MECHANISMS OF INNATE IMMUNE RESPONSES CAUSED BY SODIUM ALGINATE

BY

DONG YANG,
M.S. (OCEAN UNIVERSITY OF CHINA)

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Dong Yang, M.S.,

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AUTHOR: Dong Yang, M.S. (Ocean University of China)

SUPERVISOR: Professor Kim S. Jones

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Abstract

Alginate is a well-known naturally-derived biomaterial that has been widely used in preparing microparticles for drug delivery and in preparing scaffolds for tissue engineering. Despite desirable properties, alginate has been shown to activate inflammatory cells *in vivo*. The mechanisms are still unclear.

In this thesis, the mechanism by which alginate caused innate immune responses was investigated *in vitro* by using RAW264.7 cells, a macrophage-like cell line. The NF-κB pathway, an important signaling pathway in macrophages, has been tracked to identify cellular responses. The secretion of cytokines IL-1β, IL-6, IL-12(p40) and TNF-α was quantified to determine the activation outcomes. Also the interaction between alginate and serum was studied.

Experimental results indicated that alginate induced the activation of RAW264.7 cells with a time and dose dependent behavior. Like lipopolysaccharide, a bacterial product and known activator of innate immunity, alginate induced macrophage activation through the NF-κB pathway and eventually led to detectable IL-1β, IL-6 and TNF-α cytokine secretion. Serum influenced alginate recognition by macrophages in an unknown mechanism. Also, alginate promoted cell survival in a nutrition starvation condition. These results revealed *in vitro* alginate stimulation, and provided much information for further research.

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Chapter 1 Introduction

As more and more novel biomaterials are applied in tissue engineering, medical devices and drug delivery systems, researchers are showing an increasing concern about the relationship between biomaterials and the body's biological responses.

Until recently, researchers have particularly focused on considering the material itself. The belief was that through improving the mechanical properties of a material itself, it will perform a similar or better function as the substituted tissue or organ. Their toxicity, haemocompatibility, biodegradability and biocompatibility have been studied at length. However, in clinical practice, researchers found that when the medical device was implanted into the body, a series of host responses including injury, blood-material interactions, provisional matrix formation, inflammation, formation of granulation tissue, wounding healing and fibrosis/fibrous capsule development happened frequently, and depending on different devices and hosts, these responses can last from a few days to several years (Anderson, 2001). When used for drug delivery systems, biomaterials are often prepared into microcapsules or microparticles. The host responses to them may differ from those seen to bulk materials, but, macrophages and other phagocytes may "eat them up" (phagocytose them). These particles may be perceived as pathogens especially if a specific ligand has been recognized by membrane receptors, and some of the microparticles may arouse an innate immune response. With the increasing number of applications of biomaterials in the body, these phenomena are likely to increase, and they have attracted researchers' interests in "biomaterial immunology" as well as materials science.

Alginates are natural polysaccharides derived mostly from brown algae, and composed of both β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronate (G) residues in forming mono- or hetero-polymers (Haug *et al.*, 1966). Alginates can form hydrogels when crosslinked with divalent cations, such as Ca^{2+}, Ba^{2+}, and Sr^{2+} (Smidsrod and Skjak-Braek, 1990). The proportions of M/G residues determine the hydrogel's mechanical properties, and consequently applications in preparing microcapsules or combing with other materials to fabricate scaffold. It has been confirmed that alginates have low toxicity (Klock *et al.*, 1997), and can be hydrolyzed in normal physiological environments, such as *in vivo*. Recently, researchers have used alginate or alginate-based polysaccharide complexes to encapsulate hormone-producing cells in treating diabetes (Lim and Sun, 1980;Sun, 1987b;Sun *et al.*, 1987), to transplant myoblasts to enhance their viability (Hill *et al.*, 2006), or to control sustained drug release (Hari *et al.*, 1996;Thu *et al.*, 1996b;Thu *et al.*, 1996a).

Some of the chemical properties are undesireable, such as the unstable mechanical properties due to ion exchange from divalent cations (gel-form) to monovalent cations (water soluble). Alginate also does not easily support cell adhesion(Smetana, Jr., 1993;Rowley *et al.*, 1999). In addition, when implanted, researchers found *in vivo*, alginate capsules caused fibroblast overgrowth (Sun, 1987b), and stimulated human monocytes to secrete high levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interlerukin-6 (IL-6) (Otterlei *et al.*, 1991;Iwamoto *et al.*, 2003b). The mechanisms for such host responses are still unclear.

Protein NF-κB, a multi-subunit transcription factor, is an important factor in cellular responses. In normal conditions, this protein is inhibited by inhibitor kappa B

(IκB) and stays in the cytoplasm. When cytokines, growth factors or lipopolysaccharide (LPS) bind to specific receptors on the membrane, IκB is phosphorylated and then degrades, freeing NF-κB. The activated NF-κB moves into the nucleus and promotes gene expression including producing proinflammtory cytokines and apoptotic modulators which regulate other inflammatory cells to respond to the inflammation.

Can alginate stimulate macrophages? Does the stimulation through NF-κB pathway have the same mechanism as LPS stimulation? Or are there any interactions between serum and alginate that influence the cellular responses? All of these are still unclear and no related research work has been reported.

In this thesis, I chose sodium alginate as the object to study and used a macrophage-like cell line, RAW264.7, to track the mechanisms of immune responses, especially in studying the cellular responses to alginate stimulation *in vitro*. We hypothesized that the cellular responses will be shown in the activation of NF-κB pathway, and that serum will influence the alginate stimulation and eventually change the macrophage responses.

Chapter 2 Literature Review

2.1 Biomaterials

2.1.1 Applications of biomaterials

The term "Biomaterial" is used to indicate materials that constitute the basic framework of medical implants, extracorporeal devices and disposables utilized in medicine, surgery and dentistry, in veterinary applications, as well as in other aspects of medical care (Mahato, 2005). Biomaterials are widely employed for tissue engineering, medical devices and drug delivery.

2.1.1.1 Tissue Engineering

Tissue engineering, as one of the major approaches to regenerative medicine, is an interdisciplinary field that incorporates reactor engineering with cell biology and clinical research for the creation of new tissues and organs and the development of tissue or organ replacements (Lavik and Langer, 2004;Lewandrowski *et al.*, 2002).

There are four general challenges for tissue engineering on which research currently focuses (Langer, 2000):

1) Appropriate cells. This challenge includes appropriate cell source identification, e.g. properly differentiated and purified stem cells; or universal donor cells created by masking histocompatibility proteins on the cell surface.

2) Materials for tissue engineering. This challenge focuses on choosing of the appropriate material(s) with specific chemical and physical properties including:

mechanical strength, biodegradability and degradation time *in vivo* and *in vitro*, microstructure, permeability, and components that may regulate cell behavior such as adhesion, migration and growth.

3) Fabrication of novel polymer and structures for tissue engineering, e.g. by surface modification by grafting specific ligands, hybrids of several biomaterials, or imprint lithography.

4) Cyropreservation of larger or more complex tissues or organs.

In general, cells and materials are the key components of tissue engineered constructs. Since most biomaterials will be implanted into the body, they must have biological compatibility and must not provoke serious host rejection. Biomaterials have been used in replacement of blood, tissue, organs and even parts of the body. Currently, numerous strategies used in tissue engineering depend on employing a material scaffold, but according to the specific application or the purpose of the desired tissue, the choice of a suitable scaffold material will be quite different. The selection and synthesis of the materials is governed by physical properties, mass transport properties and biological properties and also by the intended application and the environment in which the scaffold will be placed (Drury and Mooney, 2003).

2.1.1.2 Medical devices

Since biomaterials by themselves do not work as a clinical therapy, most of time they need to be fabricated into devices or implants. Every year numerous patients are benefited from these medical devices. The most common devices include heart valves, hip and knee prostheses, breast implants, intraocular lenses (IOL), contact lenses, vascular grafts and catheters.

Medical devices based on biomaterials must satisfy requirements of mechanical performance and durability, appropriate physical and chemical properties, and biocompatibility (Ratner and Bryant, 2004). However, for different applications, the critical points are different. For example, an articular cartilage needs to be flexible and elastomeric; in contrast, contact lenses need high oxygen permeability and low protein adhesion.

2.1.1.3 Drug Delivery

Many biomaterials have been utilized in drug delivery systems. Since too high a dose is potentially harmful to the body, an ideal drug control delivery system is considered as a useful method to control appropriate drug release at specific times and specific sites. However, such target-specific drug delivery systems have not yet been developed (Petrak, 2005).

Biomaterials that can be used in drug delivery systems may have properties including: high molecular weight; special performance with phase transitions,

conditionally controlled release (e.g. by heat, UV light, or pH); dissolution or biodegradation (Ratner, 2003).

There are many kinds of drug delivery systems classified by different control mechanisms. Some diffusion-controlled systems have a reservoir core. The drugs diffuse through a rate-controlling membrane. Water penetration-controlled system relies on dry hydrogel membranes that absorb water and form porous structures that release the drug. Responsive drug delivery systems depend on the environment changing; e.g. through altered pH and temperature. For example, one self-regulating insulin-delivery system relies on the concentration of glucose in blood. High glucose in the blood is catalyzed by glucose oxidase, and the product, gluconic acid, lowers pH. The membrane swells in response to decreasing pH and releases insulin into the body (Kost *et al.*, 1985).

2.1.2 Different types of biomaterials

People have discovered and created many biomaterials, including polymers, hydrogels, metals and biomimic materials. According to their specific properties, these materials have different applications. The following describes some examples:

2.1.2.1 Polymers

Polymers consist of small repeat units. According to their origin, they can be divided into two groups: natural and synthetic.

Polysaccharides, a type of natural polymer, exist in most living organisms. Cellulose, starches, and chitosan are polysaccharides. The abundant availablility, lack of

toxicity, promising biocompatibility and other physicochemical properties make polysaccharides an advantageous choice in many applications. For example, chitosan and its derivatives can accelerate wound healing by activating inflammatory cells and fibroblast cells (Ueno *et al.*, 1999), and also they can work as scaffolds for skin, bone, cartilage and other types of tissue reconstruction (Berthod *et al.*, 1996;Kim *et al.*, 2002;Lee *et al.*, 2004;Kim *et al.*, 2003).

In order to obtain better properties, several polysaccharides have been combined together. For example, polysaccharide gels consisting of alginate and xyloglucan have been used in drug delivery (Coviello *et al.*, 2006) and chitosan has been combined with alginate for cell microcapsulation.

Poly(D,L-lactic-co-glycolic-acid), abbreviated by PLG or PLGA, is one example of a synthetic polymer. Since the hydrolysis residues can be metabolized via the citric acid cycle, PLGA has been used for many years in producing surgical sutures and in drug delivery (Waeckerle-Men and Groettrup, 2005).

2.1.2.2 Hydrogels

Hydrogels are insoluble polymer networks that are formed by water-soluble polymers crosslinked with physical (hydrogen bonds, van der Waal interactions), ionic, or covalent bonds (Hynes, 1992;Oxley *et al.*, 1993). They are often capable of absorbing large amounts of water, and their hydrophilic properties influence protein adsorption, cell adhesion and migration. On the other hand, the properties of high tissue-like water contents and elasticity provide the possibility of minimal rejection of cell-invasive

scaffolds or constructs (Smetana, Jr., 1993). In the last two decades, this ability has been applied to encapsulate or immobilize cells in tissue replacement and drug delivery. For example, Langerhans islets from humans were encapsulated in a novel alginate-based microcapsule system that helped immunocompetent diabetic mice maintain normoglycemia for over seven months (Hynes, 1992;Schneider *et al.*, 2005).

Hydrogels vary widely in physical and chemical characteristics, such as structure, charge, pore size and mechanical properties. Hydrogel scaffolds serving as a synthetic extracellular matrix (ECM) can present continuous stimuli and organize cells to seed into a three-dimensional architecture, thus guiding the growth and formation of a desired tissue (Yang *et al.*, 2001).

Poly (ethylene glycol) (PEG) offers limited protein and cell adhesion, immunogenicity and antigenicity. It is widely used in coating medical devices to prevent protein and cell adhesion (Alcantar *et al.*, 2000). PEG is not degradable but has been modified to form degradable PEG hydrogels - PEGylation by linking specific proteins, peptide or non-peptide molecules (Veronese and Pasut, 2005).

2.1.2.3 Metals and ceramics

Metals and ceramics are non-degradable materials that have been applied in orthopedics for a long time, for example, in hip and tooth replacement. These materials include titanium alloys and zirconia.

Relying on excellent mechanical, physical and biological performance, titanium alloys are used in biomedical devices, such as titanium miniscrews (also known as micro- or mini-implants), as dental implants (Heymann and Tulloch, 2006), and for hip or joint replacement (Affatato *et al.*, 2001;Long and Rack, 1998).

In order to solve alumina brittleness and the consequence of failure of hard tissue implants, biomedical grade zirconia was introduced 20 years ago (Christel *et al.*, 1988) and thus far, over 600, 000 femoral heads have been used worldwide(Chevalier, 2006;Christel *et al.*, 1988). However, *in vivo* degradation made zirconia show strong variability and undesirable consequence of the aging process (Christel *et al.*, 1988). In 2001-2002, the failure events of Prozyr® femoral heads compelled people to re-consider the application of zirconia. Recently a suggestion of using alumina-zirconia composites instead of monoliths has been forwarded (De Aza *et al.*, 2002;Affatato *et al.*, 2001). It will partly help to solve the problem, although people need more understanding of tissue related to aging.

On the other hand, these non-degradable biomaterials have disadvantages in host acceptance. People found after early implantation, due to the microenvironment changing near the implant, macrophages and other innate immune cells were quickly activated and induced fibrous encapsulation and inflammation. Furthermore, in long term implantation, metal degradation may cause corrosion and toxicity, which will lead to implant failure and adverse immune responses (Ryan *et al.*, 2006). To improve cell recognition and reduce rejection, many methods have been used through surface modification or increasing porosity in metals. Investigators found that porous metal is much closer to

bone tissue, can increase fatigue life and also provide an appropriate environment for extensive transport of body fluid (Cameron *et al.*, 1978).

2.1.2.4 Promising biomaterials

To satisfy the needs of tissue engineering and regenerative medicine, many new biomaterials have been developed and studied, and novel techniques have also been applied in fabrications. These materials include collagen, PLGA, fibrin, gelatin, and chitosan (Andreadis and Geer, 2006). They also are called biomimetic materials because of their excellent biocompatibility. For instance, in wound healing, the novel materials can be designed as not only providing scaffolds for cell attachment, but also acting as growth factor vehicles which perform in a controlled manner (Andreadis and Geer, 2006). At the same time, they will ideally be biodegraded and easily absorbed by the body after the tissue regeneration is finished.

Surface topography and chemical properties are important factors in determining cell responses to an implant. In order to be better accepted by the tissues, some novel techniques have been applied, such as surface modification by coating or linking small peptides or polysaccharides that have recognition sites similar to extracellular matrix components and can be easily accepted by cells.

Small peptide RGD (Arginine – Glycine - Aspartic acid) sequences can be recognized by cell surface receptors called integrins (Ruoslahti, 1996) and have been found to aid cell recognition (Hersel *et al.*, 2003). Researchers also found the density of

RGD and its secondary structure can greatly influence the cell responses such as adhesion, migration, and specific integrin engagement (Ochsenhirt *et al.*, 2006).

Other peptides have been found to have the similar functions as RGD. Peptide REVD (Arg-Glu-Asp-Val) can promote vascular endothelial cell adhesion and spreading (Massia and Hubbell, 1992). The short peptide sequence PHSRN (Pro-His-Ser-Arg-Asn) from fibronectin has a similar mechanism as RGD to promote cell adhesion (Feng and Mrksich, 2004). Peptide YIGSR (Tyr-lle-Gly-Ser-Arg) from laminin can help endothelial cells adhere to a bioactive polyurethane urea modified with polyethylene glycol(PEG) (Jun and West, 2005).

Fabrication of better biocompatible materials through simple chemical methods has been investigated as well. Recently, a biomimetic phospholipid polymer has been studied as soft contact lens biomaterials to replace or improve silicone hydrogels. In order to improve anti-biofouling properties and biocompatibility while maintaining high oxygen permeability, a 2-methacryloyloxyethyl phosphorylcholine (MPC) unit was added to a representative phospholipid polymer to suppress non-specific protein adsorption and improve wettability (Hsiue *et al.*, 1998;Goda and Ishihara, 2006).

Microscale technologies, such as carbon nanotubes (CNT), have also been applied in tissue engineering. Since 1991 when CNT were discovered, they have been widely used in biosensors, drug and vaccine delivery vehicles, and as nano-fillers to improve polymer mechanical properties and electronic conductivity (Smart *et al.*, 2006). However, single-walled CNTs have potential pulmonary toxicity by inhalation exposure (Warheit *et al.*, 2004).

2.1.3 Alginate

2.1.3.1 Chemical properties of alginate

Alginates are natural linear polysaccharides derived from brown algae at the industrial level, and widely used in the food industry, tissue engineering and for medical purposes. They are block copolymers composed of β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronate (G) residues which are arranged in homo- or hetero-polymeric sequences (Haug *et al.*, 1966) and are named G-blocks, M-blocks and MG-blocks, respectively.

The proportions and lengths of these blocks are widely dependent on the algae species, types and age of the tissue used for the alginate extraction (Haug *et al.*, 1974). The M/G ratio also influences the physical properties and behaviours of alginates. Depending on the application, alginates may be prepared with a wide range of average molecular weights (50-1, 000, 000 residues).

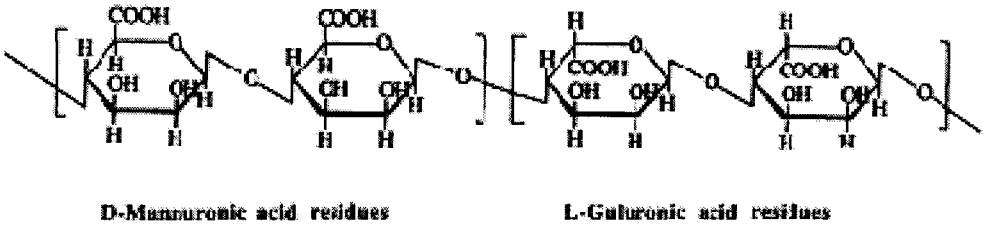


Figure 1: Chemical Structure of sodium alginate showing both β-D-mannuronic acid and α-L-guluronic acid residues.(MacGregor and Greenwood, 1980)

Generally, alginates show high water absorption and have gel-forming properties when divalent cations, such as Ca^{2+} , Ba^{2+}, and Sr^{2+} replace the hydrogen bond of

guluronate to form ionic bridges between different polymer chains(Smidsrod and Skjak-Braek, 1990). Such gels are thermally stable and can be heat-treated without melting although they may eventually degrade. By changing the M/G ratio or the divalent cations, the crosslinking density and consequently mechanical properties of gels will vary and this can be helpful in different applications such as microcapsules or biofilm preparations. The stability and water-holding capacity of alginate are also dependent on time, pH, and temperature(Haug *et al.*, 1967;Haug and Larsen, 1962;Haug and Larsen, 2006;Haug *et al.*, 2006).

2.1.3.2 Alginate applications in tissue engineering and drug delivery

Since alginates are low toxic, hydrophilic, biodegradable under normal physiological conditions and have readily available sources, they have been widely applied as a matrix in biotechnology and medicine. Examples include, in microencapsulation of hormone-producing cells to treat diabetes mellitus in animal models (Lim and Sun, 1980;Sun *et al.*, 1987;Sun, 1987a;Lim and Sun, 1980), in transplantation of myoblasts to enhance viability (Hill *et al.*, 2006), and in sustained release in drug delivery applications (Hari *et al.*, 1996;Thu *et al.*, 1996b;Thu *et al.*, 1996a).

On the other hand, alginate matrices have disadvantages, including unstable mechanical properties due to the ion exchange of crosslinked divalent cations with monovalent cations, and lack of specific cell-recognition signals that enable anchorage-dependent cells to promote the interaction with the matrix (Smetana, Jr., 1993). Moreover,

the hydrophilic nature of alginate limits the adsorption ability of serum proteins, which inhibits anchorage-sensitive cells such as hepatocytes. The introduction of anchorage sites within the scaffold is required to promote specific cell interactions and cell functions including migration, proliferation, and specific gene expression(Rowley *et al.*, 1999;Hynes, 1992;Price, 1997).

In order to enhance the mechanical properties of alginate, researchers have been using different methods such as changing the crosslinks, or combining with other biomaterials. An alginate/galactosylated chitosan scaffold has been studied for hepatocyte attachment (Chung *et al.*, 2002). A highly porous, three-dimensional sponge composed of Ca-alginate and galactosylated chitosan scaffold was prepared to provide specific hepatocyte recognition signals and enhance the mechanical property of the Ca-alginate sponge.

In addition to these chemical and physical disadvantages of alginate, researchers found alginate capsules that were injected *in vivo* caused fibroblast overgrowth (Sun, 1987b). Also they stimulated human monocytes to produce high levels of cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1(IL-1) while M-blocks and MG-blocks showed higher potency to induce cytokine production than G-blocks (Otterlei *et al.*, 1991). Similar results were found in the enzymatically depolymerized alginate oligosaccharides (Iwamoto *et al.*, 2003a). Remarkably, the work that has been reported was only based on alginate stimulation. In our experiments, we chose a macrophage-like cell line, RAW264.7 to study the mechanism of such immune responses.

2.2 Evaluation of biomaterials

2.2.1 Biocompatibility

2.2.1.1 Haemocompatibility

For those devices used in blood-contacting areas, haemocompatibility is much more important than any other properties since blood clotting must prevented. When implants are put into the body, immediately blood and its components escape from the vascular system and come into the injured tissue, and then induce activation including the extrinsic and intrinsic coagulation systems, the complement system and platelets. For the implant itself, with the Vroman effect (Vroman *et al.*, 1980;Leduc *et al.*, 1994), proteins from the blood are rapidly adsorbed onto the surface, which leads to altered thrombus and blood clot formation.

Heparin is a well-known anticoagulant used on implant surfaces to minimize thrombus formation. Such devices have emerged over the last two decades (Larm *et al.*, 1983). Another surface modification is used to increase steric repulsion by increasing the surface hydrophilicity and consequently to decrease protein adsorption and platelet adhesion. Grafting poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) chains onto the implant surface is a common method to improve biocompatibility (Mao *et al.*, 2004).

2.2.1.2 Biodegradation

Many biomaterials used in tissue engineering act as scaffolds which provide a platform for cell adhesion and growth. For most tissue engineering purposes, such scaffolds will ideally be biodegraded after the tissue reconstruction, and the biodegradability should be engineered to occur in a specific time. However, the chemical factors released from the scaffolds in situ have an obvious effect on the body's micro-environment because they may change the local pH and increase the metabolic burden. Such chemicals may be toxic. At the same time, the particles or components from the degradation may mediate many of the vascular and cellular immune responses. There are two main chemical reactions that occur in biomaterial degradation: hydrolysis and oxidation. Enzymes secreted by immune cells also participate in biodegradation: after implantation, neutrophils, macrophages and monocytes responding to chemical factors(chemotaxis) are recruited to the injured site, where they secrete enzymes and reactive oxygen to decompose the "invader" (Mikos *et al.*, 1998).

2.2.1.3 Toxicity

Toxicity includes acute toxicity, acute systemic toxicity, subchronic toxicity, and reproductive toxicity. Acute toxicity may happen in a couple of minutes to several hours. It may be due to direct chemical contact and shows cytotoxicity. Often this toxicity is first tested by using different cell lines *in vitro*. Acute systemic toxicity is a severe symptom after implantation in less than 24 hours. It may be caused by degradation of implants or debris from the materials which affect the whole body rather than a single tissue or organ(Anderson, 2001), and always has specific effects on the nervous system,

immune system and endocrine system. In addition, systemic toxicity due to the immune response may happen when the substance is antigenic such as foreign proteins, nucleoproteins and carbohydrates. The humoral immune responses by B cells and cell-mediated responses by T cells are both involved. Currently many biomaterials are used in drug delivery, and as vehicles to transport peptides, drugs or genes, so the risk of systemic toxicity is increasing.

2.2.2 Host responses to biomaterials

2.2.2.1 Brief review of innate/adaptive immunity

In vertebrates, there are two immune systems in the body to defend and destroy invading pathogens: the innate immune system and the adaptive immune system. Innate immunity is a primary host defense which is the most universal and evolutionally ancient part of the immune system, while adaptive immunity is more advanced and only exists in vertebrates. Most pathogens will be detected and destroyed by the innate immune system. Although the mechanisms for eliminating pathogens are quite different, both the primary and advanced immune systems display the same basic functions: recognition of different pathogens; elimination of the pathogens once they have been recognized; and self-tolerance which means avoiding killing of self cells (Beutler, 2004). These two immune systems are separate but interact extensively,

2.2.2.1.1 Innate immunity

Innate immunity is the first line of defense against the invading pathogens, especially microorganisms. These microbes can be recognized by their unique pathogen-associated molecular patterns (PAMPs) which are the structural features of microbes

(Janeway, Jr., 1989). According to the different forms, there are two components involved into the innate immune system: the cellular and the humoral components.

The cellular components include macrophages, neutrophils, basophils, eosinophils and mast cells, which are all derived from myeloid cells. In vertebrates, these cells have stand–alone capability and play a major part in engulfing and destroying pathogens.

At the infection site, neutrophils are the first cells to respond. They are short-lived but have many functions: they secrete chemotactic signals to attract monocytes and dendritic cells to the inflammatory site; secrete TNF to activate macrophage differentiation; and interact with lymphocytes in a bi-directional manner (Nathan, 2006).

Macrophages, or mononuclear phagocytes, are distributed throughout the body and arrive to the inflammatory site after neutrophils. They are long-lived cells and also play many functions in addition to engulfing and killing pathogens. Macrophages help to remove apoptotic cells and bodies caused by oxidative stress, secrete different cytokines to recruit other phagocytes, and present specific antigens to $CD4^+$ T cells on the surface to activate the adaptive immune responses.

Compared to the quantity of neutrophils and macrophages, the other phagocytes such as eosinophils, basophils and mast cells are much rare in the normal blood, but they can quickly increase in number in response to an allergy. Eosinophils mainly respond to large parasites that cannot be phagocytosed by releasing peroxides and toxins to kill the parasites. Activated basophils and mast cells release histamine to increase vascular permeability and release cytokines TNF-α, IL-8 and IL-5 to attract neutrophils and eosinophils to the inflammatory site (Lydyard *et al.*, 2001).

For innate immune recognition, a limited number of receptors evolved for pathogen recognition. They distinguish the molecular structure of conserved regions in pathogen products from the host. These receptors are pattern recognition receptors(PRRs) which can be expressed on the cell surface, inside of the cell or into the body fluid (Medzhitov and Janeway, Jr., 1997). Macrophage mannose receptors (MMR) on the macrophage surface can recognize a variety of microbes and mediate phagocytosis. Ten Toll-like receptors have been investigated in humans and mice thus far. Most of them are transmembrane receptors and can recognize a variety of PAMPs. For example, TLR4 can respond to lipopolysaccharide (LPS) which is expressed on the outer membrane of gram-negative bacteria; TLR9 recognizes CpG containing DNA which originates from bacteria and viruses (Akira and Takeda, 2004).

The humoral components in innate immunity include complement, lysozyme, antimicrobial peptides and cytokines, which can also kill the invaders without cell engulfment. Complement, a group of proteolytic enzymes, can be activated by an antibody-mediated pathway, the properdin pathway and the MASP (mannan-binding protein-associated serine protease) pathway. It helps to opsonize microbes, initiate phagocytosis or inactivate viruses (Beutler, 2004).

2.2.2.1.2 Adaptive immunity

The adaptive immune system appeared later than the innate immune system in the evolutionary landscape. This system is much more complex and efficient, because it helps the host to recognize specific foreign antigens due to the great variability and

rearrangement of receptor gene segments, and to have an immunological memory of infection by providing quick responses and defense to the same attack in the future.

The adaptive immune system consists of lymphocytes, lymphoid organs and tissues. T cells and B cells are the two main types of lymphocytes. They have similar morphology, but different antigen receptors and recognition sites on the surfaces. There are two kinds of T cells: helper (Th) T cells and cytotoxic (Tc) T cells. The former includes Th1 and Th2 cells with CD4 expression on the cell surface. Th cells can be activated by cytokines that are secreted by antigen presenting cells (APC) (including activated macrophages, DCs and NK cells) and can regulate the subsequent activation of both B cells and Tc cells. Some B cells secrete specific antibodies in response to foreign antigens, and the others proliferate and mature into memory B cells with T cell help. For Tc cells, with CD8 expression on the surface, some kill the infected cells by recognizing antigens presented on the surface of infected cells, and others mature into memory T cells.

2.2.2.1.3 Bridge between innate and adaptive immunity

There is still little known about the connections between innate and adaptive immunity. Pathogen recognition and antigen presentation by innate immune cells are fundamental to the activation of adaptive immune responses. Two classes of polymorphic major histocompatibility complex (MHC) molecules participate in antigen presentation: MHC I molecules are expressed on nearly all nucleated cells and can be recognized by $CD8^+$ Tc cells; MHC II molecules are expressed only on antigen presenting cells and are

recognized by $CD4^+$ Th cells. Cytokines and costimulatory molecules such as CD40, CD80 and CD86 are some of the links between T cells and APCs (Hoebe *et al.*, 2004).

2.2.2.2 Different immune responses to biomaterials

After biomaterials are implanted into the body, the host responds quickly, but due to the chemistry and topography of biomaterials, different results may appear compared to pathogen invasion. Several events occur in sequence during the host response to implantation of medical devices: injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, formation of granulation tissue, foreign body reaction, and fibrosis/fibrous capsule development (Anderson, 2001). The following will discuss some of these main reactions.

2.2.2.2.1 Non-specific protein adsorption and blood-material interactions

Non-specific protein adsorption is the primary event once the material contacts blood. The proteins from serum are mainly soluble including blood plasma and peritoneal exudates. Many proteins denature after adsorbing to the surface. pH, ionic strength, chemical properties and topography of the materials are the key factors that influence the blood-material interactions. Hydrophobic surfaces may more easily adsorb proteins than hydrophilic ones. These adsorbed proteins help platelet adhesion.

After adhesion, the platelets release ADP, then form a small amount of thrombin and generate thromboxane A_2, aggregating more platelets on the surface. The platelets induce blood coagulation sequences through activating factor X and converting

prothrombin to thrombin. Finally, a thrombus or blood clot is formed on the material surface.

2.2.2.2.2 Acute inflammation

Following implantation, inflammatory cells are recruited to the injured site in response to cytokines, chemokines and other substrates generated from the coagulation cascade (Mikos *et al.*, 1998). Neutrophils arrive first, and then macrophages and other monocytes infiltrate from the vascular system and congregate around the implant. The stimuli induce the macrophages to mature. Different phenotypes of macrophages have been found on implant surfaces (Johansson *et al.*, 1998). Proteins including complement C3 fragments, IgG and fibronectin adsorb non-specifically to the material surface and mediate neutrophil and macrophage attachment and activation (Mikos *et al.*, 1998). The activated pro-inflammatory cells up-regulate cytokine secretion to recruit more monocytes and lymphocytes to come to the site, and at the same time express reactive oxygen intermediates and proteolytic enzymes to decompose the implant. The properties of implants, such as size, ligands, surface characteristics and even shape, may cause a different intensity and duration of inflammation (Anderson, 2001).

Fibrinogen converted to fibrin also participates in the interaction between monocytes/macrophages and materials by mediating the pro-inflammatory effects. It has been found that fibrin can increase IL-1β expression by human monocytes (Perez and Roman, 1995).

Complement participates in response to infection and foreign materials. More than 20 complement components exist in plasma as enzymes or binding proteins. Many foreign substances, such as lipopolysaccharide, bacterial polysaccharide and even particles or the surfaces of biomaterials can activate complement (Gorbet and Sefton, 2004). Complement activation releases C3a, C4a and C5a (complement components) that binds to neutrophils, monocytes and macrophages, which then induces innate immune responses. In addition, complement components participate in coagulation and finally thrombus formation.

2.2.2.2.3 Chronic immune responses

Compared to acute immune responses, chronic responses mean long-lasting inflammation with persistent inflammatory stimuli. Biomaterials may cause chronic inflammation depending on their chemical and physical properties. For biodegradable materials, this inflammation may happen more often and last a longer period when in response to a constant chemical stimulation from degraded components. In the chronic inflammatory site, monocytes (especially macrophages), lymphocyctes and plasma cells are the primary cell types.

Macrophages, long lived and versatile cells, have been found as the modulators in the interactions between tissue and materials: they present antigens and secrete cytokines to recruit lymphocytes; they engulf and degrade particle materials; or they form foreign giant cells to separate non-phagocytosable materials from the host environment.

2.2.2.2.4 Foreign body reactions and granulation tissue formation

Foreign body reactions may happen weeks after implantation. While macrophages cannot remove the implant by simple digestion, they may adhere onto the surface and fuse into a giant multi-nucleated cell which isolates the implant from the body. The molecular mechanism of giant cell formation and the resulting biological responses are still unclear (Anderson, 2001). However, the formation of giant cell depends on the chemical properties and topography of biomaterials.

Granulation tissue is a highly vascularized tissue that is rich in fibroblasts and leucocytes. It replaces the initial fibrin clot in the wound. Granulation tissue formation can be regarded as the end of inflammation. Fibroblast and endothelial cells are the two critical types that participate in reconstruction of injured tissue. The activated fibroblast cells synthesize type II collagen and proteoglycans to help form the final fibrous capsules. Granulation often happens when there is a wound after implantation.

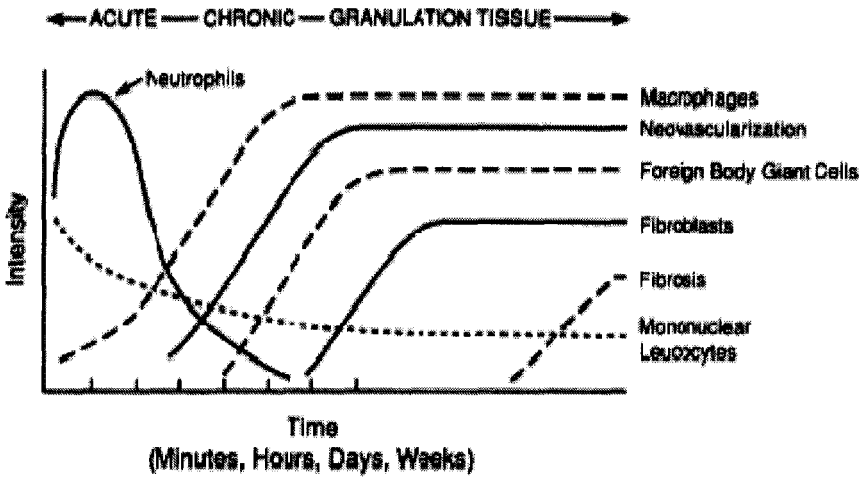


Figure 2: Series of immune responses to biomaterial when it is implanted into the body. The properties of implant such as chemical properties, size, shape and topography will influence the intensity and time of responses (Anderson, 1988).

2.2.2.3 Mechanisms of immune responses to biomaterials

2.2.2.3.1 Cellular immune responses

There are two dimensions of biomaterials that are widely used: bulk materials as devices or scaffolds seeded with specific cells for tissue replacement, or microparticles as a matrix with drugs, cells or genes encapsulated for drug delivery or gene therapy. The sizes in these two applications are quite different and the latter can be even prepared as small as nanoparticles (less or equal 20nm diameter).

The geometry of biomaterials will influence the inflammatory response. In general, biomaterial scaffolds with a large surface area will induce "frustrated phagocytosis" in which macrophages fail to engulf the materials. This eventually leads to the fusion of several macrophages into a multinucleate FBGC that damages or "walls off" the medical device. In contrast, small particle-like materials may have a different response as macrophages can easily "eat them up".

For the bulk materials, as we discussed previously, neutrophils, monocytes and lymphocytes are the main participants in the inflammation responses. Their effects are time and material dependent. The cellular activation in the interactions of biomaterial, protein and macrophages are shown in Figure 3.

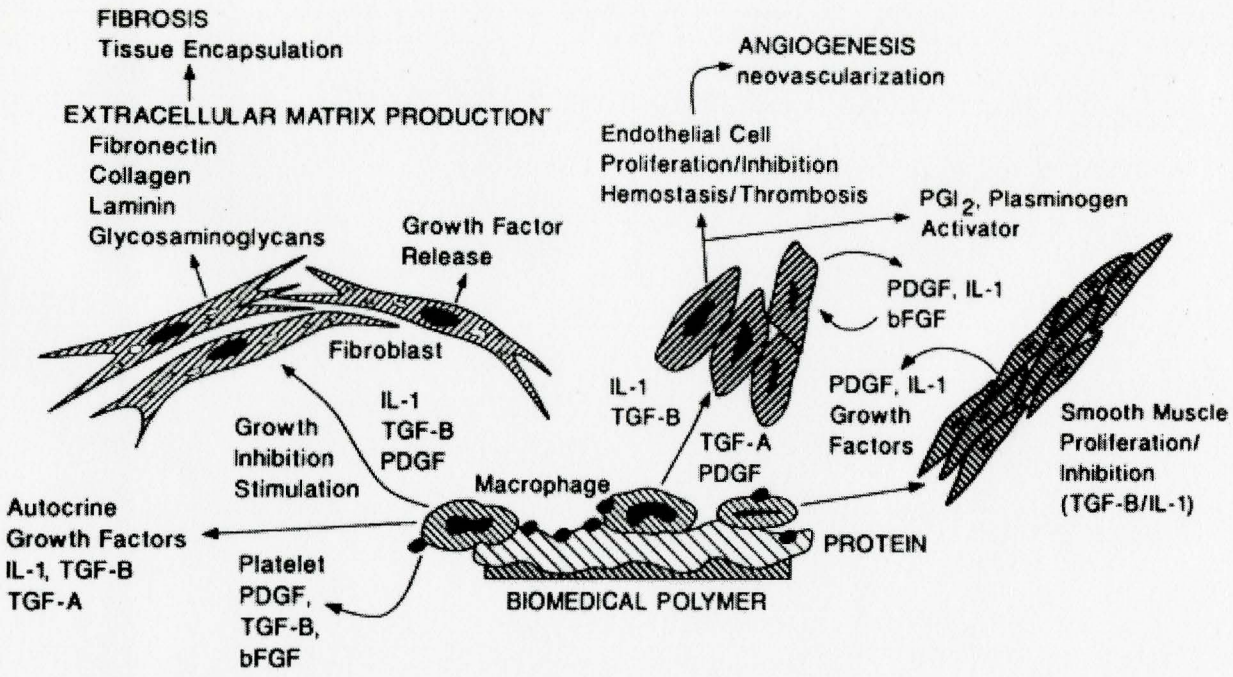


Figure 3: Polymer/protein/macrophage interfacial interactions leading to cellular activation, proliferation and synthesis. Cytokines and growth factors control cellular processes important in biocompatibility and the wound healing process (Mikos *et al.*, 1998).

For microparticle implants, we expect that host responses will be close to those after pathogen recognition. In fact, the rationale for microparticles in injectable drug delivery systems is based on the abilities of APCs to phagocytose. Researchers found the suitable size range for microparticles as delivery systems would be less than 5μm in diameter (O'Hagan *et al.*, 2004), and the maximal uptake by macrophages should be less than 2μm (Tabata and Ikada, 1988). Especially for biodegradable materials, after phagocytosis, the microparticles will be degraded by enzymes in the lysosomes which exist in phagocytic APCs. The degraded parts of small polysaccharide particles or peptides would be captured by the host Class II Major Histocompatibility Complex (MHC II) molecules and presented as antigens on the membrane surface of APCs. These

antigens can be recognized by $CD4^+$ T helper cells and induce adaptive immune responses: $CD4^+$ helper T cells recognize different antigens presented by APCs and differentiate into Th1 and Th2 cells to induce B cells to secrete different antibodies. T helper cells promote B cell growth and differentiation through direct cell surface signaling or by producing cytokines (Figure 4).

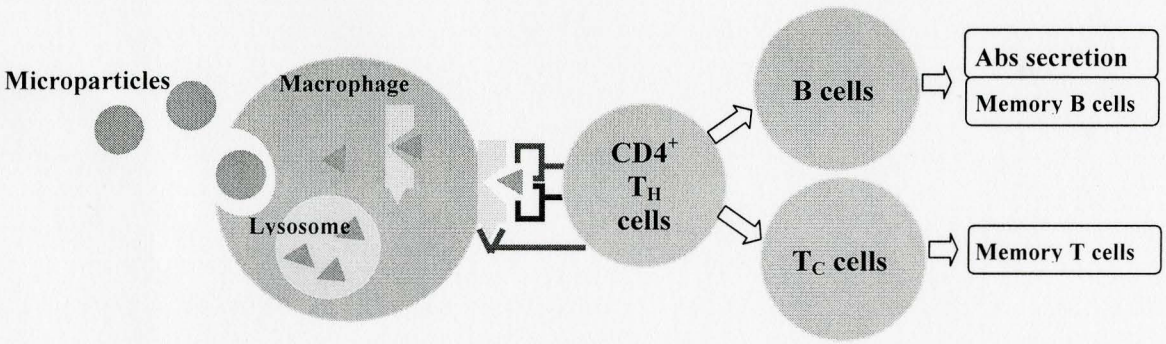


Figure 4: Cellular responses to microparticle biomaterials *in vivo*.

However, it has been found that some biomaterials can suppress the adaptive responses. For example, alginic acid oligosaccharide was shown to significantly suppress Th2 cell development and IgE production in ovalbumin specific T cell receptor transgenic mice (Yoshida *et al.*, 2004).

2.2.2.3.2 Macrophage activation in response to biomaterials

Macrophages play an important role in material responses. Firstly, macrophage receptors recognize and bind to proteins non-specifically adsorbed to the surface such as opsonins (including complement C3 fragments and IgG (Mikos *et al.*, 1998)), and

extracellular matrix(ECM) proteins (such as fibrinogen (Hu *et al.*, 2001)). Secondly, macrophages migrate and attach to the material's surface by recognition of the amino acid sequence Arginine–Glycine–Aspartic acid (RGD) contained in many ECM proteins (Ruoslahti, 1996;D'Souza *et al.*, 1991). Thirdly, cytokines (such as IL-1, IL-6, IL-12 and TNFα) and growth factors (such as TGF-α, TGF-β and PDGF) are secreted by macrophages to recruit other macrophages and lymphocytes to the implant site. Finally, activated macrophages release active oxygen intermediates to degrade foreign substances, and on the other hand, present antigens to T helper cells to help recognize foreign invaders. In most cases, if the material can not be phagocytized, several macrophages fuse to foreign body multinuclear giant cells (MNGCs or FBGCs) to separate the implant from the tissue.

The mechanisms that trigger MNGCs formation are still not clear However, compared to single macrophages, MNGCs have a reduced phagocytic activity (Papadimitriou *et al.*, 1975) but a similar cytokine secretion ability. MNGC formation depends on the properties of surface area - rough surfaces have been observed to have a high density of MNGCs while only a thin layer of MNGCs appeared on a smooth surface (Anderson, 2000). Also different lymphocytes and cytokines participate into the fusion process. For example, the cytokine IL-4 can induce human monocyte-derived macrophages to fuse into MNGCs (Anderson, 2000;McNally and Anderson, 1995); Th2 lymphocytes produce cytokines IL-4 and IL-13 to induce MNGCs formation (DeFife *et al.*, 1997;McNally *et al.*, 1996).

Activated macrophages secrete a variety of cytokines in response to infectious agents. The proinflammatory cytokines including IL-1, IL-6, IL-12 and tumor necrosis factor (TNF) can help to exaggerate the inflammatory reactions.

Along with the immunology has developed a better understanding of cytokine functions has emerged. Researchers found that most cytokines have many functions and various target cells which have specific cell membrane receptors for recognition. For example, IL-1β participates in a variety of cellular activities, including acting as an important mediator in inflammation, promoting B cell maturation and proliferation, and activating NK cells. , Interleukin-6 (IL-6) can induce acute immune responses, activate B cells to differentiate into plasma cells, promote plasma cells to secrete antibodies and help stem cell differentiation. Interleukin-12 (IL-12), working along with IL-2, can cause activated Tc cells to differentiate into cytotoxic T lymphocytes and can also activate NK cells. TNF-α can cause tumor cell death, and can feedback to macrophages by binding to the TNF receptor to exaggerate cytokine production (Janeway, Jr. *et al.*, 1999).

2.2.2.4 Macrophages studies in immune responses to biomaterials

As one the key players in immune responses caused by biomaterials, macrophages have been studied widely by *in vivo* and *in vitro* experiments. These studies include specific ligand or component recognition by macrophages receptors, behavior of macrophages, molecular mechanisms of signal transduction, cytokine production and more.

A broad range of macrophage membrane receptors, which can be divided to a series of receptor families, have many functions on self or invader recognition, cell growth, survival, migration and activation. The studies on such receptors can provide information for understanding the primary step of macrophage activation. A lot of receptor families have been studied even though the understanding is still not complete. For example, scavenger receptors, toll-like receptors and integrins have been studied for a long time. Scavenger receptors, such like CD36, SR, are nonopsonic receptors. They participate in phagocytosis of bacteria and apoptotic cells, and endocytosis of modified low density lipoprotein (LDL). Toll-like receptors, a family of pathogen recognition receptors, act as a sensor of microbes. Integrins are a family of receptors that bind to complement.

Macrophage behavior studies include cell migration, adhesion and foreign body giant cell formation. Usually the studies are combined with specific protein (mostly from serum) adsorption, receptor-ligand binding and signal transduction (Jenney and Anderson, 2000c).

For mechanisms of signal transduction studies, researchers focus on the molecular level of the signal pathway. Since there are many pathways and most of them can interact with each other, the studies are often performed through blocking or inhibiting one or several pathway to identifying the main one. Here, we chose the NF-κB pathway to illustrate how macrophages respond quickly to foreign invaders including alginate.

NF-κB pathway

There are several complex pathways to activate NF-κB translocation. The mode for NF-κB pathway is shown in Figure 5. In brief, when a variety of microbial products and inflammatory stimuli activate the inhibitor kappa B (IκB) protein kinase (IKK) complex, it phosphorylates the IκBα associated with the NF-κB p50/p65 heterodimer, which makes IκBα degrade and thus frees the NF-κB p50/p65 heterodimer to translocate into the nucleus. The free NF-κB activation cause two results: the first is to initiate the transcription of inflammatory and immune mediators such as cytokine and adhesion molecules (Zingarelli, 2005), which induce leukocyte trafficking and activation to help in immune defence. The second effect is that NF-κB also induces the transcription of apoptotic modulators which regulate cell growth, proliferation and differentiation (Zingarelli, 2005).

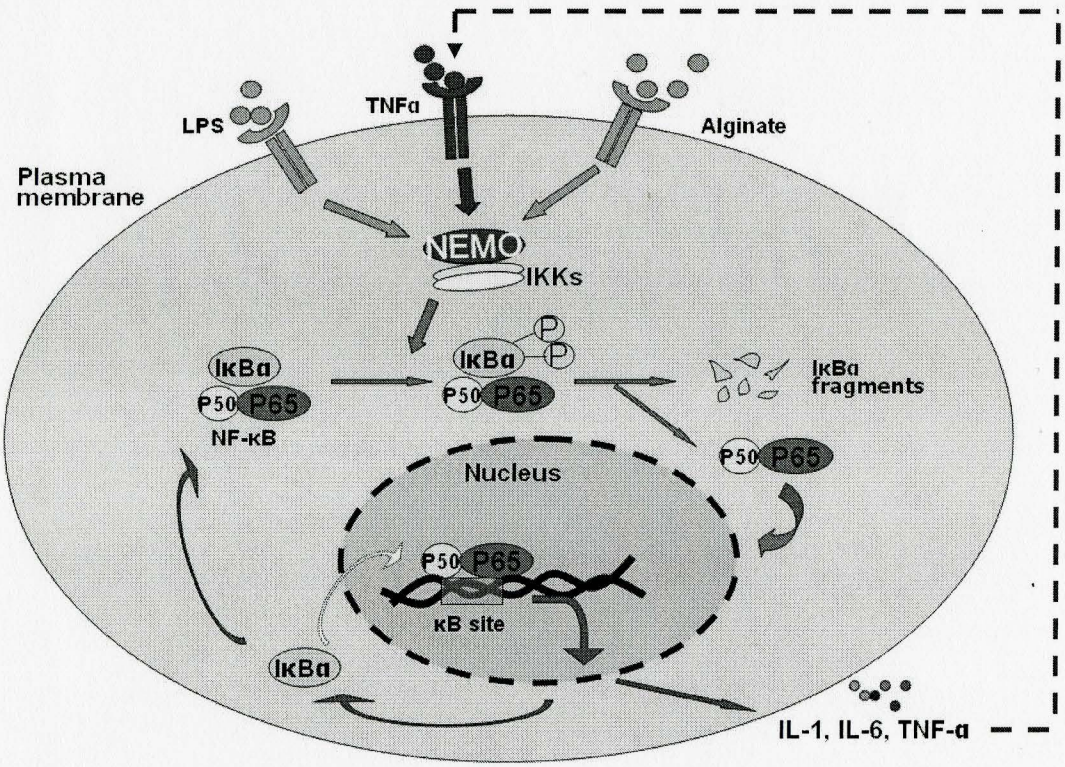


Figure 5: Model of NF-κB activation pathway in a macrophage

In normal conditions, NF-κB rapidly shuttles between the cytoplasm and nucleus, and because of the efficiency of nuclear export, most of the NF-κB stays in cytoplasm in resting cells. Recently, it has been shown that there is an equilibrium state when NF-κB stays in cytoplasm (Bartek and Lukas, 2006b;Bartek and Lukas, 2006a;Verma, 2004a;Verma, 2004b;Blackwell and Christman, 1997). In response to extracellular stimuli from bacteria, viruses or chemicals, this equilibrium state is broken and NF-κB remains a long time in the nucleus by binding to specific sites which open the expression of cell survival modulators and help the cell to return to its normal state. In work presented in this thesis, sodium alginate showed a persistent stimulus to the macrophage cells which kept the NF-κB for an extended time in the nucleus. The persistent activation of NF-κB may lead to an increase in inflammatory processes, resulting in tissue damage and potentially multi organ failure. On the other hand, a balance between cell proliferation and cell death may be broken.

Nuclear binding of NF-κB also stimulates production of IκBα and p105 which inhibits further activation of NF-κB. Increased p105 production favours the formation of p50 homodimers, which can inhibit NF-κB action by entering the nucleus and competing for NF-κB binding sites (Blackwell and Christman, 1997). Similarly, IκBα can either go into the nucleus then remove the NF-κB proteins bound to DNA or go to the cytoplasm to prevent further activation(Verma, 2004b). Several steps of the NF-κB signal transduction pathway, such as, inhibitor of NF-κBα (IκBα) phosphorylation and degradation, IκB kinase (IKK) activation, NF-κB nuclear translocation, and transcriptional activity, can be targeted by various inhibitors ((Delhalle *et al.*, 2004).

The mechanisms NF-κB activation by LPS has been studied for a long time. Toll-like receptor 4 (TLR-4) can recognize LPS and lead to a cascade of signal phosphorylation. Finally the inhibitor of NF-κB (IκB) is proteolysed and NF-κB freely transfers between nucleus and cytoplasm (Blackwell and Christman, 1997).

Cytokine production by activated macrophage

One of the important outcomes of activated macrophage is to secrete cytokines. For example, in responses to LPS, the important cytokines are produced include IL-1, IL-6, IL-8, IL-12 and TNF-α (Janeway, Jr. *et al.*, 1999). Also the NF-κB pathway participates in regulation of such cytokine secretion (Timothy S.Blackwell and John W.Christman, 1997).

Different cytokines have various functions in immune responses. Here, only such cytokines that related to this thesis are mentioned.

Cytokine IL-1, IL-6 and TNF-α are proinflammatory cytokines that help to recruit more monocytes and lymphocytes to the inflammatory site for acute immune response. The cytokine IL-1β can activate T cells by promote them to express IL-2 and IFN-γ; promote B cells maturation and proliferation; and can activate NK cells. The cytokine IL-6 causes activated B cells to differentiate into plasma cells; induces plasma cells to secrete antibodies; and helps stem cell differentiation. The cytokine TNF-α can induce local inflammatory responses and feedback locally by activating macrophages to express cell adhesion molecules and other cytokines. The cytokine IL-12 can activate NK cells;

and in cooperation with the cytokine IL-2 can cause activated Tc cells to differentiate into cytotoxic T lymphocytes (Janeway, Jr. *et al.*, 1999).

2.2.2.5 Pros and cons of immune responses caused by biomaterials

Like the two sides of a coin, the immune responses caused by biomaterials have both good and bad effects. People may overcome inflammatory effects by modification of the chemical or physical properties of implants; at the same time, the properties that cause the immune response could be used to activate the host defense in a controlled fashion for vaccine adjuvants. Of course, people are also trying to find less toxic and more biocompatible materials for tissue engineering applications.

2.2.2.5.1 Anti-inflammation modifications of biomaterials

Researchers have been studying many methods to modify the surface of biomaterials in order to reduce immune and inflammatory responses. These methods include:

1) To avoid complement activation, anti-coagulant surface modifications have been applied, such as heparin grafting to the material surfaces.

2) Hydrophobic surfaces have been changed into hydrophilic ones to avoid non-specific protein adsorption, e.g. by PEO conjugation (Han *et al.*, 1993).

3) Specific peptides have been linked to the surface in order to guide cell recognition, e.g. RGD peptide.

4) The physical properties of surface have been altered, e.g. changing rough surfaces into smooth ones (Cai *et al.*, 2005).

5) Homograft cell transplant onto the implant to acquire minimum rejection, e.g. autologous bone marrow cells living on a synthetic biodegradable scaffold can easily be accept by the body (Neuenschwander and Hoerstrup, 2004).

2.2.2.5.2 Vaccine adjuvant

Vaccines, as one of the most successful medical interventions, have been used for over two centuries. In order to get appropriate immune responses, new vaccines have two main components- antigen and adjuvant. Antigens or epitopes are whole or partial pathogens that can cause a memory immune response. Adjuvants play two roles in an advanced vaccine: one is as the antigen delivery vehicle (delivery system); the other is as the immune potentiator that triggers early innate immune responses and aims to prolong immune responses (O'Hagan and Valiante, 2003).

Delivery systems in vaccines help to maximize the antigen concentration in the lymph nodes. Several delivery systems have been tried, such as mineral salts, surface active agents, synthetic microparticles, oil-in-water emulsions and liposomes (Pashine *et al.*, 2005). However, the only licensed adjuvants thus far for use in humans are aluminum salts (Kenney and Edelman, 2003) and oil-in-water MF59 (O'Hagan, 2001). They can not be effectively used with all antigens. New adjuvants are needed for vaccine development.

Recently, people have tried to use biodegradable materials as adjuvants. Poly(lactide co-glycolide) (PLG) has been successfully applied in DNA vaccine delivery. PLG is prepared into cationic microparticles with the mean size close to 1μm. These particles with DNA adsorbed onto the surfaces can enhance the engulfment of antigen into APCs and facilitate the recognition by B cell antigen receptors, and consequently significantly enhance the immune responses compared to the immunization with naked DNA in mice (Singh *et al.*, 2001) and in non-human primates (O'Hagan *et al.*, 2001).

2.3 Hypotheses and objectives of present research

Thus far, researchers only have limited information on the mechanism of immune responses caused by alginate. In this thesis, a well-established macrophage cell line, RAW264.7, was used to investigate the cellular responses to alginate stimulation, and also to detect the cytokine production (IL-1β, IL-6, IL-12 and TNF-α) to quantify the outcomes after cell activation.

In a normal physiological environment, alginate hydrogel degradation is slow and uncontrolled. Researchers have found that in normal physiological conditions, the cross-linking by divalent cations can be broken and replaced by hydrogen bonds which results in hydrogel dissolution. But this degradation only breaks the ionic bridges between different polymer chains and has little effect on the mono-chains. When alginate hydrogel is put into the body, the amount of mono-chain alginate will increase along with degradation, and may also interact with inflammatory cells as with the hydrogel. However, there are few researchers studying in this area and no references discuss this situation. So it's valuable to investigate the interaction between mono-chains of alginate

and inflammatory cells. In this thesis, a sodium alginate which is soluble in normal conditions and mainly composed by M blocks is chosen as the focus of research.

According to the aim of this thesis, the following hypotheses are made:

1) The soluble alginate activates a macrophage cell line - RAW264.7 cells through the NF-κB pathway in the same way as LPS stimulation.

2) Cytokine production is one of the main outcomes of activated macrophages. After proteins NF-κB translocate into nucleus, cytokines such like IL-1β, IL-6, IL-12 and TNF-α will be secreted, and the production will rely on the degree of stimulation and the sorts of stimulators.

3) Serum, especially proteins in the serum, participates in macrophage activation and interacts with alginate stimulation.

The specific objective of this thesis is divided into two parts:

1) Study the effects of alginate stimulation of RAW264.7cells.

In a normal culture condition, macrophages are given different concentrations of alginate or LPS stimulation. The activation of macrophages is determined by two ways: one is with NF-κB pathway by tracking the protein distribution; the other is by quantifying different cytokine production.

2) Study the interaction of serum with alginate in stimulation of RAW264.7 cells.

After withdrawal serum from the medium, macrophages are stimulated with alginate or LPS. Cell activation is determined by protein NF-κB translocation into the nucleus and different production of cytokine. The results from these two different culture conditions will be compared to find the difference.

Chapter 3 Experimental

3.1 Materials

Soluble alginate

Low viscosity (250 cps) alginic acid sodium salt, purified from brown algae and composed primarily of anhydro-β-D-mannuronic acid residues with 1→4 linkage was obtained from Sigma (Oakville, Ontario, Canada). The ratio of M-blocks to G-blocks was 1.96 and the molecular weight was 9,500kDa.

LPS

LPS extracted from *Escherichia coli* 055:B5 (Sigma) was prepared at two concentrations in sterile PBS (100ng/ml and 1μg/ml). LPS solutions were used as positive controls in the experiments.

Chemicals and solvents for Immuno Fluorescence

- Goat serum (2% v/v in PBS) (Invitrogen, Burlington, ON, Canada)
- NF-κB p65 (C-20) rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

- Goat-anti-Rabbit IgG FITC (Fluorescein isothiocyanate) conjugate and Hoechst (blue) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA)
- Sterile round glass coverslips (Fisher Scientific, Ottawa, ON, Canada)
- Paraformaldehyde (PFA) and Triton X-100 (Sigma)

Chemicals and solvents for cell dialysis and Western Blot

- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), KCl, Phenylmethylsulphonylfluoride (PMSF), Leupeptin, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), Glycerol, Leupeptin and DTT (Sigma).
- Bradford assay reagent(Bio-Rad, Mississauga, ON, Canada)
- Acrylamide, bio-acrylamide, Tris base, HCl, sodium dodecyl sulphate (SDS), bromophenol blue and ammonium persulfate (Bioshop Canada Inc. Burlington, ON, Canada)
- NF-κB p65 (C-20) rabbit polyclonal IgG (Santa Cruz Biotechnology)
- Goat-anti-Rabbit IgG alkaline phosphatase conjugate (Bio-Rad)
- TBS (50mM Tris, 150mM NaCl, pH 7.4)
- NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Bio-Rad, USA)

- PVDF membrane (0.22μm, Bio-Rad)

Solvents and materials for ELISA

- BD OptEIATM mouse ELISA sets for IL-1β, IL-6, IL-12 and TNF-α (BD Bioscience, Mississauga, ON, Canada)
- TBS Substrate solution (BD Bioscience)

3.2 Cell culture

RAW264.7 cells were obtained from ATCC (TIB-71, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Characterized standard, Hyclone, Mississauga, ON, Canada). Cells were maintained at 37°C with 5% (v/v) CO_2 in a humidified incubator. Cell viability was determined by trypan blue staining; over 90% viability was used in all experiments.

3.3 Experimental methods

3.3.1 Endotoxin test

All solvents used in cell culture or for cell stimulation were tested by LAL test. These solvents included cell culture medium and PBS.

Soluble sodium alginate stock solutions were prepared at 1%(w/v) and 3%(w/v) in distilled water (cell culture grade, Invitrogen), and filtered with Whatman filters, then 0.45μm and 0.2 μm Supor® membrane filters (Pall Corporation, Mississauga, ON, Canada). Endotoxin tests for each alginate solution were performed before experiments by using the Limulus Amebocyte Lysate (LAL) test kit (Cape Code, Inc. MA, USA). The measured endotoxin levels were all less than 1 EU/ml (data not shown).

3.3.2 Immuno fluorescence

1) Culture: RAW264.7 cells (2×10^5) were grown to half confluence on sterile glass coverslips placed in a 12-well plate with 2ml medium each well.

2) Stimulation: The stimuli (final concentrations were 1mg/ml and 3mg/ml soluble alginate or 100ng/ml LPS) were added to cells and incubated in 37°C for 2, 6, 12, 24 and 48 hours.

2) Fixation: All the medium was aspirated, and 1.5~2 ml 4% (w/v) PFA was added to cover the whole coverslip for 10 minutes. The cells were washed with PBS once, and then incubated in 0.1% Triton-X for 10 minutes. The following procedures were all performed at room temperature.

3) Blocking: The cells were washed with PBS, then changed to PBS containing 2% (v/v) goat serum (close to 1ml/well) for 45 minutes to 1 hour.

4) Primary antibody binding: The cells were washed with PBS, then 20-40μl NF-κB P65(C-20) rabbit polycolonal IgG (1:1000 dilution with PBS containing 2% (v/v) goat serum) was added to each coverslip for 45 minutes to 1 hour.

5) Secondary antibody binding: After washing with PBS, 20-40μl Goat-anti-Rabbit IgG FITC conjugate (1:500 dilution with PBS containing 2% (v/v) goat serum) was added. The 12-well plate was kept in a dark place at room temperature for 45 minutes to 1 hour.

6) Hoechst staining: The coverslips were washed with PBS with gentle shaking, 3 times. Hoechst staining was for 5-10 minutes.

7) Mounting: The coverslips were washed with PBS with gentle shaking twice, and then changed to a ddH_2O wash once. Mounting solution helped to attach coverslips to a clean glass slide. Each slide has two duplicate coverslips. The stained cells were visualized using a Leica DM-IRE2 inverted microscope.

3.3.3 Cytosolic and nuclear extract preparation

The protocol was followed from the literature (Yu *et al.*, 2004;De *et al.*, 2005). Treated RAW264.7 cells (10^6) were scraped from Petri dishes after washing twice with ice-cold PBS to remove the influence of medium. The cells were collected by centrifugation at 180g, 10 minutes at room temperature, then resuspended in 100μl ice-cold hypotonic buffer (10mM Hepes, 10mM KCl, 0.5nM PMSF, 5μg/ml Leupeptin, 1mM DTT, 0.1mM EDTA, adjusted to pH 7.9 with KOH). The cells were lysed on ice

for 15 minutes. They were vortexed for 10 seconds, then centrifuged at 13,000g for 1minute at 4°C. The supernatant was collected as cytosolic extract and stored at -80°C.

The pellet, containing nuclear extract, was resuspended in 60µl ice-cold high salt extraction buffer (20mM Hepes, 300mM NaCl, 1mM EDTA, 25% Glycerol, 0.5mM PMSF, 5µg/ml Leupeptin, 1mM DTT, adjusted to pH 7.9 with NaOH). The solution was incubated on ice for 30 minutes, then vortexed for 10 seconds. After centrifuging at 13,000g for 15 minutes at 4°C, the supernatant was collected as nuclear extract and stored at -80°C until analysis.

3.3.4 Western Blot

The cytosolic and nuclear extracts were run on Western Blots to measure NF-κB distribution. Protein concentrations in the extracts were determined by Bradford assay (Bio-Rad) before performing the electrophoresis.

1) SDS-PAGE: Following the standard protocols for SDS-PAGE (Wiley InterScience(online service), 2005), a 12.5% separating gel and 4% stacking gel were used for separating proteins. The sample (10µl) was combined with 10µl of sample buffer and loaded in each well. PageRuler^{TM} prestained protein ladder 5µl (Fermentas, Burlington, ON, Canada) mixed with 15µl sample buffer was loaded as a prestained marker. The power pack was operated at 140-150 volts for approximately 55 minutes of electrophoresis.

2) Gel Equilibration: The separating gel was put in transfer buffer for 15 minutes.

3) Electrophoretic transfer: In a modification of the protocols prepared by Rena M. Cornelius, the proteins were transferred from the gel to a PVDF membrane for 2 hours at 80 volts in ice-cold buffer. The membrane was dried on the bench.

4) Antibody incubation: After blocking unbound membrane sites with 5% (w/v) non-fat dry milk in TBS, pH 7.4, the whole membranes were incubated with primary antibody-NF-κB P65(C-20) rabbit polycolonal IgG for one hour, then with secondary antibody-Goat-anti-Rabbit IgG alkaline phosphatase conjugate for another one hour incubation.

5) Detection: Excess and non-specific probes were washed away with 0.1% (w/v) non-fat dry milk in TBS, then incubated with substrate solution containing NBT and BCIP to develop the color reaction. The reaction was stopped by rinsing with distilled water.

6) Densitometry analysis: The stained, dry membrane was scanned and quantified by densitometry analysis software (ImageQuant 5.2 Densitometry Analysis, Molecular Dynamics, Sunnyvale, CA, USA).

3.3.5 ELISA

The medium from the stimulated 10^6 RAW264.7 cells was collected at different time periods (12 hours, 24 hours and 48 hours), and stored at -80°C before use. Four cytokines were detected and quantified including IL-1β, IL-6, IL-12, and TNF-α by using ELISA sets. The procedures all followed the manufacture's instructions.

3.3.6 Statistical analysis

The results of Western Blot and ELISA were analyzed by t-test and $p<0.05$ was noted as a significance difference compared to the negative control groups.

Chapter 4 Control Experiments

4.1 Introduction

Before tracking the immune responses to alginate stimulation, some preliminary work needed to be performed to optimize the experiments. Compared to the complex *in vivo* environment, *in vitro* studies will be a relatively simple way to get better understanding of the response mechanisms. A suitable cell line, valid positive controls and reasonable concentration of alginate solution should be determined before the stimulation experiments.

4.2 Properties of RAW264.7 cells

Macrophages play an important role in response to foreign invaders including biomaterials. The physiology of macrophages has been studied using readily-available cell lines such as RAW264.7 (Raschke *et al.*, 1978). These cells are a murine macrophage-like cell line, which was derived from a tumor induced by Abelson murine leukemia virus. They have been widely used in studying the immune responses caused by different stimuli including LPS and different biomaterials (Higuchi *et al.*, 2005;Yu *et al.*, 2004;Kang *et al.*, 2000), and also as an established macrophage model, in studying specific signal pathway in response to inflammation (Rouzer *et al.*, 2005;Steurbaut *et al.*, 2006).

There are other advantages for this cell line, including easy culture, rapid growth rate, phenotypic resemblance to primary macrophages, and some well-known signal pathways such like NF-κB activation (Yi and Krieg, 1998).

Due to these desirable properties, we chose RAW264.7 cells to study the innate immune responses caused by alginate.

4.3 Immune responses caused by different concentrations of LPS

The lipopolysaccharide (LPS) complex, also named endotoxin, is part of the outer membrane of Gram-negative bacteria. It exists everywhere. Since it has a negative charge in solution from phosphate groups, it will be easily adsorbed to hydrophobic or cationic surfaces (Hirayama and Sakata, 2002). During fabrication procedures, especially in laboratory conditions, it is hard to avoid contact with endotoxin. Following the US Food and Drug Administration (FDA) guidelines for LPS limitation, the elution from medical devices should no more than 0.5EU/ml (US department of health and human services/Public Health services/Food and Drug Administration, 1987). However, complete endotoxin elution is hard to accomplish, and endotoxin may still present on the surface of medical devices even if the extract was testified as endotoxin free (Ragab *et al.*, 1999;Daniels *et al.*, 2000). *In vivo*, it is associated with pathogens and can induce innate immune responses quickly; *in vitro*, it may stimulate a wide range of cells including leukocytes, endothelial cells and epithelial cells (Gorbet and Sefton, 2005). To add to the difficulty, endotoxin is heat stable and hard to inactivate in normal sterilizing conditions. The presence of endotoxin has been identified as endotoxin contamination, and it is very

important to measure the quantity of LPS and minimize the amount on the biomaterials before implantation.

In order to understand the levels at which LPS will stimulate the macrophages as positive controls and which concentrations of LPS will not arouse cell response, here, a series of different concentrations of LPS (055:B5) (from the lowest 0.01ng/ml to the highest 1μg/ml) were chosen to stimulate RAW264.7 cells. The measured response was the secretion of the cytokines IL-1β and IL-6.

There were 5×10^5 RAW264.7 cells treated at each concentration and the stimulation time periods were set up as 6 hours, 12 hours and 24 hours. The results are shown as follows:

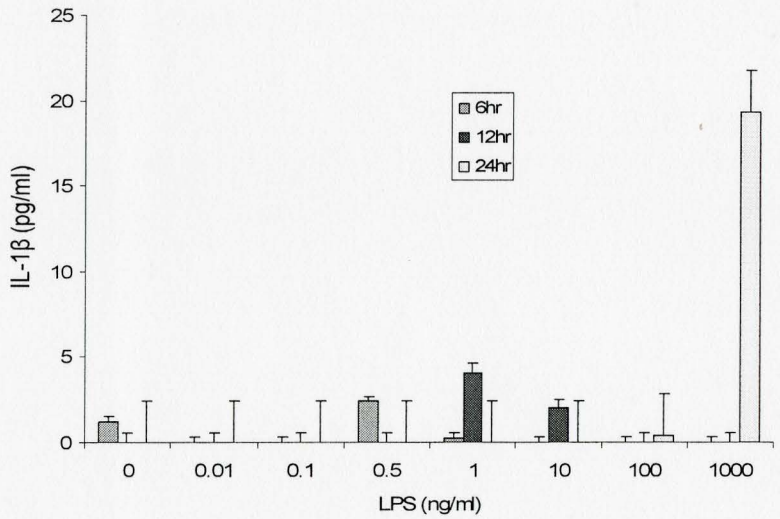


Figure 6: IL-1β secretion by RAW264.7 cells in response to different concentrations of LPS. Data are expressed as mean ± S.E.M. of two independent experiments.

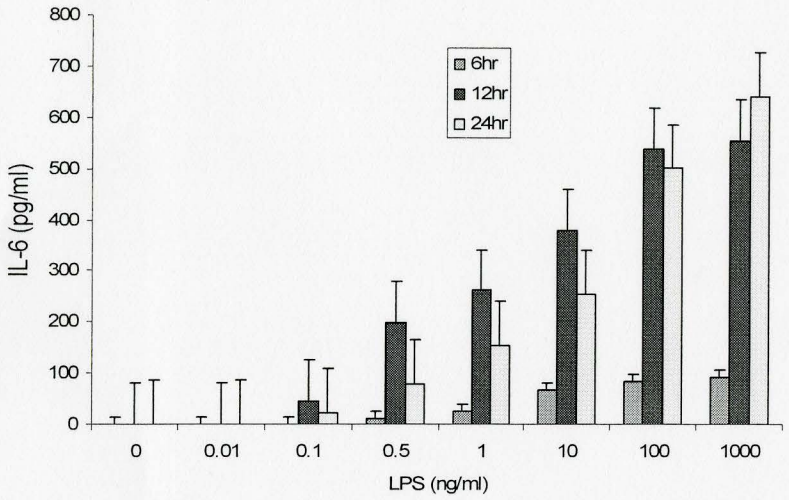


Figure 7: IL-6 secretion by RAW264.7 cells in response to different concentration of LPS. Data are expressed as mean ± S.E.M. of two independent experiments.

Production of IL-1β in response to different stimuli was very low; most secretion levels were much less than 10pg/ml. Even after 24 hours of stimulation, the 100ng/ml and 1μg/ml LPS groups had been found with less than 20pg/ml IL-1β.

There was a time and dose dependent trend in IL-6 production. In the first 6 hours, all groups secreted levels that were either undetectable or less than 100pg/ml, but after 12 hours, treatments between 0.5ng/ml and 1μg/ml had obviously increasing production. After 24 hours, the dose dependent trend was still obvious but the amount of secretion dropped a little.

It was clearly shown that high concentrations of LPS, especially 100ng/ml and 1μg/ml, remarkably stimulated RAW264.7 cells to secrete IL-6, while low concentrations of LPS (less than 0.5ng/ml) did not activate RAW264.7 cells. In fact, Wrenger found LPS (055:B5) can stimulate human peritoneal macrophages at 1ng/ml (Wrenger *et al.*, 1998).

Therefore, if the implant has lower than 0.5ng/ml LPS, the contamination effect can be ignored in our system.

According to the Product Description from Sigma (Product No. L2880), one nanogram of LPS (055:B5) is equal to 5 EU by LAL test, so one EU is equal to 0.2ng LPS. The treated sodium alginate stock solutions used in the experiments were tested for their endotoxin levels by the LAL test (see Appendix), and the data were all less than 1EU/ml. During the experiments, the stock solutions were diluted 10 times with fresh culture medium, and the actual endotoxin levels in each alginate group were less than 0.1 EU/ml (equal to 0.02ng/ml LPS) which is much lower than 0.5ng/ml.

Therefore, we determined to use 100ng/ml and 1μg/ml LPS as positive controls in the experiments. Furthermore the LPS that remained in the experimental alginate solutions after purification would not cause macrophage stimulation.

4.4 Does TNF-α stimulate the macrophages to the same levels as LPS?

TNF-α, a pro-inflammatory cytokine, has two completely opposite functions when its trimeric form binds with the TNF receptor (TNFR) which exists on the cell membrane. It has two polar functions in cell regulation: One is to promote cell death by activating caspase-8 and caspase-3 (Nicholson and Thornberry, 1997), and the other is to prevent cell death by the nuclear factor-κB (NF-κB) pathway (Karin and Ben-Neriah, 2000).

Like LPS, TNF-α can be used as a stimulus to activate different cell types, such as endothelial cells, monocytes and epithelial cells. In order to see how and whether RAW264.7 cells respond to this cytokine, we chose several different concentrations of TNF-α (from 0.1ng/ml to 100ng/ml) to stimulate the macrophage cells and compared the cytokine secretion of IL-1β and IL-6 to that elicited by high concentrations of LPS.

There were 5×10^5 RAW264.7 cells for each concentration and the stimulation time periods were set up as 0.5 hour, 1 hour, 2 hours and 24 hours. The results are shown as follows:

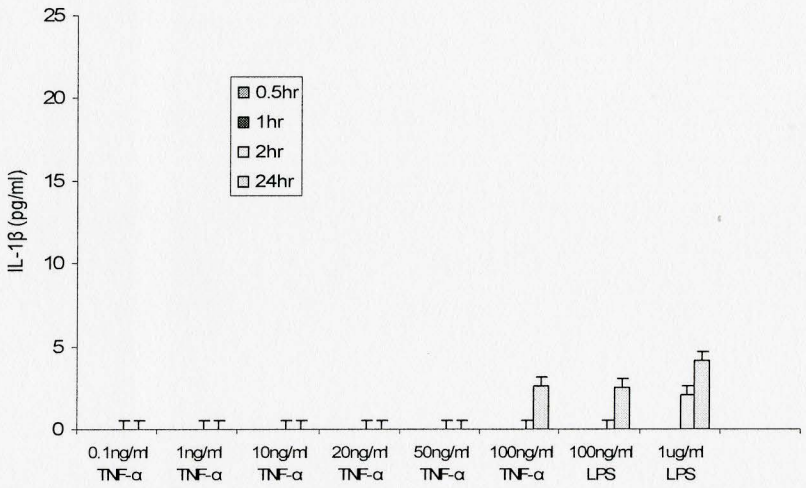


Figure 8: IL-1β secretion by RAW264.7 cells after stimulation with different concentration of TNF-α. Data are expressed as mean ± S.E.M. of two independent experiments.

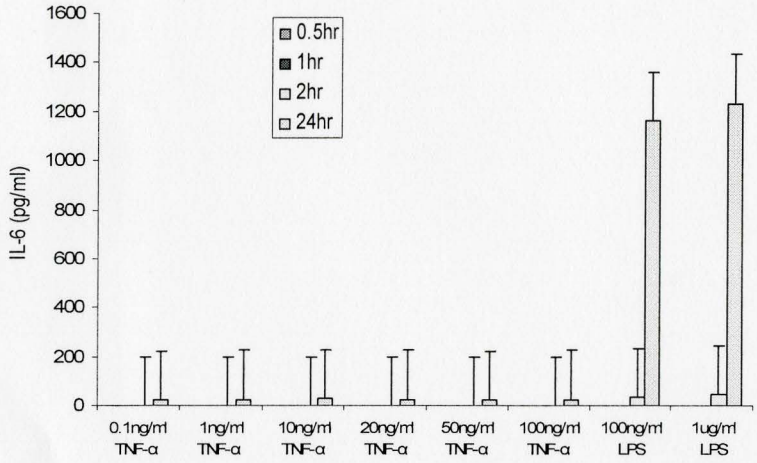


Figure 9: IL-6 secretion by RAW264.7 cells after stimulation with different concentration of TNF-α. Data are expressed as mean ± S.E.M. of two independent experiments.

Compared to LPS stimulation, IL-1β secretion stimulated by TNF-α was even harder to measure; only the highest concentration after 24 hours stimulated measurable quantities.

Unlike the time and dose dependent trend in IL-6 secretion induced by LPS, TNF-α showed low production for all treatment concentrations. After 24 hours, all TNF-α groups activated the cells to produce IL-6, but at only 21-25pg/ml and there was no dose difference for any concentrations.

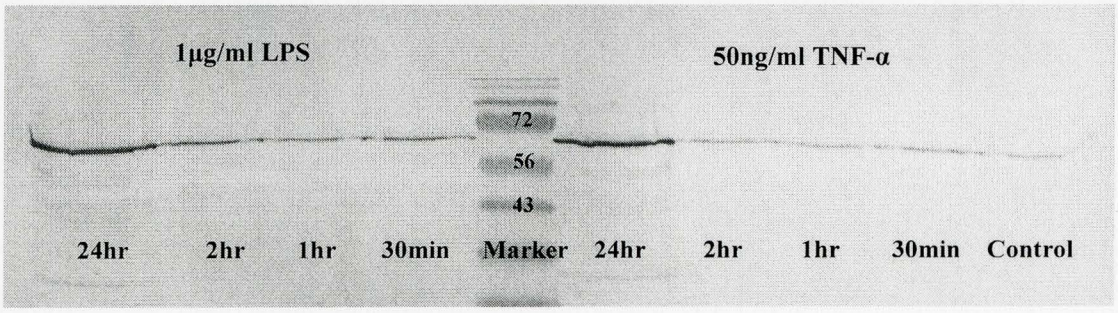


Figure 10: The NF-κB distribution in nucleus after stimulated with 1μg/ml LPS or 50ng/ml TNF-α from 30 minutes to 24 hours. The bands show protein NF-κB with a molecular weight of 65 KDa.

From the picture of Western Blot, it's easy to compare the differences of NF-κB activation with 1μg/ml LPS and 50ng/ml TNF-α stimulation separately. In a short time period (in 2 hours), LPS can promote more NF-κB to move into nucleus. After 24 hours, both TNF-α and LPS groups have an obvious increasing in NF-κB translocation.

As a macrophage-like cell, RAW264.7 cells have receptors to recognize TNF-α signals, but different pathways can result in different outcomes: One way is by NF-κB translocation to initiate pro-inflammatory cytokine gene expression and finally recruit more monocytes and lymphocytes to participate in inflammatory responses; the other way is to initiate a proapoptosis pathway which promotes cell death (Liu and Han, 2001). After NF-κB activation by TNF-α, new cytokines are produced including TNF-α and IL-1β. These cytokines can feedback to stimulate locally and eventually exaggerate cell responses (Timothy S.Blackwell and John W.Christman, 1997). Therefore, since there is competitive consumption, TNF-α accumulation will not show a stable increase with time or dose.

Since TNF-α did not produce obvious stimulation of RAW264.7 cells compared to LPS, and it may induce much more complex signal pathways which still are not clearly understood, we did not choose it as the positive control in the subsequent experiments.

4.5 Alginate solution concentration determination

Two different concentrations of sodium alginate stock solution were prepared: 10mg/ml and 30mg/ml. These two concentrations were chosen based on the following considerations: Firstly, the general concentration for alginate based-microcapsules preparation is at least 1% (w/v) in solution; secondly, according to our laboratory conditions, the alginate solution we can treat is no higher than 3% (w/v) due to the filtration methods. High concentration (over 30mg/ml) alginate solutions are very sticky thus nearly impossible to flow through 0.4 and 0.2μm filters. Also, most of the big fragments of alginate may be fouled on the membrane of the filter which will change the physical composition of alginate.

But in an *in vitro* experiment, the stimulatory solutions should not have a large volume that may influence the culture medium, so the alginate stock solutions with 10 times dilution in fresh culture medium (final concentrations of 1mg/ml and 3mg/ml) have been investigated in the following experiments.

Chapter 5 Effects of alginate stimulation of RAW264.7 cells

5.1 Introduction

As discussed in the literature review, it has been found that alginate can induce host responses, and the responses rely on different alginate compositions. However, the mechanisms of alginate stimulation remain unclear. To understand how alginate affects the cells is as important as know the ultimate outcome. In this section, the effects on macrophages caused by alginate was explored.

5.2 Experimental design

In medium containing serum, RAW264.7 cells were stimulated with two concentrations of alginate (1mg/ml and 3mg/ml) at 37°C. The stimulation time periods were set up as 2 hours, 6 hours, 12 hours, 24 hours, 48 hours and 5 days. LPS (100ng/ml and 1μg/ml) as positive controls was incubated in the same time periods as well.

The NF-κB pathway activation was illustrated by immuno fluorescence. Protein NF-κB distribution was determined by Western Blot with densitometric analysis. Levels of the secreted cytokines IL-1β, IL-6, IL-12 and TNF-α were quantified by ELISA.

5.3 Results

5.3.1 Immuno Fluorescence to track NF-κB translocation

In order to easily observe and compare the details of immunostaining within the cell, FITC staining of NF-κB and Hoechst staining of the nucleus for the same cells are shown together. Representative results are shown in Figures 11-16:

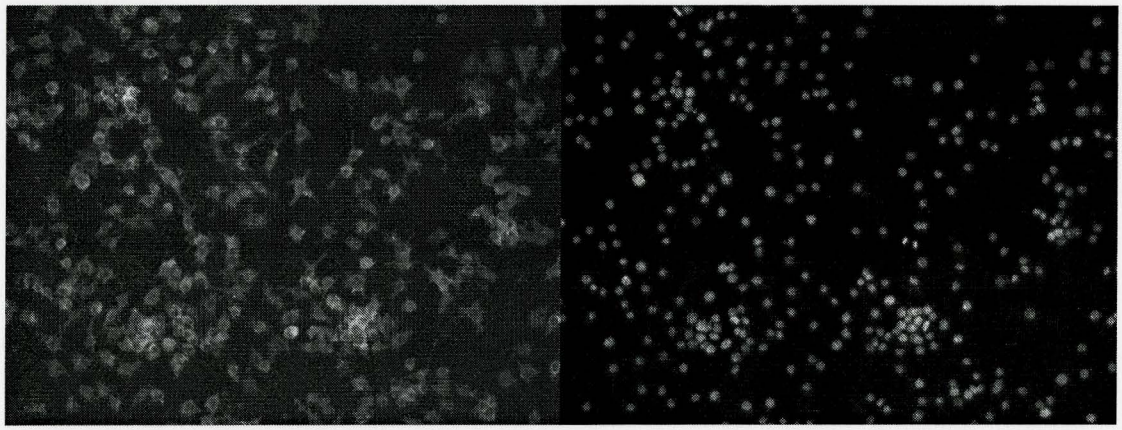


Figure 11: Control group (12 hours) (Left: FITC; Right: Hoechst)

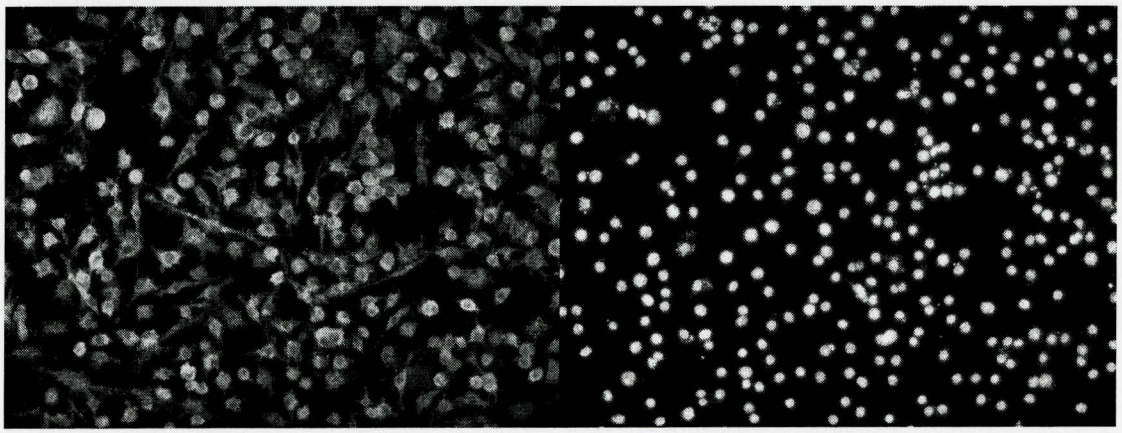


Figure 12: 100ng/ml LPS stimulation for 24 hours (Left: FITC; Right: Hoechst)

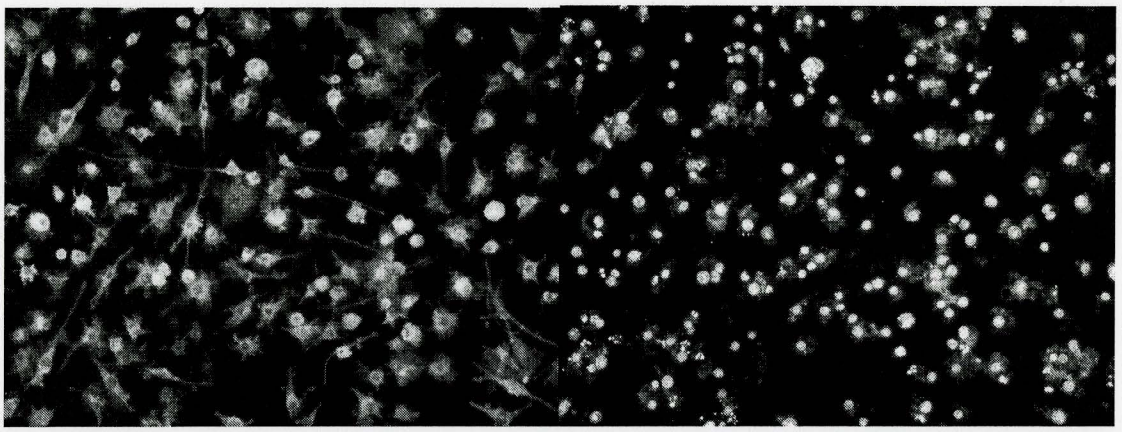


Figure 13: 1mg/ml alginate solution stimulation for 24 hours (Left: FITC; Right: Hoechst)

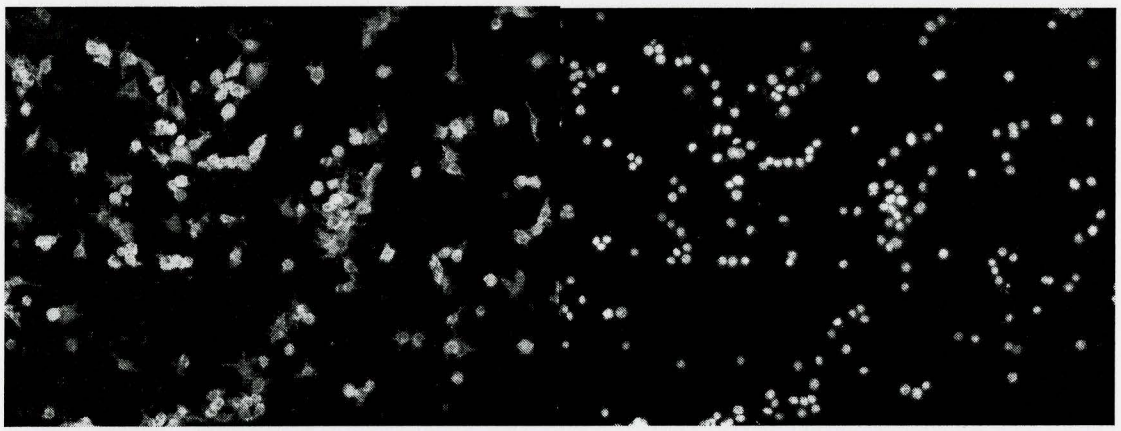


Figure 14: 3mg/ml alginate solution stimulation for 24 hours (Left: FITC; Right: Hoechst)

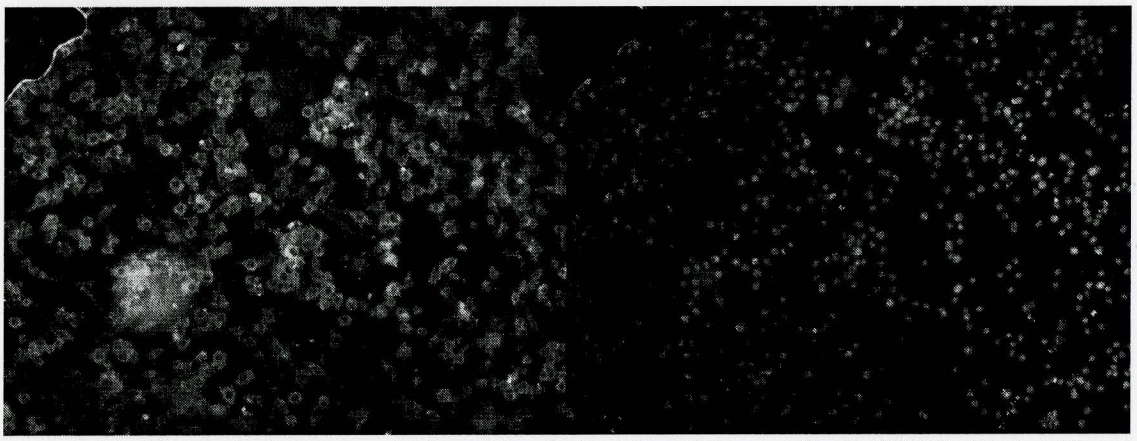


Figure 15: 1mg/ml alginate solution stimulation for 48 hours (Left: FITC; Right: Hoechst)

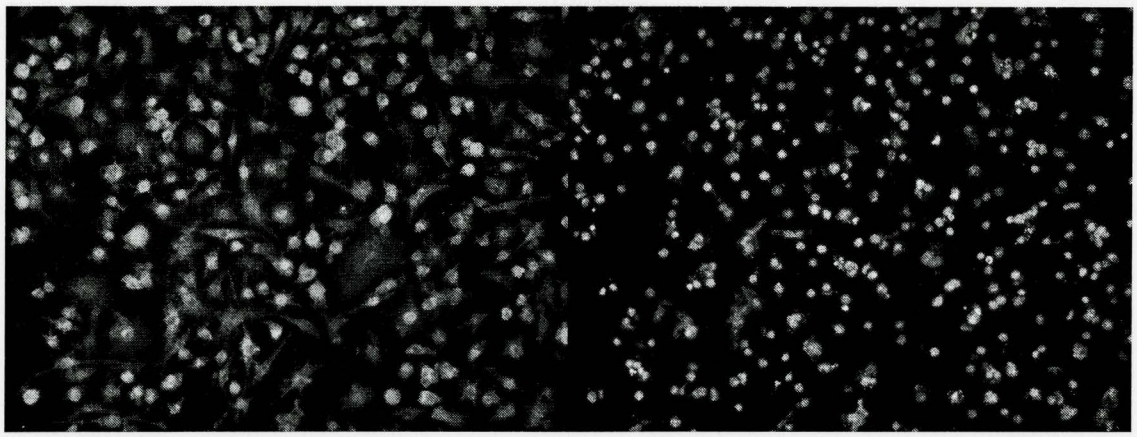


Figure 16: 3mg/ml alginate solution stimulation for 48 hours (Left: FITC; Right: Hoechst)

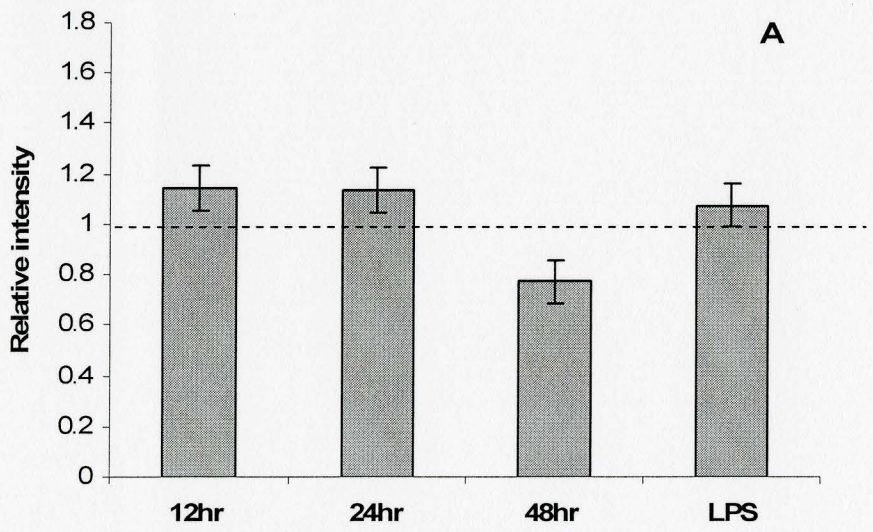
Protein NF-κB with FITC staining showed a green color and Hoechst staining showed the nucleus sites with blue color in the figures. When the cells were in a resting state, most of NF-κB was kept silent in the cytoplasm instead of the nucleus. From Figure 11, it can be easily noticed that in the control group, the green cytoplasm area circles around a dark area. When given stimuli such as LPS or alginate, NF-κB was released from its inhibitor and moved into the nuclear area that is covered by the green color. This can be confirmed by Hoechst staining which has highly specific binding to DNA.

In the control groups, most of the cells showed a normal condition with obvious green cytoplasms and clear dark nuclear areas. After incubation with 100ng/ml LPS for 24 hours, over one third of cells had green nuclei which meant they were activated. For alginate stimulation, all groups showed obvious positive results after 24 hours, and in some cells, both the cytoplasm and nucleus were shown with a green color. In addition, we found the cell morphology varied in response to alginate stimulation, especially in the

1mg/ml 24 hours group and 3mg/ml 48 hours group. In these conditions, many RAW264.7 cells grew bigger with longer "finger-like" pseudopodia than the normal cells.

5.3.2 Western Blot

Depending on the different stimuli (different concentrations of alginate or LPS), various amounts of NF-κB showed responses by translocating from cytoplasm to nucleus. Here, the ratios of NF-κB protein in the nucleus and in the cytoplasm were used to distinguish the degree of response. In order to semi-quantitatively analyse the relative amount of NF-κB in nucleus versus the cytoplasm, we arbitrarily set the measured ratio (from densitometry) of the negative control groups to one. We interpreted a ratio of nuclear versus cytoplasmic NF-κB greater than one as an indication of macrophage activation. The results were compared statistically with a t-test and shown in Figure 17:



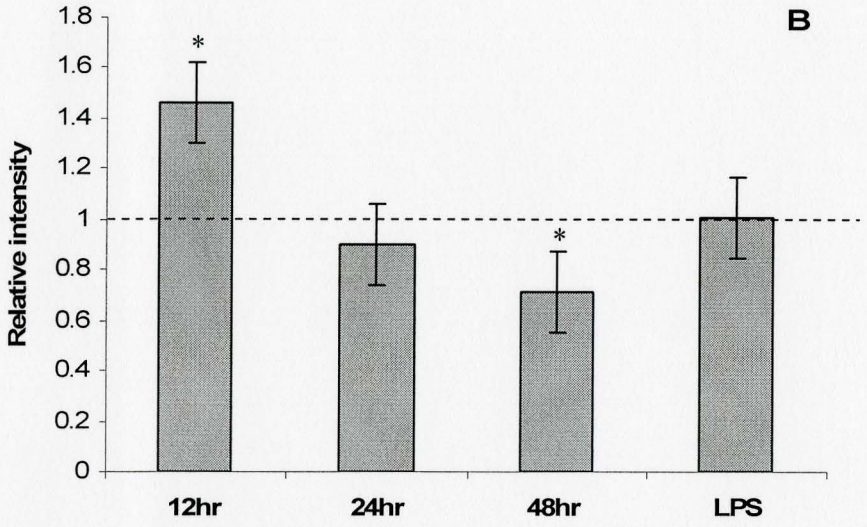


Figure 17: Western blotting along with densitometric analysis to show NF-κB translocation, stimulated by 1mg/ml and 3mg/ml alginate solution separately for 12, 24 and 48 hours, and 100ng/ml LPS individually for 12 hours. Data are expressed as mean ± S.E.M. of three independent experiments. (*$p<0.05$)
A: 1mg/ml alginate solution stimulation; *B*: 3mg/ml alginate solution stimulation.

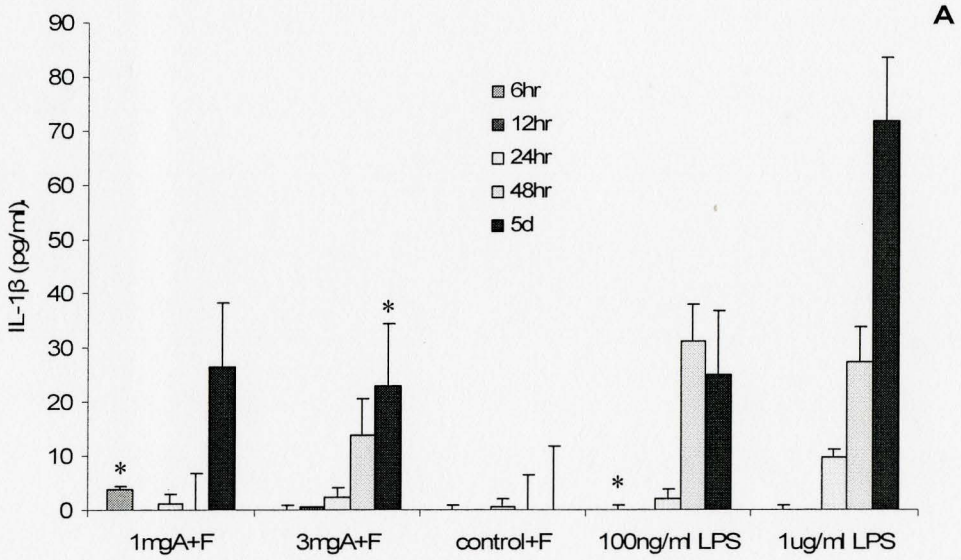
With 1mg/ml alginate solution stimulation, the NF-κB pathway was activated. The N/C ratio remained constant between 12 and 24 hours and showed the same ratio as LPS stimulation. After 48 hours, the ratio dropped, meaning that most of the NF-κB returned to the cytoplasm.

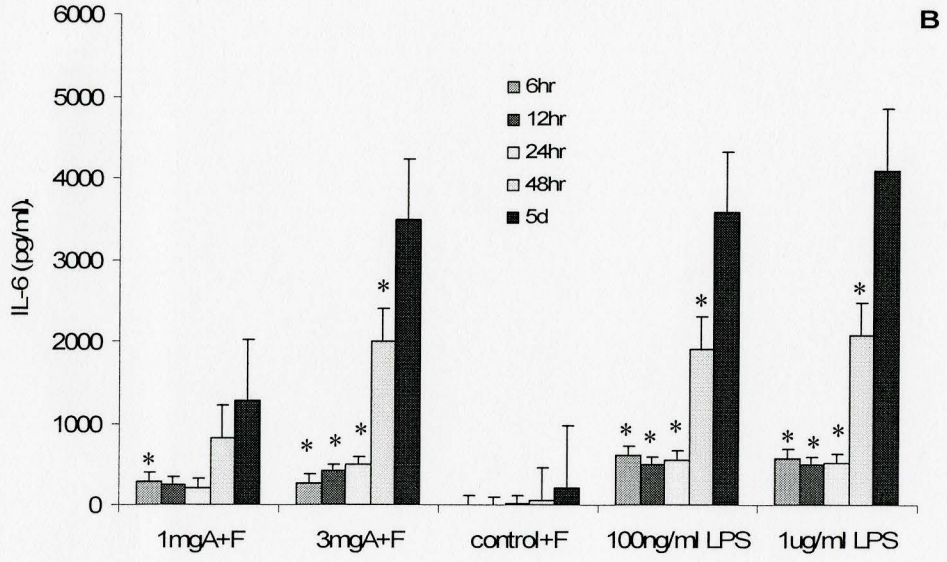
Unlike after treatment by the low concentration of alginate, with 3mg/ml alginate solution, the cells showed a quick response in the first 12 hours, but the N/C ratio dropped quickly in the following hours. Compared the stimulation caused by alginate and

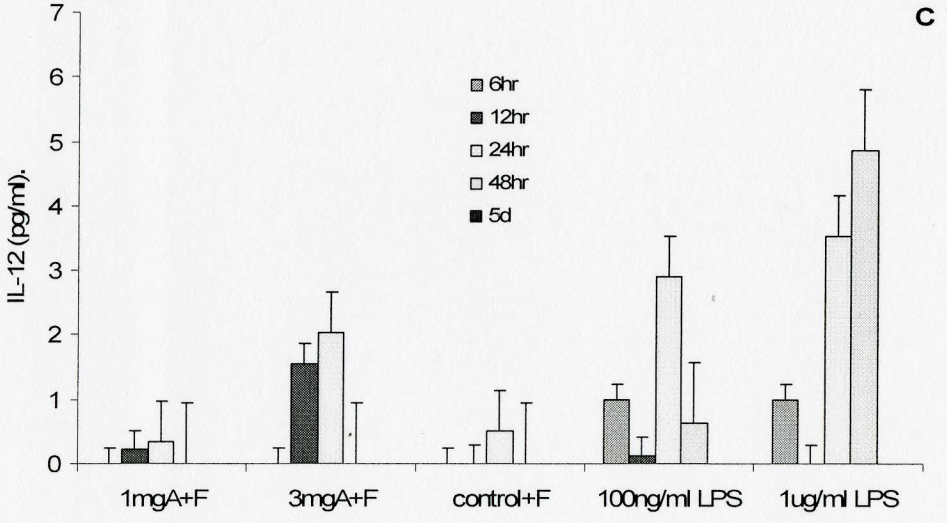
LPS, from the Figure 17, we found that the high concentration of alginate causes a greater response than LPS.

5.3.3 Cytokine secretion quantification by ELISA

One of the results of NF-κB pathway activation is to initiate gene transcription to express proinflammatory cytokines. For each group, 2.5×10^5 cells were treated with soluble alginate (1mg/ml and 3mg/ml) or LPS (100ng/ml and 1μg/ml) in 12, 24, 48 hours and 5 days. By using ELISAs, IL-1β, IL-6, IL-12(p40) and TNF-α secretion were tested separately. The results are shown in Figure 18.




B
IL-6 (pg/ml)
6000
5000
4000
3000
2000
1000
0
6hr
12hr
24hr
48hr
5d
1mgA+F
3mgA+F
control+F
100ng/ml LPS
1ug/ml LPS


C
IL-12 (pg/ml).
7
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control+F
100ng/ml LPS
1ug/ml LPS

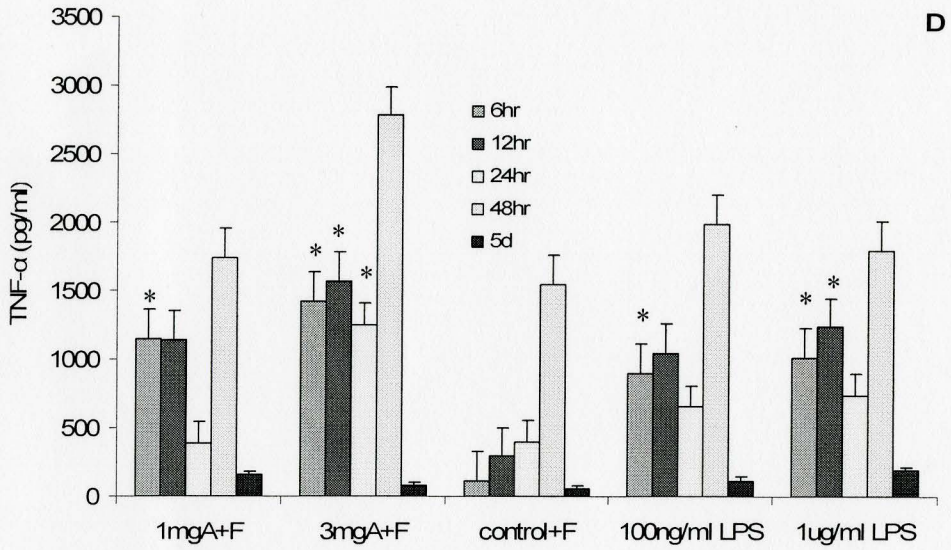


Figure 18: IL-1β, IL-6, IL-12(p40) and TNF-α secretion in response to different concentrations of alginate solutions and LPS stimulation. Data are expressed as mean ± S.E.M. of three independent experiments. Statistically significant differences (*$p<0.05$) from LPS or alginate stimulation compared to the control group.
A: IL-1β production; *B*: IL-6 production; *C*: IL-12 production; *D*: TNF-α production.
Control+F: blank control in serum medium; *1mgA+F*: 1mg/ml alginate in serum medium; *3mgA+F*: 3mg/ml alginate in serum medium.

In response to alginate solutions, the secretion of the cytokines IL-1β, IL-6 and IL-12(p40) were time and dose dependent. Especially after 24 hours, the secretions quickly increased to 2~3 times greater than the previous time-point. For LPS stimulation, there was not a dramatic difference between the 100ng/ml and 1μg/ml groups. In the negative control group, the macrophages were not measurably activated and no detectable cytokines secretion was found.

TNF-α is a quite different cytokine in that even the control groups displayed time dependent increasing secretion. Alginate groups stimulated the cells in the early 6 hours, and the secretion remained stable for 12 hours. An interesting phenomenon happened at

24 hours in that all experimental groups showed an obvious drop; however, another 24 hours later, the secretion increased to a higher level than before. Until 5 days later, TNF-α secretion in all groups dropped to minimum levels, less than 50pg/ml.

5.4 Discussion

As one of the main components of the innate immune system, macrophages recognize different chemical and biological signals from the extracellular environment through different membrane receptors. RAW264.7 cells, macrophage-like cells, are also very sensitive to the environmental changes.

From the immuno fluorescence pictures, macrophages showed a more distinct response to alginate stimulation than to LPS. In particular, this can be distinguished from the morphological changes in the activated cells. Such changes include increases in size, and more and prolonged pseudopodia. In fact, this is a result of macrophage maturing from monocytes, and the prolonged pseudopodia help to attach bacteria, which finally will be phagocytosed and destroyed. Here, we hypothesize that there are specific receptors on the membrane that recognize LPS and alginate, pass the signals to NF-κB and finally cause the cells to secrete different pro-inflammatory cytokines. The continuous stimulation by alginate, lasting for hours, may promote the cells to mature.

In previous NF-κB pathway studies, researcher found that NF-κB can unbind from IκB (the inhibitor of NF-κB) to become an active transcription factor and move into nucleus in a short time. Depending on the cell line and stimuli, the speed of NF-κB translocation is different. Wang found after TNF-α stimulation in human corneal

epithelial cells, the initial translocation happened in the first 5 minutes, and 15 minutes later reach to an maximal level that lasted for 60 minutes (Wang *et al.*, 2005). In our experiments, we chose a much longer stimulation to assay cell responses. Results showed that prolonged stimulation made the cells remain in an activated state. For NF-κB, the balance of fast entrance into the nucleus and fast retreating back to cytoplasm is broken and a new balance is set up: some NF-κB stayed in the nucleus.

Different concentrations of alginate had various influence on RAW264.7 cells. The lower concentration induced a mild but longer stimulation while the higher one induced a shorter but bigger response. It appears that on the cell membrane there are specific receptors to accept alginate signals and activate the NF-κB pathway. However, these receptors for alginate recognition are still unclear. Mannose receptors that can recognize many polysaccharides might be one such receptor.

One of the results of NF-κB activation is cytokine production. Although NF-κB activation is the primary step in causing cytokine secretion, the new cytokines can feedback locally and exaggerate the production, as with TNF and IL-1β (Siebenlist *et al.*, 2005). Of course, depending on the cell line and stimuli, the activation will be quite different. For RAW264.7 cells, IL-1β and IL-6 increased with time, possibly magnifying their own production after 24 hours, while in contrast, TNF-α was consumed just ahead of that time. In other words, TNF-α might participate into the feedback but some of it will bind with the TNF receptor (and be consumed), resulting in further NF-κB activation, thus producing more IL-1β and IL-6.

Secretion of IL-12 by RAW264.7 cells was low in response to different stimuli. We believe this is a cell-specific phenomenon, since other researchers found that from an *in vivo* experiment, given alginate stimulation, lymph node cells showed high IL-12 production (Yoshida *et al.*, 2004).

Chapter 6 Interaction of serum with alginate in stimulation of RAW264.7 cells

6.1 Introduction

In cell culture medium, serum plays an important role in providing necessary nutrition, shear protection, and in promoting cell growth and proliferation.

Like other biomaterials, alginate may interact with proteins in the aqueous phase by intermolecular forces depending on pH, ionic composition, and temperature. In fact, many researchers attribute the host responses not to the material itself, but rather to the effect of serum proteins with the material, such like non-specific protein adhesion and the following inflammatory. Especially for a biodegradable material, the material itself may participate into the host responses, so it is important to investigate how material and serum influence with each other, and the host responses caused by the co-work of material and serum.

We have shown that alginate stimulated RAW264.7 cells to translocate NF-κB to the nucleus and secrete cytokines in a serum-rich medium, but whether there is any possibility for serum proteins to participate in the interaction between alginate and macrophages is unknown. Do proteins from serum stimulate the macrophages in tandem with the alginate? Or does serum starvation alone stimulate NF-κB pathway? In order to find these answers, we chose a serum-deprived medium to treat RAW264.7 cells, and from tracking NF-κB translocation and subsequent cytokine production, we determined the influence of serum proteins in alginate-induced inflammatory responses.

6.2 Experimental design

6.2.1 RAW264.7 cell treatment in a serum deprived medium

RAW264.7 cells were cultured in normal DMEM medium with 10% FBS before the stimulation experiments were performed. When the experiments began, fresh DMEM was used after the serum-rich medium was withdrawn thoroughly, and then different stimuli were added for 2 hours till 48 hours. Two different concentrations of sodium alginate were tested: 1mg/ml and 3mg/ml. LPS (100ng/ml and 1μg/ml) groups were set up as positive controls. A blank normal group in the serum-rich medium and a blank normal group in the serum-deprived medium were cultured at the same time. The speed of cell proliferation and cell responses to serum-deprived medium by NF-κB activation was studied. The treatments and test methods were the same as section 3.1, with the exception of the serum in the medium during treatment.

6.2.2 Cells proliferation in normal and serum-deprived medium

In order to understand how the serum influenced the cells, we measured the proliferation rate of RAW264.7 cells in normal serum medium and serum-deprived medium. Firstly, we used trypan blue to ensure that cells were healthy and the viability exceeded 90%. The medium was only replaced with serum-deprived medium (pure DMEM) at the start of the treatment. Cell numbers were counted only for the living cells by trypan blue identification.

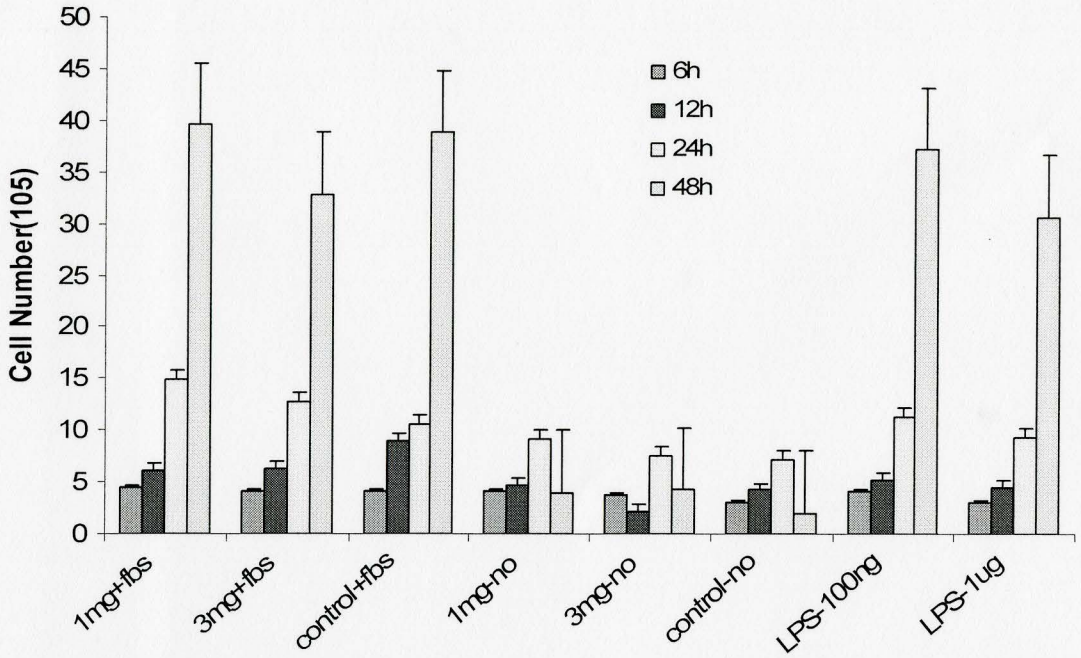


Figure 19: The RAW264.7 cells number counting after alginate (1mg/ml and 3mg/ml) and LPS (100ng/ml and 1μg/ml) stimulation in serum-rich or serum-deprived medium for 6, 12, 24, and 48 hours. Data are expressed as mean ± S.E.M. of two independent experiments. The original cell number in each group was 5×10^5.

Even with alginate and LPS stimulation, RAW264.7 maintained a normal growth rate in the serum rich condition, and the cells proliferated in 24 hours. However, after serum withdrawal from the medium, the cell proliferation was obviously inhibited. From the data shown in Figure 19, the cell numbers did not increase after 48 hours' culture. Of interest, when the nutrition was mostly consumed after five days in treatment wells (data not shown), in the normal control and LPS positive control groups, most cells were dead because of nutrition starvation, but the cells in high concentration alginate group kept alive. In the serum-deprived conditions, both in the blank normal groups and alginate treated groups in the serum-deprived conditions, the cells were still alive and even showed further proliferation in 24 hours, and also alginate groups helped to keep more

cells alive in 48 hours. It seems there are some strategies for these macrophage cells to survive that may rely on alginate stimulation. From the pictures of RAW264.7 cells stimulated with high concentration of alginate (Figure 20-23), the cells looked healthy and keep proliferation in 24 hours, and in 48 hours, the cells number decrease with morphology changing in most cells- a foam-like cell with small bubbles in the cytoplasm.

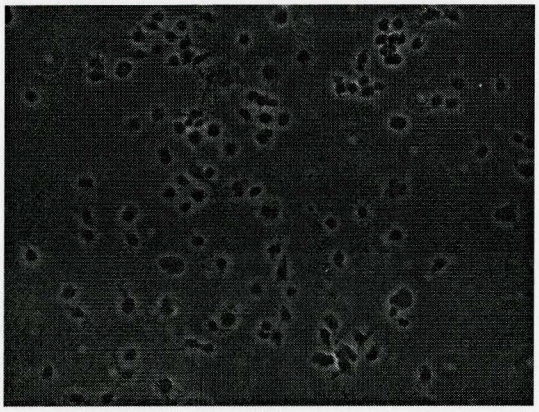


Figure 20: RAW264.7 cells with serum deprivation for 2 hours with 3mg/ml alginate stimulation

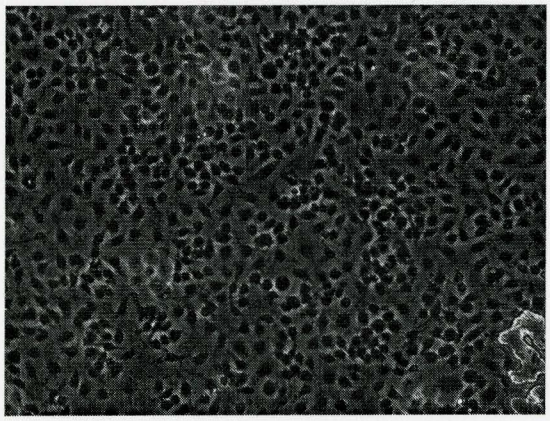


Figure 21: RAW264.7 cells with serum deprivation for 12 hours with 3mg/ml alginate stimulation

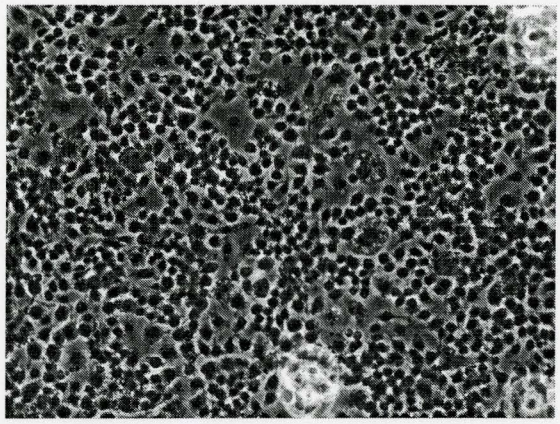


Figure 22: RAW264.7 cells with serum deprivation for 24 hours with 3mg/ml alginate stimulation

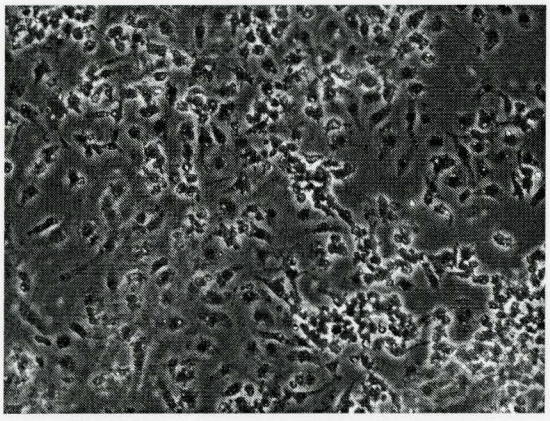


Figure 23: RAW264.7 cells with serum deprivation for 48 hours with 3mg/ml alginate stimulation

6.3 Results

6.3.1 Pictures from Immuno Fluorescence

RAW264.7 cells in serum-deprived medium showed responses to alginate as noticeably as in serum-rich medium. In fact, the responses were much quicker: the high concentration of alginate stimulated the cells in 2 hours; in 24 hours over half of the macrophages had an obvious green nucleus; and 48 hours later, almost all cells were activated in response to high concentration stimulation. Another big difference from the serum-rich medium was that the morphology of activated macrophages was more obviously changed. The cell size was enlarged and more bubble-like constructions were present in the cytoplasm. The details are shown in Figure 24-28.

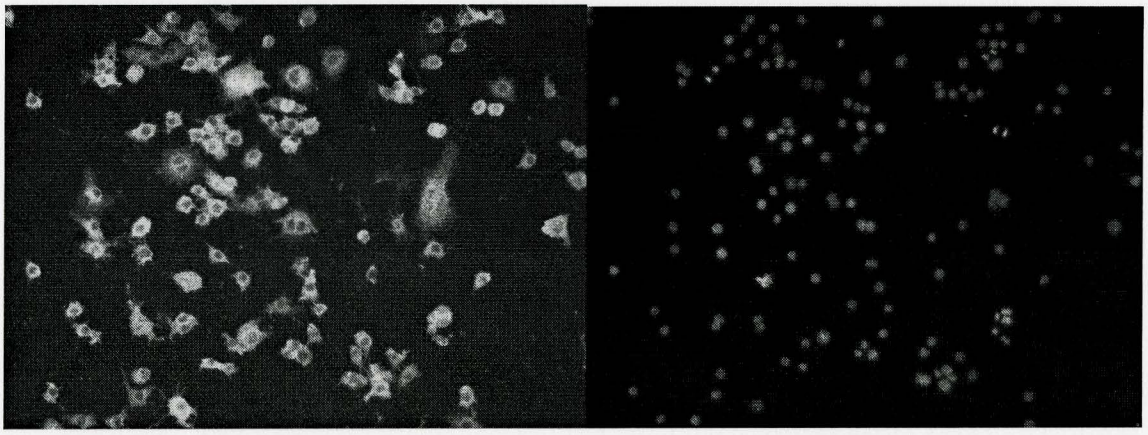


Figure 24: RAW264.7 cells with 3mg/ml alginate solution stimulation in a serum deprived medium for 2 hours(Left: FITC; Right: Hoechst)

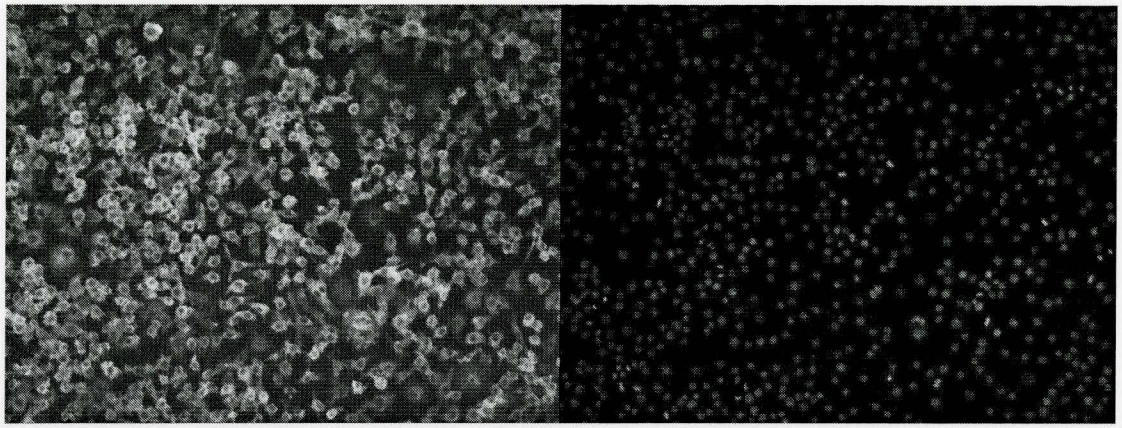


Figure 25: RAW264.7 cells with 3mg/ml alginate solution stimulation in a serum deprived medium for 6 hours (Left: FITC; Right: Hoechst)

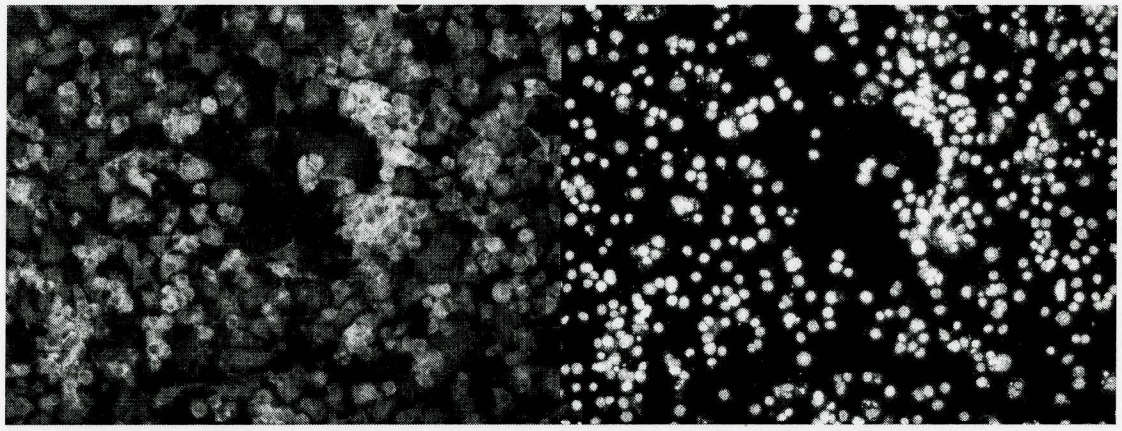


Figure 26: RAW264.7 cells with 1mg/ml alginate solution stimulation in a serum deprived medium for 24 hours (Left: FITC; Right: Hoechst)

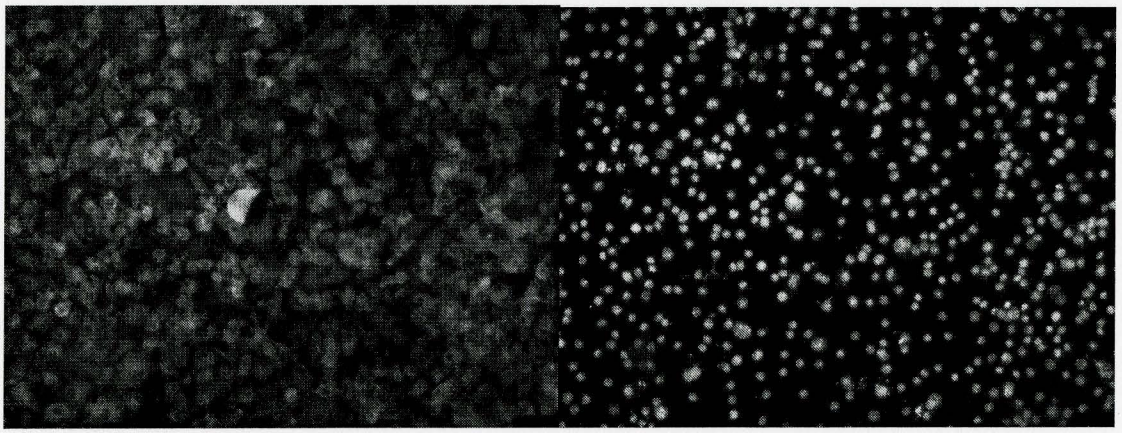


Figure 27: RAW264.7 cells with 1mg/ml alginate solution stimulation in a serum deprived medium for 48 hours (Left: FITC; Right: Hoechst)

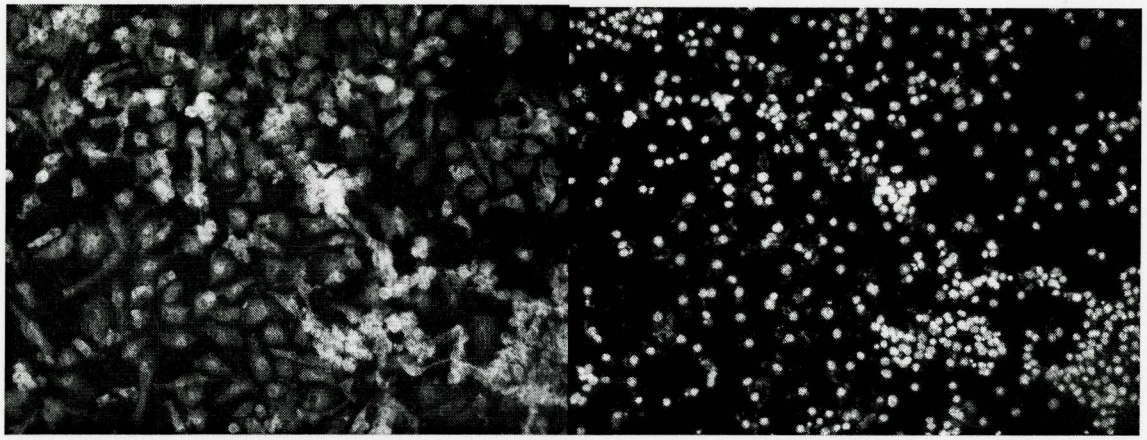


Figure 28: RAW264.7 cells with 3mg/ml alginate solution stimulation in a serum deprived medium for 48 hours (Left: FITC; Right: Hoechst)

6.3.2 Western Blot analysis of serum-deprived groups

The results are shown as ratio of quantified NF-κB distribution in the nucleus and the cytoplasm. In order to semi-quantitatively analyze the relative amount of NF-κB in the nucleus versus the cytoplasm, we set the measured ratio of the negative control groups (in a serum-deprived medium) to one.

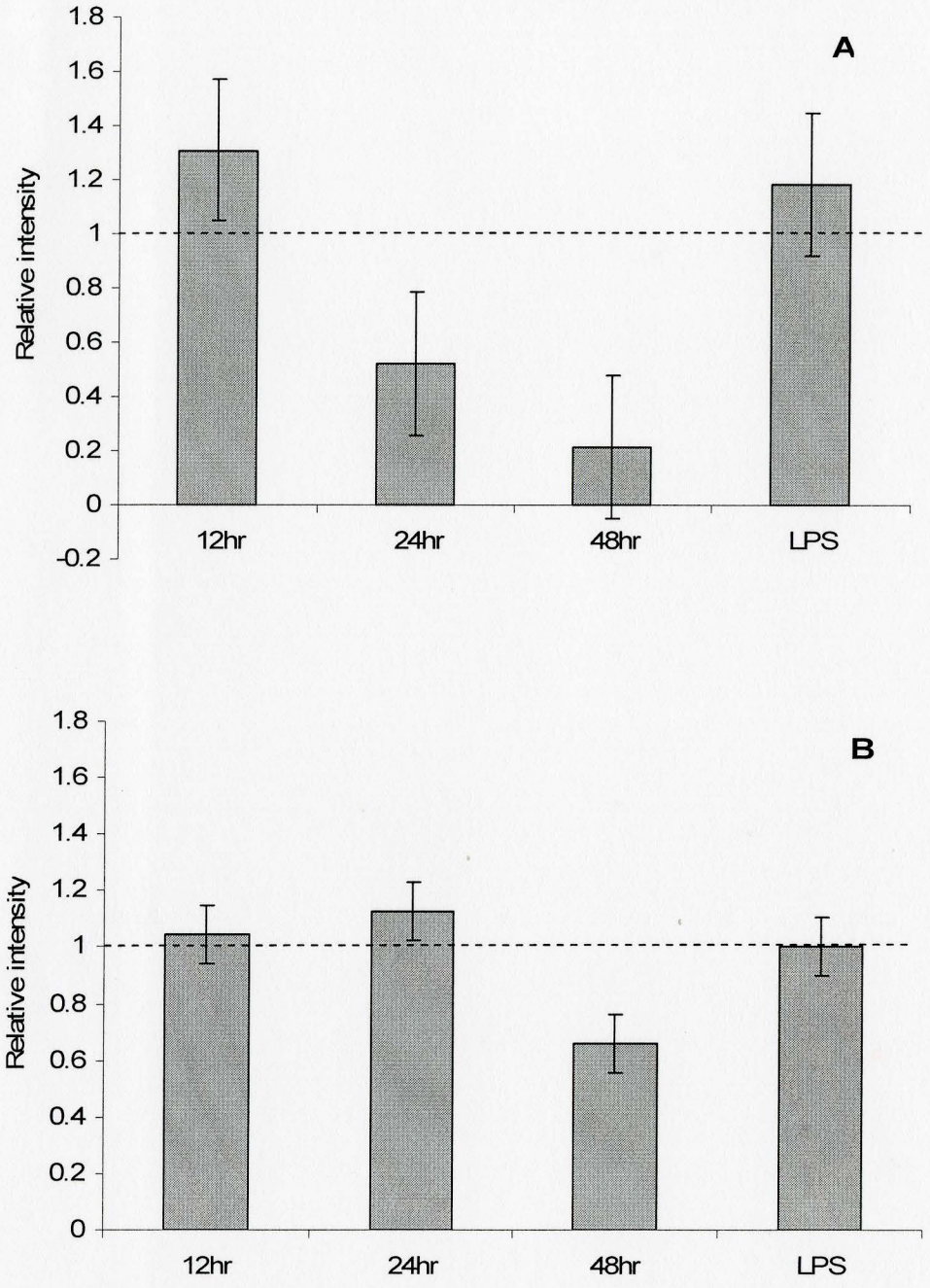
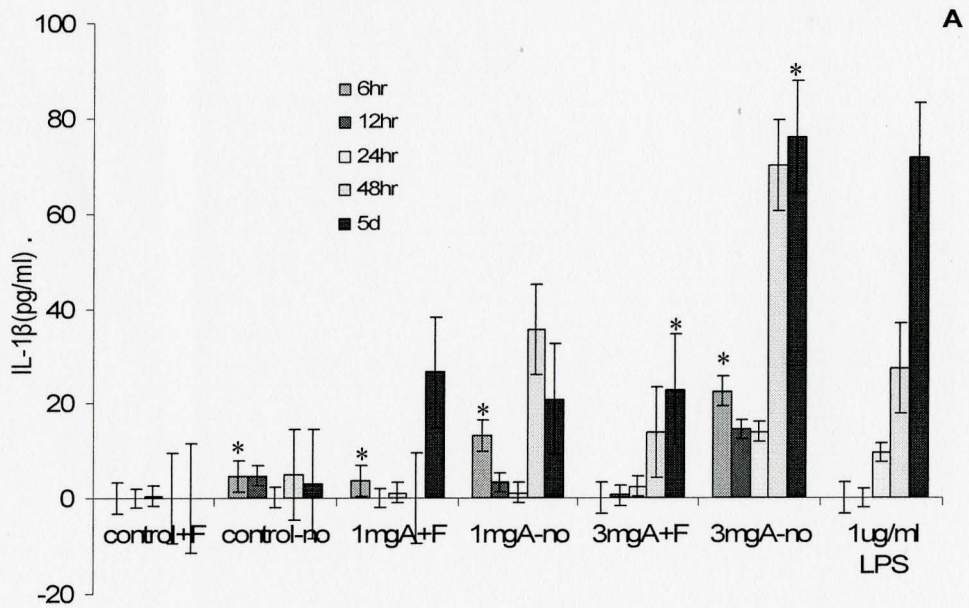


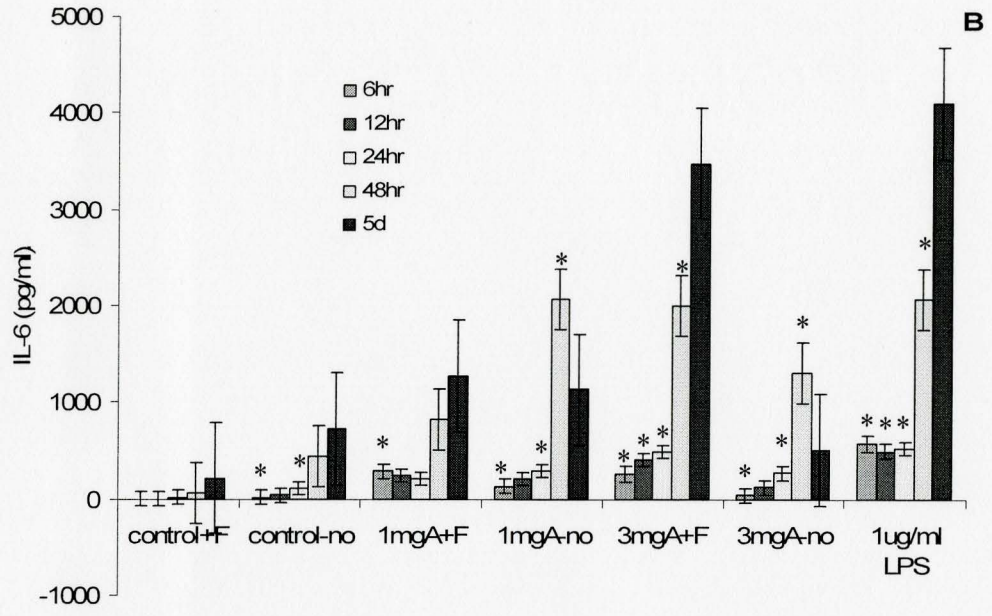
Figure 29: Alginate solution (1mg/ml and 3mg/ml) treatment with RAW264.7 cells in a serum deprived medium. The cells in control groups were cultured in normal serum medium. The group with 100ng/ml LPS in 12 hours incubation set as positive control. Data are expressed as mean ± S.E.M. of three independent experiments.
A: 1mg/ml alginate solution stimulation without serum; *B*: 3mg/ml alginate solution stimulation without serum.

Compared to the serum-rich groups, the NF-κB activation was reversed: With the lower stimuli (1mg/ml alginate), the macrophages showed a fast response. The responses only last for 12 hours, then most free NF-κB quickly returned to cytoplasm and was presumably captured by IκB. The higher stimuli (3mg/ml) provided longer stimulation which lasted for 24 hours.

6.3.3 ELISA results

According to the results obtained from the ELISAs (Figure 30), there were obvious differences between the serum groups and the serum-deprived groups.




B
5000
4000
3000
2000
1000
0
-1000
IL-6 (pg/ml)
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control-no
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1mgA-no
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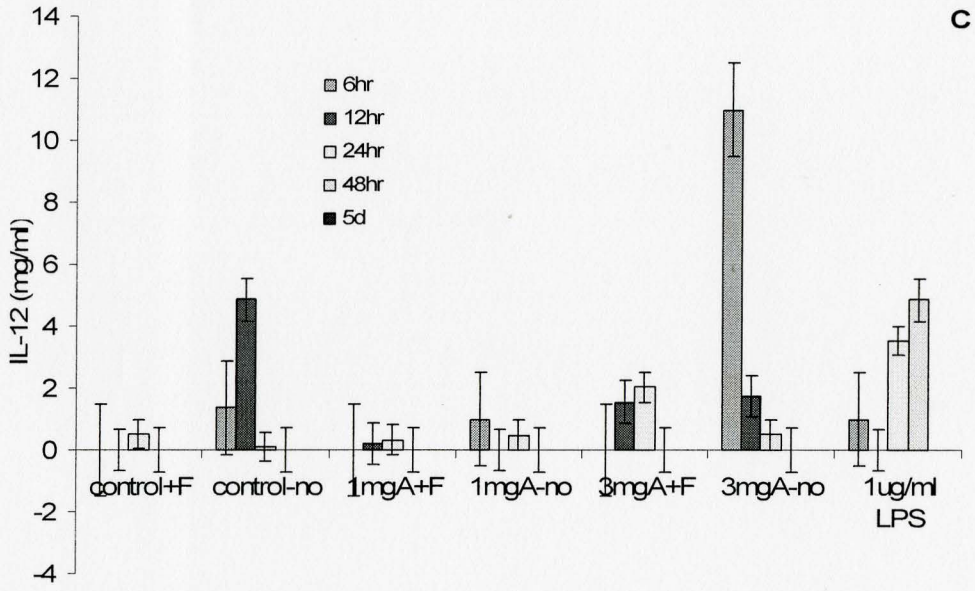
3mgA-no
1ug/ml LPS


C
14
12
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IL-12 (mg/ml)
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1ug/ml LPS

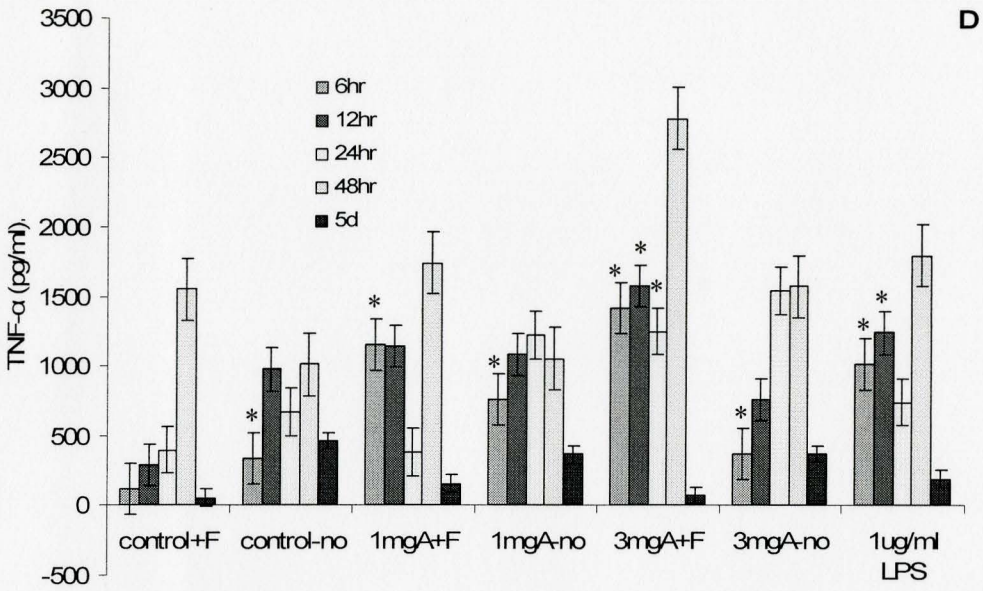

Figure 30: IL-1β, IL-6, IL-12(p40) and TNF-α secretion in response to different concentrations of alginate solutions and LPS stimulation in a serum deprived medium. Data represent as mean ± S.E.M. of three independent experiments (*p<0.05).
A: IL-1β production; *B*: IL-6 production; *C*: IL-12 production; *D*: TNF-α production. *Control+F*: blank control in serum medium; *Control-no*: blank control in serum-deprived medium; *1mgA+F*: 1mg/ml alginate in serum medium; *1mg-no*: 1mg/ml alginate in serum-deprived medium; *3mgA+F*: 3mg/ml alginate in serum medium; *3mgA-no*: 3mg/ml alginate in serum-deprived medium.

IL-1β: As opposed to the serum rich groups, the serum deprived groups had higher secretion and alginate showed an obvious influence on the cells. After withdrawal of serum, the production reached a high level after 6 hours stimulation (13.3pg/ml in 1mg/ml alginate group and 22.5pg/ml in 3mg/ml group), and then quickly dropped in the following 6 hours. The secretion remained stable for 24 hours, then a dramatic increase happened after 48 hours that lasted for 5 days. Even in the serum deprived control group, a low but stable IL-1β secretion was detected.

IL-6: The secretion did not vary much in response to serum deprivation. Nonetheless, the production did accumulate over time and showed sudden increase at 48 hours with close to three times the previous levels. However, the stimulation caused by high concentrations of alginate under serum starvation was much less than in the serum rich condition.

IL-12: There was still no obvious secretion in all groups.

TNF-α: Serum starvation showed obvious effects on the secretion of this cytokine and seemed to overpower the effect of alginate. Even in the serum deprived blank control group, TNF-α had a secretion after just 6 hours which was close to 338.9pg/ml, and the level stayed higher than 500pg/ml up to 48 hours. The high concentration of alginate showed high stimulation in the serum deprived serum for a long term; however, like the IL-6 results, the secretion was less than for the serum rich groups.

6.4 Discussion

Compared to the serum group, the results from serum deprived medium show some differences. We will try to explain these differences as follows.

Firstly, an interaction between serum proteins and alginate may influence surface receptor recognition in RAW264.7 cells.

Alginate is a polysaccharide. Researchers found that polysaccharides and proteins can interact with each other in the aqueous phase. That means the functional properties of one are often altered by the presence of the other. However, they have some common

functional properties such as solubility and gel-forming ability (Thepkunya Harnsilawat *et al.*, 2006).

In serum-deprived medium, alginate worked alone. The numbers of receptors for alginate recognition may be limited, so high concentrations of alginate will need a longer time to be fully "recognized". While in serum-rich medium, proteins are plentiful and they interact with alginate in an unclear mechanism which may influence the recognition.

One possibility is that alginate (with a negative charge in solution) combines with a positively-charged protein to form a protein-alginate complex. Only limited numbers of alginate participate into this combination since the charge sites are limited. This complex can also be recognized by receptors. For a high concentration of alginate, some alginate molecules may combine with proteins to give a persistent but slow stimulation, but the large numbers of free alginate will directly stimulate the macrophages, so finally results a big and quick stimulation. On the contrary, most of alginate molecules in the low concentration will combine with protein and slow down the recognition which finally prolongs the stimulation. Also when alginate and proteins interact, it may form gels which increase the viscosity of solution, and slows down the signal recognition.

Secondly, alginate may have induced NF-κB activation to prevent apoptosis caused by serum starvation.

In normal conditions, the macrophage is a long lived cell; during inflammation, its life span will be extended. This is controlled by survival factors such as apoptosis inhibitors and nerve growth factors secreted by macrophages or other cells (Haruta *et al.*, 2001;Garaci *et al.*, 1999). At the same time, in order to limit immune responses,

apoptosis of macrophages often happens at inflammatory sites. Apoptosis is programmed cell death controlled by a family of cysteine aspartate proteases called caspases. Many types of cells have these characteristics including activated lymphocytes. Compared to the knowledge of lymphocyte apoptosis, it still is not clear how macrophages perform such self-controlled death (Munn *et al.*, 1995;Janssen *et al.*, 2000;Donjerkovic and Scott, 2000). There are many factors which can promote apoptosis: TNF-α, nitric oxide, IFNγ and other cellular stresses. Researchers found that serum starvation can also induce apoptosis. In the human kidney epithelial carcinoma cell line 293, 6 days after omission of serum, almost all cells were apoptotic (Thepkunya Harnsilawat *et al.*, 2006;Stefan Grimm *et al.*, 1996). A similar result has been found in RAW264.7 cells which is considered to be mediated by autocrine secretion of type I IFNs (Wei *et al.*, 2006).

NF-κB activation can play an anti-apoptotic role in this process. Many anti-apoptotic genes have been expressed which are induced by the transcriptional activity of NF-κB. The products of these genes are including IAPs, c-FLIP(caspase-8/FADD-like-IL-1β-converting enzyme inhibitory protein) and the Bcl-2 family of proteins which can block the apoptotic cascade (Delhalle *et al.*, 2004).

In our experiments, all serum starvation groups stopped proliferating but remained alive: the cell numbers were very close to the original seeding density. Through the pictures of immuno fluorescence(Figure 28), the apoptosic cells can be seen very clearly: they display a shrunken cytoplasm, fragmented nuclei with condensed chromatin and blebbing on the cell membrane (Rathmell and Thompson, 1999). Of note, when compared to the serum group, the cells living in the non-serum medium control group

showed a shorter lifespan (only 24 hours), but the high alginate stimuli groups showed later blebbing (for 48 hours). It seems alginate helped the cells survive.

The mechanisms of alginate recognition by macrophages are not clear, but our experiments did confirm that the signals from alginate can be efficiently passed to activate the NF-κB pathway and finally initiate gene expression that triggers inflammation and promotes anti-apoptosis.

Thirdly, alginate may cooperate synergistically with proteins from serum which can be recognized by macrophages.

As discussed in the previous section, there are many proteins in the serum that may participate in macrophage activation: IgG, albumin, complement and other proteins. Since the experiments are performed in well plates, proteins from serum may easily adhere to the plate surface and form a unique protein layer. Since the RAW264.7 cells are also adhesion cells, this protein layer may influence the cell's behaviour and some of the exposed protein sequences may function as ligands for cell recognition (Kapur and Rudolph, 1998;Collier *et al.*, 1997). Several potentially active proteins are found in serum, including IgG, vitronectin, von Willebrand factor and others. IgG aggregation may promote long-term macrophage adhesion while von Willebrand factor may inhibit the adhesion (Jenney and Anderson, 2000a;Jenney and Anderson, 2000b).

In my experiments, with high concentration of alginate stimulation, NF-κB activation lasted for a longer time in the serum deprived group, but a quicker and bigger response in the serum rich group. But the results of low concentration stimulations were just the converse. That means alginate alone is capable of activating macrophages but that

stimulation is slow and mild, and once it cooperate with proteins from serum, the effects will be increased. There are some specific receptors of macrophage which can recognize alginate and transfer the signals to activate the whole cells. However, such receptors and the mechanisms of recognition are still unclear.

Fourthly, TNF-α and other cytokines may have cooperated in regulation of NF-κB activation.

TNF-α, FasL and other TNF-related apoptosis-inducing ligands can be recognized by a member of the TNF receptor family which resides on the cell membrane and results in activation of procaspase-8 and -10 to initiate apoptosis. On the other hand, TNF-α and IL-1β phosphorylate the cell surface signals leading to proteolysis of an inhibitory unit (IκB-α or p105) to free NF-κB which can translocate into the nucleus and open cell survival gene expression. At the same time, NF-κB activation also induces cytokine production which can feedback locally and amplify the activation (Timothy S.Blackwell and John W.Christman, 1997).

There are balances between cell survival and apoptosis, inflammation and anti-inflammation.

Chapter 7 Significance

Researchers have done a lot of work in creating novel biomaterials and surface modifications in order to improve biocompatibility. The chemical and physical properties of materials determine their final applications, especially in tissue engineering, drug delivery or for other medical purposes. For example, hydrophilic surfaces show great advantages in protein repulsion, but are not suitable for preparing scaffolds in tissue engineering because anchorage-dependent cells can not survive on them (Smetana, Jr., 1993). Biodegradable materials are very useful in drug delivery, but metabolites of the degraded materials including oligomer and monomer degradation products (Anderson, 1995) may play a role as antigens in cellular responses, or give a persistent low level stimulation which makes the body acquire immune tolerance. For materials from natural origins, the low toxicity, abundant availability, good biocompatibility, and biodegradability make material scientists believe that they will be very suitable in biological application. But they also have shortcomings: small peptides or polysaccharides can be recognized as a pathogen or epitope that can stimulate cellular responses.

Immunology is an ancient science, but only in recent decades, with the advent of molecular techniques, have researchers been able to understand 'intrinsic' immunology. Specific signals, ligand-binding receptors, transduction pathways, and gene regulation have been studied in detail.

When biomaterials enter the body, they are regarded as foreign invaders and potentially as pathogens. Therefore, the study of biomaterial immunology is as important

as the study of material science and we need immunology knowledge and methods to reveal the interaction between materials and the body.

Host responses including cellular responses can be detected by immunology techniques. There are two routes in studying the immune responses: *in vivo* and *in vitro*. The former relies on different animals (or clinical samples), and the latter depends on specific cell lines instead. Often the cell line model can provide an intuitive and relatively simple result compared to the animal model. But in fact, the animal model will be more meaningful and accurate in reflecting the host responses in humans.

In this thesis, the stimulation of alginate in a macrophage-like cell line was studied. There are several considerations for the experimental design: firstly, the material itself. Alginate is a well-known natural material that has been used in drug delivery and as a main component in preparing matrices for tissue engineering. Like other natural biomaterials, alginate has varied compositions depending on different sources and different extraction methods. It is a kind of polysaccharide, the residues of which might be recognized by specific membrane receptors and induce the cellular responses which may be like responses to oligochitosan (Feng *et al.*, 2004). Alginate can form hydrogels by crosslinking with divalent cations (Smidsrod and Skjak-Braek, 1990), and easily be degraded by proton catalyzed hydrolysis (Haug *et al.*, 1967;Haug and Larsen, 1962;Haug and Larsen, 2006;Haug *et al.*, 2006) or enzyme digestion, e.g. by alginate lyase. The debris from degradation may also stimulate the innate immune responses. Secondly, using a macrophage cell line is an easy way to explain the cellular responses. *In vitro* experiments often have relatively simple conditions, and limited factors to control, e.g. stimuli, media, and effectors. The macrophage is one of the main inflammatory cells that

respond to foreign invaders and it plays an important role in connecting the innate immune responses and the adaptive immune responses. Fundamental immunology studies have provided much information to describe the cellular responses to pathogens: membrane receptors, intracellular signal pathways, gene regulation, and cytokine or other modulator secretions. Thirdly, the *in vitro* studies with alginate can provide useful information in subsequent *in vivo* experiments.

Biomaterial science is an interdisciplinary field involving material science, chemistry, biology and immunology. For determining a good biomaterial, from the primary material selection, design, fabrication and modification, to the biological recognition, and finally host acceptance, there is a long way to go. All these processes are closely connected and we must not disregard any one.

The work in this thesis studied alginate induced cellular immunology. It not only provided further information for further investigations on *in vivo* responses and other alginate forms (e.g. hydrogels), but also set up a model for the study of other polysaccharide materials.

Alginate is a good biomaterial. The abundant availability, low toxicity, promising biocompatibility and porous hydrogel -forming properties make it a potentially useful material. There are, however, still two major problems in the application of alginate biomaterials: the first one is that alginate hydrogels are unstable in certain circumstances; and the second one is that alginate may act an immune "inducer" to activate inflammatory cells. However, these two problems can not be simply named as

shortcomings because if in appropriate conditions, these properties can be used to widen the applications of alginate in drug delivery systems.

In this thesis, we found that alginate stimulated macrophage activation, and the influence was dose-dependent: high concentrations showed a faster and greater influence in a serum environment; and the low concentration only had a relatively smooth and prolonged stimulation. These phenomena suggest that alginate may work well as an adjuvant in vaccine. It can play a role as an immune potentiator and intensify an immune response to a protein antigen. At the same time, if the degradation of alginate hydrogels could be controlled at a slow speed, which would mean a potential low concentration of alginate solution, it will be useful in controlling the release of its contents and providing suitable immune stimulation.

Recently, in order to increase alginate hydrogel stability, other polysaccharides have been combined with alginate, including chitosan. For example, chitosan-alginate multilayer beads can effectively control protein delivery through a controlled gastrointestinal passage (Anal *et al.*, 2003).

Still a lot of work needs to be done in studying the stability and degradation of alginate in an *in vivo* environment. How pH, temperature and ionic strength influences the degradation needs to be studied in detail.

Other problems for natural-origin materials are impurity and complex compositions. This is a common phenomenon to all naturally-derived materials, including alginate. Many purification methods have been developed and in most cases, the majority of impurities can be removed during these processes. However, researchers found some

unknown trace protein contents in alginate are hard to completely purify (Dusseault *et al.*, 2006), and of course, it is difficult to know how they will influence the immune responses with alginate. This may be a question for us to investigate in the future.

Chapter 8 Conclusions and Recommendations

8.1 Conclusions

In this thesis, the mechanism by alginate caused inflammatory responses was studied *in vitro* by using RAW264.7 cells, a macrophage-like cell line. Unlike the generally-used form of alginate, such as in solid microcapsules, a sodium alginate (water soluble form) was chosen to study here. Alginate will not be completely be stable in a hydrogel form in the *in vivo* environment due to the pH, ion exchange, or enzyme digestion, so it has often been combined with other polymers to increase the stability. But after degradation, alginate will be as a single actor which 'plays' with inflammatory cells. So research on soluble sodium alginate is worthwhile, as it provides more direct results and is more convenient for exploring the mechanism by which alginate causes innate immune responses.

From the observation and experimental data analysis, the following conclusions were made:

- Alginate induced RAW264.7 cell activation. The stimulation was dose and time dependent. High concentration of alginate made higher and more prolonged responses compared to the lower concentration that was tested.

- The NF-κB signal pathway in macrophages was activated by both alginate and LPS.

- The cytokines IL-1β, IL-6 and TNF-α induced obvious secretion after the stimulation by both alginate and LPS, and the secretions were also dose and time-dependent.

- Proteins in serum may interact with alginate, and influence the alginate recognition by macrophages.

- When deprived of serum from the medium, RAW264.7 cells survive but with inhibited proliferation for 48 hours. In addition, alginate, especially at the higher concentration studied, appeared to promote macrophage survival.

- Alginate and serum starvation may co-operate in activating macrophages, and eventually influence cytokine secretions. The influences were specific to different cytokine outcomes, e.g. IL-6 and TNF-α secretion were inhibited, while IL-1β secretion was promoted.

8.2 Recommendations

This is the first time that the mechanism of innate responses to a natural material has been studied. Although we have obtained many interesting and instructive results with *in vitro* alginate stimulation, much remains unexplored. Lots of work needs to be done to complete the whole idea of how biomaterials affect immune responses.

Firstly, in macrophages, different signal pathways coexist and influence one another. We have found a very useful pathway- NF-κB activation, which can be used to

track alginate stimulation, but the other pathways may also exist which have similar or opposing functions. Blocking NF-κB activation will help us to find such pathway.

Secondly, alginate can activate macrophages but the primary recognition process is still unknown. The chemical signal of alginate may bind on some specific membrane receptor which transfers the signal to the NF-κB pathway. Mannose receptors can bind chitosan derivatives (Han *et al.*, 2005), and it seems this receptor can specifically recognize specific carbohydrate residues. The work for checking its function with alginate has few reports. Of course, other receptors may participate in alginate recognition instead of mannose receptor.

Thirdly, alginate purification is an important issue. Like other naturally-derived materials, low concentrations of proteins, endotoxin and polyphenol (Dusseault *et al.*, 2006) may hard to remove, and further research needs to be done to confirm that such contamination would not influence the immune responses.

Finally, it would be very interesting to study the interaction between serum proteins and alginate further. The system is complex and it may need further effort to study the physical properties of protein and alginate in solution. In addition, we may prepare alginate microspheres and incubate them with serum to track non-specific protein adhesion on these microspheres and different proteins in the interaction with alginate and therefore study the influence in innate immune responses.

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Appendix 1: Limulus Amebocyte Lysate (LAL) Test

The protocol is prepared by Maud Gorbet with modification from Cape Code Instructions.

1) Add 100µl test solution (alginate) and 100µl endotoxin-free water into a 48 well plate separately;

2) Add 100µl of reconstituted pyrochrome to each sample;

3) Incubate the plate for 21 minutes specified in the certificate of analysis at 37°C in the dry oven;

4) Stop reaction by adding 200µl of 50% acetic acid;

5) Quickly shaking the plate;

6) Transfer supernatant of each sample in duplicate (150µl) to 96-well plate;

7) Read at 405nm.

Appendix 2: Alginate purification

Alginate is a natural-origin material. It has complex composition and undetermined components including endotoxin, proteins and polyphenol. These components can induce host immune responses when they contact the body. By different filtration techniques, we have successfully reduced the endotoxin level down to less than 0.1 EU/ml (in 1mg/ml and 3mg/ml alginate solution). However, the other two contaminants are hard to get rid of through such filtration. The following protocol modified from de Vos et al.(de *et al.*, 1997) which can effectively reduce endotoxin and polyphenol level and do some effects on protein residues (Dusseault *et al.*, 2006):

1) Prepare 1% sodium alginate in 1mM EDTA solution.

2) Add 2N HCl plus 20mM NaCl to precipitate alginate with constant stirring. Adjust pH to around 2.5 to form gel.

3) Add an equal volume of 0.01N HCl plus 20mM NaCl, shake vigorously to break gel, and filter in Buchner funnel. Repeat this washing step 3 times.

4) Re-dissolve alginate in water by slow neutralization with 0.5N NaOH and 20mM NaCl over the period of at least 1 hour.

5) Extract the alginate solution 2 times in chloroform/butanol. Use 20mL chloroform and 5mL butanol per 100mL alginate. Shake vigorously and let sit for 30 minutes.

6) Centrifuge mixture at 5 000 rpm for 30 minutes. Remove the chloroform/butanol phase with a Pasteur pipette.

7) Precipitate alginate with ethanol (200mL ethanol per 100mL alginate). Centrifuge again (5K rpm, 30 minutes).

8) Wash pellets with ethylether, freeze dry and lyophilize.

9) Re-dissolve alginate in 150mM NaCl to make 1% solution, add Trypsin (0.5 mg/ml, Sigma), and incubate for 2 hours at 37C.

10) Precipitate alginate in 2 volumes of ethanol again, centrifuge and resuspend in 150mM NaCl to make 1% solution.

11) Dialyze against distilled water (50kDa cut-off). (Tubing Prep: Boil tubing 10 min in 2% sodium bicarbonate, 1mM EDTA (pH 8); boil 10 minutes in 1mM EDTA; rinse with water).

12) To obtain crystalline alginate, freeze dry and lyophilize.

Appendix 3: Western Blot Procedure

1) SDS-PAGE

12.5% Separating Gel

Distilled H_2O 3.55ml
Separating Gel Buffer (3.0M Tris-HCl, pH8.8) 1.00ml
10% SDS 80µl
30% Acrylamide-Bis 3.33ml
10% Ammonium Persulfate (fresh) 40µl
TEMED 4µl

4% Stacking Gel

Distilled H_2O 1.20ml
Stacking Gel Buffer (0.5M Tris-HCl, pH6.8) 0.50ml
10% SDS 20µl
30% Acrylamide-Bis 0.27ml
10% Ammonium Persulfate (fresh) 10µl
TEMED 2µl

Electrophoresis Buffer (5X stock solution, pH 8.3)

Tris Base	15g
Glysine	72g
SDS	5g

Fill to 1L with water. Just before use dilute to 1X strength.

Run the gels at constant current for 25-50mA.

2) Gel Equilibration

Transfer Buffer

Tris	3.03g
Glycine	14.4g
Methanol (HPLC grade)	200mL

Fill to 1L with water. Store at 4°C.

Gel equilibration in transfer buffer for 15-20min.

3) Electrophoretic Transfer

PVDF transfer membrane cut to size ($6.8 \times 8.8 cm^2$); prewet with 100% methanol for 1-3 seconds, then distilled water for 1-2 minutes and soak in cold transfer buffer. Set up the cassette followed by Mini Trans-Blot Electrophoretic Transfer Cell Instuction Manual,

Bio-Rad). Blot for 2 hours at 80 volts in ice-cold buffer. Then dry the membrane on the bench.

4) Antibody incubation

TBS Buffer

Tris Base 50mM
NaCl 150mM
Adjust pH to 7.4

Carbonate Buffer

$MgCl_2 \cdot 6H_2O$ 20mg
$NaHCO_3$ 840mg
Fill to 100ml with distilled water, adjust pH to 9.8 with NaOH.

Substrate Solution

NBT 30mg dissolved in 300µl H2O, 700µl DMF
BCIP 15mg dissolved in 1000µl DMF
Add the above reagents to 100ml carbonate buffer just before use.

i. Prewet membrane with 100% methanol for 1-3 second, and distilled water for 1-2 minutes. Incubate the membrane with blocking buffer (5% w/v nonfat dry milk in TBS, pH7.4) for 1 hour with gentle agitation.

ii. Wash membranes with washing buffer (0.1% w/v nonfat dry milk in TBS) for 5 minutes, 3 times.

iii. Incubate with primary antibody for 1-1.5 hour (1% w/v nonfat dry milk, 0.05% v/v Tween 20 in TBS).

iv. Wash the membrane as step ii.

v. Incubate with second antibody for 1-1.5 hour (1% w/v nonfat dry milk, 0.05% v/v Tween 20 in TBS).

vi. Wash away the extra antibody as step ii.

vii. Incubate with substrate solution to develop the color reaction.