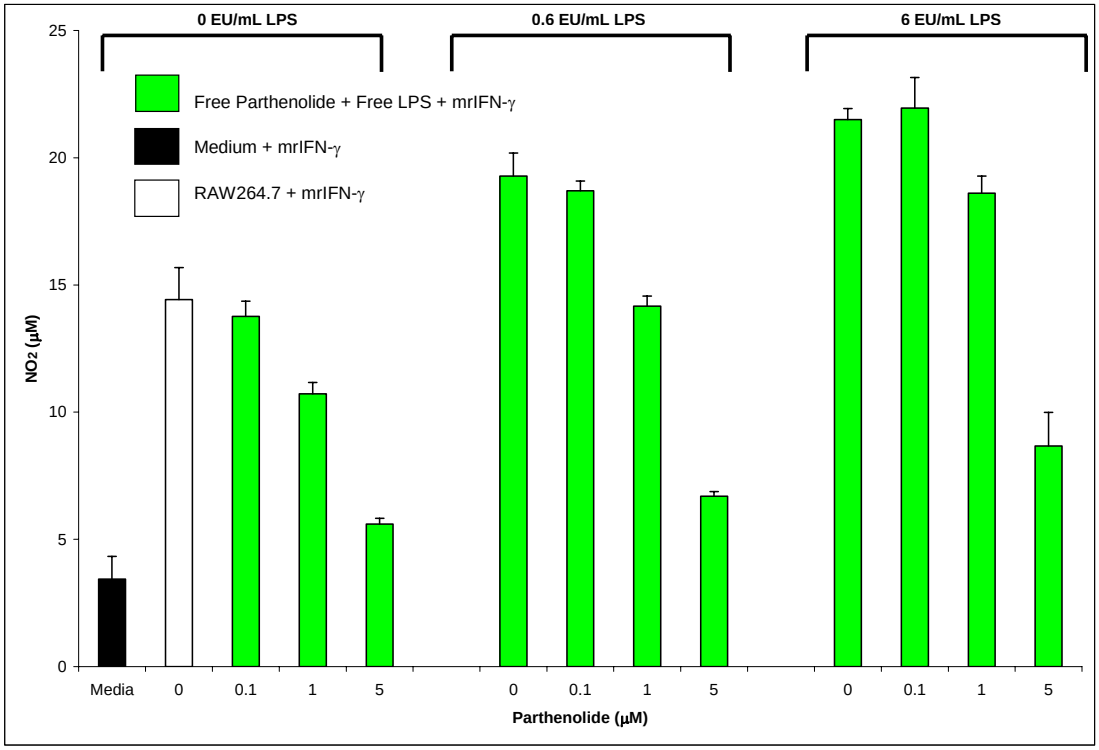

Figure 47. Parthenolide and LPS Free In Solution Super-imposed Dose Curves In the Presence of IFN-γ at 24 h.

5.3.4 Parthenolide Incorporated in 2% w/v Alginate +/-LPS +/- IFN-γ Results

Following the observation that free parthenolide significantly decreased NO_2 levels, even in the presence of LPS and IFN–γ, parthenolide was incorporated into 2% w/v alginate. Parthenolide-containing beads were dropped onto RAW +/- LPS, +/- IFN-γ, and NO_2 was measured at 20, 24, and 48 h. In Chapter 3, we demonstrated that five times the amount of LPS needed to be incorporated into alginate to give a similar NO response to free LPS. In a similar fashion, this next set of experiments demonstrated that up to forty times the amount of parthenolide needed to be incorporated into alginate to give the same inhibition of RAW cells inflammatory

response as was seen with free parthenolide. RAW was only tested, in this series of experiments, in the presence of a maximal LPS (6 EU/mL) concentration which was free in solution.

In the absence of LPS and IFN-γ (Fig. 48), RAW cells produced very little NO_2, as expected (Fig. 48). Parthenolide in beads did not change this unstimulated, background level of NO. With the addition of LPS, there was a significant increase in NO_2, indicating that RAW was reacting to LPS. Over time, the LPS-induced NO_2 increased. A striking reduction in NO_2 was observed for the two highest concentration of parthenolide in the alginate beads (100 and 200 μM) at both 20 and 24 h time points as compared to the control of RAW stimulated with only 6 EU/mL. At 20 h, even 10 μM incorporated parthenolide caused a significant decrease in NO_2. However at 48 h, the inhibition by encapsulated parthenolide was overwhelmed. No reduction in NO_2 concentration occurred in response to parthenolide-containing beads. Thus the inhibitory effect by encapsulated parthenolide on RAW264.7-produced inflammation was time and degree of severity dependent. When inflammation was induce by IFN-γ only (Fig. 52), the inhibitory effect of parthenolide –incorporated beads on the RAW –produced inflammation was completely lost. This again demonstrated dependence of the inhibition and inflammation by parthenolide-incorporated beads on the severity of the inflammation reaction present.

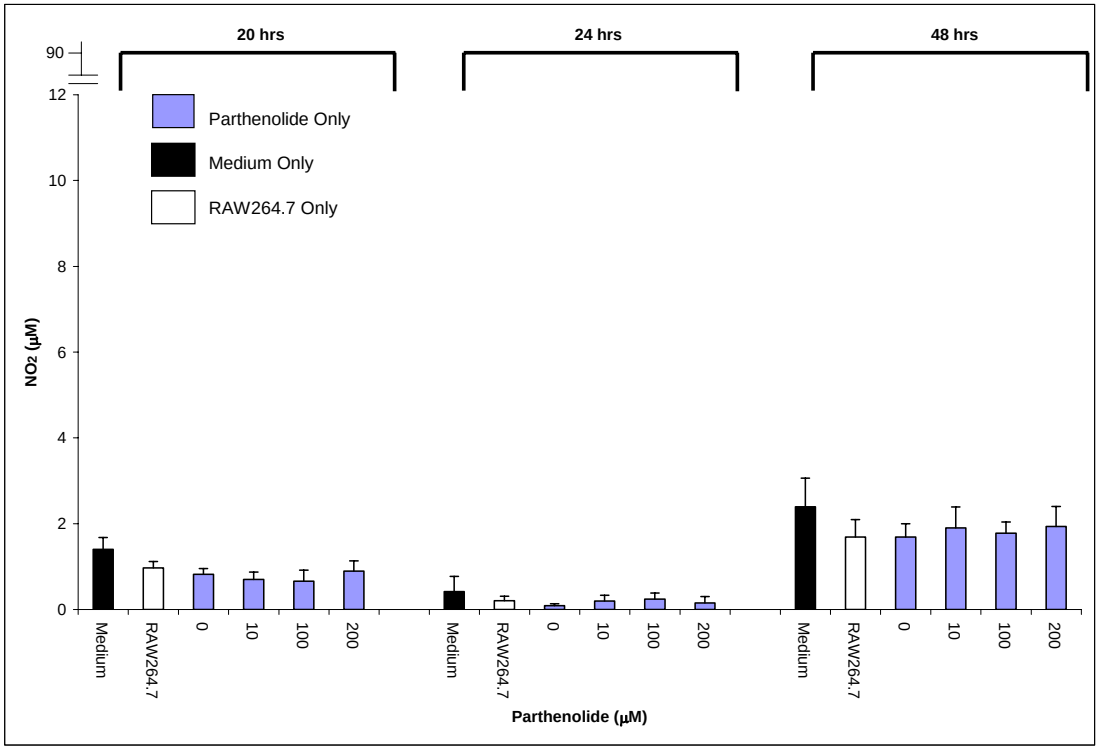

Figure 48. Inhibition of NO Production by Parthenolide Incorporated Into 2% w/v Alginate Beads (400 µm In Diameter) In the Absence of LPS and IFN-γ. There is no significant decrease in NO_2 due to increasing concentration parthenolide in the beads.

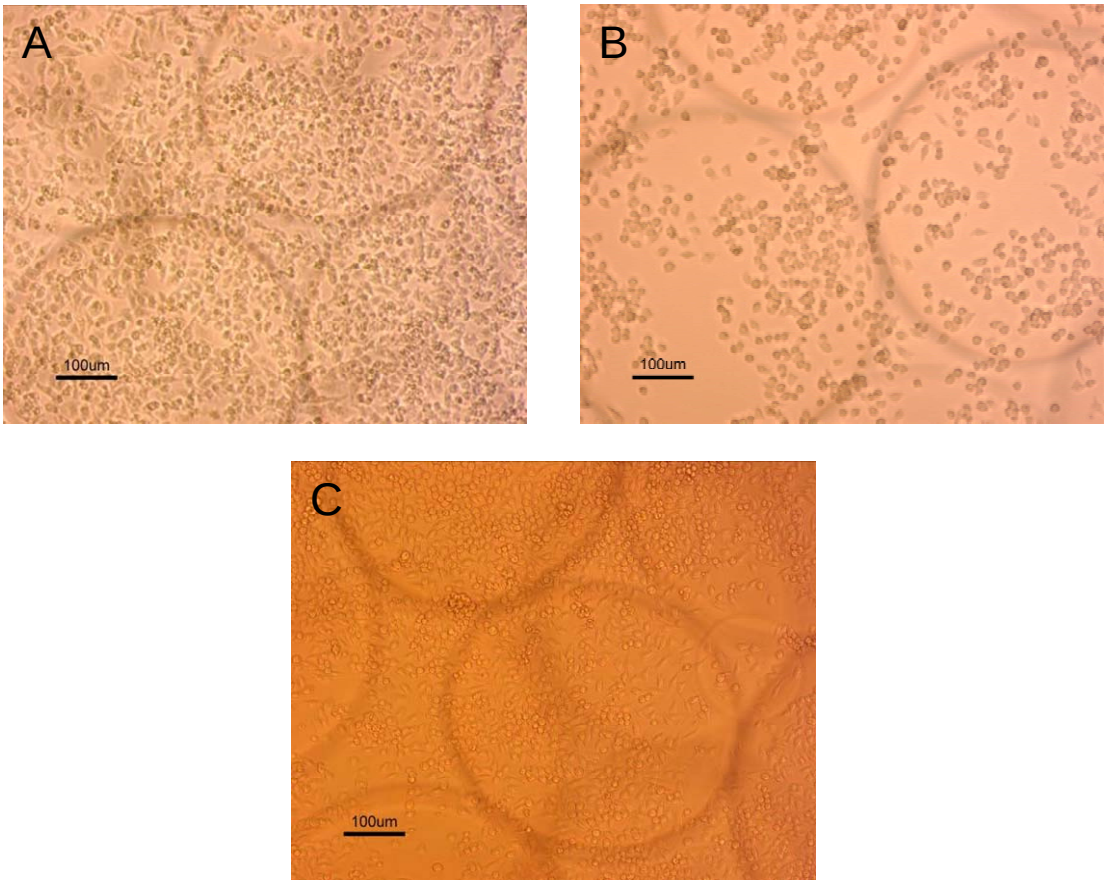

Figure 49. Comparison of Incorporated Parthenolide Into 2% w/v Alginate Beads (400 µm In Diameter) On Top of RAW264.7 with No LPS and No IFN-γ at 20 and 48 h. A) RAW at 20 h with 2% w/v alginate Beads with 0 µM parthenolide incorporated no LPS and no IFN-γ present. B) RAW at 20 h with 2% w/v alginate beads with 200 µM incorporated parthenolide no LPS and no IFN-γ present. C) RAW at 48 h with 2% w/v alginate beads with 200 µM parthenolide incorporated no LPS and no IFN-γ present.

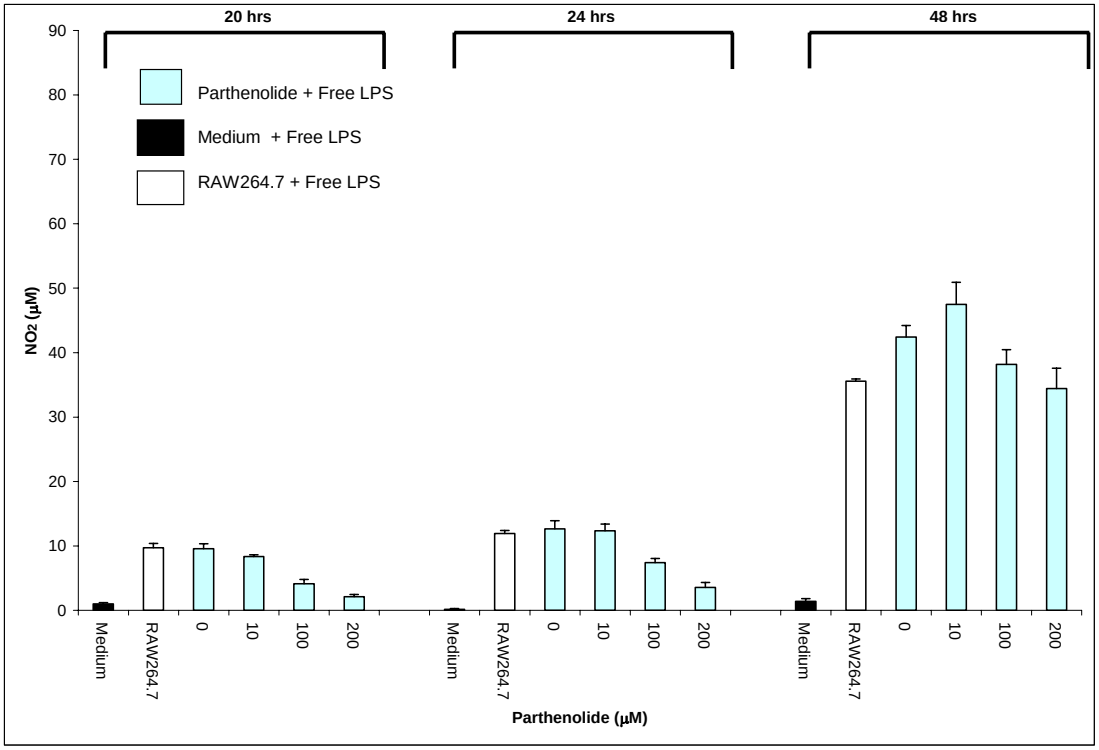

Figure 50. Inhibition of NO Production by Parthenolide Incorporated Into 2% w/v Alginate Beads (400 µm In Diameter) In the Presence of LPS and Absence of IFN-γ. RAW was stimulated with 6 EU/mL of LPS.

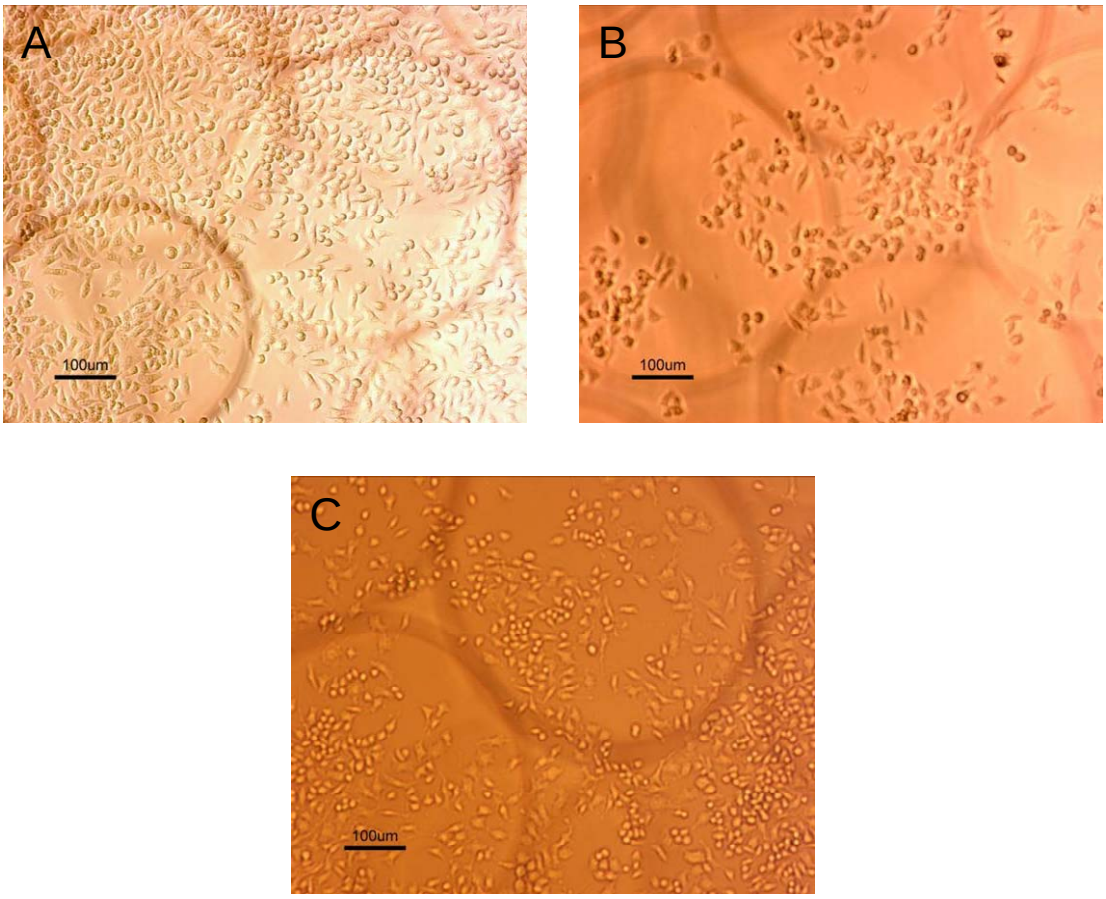

Figure 51. RAW264.7 Comparison of Incorporated Parthenolide Beads (400µm In Diameter) at 20 and 48 h with 6 EU/mL LPS. A) RAW at 20 h with 0 µM Parthenolide 2% w/v, 6 EU/mL LPS and no IFN-γ present. B). RAW at 20 h with 200 µM Parthenolide incorporated 2% w/v alginate beads, 6 EU/mL LPS and no IFN-γ present. C) RAW at 48 h with200 µM Parthenolide 2% w/v alginate beads, 6 EU/mL LPS and no IFN-γ present.

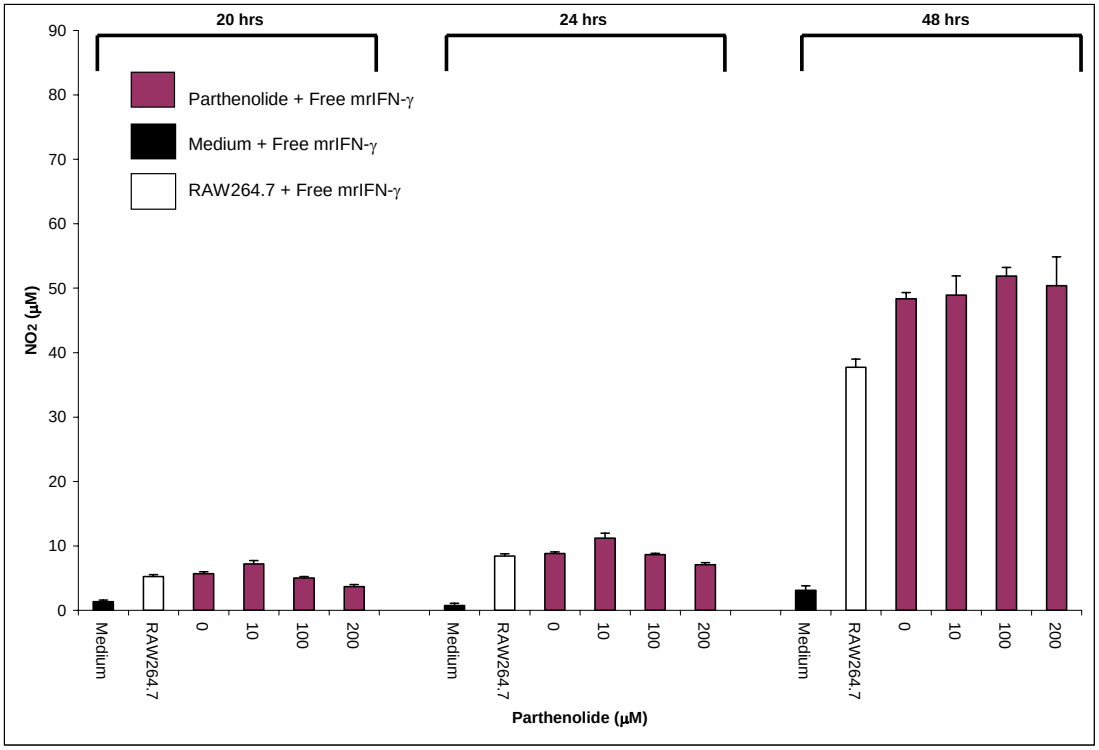

Figure 52. Inhibition of NO Production by Parthenolide Incorporated Into 2% w/v Alginate Beads (400 μm In Diameter) In the Presence of IFN-γ and No LPS. There is no significant decrease in NO_2 due to increasing concentration parthenolide in beads.

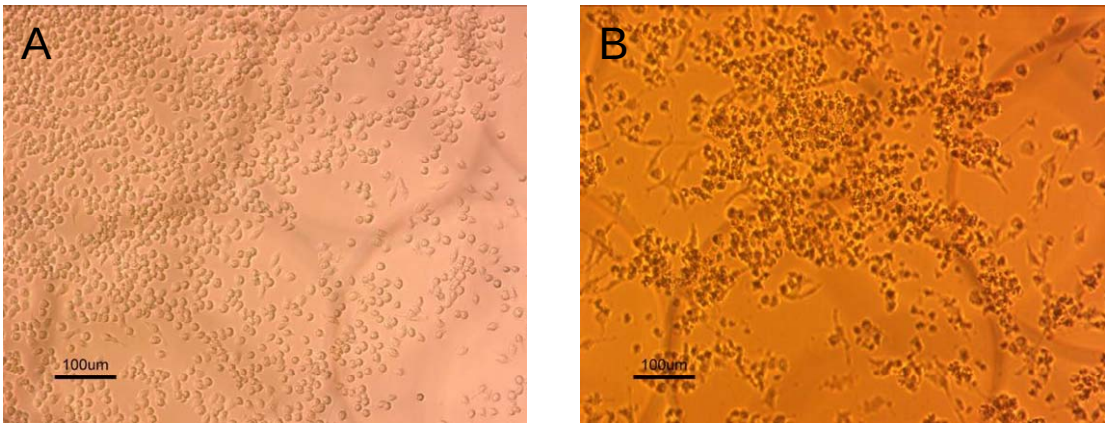

Figure 53. RAW264.7 Comparison of Incorporated 200 μM Parthenolide 2% w/v Alginate Beads (400 μm In Diameter) with IFN-γ Present at 20 h (A) and 48 h (B).

In the presence of IFN-γ (Fig. 53), RAW cells begins to appear to be flattened in appearance at 20 h even in the presence of beads with 200 μM parthenolide. At 48 h, debris is present and fewer RAW cells. The RAW cells that are present have flattened out and there are very few RAW cells still round in appearance.

However, when IFN-γ was present with LPS (Fig. 54) at every time point the inhibition effect of parthenolide incorporated into the beads was again evident. Parthenolide incorporated into alginate decreased NO_2 levels at the highest doses of parthenolide at 20 and 24 h. At 48 h though, again the parthenolide inhibition is overruled. At low levels of parthenolide (10 μM) there was a stimulatory effect with a significant increase in NO_2 compared to parthenolide-lacking beads.

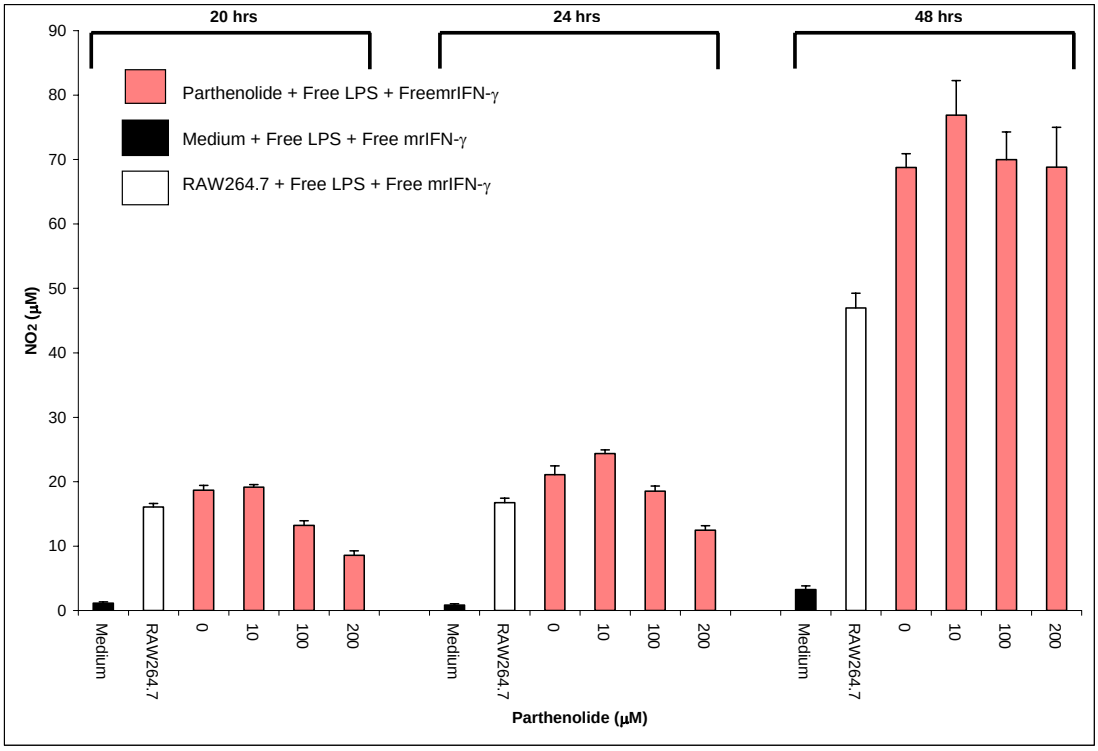

Figure 54. Inhibition of NO Production by Parthenolide Incorporated Into 2% w/v Alginate Beads (400 μm In Diameter) In the Presence of IFN-γ and LPS.

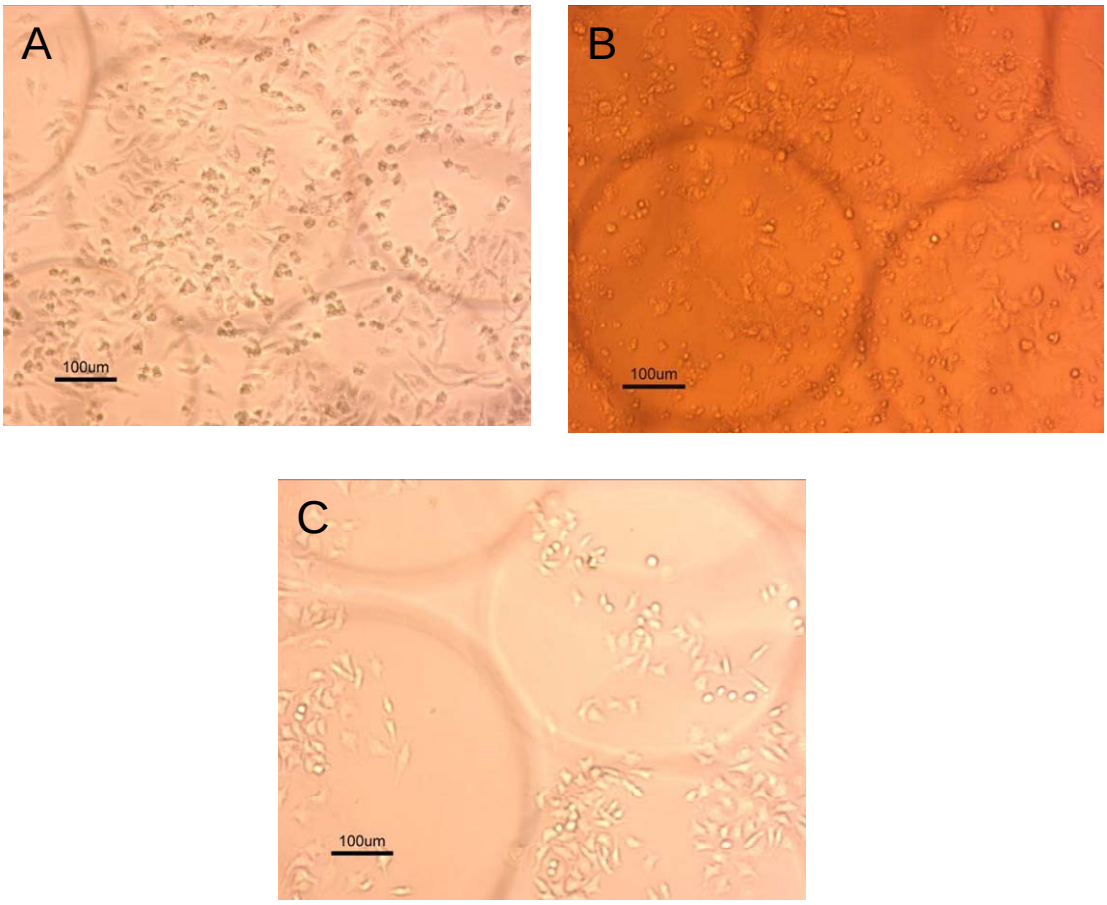

Figure 55. Comparison of RAW264.7 with Incorporated Parthenolide 2% w/v Alginate Beads (400 µm In Diameter) with LPS and IFN-γ. A) 20 h with 0 µM parthenolide. B) 48 h with 0 µM parthenolide. C) 20 h with 200 µM parthenolide

RAW in the presence of LPS and IFN-γ at 20 h, but 0 µM parthenolide beads (Fig. 55 A), exhibited the expected morphology as in Ch 3; in which the RAW cells are flattened over the surface of the well. At 48 h (Fig. 55 B), there was a lot of debris and very few RAW cells. At 20 h (Fig. 55 C), RAW, with 200 µM parthenolide beads, the cells are few in number and have the characteristic appearance due to LPS and IFN-γ.

5.4 Discussion and Conclusions

Three drugs were tested for the mitigation of NO production of RAW by being incorporated into alginate beads. Out of the three drugs, parthenolide was successful in terminating NO production. The bisphosphonates, clondronate and alendronate, have been reputed in some papers to cause macrophage apoptosis but in our hands caused little change to NO production by RAW. One published study used clondronate as a peritoneal cavity wash to eliminate any macrophages present before encapsulated rat islets were implanted (Omer *et al.* 2003). Alendronate was originally chosen as a negative control. In the literature, alendronate supposedly had no effect on macrophages or was slightly stimulatory (Makkonen *et al.* 1999). However, after observing the small inhibitory effect seen in this thesis by alendronate further literature searches brought to light some evidence that alendronate can cause macrophage apoptosis.

In our lab, little evidence was found to support using clondronate or alendronate free in solution in decreasing NO production. Since published reports indicate the increased efficacy of bisphosphonates incorporated into liposomes to protect them from being prematurely hydrolyzed in solution and encourage phagocytosis of drug (Monkkonen *et al.* 1994). This technique is called the "macrophage 'suicide' technique" and was developed by Van Roojen et al. which we attempted to replicate this methodology. The procedure requires a roto vap which Dr. DeShong at University of Maryland Chemistry Department kindly provided to me. According to the published reports about 1 % of the drug is actually incorporated into the liposomes requiring separation and recovery of drug techniques. Our attempt at

replication was terminated, given the non-sterile conditions of making the liposomes, the expense of the drugs, and the difficulty of obtaining significant concentration of encapsulated drugs precluded proceeding. Also, there was concern about the integrity of the liposome once incorporated into viscous alginate.

Parthenolide, on the other hand, was readily incorporated into our alginate beads, and did apparently decrease NO production, through inducing apoptosis of RAW. An ideal drug for incorporation into alginate would have to be non-toxic to islets at the concentration used. When parthenolide was present free in solution along with LPS and/or IFN-γ there was a dramatic decrease in NO_2; sometimes much less NO_2 compared to the control. Concomitant observations of cell morphology and number suggested that the parthenolide caused apoptosis of RAW in a dose dependent manner. Free parthenolide was capable of decreasing NO_2 at nanomolar concentrations.

With this evidence in hand, parthenolide was then incorporated into 2% w/v alginate beads which were then dropped onto RAW cells in culture. The highest possible concentration that we could be incorporate in alginate was 200 μM parthenolide. A dose curve of contamination then was done down to 10 μM parthenolide. While the results of decreasing NO_2 were not as dramatic as parthenolide free in solution, the observed decreases demonstrates promise for using Parthenolide, or some other similar acting drug, or an anti-inflammatory incorporated directly into microcapsules for inhibiting the initial inflammatory response occurring upon graft implantation. Pharmaceutical grade alginate contains LPS contamination that RAW cells can react to causing a more sustained inflammatory response.

Macrophages that are stimulated can produce high, sustained levels of NO that easily diffused through alginate causing islets to cease functioning. Using a drug like parthenolide to decrease the number of macrophages at the graft site could increase the lifespan of encapsulated islets by decreasing the overall level of NO. However, the inhibition effects were observed only at a relatively high concentration of parthenolide in the alginate, due to the sequestrating effect of the alginate gel and might limit the usefulness of this approach if islets are even slightly sensitive to the incorporated drug. Also, the inhibitory effects were only noted at early time points when a weak inflammatory response was present. Longer time of incubation when stronger NO responses were observed overwhelmed the inhibitory ability of incorporated parthenolide. Thus this adjunct to encapsulated cell therapy should be expected to be dependent on the intensity of inflammation induced by implantation, bacterial impurities are present, or complexity of endogenous inflammation encountered.

Chapter 6: Overall Conclusions and Discussion

6.1 Introduction

The aim of this thesis was to define conditions under which cell-encapsulating alginate microspheres may trigger hazardous NO production by macrophages. Pursuant of that goal, the in vitro RAW264.7/DAN assay was used to detect LPS contamination under a variety of defined circumstances. The thesis looked at different factors that could affect the production of NO by RAW cells in response to alginate microspheres. These factors included: 1) the contamination of ubiquitous LPS present in the biomaterial; 2) bead diameter and bead delivery volume; and 3) the presence of anti-inflammatory drugs in the biomaterial.

Two different classes of drugs were investigated as possible agents to mitigate NO production. Bisphosphonates are currently used in the treatment of osteoporosis and other inflammatory disease. Since macrophages come from the same origins as osteoclasts and have similar functions, this seemed to be a promising drug. Parthenolide which has been used for centuries in herbal medicine as an anti inflammatory is another drug that was studied. This drug was chosen from past experience in our lab as a potent inhibitor of NO production by RAW cells for which some published literature claims no effect on islet function.

6.2 LPS Stimulation of RAW264.7 +/- IFN-γ

Pharmaceutical grade alginate contains minute amounts of LPS (< 100 EU/g). Current purification methods cannot completely remove all traces of LPS in alginate. As a result, there is some concern that the alginate itself, due to the contamination, stimulates an immune response. This limits the applicability and lifespan of the graft with encapsulated islets. Acceptable levels of LPS contamination need to be defined for pharmaceutical grade alginate that will not instigate a significant immune response.

6.2.1 Free LPS Stimulation of RAW264.7 +/- IFN-γ

Our model macrophage cell line, RAW264.7, responded very well and in a dose dependent manner to LPS free in the cell growth medium. The NO_2 levels in the growth medium, which are proportional to NO production, increased with increasing LPS concentration. Also, over time the NO_2 levels increased for each LPS concentration up to 48 h. These results indicate that RAW response to LPS with sustained NO production similar for native macrophages. The DAN assay provided an effective and sensitive means of detecting NO_2 and could be used to detect the NOEL and LOEL levels of free LPS in solution. Overall, in the absence of INF-γ, the NOEL of LPS on RAW can be detected down to 0.006 EU/mL and the LOEL to 0.018 EU/mL.

IFN-γ is a cytokine produced by different cell types involved in the innate immune system, including macrophages. The cytokine was added to simulate a more complex inflammatory response to LPS. Adding IFN-γ, demonstrated even lower

NOEL and LOEL values. For LPS free in the growth medium stimulating RAW in the presence of IFN-γ, the LOEL value was 0.006 EU/mL, which was the lowest LPS concentration tested.

6.2.2 Stimulation of RAW264.7 +/-IFN-γ by 2% w/v Pronova Alginate Contaminated with Defined Concentration of LPS

An electrostatic encapsulator was used to create 2% w/v beads containing different amounts of LPS. The beads were used to simulate how an implanted graft would come into contact with immune cell types like macrophages.

Since LPS is one of the most ubiquitous contaminates in alginate and the other biomaterials, the most difficult to remove, it is important to determine levels of LPS in alginate that do not produce a significant increase in NO production by macrophages. LPS was added to 2% w/v highly purified alginate (<100 EU/g LPS) in defined quantities and the NO response by RAW was measured. At 20 h, in some experiments even 2% w/v Pronova alginate with no exogenously added LPS produced a significant, though small, amount of NO. As time increased so did the NO response observed to adulterated alginate, with shifts in the observed NOEL's and LOEL's. In the presence of IFN-γ, there was a synergistic increase in NO production by RAW when the cells were in the presence of beads "spiked" with LPS compared to when no IFN-γ was present.

In all of the assays, increasing LPS contamination in alginate caused a corresponding increase in NO. However, about five times the concentration of LPS was necessary in the microspheres, (Table 4) as compared to the concentration of free

LPS present in the medium, to yield comparable NO_2 levels. This indicates that assays using free LPS to determine acceptable NOEL and LOEL values for alginate are incomplete and inaccurate since much higher quantities of LPS are being sequestered in the alginate. These results also suggest tat even though inflammation can be triggered in complex immune reactions stimulated by external low levels of endotoxin, higher levels of endogenous LPS in biomaterials may be tolerated than free LPS studies might indicate.

6.3 The Effect of Bead Size on NO Production +/- IFN-γ

Besides LPS Contamination, other factors could instigate NO production in response to alginate such as beads-cell surface effects. These were studied in the form of exposing RAW cells to beads of defined diameter and volumes. The logic being that beads of smaller diameter would cover more of the bottom of the well; having more surface contact with the RAW cells. The goal was to obtain a monolayer of defined diameters beads, though using the same delivery volume of beads proved ineffectual. Beads of small diameter tended to form multiple layers at the bottom of the well while wells that were to contain larger beads sometimes had one bead or no beads at all. Therefore, different delivery volumes were employed for different sized beads to obtain a monolayer at the bottom of the well. Beads of a smaller diameter had a smaller delivery volume while beads of a larger diameter had a larger delivery volume to form monolayers on the adhered RAW cells. The appropriate volumes were determined by trial and error. The lowest volume used was 10 μL corresponding to 200 μm diameter beads.

In the absence of IFN-γ, having a lower aliquot volume of bead delivery did not correspond to lesser NO production as one might expect. This was due to the fact that there were many more beads in the bottom of the well having more contact with the RAW cells.

Since all of the bead diameters lots were contaminated with the same concentration of exogenously-added LPS it could be that with the delivery of the beads more LPS was contacting the RAW cells, instigating more NO production. Beads of smaller diameter have maximal contact with the alginate. This was confirmed by the fact that beads of approximately 1000 μm in diameter, though containing the same concentration of LPS as smaller beads, did not cause a significant increase in NO_2 compared to the control. Smaller beads give more total LPS contamination per well causing an increase in NO_2 indicating higher NO production either through direct contact or the LPS leaching out over time. This suggests that using larger beads in the encapsulation of islets might be less inflammatory. There has been much debate over the utility of larger beads versus smaller beads. Smaller beads offer better diffusion properties while larger beads give thicker alginate coverage and therefore protection from direct contact with immune cells. Further study needs to be performed to elucidate this problem. The grafts will be surrounded by tissue in humans and the effects of bead size might be irrelevant to inflammation. However, for the same concentration available to macrophages in tissue beds, per unit volume (as in the in vitro defined well volumes) a larger surface area might still result in less inflammation/unit contained is islet tissue.

In the presence of IFN-γ, though no NO trend was detected with changes in bead diameters, except for a dramatic increase in NO production that was consistently higher then the control. This demonstrates that whatever effect bead size may have on modulating the degree f LPS-induced inflammation, a more potent or complex immune reaction can quickly swamp that effect. Since IFN-γ is known to be a potent, synergistic cytokine, even though the beads have a range of sizes and therefore there are theoretical differences in the amount of LPS available to the macrophages, in the presence of IFN-γ this is sufficient to stimulate maximal reactivity.

6.4 Incorporation of Anti-Phagocytic Drugs Directly into Alginate Microspheres

The concept of incorporating drugs into medical devices to fend off inflammatory reactions and thereby increase the lifespan and effectiveness of the device is already in commercial applications. For example, drug eluting stents are coated with anti-inflammatory drugs that leach out over time, preventing scar tissue overgrowth leading to reblockage of the artery at the location of the stent. This same technique might be applied to alginate or other biomaterials when encapsulating sensitive donor tissue such as islets. A drug might be incorporated into the alginate that either kills off the surrounding macrophages or severely mitigates the production of NO while having no or little effect on islet function. Also, an ideal drug would be one that works at very low concentrations such as in the nanomolar to low micromolar range to again limit any side effects on the islets or other contained cells.

6.4.1 Bisphosphonates

Bisphosphonate are used in the treatment of a variety of bone diseases. Osteoporosis is the most well known disease where bisphosphonates are used. Since osteoclasts and macrophages have the same cellular origin and similar phagocytic activity, bisphosphonates were considered a promising candidate for an anti-inflammatory to be incorporated into alginate in this study.

As an initial study, free alendronate and clondronate were test for reduction of RAW NO reactivity in the presence of LPS +/- IFN-γ. Both drugs free in solution had little effect on NO production by RAW cells in the presence of LPS +/-IFN-γ. Since some published studies indicates that bisphosphonates may require entrapments in liposomes for maximal activity in inhibiting macrophages, we did make an effort to test this methodology.

We confirmed literature reports that this technique is far from perfect in that a very low percentage of the drug is actually incorporated into liposomes, much of the material used does not form into liposomes, there are problems in recovery of unused bisphosphonate and determination of the actual bisphosphonate concentration, it is nontrivial to remove empty of liposomes, and a slew of other problems exist.

One of the main concerns in our lab was guaranteeing sterility of the drug. The procedure described by van Roojen et al. calls for using chloroform and a roto vap. However, this type of apparatus is not easily sterilized. Given the extreme sensitivity of our assay, difficulty in obtaining a significant concentration of encapsulation bisphosphonate and concern that fragile liposomes incorporated in the

alginate might be ripped apart given the viscosity of the gel this effort was terminated.

6.4.2 Parthenolide

The other anti-inflammatory drug studied in reagents to be incorporated into the alginate gel was parthenolide, a potent inhibitor of the main intracellular signaling pathway for inflammation, the NF-κB pathway. Parthenolide has been commonly used for centuries in herbal remedies for fevers and other inflammatory aliments. Parthenolide was first tested with RAW stimulated by LPS +/- IFN-γ free in solution. Parthenolide was observed to greatly decrease NO_2 levels at low concentrations.

The effectiveness of parthenolide was tested under a number of conditions and stimuli. First, a dose curve of parthenolide was tested against a dose curve of free LPS to determine if parthenolide could overcome the stimulation of RAW by LPS +/- IFN-γ. In fact 5 μM parthenolide solutions severely decreased LPS-stimulated NO_2 levels by at least 50% even in the presence of IFN-γ. Increasing LPS, time, and the presence of IFN-γ caused an increase in NO_2 levels. However, in all cases 5 μM caused almost complete inhibition of NO production.

The decision to use up to forty times more parthenolide in the alginate was based on our experience with LPS sequestered in alginate. Much more LPS incorporated into alginate was necessary to illicit a similar NO response as free LPS. The highest concentration of drug that could be incorporated into the gel was 200 μM. Another two doses tested were 100 and 10 μM.

There were four conditions that were tested: No LPS/IFN-γ, No LPS but IFN-γ, No IFN–γ but LPS, and LPS with IFN-γ. In the case of no LPS and No IFN-γ, there was sight amount of NO production over time but as seen in previous experiments, RAW does not produce very much NO if unstimulated. With LPS to stimulate the RAW cells, there was a decrease in NO_2 with the 100 and 200 μM doses. The highest dose, 200 μM, caused the greatest decrease of NO by more then 50% compared to the control at 20 and 24 h. At 48 h, the highest dose of parthenolide, even in the presence of LPS, NO production was no higher than the control (unstimulated RAW) (Fig. 50). With IFN-γ as the only stimulator, there was a decrease in NO_2 by parthenolide-containing microcapsules, but not as dramatic as the case of only LPS. With both inflammatory stimulators, working together in a potent synergistic fashion, parthenolide caused a decrease of NO to control levels at 20 and 24 h. At 48 h, NO production in the presence of parthenolide did rise above the control level but compared to the control, possibly indicating that the LPS and IFN-γ given enough time can override the opposing parthenolide effect.

These data show that parthenolide or other anti-inflammatory drugs might be incorporated into alginate to prolong function of implanted tissue-engineered grafts. In the case of parthenolide, further studies would have to be done to confirm that has no effect on islet function, as some of the literature indicates. If successful, incorporation of anti-phagocytic drugs, like parthenolide, into alginate might be one of the factors necessary for producing a successful bioartificial pancreas device.

6.5 Conclusion

In conclusion, the data of this thesis suggest that : 1) the effectiveness of the RAW264.7/DAN assay can be very efficient in detecting LPS contamination at very low levels; 2) NOEL and LOEL concentrations for endotoxin contamination in pharmaceutical grade alginate are much lower then previously thought, particularly when IFN-γ is present; 3) correspondence of inflammation in patients implanted with biomaterial containing devices may be dependent upon the preexisting inflammatory state of the patient both systematically and at the site of implantation; 4) the bioavailability of endotoxin when contained within alginate microcapsules in the presence or absence of a T-cell mediator IFN-γ is decreased versus free endotoxin; suggests that an implanted device could safely contain more LPS then suggested by free LPS results; 5) the inflammatory effect by LPS-contaminated biomaterials is related to the amount of surface area exposed to tissues; though a strong pre-existing immune response may swamp any surface area-mitigating effects; and 6) possible modulating physical agents, like anti-phagocytic drugs such as parthenolide, may be directly incorporated into biomaterial to diminish impurity-stimulated inflammatory responses.

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