The Characterization of Chitin Microparticle Preparations: Degree of Acetylation and its Effect on Immunologic Response

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Degree of Acetylation and its Effect on Immunologic Response

A Thesis Presented
By
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I am grateful to my wife and her unwavering dedication to me throughout our lives together, and my friends for their support.
ABSTRACT

THE CHARACTERIZATION OF CHITIN MICROPARTICLE PREPARATIONS: DEGREE OF ACETYLATION AND ITS EFFECT ON IMMUNOLOGIC RESPONSE

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Studies examining the immune response upon exposure to chitin microparticles in living models have reached drastically differing conclusions, and the reason remains unclear. One notable issue between the experiments is that they have not characterized their chitin preparations for degree of acetylation. They all use different chitin processing methods prior to administration, which could potentially be the source of the variance between studies. Chitin and chitosan preparations specified in the literature and several novel preparations were analyzed for degree acetylation (DA) using High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). It was found that autoclaving and sonication processing steps do not have a significant influence on degree of acetylation. Chitin and chitosan preparations were used to create a dose-response curve of DA compared to cytokine elicitation from THP-1 monocytes, and it was found that the initial response was dominated by TNF (similar to previous studies), though after 12 hours showed a tip toward the start of an IL-1β-dominated Th17 effector response. This study also confirmed that immunostimulatory effects can occur from chitin and chitosan particles at or <1µm in size. The results suggest that very small chitin particles, which would have long residence times in air, might be implicated in initiating allergic or asthmatic processes.
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CHAPTER 1
INTRODUCTION

Chitin is a component of many parasites; hence chitin hydrolases and hydrolase-like proteins have evolved in plants and animals as a defense mechanism against chitin-containing organisms. Chitin is also the primary component of insect exoskeletons, the encasing material of dust mite droppings, and is found in nearly all fungal spores. These substances are among the primary constituents of aerosolized house dust and therefore represent a significant source of human chitin exposure on a daily basis.

One chitin hydrolase, acidic mammalian chitinase (AMCase), has been repeatedly linked to asthma and atopy (Zhu et al., 2004; Reese et al., 2007; Sohn et al., 2008; Ober et al., 2008). Increased amounts of AMCase expression were found in the bronchoalveolar lavage (BAL) fluid of ovalbumin (OVA)-sensitized and OVA-challenged mice as well as being present in the epithelial cells and alveolar macrophages in lung tissues found in human patients suffering from asthma, whereas AMCase was not found in healthy non-asthmatic humans (Zhu et al., 2004). Asthma inflammation is driven by type 2 helper T-cells (Th2)- immune response to allergenic proteins, mediated by interleukin 4, 5, and 13 (IL-4, IL-5, and IL-13) cytokines. In almost all patients, asthma is marked by an abnormally high expression of these cytokines in lung tissue. Because mammalian chitinases evolved to mount innate immune defenses against chitin-containing pathogens (Lee and Elias, 2010), chitin itself is likely a pathogen associated molecular pattern (Da Silva et al., 2009) capable of triggering asthmatic response.

Studies regarding the immunogenic reactions to chitin in living models (cell culture and murine models) have produced conflicting results. For example, Shibata
(1997a, 1997b, 1999, 2001), Strong and Clark (2002), and Da Silva et al. (2008a) all found that responses to chitin in mice are size-dependent and tend to elicit Th1-type responses. However, Bueter et al. (2011) found that chitin does not elicit an immune response at all; rather chitosan (a deacetylated form of chitin) is responsible for eliciting a Th1 response. Yet experiments by Da Silva et al. (2008b) indicated that a Th17 pathway may be involved and that the response is independent of phagocytosis, while Shibata’s research (Shibata et al. 1997a, 1997b, 2000, 2001) indicates phagocytosis both occurs and is required. Reese et al. (2007) found that in mice chitin elicits a Th2 response and triggers alternatively activated macrophages, similar to the response seen in asthmatic humans.

The reason for the difference in results is unclear. While chitin has been implicated in triggering immune response, chitosan has been utilized for therapeutic medical applications, including wound healing and antimicrobial treatments (Dutta, 2004; Dai et al., 2009). None of the studies to date have performed quantitative analyses on their samples for degree acetylation, the primary difference between chitin and chitosan and a key element in the binding of chitinases and chitinase-like proteins. One notable difference between the studies is the process by which chitin is prepared for use. Methods of preparation used by Shibata et al. (1997a, 1997b, 2000, 2001), Da Silva et al. (2008, 2009), and Strong et al. (2002) involve sonication of the chitin particles to obtain specific size classifications, including chitin microparticles (1-10µm), chitin fragments (40-70µm), or large chitin (70-100µm). Sonication of chitin in water has been shown to induce deacetylation (Cardoso et al., 2001) and depolymerization (Baxter et al., 2005). Shibata et al. (1997b, 2000, 2001) and Da Silva et al. (2008, 2009) also autoclave their
samples. However it has been shown that autoclaving of chitin decreases both molecular weight and degree of acetylation, and can increase depolymerization (No et al, 2000, 2002; Sjoholm et al, 2009). Therefore, differing processing methods may lead to chitin preparations with different characteristics that are immunologically significant. The degrees to which processing methods used in previous studies affect both particle size and the degree of acetylation of chitin have not been well characterized and it is unclear how they might have affected study outcomes. Therefore it is still unclear whether chitin plays a role in eliciting Th1- or Th2-type immune responses. Conflicting results in the literature highlights a need to better characterize these preparations in order to clarify the role of chitin in immune and allergic responses.

The objective of this study is to develop a standardized protocol for chitin preparations that controls the degree of acetylation. These preparations will be used in human cell culture to elucidate how degree of acetylation influences immune response, through cytokine production and the induction of chitinase and chitinase-like protein genes. The results of the study will provide information on the expected type of effector t-cell response to airway exposures. The primary hypothesis is that chitin particles with a high degree of acetylation will elicit cytokines associated with a Th2-type immune response indicative of the induction of an allergic response.
CHAPTER 2

CHITIN, CHITOSAN, AND CHITINASES

2.1 Chitinases

Chitinases are chitin hydrolases (enzymes that break down chitin) that are found in a variety of life forms. Chitin-containing organisms employ chitinases to control growth and molting, while organisms that do not contain chitin use chitinases to recognize and defend against chitin-containing pathogens (Gooday, 1999). Chitinase family 18 contains the largest number of glycohydrolases, and include chitinases from eukaryotes (including mammals), prokaryotes, and viruses (Bussink, 2007). These glycohydrolases contain a 150-residue conserved core that existed prior to the prokaryotic/eukaryotic split, with only minor modifications occurring since (Robertus and Monzingo, 1999), indicating the important evolutionary role for chitinases.

There are two primary categories into which chitinases can be grouped: true chitinases (AMCase and chitotriosidase) or chitinase-like proteins (YKL-40, Ym1, etc.). The functional difference between the two is that true chitinases have a C-terminal chitin binding domain and an N-terminal catalytic core domain conferring hydrolytic enzymatic activity, whereas chitinase-like proteins have no catalytic domain but can bind to chitin (Renkema et al, 1997). It is also important to note that although similar in many respects, the tissue-specific expression of human and mouse chitotriosidase is quite different. Mouse chitotriosidase is most expressed in the tongue and stomach and not in other tissues, whereas human chitotriosidase is expressed in lymph node, bone marrow, and lung, but not in the stomach. Mouse AMCase is highly expressed in the stomach, salivary glands, and lung of mice, but human AMCase is highly
expressed in the stomach, and to a lesser extent in the lung. Human alveolar macrophages express chitotriosidase, which is not found to occur in mouse lung. Instead, the mouse alveolar macrophages express AMCase. (Boot et al., 2005)

All chitinases rely on the presence of the carbonyl oxygen located on the acetyl group to initiate hydrolysis (Tews and Terwisscha, 1997; van Aalten et al., 2001), indicating that chitinase upregulation is related to the presence of chitin rather than chitosan. AMCase, chitotriosidase and chitinase-like proteins have been implicated in inflammatory response, atopy, and pathogenesis of human diseases. Their presence strongly suggests a link between pulmonary chitin exposure and disease.

2.1.1 AMCase

AMCase has been repeatedly linked to asthma and the Th2-type airway inflammatory response that causes tissue inflammation and hyperreactivity. In murine models, AMCase expression was induced in the presence of IL-13 by Th2-polarized cells, while Th1-polarized cells were unable to induce AMCase. Upon administration of anti-AMCase sera, Th2 inflammation decreased in a dose-dependent manner, but did not have any effect on mRNA levels of IL-4, IL-5, and IL-13. Increased AMCase expression was found in the bronchoalveolar lavage fluid (BAL) of OVA-sensitized and antigen-challenged mice as well as being present in the epithelial cells and alveolar macrophages in lung tissues found in human patients suffering from asthma, while AMCase was absent in healthy non-asthmatic humans (Zhu et al., 2004).

The mouse genes for AMCase and Ym1 (chitinase 3-like-3, corresponding to chitinase-3-like-1 in humans) were found to be up-regulated during Schistosoma mansoni infection in both wild type mice and Th2-polarized mice (Sandler et al., 2003). The
induction of Ym1 is a marker for alternatively activated macrophages and is dependent on IL-4 in vivo (Gordon et al., 2002). Ym1 and Ym2 have been found to be induced during Th2-type allergic inflammation in mice and are regulated by CD4+ T cells, IL-4 and IL-13 signaling via the IL-4Rα subunit (Webb et al., 2001).

Lung tissue in mice challenged by helminth infection showed that AMCase, Ym1 and Ym2 were dependent upon induction of STAT-6 (Reese et al., 2007). Zimmerman et al., 2004 also found that in OVA-sensitized mice, the genes for AMCase and Ym1 were induced within 18h of the first allergen challenge and were STAT-6-dependent. Because STAT-6 is required for IL-4 and IL-13 signaling, these results indicate that chitin may be a trigger for infiltration of IL-4- and IL-13-producing cells.

AMCase is found to be up-regulated in inflammatory cells in the nasal mucosa associated with allergic rhinitis. Expression of AMCase was also increased in persons who suffered from chronic sinusitis, an inflammation of the paranasal sinuses (Cho et al., 2010). These results suggest that chitin hydrolases may have evolutionarily developed as a defense mechanism against airborne fungal pathogens.

2.1.2 Chitotriosidase

Chitotriosidase, a true chitinase primarily expressed in the lung in humans, has various associations with disease, including Gaucher’s disease, malaria, atherosclerosis, asthma, multiple sclerosis, and was found to be elevated in the lungs of smokers (Barone et al., 2007; Lee et al., 2007; Seibold et al., 2008). Chitotriosidase is expressed by lung macrophages and also in peripheral serum and plasma by activated monocyte-derived human macrophages (Barone et al., 2007). Chitotriosidase has also been linked to asthma and atopy—Lee et al. (2007) found that subjects with Asian ancestry were more likely to
be atopic if chitotriosidase is expressed, and Wu et al. (2010) found that high fungal exposures (via aerosolized household dust) along with a single nucleotide polymorphism in the *CHIT1* intron were associated with severe asthma exacerbations.

### 2.1.3 YKL-40

YKL-40 is a chitinase-like protein in humans also known as human cartilage glycoprotein 39 (HCgp-39) or chitinase 3-like-1 (CHI3L1). It is found in increased quantities in the circulation and lungs of some subpopulations of patients with asthma, and can correlate with the severity of symptoms and degree of sub-epithelial basement membrane thickening (Chupp et al., 2002).

In a cohort of Korean children, variations in two SNPs within the *CHI3L1* gene were strongly associated with atopy, increased circulating YKL-40 levels, and increased IgE levels, but were unable to be correlated with asthma (Sohn et al., 2008). Contrastingly, Ober et al., (2008) found that within a population of European descent, *CHI3L1* may be an asthma susceptibility locus, as a promoter within the gene was significantly associated with serum YKL-40 levels, asthma, and bronchial hyperresponsiveness. In murine models, chitinase 3-like-3 (Ym1 in mice), Ym2, AMCase, Fizz1 proteins (found in inflammatory zone 1, a marker for alternatively activated macrophages) in BAL were found to be increased in OVA-challenged mice as compared to a control group, and the same proteins were increased in the lung tissue of murine asthma models. Upon treatment with N-acetylcysteine (NAC), expression of Ym1 and Ym2 were inhibited, indicating that chitinases can serve as oxidative stress proteins, and are involved in the pathogenesis of asthma (Zhang et al., 2009).

### 2.2 Chitin
Found widely in nature, chitin is a nitrogenous cellulose-like polysaccharide composed of repeating $\beta(1\rightarrow4)$-linked N-acetyl-D-glucosamine units. As one of the most abundant natural polysaccharides, it is found in substances including crustacean shells, helminth eggs, insect exoskeletons, dust mite droppings, and in the cell walls of fungi (Nathan et al., 2009; Van Dyken et al., 2011). Chitin is insoluble in water and other organic solvents, though it begins to show signs of solubility as it approaches 40% deacetylation, at which point it is generally referred to as chitosan (Sorlier, 2001; Dutta, 2004; Rinaudo, 2006), though some disagreement exists. The changeover from chitin to chitosan has been cited at 70-100% deacetylated (Aiba, 1992) or at 50% (Chatelet et al., 2001). Chitin is found naturally in three different crystallographic forms, known as $\alpha$-chitin, $\beta$-chitin, and $\gamma$-chitin. The forms differ in the organization and polarity of their polysaccharide chains within sheets, the interaction of which beget distinct crystallographic traits. The $\alpha$- form of chitin is found abundantly, whereas $\beta$-chitin is found primarily in squid pens, pogonophoran tubes, and the spines excreted by certain diatoms (Rinaudo, 2006).

In nature, chitin acetylation is typically around 90% with a nitrogen content of approximately 7% (Meyers, 1995). Source material, purification techniques, and processing methodology contribute to differing degrees of acetylation, polymer structure, and nitrogen content in the final usable product (Meyers, 1995; Percot, 2003; Brine and Austin, 1981)

2.2.1 Source Material

Commercial chitin from crustacean sources is typically derived from crab, shrimp or lobster. Different sources contain differing amounts of crude protein, crude fat, ash,
calcium, and magnesium as impurities, which greatly influence the purification process that must be used (Percot, 2003; Meyers, 1995). Brine and Austin (1981) reported chitin content between different crab species ranged from 14.8% to 27.6%, while average acetylation and molecular weight did differ, but depended more on purification technique than source.

Nitrogen content in chitin varies by species, which is subsequently a relic of the purification method required for extraction. The theoretical value for nitrogen content in pure chitin is 6.89% by mass—higher values indicate deacetylation (N% of up to 8.69) or remaining protein in the sample, while lower values are indicative of deamination (by hydrolysis) or a contamination of the sample (Rutherford and Austin, 1978).

2.2.2 Purification Techniques for Source Material

The purpose of purification is to go from crustacean source material to pure chitin. This involves deproteinization, demineralization, and optional decolorization. Typically deproteinization occurs from a treatment of ground shell material with 1-10% NaOH at elevated temperature (65-100°C) to dissolve the protein, though dilute KOH solution can be used depending on the species of source material (No and Meyers, 1995). Demineralization serves to dissolve any calcium carbonate in the source material. This is conventionally done over a short time (1-3 hours) using a dilute HCl solution at room temperature, though dilute acetic acid can also be used (Brine and Austin, 1981). Decolorization can be achieved by a variety of techniques, most of which involve acetone, chloroform, and/or ethanol (No and Meyers, 1995).

2.2.3 Processing Methodology Used in Experiments
Brine and Austin (1981) found that there is a parallel relationship between the degree of depolymerization and the degree of deacetylation, and that preparation method strongly influences both values. Other techniques used for sterilization, to induce solubility, or to reduce particle size can also affect the chemical and physical properties of chitin through deacetylation, depolymerization, or change in crystal structure (Brine and Austin, 1981).

2.2.3.1 Autoclaving

Chitin is sensitive to heat and begins to denture at temperatures as low as 50°C (Muzzarelli, 2005). Autoclaved chitin in 45% NaOH solution can decrease degrees of acetylation (DA) to between 17.1±0.1 % after 10 minutes and 9.6±0.1% after 30 minutes. At 50% NaOH, 30 minutes of autoclaving results in a DA of 8.2±2.9%. It should be noted that commercial chitosan averages a DA of 12.4±0.1% (No et al., 2000). No et al. (2002) found that autoclaving chitosan (deactylated chitin) with 1% acetic acid caused depolymerization; molecular weight decreased by 51% after 15 minutes and 61% after 60 minutes. Sjoholm et al. (2009) determined that autoclaving chitosan in the presence of strong NaOH causes both depolymerization and even further deacetylation (from 80% to 98% deacetylation over 30 minutes). Taken together, these results indicate that autoclaving can deacetylate and depolymerize chitin.

2.2.3.2 Sonication

Sonication used to decrease particle size has physical effects on the properties of chitin, as well. Sonication of chitin in water caused aggregation of chitin particles, changes in x-ray diffraction patterns (likely due to the size reduction of crystals rather than structural changes), grain-like surface protrusions that increase surface area, and
activation of chitin for deacetylation reactions (Cardoso et al., 2001). Sonication of 3g chitin/100mL of chilled water over 180 minutes was sufficient to show a time-dependent creation of oligosaccharides, with peaks of formation at 60 minutes and a significant increase at 120 minutes (Takahashi et al., 1994). Machova et al. (1999) showed that sonication was able to significantly depolymerize a chitin-glucan complex found in Aspergillus niger cell walls, indicating that sonication causes changes on the molecular level.

2.2.3.3 Acid/base treatment

Acids readily hydrolyze chitin, and concentrated acids can react with chitin to form colloidal chitin (a suspension of nanoparticles) (Rupley, 1964). Concentrated HCl reacts with β-chitin to decrystalize its structure and completely convert it to α-chitin (Saito et al., 1997). Concentrated HCl also depolymerizes chitin though hydrolysis, during which the glycosidic linkage is broken, causing both lower molecular weight and decrease in acetylation (Brine and Austin, 1981; BeMiller and Whistler, 1962). Prolonged time in even dilute HCl solution can also cause depolymerization (Hirano, 1982).

Concentrated NaOH reacts with chitin to deacetylate it up to 96% after four hours at 110°C (Mima et al., 1983), though 78% deacetylation occurred in only 2 hours. In an oxygenated environment, exposure to concentrated NaOH can result in both deacetylation and depolymerization (Sannan et al., 1976), though in dilute alkali solutions it remains relatively stable (Varum et al., 2004).

3. Chitosan

Deacetylated chitin is known as chitosan (less than 40% degree acetylation, though fungal chitosans are often in the 10% DA range (Aranaz et al., 2009), and has
reactive amino and hydroxyl groups. The free amine is one of the primary causes for the solubility of chitosan, another key difference between chitin and chitosan (Aranaz et al., 2009). The cationic charge responsible for the solubility of chitosan in mild acid solution is also responsible for its antimicrobial properties, as the charged amino group is able to bind to bacterial membranes and affect nutrient transport to the bacterial cell (Rabea et al., 2003). It has also been established that the degree of cell adhesion is directly proportional to the degree of acetylation (Mao et al., 2004), further illustrating the importance of acetylation. Nitrogen content is generally greater than 7% of the total structure because of the lack of acetyl groups.

Chitosan solutions are extremely sensitive to aging, primarily due to depolymerization, aggregate formation, and microbial degradation (Zhao, 2010). It is a natural, biodegradable, non-toxic polymer that binds to mammalian and microbial cells, and is currently being researched for medical applications (Dutta, 2004). Mao et al. (2010) reference chitosan as being used as nonviral carriers for plasmids and drugs, and chitosan nanoparticles have been found to protect drugs from enzymatic degradation and enhance intestinal absorption (Thanou et al., 2000). Chitosan has been used in burn wound dressings to prevent infection and systemic sepsis, and it has been proven to have antimicrobial properties (Dai, 2009). Chitosan has also been employed in sutureless tissue fixation because it has proven to be biocompatible (Lauto, 2009).
CHAPTER 3

PREVIOUS STUDIES

3.1 Findings

3.1.1 Th1-type Response

Shibata et al. (1997a) found that chitin microparticles (CMP, particle size 1-10µm) administered intravenously both primed alveolar macrophages and stimulated reactive oxygen species (ROI) in SCID and C57BL/6 mouse models. Soluble chitin/chitosan oligosaccharides administered similarly did not prime the alveolar macrophages. Decreased priming resulted from CMP administered to C57BL/6 mice co-administered with monoclonal antibodies (mAb) to either IFN-ϒ or NK1.1 (natural killer cells). Spleen cells from C57BL/6 or SCID mice exposed to CMP expressed IFN-ϒ, whereas cells administered mAb to NK1.1 and CMP resulted in inhibited IFN-ϒ production. Cells exposed to chitosan or phagocytosable latex beads produced no IFN-ϒ. These results indicate that macrophage priming is, in part, a result of the production of endogenous IFN-ϒ by NK1.1+CD4+ cells, which is up-regulated by IL-12 and TNF-α. Because the activation also occurred in SCID mice which lack functional T and B cells, the priming is a result of innate immune response.

Shibata et al. (1997b) explored the mechanism responsible for CMP-induced immune response. Splenic macrophage cultures obtained from BALB/c mice were administered combinations of soluble mannan, CMP, phagocytosable beads coated with either chitin, mannan, or laminarin, or non-phagocytosable agarose beads cross-linked with either mannan or N-acetyl-D-glucosamine, or cytochalasin D (an inhibitor of phagocytosis). Soluble mannan inhibited the interaction between macrophages and CMP,
but does not inhibit IL-12-mediated IFN-ϒ production. The non-phagocytosable beads elicited no IFN-ϒ whereas the phagocytosable beads coated in carbohydrate did. These results indicate that IFN-ϒ/IL-12 production induced by CMP is reliant upon the mannose receptor-mediated phagocytosis.

Using both C57BL/6 and BALB/c murine models and splenic culture, Shibata et al. (1998) found that IL-10 downregulates chitin, BCG and LPS-induced IFN-ϒ production, but it remained unclear as to which part of the innate response induced by chitin was inhibited by IL-10 though the inhibition is, in part, derived by NK cells and macrophages.

Shibata et al. (2000) used ragweed-sensitized spleen cells and ragweed-sensitized BALB/c and C67BL/6 mice to explore the effects of orally-administered CMP on the regulation of Th2 immune response. When CMP were applied to ragweed-sensitized spleen cells, IFN-ϒ and IL-10 were secreted, but there was no IL-4 or IL-5 (indicating CMP induce a Th1-type response rather than an allergic Th2-type). Chitin microparticles and neutralizing Abs to IL-10 or IFN-ϒ were administered and an enhanced IL-4 and IL-5 response was observed, indicating that IFN-ϒ (later determined to be from NK cells) and IL-10 are at least partly responsible for inhibiting the Th2 response to chitin. Prophylactic oral administration of CMP to ragweed-sensitized mice led to reduced serum IgE, reduced ragweed-specific IgE and increased ragweed-specific IgG2a, which indicates isotype switching of B cells. BAL cells showed reduced eosinophil and lymphocyte recruitment to the lung and no IL-4 or IFN-ϒ.

Shibata et al. (2001) then explored the Th1 adjuvant capabilities of CMP. In splenic macrophage cultures of WT mice immunized with MBP-59 (a mycobacterium
protein), subsequent exposure to MBP-59 induced a Th2 response (IL-4, IL-5, IL-10 and no IFN-ϒ) whereas exposure to MBP-59 and CMP showed a reduced Th2 response and increased IFN-ϒ production, indicating that CMP down-regulates Th2-type responses and up-regulates Th1-type responses. In the WT murine model, exposure to MBP-59 and CMP significantly reduced levels of IgG1 and IgE. Administration of MBP-59 alone increased total IgE and MBP-59-specific IgG1. In an IL-10 knockout mouse, exposure to MBP-59 induced a stronger Th2 response as compared to the WT with enhancement of total serum IgE, MBP-59-specific IgE, and increased IgG1. The administration of MBP-59 and CMP elicited higher IFN-ϒ, reduced IL-4 and IL-5 and IgG2a, indicating B-cell isotype switch. These results indicate that endogenous IL-10 down-regulates the IL-4-dependent IgG1 and IgE production, in addition to the IFN-ϒ-dependent production IgG2a.

In an effort to determine whether chitin could act as a Th1 adjuvant without eliciting a Th2-type response, Nishyama et al. (2006) from the Shibata lab administered CMP to RAW264.7 macrophage-like cells and found that there was minimal IL-10 produced and a large TNF-α response. The mitogen-activated protein kinase (MAPK) pathway is phosphorylated upon phagocytosis of CMP, and stimulated cells to also express COX-2 protein and PGE2. It was also found that there was no direct relationship between the inhibition of COX-2 (regulated by the MAPK pathway) and the production of TNF-α. Nishyama et al. (2008), using RAW264.7 cells and cellular proteins from the RAW264.7 cells associated with exposure to CMP found that depletion of cholesterol from the plasma membrane results in minimal alteration of recognition and phagocytosis of CMP, as there are different membrane structure requirements for binding and
phagocytosis than MAPK activation, cytokine production and PGE$_2$ production. MAPK and PGE$_2$ activation indicates a Th1 response, and although also typically associated with IL-10 production, this study failed to find any IL-10 production, in contrast to their earlier findings (Shibata et al., 1998).

3.1.2 Th2-type response

In investigating host responses to allergic and parasitic challenge, Reese et al. (2007) found that intranasally-administered chitin in mice caused recruitment of eosinophils and basophils to lung tissue, and as demonstrated by intraperitoneal (i.p.) injection of chitin, the response was not only limited to the lung. Chitin pre-treated with active AMCase and intranasally administered to mice led to loss of cell recruitment in the lung tissues. Mice overexpressing AMCase had attenuated inflammatory responses, indicating that chitin elicits infiltration of lung tissue by IL-4 competent cells, and AMCase halts this activity. Although AMCase is dependent on the Stat-6 and Rag pathways, eosinophil recruitment by chitin is not, indicating that the immune response to chitin occurs very early. Chitin exposure also causes macrophages to produce eosinophil chemoattractant, though chitin ceases to do so if pretreated with AMCase. Basophil and eosinophil recruitment by chitin exposure was also attenuated in mice with the macrophage-produced chemoattractant leukotriene B4 receptor (BLT-1) knocked out. Further experiments revealed that exposure to chitin elicits accumulation of arginase I-positive macrophages, and that inflammatory macrophages did not acquire arginase I expression until later in the response. This indicates that chitin may be a PAMP recognized in tissues and implicated in inducing early innate immune responses.
Noticing disparities between study results regarding immune response, Kogiso et al. (2011) from the Shibata lab investigated the immune response of mice that were given i.p. injections of latex beads of various sizes coated in various carbohydrates and compared them to their CMP and chitosan microparticles. They found that large, non-phagocytosable beads (LCB, chitin-coated beads >40µm, analogous to the material used by Reese et al., 2007 but differing in processing methodology) induce local eosinophilia regardless of carbohydrate composition. They then found that CMP and LCB do not significantly influence levels of CHI3L1 nor do they induce IL-10. It was further found that CMP actually reduce IL-10 in the presence of inflammatory conditions. In RAW265.7 macrophage cell culture, CMP elicited an M1 phenotype (classically activated macrophages) whereas LCB did not, and neither elicited arginase I. This differs from the in vivo result in which CMP elicited slight ArgI\textsuperscript{low} (indicating TLR-dependent, STAT6-independent) and LCB and Sephadex (uncoated) beads had greater expression of ArgI\textsuperscript{high}, independent of CRTH2 (receptor for PGD\textsubscript{2}, responsible for recruitment of Th2 cells). Taken together, these results indicate that large non-phagocytosable beads, regardless of carbohydrate coating, will result in an innate response with alternatively activated macrophages.

3.1.3 Other Responses

Da Silva et al. (2008) suspected that immune responses to chitin were size dependent and used big chitin (70-100µm) and intermediate chitin fragments (40-70µm) first on macrophages derived from either peritoneal or bone marrow and found that the big chitin had no effect on IL-17 or IL-17AR expression. The fragments, however, stimulated IL-17, IL-17AR mRNA and protein expression, IL-12/23p40, IL-23, and
TNF-α. Using macrophage cultures from a variety of knockout mice (TLR-2, TLR-4, MyD88, IL-17), it was found that chitin fragments stimulate macrophages via a MyD88-dependent pathway that involves TLR-2 but not TLR-4, and through mechanisms that are IL-17-dependent and independent. WT mice given intermediate chitin fragments intranasally had acute inflammation and accumulation of IL-12 that are induced by a pathway dependent on MyD88, TLR-2 and IL-17. Rag2−/− mice administered intranasal chitin fragments produced IL-17, indicating that the response to chitin is innate. Taken together, these results imply that chitin is a size-dependent pathogen associated molecular pattern (PAMP) that activates TLR-2 and MyD88. The inflammatory response is initially neutrophilic and becomes eosinophil-dominated over time.

Using peritoneal macrophages derived from C57BL/6 mice, Da Silva et al. (2009) found that the size of the particle greatly influenced the response of macrophages, as large chitin and chitin oligomers had no effect, intermediate chitin (40-70µm) stimulated TNF-α through TLR-2-, NFκB- and dectin-1-dependent, phagocytosis-independent mechanisms. Small chitin (<40µm, primarily 2-10µm) stimulated TNF-α primarily via Syk and dectin-1 (and to some extend the mannose receptor), but unlike intermediate chitin, also stimulated IL-10 production. These results indicate that size-dependent elicitation of inflammation may be mimicking the response to parasite/pathogen elimination, with intermediate and small chitin serving as a PAMP to signal the need for destruction and elimination.

Due to the varying outcomes from other groups, Da Silva et al. (2010) explored whether chitin could be an adjuvant for a variety of adaptive immune responses. Using both in vivo and in vitro models, it was determined that chitin (a PAMP) is an adjuvant
for Th1, Th2, and Th17-type responses and antigen-specific IgE induction, mediated by TLR-2 and MyD88-dependent pathways. They also found in the presence of OVA- and chitin-sensitized mice (in vivo and with CD4+ T cells in the presence of bone marrow derived macrophages), the presence of IL-17 downregulates IFN-γ and can cause a tip toward a Th2-dominant cytokine profile.

Amidst the various results of previous chitin and chitinase studies, Bueter et al. (2011) investigated the effects of chitin and chitosan on the NLRP3 inflammasome, which is triggered by fungal pathogens and is responsible for modulating the release of IL-1β, IL-18, and IL-33. The study was conducted on bone marrow macrophages in culture and found that, contrary to the previous studies, chitosan and chitosan-coated beads elicited a dose-dependent IL-1β response that was dependent on the NLRP3 inflammasome, was size dependent (particles <20µm) as well as phagocytosis-dependent. Chitin, however, did not elicit any significant IL-1β response nor was it able to inhibit IL-1β release from exogenous stimuli. This indicates that chitosan exposure leans toward a Th1-type response, as IL-18 acts to increase IFN-γ release, which downregulates the Th2 response, though the presence of IL-1β indicates alternatively-activated macrophages.

In an effort to determine how chitin modulates immune response, Wagner et al. (2010) found that “small” chitin (20-40 µm), administered intranasally simultaneously with OVA attenuated antigen-specific CD4+ cell expansion in the lung, but had no effect on Th2-cell polarization. Chitin administered with soluble mannan (which competitively binds to the mannose receptor) to cultured CD4+ T-cells had no effect on the inhibitory effect of chitin, indicating the effect of chitin is mediated by a different cell type. Splenocytes cultured with chitin did not express Fizz1, though Arginase 1 [Arg1], a
protein expressed in human and murine asthmatic lung cells (Zimmerman et al., 2003)] was expressed at low levels. Chitin upregulated B7-H1, an inhibitory ligand on macrophages that binds to PD-1 and blocks the PI3K pathway, which inhibits T-cell proliferation. The upregulation was independent of MyD88, TLR2, TLR3, TLR4, and STAT6.

3.2 Chitin Processing Methods

Studies conducted to date on the immunological response to chitin have used a variety of methods in the preparation of the chitin material without proper characterization of the resulting degree of acetylation. Table 1 categorizes studies by chitin processing method and resulting immune pathway response. Since no clear association is evident, it will be important to replicate some of these preparation methods and determine degree of acetylation.

3.2.1 Sonication Without Autoclave

Shibata et al. (1997a) used purified (C3641) and practical (C7170) grades of chitin preparations from Sigma-Aldrich. To make chitosan, chitin particles were treated with 1 - 2M NaOH at 37°C for 4-6 hours. In the case of C7170 further extraction from the crude chitin was performed using concentrated HCl, neutralized with NaOH and the extracted chitin was then dried. The chitin powder was then suspended in saline and sonicated at 25% output power in a glass vessel with a Branson sonicator (Sonifier 450; Branson Ultrasonics, Danbury, Conn.). Sonication occurred for 5 minutes, with a 5-minute rest on ice and was repeated 5-10 times. This was followed by light centrifugation (50 x G, 10 min). Small particles (1 to 10 µm in diameter) in the supernatant were removed, filtered through a 400/2800 stainless steel mesh, and further centrifuged to collect the
particles at 1,400 x G for 10 min. One milligram of chitin/chitosan particles (1 to 10 µm) contained 2x10^8 to 3x10^8 particles. Particle sizes and size distribution were flow cytometrically determined by comparing chitin particles to several sizes of standardized latex bead (1.1, 10.0, and 47.8 µm in diameter; Polysciences, Warrington, Pa.).

In the study by Bueter et al. (2011) chitosan (Primex, 76% deacetylated) was deacetylated with NaOH, washed with PBS until the pH was neutralized, at which point half of the chitosan was reacetylated with NaHCO_3 and 97% acetic anhydride, and washed with PBS to reach neutral pH. The chitin and chitosan were then passed through a 100µm nylon mesh filter basket (BD Falcon) and treated with 0.1M NaOH at 22°C for 30 minutes, followed by two washes with PBS. The particles were then stored at 4°C in PBS. The particles were sonicated at 30% output for 5 minutes and fractioned off using filtration. Additional sonication occurred for some portions of the study, in which particles were suspended in 200µl PBS (at a concentration of 10 mg/ml) in 1.5ml microcentrifuge tubes. Sonication occurred for 5 minutes using a horn sonicator at 20% output. Degree of acetylation was performed by first digesting the chitin or chitosan to monosaccharides. The DA was determined using epi-UV illumination and a FluorChem HD2 digital imaging system, and quantified using ImageJ pre-sonication.

3.2.2 Sonication With Autoclave

Shibata et al. (1997b, 2000, 2001), Kogiso et al. (2011) and Nishiyama et al. (2006, 2008) prepared chitin particles (1–10 µm diameter) from purified chitin powders (Sigma, St. Louis, MO), as described in Shibata et al., 1997. The particles were suspended in saline at a concentration of 10–16 mg/ml, autoclaved (121°C 30 min), and
stored at 4°C until use. The chitin preparations contained undetectable levels of endotoxin (0.03EU/ml), as determined by the Limulus amebocyte lysate assay (Sigma).

Da Silva et al. (2008, 2009, 2010) used chitin powder (Sigma-Aldrich) that was suspended in sterile PBS (LifeTechnologies) and sonicated at 25% output power three times for 5 min with a Branson sonicator (Sonifier 450, Branson Ultrasonics). The suspension was then filtered with 100, 70, and 40 µm sterile cell strainers (BD Biosciences). Following centrifugation (2800 x g, 10 min), chitin pellets of different sizes (big chitin 70–100 µm, or chitin fragments 40–70 µm) were suspended in the desired volume of sterile PBS and autoclaved for 90 minutes. Afterward, 200µl was run on speed vac in a pre-weighed tube. Dry chitin was then weighed and calculations were made in terms of powder versus initial volume. Particle sizes and size distribution were evaluated by flow cytometry by comparing the chitin to different-sized latex bead controls (0.085, 11.156, and 42.0 µm in diameter; Polysciences). Endotoxin levels were below the limits of detection in a Limulus amebocyte lysate assay (Sigma-Aldrich). All work was performed under a cell culture hood (even the sonication) to avoid any contamination.

3.2.3 Centrifugation or Settling Only

Strong et al. (2002) prepared CMP from pure chitin (Sigma-Aldrich, Poole, UK) by sonication of a suspension of chitin in sterile, endotoxin-free phosphate-buffered saline (PBS). The particles were collected by centrifugation, washed with 20% (v/v) ethanol to ensure sterility and washed five times with sterile PBS to remove soluble chitin. The suspension of CMP was examined by fluorescence-activated cell sorting (FACS) and compared with 1 µm and 20 µm standardized beads (Polysciences, Inc., Warrington, PA, USA). Ninety-eight per cent of the particles were smaller than 20 µm.
and 33% were less than 1 µm in size. Sterility was confirmed by absence of colony-forming units after plating an aliquot on an agar plate and endotoxin was absent.

A chitin microbead preparation (New England Biolabs #S6651S) was utilized in the study by Reese et al. (2007). The preparation was diluted in a 1:4 ratio in sterile PBS, washed three times, and large aggregates were allowed to settle for 2 min. Suspended chitin was collected and diluted 1:4 in PBS. The supernatant, which consisted of a bead size 10-70µm at 10^5 beads/ml, as verified by nylon mesh filtering and microscopy, was collected and administered (Reese et al., 2007).

Wagner et al. (2010) used purified chitin from crab shells (Sigma-Aldrich C9752). The chitin was suspended in PBS at room temperature and allowed to stand for 2 minutes to settle large particles. The supernatant was collected, centrifuged at 14000 rpm, and the pellet was resuspended in PBS, and no LPS contamination was found. The suspensions were stored at 4°C. The particle size was assumed to be 20-30 µm.

### Table 1: Immune Responses Associated With Various Chitin Treatment

<table>
<thead>
<tr>
<th>Chitin Treatment</th>
<th>Th1-type response</th>
<th>Th2-type response</th>
<th>Other response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sonication, no autoclaving</strong></td>
<td>Shibata et al., 1997a</td>
<td></td>
<td>Bueter et al., 2011*</td>
</tr>
<tr>
<td><strong>Sonication, plus autoclaving</strong></td>
<td>Shibata et al., 1997b, 2000, 2001 Nishiyama et al., 2006, 2008 Da Silva et al., 2010</td>
<td>Da Silva et al., 2010</td>
<td>Da Silva et al. 2006, 2009, 2010; Kogiso et al., 2011</td>
</tr>
<tr>
<td><strong>Centrifugation/settling only</strong></td>
<td>Strong et al., 2002</td>
<td>Reese et al., 2007</td>
<td>Wagner et al., 2010</td>
</tr>
</tbody>
</table>
CHAPTER 4

METHODOLOGY

4.1 Objective

The primary goals were to optimize protocol methods to obtain chitin preparations with varying degrees of acetylation and particle size and to then identify the influence of degree of acetylation of chitin on cellular response. This was achieved by using previously-developed protocols and subsequently quantifying the degree of acetylation and particle size of chitin resulting from these methods. This then allowed for the development of a protocol that ensures reproducible creation of chitin with small particle size and either a high, medium or low degree of acetylation. Since previous studies have shown that smaller sized chitin is more immunologically relevant, and it is the most relevant to airborne exposure to the lung, my studies will focus on small sized chitin particles (1-10µm). To determine which steps may be responsible for the drastically differing immunologic responses, High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) will be performed on sonicated samples before autoclave and at 30 and 90 minutes post-autoclave and after various chemical manipulations of degree acetylation. All preparations will be done in triplicate.

4.2 Previous Chitin Preparation Methods

4.2.1 Shibata Method

This technique has been used fairly extensively in chitin preparations (Shibata et al., 1997a, 1997b, 1999, 2001; Da Silva et al., 2008, 2009; Koller et al., 2011; Kogiso et al., 2011, Nishiyama et al., 2006, 2008). Purified chitin from crab shells (Sigma Aldrich
C3641) is suspended in 30 ml saline at a concentration of 10 mg/ml in a 50 ml Falcon tube. The chitin is then sonicated twice, which includes the glass vessel being chilled, put in an ice bath, and sonicated at 25% output (Sonifer 450; Branson Ultrasonics, Danbury, CT) for 5 minutes. The preparation is centrifuged (50 x g, 10 minutes), supernatant collected, filtered through a 400/2800 stainless steel mesh (or 10µm nylon mesh), and further centrifuged (1400 x g, 10 min) to collect the chitin particles. The chitin was then suspended in saline at a concentration of 10 mg/ml and autoclaved at 121°C 15 psi for 30 minutes.

4.2.2 Da Silva Method

See Shibata through filtering step. The desired size fraction was autoclaved for 90 minutes at 121°C, 15 psi.

4.3 Novel Protocol for Preparation of Pure Chitin and Chitosan

4.3.1 Particle size fractionation (1-10 µm)

Purified chitin from shrimp shells (Sigma Aldrich C9752) is suspended in PBS at a concentration of 10 mg/ml in a 50mL conical tube (Eppendorf). The tube of chitin is placed into an ice bath and undergoes sonication at 20% output (Fisher Scientific Sonic Dismembrator) for four periods of five minutes each. The suspension is then centrifuged at 50 x g for 10 minutes. The supernatant is collected and sieved through a stainless steel #400 mesh and collected in a 15 mL conical tube (BD Falcon). The resulting suspension of CMP is then centrifuged at 1400 x g for 10 minutes. The supernatant is removed and the remaining pellet is then resuspended in endotoxin-free PBS at the desired concentration. The Shibata, fully acetylated chitin, and partially deacetylated chitin preparations were dried and reconstituted with PBS to the desired concentration. All
chitosan preps were kept wet, centrifuged three times at 1600 x g for 10 minutes with excess water removed between centrifugation. The mass was determined, approximated that 90% of the mass was water, and reconstituted to the desired concentration.

4.3.2 Deacetylated Chitin

Deacetylation was performed by exposing the chitin microparticle sample to 1.0M NaOH at the concentration of 6 mg chitin/0.08 ml NaOH at 60°C for four hours, then washed with PBS until the sample returned to neutral pH.

4.3.3 Acetylated Chitin

To acetylate, 30mg sonicated and dried chitin was suspended in 200µl 1.0M sodium bicarbonate (NaHCO₃), to which 10 µl of 97% acetic anhydride (C₄H₆O₃) was added. This reaction was performed for 20 minutes at 22°C with periodic mixing. The chitin was then collected by centrifugation at 1400 x g for 15 minutes, and resuspended in fresh sodium bicarbonate and acetic anhydride. The reaction was performed for 20 minutes at 22°C followed by 10 minutes at 100°C. The preparation was centrifuged to collect the chitin particles, which were then washed twice with PBS. Degree of acetylation was assessed using HPAEC-PAD conducted by the CCRC.

4.3.4 Partially-acetylated Chitosan

Crab chitosan (Sigma) was suspended at 10 mg/mL in 30 mL RO/DI water. The size fractionation occurred in the same manner as chitin particles. The chitosan was then treated with acetic anhydride using the same protocol used for acetylingating chitin, without the second acetic anhydride exposure and heating.

4.3.5 Commercial Chitosan
Crab chitosan (Sigma) was suspended at 10 mg/mL in 30 mL RO/DI water. The size fractionation occurred in the same manner as chitin particles. The chitosan was not treated chemically, and was autoclaved for 30 minutes.

4.3.6 Purified Chitosan

Crab chitosan was suspended at 10 mg/mL in 30 mL RO/DI water. The size fractionation occurred in the same manner as chitin particles. The deacetylation procedure followed the same protocol of the deacetylation of chitin particles where the sample was exposed to 1.0M NaOH at 60°C for four hours, then washed with PBS.

4.4 Determination of Degree Acetylation of Samples

Many techniques exist for determination of degree acetylation of chitin and chitosan. Of interest are nuclear magnetic resonance (NMR) techniques and mass spectrometry, particularly $^1$H NMR and High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). After consulting with Dr. Parastoo Azadi of the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, the industry standard and leading analytic tool for determining degree of acetylation chitin is via HPAEC-PAD. Dried samples (0.5-1.0g) were sent out to the CCRC for analysis, and they digested it using a chitinase from T. viridae (C8241, Sigma) and chitosanase from S. griseus (C9830, Sigma) to create analyzable monomers, and finally it was analyzed using a Dionex ICS3000 system equipped with a gradient pump, an electrochemical detector, and an autosampler.

4.5 Particle Size Determination

A fluorescence-activated cell sorting (FACS) analysis using Spherotech polystyrene beads of known sizes and concentration was run to assess size using a BD
LSR II flow cytometer. Spherotech kits used were NPPS-4k (sizes 0.1-0.3µm, 1.0-1.9µm) and PPS-6K (sizes 5.0-5.9µm, 8.0-12.9µm).

4.6 Endotoxin Screen

All replicates were tested for the presence of endotoxin using a GenScript ToxinSensor™ Single Test gel clot endotoxin screen kit at the sensitivity of 0.03 EU/mL used according to manufacturer guidelines. Using Eppendorf BioPur pipette tips, 200µl of sample was transferred into each vial, shaken to mix properly, and incubated at 37°C in a water bath for 60 ± 2 minutes. Each vial was then inverted to check whether gelation had occurred (a +ve reaction indicating >0.03 EU).

4.7 Cell Culture Protocol

4.7.1 THP-1 Cell Line

The THP-1 cell line is a human monocytic cell line that expresses pattern recognition receptors, including TLRs. THP-1 cells also express YKL-40, chitotriosidase (CHIT-1) and chitinase 3-like-1 (CHI3L1) on the cell surface and can actively bind to chitin (Vega et al., 2010; Salah et al., 2012). Drago et al. (2010) have reported that stimulation with various strains of Lactobacillus elicited measurable Th1- and Th2-type cytokine response, including IL-12, IFN-γ, IL-4 and IL-5 while Auwerx (1991) reported that THP-1 cells can also secrete IL-1α, IL-1β, and TNF-α. Additionally, IL-6 and IL-8 have been reported to be elicited in PMA-stimulated cells (Daigneault et al., 2010). The addition of phorbol-12-myristate-13-acetate (PMA) to the THP-1 cells causes the cells to cease proliferating and differentiate into macrophages (Auwerx, 1991) and can be used as a positive control.

4.7.2 Maintenance and Storage of Cell Line
The THP-1 cell line was obtained from ATCC and stored in liquid nitrogen until thawed. As recommended by ATCC, the growth medium used consisted of the recommended ATCC formulation of RPMI-1640 medium (Gibco), 10% triple 0.1μm filtered fetal bovine serum (JR Scientific). This will hereafter be referred to as complete RPMI-1640 growth medium. All cell work was performed under sterile conditions in laminar flow.

Cells were thawed and the contents were transferred using a serological pipette to a sterile 15ml Falcon tube containing 6mL complete RPMI-1640 growth medium. The tube was centrifuged at 200 x g for 5 minutes until a pellet formed. The supernatant was aspirated, and the pellet was resuspended in 6mL fresh complete RPMI growth medium and subsequently placed in a sterile 250ml Falcon tissue culture flask with a 0.2μm microporous hydrophobic membrane filter. The cells were then incubated at 37°C in 5% CO₂. After three days, cell viability was assessed using trypan blue stain, and found to be approximately 95% viable.

Cell concentration was assessed using a hemacytometer and light microscope, and once concentrations reached 2-4 x 10⁸ cells/ml, indicating log-phase growth (Freshney, 1987), the cells were split in a 1:2 ratio. This occurred every 3-4 days. When splitting, cells were aspirated from the Falcon flasks, gently placed into 15ml Falcon tubes, and centrifuged at 200 x g for 5 minutes. The supernatant was aspirated and discarded, the pellet was resuspended in 9ml of fresh complete RPMI-1640 growth medium pre-warmed to 37°C, and transferred into Falcon flasks. The flasks were then immediately placed back into the incubator.
On the third passage, 18 vials of cells were frozen. At the time of freezing, the cells were at a density of \(1 \times 10^6\) cells/ml. The freezing medium consisted of complete RPMI-1640 with 20% glycerol as a cryoprotectant. The cells were transferred to a 15mL Falcon tube and centrifuged at 200 \(\times\) g for 5 minutes. The supernatant was aspirated and discarded, and the cells were resuspended in 6 ml of the freezing medium. The cells were then aliquoted into 1.2 ml sterile cryovials to a volume of 1.0 ml. The vials were then placed in a Mr. Frosty (Nalgene) in an -80°C freezer overnight, and subsequently transferred to liquid nitrogen storage.

### 4.7.3 Administration of Chitin Preparations and Collection of Supernatant

Prior to administration, all preparations were sonicated between 30 seconds to one minute at 70% output using a probe that was sanitized using 70% ethanol. Then, 6.5mL of the desired microparticle preparation, at a concentration of \(1 \times 10^6\) particles/mL, was transferred into the wells of a 24-well plate using Eppendorf BioPur pipette tips. An equal volume of THP-1 cells at a concentration of \(1 \times 10^6\) cells/ml was added to each active well and the time point began. PMA was used as a positive control and sterile endotoxin-free PBS was used as a vehicle control. The plates were then incubated at 37°C with 5% CO₂. Time points for analysis were 6, 12, 18 and 24 hours for each replicate.

After the time points had been reached, the supernatant was collected using Eppendorf BioPur pipette tips and placed into Axygen pyrogen-free 1.6-mL snap-top tubes. The tubes were centrifuged at 900 \(\times\) g for 5 minutes. The supernatant was collected into fresh Axygen pyrogen-free tubes using Eppendorf BioPur pipette tips and then subsequently frozen. The cell material remaining was treated with Qiagen RNPProtect in
accordance with manufacturer guidelines and then frozen. Briefly, 5-10 volumes of RNA Protect were added per volume of cells, and then vortexed to resuspend.

4.8 Analysis

4.8.1 Cytokines

To give as robust an analysis as possible, cytokines from both Th1- and Th2-type responses will be analyzed. A BD Biosciences cytometric bead assay (CBA) flex set and a BD Biosciences flow cytometer (LSR II) was used to analyze the supernatant samples for the presence of IFN-γ, TNF, GM-CSF, IL-1β, IL-6, IL-10, IL-12, IL-2, IL-17a, IL-13, and IL-4. Serial dilutions of all lyophilized standards were reconstituted using assay buffer. The capture beads were vortexed prior to mixing, and were diluted in an appropriate amount of capture bead diluent to prepare enough for 97 tests. The Phycoerythrin (PE) detection reagent was prepared in a similar way. The assay was prepared by adding 50μl of each sample to a 12x75 Falcon round-bottomed tube to which 50μl of the mixed capture beads were added. The tubes were incubated for one hour at room temperature, after which 50μl of the mixed PE detection reagent was added and incubated for an additional two hours. Wash buffer was added to each assay tube, centrifuged at 200 x g, washed with wash buffer, and resuspended for analysis in 300μl wash buffer.

4.8.2 Morphological Changes

Since it is thought that chitin induces innate immune responses very early in the immune response, exposure of monocytes to chitin could lead to differentiation into macrophages. Differentiation of THP-1 cells is quite evident under the microscope. The primary visual cue is that the cells transform from being in suspension to being adherent
Markers of macrophage polarization (M1 classically-activated or M2 alternatively activated) would give further insight as to potential polarization toward parasitic pathogen response (Martinez et al., 2009). Macrophage polarization can be determined using real-time PCR and several markers: TNF-α and CXCL11 for M1 macrophages, and CCL18 and CD206 for M2 macrophages.

4.8.3 Statistical Methods and Data Analysis

All statistics were performed using StatPlus (AnalystSoft), XLSTAT (Addinsoft) and Microsoft Excel 2011 for Mac. To analyze whether autoclaving and sonication had a significant effect on degree of acetylation as compared to sonication alone, two-tailed t-tests were used with a p=0.05. To determine whether source species influenced degree of acetylation post-sonication, a two-tailed t-test was used with a p=0.05. To assess statistical significance between chitin and chitosan preparations, pairwise comparisons were made using Tukey’s test.

To analyze flow cytometry data, FCAP Array 3.0 (BD Biosciences) was used to create a standard curve and interpolate the presence and concentration of cytokines.
CHAPTER 5

RESULTS

5.1 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was used to quantify the number of moles of both N-acetyl-D-glucosamine (GlcNAc) and glucosamine (GlcN). As shown in Table 4, the acetylated samples (A1, A3, A4) had 3.12, 2.20 and 2.88 nMoles of GlcNAc, respectively and 0.00 nMoles of GlcN, with a degree of acetylation of 100.00%. The Da Silva samples D1, D3 and D4 remained fairly highly acetylated (degree acetylation between 97.55 and 100.05 with mean acetylation 98.72 ± 1.26) as seen in Table 5. Chitin processed using the Shibata method (Table 6) yielded an average degree of acetylation of 97.72 ± 0.59. Deacetylating chitin resulted in an average degree of acetylation of 89.33 ± 1.20 (Table 7). The degree of acetylation of two differing sources of chitin (shrimp and crab) was compared. The sonicated and otherwise untreated shrimp chitin averaged 96.42 ± 0.43 acetylated, and the crab chitin subjected to the same treatment averaged 96.51 ± 0.79 acetylated (Table 8).

Samples of chitosan that underwent the partial-acetylation procedure (acetylated chitosan 1, 5, and 6) had degrees of acetylation between 79.00% and 95.3% with a mean acetylation of 86.00 ± 8.39 (Table 9). Chitosan samples that were sonicated and autoclaved for 30 minutes (Commercial Chitosan samples 7, 8, and 9) had a degree acetylation of 56.3, 56.2, and 70.6, respectively. The average degree acetylation was 61.03% ± 8.29 (Table 10). Chitosan samples that were purified (Purified Chitosan 2, 3,
and 4) had degrees of acetylation ranging from 9.5 to 20.7, with a mean of $14.97 \pm 5.60$ (Table 11).

I compared means between the sonicated crab sample and the Shibata preparation (which includes sonication and a 30 minute autoclave treatment) with the null hypothesis being that there is no difference in degree acetylation between samples that were sonicated alone versus samples that were sonicated and then autoclaved. A two-tailed t-test was used and a p-level of 0.13982 (i.e. $p>0.05$) was found. For the Da Silva method (sonication and a 90 minute autoclave treatment), the p-level was 0.12076 (Table 12). Similarly, a two-tailed t-test was used to determine whether there was a significant difference in the degree of acetylation between chitin sources and a p-value of 0.89524 was obtained (Table 12). These results indicate that the null hypothesis cannot be rejected, therefore autoclaving does not induce a statistically significant change in degree of acetylation, nor does the source from which the chitin is derived. I used a one-way analysis of variance (ANOVA) to compare the means of all of the groups of degree acetylation with their respective within-group variability, and a $p<<0.05$ indicates that the groups differ significantly in their degree of acetylation (see table 15).

5.2 Endotoxin Screen

Two days after the chitin samples were used in cell culture experiments, all samples were tested for the presence of $>0.03$ EU/mL of endotoxin. All samples were stored at 4°C until testing. Sample CC7 was the only sample to test positive for endotoxin. All other samples were below 0.03 EU/ml.

5.3 Size Determination
The Spherotech beads were used to gate the flow cytometer on 1.34μm, 5.1μm, and 10.1μm. The histogram display indicated that most particles were below 1.34μm (see Appendix A).

5.4 Cytokine Bead Array

Due to technical difficulties in establishing a well-fit standard curve, standard curves had to be fit for each cytokine individually. The data was still somewhat limited and several cytokines (IL-4, IL-12p70, IL-13, IFN-γ, GMCSF, IL-2, and IL-17a) were extrapolated to concentrations well below the standard curve or were not able to be calculated. These were omitted from analysis and IL-1β, TNF, IL-10, and IL-6 were used. Cytokine profiles had a large variability between preparations and standard curves were truncated (see Appendix B), therefore cytokines present at very low concentrations may not have been accurately reported.

The CBA assay indicated that IL-1β was present in the 61% acetylated commercial chitosan sample at 6 hours (mean concentration 34.4 pg/mL ± 55.0), 12 hours (mean concentration 34.4 pg/mL ± 55.0), 18 hours (mean concentration 170.7 pg/mL ± 84.5) and 24 hours (mean concentration 95.2 pg/mL ± 86.1). IL-1β was also present in the 97.7% acetylated Shibata preparations at 12 hours (mean concentration 16.7 pg/mL ± 12.1), 18 hours (mean concentration 16.9 pg/mL ± 24.7) and 24 hours (mean concentration 9.6 pg/mL ± 12.1) as well as the 15% acetylated purified chitosan preparations at 18 hours (mean concentration 58.0 pg/mL ± 49.4) and 24 hours (mean concentration 62.8 pg/mL ± 70.4) (see Table 13, Figures 3-7).

TNF was present in all samples at 6 hours, and all samples except partially deacetylated chitin (89.5% acetylated) at 12 and 24 hours. At 18 hours, it was again
present in all samples except purified chitosan. At 24 hours, it was present in the Shibata prep (mean concentration 47.5 pg/mL ± 34.5) and commercial chitosan (mean concentration 69.0 pg/mL ± 71.8) (see Table 14). IL-10 was found only in the Shibata method at 12 hours (mean concentration 9.7 pg/mL ± 14.0) (see Table 15). IL-6 was present in deacetylated chitin at 6 hours (mean concentration 14.6 pg/mL ± 6.3) and in the Shibata method at 24 hours (mean concentration 14.6 pg/mL ± 6.3). This indicates a shift from an early TNF-dominated response to an IL-1β response. IL-10 appeared at the 12-hour time point, after which IL-1β is present at higher concentrations (see Tables 15-18, Figures 3-7).

The degree of acetylation seems to affect cytokine production/presence at later time points, particularly for IL-1β. Mid-acetylated chitin/chitosan seems to have the greatest IL-1β elicitation at all time-points, and would seem to cause a time-dependent Th2-polarizing tip from an initially Th1-polarizing response, as Th17 cytokines (IL-1β and IL-6) are present and Th1 cytokines (TNF) become less predominant. The presence of TNF at the earliest time point indicates an initial Th1-polarizing response that is either reduced or eliminated over time. Because IL-10 inhibits Th1 polarization, the switch between the two may be occurring around the 12-hour time point (Figures 3-7; Tables 12-17). The presence of endotoxin does not seem to have greatly affected the cytokine elicitation within the CC7 sample, and it appears to have much lower concentrations of cytokines than both CC8 and CC9 (Figures 10-12). Although endotoxin was present, the exact value above 0.03 EU/mL is unknown, however, IFN-γ would have been expected as a cytokine elicited in a significant contamination (Shibata et al., 1997a; Duramad et al.,
Both the vehicle control and positive control were negative for cytokine elicitation (Figures 8 and 9).

5.5 Morphological Changes

Little to no visual change was observed in cells at the 6-hour time point. At the 12 and 18-hour time point, cells exposed to acetylated chitin (100% DA), the Shibata method (97.7% DA), and commercial chitosan (61.0% DA) appeared slightly granular and began to look misshapen, while all others appeared to be mostly hyaline and round. Little was observed between 18 and 24-hours. From visual morphological changes, it would appear that the THP-1 monocytes did not begin to differentiate until 12-18 hours. It does not appear that the monocytes fully differentiated even at the 24-hour point.

Table 2: Summary, Degree Acetylation of Chitin Preparations (HPAEC-PAD)

<table>
<thead>
<tr>
<th>% Acetylation</th>
<th>Acetylated chitin</th>
<th>Deacetylated chitin</th>
<th>Shibata</th>
<th>Da Silva</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.00 ± 0</td>
<td>89.72 ± 1.26</td>
<td>97.72 ± 0.59</td>
<td>98.33 ± 1.20</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Summary, Degree Acetylation of Chitosan Preparations (HPAEC-PAD)

<table>
<thead>
<tr>
<th>% Acetylation</th>
<th>Acetylated chitosan</th>
<th>Commercial chitosan</th>
<th>Purified chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>86.00 ± 8.39</td>
<td>61.03 ± 8.28</td>
<td>14.97 ± 5.60</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Degree Acetylation of Acetylated Chitin (HPAEC-PAD)

<table>
<thead>
<tr>
<th>GlcN</th>
<th>GlcNAc</th>
<th>GlcNAC:GlcN</th>
<th>°DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>3.12</td>
<td>2.20</td>
<td>100.00 ± 0</td>
</tr>
</tbody>
</table>

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Table 5: Degree Acetylation of Da Silva Method (HPAEC-PAD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>D1</th>
<th>D3</th>
<th>D4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.09</td>
<td>0.04</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>3.77</td>
<td>2.47</td>
<td>3.80</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>39.78</td>
<td>68.15</td>
<td>100.05</td>
<td>98.72 ± 1.26</td>
</tr>
<tr>
<td>^°DA</td>
<td>97.55</td>
<td>98.55</td>
<td>100.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Degree Acetylation of Shibata Method (HPAEC-PAD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>S1</th>
<th>S3</th>
<th>S4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>3.31</td>
<td>3.10</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>49.83</td>
<td>50.99</td>
<td>32.80</td>
<td></td>
</tr>
<tr>
<td>^°DA</td>
<td>98.03</td>
<td>98.08</td>
<td>97.04</td>
<td>97.72 ± 0.59</td>
</tr>
</tbody>
</table>

Table 7: Degree Acetylation of Deacetylated Chitin (HPAEC-PAD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Da1</th>
<th>Da3</th>
<th>Da4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.21</td>
<td>0.06</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>1.76</td>
<td>0.60</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>8.56</td>
<td>9.43</td>
<td>7.36</td>
<td></td>
</tr>
<tr>
<td>^°DA</td>
<td>89.54</td>
<td>90.41</td>
<td>88.03</td>
<td>89.33 ± 1.20</td>
</tr>
</tbody>
</table>

Table 8: Degree Acetylation of Sonicated Shrimp and Crab Chitin (HPAEC-PAD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shrimp1</th>
<th>Shrimp2</th>
<th>Crab3</th>
<th>Crab4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.10</td>
<td>0.15</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>2.91</td>
<td>3.76</td>
<td>3.97</td>
<td>4.52</td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>29.52</td>
<td>24.67</td>
<td>33.12</td>
<td>23.72</td>
</tr>
<tr>
<td>^°DA</td>
<td>96.72</td>
<td>96.11</td>
<td>97.07</td>
<td>95.95</td>
</tr>
</tbody>
</table>

Table 9: Degree Acetylation of Acetylated Chitosan (HPAEC-PAD)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetylated Chitosan1</th>
<th>Acetylated Chitosan 5</th>
<th>Acetylated Chitosan 6</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.12</td>
<td>0.48</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>2.4</td>
<td>2.47</td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>20.4</td>
<td>5.1</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>95.3</td>
<td>83.7</td>
<td>79</td>
<td>86.00 ± 8.39</td>
</tr>
</tbody>
</table>
Table 10: Degree Acetylation of Commercial Chitosan (HPAEC-PAD)

<table>
<thead>
<tr>
<th></th>
<th>Commercial Chitosan 7</th>
<th>Commercial Chitosan 8</th>
<th>Commercial Chitosan 9</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>1.11</td>
<td>1.07</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>1.43</td>
<td>1.37</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>1.3</td>
<td>1.3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>56.3</td>
<td>56.2</td>
<td>70.6</td>
<td>61.03 ± 8.28</td>
</tr>
</tbody>
</table>

Table 11: Degree Acetylation of Purified Chitosan (HPAEC-PAD)

<table>
<thead>
<tr>
<th></th>
<th>Purified Chitosan 2</th>
<th>Purified Chitosan 3</th>
<th>Purified Chitosan 4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>1.22</td>
<td>1.7</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>0.32</td>
<td>0.18</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>GlcNAC:GlcN</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>20.7</td>
<td>9.5</td>
<td>14.7</td>
<td>14.97 ± 5.60</td>
</tr>
</tbody>
</table>

Table 12: Sonicated Autoclaved Preparations and Species Statistics

<table>
<thead>
<tr>
<th></th>
<th>90 Minute Autoclave Compared to Sonication Alone</th>
<th>30 90 Minute Autoclave Compared to Sonication Alone</th>
<th>Crab Chitin Compared to Shrimp Chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees Freedom</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Test Statistic</td>
<td>2.14956</td>
<td>1.99633</td>
<td>0.14898</td>
</tr>
<tr>
<td>p-level</td>
<td>0.12076</td>
<td>0.13982</td>
<td>0.89524</td>
</tr>
<tr>
<td>t critical value (5%)</td>
<td>3.18245</td>
<td>3.18245</td>
<td>4.30265</td>
</tr>
<tr>
<td>Pooled variance</td>
<td>1.26461</td>
<td>0.43842</td>
<td>0.40663</td>
</tr>
</tbody>
</table>
### Table 13: One-Way Analysis of Variance of Degree Acetylation Among Preparations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample size</th>
<th>Sum</th>
<th>Mean</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylated</td>
<td>3</td>
<td>300.</td>
<td>100.</td>
<td>0.E+0</td>
</tr>
<tr>
<td>Shibata</td>
<td>3</td>
<td>293.15</td>
<td>97.7167</td>
<td>0.34403</td>
</tr>
<tr>
<td>Deacetyl</td>
<td>3</td>
<td>267.98</td>
<td>89.3267</td>
<td>1.45023</td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>183.1</td>
<td>61.0333</td>
<td>68.64333</td>
</tr>
<tr>
<td>PC</td>
<td>3</td>
<td>44.9</td>
<td>14.9667</td>
<td>31.41333</td>
</tr>
</tbody>
</table>

### ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p-level</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>15,350.33</td>
<td>4</td>
<td>3,837.58</td>
<td>188.39</td>
<td>&lt;&lt;0.05</td>
<td>4.81564</td>
</tr>
<tr>
<td>Within Groups</td>
<td>203.70</td>
<td>10</td>
<td>20.37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 14: Cytokine Bead Array Assay Limits of Detection

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Lower Limit of Detection (pg/mL)</th>
<th>Upper Limit of Detection (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5.30</td>
<td>301.07</td>
</tr>
<tr>
<td>TNF</td>
<td>55.06</td>
<td>294.90</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.22</td>
<td>295.62</td>
</tr>
<tr>
<td>IL-6</td>
<td>21.91</td>
<td>289.78</td>
</tr>
<tr>
<td>IL-17a</td>
<td>104.46</td>
<td>242.43</td>
</tr>
<tr>
<td>IL-4</td>
<td>66.89</td>
<td>283.05</td>
</tr>
<tr>
<td>IL-2</td>
<td>5.50</td>
<td>285.08</td>
</tr>
<tr>
<td>IL-13</td>
<td>17.41</td>
<td>314.76</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>5.82</td>
<td>310.30</td>
</tr>
<tr>
<td>IFN-Y</td>
<td>47.19</td>
<td>303.37</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>8.89</td>
<td>269.48</td>
</tr>
</tbody>
</table>

Table 15: Mean (and standard deviation) cytokine concentrations of IL-1β (pg/mL) by mean degree of acetylation of various chitin/chitosan preparations

<table>
<thead>
<tr>
<th></th>
<th>100% acetylated</th>
<th>97.7% acetylated</th>
<th>89.3% acetylated</th>
<th>61% acetylated</th>
<th>15% acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hour</td>
<td>2.6 (0.00)</td>
<td>2.6 (0.00)</td>
<td>2.6 (0.00)</td>
<td>34.4 (54.95)</td>
<td>2.6 (0.00)</td>
</tr>
<tr>
<td>12 hour</td>
<td>2.6 (0.00)</td>
<td>16.7 (12.1)</td>
<td>2.6 (0.00)</td>
<td>34.4 (55.0)</td>
<td>2.6 (0.00)</td>
</tr>
<tr>
<td>18 hour</td>
<td>2.6 (0.00)</td>
<td>16.9 (24.7)</td>
<td>2.65 (0.00)</td>
<td>170.7 (84.5)</td>
<td>58.0 (49.4)</td>
</tr>
<tr>
<td>24 hour</td>
<td>2.6 (0.00)</td>
<td>9.6 (12.1)</td>
<td>9.6 (12.2)</td>
<td>95.2 (86.1)</td>
<td>62.8 (70.4)</td>
</tr>
</tbody>
</table>

40
Table 16: Mean (and standard deviation) cytokine concentrations of TNF (pg/mL) by mean degree of acetylation of various chitin/chitosan preparations

<table>
<thead>
<tr>
<th></th>
<th>100% acetylated</th>
<th>97.7% acetylated</th>
<th>89.3% acetylated</th>
<th>61% acetylated</th>
<th>15% acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hour</td>
<td>80.2 (91.2)</td>
<td>152.1 (215.8)</td>
<td>82.0 (91.2)</td>
<td>156.5 (119.8)</td>
<td>67.4 (34.5)</td>
</tr>
<tr>
<td>12 hour</td>
<td>58.9 (52.9)</td>
<td>125.8 (122.2)</td>
<td>27.5 (0.0)</td>
<td>120.1 (56.8)</td>
<td>47.5 (34.5)</td>
</tr>
<tr>
<td>18 hour</td>
<td>47.5 (34.5)</td>
<td>78.0 (46.6)</td>
<td>47.5 (34.5)</td>
<td>47.5 (34.5)</td>
<td>27.5 (0.0)</td>
</tr>
<tr>
<td>24 hour</td>
<td>327.5 (0.0)</td>
<td>47.5 (34.5)</td>
<td>27.5 (0.0)</td>
<td>69.0 (71.8)</td>
<td>27.5 (0.0)</td>
</tr>
</tbody>
</table>

Table 17: Mean (and standard deviation) cytokine concentrations of IL-10 (pg/mL) by mean degree of acetylation of various chitin/chitosan preparations

<table>
<thead>
<tr>
<th></th>
<th>100% acetylated</th>
<th>97.7% acetylated</th>
<th>89.3% acetylated</th>
<th>61% acetylated</th>
<th>15% acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hour</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
</tr>
<tr>
<td>12 hour</td>
<td>1.6 (0.00)</td>
<td>9.7 (14.0)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
</tr>
<tr>
<td>18 hour</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
</tr>
<tr>
<td>24 hour</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
<td>1.6 (0.00)</td>
</tr>
</tbody>
</table>

Table 18: Mean (and standard deviation) cytokine concentrations of IL-6 (pg/mL) by mean degree of acetylation of various chitin/chitosan preparations

<table>
<thead>
<tr>
<th></th>
<th>100% acetylated</th>
<th>97.7% acetylated</th>
<th>89.3% acetylated</th>
<th>61% acetylated</th>
<th>15% acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hour</td>
<td>16.1 (89)</td>
<td>11.0 (0.00)</td>
<td>14.6 (6.3)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
</tr>
<tr>
<td>12 hour</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
</tr>
<tr>
<td>18 hour</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
</tr>
<tr>
<td>24 hour</td>
<td>11.0 (0.00)</td>
<td>14.6 (6.3)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
<td>11.0 (0.00)</td>
</tr>
</tbody>
</table>

Figure 1: Average Degree Acetylation of Chitin and Chitosan Microparticle Preparations Analyzed Using HPAEC-PAD ± Standard Error, Bars With Different Superscripts Are Significantly Different (Tukey’s Test, p<0.05)
Figure 2: Degree Acetylation of Applied Chitin and Chitosan Microparticle Preparations Analyzed Using HPAEC-PAD

Figure 3: Cytokine Elicitation of THP-1 Cells by Fully Acetylated Chitin (DA 100%) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry, With Standard Error Bars
Figure 4: Cytokine Elicitation of THP-1 Cells by Chitin Prepared Using the Shibata Method (DA 97.7%) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry, With Standard Error Bars

Figure 5: Cytokine Elicitation of THP-1 Cells by Partially Deacetylated Chitin (DA 89.3%) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry, With Standard Error Bars
Figure 6: Cytokine Elicitation of THP-1 Cells by Commercial Chitosan (DA 61%) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry, With Standard Error Bars

Figure 7: Cytokine Elicitation of THP-1 Cells by Purified Chitosan (DA 15%) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry With Standard Error Bars
Figure 8: Cytokine Elicitation of THP-1 Cells by Vehicle Control (Phosphate Buffered Saline) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry

Figure 9: Cytokine Elicitation of THP-1 Cells by Positive Control (PMA) Over 24 Hours Analyzed Using a Cytokine Bead Array and Flow Cytometry
Figure 10: Cytokine Elicitation of THP-1 Cells by Commercial Chitosan 7 (DA 61%) Analyzed Using a Cytokine Bead Array and Flow Cytometry

Figure 11: Cytokine Elicitation of THP-1 Cells by Commercial Chitosan 8 (DA 61%) Analyzed Using a Cytokine Bead Array and Flow Cytometry
Figure 12: Cytokine Elicitation of THP-1 Cells by Commercial Chitosan 9 (DA 61%) Analyzed Using a Cytokine Bead Array and Flow Cytometry
CHAPTER 6

DISCUSSION

The primary hypothesis of this study, that chitin particles with a high degree of acetylation will elicit cytokines associated with a Th2-type immune response, is not currently supported. By replicating the processing methods and better characterizing the chitin preps with regard to degree of acetylation, I attempted to fill in gaps in other studies and gain a clearer picture of immunological response to chitin exposure.

My study indicates that autoclaving and sonication processing steps do not have a significant influence on degree of acetylation as suggested by various studies (No et al., 2000; Muzzarelli, 2005; Sjoholm et al., 2000; Takahashi et al., 1994). Autoclaving does not have a statistically significant effect on degree acetylation when comparing the mean degrees of acetylation between autoclaved and un-autoclaved sonicated chitin samples (p>0.05). Sonication also did not reduce DA to the point of causing chitin to turn to chitosan, as evidenced by the average DA of the Shibata prep and the Da Silva prep being 97.72 ± 0.59 and 98.33 ± 1.20, respectively, which included sonication steps. The physical treatments used in the Shibata and Da Silva methodology do not cause a drastic deacetylation, and the particles produced are chitin. There is also not a statistically significant difference in DA between chitin sources, as comparing the DA of crab and shrimp chitin resulted in a p-value of 0.12076. Chitosan was markedly different to work with and sonication may have converted some of the chitosan into soluble monomers or dimers as there was a large reduction in yield of chitosan particles during processing.

Analysis by flow cytometry indicated that the preparations used in this study, although replicating methodology of previous studies, had particle sizes below the 1.0µm
range that was frequently found in literature to be the lowest size limit tested for immunogenicity of chitin particles [Shibata et al. (1997a, 1997b, 2000, 2001); Strong and Clark (2002); Da Silva et al. (2008a); Kogiso et al. (2011)]. Overall particle size was well below the anticipated 1-10µm size distribution. This may have been due to oversonation, possibly resulting from use of a different size or shape of sonication vessel. Because ultrasonic energy is produced from the tip of the sonicator and directed downward, a large or wide vessel would result in less mixing and an overall decrease in the amount of sample being subjected to the treatment and subsequently produce a larger particle size. These results indicate that the parameters of sonication (time, intensity, and vessel size and shape) can significantly alter the distribution of chitin particle size and can easily skew it toward a much smaller size. The development of standardized protocols for the preparation of chitin particles in future studies must take this into account in achieving chitin particles of differing size ranges.

My study makes a unique contribution by establishing that immunostimulatory effects can occur from particles at or <1µm in size. This is relevant to asthma and allergy as these particles are well within the respirable range (Brown et al., 2013). A 0.5µm particle can take upwards of 41 hours to settle five feet and in a turbulent environment may never settle (Baron, 2010). Chitin is present in several components of house dust including fungal cell walls, insect exoskeletons, and dust mite droppings (Nathan et al., 2009; Van Dyken et al., 2011), therefore fragments of chitin may remain suspended indefinitely, and the likelihood of exposure to particles containing chitin within this size range is quite likely.
Although our cytokine analysis had a high variability, particles with a high degree of acetylation did not seem to elicit a Th2-type effector cytokine profile. The DA does seem to affect cytokine production/presence at later time points (12- through 24-hours), though I found that it is the mid-acetylated chitin/chitosan preparations that elicited the greatest cytokine response, particularly for IL-1β. Bueter et al. (2011) found that chitin microparticles elicited IL-1β at 6 hours in bone marrow-derived macrophages at concentrations of around 25 pg/ml and chitosan elicited concentrations around 75 pg/ml, whereas I found that at early time points, IL-1β concentrations were quite a bit lower in all samples. IL-1β production at later time points (18 and 24 hours) was at higher concentrations with the commercial chitosan (61% acetylated), which indicates that the THP-1 cell line has a greater response to mid-acetylated chitin with an initial Th1-like response that changes to a Th17-dominated response with the production of IL-1β (Acosta-Rodriguez et al., 2007).

My results also indicate a change from a TNF-dominated response to an IL-1β-dominated response, with IL-10 (a Th1 inhibitor) occurring at the 12-hour time point. The earliest time point is similar to results seen by Shibata (1997a) and Da Silva et al. (2009) with TNF production being dominant, however this changed over time. Da Silva et al. (2009) found that both TNF and IL-10 response was greatest at 6 and 18 hours, with peaks of 1500 pg/mL and 150 pg/mL, respectively, decreasing over 24 hours. This pattern was true for TNF release associated with all preparations except the fully acetylated (DA 100%), though the commercial chitosan (DA 61%) had the highest TNF elicitation (156 pg/mL) was almost a full order of magnitude less than what Da Silva et al. (2009) reported from peritoneal macrophages. I found that IL-10 peaked in chitin
samples (DA 100% and 97.7%) at 24 hours and 12 hours, respectively, also at a full magnitude lower than the response observed in Da Silva et al. (2009). Taken together, these results show that the magnitude of the initial Th1-type effector response of THP-1 monocytic cells in response to chitin microparticle stimulation is of a much smaller magnitude than those in previous studies, and may be indicative of what happens very early on in the immune response associated with exposure to chitin and chitosan. This is in agreement with the observations made by Lee et al. (2012) who found that monocytes exposed to Active Hexose Correlated Compound, a natural extract composed of oligosaccharides (including α- and β-glucans) produced IL-1β at 18 hours post-exposure.

The results also indicate that in contrast to Bueter et al. (2011), the sample with the lowest DA was not the most immunologically active, rather it was the sample averaging 61% DA (Figures 3-7). While further work is needed to reduce variability in the cytokine analysis, we have remaining cell culture supernatant and additional cell culture response replicates such that this can be further refined.

My study has created a dose-response curve of DA as compared to cytokine elicitation using preparations that were replicated as closely as could be achieved and then characterized for their DA, which has never been previously attempted. The results initially were similar to previous studies, though showed a tip toward the start of an IL-1β-dominated Th17 effector response. This is likely a result of both degree acetylation and particle size, and cell type. This study also revealed that monocytes, the precursors to macrophages, are both activated by and elicit cytokines in response to chitin particles <1μm, indicating that chitin and chitosan fragments stimulate the innate immune system.
to recruit antigen presenting cells and activate T lymphocytes in a Th17 manner, similar to the results seen in Lee et al. (2012).

Although this study addressed only the degree of acetylation of samples, there are several other characteristics of chitin and chitosan that may also influence its behavior, including protein and mineral content as well as its association with polysaccharides. Shibata et al. (1997b) found that chitin binds through the mannose receptor, and because N-glycans can have high concentrations of mannose (Kang et al., 2005), competitive binding may be occurring. Fully characterizing the preparations with polysaccharide composition as well as degree acetylation may provide fuller insight into the effects of various processing methods of chitin and chitosan as well as the immunologic response resulting from them. This may ultimately shed light onto some of the discrepancies between studies if there is a mechanism that is currently unaccounted for in the chitin and chitosan preparations currently being employed by researchers.

Future studies should also include the use of different cell lines, particularly the analysis of cytokine production by macrophages exposed to chitin and chitosan, as they are more likely to be exposed to chitin and chitosan in the lung. Studies involving cell co-culture with an antigen presenting cell (such as a dendritic cell) and a CD4+ T-cell population would give a much better indication as to any T-helper cell polarization that results from exposure to chitin and chitosan.
APPENDIX A

SIZE DISTRIBUTION HISTOGRAMS

Histogram 2: Acetylated Chitin 1

Histogram 3: Acetylated Chitin 3

Histogram 4: Acetylated Chitin 4

Histogram 5: Shibata Method 1

Histogram 6: Shibata Method 3

Histogram 7: Shibata Method 4

Histogram 8: Partially Deacetylated Chitin 1
Histogram 9: Partially Deacetylated Chitin 3

Histogram 10: Partially Deacetylated Chitin 4

Histogram 11: Commercial Chitosan 7

Histogram 12: Commercial Chitosan 8

Histogram 13: Commercial Chitosan 9

Histogram 14: Purified Chitosan 2

Histogram 15: Purified Chitosan 3

Histogram 16: Purified Chitosan 4
APPENDIX B

STANDARD CURVES FOR CYTOKINE ANALYSIS

**Standard Curve: 1 TNF**

**Standard Curve: 2 IL-1β**

**Standard Curve: 3 IL-10**

**Standard Curve: 4 IL-6**

**Standard Curve: 5 IFN-γ**

**Standard Curve: 6 GM-CSF**


Brown, James; Gordon, Terry; Price, Owen; Asgharian, Bahman. 2013. Thoracic and respirable particle definitions for human health risk assessment. *Particle and Fibre Toxicology* 10 (1).


Nathan, Amy T., Elizabeth A. Peterson, Jamila Chakir, and Marsha Wills-Karp. 2009. Innate immune responses of airway epithelium to house dust mite are mediated through β-glucan–dependent pathways. *Journal of Allergy and Clinical Immunology* *Journal of Allergy and Clinical Immunology* 123 (3): 612-8.


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