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Disparate Activation of the Inflammasome by Chitin and Chitosan: A Dissertation

Chelsea L. Bueter
University of Massachusetts Medical School

DISPARATE ACTIVATION OF THE INFLAMMASOME BY CHITIN AND CHITOSAN

A Dissertation Presented

By

Chelsea Linnay Bueter

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The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Disseratation

Stuart M. Levitz, Thesis Advisor

Beth A. McCormick, Member of Committee

Christopher M. Sassetti, Member of Committee

Jatin M. Vyas, Member of Committee

Jennifer P. Wang, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Katherine A. Fitzgerald, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

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Abstract

Chitin is an abundant polysaccharide found in fungal cell walls, crustacean shells, and insect exoskeletons. The immunological properties of both chitin and its deacetylated derivative chitosan are of relevance due to frequent natural exposure and their increasing use in translational applications. Depending on the preparation studied and the endpoint measured, these compounds have been reported to induce allergic responses, inflammatory responses, or no response at all. Highly purified chitosan and chitin were prepared and the capacity of these glycans to stimulate the release of the inflammasome-associated cytokine IL-1β was examined. Chitosan was shown to be a potent inflammasome activator in mouse bone marrow macrophages, macrophages polarized towards a M1 or M2 phenotype, dendritic cells, peritoneal cells, and human PBMCs. Acetylation of the chitosan to chitin resulted in a near total loss of IL-1β activity in all cell types tested. The size of the chitosan particles played an important role, with small particles eliciting the greatest activity. An inverse relationship between size and stimulatory activity was demonstrated using chitosan passed through size exclusion filters as well as with chitosan-coated beads of defined size. Partial digestion of chitosan with pepsin resulted in a larger fraction of small phagocytosable particles and more potent inflammasome activity. Inhibition of phagocytosis with cytochalasin D abolished the IL-1β stimulatory activity of chitosan, offering an explanation for why the largest particles were nearly devoid of activity. Thus, the deacetylated polysaccharide chitosan potently activates the NLRP3 inflammasome in a phagocytosis-dependent manner. The reason for

chitin's inability to elicit IL-1β is unknown, but it does not appear to be due to active inhibition of the inflammasome and while chitin appears to be more readily digested by macrophage cell lysates, it does not occur at a rate which would likely impact inflammasome activation. There are three proposed mechanisms for NLRP3 inflammasome activation: K^+ efflux, ROS, and lysosomal destabilization. The contributions of these mechanisms were tested and it was revealed that each of these pathways participated in optimal NLRP3 inflammasome activation by chitosan. Finally, the laminin receptor was evaluated as a potential chitin receptor. These studies provide insight into the activating properties of chitin and chitosan and highlight the importance of matching particle size and degree of acetylation to the level of activity desired for translational applications.

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List of Abbreviations

AIM2	Absent in melanoma 2
AMCase	Acidic mammalian chitinase
ASC	Apoptosis-associated speck-like protein containing a CARD
BMMΦ	Bone marrow-derived macrophages
CARD	Caspase recruitment domain
CEBiP	Chitin elicitor-binding proteins
CFTR	Cystic fibrosis transmembrane conductance regulator Cl^- channel
DAMP	Danger associated molecular pattern
DC	Dendritic cell
FIBCD1	Fibrinogen C domain containing 1
IFI16	Interferon γ-inducible protein 16
IFN	Interferon
IL-1β	Interleukin-1β
IL-1R	Interleukin-1 receptor
IL-1RA	Interleukin-1 receptor agonist
IPAF	Interleukin-converting enzyme protease-activating factor
GBP5	Guanylate binding protein 5
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte macrophage colony-stimulating factor

KO	Knock-out
LPS	Lipopolysaccharide
LysM	Lysine motif
MAC-1	Macrophage-1 antigen
MAVS	Mitochondrial antiviral signaling protein
M-CSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
MSU	Monosodium urate
NADPH	Nicotinamide adenine dinucleotide phosphate
NAIP5	NLR family, apoptosis inhibitory protein 5
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding domain and leucine-rich repeat containing
NLRC4	NLR family CARD domain-containing protein 4
NLRP	NLR family, pyrin domain containing
P2X7	Purinoceptor 7
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
POP	PAAD/PYRIN-only protein
PYD	Pyrin domain
ROS	Reactive oxygen species

SLO	Streptolysin O
TLC	Thin-layer chromatography
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TXN	Thioredoxin
TXNIP	Thioredoxin-interacting protein
YGP	Yeast glucan particle

Preface to Chapter I

A portion of this chapter, including Figure 1.1, has been published in PLoS Pathogens

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Chapter I: Introduction

Chitin and Chitosan

Chitin, a β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc) and its deacetylated derivative chitosan, a β-(1,4)-linked polymer of glucosamine (GlcN), are two predominant, naturally occurring polysaccharides (Figure 1.1). Although not present in vertebrates, chitin, as a major component in crustacean shells and insect exoskeletons, is the second most abundant natural polysaccharide after cellulose (Boot et al., 2001; Boot et al., 1998; Neville et al., 1976). It is also an essential component of fungi and some parasites, including helminths and protozoa (Araujo et al., 1993; Debono and Gordee, 1994; Fuhrman and Piessens, 1985; Shahabuddin and Kaslow, 1994). Chitosan is not as prevalent naturally, although some medically important fungi, particularly *Cryptococcus neoformans* and members of the Zygomycetes, contain chitin deacetylases which promote conversion of chitin to chitosan (Banks et al., 2005; Bartnicki-Garcia, 1968).

Chitin is a key structural component of the fungal cell wall (Figure 1.2). In fungi, chitin is produced by chitin synthases, membrane integral glycosyltransferases that catalyzes the chitin polymerization reaction and then extrudes chitin (Merzendorfer, 2011). Without chitin, fungal cells are nonviable as it is required in both yeast and hyphal cells for septum formation and lateral cell wall integrity (Munro et al., 2001). Beyond structural integrity, chitin has also been proposed to have a role in epithelial adhesion (Gottlieb et al., 1991; Segal et al., 1988), linkage between cell wall and capsule

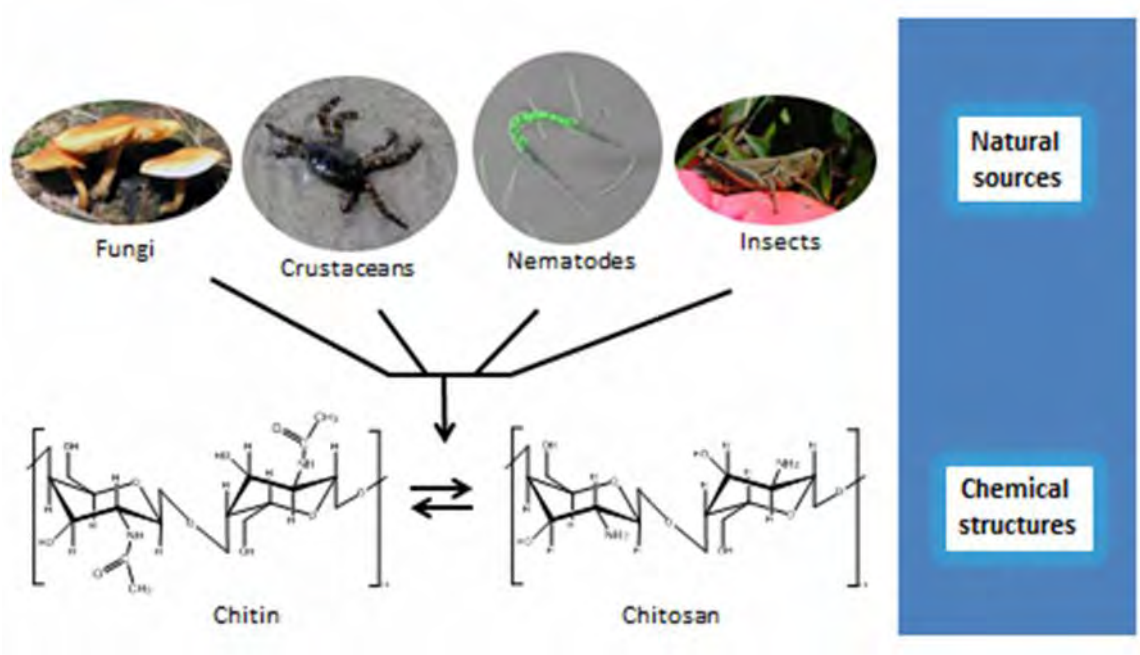

Figure 1.1. The sources and structures of chitin and chitosan. Chitin and chitosan are naturally found in fungal cell walls, crustacean shells, nematodes eggs and gut linings, and insect exoskeletons. These polymers consist of long chains of N-acetylglucosamine (chitin) or glucosamine (chitosan). Conversion between the two polysaccharides can be performed chemically or happen within the organisms where chitin deacetylases catalyze conversion of chitin to chiotsan.

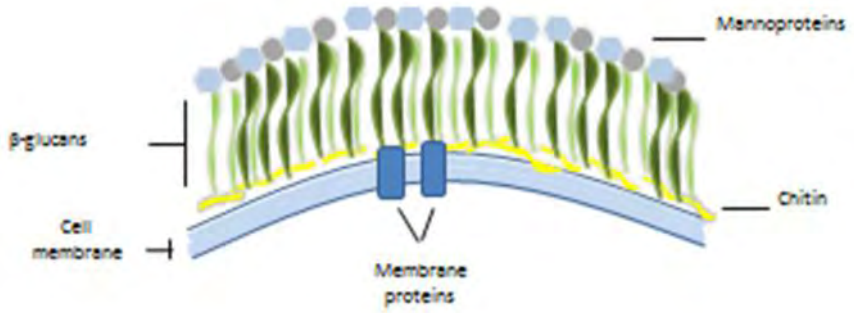

Figure 1.2 Simplified schematic of the structure of the fungal cell wall. The fungal cell wall is composed of a base layer of chitin along the cell membrane. A second layer is composed of β-glucans and finally mannoproteins make up the outermost layer of the fungal cell wall.

(Rodrigues et al., 2008), and antifungal resistance (Lee et al., 2012), making it a polysaccharide of vital importance to fungi.

Like chitin, chitosan is also essential for cell wall integrity. Fungi that contain chitin deacetylases convert a portion of their chitin to chitosan resulting in chitosan occupying the same location as chitin in the cell wall structure. Much like for chitin, chitosan deficiency results in slow growth and decreased cell integrity (Baker et al., 2011). Furthermore, chitosan has been shown to be necessary for cryptococcal virulence and persistence in mammalian hosts (Baker et al., 2011), highlighting the importance of these polysaccharides in fungi.

Although chitin and chitosan are of immense structural importance in the fungal cell wall, they generally compose only a small fraction of it with other polysaccharides such as β-glucans making up the major content of fungal cell walls. Chitin content of the cell wall is commonly around 3-5% of the cell wall content, although it varies and fungi such as *Allomyces macrogynus* have up to 60% of the cell wall composed of chitin (Bartnicki-Garcia, 1968). Chitosan cell wall content is similar to chitin. Zygomycetes generally have a chitosan content anywhere from 9-32% of the cell wall, which varies based on the stage of the life-cycle (Bartnicki-Garcia, 1968). Among fungi with chitin deacetylases, generally only a portion of the chitin is converted to chitosan and there is significant variation among different fungi in the amount of chitin converted to chitosan. *Candida albicans* converts less than 5% of its chitin to chitosan, while some of the zygomycetes may convert more than two-thirds (Lenardon et al., 2010).

Receptor-mediated recognition of chitin and chitosan

Mammalian cells lack chitin and chitosan, while infectious and allergic agents such as fungi, parasitic nematodes, and dust mites all contain these foreign polysaccharides, making them potential targets for recognition by the immune system. Unfortunately, exactly how the immune system recognizes these polymers is unknown. The search for the chitin receptor(s), has identified a number of candidates including: fibrinogen C domain containing 1(FIBCD1), natural killer cell receptor protein 1 (NKR-P1), RegIIIγ, and galectin-3 (Cash et al., 2006; Schlosser et al., 2009; Seetharaman et al., 1998; Semenuk et al., 2001). However, none of these have yet to be shown to act as a receptor as opposed to a protein that binds chitin. Other receptors including TLR2, mannose receptor and dectin-1 have also been implicated in chitin signaling, but none of these receptors have been shown to bind chitin (Da Silva et al., 2008; Koller et al., 2011; Mora-Montes et al., 2011a). Neither has a chitosan receptor(s) been identified.

Immunological activity of chitin and chitosan

Despite the prevalence of chitin and chitosan, their immunostimulatory properties are poorly understood. Varying reports have characterized these polysaccharides as relatively inert, proinflammatory, and pro-allergenic (Lee, 2009; Lee et al., 2008; Reese et al., 2007; Shibata et al., 1997; Wagner et al., 2010). Most commercial preparations of chitin and chitosan are derived from crustacean shell waste. Unfortunately this introduces a number of factors that contribute to some of the confusion seen in the field of chitin and chitosan. In crustaceans, chitin is covalently linked to proteins and tanned

by quinones, creating multiple contamination possibilities depending upon the isolation procedures used (Muzzarelli, 2010). In addition to putative contaminants, these polymers are unlikely to be homogeneous and may vary from batch to batch. Methods to isolate and purify chitin generally result in partial deacetylation of the polymer, while some acetylation remains following the heat-alkali treatment typically used to produce chitosan (Aranaz et al., 2009). Thus, most marketed chitin and chitosan consist of both acetylated (GlcNAc) and deacetylated (GlcN) residues. Generally chitosan polymers are at least 60% deacetylated and soluble in weak acid, whereas chitin is insoluble in both acid and base (Aranaz et al., 2009). Different isolation and preparation procedures of chitin and chitosan have significant impact on the polymer produced and consequently on the activity of the polymer.

Additional possible explanations for these disparate findings include different sources (e.g., shrimp, crab, fungal) and variability in the tertiary structure of the polymers due to manufacturing process differences (Aranaz et al., 2009; Mora-Montes et al., 2011b). Another possible explanation for the varied immunological response is particle size. The importance of size has been suggested by studies demonstrating differential stimulation of TNFα and IL-10 by size-fractionated chitin. Particles of intermediate size (40-70 μm) induced just TNFα whereas smaller particles (<40 μm) induced both TNFα and IL-10 (Da Silva et al., 2009). Altogether there are many different aspects of chitin and chitosan structure and chemistry that are likely to impact the results of those who

study them. Greater understanding of the polymers themselves may help clear up some of the confusion seen in the field.

How are these polymers being used translationally?

Despite the varying reports on the immunological activity of chitin and chitosan, there has been increasingly intense interest in these polymers for use in translational applications. Pharmaceutical and commercial applications such as gene and drug delivery constructs, tissue scaffolds, wound dressings, absorption of toxic metals such as mercury, cadmium and lead, and serving as biosensors through the immobilization of enzymes, have utilized chitin and chitosan with promising results (Agnihotri et al., 2004; Jayakumar et al., 2010; Morganti and Morganti, 2008; Nakagawa et al., 2003; Read et al., 2005). Both chitin and chitosan are nontoxic, easy to conjugate with other polymers, and they break down to harmless amino sugars that are easily and completely absorbed by the human body (Agnihotri et al., 2004; Pillai et al., 2009).

One characteristic of chitin and chitosan that is being utilized for translational applications is its antimicrobial properties. Chitosan oligosaccharides have been shown to be antimicrobial; acting by disrupting/permeabilizing cell membranes of bacteria and fungi while leaving mammalian cells relatively unaffected (Jaime et al., 2012). Therefore, chitosan may be a potentially effective fungicide for drug-resistant fungal pathogens (Jaime et al., 2012). This would be particularly welcome to a field where there is a definite need for new anti-fungal treatments. While chitin does not appear to exhibit the same direct antimicrobial properties, it is capable of resisting infection as well as

degradation. Chitin fibrils have been used in sutures as it resists attack from bile urine and pancreatic secretions better than other available options, all while being a poor substrate for microbial colonization (Austin et al., 1981). Although the polymers generally leave mammalian cells unaffected, chitosan has been shown to actually have an inhibitory effect on cancer cells, causing DNA fragmentation and elevated caspase-3 activity in tumor cells (Hasegawa et al., 2001; Qi et al., 2007). It is not understood how this occurs, but chitosan may one day be a useful, non-toxic anti-cancer therapy.

The unique structural and biological properties of chitin and chitosan are increasingly being exploited for use in biomedical applications. This has been facilitated by advances in technology to produce purified polymers with the desired physical properties. Building on the antimicrobial properties of these polymers, another promising area of research has focused on using chitin and chitosan to create tissue scaffolds. Chitin has been used as a growth substrate for human keratinocytes and fibroblasts (Tamura et al., 2004), and chitosan has been shown to induce and stimulate connective tissue-rebuilding (Muzzarelli et al., 1988). The polycationic properties of chitosan are also being developed for use in wound dressings to induce cell migration and proliferation at the wound site (Jayakumar et al., 2010). Particle size can be manipulated to control the resulting inflammatory response induced by these polymers and the large particles used in scaffolds are relatively inert, providing a perfect base in which to grow cells upon.

Smaller chitin and chitosan particles have been shown to act as an adjuvant, boosting the immune response to vaccines. Chitosan as an adjuvant induces macrophage NO production and chemotaxis (Peluso et al., 1994). Mucosal vaccines adjuvanted with chitosan have been shown to elicit a robust antibody response, enhanced helper T cell function, and generation of alloreactive cytotoxic T cells and NK cells (Nishimura et al., 1985; Read et al., 2005). Chitin microparticles have been shown to have adjuvant-like properties by significantly reducing influenza virus loads and clinical symptoms when given prior or soon after exposure to the virus (Baaten et al., 2010). Antigen encapsulated in chitosan particles was shown to be effectively processed and presented via both MHCI and MHCII pathways, presenting a delivery system which enhances the vaccine response to weakly immunogenic subunit and polypeptide antigens (Koppolu and Zaharoff, 2013). The molecular weight, oligomer side chain length degree of deacetylation, particle size, viscosity, and impurities all impact adjuvant activity (Lu et al., 2009; Scherliess et al., 2013). The studies conducted so far show that both chitin and chitosan appear quite effective at boosting an immune response as an adjuvant.

Finally, an area that has garnered a lot of attention is the use of chitosan as an efficient delivery system for conjugated drugs and genes. The polycationic and biodegradable properties of chitosan make it attractive as a controlled delivery system for conjugated materials. It is safe and biocompatible, provides controlled release of conjugated substances, is mucoadhesive and easily conjugated with other materials, such as carboxy-methyl cellulose, diethylenetriamene, triethylenetetramine,

tetraethylenepentamine, or linear polyethylenimine which present additional beneficial properties such as boosted transfection efficiency (Bowman and Leong, 2006; Ichikawa et al., 2005; Lu et al., 2009; Werle et al., 2009). These properties allow chitosan to easily bind, protect and then deliver their conjugates.

Chitosan that has bound and condensed plasmid DNA is able to efficiently transfect cells, even more efficiently than PEI, with gene expression gradually increasing over time (Erbacher et al., 1998; Lu et al., 2009). It has also been applied in vivo for oral administration of plasmid DNA delivered to the intestine where there was successful transfection of intestinal cells and a sustainable increase in the target protein (Li et al., 2009a). The conjugation of chitosan to its cargo also helps protect it and enables oral delivery of hydrophilic drugs such as peptides, which would otherwise be degraded long before they reached the targeted area (Werle et al., 2009). Along those lines, the chitosan microparticles have been shown to be quite stable, and have been used as a controlled release system for the delivery of hormones over long periods of time (Illum, 1998). Further reducing the particle size to nanoparticles enables them to enter the nuclear membrane and deliver their cargo directly to the nucleus (Tan et al., 2009). Micro and nanoparticle chitin and chitosan conjugates are showing great promise in a wide variety of applications and additional new potential applications all the time.

Although there appears to be promising future applications for these polymers, currently, chitin and chitosan are approved by the US Food and Drug Administration only

for use as food additives. However, there are a number of promising ongoing clinical trials looking to expand their approved roles.

The inflammasomes

IL-1β is an important pro-inflammatory mediator generated at sites of injury or immunological challenge to attract cellular influx to these sites. Processing and release of IL-1β and IL-18 is controlled by inflammasomes. Inflammasomes are cytosolic complexes containing caspase-1, which is responsible for the cleavage of pro-IL-1β and pro-IL-18 to their active forms. Most of the described inflammasomes complexes also consist of a nucleotide-binding domain and leucine-rich repeat containing (NLR) and the adaptor molecule ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (Martinon et al., 2002). ASC associates with the NLRs via homotypic pyrin domain (PYD) interactions and then recruits caspase-1 via a homotypic CARD interaction (Vajjhala et al., 2012). The ASC PYD contains two distinct binding sites which allow recruitment of the NLR as well as self-association which promotes oligomerization thereby inducing activation of caspase-1 (Vajjhala et al., 2012). Of the 23 described NLRs in humans and 34 in mice, only a few so far have been shown to pair with caspase-1 to form an inflammasome (Bryant and Fitzgerald, 2009). There are four well-established inflammasomes, and three more recently established, less well understood inflammasomes (Table 1.1). Of the well-established inflammasomes, three are NLR-containing inflammasomes: NLR family, pyrin domain containing 1 (NLRP1), NLR family CARD domain-containing protein 4 (NLRC4, also known as IPAF), and the

NLR family, pyrin domain containing 3 (NLRP3). The fourth well-established inflammasome does not contain an NLR, instead it contains a HIN200 domain, and it is known as the absent in melanoma 2 (AIM2) inflammasome. The three less well understood inflammasomes include: interferon gamma-inducible protein 16 (IFI16), the NLR family, pyrin domain containing 6 (NLRP6), and the NLR family, pyrin domain containing 12 (NLRP12).

Table 1.1. List of the inflammasomes and their activators

Inflammasome	**Activator**
AIM2	cytosolic DNA
IFI16	viral DNA
NLRC4 (IPAF)	flagellin, bacteria with type III or IV secretion systems
NLRP1	anthrax lethal toxin
NLRP3	alum, chitosan, MSU, cholesterol crystals, hemazoin, amyloid-β, silica, ATP, nigericin, fungi, viruses, bacteria
NLRP6	unknown
NLRP12	Yersinia pestis

NLRP1

The NLRP1 inflammasome is a complex formed of NLRP1, ASC, caspase-1 and caspase-5 (Martinon et al., 2002). It has been shown to be activated by anthrax lethal toxin and the bacterial cell wall component, muramyl dipeptide (MDP) (Newman et al., 2010; Wickliffe et al., 2008). The NLR contains both a PYD domain and a CARD domain (Hlaing et al., 2001) which allows for interaction with the adaptor molecule ASC, as well as direct interaction between the NLR and caspase. Although ASC is not required for activation of NLRP1, it has been shown to enhance activation in response to MDP (Faustin et al., 2007). Additionally, NLRP1 contains a function to find domain (FIIND), of which autolytic cleavage is required for activation (Finger et al., 2012).

NLRP1 has multiple isoforms, not all of which are conserved across species. Mice express NLRP1b which responds to anthrax lethal toxin, however human NLRP1 is unable to respond to anthrax lethal toxin (Boyden and Dietrich, 2006; Finger et al., 2012). Both human and mouse NLRP1 will respond to MDP though (Franchi et al., 2009a). In humans, mutations in NLRP1 have been shown to be associated with a number of autoimmune and autoinflammatory diseases such as: vitiligo, rheumatoid arthritis and Crohn's disease (Finger et al., 2012; Jin et al., 2007).

NLRC4

The NLRC4 inflammasome is activated by bacteria such as: *Salmonella enterica serovar Typhimurium*, *Shigella flexneri*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* (Case et al., 2009; Franchi et al., 2006; Lara-Tejero et al., 2006; Warren et al., 2008). This inflammasome has been shown to be specifically activated by bacteria with

flagellin and that have type III or IV secretion systems, such as PrgJ in Salmonella (Miao et al., 2010b) that enable the bacteria to escape to the cytosol. For some NLRC4 stimuli, Legionella and minimal C-terminal flagellin peptide, activation requires the presence of NLR family, apoptosis inhibitory protein 5 (NAIP5) (Lightfield et al., 2008; Ren et al., 2006), although others such as PrgJ and full length flagellin are able to activate NLRC4 without NAIP5 (Lamkanfi et al., 2007; Lightfield et al., 2011).

Unlike the other two NLR inflammasomes, NLRC4 lacks a PYD domain, but it does contain a CARD domain. This allows for direct interaction between the NLRC4 and caspase-1 CARD domains, which would suggest this inflammasome does not require the adaptor molecule ASC (Case et al., 2009). However, multiple studies have shown abrogated IL-1β release with NLRC4 stimuli in the absence of ASC (Franchi et al., 2006; Mariathasan et al., 2004), suggesting the adaptor does have a role in activation of the NLRC4 inflammasome.

NLRP3

The NLRP3 inflammasome contains NLRP3, ASC and caspase-1. This inflammasome is perhaps the most well studied of the inflammasomes, because unlike the other described inflammasomes with more specific stimuli, the NLRP3 inflammasome has been shown to be activated by an incredibly wide variety of stimuli. NLRP3 activators include particulates such as alum, chitosan, uric acid crystals, cholesterol crystals, hemazoin, amyloid-β, and silica (Dostert et al., 2009; Dostert et al., 2008; Grebe and Latz, 2013; Halle et al., 2008; Li et al., 2007; Li et al., 2008; Martinon

et al., 2006), soluble stimuli such as ATP (Hogquist et al., 1991) and the pore-forming toxin nigericin (Perregaux et al., 1992), as well as a variety of fungi, bacteria and viruses (Mariathasan et al., 2006; Tschopp and Schroder, 2010). NLRP3 has been also been shown to be activated by necrotic but not apoptotic cells (Li et al., 2009b), and activation is most likely triggered by sensing released cellular components, specifically ATP (Iyer et al., 2009). As there are so many different stimuli responsible for activation of this inflammasome, it is thought to be a sensor of cellular stress more than it is likely to be directly activated by all of the varied stimuli.

There are a number of IL-1β diseases associated with NLRP3 defects including familial Mediterranean fever, familial cold autoinflammatory syndrome and Muckle-Wells syndrome (Case, 2011). Additionally, accumulation of crystalline stimuli in diseases such as gout (MSU crystals), and atherosclerosis (cholesterol crystals) are also attributed to NLRP3 activation and consequently disease.

AIM2

The fourth well established inflammasome is the AIM2 inflammasome. This inflammasome contains HIN200 and the PYD domain protein AIM2, ASC and caspase-1, and is activated by cytosolic double-stranded DNA (Bryant and Fitzgerald, 2009; Hornung et al., 2009). The AIM2 inflammasome is able to bind directly to cytosolic DNA, which thereby induces IL-1β processing and pyroptosis (Rathinam et al., 2010). It has also been shown to be essential for in vivo response to *Francisella tularensis*, vaccinia virus and mouse cytomegalovirus (Rathinam et al., 2010).

IFI16, NLRP6, and NLRP12

In addition to the four more well studied inflammasomes, there are three additional inflammasomes that have recently been discovered. Although they have been identified as interacting with caspase-1 to form an inflammasome, not much is yet known about what activates them. The interferon gamma-inducible protein 16 (IFI16) is a PYD containing HIN200 protein expressed in the nuclei of endothelial cells (Kerur et al., 2011). This inflammasome complexes with ASC and caspase-1 in the nucleus where it has been shown to be activated by the Kaposi Sarcoma-associated herpesvirus (Kerur et al., 2011). IFI16 appears to be activated by directly binding IFN-β-stimulating viral DNA through its HIN200 domain, much like AIM2 (Unterholzner et al., 2010). While AIM2 recognizes DNA in the cytosol, IFI16 acts as a sensor of foreign DNA in the nucleus.

The stimulus of the NLRP6 inflammasome is unknown, however it has been shown to be important for regulating the gut microbial ecology (Elinav et al., 2011). NLRP6 KOs in mouse colonic epithelial cells results in reduced IL-18 levels and altered fecal microbiota (Elinav et al., 2011). Unlike the other described inflammasomes, it has actually been proposed to act as a negative regulator of inflammatory signaling, as NLRP6 KOs are highly resistant to a number of infections including: *L. monocytogenes*, *S. typhimurium*, and *Escherichia coli* (Anand et al., 2012). These NLRP6-deficient mice show increased cytokine and chemokine activation when compared to WT (Anand et al., 2012), suggesting NLRP6 is suppressing the inflammatory response to these pathogens.

The third newly described inflammasome is the NLRP12 inflammasome. It was the first inflammasome discovered by biochemical assay where the NLR was shown to associate with ASC to form an inflammasome (Wang et al., 2002). The activator of the NLRP12 inflammasome is unknown, but it has been shown to be important in *Yersinia pestis* infection (Vladimer et al., 2012). Like many of the other inflammasomes, mutations in NLRP12 have been linked to inflammatory disease, namely hereditary periodic fever syndrome (Jeru et al., 2008). Independent of an inflammasome role, NLRP12 has also been shown to be important in maintaining neutrophils and dendritic cells in a migration-competent state, without it they have reduced capacity to migrate to draining lymph nodes (Arthur et al., 2010).

Activation of the inflammasomes

Activation of the inflammasomes involves a two-signal process (Figure 1.3). The first signal is generally provided through activation of a Toll-like receptor (TLR), such as LPS via TLR4. This priming step induces upregulation of pro-IL-1β and pro-IL-18. Priming also results in an upregulation of NLRP3, offering additional regulation for the widely activated NLR, while the other NLRs appear to be constitutively expressed (Bauernfeind et al., 2009). After priming, the second signal, the inflammasome activator, induces oligomerization of the inflammasome complex. ASC, which is localized primarily in the nucleus rapidly translocates to the cytosol (Bryan et al., 2009), complexing with the NLR through its PYD domains and caspase-1 through its CARD domains, enabling inactive pro-caspase-1 to autocleave to active caspase-1. The

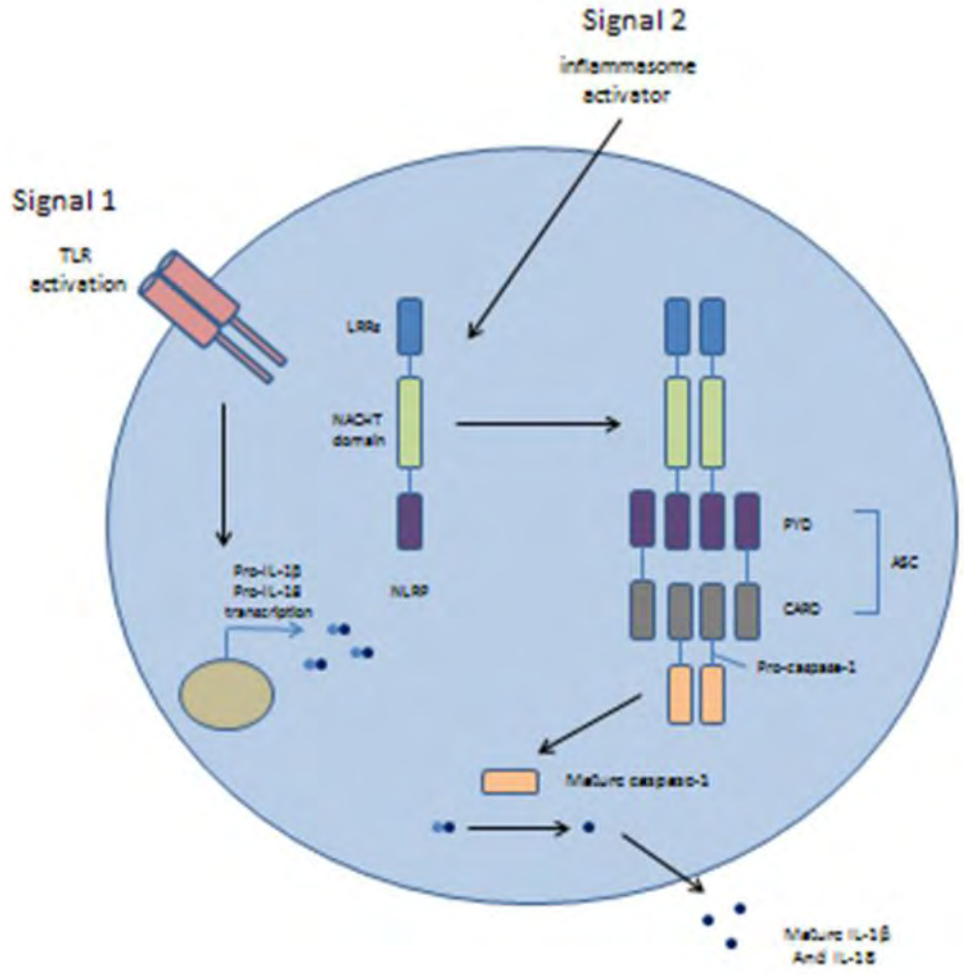

Figure 1.3 Inflammasome activation. Inflammasome activation is a two-step process. The first signal is delivered through a TLR and induces transcription of pro-IL-1β and pro-IL-18. Next the inflammasome stimuli activates oligomerization of the inflammasome and autocatalytic processing of caspase-1 which is then free to process pro-IL-1β and pro-IL-18 to their mature forms which are then released from the cell.

mature caspase-1 can then cleave pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18, which are then released from the cell.

Though processing of IL-1β through caspase-1 and the inflammasome is predominant, there is some evidence of IL-1β cleavage in caspase-1 KO cells, so there may also be a caspase-1-independent pathway (Mayer-Barber et al., 2010). A number of enzymes including: granzyme A, neutrophil-derived proteinases: NE and CatG, and human mast cell chymase, have been shown to cleave recombinant pro-IL-1β in vitro (Coeshott et al., 1999). However, as of yet exactly how, or even if, this occurs in vivo is unknown.

Effects of IL-1β and IL-18 inflammasome activation

IL-1β is an important mediator of the inflammatory response. It is involved in cell proliferation, differentiation and apoptosis (Bryant and Fitzgerald, 2009). It enhances expression of adhesion molecules on endothelial cells, promotes extravasation of leukocytes, modulates muscle metabolism and induces fever (Allan et al., 2005; Dinarello, 2002). IL-1β also induces a number of other proinflammatory cytokines such as IL-6 and CSF, as well as chemokines which will serve to attract cells to sites of infection (Dinarello, 1997), while release of IL-1β itself will attract and engage cells harboring the IL-1R (Martinon, 2010). IL-1β also induces increased expression of adhesion molecules such as ICAM-1 and VCAM-1 which promotes infiltration of proinflammatory and immunocompetent cells into extravascular space (Dinarello, 2002). Beyond its immediate pro-inflammatory effects, IL-1β has also been shown to promote

differentiation of Th-17 cells and antigen-specific cellular immunity (Dostert et al., 2013; Sharp et al., 2009). Exhibiting how important this cytokine is, animals with diminished IL-1β, such as NLRP3-\-, have fewer lymphocytes, eosinophils and neutrophils recruited to the lung during challenge (Dostert et al., 2008; Shimada et al., 2010).

IL-18 is not an endogenous pyrogen, but stimulates proinflammatory cytokines (such as Il-6, IL-8, TNFα, IL-1β, and IFNγ) and chemokines, upregulation of adhesion molecules and activation of natural killer cell activity (Dostert et al., 2013; Ferrari et al., 2006; Gracie et al., 2003). Although both IL-1β and IL-18 are processed by caspase-1 through the inflammasome, there appears to be distinct licensing requirements for processing of IL-1β and IL-18 by NLRP3. Inhibition of ROS or caspase-11 blocked IL-1β production but had no effect on IL-18 (Schmidt and Lenz, 2012), suggesting the inflammasome complex requires different signals for the processing of the pro-forms of these two cytokines.

Regulation of IL-1B, IL-18 and the inflammasome

The cytokines produced by the inflammasome are powerful inflammatory mediators, and as such their regulation is tightly controlled by multiple pathways. The activity of IL-1β is controlled through the endogenous IL-1Ra, which regulates IL-1β action by competing with IL-1β for the receptor site and is secreted in response to the same stimuli that induce IL-1β (Arend and Gabay, 2000; Schroder and Tschopp, 2010). IL-18 is directly regulated through the IL-18 binding protein (IL-18BP) which binds and neutralizes IL-18 (Dinarello, 2002).

There are many layers of regulation of the inflammasome itself as well, helping to control the system before IL-1β or IL-18 are even processed and released. Proteins such as pyrin, PAAD/PYRIN-only protein (POP1, also known as PYDC1), and POP2 (PYDC2) have been proposed to suppress the inflammasome by binding the PYD domains present on NLRs and ASC, thereby blocking component recruitment (Rathinam et al., 2012; Schroder and Tschopp, 2010). POP1 is present in macrophages and granulocytes where it associates with ASC to modulate pro-caspase-1 regulation (Stehlik et al., 2003). POP2 is expressed mostly in peripheral blood leukocytes and colocalizes with ASC in perinuclear specks thereby preventing formation of specks with NLRP3 (Bedoya et al., 2007). Conversely, the guanylate binding protein 5 (GBP5) has been shown to promote NLRP3 inflammasome assembly by binding the NLRP3 pyrin domain and promoting oligomerization for pathogenic bacteria and soluble stimuli, but not crystalline stimuli (Caffrey and Fitzgerald, 2012; Shenoy et al., 2012).

Additional inflammasome regulation has been shown in the form of the human serpin analogue proteinase inhibitor 9 (PI9). It acts in a similar way to the POP proteins, in that it binds the active site of caspase-1 thereby preventing hydrolysis of IL-1β (Annand et al., 1999). There is even miRNA regulation of the inflammasome, with recent reports of miRNA regulation of NLRP3 through miR-233 (Bauernfeind et al., 2012; Haneklaus et al., 2012). This miRNA targets the mitochondrial uncoupling protein 2 (UCP2), however it is unknown what role UCP2 plays in inflammasome activation (Bandyopadhyay et al., 2013).

While the major role for caspases outside caspase-1 is apoptosis, a number of caspases have also been shown to have a role in regulating inflammasome activation. Caspase-8 and caspase-12 have both been shown to have an inhibitory effect on IL-1β and IL-18 production by associating and inhibiting caspase-1inflammasome activation (Kang et al., 2013; Saleh et al., 2006). Caspase-11 has also been shown to be involved, though it appears to actually promote caspase-1 activation instead of inhibit like the other two (Ng and Monack, 2013).

There are also mechanisms in place to shut down the inflammatory response once it has served its purpose thereby preventing damage to the host. Effector and memory $CD4^+$ T cells can attenuate processing of caspase-1 and IL-1β through direct cell to cell contact (Guarda et al., 2009). Both type I and type II interferons have been shown to have a suppressive effect on inflammasome activation. Type I interferons restrain IL-1β production by diminishing the pro-IL-1β or inhibiting caspase activation (Guarda et al., 2011). IFNγ has also been shown to transiently inhibit pro-IL-1β in mouse cells, though human cells show an increase in IL-1β following IFNγ addition (Masters et al., 2010). Additionally, IL-13 induces upregulation of IL-1Ra, while downregulating caspase-1 (Scotton et al., 2005).

Sterile inflammasome activation

The sterile inflammatory response is characterized by inflammation triggered by tissue damage in the absence of infection (Li et al., 2009b). It has been implicated in a number of diseases including gout, Alzheimer's disease, silicosis, type 2 diabetes and

atherosclerosis (Cassel et al., 2008; Halle et al., 2008; Haneklaus et al., 2013; Strowig et al., 2012). In gout, MSU crystals collect in joints, and in Alzheimer's disease amyloid-β collects in the brain, both of which activate the inflammasome and cause chronic inflammation (Halle et al., 2008; Martinon et al., 2006). Similarly, inhaled silica particles can induce chronic inflammation known as silicosis (Cassel et al., 2008). In diabetes, inflammasome activation in hematopoietic cells impairs insulin signaling in several target tissues, reducing glucose tolerance and insulin sensitivity (Wen et al., 2011). NLRP3 has also been shown to sense obesity-associated danger signals and contributes to obesity-induced inflammation and insulin resistance (Vandanmagsar et al., 2011). Inflammasome activation and IL-1β production has also been shown to be involved in atherosclerosis. IL-1β has been shown to drive atherogenesis (Grebe and Latz, 2013), which is thought to be caused by cholesterol crystals activating the NLRP3 inflammasome early in those developing atherogenesis (Duewell et al., 2010b). For some of these diseases it has been shown that soluble endogenous ligands (oxidized low-density lipoprotein (LDL), amyloid-β, and amylin) begin to collect, they are then endocytosed via a pathway mediated by CD36 and converted intracellularly to crystals or fibrils (Sheedy et al., 2013). The crystals/fibrils are then responsible for inflammasome activation, causing chronic sterile inflammation without infection.

Pyroptosis

There is a fine line between inflammasome activation and pyroptosis. Pyroptosis is characterized by water influx leading to cellular swelling, osmotic lysis, plasma

membrane rupture, and release of proinflammatory stimulants (Schroder and Tschopp, 2010). Unlike other forms of cell death, which utilize other caspases, pyroptosis is dependent upon caspase-1 (Fink and Cookson, 2005). There is also evidence that ASC plays a central role in pyroptosis, as it is critical for the formation of the pyroptosome. In the pyroptosome, ASC oligomerizes and rapidly recruits caspase-1, thereby activating it and inducing pyroptosis (Fernandes-Alnemri et al., 2007). This pyroptosome is unique and distinct from the inflammasome (Fernandes-Alnemri et al., 2007).

NLRP1, NLRP3, NLRC4 and AIM2 can all trigger pyroptosis and the extent of pyroptosis increases with more potent inflammasome stimulation (Bryant and Fitzgerald, 2009). Though in an effort to control pyroptosis, caspase-1 promotes autophagy and autophagy protects the cell from pyroptosis (Byrne et al., 2013). However the strongest inflammasome activators will still induce pyroptosis, thereby alerting surrounding cells to the danger.

One of the major reasons for pyroptosis appears to be a defense against intracellular bacteria. Intracellular bacteria that activate caspase-1 and ultimately pyroptosis allows for clearance of those bacteria through a mechanism independent of IL-1β signaling (Miao et al., 2010a). Once intracellular bacteria have caused a cell to undergo pyroptosis, they are expelled from the cell along with the cellular contents and inflammatory cytokines which attract cells such as neutrophils which can then take up the bacteria and kill them by ROS (Miao et al., 2010a).

Thesis Overview

The immunological activity attributed to chitin and chitosan is full of apparent contradictions. It has been shown to be pro- and anti-allergenic, pro- and anti-inflammatory as well as inert. The first step in this study was to purify the chitin and chitosan polymers before assessing their immunological activity. The chitosan was also used as a base to convert to chitin to maintain as many similarities between the polymers as possible, enabling comparison of polymers that differed solely by degree of acetylation. After this purification and acetylation, the immunological activity of the resulting preparations was assessed, using inflammasome activation as the major readout. It was found that chitosan, but not chitin, is a potent NLRP3 inflammasome activator. Through characterization of the particles it was shown that small, phagocytosable, chitosan particles are necessary for inflammasome activation. Chitin does not actively inhibit the inflammasome, though the specific reason for its lack of activity is unknown. Chitin is more readily digested by macrophage cell lysates, but not at a rate that would likely impact inflammasome activation. Chitosan activation of the NLRP3 inflammasome is dependent upon all three proposed activation mechanisms: K+ efflux, ROS and lysosomal destabilization. Finally, while both polysaccharides are readily phagocytosed by macrophages, a cell receptor(s) responsible for the recognition and internalization has not been identified for either. There has not been a lot of work to identify a chitosan receptor, perhaps due to the positive charge leading to many possible interactions. The chitin receptor has been sought for a long time, though as of yet no one

has identified it. Here, the laminin receptor is analyzed as a potential chitin receptor. Though it does not appear to be the sole chitin receptor, there is evidence it may serve as one of multiple chitin receptors.

Aim 1: Purify the chitin and chitosan preparations and analyze those purified preparations for immunological activity.

Aim 2: Characterize the activating particles.

Aim 3: Determine the mechanism of inflammasome activation by chitosan.

Aim 4: Analyze the laminin receptor as a potential chitin receptor.

Preface to Chapter II

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Chrono K. Lee performed BMMΦ isolations

Chelsea L. Bueter performed the experiments

Chelsea L. Bueter, Charles A. Specht and Stuart M. Levitz designed the experiments and wrote the manuscript

Chapter II: Purification of Chitin and Chitosan

Abstract

The immunological activity of chitin and chitosan is somewhat controversial as they have been shown to induce pro-allergenic, pro-inflammatory or inert responses in different studies. Commercial preparations available vary by source, contamination, polymer lengths, and sizes. Four different commercial sources of chitin were tested for TNFα activity and showed significantly different activity levels. To purify chitosan, a procedure was developed using NaOH. Half the chitosan was then converted to chitin to allow for a direct comparison of the two polymers differing only through their degree of acetylation. After the purification and acetylation, neither the chitosan nor the chitin elicited any TNFα, resulting in purified preparations suitable for further experiments.

Introduction

The immunostimulatory properties of chitin and chitosan remain poorly understood. Most of the immunological work has focused on chitin, which has been characterized as both pro- and anti-allergenic, pro- and anti-inflammatory, as well as relatively inert in different reports. These conflicting reports raise a lot of questions as to what the actual immunological activity of chitin and chitosan really is.

The allergic response is characterized by elevated serum IgE, epithelial cell differentiation to mucus-secreting, hyperproliferative states, alternatively activated macrophages with high expression levels of chitinases and chitinase-like proteins, and infiltration of affected tissues by Th2 cells, eosinophils, and basophils (Dasgupta and Keegan, 2011; Voehringer et al., 2006). The alternatively activated macrophages are one of the hallmarks of an allergic response and their production of chitinases makes them especially important for the immunological response to chitin. Chitin containing Aspergillus (Bhatia et al., 2011) and house dust mites (Lee et al., 2009) have both been shown to induce alveolar macrophages towards an alternatively activated macrophage phenotype and thereby raised chitinase levels. However, it has been shown that there are elevated levels of chitinase in human serum during non-chitin containing infections (Labadaridis et al., 2005) suggesting elevated chitinase levels may be a part of the general host inflammatory response, rather than a direct defense against chitin (Lee, 2009; Vega and Kalkum, 2012).

In allergy chitinases and chitinase-like proteins appear to have a role, although there is some debate whether they help or exacerbate an allergic response. Ym1, Ym2 (two chitinase-like proteins) and acidic mammalian chitinase (AMCase) are all induced in asthma (Webb et al., 2001; Zhang et al., 2009), and AMCase has been shown to reduce or inhibit allergic inflammation induced by chitin (Lee et al., 2011; Reese et al., 2007), perhaps by breaking down the presumed allergin chitin. However, it has also been shown that blocking AMCase or knocking out BRP-39 (chitinase-like protein) results in decreased inflammation and eosinophilia (Dasgupta and Keegan, 2011; Zhu et al., 2004), suggesting their presence actually exacerbates the allergic reaction and confusing the role of chitinases and chitinase-like proteins in the allergic response.

Whereas fungal chitinases are involved in cell wall remodeling (Vega and Kalkum, 2012), in plants chitinase is the main inhibitor of fungal growth (Schlumbaum et al., 1986), and mammalian chitinase has also been shown to inhibit fungal growth *in vitro* and *in vivo* (Chen et al., 2009; van Eijk et al., 2005). It degrades chitin to small chitin-oligomers that then enhance macrophage stimulation and result in more chitinase production (Vega and Kalkum, 2012).

Though the average person is exposed to millions of fungal spores daily and are relatively unaffected by the exposure, increases in atmospheric fungal spore levels due to climate change (Gange et al., 2007) have been associated with increases in asthma exacerbations and patients sensitized to fungi (Roy and Klein, 2013). Those with occupations predicted to have high environmental chitin levels, such as shellfish

processors, have also shown a high incidence of asthma (Reese et al., 2007). Chitin has been shown to induce an allergic response consisting of an accumulation of eosinophils and basophils expressing IL-4 as well as alternatively activated macrophages (Reese et al., 2007; Satoh et al., 2010; Van Dyken et al., 2011). It has also been shown to increase alveolar epithelial type II cells that produce IL-33, inducing a Th2 response (Yasuda et al., 2012). The allergic inflammation induced by chitin has been shown to be dependent upon chitin-induced CCL2 in airway epithelial cells (Roy et al., 2012). However, there are some that believe the chitin-induced allergic response is actually an artifact. One proposed explanation for the allergic response to chitin actually attributes the activity to a contaminant in the crustacean derived chitin. The allergic response seen may actually be a response to the known crustacean allergen, muscle protein tropomyosin, which depending upon the purification procedure, may not be fully removed (Muzzarelli, 2010).

Regardless of the source of the allergic response, chitin or a contaminant in the chitin, others have shown chitin to induce the opposite response. The allergic response to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in mice has also been shown to be downregulated in the presence of chitin (Strong et al., 2002). This downregulation of the allergic response has shown to work by preventing and treating histopathologic changes in the airways of asthmatic mice, showing lowered serum IgE levels and lung eosinophil numbers, apparently inducing a redirecting of the response from Th2 to Th1 (Ozdemir et al., 2006; Shibata et al., 2000b). This may occur through chitin inducing

production of IL-12, IL-18 and TNF, leading to the production of IFNγ by NK cells, helping downregulating the allergic response (Shibata et al., 1997).

There are multiple reports supporting chitin as more of a Th1 agonist, inducing pro-inflammatory cytokines such as TNFα, RANTES, MIP-2 and IL-18 (Da Silva et al., 2009; Da Silva et al., 2008; Lee et al., 2008; Shibata et al., 2001). It has been shown to induce IL-1 while also inducing increased antibody production and antitumor activity (Nishimura et al., 1986a; Nishimura et al., 1986b) . However, there are also reports where chitin is shown to be anti-inflammatory. It has been shown to induce anti-inflammatory IL-10 (Da Silva et al., 2009), to inhibit T cell proliferation (Wagner et al., 2010) and block dectin-1 mediated inflammation (Mora-Montes et al., 2011a). Unfortunately the literature on chitin and chitosan is rife with many contradictory immunological responses.

Possible explanations for the disparate findings include the sources of chitin, the manufacturing processes and the presence of contaminants (Aranaz et al., 2009; Bueter et al., 2013; Mora-Montes et al., 2011b). The structure of chitin microfibrils has been show to vary widely in different organisms (major sources include shrimp, crab and fungi) and even in different parts of the cell wall of fungi (Lenardon et al., 2007). Different manufacturing processes can affect the degree of deacetylation and tertiary structure as well as impact the presence of contaminants. Variation in molecular weight has also been shown to impact the immunological profile (Otterlei et al., 1994). Chitin is frequently isolated from crustacean waste products and is naturally associated with

protein and minerals that are difficult to remove, while purification by deproteinization followed by demineralization will likely destroy the native structure (Aranaz et al., 2009). Each of these explanations could result in a slightly different polymer which may help explain why groups find contrasting results.

Another possible explanation for the varied immunological response is particle size. Interestingly, the size of the chitin particles determined the type of response observed: smaller fragments (<40 μm) induced cytokines that inhibited tissue inflammation, modest-sized fragments (40-70 μm) induced a strong pro-inflammatory response and larger fragments were relatively inert (Da Silva et al., 2009).

Before the immunological activity of chitin and chitosan was assessed, a purification procedure was developed. The goal was to start with chitosan and destroy potential contaminants, and then convert a portion of the chitosan to chitin. This way any remaining contaminants would be present in both the chitin and chitosan preparations, allowing a direct comparison of the two polymers while keeping potential contaminants, polymer length and structure the same.

Results

Commercial preparation variability.

Four different commercial preparations of chitin were analyzed for TNFα activity (Fig. 2.1). There were large variations in TNFα activity across all four preparations. Chitin preparations #1 and #2 were both from sigma with the same catalog number, but different lot numbers and yet they exhibited quite different TNFα activities. Chitin #3 was also from sigma, but had a different catalog number from the first two. Chitin #3 also happens to supposedly be the least pure of the sigma chitins and yet it showed the lowest TNFα of all the chitins tested. The fourth chitin tested was derived from squid (Seikagaku), whereas the three sigma chitins were derived from crab. Despite being derived from a different species, it showed similar TNFα activity to chitin #2.

Chitin and chitosan purification.

The variable TNFα activity showcased in figure 2.1 highlighted the need for a purification procedure for these glycans. To remove possible contaminants chitosan was suspended in 1.0 M NaOH at 90^{o}C for 1 h (Fig. 2.2A). In particular, NaOH destroys bacterial endotoxin while having no effect on the chitosan polymer itself (Aranaz et al., 2009; Sofer and Hagel, 1997). After this purification step, half the chitosan sample was suspended in 1M sodium bicarbonate and acetic anhydride to acetylate the chitosan to chitin (Banks et al., 2005), resulting in preparations that only differ through their degree of acetylation. Both glycan preparations were then incubated with NaOH (0.1 M) to destroy any endotoxin that might have been introduced during the conversion or handling

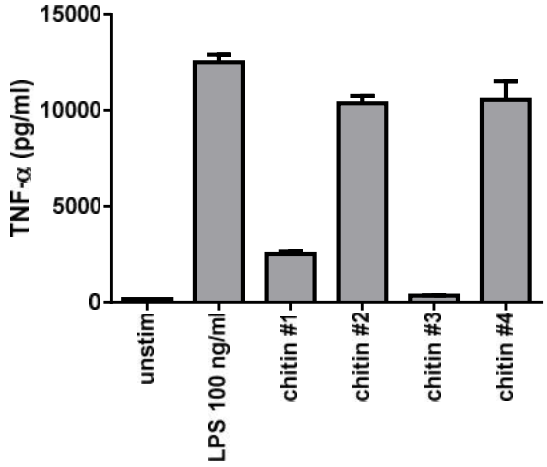

Figure 2.1. TNFα activity of commercial chitin preparations.

BMMΦ (1 x 10^5/well) were stimulated with LPS (100 ng/ml) or chitin (0.1 mg/ml) for 6 hours. Chitin #1 (sigma: C9752-1G, lot 107K7005), chitin #2 (sigma: C9752-1G, lot 114K7032), chitin #3 (sigma: C-7170-100G, lot 040M7000V), and chitin #4 (Seikagaku: 400629, lot 0804170). Supernatants were collected and analyzed by ELISA.

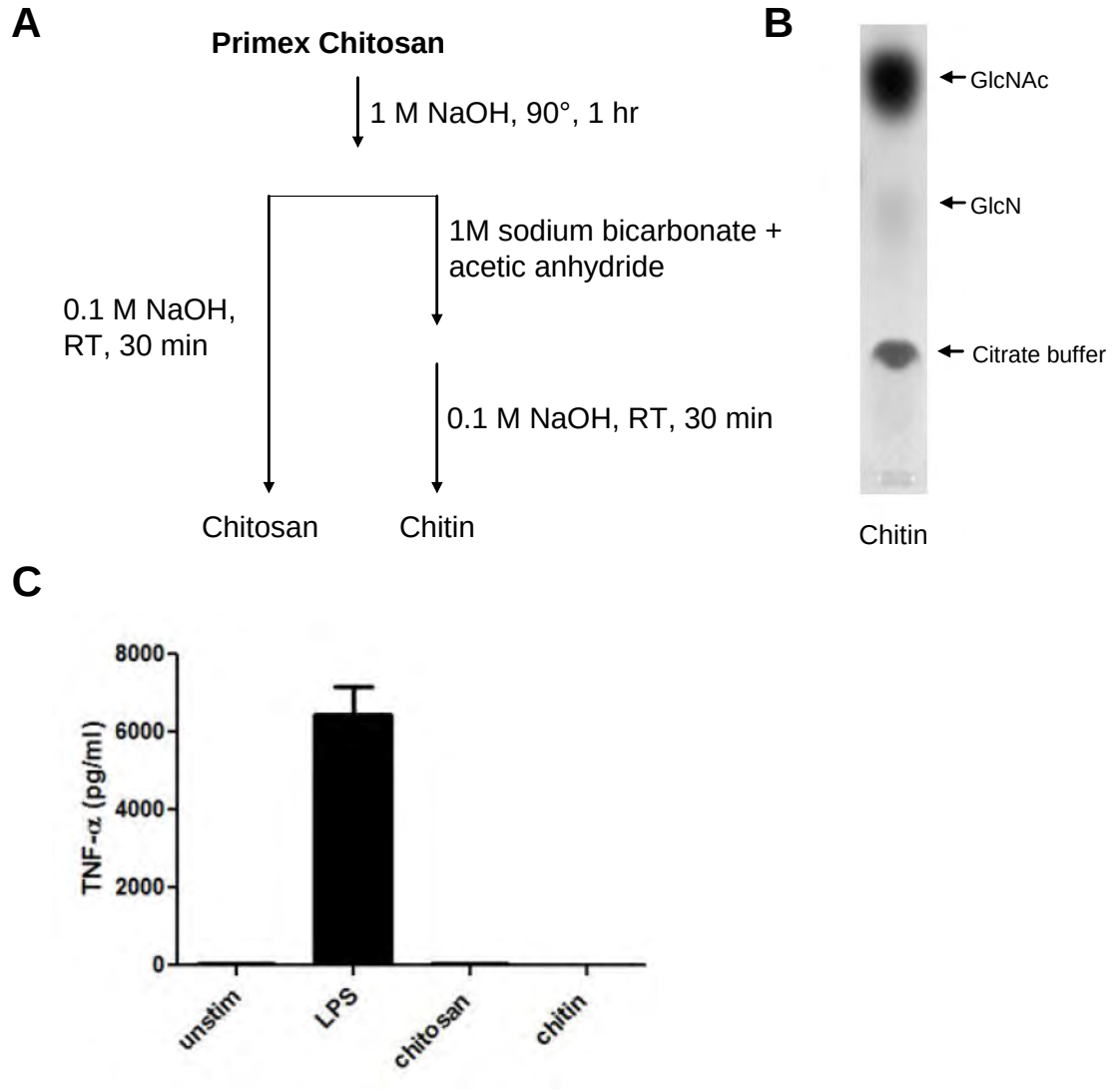

Figure 2.2. Chitin and chitosan purification and TLC analysis.

A, Commercial chitosan was treated with 1 M sodium hydroxide. A portion of the chitosan was then converted to chitin, by suspension in sodium bicarbonate with acetic anhydride to drive the acetylation reaction. Both preparations were then further purified in 0.1 M sodium hydroxide. B, To assess the efficacy of the acetylation reaction, the chitin was digested to monomers by *Trichoderma viride* chitinase and analyzed by TLC. C, BMMΦ (1×10^5/well) were stimulated with LPS (100 ng/ml), purified chitosan (0.1 mg/ml) or purified chitin (0.1 mg/ml) for 6 hours. Supernatants were collected and analyzed by ELISA.

processes and to remove any O-acetyl groups added during acetylation. To analyze the efficiency of the acetylation reaction, the chitin was digested with chitinase followed by separation of GlcNAc and GlcN by TLC (Fig. 2.2B). The acetylation proved successful with only about 7% of the residues remaining deacetylated. After the purification procedure and successful acetylation, neither the chitosan nor the chitin exhibited any TNFα activity (Fig 2.2C). This suggests the purification process at the very least removed endotoxin contaminants capable of inducing TNFα.

Discussion

The immunological properties of chitin and chitosan have been the subject of much investigation. However, previously reported studies generally used partially purified preparations and/or did not compare the two glycans side by side. In analyzing the TNFα activity of commercial chitin preparations, there was significant variability in activity levels between chitins that differed by as little as lot number and by as much as organism source. This variable profile highlights a major problem of the field of chitin and chitosan, with the choice of starting material having a significant impact on the potential results of any given study.

The seemingly contradictory literature on the immunostimulatory properties of chitin and chitosan and the variable TNFα activation profile of chitin seen above, are likely due to many factors including: differences in the sources of the material, procedures used for purification, readouts for inflammatory responses and the size of the glycan particles. Most published studies on chitin and chitosan used preparations derived from crustacean sources (Da Silva et al., 2009; Wagner et al., 2010), although some have used chitin isolated from fungi (Gow et al., 1980; Mora-Montes et al., 2011b). There are known structural differences both between and within crustacean and fungal sources (Lenardon et al., 2007) which could have an impact on how the particles effect the immunological response. Chitin and chitosan are able to withstand many harsh purification procedures (Hunsley and Burnett, 1968), however, some of these procedures may affect the tertiary structure of the polymers (Aranaz et al., 2009) which may also

have an effect on their immunological activity. Additionally, methods of purification are often proprietary and different preparations may contain endotoxin, glucans, proteins and other contaminants that may impact the results obtained. Starting with relatively pure chitosan and then performing a series of further purification steps, such as NaOH treatment to destroy possible endotoxin contaminants (Sofer and Hagel, 1997), allowed for preparations with presumably little contamination. Finally, for each experiment, half of the chitosan was acetylated to chitin, and therefore one would expect that had contaminants remained after the purification procedure, they would be present in both of the preparations, thereby allowing direct comparison of the two glycans which differ only by their degree of acetylation.

Materials and Methods

Reagents and cell culture- All materials were obtained from Sigma-Aldrich unless otherwise stated. Ultrapure LPS (free of TLR2-stimulating lipopeptides) was purified from the original Sigma stock (L2630) by two treatments with deoxycholate followed by phenol extraction and ethanol precipitation (Hirschfeld et al., 2000). Chitin #1 (sigma: C9752-1G, lot 107K7005), chitin #2 (sigma: C9752-1G, lot 114K7032), chitin #3 (sigma: C-7170-100G, lot 040M7000V), and chitin #4 (Seikagaku: 400629, lot 0804170). Chitosan (76% deacetylated) was obtained from Primex. Complete media is defined as RPMI 1640 media (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 ug/ml streptomycin. Cell culture was at 37°C in humidified air supplemented with 5% CO_2. All experiments were performed under conditions designed to minimize endotoxin contamination.

Bone marrow-derived macrophages- Bone-marrow derived macrophages (BMMΦ) were generated as described (Johnson et al., 1983). Briefly, bone marrow was extracted from the femurs and tibiae of WT C57BL/6 mice (The Jackson Laboratory). Cells were cultured in complete media supplemented with supernatant from macrophage colony–stimulating factor (M-CSF)-secreting L929 fibroblasts at a final concentration of 20% and fed on days 4 and 7 with fresh media containing M-SCF. On day 8, macrophages were treated with 0.05% trypsin-EDTA, harvested and washed once in complete media before use in experiments.

Chitosan purification and conversion to chitin- Chitosan was suspended (6g/ 80 ml) in 1.0 M sodium hydroxide and heated at 90^{o}C for 1 h. The chitosan was collected by centrifugation and washed with PBS until the pH was neutralized. Half the purified chitosan was converted to chitin by suspending in 20 ml of 1.0 M sodium bicarbonate, followed by addition of 1 ml 97% acetic anhydride (Acros). The acetylation reaction was performed at 22°C for 20 min with periodic mixing. The acetylated glycan was collected by centrifugation and further acetylated by suspension in fresh sodium bicarbonate and acetic anhydride (as described above) for 20 min at 22°C followed by 10 min at 100°C. The particles were collected by centrifugation and washed 3x with PBS. Both the chitin and chitosan preparations were then passed through a 100 µm nylon mesh filter basket (BD Falcon) to remove the largest particles. The preparations were further treated in 0.1 M sodium hydroxide at 22°C for 30 min as a final purification procedure, followed by washing twice with PBS. Samples were stored at 4°C in PBS.

Determination of the degree of glycan acetylation- Reacetylated chitosan was digested to monosaccharides with chitinase followed by their separation using TLC. To 1 mg of chitin suspended in 200 µl of MacIlvaine's citrate phosphate buffer, pH 6.0, were added 10 µl *Trichoderma viride* chitinase (5 mg/ml in PBS). Following incubation at 30^{o}C for 5 days, samples and standards (5 µl) were spotted on a silica gel 60 glass-backed plate (EMD Chemicals) and developed using n-butanol: ethanol: water: acetic acid (5:4:3:1). Standards were GlcNAc and GlcN at various concentrations ranging from 1-20 mM. Plates were sprayed with 30% (w/v) ammonium hydrogen sulfate (Acros Organics) in

water and then baked at 140^{o}C for 30 min. The spraying and baking were repeated two more times (Gal, 1968). Visualizing the separated sugars was done with epi-UV illumination and a FluorChem HD2 digital imaging system (Alpha Innotech). Quantifications of the digital images were done with ImageJ (imagej.nih.gov).

Preface to Chapter III

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Chrono K. Lee performed BMMΦ isolations

Chelsea L. Bueter performed the experiments

Chelsea L. Bueter, Charles A. Specht and Stuart M. Levitz designed the experiments and wrote the manuscript

Chapter III: Cellular Response to Chitin and Chitosan

Abstract

The purification procedure devised in chapter one resulted in preparations derived from a common source that differ solely by their degree of acetylation. The immunological activity of the resulting preparations was assessed using inflammasome activation as the readout, ascertaining what impact acetylation had on inflammasome activation. While chitosan potently activates the inflammasome in mouse bone marrow macrophages, macrophages polarized towards a M1 or M2 phenotype, peritoneal macrophages, dendritic cells and human PBMCs, chitin is only a very weak stimulator. Both polymers were analyzed for other cytokine and chemokine activity, though neither chitosan nor chitin stimulated significant release from unprimed BMMΦ of any of the 22 cytokines and chemokines assayed.

Introduction

Fungal infections and the inflammasome

Between inhalation of fungal spores and potential exposure to chitin and chitosan through newly developed translational applications, there is frequent everyday exposure to these polymers. IL-1β has been shown to control antifungal immunity in vivo. The NLRP3 inflammasome, IL-1β, and IL-1α have been shown to be essential for defense against *Candida albicans* fungal infections, as KOs had significantly reduced survival compared to WT (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009). IL-1β release appears to be reliant on recognition of N-mannan-linked residues, chitin and β-glucan components of the cell wall (van de Veerdonk et al., 2009).

While BMMΦ have been the most often studied cell type by inflammasome researchers, other pro-inflammatory cell types have also been investigated. Macrophages are polarized between classically activated macrophage (M1) and alternatively activated macrophage (M2) phenotypes. The functional skewing of macrophages in vivo occurs in multiple pathologies such as: allergic and chronic inflammation, tissue repair, infection and cancer (Sica and Mantovani, 2012). M1 macrophages are generally considered pro-inflammatory with high levels of ROS, NO and Th1 inflammatory cytokines, while M2 macrophages are considered anti-inflammatory with high levels of scavenger, mannose and galactose-type receptors, inducing a Th2 response; however, there is reversible plasticity between the phenotypes (Gordon, 2003; Mantovani, 2006; Mosser and Edwards, 2008; Stout and Suttles, 2004). Alternatively activated macrophages are

actually multiple populations, but every macrophage state is rapidly and fully reversible (Porcheray et al., 2005). M1 macrophages have been shown to have a strong inflammasome response, which diminishes as macrophages become polarized towards the M2 phenotype (Pelegrin and Surprenant, 2009). Similar to cultured cells, primary cells such as peritoneal macrophages have also been shown to have strong inflammasome responses (Mariathasan et al., 2006). Activation of the inflammasome in murine dendritic cells (DC) may be an important intermediary between the innate immune response and the adaptive immune response. DC activation is crucial for vaccine adjuvants to stimulate protective adaptive immunity (Kool et al., 2008) and the IL-1β produced by DCs is required for the optimal priming of T cells (Ghiringhelli et al., 2009). Many parallels exist between mouse and human cell inflammasome activation. However, one important difference is that human blood monocytes have constitutively active caspase-1 and can be stimulated by LPS alone to secrete IL-1β (Netea et al., 2009).

Chitosan-induced IL-1β release was assayed in mouse bone marrow macrophages, macrophages with either a M1 or M2 phenotype, dendritic cells, and peritoneal cells, as well as human PBMCs. For all cell types tested, chitosan, but not chitin, induced IL-1β release. Finally, with use of a multiplex assay, we determined that purified chitosan and chitin are relatively weak inducers of cytokines and chemokines from unprimed BMMΦ.

Results

Chitosan stimulates the inflammasome, chitin does not. Mouse BMMΦ were primed for 3 h with 100 ng/ml ultrapure LPS and then stimulated with the pure chitosan and chitin preparations generated as in Figure 1A. Supernatants were assayed for IL-1β as a measure of inflammasome activation. As previously reported (Li et al., 2008), chitosan stimulated IL-1β (Fig. 3.1A). However, surprisingly, macrophages released only scant amounts of IL-1β when stimulated with chitin. IL-1β stimulation by chitosan was dose-dependent, with peak stimulation seen at a concentration of 0.3 mg/ml (Fig. 3.1B). Inflammasome activation by chitosan was dependent upon the NLRP3 inflammasome as IL-1β was not detected in supernatants of chitosan-stimulated macrophages from NLRP3-/- mice (Fig. 3.1C). IL-1β release from NLRP3-deficient macrophages was severely reduced in response to alum, which is known to predominantly activate this inflammasome, but remained intact in response to the AIM2 inflammasome activator, poly(dA:dT).

Chitosan, but not chitin, induces IL-1β in all cell types tested.

Chitosan elicited a robust NLRP3 inflammasome-dependent IL-1β response in BMMΦ, while little IL-1β was elicited by chitin. To characterize the spectrum of cells that release IL-1β in response to chitosan and chitin, a variety of cultured and primary cell types from mice and humans were studied. First mouse bone marrow cells were cultured in GM-CSF and M-CSF to promote classically activated (M1) and alternatively activated macrophage (M2) phenotypes, respectively (Fleetwood et al., 2009). An IL-1β response

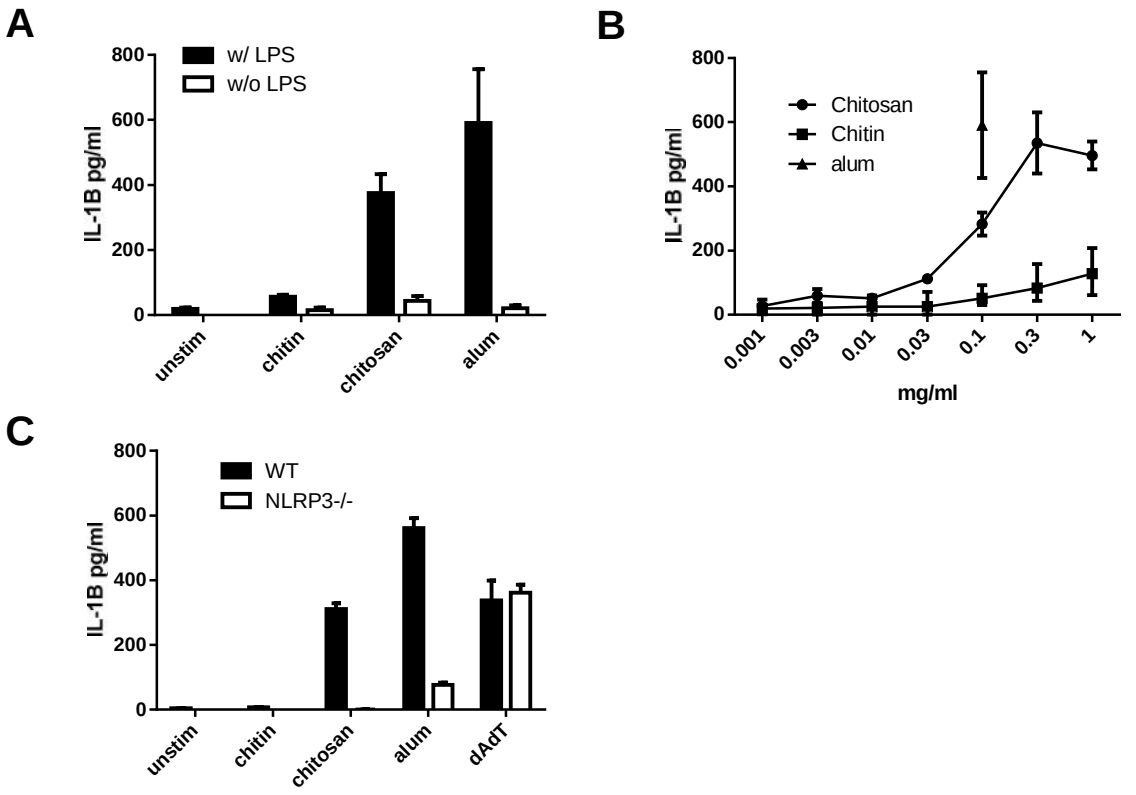

Figure 3.1. Inflammasome activation stimulated by chitin and chitosan.

A, BMMΦ (1 x 10^5/well) were primed for 3 h with 100 ng/ml LPS, or left unprimed, and then stimulated for 6 h with alum (0.1 mg/ml), or with the chitosan and chitin (0.1 mg/ml) preparations generated as in Figure 1. Supernatants were assayed for the inflammasome cytokine IL-1β by ELISA. Data are means ± SE of four independent experiments, each performed in triplicate. $p < 0.001$ comparing primed chitin to primed chitosan, unprimed alum to primed alum and unprimed chitosan to primed chitosan, as analyzed by 2-way ANOVA. B, Dose response curve of chitin and chitosan stimulating BMMΦ (1 x 10^5/well) after they were primed for 3 h with 100 ng/ml LPS. Data are means ± SE of four independent experiments, each performed in triplicate. C, IL-1β production from stimulated WT and NLRP3-/- macrophages was compared. Chitin and chitosan were used at 1 mg/ml. Alum (1 mg/ml) and poly(dA:dT) (2 μg/ml), which stimulate the NLRP3 and AIM2 inflammasomes, respectively, served as controls. IL-1β release was significantly reduced in NLRP3-/- macrophages stimulated with alum and chitosan. Data are means ± SE of a representative of two independent experiments, each performed in triplicate. $p < 0.001$ comparing WT macrophages and NLRP3-/- macrophages stimulated by chitosan or alum as analyzed by 2-way ANOVA.

was induced by chitosan in both cell types (Fig. 3.2A). The response to chitosan was more pronounced in the M1 phenotype, which is consistent with the response to other stimuli (Pelegrin and Surprenant, 2009). It was also observed a robust IL-1β response to chitosan in the primary peritoneal macrophages (Fig. 3.2B) and BMDCs (Fig. 3.2C). Finally, human PBMCs were tested to see if they respond to chitosan similarly as the mouse cells tested, and once again we saw a strong IL-1β response (Fig. 3.2D). Significant amounts of IL-1β were not released in response to chitin for any of the cell types tested.

Spectrum of cytokines and chemokines elicited by chitosan and chitin.

The above studies examined IL-1β release from primed BMMΦ. To examine the spectrum of cytokines and chemokines stimulated by chitosan and chitin, a multiplex assay was run on the supernatants from unprimed BMMΦ stimulated with these polysaccharides (Table 3.1). None of the 22 cytokines and chemokines assayed was significantly induced by either chitosan or chitin. Trends of higher responses for chitosan compared to chitin were observed for some of the cytokines and chemokines, but these did not achieve statistical significance following corrections for multiple comparisons. The positive control, LPS, stimulated significant amounts of all the cytokines and chemokines tested except MCP-1 and RANTES.

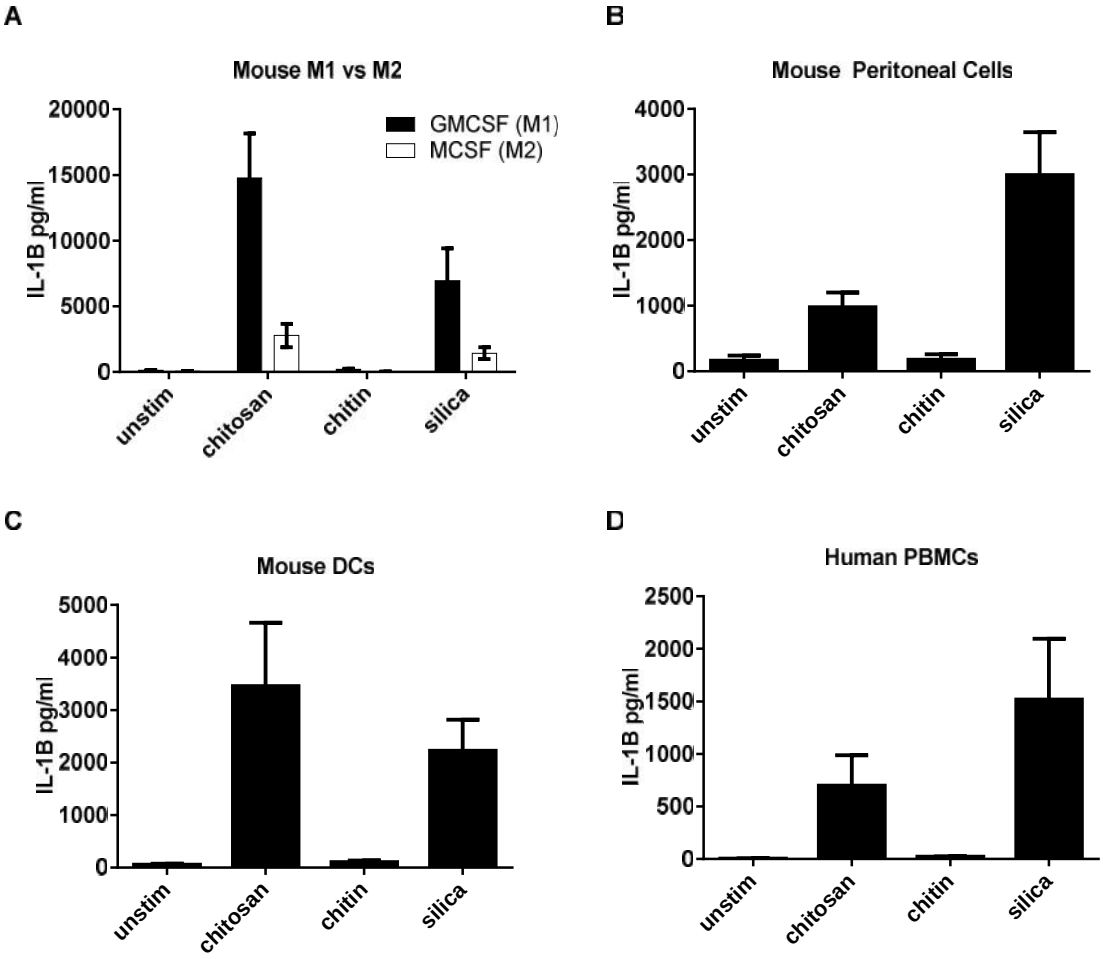
A
Mouse M1 vs M2
GMCSF (M1)
MCSF (M2)
IL-1B pg/ml
20000
15000
10000
5000
0
unstim
chitosan
chitin
silica
B
Mouse Peritoneal Cells
IL-1B pg/ml
4000
3000
2000
1000
0
unstim
chitosan
chitin
silica
C
Mouse DCs
IL-1B pg/ml
5000
4000
3000
2000
1000
0
unstim
chitosan
chitin
silica
D
Human PBMCs
IL-1B pg/ml
2500
2000
1500
1000
500
0
unstim
chitosan
chitin
silica

Figure 3.2. Chitosan, but not chitin, induces IL-1β release from all cell types tested

A. BMMΦ were cultured for 8 days in GM-CSF to promote the M1 phenotype or M-CSF to promote the M2 phenotype, and then plated at 1 x 10^5 cells/well. Cells were primed for 3 h with 100 ng/ml LPS, and then stimulated for 6 h with silica, chitosan, or chitin (all at 0.1 mg/ml). Supernatants were collected and analyzed by ELISA for IL-1β. $p < 0.0001$ comparing M1 chitosan to unstimulated (unstim), and $p < 0.01$ comparing M1 silica to unstimulated, as analyzed by 2-way ANOVA. In B, mouse peritoneal macrophages and in C, BMDCs were plated, primed and stimulated as in A. For peritoneal cells $p < 0.05$ comparing chitosan to unstimulated and silica to unstimulated as analyzed by 1-way ANOVA. For DCs $p < 0.01$ comparing chitosan to unstimulated as analyzed by 1-way ANOVA. D, human PBMCs were plated at 5 x 10^6/well in a 24-well plate, primed with 50 pg/ml LPS, and then stimulated overnight (same stimuli concentrations as in A). Supernatants were collected and analyzed by ELISA for IL-1β. $p < 0.001$ comparing unstimulated to chitosan and silica as analyzed by Kruskal-Wallis test. Data are means ± SE of three independent experiments, each performed in triplicate.

Table 3.1. Induction of cytokines and chemokines by chitosan and chitin. Unprimed BMMΦ (1 x 10^5/well) were left unstimulated (unstim) or stimulated with chitosan (0.1 mg/ml), chitin (0.1 mg/ml) or LPS (100 ng/ml) for 6 h. Supernatants were analyzed by multiplex assay. *$p < 0.01$ by the Kruskal-Wallis test. Data are means (pg/ml) ± SE of two independent experiments, each with quadruplicate determinations. Shaded boxes indicate that the mean value was below the lower limit of detection for the assay.

	unstim	chitin	chitosan	LPS
IL-1α	0.2 ± 0.1	1.6 ± 0.7	4.5 ± 1.2	60.5 ± 10.2*
IL-2	1.3 ± 0.6	3.9 ± 1.4	4.7 ± 1.6	19.3 ± 3.9*
IL-3	0.4 ± 0.2	1.3 ± 0.2	2.3 ± 0.5	19.2 ± 3.5*
IL-4	0.6 ± 0.2	1.6 ± 0.3	3.0 ± 0.6	26.9 ± 4.8*
IL-5	0 ± 0	0 ± 0	0 ± 0	6.9 ± 1.0*
IL-6	0.9 ± 0.7	1.7 ± 1.1	6.5 ± 2.9	489.7 ± 85.9*
IL-9	9.6 ± 6.3	23.6 ± 9.1	52.2 ± 14.6	517.2 ± 89.8*
IL-10	2.9 ± 1.3	5.4 ± 1.4	10.3 ± 2.1	145.8 ± 22.6*
IL-12(p40)	3.5 ± 1.3	5.3 ± 0.9	5.4 ± 1.2	1002.2 ± 322.7*
IL-12(p70)	2.9 ± 2.0	11.4 ± 2.8	23.9 ± 4.2	221.9 ± 36.8*
IL-13	2.5 ± 1.2	23.3 ± 7.0	53.9 ± 13.5	774.6 ± 74.1*
IL-17	0 ± 0	0.2 ± 0.1	1.3 ± 0.3	17.7 ± 2.2*
Eotaxin	6.8 ± 6.8	124.2 ± 49.2	251.9 ± 63.9	2359.3 ± 370.0*
G-CSF	2.9 ± 1.6	5.4 ± 2.5	5.2 ± 1.5	316.7 ± 110.9*
GM-CSF	0 ± 0	4.3 ± 2.8	8.3 ± 4.2	135.6 ±22.5*
IFN-γ	0.9 ± 0.9	2.8 ± 1.1	10.8 ± 1.8	162.9 ±29.4*
KC	55.8 ± 12.5	68.7 ± 13.6	80.3 ± 15.3	910.8 ± 147.6*

MCP-1	191.0 ± 50.8	263.4 ± 52.3	362.2 ± 68.9	1026.6 ± 193.1
MIP-1α	34.3 ± 10.6	106.9 ± 23.7	269.7 ± 57.7	22089.2 ± 5358.4*
MIP-1β	280.4 ± 57.2	832.6 ± 137.9	1295.5 ± 139.7	30927.4 ± 6449.7*
RANTES	1261.7 ± 254.5	1841.9 ± 367.2	2044.7 ± 329.7	10783.2 ± 1222.5
TNFα	0.2 ± 0.2	0.5 ± 0.3	1.2 ± 0.7	709.5 ± 340.6*

Discussion

The immunological properties of chitin and chitosan have been the subject of much investigation. However, previously reported studies generally used partially purified preparations and/or did not compare the two glycans side by side. Here preparations that were derived from a common source and differed solely by their degree of acetylation were utilized to ascertain what impact acetylation had on inflammasome activation. It was shown that while chitosan potently activates the inflammasome, chitin is only a very weak stimulator. With both polymers being derived from a common source and passing through the same purification process, the inflammasome activating activity shown by chitosan is unlikely to be due to a contaminant but from the polysaccharide itself.

After examining the inflammasome activation in BMMΦ, the activation profiles of other cell types were also examined. Chitosan induced a significant IL-1β response across a broad range of cell populations and across species, while chitin only induced a significant response from the human PBMCs. While macrophages polarized towards both a M1 phenotype and a M2 phenotype were stimulated by chitosan, the M1 response was much greater, consistent with their more pro-inflammatory phenotype. The IL-1β response seen from the M2 macrophages may be explained by the plasticity of macrophages and a switch towards a more M1 phenotype caused by the LPS priming step (Gordon, 2003). It has been shown with LPS and MDP that both M1 and M2 macrophages produce IL-1β suggesting macrophages can be quickly repolarized to react

to exogenous danger signals (Gratchev et al., 2006). The finding that chitosan stimulates IL-1β secretion in human cells has implications for the translational use of this polysaccharide. For example, the use of chitosan as a drug or vaccine delivery system could result in a potent, targeted inflammatory response.

The preparations of chitosan and chitin that were used in our studies were extensively purified to remove potential contaminants including proteins, nucleic acids and lipopolysaccharides. The purification steps included solubilization, chloroform:isoamyl alcohol extraction, reprecipitation, hot-alkali treatment and extensive washing. Using the resulting ultrapure preparations, chitin and chitosan did not stimulate unprimed macrophages to release statistically significant quantities of any of the 22 cytokines and chemokines analyzed by multiplex assay. However, due to our use of stringent criteria to correct for the large number of comparisons, the possibility of a Type II error causing us to miss significant associations cannot be ruled out. Indeed some of the cytokines and chemokines trended higher, with chitosan being a more potent stimulus compared with chitin. It should also be noted that the concentrations of cytokines and chemokines stimulated by chitosan and chitin were relatively low compared to LPS.

Others have reported that chitin can elicit IL-10, TNFα, IL-17A, IL-12, or IL-18 (Da Silva et al., 2009; Da Silva et al., 2008; Shibata et al., 2000a). It has also been shown that Candida activation of the inflammasome is significantly reduced in mutants with 70% less chitin than WT, suggesting a role for chitin in inflammasome activation (van de Veerdonk et al., 2009). The explanation for discrepancies between these published data

and our is speculative but may be due to differences in chitin source, contaminants, tertiary structures and/or particle size. Less work has been done on the immunological activity of chitosan, although our inflammasome and multiplex assays suggest that chitosan is the more immunostimulatory of the two polymers. Defining the conditions under which chitin and chitosan trigger or fail to trigger immune responses has translational relevance given frequent natural exposure and the increasing use of these polymers in biomedical applications.

Materials and Methods

Reagents and cell culture- All materials were obtained from Sigma-Aldrich unless otherwise stated. Ultrapure LPS (free of TLR2-stimulating lipopeptides) was treated with deoxycholate twice followed by phenol extraction and ethanol precipitation (Hirschfeld et al., 2000) to further purify the original Sigma-Aldrich stock (catalog # L2630). Chitosan was obtained from Primex (ChitoClear, high molecular weight shrimp chitosan, 76% deacetylated). Complete media is defined as RPMI 1640 media (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell culture was at 37°C in humidified air supplemented with 5% CO_2. All experiments were performed under conditions designed to minimize endotoxin contamination.

Cell populations -BMMΦ were generated as described (Johnson et al., 1983). Briefly, bone marrow was extracted from the femurs and tibiae of wild type C57BL/6 mice (The Jackson Laboratory). Cells were cultured in complete media supplemented with 10 ng/ml recombinant M-CSF (eBiosciences) and fed on days 4 and 7 with fresh media containing M-CSF. On day 8, non-adherent cells were washed away and the adherent macrophages were treated with 0.05% trypsin-EDTA, harvested and washed once in complete media before use in experiments. For M1/M2-like macrophages bone marrow was harvested and cultured as above for M2-like macrophages while M1-like macrophages were cultured with 5 ng/ml recombinant GM-CSF (Miltenyi Biotec) instead

of M-CSF (Fleetwood et al., 2009; Martinez et al., 2006). On day 8, the adherent macrophages were harvested as above and used in experiments. Bone marrow-derived dendritic cells (BMDCs) were generated as described (Huang et al., 2012). Briefly, bone marrow was extracted and cultured as described for M1-like macrophages except on day 8 non-adherent cells were collected for use in experiments. Resident peritoneal cells were harvested by lavaging the peritoneal cavity of C57BL/6 mice with 10 ml of PBS. Experimental protocols involving animals were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. Human PBMCs were isolated from the blood of adult, healthy donors under a protocol approved by the University of Massachusetts Medical School Institutional Review Board using Ficoll-Hypaque density centrifugation.

Chitosan digestion, purification, and conversion to chitin- Chitosan was cleaved by pepsin to reduce the polymer length, purified and converted to chitin as previously described (Bueter et al., 2011). Briefly, chitosan (250 mg) was dissolved in 25 ml 0.1 M sodium acetate, pH 4.5. Pepsin (Sigma, P7000) was added (100 U/ml) for 18 h at 37^{o}C to partially digest the chitosan (Roncal et al., 2007). This was followed by extraction with chloroform:isoamyl alcohol (24:1) then mixing the recovered aqueous layer with an equal volume of 12% potassium hydroxide and heating at 80^{o}C for 90 min. Precipitated chitosan was collected by centrifugation and washed 3x with water followed by PBS to neutralize. Half of the chitosan was converted to chitin by suspending in 20 ml 1.0 M sodium bicarbonate, followed by addition of 1 ml acetic anhydride (Acros) and

incubation at 22°C for 20 min with periodic mixing. The acetylation reaction was repeated, and terminated with heating at 100°C for 10 min. Chitin was collected by centrifugation and washed 3x with PBS. Chitin and chitosan suspensions were passed through a 100 μm filter (BD Falcon) to remove the largest particles then treated in 0.1 M sodium hydroxide at 22°C for 30 min, followed by washing with PBS and storage at 4°C.

Stimulation of IL-1β release- BMMΦ, M1-like macrophages, M2-like macrophages, DCs, or peritoneal cells were plated at 1 x 10^5 cells/well in a 96-well plate. PBMCs were plated at 5x10^6/well in 24 well plates and after 1 h, non-adherent cells were washed away. Mouse cells were primed with 100 ng/ml ultrapure LPS, while PBMCs were primed with 50 pg/ml ultrapure LPS for 3 h (control cells were left unprimed), followed by incubation with the stimuli for 6 h (18 h for PBMCs). Positive stimuli controls included silica (topsize 15 microns, US Silica, MIN-U-SIL-15, used as described (Hornung et al., 2008)), synthetic double stranded DNA: poly(dA:dT), ATP, and Streptolysin O (SLO) or SLO + flagellin (FLA-ST, InvivoGen). Supernatants were collected for cytokine measurement and assayed by IL-1β ELISA (eBiosciences).

Multiplex Assay- BMMΦ were plated as described above and stimulated for 6 h with 0.1 mg/ml chitin or chitosan. Supernatants were collected and analyzed by Bio-Plex Pro Assays (Bio-Rad).

Statistical Analysis- Data were analyzed and figures prepared using GraphPad Prism. Significance was assessed by 2-way ANOVA with Bonferroni post-hoc test, 1-way ANOVA with Dunnett post-hoc test, or Kruskal-Wallis 1-way ANOVA by ranks with

Dunn post-hoc tests, as indicated. *p*-values of <0.05 after correction for multiple comparisons were considered significant.

Preface to Chapter IV

A portion of this chapter has been published in the Journal of Biological Chemistry

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Chrono K. Lee performed BMMΦ isolations

Vijay A. Rathinam performed the westerns

Chelsea L. Bueter performed the rest of the experiments

Chelsea L. Bueter, Charles A. Specht and Stuart M. Levitz designed the experiments and wrote the manuscript

Chapter IV: Characterization of Chitin and Chitosan Particles

Abstract

Chitosan activates the NLRP3 inflammasome, while chitin does not. In an effort to understand the activation profiles of chitosan and chitin, particle size, solubility, phagocytosis and digestibility were examined. The size of the chitosan particles played an important role, with small particles eliciting the greatest activity. An inverse relationship between size and stimulatory activity was demonstrated using chitosan passed through size exclusion filters as well as with chitosan-coated beads of defined size. Partial digestion of chitosan with pepsin resulted in a larger fraction of small phagocytosable particles and more potent inflammasome activity. Inhibition of phagocytosis with cytochalasin D abolished the IL-1β stimulatory activity of chitosan, offering an explanation for why the largest particles were nearly devoid of activity. Thus, the deacetylated polysaccharide chitosan potently activates the NLRP3 inflammasome in a phagocytosis-dependent manner. In contrast, chitin is relatively inert. The reason for chitin's inability to elicit IL-1β is unknown, but it does not appear to be due to active inhibition of the inflammasome and while chitin appears to be more readily digested by macrophage cell lysates, it does not occur at a rate which would likely impact inflammasome activation.

Introduction

In chapter 3 chitosan was shown to be a strong activator of the inflammasome, while chitin was shown to be a weak stimulator. The purification procedure devised in chapter 2 allowed for a direct comparison of the two polymers, however beyond acetylation the particles remained uncharacterized. The major difference between chitin and chitosan come from the degree of acetylation. It is an extremely important component of chitin and chitosan and the response they garner. While chitin is considered acetylated and chitosan deacetylated, it is rare for a polymer to ever be fully one or the other, especially natural polymers. In fact, a mixture of acetylated and unacetylated residues is generally more immunostimulatory than a pure polymer: A mixture of 30% chitin and 70% chitosan was found to have a greater ability to induce macrophage activation that pure polymers of either chitin or chitosan or other ratios tested (Lee et al., 2008). The chitosan from primex was listed as 76% deacetylated, and after the acetylation procedure the chitin was about 7% deacetylated.

As stated previously the size of chitin and chitosan particles has been shown to be very important for the stimulation of other cytokines (Da Silva et al., 2009). It has also been reported that the texture of particles has a significant impact on inflammasome activation with textured particles inducing a much more robust IL-1β response than smooth particles (Vaine et al., 2013). Chitin and chitosan polymers are likely quite textured as the chains can have extensive branching.

In addition to the importance of size and texture of the particles, it has also been shown that phagocytosis of particulate inflammasome activators is important. There are multiple reports showing phagocytosis is necessary for inflammasome activation with particles such as silica, alum, and amyloid-β (Halle et al., 2008; Hornung et al., 2008). However, it has also been reported that particles too large to be phagocytosed induce "frustrated phagocytosis" which stimulates activation of NADPH oxidase and subsequent activation of the NLRP3 inflammasome (Dostert et al., 2008). Chitin and chitosan particles exist in a broad range of sizes, from nanoparticles able to enter a cell independent of phagocytosis, microparticles which would need to be phagocytosed, and finally particles too large to phagocytose that may induce "frustrated phagocytosis".

Assuming the particles have been phagocytosed, the question remains: what happens to the foreign polymers? Are they readily digested to subunits that are responsible (or not) for the inflammasome activation? Or are the particulates themselves the activators? Mammalian cells have developed a number of methods to degrade the polysaccharides chitin and chitosan. The most prominent being the chitinases: AMCase, chitotriosidase and hexosaminidase. While chitinases are not extremely efficient at degrading chitin, and really inefficient at degrading chitosan, AMCase overexpression resulted in an attenuated inflammatory response when challenged with chitin (Reese et al., 2007), suggesting that the chitinase was able to make a marked decrease on the chitin levels. In addition to chitinases, there are other enzymes proposed to have the ability to depolymerize chitosan such as: cellulose, lipase, amylase and pectinase (Aranaz et al.,

2009). Also both chitin and chitosan can be degraded by lysozyme, papain and pepsin, with the degradation kinetics inversely related to the degree of acetylation (Aranaz et al., 2009). Mammalian cells are capable of digesting these polymers, although it is unknown if the particulates or their digested products are involved in inflammasome activation.

In this chapter, the activating chitosan and the non-activating chitin are characterized. First the importance of small particles is shown through size fractionated preparations and 3 and 50 μM beads. However, too small or soluble chitosan is unable to activate. Supporting that the particles must be the proper size to be phagocytosed is shown with the use of cytochalasin D inhibition of phagocytosis blocking IL-1β release. Chitin was then examined for its inactivity, and found that it does not actively inhibit the inflammasome, it simply does not activate. Finally, the digestion profile of both polymers was analyzed. Chitosan remained undigested while chitin showed significant degradation, however at a slow enough rate to be unlikely to have any impact on inflammasome activation in the amount of time the assay took.

Results

IL-1β release is size-dependent

Next we examined the influence of particle size on the capacity of chitin and chitosan to stimulate IL-1β. Accordingly, we sonicated the chitosan and chitin preparations to generate smaller particles, and then size-fractionated the preparations by sequential passage through 100 µm and 20 µm filters. This resulted in fractions with predicted sizes of <20 µm, 20-100 µm, and >100 µm. These three fractions were then compared with the sonicated but unfractionated polysaccharides for their ability to stimulate IL-1β release from primed macrophages (Fig. 4.1A). Chitosan induced the most IL-1β from the <20 fraction, with the >100 fraction eliciting only low amounts of IL-1β. This suggests that smaller chitosan particles are primarily responsible for inducing IL-1β. The low amount of activity in the >100 fraction may have been due, at least in part, to the presence of some smaller particles that were retained by the filter despite washing. The photomicrographs in Fig 4.1B and C highlight the problems with the size fractions. The <20 µM fraction has aggregates larger than 20 µM and the 20-100 µM fraction has particles that are both larger than 100 µM and smaller than 20 µM. An additional problem with the size fractions was that the chitin induced some IL-1β, although it was greatly reduced compared with chitosan and the positive control alum. One possible explanation for this activity is that sonication broke apart large particles of chitin that had cores of chitosan that were inaccessible to the acetylation reaction. Upon sonication,

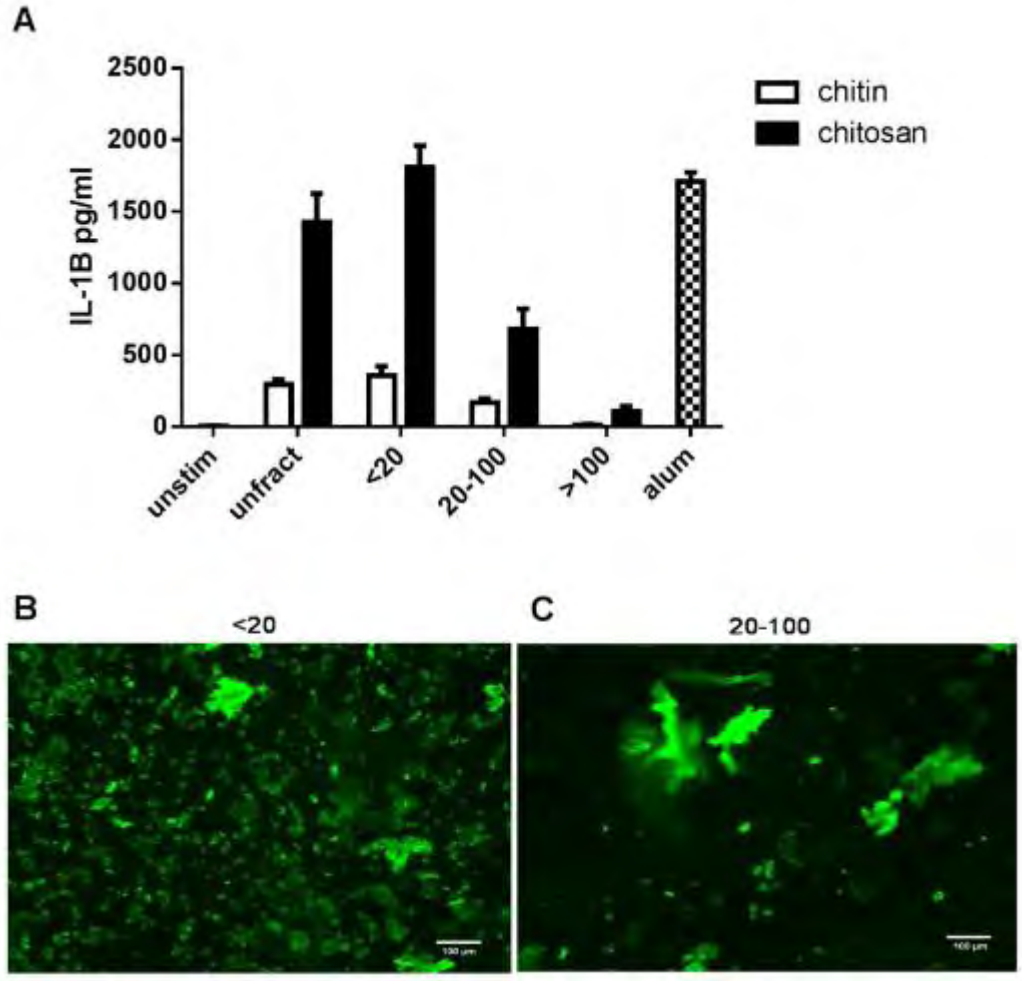


Figure 4.1. The effect of particle size on inflammasome activation.

A, Chitosan and chitin preparations prepared as in Figure 1 were sonicated and then size-fractionated through 100 μm and 20 μm filters. BMMΦ (1 x 10^5/well) were primed with LPS and then stimulated with chitosan or chitin particles (1 mg/ml) that were left unfractionated (unfract) or size-fractionated as indicated. IL-1β was analyzed by ELISA. Data are means ± SE of three independent experiments, each performed in triplicate. $p < 0.001$ comparing unfractionated chitosan to 20-100 chitosan and >100 chitosan fractions, and between the <20 chitosan fraction and the 20-100 and >100 chitosan fractions, analyzed by 2-way ANOVA. Photomicrographs of the <20 fraction of chitosan (B) and the 20-100 fraction of chitosan (C) stained with solophenol.

these particles were broken apart, exposing their inner chitosan which then were able to activate IL-1β.

In order to address the problems arose with the fractionation experiments and further study the influence of particle size, 3 and 50 μm polystyrene beads that were coated with chitin or chitosan were used. These provided a uniform particle size and glycan surface. Both the chitin and chitosan 3 μm beads were readily phagocytosed by macrophages (Fig. 4.2A & B) whereas the 50 μm beads were too large to be phagocytosed (data not shown). Uncoated beads and chitin-coated beads stimulated little IL-1β activity (Fig. 4.2C). However, the 3 μm beads coated with chitosan elicited a strong IL-1β response. None of the 50 μm beads, regardless of their surface, stimulated macrophage IL-1β release. These data provide further support for the concept that chitosan, but not chitin, potently stimulates IL-1β via a size-dependent mechanism.

Pepsin-digestion of Chitosan

As an alternative way to examine the effect of particle size, the ability of pepsin to partially digest chitosan was utilized (Roncal et al., 2007). Following the procedure outlined in Fig 4A, chitosan was digested with pepsin. Upon digestion, the thick, viscous chitosan solution became much less viscous, suggesting a successful digestion. After the digestion, the chitosan was chloroform extracted to remove the pepsin and other possible contaminants. The chitosan was then precipitated and half was converted to chitin. Photomicrographs comparing the glycans as prepared in figure 2.2A (NaOH) and the result of figure 4.3A (pepsin) show the NaOH preparation consists of many large

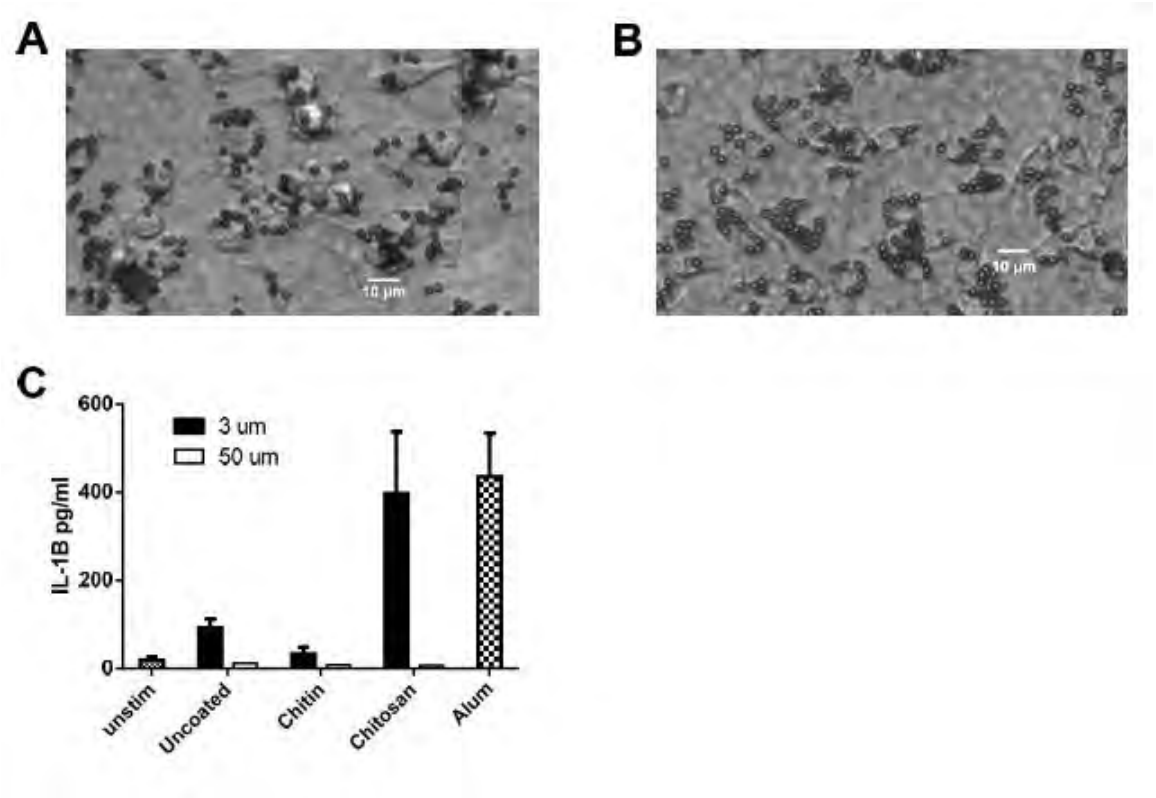


Figure 4.2. Phagocytosis and inflammasome activation by chitin and chitosan beads.

Representative photomicrographs of BMMΦ following 30 min incubation with 3 µm chitin-coated (A) and chitosan-coated (B) beads demonstrating robust phagocytosis of both types of glycan-coated beads. C, LPS-primed BMMΦ (1 x 10^5/well) were left unstimulated (Unstim) or incubated for 6 h with the indicated size and type of beads (1 mg/ml). Alum (1 mg/ml) served as a positive control. Supernatants were analyzed for IL-1β by ELISA. Data are means ± SE of three independent experiments, each performed in triplicate. $p < 0.01$ comparing 3 µm chitosan beads and 50 µm chitosan beads by 2-way ANOVA.

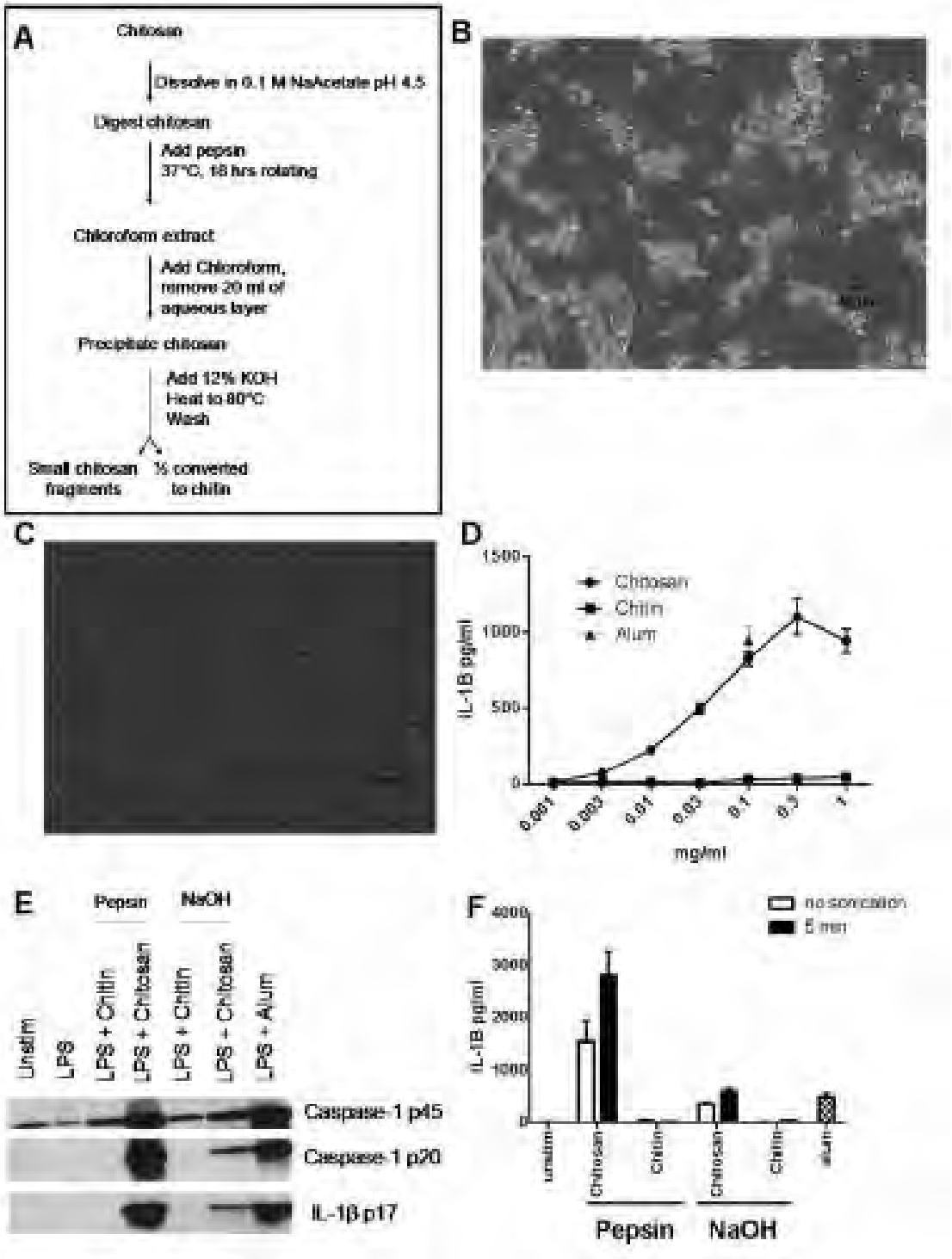

A
Chitosan
Dissolve in 0.1 M NaAcetate pH 4.5
Digest chitosan
Add pepsin
37°C, 18 hrs rotating
Chloroform extract
Add Chloroform,
remove 20 ml of
aqueous layer
Precipitate chitosan
Add 12% KOH
Heat to 80°C
Wash
Small chitosan fragments
½ converted to chitin
B
C
D
IL-1B pg/ml
1500
1000
500
0
Chitosan
Chitin
Alum
0.001
0.003
0.01
0.03
0.1
0.3
1
mg/ml
E
Pepsin
NaOH
Unstim
LPS
LPS + Chitin
LPS + Chitosan
LPS + Chitin
LPS + Chitosan
LPS + Alum
Caspase-1 p45
Caspase-1 p20
IL-1β p17
F
IL-1B pg/ml
4000
3000
2000
1000
0
no sonication
5 min
unstim
Chitosan
Chitin
Chitosan
Chitin
alum
Pepsin
NaOH

Figure 4.3. Effect of pepsin digestion of chitosan on inflammasome activation.

Following the procedure outlined in A, chitosan was digested with pepsin then half was converted to chitin. B, dose curve of the pepsin-treated chitin and chitosan stimulating BMMΦ (1 x 10^5/well) after they were primed for 3 h with 100 ng/ml LPS. Data are means ± SE of four independent experiments, each performed in triplicate. $P<0.01$ comparing chitin and chitosan at any concentration $\geq$ 0.1 mg/ml, as analyzed by unpaired t-test. C, BMMΦ (1.5 x 10^6/well) were primed for 3 h with 100 ng/ml LPS then stimulated with alum (0.1 mg/ml), or chitin and chitosan derived from the procedure outlined in Figure 4A (pepsin), or the procedure outlined in Figure 1A (NaOH). Supernatants were then collected and analyzed for caspase-1 and IL-1B by immunoblot. Caspase-1 p20 and IL-1B p17 represent the mature forms and indicate an active inflammasome, while capspase-1 p45 is an inactive pro-form of caspase-1. D, BMMΦ (1 x 10^5/well) were primed as in B then stimulated with alum or chitin and chitosan derived from the procedure outlined in Figure 4A (pepsin), or the procedure outlined in Figure 1A (NaOH). The chitin and chitosan preparations were left unsonicated (no sonication) or sonicated for 5 min (5 min). All stimuli were added at a concentration of 0.1 mg/ml. Supernatants were analyzed by ELISA for IL-1β. Data are means ± SE of three independent experiments, each performed in triplicate. $p < 0.001$ comparing no sonication and 5 min sonication of pepsin chitosan by 2-way ANOVA. Data are means ± SE of two independent experiments, each performed in triplicate. Data are means ± SE of two independent experiments, each performed in triplicate. $p < 0.01$ comparing insoluble with soluble chitosan by two-tailed unpaired t-test. Photomicrographs of the NaOH (E) and the pepsin (F) preparations, highlighting the size distributions.

particulates, whereas the pepsin preparation is composed of most small particles (Fig 4.3B and C). Both glycans were then assayed for their ability to activate the inflammasome by measuring release of IL-1β activity (Fig 4.3D). The digested chitosan was a potent IL-1β activator, with peak activity again seen at 0.3 mg/ml. Once again, chitin was nearly inert. Chitosan, but not chitin, stimulated cleavage of procaspase-1 to active caspase-1 and pro- IL-1β to the mature form of IL-1β (Fig 4.3 E).

Regardless of whether prepared as in figure 2.2A (NaOH) or figure 4.3A (pepsin), both chitin and chitosan particles aggregate when left to stand over time, although average size of the glycans produced through the method used in figure 4.3A was smaller. We next studied whether breaking up the aggregates with sonication affected the ability of the glycans to stimulate the inflammasome. Mild sonication in a horn sonicator did not significantly affect IL-1β release in response to the NaOH preparations, but there was a significant increase in IL-1β activity after sonication of the pepsin-digested chitosan preparation (Fig 4.3F). Neither chitin preparation induced more IL-1β after mild sonication.

Effect of solubilizing the chitosan

We next studied whether soluble chitin and chitosan hexamers stimulate the inflammasome. After a 6 h stimulation of LPS-primed BMMΦ with chitin and chitosan hexamers, IL-1β concentrations in the supernatants were below the limits of detection (Fig 4.4A). To examine whether the lack of stimulation by the chitosan hexamers was a general property of soluble chitosan, we solubilized chitosan by dissolving it in dilute

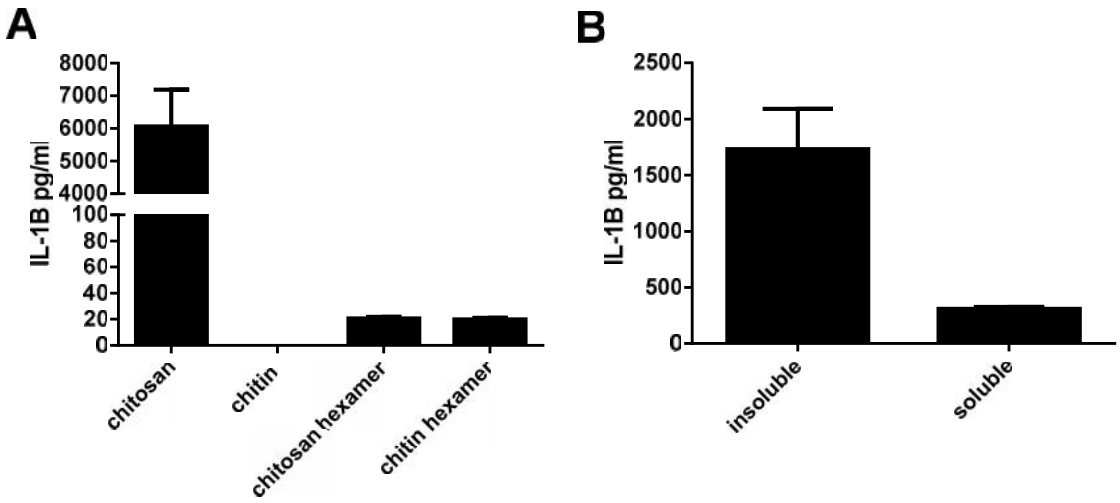


Figure 4.4. Effect of soluble chitosan on inflammasome activation.

BMMΦ (1 x 10^5/well) were primed as in B. A, insoluble suspended chitosan and chitosan that had been solubilized in acetic acid were diluted in media and added to cells. Supernatants were analyzed by ELISA for IL-1β. Data are means ± SE of two independent experiments, each performed in triplicate. $p < 0.01$ comparing insoluble with soluble chitosan by two-tailed unpaired t-test. B, BMMΦ were plated and primed as in Fig. Cells were then stimulated with chitosan, chitin, chitosan hexamer or chitin hexamer (all at 0.1 mg/ml) for 6 hrs. Supernatants were analyzed by ELISA for IL-1β. Data are means ± SE of one experiments performed in triplicate.

acetic acid. While the soluble chitosan induced IL-1β release, the levels were less than 20% that seen when insoluble particulate chitosan served as the stimulus (Fig 4.4B).

Effect of "complete" deacetylation

The primex chitosan is listed as 76% deacetylated. To analyze the effect of full deacetylation, the chitosan was suspended in 40% KOH for 18 h at 100 °C (Fig 4.5). There was no significant difference between the untreated chitosan and the chitosan that had undergone the deacetylation procedure.

Effect of cytochalasin D on IL-1β activation.

The inverse association of size of the chitosan particles with inflammasome activity suggests that phagocytosis is necessary for inflammasome activation. In order to test this supposition further, the effect of cytochalasin D, an inhibitor of actin polymerization and phagocytosis, on stimulated release of IL-1β was examined (Fig. 4.6). For the particulates, alum and chitosan, pretreatment of macrophages with cytochalasin D significantly reduced the amount of IL-1β produced. The small amount of IL-1β stimulated by the chitin was also inhibited by cytochalasin D. However, IL-1β production in response to soluble nigericin was unaffected. Taken together, the data demonstrate that phagocytosis is required for IL-1β activation by chitosan.

Chitin does not actively inhibit the inflammasome

The inability of chitin to activate the inflammasome may be because it does not activate the inflammasome or because it actively suppresses the inflammasome. Macrophages

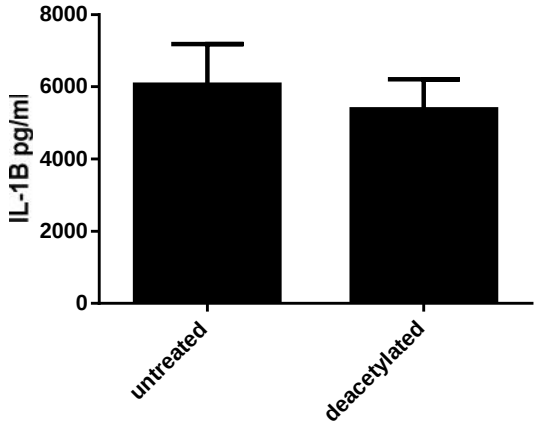


Figure 4.5. Effect of "complete" deacetylation on inflammasome activation.

BMMΦ were plated and primed as above, then stimulated with untreated chitosan or deacetylated chitosan (0.1 mg/ml). Deacetylated chitosan was suspended in 40% KOH for 18 h at 100 °C. Supernatants were then collected and analyzed by ELISA. Data are means ± SE of one experiment performed in triplicate.

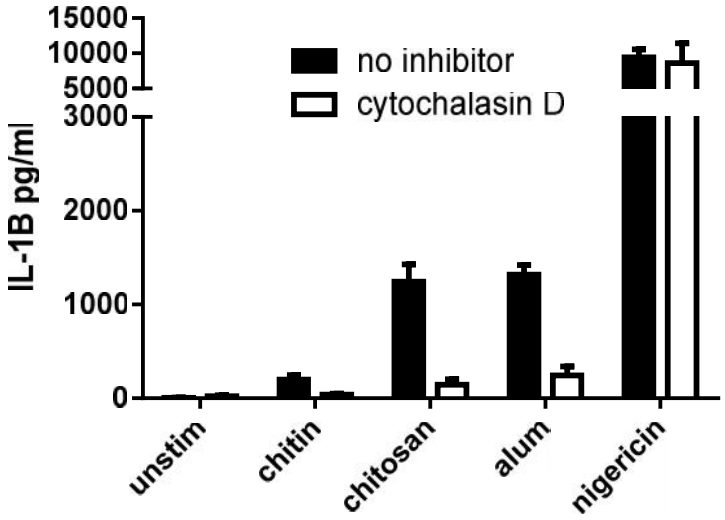


Figure 4.6. Inhibition of phagocytosis blocks inflammasome activation.

BMMΦ (1 x 10^5/well) were primed for 3 h with 100 ng/ml LPS. The BMMΦ were treated with 1 μg/ml cytochalasin D to inhibit phagocytosis 10 min prior to addition of stimuli. After 6 h stimulation with nigericin (2.5 μM), alum (0.1 mg/ml), chitin (0.1 mg/ml) and chitosan (0.1 mg/ml), supernatants were collected and analyzed by ELISA. Data are means ± SE of three experiments performed in triplicate. $P<0.001$ comparing cytokine concentrations with and without cytochalasin D following stimulation with alum and chitosan, analyzed by two-tailed unpaired t-test.

were preincubated with 0.1 mg/ml pepsin-digested chitin and then stimulated with alum, chitosan or nigericin (Fig 4.7). Chitin was unable to inhibit IL-1β release by any of these stimuli, showing chitin does not actively suppress the inflammasome but simply doesn't activate.

Digestion of chitosan and chitin hexamers

To analyze the fate of chitin and chitosan after uptake, crude lysates of BMMΦ were incubated with hexamers of chitosan (Fig 4.8A) or chitin (Fig 4.8B). There was no digestion seen of the chitosan hexamer at any time point tested, suggesting the BMMΦ lysates were unable to degrade the polymer. However, the chitin hexamer showed evidence of digestion in as little as 1 hour, and by 24 hours much of the hexamer had been digested. The enzymes present in the lysates were much more readily able to digest the chitin than the chitosan.

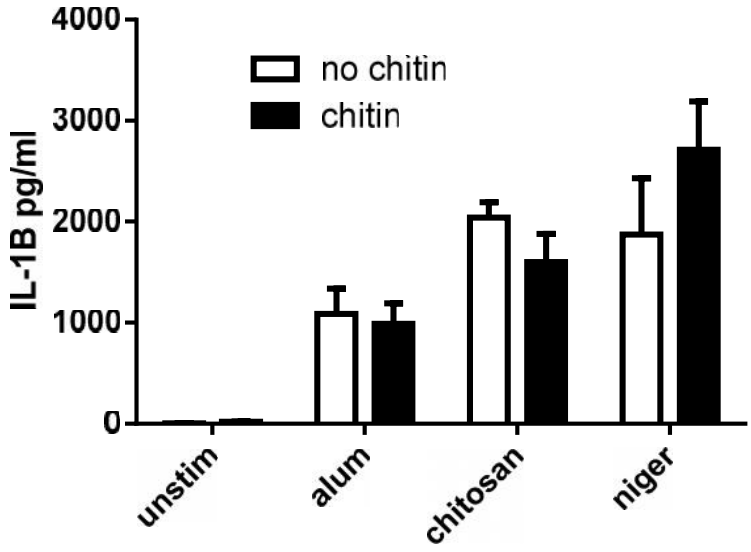
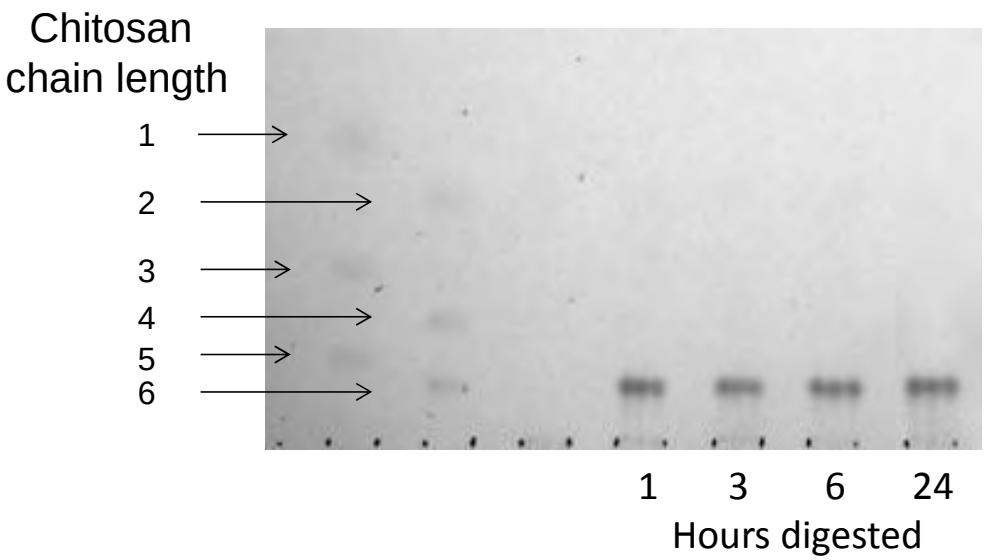


Figure 4.7. Chitin does not inhibit inflammasome activation.

BMMΦ (1 x 10^5/well) were primed as in B. Two h later, wells either received 0.1 mg/ml chitin or were left without chitin treatment (no chitin). One h later, cells were left unstimulated (unstim) or stimulated for 6 h with alum (0.1 mg/ml), chitosan (0.1 mg/ml) or nigericin (2.5 μM). Supernatants were analyzed by ELISA for IL-1β. Data are means ± SE of two independent experiments, each performed in triplicate. Data are means ± SE of two independent experiments, each performed in triplicate. $p < 0.01$ comparing insoluble with soluble chitosan by two-tailed unpaired t-test.

A


Chitosan
chain length
1
2
3
4
5
6
1 3 6 24
Hours digested

B

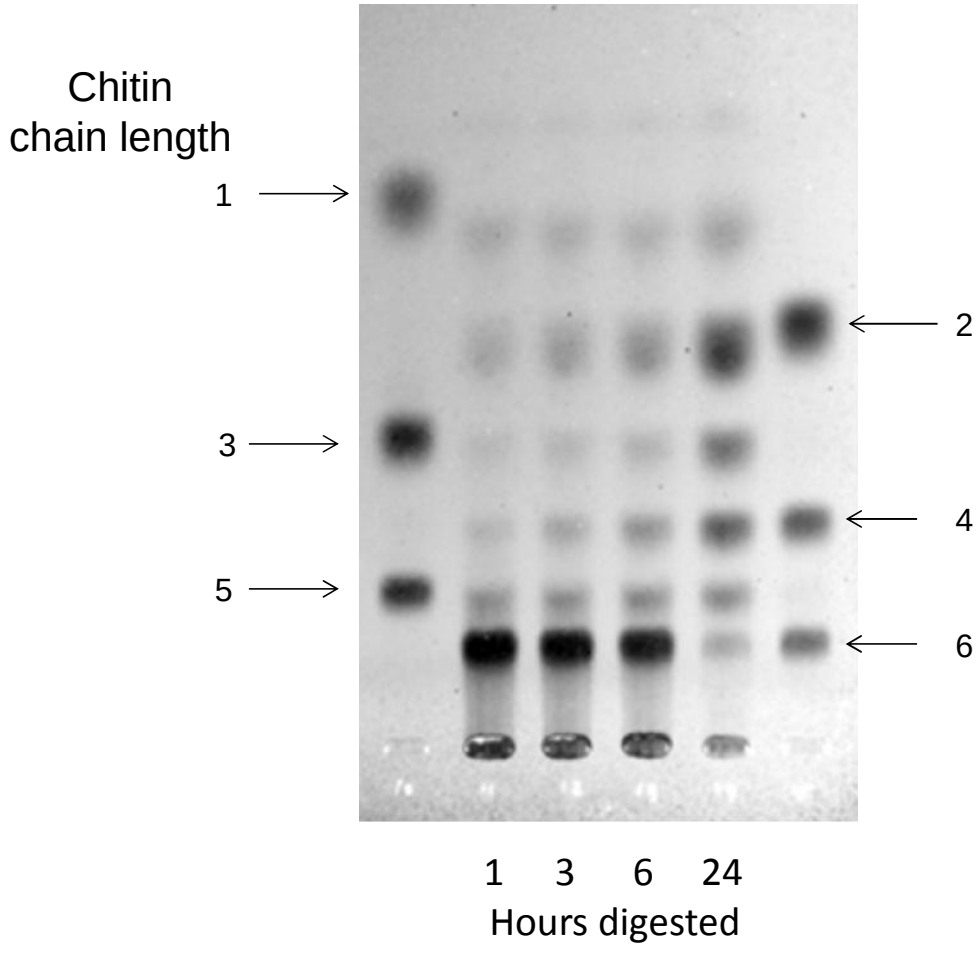

Chitin
chain length
1
2
3
4
5
6
1 3 6 24
Hours digested

Figure 4.8. Digestion of chitin and chitosan hexamers by bone marrow lysates.

BMMΦ lysates were generated via sonication then incubated with 1 mg/ml chitosan hexamer (A) or 1 mg/ml chitin hexamer (B) for 1, 3, 6, or 24 hours. They were then run on a TLC gel.

Discussion

In addition to contaminants, disparities in the size of the chitin and chitosan preparations may account for some of the seemingly contradictory results reported in the literature. In a prior study, particle size was reported to impact the capacity of chitin to stimulate macrophage TNFα and IL-10 production (Da Silva et al., 2009). However, these studies showed chitin was a poor activator of the inflammasome regardless of particle size. In contrast, size had a major influence on the immunostimulatory properties of chitosan.

Several lines of evidence support the inverse relation between size and the ability of chitosan to stimulate the inflammasome. First, when the particulate glycans were passed through filters of defined size, the smallest size fraction (<20 μm) induced the most cleavage of pro-IL-1β and release of the mature cytokine. The larger sized fractions also had some bioactivity, which may have been due to some smaller particles that failed to pass through the filters. Second, when macrophages were challenged with chitosan-coated beads, the 3 μm but not the 50 μm diameter chitosan beads were stimulatory. Third, partial digestion of chitosan with pepsin boosted the ability of the glycan to activate the inflammasome. Finally, mild sonication, which broke up aggregated particles, resulted in a boost in the IL-1β signal.

Generally, small particles or soluble compounds have been found to be the best activators of the inflammasome, although inflammasome stimulation following "frustrated phagocytosis", defined as the process whereby phagocytes attempt to

phagocytose particles too large to be ingested, has been described (Dostert et al., 2008). With chitosan though, inflammasome activation did not occur via frustrated phagocytosis, as the 50 µm chitosan-coated beads were not stimulatory. Rather phagocytosis appeared to be required for inflammasome activation as particles that were small enough to be phagocytosed were the best activators. Moreover, treatment of macrophages with cytochalasin D, which inhibits phagocytosis, abolished chitosan-induced IL-1β release. Similarly, for other particulate activators of the inflammasome, inhibition of phagocytosis also abrogates inflammasome activation (Dostert et al., 2008; Sharp et al., 2009).

In addition to a requirement that chitosan particles be small enough to be phagocytosed, optimal inflammasome stimulation required that the chitosan be in a particulate form. Soluble chitosan hexamers failed to stimulate IL-1β release while soluble chitosan stimulated greatly reduced amounts of IL-1β compared with particulate chitosan. An analogous situation exists for β-glucans where Dectin-1 signaling and cytokine release is activated by particulate, but not soluble, β-glucans (Goodridge et al., 2011). Peptidoglycan has also been shown to need to be particulate to activate (Shimada et al., 2010). Taken together, these data emphasize that the level of stimulation seen with glycans will vary as a function of their physicochemical properties, including size, solubility and tertiary structure.

Full deacetylation of a chitosan polymer has not been shown. In fact, the maximum degree of acetylation achieved was only 93%, and there is also polymer

degradation under the conditions needed for deacetylation to occur (Chang et al., 1997). As the degree of deacetylation was not confirmed as complete, the "fully" deacetylated chitosan polymer may not be fully deacetylated. No real conclusions can be drawn from this polymer, especially since the deacetylation process also results in polymer degradation which will change the polymer length perhaps to an extent that would affect activation. Another caveat of the deacetylation process is there was a distinct color change after the deacetylation process. The coloration is likely due to the presence of hydroxymethyl-furfurals which accumulate when chitosan is subjected to high temperatures and cause discoloration (Rajpal, 2007). The presence of furfurals has an unknown effect on inflammasome activation.

The inflammasome is an important component of the immune response to fungal infections. IL-1β has been shown to be essential for host defenses against fungal pathogens (Netea et al., 2004), and several fungal pathogens have been shown to activate the NLRP3 inflammasome (Gross et al., 2009; Hise et al., 2009; Kumar et al., 2009). Our data suggest that cell wall chitin is unlikely to contribute greatly to the IL-1β release seen in response to fungal stimulation. A more likely stimulator is β-glucans, which are abundant components of the fungal cell wall and have recently been shown to be activators of the NLRP3 inflammasome (Kumar et al., 2009). While a mutant strain of *C. albicans* with reduced chitin content stimulated less IL-1β release compared to the wild-type parent (van de Veerdonk et al., 2009), compensatory structural changes in the cell wall could have been responsible for the results. However, for those fungi such as *C.*

neoformans that contain significant amounts of chitosan, that glycan could contribute to inflammasome activation. While chitin and chitosan are part of the inner cell wall and therefore not surface exposed, following phagocytosis and phagolysosomal fusion, digestion by lysozyme and chitinase could result in release of fragments of chitin and chitosan.

Chitosan has been demonstrated to have adjuvant properties, leading some to propose its use as a vaccine adjuvant (Kang et al., 2006; McNeela et al., 2004; Read et al., 2005; van der Lubben et al., 2003; van der Lubben et al., 2001). If future studies determine that chitosan's adjuvant properties are inflammasome-dependent, then formulations consisting of particles small enough to be phagocytosed would likely lead to maximum effectiveness. Interestingly, the commonly used adjuvant, alum, also stimulates the NLRP3 inflammasome (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008), although recent studies have suggested that the ability of alum to activate the inflammasome is not required for its adjuvanticity (Flach et al., 2011). Chitosan has also been utilized to encapsulate DNA (Borchard, 2001) and the primary amines of chitosan can be exploited to conjugate antigens, thus allowing direct delivery into cells. The efficiency of polycation/DNA particles relies on endosomal swelling and rupture to provide an escape mechanism (Behr, 1997). Conversely, in biomedical applications where inflammatory responses are not desired, such as in bioprostheses, our data suggest that non-phagocytosable (e.g., >50 μM) chitosan and/or acetylation to chitin should be considered.

The mechanistic basis for why chitosan activates the inflammasome but chitin does not is speculative. Chitin does not play an inhibitory role as incubation of macrophages with chitin does not prevent IL-1β release by known activators of the inflammasome. While chitosan is charged due to its free amine, the presence of N-acetylation results in chitin lacking charge. Thus, activation could be dependent, at least in part, on a charge-charge interaction, although a large variety of compounds activate the NLRP3 inflammasome, including β-glucan, which is uncharged (Kumar et al., 2009). Another possible explanation for inflammasome activation by chitosan but not chitin may be related to differences that occur in the phagolysosome after uptake. While chitin and chitosan particles are readily phagocytosed, the environment of the phagolysosome is likely to have very different effects on the two glycans. Acid soluble chitosan may become soluble in the phagolysosome, whereas chitin will remain particulate. Conversely, mammalian white blood cells contain chitinases and lysozyme (Boot et al., 2001; Escott and Adams, 1995) that can act on both chitosan and chitin, but are more effective on chitin (Gorzelanny et al., 2010; Hirano et al., 1989). These properties may better enable chitosan to translocate from the phagolysosome to the cytosolic compartment and activate the NLRP3 inflammasome.

Lysozyme able to hydrolyze β(1-4) linkages between GlcNac and GlcN, which leads to greater degradation of chitin as it has a greater number of GlcNac residues (Han et al., 2012). This is likely why the pure chitosan hexamer was not digested by the lysozyme in the BMMΦ lysates. Although if a GlcNac residue and a GlcN residue is

required for lysozyme to hydrolyze the linkage, the pure chitin hexamer was unlikely to be digested by the lysozyme either.

In summary, it was demonstrated that chitosan potently activates the inflammasome while chitin does not. Moreover, stimulation of IL-1β release by chitosan is dependent on both phagocytosis and assembly of the NLRP3 inflammasome. It is possible that the inflammasome response elicited by exposure to naturally occurring versions of these glycans may vary, due to the variable degrees of acetylation found in natural chitin and chitosan. However, our findings have important implications for the formulation of chitin and chitosan for use in biomedical applications, both in situations where an inflammatory response is desirable (e.g., enhancing adjuvanticity) and in those where it is not (e.g., bioprostheses). Finally, our improved methodology for purification of chitin and chitosan will be useful to those studying or preparing these ubiquitous glycans.

Materials and Methods

Reagents and cell culture- All materials were obtained from Sigma-Aldrich unless otherwise stated. Ultrapure LPS (free of TLR2-stimulating lipopeptides) was purified from the original Sigma stock (L2630) by two treatments with deoxycholate followed by phenol extraction and ethanol precipitation (Hirschfeld et al., 2000). Chitosan (76% deacetylated) was obtained from Primex. Chitin and chitosan hexamers were purchased from Associates of Cape Cod and used at a final concentration of 0.1 mg/ml. Complete media is defined as RPMI 1640 media (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell culture was at 37°C in humidified air supplemented with 5% CO_2. All experiments were performed under conditions designed to minimize endotoxin contamination.

Bone marrow-derived macrophages- Bone-marrow derived macrophages (BMMΦ) were generated as described (Johnson et al., 1983). Briefly, bone marrow was extracted from the femurs and tibiae of WT C57BL/6 mice (The Jackson Laboratory). Cells were cultured in complete media supplemented with supernatant from macrophage colony–stimulating factor (M-CSF)-secreting L929 fibroblasts at a final concentration of 20% and fed on days 4 and 7 with fresh media containing M-SCF. On day 8, macrophages were treated with 0.05% trypsin-EDTA, harvested and washed once in complete media before use in experiments.

Stimulation of IL-1β- BMMΦ were plated 1 x 10^5 cells/well in a 96-well plate. Cells were primed with 100 ng/ml ultrapure LPS for 3 h (control cells were left unprimed), followed by incubation with the stimuli for 6 h. Stimuli controls were alum (Imject), synthetic double stranded DNA: poly(dA:dT), and nigericin. Supernatants were collected for cytokine measurement, assayed by IL-1β ELISA (eBiosciences). Cytochalasin D (1 mg/ml stock solution dissolved in chloroform) was added 30 min prior to stimuli addition for the phagocytosis inhibition assays and utilized at a final concentration of 1 µg/ml.

Immunoblot analysis- BMMΦ were plated at 1.5 x 10^6 cells/well in a 12-well plate. Cells were primed with 200 ng/ml LPS for 3 h, followed by incubation with the indicated stimuli for 6 h. Supernatants were collected and proteins precipitated by methanol-chloroform extraction as described (Hornung et al., 2008). Immunoblot analysis was performed as described (Hornung et al., 2008) using anti-mouse caspase-1 (clone 5B10; eBioscience) and anti-mouse IL-1β (AF-401-NA; R&D Systems).

Chitosan digestion, purification, and conversion to chitin- Chitosan was cleaved by pepsin to reduce the polymer length, purified and converted to chitin as previously described (Bueter et al., 2011). Briefly, chitosan (250 mg) was dissolved in 25 ml 0.1 M sodium acetate, pH 4.5. Pepsin (Sigma, P7000) was added (100 U/ml) for 18 h at 37^{o}C to partially digest the chitosan (Roncal et al., 2007). This was followed by extraction with chloroform:isoamyl alcohol (24:1) then mixing the recovered aqueous layer with an equal volume of 12% potassium hydroxide and heating at 80^{o}C for 90 min. Precipitated chitosan was collected by centrifugation and washed 3x with water followed by PBS to

neutralize. Half of the chitosan was converted to chitin by suspending in 20 ml 1.0 M sodium bicarbonate, followed by addition of 1 ml acetic anhydride (Acros) and incubation at 22°C for 20 min with periodic mixing. The acetylation reaction was repeated, and terminated with heating at 100°C for 10 min. Chitin was collected by centrifugation and washed 3x with PBS. Chitin and chitosan suspensions were passed through a 100 µm filter (BD Falcon) to remove the largest particles then treated in 0.1 M sodium hydroxide at 22°C for 30 min, followed by washing with PBS and storage at 4°C.

Size fractionation- Chitosan and chitin particles in PBS were subjected to 3 rounds of sonication using a microtip probe at 30% power for 5 mins in PBS. Particles were first filtered through a 100 µm nylon mesh basket filter (BD Falcon). Particles that did not pass through that filter were collected for the >100 µm fraction. The filtrate was then further fractionated through a 20 µm nylon mesh filter (Millipore) to create the <20 µm fraction. Particles retained on the filter were collected and designated the 20-100 µm fraction. Uncoated, chitin-coated and chitosan-coated polystyrene beads with diameters of 3 µm and 50 µm were from Micromod and New England Biolabs. The chitosan beads were converted to chitin beads with acetic anhydride. Briefly, the beads were washed sequentially with water:methanol; first with 70:30 (v/v), then 50:50, then 30:70, and finally 0:100. An equal volume of acetic anhydride:methanol (50:50, v/v) was added to the beads followed by rocking for 2h at 22°C. The beads were washed in reverse order with the water:methanol mixtures and finally 5 times with water. All beads were stored at 4°C.

Soluble chitosan- Chitosan that had been digested with pepsin as above was dissolved in 10 mM acetic acid (2 mg/ml), then diluted 1:100 in complete media and immediately added to cells at a final concentration of 0.01 mg/ml. Undissolved (insoluble) chitosan was used as a control.

"Complete deacetylation"- Chitosan that had been digested with pepsin as above was suspended in 40% KOH for 18 h at 100 °C. After 18 h the chitosan had turned from white to a more browned color which remained through multiple washes with PBS.

Sonication- Where indicated, chitin and chitosan preparations were suspended in 200 µl PBS at a concentration of 10 mg/ml in 1.5 ml microcentrifuge tubes. The tubes were then sonicated for 5 min using a horn sonicator (S-4000, Misonix Inc) at 20% amplitude.

Digestion of hexamers with bone marrow lysates- 10^6 BMMΦ were lysed using a horn sonicator (S-4000, Misonix Inc). Chitin and chitosan hexamers were then incubated with the lysates for 1, 3, 6, or 24 hrs. TLC was then used to analyze the digestion profile, the samples along with standards (5 µl) were spotted on a silica gel 60 glass-backed plate (EMD Chemicals) and developed using n-butanol: ethanol: water: acetic acid (5:4:3:1). Standards were GlcNAc and GlcN at various concentrations ranging from 1-20 mM. Plates were sprayed with 30% (w/v) ammonium hydrogen sulfate (Acros Organics) in water and then baked at 140^{o}C for 30 min. The spraying and baking were repeated two more times (Gal, 1968). Visualizing the separated sugars was done with epi-UV illumination and a FluorChem HD2 digital imaging system (Alpha Innotech). Quantifications of the digital images were done with ImageJ (imagej.nih.gov).

Statistical Analysis- Data were analyzed using GraphPad Prism. Significance was assessed by either 2-way-ANOVA or two-tailed unpaired t test as indicated. p-values of <0.05 after the Bonferroni correction were considered significant.

Preface to Chapter V

Chrono K. Lee performed BMMΦ isolations

Chelsea L. Bueter performed the experiments

Chelsea L. Bueter, Charles A. Specht and Stuart M. Levitz designed the experiments

Chapter V: Mechanisms of NLRP3 Activation by Chitosan

Abstract

Chitosan activates the NLRP3 inflammasome. As the NLRP3 inflammasome is activated by such a wide variety of stimuli, direct interaction between NLRP3 and individual stimuli is unlikely to occur. Activation of this inflammasome likely occurs through indirectly. There are three proposed mechanisms for NLRP3 inflammasome activation: K^+ efflux, ROS, and lysosomal destabilization. The K^+ efflux pathway was blocked with the inhibitor glibenclamide or high extracellular potassium. The ROS pathway was blocked with the inhibitors Mito-TEMPO and DPI. Finally, the lysosomal destabilization pathway was blocked by blocking acidification through use of Bafilomycin A and chloroquine or direct inhibition of cathepsin B by CA-074-me. It was revealed that each of these pathways participated in optimal NLRP3 inflammasome activation by chitosan. The role of CFTR was also analyzed as it has been proposed to be necessary for phagosomal acidification, and although the CFTR-inh showed reduced IL-1β activity, the CFTR KO showed no phenotype. All three proposed mechanisms for NLRP3 are shown have a role in chitosan activation of the inflammasome.

Introduction

The NLRP3 inflammasome has been shown to be activated by a wide variety of stimuli such as particulates including alum, amyloid β, cholesterol and silica (Dostert et al., 2008; Grebe and Latz, 2013; Li et al., 2008), soluble stimuli such as ATP and the pore-forming toxin nigericin (Mariathasan et al., 2006), and bacterial, fungal, and viral pathogens such as *Stapholococcus aureus*, *L. monocytogenes*, *C. albicans*, and Influenza A virus (Allen et al., 2009; Gross et al., 2009; Mariathasan et al., 2006). This variation in NLRP3 activating particles makes direct interaction between NLRP3 and its various activators extremely unlikely. The NLRP3 inflammasome is instead proposed to act as a sensor of cytosolic stress induced by the various activators. This theory has led to the development of three proposed, non-mutually exclusive, mechanisms for NLRP3 inflammasome activation: K^+ efflux, reactive oxygen species (ROS), and lysosomal destabilization

The K+ efflux model

K^+ efflux has been shown to be required for NLRP3 inflammasome activation by many different stimuli. Some of the most potent NLRP3 inflammasome inducers are in fact K^+ channels: nigericin, gramicidin, maitotoxin and α-toxin (Petrilli et al., 2007), highlighting the importance of K^+ efflux on NLRP3 activation. Additionally, stimuli such as MSU that induce macrophage cell swelling results in a decrease in intracellular K+ concentration and NLRP3 activation (Compan et al., 2012). However, K^+ efflux alone is not enough to activate (Pelegrin and Surprenant, 2009). The K^+ efflux model

was first described for ATP. Extracellular ATP activates P2X7, the ATP-gated ion channel, thereby triggering rapid K^+ efflux (Ferrari et al., 2006) and recruitment of the pannexin 1 hemichannel (Kanneganti et al., 2007). Involvement of pannexin-1 led to the theory that the creation of pores allowed the NLRP3 stimuli access to the cytoplasm whereby they could potentially interact directly with NRLP3. However now, given the variety of stimuli known to activate NLRP3, direct interaction between NLRP3 and its stimuli is unlikely.

Instead of activation through direct interaction with stimuli, NLRP3 activation may occur by sensing the K^+ efflux itself somehow. Although this mechanism does not appear to be unique to NLRP3, as there is evidence that NLRP1 requires K^+ efflux for activation (Muruve et al., 2008), NLRC4 activators such as *S. typhimurium* are able to activate NLRC4 independently of K^+ efflux (Lamkanfi et al., 2009).

The ROS model

The second proposed mechanism of activation involves NLRP3 acting as general sensor of cellular stress by recognizing and being activated by ROS. This mechanism appears to be important for all NLRP3 stimuli as so far, all stimuli tested have shown ROS involvement in their activation of the NLRP3 inflammasome (Tschopp and Schroder, 2010). Inhibitors of ROS or ROS scavengers strongly inhibit NLRP3 activation (Cassel et al., 2008; Dostert et al., 2008; Liao et al., 2013). However, understanding of how exactly ROS activates NLRP3, or even the source of the activating ROS, has not yet been established.

There is debate on what type of ROS is important for NLRP3 activation, as both NADPH-derived ROS and mitochondrial ROS have been shown to be important. Evidence for NADPH-derived ROS has been shown with knock-downs of the $p22^{phox}$ subunit of the NOX 1-4 subfamily of NADPH oxidases, or $gp91^{phox}$, another essential component of NADPH oxidase, both instances resulting in impaired NLRP3 activation (Dostert et al., 2008; Liao et al., 2013). Conversely, it has been shown that macrophages deficient in NOX 1, 2, or 4 respond normally to inflammasome stimuli (Zhou et al., 2011). There are also multiple studies using cells from chronic granulomatous disease (CGD) patients, a disease which exhibits defects in NADPH oxidase, but these cells show no NLRP3 defects (Meissner et al., 2010; van Bruggen et al., 2010; van de Veerdonk et al., 2010). There appears to be evidence both for and against NADPH oxidase-derived ROS having an important role in NLRP3 inflammasome activation.

Recently mitochondrial ROS has been suggested to be the important ROS for NLRP3 inflammasome activation. While the bulk of mitochondrial ROS production occurs through the electron transport chain as a by-product of respiration (Kowaltowski et al., 2009), multiple reports have shown mitochondria being central to NLRP3 activation (Nakahira et al., 2011; Zhou et al., 2011). After stimulation NLRP3 colocalizes with ER and mitochondria at mitochondria-associated ER membranes (MAMs) (Zhou et al., 2011). This recruitment to mitochondria is dependent on the mitochondrial antiviral signaling protein (MAVS) (Subramanian et al., 2013). It has also been shown that inhibition of voltage-dependent ion channel (VDAC) 1 and 2, which are

important regulators of mitochondrial metabolic activity and ultimately ROS production, results in impaired IL-1β release and highly diminished mitochondrial ROS (Zhou et al., 2011). Autophagy is also implicated in this pathway, as a deficiency in autophagy results in a greater abundance of damaged mitochondria and consequently mitochondrial ROS and correlates with caspase-1 activation (Nakahira et al., 2011).

ROS activation of the NLRP3 inflammasome is not well understood, however there are a few proposed mechanisms. The first proposed mechanism relies on the thioredoxin-interacting protein (TXNIP), which is constitutively bound and inhibited by oxidoreductase thioredoxin (TXN). The presence of TXN pushes macrophages towards an M2, anti-inflammatory phenotype (El Hadri et al., 2012). An increase in cellular ROS induces dissociation of TXNIP from TXN, thereby allowing TXNIP to bind NLRP3 and thereby activate (Franchi et al., 2009b; Zhou et al., 2010). Another proposed mechanism relies on mitochondrial ROS oxidizing mitochondrial DNA. In this activation mechanism the oxidized mitochondrial DNA acts as a NLRP3 agonist, as it has been shown to bind NLRP3 thereby activating the inflammasome (Shimada et al., 2012).

The lysosomal destabilization model

The final proposed model involves phagocytosed particulate stimuli. After internalization, phagosomal maturation and lysosomal fusion, ultimately phagosomal destabilization and lysosomal rupture occur. This rupture may be due to inefficient clearance of activating particles or from phagocytosis of particles with jagged edges puncturing a hole in the lysosomal membrane. Or in the case of phagocytosed, live

organisms, phagosome rupture may also be caused by a mechanism enabling escape from the lysosome such as the formation of fungal hyphae after phagocytosis causing membrane disruption and phagosome escape (Joly et al., 2009), or *Mycobacterium tuberculosis* ESAT-6, an ESX-1 substrate implicated in membrane damage (Mishra et al., 2010). Upon destabilization of the lysosome, the stimuli as well as the lysosomal contents are released. Among them, the lysosomal protein cathepsin B has been shown to be particularly important, somehow inducing the activation of the NLRP3 inflammasome (Hornung et al., 2008). However, it is uncertain whether cathepsin B is the sole activator as knock-down studies have shown conflicting results (Dostert et al., 2009; Halle et al., 2008), suggesting more than one cathepsin may be involved.

Prior to release of lysosomal contents, acidification of the lysosome is required (Hornung et al., 2008). Blocking lysosomal acidification completely abrogates the release of IL-1β in response to a number of phagocytosed stimuli such as silica or alum (Hornung et al., 2008). This acidification process is important for activation of the lysosomal proenzyme cathepsin B (Hornung et al., 2008). Also supporting the importance of lysosomal acidification in inflammasome activation, it has been shown that caspase-1 localizes to phagosomes where it modulates pH buffering by NOX2, counteracting the inhibitory effect of intracellular gram-positive bacteria that actively neutralize the pH of the phagosomes they are in, such as *S. aureus*, and allowing for inflammasome activation (Sokolovska et al., 2013).

In addition to blocking lysosomal acidification by inhibiting the vaculoular ATPase with Bafilomycin A or by adding chloroquine , a weak base that collects in acidic compartments and raises pH, there is evidence that the cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR), which is localized to endosomes (Lukacs et al., 1992), is also important in lysosomal acidification (Deriy et al., 2009; Di et al., 2006; Gottlieb and Dosanjh, 1996). Mutation in CFTR lead to alveolar macrophages with lysosomes that fail to acidify and are therefore unable to kill internalized bacteria (Deriy et al., 2009; Di et al., 2006). The role of the inflammasome in cystic fibrosis is relatively untested, however the lysosomal acidification defect may suggest an inflammasome defect as well, thereby exacerbating disease in cystic fibrosis patients.

Combining all three models

In an effort to combine all three mechanisms, mitochondria have increasingly been shown to be central to NLRP3 inflammasome activation. They are complex regulators of cytosolic homeostasis, sensing and responding to changes in intracellular K^+ and ROS. As perturbation of intracellular ROS, K^+ or lysosomal stability can result in mitochondrial dysfunction and apoptosis, this positions mitochondria as a potential control hub for the integration of the diverse signals sensed by the NLRP3 inflammasome (Shimada et al., 2012). A potential mechanism combing the K^+ efflux model and the ROS model suggest that the loss of mitochondrial membrane potential involved in NLRP3 activation may be triggered by K^+ efflux functioning upstream of inflammasome

assembly (Shimada et al., 2012). K^+ transport has three roles in mitochondria: volume homeostasis to prevent excess matrix swelling, volume homeostasis to prevent excess matrix contraction, and cell signaling (via regulation of mitochondrial ROS production) (Garlid and Paucek, 2003). Also ROS generation is frequently accompanied by K^+ efflux, one may trigger the other, combining these two proposed mechanisms (Kowaltowski et al., 2009). Also supporting the importance of mitochondria, autophagy KO have an accumulation of swollen/injured mitochondria and mitochondrial superoxide anion radical (0_2^-) which correlates with increased amounts of cleaved caspase-1 and IL-1β (Nakahira et al., 2011).

The mechanisms by which chitosan activates the NLRP3 inflammasome was analyzed. Here it was shown that all three mechanisms: K^+ efflux, ROS, and lysosomal destabilization have an important role.

Results

Importance of K^+ efflux chitosan stimulated IL-1β release

To understand how chitosan stimulates the NLRP3 inflammasome, we analyzed each of the three proposed mechanisms mentioned above. To examine the contribution of cellular K^+ efflux, we blocked K^+ efflux using the K^+ ion channel inhibitor glibenclamide (Fig. 5.1A). The release of IL-1β in response to both chitosan and silica was significantly inhibited in a dose-dependent manner by glibenclamide. However, the NLRC4 inflammasome activator flagellin (delivered to the cytosol by SLO) still induced IL-1β release even at the highest dose of inhibitor tested. To further confirm a role for K^+ efflux, we destroyed the gradient required for K^+ efflux by replacing the extracellular media with a buffer containing 150 mM K^+, which is approximately equal to the intracellular K^+ concentration (Fig. 5.1B). As a control we used a buffer containing 150 mM Na^+ which preserves the K^+ gradient. While all three stimuli induced IL-1β in the Na^+ buffer, neither chitosan nor silica induced significant quantities of IL-1β in the high K^+ buffer. Once again, flagellin delivered with SLO induced IL-1β even when the K^+ gradient was collapsed. Thus, K^+ efflux appears to be required for chitosan inflammasome activation.

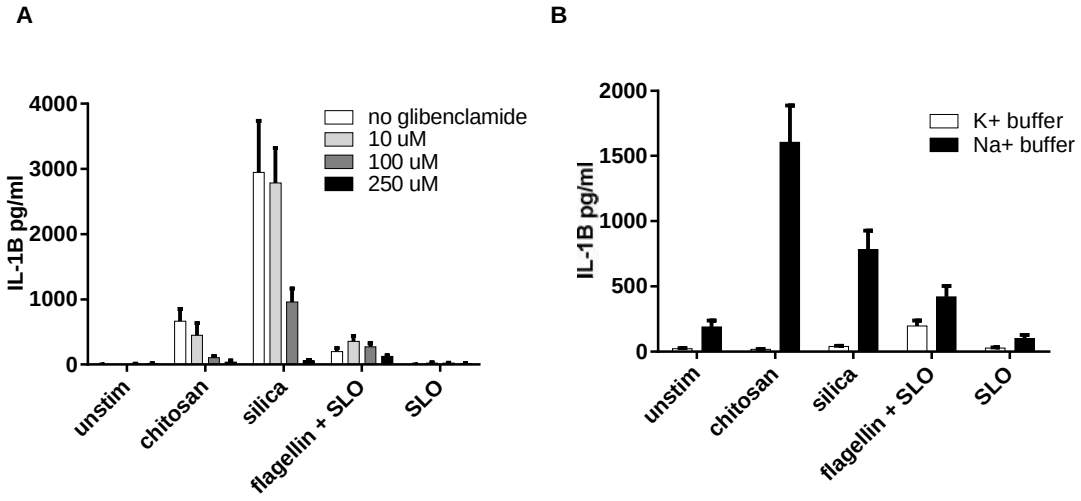


Figure 5.1. K^+ efflux is required for NLRP3 inflammasome activation by chitosan

A, BMMΦ (1 x 10^5/well) were primed for 2 h with 100 ng/ml LPS. Glibenclamide (10 µM, 100 µM, or 250 µM) was then added to the wells receiving inhibitor 1 h prior to addition of the following stimuli: chitosan (0.1 mg/ml), silica (0.1 mg/ml), SLO (5 µg/ml), and flagellin (1 µg/ml) + SLO. Control wells were left unstimulated (unstim). After 6 h, supernatants were collected and analyzed for IL-1β by ELISA. $p < 0.0001$ comparing 100 µM and 250 µM to no inhibitor for both chitosan and silica, $p < 0.05$ comparing 10 µM to no inhibitor for chitosan as analyzed by 2-way ANOVA. B, BMMΦ were primed for 3 h with 100 ng/ml LPS. After priming, the media was replaced with K^+ buffer or Na^+ buffer followed by the addition of stimuli as above. $p < 0.001$ comparing chitosan and silica to unstim in Na^+ buffer and comparing K^+ buffer to Na^+ buffer in the presence of chitosan and silica as analyzed by 2-way ANOVA. Data are means ± SE of three independent experiments, each performed in triplicate.

Effect of ROS on chitosan induced IL-1β release

The mitochondrial ROS inhibitor, Mito-TEMPO was used to analyze the role of mitochondrial ROS. Using an inhibitor concentration of 100 μM, there was a significant reduction in IL-1β release induced by both chitosan and silica (Fig 5.2A). To demonstrate specificity, the effect of Mito-TEMPO on IL-1β release stimulated by the ROS-independent AIM2 inflammasome activator poly(dA:dT) was examined (Bauernfeind et al., 2011). No significant inhibition of poly(dA:dT)-stimulated IL-1β was observed in the presence of Mito-TEMPO. In addition to Mito-TEMPO, the less specific ROS inhibitor DPI was utilized (Fig 5.2B). DPI will inhibit both NADPH ROS and mitochondrial ROS (Li and Trush, 1998). It blocked IL-1β release in response to both NLRP3 and AIM2 stimuli. This is either because DPI has off target effects which impact AIM2 inflammasome activation, or that NAPDH ROS has a role in AIM2 inflammasome activation. These data suggest that mitochondrial ROS is required for optimal NLRP3 inflammasome stimulation by chitosan, however the inhibition was incomplete and the broader ROS inhibitor also affected IL-1β release, pointing to a possible role for NADPH ROS as well.

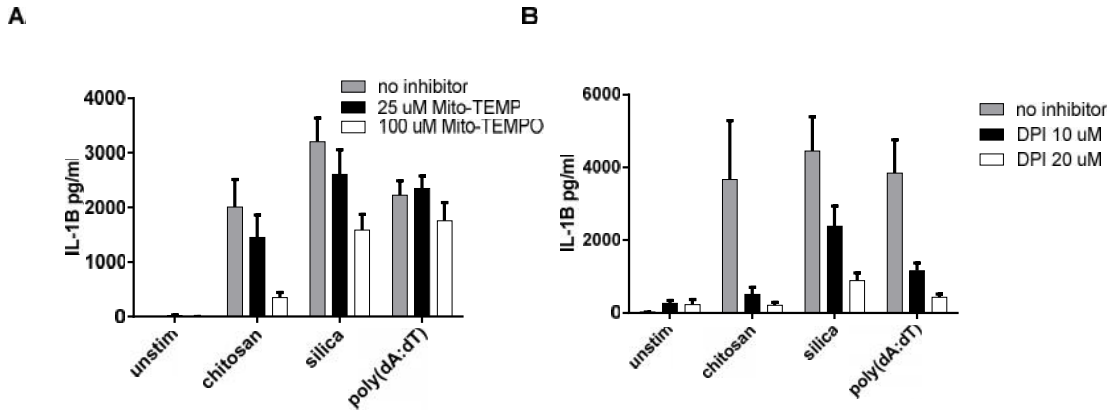


Figure 5.2. ROS is required for NLRP3 inflammasome activation by chitosan

BMMΦ (1 x 10^5/well) were primed for 2 h with 100 ng/ml LPS. In A, Mito-TEMPO (25 µM, or 100 µM) and B, DPI (10 µM or 20 µM) was added to the indicated wells 1 h prior to addition of the following stimuli: chitosan (0.1 mg/ml), silica (0.1 mg/ml), and poly(dA:dT) (2 µg/ml). Control wells were left unstimulated (unstim). After 6 h, supernatants were collected and analyzed for IL-1β by ELISA. $p < 0.01$ comparing 100 µM Mito-TEMPO to no inhibitor for both chitosan and silica, and comparing 10 µM DPI to no inhibitor for chitosan and poly(dA:dT), $p < 0.001$ comparing 20 µM DPI to no inhibitor for chitosan, silica and poly(dA:dT) as analyzed by 2-way ANOVA. Data are means ± SE of three independent experiments, each performed in triplicate.

The role of lysosomal destabilization in chitosan stimulated IL-1β release

Upon phagolysosomal fusion, acidification occurs which has been shown to be necessary for NLRP3 inflammasome activation through the lysosomal destabilization pathway (Hornung et al., 2008). Lysosomal acidification was inhibited with either bafilomycin A1 (Fig. 5.3A) or chloroquine (Fig. 5.3B). IL-1β release from both chitosan and silica was significantly inhibited by bafilomycin A concentrations as low as 10 nM. Chloroquine concentrations as low as 1 μM significantly blocked IL-1β release induced by silica. A trend, albeit not statistically significant, towards a reduction in IL-1β release induced by chitosan in the presence of chloroquine was noted. To further explore the lysosomal destabilization pathway, we inhibited cathepsin B using CA-074-me (Fig. 5.3C). Upon lysosomal destabilization, cathepsin B is likely released into the cytosol where it activates the NLRP3 inflammasome (Hornung et al., 2008). IL-1β release by the particulate stimuli chitosan and silica was significantly inhibited at CA-074-me concentrations of 10 μM. IL-1β release induced by the soluble inflammasome stimulator ATP was unaffected by these inhibitors, as expected for a phagocytosis-independent stimulus.

Role of CFTR in inflammasome activation

The cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR) has been suggested to have a role in lysosomal acidification. To test if this role was enough to impact inflammasome activation through the lysosomal destabilization pathway, CFTR-inh-172 was used to block CFTR. Upon addition of the inhibitor, there was significant

inhibition of IL-1β release for both NLRP3 stimuli alum and chitosan (Fig 5.4A). The AIM2 stimulator, poly(dA:dT), was unaffected. To confirm CFTR had a role in IL-1β release, macrophages from WT and CFTR KO mice were utilized (Fig 5.4B). However, the CFTR KO showed no phenotype differences compared to WT.

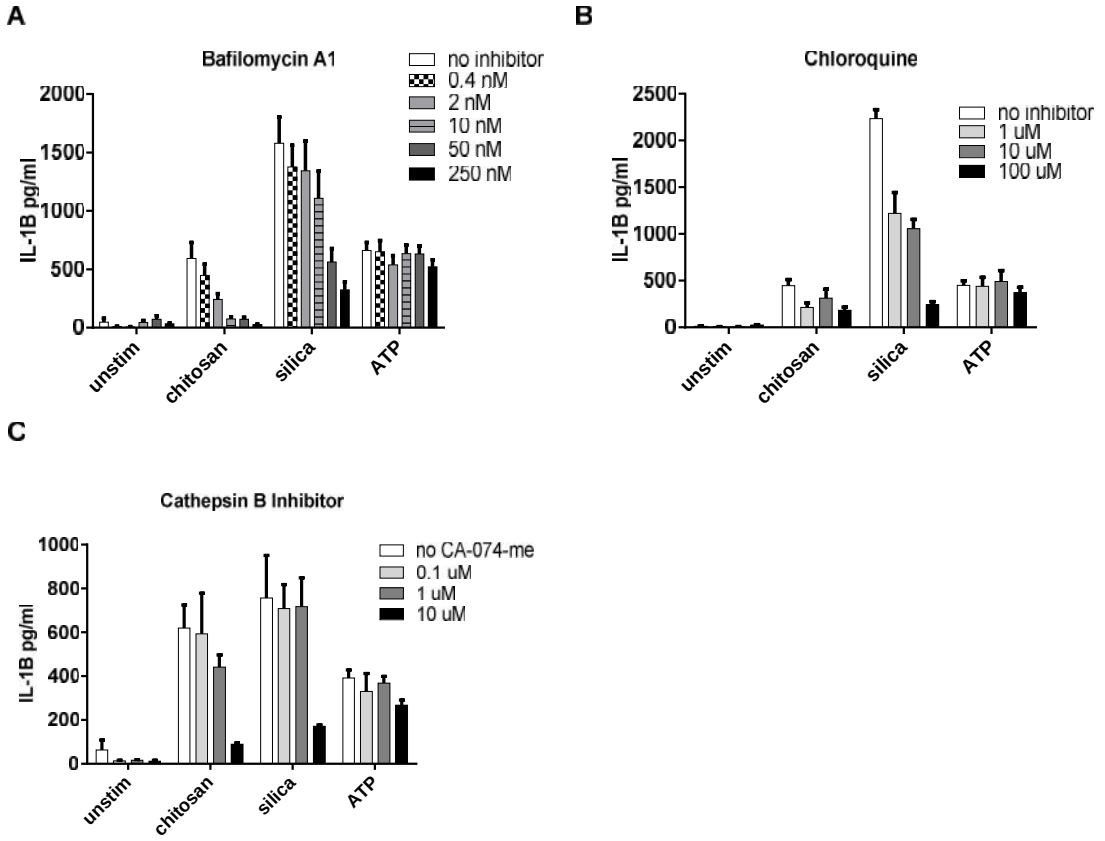


Figure 5.3. Lysosomal destabilization is required for NLRP3 inflammasome activation by chitosan

BMMΦ (1 x 10^5/well) were primed for 2 h with 100 ng/ml LPS. A, Bafilomycin A1 (0.4 nM, 2 nM, 10 nM, 50 nM, or 250 nM) B, Chloroquine (1 μM, 10 μM, or 100 μM) and C, Ca-074-me (0.1 μM, 1 μM, or 10 μM) were added to the wells receiving inhibitor 1 h prior to addition of the following stimuli: chitosan (0.1 mg/ml), silica (0.1 mg/ml) and ATP (5 mM). Control wells were left unstimulated (unstim). After a 6 h stimulation period, supernatants were collected and analyzed for IL-1β by ELISA. For bafilomycin A1, $p < 0.01$ comparing 10 μM, 50 μM, and 250 μM to no inhibitor with chitosan stimulation, $p < 0.05$ comparing 10 μM to no inhibitor with silica stimulation, and $p < 0.0001$ comparing 50 μM and 250 μM to no inhibitor with silica stimulation. For chloroquine, $p < 0.0001$ comparing any inhibitor concentration to no inhibitor in the presence of silica. For CA-074-me, $p < 0.001$ comparing 10 μM to no inhibitor in the presence of chitosan or silica. Comparisons are by 2-way ANOVA. Data are means ± SE of three independent experiments, each performed in triplicate.

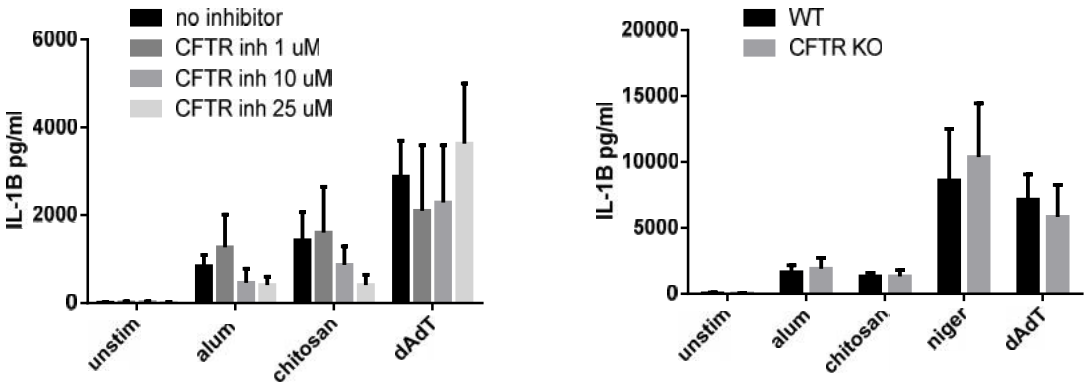


Figure 5.4. Role of CFTR in inflammasome activation.

A, BMMΦ (1 x 10^5/well) were primed for 2 h with 100 ng/ml LPS, followed by addition of the CFTR-inh-172 (1 μM, 10 μM, or 25 μM). After a 6 h stimulation period, supernatants were collected and analyzed for IL-1β by ELISA. B, BMMΦ (1 x 10^5/well) from WT BalbC or CFTR KO BalbC mice were primed for 3 h with 100 ng/ml LPS, followed by a 6 h stimulation period. Supernatants were then collected and analyzed for IL-1β by ELISA.

Discussion

In analyzing the mechanistic basis for NLRP3 inflammasome activation, there was evidence that K^+ efflux, mitochondrial ROS, and lysosomal destabilization each contribute to chitosan activation of the NLRP3 inflammasome. K^+ efflux from the cytosol has been shown to be required for NLRP3 inflammasome activation by many different stimuli, therefore NLRP3 activation may be through sensing the K^+ efflux by an unknown mechanism. One possibility is that the physiological intracellular K+ concentrations block the recruitment of caspase-1 to ASC, possibly by interfering with NLRP oligomerization (Petrilli et al., 2007). Inhibition of chitosan-induced IL-1β release through blocking K^+ efflux is consistent with previous published reports for other NLRP3 inflammasome stimuli (Petrilli et al., 2007; Tschopp and Schroder, 2010), showing that chitosan also requires K^+ efflux to activate the NLRP3 inflammasome.

The second proposed model for NLRP3 inflammasome activation involves ROS-induced activation of NRLP3. There is some debate over what type of ROS is important for NLRP3 activation, with evidence for both NADPH-oxidase and mitochondrial-derived ROS being important (Dostert et al., 2008; Nakahira et al., 2011; Zhou et al., 2011). Here it was demonstrated that blocking mitochondrial ROS inhibited chitosan induced IL-1β release. The targeted mitochondrial ROS inhibitor had no significant effect on the AIM2 inflammasome activator poly(dA:dT), whereas the less specific ROS inhibitor, DPI blocked IL-1β for both NLRP3 and AIM2 stimuli. It has recently been suggested that ROS may not only be involved in the NLRP3 activation but may also be

important for the priming step: upregulation of pro-IL-1β and NLRP3 (Bauernfeind et al., 2011; Liao et al., 2013). ROS is known to induce activation of NF-κB and has been shown to have a distinct role in upregulation of mRNA for inflammatory cytokines such as IL-1β and TNFα (Takada et al., 2003; van de Veerdonk et al., 2010). If inhibition of all ROS reduces the pro-IL-1β level, this could explain why DPI has more far reaching effects than the more targeted Mito-TEMPO.

Acidification-dependent lysosomal destabilization resulting in release of cathepsin B and subsequent activation of the NLRP3 inflammasome has been demonstrated following phagocytosis of particulate stimuli including silica and alum (Hornung et al., 2008). We hypothesized that the polycationic properties of chitosan would enable its escape from lysosomes by sponging protons delivered by the vacuolar-ATPase. This in turn would lead to the retention of Cl^- ions and water molecules resulting in lysosome swelling, leakage and eventual rupture (Kumar et al., 2004; Nel et al., 2009). Indeed, we found that inhibition of lysosomal acidification (which is required for the proton sponge effect), prevented chitosan from activating the inflammasome. The neutral charge of chitin due to acetylation of the polymer is a possible reason for its inability to activate the inflammasome. We were also able to block chitosan-induced NLRP3 inflammasome activation by blocking the downstream proposed activator of the lysosomal destabilization mechanism, cathepsin B, with the cathepsin B specific inhibitor Ca-074-me. Inhibition was incomplete which may be due to stimulation of the NLRP3

inflammasome by other lysosomal contents, such as cathepsin L or cathepsin D (Dostert et al., 2009; Duewell et al., 2010a; Halle et al., 2008).

The role of CFTR in lysosomal acidification is a bit controversial. Here the CFTR-inh-172 was shown to inhibit IL-1β release, though it was not confirmed to act through an acidification mechanism. It may have been an off target effect of the inhibitor rather than actual blocking of lysosome acidification. The results obtained with the CFTR knock-out mice suggest that CFTR is not involved in inflammasome activation, as there was no difference between the knock-out and WT. Though the knock-out mice are known to be leaky, so there could be some CFTR compensation or even another lysosomal acidification process compensating for the loss of CFTR. However, it appears that CFTR is not involved in inflammasome activation, which is supported by the report finding no inflammasome activation defect in cystic fibrosis patients (Tang et al., 2012). In fact, cystic fibrosis airway epithelial cells produced an exaggerated pro-inflammatory cytokine response (Venkatakrishnan et al., 2000; Weber et al., 2001).

Materials and Methods

Reagents and cell culture- All materials were obtained from Sigma-Aldrich unless otherwise stated. Ultrapure LPS (free of TLR2-stimulating lipopeptides) was treated with deoxycholate twice followed by phenol extraction and ethanol precipitation (Hirschfeld et al., 2000) to further purify the original Sigma-Aldrich stock (catalog # L2630). Chitosan was obtained from Primex (ChitoClear, high molecular weight shrimp chitosan, 76% deacetylated). Complete media is defined as RPMI 1640 media (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell culture was at 37°C in humidified air supplemented with 5% CO_2. All experiments were performed under conditions designed to minimize endotoxin contamination.

Cell populations -BMMΦ were generated as described (Johnson et al., 1983). Briefly, bone marrow was extracted from the femurs and tibiae of wild type C57BL/6 mice (The Jackson Laboratory) WT BalbC, or CFTR-/- BalbC (generous gifts from Dr. Terence R. Flotte). Cells were cultured in complete media supplemented with 10 ng/ml recombinant M-CSF (eBiosciences) and fed on days 4 and 7 with fresh media containing M-CSF. On day 8, non-adherent cells were washed away and the adherent macrophages were treated with 0.05% trypsin-EDTA, harvested and washed once in complete media before use in experiments.

Chitosan digestion, purification, and conversion to chitin- Chitosan was cleaved by pepsin to reduce the polymer length, purified and converted to chitin as previously described (Bueter et al., 2011). Briefly, chitosan (250 mg) was dissolved in 25 ml 0.1 M sodium acetate, pH 4.5. Pepsin (Sigma, P7000) was added (100 U/ml) for 18 h at 37^{o}C to partially digest the chitosan (Roncal et al., 2007). This was followed by extraction with chloroform:isoamyl alcohol (24:1) then mixing the recovered aqueous layer with an equal volume of 12% potassium hydroxide and heating at 80^{o}C for 90 min. Precipitated chitosan was collected by centrifugation and washed 3x with water followed by PBS to neutralize. Half of the chitosan was converted to chitin by suspending in 20 ml 1.0 M sodium bicarbonate, followed by addition of 1 ml acetic anhydride (Acros) and incubation at 22°C for 20 min with periodic mixing. The acetylation reaction was repeated, and terminated with heating at 100°C for 10 min. Chitin was collected by centrifugation and washed 3x with PBS. Chitin and chitosan suspensions were passed through a 100 μm filter (BD Falcon) to remove the largest particles then treated in 0.1 M sodium hydroxide at 22°C for 30 min, followed by washing with PBS and storage at 4°C.

Stimulation of IL-1β release- BMMΦ were plated at 1 x 10^{5} cells/well in a 96-well plate. The cells were primed with 100 ng/ml ultrapure LPS for 3 h (control cells were left unprimed), followed by incubation with the stimuli for 6 h (18 h for PBMCs). Positive stimuli controls included silica (topsize 15 microns, US Silica, MIN-U-SIL-15, used as described (Hornung et al., 2008)), synthetic double stranded DNA: poly(dA:dT), ATP, and Streptolysin O (SLO) or SLO + flagellin (FLA-ST, InvivoGen). Supernatants were

collected for cytokine measurement and assayed by IL-1β ELISA (eBiosciences). For K^+ efflux, ROS and lysosomal inhibition assays, the inhibitors were added 1 h prior to secondary stimuli addition. K^+ efflux was inhibited with Glibenclamide, mitochondrial ROS was inhibited with Mito-TEMPO (Enzo Life Sciences), total ROS inhibited with diphenyleneiodonium chloride (DPI), lysosomal acidification was inhibited with bafilomycin A1, chloroquine or CFTR-inh-172, and cathepsin B was selectively inhibited with CA-074-Me (Enzo Life Sciences). All inhibitors were assayed for cytotoxicity using the cytotoxicity detection kit (LDH) from Roche and none of the inhibitors at the concentrations used induced significant cell death above background (data not shown).

Inhibition of K^+ efflux with high extracellular potassium- BMMΦ were plated and primed as described above. Ten min after stimuli were added, cells given Streptolysin O (SLO) or SLO + flagellin were washed three times with fresh media. Supernatants were collected for all stimuli after 2 h and analyzed by ELISA. For the K^+ and Na^+ buffer experiments, after the priming step the media was replaced with either K^+ buffer (150 mM KCl, 5 mM NaH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% BSA) or Na^+ buffer (150 mM NaCl, 5 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% BSA). SLO and SLO + flagellin treated cells were again washed three times after 10 minutes incubation and fresh buffer added, then supernatants for all stimuli were collected after 2 h and analyzed by ELISA for IL-1β.

Multiplex Assay- BMMΦ were plated as described above and stimulated for 6 h with 0.1 mg/ml chitin or chitosan. Supernatants were collected and analyzed by Bio-Plex Pro Assays (Bio-Rad).

Statistical Analysis- Data were analyzed and figures prepared using GraphPad Prism. Significance was assessed by 2-way ANOVA with Bonferroni post-hoc test, 1-way ANOVA with Dunnett post-hoc test, or Kruskal-Wallis 1-way ANOVA by ranks with Dunn post-hoc tests, as indicated. *p*-values of <0.05 after correction for multiple comparisons were considered significant.

Preface to Chapter VI

A portion of this chapter's introduction has been published in PLoS Pathogens

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The data in this chapter has not been published

Chrono K. Lee performed BMMΦ isolations

Rajesh Velagapudi performed the mass-spectrometry identification of chitin bound proteins

Chelsea L. Bueter performed the experiments

Chelsea L. Bueter, Charles A. Specht and Stuart M. Levitz designed the experiments

Chapter VI: The Search for the Chitin Receptor

Abstract

The search for the chitin receptor has been going on for decades, however as of yet it has not been discovered. The role of two proposed chitin receptors, FIBCD1 and galectin-3 was first analyzed utilizing chitin beads. However, neither of the receptors appeared to have a significant role in chitin bead uptake. To generate potential chitin receptor candidates, a mass spectrometry analysis of proteins that bound to chitin beads was performed. Among the proteins identified was the laminin receptor. To analyze the potential role of the laminin receptor as a chitin receptor, the natural laminin receptor substrate laminin was used to block the receptor. Laminin was able to block uptake of chitin beads while YGPs were unaffected, suggesting it may have a role as a chitin receptor. However, laminin was unable to block particulate chitin and antibody to the laminin receptor was also unable to block chitin bead uptake. The potential chitin receptor that is inhibited by laminin appears to be unique to macrophages (or hematopoietic cells) as HEK and HeLa cells were able to uptake chitin beads regardless of the presence of laminin. The laminin receptor does not appear to be the chitin receptor, however laminin is able to bind other receptors as well, perhaps one of these serves as a chitin receptor.

Introduction

Recognition of chitin and chitosan by mammals

The lack of chitin or chitosan in mammalian cells, and their presence in dust mites, parasitic worms and fungi, makes these polymers potential targets for recognition by the innate immune system. In fungi, chitin and possibly chitosan are not readily accessed by protein-sized probes, though they can be stained with low molecular weight dyes. Exposure of fungal chitin and chitosan requires some degree of degeneration of their surrounding architecture, as occurs in damaged cells or fungal/crustacean detritus (Mora-Montes et al., 2011a). Upon exposure chitin can be recognized by mammalian chitinases, which bind and actively degrade chitin, and chitinase-like proteins, which also bind chitin but are catalytically inactive. It has become evident that this family of chitin binding proteins (Glycosyl Hydrolase Family 18) plays an active role in inflammation and innate and adaptive immunity based on their up-regulation during various disease states (Lee et al., 2011).

Three innate immune receptors, Toll-like receptor (TLR) 2, Dectin-1, and mannose receptor, have been implicated in mediating a variety of immune responses to chitin. However, how this occurs is not well understood. Direct binding to chitin has not been demonstrated and the possibility that contaminants are responsible for some of these effects cannot be excluded. One study showed chitin, acting via an apparent Dectin-1-dependent, but mincle (a C-type lectin), TLR2, and TLR4-independent mechanism, can partially block cytokine production in response to *C. albicans*, although chitin did not

directly interact with Dectin-1 (Mora-Montes et al., 2011a). However, TLR2 was found to contribute to sensing of chitin by keratinocytes (Koller et al., 2011) and chitin induced expression of IL-17A and IL-17AR (Da Silva et al., 2008). Moreover, TNFα and IL-10 induced by chitin appeared to be mediated by TLR2, Dectin-1 and mannose receptor.

Both chitin and chitosan particles are readily phagocytosed (Bueter et al., 2011), supporting a role for recognition via specific receptor(s) mediating phagocytosis. Receptors on myeloid cells that bind chitin or chitosan and induce a phagocytic response have yet to be definitively identified. However, several receptors have been shown to have an affinity for chitin or chitin oligosaccharides including: fibrinogen C domain containing 1(FIBCD1), a homotetrameric 55 kDa type II transmembrane protein expressed in the gastrointestinal tract that is known to recognize acetylated residues (Schlosser et al., 2009), natural killer cell receptor protein 1 (NKR-P1), an activating receptor on rat natural killer cells (Semenuk et al., 2001), RegIIIγ, a secreted C-type lectin (Cash et al., 2006), and galectin-3, a lectin with affinity for β-galactosides (Seetharaman et al., 1998). However, none of these have yet to be shown to act as a receptor as opposed to a protein that binds chitin. Also, receptors that recognize soluble oligosaccharides, as by-products of chitinase digestion, may not recognize full length, insoluble chitin.

Recognition of chitin and chitosan by plants

Fungi are major crop pathogens. Plants are readily able to recognize chitin and chitosan and respond rapidly, within 6 hours (Roby et al., 1987). It is not surprising then that

plants exhibit a wide variety of defense responses to chitin and chitosan following fungal infestation, including increases in chitinase expression, proteinase inhibitors, reactive oxygen species (ROS), cytoplasmic acidification, and expression of early responsive genes and defense genes (Shibuya and Minami, 2001). Presumably, most of these responses have developed to fight fungal infections, though chitin-binding lectins have also been shown to have insecticidal activity (Vandenborre et al., 2011). Likewise, fungi have developed methods to avoid recognition of chitin and thereby prevent the effective anti-fungal response, such as by masking chitin with α-1,3-glucan, a compound plants are unable to digest (Fujikawa et al., 2012). Conversely, recognition of modified chitin oligosaccharides is important for symbiotic relationships between leguminous plants and rhizobial bacteria (Nakagawa et al., 2011).

A number of receptors in plants that bind directly to chitin or mediate the response to chitin have been identified. Chitin elicitor-binding proteins (CEBiP) containing an extracellular lysine motif that binds chitin directly are conserved across multiple plant species. CEBiP knockdown in suspension-cultured rice cells results in an absence of ROS generation in response to chitin (Kaku et al., 2006). CERK1, the Arabidopsis CEBiP homolog, is essential for chitin elicitor signaling; dimerization upon binding through the lysine motif (LysM) is critical for MAPK activation, ROS generation and gene expression in response to chitin (Liu et al., 2012; Miya et al., 2007). In contrast, chitosan appears to elicit activity from plant cells via charge-charge interactions with negatively charged phospholipids instead of via a receptor-specific interaction

(Kauss et al., 1989). Whether analogous charge-based, receptor-free interactions between mammalian cells and this highly positively charged polymer occur is speculative.

Though chitin receptors have been identified in plants, the work is still ongoing in mammalian cells. Though there have been a number of receptors proposed to be involved in chitin recognition, none of them have yet been proven to be a chitin receptor. Here FIBCD1 and galectin-3 are first analyzed for their potential as a chitin receptor. Neither of them appears to be a chitin receptor. Next, the protein identified by a mass spectrometry analysis, the 67 kD laminin receptor, is analyzed as a potential chitin receptor. The 67 kD receptor is a nonintegrin membrane protein and has been identified on macrophages, neutrophils, endothelial cells, epithelial cells, hepatocytes, tumor cells, muscle cells, many types of interstitial cells and neuronal cells (Chen et al., 2003; Mecham, 1991). It has been shown to recognize laminin, fibronectin, and type IV collagen, as well as neurotropic viruses, such as sindbis virus and dengue virus, the cellular prion protein (PrP), the secreted cytotoxic necrotizing factor toxin of *E. coli*, neuroinvasive bacteria such as pneumococcus, *Haemophilus influenzae*, and meningococcus, and finally epigallocatechin-e-gallate (EGCG), a component of green tea shown to protect mice against lethal endotoxemia induced by LPS (Byun et al., 2010; Chen et al., 2003; Orihuela et al., 2009). It addition to the 67 kD laminin receptor's role as a membrane receptor, it's 37 kD precursor protein is an integral ribosomal component, which makes study of this particular receptor difficult (Ardini et al., 1998). There seems to be some evidence supporting a role for the laminin receptor as a chitin receptor, though

it may only be an off target blocking effect of laminin, and the actual chitin receptor is another receptor that is able to bind laminin. Unfortunately a definite chitin receptor was not identified, and more work is needed in this area.

Results

Neither FIBCD1 nor Galectin-3 appear to act as a chitin receptor

Both FIBCD1 and galectin-3 have been proposed as possible chitin receptors. FIBCD1 recognition of acetylated residues has been shown to be inhibited by GlcNAc (Schlosser et al., 2009). To test whether FIBCD1 was involved in recognition of chitin beads and their uptake, macrophages were incubated with GlcNAc prior to the addition of chitin beads. Neither GlcNAc nor the unacetylated control GlcN inhibited chitin bead uptake by macrophages (Fig 6.1 A & B) suggesting FIBCD1 is not facilitating their uptake. To analyze the involvement of Galectin-3 in chitin bead uptake, BMMΦ from both WT and Galectin-3 KO mice were used. Both the Galectin-3 KO and WT macrophages were readily able to phagocytose chitin beads (Fig. 6.1 C & D), suggesting that Galectin-3 is not the sole chitin receptor either.

Proposing laminin as a potential chitin receptor

Membrane preparations of BMMΦ were passed over chitin affinity columns, those proteins that stuck to the column were identified by mass spectrometry. The 67 kD laminin receptor was one of the proteins identified. To test whether the laminin receptor was also acting as a chitin receptor, BMMΦ were incubated with no laminin (Fig 6.2A), 30 μg/ml laminin (Fig 6.2B) or 100 μg/ml laminin (Fig 6.2C) prior to the addition of chitin beads. The macrophages which did not receive laminin prior to chitin beads

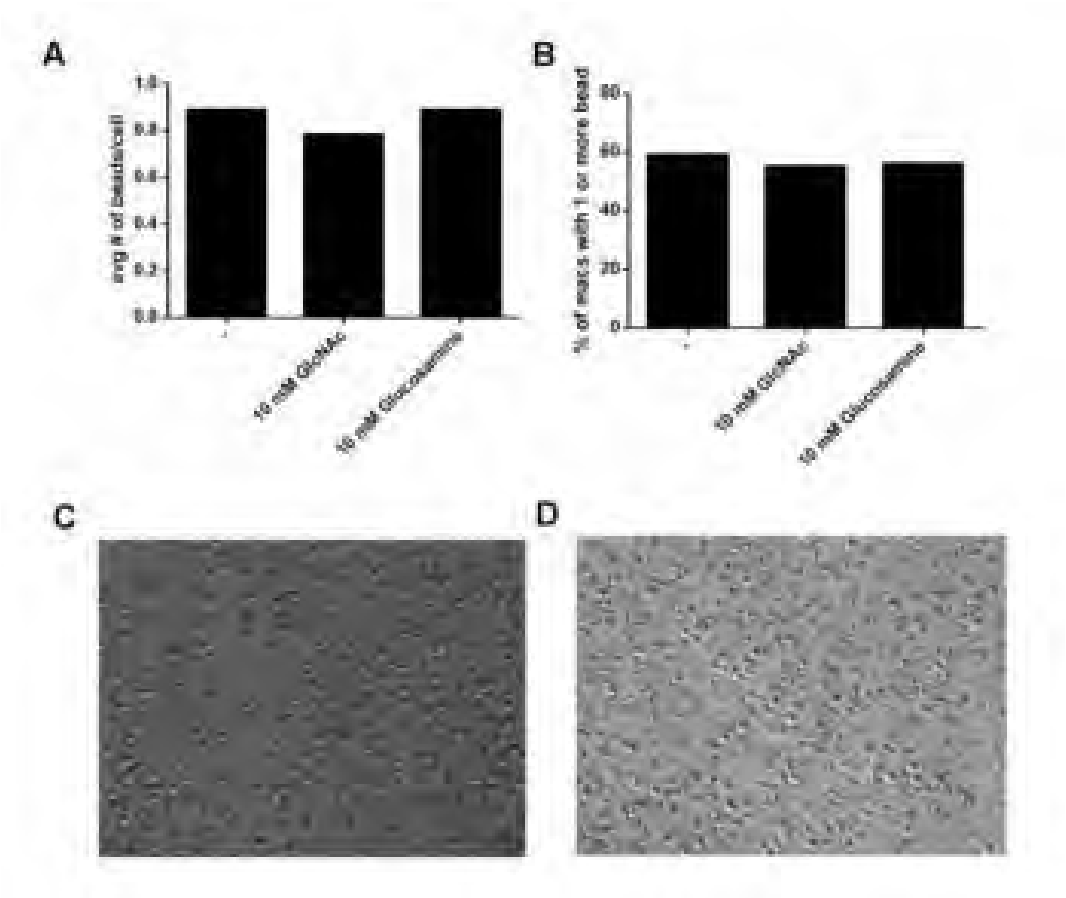
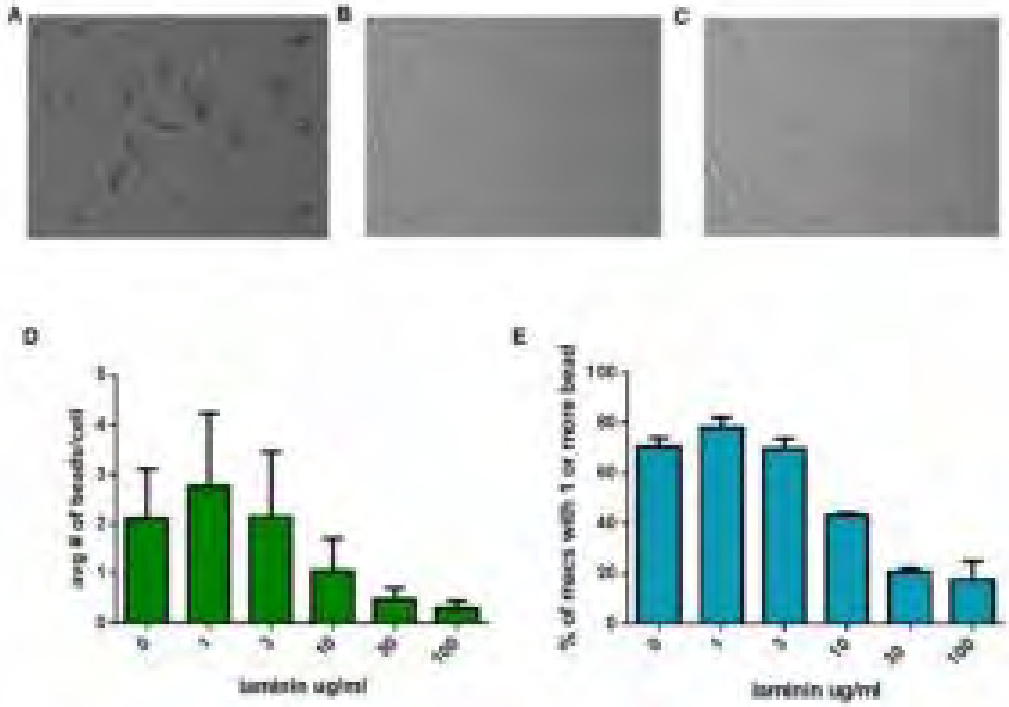


Figure 6.1. Neither FIBCD1 or Galectin-3 appear to act as chitin receptors.

A and B, BMMΦ (2 x 10^5/well) were incubated with no sugar, 10 mM GlcNac, or 10 mM Glucosamine for 30 minutes. The cells were then given 1 x 10^6 chitin beads/well and incubated for another 30 min at 37° C. Cells were then washed 3x with PBS and analyzed for phagocytosis under a microscope. A, The average number of beads per cell. B, the percentage of macrophages with 1 or more phagocytosed beads. C and D BMMΦ (2 x 10^5/well) from galectin-3 KO mice were incubated with 0 µg/ml laminin (C), or 100 µg/ml laminin (D). Cells were then washed 3x with PBS and analyzed for phagocytosis under a microscope.


A
B
C
D
avg # of beads/cell
laminin ug/ml
E
% of nscs with 1 or more bead
laminin ug/ml

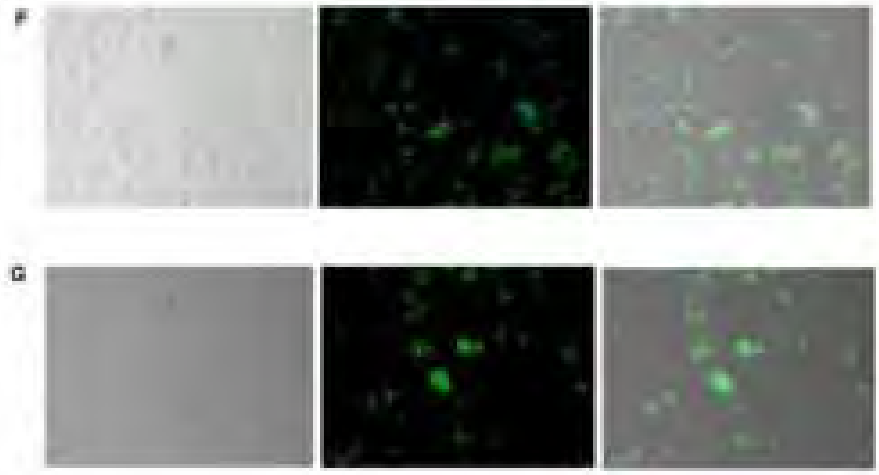

F
G

Figure 6.2. Laminin inhibits chitin bead uptake.

BMMΦ (2 x 10^5/well) were incubated with 0 μg/ml laminin (A), 30 μg/ml laminin (B), or 100 μg/ml laminin (C) for 30 minutes. The cells were then given 1 x 10^6 chitin beads/well and incubated for another 30 min at 37° C. The cells were then washed 3x with PBS and analyzed for phagocytosis under a microscope. D and E, Cells were treated as above with 0, 1, 3, 10, 30, or 100 μg/ml laminin. The average number of beads per cell was calculated for 3 individual wells in D. E, the percentage of macrophages with 1 or more phagocytosed beads, calculated for 3 individual wells. Fluorescent yeast glucan particles (F-YGPs) were incubated with macrophages given 0 μg/ml laminin (F) or 100 μg/ml laminin (G) following the same procedure as in A.

readily phagocytosed the beads. However, in the presence of either concentration of laminin, phagocytosis was almost completely abolished. To quantify the effect of laminin blocking chitin bead phagocytosis, macrophages were treated with multiple concentrations of laminin and the number of beads phagocytosed per macrophage were counted (Fig 6.2D) as well as the number of macrophages containing more than one bead (Fig 6.2E). As the concentration of laminin increases, both the average number of beads per macrophage and the % of macrophages with at least one bead decrease. Yeast glucan particles (YGPs) were used as a control to show that laminin was not blocking general phagocytosis, and as shown in Figures 6.2F and G, YGPs are phagocytosed in the absence or presence of laminin.

To further test whether the 67 kD laminin receptor was acting as a chitin receptor, BMMΦ were incubated with no laminin (Fig 6.3A), or 100 μg/ml (Fig 6.3B) laminin prior to the addition of chitin particles. Unlike what was seen with the chitin beads, both macrophages that received no laminin and those that did readily phagocytosed chitin particles.

Laminin is a large protein, 1 MDa, and it is quite possible that it is blocking more than just the 67 kD laminin receptor when added to cells. To address this, polyclonal antibody to the 67 kD laminin receptor was used to try and replicate the results seen with laminin (Fig 6.4A and B). Unfortunately not even the highest concentration of polyclonal antibody only had a significant inhibitory effect on chitin bead uptake.

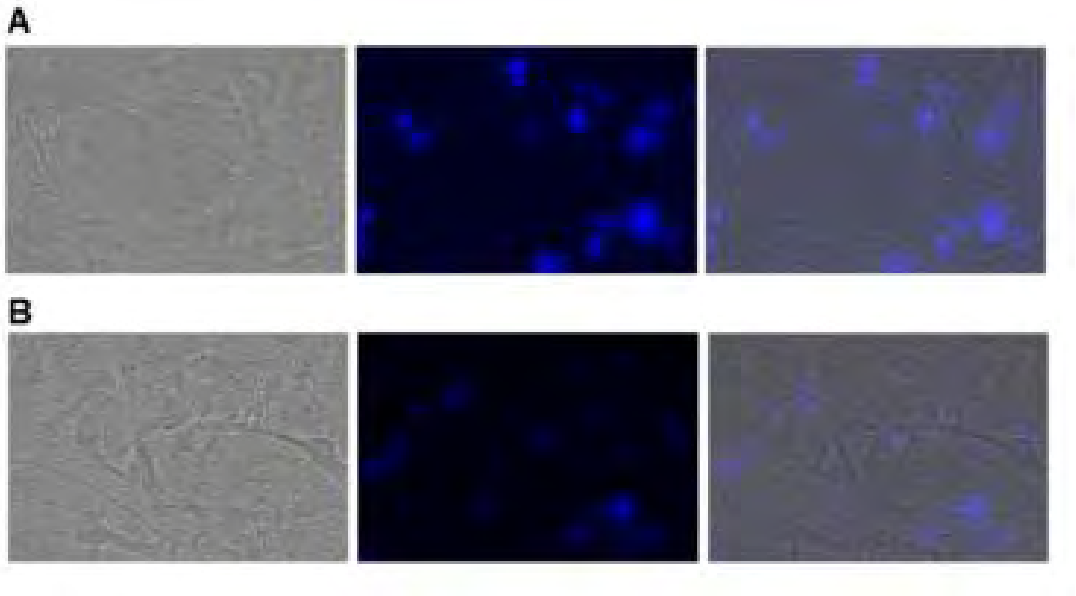


Figure 6.3. Laminin does not inhibit chitin particle uptake.

BMMΦ (2 x 10^5/well) were incubated with 0 µg/ml laminin (A), 30 µg/ml laminin (B), or 100 µg/ml laminin (C) for 30 minutes. The cells were then given 0.1 mg/ml chitin particles and incubated for another 30 min at 37° C. The cells were then washed 3x with PBS, stained for chitin with UVtex, and analyzed for phagocytosis under a microscope (100x).

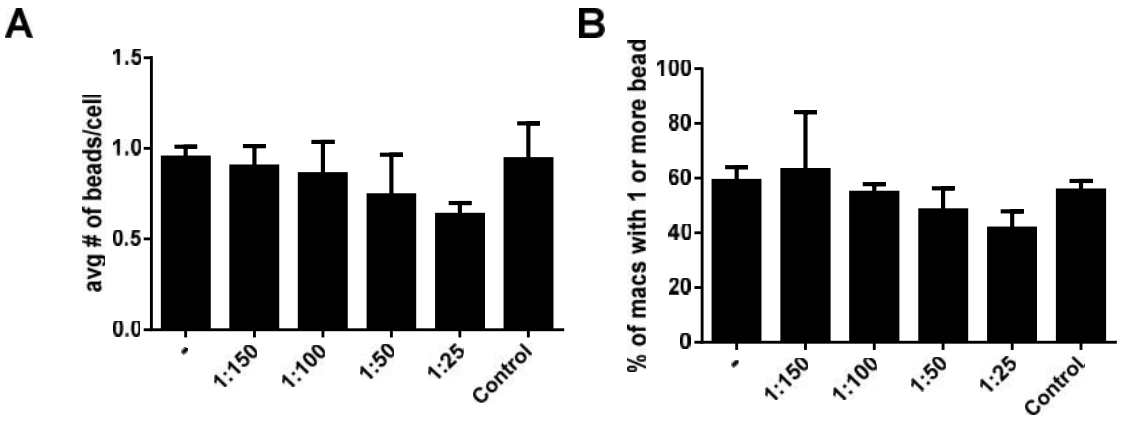


Figure 6.4. Polyclonal antibody against the 67 kD laminin receptor does not inhibit chitin bead uptake.

BMMΦ (2 x 10^5/well) were incubated with polyclonal antibody against the 67 kD laminin receptor (no antibody, dilutions of 1:150, 1:100, 1:50 or 1:25, or with 1:25 dilution of control antibody) for 30 minutes. The cells were then given 1 x 10^6 chitin beads/well and incubated for another 30 min at 37° C. Cells were then washed 3x with PBS and analyzed for phagocytosis under a microscope. A, the average number of beads per cell was calculated for 3 individual wells. B, the percentage of macrophages with 1 or more phagocytosed beads, calculated for 3 individual wells. One-way ANOVA with Holm-Sidak's multiple comparisons test showed no significant differences between no antibody and any of the polyclonal antibody dilutions.

Unique receptor for macrophages?

The above experiments were all done using BMMΦ, and the laminin receptor may be able to act as a chitin receptor in these cells. Does this also hold true for other cell types though? BMMΦ, HeLa, and HEK cells were all tested for chitin bead phagocytosis in the absence and presence of 100 μg/ml laminin (Fig 6.5A & B). Chitin bead phagocytosis in BMMΦ was once again inhibited by laminin, however, neither the HeLa cells nor the HEK cells appear to be affected by the presence of laminin. This may suggest multiple chitin receptors, the expression which varies on different cell types.

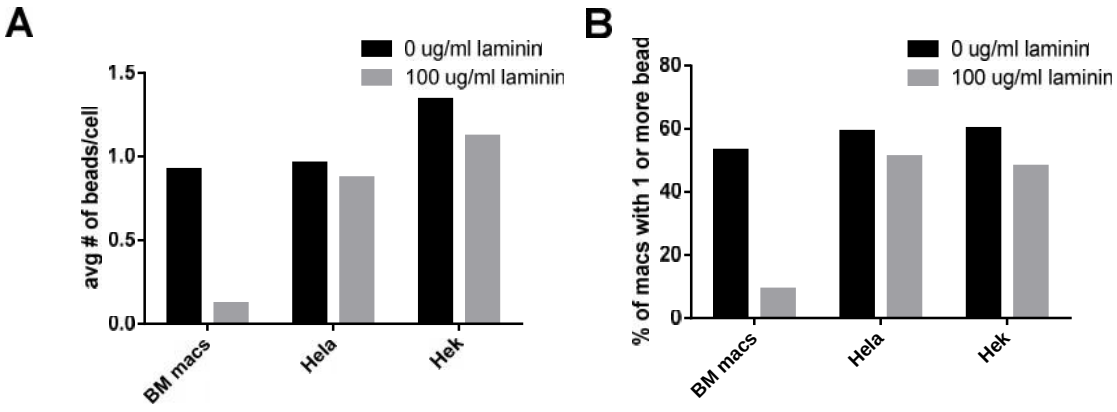


Figure 6.5. Laminin does not inhibit chitin bead uptake in HeLa or HEK cells.

BMMΦ, HeLa cells or HEK cells (2 x 10^5/well) were incubated with 0 μg/ml laminin or 100 μg/ml laminin for 30 minutes. The cells were then given 1 x 10^6 chitin beads/well and incubated for another 30 min at 37° C. Cells were then washed 3x with PBS and analyzed for phagocytosis under a microscope. A shows the average number of beads per cell and in B, the percentage of macrophages with 1 or more phagocytosed beads.

Discussion

Identification of a mammalian chitin receptor has been unsuccessful despite many groups working towards its identification. Though many receptors have been proposed to have a role in chitin signaling or potentially be chitin receptors, there has yet to be any definitive evidence of an actual receptor, as opposed to a protein that binds chitin. Two proposed potential chitin receptors were tested for their involvement in chitin bead uptake: FIBCD1 and Galectin-3. Binding of FIBCD1 to acetylated residues can be blocked by acetylated monomers such as N-acetylglucosamine and N-acetylgalactosamine (Schlosser et al., 2009). However, use of N-acetylglucosamine had no effect on chitin bead uptake by macrophages. Galectin-3 has been shown to have an affinity for β-galactosides (which are composed of lactose, and N-acetyllactosamine) and are abundantly expressed on macrophages (Seetharaman et al., 1998), however chitin bead uptake in the Galectin-3 KO macrophages was not inhibited either. Neither of these proposed chitin receptors appear to have a dominant role in chitin recognition and uptake.

The 67 kD laminin receptor, identified by Rajesh Velagapudi in a mass spectrometry screen, is another potential chitin receptor candidate. Use of the known agonist, laminin, blocked chitin bead uptake efficiently. However, these results were not replicated with the chitin particles. Differences between the chitin beads and the chitin particles include size and chitin content. The beads are smaller than most of the chitin particles, and contain a significantly less chitin than the pure chitin particles, perhaps making them easier to block with laminin compared to the larger particles. However, the

polyclonal antibody to the 67 kD laminin receptor did not block chitin bead uptake either. The blocking ability of the antibody may be compromised though, as the laminin receptor is a highly conserved receptor across mammals and generation of antibodies against it may be difficult if it is recognized as self. If the antibody is blocking properly though, it is possible that a receptor associated with the laminin receptor. At 1 MDa, laminin is quite a large protein and would potentially easily block both the laminin receptor and a receptor associated with it. It is also possible a different receptor that recognizes laminin may actually be the chitin receptor. MAC-1 (also known as $\alpha_M\beta_{2,}$ CR3, CD11b/CD18) is known to recognize many different cellular and foreign components including: fibronectin, laminin, collagen, elastase, ICAM-1, ICAM-2, fibrogen, Factor X, kininogen and iC3b, as well LPS, β-glycans, heparins and proteoglycans (Plow and Zhang, 1997). When MAC-1 binds laminin it is known to pair with α6β4 integrin, and perhaps the assay which showed laminin blocked chitin bead uptake actually went through this pair of receptors (Ardini et al., 1997). The MAC-1 receptor is also expressed on multiple hematopoietic cells but is not expressed on HeLa or HEK cells, offering a potential explanation for why laminin inhibits chitin bead uptake in macrophages but is ineffective blocking chitin uptake in HeLa or HEK cells.

The data presented here are not definitive. Chitin beads phagocytosis is inhibited by laminin, but chitin particles are not and the polyclonal antibody does not block chitin bead uptake. Differences between chitin content and particle size and the potential problems with the antibody do not completely discount the possibility that the 67 kD

laminin receptor is able to act as a chitin receptor. There is also a significant possibility that there is more than one chitin receptor and even if the 67 kD laminin receptor is able to act as a chitin receptor, other chitin receptors may compensate when it is blocked. More studies are needed, perhaps finding a blocking antibody with confirmed ability to block, or if possible developing a knockout that only affects the 67 kD laminin receptor, while leaving the 37 kD ribosomal protein intact. Additional experiments could focus on analyzing other receptors that can recognize laminin such as MAC-1, and testing if blocking multiple receptors has a significant impact on chitin bead and particle uptake.

Materials and Methods

Reagents and cell culture- All materials were obtained from Sigma-Aldrich unless otherwise stated. Chitosan (76% deacetylated) was obtained from Primex. Complete media is defined as RPMI 1640 media (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 ug/ml streptomycin. Cell culture was at 37°C in humidified air supplemented with 5% CO_2. All experiments were performed under conditions designed to minimize endotoxin contamination.

Bone marrow-derived macrophages- Bone-marrow derived macrophages (BMMΦ) were generated as described (Johnson et al., 1983). Briefly, bone marrow was extracted from the femurs and tibiae of WT C57BL/6 mice or Galectin3 -/- mice (The Jackson Laboratory). Cells were cultured in complete media supplemented with 10 ng/ml recombinant M-CSF (eBiosciences) and fed on days 4 and 7 with fresh media containing M-CSF. On day 8, non-adherent cells were washed away and the adherent macrophages were treated with 0.05% trypsin-EDTA, harvested and washed once in complete media before use in experiments.

Phagocytosis inhibition assays- BMMΦ were plated at $2x10^5$ cells/well in a 48-well plate. Cells were then incubated for 30 minutes with 10 mM GlcNAc, 10 mM GlcN to test the role of FIBCD1, or 0, 1, 3, 10, 30, or 100 ug/ml laminin (Invitrogen: natural mouse, Cat no. 23017-015) or 1:150, 1:100, 1:50 or 1:25 dilution of the polyclonal antibody to the 67 kD laminin receptor (sc-21534, Santa Cruz Biotechnology) to test the

role of the laminin receptor. Next, 10^6 chitin beads, 10^6 YGPs, or 0.1 mg/ml chitin particles were added for a 30 minutes incubation. Cells were then washed three times with PBS. Then 100 cells for each condition were counted, recording the number of cells with one or more bead and the number of beads phagocytosed. Photomicrographs were also taken.

Chapter VII: Conclusions

Recent research has begun to clarify when and how mammals and plants recognize and respond to exposure to chitin and chitosan. Nevertheless, there are still a lot of unanswered questions. Disparities in the literature regarding the immunological activity of chitin and chitosan are likely due in large part to the purity and heterogeneity of the glycan preparations used as stimuli. The procedures developed here resulted in preparations that did not induce TNFα or any of the other cytokines or chemokines tested in a multiplex assay, suggesting a lack of contaminants. The chitin was also derived from the chitosan, allowing for both polymers to presumably retain similar structures and polymer lengths. The major difference between the two polymers, degree of acetylation, had a significant impact on the ability of the polymer to activate the NLRP3 inflammasome. This may be of importance in fungal and parasitic immunology, allergy, and in translational applications. Fungi with only chitin in their cell walls are unlikely to induce inflammasome activation through chitin. Fungi with chitosan however, are likely to activate. Other components of the fungal cell wall such as β-glucans will activate the inflammasome though (Kankkunen et al., 2010), so it is unknown if the presence or absence of chitosan makes a significant difference in the inflammasome activation profile of any particular fungus. Where the difference in inflammasome activation between chitin and chitosan may have a greater impact is through their use in translational applications. If inducing an inflammatory response would enhance a particular translational application, such as encouraging cellular influx to a site, chitosan would be ideal, but if inflammation is not desired, the acetylated chitin would be better. This allows for diversity among the applications in which these polymers can be useful.

The biodegradable and physicochemical properties of chitin and chitosan make these glycans ideal for a wide range of translational applications. Recent studies, including this one, have demonstrated an inverse relationship between particle size and immunological activity. Matching the goal of a particular translational application with the particle size allows for a diverse range of options. Small particles can be used to induce an immunological response, with chitosan microparticles readily internalized and nanoparticles trafficking rapidly to the lymph nodes (Al Kobiasi et al., 2012), while larger particles will remain inert. Large particles of either polysaccharide are generally considered inert, making them perfect for tissue scaffolds or immobilization of a biosensor. These polysaccharides have proven to be incredibly versatile and will likely have many more translational applications discovered in the future as they become more well understood.

The NLRP3 inflammasome has been shown to be activated by a diverse variety of stimuli. Chitosan also activates the NLRP3 inflammasome, whereas chitin does not. Deacetylation of chitin is enough to induce activation of the inflammasome, perhaps because this results in positively charged residues which can lead to charge-induced lysosomal swelling. If the lysosomal destabilization induced by chitosan is the major difference between chitin and chitosan, it would suggest the lysosomal destabilization mechanism of NLRP3 inflammasome activation is central for chitosan activation. However, all three proposed mechanisms for NLRP3 inflammasome activation were shown to be important for chitosan-induced activation of the NLRP3 inflammasome.

While much progress has been made in elucidating how plants recognize chitin and chitosan, the principal receptor(s) responsible for mammalian recognition remain to be determined. A potential chitin receptor, the 67 kD laminin receptor was identified through a mass spectrometry screen of proteins bound to chitin. Though it was shown that laminin is able to block chitin bead uptake suggesting the 67 kD laminin receptor may be a chitin receptor, this observation was not observed for chitin particles or through the use of the polyclonal antibody against the 67 kD laminin receptor. Perhaps it was an indirect effect of the large laminin protein blocking an unrelated receptor, or binding to another of the receptors known to bind laminin. Unfortunately it appears that identification of definitive mammalian chitin receptor(s) remains elusive for now.

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