



# **Effect of chitin derivatives on macrophages**

The role of chitinases and chitinase-like proteins

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**HÁSKÓLI ÍSLANDS**

**Áhrif kítín afleiða á átfrumur**  
***Hlutverk kítínasa og kítínasa líkra prótína***

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Ritgerð til meistaragráðu í líf- og læknávisindum

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## Ágrip

Eiginleikar kítíns gera það að áhugaverðum valkosti þegar kemur að notkun í heilbrigðisverkfræði og líftækni almennt. Vegna takmarkaðs vatnsleysanleika kítíns hafa afasetíleraðar afleiður þess, kítósan og kítósan fásýkrur (ChOS), frekar verið notaðar. Lífvirkni slíkra sameinda er oft óljós. Breyting á kítíni yfir í kítósan og ChOS með ýmsum afasetíleringum aðferðum mynda fjölliður eða fálíður sem eru misstórar og hafa mismunandi afasetíleringarstig. Lífvirkni-rannsóknir á kítósan og ChOS standa því oft frammi fyrir því vandamáli að illa skilgreind efni er notuð sem veldur því að erfiðara er að finna út sameindalega ferla sem liggja að baki lýstrar lífvirkni.

Í þessu verkefni voru notaðar fimm vel skilgreindar afleiður af kítíni (ChOS lactate, kítósan <30  $\mu\text{m}$ , kítósan, ChOS og kítín sexliður) til að rannsaka m.a. hlutverk afasetíleringar og stærðar kítínafleiðanna á viðbragð stórátsumna, frumna ósérhæfða ónæmiskerfisins. Svörun stórátsumna eftir örvun með kítín afleiðum var metin út frá breytingum í seytingu á virka kítínasans chitotriosidase (Chit1) og kítínasa-líka próteinsins YKL-40, ásamt bólgusvörun og eitrunaráhrifum í tveimur stórátsumu líkönum; THP-1 stórátsumulínunni og einkjörnungaafleiddum stórátsumum (MDMs).

100  $\mu\text{g/mL}$  af kítósan, kítósan <30  $\mu\text{m}$  og ChOS lactate lækkuðu marktækt seytingu á YKL-40 hjá MDMs, en hafði engin áhrif á seytingu Chit1. THP-1 frumur seyttu aðeins lægra magni af YKL-40 þegar þær voru meðhöndlaðar með ChOS lactate. ChOS lactate dró úr YKL-40 seytingu við lægri styrkleika en allar hinar kítósan afleiðurnar sem bendla má við eitrunaráhrif efnisins sem sást í styrkleikum niður í 20  $\mu\text{g/mL}$ . Lækkunin á YKL-40 seytingu hélst í hendur við aukna IL-1 $\beta$  seytingu (inflammasóm virkjun), sem er háð upptöku á ChOS lactate sameindunum og virkjun caspase-1. Engin hinna kítín afleiðanna hafði eitrunaráhrif, hins vegar virkjuðu bæði kítósan og kítósan <30  $\mu\text{m}$  líka inflammasómið, en höfðu vægari áhrif en ChOS lactate. ChOS og kítín sexliðurnar höfðu engin áhrif á þá þætti sem skoðaðir voru í þessari rannsókn.

Breytileikinn í lífvirkni kítósan afleiðanna má rekja til mismunandi efnasamsetningu þeirra, þ.e. stig afasetíleringar, stærð og lengd fjölsykrukeðju. ChOS lactate er almennt minna en kítósan efnin og afasetíleraðra. ChOS er aftur á móti minnsta afleiðan sem notuð var í rannsókninni, en þar sem það er minna afacetylarað er það líkara kítíni sem gæti útskýrt hve óvirkt það er.



## Abstract

Chitin is considerably attractive for the use in clinical and biomedical research based on its properties. However, due to chitin's insolubility in aqueous solutions, the deacetylated derivatives, chitosan and chitosan oligosaccharide (ChOS) have been attracting more interest in the biomedical setting. The bioactivity of these derivatives is often unclear. Conversion of chitin to chitosan and ChOS by various deacetylation techniques, gives a heterogeneous product of variable acetylation and size. The usage of poorly defined chitosan and ChOS is a recurrent problem in bioactivity studies, which makes it difficult to find the molecular mechanism behind the described bioactivity.

This study uses five relatively well defined chitin derivatives (ChOS lactate, chitosan <30  $\mu$ m, chitosan, ChOS and chitin hexamer) to examine the role of deacetylation and size of chitin derivatives on the responses of macrophages, cells of the innate immune system. Macrophage responses were evaluated by examining changes in secretion of the active chitinase chitotriosidase (Chit1) and the chitinase-like protein YKL-40, as well as pro-inflammatory cytokines and cytotoxicity following chitin derivative stimulation in two macrophage cell culture models; THP-1 macrophages and monocyte-derived macrophages (MDMs).

100  $\mu$ g/mL chitosan, chitosan <30  $\mu$ m or ChOS lactate significantly decreased YKL-40 secretion in MDMs while having no effect on Chit1 secretion. THP-1 only decreased YKL-40 secretion in response to ChOS lactate. ChOS lactate displayed an inhibitory effect on YKL-40 secretion at lower concentrations than any of the other chitosan derivatives, which was attributed to the cytotoxic effect of the material that could be observed at concentrations as low as 20  $\mu$ g/ml. The downregulation of YKL-40 secretion via ChOS lactate may be linked to the increased IL-1 $\beta$  secretion (inflammasome activation) via caspase-1 after successful phagocytosis of the ChOS lactate particles. None of the other chitin derivatives displayed any cytotoxicity, however, both chitosan and chitosan <30  $\mu$ m did cause inflammasome activation, comparable though much milder than was seen in the presence of ChOS lactate.

The differences in bioactivity of the chitosan derivatives can most likely be attributed to the chemical differences of the materials, i.e., deacetylation degree, molecular weight, and polysaccharide chain length. Generally, the ChOS lactate used in this study was smaller than the chitosan preparations and more deacetylated; whereas ChOS was the smallest of the derivatives tested, but had a higher degree of acetylation that most likely explains its inertia.



## **Þakkir**

Rannsóknin var framkvæmd á Rannsóknarstofu í Taugalíffræði (RTL) á Lífvísindasetri Læknagarðs og í Blóðbankanum Landspítala Háskólasjúkrahúsi á árunum 2012-2013. Verkefnið var styrkt af Vísindasjóði Landspítala Háskólasjúkrahúss og Nýsköpunarsjóði námsmanna. Verkefnið er síðasti hluti stærra verkefnis um lífvirkni kítínfásykra. Það verkefni var styrkt af Tækniþróunarsjóði og var samvinna HÍ, Blóðbankans og Genís ehf.

Ég vil þakka Pétri Henry Petersen leiðbeinanda mínum fyrir að veita mér tækifæri og aðstöðu til að vinna að þessu verkefni, fyrir handleiðslu, góðar umræður og hvatningu. Einnig vil ég þakka Ólafi Eysteini Sigurjónssyni fyrir faglega ráðgjöf, góðar ábendingar og fyrir að veita mér aðgang að allri fagþekkingu, tækjum og aðstöðu Blóðbankans. Guðmundi Hrafn Guðmundsyni vil ég þakka fyrir að hafa tekið að sér að vera þriðji maður í meistaranámsnefndinni minni, fyrir yfirlestur á ritgerðinni og góðar ábendingar.

Ramona Lieder vil ég sérstaklega þakka fyrir að taka þátt í þróun verkefnisins með faglegum umræðum, góðum ábendingum, aðstoð við útfærslu og framkvæmd real time qPCR og notkun tækjabúnaðar sem tengdist því. Ramona fær einnig þakkir fyrir ítarlegan yfirlestur á ritgerðinni.

Ég vil þakka Leifi Þorsteinssyni fyrir kennslu, handleiðslu og hjálp við frumurækt á MDMs. Annað starfsfólk Blóðbankans fær þakkir fyrir liðlegheit, blóðgjöf og að veita hjálp og leiðsögn eftir þörfum. Einnig Genís ehf, fyrir aðgang að kítínfásýkrum þróuðum af fyrirtækinu.

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Þá vil ég einnig þakka starfsmönnum og nemendum Lífvísindaseturs Læknagarðs fyrir góðan félagsskap.

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## Abbreviations

<b>AKT:</b>	Ak thymoma, also known as Protein Kinase B
<b>AMCase:</b>	Acidic mammalian chitinase
<b>ANOVA:</b>	Analysis of variance
<b>APS:</b>	Ammonium persulfate
<b>ASC:</b>	Apoptosis-associated speck-like protein containing a CARD
<b>ATCC:</b>	American type culture collection
<b>ATP:</b>	Adenosine triphosphate
<b>bFGF:</b>	Basic fibroblast growth factor
<b>BAX:</b>	Bcl-2-associated X protein
<b>BME:</b>	β mercaptoethanol
<b>BSA:</b>	Bovine serum albumin
<b>CARD:</b>	C-terminal caspase-recruitment domain
<b>CASP1:</b>	Caspase-1
<b>CD14:</b>	Cluster of differentiation 14
<b>cDNA:</b>	Complementary deoxyribonucleic acid
<b>ChOS:</b>	Chitosan oligosaccharide
<b>ChOS L:</b>	Chitosan oligosaccharide lactate
<b><i>Chi3L1</i>:</b>	Chitinase 3-like 1, YKL-40 gene
<b><i>Chi3L2</i>:</b>	Chitinase 3-like 2, also known as YKL-39
<b><i>Chi3L3</i>:</b>	Chitinase 3-like 3, also known as Ym1
<b><i>Chi3L4</i>:</b>	Chitinase 3-like 4, also known as Ym2
<b>Chit1:</b>	Chitotriosidase
<b>CLPs:</b>	Chitinase-like proteins
<b>CT:</b>	Threshold cycle
<b>DAMPs:</b>	Damage-associated molecular patterns
<b>ddH<sub>2</sub>O:</b>	Double distilled water
<b>DMSO:</b>	Dimethyl sulfoxide
<b>dNTP:</b>	Deoxynucleotide triphosphate
<b>DP:</b>	Degree of polymerization
<b>ECM:</b>	Extracellular matrix
<b>EDTA:</b>	Ethylenediaminetetraacetic acid
<b>ELISA:</b>	Enzyme-linked immunosorbent assay
<b>ERK1/2:</b>	Extracellular-signal-regulated kinase 1 and 2
<b>FACS:</b>	Fluorescence-activated cell sorting
<b>Faim 3:</b>	Fas apoptotic inhibitory molecule 3
<b>FAK:</b>	Focal adhesion kinase
<b>FBS:</b>	Fetal bovine serum
<b>GAPDH:</b>	Glyceraldehyde 3-phosphate dehydrogenase
<b>GlcNAc:</b>	N-acetyl-D-glucosamine

<b>H<sub>2</sub>SO<sub>4</sub>:</b>	Sulfuric acid
<b>HcGP-39:</b>	Human cartilage glycoprotein 39, also known as YKL-40
<b>HPLC:</b>	High-performance liquid chromatography
<b>IFN-<math>\gamma</math>:</b>	Interferon $\gamma$
<b>IGF-1:</b>	Insulin-like growth factor 1
<b>IgG1:</b>	Immunoglobulin G 1
<b>IL-10:</b>	Interleukin 10
<b>IL-12:</b>	Interleukin 12
<b>IL-13:</b>	Interleukin 13
<b>IL-18:</b>	Interleukin 18
<b>IL-1<math>\beta</math>:</b>	Interleukin 1 $\beta$
<b>IL-4:</b>	Interleukin 4
<b>IL-6:</b>	Interleukin 6
<b>kDa:</b>	Kilodalton
<b>LAMP2:</b>	Lysosome-associated membrane protein 2
<b>LPS:</b>	Lipopolysaccharide
<b>LRR:</b>	Leucine-rich repeats
<b>LTB:</b>	Lower tris buffer
<b>MACS:</b>	Magnetic-activated cell sorting
<b>MAP kinase:</b>	Mitogen-activated protein kinase
<b>MDM:</b>	Monocyte-derived macrophages
<b>MeOH:</b>	Methanol
<b>M<sub>n</sub>:</b>	Number average molar mass
<b>MS:</b>	Multiple sclerosis
<b>MSU:</b>	Monosodium urate
<b>MW:</b>	Molecular weight
<b>NaCl:</b>	Sodium chloride
<b>NACHT:</b>	Central nucleotide binding and oligomerization domain
<b>NADPH:</b>	Nicotinamide adenine dinucleotide phosphate (reduced)
<b>NaF:</b>	Sodium fluoride
<b>NaOH:</b>	Sodium hydroxide
<b>NAVO<sub>3</sub>:</b>	Sodium orthovanadate
<b>NF<math>\kappa</math>B:</b>	Nuclear factor kappa-light-chain-enhancer of activated B cells
<b>NGS:</b>	Normal goat serum
<b>NH<sub>2</sub>:</b>	Amide
<b>NK cell:</b>	Natural killer cell
<b>NLR:</b>	NOD-like receptors
<b>NLRC4:</b>	NLR card domain-containing 4
<b>NLRPs:</b>	NLR pyrin domain
<b>NLRP3:</b>	NLR pyrin domain-containing 3

<b>NO:</b>	Nitric oxide
<b>NOD:</b>	Nucleotide-binding oligomerization domain
<b>N.P.D.:</b>	Niemann-Pick disease
<b>P2X7R:</b>	P2X purinoceptor 7
<b>PAMPs:</b>	Pathogen-associated molecular patterns
<b>PBS:</b>	Phosphate buffered saline
<b>PBST:</b>	Phosphate buffered saline with tween
<b>PCR:</b>	Polymerase chain reaction
<b>PI-3K:</b>	Phosphatidylinositide 3-kinase
<b>PMA:</b>	Phorbol 12-myristate 13-acetate
<b>PMSF:</b>	Phenylmethylsulfonyl fluoride
<b>PRR:</b>	Pattern recognition receptors
<b>PVDF:</b>	Polyvinylidene fluoride
<b>PYD:</b>	Pyrene domain
<b>RIPA:</b>	Radioimmunoprecipitation buffer
<b>RNA:</b>	Ribonucleic acid
<b>ROS:</b>	Reactive oxygen species
<b>RPM:</b>	Rotations per minute
<b>RPMI:</b>	Roswell Park Memorial Institute
<b>RT:</b>	Reverse transcriptase
<b>RT PCR:</b>	Real time polymerase chain reaction
<b>SDS:</b>	Sodium dodecyl sulfate
<b>SEM:</b>	Standard error of the mean
<b>SI:</b>	Secretion index
<b>Sp1:</b>	Specificity protein 1
<b>Syk:</b>	Spleen tyrosine kinase
<b>TBS:</b>	Tris buffered saline
<b>TBST:</b>	Tris buffered saline with tween
<b>Th:</b>	T helper cell
<b>THP-1:</b>	The human acute monocytic leukemia cell line
<b>TIMP-1:</b>	Tissue inhibitor of metalloproteinases
<b>TLR:</b>	Toll-like receptor
<b>TMB:</b>	3,3',5,5'-Tetramethylbenzidine
<b>TNF-<math>\alpha</math>:</b>	Tumor necrosis factor $\alpha$
<b>UTB:</b>	Upper tris buffer
<b>UVB:</b>	Ultraviolet B
<b>VEGF:</b>	Vascular endothelial growth factor
<b>XTT:</b>	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
<b>YKL-40:</b>	Tyrosine (Y), lysine (K), leucine (L) 40 kDa



# 1 Introduction

Regenerative medicine and tissue engineering aim at restoring normal function of diseased or injured tissue. This is accomplished by replacing nonfunctional tissue and/or to stimulate the body's own repair mechanisms. Regenerative medicine faces a problem when introducing foreign material into the body; it can be sensed as a dangerous entity and attacked by the body's defenses. Therefore, developing safe, biocompatible and even biodegradable materials is important.

Chitin and its derivatives have been extensively studied as potential candidates for such materials in regenerative medicine, due to their many favorable characteristics, such as biodegradability, bioabsorbability and their positive charge (1, 2). However, chitin derivatives have been reported to have immunomodulatory effects, which could be beneficial or harmful (3). The nature of these immunomodulatory effects is often unclear, and it was the goal of this study to examine the role of size and acetylation on the immunomodulatory effect of chitin derivatives.

## 1.1 Chitin

Chitin, the second most abundant polysaccharide in nature after cellulose, is a polymer of  $\beta$ -(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine (GlcNAc) (4). During synthesis, chitin polymers anneal to one another in order to form fibers of high tensile strength (5). The chitin fibers are then cross-linked with proteins resulting in the exoskeleton of various invertebrates, e.g., shrimps and beetles (6), or interact with glycans to form the meshwork reinforcing the cell wall of fungi (7). High-molecular weight chitin has long been considered an attractive option for various clinical and biomedical applications due to it being nontoxic, non-allergenic, biocompatible, biodegradable, and bioabsorbable (8). However, chitin is insoluble in aqueous solutions due to intermolecular hydrogen bonds but can be dissolved in extremely alkaline conditions and low temperatures (4, 9). This makes the polymer, despite its interesting properties, an impractical candidate for biomedical application.

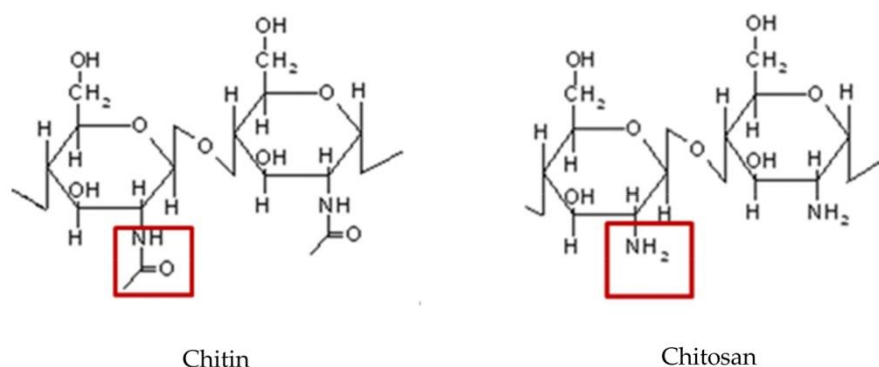
### 1.1.1 Chitosan and ChOS

Many applications require chitin derivatives with increased solubility. The removal of 50% or more of the acetyl groups in the chitin polymer renders the resulting material (chitosan) soluble in dilute acidic solutions (10) (Figure 1).

The chemical composition of chitosan is inherently more complex than chitin. Chitosan can be defined by the length of the polymer and the degree of acetylation, which strongly affect the biological and chemical properties of the polymer. By deacetylating chitin, positively charged amine groups are revealed making chitosan positively charged. The charged amine groups are able to form stable complexes with anions by electrostatic interactions in acidic conditions and interact with metal ions in neutral to alkaline conditions (11). Depending on the application, chitosan can be shaped into different forms, such as fibers, hydrogels, beads, sponges, membranes and nanoparticles. Chemically modified

chitosan in various formulations show great potential as drug carriers, bandages and transfection agents (2).

Chitin and chitosan can be further degraded into smaller polymers by hydrolysis or enzymatic reactions; the resulting polymers are called chitin oligosaccharides or chitosan oligosaccharides (ChOS) (10). ChOS are smaller and more soluble than chitosan and have other biological properties, such as anti-tumor activity (12, 13).



**Figure 1: Chemical structure of chitin and chitosan.**

Chitosan is obtained by deacetylating chitin; red box: side group that is modified. Figure obtained from Stamford et al. (14).

### 1.1.2 Biological activity of chitosan and ChOS

Both chitosan and ChOS have been shown to have biological activity (2, 10). At least part of the bioactivity of chitosan and its derivatives stems from the physico-chemical properties of the polymer, especially the positive charge. This is probably true in the case of the supposed cholesterol lowering ability of chitosan where the highly cationic polymer interacts with anionic bile and fatty acids and hinders emulsification of lipids (15). This also applies to the reported anti-bacterial activity of chitosan. Positively charged amino groups of chitosan interact with negatively charged components of the microbial cell wall and alter its properties and hence this could prevent normal nutrient absorption or cause cellular leakage (16).

ChOS have been described to have either inflammatory (17) or anti-inflammatory activities (18). The differences in the biological activity most likely results from the stark variation in structural and chemical properties, i.e., degree of deacetylation, degree of polymerization, molecular weight, contamination, and salt form. Several studies show that polymer size and acetylation percentage are the key factors that influence its bioactivity (19). Generally, ChOS with a higher degree of polymerization (DP 5-7) are more active than polysaccharides with a DP of 1-4 (20). Furthermore, the biological properties are not only dependent on the chemical and structural properties of the polymer, but are also strongly affected by the cell models used and the disease phenotype studied.

#### 1.1.2.1 Chitin and chitin hexamer

Interpreting studies on chitin bioactivity can be difficult, due to contradictory results utilizing seemingly similar polymers. Shibata et al. (21, 22) showed that intravenous administration of 1-10  $\mu$ m chitin

particles in mice induced alveolar macrophage production of TNF- $\alpha$ , IL-12 and IL-18 and NK cell production of IFN- $\gamma$ , a pathway that is mediated by phagocytosis of chitin via mannose receptor. The same group also showed that oral administration of chitin downregulated Th2 immunity in allergic mice (23). Chitin particles <20  $\mu\text{m}$  elicited the same pro-inflammatory, anti-allergic effect when administered intranasal to allergy challenged mice (24).

In contrast, Reese et al. (25) showed that intranasal administration of chitin particles (of undetermined size) activated macrophages and increased tissue infiltration of IL-4 expressing immune cells connected to allergy and responses to helminth infections, such as eosinophils and basophils. This effect was not seen in mice overexpressing acidic mammalian chitinase (AMCase), an active chitinase, or in mice that received AMCase treated chitin particles (25).

Insight into the biological effect of chitin was obtained by Da Silva et al. (26) who found that large chitin fractions (>70  $\mu\text{m}$ ) and very small fractions (<2  $\mu\text{m}$ ) were inert, while intermediate sized chitin (40-70  $\mu\text{m}$ ) and small chitin (<40  $\mu\text{m}$ , usually 2-10  $\mu\text{m}$ ) stimulated TNF- $\alpha$  secretion of bronchoalveolar and peritoneal macrophages. Small chitin also stimulated IL-10 secretion, an effect that was not seen in intermediate sized chitin. The authors concluded that chitin contains size-dependent pathogen-associated molecular pattern (PAMP) that stimulate TLR2, dectin-1 and the mannose receptor that differentially activate NF- $\kappa\text{B}$  and Syk (26). The same group also showed that intermediate sized chitin is a potent multifaceted adjuvant that induces adaptive Th2, Th1 and Th17 responses (27).

Hence, specifying the size of the chitin polymers that are used is important for correct interpretation of results in chitin research. Chitin hexamers are one example of commercially available chitin derivatives and have been shown to increase the expression of osteogenic differentiation markers and the pro-inflammatory cytokines IL-6 and IL-8 in short term expansion of bone-marrow derived, human mesenchymal stem cells (28). Chitin hexamer application on murine macrophages and human monocytes on the other hand has been reported to increase markers connected to alternative activation and downregulate LPS-induced inflammation (29). These seemingly contradictory results show the differences in responses in different experimental models or approaches.

### **1.1.2.2 Chitosan**

Chitosan has been shown to have immunomodulating effect, such as boosting nitric oxide (NO) production in IFN- $\gamma$  stimulated macrophages and activating the complement system, which leads to increased infiltration of polymorphonuclear cells (30, 31). Chitosan and not chitin has been shown to have a direct effect on cellular activity by activating the inflammasome via phagocytosis-dependent pathway (32). These data show that chitosan could serve as an adjuvant and boost immunological responses. Liu et al. (33) showed that chitosan microparticles enhanced antigen specific IgG titers, cytokine secretion and complement activation in mice. The immunomodulatory effect of chitosan is further supported by increased survival and immune responses of the fish *Epinephelus bruneus* (Kelp Grouper) against the protozoan parasite *Philasterides dicentrarchi*, when on chitosan supplemented diet (34).

Chitosan has been used in wound bandages, with good results, because of its hemostatic effect, inhibition of excessive scar formation and accelerated healing (2, 35). Chitosan has also shown

promise in stimulating healthy regeneration of cartilage by modulating the healing process; inhibiting fibrocartilage scar formation, increasing bone remodeling and modulating angiogenesis (36, 37). Taken together chitosan shows great promise in regenerative medicine.

### **1.1.2.3 Chitooligosaccharides (ChOS)**

ChOS have been reported to have a wide range of biological activities, some only reported once. Lack of proper characterization of ChOS used in biological research has often made comparison of studies using ChOS difficult. One of the biological effect of ChOS that has received much attention is the anti-tumor activity, which was first described in the 1970's (38). Several mechanisms for the anti-tumor activity have been proposed. ChOS is thought to mediate anti-tumor effect by inducing apoptosis via upregulation of the pro-apoptotic protein Bax and subsequent release of cytochrome c (39). The hypothesis that the anti-tumor effect of ChOS could be by inhibiting angiogenesis has also received some attention (12, 40). Xiong et al. (12) showed that fully deacetylated hexamers inhibited angiogenesis via a pathway that resulted in downregulation of vascular endothelial growth factor (VEGF) and upregulation of tissue inhibitor of metalloproteinases (TIMP)-1. Fully acetylated ChOS (DP 2-7) have also been shown to have pro-apoptotic anti-angiogenesis effect (13). ChOS has been shown to enhance natural killer activity in intestinal intraepithelial lymphocytes; ChOS could, therefore, inhibit tumors indirectly by activating innate immune responses (41).

ChOS of variable acetylation have been shown to function as inhibitors to family 18 chitinases (42), making ChOS an attractive therapeutic option in diseases such as asthma, where chitinases are over-expressed (10). ChOS have also been suggested as a control strategy in malaria, where chitinases play an essential role in the life cycle of the parasite (10). Other possible uses of ChOS include anti-fungal usage (10), as vectors in gene delivery and as drug delivery agents (2).

In this study ChOS (also known as Therapeutic ChOS, TChOS™) and ChOS lactate. TChOS has been shown to stimulate chondrocyte growth *in vitro*, an affect that is abolished by adding chitin trimer to the culture (43). In contrast, it did not improve osteogenic differentiation or calcium deposition during osteogenic differentiation of mesenchymal stem cells (44). Little is known about ChOS lactate, and its immunomodulating effect has not been studied. It has been shown to have a protective role in human gingival fibroblasts against double strand breaks induced by methacrylates used in dentistry (45). It has also been shown to have low anti-fungal effect (46).

## **1.2 Chitinases and chitinase-like proteins**

Chitin synthesizing organisms produce enzymes that bind to and digest chitin, which ensures proper turnover of chitin in processes such as molting in insects. These enzymes, called chitinases, prevent the buildup of chitin in the ecosystem (47). Chitinases are evolutionary conserved and have only recently been detected in organisms which do not synthesize chitin, such as mammals (48). On the basis of sequence homology chitinases can be categorized into two families, glycosyl hydrolase families 18 and 19 (49). Mammalian chitinases belong to the glycosyl hydrolase family 18 and so far two enzymatically active chitinases able to hydrolyze natural chitin have been discovered:

chitotriosidase (Chit1) and AMCase. In addition to true chitinases mammals express enzymatically inactive chitinases, termed chitinase-like proteins (CLP), such as YKL-40 (also known as chitinase 3-like 1 and HcGP-39), YKL39 (Chi3L2), Ym1 (Chi3L3), Ym2 (Chi3L4), stabilin-1-interacting chitinase-like protein and oviductin (50).

### **1.2.1 Chitotriosidase**

Chitin has been identified as a structural component of various pathogens, including fungi, which would attribute human chitinases a fungistatic activity that is comparable to plant chitinases (51). Supporting this notion, a 24 base pair duplication in the Chit1 gene, which results in a Chit1 deficiency that has varying frequencies (0-9%) in different populations, has been suggested to render the host more susceptible to infections by chitin containing parasites (52, 53). The exact biological role of Chit1 in humans remains elusive but the fact that it is produced and secreted by neutrophils and macrophages (54) suggests a role in the innate immune system. The upregulation of Chit1 in several pathological disease involving activated macrophages, e.g., Gaucher's disease, Niemann-Pick disease, sarcoidosis, multiple sclerosis and atherosclerosis, highlight the importance of this enzyme in inflammatory process (55-59). Chit1 has even been indicated in the pathology of cerebrovascular dementias, in which the inflammatory processes is activated (60).

Therefore, overproduction of Chit1 might exert deleterious effects, e.g. by altering the normal inflammatory process. This concept is supported by a study where human longevity and several phenotypes of healthy aging was seen in heterozygous carriers of the genetic variations in the Chit1 gene, namely the 24 base pair duplication that results in Chit1 deficiency (61).

### **1.2.2 YKL-40**

YKL-40, originally named after its three NH<sub>2</sub>-terminal amino acids tyrosine (Y), lysine (K), leucine (L) and its molecular weight of 40 kDa, is the most studied CLP in humans. YKL-40 lack of chitinolytic activity is attributed to the substitution of an essential glutamic acid residue by leucine in the active site. However, despite the lack of enzymatic ability, YKL-40 is still able to bind chitin and chitin-derived oligosaccharides with high affinity (62, 63).

The exact biological role of YKL-40 has remained elusive, but recent years have uncovered new insights into its nature. Similar to Chit1, it is secreted by macrophages and is considered a marker for fully differentiated macrophages (64). Interestingly, Chit1 and YKL-40, despite belonging to the same family and the genes being located next to one another in the same orientation are not regulated in the same manner, and YKL-40 is thought to play a more significant role during initial innate immune response (64). YKL-40 transcription has been studied in monocyte to macrophage differentiation processes and binding sites for several known transcription factors were identified in the promoter region. Especially, the transcription factor Sp1 was found to have a predominating role in controlling YKL-40 promoter activity (65). YKL-40 expression can be regulated by various cytokines and hormones, including IL-6, IL-13, IFN- $\gamma$ , vasopressin and parathyroid hormone-related protein (66). IL-1 $\beta$  and TNF- $\alpha$  were also shown to stimulate YKL-40 expression in articulate chondrocytes (67).

YKL-40 is secreted by a number of cells apart from macrophages, including neutrophils (68), chondrocytes (69, 70), fibroblast-like synovial cells (70), giant cells, vascular smooth muscle cells (71), endothelial cells (72), astrocytes (73) and malignant cells from a variety of carcinomas (74-77). The increase of YKL-40 secretion in pathological conditions that involve acute or chronic inflammation indicates a role for the protein in remodeling of the extracellular matrix (ECM), fibrosis and the reduction of the negative effects of pro-inflammatory cytokines. This increase in YKL-40 secretion is met by an equivalent increase in its serum concentrations, sparking the intense interest of the use of YKL-40 as a biomarker in various inflammatory and degenerative diseases (66). Diseases under investigation for the application of YKL-40 as distinctive biomarker include rheumatoid arthritis, osteoarthritis (69), asthma (78), liver fibrosis (79), inflammatory bowel disease (80) and several cancers (74-77). High YKL-40 serum concentrations in these patients have been linked to shorter life expectancy, disease severity, and poorer prognosis.

The biological pathways conveying the activation of YKL-40 and the induction of conformational changes upon ligand binding are under investigation. Crystallographic analyses indicate that some of YKL-40's activity may be conveyed via heparin binding, comparable to the interaction of the ECM protein vitronectin and the angiogenic factors basic fibroblast growth factor (bFGF) and VEGF (81, 82).

In addition, YKL-40 has been shown to promote angiogenesis by inducing the downstream effectors focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAP) ERK1/2 signaling cascades. The above mentioned signaling cascades have been associated with the mediation of cell adhesion, spreading, survival, and migration in vascular endothelial cells, again through heparin binding capabilities (82). On a different note, Recklies et al. (83) showed that YKL-40 stimulates proliferation in fibroblasts, similar to the growth factor IGF-1, via MAP kinase and PI-3K signaling cascades leading to the phosphorylation of ERK1/2 and AKT, respectively. Interestingly the activation of AKT, and not ERK, has been shown to be dependent on the chitin-binding motif of YKL-40 (84). The modulation of fibroblast growth is represented in the severity of diseases that result from the excess production of connective tissue, further related to increased serum concentrations of YKL-40 (79, 85).

Other biological effects of YKL-40 include recruitment of macrophages (75) and control of inflammatory responses in bacterial infections (86). It has been shown that YKL-40 knockout mice have substantially lower survival rates compared with wild-type mice following *Streptococcus pneumoniae* infection. YKL-40 augments macrophage *S. pneumoniae* killing by inhibiting caspase-1 dependent macrophage pyroptosis and augments host tolerance by controlling inflammasome activation, ATP accumulation, expression of ATP receptor P2X7R, and the production thymic stromal lymphopoietin and cytokines associated with Th1, Th2 and Th17 cells (86). Taken together, YKL-40 appears to have multiple functions connected to stress responses, injury, infections and innate immune reactions.

### 1.3 Immune system

The mammalian immune system is very efficient at dealing with foreign entities or endogenous danger signals. It is comprised of two systems that interact to deal with threats, the innate and adaptive immune system. The innate immune system is the body's first line of defense against pathogens. It is comprised of macrophages that reside in tissues, where they clear apoptotic cells and general cellular debris, the results of tissue turnover or remodeling, until they come in contact with pathogens that result in their activation. Activated innate immune cells eliminate pathogens through unspecific methods that do not change throughout the individual's lifetime and help the maturation of an adaptive immune response. The adaptive immune system, which is comprised of lymphocytes, on the other hand eliminates pathogens through antigen-specific methods but it takes several days for the response to develop in contrast to the immediate innate response. Once an adaptive response has formed, subsequent exposures of that particular pathogen result in a more robust and swift response, because of immunological memory (87).

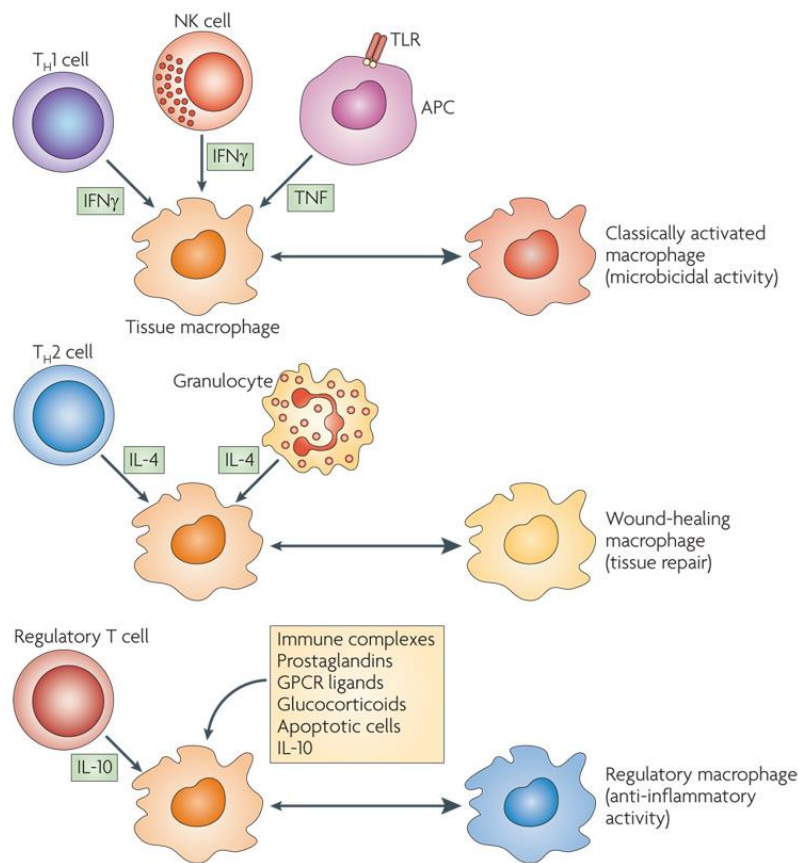
### 1.4 Macrophages

Macrophages are derived from monocytes and play an important role in the innate immune system by participating directly in the immune response and controlling the course of inflammatory reactions (88, 89). Monocytes originate in the bone marrow, from a common myeloid progenitor, and are released into the peripheral blood where they circulate throughout the body (90). Monocytes are thought to migrate into tissues in the steady state to replenish the tissue macrophage populations (90, 91). Infection or other danger signs in tissue increases recruitment of monocytes by inducing the expression of adhesion molecules on vascular endothelial cells which 'hook' the monocytes and help them migrate through the endothelium and enter into the tissue (92). Once they start to migrate they also start to differentiate into macrophages, assisted by macrophage colony stimulating factor (M-CSF), but are still inactive until a chain of events results in their activation (90).

Inactive macrophages are very efficient phagocytic cells that clear cellular debris, apoptotic cells and short-lived erythrocytes, which contain hemoglobin that can subsequently be recycled. Phagocytosis of cellular debris and apoptotic cells does not lead to the activation of the macrophages. In contrast, phagocytosis of necrotic cellular debris, which contains many endogenous danger signals, results in activation, making macrophages one of the primary danger sensors of the host (91).

There are several ways for macrophages to be activated and different stimuli result in differently activated cells. Activated macrophages can roughly be classified into three groups: classically activated (type-1), alternative activated (type-2) and regulatory macrophages. Classical activation results in macrophages with high microbicidal activities that secrete reactive oxygen species and pro-inflammatory cytokines to aid in the clearance of invading pathogens and stimulate the acquired immune response. Alternative activated macrophages, on the other hand, are connected with tissue repair and regulatory macrophages with resolution of inflammation (91). Insufficient activation or errors in activation can impair the body's defenses against infection, excessive activation or impaired

resolution of inflammation can also be harmful and cause chronic inflammation or tissue fibrosis (93). It is, therefore, of great importance to understand what activates macrophages, thus yielding methods to influence their activation state, for instance in chronic inflammatory diseases or difficult infections.



**Figure 2: Activation of macrophages.**

Macrophage activation pathways: classical activation, wound healing/alternative activation and regulatory activation. Figure obtained from Mosser et al. (91).

Activated macrophages express specific proteins that are connected to the new roles the macrophage have after activation. These proteins can be used to identify the activation state of the macrophage (94). Cytokine secretion profile can be used to determine the activation state of the cells. The cytokines are generally categorized as pro-inflammatory or anti-inflammatory, even though many can take part in both. Pro-inflammatory cytokines like  $\text{TNF-}\alpha$ , IL-1 and IL-12 are usually connected to classically activated cells (95) while anti-inflammatory cytokines like IL-10 are connected with regulatory cells (91).

The proteins Chit1 and YKL-40, discussed in sections 1.2.1 and 1.2.2, are secreted by fully differentiated macrophages *in vitro* (64, 96) and were recently described to be upregulated in classically activated macrophages and downregulated in alternative activated macrophages (73, 97), suggesting a role for them in activated macrophages. *In vivo* macrophages, on the other hand, seem to have a stricter regulation of Chit1 and YKL-40 as only a small subset of macrophages in

atherosclerotic lesions express Chit1 and YKL-40 (59), and in neuroinflammatory conditions astrocytes and not macrophages express abundant YKL-40 (73).

#### **1.4.1 THP-1 cells**

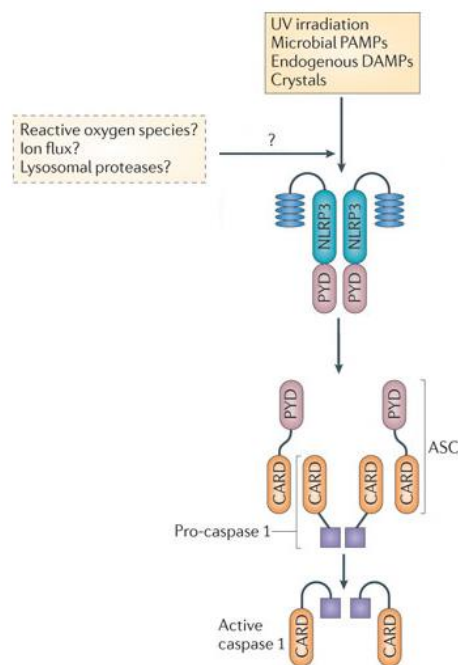
Studies of human monocytes and/or macrophages are often restricted due to limited accessibility of primary human monocytes. Donor variation can also affect experiments when using primary cells. Cell lines, on the other hand, are more stable, easier to culture and not restricted by donor variation. The monocytic leukemia cell line, THP-1, is a monocyte-like cell line, originally isolated by Tsuchiya et al. in 1980 from a boy suffering from acute monocytic leukemia (98). It is widely used as a model for human monocyte and macrophage functions, due to its close resemblance to primary monocytes. THP-1 cells can be differentiated into macrophages with phorbol-12-myristate-13-acetate (PMA), which induces changes which are hallmarks of macrophages, for example: cell adhesion, spreading and enhanced phagocytic ability (99). Caution needs to be taken when deciding on PMA concentration for differentiation, as too high concentration (such as 100 ng/mL) results in transcription of pro-inflammatory genes, such as IL-18, IL-1 $\beta$  and TNF- $\alpha$ , that could mask responses of a weak stimuli (100). It is also of importance to keep in mind that the differentiation pathway of PMA-THP-1 cells vs. primary human monocyte-derived macrophages diverges to a certain degree, e.g. IL-1 $\beta$  transcription is downregulated in monocyte to macrophage differentiation but upregulated in PMA-THP-1 differentiation (101). Extrapolation of results needs to be carefully considered due to the use of a cell line instead of primary cells.

#### **1.4.2 The inflammasome**

Cells of the innate immune system make up the first line of defense against pathogens, they express pattern recognition receptors (PRR) that recognize and bind distinct conserved PAMPs which are predominantly found in microbes. The outcome of PRR stimulation depends on the responding cell type and the pathogen involved. One set of intracellular PRRs make up a family called the NOD-like receptors (NLR). They sense host derived danger signals (damage-associated molecular patterns, DAMPs) that are released when tissue homeostasis is disturbed by microbial or non-microbial insults, in addition to pathogen motifs. Phylogenetic analysis revealed 3 distinct subfamilies within the NLR; NODs, NLRC4 and NLRPs, which is supported by similarities in structure (102).

Certain members of the NLR family form cytosolic protein complexes, known as inflammasomes, upon stimulation. Inflammasomes control maturation and secretion of inflammatory cytokines, such as IL-1 $\beta$  and IL-18, by serving as caspase-1 activation platform. The most studied inflammasome is the NLR pyrin domain-containing 3 (NLRP3, also known as NALP3) inflammasome (Figure 3). It consists of 3 domains; C-terminal leucine-rich repeats (LRR), central nucleotide binding and oligomerization domain (NACHT) and N-terminal pyrine domain (PYD) (102). Under normal conditions the NLRP3 remains inactive due to internal interaction between the LRR- and NACHT domains (103). The precise mechanism by which the auto-inhibition is relieved is not known (104). It is possible that some of the activators interact directly with NLRP3 and relieve the suppression, but the structural variety of NLRP3 activators makes it unlikely that this is true for all activators. NLRP3 can be activated by various

signals associated with pathogens (whole pathogens, PAMPs and bacterial toxins), but also signals for cellular danger or stress (K<sup>+</sup> efflux, elevated extracellular ATP, glucose or monosodium urate crystals) and environmental irritants (silica, asbestos and UVB irradiation) (105). Three nonexclusive models of NLRP3 activation have been proposed, reviewed in (105). The channel model proposes that the activators induce pore formation in the cell membrane that lead to K<sup>+</sup> efflux, which is a requirement for inflammasome activation. Another model, the lysosome rupture model, proposes lysosome rupture following phagocytosis of large particles the cell is unable to process normally. The lysosomal protein cathepsin B, which is normally contained in the lysosome, is released into the cytoplasm and triggers inflammasome activation directly or indirectly through an uncharacterized pathway (106). The last model, the ROS (reactive oxygen species) model, proposes that NLRP3 does not distinguish between different activators, instead it is activated by ROS generated in proximity to the inflammasome, serving as a general sensor of cellular stress (105).



**Figure 3: NLRP3 inflammasome assembly.**

Various insults trigger the assembly of the inflammasome. Autorepression of NLRP3 is removed leading to self oligomerization and subsequent oligomerization of the PYD domain to PYD containing CARD. CARD recruits pro-caspase 1 which is cleaved into active caspase 1. Figure adapted from Lamkanfi (107).

Removal of auto-repression between the LRR and NACHT domains, by any of the pathways discussed above, activates the NLRP3. The unbound NACHT domains are now free to interact with other NLRP3 NACHT domains, creating high molecular weight complexes. These complexes are then able to recruit PYD- and CARD-containing ASC (apoptosis-associated speck-like protein containing a CARD) via interaction between the PYD domains. The CARD domains in turn recruit pro-caspase 1, which results in cleavage of the pro-caspase 1 into the active form (Figure 3). Activated caspase 1 is then able to process cytoplasmic targets, such as IL-1 $\beta$  and IL-18 or under certain situations causes the cell to go through pyroptosis, a pro-inflammatory type of cell death (102, 104).

Inflammasome activation is an important innate immune response. Its secretion products, IL-1 $\beta$  and IL-18, are potent signaling molecules that help eliminating pathogens by stimulating fever, attracting leukocytes to the site of infection and promoting adaptive immune responses. Excessive activation can lead to pyroptosis, which is also an important innate response to eliminate intracellular pathogens (108). This potent innate pathway is manipulated in vaccination, by using adjuvants such as alum that activate the inflammasome and therefore increase the immunological response towards the inactive pathogen in the vaccine. Biomaterials that affect the inflammasome are therefore of great interest as a possible therapeutic options where immunomodulation is needed.



## **2 Aims**

Chitin and its derivatives have been shown to have immunomodulatory effect. Different chitin derivatives do not necessarily have the same biological effect. Degree of acetylation and size seem to play a large role in terms of biological effect. Chitosan and not chitin has for example been shown to activate the inflammasome (32).

YKL-40 and Chit1 are an inactive and an active chitinase, respectively. These proteins, in addition to bind to (YKL-40 and Chit1) and degrade chitin (Chit1), have a role in innate immunity, as they are secreted by macrophages. They have both been found to be highly upregulated in conditions that involve activated and even uncontrolled innate immune responses.

The aim of this project was to examine whether chitin derivatives have an effect on the secretion of Chit1 and YKL-40 by macrophages. Chitin derivatives could induce, as has been suggested for Chit1, or even inhibit YKL-40 and Chit1 secretion. Changes in YKL-40 secretion were of special interest as chitosan has been shown to activate the inflammasome and YKL-40 has been shown to be an important inhibitor of the inflammasome.

### **2.1 Specific aims**

- 1. Evaluate changes in secretion of YKL-40 and Chit1 in macrophages after chitin derivative stimulation**
- 2. Evaluate the pro-inflammatory effect by measuring TNF- $\alpha$  and IL-1 $\beta$  in macrophages after chitin derivative stimulation**
- 3. Evaluate cytotoxicity of each chitin derivative on macrophages**
- 4. Compare results from two different human cell models; THP-1 macrophages and monocyte-derived macrophages**



### **3 Materials and methods**

#### **3.1 Chitin derivatives**

##### **3.1.1 ChOS**

Chitosan oligosaccharide (ChOS) production has been described previously (44). Briefly, ChOS was derived from shrimp shell and produced by the Icelandic company Genis. ChOS is 60% deacetylated and 78.5% of the material has a DP between 6-12. Before use, ChOS was endotoxin cleaned with Detoxi-Gel Endotoxin Removing Gel (Thermo). It was stored in aliquots at -20°C.

##### **3.1.2 ChOS lactate**

Chitosan oligosaccharide lactate salt (Sigma-Aldrich) was dissolved in PBS, vortexed and sterile filtered (0.45 µm). It has been characterized as a 90% deacetylated ChOS with 75% of the material having a DP between 3-5 (109), with average molecular weight of  $M_n$  5,000 (Sigma-Aldrich). It was stored at 4°C.

##### **3.1.3 Chitosan**

Chitosan (Sigma) was dissolved in 30 mL 1 M NaOH solution and kept at 90°C for 1 hour to remove possible endotoxins (32). Solution was centrifuged, washed once in ddH<sub>2</sub>O and five times in sterile endotoxin free PBS (Gibco). The material is reported to be ≥75% deacetylated by the manufacturer.

“Small” chitosan (<30 µm) was made from the original chitosan solution by sonicating the solution at full power for 15 minutes in Bioruptor sonicator, after which it was filtered with a 30 µm filter.

##### **3.1.4 Chitin hexamer**

Chitin hexamer (Chitohexaose, IsoSep AB) has the molecular formula of C<sub>48</sub>H<sub>80</sub> N<sub>6</sub>O<sub>31</sub> (DP of 6), molecular weight of MW 1237.1 g/mol and a purity of 95% (determined by HPLC), as reported by manufacturer. It was endotoxin cleaned with Detoxi-Gel Endotoxin Removing Gel (Thermo).

#### **3.2 Cell culture**

##### **3.2.1 THP-1 cells**

The human acute monocytic leukemia cell line (THP-1) (American type culture collection, ATCC) was grown in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 100 Units/mL Penicillin and 100 µg/mL Streptomycin (Gibco) and 10% fetal bovine serum (FBS, Gibco) and kept at 37°C in 95% humidified 5% CO<sub>2</sub> atmosphere. Cells were split every 3-4 days to maintain a cell density between 2·10<sup>5</sup> and 1·10<sup>6</sup> cells/mL. Cells were routinely counted using the automated cell counter Countess (Invitrogen) by mixing 8 µL of cells with 8 µL of 0.4% trypan blue (Invitrogen) and loading 10 µL of the trypan blue stained cells into a Countess counting chamber slide (Invitrogen). Then, cells were spun down at 1200 rpm for 10 minutes, the old medium removed and cells resuspended with fresh medium at a concentration of 2·10<sup>5</sup> - 2.5·10<sup>5</sup> cells/mL.

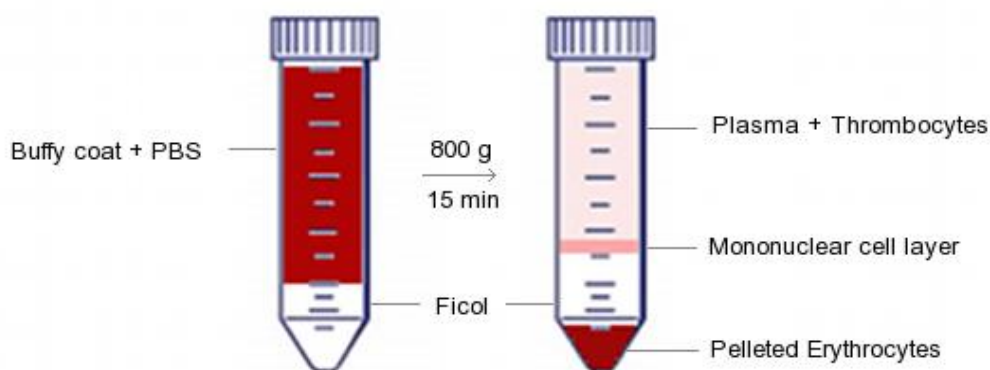
One passage before cells were used in experiments, they were split into medium containing 2% FBS. During the experiments, the cells were seeded at a density of  $5 \cdot 10^5$  cells/mL into medium containing 2% FBS and 50 ng/mL of PMA (Calbiochem) (day 0). Two days later (day 2), cells were washed gently with phosphate buffered saline (PBS) and re-incubated in PMA free medium. THP-1 macrophages have been shown to be most similar to primary monocyte-derived macrophages when given a few days to rest after PMA stimulation (110). They were, therefore, rested for two days (day 4). On day 4 the cells were stimulated with the test substances (chitin derivatives (see 3.1), lipopolysaccharide (LPS) (Sigma, from *E. coli* 055:B5), cytochalasin D (see 3.2.3), IL-6 (Peprotec), TNF- $\alpha$  and IFN- $\gamma$  (R&D Systems)) in medium supplemented with 2% FBS and either 24 hours later (day 5), cell culture medium supernatants were collected for western blot (see 3.5) and ELISA (see 3.6) analysis and stored in aliquots at -20°C, or the cells were incubated for 1-4 hours and then lysed with RIPA lysing buffer (see 3.4) for intracellular western blot analysis.

These experiments were performed on cells that had not reached passage 30, usually around passage 20, where one passage is defined as sub-culturing of the cells for 3-4 days.

### **3.2.2 Monocyte-derived macrophages (MDMs)**

Primary human monocytes were isolated from buffy coat blood (plasma free blood) of healthy human volunteers, which was obtained from the Blood Bank, Landspítali – The National University Hospital of Iceland (day 0). Monocyte isolation was initiated by isolating mononuclear cells with density gradient centrifugation using Ficoll-Paque™ (Sigma). Briefly, 10 mL buffy coat blood and 30 mL PBS (Gibco) were mixed in a 50 mL test tube. Then, 10 mL of Ficoll-Paque was slowly injected to the bottom of the tube with a syringe. After 15 min centrifugation at 800 g, the mononuclear cell layer was collected (Figure 4), diluted with PBS and spun down again at 800 g for 15 minutes.

After centrifugation, the supernatant was removed completely and cells incubated with 10 mL FACS lysing-solution (BD Biosciences) for 10 minutes, after which PBS was topped up to 50 mL and cells spun down at 1200 rpm for 10 minutes. The resulting cell pellet was re-suspended in 10-20 mL PBS and counted in Cell Dyn Ruby (Abbot Diagnostics). Then, PBS was added to the 50 mL mark and the cells spun down again at 1200 rpm for 10 minutes. The cell pellet was re-suspended in 80  $\mu$ L of MACS buffer (PBS supplemented with 2 mM EDTA (Merck) and 0.5% Bovine serum albumin (BSA, Sigma)) and 20  $\mu$ L of MACS CD14 MicroBeads (Miltenyi Biotec) per  $10^7$  total cells. This mixture was then thoroughly mixed and incubated at 4°C for 15 minutes. After incubation, cells were washed by adding 10-20x the labeling volume of buffer and centrifuged at 300 g for 10 minutes. The supernatant was removed and cells re-suspended in 500  $\mu$ L of MACS buffer per  $10^8$  cells. CD14 positive cells were then isolated by magnetic separation with a positive selection column. LS+/VS+ column (Miltenyi Biotec) was placed on a magnetic field separator and washed with 3 mL of MACS buffer. The cell suspension was then applied on to the column after which the column was washed 3 times with 3 mL



**Figure 4: Ficoll density gradient centrifugation.** 10 mL of buffy coat from healthy individuals and 30 mL of PBS is mixed in a 50 mL test-tube and 10 mL of Ficoll is injected at the bottom of the tube. The test tube is then spun down at 800g for 15 minutes, the result are four different layers of which the mononuclear cell layer is collected.

of MACS buffer. Finally, the column was removed from the magnetic field, washed with 5 mL of buffer and the CD14 positive cells collected. Cells were again counted in Cell Dyn Ruby, washed with PBS and seeded at  $2\text{--}2.5 \cdot 10^6$  monocytes/well in a 6 well plate or  $1 \cdot 10^6$  monocytes/well in a 12 well plate in RPMI 1640 Glutamax (Gibco) medium supplemented with 100 Units/mL Penicillin, 100  $\mu\text{g/mL}$  Streptomycin and 10% pooled normal human serum (see 3.2.2.1). After 3 days half of the medium was replaced (day 3). On day 6, cells were washed with PBS and medium completely replaced. On day 9, cells were stimulated with test substances (chitin derivatives (see 3.1), LPS, cytochalasin D (see 3.2.3)) in serum free medium and 24 hours later the medium was collected from the cells for western blot (see 3.5) and ELISA (see 3.6) analysis and stored in aliquots at  $-20^\circ\text{C}$  and the cells stained with crystal violet (see 3.3).

The challenge with using primary cells is donor variation regarding final yield of macrophages, i.e. some donors gave a very good layer of macrophages, while others did not. There also seemed to be some variation between wells containing cells from the same donor. This created problems in western blot analysis on medium because the western blot requires same cell numbers to be present in each well. If there are fewer cells in one well it creates a secretion bias as there are fewer cells secreting proteins into the medium. If this is not adjusted for one might interpret the results wrongly. It was therefore decided to stain the cells with crystal violet after medium had been harvested to get an estimate on cell numbers and adjust the western results according to the cell numbers.

### **3.2.2.1 Human serum**

Blood was drawn from a minimum of 6 healthy volunteers into Vacuette z serum clot blood collection tubes (Greiner Bio One). Blood was incubated at room temperature for 1-2 hours, for sufficient clotting. Tubes were centrifuged at 1200 g for 10 min, serum collected and pooled into a fresh tube/s and centrifuged again at 1800 g for 10. Serum was sterile filtered, complement inactivated by incubating it at  $65^\circ$  for 30 minutes, aliquoted into 4 mL tubes and stored at  $-20^\circ\text{C}$  until used in cell culture.

### **3.2.3 Phagocytosis inhibition**

Cytochalasin D (Gibco) was dissolved in DMSO at a concentration of 10 mg/mL and stored in aliquots at -20°C. Phagocytosis inhibition was obtained by incubating THP-1 macrophages or monocyte-derived macrophages with a final concentration of 1 µg/mL Cytochalasin D 30 minutes prior to chitin derivative stimulation.

### **3.2.4 Cell proliferation assay**

100 µL of  $6 \cdot 10^5$  primary monocytes were seeded into a 96 well plate, treated as described above (see 3.2.1 and 3.2.2) and incubated with test substances for 24 h. Then, 50 µL XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, ATCC) was added to each well and incubated for 4 h. Plates were analyzed in SpectraMax Plus 384 absorbance reader (Molecular devices) at 450 nm and 360 nm after appearance of orange color change. The XTT assay measures the cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes that reduce the tetrazolium dye, XTT, to formazan that gives an orange color. Proliferation was determined after correction for background (media without cells) and nonspecific noise (360 nm measurement).

### **3.3 Crystal violet staining**

Cells were washed with PBS at the end of experiments and fixed with 4% formaldehyde (Sigma) for 10-15 minutes. Then cells were washed with PBS and dyed with crystal violet solution (PBS w/ 10% v/v ethanol, 0.1% w/v crystal violet) for 20 minutes. Then, cells were washed three times with dH<sub>2</sub>O and the cell density determined by dissolving the dye with 30% acetic acid and measuring optical density at 570 nm. Crystal violet accumulates in the nuclei, the optical density correlates therefore with the nuclear DNA content and thus cell numbers.

### **3.4 Intracellular protein extraction**

Cells were rinsed with ice cold PBS, RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX 100, supplemented with the protease inhibitors: NAVO<sub>3</sub>, PMSF, NAF and protease inhibitor cocktail) pipetted onto cells (75 µL for each well on a 6 well plate or 50 µL for a 12 well plate) and incubated on cells for 10 minutes on ice with occasional tilting of the plate. Then, the surface was scraped with a cell scraper or the wide end of a pipette tip and cell lysates transferred to an eppendorf tube. The lysate was then sonicated for 3 cycles at medium setting (about 2 minutes) in a Bioruptor sonicator, after which the lysate was kept on ice for 10 minutes and finally centrifuged at 12,000g for 20 minutes at 4°C. Lysates were stored at -20°C.

### 3.5 Western blot

#### 3.5.1 Sample preparation

Media supernatants from THP-1 and primary macrophages were acetone precipitated by mixing 300  $\mu$ L sample with 1200  $\mu$ L ice-cold acetone to increase protein concentration. Then, the acetone cell culture media mixture was vortexed and incubated at -20°C for at least 1 hour. After the incubation, the solution was centrifuged at 15,000 g for 10 minutes. The resulting pellet was air dried for 10-20 minutes, resuspended in 30  $\mu$ L 2x SDS loading buffer (4% w/v SDS, 20% v/v Glycerol, 120 mM Tris, 5% v/v BME) and incubated at 80°C for 20 min with vigorous shaking (1400 rpm). Cell lysates were mixed with equal volumes of 2x SDS loading buffer and incubated at 90°C for 10 minutes.

#### 3.5.2 Electrophoresis and transfer

Samples were loaded on a 12.5% SDS-polyacrylamide gel (Table 1) and electrophorized at 110 V for 90 minutes in running buffer (200 mM Glycine, 0.1% w/v SDS, 25 mM Tris). The gels were then transferred to a methanol pre-activated PVDF membrane (Immobilon-FL, Millipore) or a nitrocellulose membrane (Macherey-Nagel) at 400 mA for 60 minutes in transfer buffer (25 mM Tris, 200 mM Glycine, 20% v/v MeOH).

**Table 1: Recipe for two 12.5% SDS-polyacrylamide gels.**

Acrylamide and ammonium persulfate (APS) was purchased from AppliChem. Lower tris buffer (LTB): 3 M Tris, 0.8 % w/v SDS. Upper tris buffer (UTB): 500 mM Tris, 0.4% w/v SDS.

<i><b>Materials</b></i>	<i><b>Lower/separating gel</b></i>	<i><b>Upper/stacking gel</b></i>
<b>Water</b>	4.15 mL	3.61 mL
<b>40% Acrylamide</b>	3.15 mL	0.62 mL
<b>LTB</b>	2.6 mL	-
<b>UTB</b>	-	0.68 mL

#### 3.5.3 Immunoblotting

Membranes were blocked in TBS (Tris buffered saline, 20 mM Tris, 137 mM NaCl) with 5% w/v skimmed milk powder (Mjólkursamsalan) for 30-60 minutes, incubated either for 3 hours at room temperature or overnight at 4°C with the primary antibody solution (TBS w/ 5% w/v milk, 0.1% v/v Tween (Sigma) and either: anti-YKL-40 (1:50, Quidel), anti-Chit1 (1:500, Sigma), anti-Caspase-1 (1:1000, Abcam), or anti- $\alpha$ -actin (1:10,000, Millipore)). Then, the membrane was washed four times for 5 minutes with TBST (TBS w/ 0.1% v/v Tween) and incubated with IRdye 800 rabbit or IRDye 700 mouse secondary antibodies (1:20,000, Li-cor) for 1 hour at room temperature. After incubation, the membrane was washed four times for 5 minutes in TBST, and then scanned and analyzed in the Odyssey imaging system (Li-cor). Absolute fluorescent values were normalized with crystal violet optical density values, to adjust for cell numbers. Secretion index (SI) was obtained as follows: A

mean, calculated from all untreated normalized replicates, was used as a baseline for the SI, independently for every donor. Values from all treatments (within each donor) were then divided by the mean which gave a relative secretion value.

### 3.6 ELISA

Enzyme-linked immunosorbent assay (ELISA) for TNF- $\alpha$ , IL-1 $\beta$  (DuoSet ELISA, R&D Systems) and YKL-40 (MicroVue ELISA, Quidel) was performed on cell culture media supernatants of stimulated cells. YKL-40 ELISA was performed according to manufacturer's instructions. For TNF- $\alpha$  and IL-1 $\beta$ , MaxiSorp (Nunc) flat bottom 96 well plates were coated with 100  $\mu$ L of 4  $\mu$ g/mL TNF- $\alpha$  or IL-1 $\beta$  in PBS overnight. The next day, plates were washed 3 times with PBST (PBS w/ 0.05% v/v Tween) using BioTek ELx405 plate washer, and blocked for 1 hour with reagent diluent (PBS with 1% w/v BSA). Plates were washed 3 times with PBST and incubated for 2 hours with a standard curve for each cytokine and the media supernatant; diluted according to needs. After washing the plates 3 times with PBST, the detection antibody (100  $\mu$ L of 0.2  $\mu$ g/mL stock) was incubated for 2 hours and then the plates were washed again. Streptavidin conjugated horseradish peroxidase (mixed 1:200 with reagent diluent) was added for 20 minutes. Finally, plates were washed again, TMB one (3,3', 5, 5'-tetramethylbenzidine, Kem En Tec diagnostics or Thermo) added, and the reaction stopped after 15-20 minutes with 2 M H<sub>2</sub>SO<sub>4</sub>. Plates were read at 450 nm in SpectraMax Plus 384. Sample concentration was calculated using a standard curve for each cytokine. Detection limits of each ELISA were as follows: YKL-40 15.6 ng/mL, TNF- $\alpha$  15.5 pg/mL, IL-1 $\beta$  3.91 pg/mL.

### 3.7 Immunocytochemistry

Cells were incubated with each of the chitin derivatives for 2 hours, then fixed with 4% formaldehyde (Sigma) for 10 minutes and washed three times with PBS. Unspecific binding was blocked and cell membrane perforated by incubating fixated cells with PBS containing 5% NGS (normal goat serum, Sigma) and 0.1% TritonX-100 (Merck) for 20 minutes. Cells were then incubated overnight at 4°C with anti-LAMP-2 (1:200, DSHB) in PBS with 5% NGS. The next day, cells were washed three times with PBS and incubated for 30 minutes with Alexa fluor 546 anti-mouse IgG1 (1:1000, Invitrogen) and TO-PRO 3 (1:1000, Invitrogen) in PBS with 5% NGS. Lastly, cells were washed three times with PBS and mounted with fluormount (Sigma) onto a glass slide.

Cells that were treated with Lysotracker DND-99 (Invitrogen) were treated for 30 minutes with 5 nM Lysotracker, 1.5 hour after chitin derivative activation, after which they were mounted onto glass slides with fluormount and live cell imaged. Cells were imaged with LSM 5 Pascal from Zeiss. Images were resized and arrows were added with the image manipulation software Gimp version 2.6.11.

## 3.8 Real time PCR

### 3.8.1 RNA isolation

At the end of the experiments, cells were lysed with RLT lysis buffer (Quiagen). RNA isolation was performed with Quiagen BioRobot workstation and the EZ-1 RNA Cell Mini Kit (Quiagen), following manufacturer's instructions.

### 3.8.2 cDNA transcription

RNA was transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Mastermix preparation is described in Table 2. Ten  $\mu\text{L}$  sample RNA and 10  $\mu\text{L}$  master mix were transferred together into PCR tubes and then centrifuged for a few seconds at 2500 rpm to remove air bubbles. Then PCR tubes were transferred to a thermal cycler with the following cycling conditions: 25°C for 10 minutes (enzyme activation), 37°C for 120 minutes (cycling), 85°C for 5 seconds (enzyme deactivation), 4°C until transferred to a -20°C freezer.

**Table 2: Mastermix for cDNA transcription.**

<i><b>Material</b></i>	<i><b>Volume</b></i>	<i><b>Manufacturer</b></i>
<b>10x RT buffer</b>	2.0	Applied Biosystems
<b>25x dNTP</b>	0.8	Applied Biosystems
<b>10x Random Primers</b>	2.0	Applied Biosystems
<b>Multiscribe RT</b>	1.0	Applied Biosystems
<b>RNAse Inhibitor</b>	1.0	Applied Biosystems
<b>Nuclease-free H<sub>2</sub>O</b>	3.2	Fermentas

### 3.8.3 Real-time PCR

Real-time PCR was performed in the 7500 Real Time PCR System (Applied Biosystems) utilizing the StepOne software. Assays were performed as following:

cDNA: 1  $\mu\text{L}$  cDNA + 9  $\mu\text{L}$  sterile H<sub>2</sub>O

Assay: 1  $\mu\text{L}$  Taqman Assay (Chi3L1 (Hs00609691\_m1) and GAPDH) + ready-made master mix (Applied Biosystems)

Nine  $\mu\text{L}$  of cDNA dilution was mixed with 11  $\mu\text{L}$  of assay preparation in a 48-well PCR plate, adhesive foil was applied and plate spun down for a few seconds to get rid of bubbles. Samples were run with a quantification protocol. Data was analyzed with GenEx 5.3.2.13 software (MultiD) using GAPDH as a housekeeping gene. Fold changes were calculated with the deltadelta CT method and normalized to the control samples.

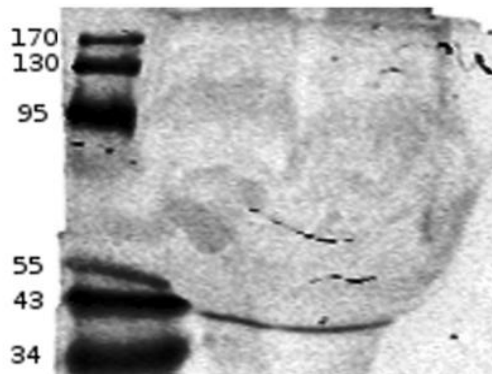
### **3.9 Statistical analysis**

Primary analysis and normalization of data was performed in Microsoft Excel 2008 (Microsoft Corporation). Data are presented as mean  $\pm$  standard error or individual value presented in a dot-plot along with grand mean of each group. Figures were generated and statistical analysis was performed in Prism 5.01 software (GraphPad Software Inc.). One-way ANOVA with Tukey's multiple comparison test was used to determine statistical difference between groups, two-way ANOVA between paired groups with Bonferroni post-test or paired student's t-test for paired samples. Difference was considered significant when  $p < 0.05$ .

## 4 Results

### 4.1 Verification of detection methods

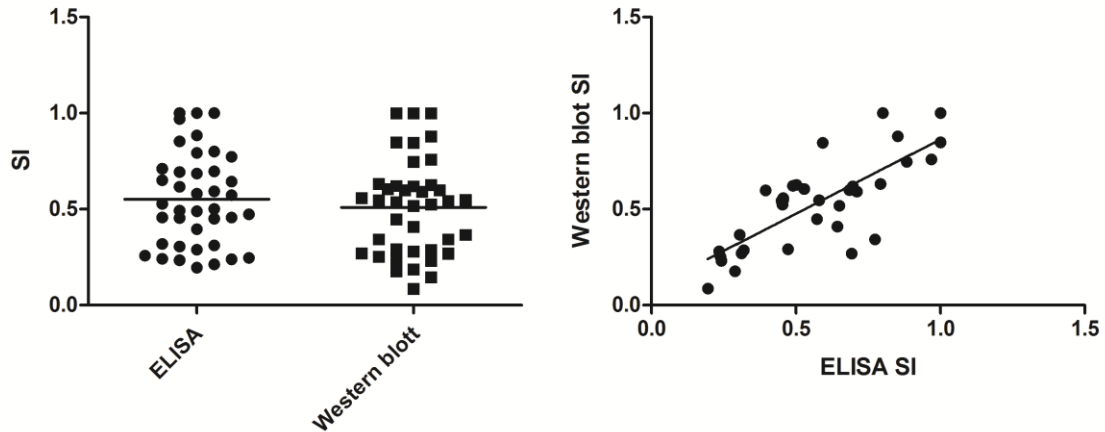
Chit1 and YKL-40 are secreted by differentiated macrophages into the extracellular matrix. Measurement of secreted YKL-40 and Chit1 depend on detection in media or plasma. In the media supernatants, both proteins are present in too low concentrations to be detected directly via western blotting. Therefore, acetone precipitation was used to increase the protein concentration. Bands for YKL-40 and Chit-1 showed the appropriate MW, that is 40 and 50 kDa, respectively. However, for experiments performed in the presence of 10% serum the protein pellet could hardly be dissolved in the loading buffer and affected the running of the gel (Figure 5). Therefore, chitosan derivatives were administered in low serum (2%, THP-1 macrophages) or serum free experiments (monocyte-derived macrophages).



**Figure 5: Using medium supplemented with 10% serum in experiments is inconvenient when medium samples are to be acetone precipitated and western blotted.**

Western blot for YKL-40 (~40 kDa) on acetone precipitated medium from cell culture supplemented with 10% serum. Samples loaded into two adjacent wells do not form two distinct bands, one for each well, but merge because of excess protein amount due to high serum content.

To evaluate the detection sensitivity of western blotting, samples were analyzed for YKL-40 with western blotting and also with a commercial YKL-40 ELISA, which is a more sensitive detection method, but a more expensive choice. The methods proved to give similar results; difference was not significant as measured with paired student's t-test (Figure 6).



**Figure 6: Evaluation of western blotting sensitivity**

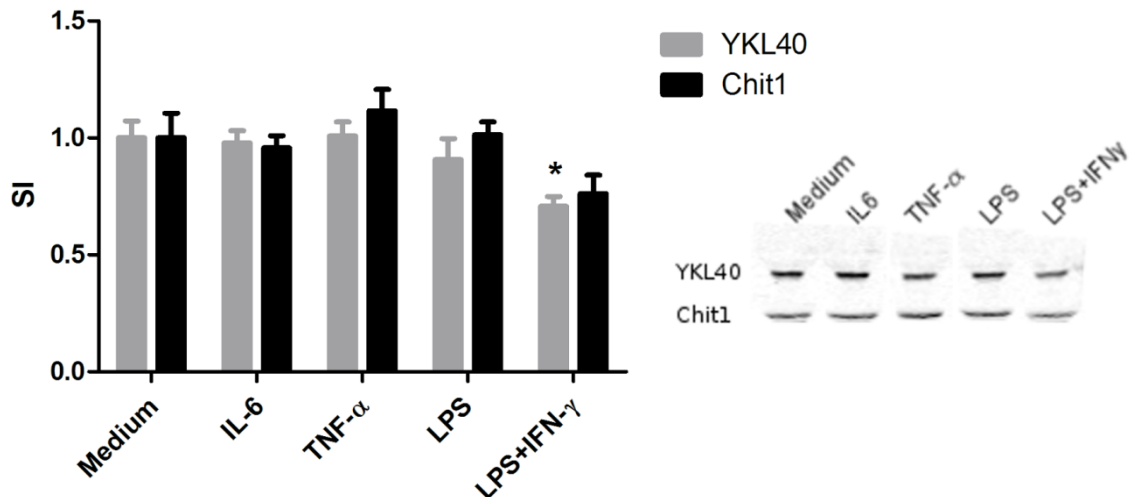
YKL-40 was measured in paired samples with western blot analysis and ELISA and sensitivity was evaluated. Dotplot (left). Linear regression (right) shows a  $R^2=0.6$ . Secretion index (SI): measurements normalized with cell numbers (crystal violet staining) and untreated cells (medium) represent the baseline secretion of 1.0.

## 4.2 Chitin derivative effect on THP-1 macrophages

During the differentiation process of THP-1 cells to macrophages, their morphology changes drastically from round cells in suspension, towards flat adherent cells with various processes, which form tight colonies where the cell density is high.

### 4.2.1 Positive control for YKL-40 and Chit1 protein secretion in THP-1 macrophages

Recent studies indicate that YKL-40 and Chit1 are markers for classical activation (73, 97). Several substances have been reported to increase YKL-40 in different cell types and/or Chit1 gene and protein expression, including: IL-6 (111), TNF- $\alpha$  (67), LPS (44, 112), and LPS + IFN- $\gamma$  (73). All of the mentioned substances were tested on THP-1 macrophages for 24 hours and YKL-40 and Chit1 secretion was evaluated with western blotting on medium from treated cells. None of the substances led to an increase in YKL-40 or Chit1 secretion, and surprisingly stimulating the cells with LPS and IFN- $\gamma$  decreased YKL-40 secretion slightly (Figure 7).



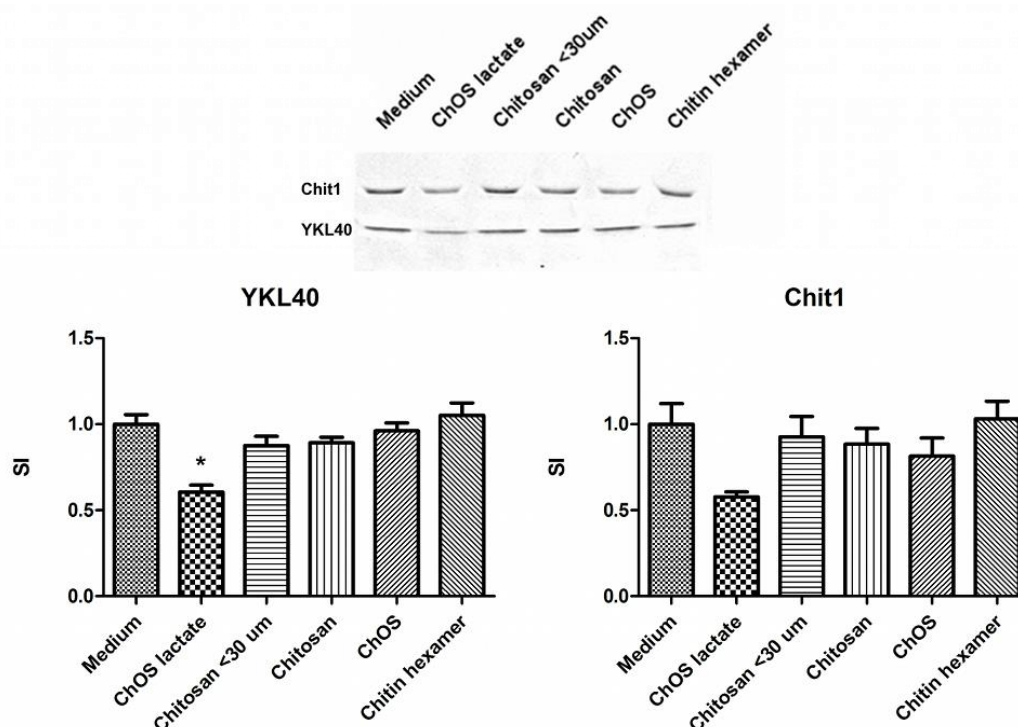
**Figure 7: IL-6, TNF- $\alpha$ , LPS and LPS+IFN $\gamma$  do not stimulate YKL-40 or Chit1 secretion of THP-1 macrophages**

Western blot analysis for YKL-40 and Chit1 in medium from THP-1 macrophages (N=1, single experiment with 6 repeats) after 24 hour stimulation with 20 ng/mL IL-6, 50 ng/mL TNF- $\alpha$ , 100 ng/mL LPS, 100 ng/mL LPS + 20 ng/mL IFN- $\gamma$  or left untreated (medium).

Secretion index (SI): untreated cells (medium) represent the baseline secretion of 1.0. Bars represent SEM. \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

#### 4.2.2 YKL-40 and Chit1 protein secretion in THP-1 macrophages after chitin derivative stimulation

Chitosan and ChOS have the potential to inhibit chitinases and chitinase-like proteins by blocking the active site (113). It was unclear whether chitin derivatives lead to changes in YKL-40 and Chit1 secretion. To study this, THP-1 macrophages were stimulated with 100  $\mu$ g/mL of ChOS lactate, chitosan <30  $\mu$ m, chitosan, ChOS or chitin hexamer for 24 hours and YKL-40 and Chit1 protein secretion was evaluated with western blotting. ChOS lactate was the only chitin derivative to have an effect on YKL-40 protein levels, by decreasing YKL-40 amount by about 40%. Chit1 secretion showed a similar trend as YKL-40 protein secretion, yet the decrease was not found to be significant (Figure 8).



**Figure 8: ChOS lactate decreases YKL-40 secretion in THP-1 macrophages**

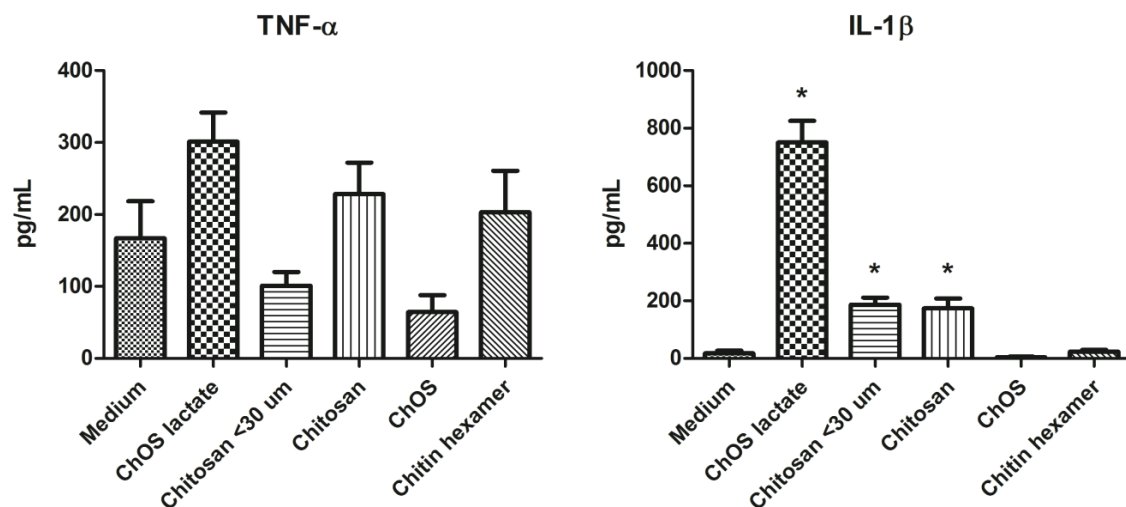
Western blot analysis (top) for YKL-40 (left) and Chit1 (right) in medium from THP-1 macrophages (N=1, single experiment with 6 replicates) after 24 hour stimulation with 100 µg/mL ChOS lactate, chitosan <30 µm, chitosan, ChOS, chitin hexamer or untreated (medium). Secretion index (SI): untreated cells (medium) represent the baseline secretion of 1.0. Bars represent SEM. \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

#### 4.2.3 IL-1 $\beta$ and TNF- $\alpha$ response in THP-1 macrophages after chitin derivative stimulation

TNF- $\alpha$  and IL-1 $\beta$  are pro-inflammatory markers indicative of classical activation (95). The secretion of these cytokines was measured after chitin derivative stimulation of THP-1 macrophages to see if the chitin derivatives had an immunomodulatory effect. TNF- $\alpha$  levels fluctuated to some extent between treatments but were not statistically different from untreated cells (Figure 9, left). IL-1 $\beta$  levels were significantly increased after ChOS lactate ( $750.7 \pm 74.75$  pg/mL,  $p < 0.0001$ ), chitosan <30 µm ( $187.0 \pm 24.19$  pg/mL,  $p < 0.05$ ) and chitosan ( $173.8 \pm 33.86$  pg/mL,  $p < 0.05$ ) treatment, indicating that these materials are inflammasome activators; especially ChOS lactate (Figure 9, right).

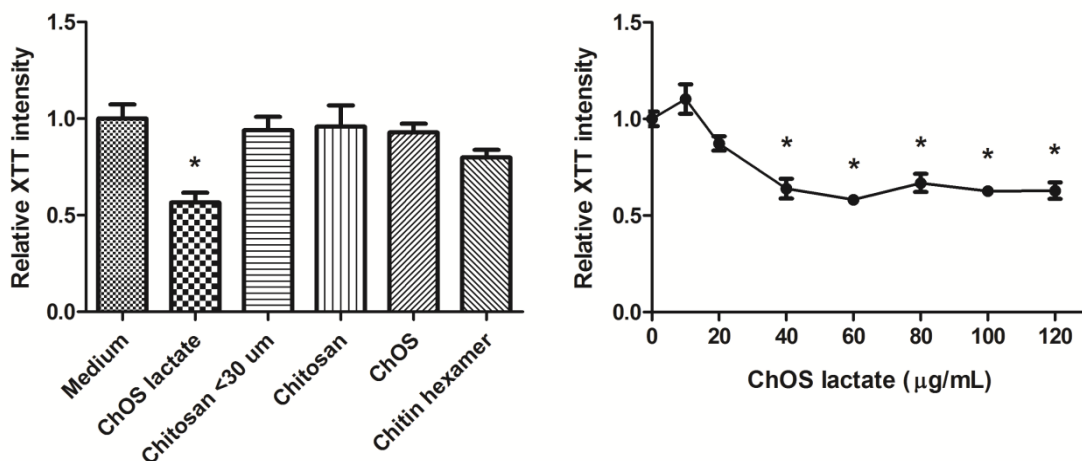
#### 4.2.4 Cytotoxicity of the chitin derivatives on THP-1 macrophages

To ascertain that the changes observed in YKL-40 and the cytokines were not due to changes in cell viability or metabolic activity, an XTT assay was performed. The results (Figure 10) showed a clear effect of ChOS lactate at 100 µg/mL (Figure 10, left), the concentration used in previous experiments. This cytotoxic effect of ChOS lactate was seen in even lower concentrations (Figure 10, right). This suggests that the decrease in YKL-40 secretion was in fact connected to reduced survival or toxicity.



**Figure 9: ChOS lactate and chitosan are stimulators of the inflammasome in THP-1 macrophages.**

TNF- $\alpha$  (left) and IL-1 $\beta$  (right) concentration in medium from THP-1 macrophages (N=1, single experiment with 6 replicates) after 24 hour stimulation with 100  $\mu$ g/mL ChOS lactate, chitosan <30  $\mu$ m, chitosan, ChOS, chitin hexamer or untreated (medium). Bars represent SEM. \* Statistically significant difference compared with untreated cells (medium), measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).



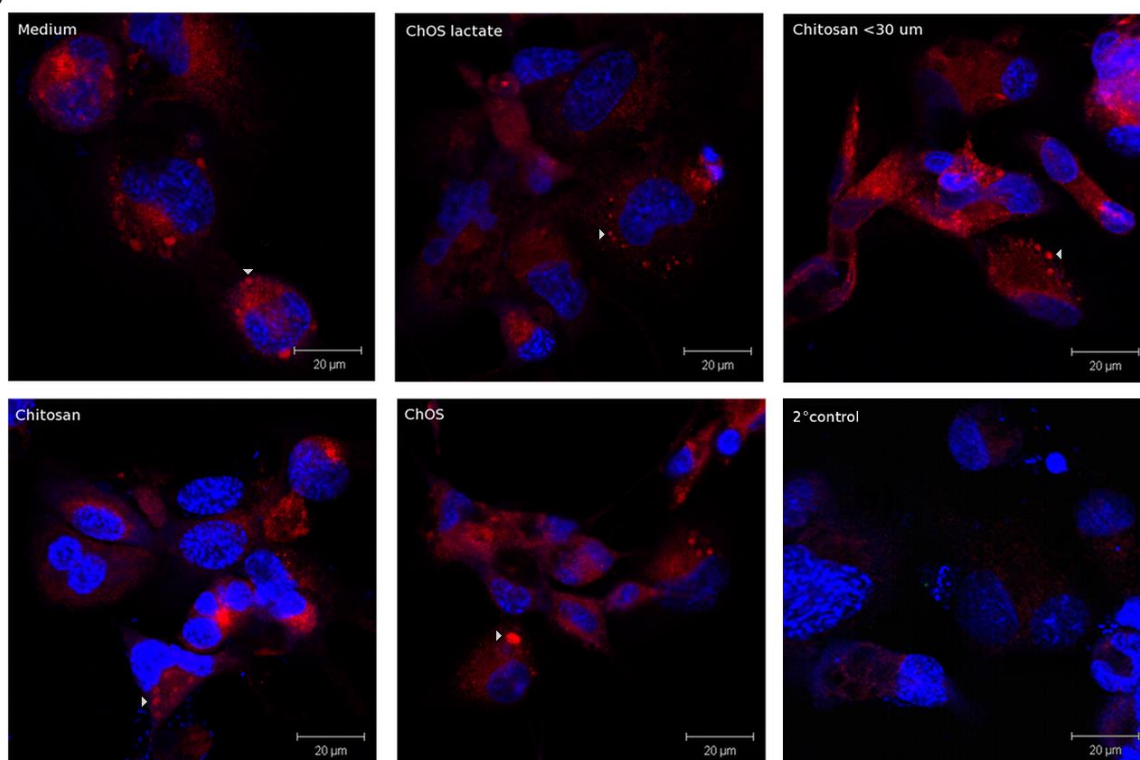
**Figure 10: ChOS lactate is cytotoxic to THP-1 macrophages from 40  $\mu$ g/mL**

XTT test on THP-1 macrophages after 24 hour stimulation with indicated chitin derivatives. Left: 100  $\mu$ g/mL ChOS lactate, chitosan <30  $\mu$ m, chitosan, ChOS, chitin hexamer or untreated (medium) (N=2, two experiments with minimum 3 replicates). Right: ChOS lactate concentration curve ranging from 10  $\mu$ g/mL – 120  $\mu$ g/mL (N=1, single experiment with 3 replicates). Bars represent SEM. \* Statistically significant difference compared with untreated cells (medium), measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

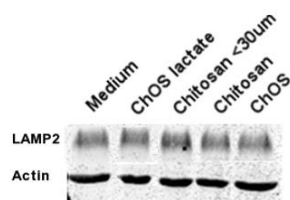
### 4.2.5 Role of phagocytosis in inflammasome activation of THP-1 macrophages

To evaluate whether the chitin derivatives were phagocytosed to a different degree, which could explain different biological effect, THP-1 macrophages were incubated with each of the chitin derivatives at a concentration of 100  $\mu\text{g/mL}$  for 2 hours. Cells were then fixed and immunostained for Lysosome-associated membrane protein 2 (LAMP2) (114) and staining intensity and signal location evaluated. All of the treatments, including media only, showed uniform cytosolic staining. Few cells in each treatment showed a staining pattern indicative of cytosolic vesicles; these vesicles are most likely lysosomes. None of the treatments showed an increase (evaluated by visual examination) in these cytosolic vesicles (Figure 11a), which either indicates no increase in phagocytosis or that LAMP2 is not suitable for phagocytosis evaluation. To verify that there is no increase in LAMP2 protein between treatments a western blot for LAMP2 on whole cell lysates was performed. Western blotting showed no changes in protein amount between treatments (Figure 11b).

a)



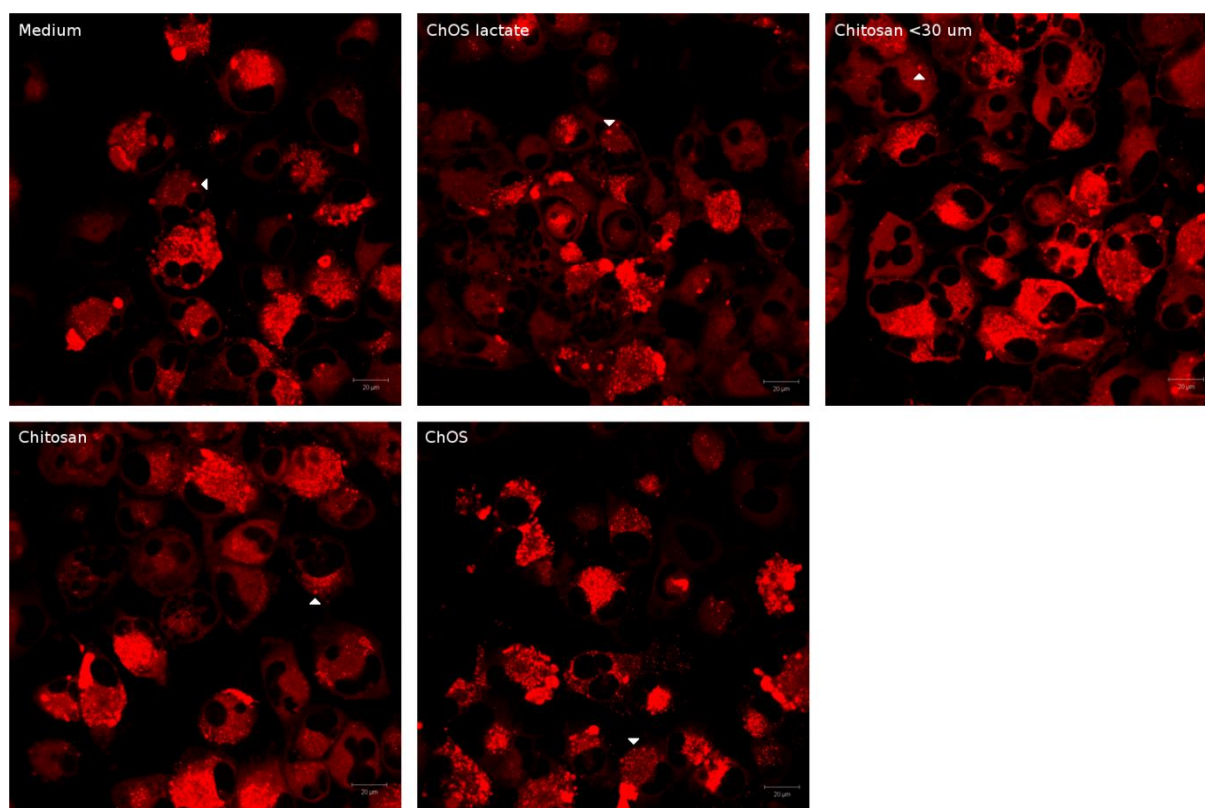
b)



**Figure 11: Changes in phagocytosis in THP-1 macrophages is not detected with LAMP2 immunocytochemistry and western blotting**

THP-1 macrophages were stimulated with 100  $\mu\text{g/mL}$  ChOS lactate, chitosan <30  $\mu\text{m}$ , chitosan, ChOS or untreated (medium) for 2 hours after which they were a) immunostained with LAMP2 (red) and TO-PRO 3 (blue) or b) western blotted for LAMP2 and actin. White arrowheads: possible lysosomes.

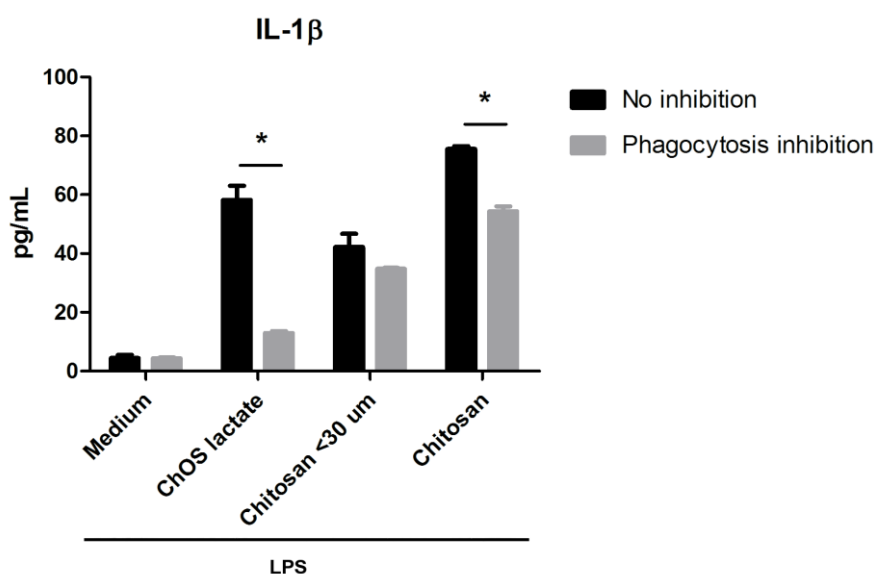
A second attempt was made to stain the lysosomes, this time with lysotracker, a fluorophore linked to a weak base that freely permeates cell membranes and typically concentrates in spherical organelles, especially acidic organelles such as lysosomes. Lysotracker is usually used with live cell imaging because fixing the cells can distort the staining, which means that images have to be gathered quickly and without the possibility of co-staining with antibodies. Lysotracker staining showed very similar pattern between different treatments. All cells showed faint uniform cytosolic staining, which is most probably background signal. Many cells in all treatments groups had one or more concentrated signals that indicate the presence of lysosomes (Figure 12), but there was no difference between the groups in intensity or amount of lysosomes (as determined with visual examination).



**Figure 12: Changes in phagocytosis in THP-1 macrophages is not detected with Lysotracker**

THP-1 macrophages were stimulated with 100 µg/mL ChOS lactate, chitosan <30 µm, chitosan, ChOS or untreated (medium) for 1.5 hours after which they were treated with 50 nM Lysotracker for 30 minutes. White arrowheads: possible lysosomes.

Inflammasome activation by chitosan has been shown to be phagocytosis dependent (32), it was therefore decided to see if the same applied to the chitin derivatives studied here (Figure 9). Actin polymerization was inhibited by cytochalasin D, prior to chitin derivative stimulation, which resulted in a dramatic decrease in IL-1 $\beta$  secretion in ChOS lactate stimulated cells ( $13.0 \pm 0.7$  vs.  $58.2 \pm 4.8$  pg/mL,  $p < 0.001$ ) (Figure 13). Hence ChOS lactate needs to be phagocytosed in order to mediate its activity. Chitosan <30 µm and chitosan seem to be less dependent on phagocytosis to mediate their effect on inflammasome activation.

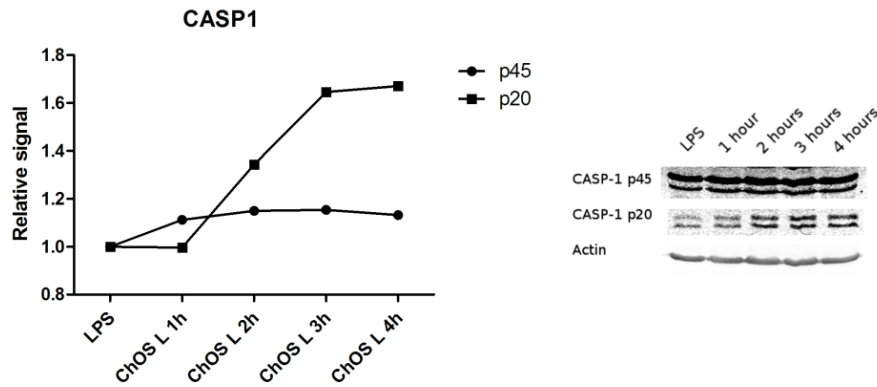


**Figure 13: ChOS lactate inflammasome activation is more dependent on phagocytosis than chitosan**

IL-1 $\beta$  concentration in medium from THP-1 macrophages (N=1, single experiment in duplicate) after 24 hour stimulation with 20  $\mu$ g/mL ChOS lactate, 100  $\mu$ g/mL chitosan <30  $\mu$ m, chitosan or untreated (medium). Cells were primed with 100 ng LPS for 3 hours and phagocytosis was inhibited by adding 1  $\mu$ g/mL Cytochalasin D for 30 minutes prior to chitin derivative stimulation. Bars represent SEM. \* Statistically significant difference compared to untreated cells, measured with two way ANOVA and Bonferroni post-test ( $p < 0.05$ ).

#### 4.2.6 ChOS lactate induces inflammasome activation of THP-1 macrophages via Caspase-1

IL-1 $\beta$  secretion is closely linked to inflammasome assembly. Inflammasomes serve as an activation platform for caspase-1, cleaving it into the active 20 kDa form (p20), which in turn is able to cleave pro-IL-1 $\beta$  into the final and active form that is secreted along with the activated caspase-1. Intracellular caspase-1 levels were studied in THP-1 macrophages that had been primed with LPS for 3 hours and stimulated with 100  $\mu$ g/mL ChOS lactate for 1, 2, 3 or 4 hours to see if the IL-1 $\beta$  response was due to activation of caspase-1. The 45 kDa inactive caspase-1 (p45) did not change with ChOS lactate treatment, but the active form (p20) steadily accumulated with increased ChOS lactate incubation time (Figure 14), indicating the involvement of caspase-1 in the observed responses.

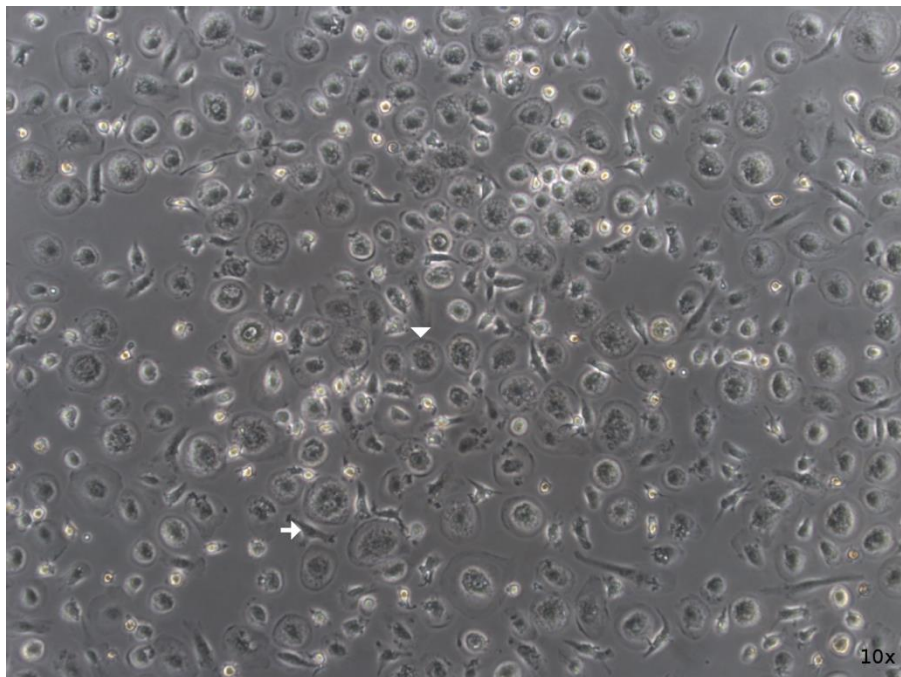


**Figure 14: ChOS lactate stimulates cleavage of Caspase-1 into its active form**

Western blot analysis for Caspase-1 (CASP1) of whole cell lysates from THP-1 macrophages primed with 100 ng/mL LPS for 3 hours and stimulated with 100  $\mu$ g/mL ChOS lactate for 1, 2, 3 or 4 hours.

### 4.3 Chitin derivative effect on human monocyte-derived macrophages

It was of interest to investigate the responses of primary macrophages to chitin derivative stimulation to get a better insight into the possible *in vivo* responses because cell lines like THP-1 do not always reflect the *in vivo* cellular reaction. Therefore, monocytes from peripheral blood were isolated and cultured under neutral conditions to obtain macrophages, as described in 3.2.2. After 9 days of culture in 10% human serum, two different types of macrophages could be distinguished: round sessile type cells with elongated cells in between.

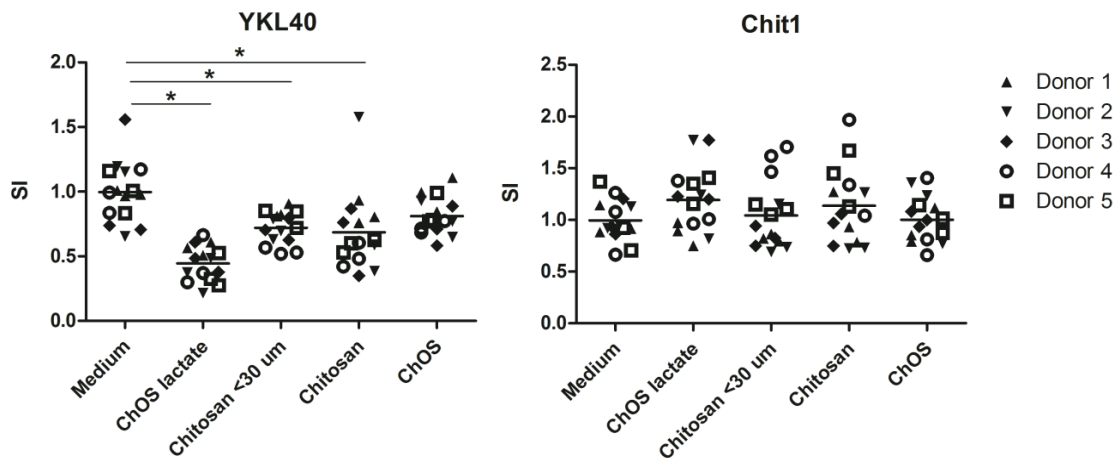


**Figure 15: Human monocyte-derived macrophages after 9 days of culture.**

Monocytes cultured under neutral conditions for 9 days. White arrowhead: round sessile macrophage; white arrow: elongated macrophage. 10x magnification.

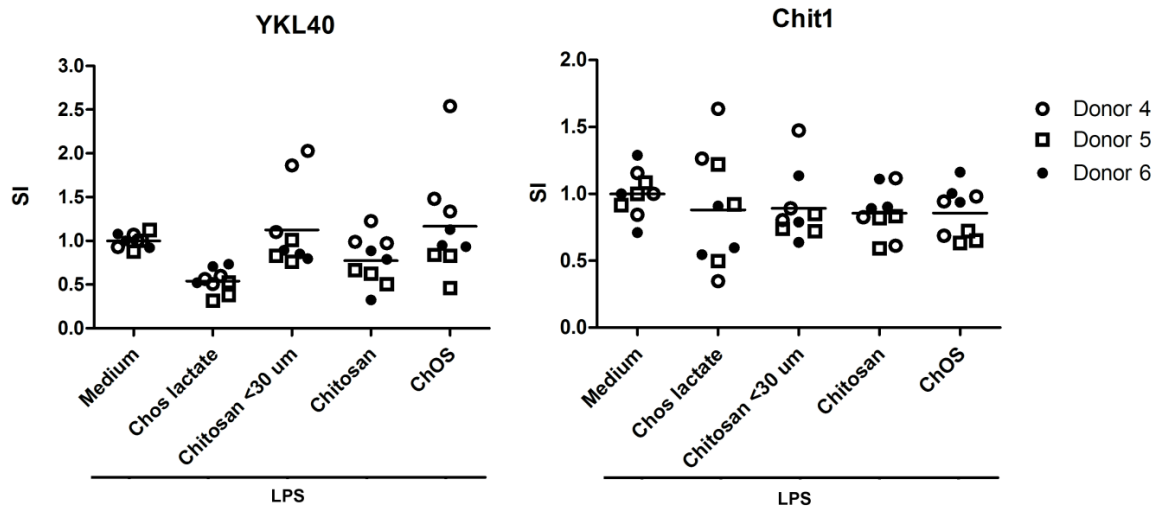
#### 4.3.1 YKL-40 and Chit1 protein secretion in monocyte-derived macrophages after chitin derivative stimulation

Nine days old MDMs from five different donors were stimulated with 100  $\mu\text{g/mL}$  of each of the chitin derivatives for 24 hours after which YKL-40 and Chit1 protein secretion was assessed with western blotting. ChOS lactate and chitosan of both sizes decreased YKL-40 secretion, with the most decrease seen in ChOS lactate (ChOS lactate:  $0.44 \pm 0.04$  SI,  $p < 0.0001$ ; chitosan  $<30 \mu\text{m}$ :  $0.72 \pm 0.03$  SI,  $p < 0.001$ ; chitosan:  $0.69 \pm 0.08$  SI,  $p < 0.0001$ ) (Figure 16, left). None of the chitin derivatives tested had an effect on Chit1 secretion (Figure 16, right). Priming cells with 100 ng/mL LPS for 3 hours prior to chitin derivative stimulation abolished the YKL-40 decrease seen in chitosan  $<30 \mu\text{m}$  ( $1.13 \pm 0.16$  SI) stimulated cells (Figure 17, left). ChOS lactate concentration was decreased to 20  $\mu\text{g/mL}$  in this experiment, below the cytotoxic limit seen in THP1 macrophages (Figure 10). YKL-40 levels in ChOS lactate and chitosan stimulated cells show a trend of decreasing (ChOS lactate:  $0.54 \pm 0.05$  SI; chitosan:  $0.78 \pm 0.09$  SI), but the data did not meet statistical significance (Figure 17, left). Later experiments with a ChOS lactate concentration curve showed that concentrations below 20  $\mu\text{g/mL}$  affected YKL-40 secretion less (Figure 18). Chit1 secretion in LPS primed MDMs was not affected by treatments (Figure 17, right).



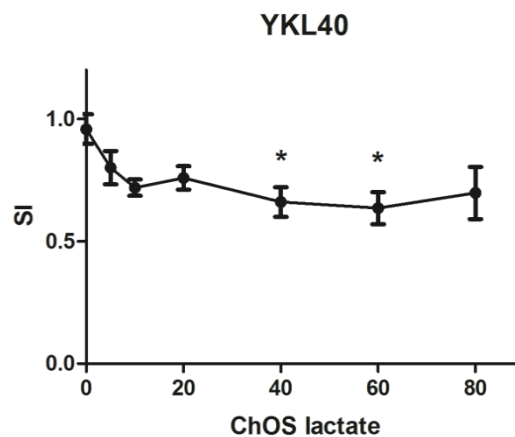
**Figure 16: ChOS lactate and chitosan decreases YKL-40 secretion in MDMs**

Western blot analysis for YKL-40 (left) and Chit1 (right) in medium from monocyte-derived macrophages (N=5, five separate donors with 3 replicates) after 24 hour stimulation with 100  $\mu\text{g/mL}$  ChOS lactate, chitosan  $<30 \mu\text{m}$ , chitosan, ChOS or left untreated (medium). Secretion index (SI): western blot values normalized with cell numbers (crystal violet staining) and untreated cells (medium) represent the baseline secretion of 1.0. \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).



**Figure 17: YKL-40 secretion is less affected in LPS primed MDMs**

Western blot analysis for YKL-40 (left) and Chit1 (right) in medium from monocyte-derived macrophages (N=3, three separate donors with 3 replicates) after 3 hour priming with 100 ng/mL LPS and 24 hour stimulation with 20  $\mu$ g/mL ChOS lactate and 100  $\mu$ g/mL chitosan <30  $\mu$ m, chitosan, ChOS or left untreated (medium). Secretion index (SI): western blot values normalized with cell numbers (crystal violet staining) and untreated cells (medium) represent the baseline secretion of 1.0. \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).



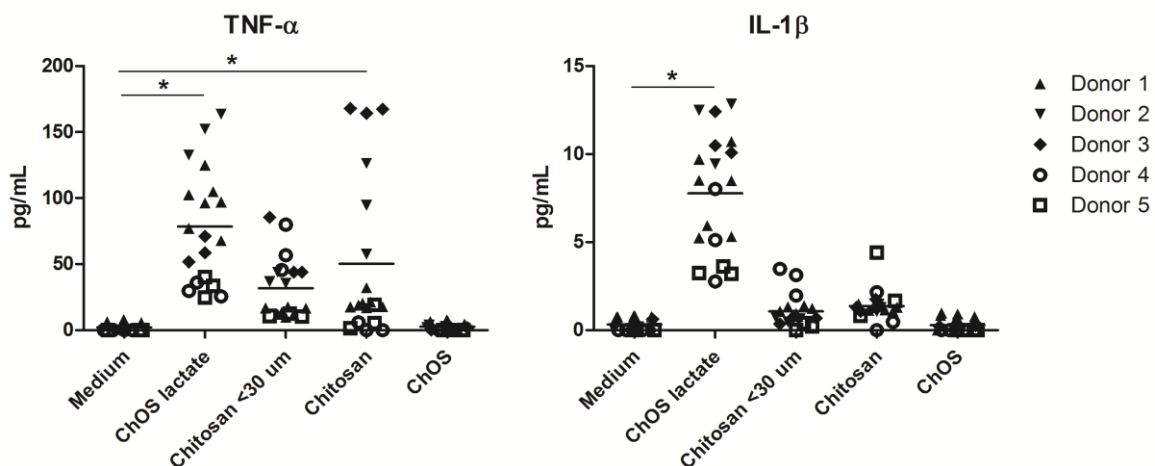
**Figure 18: YKL-40 secretion is affected at 40  $\mu$ g/mL ChOS lactate**

Western blot analysis for YKL-40 in medium from monocyte-derived macrophages (N=3, three separate donors with minimum of 3 replicates) after 24 hour stimulation with ChOS lactate concentration curve ranging from 5  $\mu$ g/mL – 80  $\mu$ g/mL. Secretion index (SI): western blot values normalized with cell numbers (crystal violet staining) and untreated cells (medium) represent the baseline secretion of 1.0. Bars represent SEM. \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

As these changes are seen at the protein level, it was of interest to analyse whether this response is regulated at the transcriptional level, i.e. due to decreased expression of the *Chi3L1* gene. MDMs were therefore stimulated with a concentration curve of ChOS lactate ranging from 0.5-40 µg/mL and the expression of *Chi3L1* was measured with real time PCR. Gene expression of two donors was analyzed but the two donors gave very opposite results, which are not shown, as they cannot be interpreted the way they are.

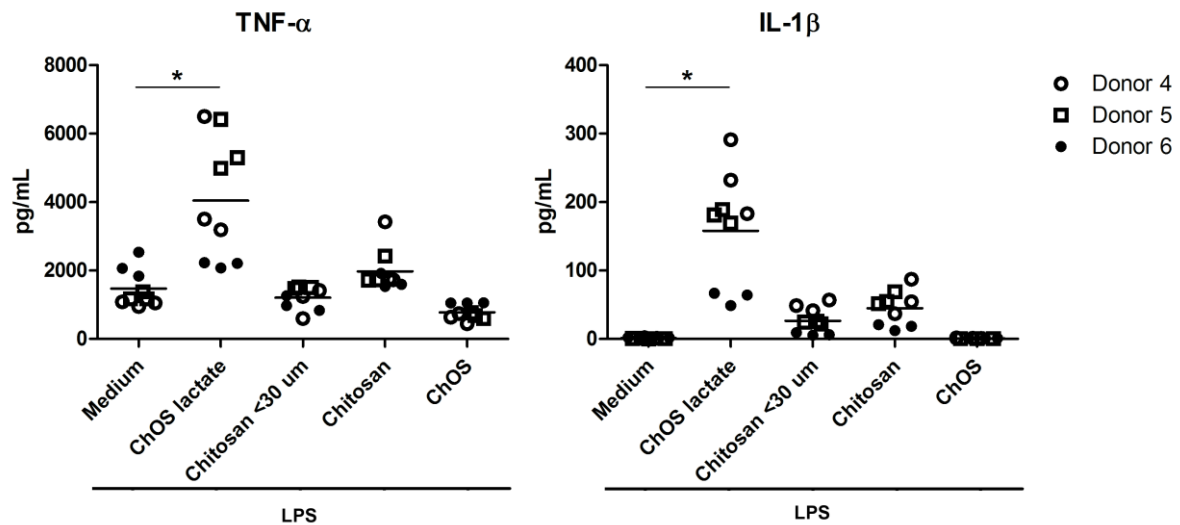
#### 4.3.2 IL-1β and TNF-α response in monocyte-derived macrophages after chitin derivative stimulation

Inflammatory response of MDMs after 24 hour chitin derivative stimulation was evaluated by measuring the pro-inflammatory cytokines TNF-α and IL-1β with ELISA. ChOS lactate and chitosan increased TNF-α secretion (ChOS lactate:  $78.4 \pm 10.0$  pg/mL,  $p < 0.0001$ , chitosan:  $50.3 \pm 14.0$  pg/mL,  $p < 0.0001$ ), but there was substantial donor variation. The increase seen after chitosan stimulation is mostly due to the responses of donor 2 and 3 (Figure 19, left). ChOS lactate was the only derivative to have an effect on IL-1β secretion, by increasing it moderately ( $7.8 \pm 0.8$  vs.  $0.3 \pm 0.08$  pg/mL,  $p < 0.0001$ ) (Figure 19, right). By priming the cells with 100 ng/mL LPS for 3 hours prior to chitin derivative stimulation it is clear that ChOS lactate is a potent inflammasome activator, seen as a large increase in IL-1β secretion ( $158.5 \pm 27.5$  vs.  $1.8 \pm 0.5$  pg/mL,  $p < 0.0001$ ) (Figure 20, right), even though the concentration of ChOS lactate was decreased to 20 µg/mL. Chitosan elicited a moderate increase in IL-1β secretion, the increase was not statistically significant (chitosan <30 µm:  $26.7 \pm 6.3$  pg/mL, chitosan:  $45.1 \pm 8.3$  pg/mL). ChOS lactate was also the only derivative to show synergy to the LPS stimulated TNF-α secretion ( $4045 \pm 597.1$  vs.  $1472 \pm 183.0$  pg/mL,  $p < 0.0001$ ) (Figure 20, left).



**Figure 19: ChOS lactate induces proinflammatory responses in MDMs**

TNF-α (left) and IL-1β (right) concentration from medium of monocyte-derived macrophages (N=5, five separate donors with minimum of 3 replicates) after 24 hour stimulation with 100 µg/mL ChOS lactate, chitosan <30 µm, chitosan, ChOS or left untreated (medium). \* Statistically significant difference compared with untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

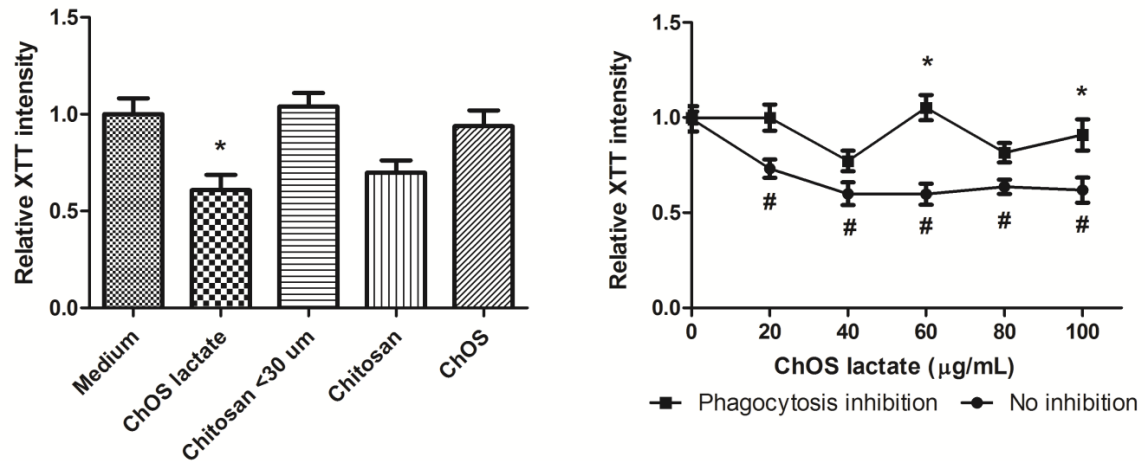


**Figure 20: ChOS lactate is a potent inflammasome activator in MDMs**

TNF- $\alpha$  (left) and IL-1 $\beta$  (right) concentration from medium of monocyte-derived macrophages (N=3, three separate donors with 3 replicates) after 3 hour priming with LPS and 24 hour stimulation with 20  $\mu$ g/mL ChOS lactate and 100  $\mu$ g/mL chitosan <30  $\mu$ m, chitosan, ChOS or left untreated (medium). \* Statistically significant difference compared to untreated cells, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

#### 4.3.3 Cytotoxicity of the chitin derivatives on monocyte-derived macrophages

Cytotoxicity of the chitin derivatives on MDMs was assessed via XTT assay. ChOS lactate was the only derivative to have a negative effect on cellular metabolism at a concentration of 100  $\mu$ g/mL (Figure 21, left). ChOS lactate was also found to have detrimental effect on MDMs from 20  $\mu$ g/mL, this effect could be decreased by treating the cells with the phagocytosis inhibitor Cytochalasin D prior to ChOS lactate stimulation (Figure 21, right).



**Figure 21: ChOS lactate cytotoxicity to MDMs is phagocytosis dependent**

XTT test on monocyte-derived macrophages after 24 hour stimulation with indicated chitin derivatives. Left: 100 µg/mL ChOS lactate, chitosan <30 µm, chitosan, ChOS or untreated (medium) (N=3, three separate donors with minimum of 3 replicates). Right: ChOS lactate concentration curve ranging from 20 µg/mL – 100 µg/mL without phagocytosis inhibition (circles, N=4, four separate donors with minimum of 3 replicates) or with phagocytosis inhibition (1 µg/mL Cytochalasin D) (boxes, N=2, two separate donors with 4 replicates). Bars represent SEM. # Statistically significant difference compared to untreated cells (medium), \* statistically significant difference compared to cells receiving same amount of ChOS lactate without inhibition, measured with one way ANOVA and Tukey's multiple comparison test ( $p < 0.05$ ).

## 5 Discussion

This study aimed at determining the effect that chitin derivatives, differing in size and acetylation, have on the secretion of YKL-40 and Chit1, an inactive and active chitinase respectively, in macrophages. The pro-inflammatory effect of the chitin derivatives was also studied by measuring changes in TNF- $\alpha$  and IL-1 $\beta$  secretion. This was accomplished by using two different macrophage experimental models; THP-1 macrophages and primary MDMs. Macrophages were stimulated with 100  $\mu\text{g/mL}$  of each chitin derivative and their responses were evaluated by measuring changes in the aforementioned markers and cytokines. A western blotting detection method for YKL-40 and Chit1 was set up, verified and optimized. At 100  $\mu\text{g/mL}$  each of the chitin derivatives elicited different inflammatory responses. ChOS lactate, a 90% deacetylated derivative with DP of 3-5, was highly cytotoxic both to THP-1 macrophages and MDMs. The cytotoxicity of ChOS lactate was closely linked to a decrease in YKL-40 secretion, inflammasome activation via caspase-1 and increased TNF- $\alpha$  secretion. Chitosan and chitosan <30  $\mu\text{m}$ , 75% deacetylated derivatives, were not cytotoxic to macrophages, yet they decreased YKL-40 secretion in MDMs and activated the inflammasome. The inflammasome activation elicited by chitosan was less than ChOS lactate induced activation. Chitin hexamer, a fully acetylated derivative with DP of 6, and ChOS, a 60% deacetylated derivative with DP of 6-12, did not induce any response in the measured factors.

### 5.1 Cell culture and detection methods

Detection methods for the proteins that were of interest, namely YKL-40 and Chit1, were tested and verified. There are commercial ELISAs available for the detection of these proteins in media, but these ELISAs are costly. Western blotting is a cost efficient alternative but first it had to be tested to see whether the proteins were detectable with this method. Detection by western blotting on media samples proved to be only possible if the proteins in the media were precipitated prior to blotting for sufficient signal. High serum content in the media caused problems when blotting precipitated samples. Serum content of the medium, when the cells were stimulated with the chitin derivatives, was thus lowered to resolve this. THP-1 macrophages received 2% FBS content throughout the culture but the MDMs were cultured in 10% NHS and in serum-free medium the last 24 hours when they were stimulated with the chitin derivatives. Even the 2% serum content in the THP-1 macrophage culture smeared the YKL-40 and Chit1 signal to some extent.

Serum deprivation is generally not considered an optimal culture environment, causing cellular stress and even apoptosis. We concluded that 24 hours of serum deprivation would not be a deciding factor in this experimental setup. Munn et al. (115) looked into the effect of serum deprivation on MDMs and concluded that newly isolated monocytes were susceptible to apoptosis resulting from serum deprivation, but macrophages tolerated serum deprivation better.

There are several factors that could influence western blotting sensitivity, those include pipetting errors when measuring samples to be acetone precipitated (i.e. it is important to measure exactly the same amount in samples to be compared because there is no loading control), possible loss of protein pellet when discarding the acetone supernatant and pipetting errors when adding sample buffer to dissolve the protein pellet. This matter was addressed by comparing YKL-40 western blotting values with YKL-40 ELISA values. The ELISA proved to be a more sensitive detection method, yet western blotting was still sensitive enough to be applicable despite fluctuations in values.

## 5.2 THP-1 macrophages

Cell lines are generally easier to work with than primary cells. Many of them, like THP-1, have been around for decades and have been studied extensively. They can be a good choice when deciding on a well-defined experimental model.

YKL-40 and Chit1 have been implicated as activation markers for macrophages, this was tested by stimulating THP-1 macrophages with the materials that have been shown to increase YKL-40 secretion, as a positive control. Bonnef-Barkay et al. (73) observed a quadruple increase in YKL-40 expression when stimulating 3 days old MDMs for 24 hours with the classical activators LPS and IFN- $\gamma$ . In contrast, THP-1 macrophages had decreased YKL-40 secretion after 24-hour stimulation with the same concentration of LPS and IFN- $\gamma$ . Studies on articular chondrocytes indicate that LPS and TNF- $\alpha$  are potent stimulators of YKL-40 expression and secretion (67, 112). Yet, neither LPS nor TNF- $\alpha$  increased YKL-40 secretion in THP-1 macrophages in this study. This discrepancy in results may have several explanations, including; differential regulation of YKL-40 in THP-1 macrophages compared to MDMs and chondrocytes, insufficient concentration or low quality/degradation of cytokines and LPS, insufficient time for changes in secretion to be measurable or that THP-1 macrophages are already classically activated and have reached maximum YKL-40 secretion. Maeß et al. (116) discussed the issue of activation of THP-1 macrophages after differentiation with high concentrations of PMA. They concluded that PMA concentrations above 10 ng/mL resulted in transcription of genes related to classical activation (116). In light of that knowledge the 50 ng/mL of PMA used in this study might have skewed the THP-1 macrophages towards classical activation prior to stimulation with indicated substances. On the other hand YKL-40 and Chit1 might not be reliable activation markers in *in vitro* macrophages, and rather be differentiation marker. DiRosa et al (64) studied the gene expression of 7 days old *in vitro* MDMs and did not see difference in expression of these markers between M1 and M2 polarized MDMs. The previous results indicating YKL-40 and Chit1 as activation markers could therefore possibly reflect faster differentiation of monocytes to macrophages *in vitro*. Although it is important to keep in mind that *in vivo* pathways are undoubtedly more complex and have a stricter regulation, this is reflected in increased serum concentration of YKL-40 and Chit1 in various inflammatory conditions discussed in the introduction.

THP-1 macrophages did not show any change in YKL-40 or Chit1 secretion in response to stimulation with 100  $\mu$ g/mL chitosan, chitosan 30 $\times$   $\mu$ m, ChOS or chitin hexamer. ChOS lactate on the other hand decreased YKL-40 and Chit1 secretion by about 40%, but only the change in YKL-40 secretion reached significance most likely due to small sample size. Cytokine secretion profile of

ChOS lactate treated cells indicates classical activation, which supports the notion that YKL-40 may not be a reliable marker for classical activation in *in vitro* macrophages.

A possible explanation for the YKL-40 decrease is that it reflects decreased survival, as ChOS lactate was cytotoxic at concentrations far below the ones used in these experiments. YKL-40 has been shown to inhibit inflammatory cell apoptosis by enhancing the expression and activation of protein kinase B/AKT and Fas apoptosis-inhibiting molecule (Faim) 3 (117), thus a decrease in YKL-40 would alleviate this inhibition.

YKL-40 has also been shown to be a vital inflammasome regulator (86). These results show that ChOS lactate lowers YKL-40 secretion and is a potent inflammasome activator via caspase-1. These responses could be linked because of YKL-40s binding affinity to ChOS (10). Yet, a recent study on YKL-40 binding affinity to differently acetylated ChOS showed that fully deacetylated chitohehexose had no affinity for YKL-40 (43), making it unlikely that ChOS lactate, which is 90% deacetylated, is mediating its effect through YKL-40 binding. Chitosans with deacetylation of 70% or lower have been shown to be more susceptible to degradation by chitonolytic enzymes than chitosans with deacetylation higher than 70%, which was explained by the essential sequential arrangement of the N-acetyl units for recognition by the chitinolytic enzymes (118). In light of that knowledge ChOS lactate is probably affecting the cells through a pathway that affects YKL-40 transcription or secretion independent of direct binding. One explanation could be lysosomal rupture after phagocytosis (106).

Chitosan and chitosan <30  $\mu$ m also activated the inflammasome, but to a lesser degree, without lowering YKL-40 secretion in THP-1 macrophages. The inflammasome activation results are in line with the results of Bueter et al. (32), which showed that chitosan (same chitosan as used in this study, 76% deacetylated) and not chitin activates the NLRP3 inflammasome. ChOS, which is 60% deacetylated, and chitin hexamer did not influence the inflammasome. This means the inflammasome activation is very sensitive towards the degree of deacetylation; 60% deacetylated chitosan is inert; 76% deacetylated chitosan exerts mild inflammasome activation without cytotoxic effects; and 90% deacetylated chitosan is a potent inflammasome activator and is cytotoxic. The cytotoxicity of ChOS lactate is most likely because of uncontrolled inflammasome activation.

Chitin derivatives have not previously been reported to influence YKL-40 secretion of macrophages. The possibility that chitosan and ChOS might influence inflammasome activation through YKL-40 is intriguing. One possible explanation to why ChOS lactate elicits a stronger effect than chitosan might lie in differences in phagocytosis. ChOS lactate has a DP of 3-5, while chitosan is of undefined DP but is larger. A smaller polymer is expected to be more readily phagocytosed (119), it was therefore examined whether different derivatives were phagocytosed to different degrees. No change in phagocytosis was detected using LAMP2 antibody staining nor markers of acidic organelles, however inhibiting phagocytosis had an effect on cellular responses, suggesting that phagocytosis is important for the cellular response, as has been previously reported (32). Other methods for measuring phagocytosis such as coating of latex or polystyrene beads with the chitin derivatives and observing their uptake with light microscopy or fluorescently tagging the derivatives and measuring their uptake with confocal microscopy or flow cytometry might have been better as they would have provided a more quantitative measurement of phagocytosis.

### 5.3 Monocyte-derived macrophages

Tissue macrophages, which are derived from circulating monocytes, are a highly heterogeneous group. The heterogeneity reflects the specialized functions of macrophages in different anatomical locations (90). *In vitro* macrophage studies face a choice; either they can isolate tissue macrophages or culture monocytes into macrophages. Both methods have their benefits and disadvantages. Isolation of tissue macrophages is possible in mouse models, but is too invasive in human subjects to be practical. Isolated tissue macrophages would be ideal in studies where certain macrophage subsets are to be studied. Peripheral monocytes on the other hand are relatively easy to obtain in large quantities, but then again are more difficult to differentiate into specific subsets that reflect *in vivo* macrophages. This study aimed at discerning the general responses of macrophages, as a group, to chitin derivatives. For this, peripheral monocytes were cultured in a neutral environment, with no added cytokines or stimulatory materials except for the ones contained in the pooled human serum. This method gives M0 macrophages – a non-activated mixed macrophage population. In contrast to M1 macrophages (classically activated) obtained by IFN- $\gamma$  and/or LPS stimulation or M2 macrophages (alternative activated) obtained by IL-13 and/or IL-4 stimulation (120). This classification of M0, M1 and M2 macrophages is simplified to what is found *in vivo*, but is often used in *in vitro* macrophage studies.

The mixed population obtained at day 9 is reflected in different morphology observed. Unfortunately these macrophages were not characterized with flow cytometry, so little can be said about their classification. A recent study by Eligini et al. (121) showed that MDMs cultured in autologous serum for 7 days showed the same morphology as seen here; flat round cells and elongated cells. The round MDMs showed functional traits similar to the non-inflammatory and reparative M2 phenotype, whereas the spindle MDMs exhibited a pro-inflammatory profile similar to the M1 phenotype (121).

The MDMs responded to ChOS lactate in a similar manner as THP-1 macrophages, by decreasing YKL-40 secretion, activating the inflammasome and showed signs of cytotoxicity from 20  $\mu\text{g/mL}$ , which was phagocytosis dependent. Unlike THP-1 macrophages, MDMs also responded to chitosan and chitosan <30  $\mu\text{m}$  by decreasing YKL-40 secretion. This reflects the difference between the two cell culture models; even though there is considerable donor variation in MDMs, it can be more sensitive to small differences. Then again THP-1 macrophages showed, in addition to YKL-40, a decrease in Chit1 secretion that is not seen in MDMs. The transcriptional regulation of *CHIT1* and *Chi3l1* has been shown to be very complex and involving many control elements (64, 65) reflecting tissue specific responses, which could explain the discrepancy in THP-1 macrophages vs. MDMs – these are probably not the same macrophage subsets. Yet the implications of this study are interesting, even though the cell models might not be reflecting the same macrophage subsets.

Chitosan has previously been shown to activate the inflammasome (32) but the reason it does so is not known. This study showed that inflammasome activation could possibly be because of decreased levels of YKL-40, an important inflammasome regulator. Phagocytosis of chitosan is a prerequisite for its inflammasome effect, indicating that chitosans are mediating their effect inside the cell. This could

be because of lysosomal rupture following phagocytosis similar to silica (106), signaling through an extracellular receptor that is dependent on uptake similar to TLR9 (122), binding to an unknown factor that could inhibit YKL-40 transcription or through some uncharacterized pathway. Unfortunately *Chi3l1* gene expression analysis following ChOS lactate stimulation was inconclusive and would be needed to be repeated to be able to answer if the responses were happening on a gene or protein level. It would also be very interesting to see if adding exogenous YKL-40 to the cultures, at the same time as chitosan and ChOS lactate, would decrease the observed inflammatory and cytotoxic responses.

It is well known that highly cationic polymers used as vectors for gene- and drug delivery can be very cytotoxic, posing a problem for their usage *in vivo* (123). ChOS lactate, being 90% deacetylated, has many free primary amine groups ( $\text{NH}_2$ ), making the polymer positively charged. Chitosan on the other hand has a lower positive charge because it is more acetylated. Studies on cationic polymers have shown that linear and branched polymers with high flexibility are more toxic than globular ones, owing to increased interaction with the negatively charged cell membrane (123). Cationic polymers are thought to escape degradation by lysosomes by acting as proton sponges; when the pH decreases the cationic polymers become more protonated leading to  $\text{Cl}^-$  influx, triggering osmotic stress with increased water influx that finally end in endo-/lysosomal rupture (124). This pathway could also apply to ChOS lactate because of its cationic properties and explain why it is such a potent inflammasome activator. It could also explain why chitin, which has a neutral charge, does not activate the inflammasome.

Chitosan and ChOS have been thought to be an attractive biomaterial in regenerative medicine. Chitosan and various chitosan derivatives have been used as wound dressings with good results; they have improved healing and decreased the formation of scar tissue (2). Chitosan has also been shown to modify cartilage healing by modifying angiogenesis and inhibiting the formation of fibrotic connective tissue (36, 37). Interestingly YKL-40 has been implicated in both angiogenesis and formation of connective tissue (82, 83), a lowering in YKL-40 secretion could thus provide a molecular mechanism for this effect.



## 6 Conclusion

Chitin and chitosan do not have the same effect on macrophages. Degree of acetylation is a major factor in determining the bioactivity. Materials that resemble chitin, chitin hexamer and ChOS (which is 60% deacetylated) are possibly taken up by the macrophages (32) but subsequent events show no effect on the inflammatory status of the macrophages. 75% deacetylated chitosan on the other hand decrease YKL-40 secretion of macrophages and have a mild inflammasome activating effect. ChOS lactate, which is a very small (DP 3-5), 90% deacetylated ChOS, shows the most effect on macrophages. It decreased YKL-40 secretion the most and is a very potent inflammasome activator, it is also highly cytotoxic. The relationship between decreased YKL-40 secretion, inflammasome activations and cytotoxicity suggest a causal relationship. YKL-40 has been shown to be an inflammasome regulator (86), which explains why the inflammasomes are most activated where YKL-40 is down regulated the most. ChOS lactate is a highly cationic polymer, which could explain its cytotoxicity. Cationic polymers are thought to disrupt lysosomes by osmotic stress induced by its charge (124). Lysosomal disruption has been shown to activate inflammasomes and excessive inflammasome activation leads to pyroptosis, an inflammatory cell death.

This type of response is generally not acceptable *in vivo*, except in cancer therapy where cytotoxicity and immunomodulatory effects can be beneficial. ChOS lactate could therefore be an interesting candidate for cancer therapy, either alone or as a drug carrier that could act in synergy with other cancer drugs. The mild effect of chitosan would be more beneficial in situations where cytotoxicity is not acceptable but immunomodulatory effects and controlled tissue remodeling are needed, such as in wound dressings and implants in regenerative medicine.

Chitosan and ChOS have been reported to have various bio-activity, the molecular mechanism behind the reported mechanisms have often been poorly defined. This study reports a decrease in YKL-40 secretion that is dependent on highly deacetylated polymers, an effect that could provide a molecular mechanism behind some of the biological activities reported.



## References

1. Khoushab F, Yamabhai M. Chitin research revisited. *Marine drugs*. 2010;8(7):1988-2012. Epub 2010/08/18.
2. Kumar MN, Muzzarelli RA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. *Chem Rev*. 2004;104(12):6017-84. Epub 2004/12/09.
3. Muzzarelli RA. Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine drugs*. 2010;8(2):292-312. Epub 2010/04/15.
4. Rinaudo M. Chitin and chitosan: Properties and applications. *Progress in Polymer Science*. 2006;31(7):603-32.
5. Pillai CKS, Paul W, Sharma CP. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science*. 2009;34(7):641-78.
6. Vincent JFV, Wegst UGK. Design and mechanical properties of insect cuticle. *Arthropod Structure & Development*. 2004;33(3):187-99.
7. Adams DJ. Fungal cell wall chitinases and glucanases. *Microbiology*. 2004;150(Pt 7):2029-35.
8. Nakajima M, Atsumi K, Kifune K, Miura K, Kanamaru H. Chitin is an effective material for sutures. *Jpn J Surg*. 1986;16(6):418-24. Epub 1986/11/01.
9. Minke R, Blackwell J. The structure of  $\alpha$ -chitin. *Journal of Molecular Biology*. 1978;120(2):167-81.
10. Aam BB, Heggset EB, Norberg AL, Sorlie M, Varum KM, Eijsink VG. Production of chitooligosaccharides and their potential applications in medicine. *Marine drugs*. 2010;8(5):1482-517. Epub 2010/06/19.
11. Franca EF, Lins RD, Freitas LCG, Straatsma TP. Characterization of Chitin and Chitosan Molecular Structure in Aqueous Solution. *Journal of Chemical Theory and Computation*. 2008;4(12):2141-9.
12. Xiong C, Wu H, Wei P, Pan M, Tuo Y, Kusakabe I, et al. Potent angiogenic inhibition effects of deacetylated chitohexaose separated from chitooligosaccharides and its mechanism of action in vitro. *Carbohydr Res*. 2009;344(15):1975-83. Epub 2009/07/28.
13. Harish Prashanth KV, Tharanathan RN. Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. *Biochim Biophys Acta*. 2005;1722(1):22-9. Epub 2005/02/18.
14. Stamford TCM, Stamford-Arnaud, T. M., Cavalcante, H. M. M., Macedo, R. O., Camposs-Takaki, G. M. Microbiological Chitosan: Potential Application as Anticariogenic Agent. Andrade A, editor: InTec; 2013.
15. Santas J, Espadaler J, Mancebo R, Rafecas M. Selective in vivo effect of chitosan on fatty acid, neutral sterol and bile acid excretion: a longitudinal study. *Food Chem*. 2012;134(2):940-7. Epub 2012/10/31.
16. Tsai GJ, Su WH. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J Food Prot*. 1999;62(3):239-43. Epub 1999/03/25.
17. Wu GJ, Tsai GJ. Chitooligosaccharides in combination with interferon- $\gamma$  increase nitric oxide production via nuclear factor- $\kappa$ B activation in murine RAW264.7 macrophages. *Food and Chemical Toxicology*. 2007;45(2):250-8.
18. Fernandes JC, Spindola H, de Sousa V, Santos-Silva A, Pintado ME, Malcata FX, et al. Anti-inflammatory activity of chitooligosaccharides in vivo. *Marine drugs*. 2010;8(6):1763-8.
19. Mellegård H, Strand SP, Christensen BE, Granum PE, Hardy SP. Antibacterial activity of chemically defined chitosans: Influence of molecular weight, degree of acetylation and test organism. *International Journal of Food Microbiology*. 2011;148(1):48-54.
20. Kendra DF, Hadwiger LA. Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. *Experimental Mycology*. 1984;8(3):276-81.

21. Shibata Y, Foster LA, Metzger WJ, Myrvik QN. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. *Infect Immun*. 1997;65(5):1734-41. Epub 1997/05/01.
22. Shibata Y, Metzger WJ, Myrvik QN. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *Journal of immunology (Baltimore, Md : 1950)*. 1997;159(5):2462-7. Epub 1997/09/01.
23. Shibata Y, Foster LA, Bradfield JF, Myrvik QN. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. *Journal of immunology (Baltimore, Md : 1950)*. 2000;164(3):1314-21. Epub 2000/01/21.
24. Strong P, Clark H, Reid K. Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in murine models of allergy. *Clin Exp Allergy*. 2002;32(12):1794-800. Epub 2003/03/26.
25. Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature*. 2007;447(7140):92-6. Epub 2007/04/24.
26. Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *Journal of immunology (Baltimore, Md : 1950)*. 2009;182(6):3573-82. Epub 2009/03/07.
27. Da Silva CA, Pochard P, Lee CG, Elias JA. Chitin particles are multifaceted immune adjuvants. *Am J Respir Crit Care Med*. 2010;182(12):1482-91. Epub 2010/07/27.
28. Lieder R, Thormodsson F, Ng CH, Einarsson JM, Gislason J, Petersen PH, et al. Chitosan and Chitin Hexamers affect expansion and differentiation of mesenchymal stem cells differently. *International Journal of Biological Macromolecules*. 2012;51(4):675-80.
29. Panda SK, Kumar S, Tupperwar NC, Vaidya T, George A, Rath S, et al. Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia. *PLoS Pathog*. 2012;8(5):e1002717. Epub 2012/06/02.
30. Minami S, Suzuki H, Okamoto Y, Fujinaga T, Shigemasa Y. Chitin and chitosan activate complement via the alternative pathway. *Carbohydrate Polymers*. 1998;36(2-3):151-5.
31. Jeong HJ, Koo HN, Oh EY, Chae HJ, Kim HR, Suh SB, et al. Nitric oxide production by high molecular weight water-soluble chitosan via nuclear factor-kappaB activation. *International journal of immunopharmacology*. 2000;22(11):923-33. Epub 2000/11/25.
32. Bueter CL, Lee CK, Rathinam VA, Healy GJ, Taron CH, Specht CA, et al. Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis. *J Biol Chem*. 2011;286(41):35447-55. Epub 2011/08/25.
33. Liu Y, Yin Y, Wang L, Zhang W, Chen X, Yang X, et al. Engineering Biomaterial-Associated Complement Activation to Improve Vaccine Efficacy. *Biomacromolecules*. 2013;14(9):3321-8.
34. Harikrishnan R, Kim JS, Balasundaram C, Heo MS. Dietary supplementation with chitin and chitosan on haematology and innate immune response in *Epinephelus bruneus* against *Philasterides dicentrarchi*. *Experimental parasitology*. 2012;131(1):116-24. Epub 2012/04/06.
35. Klokkevold PR, Fukayama H, Sung EC, Bertolami CN. The effect of chitosan (poly-N-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. 1999;57(1):49-52. Epub 1999/01/23.
36. Mathieu C, Chevrier A, Lascau-Coman V, Rivard GE, Hoemann CD. Stereological analysis of subchondral angiogenesis induced by chitosan and coagulation factors in microdrilled articular cartilage defects. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2013;21(6):849-59. Epub 2013/03/26.
37. Guzmán-Morales J, Lafantaisie-Favreau CH, Chen G, Hoemann CD. Subchondral chitosan/blood implant-guided bone plate resorption and woven bone repair is coupled to hyaline cartilage regeneration from microdrill holes in aged rabbit knees. *Osteoarthritis and Cartilage*. 2013(0).

38. Muzzarelli RAA. Chitin. London, UK: Oxford Pergamon Press; 1977.
39. Xu Q, Dou J, Wei P, Tan C, Yun X, Wu Y, et al. Chitooligosaccharides induce apoptosis of human hepatocellular carcinoma cells via up-regulation of Bax. *Carbohydrate Polymers*. 2008;71(4):509-14.
40. Wu H, Aam BB, Wang W, Norberg AL, Sørli M, Eijsink VGH, et al. Inhibition of angiogenesis by chitooligosaccharides with specific degrees of acetylation and polymerization. *Carbohydrate Polymers*. 2012;89(2):511-8.
41. Maeda Y, Kimura Y. Antitumor effects of various low-molecular-weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice. *J Nutr*. 2004;134(4):945-50. Epub 2004/03/31.
42. Henning Cederkvist F, Parmer MP, Vårum KM, Eijsink VGH, Sørli M. Inhibition of a family 18 chitinase by chitooligosaccharides. *Carbohydrate Polymers*. 2008;74(1):41-9.
43. Einarsson JM, Bahrke S, Sigurdsson BT, Ng C-H, Petersen PH, Sigurjonsson OE, et al. Partially acetylated chitooligosaccharides bind to YKL-40 and stimulate growth of human osteoarthritic chondrocytes. *Biochemical and Biophysical Research Communications*. 2013;434(2):298-304.
44. Lieder R, Gaware VS, Thormodsson F, Einarsson JM, Ng CH, Gislason J, et al. Endotoxins affect bioactivity of chitosan derivatives in cultures of bone marrow-derived human mesenchymal stem cells. *Acta Biomater*. 2013;9(1):4771-8. Epub 2012/09/06.
45. Szczepanska J, Pawlowska E, Synowiec E, Czarny P, Rekas M, Blasiak J, et al. Protective effect of chitosan oligosaccharide lactate against DNA double-strand breaks induced by a model methacrylate dental adhesive. *Med Sci Monit*. 2011;17(8):BR201-8. Epub 2011/08/02.
46. Seyfarth F, Schliemann S, Elsner P, Hipler UC. Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and N-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*. *Int J Pharm*. 2008;353(1-2):139-48. Epub 2008/01/01.
47. Arakane Y, Muthukrishnan S. Insect chitinase and chitinase-like proteins. *Cell Mol Life Sci*. 2010;67(2):201-16. Epub 2009/10/10.
48. Hollak CE, van Weely S, van Oers MH, Aerts JM. Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. *J Clin Invest*. 1994;93(3):1288-92. Epub 1994/03/01.
49. Henrissat B. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J*. 1991;280 ( Pt 2):309-16. Epub 1991/12/01.
50. Bussink AP, Speijer D, Aerts JM, Boot RG. Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. *Genetics*. 2007;177(2):959-70. Epub 2007/08/28.
51. Gonzalez-Teuber M, Eilmus S, Muck A, Svatos A, Heil M. Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. *Plant J*. 2009;58(3):464-73. Epub 2009/01/16.
52. Choi EH, Zimmerman PA, Foster CB, Zhu S, Kumaraswami V, Nutman TB, et al. Genetic polymorphisms in molecules of innate immunity and susceptibility to infection with *Wuchereria bancrofti* in South India. *Genes Immun*. 2001;2(5):248-53. Epub 2001/08/31.
53. Piras I, Melis A, Ghiani ME, Falchi A, Luiselli D, Moral P, et al. Human CHIT1 gene distribution: new data from Mediterranean and European populations. *Journal of human genetics*. 2007;52(2):110-6. Epub 2006/11/16.
54. Boot RG, Renkema GH, Strijland A, van Zonneveld AJ, Aerts JM. Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J Biol Chem*. 1995;270(44):26252-6. Epub 1995/11/03.
55. Bussink AP, van Eijk M, Renkema GH, Aerts JM, Boot RG. The biology of the Gaucher cell: the cradle of human chitinases. *Int Rev Cytol*. 2006;252:71-128. Epub 2006/09/21.
56. Brinkman J, Wijburg FA, Hollak CE, Groener JE, Verhoek M, Scheij S, et al. Plasma chitotriosidase and CCL18: early biochemical surrogate markers in type B Niemann-Pick disease. *J Inherit Metab Dis*. 2005;28(1):13-20. Epub 2005/02/11.

57. Bargagli E, Bennett D, Maggiorelli C, Di Sipio P, Margollicci M, Bianchi N, et al. Human chitotriosidase: a sensitive biomarker of sarcoidosis. *J Clin Immunol.* 2013;33(1):264-70. Epub 2012/08/11.
58. Comabella M, Dominguez C, Rio J, Martin-Gallan P, Vilches A, Vilarrasa N, et al. Plasma chitotriosidase activity in multiple sclerosis. *Clin Immunol.* 2009;131(2):216-22. Epub 2009/01/30.
59. Boot RG, van Achterberg TA, van Aken BE, Renkema GH, Jacobs MJ, Aerts JM, et al. Strong induction of members of the chitinase family of proteins in atherosclerosis: chitotriosidase and human cartilage gp-39 expressed in lesion macrophages. *Arterioscler Thromb Vasc Biol.* 1999;19(3):687-94. Epub 1999/03/12.
60. Di Rosa M, Dell'Ombra N, Zambito AM, Malaguarnera M, Nicoletti F, Malaguarnera L. Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. *Eur J Neurosci.* 2006;23(10):2648-56. Epub 2006/07/05.
61. Malaguarnera L, Ohazuruike LN, Tsianaka C, Antic T, Di Rosa M, Malaguarnera M. Human chitotriosidase polymorphism is associated with human longevity in Mediterranean nonagenarians and centenarians. *Journal of human genetics.* 2010;55(1):8-12. Epub 2009/11/03.
62. Renkema GH, Boot RG, Au FL, Donker-Koopman WE, Strijland A, Muijsers AO, et al. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur J Biochem.* 1998;251(1-2):504-9. Epub 1998/03/10.
63. Houston DR, Recklies AD, Krupa JC, van Aalten DM. Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. *J Biol Chem.* 2003;278(32):30206-12. Epub 2003/05/31.
64. Di Rosa M, Malaguarnera G, De Gregorio C, Drago F, Malaguarnera L. Evaluation of CHI3L-1 and CHIT-1 expression in differentiated and polarized macrophages. *Inflammation.* 2013;36(2):482-92. Epub 2012/11/15.
65. Rehli M, Niller HH, Ammon C, Langmann S, Schwarzfischer L, Andreessen R, et al. Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J Biol Chem.* 2003;278(45):44058-67. Epub 2003/08/23.
66. Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull.* 2006;53(2):172-209. Epub 2006/11/08.
67. Recklies AD, Ling H, White C, Bernier SM. Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. *J Biol Chem.* 2005;280(50):41213-21. Epub 2005/10/20.
68. Volck B, Price PA, Johansen JS, Sorensen O, Benfield TL, Nielsen HJ, et al. YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. *Proc Assoc Am Physicians.* 1998;110(4):351-60. Epub 1998/08/01.
69. Volck B, Johansen JS, Stoltenberg M, Garbarsch C, Price PA, Ostergaard M, et al. Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2001;9(3):203-14. Epub 2001/04/13.
70. Hakala BE, White C, Recklies AD. Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *J Biol Chem.* 1993;268(34):25803-10. Epub 1993/12/05.
71. Johansen JS, Baslund B, Garbarsch C, Hansen M, Stoltenberg M, Lorenzen I, et al. YKL-40 in giant cells and macrophages from patients with giant cell arteritis. *Arthritis Rheum.* 1999;42(12):2624-30. Epub 2000/01/01.
72. Kawada M, Hachiya Y, Arihiro A, Mizoguchi E. Role of mammalian chitinases in inflammatory conditions. *Keio J Med.* 2007;56(1):21-7. Epub 2007/03/30.
73. Bonne-Barkay D, Bissel SJ, Kofler J, Starkey A, Wang G, Wiley CA. Astrocyte and Macrophage Regulation of YKL-40 Expression and Cellular Response in Neuroinflammation. *Brain Pathol.* 2011. Epub 2011/11/15.

74. Jensen BV, Johansen JS, Price PA. High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. *Clin Cancer Res.* 2003;9(12):4423-34. Epub 2003/10/14.
75. Kawada M, Seno H, Kanda K, Nakanishi Y, Akitake R, Komekado H, et al. Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer. *Oncogene.* 2012;31(26):3111-23. Epub 2011/11/08.
76. Hogdall EV, Johansen JS, Kjaer SK, Price PA, Christensen L, Blaakaer J, et al. High plasma YKL-40 level in patients with ovarian cancer stage III is related to shorter survival. *Oncol Rep.* 2003;10(5):1535-8. Epub 2003/07/29.
77. Yan C, Ding X, Wu L, Yu M, Qu P, Du H. Stat3 Downstream Gene Product Chitinase 3-Like 1 Is a Potential Biomarker of Inflammation-induced Lung Cancer in Multiple Mouse Lung Tumor Models and Humans. *PLoS One.* 2013;8(4):e61984. Epub 2013/04/25.
78. Ober C, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R, et al. Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. *N Engl J Med.* 2008;358(16):1682-91. Epub 2008/04/12.
79. Johansen JS, Christoffersen P, Moller S, Price PA, Henriksen JH, Garbarsch C, et al. Serum YKL-40 is increased in patients with hepatic fibrosis. *J Hepatol.* 2000;32(6):911-20. Epub 2000/07/18.
80. Vind I, Johansen JS, Price PA, Munkholm P. Serum YKL-40, a potential new marker of disease activity in patients with inflammatory bowel disease. *Scand J Gastroenterol.* 2003;38(6):599-605. Epub 2003/06/27.
81. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, et al. Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem.* 1999;68:729-77. Epub 2000/06/29.
82. Shao R, Hamel K, Petersen L, Cao QJ, Arenas RB, Bigelow C, et al. YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene.* 2009;28(50):4456-68. Epub 2009/09/22.
83. Recklies AD, White C, Ling H. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. *Biochem J.* 2002;365(Pt 1):119-26. Epub 2002/06/20.
84. Chen CC, Llado V, Eurich K, Tran HT, Mizoguchi E. Carbohydrate-binding motif in chitinase 3-like 1 (CHI3L1/YKL-40) specifically activates Akt signaling pathway in colonic epithelial cells. *Clin Immunol.* 2011;140(3):268-75. Epub 2011/05/07.
85. Berres ML, Papen S, Pauels K, Schmitz P, Zaldivar MM, Hellerbrand C, et al. A functional variation in CHI3L1 is associated with severity of liver fibrosis and YKL-40 serum levels in chronic hepatitis C infection. *J Hepatol.* 2009;50(2):370-6. Epub 2008/12/17.
86. Dela Cruz CS, Liu W, He CH, Jacoby A, Gornitzky A, Ma B, et al. Chitinase 3-like-1 promotes *Streptococcus pneumoniae* killing and augments host tolerance to lung antibacterial responses. *Cell Host Microbe.* 2012;12(1):34-46. Epub 2012/07/24.
87. Parkin J, Cohen B. An overview of the immune system. *The Lancet.* 2001;357(9270):1777-89.
88. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science.* 2010;327(5966):656-61. Epub 2010/02/06.
89. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci.* 2008;13:453-61. Epub 2007/11/06.
90. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol.* 2005;5(12):953-64.
91. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008;8(12):958-69.
92. Imhof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol.* 2004;4(6):432-44. Epub 2004/06/03.

93. Barron L, Wynn TA. Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. *Eur J Immunol.* 2011;41(9):2509-14.
94. Loke P, Nair MG, Parkinson J, Guiliano D, Blaxter M, Allen JE. IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol.* 2002;3:7. Epub 2002/07/06.
95. Dinarello CA. Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. *J Biol Regul Homeost Agents.* 1997;11(3):91-103. Epub 1997/07/01.
96. Krause SW, Rehli M, Kreutz M, Schwarzfischer L, Paulauskis JD, Andreesen R. Differential screening identifies genetic markers of monocyte to macrophage maturation. *J Leukoc Biol.* 1996;60(4):540-5. Epub 1996/10/01.
97. Di Rosa M, Musumeci M, Scuto A, Musumeci S, Malaguarnera L. Effect of interferon-gamma, interleukin-10, lipopolysaccharide and tumor necrosis factor-alpha on chitotriosidase synthesis in human macrophages. *Clin Chem Lab Med.* 2005;43(5):499-502.
98. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer.* 1980;26(2):171-6. Epub 1980/08/01.
99. Schwende H, Fitzke E, Ambs P, Dieter P. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J Leukoc Biol.* 1996;59(4):555-61. Epub 1996/04/01.
100. Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res.* 2007;56(1):45-50. Epub 2007/03/06.
101. Kohro T, Tanaka T, Murakami T, Wada Y, Aburatani H, Hamakubo T, et al. A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. *J Atheroscler Thromb.* 2004;11(2):88-97. Epub 2004/05/22.
102. Schroder K, Tschopp J. The inflammasomes. *Cell.* 2010;140(6):821-32. Epub 2010/03/23.
103. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol.* 2007;8(5):405-13. Epub 2007/03/23.
104. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol.* 2009;7(2):99-109. Epub 2009/01/17.
105. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol.* 2010;10(3):210-5. Epub 2010/02/20.
106. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol.* 2008;9(8):847-56. Epub 2008/07/08.
107. Lamkanfi M. Emerging inflammasome effector mechanisms. *Nat Rev Immunol.* 2011;11(3):213-20. Epub 2011/02/26.
108. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol.* 2010;11(12):1136-42. Epub 2010/11/09.
109. Marudova M, MacDougall AJ, Ring SG. Pectin-chitosan interactions and gel formation. *Carbohydr Res.* 2004;339(11):1933-9. Epub 2004/07/21.
110. Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One.* 2010;5(1):e8668. Epub 2010/01/20.
111. Nielsen AR, Plomgaard P, Krabbe KS, Johansen JS, Pedersen BK. IL-6, but not TNF-alpha, increases plasma YKL-40 in human subjects. *Cytokine.* 2011;55(1):152-5. Epub 2011/04/12.

112. Haglund L, Bernier SM, Onnerfjord P, Recklies AD. Proteomic analysis of the LPS-induced stress response in rat chondrocytes reveals induction of innate immune response components in articular cartilage. *Matrix Biol.* 2008;27(2):107-18. Epub 2007/11/21.
113. Henning Cederkvist F, Parmer MP, Vårum KM, Eijssink VGH, Sjörlie M. Inhibition of a family 18 chitinase by chitoooligosaccharides. *Carbohydrate Polymers.* 2008;74(1):41-9.
114. Harter C, Mellman I. Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J Cell Biol.* 1992;117(2):311-25. Epub 1992/04/01.
115. Munn DH, Beall AC, Song D, Wrenn RW, Throckmorton DC. Activation-induced apoptosis in human macrophages: developmental regulation of a novel cell death pathway by macrophage colony-stimulating factor and interferon gamma. *J Exp Med.* 1995;181(1):127-36. Epub 1995/01/01.
116. Maeß MB, Wittig B, Cignarella A, Lorkowski S. Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli. *Journal of Immunological Methods.* (0).
117. Lee CG, Hartl D, Lee GR, Koller B, Matsuura H, Da Silva CA, et al. Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *J Exp Med.* 2009;206(5):1149-66. Epub 2009/05/06.
118. Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. *Biomaterials.* 1997;18(7):567-75. Epub 1997/04/01.
119. Champion JA, Walker A Fau - Mitragotri S, Mitragotri S. Role of particle size in phagocytosis of polymeric microspheres. (0724-8741 (Print)).
120. Pajarinen J, Kouri V-P, Jämsen E, Li T-F, Mandelin J, Kontinen YT. The response of macrophages to titanium particles is determined by macrophage polarization. *Acta Biomater.* 2013;9(11):9229-40.
121. Eligini S, Crisci M, Bono E, Songia P, Tremoli E, Colombo GI, et al. Human monocyte-derived macrophages spontaneously differentiated in vitro show distinct phenotypes. *J Cell Physiol.* 2013;228(7):1464-72. Epub 2012/12/21.
122. Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M, et al. Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *Journal of immunology (Baltimore, Md : 1950).* 2001;167(7):3555-8. Epub 2001/09/21.
123. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials.* 2003;24(7):1121-31.
124. Khalil IA, Kogure K Fau - Akita H, Akita H Fau - Harashima H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. 2006(0031-6997 (Print)).