Host chitinase CHIT1 generates oligomeric chitin MAMPs from pathogenic fungi for CD14-TLR1-TLR2 activation

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List of abbreviations

4-MU:	4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside
AMCase:	Acidic mammalian chitinase
APC:	Antigen presenting cells
BiFC:	Bimolecular complementary
BMDMs:	Bone marrow derived macrophages
CAPS:	Cryopyrin-associated periodic syndrome
CBM:	C-terminal carbohydrate binding module
CDS:	Cytosolic DNA sensors
CHIT1:	Chitotriosidase
CLPs:	Chitinase-like proteins
CLRs:	C-type lectin receptors
ConA:	Concanavalin A
COPD:	Chronic obstructive pulmonary disease
DA:	Degree of acetylation
DAMPs:	Damaged-associated molecular patterns
DP:	Degree of polymer
dPBS:	Dulbecco's Phosphate Buffered Saline
ECD:	Ectodomain
ELISA:	Enzyme-linked immunosorbent assay
FCS:	Fetal calf serum
FIBCD1:	Fibrinogen C domain-containing protein 1
GH:	Glycosyl hydrolase
GlcNAc:	N-acetylglucosamine
h:	Hour, hours
HDM:	House dust mite
HSC:	Hematopoietic stem cells
IL-6:	Interleukiin-6
LBP:	LPS-binding protein

LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
LTA:	Lipoteichoic acid
MAMPs:	Microbe-associated molecular patterns
MAPK:	Mitogen-activated protein kinases
MHC:	Major histocompatibility complex
NF-κB:	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells
Pam2:	Pam_2CSK_4
Pam3:	Pam_3CSK_4
PRRs:	Pattern recognition
RLRs:	Retinoic acid-inducible gene-I-like receptors
RT:	Room temperature
Sap:	Secreted aspartic protease
SEC:	Size exclusion chromatography
TIR:	Toll-interleukin receptor domain
TLRs:	Toll-like receptors
TNF:	Tumor necrosis factor
WGA:	Wheat germ agglutinin

Chapter 1: Introduction

1.1 Overview of the immune system

The mammalian body is protected from various infectious microbes, damaging toxins and foreign molecules by natural physical barriers and a variety of effector cells that build up the immune system. The immune system is comprised of two parts: innate immunity and adaptive immunity. Both arms of the immune system rely on the activities of white blood cells or leukocytes which arise from hematopoietic stem cells (HSC) of the bone marrow where the two main categories of cell groups, the lymphoid and myeloid lineages, are generated and differentiated. Terminally differentiated cells like granulocytes (e.g. neutrophils, eosinophils and basophils), mast cells, macrophages and dendritic cells are from myeloid progenitors; and cells like T cells, B cells, plasma cells and NK cells are from lymphoid progenitors, also called lymphocytes. Although non-hematopoietic tissue cells contribute to innate immune functions, myeloid cells are the major innate effector immune cells that immediately respond to invading microbes and initiate an innate immunity. The activation of innate immune cells can subsequently bridge the adaptive immune response via interaction with lymphocytes. This interplay of innate and adaptive immunity completes the immune system to enable effective defense against pathogen infection (Parkin and Cohen, 2001).

1.1.1 Innate immunity

The innate immune system, in addition to physical and chemical barriers (e. g. skin, mucous membrane and pH), is the first line of host defense against pathogens and includes host soluble molecules (e. g. complement system, antimicrobial peptides and antimicrobial enzymes) and immune cell-mediated sensing and effector responses (Turvey and Broide, 2010). As aforementioned, myeloid cells like macrophages, neutrophils and dendritic cells are key immune cells to initiate innate immunity. The different cell types possess different strategies to protect the host from infectious microbes like phagocytosis, pro-inflammatory cytokine, chemokine or type I interferon production, and finally destruction of pathogens through phagocytosis, or in the process

of programmed cell death of infected cells via apoptosis or pyroptosis. More in detail, the process of phagocytosis is initiated when surface receptors (also known as "pattern recognition receptors", PRRs, which will be described in section 1.2) interact with the particles or ligands from the microbial surface (Turvey and Broide, 2010), so-called microbe-associated molecular patterns (MAMPs). The bound pathogen is subsequently internalized into a phagosome. The phagosome then fuses with lysosomes to digest and the microbes are killed via hydrolytic enzyme or reactive oxygen or nitrogen species (Uribe-Querol and Rosales, 2020). Cells which exhibit phagocytosis like neutrophils, macrophages and dendritic cells are also called phagocytes. Dendritic cells in this context, preserve "useful information" from degraded microbes in the form of fragments of peptides and can then present them to the surface via major histocompatibility complex (MHC) II molecules (Savina and Amigorena, 2007) as so called antigens. Dendritic cells and macrophages are also called antigen presenting cells (APC) and represent the key innate immune cells to bridge and develop antigen-specific adaptive immune response.

1.1.2 Adaptive immunity

In contrast to innate immunity, adaptive immunity exhibits high specificity to certain pathogens via activation of antigen-specific T- and B- lymphocytes and generally takes several days to establish. The buildup of antigen-specific lymphocytes requires clonal selection of naïve lymphocytes into mature T cells and B cells which are distinguished by their expression of surface antigen receptors, so called T-cell and B-cell receptors and immunoglobulins (Murphy & Weaver, 2016; Taniuchi, 2018). These receptors are generated by somatic recombination of gene segments which gives rise to an extensive diversity to recognize antigens. During the selection process, self-reactive receptors or immature cells are eliminated. Through recognizing peptide fragments on MHC molecules, immature T cells become effector T cells which are classified into two major groups: $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells (Taniuchi, 2018). Cytotoxic T cells directly kill the cells that are infected with viruses or bacteria. The function of helper T cells, based on their different subsets (e.g. Th1, Th2 and Th17) exhibit multiple

ways of directing and polarizing the immune response in an appropriate manner (Taniuchi, 2018). Helper T cells also activate B cells to produce antibodies which function by neutralizing specific toxins, preventing microbe adhesion, activating complement and triggering antibody-dependent cytotoxicity by killer cells (Pasare and Medzhitov, 2005). The interaction between T cells and B cells also provides them the long-lasting memory of protective effect to rapidly respond with repeatedly invading pathogens.

1.2 The role of pattern recognition receptors in the innate immune system

1.2.1 Patten recognition receptors

Innate immune responses are typically initiated when cells detect microbes through innate recognition receptors, so called pattern recognition receptors (PRRs). They are germline-encoded receptors extensively expressed in multiple cell types including leukocytes, nerve cells, epithelial cells and others on the cell surface or within cytoplasm. Innate immune myeloid cells are the major populations that express high levels of PRRs. They recognize and sense simple molecules or structures that are part of microorganisms or damaged cells or tissues in the host (Janeway, 1989). The molecules like lipopolysaccharide (LPS), acylated lipoproteins, mannose-rich oligosaccharides, β-glucans as well as DNA or RNA coming from bacteria, virus and fungi are called microbe-associated molecular patterns (MAMPs). Other structures like ATP, uric acid and mitochondria DNA appearing from tissue damage or cellular stress are known as damaged-associated molecular patterns (DAMPs) and can also trigger PRRs. A cascade of signaling events take place following MAMP and DAMP recognition by PRRs, which activate different downstream signaling pathways to initiate innate host defense immune responses to prevent or fight against infections and then elicited a subsequent adaptive immune response (Newman *et al.,* 2013; Rivera *et al*., 2016; West *et al*., 2006).

PRRs can be categorized into different subgroups. Based on their localization, PRRs are divided into soluble, membrane-bound and cytosolic PRRs. According to their ligand specificity, structure and function, PRRs are classified into five major families. The membrane-bound receptors are Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). The cytosolic receptors are NOD-like receptors (NLRs), Retinoic acid-inducible gene-I-like receptors (RLRs) and cytosolic DNA sensors (CDSs). TLRs cover a wide range of MAMPs from different types of microbes, including LPS or flagellin from bacteria, or double-stranded or single-stranded RNA from viruses (Brubaker *et al*., 2015). CLRs majorly sense fungi components based on the cell wall structure of fungal cell walls like β-glucans for Dectin-1 or α-mannans for Dectin-2 and play a role in the complement system and as cellular receptors (Hoving *et al*., 2014). NLRs are known to detect a variety of ligands or cellular damages like NOD1 for γ-D-glutamyl-meso-diaminopimelic acid, NLRP1 for muramyl dipeptide and viral ssRNA and NLRC4 for flagellin (Kim *et al*., 2016). RLRs like RIG-I or MDA5 identify viral double-stranded or single-stranded RNA (Rehwinkel and Gack, 2020). CDSs like cGAS sense viral double-stranded DNA (Brubaker *et al*., 2015; Gay *et al*., 2014; Yu and Liu, 2021). The malfunction of PRRs might cause aberrant inflammatory cytokine production such as inflammatory bowel disease or cryopyrin-associated periodic syndrome (CAPS), and uncontrolled growth of invading microbe such as sepsis or candidiasis (de Torre-Minguela *et al*., 2017; Hardison and Brown, 2012; Lu *et al*., 2018; Tsujimoto *et al*., 2008).

1.2.2 Toll-like receptors and their corresponding ligands

From the TLR family, the first *Toll* gene was identified for controlling the dorsal-ventral patterning establishment of a *Drosophila melanogaster* embryo (Anderson *et al*., 1985). In 1996, the group of Jules Hoffmann discovered that Toll signaling in adult fly induces expression of antimicrobial peptides like drosomycin which is important for host defense against bacterial and fungal infection (Lemaitre *et al*., 1996). One year after, the first mammalian Toll was identified by the group of Charles Janeway (Medzhitov *et al*., 1997). Nowadays, 10 members of the *TLR* genes in human and 12 in

mice have been identified (O'Neill *et al*., 2013). TLR4, TLR5, TLR11, and the heterodimers of TLR2/TLR1 or TLR2/TLR6 bind to their respective ligands at the cell surface. TLR3, TLR7/TLR8, TLR9 and TLR13 localize and sense microbial ligands in the endosomes (O'Neill *et al.*, 2013). The mammalian TLRs can recognize MAMPs from most of the microbes and are expressed by many types of cells including macrophages, neutrophils, dendritic cells, stromal cells and epithelial cells. Once TLRs sense and bind their relative ligands, they can initiate the downstream signaling events to trigger several innate immune responses including pro-inflammatory cytokine or chemokine production, and antimicrobial peptides expression. The latter, combined with cytokine production subsequently bridges to the adaptive immune system to fight against pathogen infection. In this study, I focus on TLRs and the chapters below will give more insights and detailed roles of TLRs.

TLRs are composed of a single-pass transmembrane protein and an extracellular region of 18 – 25 copies of the leucine-rich repeat (LRR) motif. Each LRR normally contains 20 – 25 amino acids and multiple LRRs fold into a horseshoe-shaped protein scaffold. For TLRs, ectodomains composed of 16 – 28 diverse LRR modules are a major region responsible for ligand binding and recognition (Akira *et al*., 2006). All the TLR proteins also contain one cytoplasmic domain called the Toll-IL-1 receptor (TIR) domain, which is responsible for interacting with other TIR domain-containing proteins to activate downstream signaling events (see section 1.2.3). Each TLR recognizes one or more microbial ligands. Some TLRs which were mentioned earlier like TLR2 and TLR1 can form heterodimers in response to their relative ligands. The following paragraphs will describe the diverse ligand specificity of TLRs recognition **(Table 1)**.

TLR1, TLR2 and TLR6, which are the focus of this project and hence are described in greater detail in the next section, are activated by various hydrophobic ligands, such as lipoteichoic acid (LTA), diacyl and triacyl lipoproteins of Gram-negative bacteria, lipomannans of mycobacteria and cell wall components like β-glucan from fungi. Ligand binding triggers the heterodimerization of TLR2 and TLR1 by triacyl

lipopeptides, or of TLR2 and TLR6 by diacyl lipopeptides. Dimerization brings the two TIR domains of TLR1/TLR2 or TLR2/TLR6 close enough to initiate downstream signaling events (Jin *et al*., 2007).

TLR4 is well known to recognize lipopolysaccharide (LPS) from Gram-negative bacteria. To recognize LPS, the ectodomain of TLR4 associates with an accessory protein, MD-2 (Kim *et al*., 2007). MD-2 firstly binds to TLR4 in the endoplasmic reticulum lumen and facilitates their trafficking to the cell surface, making it ready for the recognition of LPS. The acyl chains of LPS can bind to a hydrophobic pocket in MD-2 within one MD-2-TLR4 complex (Park *et al*., 2009). Besides MD-2, two other proteins are also involved in LPS delivery to facilitate the activation of TLR4; LPS-binding protein (LBP) is known as a lipid binding protein that is abundant in the blood and in the fluid in tissues, and that shows a high affinity to LPS. Once LPS and LBP form a complex, LBP delivers LPS to CD14, a GPI-anchor protein which is also present on the surface of myeloid cells. CD14 can facilitate the transfer of LPS to TLR4-MD-2 and thereby enhances downstream signaling events (Ryu *et al*., 2017).

TLR5 is known to recognize flagellin, a protein subunit of the bacterial flagellum. TLR5 only recognizes a highly conserved site on monomeric flagellin, which is hidden in the flagellar filament. Interestingly, mice additionally express TLR11 and TLR12 which have the ability to recognize intact flagellin (Song *et al*., 2017). In addition, TLR11 and TLR12 have also shown an overlapping function to recognize protozoan parasites like *Toxoplasma gondii* via its actin-binding protein profilin (Yarovinsky *et al*., 2005).

The other TLRs like TLR3, TLR7, TLR8 and TLR9 are known as endosomal nucleic acid sensors, especially for virus recognition. TLR3 recognizes double-stranded RNA (dsRNA) and the synthetic polymer composed of inosinic and cytidylic acid, poly I:C. TLR7 and TLR8 are activated by breakdown products of single-stranded RNA (ssRNA)

(Heil *et al*., 2004; Liu *et al*., 2008). TLR9 recognizes DNA with unmethylated CpG dinucleotide (Lund *et al*., 2003).

Table 1. List of TLR-ligands

TLRs	**Ligands**
TLR2	Lipopeptides, lipoteichoic acid (LTA) and peptidoglycans (bacteria), lipomannans (mycobacteria), β-glucan and zymosan (fungi)
TLR1/TLR2	Triacylated lipopeptides (bacteria), Pam_3CSK_4 (synthetic)
TLR2/TLR6	Diacylated lipopeptides (bacteria), Pam_2CSK_4 and FSL-1 (synthetic)
TLR3	Double-stranded RNA (viruses), poly I:C (synthetic)
TLR4	LPS (Gram-negative bacteria), LTA (Gram-positive bacteria)
TLR5	Flagellin (bacteria)
TLR7	Single-stranded RNA (viruses)
TLR8	Single-stranded RNA (viruses)
TLR9	DNA with unmethylated CpG (bacteria and herpesviruses)
TLR10 (human only)	*Listeria monocytogenes* (the specific ligand is still unknown)
TLR11(mouse only)	Profilin and profilin-like proteins (*Toxoplasma gondii*)
TLR12 (mouse only)	Profilin (*Toxoplasma gondii*)
TLR13 (mouse only)	Single-stranded ribosomal RNA (bacteria)

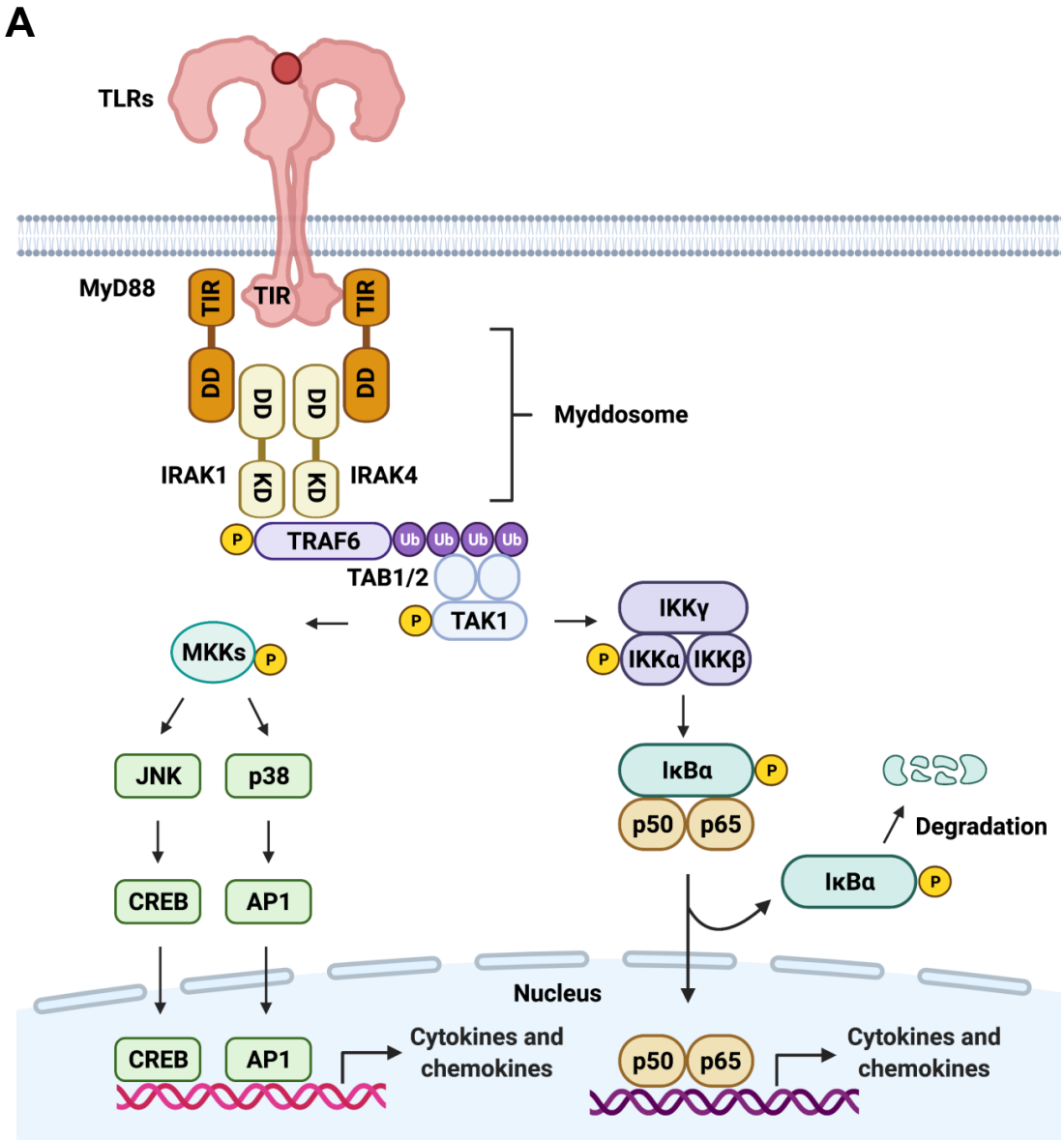
Table is modified and adapted from Murphy K & Weaver C, 2016 Janeway's Immunobiology, Ninth Edition.

1.2.3 TLR downstream signaling pathway

Ligand-induced dimerization of TLRs ectodomains allows the cytoplasmic TIR domains to come close together. This activates TIR domains to interact with cytoplasmic adaptor proteins to initiate intracellular signaling **(Figure 1).** Myeloid differentiation primary response 88 (MyD88) and MyD88 adaptor-like (MAL) are two major adaptor proteins interacting with almost all TLR-TIR domains, except for TLR3, which interacts only with TIR domain-containing adaptor-inducing IFN-β (TRIF). The first step of signaling cascade is that the TLRs TIR domains interact with TIR domains of MyD88/MAL, where MyD88 molecules form a scaffold to enable interaction with other proteins. This takes place in the context of a multi-protein complex termed the Myddosome. The N-terminal of MyD88, so called death domain, recruits the serine-threonine kinases, IL-1-receptor associated kinase (IRAK)4 and IRAK1. IRAKs recruit and phosphorylate the E3 ubiquitin ligase, TNFR-associated factor 6 (TRAF6). The activated TRAF6 is polyubiquitinated and recruits the transforming growth factor-b-activated kinase 1 (TAK1) and two adaptor proteins, TAK1-binding protein 1 (TAB1) and TAB2. TAB1 and TAB2 bind to polyubiquitin that brings TAK1 to the Myddosome to be phosphorylated. The activated TAK1 then phosphorylates the IκB kinase complex (IKK) which is composed of IKKα, IKKβ and IKKγ (also known as NEMO, NF-κB essential modifier). The activated NEMO complex leads to the final step of degradation of IκBα which allows the two subunits of the transcription factor, p50 and p65, to translocate to the nucleus. In parallel, activated TAK1 can also activate the MAP kinase JNK and p38 cascade, which subsequently leads to AP-1 or CREB activation and translocation to the nucleus. Those transcription factors NF-κB, AP-1 and CREB can drive pro-inflammatory cytokine gene transcription such as IL-6, TNF and pro-IL-1β (Häcker *et al*., 2006; Kawai and Akira, 2007).

Besides the surface TLRs, the endosomal nucleic-acid sensing TLRs like TLR3, TLR7, TLR8 and TLR9 activate the IRF (interferon regulatory factor) family. For TLR3, the TIR domain interacts with TRIF adaptor protein. TRIF interacts with the E3 ubiquitin ligase TRAF3 to generate a polyubiquitin tail which allows to recruit NEMO kinases

containing IKKε and TBK1 (TRAF family member-associated NF-κB activator binding protein 1). The activated TBK1 phosphorylates transcription factor IRF3, and then IRF3 enters the nucleus and induces expression of type I interferon genes (Honda and Taniguchi, 2006). For TLR7 and TLR9 signaling, it is uniquely through MyD88. The same as mentioned earlier, MyD88 together with IRAK4 and IRAK1 form a Myddosome complex. In contrast to recruit TRAFs, here the Myddosome complex recruits IRF7. This leads IRF7 to be phosphorylated by IRAK1 and then allows IRF7 to enter the nucleus to induce Type I interferon (Kawai *et al*., 2004).



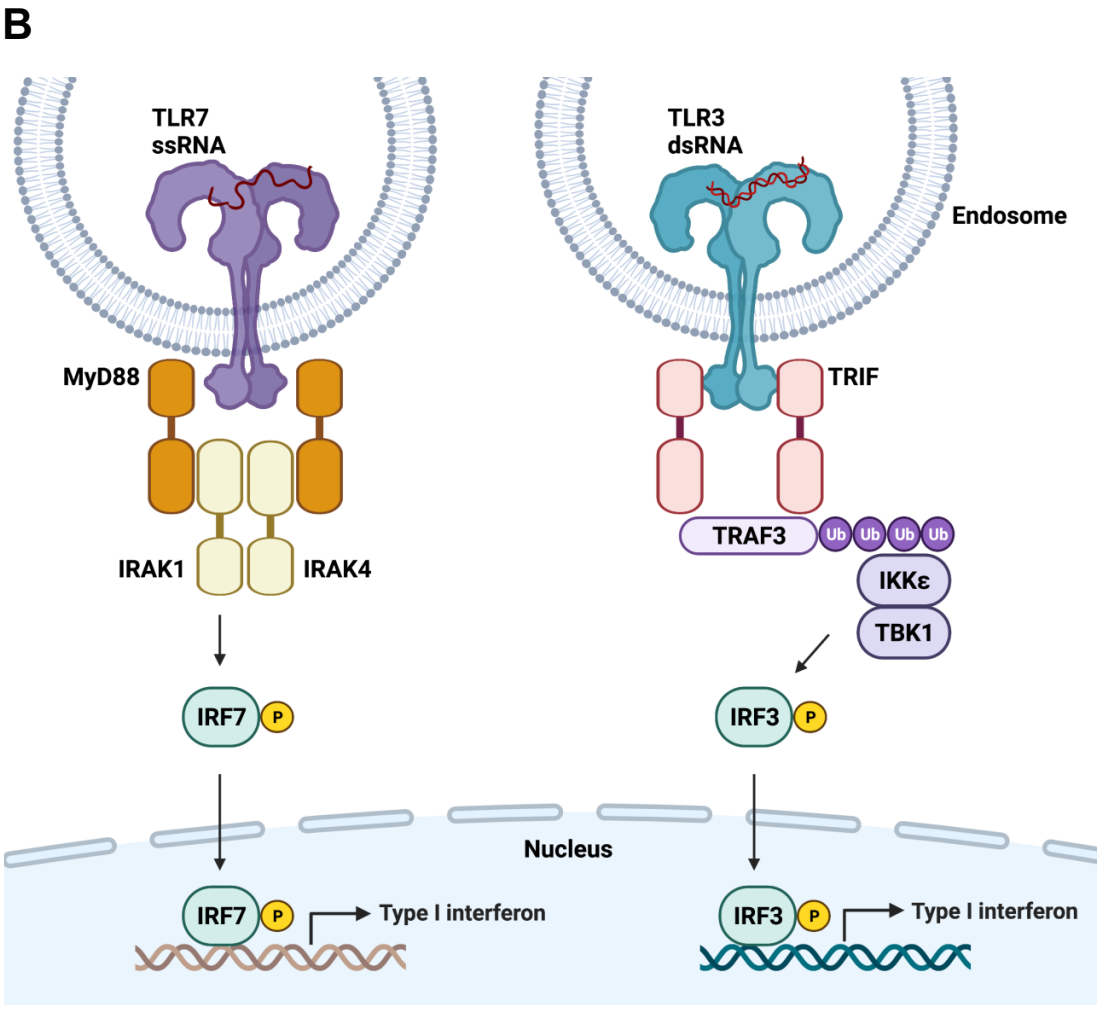


Figure 1. TLRs downstream signaling pathway.

The pictures were created with BioRenders.com. DD, death domain; TIR, Toll-IL-1 receptor; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; Ub, ubiquitination; P, phosphorylation; TAK1, TGFβ - activated kinase 1; TAB, TAK1-binding protein; MKK, mitogen activated protein kinase kinase; AP-1, activator protein 1; CREB cAMP-responsive element-binding protein; IKK, inhibitor of NF-κB kinase; IκBα, inhibitor of NF-κBα; TRIF, TIR domain-containing adaptor protein inducing IFNβ; TBK1, TANK-binding kinase 1; IRF, interferon regulatory factor

1.3 Toll-like receptor 2

1.3.1 Toll-like receptor 2 and its co-receptors

TLR2 is the only TLR described so far to form functional heterodimers with another type of TLRs, primarily TLR1 and TLR6. TLR2 is also known to interact with non-TLR molecules such as Dectin-1, allowing the recognition of a great number and variety of MAMPs (Netea *et al*., 2006). In this study **Table 2** summarizes the diversity of TLR2 ligands, their co-receptors and accessory proteins. In the following sections, I will first state the role of co-receptors and accessory proteins for TLR2; and then give some examples to delineate the role of TLR2 for these diversities of ligands recognition.

As mentioned earlier, the TLR2/TLR1 and TLR2/TLR6 heterodimers are known to specifically bind lipoproteins like synthetic tri- or diacylated lipopeptides, respectively (Jin *et al.*, 2007; Takeuchi *et al*., 2001). This distinctive binding is due to structural differences. The binding of the triacyl lipopeptide, Pam3, induces a "m" shaped formation of a heterodimer of TLR1 and TLR2 whereas binding of diacylated lipopeptide, Pam_2CSK_4 (Pam2) does not. The three lipid chains of Pam_3CSK_4 (Pam3) mediate the TLR1/TLR2 heterodimerization: two ester-bound lipid chains are inserted into the ectodomain (ECD) of TLR2, while the one amide-bound lipid chain is inserted into the hydrophobic channel in TLR1 (Jin *et al.*, 2007). In contrast, TLR6 lacks the lipid binding channel where the TLR1 is located (Kang *et al*., 2009) so that the amide-bound lipid chain of Pam3 cannot bind to TLR6. However, the interface of TLR2/TLR6 hydrophobic heterodimerization compensates for the lack of amide-bound lipid interaction between the Pam2 and TLR2/TLR6 (Kang *et al.*, 2009).

Table 2. TLR2 microbial activators and their co-receptor and accessory proteins

Ligand	Original microbe	TLRs	Ligand delivery	References
	Bacterium			
Diacyl lipopeptides (Pam2/FSL-1)	Mycoplasma	TLR2/TLR6	CD14/CD36	(Jimenez-Dalmaroni *et al*., 2009)
Triacyl lipopeptides (Pam3)	Bacteria	TLR2/TLR1	CD14/LBP	(Beutler *et al*., 2006)
Lipomannan	Mycobacterium	TLR2/TLR1	CD14/CD36	(Jimenez-Dalmaroni *et al.*, 2009)
Lipoteichoic acid (LTA)	Gram-positive bacteria	TLR2/TLR6	CD14/CD36/MBL	(Ip *et al*., 2008; Jimenez-Dalmaroni *et al.*, 2009)
Peptidoglycan	*Staphylococcus aureus*	TLR2/?	CD14	(Natsuka *et al*., 2008)
Porins	*Neisseria meningitidis*	TLR2/TLR1	Unknown	(Massari *et al*., 2006)
Lipopolysaccharide	Gram-negative bacteria	TLR2/TLR4	TLR10	(Nagashima *et al*., 2015)
	Fungus			
Zymosan	*Saccharomyces Cerevisiae*	TLR2/TLR6	CD14/Dectin-1	(Ikeda *et al*., 2008)
Peptidoglycan	*Saccharomyces Cerevisiae*	TLR2/?	MBL	(Wang *et al*., 2019a)
Phospholipomannan	*Candida albicans*	TLR2/TLR6	CD14/Dectin-1	(Netea *et al.*, 2006)
β-glucan	*Candida albicans,*	TLR2/TLR4	Dectin-1	(Ferwerda *et al*., 2008)
	Histoplasma capsulatum	TLR2/?	Dectin-1/CD18	(Sorgi *et al*., 2009)

Ligand	Original microbe	TLRs	Ligand delivery	References
	Fungus			
N-acetyl glucosamine (chitin)	*Candida albicans*	TLR2? /?	Unknown	(Fuchs *et al*., 2018)
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2/TLR1	CD14	(Fonseca *et al*., 2010)
	Virus			
EBV-encoded dUTPase	Epstein-Barr	TLR2/TLR6	Unknown	(Ariza *et al*., 2009)
Glycoprotein B	Cytomegalovirus	TLR2/?	CD14	(Barbalat *et al*., 2009)
NS1	Dengue virus	TLR2/TLR6	CD14	(Chen *et al*., 2015)
Core and NS3 protein	Hepatitis C	TLR2/TLR6	Unknown	(Chang *et al*., 2007)
Envelope protein	SARS-CoV-2	TLR2/?	Unknown	(Zheng *et al*., 2021)
	Protozoan/helminth			
GPI anchors	*Trypanosoma, Toxoplasma*	TLR2/TLR4	CD14/CD36	(Debierre-Grockiego *et al*., 2007)
Lipophosphoglycan	*Leishmania*	TLR2/?	Unknown	(Kavoosi *et al*., 2010)

Table modified and adapted from Oliveria-Nascimento, 2012.

1.3.2 Toll like receptor 2 ligand delivery-associated proteins

As shuttles of hydrophobic MAMPs, LBP and CD14 not only interact with LPS-TLR4 signaling but play roles for ligand delivery to TLR2. LBP directly interacts with lipopeptides through binding with its C-terminal domain containing a negatively charged groove and a hydrophobic core (Eckert *et al*., 2013). CD14 is also known to directly bind Pam3 and deliver it to TLR2 (Vasselon *et al*., 2004). Ranoa *et al*. also reported that CD14 or LBP independently delivers Pam3 to TLR1 and TLR2 to facilitate the heterodimerization and enhance the ternary complexes (Ranoa *et al*., 2013).

Mannose binding lectin (MBL), which shares a similar role as LBP, binds the LTA from *Staphylococcus aureus* and synergizes with TLR2/TLR6 to enhance inflammatory responses (Ip *et al.*, 2008). This enhancement by MBL also occurs through the binding of peptidoglycan, lipoarabinomannan, and lipophosphoglycan (Ip *et al*., 2009). However, recently, one study showed that the MBL association with both TLR2 and peptidoglycans suppresses the inflammatory cytokine production by peptidoglycan derived from *Saccharomyces cerevisiae* (Wang *et al.*, 2019a). These results point to a role of ligand delivery by MBL to TLR2, but this still seems controversial.

CD36, a glycoprotein which is known to function as a scavenger receptor, also participates in the ligand delivery to TLRs. CD36 binds ligands and transfers them to the accessory protein, CD14. This loads the ligands onto TLR1/TLR2 and TLR2/TLR6 heterodimers (Jimenez-Dalmaroni *et al.*, 2009). Besides, not only to TLR2, CD36 also has been suggested to cooperate with TLR4 in several studies. Steward *et al*. reported that CD36 facilitates the LDL and β-amyloid recognition of TLR4/TLR6, as novel heterodimerization, in atherosclerosis and Alzheimer's disease model (Stewart *et al*., 2010). In addition, Cao *et al*. reported recently that CD36 plays a role in cooperation with TLR4 for LPS delivery (Cao *et al*., 2016). It seems that CD36 is as similar as CD14 and mediates ligand delivery to several TLRs.

Above all, it is worth to mention that, even though CD14, LBP and CD36 are not necessary for TLR2 or TLR4 signaling, the role of these proteins is to enhance and facilitate the ligand binding which reduces the threshold of ligand concentrations for TLRs recognition and signaling (Hoebe *et al*., 2005; Nakata *et al*., 2006; Ranoa *et al.*, 2013)

An additional example is TLR10, which is the only TLR that exhibits anti-inflammatory properties. TLR10 can form a heterodimer with TLR2 in response to Pam3 stimulation and thereby dampens the inflammatory response (Oosting *et al*., 2014). LPS has been also identified as potential ligands for the TLR2/TLR10 heterodimer in *Helicobacter pylori* infection (Nagashima *et al.*, 2015). On the other hand, Ragan *et al*. indicated that *Listeria monocytogenes* can be recognized by TLR2/TLR10 and subsequently causes the activation of NF-κB (Regan *et al*., 2013). It is currently unclear whether there is also a cognate activating TLR10 ligand.

1.3.3 Role of TLR2 in infectious diseases

In general, the uncontrolled activation of TLRs signaling by excessive ligand exposure or gain-of-function mutation leads to hyper-inflammation and tissue damage. Whereas the early inflammatory response is an important step to initiate innate immunity for microbe clearance, the malfunction of TLRs recognition and signaling events can cause uncontrolled growth of microbe in infectious diseases. This is evidenced in patients with MyD88 or IRAK4 deficiency (Picard *et al*., 2011). In complement to the list of TLR2 ligands, the following sections will state the role of TLR2 in infectious diseases by bacteria, viruses and fungi.

1.3.3.1 Bacteria

The cell wall of Gram-positive bacteria contains a thick peptidoglycan layer composed of lipoproteins, peptidoglycans and lipoteichoic acid, which can activate TLR2 (Müller-Anstett *et al*., 2010; Ryu *et al*., 2009). Generally, in the absence of TLR2,

murine immune cells showed a reduction of IL-6 and TNF production upon Gram-positive bacteria or their cell wall components stimulation (Oliveira-Nascimento *et al*., 2012). Mycobacterium infection in TLR2 knockout mice shows increased bacterial burden, more severe tissue damage and lower survival rates than WT mice (Yim *et al*., 2006). A human TLR2 polymorphism study showed that patients with decreased TLR2 expression easily develop tuberculosis (Yim *et al.*, 2006) and non-tuberculous mycobacterial lung disease (Yim *et al*., 2008).

Regarding Gram-negative bacteria, like *Escherichia*, *Salmonella* and *Klebsiella*, though LPS recognition by TLR4 is essential, TLR2 can also sense Gram-negative bacteria and plays a role in host protection and pathology. In the absence of TLR2, mice have higher bacterial burdens during infection with *Klebsiella* and *Salmonella* compared to WT mice (Seibert *et al*., 2010; Spiller *et al*., 2008). TLR2/TLR4 double-knockout mice have an even higher rate of susceptibility to *Salmonella* infection compared to TLR4 single knockout mice (Spiller *et al*., 2008). Above all, these studies demonstrate an important role of TLR2 recognition, alone or synergistically with the co-receptor like TLR4, to mediate the control of bacterial infection.

1.3.3.2 Viruses

Intracellular TLRs like TLR3, TLR7 and TLR8 can recognize nucleic acid, especially from viral pathogens to induce IFN gene induction. However, unlike intracellular TLRs, TLR2 recognizes viral proteins to mediate innate immune responses (Lester and Li, 2014). TLR2 has been shown to recognize the glycoproteins B of cytomegalovirus to produce type I IFN by murine monocytes (Barbalat *et al.*, 2009). Although this has not been shown in more physiological systems so far, dUTPase from Epstein-Barr virus can be recognized by TLR2 and induces IL-6 production in HEK293T cells which over-expressed TLR2 (Ariza *et al.*, 2009). Dengue virus nonstructural protein 1 (NS1) can be sensed by TLR2 and TLR6 to induce IL-6 and TNF production in human PBMCs (Chen *et al.*, 2015). In the absence of TLR6, mice exhibit more susceptibility to Dengue virus infection (Chen *et al.*, 2015). Recently, Zheng et al reported that the gene

expression of *TLR2* and *MYD88* are correlated with COVID-19 disease severity (Zheng *et al.*, 2021). They showed that the envelop protein of SARS-CoV-2 can be recognized by TLR2 which leads to the production of inflammatory cytokines in human macrophages. Consequently, mice treated with TLR2 inhibitors showed a higher survival rate than the untreated mice by SARS-CoV-2 infection. They indicated that blocking TLR2 can be used as a therapeutic target for COVID-19 infection (Zheng *et al.*, 2021).

1.3.3.3 Fungi

Fungal infection generally most often be controlled in immunocompetent individuals, while systemic dissemination can occur when the host is immunocompromised that still caused approximately 1.7 million death per year. Because of the different morphologies of most fungal species, the cell wall composition of the fungal pathogen can change between different forms like yeast, conidia and hyphae which lead to different consequences on the immune response by the host. *Candida albicans* is the most common opportunistic fungal pathogen that causes superficial and disseminated infections in humans (Brown *et al*., 2012). As described earlier, TLR2 plays a role to synergize with Dectin-1 to sense one of the most prevalent fungal pathogens, *Candida albicans* (Ferwerda *et al.*, 2008). TLR2 and Dectin-1 also both recognize zymosan from *Saccharomyces cerevisiae* and *C. albicans*, which is mainly composed of β-1,3-glucans (Ikeda *et al.*, 2008) but also contains chitin (Fuchs *et al.*, 2018). TLR2 also recognizes phospho-lipomannan in both hyphae and yeast forms of *C. albicans* and induces production of IL-6 and IL-8 in keratinocytes (Li *et al*., 2009). In the absence of TLR2, macrophages show an inability of fungal clearance, and the *Tlr2*-deficient mice are more susceptible to systemic fungal infection (Drage *et al*., 2009; Hise *et al*., 2009). On the other hand, one recent study showed that TLR2 and TLR4 can recognize the small secreted protein so called Sel1 from *C. albicans* yeast to trigger inflammatory responses (Wang *et al*., 2019b). This Sel1 recognition is critical for the host to facilitate fungal clearance (Wang *et al.*, 2019b). Taken together, TLR2 is characterized to recognize various ligands from the fungal cell walls and plays a critical role in fungal clearance.

1.4 Chitin

1.4.1 Chitin general features and properties

Chitin is the second most abundant polysaccharide after cellulose. It is a polymer composed of β-(1, 4)-linked N-acetylglucosamine (GlcNAc). The deacetylated derivative of chitin is called chitosan, a cationic polymer of glucosamine. In natural preparations, chito-materials with a degree of acetylation of < 50% are generally called chitosan, those > 50% chitin. Thus, the designation as "chitosan" or "chitin" does not mean that acetylation/de-acetylation are 0 vs 100%, unless this was verified experimentally. Chitin is also a major structural component in the cell walls of mushrooms and fungi, exoskeleton of arthropods, insects and nematodes (Araujo *et al*., 1993; Debono and Gordee, 1994; Fuhrman and Piessens, 1985; Tharanathan and Kittur, 2003). Those chitin-containing life forms synthesize and use chitin as a protection against harsh conditions in their environment and, for human pathogens, as protection against the host's immune response. Chitin polymers are synthesized intracellularly by chitin synthases. They can be translocated across the plasma membrane and then form rigid crystallites (Cohen, 2001). Chitin does not accumulate in the environment because of many chitinolytic enzymes, so called chitinases, which mediate the degradation and recycling of chitin polymers to small GlcNAc units in nature (Burton and Zaccone, 2007). However, chitinases are also critical for fungal cell wall biogenesis and plasticity (Sahai and Manocha, 1993), as they allow for precise shaping of its composition. Although its ubiquity and abundancy in the lower life forms, chitin does not exist and is not expressed by mammals and plants (Lee *et al*., 2008). Thus, in mammals and plants, chitin is an ideal MAMP as evidenced by the fact that immune responses through PRR activation and chitinase induction triggered by chitin exposure aid in defense against chitin-containing pathogens such as pathogenic fungi.

1.4.2 Chitin-induced immune responses

Chitin from allergens or pathogens has been known to be a potential ligand recognized by PRRs and subsequently trigger the innate immune response. The accumulation of chitin in the human respiratory tract will drive inflammation, remodeling to chronic lung diseases like asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (Mack *et al*., 2015). Recently, the issue of the size-dependent effect of chitin has attracted attention and is crucial to determine PRR recognition and possibly reconcile conflicting findings relating to the use of different chitin preparations. Da Silva *et al* reported that large chitin fragments are inactive, whereas the intermediate size of chitin (40 – 70 μm) and small chitin (2 – 10 μm) can stimulate TNF and IL-17 production, while the IL-10 production can only be observed by small chitin stimulation (Da Silva *et al*., 2009; Da Silva *et al*., 2008). They suggested that different size of chitin stimulates different activation of TLR2, Dectin-1 and mannose receptor (Da Silva *et al.*, 2008). In contrast, the *in vivo* study of different sizes of chitin showed that "large" chitin induces eosinophil infiltration and leads to M2 macrophages activation in the peritoneal cavity whereas the small size of chitin is prone to activate M1 macrophages (Kogiso *et al*., 2011). Of note, "small" in this context should be considered with caution as macrophages are 10 μm in diameter. These dimensions of chitin particles are actually difficult to evaluate the chitin receptor at the molecular level since aforementioned the binding size of the ectodomain is on average 8 nm (Gutmann *et al*., 2018). However, studies in plants used more defined chitin oligomers and identified CEBiP and CERK1 as receptors for chitin oligosaccharides $(GlcNAc)_8$ that mediate plants' innate immune signaling (Hayafune *et al*., 2014; Miya *et al*., 2007). It seems that this oligomeric size of chitin would be ideal for PRR to recognize. Moreover, it should be noted that chitin oligomers above the size of 8 units (octamer) are naturally insoluble and therefore difficult to synthesize, purify and be commercially available (Tharanathan and Kittur, 2003). In our previous study, we showed that chitin oligomers in the range of 10 – 15 units are the ideal size of oligomers to induce pro-inflammatory cytokine response through TLR2 recognition (Fuchs *et al.*, 2018). These observations all indicate that large chitin polymers are generally inactive, whereas small fragments or especially

oligosaccharides chitin are prone to be sensed by PRRs and more immunogenic to elicit an inflammatory response.

1.4.3 Immune response by fungal chitin

PRRs recognition to fungal pathogens by immune cells is the first essential event to establish an innate immune system to protect the host from mycosis. The fungal cell wall is not only essential for maintaining the fungal integrity and viability, but also the first part contacting with the host cells and tissues. Furthermore, most cell wall components are immunogenic and potent to trigger host cellular and humoral responses during infection (Erwig and Gow, 2016). The *Candida albicans* cell wall, for example, from outer layer to inner layer, is composed of mannan (N- and O-linked glycosylated protein), β-1,6- and β-1,3-glucan, and chitin (Hardison and Brown, 2012) **(Figure 2)**. It has been reported that mannan can be recognized by mannose receptor, DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) and Dectin-2 (Erwig and Gow, 2016). Galectin-3 is also involved in sensing α-1-2-mannose (Kohatsu *et al*., 2006). As mentioned earlier, the most abundant sugar polymer, β-1,3-glucan, is known to be recognized by TLR2 and Dectin-1 (Netea *et al.*, 2006). However, which receptor mediates chitin recognition on *C. albicans* has not been well defined.

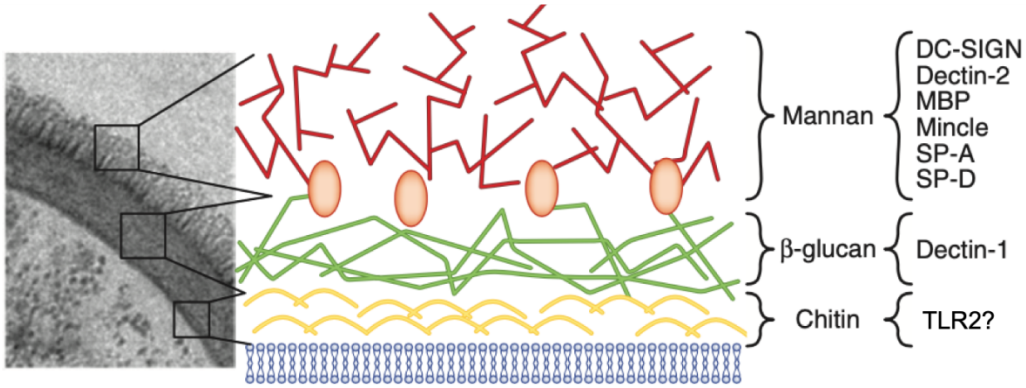


Figure 2. Structure of *Candida albicans* cell wall.
Figure was adapted from Hardison and Brown (Hardison and Brown, 2012). Electronic micrograph of the *C. albicans* cell wall. From outer to inner layer, this majorly composed with thick polysaccharides which highlighted: mannan (α-mannosylated proteins), β-glucan and chitin. Several C-type lectin receptors (CLRs) have been identified that specifically recognize these cell-wall structures.

What complexity of these studies is the diverse and dynamic nature of the fungal cell wall. One study comparing different strains of *C. albicans*, Marakalala showed that the differences of cell wall composition affect the role of Dectin-1 to control systemic candidiasis (Marakalala *et al*., 2013). They indicated that the chitin content in different strains of *C. albicans* determines the fate of the host defense against *C. albicans* infection (Marakalala *et al.*, 2013). Furthermore, this study showed that *in vivo C. albicans* adapts its cell wall composition (Marakalala *et al.*, 2013). Recently, some studies investigated the potential receptor and the immune response signaling cascade by fungal chitin. Mora-Montes *et al*. showed that extracted chitin from *C. albicans* blocks and inhibits cytokine production or produces anti-inflammatory cytokines (Mora-Montes *et al*., 2011). They suggested that fungal chitin does not directly interact with TLR2, TLR4 and Dectin-1 while it masks the immune recognition of Dectin-1 to β-1,3-glucans from *C. albicans* (Mora-Montes *et al.*, 2011). In addition, Wagener *et al*, based on KO mice proposed that NOD2 and TLR9 mediate fungal chitin recognition which leads to anti-inflammatory cytokine IL-10 production (Wagener *et al*., 2014). Above all, these studies indicate that fungal chitin from *C. albicans* might be inert to trigger inflammatory cytokine production. However, the direct binding was not shown and the involvement of NOD2 and TLR9 subsequently challenged (Fuchs *et al.*, 2018). In other studies, fungal chitin isolated from house dust-derived *Aspergillus* induces robust recruitment of inflammatory eosinophils and then leads to Th2 cell activation in mouse lung (Van Dyken *et al*., 2011). Furthermore, in a previous study from our group, we showed that the recombinant human TLR2 can directly bind to the chitin on *C. albicans* yeast (Fuchs *et al.*, 2018). Thus, the fact that fungal chitin can trigger an inflammatory immune response and by which receptor is still controversial, probably due to the high heterogeneity of the used material. Approaches that use well-defined chitin ligands are therefore highly necessary to decipher the immunogenic properties of chitin more accurately.

On the other hand, the group of Holmskov has identified fibrinogen C domain-containing protein 1 (FIBCD1) as an acetyl group-binding receptor that can bind chitin or chitin fragments through a conserved hydrophobic funnel (S1) binding site (Schlosser *et al*., 2009; Thomsen *et al*., 2010). FIBCD1 is majorly expressed in intestine and lung epithelial cells that plays role in regulating *Aspergillus*-mediated lung pathology (Jepsen *et al*., 2018) and controlling fungal dysbiosis and intestinal inflammation (Moeller et al., 2019). Since we have identified that TLR2 is a major receptor for chitin oligomers, whether epithelial cells also respond with oligomeric chitin through recognition of FIBCD1 or TLR2 is intriguing to investigate.

1.4.4 Immune response by house dust mite chitin

House dust mites (HDMs), *Dermatophagoides farina* and *Dermatophagoides pteronyssinus*, are two well-known inhaled allergens that commonly cause Th2-type airway inflammation diseases like allergic asthma (Gregory and Lloyd, 2011). Asthma pathogenesis includes eosinophil infiltration, epithelium cells activation, bronchial hyperactivity and goblet cell metaplasia are mostly initiated by contacting the HDM allergens (Lambrecht and Hammad, 2012; Palm *et al*., 2012). The initiation of the HDM allergic response is dependent on skin/mucosal innate immune receptors which is a central step for Th2-mediated sensitization by proinflammatory cytokines and chemokines production. Ryu *et al* have reported that HDM-derived β-glucans and LPS can induce nose and lung epithelial cells to produce CCL-20 and IL-33, respectively (Ryu *et al*., 2013). The TLR2 and TLR4 activation by HDM β-glucans and LPS elicits oxidase 2-mediated reactive oxygen species (ROS) to regulate innate immunity.

However, most studies have focused on HDM extracts that contain the actual T cell allergens, i.e. proteins. Since chitin is the major exoskeleton component of HDM, HDM preparations (but not extracts) contain chitin, which may impact the allergic responses. Whether HDM-derived chitin can be sensed by the innate immune system is still largely unknown.

1.5 Chitinases

1.5.1 Chitinases and their glycosyl hydrolase families

Chitinases (E.C. 3.2.1.14) are glycosylated hydrolytic enzymes which majorly cause the degradation of chitin into N-acetylglucosamine monomers or dimers, and are also called endo-β-1,4-N acetylglucosamidases. These enzymes are widely expressed and can be found in plants, fungi, bacteria, actinomycetes, insects and human beings (Rathore and Gupta, 2015). The first evidence of chitinases was found in 1921, when Folpmers observed the decomposition of chitin and its derivates in dishes by adding two strains of bacteria together with chitin (Folpmers *et al*, 1921). In 1929, Karrer and Hofmann extracted the first chitinase from the snail *Helix pomatia* (Karrer *et al*, 1929). According to their homology of amino acids, chitinases are classified into several gene groups including 18-, 19- and 20-glycosyl hydrolase families (Henrissat, 1991; Patil *et a*l., 2000; Rathore and Gupta, 2015). Families of 18- and 19-glycosyl hydrolase are the most common families which can be found in bacteria, fungi, plants, insects and animals (Henrissat, 1991). Family 20-glycosyl hydrolase enzymes are found in certain bacteria and fungi (Duan *et al*., 2018). In lower life forms like bacteria and fungi, glycosyl hydrolases play a role in regulating the deposition of chitin and maintaining the balance of chitin synthesis and degradation. In the eukaryotic cells like in plants and animals, the production of glycosyl hydrolase apparently aids host defense against infection of chitin-containing organisms (Burton and Zaccone, 2007). The role of chitinases in plants' defense against pathogen infection is also widely investigated *(Collinge et al., 1993; Grover, 2012; van Loon et al*., 2006). Plants chitinases possess the ability to majorly hydrolyze the hyphal tips of fungal in order to limit the growth and prevent evasion of the fungal pathogen (Mauch *et al*., 1988). Although the catalytic function of the glycosyl hydrolase family to degrade chitin is extensively studied in prokaryote and plant kingdom, in mammalian the role of chitinases is still largely unknown.

1.5.2 Mammalian chitinases and chitinase like proteins

In mammals, regardless of the absence of endogenous chitin, they keep the evolutionarily conserved proteins and retain the function as active chitinase and chitin binding property to natural chitin substrates. In humans, chitinases and chitinase-like proteins (CLPs) are both classified to belong to the conserved family of 18 glycosyl hydrolases (Bussink *et al*., 2007). Based on their chitinase catalytic activity, they can be further divided into: (1) Active chitinases, such as chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase). (2) Inactive chitinases, or chitinase-like proteins such as YKL-40/CHI3L1, YKL-39/CHI3L2 and Stabilin-1-interacting chitinase-like protein (SI-CLP). While both inactive and active chitinases and chitinase like proteins can bind to chitin, only the active chitinases, like CHIT1 and AMCase exhibit catalytic activity to cleave chitin (Lee *et al*., 2011). The following **Table 3** lists and categorizes the name and function of different human chitinases.

Table 3. Members of the 18-glycosyl hydrolase family and their immune function

Name	Chitinolytic activity	Major immune function	References
Acidic mammalian chitinase (AMCase)	Yes	Ameliorate chitin-, *Aspergillus*- or house dust mite-induced type 2 inflammatory response	(Kim *et al*., 2015)
		Mediate IL-13-induced pulmonary inflammation and cystic fibrosis	(Zhu *et al*., 2004)
Chitotriosidase (Chitinase 1, CHIT1)	Yes	Defense against infection by organisms	
		Plasmodium falciparum	(Barone *et al*., 2003)
		Candida albicans	(van Eijk *et al*., 2005)
		Cryptococcus neoformans	(Wiesner *et al*., 2015)
		Elevated CHIT1 serves as biomarker for several human diseases	
		Lysosomal storage diseases, such as Gaucher disease	(Šumarac *et al*., 2011)
		Respiratory diseases, such as fungal-associated cystic fibrosis, COPD	(Hector *et al*., 2016)
		Diabetes	(Żurawska-Płaksej *et al*., 2015)
		Neurological diseases, such as Alzheimer, multiple sclerosis	(Steinacker *et al*., 2018)
YKL-40 (CHI3L1)	No	Trigger aeroallergen-induced adaptive Th2 inflammation	(Zhao *et al*., 2020)
		Play roles in infection diseases, such as pneumonia and purulent meningitis	
YKL-39 (CHI3L2)	No	Not well defined	(Lee *et al*., 2011)
Stabilin-1-interacting chitinase-like protein (SI-CLP)	No	Serves as biomarker response to corticosteroid treatment	(Kzhyshkowska *et al*., 2006)

1.5.3 Chitotriosidase and its isoforms

CHIT1 was the first chitinase to be isolated and characterized in mammals (Renkema *et al*., 1995). Immune cells such as neutrophils and macrophages or epithelial cells were shown to secret CHIT1 (Lee *et al*., 2012; Renkema *et al.*, 1995; van Eijk *et al.*, 2005). CHIT1 has two major forms: a 50 kDa form, which is mainly found in the bloodstream; and a 39 kDa form, which is dominant in the tissues. These two both express chitinolytic activities (Elmonem *et al*., 2016). CHIT1 50 kDa consists of two domains: The N-terminal, catalytic glycosylase-18 domain and a C-terminal carbohydrate binding module (CBM), which is important for extended chitin binding (Fadel *et al*., 2016). These two domains are linked with a proline-rich hinge region, which is flexible for randomly placing CBM to the chitin substrate (Fadel *et al.*, 2016). The 39 kDa form of CHIT1 contains the glycosylase-18 domain but lacks the CBM domain, after its processing from CHIT1 50 kDa in lysosomes by proteolytical cleavage of the C-terminus (Renkema *et al*., 1997). In a recent study, Kuusk *et al* demonstrated that both forms of CHIT1 have endochitinase processing ability (Kuusk *et al*., 2017), i.e. they do not only remove small GlcNAc units from the ends of chitin strands but are able to cleave within fibrils. On the other hand, in contrast to hydrolysis of endochitinase activity, CHIT1 also possesses transglycosylation activity which is the glycosylation processivity to add on the glucose residues *(Eneyskaya et al*., 1997) **(Figure 3)**. This transglycosylation activity naturally occurs when the chitin substrates surpass the loading of endochitinase activity of CHIT1 (Aguilera *et al*., 2003). Other studies showed that CHIT1 50 kDa exhibits stronger transglycosylation activity compared with CHIT1 39 kDa due to its CBM domain (Stockinger *et al*., 2015). Since these distinct features of CHIT1, it is intriguing to investigate how the enzymatic activity of CHIT1 plays role in the mammalian innate immune response.

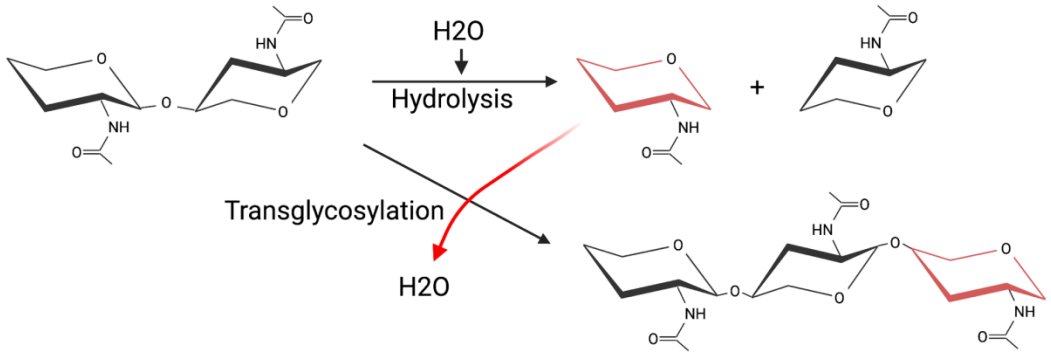


Figure 3. Hydrolysis and transglycosylation model.
The picture was created with BioRender.com. Hydrolysis and transglycosylation are catalyzed by chitotriosidase.

1.5.4 Modulation of immune responses to processed chitin by chitinases

Most of the studies on chitinases are focusing on the chitin degradation to ameliorate the chitin-induced allergy response or chronic inflammation. Chitin induces the accumulation of innate immune cells including IL-4 expression eosinophils and basophils in *in vivo* lung challenging (Reese *et al.*, 2007). Mice which is overexpressed AMCase ameliorate the inflammatory response caused by recruited innate immune cells in the lung (Reese *et al.*, 2007). In a recent study, van Dyken *et al.* indicated that inhaled chitin induces lung innate lymphoid type 2 cells activation and subsequently triggers the accumulation of eosinophils and alternatively activated macrophages (Van Dyken *et al.*, 2014). These two studies suggested that chitin accumulation initiates innate cytokines response and contributes to the development of Th2 cells activation. Moreover, van Dyken *et al.* further demonstrated that AMCase secreted by lung epithelial cells plays an important role in chitin clearance to ameliorate type 2 inflammations (Van Dyken *et al.*, 2017). Thus, they suggested that the persistent immune activation and age-related fibrosis caused by the absence of AMCase depends on its critical endochitinase activity to eliminate the chitin accumulation (Van Dyken *et al.*, 2017).

Besides, the elicitation of chitinase activity by HDM-derived chitin might also influence the allergy response. In line with this, Kim *et al* showed that AMCase serves as an important type 2 immune regulator by inhaled HDM. By using enzymatic mutant AMCase knock-in mice, they indicated that AMCase cleaves chitin and regulates the development of Th2 response by HDM chitin (Kim *et al.*, 2015). In addition, Choi *et al* further supported this observation by showing that HDM chitin enhances OVA-specific Th2 cell response via a TNF-dependent pathway. This Th2 enhancement is abolished by the treatment of chitinases (Choi *et al*., 2016). These studies all indicate the important role of elicitation of chitinase activity to ameliorate the HDM-induced allergy response by chitin degradation. Recently, Hong *et al* further confirmed these observations. By using chitotriosidase null mutation mice, they showed that Th2 cytokine and IgE responses are enhanced in chitotriosidase null mutation mice to HDM sensitization and challenge (Hong *et al*., 2018). They demonstrated that CHIT1 plays a protective role in the pathogenesis of allergic asthma (Hong *et al.*, 2018). However, since the above studies all indicate the important role of chitinases by its catalytic activity to cleave chitin from HDM allowing chitin fractions to be exposed or released and subsequently to regulate allergy or type 2 responses, there is still a missing link for the cellular response upon these cleaved chitin fractions or even small chitin oligomers.

Chitinase activity has been known to break down chitin and function as a role of host defense against fungal infections. Van Eijk *et al* firstly investigated the characteristic of antifungal response from chitotriosidase produced by GM-CSF-differentiated macrophages (van Eijk *et al.*, 2005). They showed that chitotriosidase inhibits the growth of the hyphal form of *C. albicans* and can further protect the mice from systemic candidiasis (van Eijk *et al.*, 2005). Gordon-Thomson *et al* further confirmed that transfected chitotriosidase to hamster ovary cells restricts the growth of *Aspergillus niger*, *C. albicans* and *Crytococcus neoformans* (Gordon-Thomson *et al*., 2009). These two studies both implicated an important role of chitotriosidase in fighting against infection by chitin-containing pathogens. In addition, Wiesner *et al*. showed that chitin binding via chitotriosidase initiates the Th2 cell differentiation by $CD11b^{+}$ lung-

resident dendritic cells in response to *Cryptococcal* pulmonary infection (Wiesner *et al.*, 2015). They also showed that fungal infections elevated chitotriosidase expression and this influences Th2 development. A study by Hector *et al*. further reported that patients with CHIT1 deficiency possess a higher risk of colonization with *C. albicans* in cystic fibrosis patient (Hector *et al.*, 2016). That study implicates the association of chitinase induction by chitin-containing pathogens and the potential role of T cells response. However, the detailed mechanism of chitinase induction and the correlated immune response by chitinases' processed oligomeric chitin, especially from fungal chitin, remain incompletely understood.

1.6 Hypothesis and aims of study

Since chitin is highly abundant in nature and specifically in human pathogens, it is not possible for mammals and plants to avoid contact with chitin. Once exposure to chitin takes place, degradation occurs by chitinases. Studies on chitinases mostly focused on the defense mechanism of chitin degradation or allergic responses associated with chitinases that bind and process chitin-containing pathogens. None of the studies investigate whether breakdown products, e.g. chitin oligomers, could be potential ligands recognized by a receptor and subsequently trigger an innate immune response. However, this has not been formally proven.

From our previous study, by using electron microscopy, we showed that the chitin particles used in earlier studies are generally larger than the normal size of macrophages (Fuchs *et al.*, 2018). This huge particle is not an ideal MAMP for a PRR to recognize. However, by using different sizes of small units of chitin oligomers, we revealed that the oligomers larger than 6 units could gradually induce inflammatory cytokine response in macrophages. We further identified that TLR2 is the major receptor to recognize these chitin oligomers. Moreover, data from protein structure *in silico* docking analysis showed that the protruding end of chitin oligomers out of TLR2 ectodomain (ECD) might be possible to contact another co-receptor. Preliminary results of the receptor blocking on TLR2-HEKs also suggested that TLR1 could be a potential

co-receptor with TLR2. However, evidence of heterodimer formation as a result of chitin binding has not been gained.

Collectively, I hypothesized that chitin oligomers can be generated by chitinase degradation from chitin-rich organisms or chitin particles. These chitin oligomers might possess immunogenicity to trigger an innate immune response and the co-receptor with TLR2 would be also involved. Here, I stated three major aims of studies and the approach methods:

1) Whether chitin oligomers from chitin particles or chitin-containing organisms released by chitinase degradation induce innate immune responses?

To address this, I used human chitotriosidases and their isoforms to digest chitin particles and chitin-rich organisms to evaluate the innate immune response.

2) Does the catalytic activity of chitinase render chitin oligomers diffusible and immunogenic from macroscopic chitin?

Here I applied site-directed mutagenesis on the chitinase catalytic site to investigate the role of chitinolytic activity in the innate immune response. In addition, a transwell setting was used here to evaluate the possibility of releasing diffusible chitin oligomers from chitinase degradation.

3) Which co-receptors are involved in oligomeric chitin sensing?

In order to assess this, I applied bimolecular complementary fluorescence (BiFC) assays and cells from TLRs receptor deficient mice to investigate which co-receptor(s) participate in oligomeric chitin sensing.

Chapter 2: Materials and methods

2.1 General maintenance and preparation

2.1.1 Cell maintenance

The general medium we used here were Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 from Sigma Aldrich. Some supplements like heat-inactivated fetal calf serum (FCS) were from Biowest; penicillin, streptomycin, Zeocin™, hygromycin and L-glutamine were from Gibco. For washing and detaching the cells, Dulbecco's Phosphate Buffered Saline (dPBS), Trypsin and ethylenediaminetetraacetic acid (EDTA) from Gibco were used. All cell lines or primary cells were maintained at 37℃ and 5% CO_2 in a humidified incubator.

Table 4. Cell culture medium

Cell type	Medium	Supplements
HEK 293T	DMEM	10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine
HEK-Dual™ hTLR2 cells	DMEM	10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (For selection, add 100 μg/ml hygromycin and 50 μg/ml Zeocin™)
Bone marrow-derived macrophages	RPMI-1640	10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine

2.1.2 Mice and bone marrow-derived macrophages

C57BL/6 wild type and *Tlr2* KO mice were maintained and sacrificed by using CO_2 in the local animal facility (IFIZ, Department of Immunology, University of Tübingen)

which followed the institutional guidelines and protocols (§4Mitteilung 18.12.2015 (Chitin)). Bone marrow cells were isolated from femurs and tibias which were cut and flushed out by using 24-gauge syringe resuspended in RPMI-1640 medium. 1,2 x 10^7 of cells were seeded in 10-cm petri dish in RPMI-1640 complete medium containing with 10% mGM-CSF cultured supernatant. Additional 5 ml of fresh culture medium was added on day 3. On day 7 after differentiation, adherent cells were harvested and plated in 96 well plates (1 x 10^5 cells/well) or 12 well plates (2 x 10^6 cells/well) in RPMI-1640 complete medium without mGM-CSF. After one day resting, cells were stimulated for 18 h with C10-15, Pam2, Pam3, FSL-1, LPS, *C. albicans* and house dust mite.

Bone marrow cells from WT, *Tlr2*, *Tlr1* and *Tlr6* KO mice, were a gift from T. Roger (Lausanne University Hospital, Lausanne, Switzerland). All mice were on a C57BL/6 background.

2.1.3 Reagents and the quality control of chitin

All chemicals used in the lab were from Sigma-Aldrich unless otherwise stated. The preparation of C10-15 chitin oligomers was described before (Fuchs *et al.*, 2018) and more details are provided below. C10-15 chitin oligomers were generated from C10-15 chitosan (2000-3000 MW, equivalent to 10-15 subunits, Carbosynth), which was derived from crab shells, chemically hydrolyzed and HPLC-fractionated to >95% purity as confirmed by HLPC and mass spectrometry analysis. By using sodium bicarbonate and acetic anhydride acetylation (Bueter *et al.*, 2011), chitosan was allowed to be acetylated to chitin. The resulting degree of acetylation was assessed either directly (C4 through C7 in water) or upon trifluoracetic acid hydrolysis (for C10-15, 2 h at 100 °C) by ESI and MALDI mass spectrometry. Batches with up to 90% of acetylation were used here. Prior to use for sterile stimulation, acetylated chitin oligomers were suspended in endotoxin free water and tested for endotoxin level by using the limulus amebocyte lysate (LAL) assay (Lonza, CH). Levels below 0,25 EU/ml (< 25 pg/ml LPS) in final dilutions were considered acceptable. For levels >

0,25 EU/ml, the chitin preparation was incubated for 3 h with 10 µg/ml Polymyxin B (Thermo Fisher), washed by centrifugation and re-assessed.

2.1.4 Production of chitin oligomers of defined degree of polymer

The chitin oligomers of defined degree of polymer (DP) were prepared by preparative size exclusion chromatography (SEC) followed by chemical full acetylation. Chitosan with a molecular weight of 2000 - 3000 g/mol (Carbosynth) was dissolved in SEC buffer (0.15 M ammonium acetate, pH 4.5) at a concentration of 10 mg/mL and filtered through a 0.45 µm pore size filter (Millipore). 5 mL were separated via preparative SEC (SECcurity GPC System, 1200 Series, Agilent Technologies) using three successive HiLoad™ 26/600 Superdex™ 30 prep grade columns (GE Healthcare Europe GmbH, Freiburg, Germany) with overall dimension of 2.60 x 180 cm. An isocratic flow of 0.8 mL/min of the SEC buffer was applied and fractions of 8 mL were collected between minute 500 - 1000. The elution of the oligomers was monitored with a refractive index detector (1260 Infinity Refractive Index Detector, Agilent Technologies Deutschland GmbH, Böblingen, Germany).

Fractions of chosen DPs from three subsequent SEC runs **(Figure 5A)** were pooled **(see Table 11)**, diluted 1:1 with water, frozen and dried via lyophilization. To confirm, that the fractions contained nearly exclusively the desired DPs, mass spectrometry with electrospray ionization coupled to liquid chromatography was used (UHPLC-ESI-MS) as described previously. The hydrophilic interaction liquid chromatography (HILIC) was performed with a Dionex Ultimate 3000RS UHPLC system (Thermo Scientific, Milford, USA) using an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 50 mm; Waters Corporation, Milford, MA, USA) combined with a VanGuard precolumn (1.7 µm, 2.1 mm × 5 mm; Waters Corporation, Milford, MA, USA). The HILIC run was performed at a flow rate of 0.4 mL/min and at 45 °C oven temperature over 8.5 min with the following gradient elution profile: 0 - 0.5 min, isocratic 100% A (80:20 ACN/ H_2O with 10 mM NH_4HCO_2 and 0.1% (v/v) HCOOH); 0.5 - 6 min, linear from 0% to

100% (v/v) B (20:80 ACN/H_2O with 10 mM NH_4HCO_2 and 0.1% (v/v) HCOOH); 6 - 7 min, isocratic 100% (v/v) B; column re-equilibration: 7 - 7.5 min, linear from 0% (v/v) to 100% A; 7.5−8.5 min, isocratic 100% A. The UHPLC system was coupled to an amaZon speed ESI-MS^n detector (Bruker Daltonik, Bremen, Germany) in positive mode.

Fully deacetylated to partially acetylated chitosan oligomers of defined DPs were obtained on a milligram scale and chemically fully acetylated as described previously. Briefly, the oligomers were dissolved at a concentration of 1 mg/mL in 1:1 50 mM $NaHCO_3$/MeOH and re-acetylated in two steps with 30 min incubation with 1 µL acetic anhydride per 30 µg of oligomers each. After lyophilization, water was added to prepare stock solutions of 2 mM of the fully acetylated oligomers to be used in cell assays.

2.1.5 Plasmids preparation

The used plasmids in this study were listed in **Table 6**. The Flag-tagged TLR2 plasmid was gift from I. Bekeredjian-Ding (Medical Microbiology, Heidelberg University, Germany). The split-mLumin backbone plasmids for bimolecular complementary assay were gifts from Stefan Pusch (Heidelberg University, Germany). hTLR1 and hTLR2 plasmids opened for Gateway system were ready to use. About TLR6 plasmids, the full length hTLR6 flanked with attB site was synthesized by GENEWIZ then the BP reaction was performed in order to obtain hTLR6 associated with attL site. To clone hTLR1, hTLR2 and hTLR6 in the split-mLumin N-terminal and C-terminal, respectively destination vectors, the LR reactions were performed by using the Gateway LR Clonase II Enzyme mix kit (Thermo Fisher). After the LR reaction, the destination vectors were transformed in the DH5α *E.coli* strain. The successful growth colonies were picked up and the corrected insertion of the hTLR was proved by restriction enzyme BsgI (New England Biolabs) digestion (200 – 300 ng plasmid and 5 units enzyme in 1X Tango Buffer, 1 h at 37°C water bath). The colonies showed the correct size of DNA in 1% agarose gel were sent to Sanger sequencing (GATC Biotech). After the sequencing confirmation, the protein expression test was performed by Western

blotting. Plasmids were propagated and prepared according to standard procedures (Promega PureYield Midiprep). The chitinase catalytic mutant sites, D138A E140L, were introduced to both CHIT1 50 kDa and 39 kDa plasmids by using the QuickChange II XL site-directed mutagenesis kit (Agilent). The mutagenesis primers were designed following by the manual instructions stated in the kit and monitoring in Geneious R6 software 6.1.8 version. The desired mutation was confirmed by Sanger sequencing (GATC Biotech). The secreted protein expressions were confirmed by Western blotting (section 2.2.5) and the malfunction of catalytic activity was checked by the measurement of the chitinase activity (section 2.2.6).

2.1.6 *Candida albicans* maintenance and growth conditions

C. albicans strain SC5314 was used in this study. Cells were firstly obtained from Dr. Anurag Singh (Universitätsklinikum Tübingen) and picked a colony immediately to save a frozen stock at -80°C in RPMI medium only (W/O FCS) containing 10% glycerol. In normal preparation, cells were taken up from frozen stock and were grown at 30℃ in yeast extract-peptone-dextrose (YPD) agar medium (1% [w/v] BactoYeast extract, 2% [w/v] BactoPeptone, 2% [w/v] Dextrose, 2% [w/v] agar) on 10-cm dish. After overnight incubation, cells were stored in the 4℃ fridge for maximum one month. Before any experiment or treatment, cells were freshly prepared by sub-culturing from 10-cm dish to glass slant tube. Cells were harvested by picking up a smear and re-suspending in RPMI-1640 medium.

To expose chitin content on *C. albicans*, yeast cells were incubated in RPMI-1640 medium supplemented with 0.032 μg/ml caspofungin (Sigma-Aldrich) for 6 h at 30°C. After incubation, yeast cells were washed twice with dPBS and tested once for viability. For hyphae induction, live yeast cells were counted for $1x10^6$ and re-suspended in YPD medium with 20% FCS. Cells were incubated at 37°C for at least 3.5 h until 90-95% filamentation was observed. The hyphae were collected by cell scraper and then washed twice with dPBS. For heat-killed treatment, both *C. albicans* yeast and hyphae were

prepared by incubation at 65℃ in water bath for one hour, with killing confirmed by plating on YPD agar slant tube.

2.1.7 House dust mite preparation

House dust mite, *Dermatophagoides pteronyssinus*, the whole culture and mite body were bought from CITEQ Biologics. The powder of whole culture and mite body were weight and re-suspended in dPBS then applied polymyxin B (Thermo Fisher) at 100 units/ml for working concentration to remove the endotoxin. After incubation at least 1 h at RT, whole culture and mite body were re-suspended in dPBS to 10 mg/ ml for the final stock concentration and stored at -20 °C. 100 μg/ml of whole culture and mite body were used as working concentration.

2.1.8 Chitin flakes preparation

Chitin flakes from shrimp shell was bought from Sigma-Aldrich. The flakes were firstly sieved by 2 mm or 1 mm pore size of steel sieve (Amazon) to sort small pieces of flakes which would be ideal size for 24-well cell culture plate. The obtained flakes were picked up to the sterile 1.5 ml Eppendorf and next applied polymyxin B washing for 1 h at RT. The flakes were washed 3 times with dPBS and the final re-suspended into 1 ml dPBS and stored at 4 °C.

2.2 Biochemical and biological method

2.2.1 Chitin and chitinase staining on *C. albicans*

2 x 10^6 *C. albicans* yeast cells and hyphae solution were transferred to 96-well v-bottom plate. Plates were centrifuged at 5000 g 10 min and then the supernatants were removed. Cells were resuspended in 100 μl dPBS containing 5 μg/ml of wheat germ agglutinin Alexa Fluor® 647 (WGA, 1:200, Thermo Fisher) and 50 μg/ml Concanavalin A Alexa Fluor® 488 (ConA, 1:200, Thermo Fisher), 1 h at 4°C in the dark. For chitinase staining after recombinant CHIT1 incubation, cells were stained with anti-His Alexa Fluor® 594 in 100 μl dPBS overnight at 4°C. After staining, cells were centrifuged and washed

twice with cold dPBS. Cells were resuspended in 5 µl ProLong™ Diamond Antifade Mountant mounting solution (Thermo Fisher). The mounting solution were then transferred to glass slides and covered with coverslip. Store the slides at RT in the dark until the mounting solution hardens. The samples were ready to image by Zeiss LSM 800 Inverted Confocal Microscope, 630 X magnification, Airyscan mode, 1.5 X zoom.

2.2.2 Chitotriosidase (CHIT1) digestion

Chitin flakes, *Candida albicans*, house dust mite whole culture and mite body were applied to recombinant CHIT1 digestion. For recombinant CHIT1 digestion, both 50 kDa and 39 kDa (stock solution 1 µM) were diluted to 4 nM (1:250) for working concentration. For transfected-CHIT1 digestion, 250 ng of CHIT1 plasmids were transfected to HEK 293T cells supplemented with 250 µl DMEM complete medium. After 48 h transfection, the collected cultured medium was used for chitinase digestion. To digest chitin flakes, *Candida albicans*, house dust mite whole culture and mite body, those were incubated with recombinant or transfected-CHIT1 into 1.5 ml Eppendorf. The incubation took place at rotation wheel for 18 h at RT.

For some experiments, transwell setting was applied after chitinase digestion. TLR2-transfected HEK cells or BMDMs were seeded at 24-well plate supplemented with 250 µl medium. The transwell insert for 24-well plate (8 µm pore size, Nunc) was filled with 250 µl of the chitinase digested-chitin flakes, -*C. albicans* or -house dust mite cultured medium. In the meantime, the transwell was placed into the 24-well cell seeding plate. After 18 h incubation, almost 2/3 of culture medium from the layer of transwell was passing through to the cell culture plate. The transwell was removed and checked by microscope. Almost all the large particle like chitin flakes, hyphal form of *C. albicans* and house dust mite were trapped into the transwell.

2.2.3 HEK-Dual™ hTLR2 (NF/IL8) reporter cells assay

HEK-Dual™ hTLR2 cells were bought from Invivogen and maintained several stocks frozen in liquid nitrogen. Cells were generated from the human embryonic kidney 293 (HEK 293)-derived cell line stably transfected with hTLR2 gene, NF-κB/AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct and Lucia luciferase, a secreted luciferase inserted under the control of the endogenous IL-8 promoter. Cells were kept under the antibiotic selection of Hygromycin B and Zeocin. Before the test of stimulation, 5 x 10^4 cells were seeded in 96-well plate without antibiotic selection. After overnight of resting, the culture medium was exchanged with fresh DMEM complete medium with or without the ligands to test. Cells were stimulated for 18 h and the culture supernatant can be assessed by monitoring NF-κB/AP-1-induced SEAP production and IL-8-dependent expression of Lucia luciferase.

These two secreted reporter proteins can be measured by using QUANTI-Blue™ Solution and QUANTI-Luc ™, separately. For QUANTI-Blue™ measurement, 20 µl of cell culture supernatant was added to 180 µl QUANTI-Blue™ solution at 96-well flat plate. The plate was incubated at 37 °C incubator for half hour. The SEAP levels were determined by SpectraMax® plate reader at 650 nm. For QUANTI-Luc ™ measurement, 10 µl of cell culture supernatant was pipetted in 96-well white plate. The measurement of Lucia luciferase activity was performed by FluoStar plate reader (BMG Labtech) which automatically added 50 µl of QUANTI-Luc ™ solution.

2.2.4 Dual NF-κB luciferase assay in HEK 293 T cells

5 x 10^4 HEK 293T cells were seeded in 24-well plate with 500 µl DMEM complete medium and incubated overnight. On the next day, cells were transfected with the following amounts of plasmid DNA per well: 100 ng NF-κB firefly reporter luciferase, 10 ng of *Renilla* luciferase under a constitutive promoter. For measuring TLR2 response, cells were further transfected with 100 ng of human TLR2 or one 100 ng backbone as an empty vector control. Transfection was performed by using 1 µl of X-

treme GENE^{TM} HP DNA Transfection Reagent (Sigma-Aldrich) mixed into total 50 µl Opti-MEM together with above indicated plasmids. After 15 min incubation at RT, dropped slowly 50 µl Opti-MEM-X-treme-plasmids mixed to the cells. After 48 h incubation, the medium was replaced by fresh DMEM complete medium with or without the TLR ligands or stimuli. Cells were stimulated for 18 h and cell lysates were harvested immediately. The concentrations of all stimuli and ligands are listed in **Table 8**. To analyze luciferase activity, the culture supernatants were firstly removed and the cells were washed with 350 µl dPBS. Subsequently, cells were lysed in 60 µl passive lysis buffer (Promega). After plating the cells to shaker for 5 min, cells were frozen in -80 °C for at least 15 minutes. Lysates were harvested by centrifugation and 10 µl of lysate was used to measure the luciferase activity on FluoStar plate reader (BMG Labtech). In the plate reader, the corresponding substrates for firefly and *Renilla* were automatically added. Luciferase Assay reagent II (Promega) is for first firefly luciferase and the Stop & Glo reagent (Promega) is to quench the firefly and initiate *Renilla* luciferase. The analysis settings were used as recommended in the luciferase reporter system by Promega. The results were calculated by the sum of firefly luminescence divided with the sum of *Renilla* luminescence using OPTIMA-Data analysis software. Graphs were generated and statistics analysis applied in GraphPad Prism 8 version 8.3.1.

2.2.5 Immunoblotting

HEK cells transfected with chitotriosidase plasmids were checked for their protein expression. In order to detect clearly of secreted chitotriosidases, cells after 24 h transfection, the culture medium was replaced with Opti-MEM without any FCS. After 24 h further incubation, both whole cells lysates (WCL) and culture supernatants were harvested. For obtaining WCL, cells were washed once with dPBS and then lysed with 60 µl RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% SDS, 1% Triton X-100 and 0.5% deoxycholate) supplemented with EDTA-free protease inhibitor (Roche) and 0.1 µM PMSF. After maximum speed of centrifugation, WCL were mixed with reducing reagent and sample loading buffer (Novex, Thermo Fisher) and then boiled for 5 min at 95 °C to denature the protein. For

obtaining the supernatants, 60 µl of supernatants was applied to the same methods as WCL. Samples (10 µl of WCL and 20 µl of supernatant) were subjected to electrophoresis at 10% Tris-glycine gels with SDS buffer (25 mM Tris-base, 250 mM glycine and 0.1% SDS) and transferred to a 0.45 µm nitrocellulose membrane (GE Healthcare). The membrane was blocked with 5% bovine serum albumin (BSA) (w/v) in Tris-buffered saline solution with 0.1% (v/v) Tween-20 (TBS-T) for 1 h at RT and then left in 5 ml buffer containing primary antibodies in 5% BSA in TBS-T overnight at 4 °C with rotation. Next day, the membranes were washed 3 times with TBS-T for at least 5 min and then incubated with the 10 ml TBS-T buffer containing HRP-conjugated secondary antibodies in 5% non-fat milk for 1 h at RT with rotation. After incubation with secondary antibodies, membranes were washed 3 times with TBS-T at least 5 min each wash. ECL reagent (Peqlab) was used to detect chemiluminescence and the development was using Licor camera. Pictures were analyzed and edited in Image Studio Lite software. The information of antibodies and reagents can be found in **Table 5. List of antibodies**

2.2.6 Measurement of chitinase activity

Chitinase activity in the culture supernatant form CHIT1 plasmids transfected-HEK cell was measured by using a fluorescence assay kit (Sigma-Aldrich). The 10 µl supernatant sample was mixed with 90 µl substrate solution containing 0.5 mg/ml of 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside in the assay buffer at 96-well plate (black/clear bottom plate, Thermo Fisher). The reaction was taking place at 37 °C for 30 min and then was stopped by adding 100 µl stop solution. The fluorescence was measured at excitation of 360 nm and emission of 450 nm by FluoStar plate reader (BMG Labtech).

2.3 Molecular biology method

2.3.1 ELISA (enzyme-linked immunosorbent assay)

Cytokine production in culture supernatants from BMDMs were collected and stored in -80 °C. ELISA kits (Biolegend) were used to quantify IL-6 and TNF production. Firstly, coating antibody was added to high binding 96 well plates (half area, Greiner Bio-One) and incubated overnight at 4 °C. Next day, after three times washing with PBS, plates were blocked with assay diluent for 1 h at RT. Next, after applied the third washed, the supernatants were diluted (the ratio listed below) and added to the plate for 2 h incubation at RT. After applied fourth washed step, the biotinylated detection antibody was added for 1 h incubation at RT. Fifth washed again, Avidin-HRP was added for 30 min at RT. After applied the final washed, the TMB substrate solutions were added and incubated for 5 – 20 min then the stop solution was applied according to visible color change of the lowest standard (15.625 μg/ml). The 450 nm absorbance was measured by SpectraMax® plate reader.

For murine TNF and IL-6 ELISA, the dilution ratio of supernatants as followed: for C10-15 stimulation 1:3; for Pam2, Pam3, FSL-1 and LPS stimulations 1:50; for *C. albicans* and HDM stimulation 1:5; and unstimulated conditions were diluted to 1:3.

2.3.2 Bimolecular complementary (BiFC) assay

2 x 10^4 HEK 293T cells were seeded at 8 wells 1.5H glass chamber slide (Ibidi). After one day resting, cells were co-transfected with split-mLumin plasmids as the following two listed combinations: (1). hTLR2-LC151 (200 ng) + hTLR2-LN151 (200 ng); (2). hTLR2-LN151 (100 ng) + hTLR6-LC151 (300 ng). After 48 h transfection, cells were stimulated with or without C10-15, Pam3 or Pam2 by replacing the culture medium. Next day after 18 h stimulation, HEK cells were gently washed once with 200 μl dPBS and then fixed with 4% paraformaldehyde in 150 μl dPBS for 10 min at RT. Cells were applied second washed and then stained with Hoechst 33342 (Invitrogen), 1:10,000 dilution in dPBS for 8 min at RT. After applying the last wash, cells were mounted with

Mounting Medium (Ibidi) and ready to image by using the Zeiss LSM 800 Inverted Confocal Microscope, 400 X magnification.

2.3.3 qPCR analysis

2 x 10^6 BMDMs after 7 days of differentiation were seed at 12 well plates resting for the experiments. After the stimulation, cells were washed with dPBS and lysed in 350 µl RLT buffer (Qiagen) containing 1% (v/v) β-mercaptoethanol. Total RNA isolation was performed by using reagents from the RNeasy Mini kit (Qiagen) following DNA digestion (RNase-free DNase set, Qiagen) in a Qiacube instrument. The concentration of total RNA was measured by NANO drop. mRNA reverse transfection to cDNA was performed by using High Capacity RNA-to-cDNA Kit (Thermo Fisher) following with the cycle: 1 h/37 °C; 5 min/95 °C; cool for storage. Quantitative PCR was performed in 10 µl mixed containing 1 to 10 diluted cDNA, TaqMan Universal MasterMix II and 0.3 µM TaqMan primer and RNA-free water. Each sample was done in triplicates in a real-time cycler (Thermo QuantStudio 7 Flex, Thermo Fisher). The following cycle was: 10 min/ 95 °C; 15 s/ 95 °C and 1 min/60 °C for 40 cycles; cool and save. The used of primers are listed in **Table 9**.

2.4 Statistical analysis

Experimental data were analyzed in GraphPad Prism 8.2.0 (GraphPad Software, Inc.) using 2-tailed Student *t* tests or one-way or two-way ANOVA tests. Microscopy data were acquired in Zeiss ZEN blue 3,0 software and analyzed with ImageJ and Fiji. *p*-values < 0.05 were generally considered statistically significant and were denoted by one asterisk throughout the figures, even if considerably lower.

2.5 Reagents and chemicals

Table 5. List of antibodies

Antibodies	Application	Company	Specious	Catalog No.	Reactivity	Dilutions	storage
Anti-TLR1	WB (Primary)	CST	Rabbit	#2209	Human	1 to 1000	-20 °C
Anti-TLR2 (D7G9Z)	WB (Primary)	CST	Rabbit	#12276	Human	1 to 1000	-20 °C
Anti-TLR6 (D1Z8B)	WB (Primary)	CST	Rabbit	#12717	Human and mouse	1 to 1000	-20 °C
Anti-GAPDH (GA1R)	WB (Primary)	Thermal Fisher	Mouse	MA5-15738	Human	1 to 5000	-20 °C
Anti-Penta His	WB (Primary)	Qiagen	Mouse	34660	-	1 to 2000	4°C
Anti-Mouse HRP conjugated	WB (Secondary)	Promega	Goat	W4028	Mouse	1 to 10000	4°C
Anti-Rabbit HRP conjugated	WB (Secondary)	Vector	Goat	PI-1000	Rabbit	1 to 5000	4°C
Anti-His Alexa Fluor® 594 conjugated	Confocal	Biolegend	Mouse	362609	-	1 to 100	4°C

Table 6. List of plasmids

Insert	Tag	Backbone	Resistance	Origin	Internal ID
Empty	N.A.	pcDNA3	Ampicillin	Addgene	pEx 021
hTLR2	FLAG	pcDNA3	Ampicillin	Gift from I. Bekeredjian-Ding, Paul-Ehrlich-Institute, Langen	pEx 073
Empty (for BP reaction)	N.A.	pDONR207	Gentamycin	Joschka Willemsen, DKFZ, Heidelberg	pEx 234
hTLR2 (open for gateway)	N.A.	pDONR221	Kanamycin	Harvard Plasmids	pEx 636
hTLR1 (open for gateway)	N.A.	pENTR223	Spectinomycin	Harvard Plasmids	pEx 640
hCHIT1 50 kDa	His	pTT5V5H8Q	Ampicillin	Morten Sorlie, Norwegian University Anne Tondervik, SINTEF, Trondheim Norway	pEx 738
hCHIT1 39 kDa	His	pTT5V5H8Q	Ampicillin	Morten Sorlie, Norwegian University Anne Tondervik, SINTEF, Trondheim Norway	pEx 739
Empty	Myc	pDEST_Myc-LC151	Ampicilln	Stefan Pusch, University Hospital Heidelberg	pEx 744
Empty	HA	pDEST_HA-LN151	Ampicilln	Stefan Pusch, University Hospital Heidelberg	pEx 745
hTLR2-LC151	Myc	pDEST_Myc-LC151	Ampicillin	This study	pTHC 022
hTLR2-LN151	HA	pDEST_HA-LN151	Ampicillin	This study	pTHC 023

Insert	Tag	Backbone	Resistance	Origin	Internal ID
hTLR1-LC151	Myc	pDEST_Myc-LC151	Ampicillin	This study	pTHC 024
hTLR1-LN151	HA	pDEST_HA-LN151	Ampicillin	This study	pTHC 025
attB-hTLR6	N.A.	pUC57	Kanamycin	Synthesis by GENEWIZ	pTHC-028
attL-hTLR6 (open for gateway)	N.A.	pDONR207	Genramycin	This study	pTHC 029
hTLR6-LC151	Myc	pDEST_Myc-LC151	Ampicilln	This study	pTHC 030
hTLR6-LN151	HA	pDEST_HA-LN151	Ampicilln	This study	pTHC 031
hCHT1 (50kDa) D138AE140L	His	pTT5V5H8Q	Ampicillin	This study	pTHC 034
hCHT2 (39kDa) D138AE140L	His	pTT5V5H8Q	Ampicillin	This study	pTHC 035
NF-κB firefly luciferase reporter	N.A.	pNF-κB	Ampicillin	Stratagene	None
Renilla luciferase	N.A.	pRL-TK	Ampicillin	Promega	pEX 351

Table 7. Mutagenesis primers for catalytic mutant chitinases

Primer name	Sequence (5' to 3')	Internal ID	Designed by
CHIT1 D138AE140L Fwd	5‘-CTTGACCTTGCCTGGCTGTACCCAGGAAGC -3’	AWm632	T.-Z.(Austin) Chang
CHIT1 D138AE140L Rev	5’- GCTTCCTGGGTACAGCCAGGCAAGGTCAAG -3’	AWm633	T.-Z.(Austin) Chang

Table 8. List of ligands, recombinant proteins and inhibitors

Receptors	Ligands	Concentration	Catalog ID	Origin
TLR2	C10-15 (chitosan)	20 μM	OC28900	Carbosynth Self-acetylated
TLR2	Zymosan	100 μg/ml	tlrl-zyn	Invivogen
TLR2-TLR1	Pam_3CSK_4	40 nM to 1 μM	tlrl-pms	Invivogen
TLR2-TLR6	Pam_2CSK_4	40 nM to 1 μM	tlrl-pm2s-1	Invivogen
TLR2-TLR6	FSL-1	40 nM to 1 μM	tlrl-fsl	Invivogen
TLR4	LPS	100 ng/ml	tlrl-eklps	Invivogen
TLR7 & TLR8	R848	5 μg/ml	tlrl-r848-5	Invivogen
TLR9	CpG	2,5 μM	-	TIM MOLBIOL
TLR3	Poly I:C	20 μg/ml	tlrl-picw	Invivogen
TLR7 & TLR8	ssRNA	0,1 nM	-	TIM MOLBIOL
	Proteins	**Concentration**	**Catalog ID**	**Origin**
	rCHIT1 50 kDa & rCHIT1 39 kDa	1 μM	-	Group of Morten Sørlie, Norwegian University
	Zymolase	25 nM	tlrl-zyn	Invivogen
	hLBP	5 nM to 25 nM	ab151656	Abcam
	rYKL-40	0,5 μM to 5 μM	Ab140057	Abcam
	LL37	20 μg/ml	tlrl-l37	Invivogen
	Inhibitors	**Concentration**	**Catalog ID**	**Origin**
	Polymyxin B	10 μg/ml	21850029	Thermo Fisher
	Caspofungin	0,032 μg/ml	SML0425	Sigma-Aldrich

Table 9. qPCR primers

Gene	Primer number	Catalog number
Tbp	Mm00446971_m1	4331182
Il6	Mm00446190_m1	4331182
Chit1	Mm01291360_m1	4331182

Table 10. Buffer solutions that were used in this study

Buffer name	Recipe
PBS (phosphate buffered saline)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, add ddH_2O to a final volume 1 L, adjust to pH 7.4
TBS (Tris buffered saline)	50 mM Tris HCl, pH 7.4 and 150 mM NaCl
TBS-Tween 0.1% (immunoblot washing buffer)	TBS, 0.1% Tween-20
ELISA stop solution	2N H_2SO_4
SDS running buffer	Tris 25 mM, glycine 250 mM, SDS 0.1%
RIPA lysis buffer	50 mM HEPES, 150 mM NaCl, 0.1% NP-40, 20 mM β-glycerophosphate, 2 mM DTT, adjust to PH 6.9, freshly supplemented with Roche inhibitor tablets Complete Mini Protease Inhibitor
Yeast extract-peptone-dextrose (YPD) medium	1% [w/v] BactoYeast extract, 2% [w/v] BactoPeptone, 2% [w/v] Dextrose, 2% [w/v] agar
YPD agar medium	YPD medium, 2% [w/v] agar
Yeast aspartic protease induction medium	2 mM $MgSO_4$, 7.3 mM KH_2PO_4, 1% glucose, 0.5% bovine serum albumin, 1% 100× HEPES, adjust pH to pH 4.0

Chapter 3: Results

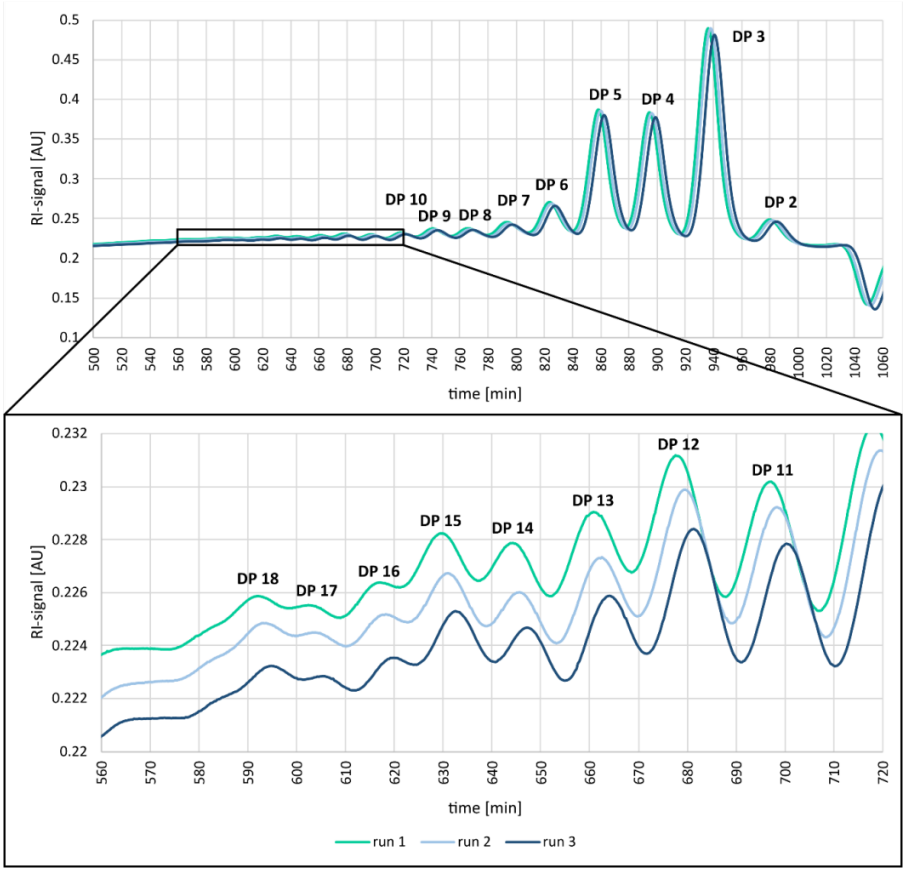
3.1 TLR2 recognition shows a preference for DP16 chitin oligomers

In our previous study, we showed that oligomeric chitin larger than 6 GlcNAc units could induce inflammatory cytokine production in macrophages (Fuchs *et al.*, 2018). However, the chitin oligomers, C10-15, which showed the highest immunogenicity were a mixed population of an MW of 2000 to 3000, corresponding to a degree of polymerization (DP) of 10-15 with unknown relative abundances meaning not a pure and specific size of a chitin oligomer. This preparation of chitin had been generated from a chitosan mixture. First of all, we determined to reanalyze the TLR2 of specific chitin oligomers with defined DP. Our collaborators at Münster University, Margareta Hellmann and Prof. Bruno Moerschbacher separated the aforementioned chitosan by using size exclusion chromatography (SEC) and mass spectrometric (MS) to prepare purified chitosan oligomers of specific but different DP **(Figure 4A, Table 11)**. These fractionated chitosan oligomers were further acetylated to chitin, yielding chitin oligomers of DP 2 to 22. The degree of acetylation (DA) was also checked by Margareta (Cord-Landwehr *et al*., 2017; Hamer *et al*., 2015). To confirm, the samples were enzymatically degraded by bacterial chitinase and the products were detected via UHPLC-ESI-MS. For all samples, the MS signal of the fully acetylated dimer (A2) formed the biggest proportion of all signals and in total, A2, and A3 together make up at least 84% of all MS signals **(Figure 4B)**. The degree of acetylation close to 100% was hereby confirmed. As expected, only the undigested controls of DP 4+5, DP 6+7, and DP 8+9 showed signals of the respective fully acetylated intact oligomers, because chitin oligomers of higher DPs are not soluble and therefore not detectable **(Figure 4C)**. Finally, all the DP samples were ready to test the cellular response.

Table 11. Pooled fractions from SEC with oligomers of defined DP.
The start and end points of the collected fractions are indicated as well as the fraction numbers.

sample	DP 4+5		DP 6+7		DP 8+9		DP 10+11			DP 12	DP 13	DP 14	DP 15	DP 16	DP 17+18		DP 19-22		
DP	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
start [min]	880	840	810	780	750	730	710	690	670	650	640	620	610	600	590	570	550	530	510
end [min]	910	880	840	810	780	750	730	710	690	670	650	640	620	610	600	590	570	550	530

A



B

C

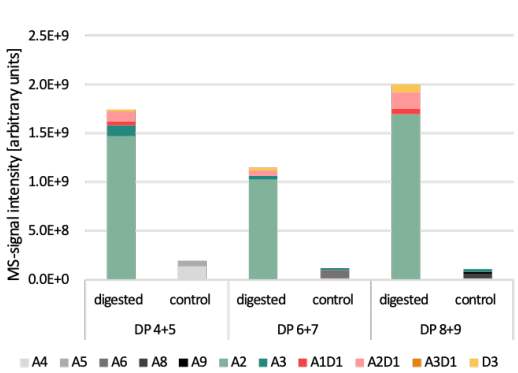


Figure 4. Production of chitin oligomers of defined DP.
(A) Chromatograms of three consecutive SEC runs to prepare chitin oligomers of defined DP. 50 mg of chitosan with a molecular weight of < 3000 Da were separated using an isocratic flow of 0.8 ml/min of SEC buffer (0.15 M ammonium acetate, pH 4.5). A refractive index detector was used to monitor the separation and fractions of 8 ml were collected between minutes 500-1000. (B) Proportion of the MS-signals of the enzyme products for each sample. (C) MS-signal intensity of the enzyme products and the non-digested controls. A: GlcNAc, D: GlcN.

To examine the TLR2 effect of these different purified oligomeric chitin DPs, here we used HEK-Dual™ hTLR2 cells, a reporter cell line stably expressed NF-κB/AP-1-induced SEAP reporter and Lucia luciferase under the endogenous IL-8 promoter, to assess what range of DPs can trigger TLR2 response at equimolar concentrations by quantifying NF-κB activity and IL-8 production (see Methods 2.2.3). The results showed that the stimulation status of the TLR2-HEK-Dual gradually increased the NF-κB activity and IL-8 production in the range of DP 13 – 16 **(Figure 5A and 5B)**. The TLR2 response peaked at DP 16 and went down at the range of DP 17 – 22 **(Figure 5A and 5B)**. In the range of DP 4 – 7 generally had no immunogenic while DP 8 – 13 induced mild but non-significant TLR2 activation **(Figure 5A and 5B)**. These data prove and fit our previous observation that the specific size of oligomeric chitin, especially the DPs larger than 10 and smaller than 18, can be recognized by TLR2.

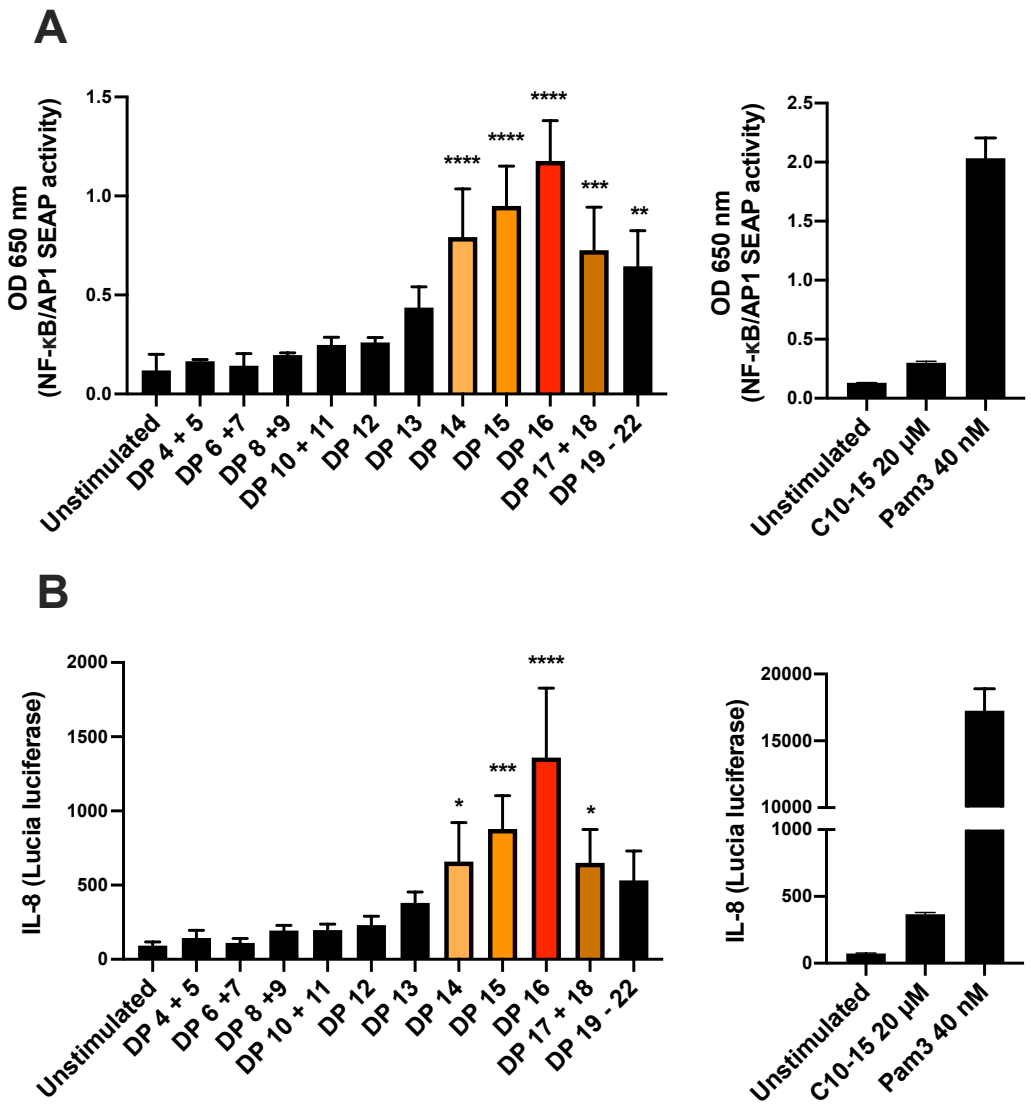


Figure 5. Fractionated chitin oligomers induce size-dependent TLR2 response.
Measurement of NF-κB activity in HEK-Dual™ hTLR2 after stimulation with defined DPs fractions. Stimulation of chitin C10-15 and Pam3 were used as positive controls for TLR2-dependent responses. (A) The NF-κB/AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) was measured with the SEAP detection reagent (Quanti-blue™ solution). The SEAP level was determined by the plate reader at 650 nm. (B) The IL-8 production was determined by Lucia luciferase activity (Quanti-luc™). Data are from one representative of three independent experiments. Error bars indicate standard deviation of the mean. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ [one-way ANOVA with follow up Dunnett's multiple comparisons test]

Next, to investigate whether myeloid cells also behave in a similar pattern compared to TLR2-HEK cells, we moved to a more physiological model by employing mouse bone marrow-derived macrophages (BMDMs) to test inflammatory cytokine production by oligomeric chitin. In this study, we employed murine WT and *Tlr2* KO BMDMs to stimulate some selected oligomeric chitin DPs. The results showed that BMDMs in the absence of *Tlr2* showed a significant reduction of IL-6 production upon stimulation with oligomers of DP 14 and DP 16 **(Figure 6A)** while oligomers of other DPs induced lower IL-6, there was no significant difference compared between WT and *Tlr2* KO cells **(Figure 6A)**. Unexpectedly, TNF production was generally low in this experimental setting and it was not ideal to make a comparison **(Figure 6B)**. These data demonstrate that chitin in a DP range of 14 – 16 has the highest capacity to induce a TLR2-dependent IL-6 response in macrophages.

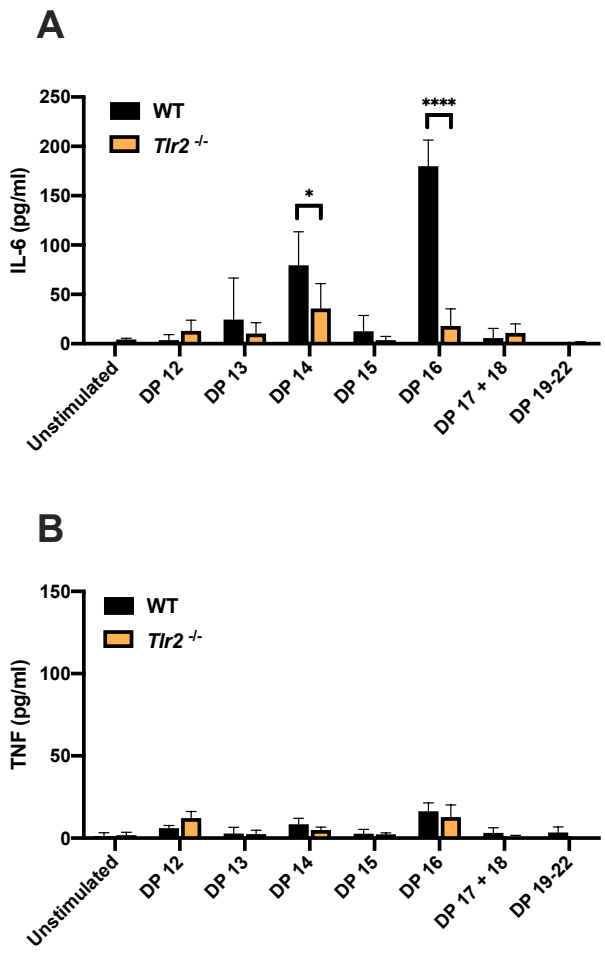


Figure 6. Chitin oligomers from size specific chitin DPs induce TLR2-dependent IL-6 response.

(A & B) Murine IL-6 and TNF production in WT and *Tlr2* KO BMDMs upon 18 h stimulation with selected sizes of chitin DPs were measured by ELISA. (A & B) Data are from one representative of four independent experiments. * $p < 0.05$, **** $p < 0.0001$ [two-way ANOVA with follow up Sidak's multiple comparisons test]

3.2 Chitotriosidase digestion on chitin particle induces TLR2 response

Polysaccharide chitin in the environment is widely distributed by degradation and recycling by organisms' chitinases. The microbiota express multiple chitinases and also mammals express proteins of the aforementioned conserved 18-GH chitinase family to mediate degradation of polysaccharide chitin into small size of oligomeric chitin even though chitin itself is a biomolecule not found in mammals (Bussink *et al,* 2007). Whilst the immunological influence of both human chitinases, AMCase and CHIT1, has been widely documented, no studies have shown that oligomeric chitin from human chitinase degradation can directly trigger an innate inflammatory response. Therefore, as CHIT1 is supposed to act as an endochitinase (Kuusk *et al.*, 2017) that could release oligomers rather than only terminal disaccharide units, we hypothesized that the oligomeric chitin can be generated by chitinase digestion to trigger a TLR2 response. To address this, we used recombinant forms of the human chitotriosidases (CHIT1), one full-length 50 kDa form, and a smaller 39 kDa isoform. Both were used to degrade (and thereby possibly release oligomers from) large, macroscopic chitin particles, namely chitin flakes isolated from shrimp shells. To obtain the ideal size approximately in 1mm, chitin flakes were firstly filtered by the 1 mm pore size of steel sieve and were subsequently washed with polymyxin B extensively to remove any contaminating oligomers. After washing with dPBS several times to remove the polymyxin B, chitin flakes were ready to stimulate the cells. Indeed, chitin flakes prepared in this way were still too big and cannot induce any TLR2 response **(Figure 7A)**. Cells stimulation with Pam3 and C10-15 were as the same before and applied as positive control **(Figure 7A, right panel)**. Surprisingly, chitin flakes with CHIT1 degradation, especially with the 39 kDa isoform, elicited significant TLR2-NF-κB activity **(Figure 7A, left panel)**. One explanation could be that the chitinase rendered the macroscopic chitin active, like generating chitin free ends, possibly not be releasing chitin oligomers but rather direct contact with the cells. Next, I therefore applied a transwell setting to trap the chitin flakes in the upper reservoir to avoid direct contact to the cells (i.e. TLR2-HEK cells) and only allow CHIT1 digestion products – supposedly the diffusible oligomeric chitin released by CHIT1 degradation – of < 8 μm to pass through to the cells layer. Interestingly, with

this transwell setting, chitin flakes digestion by CHIT1 39 kDa also significantly increased TLR2-NF-κB response compared to chitin flakes without CHIT1 digestion **(Figure 7B)**. These data suggest that CHIT1 degradation on chitin particle could generate diffusible oligomeric chitin to trigger TLR2 activity.

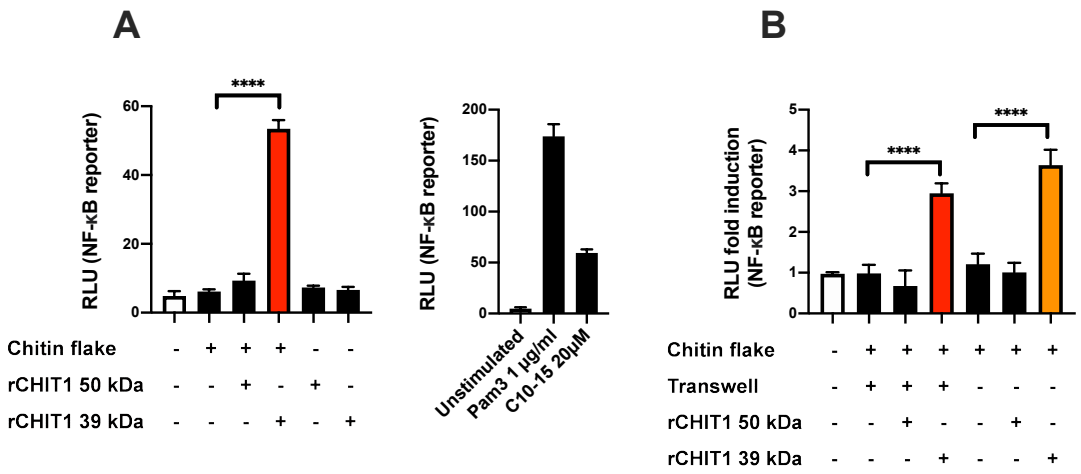


Figure 7. Chitin oligomers released from CHIT1 digestion of chitin flakes induce TLR2 response.

(A & B) DLA measurement of NF-κB response in TLR2-transfected HEK 293T cells upon 18 h chitin flakes stimulation with or without recombinant CHIT1 (rCHIT1) digestion. (B) Transwell setting was applied to separate big particle of chitin flakes resulted by the digestion and to avoid direct contact with the cells. The recombinant CHIT1-generated supernatant was allowed to pass through the transwell to stimulate HEK 293T cells for 18 h. Data are from (A) one representative or (B) pooled of three independent experiments. Error bars indicate standard deviation of the mean. **** $p < 0.0001$ [one-way ANOVA with follow up (A) Dunnett's or (B) Sidak's multiple comparisons test]

In human chitinase 18 – GH family, chitinase activity depends on a catalytic site which is located at positions 136 to 140 in the primary amino acid sequence of glycol-18_domain (Kzhyshkowska *et al*., 2007). To confirm that the effect of CHIT1 on the TLR2 response was truly dependent on chitinase catalytic activity, I applied mutagenesis to expression constructs for both the CHIT1 50 and 39 kDa isoforms, targeting the chitinase catalytic site by corresponding D138A and E140L mutation **(Figure 8A and 8B).** The protein expression and secretion into the cell culture media of both WT and catalytic mutant CHIT1 were confirmed by Western blotting **(Figure 8C)**. Results showed that both WT and mutant CHIT1 proteins can be secreted from transfected-HEK 293T cells **(Figure 8C)**. By using 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside (4-MU) to test their chitinase activity, the results confirmed that both WT transfected- and recombinant 50 kDa and 39 kDa CHIT1s showed chitinase activity while the catalytic mutant CHIT1s failed to show detectable chitinase activity **(Figure 8D)**. Of note, the 39 kDa isoform, from both transfectants and as a recombinant protein, showed higher chitinase activity compared to the 50 kDa isoform **(Figure 8D)**. Next, supernatants containing the secreted 50 kDa and 39 kDa CHIT1 isoforms were harvested from transfected HEK cells and used to digest chitin flakes. The digestion supernatant together with chitin flakes was then subjected to stimulate TLR2-HEK cells. The results showed that transfected-CHIT1 39 kDa but not 50 kDa incubated with chitin flakes also led to a TLR2 response in a dose-dependent manner **(Figure 8E)**. In contrast, the catalytic mutant CHIT1, m50 kDa and m39 kDa, were both resulted in an inability to induce TLR2 response, presumably by failing to generate chitin oligomers **(Figure 8F)**. Overall, these data suggest that CHIT1 39 kDa isoform can digest chitin flakes to release oligomeric chitin which can be sensed by TLR2. Conversely, the CHIT1 protein or the undigested chitin flakes alone cannot elicit any TLR2 response. It appears that the oligomeric chitin-mediated TLR2 response relies on the chitinolytic activity from the CHIT1 to degrade chitin particles.

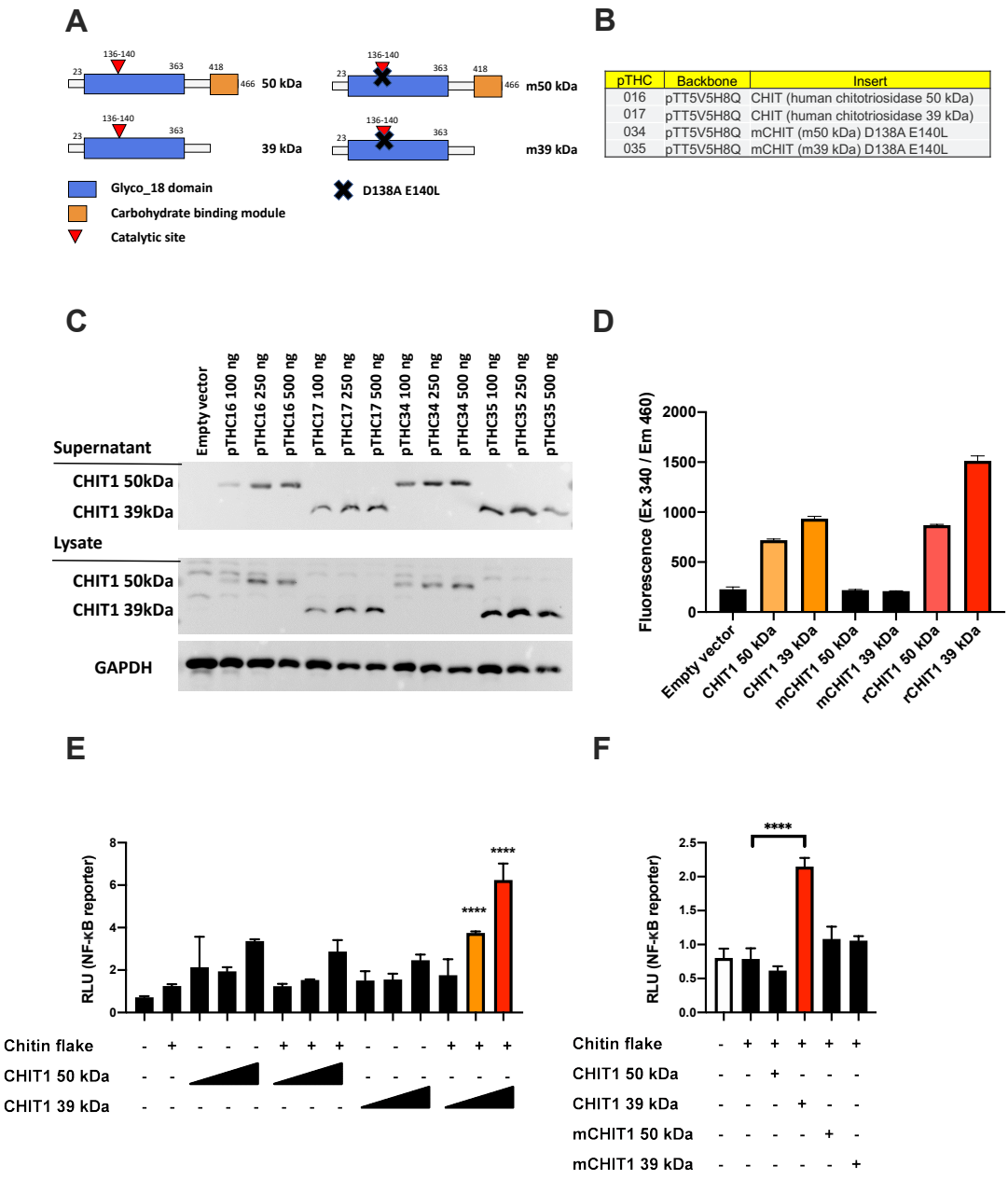


Figure 8. Catalytic mutant chitotriosidases fail to digest chitin flakes to release diffusible chitin oligomers.

(A) Scheme of WT and catalytic mutant of CHIT1 protein (mCHIT1) with glycol_18 domain or carbohydrate binding module. The chitinase catalytic site is located at glycol_18 domain. The black cross indicates the introduction of mutant amino acids to catalytic site. (B) Detailed information of WT and mutant CHIT1 expression plasmids. (C) CHIT1 WT and catalytic mutant protein expressions were assessed by immunoblot with anti-His antibody. (D) Measurement of chitinase activity by the hydrolysis of 4-Methylumbelliferyl N,N'-diacetyl-β-D-chitobioside (4-MU-DAC) releasing fluorescent 4-MU. (E) DLA measurement of NF-κB response in HEK 293T cells upon transient TLR2 transfection after stimulation with the supernatant from dose titrated CHIT1 50 kDa or 39 kDa incubated

with chitin flakes. (F) DLA measurement after stimulation with WT or catalytic mutant CHIT1 (mCHIT1) incubated with chitin flakes. Data are from one representative of (C) two, (D & E) three or (F) four independent experiments. Error bars indicate standard deviation of the mean. **** $p < 0.0001$ [one-way ANOVA with follow up (E) Sidak's and (F) Dunnett's multiple comparisons test]

3.3 Chitin oligomers released from chitin-rich organisms trigger TLR2 response

House dust mite (HDM), *Dermatophagoides pteronyssinus,* is well known as allergy-triggering organism. It has been shown that the HDM-derived chitin induced TNF production to enhance Th2 cells response (Choi *et al.*, 2016), with a focus on HDM allergens rather than adjuvant-like constituents like chitin. On the other hand, studies on the pathogenic fungus *C. albicans* paid attention to its chitin content which was observed to promote IL-10 production and to suppress an inflammatory response (Wagener *et al.*, 2014). However, whether oligomeric chitin from these chitin-rich organisms could be released by host chitinases digestion like CHIT1 and whether released oligomers play a role in the innate immune response are still unknown. Based on our aforementioned results, we hypothesized that CHIT1 degradation might also release chitin oligomers from these chitin-rich pathophysiologically relevant organisms that could also serve as TLR2 ligands. Firstly, HDM whole culture and mite body were commercially available and were also applied polymyxin B washing before stimulation. On the other hand, it has been known that *C. albicans* treated with low doses of the antifungal drug, caspofungin, can expose chitin from the inner fungal cell wall (Walker *et al*., 2013). *C. albicans* hyphae transition also coincides with pronounced chitin exposure (Garcia-Rubio *et al*., 2019). Thus, secondly, I applied caspofungin treatment on *C. albicans* yeast to expose chitin and induced *C. albicans* hyphae transition by culturing at 37 °C in the presence of FCS. Next, to examine the TLR2 effect from those chitin-rich organisms, commercially available HDM whole cultures, isolated mite bodies, or *C. albicans* caspofungin-treated yeast and hyphae were subjected to CHIT1 degradation and then the resulted presumably exposed or released chitin oligomers-containing supernatants were tested for TLR2-NF-κB activity. NF-κB activity for HDM showed that with CHIT1 39 kDa digestion, both HDM whole culture and mite body triggered a significantly increased TLR2 response compared to the group without CHIT1 digestion **(Figure 9A)**. Interestingly, not only CHIT1 39 kDa but also 50 kDa digestion on the mite body showed a significant increase of TLR2 response **(Figure**

9A). On the other hand, results for *C. albicans* showed that CHIT1 39 kDa digestion on both yeast and hyphal *C. albicans* further enhanced TLR2 activity **(Figure 9B).** *C. albicans* yeast with zymolase digestion also showed a significant increase in TLR2 response, even though the increasing level was not comparable to the CHIT1 digestion group **(Figure 9B)**. These data indicate that exposed chitin free ends or diffused oligomeric chitin from HDM and *C. albicans* can be recognized by TLR2.

Because of this distinct feature of CHIT1 isoform, again, I speculated that CHIT1 39 kDa could digest and release diffusible chitin oligomers in chitin-rich organisms to trigger an inflammatory response. To investigate this cutting and releasing hypothesis, I used again the transwell setting, to test whether diffusible chitin oligomers released from HDM and *C. albicans* by CHIT1 digestion can be sensed independent of direct contact, and enhance a TLR2 response. As expected, the supernatant passing through the transwell from CHIT1-digested HDM mite body enhanced TLR2-NF-κB response **(Figure 9C)**, probably via diffusible oligomers. Interestingly, both CHIT1 50 kDa and 39 kDa digestion of HDM could boost TLR2 activity. It seems that even with CHIT1 50 kDa digestion some small oligomers could be generated from HDM. In addition, results in *C. albicans* hyphae also showed that CHIT 39 kDa digestion led to more TLR2-NF-κB activity **(Figure 9D)**. It should be noted that zymolase digestion, which annihilated the outer layer of β-glucan and α-mannan and facilitated the exposure of inner chitin on *C. albicans,* did not further increase TLR2 response within this transwell setting **(Figure 9D)**. This suggests that only with CHIT1 39 kDa isoform degradation, presumably, the specific size of chitin oligomers could be released from *C. albicans* to activate TLR2. On the whole, these data indicate that stimulation of TLR2 sensing cells with chitin-rich organisms can trigger a response even without direct contact albeit only upon CHIT1 digestion, especially by its 39 kDa isoform. This indicates that CHIT1 can generate diffusible chitin oligomers from chitin-containing organisms to elicit distal TLR2 immune activation.

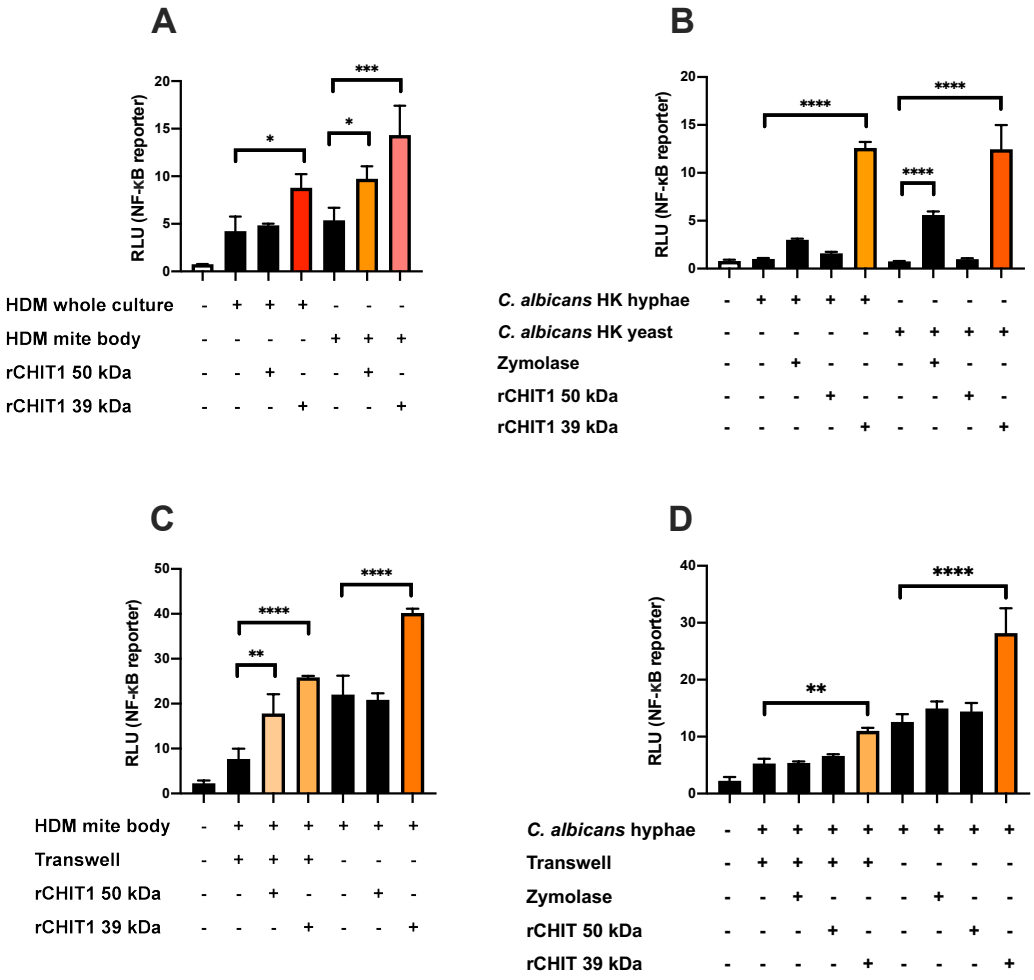


Figure 9. Diffusible chitin oligomers released from chitin-rich organisms can be sensed by TLR2.

(A – D) DLA measurement of NF-κB response in TLR2-transfected HEK 293T cells after 18 h stimulation with (A & C) house dust mite (HDM) whole culture or mite body and (B & D) *C. albicans* heat-killed yeast or hyphae with or without recombinant CHIT1 or zymolase digestion. (C & D) Transwell separation was applied for retaining big particle of HDM mite body and *C. albicans* hyphae. Collected culture supernatant passing through the 8 µm transwell was used for stimulation. Data are from one representative of (A, B & D) two or (C) three independent experiments. Error bars indicate standard deviation of the mean. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$ [one-way ANOVA with follow up Sidak's multiple comparisons test]

From a previous study, Wagener J *et al* reported that fungal chitin extracted from *C. albicans* could not induce a pro-inflammatory cytokine response (Wagener *et al.*, 2014). They suggested that TLR4 recognition by LPS was masked by fungal chitin and then inhibited the inflammatory signaling event (Wagener *et al.*, 2014). However, the extracted fungal chitin they used was in the range of 1 – 10 μm which is, according to our previous work, supposedly too big to be directly recognized by TLR2 (Wagener *et al.*, 2014). To clarify this discrepancy, thanks to the group of Prof Neil Gow who kindly provided us the fungal chitin, I aimed to investigate the TLR2 response on fungal chitin after CHIT1 degradation. Firstly, consistent with Wagener *et al* previous observation, fungal chitin alone failed to trigger a TLR2 response even at a concentration as high as 100 μg/ml **(Figure 10A and 10B)**. However, when applying CHIT1 digestion, not only the CHIT1 39 kDa significantly increased TLR2-NF-κB activity and IL-8 production considerably, but also the CHIT1 50 kDa triggered a mild TLR2 response **(Figure 10A and 10B)**. These data indicate that immunostimulatory, oligomeric chitin can be produced by CHIT1 digestion from the extracted chitin from HDM or fungal cell walls. It appears that large macroscopic chitin generally fails to be detected by TLR2, even though the chitin source is an immunogenic pathogen.

Furthermore, WT and *Tlr2* KO BMDMs were again employed to test the pro-inflammatory cytokine production by fungal chitin with or without CHIT1 two isoforms degradation. As expected, the result was as similar as I observed in **Figure 10A** and **10B**. Fungal chitin alone did not trigger IL-6 and TNF response **(Figure 10C and 10D)**. Conversely, fungal chitin with 39 kDa CHIT1 digestion stimulated BMDMs to produce IL-6 and was confirmed to be TLR2 dependent **(Figure 10C)**. In contrast, there was no TNF production by fungal chitin even with 39 kDa CHIT1 digestion **(Figure 10D)**. Interestingly, it is worth noting that 50 kDa CHIT1 digestion of fungal chitin could also induce mild IL-6 production even though it had no significant difference compared between WT and *Tlr2* KO **(Figure 10C)**. Taken together, these data are strongly consistent with the above results and demonstrate that CHIT1 is able to generate a diffusible TLR2 agonist, presumably chitin oligomers, to induce TLR2-

dependent inflammatory cytokine response. The production of inflammatory cytokine by such oligomeric fungal chitin is more prominent for IL-6 than TNF.

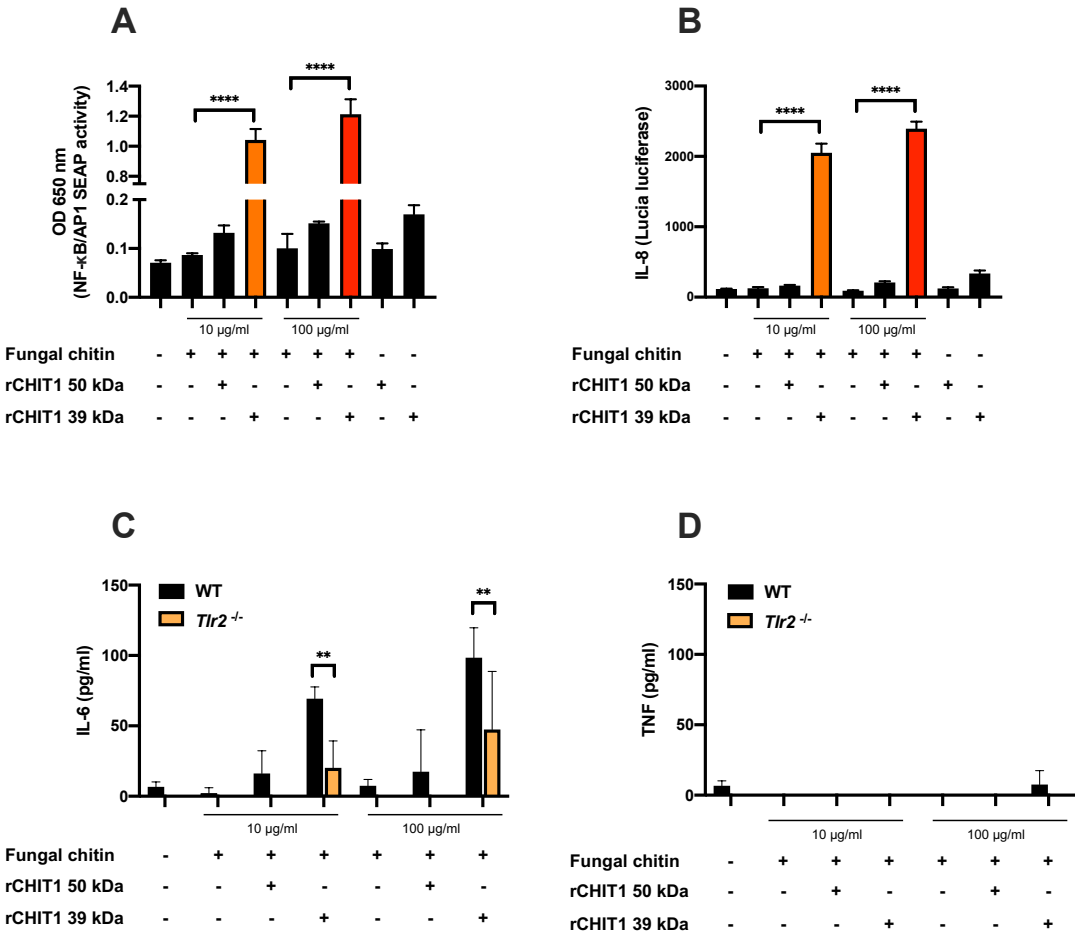


Figure 10. Chitin oligomers from CHIT1 digested fungal chitin induce TLR2-dependent inflammatory response.

(A – D) Fungal chitin treated with or without CHIT1 digestion were applied to stimulate (A & B) HEK-Dual ™ hTLR2 or (C & D) WT and *Tlr2* KO BMDMs for 18 h. (A) The NF-κB/AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) was reacted with the SEAP detection reagent (Quanti-blue^{TM} solution). The SEAP levels were determined by the plate reader at 650 nm. (B) The IL-8 production was determined by Lucia luciferase activity (Quanti-luc^{TM}). (C – D) The production of murine IL-6 and TNF were measured by ELISA. Data are from one representative of (A & B) four independent experiments. (C & D) Data are from one experiment which is still a preliminary result. Error bars indicate standard deviation of the mean. ** $p < 0.01$, **** $p < 0.0001$ [two-way ANOVA with follow up Sidak's multiple comparisons test]

3.4 Chitotriosidase digestion unmasks chitin on *C. albicans*

Since it has been shown that caspofungin treatment can destroy the layer of β-glucan and further expose chitin from the inner layer of the cell wall (Walker *et al.*, 2013), it might be possible that *C. albicans* treated with caspofungin allows subsequently CHIT1 digestion more effortless to generate chitin oligomers. In this study, since earlier we showed CHIT1 39 kDa expressed stronger chitinase activity than 50 kDa **(Figure 8D)** and their generated products are more immunogenic **(Figure 9 and Figure 10)**, we suggested that 39 kDa digestion on *C. albicans* can expose or release more chitin content from the inner layer of the cell wall in reasoning to enhance TLR2 response. To address this, *C. albicans* which is treated with caspofungin and next applied CHIT1 digestion was stained with wheat germ agglutinin that indicates the staining of surface chitin and then subjected to a confocal microscope. The results showed that *C. albicans* heat-killed yeast without caspofungin treatment displayed almost no chitin exposure on the surface while treated *C. albicans* yeast with caspofungin can observe a bit surface chitin **(Figure 11A)**. Surprisingly, with additional CHIT1 digestion, *C. albicans* was dramatically exposed the surface chitin **(Figure 11A)**. However, not only with CHIT1 39 kDa digestion but also with CHIT1 50 kDa exposed a lot of surface chitin (**Figure 11A**). By using Fiji ImageJ to quantify the WGA intensity of every single yeast, the results showed that both CHIT1 50 kDa and 39 kDa digestion significantly increased surface chitin exposure **(Figure 11B)**. It is worth to mention that chitin exposure was more efficient by 39 kDa digestion than 50 kDa **(Figure 11B)**. Regarding the differences of CHIT1 isoform, it has been shown that CHIT1 39 kDa lacks a chitin-binding module (CBM) compared to CHIT1 50 kDa, supposedly CHIT1 39 kDa cannot bind to the chitin but freely disseminate in the media (Kuusk *et al.*, 2017). Therefore, by using anti-His tag to directly stain with these two CHIT1s on *C. albicans*, it showed that 50 kDa was overlapping with chitin signal while there was no 39 kDa that can be detected **(Figure 11C)**. Taken together, these data demonstrate that CHIT1 50 kDa can digest and, in the meantime, firmly bind to the chitin; whereas, CHIT1 39 kDa isoform can digest and leave the chitin immediately. This characteristic of CHIT1 39 kDa might

be possible to freely degrade the chitin particle which leads to generating the abundant and various sizes of oligomeric chitin.

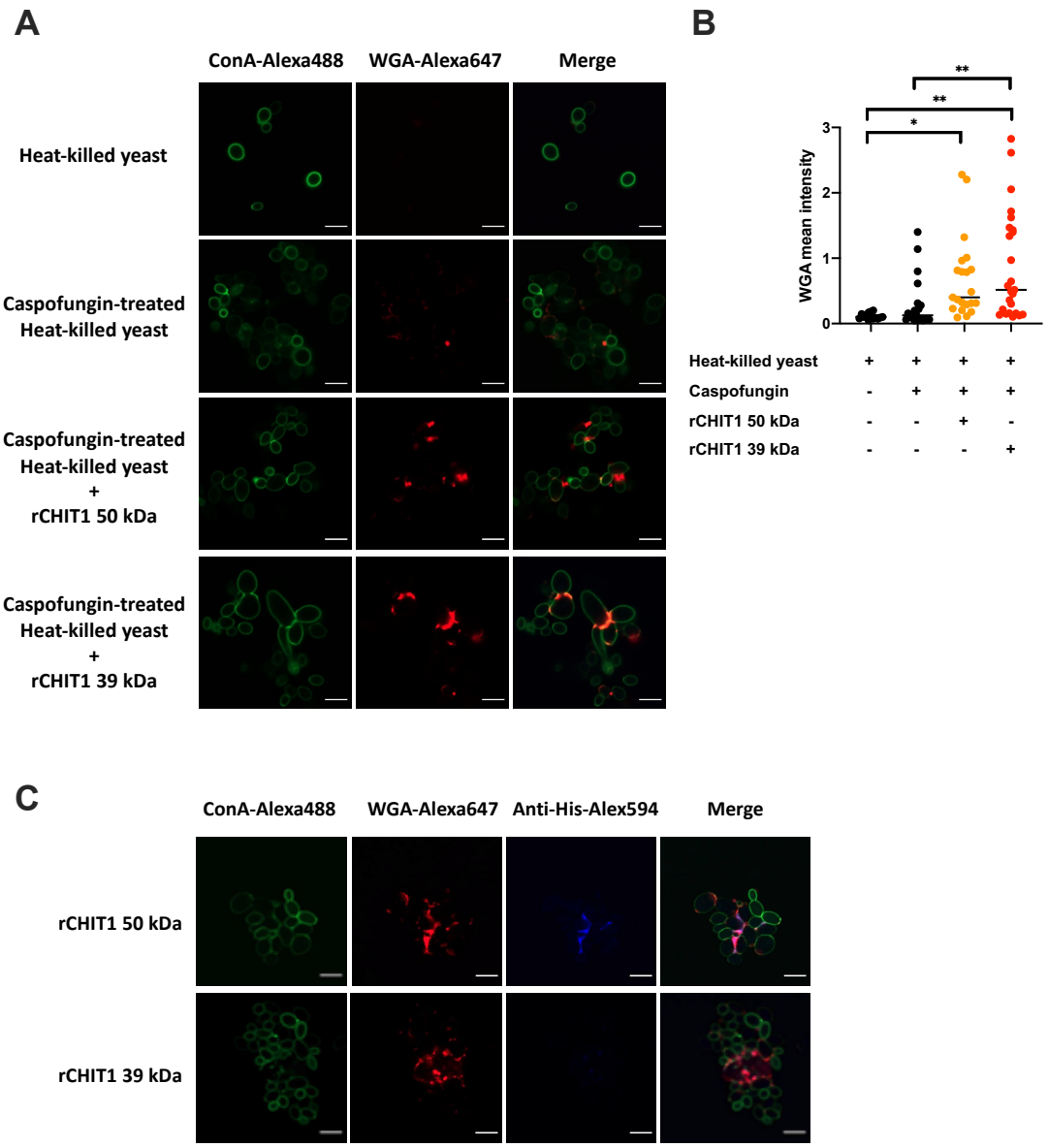


Figure 11. CHIT1 digestion increases chitin exposure on *C. albicans* yeast.
(A) Heat-killed *C. albicans* yeast cells were treated with caspofungin and CHIT1. Cells were then stained with ConA to recognize the α-mannan from outer layer of fungal cell wall and WGA to stain the surface chitin. Cells were viewed under Zeiss LSM800 confocal microscope. (B) The WGA fluorescent intensity was quantified by ImageJ. The ConA and WGA signal from each single yeast were threshold and measured by using Otsu method. The mean intensity of WGA signal was normalized with ConA. (C) *C. albicans* yeast cells were digested with CHIT1 and then subjected to ConA, WGA and anti-His staining to detect recombinant CHIT1. The white scale bars represent 6

μm. Data are from one representative of (A & B) three or (C) one independent experiments. Error bars indicate standard deviation of the mean. * $p < 0.05$, ** $p < 0.01$ [one-way ANOVA with follow up Tukey's multiple comparisons test]

3.5 Diffusible chitin oligomers released from pathogen elicit TLR2-dependent inflammatory cytokine response

To examine whether chitin oligomers released from chitin-rich organisms can also induce inflammatory cytokine production, the WT BMDMs were firstly applied to test the effect of CHIT1 degradation. The results showed that house dust mite whole culture and mite body alone can already trigger IL-6 and TNF production **(Figure 12A)**. Whereas, HDM whole culture with both CHIT1 50 kDa and 39 kDa isoform digestion showed enhanced IL-6 production; mite body with CHIT1 39 kDa but not 50 kDa digestion significantly impacted on the release of IL-6 **(Figure 12A)**. Furthermore, CHIT1 39 kDa digestion on both whole culture and mite body also significantly increased TNF production **(Figure 12B)**. On the other hand, results in *C. albicans* also showed that *C. albicans* hyphae digested by CHIT1 39 kDa isoform increased IL-6 release while there was no significant difference in TNF **(Figure 12C and 12D)**. In slight summary, these data demonstrate that oligomeric chitin can be generated by CHIT1 digestion on chitin-rich organisms like house dust mite and *C. albicans*. The released or exposed oligomeric chitin can be sensed by TLR2 to trigger NF-κB activity then lead to producing pro-inflammatory cytokine.

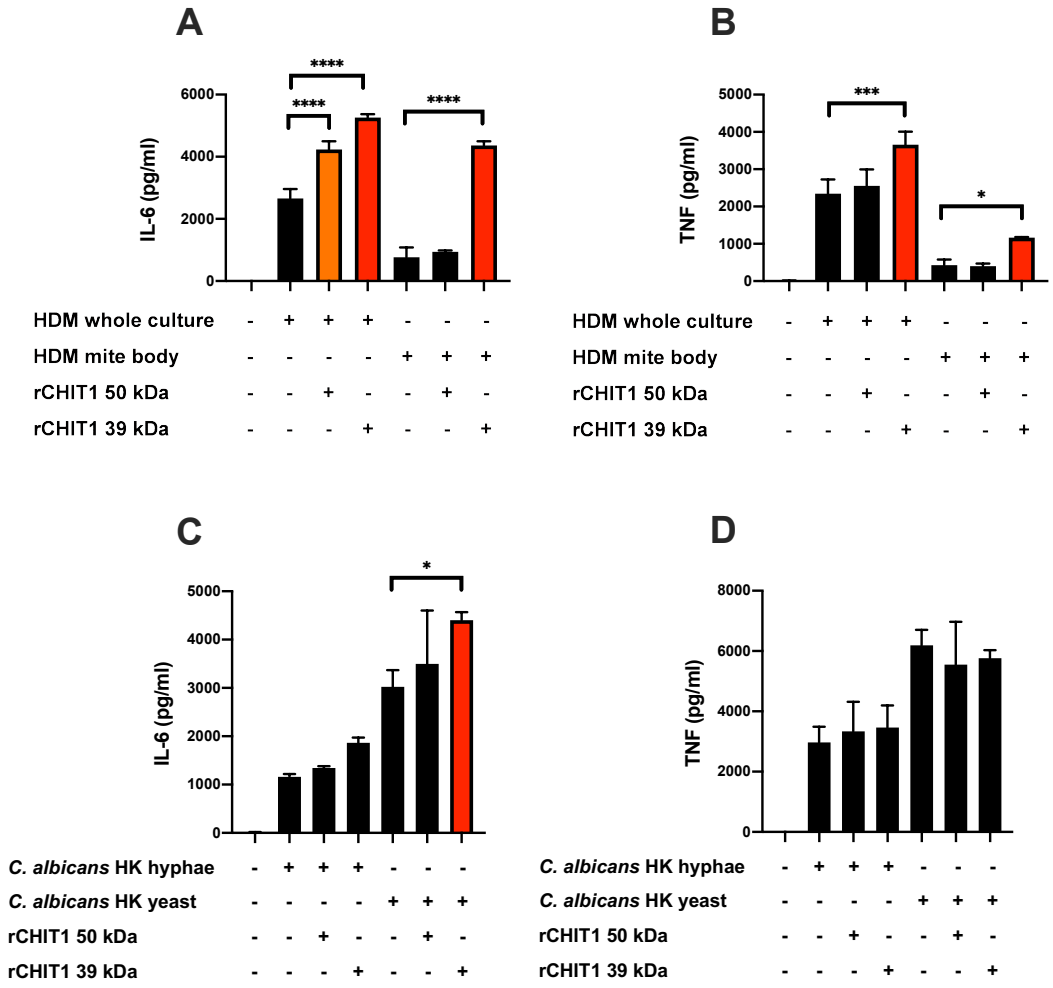


Figure 12. Chitin oligomers released from chitin-rich organisms increase pro-inflammatory cytokine production in murine WT BMDMs.

(A – D) Murine IL-6 and TNF production in BMDMs upon 18 h stimulation with stated organisms measured by ELISA. BMDMs were differentiated with mGM-CSF for 7 days. (A & B) HDM mite body and (C & D) *C. albicans* heat-killed hyphae and yeast were firstly incubated with or without CHIT1 then subjected to stimulate BMDMs. Data are from one representative of two independent experiments. Error bars indicate standard deviation of the mean. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ [one-way ANOVA with follow up Sidak's multiple comparisons test.]

To further confirm whether the effect of CHIT1-generated chitin oligomers on the activation of an inflammatory response is truly TLR2-dependent and follows the same principles even in cells expressing multiple other PRRs, I applied the aforementioned transwell setting of CHIT1-digested organisms for the evaluation of inflammatory cytokine production from WT and *Tlr2* KO BMDMs. Firstly, CHIT1 digestion of HDM mite body showed that, even in the transwell setting, there was no increase of IL-6 or TNF production while the direct contact of mite body triggered TLR2-dependent IL-6 and TNF production **(Figure 13A and 13B)**. To simply explain this contradiction comparing with previous results, it could be that CHIT1 did not generate sufficiently diffusible chitin oligomers from HDM to activate TLR2 on murine BMDMs. In contrast, in the transwell setting, results of CHIT1 degradation on *C. albicans* hyphae showed that digestion of *C. albicans* hyphae by both CHIT1 50 kDa and 39 kDa isoforms significantly induced TLR2-dependent IL-6 production **(Figure 13C left panel)**. Nevertheless, the digestion of *C. albicans* hyphae synergized the IL-6 production from its direct contact with the murine BMDMs was TLR2 independent **(Figure 13C right panel)**. Interestingly, in the transwell setting, there was no TNF production **(Figure 13D left panel)**. However, the direct contact of *C. albicans* hyphae induced partially TLR2 dependent TNF production, suggesting TNF induction strictly requires direct contact and hence probably co-engagement of other PRR and/or phagocytosis, and cannot be triggered by diffusible oligomers. In line with this notion, there was no adding effect of CHIT1 digestion **(Figure 13D right panel)**. Taken together, these data indicate that CHIT1 digestion is able to induce TLR2-dependent inflammatory cytokine response, most likely by diffusible chitin oligomers. However, it might be possible that different organisms, depending on their surface chitin distribution or cell wall component, cause different responses with TLR2 in the context of other PRRs typically expressed by such macrophages.

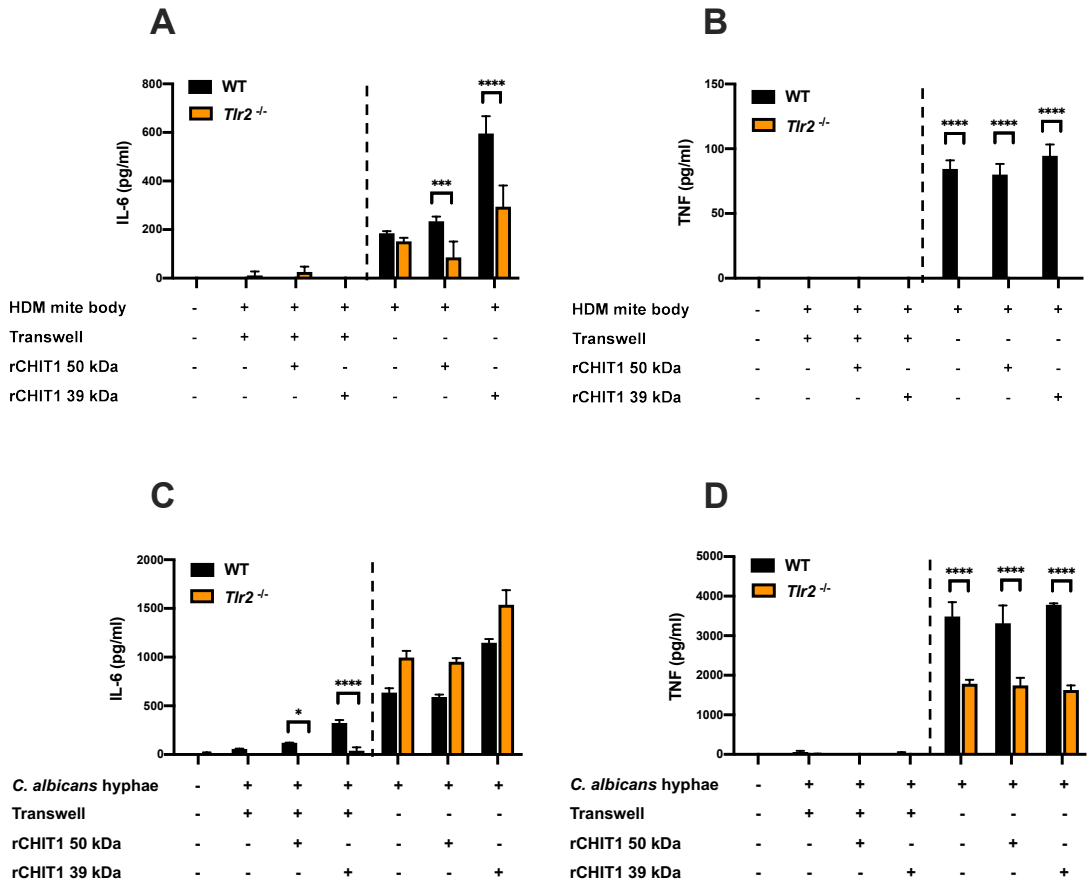


Figure 13. Diffusible chitin oligomers released from *C. albicans* hyphae increase TLR2-dependent IL-6 production in BMDMs.

(A - D) Murine IL-6 and TNF production in WT and TLR2 KO BMDMs upon 18 h stimulation with stated organisms measured by ELISA. (A & B) HDM body and (C & D) *C. albicans* heat-killed hyphae were firstly incubated with or without the two CHIT1 isoforms. The culture supernatants were passing through the 8 μm pore size of transwell and then subjected to stimulate BMDMs. Data are from one representative of three independent experiments. Error bars indicate standard deviation of the mean. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ [two-way ANOVA with follow up Sidak's multiple comparisons test]

3.6 *Chit1* gene induction by *C. albicans* hyphae is TLR2-independent

CHIT1 has been reported to inhibit the hyphae growth of *C. albicans* and rescue the mice from systemic *C. albicans* infection (van Eijk *et al.*, 2005; Vendele *et al.*, 2020). Van Eijk *et al* reported that neutrophils and macrophages are the major CHIT1 producing cells and macrophages can constitutively express CHIT1 under stimulation of GM-CSF. In our previous study, we have been reported that crude chitin particle induces chitinase activity in human whole blood and murine bronchoalveolar lavage fluid (BALF) (Fuchs *et al.*, 2018). Though CHIT1 serves as a conserved and highly regulated protein by stimuli in mammals (Lee *et al.*, 2011), it is still unclear what specific ligands and their correspondent receptors mediate CHIT1 expression. Moreover, chitin oligomers released from CHIT1 could possibly further enhance CHIT1 expression that becomes a positive feedback cycle leading to more chitin degradation. To investigate whether the treatment with a chitin-containing entity prompted *Chit1* transcription induction, BMDMs were stimulated with HDM mite body, *C. albicans* hyphae (i.e. entities containing macroscopic chitin) or Pam3 (small molecular TLR2 agonist) and C10-15 (already oligomeric chitin) and subjected to RNA isolation and then qPCR analysis. The results showed that only *C. albicans* hyphae induced 2-3 fold of *Chit1* mRNA levels **(Figure 14A)**. Conversely, other stimuli like HDM, Pam3, and C10-15, all failed to induce *Chit1* even though they exhibited strong induction of *Il6* **(Figure 14B)**. To further investigate whether *Chit1* induction by *C. albicans* hyphae is TLR2 dependent, I again employed *Tlr2* KO BMDMs. However, the results showed that there was no significant difference in *Chit1* induction between WT and *Tlr2* KO BMDMs **(Figure 14C)**. In contrast, in the absence of TLR2, the cells exhibited a reduction of *Il6* upon stimulation with *C. albicans* and C10-15, and *Il6* induction was also completely abolished by Pam3 stimulation compared to WT **(Figure 14D)**. These data suggest that other factors of *C. albicans* hyphae like cell wall components such as β-glucan or α-mannan instead of chitin might be responsible for mediating *Chit1* induction.

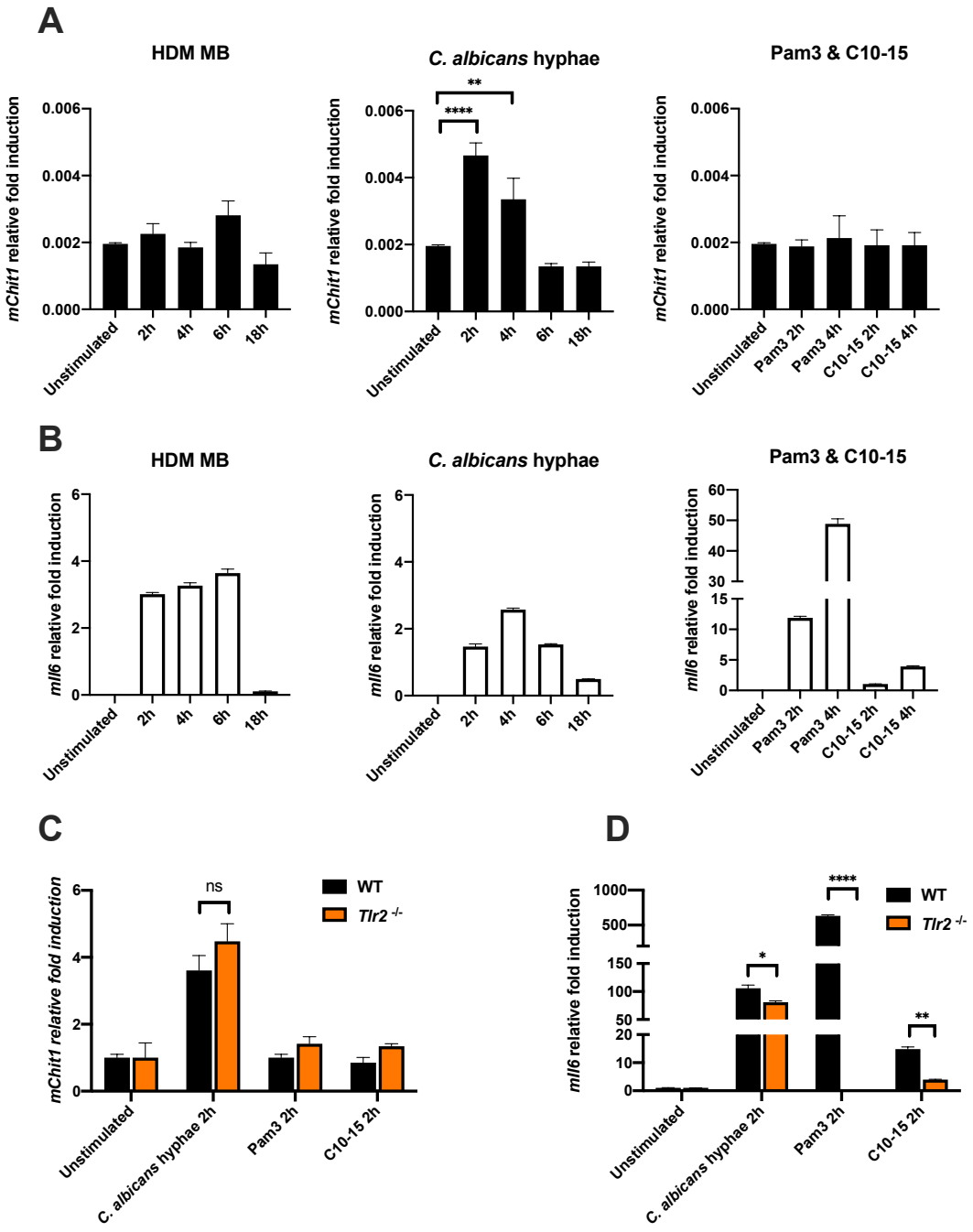


Figure 14. *C. albicans* hyphae induces TLR2-independent murine *Chit1* expression. (A & B) Relative fold induction of murine *Chit1* and *Il6* mRNA in BMDMs upon stimulation with 100 µg/ml HDM MB (house dust mite, mite body), *C. albicans* hyphae, 40 nM Pam3 and 20 µM C10-15 as indicated time course. (C & D) Relative fold induction (normalized with unstimulated) of murine *Chit1* and *Il6* mRNA in WT and TLR2 KO BMDMs upon stimulation with *C. albicans* hyphae, Pam3 and C10-15 for 2h. (A & B) Data are from one representative of two independent experiments. (C & D) Data are from one experiment which is still a preliminary result. Error bars indicate standard deviation of the mean. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ [(A & B) One-way ANOVA with follow up Dunnett's multiple comparisons test; (C & D) Two-tailed Student's *t*-test]

3.7 Co-receptor of chitin sensing

After confirming and testing other models to check TLR2 dependency, I aimed to assess the involvement of a co-receptor in the detection of chitin. TLR2 is known to work together with TLR1 and TLR6 to form heterodimers upon their relative ligand stimulation (Oliveira-Nascimento *et al.*, 2012). From our previous study, we have shown that blocking TLR1 and TLR2 but not TLR6 on HEK-Dual ™ hTLR2 inhibited the NF-κB activity upon C10-15 stimulation (Fuchs *et al.*, 2018). This suggests that TLR1 but not TLR6 plays a potential role as a TLR2 co-receptor to recognize oligomeric chitin. To further investigate and to prove that TLR1 directly interacts with TLR2 for chitin sensing, I applied bimolecular fluorescence complementary (BiFC) assay to examine the TLR receptor interaction. The idea for BiFC assay is to fuse different TLR receptors (e.g. supposed heterodimeric TLR1 and TLR2) to the two halves of a "split" fluorescence protein, e.g. the N-terminal and C-terminal half of the mLumin fluorescent protein, a derivative of far red fluorescent protein **(Figure 15A)** (Christians *et al*., 2019). If the co-transfected split-mLumin tagged TLRs form a heterodimer in response to chitin or other cognate ligands, the two halves of the mLumin protein can be close enough to form a complete mLumin fluorophore and hence generate a fluorescent signal **(Figure 15B)** (Christians *et al.*, 2019; Kerppola, 2006). In this experiment, I firstly cloned His and Myc tagged TLR1, TLR2 and TLR6 receptors and checked the protein expression of these receptors by immunoblot and their functionality to the corresponding ligands using dual luciferase assays **(Figure 15C)**. The results of immunoblotting confirmed that the generated split-mLumin plasmids indeed encoded full-length TLR1, TLR2 and TLR6 proteins **(Figure 15C)**. The single- or co-transfection of TLR1 and TLR2 responded and enhanced NF-κB activity by C10-15 and Pam3 stimulation while there was no increased NF-κB response without ligand stimulation **(Figure 15D)**. Next, I moved to test the heterodimerization of TLR1/TLR2 and TLR2/TLR6 upon stimulation with Pam2, Pam3 and C10-15 using confocal fluorescence microscopy. Apart from the mLumin channel, cells were enumerated using Hoechst DNA staining. Representative confocal images shown in **Figure 16A** indicate that TLR1/TLR2 co-transfected HEK cells, without any ligand stimulation,

already expressed a low mLumin fluorescence, indicative of some ligand-independent preformed dimer formation. Conversely, in TLR1/TLR2-transfected HEK cells stimulated with Pam3 and C10-15 but not Pam2 the mLumin signal was greatly enhanced **(Figure 16A)**, whereas, TLR2/TLR6-transfected HEK cells more pronouncedly responded to Pam2 but not Pam3 and C10-15 stimulation **(Figure 16B)**. By using Fiji ImageJ to quantify the mLumin signal across experiments and multiple images, the results showed that stimulation of C10-15 and Pam3 significantly enhanced mLumin intensity in TLR1/TLR2 HEK cells, while TLR2/TLR6 HEK cells only significantly responded to Pam2 **(Figure 16C and 16D)**. Though TLR2/TLR6 co-transfected HEK cells showed a slight increase of mLumin intensity by C10-15 and Pam3 stimulation, it did not reach statistical significance **(Figure 16D)**. Collectively, and in line with the earlier study from the use of blocking antibodies, these data indicate that chitin oligomers induced TLR1/TLR2 but not TLR2/TLR6 heterodimerization.

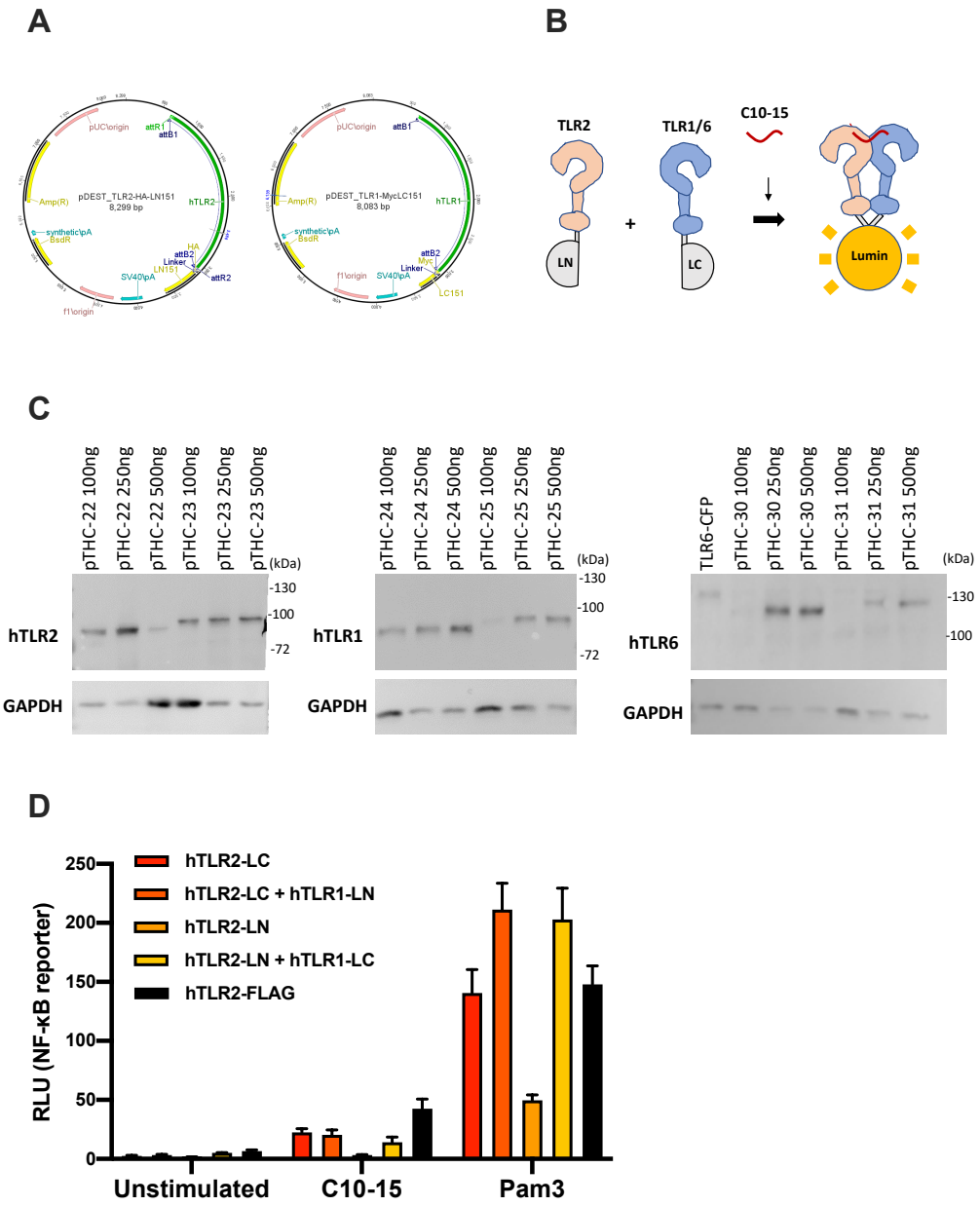


Figure 15. The cloning strategy and the receptor functional check for split-mLumin plasmids.

(A) Plasmid maps from split-mLumin destination vectors after cloning of the hTLR1 and hTLR2. Maps are generated in Geneious Pro version 6.4.5. (B) Working model of bimolecular complementary assay with TLR2- and TLR1/TLR6-split-mLumin plasmids. (C) Protein expression of the split-mLumin-TLR2, -TLR1 and -TLR6 plasmids transfected in HEK 293T cells were assessed by immunoblotting using anti-hTLR2, -hTLR1 and -hTLR6 antibodies. GAPDH was internal control. (D) DLA measurement of NF-κB response in split-mLumin single- or co-transfected HEK 293T cells after 18 h stimulation with C10-15 or Pam3. Data are from one representative of two independent experiments. Error bars indicate standard deviation of the mean.

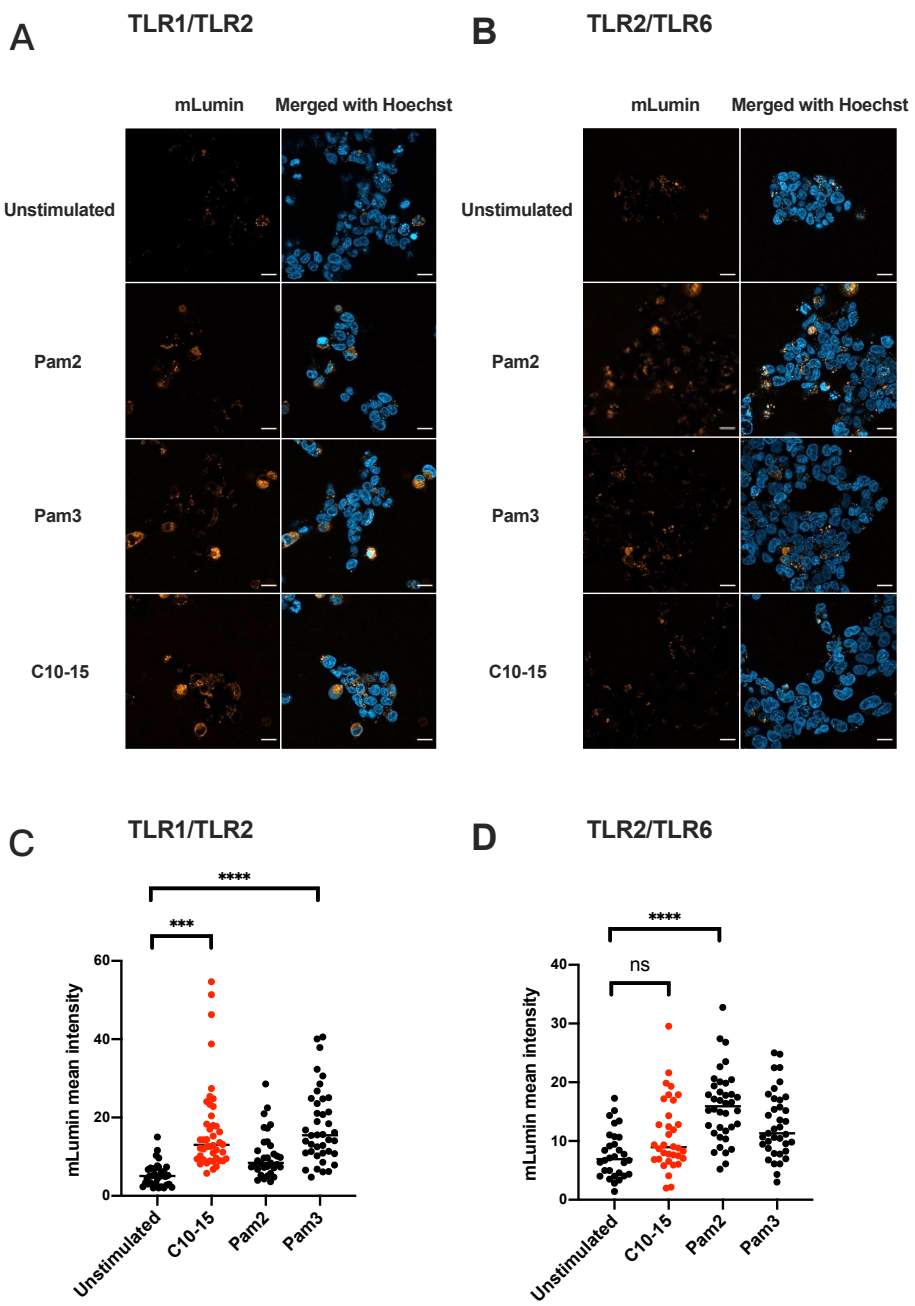


Figure 16. TLR1 but not TLR6 serves as a co-receptor of TLR2 to form heterodimer in response to chitin stimulation.

HEK 293T cells were co-transfected with (A) split-mlumin-TLR1 and -TLR2 or (B) split-mlumin-TLR2 and -TLR6. After 18 h stimulation Pam2, Pam3 or C10-15, cells were stained with Hoechst then viewed under Zeiss LSM800 confocal microscope. The white scale bars represent 20 μm. (C & D) The mLumin fluorescent intensity was quantified by ImageJ. The fluorescent signal from each single cell was threshold and measured by using Otsu method. The mean intensity of mLumin was normalized with Hoechst. Data are (A & B) from one representative of three independent experiments or (C & D) from pooled with two independent experiments. Error bars indicate standard deviation of the mean. *** $p < 0.001$, **** $p < 0.0001$ [one-way ANOVA with follow up Tukey's multiple comparisons test]

Next, I sought to further investigate whether C10-15 induced TLR1/TLR2-dependent inflammatory cytokine production on KO macrophages. BMDMs from WT, *Tlr1* KO, *Tlr2* KO and *Tlr6* KO mice were stimulated with C10-15 and several TLRs ligands as receptor control. The results showed that BMDMs in the absence of TLR1 and TLR2, but not TLR6, significantly reduced IL-6 and TNF production upon C10-15 stimulation **(Figure 17A and 17B)**. The results of TLRs ligand control also showed that in the absence of their correlated TLR receptors failed to produce inflammatory cytokine **(Figure 17C and 17D)**. Taken together, these data demonstrate that chitin oligomers induce TLR1/2 heterodimerization to produce inflammatory cytokine production on macrophages.

On the other hand, preliminary data from my colleague Carsten Leo Greve applied immortalized keratinocyte cell line, N/TERT-1 cells, to stimulate different TLR ligands and then test the pro-inflammatory cytokine response. Interestingly, the results showed that N/TERT-1 cells stimulation of chitin C10-15, Pam3 and *C. albicans* cannot induce IL-8 and IL-6 production while the other TLRs ligands like poly I:C, Pam2, FSL-1, zymosan and the ssRNA plus LL37 (which knowns to activate TLR8 and trigger NETosis in neutrophils (Herster *et al*., 2020)) can trigger various level of IL-8 and IL-6 secretion **(Figure 18A and 18B,** C. L. Greve, personal communication**)**. It seems that N/TERT-1 cells did not or expressed less of TLR1 thus the heterodimerization of TLR1/TLR2 could not be triggered by those cognate ligands like C10-15 and Pam3. However, N/TERT-1 cells did express TLR2 and TLR6 according to the amount of cytokine response by Pam2, zymosan and FSL-1 stimulation **(Table 2)**. These data implicate that cells which express TLR1 and TLR2 and their heterodimerization are essential for chitin sensing.

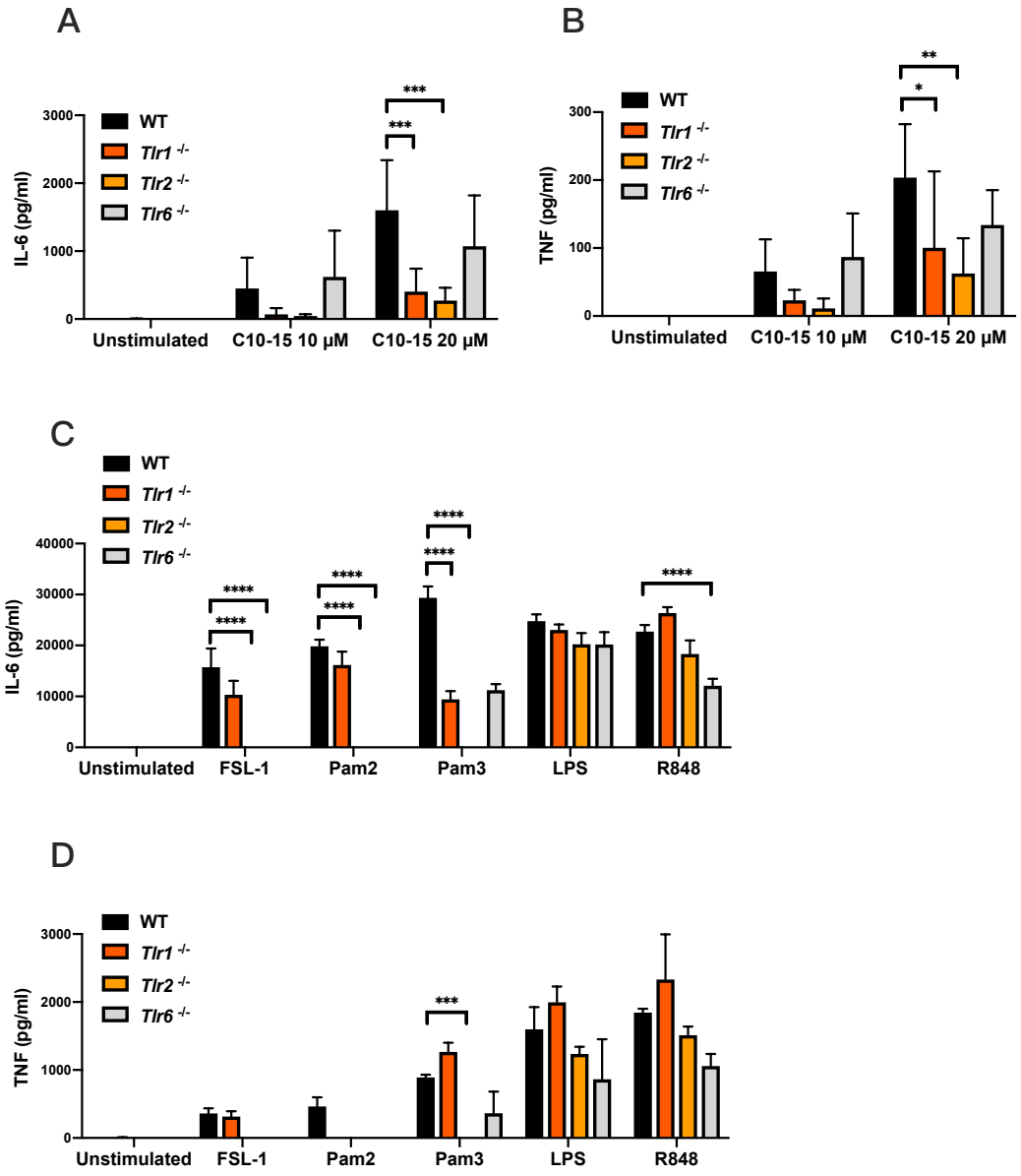


Figure 17. TLR1 but not TLR6 serves as a co-receptor of TLR2 to mediate the inflammatory cytokine production in response to chitin stimulation in BMDMs. (A - D) Murine IL-6 and TNF production in WT, *Tlr1* KO, *Tlr2* KO and *Tlr6* KO BMDMs upon 18 h stimulation with stated ligands measured by ELISA. (A & B) Chitin C10-15 was used to stimulate mGM-CSF differentiated BMDMs. (C & D) FSL-1, Pam2, Pam3 LPS and R848 were used as ligand control to check BMDMs receptor specificity. (A & B) Data are pooled with four individual means of triplicates from two independent experiments. (C & D) Data are from one representative of three independent experiments. Error bars indicate standard deviation of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ [two-way ANOVA with follow up Sidak's multiple comparisons test]

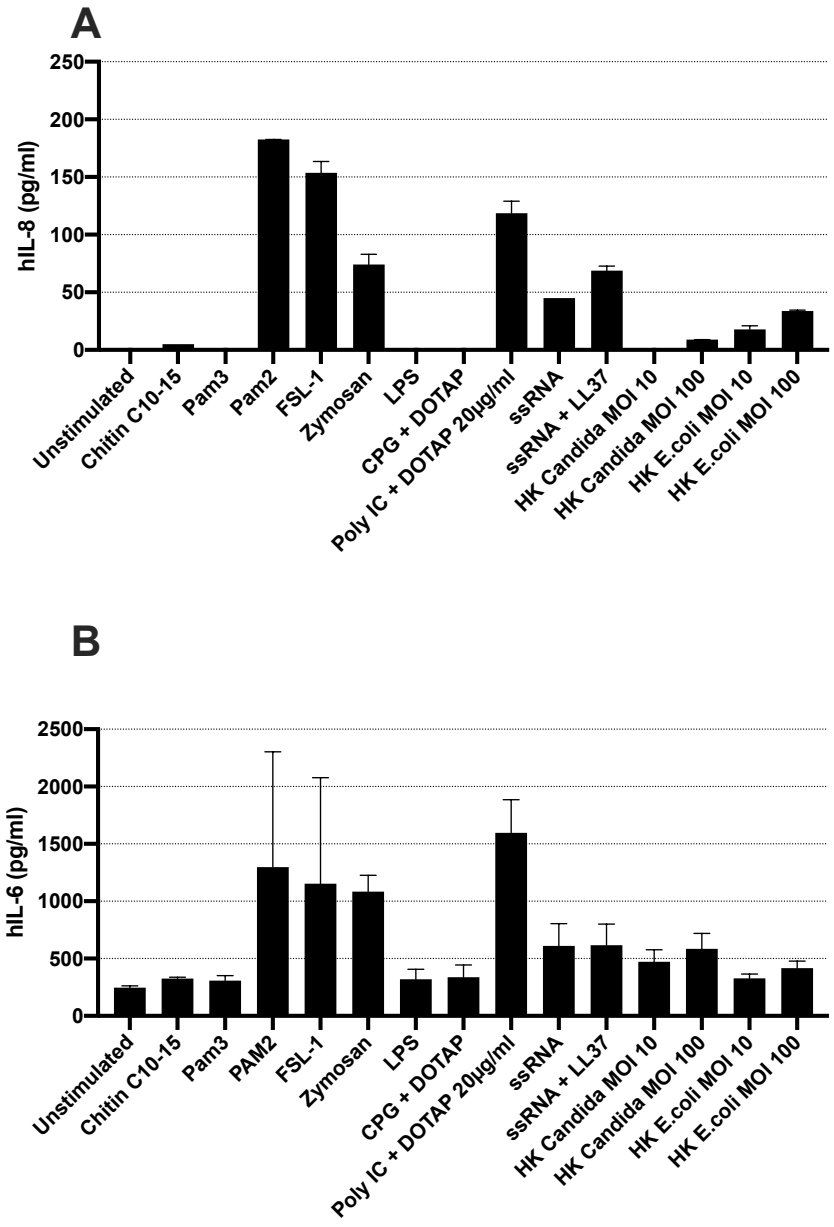


Figure 18. N/TERT-1 cells exhibit TLRs ligands specificity to produce pro-inflammatory cytokines.

(A & B) Human IL-8 and IL-6 production in N/TERT-1 cells upon 24 h stimulation with stated ligands measured by ELISA. The use of TLRs ligands and their concentrations for stimulation were listed in **Table 8**. (A) Data are from one representative of four independent experiments. (B) Data are preliminary results. Error bars indicate standard deviation of the mean.

3.8 Accessory proteins are involved in oligomeric chitin-induced TLR2 activation

It has been known for some time that CD14 plays an essential role as a co-receptor for TLR2 and TLR4 (Ranoa *et al.*, 2013). Moreover, LBP can bring the LPS to CD14 and ultimately the MD-2/TLR4 to form a ternary complex (Ryu *et al.*, 2017). This complex facilitates the LPS to bind to TLR4 to trigger and enhance the downstream signaling (Ryu *et al.*, 2017). Similar results were obtained by LBP for the sensing of TLR2 ligands (Ranoa *et al.*, 2013). In this study, I therefore hypothesized that CD14 and LBP protein also participate in TLR2 chitin sensing. To examine the role of CD14 and LBP, I co-transfected CD14 and TLR2 to HEK 293T NF-κB reporter system and then added different doses of recombinant LBP plasmid together with stimulation of C10-15 and TLR2 specific ligands. The results showed that CD14 transfection increased the TLR2-NF-κB response mediated by C10-15, Pam3 and Pam2 **(Figure 19A, 19B and 19C)**. Surprisingly, adding LBP enhanced TLR2-NF-κB activity upon C10-15 and Pam3 stimulation even further **(Figure 19A and 19B)**. By using three different doses of LBP, it was observed that only an LBP concentration of 25 nM can significantly boost the TLR2 response **(Figure 19A and 19B)**. Interestingly, adding LBP did not increase the Pam2 sensing at any dose **(Figure 19C)**. It thus seems that LBP might have a preference for a certain heterodimer, namely TLR1/2. It should be noted that in the presence of CD14 and LBP the background of TLR2-NF-κB activity was also increased; however, this background did not influence the overall outcome **(Figure 19D)**. Together, these data demonstrate that CD14 and LBP protein can facilitate chitin sensing by TLR2. Additionally, this CD14 and LBP effect might be specific to the ligand of TLR1/TLR2 heterodimerization.

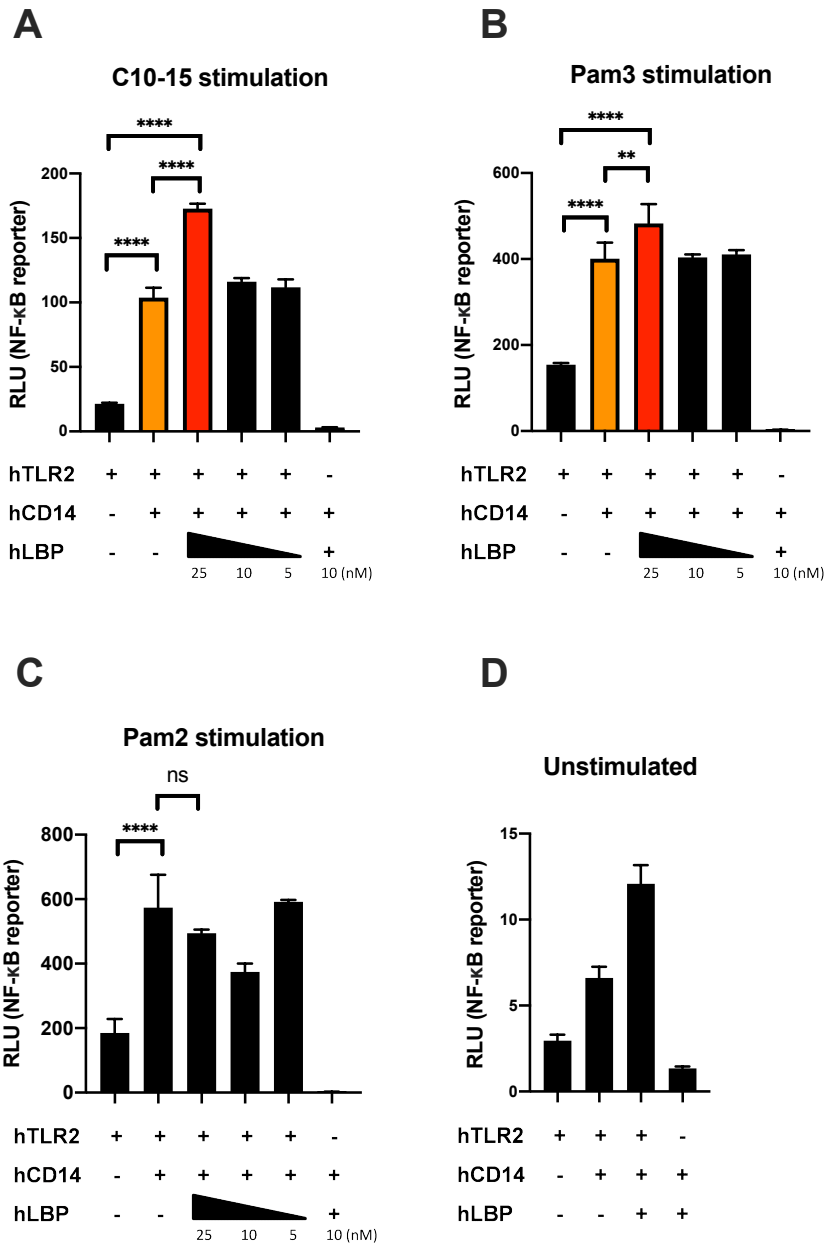


Figure 19. LBP and CD14 enhance TLR2-NF-κB response to chitin.

(A - D) DLA measurement of NF-κB response in TLR2 or TLR2/CD14 co-transfected HEK 293T cells after 18 h stimulation. Dose titrated recombinant LBP protein was added together with the stimuli. Data are from one representative of four independent experiments. Error bars indicate standard deviation of the mean. ** $p < 0.01$, **** $p < 0.0001$ [one-way ANOVA with follow up Sidak's multiple comparisons test]

3.9 Summary of the main findings

For summary, we firstly showed that the unique fractionated chitin oligomers in a specific size of DP 10 – 18 could induce TLR2 dependent inflammatory cytokine response. To provide the evidence that the chitin oligomers exist and could release from chitin particles that become immunogenic, I applied CHIT1 as a tool of MAMPs generator to digest different chitin sources and subsequently assess the TLR2 response. Surprisingly, the results showed that CHIT1 isoform 39 kDa degradation on chitin flakes, HDM and *C. albicans* could allow them to become more immunogenic to stimulate TLR2 activation. Furthermore, even though cells were without direct contact with chitin-rich organisms or particles, preassembly of the diffusible chitin oligomers from CHIT1 degradation elicited TLR2 activation. Finally, we further showed that TLR1 but not TLR6 serves as a co-receptor with TLR2 to form a heterodimer in response to chitin stimulation. The accessory proteins, CD14 and LBP, both play important roles in chitin sensing with TLR2. In overall, we provide new insight into the role of CHIT1 as a MAMPs generator and unravel the detailed mechanism of oligomeric chitin sensing with TLR2. Based on our findings, here **Figure 20** is the proposed working model.

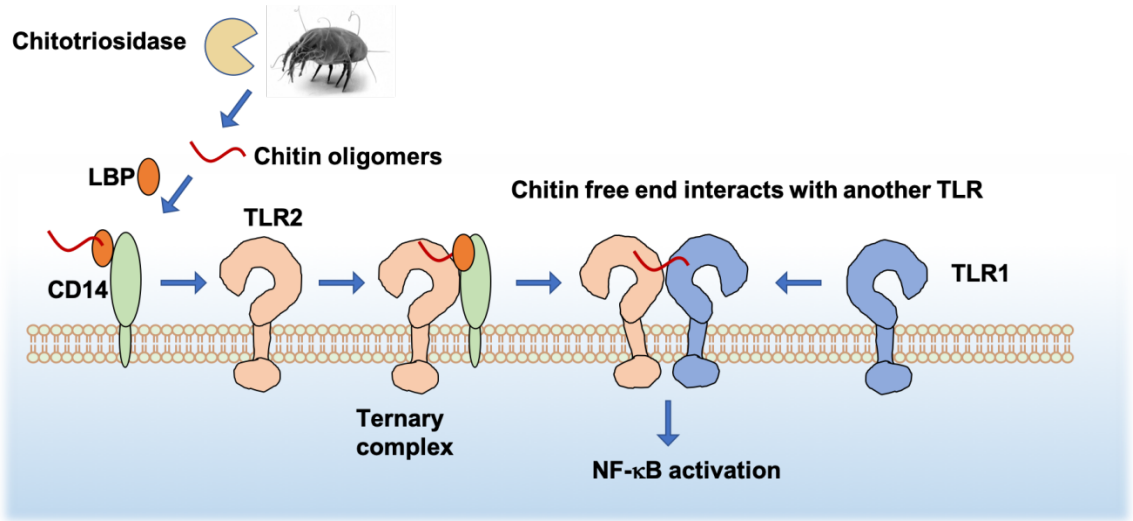


Figure 20. Human chitotriosidase generates MAMPs from chitin-rich organisms to activate TLR1/TLR2-NF-κB pathway.
Chitotriosidase can digest the chitin rich organisms to release the small size of chitin

oligomers. LBP protein helps to bring the chitin to CD14. Then CD14 can transfer the oligomeric chitin to TLR2 to form a ternary complex. In the meantime, the chitin free end interacts with TLR1 to form a heterodimer inducing NF-κB activation.

Chapter 4: Discussion

Chitin recognition by innate immune cells is a long-term controversial issue since many factors including purity, size, solubility, and origin can influence differently and lead to disputed conclusions. Until recently, pure and defined chitin oligomers in the specific range of a size (10 – 15 units of GlcNAc) could be generated, which can trigger innate immune responses via TLR2 recognition (Fuchs *et al.*, 2018). In this study, we sought to unearth the immune response to natural sources of chitin oligomers. Chitin oligomers can be generated from the degradation of chitin particles and chitin-containing organisms by chitinolytic enzymes, chitinases. We showed that human chitotriosidase (CHIT1), especially CHIT1 39 kDa isoform, possessed the ability to generate immunogenic chitin oligomers to activate TLR2. We further demonstrated that not only commercial chitin particles from shrimp shelves, but also natural house dust allergens and pathogenic fungi, which are allowed to be catalyzed by CHIT1 to expose or release their "MAMPs" as oligomeric chitin, can be recognized by TLR2. To this end, we further identified TLR1 as a co-receptor to TLR2, and CD14 and LBP as ligand delivery-accessory proteins that contribute together to the chitin-mediated inflammatory cytokine response.

In this chapter, I will mainly discuss (1) size and co-receptor dependent recognition of oligomeric chitin, (2) the role of CHIT1 as a novel MAMP generator enzyme, (3) effects of CHIT1 on known chitin-containing organisms, (4) overall similarities and differences of the chitin-chitinase-receptor sensing system in plants and invertebrates, and (5) therapeutic implications.

4.1 Size and co-receptor dependent recognition of oligomeric chitin

4.1.1 Different chitin DPs induce size dependent TLR2 activation

Several studies have indicated that chitin can be recognized by innate PRRs and subsequently induce inflammatory cytokine response. However, because of the different source, size, and purity of the used preparation, the actual PRRs, and precise signaling events for chitin recognition still remained largely elusive. Size-dependent

effect of chitin recognition has been a long-term controversial issue. The chitin particles prepared from shrimp shelve or shellfish waste are generally larger than 10 μm in dimension, and the immune-stimulatory effect seems incompletely clear (Da Silva *et al.*, 2009; Da Silva *et al.*, 2008). Notably, Alvarez et al pointed that the "very small" chitin (less than 2 μm) isolated from *C. albicans* and *A. fumigatus* can significantly induce IL-6 production (Alvarez, 2014). It seems that chitin either in a range of specific small size or from different origins can be immunostimulatory or inert. In this present study, we successfully isolated defined-size of fractionated DPs from chitosan (MW < 3000 Da) by SEC and subsequently performed full chemical acetylation to obtain pure, size-specific chitin oligomers. The stimulation results showed that these individual chitin oligomers induce size-specific TLR2-dependent inflammatory responses. These fractionated chitin oligomers in the range of DP 14 – 18, at equimolar concentrations, showed an increasing ability to trigger TLR2 activity and a trend of going down after DP 18. Accordingly, these data are consistent with our previous findings as comparing with chitin oligomers DP10-15 (Fuchs *et al.*, 2018) but also go beyond. We proved that the activation ability does not simply keep increasing the longer chain length, but rather it reaches an optimal stimulatory potential at around DP 16, and longer chitin oligomers decrease the potential. Additionally, according to Hamley *et al*, the length of Pam3 is 3.6 nm that is known as the ideal length to induce and stabilize TLR2/TLR1 dimerization (Hamley *et al*., 2014). In line with our previous study in *in silico* docking model, we showed that 10 GlcNAc units long chitin oligomer is approximately 4 nm which 5 GlcNAc units are located inside the TLR2 hydrophobic pocket and the other GlcNAc units are protruding out (Fuchs *et al.*, 2018). Therefore, it is plausible that the chitin oligomers with 10-16 DPs would be the ideal length to dock in the TLR2 hydrophobic pocket and bridge the TLR1 to form a heterodimer. The detailed mechanism of TLR1/TLR2 heterodimerization will be discussed in the following section.

4.1.2 Chitin induces TLR1/TLR2 heterodimerization but not TLR2/TLR6. The similarity to other TLRs heterodimerization interaction with MAMPs

TLR2 is involved in recognizing a variety of MAMPs from pathogens, including fungi, viruses, bacteria, and parasites (Oliveira-Nascimento *et al.*, 2012). The diversity of ligand recognition through TLR2 can be accounted to its ability to form heterodimers with TLR1 or TLR6, or synergize with another co-receptor like TLR4 or Dectin-1 (Oliveira-Nascimento *et al.*, 2012). Crystallographic evidence shows that binding of the Pam3 induces the hydrophobic interaction of the TLR1 and TLR2 ectodomains whereas binding of the Pam2 does not (Jin *et al.*, 2007). In our previous study, chitin oligomers GlcNAc 10 units perfectly accommodate into the TLR2 ectodomain of hydrophobic pocket, which is as similar as TLR2 binding to the lipopeptides Pam2 and Pam3 through validating by SSL3 blocking assay (Fuchs *et al.*, 2018; Koymans *et al.*, 2015). Thus, we speculated that the protruding end of chitin oligomers supposedly interacts with another co-receptor (Fuchs *et al.*, 2018). In this study, chitin oligomers induced TLR1/TLR2 but not TLR2/TLR6 heterodimerization to trigger downstream NF-κB signaling and subsequently produce inflammatory cytokines. These results not only fit our previous *in silico* docking model suggesting a co-receptor but confirmed and supported the previously shown receptor blocking assay on TLR2-HEK dual cells, which showed a significant reduction of C10-15-mediated NF-κB activity by blocking with anti-TLR1 and anti-TLR2 but not anti-TLR6 antibodies (Fuchs *et al.*, 2018). It appears that oligomeric chitin recognition by TLR2 is as similar as Pam3 recognition which features in TLR1/TLR2 heterodimerization. As we earlier described that DP 16 would be the optimum size of chitin oligomers to activate TLR2, this specific length of oligomeric chitin might be critical to promote TLR1/TLR2 heterodimerization (Fuchs *et al.*, 2018). Besides, this TLR1/TLR2 heterodimerization also occurs in recognition of lipomannan from *Mycobacterium* (Jimenez-Dalmaroni *et al.*, 2009), porins from *Neisseria meningitidis* (Massari *et al.*, 2006), and glucuronoxylomannan from

Cryptococcus neoformans (Fonseca *et al.*, 2010) that presumably ration the similar mechanism as chitin recognition.

4.1.3 The role of CD14 and LBP to facilitate the chitin sensing by TLR2

The accessory proteins have been shown to participate in ligand delivery and enhancement of TLR2 activation (Oliveira-Nascimento *et al.*, 2012). In the present study, our data demonstrated that CD14 and LBP can facilitate and enhance TLR2 activation through oligomeric chitin recognition. By comparing with Pam2 and Pam3 stimulation, it appears that CD14 and LBP exert possibly a preference for TLR1/TLR2 heterodimerization. Since we described earlier that chitin oligomers can dock into TLR2 hydrophobic pocket and can form a heterodimer with TLR1 which is similar to Pam3 interaction to TLR2 (Fuchs *et al.*, 2018; Jin *et al.*, 2007), the strategy of ligand delivery of CD14 and LBP might therefore prefer this TLR1/TLR2 hydrophobic interaction rather than interface interaction of TLR2/TLR6 heterodimerization with Pam2 (Kang *et al.*, 2009). Additionally, CD14 and LBP are known as accessory and ligand delivery proteins from LPS to TLR4-MD2 which also has a hydrophobic binding pocket (Ryu *et al.*, 2017). In this context along with the previous description, chitin oligomers in the range of around 16 units of GlcNAc could facilitate the binding with CD14 and LBP to reinforce the TLR1/TLR2 hydrophobic interaction (Jin *et al.*, 2007). Further investigations like immunoprecipitation assay and protein crystallographic analysis are needed to confirm the formation of chitin-TLR2/TLR1 and its ternary complex with accessory proteins.

4.1.4 Chitin recognition on epithelial cells

In this study, we showed that the keratinocyte cell line, N/TERT-1 cells, did not respond to the stimulation of chitin C10-15, Pam3 and *C. albicans* while it reacted with zymosan, Pam2 and FSL-1 to produce IL-6 and IL-8. It could be reasoned that N/TERT-1 cells lack TLR1 expression thus the TLR1/TLR2 heterodimerization cannot be triggered via

its correspondent agonists. However, one recent study showed that FIBCD1-overexpressed A549 cell line suppresses TLR2 and TLR4 agonist-induced IL-8 and TNF production (Jepsen et al., 2018). In partially consistent with our results, N/TERT-1 cells also showed no response to LPS (TLR4 agonist) and Pam3 (TLR1/TLR2 agonist) stimulation. Since FIBCD1 has been identified as a chitin binding receptor and is mainly expressed in human epithelial cells (Thomsen et al., 2010), it could be possible that N/TERT-1 cells also express FIBCD1 which binds to chitin and meanwhile inhibits TLR1/TLR2 and TLR4 downstream signaling events. These overall observations can also support one recent study that showed the role of FIBCD1 in regulating the balance of the mycobiome to prevent the uncontrolled growth of fungi and dampen the intestine inflammation (Moeller et al., 2019). Our future work could firstly check the TLRs and FIBCD1 expressions on N/TERT-1 cells and validate the receptor binding effect for those cognate ligands. Besides, the detailed mechanism of a negative regulator of FIBCD1 in fungal chitin sensing would be interesting for further investigation.

4.2 CHIT1 as a novel MAMP generating enzyme

4.2.1 Chitin-containing organisms are actually inert when it comes to TLR2, and CHIT1 renders them immunostimulatory

CHIT1 has been identified as an innate immune activation marker in several studies. The increased expression of CHIT1 has been associated with various disease models like cystic fibrosis, COPD, and systemic candidiasis (Mack *et al.*, 2015). Since the previous studies of endochitinase activity of CHIT1 showed efficient degradation to chitin substrates (Kuusk *et al.*, 2017; Stockinger *et al.*, 2015), it is possible that CHIT1 could be a "MAMPs generator" leading to innate immune receptor recognition and subsequently trigger an inflammatory response. However, not so many studies directly link the biological role of the chitinolytic activity of chitinases to the immunostimulatory effect. One interesting study by Gorzelanny *et al* showed that a chitin hexamer could not significantly induce TNF or IL-6 production in THP-1 cells (Gorzelanny *et al*., 2010). While, intriguingly, chitin particles incubated with CHIT1

potentially exhibits macrophages activation leading to more CHIT1 secretion and chitin degradation (Gorzelanny *et al.*, 2010). They indicated that small chitin oligomers released from CHIT1 degradation augment the activation cycle of CHIT1 secretion and processing (Gorzelanny *et al.*, 2010). However, the link between CHIT1 degradation and innate immune responses regarding its receptor recognition was still missing. Thus, we speculated that CHIT1 could degrade chitin-containing components and supposedly the releasing chitin oligomers could subsequently be recognized by TLR2 and elicit an innate immune response. In the present study, we showed that CHIT1 degradation on chitin flakes trigger TLR2 activation. Notably, chitin flakes alone show no response to the TLR2-HEK cells whereas the CHIT1 degradation gives chitin flakes a potentially immunogenic effect to stimulate TLR2 activation. This might be that CHIT1 promptly generates chitin free ends on chitin flakes which are accessible for TLR2 recognition. Unexpectedly, even though without direct contact, CHIT1 degradation might further produce diffusible chitin oligomers which are freely disseminated to activate TLR2. Presumably, these diffusible chitin oligomers might contain a similar extent of DPs, as mentioned earlier like DP 10 – 18, which display the ability to stimulate TLR2. However, because of the insoluble nature of chitin, so far by our limited knowledge and the techniques we have, we could not successfully detect and prove CHIT1 digestions contain chitin oligomers in the range of DP 8 – 18. Once the methodology is improved, the detection of chitin oligomers will be our major interest to further investigate for *in vitro* digestions as well as biologically relevant settings.

4.2.2 Chitinolytic activity of CHIT1 and the comparison with AMCase

More importantly, we confirmed that the immunogenic effect given by CHIT1 degradation accounts for its catalytic activity but not CHIT1 protein itself. Using site-directed mutagenesis, we modified the chitinase catalytic site to become an inactive domain which mimics the domain similar to the chitinase-like protein YKL-40 (Kzhyshkowska *et al.*, 2007). Indeed, the preliminary data of chitin flakes digestion by dose-titrated YKL-40 showed an inability to activate a TLR2 response **(Figure S1)**. Besides, AMCase also has been known for its similar endochitinase activity as CHIT1

(Lee *et al.*, 2011). AMCase is mainly expressed in pulmonary epithelial cells whereas CHIT1 expression is restricted to myeloid cells like macrophages and dendritic cells (Garth *et al*., 2018; Homer *et al*., 2006; Kim *et al.*, 2015). Van Dyken *et al* reported that mice in the absence of AMCase cause environmental chitin spontaneously accumulated in the airways (Van Dyken *et al.*, 2017). They also indicated that epithelial cells which express AMCase mediate the endochitinase activity to digest the inhaled chitin in the airway (Van Dyken *et al.*, 2017). However, whether AMCase degradation on chitin particles can promote inflammatory response is still unknown. It is conceivable that the role of AMCase might be only to clear chitin that mammalian hosts are abundantly exposed to. CHIT1, on the other hand, may be geared towards generating chitin ligands for TLR2 detection and response to the chitin-containing organism. In contrast to previous studies assessing the role of chitinases, we demonstrated that CHIT1 chitinolytic activity renders the inert chitin particle to become immunogenic and highlighted the important role of the chitinolytic activity of chitinases in innate immune responses. Furthermore, we would expect that AMCase endochitinase activity degrades chitin to generate products that are not TLR2-active or in general not immunogenic. Thus, in tissues where both AMCase and CHIT1 are present, AMCase may counteract CHIT1's generation of oligomeric chitin MAMPs. Thus, the role of AMCase in innate immunity is worth future investigation.

4.2.3 Differences between CHIT1 50 kDa and 39 kDa.

It has earlier been reported that human CHIT1 has endochitinase activity (Lee *et al*., 2011). This endochitinase activity is suggested to be more efficient to digest chitin compared to enzymes with exclusive exochitinase activity, like chitinases found in bacteria (Gorzelanny *et al.*, 2010). Endochitinases randomly cleave chitin within the string-like chitin structure (Sahai and Manocha, 1993). This is expected to generate multiple sizes of chitin oligomers in comparison to exochitinase which can only produce dimers or monomers (Sahai and Manocha, 1993). In the present study, our data showed that the CHIT1 39 kDa isoform exhibits higher catalytic activity and leads to a significantly higher inflammatory immune response compared to CHIT1 50 kDa. One

explanation could be that the CHIT1 39 kDa isoform has higher endochitinase activity than the 50 kDa form. However, until now there is insufficient evidence to support such a notion. Recently, Kuusk *et al* confirmed that both the 50 kDa and 39 kDa CHIT1 have similar endochitinase activity (Kuusk *et al.*, 2017). The chitin-binding module (CBM), which is present in the CHIT1 50 kDa form but not in the CHIT1 39 kDa form, does not seem to affect the endochitinase kinetic properties (Kuusk *et al.*, 2017). Thus, it seems that there are not so many differences in their chitinolytic activity in between these two CHIT1 isoforms. However, Stockinger et al reported that by using chitosan as a substrate to test the processivity of CHIT1 two isoforms, CHIT1 39 kDa degradation exhibits a faster appearance of degraded chitosan oligomers than CHIT1 50 kDa dose (Stockinger *et al.*, 2015). These distinct lengths of degraded-oligomers were attributed to the different transglycosylation activity between these two isoforms (Stockinger *et al.*, 2015). In this context, the CBM domain and the transglycosylation activity might be a better explanation for the differences between CHIT1 50 kDa and 39 kDa in the notion of being an effective MAMPs' generator. According to our data, the CBM domain in the CHIT1 50 kDa strongly increases the likelihood of an initially generated oligomer to be cleaved again, resulting in a higher proportion of smaller oligomers compared to what would be expected for the 39 kDa isoform. The inability to "hang on" to the chitin strand would mean a preference to generate multiple and longer oligomers. As discussed above, TLR2 is optimally activated by 16 GlcNAc units long oligomers. These probably arise more in CHIT1 39 kDa digestions. Moreover, the transglycosylation activity of CHIT1 50 kDa might be likely to synthesize oligomers that are not favorable to be sensed by TLR2. This is in line with the observation that CHIT1 39 kDa digestion generates more TLR2 activity from the same macromolecular chitin than the CHIT1 50 kDa. As mentioned earlier, because of the insolubility of chitin, we cannot provide experimental evidence yet that CHIT1 39 kDa can generate suitable sizes of chitin oligomers. The observations that various chitin sources such as chitin flakes, HDM, and *C. albicans* yield more TLR2-dependent inflammatory responses with 39 kDa CHIT1 degradation support this notion.

4.3 Effects of CHIT1 on known chitin-containing organisms

4.3.1 House dust mite-induced allergy response and the correlation with CHIT1 degradation

HDM is a well-known allergy-triggering organism. The role of HDM in triggering Th2 immunity has been extensively studied. Choi *et al* reported that HDM chitin enhances OVA-specific Th2 cell response (Choi *et al.*, 2016). This response was abolished by the treatment of chitinase (Choi *et al.*, 2016). Kim *et al* showed that AMCase is a critical regulator for type 2 allergy response by HDM (Kim *et al.*, 2015). They indicated that AMCase degrades large chitin from HDM and then the degraded chitin could be phagocytosed and subsequently activates caspase-1 in mouse macrophages (Kim *et al.*, 2015). In the absence of AMCase, the type 2 immune response is promoted and sustained by HDM inhalation (Kim *et al.*, 2015). These studies both indicate that chitinase degradation on HDM can generate the small chitin and that degradation ameliorates the HDM-induced allergic response. Intriguingly, the degraded HDM chitin might be potent to trigger an innate immune response (Kim *et al.*, 2015). In the present study, we showed that HDM by CHIT1 degradation enhances TLR2 activation. Without direct contact, CHIT1 degradation on HDM also renders a stimulatory effect to TLR2-HEK cells. Unexpectedly, this immunogenic effect of diffusible HDM oligomeric chitin does not induce IL-6 or TNF production in BMDMs. Only direct contact of CHIT1-digested HDM could stimulate BMDM to produce inflammatory cytokine. There are three possible explanations: (1) Unlike TLR2-overexpressing HEK293T cells, BMDMs probably have lower expression of TLR2 compared to other PRRs, and possibly other MAMPs from HDM could influence these receptors' signaling on BMDMs, masking TLR2 effects. (2) The chitin contents from HDM or, presumably, disseminating chitin oligomers by CHIT1 degradation are probably not sufficient to be recognized by TLR2 on BMDMs. (3) In line with a previous study (Kim *et al.*, 2015), since AMCase is the targeted enzyme in HDM challenging model, it might be possible that AMCase exhibits a preference to HDM and could possibly generate immunogenic chitin on HDM degradation compared with CHIT1.

4.3.2 CHIT1 digestion on fungal chitin and *C. albicans* to trigger innate immune response

In spite of HDM, studies on *C. albicans* give us more evidence to support the concept of releasing diffusible chitin oligomers. Firstly, the surprising finding was that purified fungal chitin alone plays no role in TLR2 activation while CHIT1 degradation appears necessary and sufficient to render fungal chitin capable of triggering a TLR2-dependent innate immune response. Secondly, CHIT1 degradation on both *C. albicans* yeast and hyphae enhanced the TLR2 activation. Remarkably, without direct contact, CHIT1 degradation possibly generates diffusible chitin oligomers from *C. albicans* hyphae to elicit TLR2-dependent IL-6 production. It is noteworthy that these diffusible oligomeric fungal chitins are prone to induce IL-6 secretion in a pattern similar to what was observed for the individually fractionated chitin oligomers. The next four following paragraphs will provide evidence from several studies which can support, discuss and extend these findings.

4.3.2.1 Fungal chitin and CHIT1 degradation

One previous study showed that extracted fungal chitin from *Aspergillus* can induce robust recruitment of eosinophils (Van Dyken *et al.*, 2011). Mice constitutively overexpressed enzymatic activity of AMCase attenuates the eosinophil influx and eases the lung pathology by *Aspergillus* challenge (Van Dyken *et al.*, 2011). However, the cellular event of innate immune response by *Aspergillus*-derived fungal chitin has not been elucidated (Van Dyken *et al.*, 2011). The group of Neil Gow, who provided us the fungal chitin used here, reported that *C. albicans*-extracted fungal chitin cannot induce inflammatory response while it triggers anti-inflammatory IL-10 secretion (Wagener *et al.*, 2014). They further showed that this anti-inflammatory response by fungal chitin is mediated by NOD2, TLR9, and mannose receptor (Wagener *et al.*, 2014). In our present study, consistent with Wagener's findings, fungal chitin alone cannot induce TLR2 activation and stimulate cells to secrete inflammatory cytokines. In contrast, CHIT1 degradation confers immunogenic properties to fungal chitin, namely the ability

to induce TLR2-dependent IL-6 production. This phenomenon is similar to the effect of CHIT1 degradation on chitin flakes. This implicates that no matter what the chitin sources are, once oligomeric chitin is released, TLR2 activation is enabled. On the other hand, Wiesner *et al* reported a pulmonary infection model of *Cryptococcus*-elicited Th2 response via the activation of CHIT1 (Wiesner *et al.*, 2015). In parallel with our observations of CHIT1 in innate immune response, they suggested that chitin fragments released from *Cryptococcus* by CHIT1 cleavage lead to Th2 cell accumulation and pathology (Wiesner *et al.*, 2015). Taken together, theoretically, it could happen that during fungal infection, MAMPs like oligomeric chitin could be generated by chitinolytic CHIT1 that renders the activation of immune cells not only occurring locally but also the distal site of tissues or resident cells.

4.3.2.2 TLR2 recognition on *C. albicans* and oligomeric fungal chitin

Dectin-1 and TLR2 are known to synergize together to sense fungal infection. Unlike Dectin-1, which is already known specificity for β-glucan recognition, in this context what ligands specificity for TLR2 on *C. albicans* still be controversial (Hise *et al.*, 2009; Mora-Montes *et al.*, 2011; Netea *et al.*, 2006). Finally, we previously demonstrated that recombinant TLR2 can directly bind fungal chitin on *C. albicans* (Fuchs *et al.*, 2018). This intrigued us to go deep into a more detailed TLR2 cellular response upon *C. albicans* stimulation. In the present study, we showed that CHIT1 degradation on *C. albicans* yeast and hyphae both increase the TLR2 activation and can enhance the subsequent IL-6 production. We speculated that CHIT1 degradation generates more free ends of chitin oligomers on *C. albicans* surface which is accessible for TLR2 binding. Moreover, CHIT1 might produce diffusible chitin oligomers that allow TLR2 can directly sense them even though without direct contact with *C. albicans.* However, we did not observe TNF production from possibly *C. albicans*-derived chitin oligomers. While the direct contact with *C. albicans* did produce TNF and was partially TLR2 dependent. The possible explanation could be that β-glucan recognized by Dectin-1 majorly produces TNF whereas oligomeric chitin sensed by TLR2 accounts for IL-6 production. This provides new evidence to support and explain the synergistic effect of

Dectin-1 and TLR2: Dectin-1 and TLR2 might recognize β-glucan and chitin, respectively on *C. albicans*, to trigger two distinct signaling cascades leading to different pro-inflammatory cytokine production.

4.3.2.3 Fungal drug resistance and the correlation with chitin exposure

The antifungal drug, caspofungin, also called echinocandin, inhibits the catalytic function of β-(1, 3)-glucan synthases (Adams *et al*., 2008; Wheeler *et al*., 2008). It has been reported that treatment with this drug alters the content of the fungal cell walls including the level of β-glucan and chitin content *(Walker and Munro, 2020; Walker et al*., 2008). Here in this study we also applied caspofungin to *C. albicans* before CHIT1 degradation. Notably, although caspofungin treatment exposed a bit of surface chitin on *C. albicans* yeast, it still cannot induce TLR2 activation. Whereas, the pre-exposure of surface chitin by caspofungin might facilitate CHIT1 degradation. Furthermore, while zymolase digestion on *C. albicans* drastically exposed the surface chitin, the direct contact with the cells only induced lower TLR2 activation compared to *C. albicans* with CHIT1 degradation. Accordingly, these data indicate that only if fungal chitin on *C. albicans* is undergoing sufficiently exposed and catalytic digested, it can become immunogenic and be directly recognized by TLR2.

CHIT1 has been known to play a protective role to restrict the growth of *C. albicans* and serve as a biomarker for cystic fibrosis which is also correlated with *C. albicans* colonization in the lung (Hector *et al.*, 2016; van Eijk *et al.*, 2005; Vandevenne *et al*., 2011). From an opposite perspective, recently Schmitz *et al* reported a counterproductive role of mouse Chit1, showing that mice in the absence of Chit1 significantly reduce kidney fungal burden in systemic *C. albicans* infection (Schmitz *et al*., 2021). Interestingly, using chitinase inhibitor, bisdionin C, it facilitated the neutrophil killing of *C. albicans* hyphae (Schmitz *et al.*, 2021). Collectively, it seems that the role of CHIT1 in *C. albicans* infection and the detailed cellular response of chitinolytic degradation on *C. albicans* are still enigmatic. In addition, the perspective of chitin exposure on *C. albicans* is also a critical event. The increasing level of chitin

not only affects the role of Dectin-1 in the determination of host defense against *C. albicans* infection (Marakalala *et al.*, 2013), but also influences the function of phagocytosis and inflammatory response in macrophages (Walker *et al.*, 2013; Walker and Munro, 2020). Although none of the studies assess the association of releasing chitin and CHIT1, it is possible that in accordance with the chitin exposure on the fungal pathogen, the elevated expressed CHIT1 can digest and release more abundant oligomeric fungal chitin. Besides, as mentioned above, the use of antifungal drugs would be also a severe issue. Once the patients are controlled with echinocandins treatment, they might carry survived *C. albicans* in the tissues where the fungal chitin is possibly kept exposed and then facilitates CHIT1 degradation resulting in more and more disseminating chitin oligomers to the bloodstream (Brown *et al.*, 2012; Walker *et al.*, 2013; Walker and Munro, 2020). Here in this study, we identified the important role of CHIT1 as MAMPs generator to *C. albicans* stimulation that activates the TLR2-mediated inflammatory cytokine response. We suggest CHIT1 as a potential therapeutic target for fungal infectious diseases.

4.3.2.4 Mouse *Chit1* induction by chitin-containing organisms

CHIT1 is known as or to be constitutively expressed in M1 macrophages and the GM-CSF is a potential inducer for CHIT1 induction (van Eijk *et al.*, 2005). However, none of the studies have shown which ligand(s) and the precise cognate receptor(s) mediate CHIT1 expression. Given that CHIT1 is essential for enabling distal sensing of MAMPs, it would make sense for it to be induced when fungi or other chitin-containing particles are detected, for example by phagocytic receptors. In the present study, we showed that mouse *Chit1* gene expression can only be induced by *C. albicans* hyphae in mouse BMDMs while this induction is TLR2-independent. Unexpectedly, neither TLR ligands such as LPS, Pam3, and chitin nor HDM mite body can induce *Chit1* expression. It appears that chitin oligomers in this context cannot directly trigger mouse *Chit1* expression while the direct contact with macroscopic chitin on *C. albicans* probably dose (but not through TLR2). In consistent with the previous study on *Cryptococcus*, the stimulation of macroscopic chitin of *Cryptococcus* can also elicit CHIT1 activity

while the receptor is still unknown (Wiesner *et al.*, 2015). Supposedly, other MAMPs like β-glucans or α-mannans on *C. albicans* hyphae could play a role to trigger *Chit1* induction through recognition of Dectin-1 or Dectin-2 or other phagocytic receptors (Hardison and Brown, 2012). Future studies will firstly employ Dectin-1 deficient immortalized-macrophages to test whether *Chit1* induction is thorough Dectin-1 recognition upon *C. albicans* hyphae stimulation.

In line with this observation, *CHIT1* induction by *C. albicans* hyphae might provide a way to explain why the inflammation keeps arising during systemic *C. albicans* infection even though the *C. albicans* is not found in the bloodstream (Brown *et al.*, 2012). As described above, the survived *C. albicans* might reside in the tissue where it keeps inducing *CHIT1* induction by resident macrophages. Sequentially, CHIT1 might generate oligomeric chitin and then they both release into the bloodstream to cause a positive feedback cycle for deteriorating inflammatory response **(Figure 21)**. In this notion, CHIT1 could be an ideal therapeutic target to ease the aberrant inflammation in systemic candidiasis.

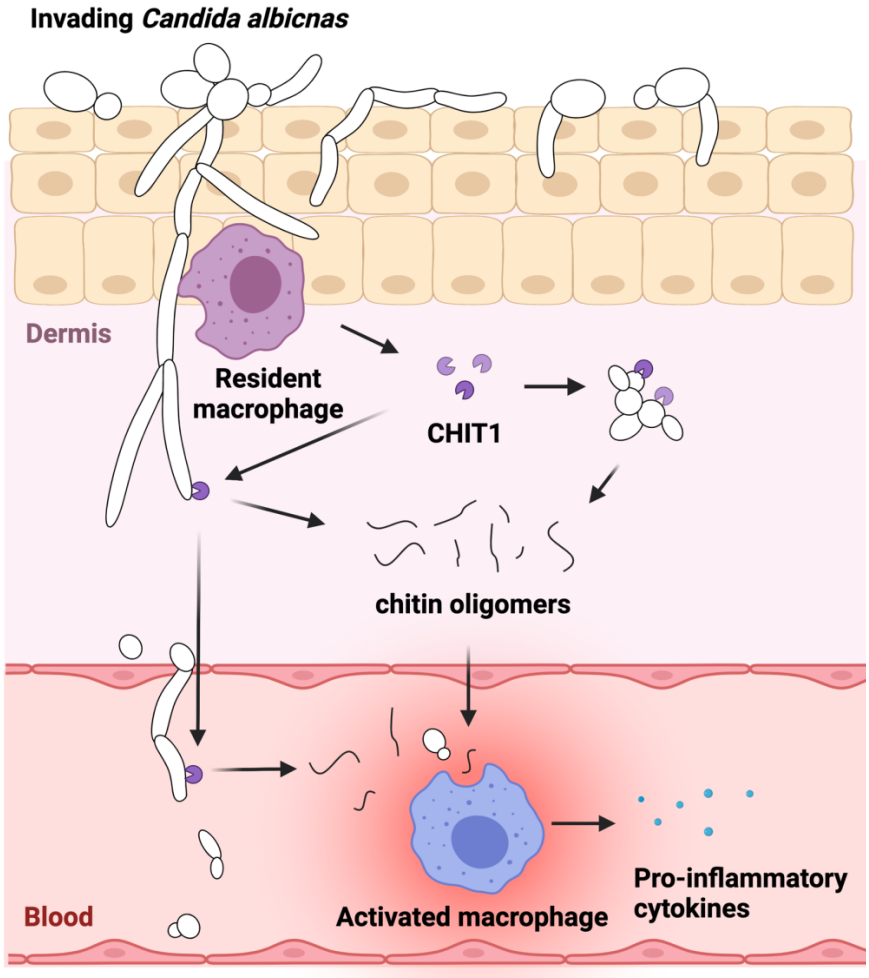


Figure 21. A working hypothesis of CHIT1 as a MAMPs generator in a model of invading *Candida albicans* infection.
The picture was created with BioRender.com. Tissue resident macrophage senses invading *C. albicans* hyphae and subsequently expresses CHIT1 into the dermis. CHIT1 then digests both *C. albicans* yeast and hyphae to release chitin oligomers. Sequentially, chitin oligomers and CHIT1 together with *C. albicans* disseminating to the bloodstream further stimulate macrophages to secrete pro-inflammatory cytokines.

4.3.3 Counter strategies of *C. albicans* against the CHIT1-TLR2 sensing system

During infection, *C. albicans* has developed several efficient strategies to evade attack by the host immune system (Mayer *et al*., 2013). To avoid the attack by the complement system, *C. albicans* displays surface proteins such as phosphor-glycerate-mutase (Gpm1p) (Poltermann *et al*., 2007) and pH-regulated-antigen 1 (Pra1) (Luo *et al*., 2009) to bind the host factor H, FHL1 and plasminogen to inactivate the C3 convertase (Luo

et al., 2009; Poltermann *et al.*, 2007). *C. albicans* also secretes aspartic proteases (Sap) to degrade complement convertases and evade the attack (Gropp *et al.*, 2009). In this study, we hypothesized that *C. albicans* can also exhibit counteractivity against the degradation of CHIT1. Indeed, preliminary data showed that *C. albicans*, in an acidic environment, secrete factors that degrade the CHIT1 **(Figure S2)**. However, the detailed role of Sap proteases and the interaction with CHIT1 still await further investigation.

4.4 Overall similarities and differences of chitin-chitinase-receptor sensing system in plants and invertebrates

4.4.1 Comparison with plants MAMPs sensing systems

In comparison to the study of mammalian chitinases and chitin immune response, the physiological role of chitin and chitinases in plants has been widely described (Grover, 2012; Sahai and Manocha, 1993). The role of chitinases or in general hydrolases in plants are like MAMPs generators, which mostly are endochitinases to randomly cut chitin polymer (Grover, 2012). For instance, the degraded chitin oligomers can be recognized by plants' chitin receptors like CEBiP (Hayafune *et al.*, 2014) and AtCERK1 (Liu *et al*., 2012) through dimerization to initiate plant innate immunity against the invading pathogen (Wan *et al*., 2008). This overall pathway is remarkably similar to what we describe here. In our study, by employing common mammalian pathogens, we demonstrated that CHIT1 degradation on HDM and *C. albicans* also enhances the TLR2 activation. Like dimerization of CEBiP and AtCERK1 in plants, mammalian TLR2 also forms heterodimer with TLR1 upon chitin stimulation. This cross-kingdom of plants and mammalians share a similar mechanism of dimerization in response to chitin oligomers that are both crucial to trigger subsequent innate immunity to defense against pathogen infection. In addition, fungal infection in plants also elicits chitinases upregulation to restrict the growth of fungal pathogen by causing lysis of hyphal tips (Wang *et al*., 2008). This phenomenon is resembling what we observed in *Chit1* induction by *C. albicans* hyphae and also corresponding with CHIT1

in mammalian antifungal strategy (van Eijk *et al.*, 2005). Interestingly, unlike human chitinases, some plants of chitinases possess exochitinase activity as well as the activity of lysozyme (EC 3.2.1.17) that can hydrolyze N-acetylmuramic acid residues in peptidoglycans of bacteria for being an approach to keep from bacterial infection (Collinge *et al.*, 1993). Since up to now, studies in mammalian chitinases are mostly focusing on the chitin-containing organisms, whether mammalian chitinases also share a similar catalytic function to non-chitin organisms are worth future investigation.

4.4.2 Comparison the function of chitinases with *Drosophila*

Chitinases in insects like *Drosophila* also belong to the family of 18-GH that has endochitinase activity as similar as in plants and mammals (Henrissat, 1991). The major function of *Drosophila* chitinases has been known in the turnover of the chitin-containing extracellular matrix such as cuticle and the peritrophic matrix during molting (Arakane and Muthukrishnan, 2010). Interestingly, there is published evidence investigating the immune function of insects' chitinases. Yan *et al*. reported that 50 kDa chitinase gene can be detected in the fat body of the pregnant female of the tsetse fly (*Glossina morsitans*) (Yan *et al*., 2002). They demonstrated that this chitinase gene expression might play role in immune defense against chitin-containing pathogens and also provides protection for larvae and pupae (Yan *et al.*, 2002). On the other hand, one recent study also showed that chitinase-like protein (CLP) in *Drosophila*, IDGF3, plays role in immune protection during entomopathogenic nematode infections (Kucerova *et al*., 2016). Unlike human CHIT1 which utilizes its catalytic activity to restrain the overgrowth of chitin-containing pathogens (van Eijk *et al.*, 2005), *Drosophila* IDGF3 modifies Wingless (Wg) and Jak/STAT signaling for wound healing as well as regenerative processes during nematode infection (Kucerova *et al.*, 2016). Their findings are resembling one study that showed the role of mammalian CLPs in mediating neutrophil recruitment to kill the nematode (Sutherland *et al*., 2014). Collectively, together with our findings that showed the role of mammalian CHIT1 in eliciting an inflammatory response, those conserved 18-GH family of chitinases and

chitinase-like proteins in vertebrates and invertebrates share the analogous setting and function to impact innate immune response and pathogen infections.

4.5 Therapeutic implications

In clinical diagnosis, CHIT1 was originally described in the patient with dysfunction of lysosomal storage, also called Gaucher disease (Bussink *et al*., 2006). In a healthy patient, CHIT1 activity is generally low in the serum. Whereas patients with Gaucher disease, the abnormal activation of macrophages leads to excessive production of CHIT1 (Bussink *et al.*, 2006). In this context, the elevation of CHIT1 serves as a biomarker in many disorders like cystic fibrosis, COPD, allergic asthma and atherosclerosis (Kanneganti *et al*., 2013). In contrast, as described earlier, CHIT1 has been known to play a pivotal role in defense against chitin-containing pathogens infection (Gordon-Thomson *et al.*, 2009). It seems that CHIT1 has "double-edged" roles (pathogenic and protective) depending on the different types of diseases in inflammatory conditions. In earlier section 4.2, we described that CHIT1 plays a role in MAMPs generator to produce immunogenic chitin oligomers during *C. albicans* infection. Theoretically, the elevation of CHIT1 by *C. albicans* stimulation could possibly exacerbate the inflammatory pathology. Although it's been stated the protective role of CHIT1 in systemic candidiasis, the treatment of CHIT1 should be seriously considered for patients bearing an overt or chronic inflammatory condition. Until now two of the known chitinase inhibitors, allosamidin and bisdionin C, have been described and used in several studies (Sakuda S Fau - Isogai *et al*., 1987; Schüttelkopf *et al*., 2011). However, their complex structure and unspecific feature to distinct chitinases make them challenging to apply in clinical use (Gloeckner *et al*., 2010). Thus, an ideal drug specifically targeting CHIT1 still awaits development.

On the other hand, we newly described that TLR1/TLR2 heterodimerization is crucial for chitin sensing. Since so far not so many known TLR2 inhibitors show successful efficacy in treating aberrant TLR2 activation, targeting both TLR1 and TLR2 could be

considered as an alternative therapeutic strategy for chitin-associated diseases and fungal infection.

4.6 Conclusion

In conclusion, we demonstrated a complex system of oligomeric chitin sensing via identifying the potent role of CHIT1 as a MAMP generator and delineating the detailed mechanism of TLR1/TLR2-CD14-LBP-mediating inflammatory cytokine response. Furthermore, based on the results of CHIT1 degradation on HDM and *C. albicans*, we provided a possible explanation for the overt or chronic inflammatory response in chitin-related diseases and fungal infections. The next steps, establishing an *in vivo* model to sense distributed chitin oligomers underlying CHIT1 elevation in *C. albicans* infection or house dust mite challenge would be an advanced leap for better understanding the role of CHIT1 in innate immunity. Finally, our study highlights CHIT1 as a valuable therapeutic target as the primary step to restrict the possibly disseminating chitin oligomers in chitin-related pathologies and systemic fungal infection.

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Appendix

Supplementary figures

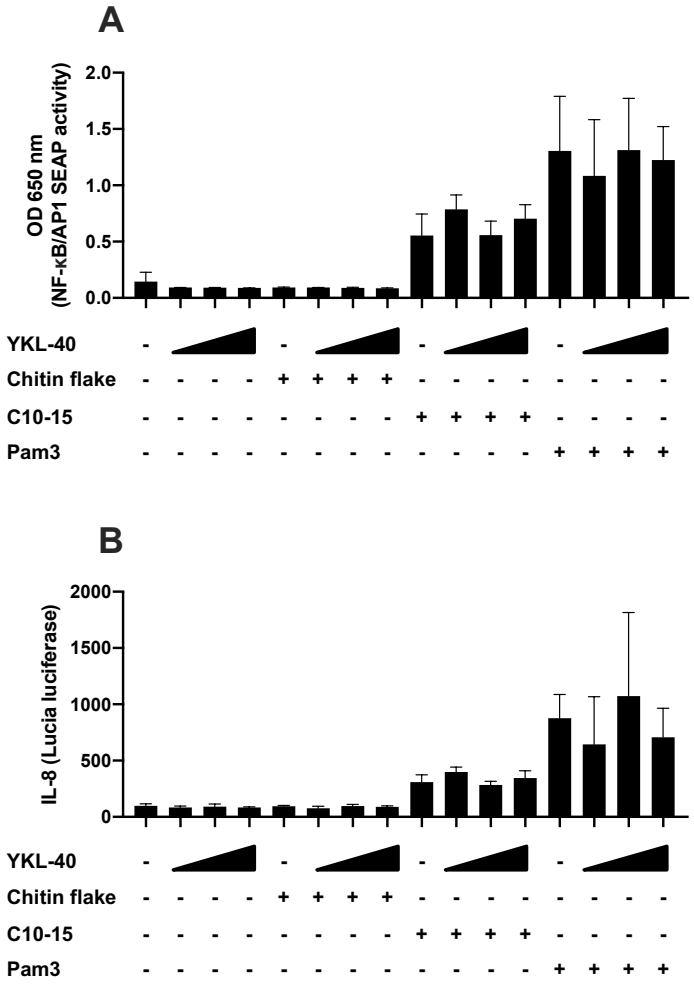

Figure S1. YKL-40 fails to induce TLR2 activation.
Measurement of NF-κB activity in HEK-Dual™ hTLR2 after stimulation with dose-titrated YKL-40 alone or chitin flakes, C10-15 and Pam3 incubated with or without YKL-40. (A) The NF-κB/AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) was reacted with the SEAP detection reagent (Quanti-blue™ solution). The SEAP levels were determined by the plate reader at 650 nm. (B) The IL-8 production was determined by Lucia luciferase activity (Quanti-luc™). Data are preliminary from one experiment. Error bars indicate standard deviation of the mean.

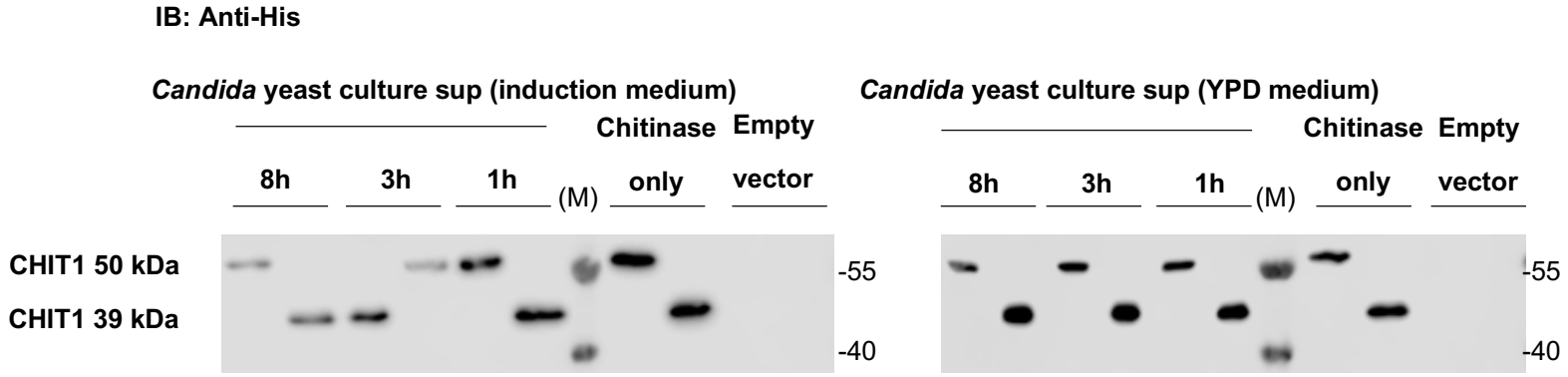

Figure S2. CHIT1 two isoforms are degraded by conditioned *C. albicans* culture supernatants.

C. albicans was cultured in protease induction medium and YPD medium, separately. The culture supernatants were collected and subsequently incubated with CHIT1 50 kDa and 39 kDa from CHIT1-transfected HEK cells respectively. The protein samples were harvested at incubated time point and then applied heat-inactivation. CHIT1 two isoforms expression were assessed by immunoblot with anti-His antibody. Chitinase only was used as CHIT1 expression positive control. "M" is used as marker.

Summary

Chitin (N-acetyl-glucosamine) is a highly abundant polysaccharide in nature and a major component of fungal cell walls, insects and common house dust allergens. Chitin also serves as microbe-associated molecular patterns (MAMPs) which is recognized by pattern recognition receptors (PRRs) of the innate immune system. In our previous study, we demonstrated that chitin oligomers longer than 6 units induce an inflammatory response via the PRR Toll-like receptor (TLR) 2. However, it remained unclear whether the co-receptors and accessory proteins are required for sensing and how chitin oligomers could be released from organisms containing polymeric chitin, which is considered immunologically inert. Interestingly, mammals express chitotriosidase-1 (CHIT1), namely chitinase, which has been determined as a biomarker constitutively expressed in fungal infection, cystic fibrosis and allergic asthma. However, the detailed role of CHIT1 in mediating innate immunity that correlates with its chitinolytic function has not been well understood. Thus, with this knowledge, it was hypothesized that host-chitinases like CHIT1 mediate the degradation of chitin-rich organisms to generate immunogenic oligomeric chitin, whose recognition is mediated by TLR2-expressing innate immune cells. Firstly, by examining two human chitotriosidase isoforms of 50 kDa and 39 kDa, we found that CHIT1 39 kDa isoform degradation products of chitin flakes, *Candida albicans* and house dust mites could induce TLR2-dependent NF-κB activation. Importantly, this immunogenic effect of CHIT1 39 kDa isoform required its intact catalytic activity. Furthermore, we found that chitin sensing of chitin particles and chitin-rich organisms might not be strictly dependent on direct contact, but could be mediated by diffusible oligomers generated in the presence of CHIT1. In addition, by using size exclusion chromatography (SEC) and mass spectrometric (MS) to prepare defined units of chitin oligomers, we found that optimally 14 - 16 units long oligomers triggered TLR2-NF-κB dependent inflammatory responses. To elucidate the involvement of TLR2 co-receptors, analysis in bimolecular complementary fluorescence (BiFC) assays and *Tlr*-deficient murine macrophages were performed and showed that chitin oligomers induce TLR1 and TLR2 heterodimerization to trigger NF-κB signaling and subsequently the

production of pro-inflammatory cytokines. Finally, we demonstrated that CD14 and lipopolysaccharide binding protein (LBP), known mediators in the sensing of other hydrophobic MAMPs, enhanced and facilitated TLR2-NF-κB dependent chitin sensing. In overall, our study not only highlights CHIT1 as a novel therapeutic target for chitin-related inflammation disease but reveals an intricate system of MAMP generation, PRR sensing and induction of an enzymatic MAMP "generase" that bears resemblance to MAMP sensing in invertebrates.

Zusammenfassung

Chitin (N-Acetyl-Glucosamin, GlcNAc) ist in der Natur ein sehr häufig vorkommendes Polysaccharid und ein Hauptbestandteil von Pilzzellwänden, Insekten und gängigen Hausstauballergenen. Chitin-haltige Organismen zerlegen dieses in kleine Fragmente (Oligomere), welche als Mikroben-assoziierte molekulare Muster (MAMPs) dienen können, die von Mustererkennungsrezeptoren (PRRs) erkannt werden. Dieser Abbau von Chitin wird hauptsächlich durch glykosylierte hydrolytische Enzyme, die sogenannten Chitinasen, bewirkt. Eine der menschlichen Chitinasen, die Chitotriosidase-1 (CHIT1), wurde als Biomarker bestimmt, welcher bei Pilzinfektionen, zystischer Fibrose und allergischem Asthma konstitutiv exprimiert wird. Die genaue Rolle von CHIT1 bei der Aktivierung der angeborenen Immunität, die mit dessen chitinolytischen Funktion korreliert, ist jedoch noch nicht ausreichend geklärt. In dieser Arbeit wird gezeigt, dass das oligomere Chitin durch den Chitinasen-Abbau von Chitin-Partikeln oder Chitin-reichen Organismen erzeugt werden könnte, welches von TLR2 als immunogenes MAMP erkannt wird. In einer früheren Studie wurde festgestellt, dass Chitin-Oligomere, die länger als sechs Einheiten sind, eine Entzündungsreaktion über den angeborenen Rezeptor Toll-like receptor (TLR) 2 auslösen. In einem *In-silico*-Docking-Assay Experiment wurde außerdem beobachtet, dass Chitin-Oligomere mit fünf Einheiten in der hydrophoben Tasche des TLR2 untergebracht werden können. Die freien Enden von Chitin-Oligomeren länger als fünf Einheiten, die aus dem TLR2 herausragen, deuteten auf die Möglichkeit einer Beteiligung von Co-Rezeptoren hin. Daraus resultiert der zweite Schwerpunkt der hier vorliegenden Arbeit, nämlich die Entdeckung möglicher beteiligter Co-Rezeptoren an der TLR2-vermittelten Chitin-Detektion. In dieser Studie wurden mit Hilfe der Größenausschlusschromatographie und der Massenspektrometrie definierte Fraktionen von Chitin-Oligomeren bestimmter Längen hergestellt. Mit diesen wurde zunächst die Annahme bestätigt, dass solche Chitin-Oligomere, im Idealfall mit einer Länge von 14-16 Einheiten, die TLR2-NF-κB-abhängige inflammatorische Zytokin-Produktion auslösen. Anschließend wurden zwei Isoformen des menschlichen CHIT1, 50 kDa und 39 kDa, untersucht und festgestellt, dass der Abbau von Chitinflocken, *Candida albicans* und Hausstaubmilben durch die

39 kDa Isoform von CHIT1 eine TLR2-abhängige NF-κB-Aktivierung auslösen kann. Wichtig ist, dass diese immunogene Wirkung der CHIT1 39 kDa-Isoform auf ihrer katalytischen Aktivität beruhte. Darüber hinaus wurde beobachtet, dass die Chitin-Erkennung von Chitin-Partikeln und Chitin-reichen Organismen nicht strikt vom direkten Kontakt abhängt, sondern durch diffusionsfähige Oligomere vermittelt werden könnte, die von der CHIT1 39 kDa-Isoform gebildet werden. Schließlich zeigten Analysen in bimolekularen komplementären Fluoreszenz-Assays (BiFC) und TLR-defizienten Mausmakrophagen, dass Chitin-Oligomere eine TLR1- und TLR2-Heterodimerisierung induzieren, um NF-κB-Signale auszulösen was zur anschließenden Produktion von pro-inflammatorischen Zytokinen führt. Zuletzt wurde gezeigt, dass CD14 und lipopolysaccharide binding protein (LBP) wichtige Zusatzproteine sind, welche die TLR2-NF-κB-abhängige Chitin-Detektion verstärken und erleichtern. Insgesamt hebt diese Studie nicht nur CHIT1 als neuartiges therapeutisches Ziel für Chitin-bedingte entzündliche Erkrankungen hervor, sondern offenbart auch ein kompliziertes System aus MAMP-Generierung, PRR-Erkennung und Induktion eines enzymatischen MAMP-"Erzeugers".

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Contribution declaration

The dissertation work was carried out at the IFIZ, Department of Immunology, under the supervision of Prof Alexander Weber.

Contribution of others:

1. Figure 4: the size exclusion chromatography and mass spectrometric to prepare the purified size specific chitosan oligomers and make them fully acetylation were done by Margareta Hellmann at Münster University.

2. Figure 18: the measurement of cytokine production in N/TERT-1 cells upon different TLRs ligands stimulation was done by our colleague, Carsten Leo Greve.

Own contribution:

Experimental work that is not indicated above, data acquisition, analysis of data and preparation of the figures presented in the results of this thesis.

I hereby confirm that I wrote the manuscript myself (under the supervision of Prof Alexander Weber and Dr. Yamel Cardona) and that any additional sources of information have been duly cited.

Signed_____

on [date] _____ in Tübingen