Identification and Characterization of LYSMD3, a Novel Epithelial Cell Pattern Recognition Receptor for Chitin

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biological Sciences

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September 30, 2019
Blacksburg, VA

Keywords: allergy, Alternaria, asthma, chitin, innate immunity, LYSMD3, pattern recognition receptor

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ABSTRACT

LysM-domain containing (LysMD) proteins are widespread in nature and associated with host-pathogen interactions, often-binding peptidoglycan and chitin. However, the functions of mammalian LysMD proteins have not been fully defined. Chitin, a major component of fungal cell walls, has been associated with allergic disorders such as asthma. However, chitin recognition by mammals remains enigmatic at best. The principal receptor(s) on epithelial cells for chitin recognition remain to be determined. In this study, we demonstrate that LYSMD3 is expressed on the surface of human airway epithelial cells. Interestingly, LYSMD3 is able to bind chitin and β-glucan as well as fungal spores. Knockdown and knockout of LYSMD3 markedly impaired chitin and fungi-induced inflammatory cytokine production in lung epithelial cells. Antagonization of LYSMD3 ectodomain by soluble LYSMD3 protein, multiple ligands, or antibody against LYSMD3 all significantly blocked chitin signaling. Taken together our study identifies LYSMD3 as a mammalian pattern recognition receptor (PRR) for chitin and is required for the epithelial inflammatory response to chitin and fungal spores.

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Pattern Recognition Receptor for Chitin

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GENERAL AUDIENCE ABSTRACT

Chitin is the main ingredient in the crustacean shells (e.g. crab, shrimp, lobster). It can also be found in fungal cell walls and insect exoskeletons like house dust mites and cockroaches. Many people are allergic to seafood, fungal spores, house dust mites, and cockroach. These allergies are thought to be driven at least partially by a response to chitin. However, how mammals sense and response to chitin is largely unknown. In plants, LysM-domain (LysMD, chitin binding domain) containing receptors are the primary receptors for chitin. These receptors can bind directly to chitin and/or mediate the innate immune response against chitin-containing pathogens such as fungi. Mammals also have LysMD containing proteins, but the functions of these proteins are unclear. In this study, we demonstrate that human LYSMD3 is a novel receptor for chitin. LYSMD3 is essential for chitin recognition and chitin induced inflammatory responses by airway epithelial cells. Our characterization of LYSMD3 as the elusive human epithelial cell receptor for chitin, resolves a long-standing mystery and provides a new insight into the context of innate immunity in mammals against chitin-containing organisms and allergic inflammation.

DEDICATION

I dedicate this work to Miss Ayumi Hamasaki, who accompanied me during the days and nights of writing this dissertation. You gave me strength, you helped me endure, and you set my soul alight. As you have told me, I wish to walk with firm steps, facing forward as you have done. My wish for you is eternal youth, beauty, and health. May your voice carry on throughout the ages.

ACKNOWLEDGMENTS

First and foremost I'd like to thank my advisor, Dr. Christopher Lawrence for the mentorship, emotional and social support, as well as a numerous other ways he has aided. He always tries his best to give whatever help I need. We shared the excitement of every new discovery on my research.

Secondly, I'd like to thank my committee members, Dr. Liwu Li, Dr. Virginia Buechner-Maxwell, And Dr. Caroline N. Jones for their wisdom, kindness, encouragement, patience, and support throughout this project. I am very lucky that my committee members are all extremely nice people. Dr. Li can always solve my questions in a very short time and also provide new insights at the same time. Talking to him is a great enjoyment. Dr. Maxwell and Dr. Jones have provided a lot of timely and valuable feedback on my manuscript. I would also like to thank people in Dr. Jones lab, Udaya Datla and Nidhi Menon, who have offered great support to my research.

Next, I must thank people in my lab, Brad Howard, Dr. Tristan Hayes, Mengyao Luo, and Tiffany Roach, for making my days in the lab easier and happy. I would especially like to thank Tiffany, who is an excellent research assistant, manuscript editor, lab manager, and cookie maker. I entered the most productive period of research life when she came to the lab.

I'd like to thank Dr. Shiv Kale for in depth evaluation and suggestions on my manuscript.

I'd like to thank my old lab mates and college classmate in China, Dr. Hongyan Li, Dr. Yang Liu, Dr. Bo Wang, and Kang Cui, who continuously provide support on my research and career development.

I thank people I met in Blacksburg. Feiran Chen, Zhitao Fan, Yecheng Zhao, Biao Li, Dr. Zhixiong Sun, Dr. Xiaolin Zhao, Ye fan, Xinxuan Hao, and Yaxuan Zhang are all great people who have given me a lot of good memories during my time at Virginia Tech.

I would like to thank Sue Rasmussen and Rebecca Zimmerman for their continuous administrative supports for years. I thank Annette Fluri and Pamelia Stolte, for offering whatever assistance I need in LS1. I thank Kristen Talley, Stacie Quesenberry, and Michelle Caldwell-Sadler for handling hundreds of my purchase orders. I'd like to thank Wooram Li, Sharon Sible, Tina Keesee, and Catherine Sarmadi for their support to my work as a teaching assistant. I thank Dennie Munson for her kind guidance on graduation Stuff.

And ultimately, I would like to thank my parents Xiushan He, and Xiangyun Song, who have offered unlimited support and love to me for decades and have not yet got any return actually. I owe a great to them.

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CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1 What is chitin?

Chitin, a derivative of glucose, is the second most abundant form of polysaccharide in nature after cellulose. The structure of chitin is a linear polymer made up of β-(1,4)-linked N-acetylglucosamine (GlcNAc) (Fig. 1-1). It is present in cell walls or exoskeletons of organisms such as fungi, crustaceans, parasites and insects, but not in mammals (Bueter, Specht, & Levitz, 2013; C. G. Lee, 2009). In nature, chitin exists in two different crystalline forms, α-chitin and β-chitin. The former is the dominant form which consists of linear chains of GlcNAc arranged in an antiparallel manner. In contrast, β-chitin is composed of parallel chains (Adrangi & Faramarzi, 2013). Chitin is an important component of the fungal cell wall. It functions to maintain the strength and integrity of cell wall of fungi.

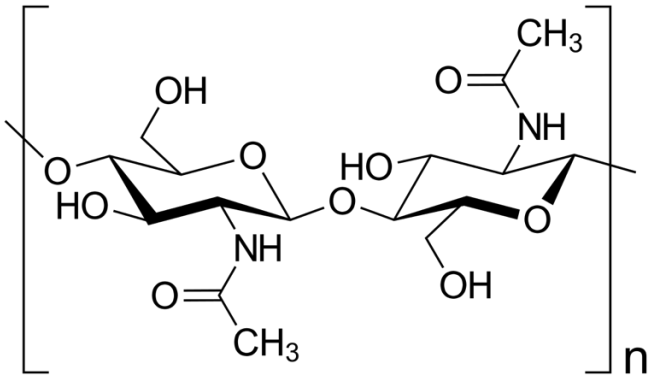

Figure 1-1. Structure of the chitin molecule.

1.2 The immunological characteristics of chitin

Despite the prevalence of chitin, its immunological characteristics are not well understood. There are a great number of reports concerning the immunological properties of chitin in mammals, but the conclusions are very controversial. Some reports have shown chitin as inert, proinflammatory/antiinflammtory, or proallergenic. In general, chitin is known to be non-toxic and non-allergenic. However, at the same time, chitin is also a common component that could be found in many organisms that trigger allergies, including shrimp, crab, house dust mite and fungi (Chun Geun Lee, Da Silva, Lee, Hartl, & Elias, 2008). The effects of chitin on immune cell function are considered size-dependent and pathway specific. Smaller fragments (<40 μm) induced anti-inflammatory IL-10 production via Dectin-1 and Syk pathway; moderate-sized particles (40-70 μm) triggered a strong pro-inflammatory response, including production of IL-17, TNF and IL-23 through TLR-2 and the NF-κB signaling pathway. Larger fragments were relatively inert (Bueter et al., 2013; Chun Geun Lee et al., 2008). This tricky way of balancing may be able to be explained in mammals by the existence of a kind of enzyme, Acidic mammalian chitinase (AMCase), however the function(s) of this protein in humans that do not contain chitin has been studied only recently. Chitinase enzymes also exist in chitin-containing fungi in order to construct and reshape their own chitin. Chitinases are also found in plants as an important means to defend themselves against infectious agents (Punja & Zhang, 1993). However, chitinase in humans and other mammals have only been studied for the past two decades (Boot et al., 2001). AMCase can cleave large polymers of chitin into intermediate size chitin fragments, and these fragments are capable of promoting the inflammatory response, which in turn, triggers the production of

increased levels of AMCase. AMCase can then further degrade the intermediate-sized chitin fragments into smaller fragments, which can prevent the spread of inflammation through another kind of mechanism. Those chitin molecules can become even smaller and inert under the effect of AMCase which likely contribute to the resolution of the local inflammatory response after the pathogen has been controlled (Fig. 1-2) (Chun Geun Lee et al., 2008).

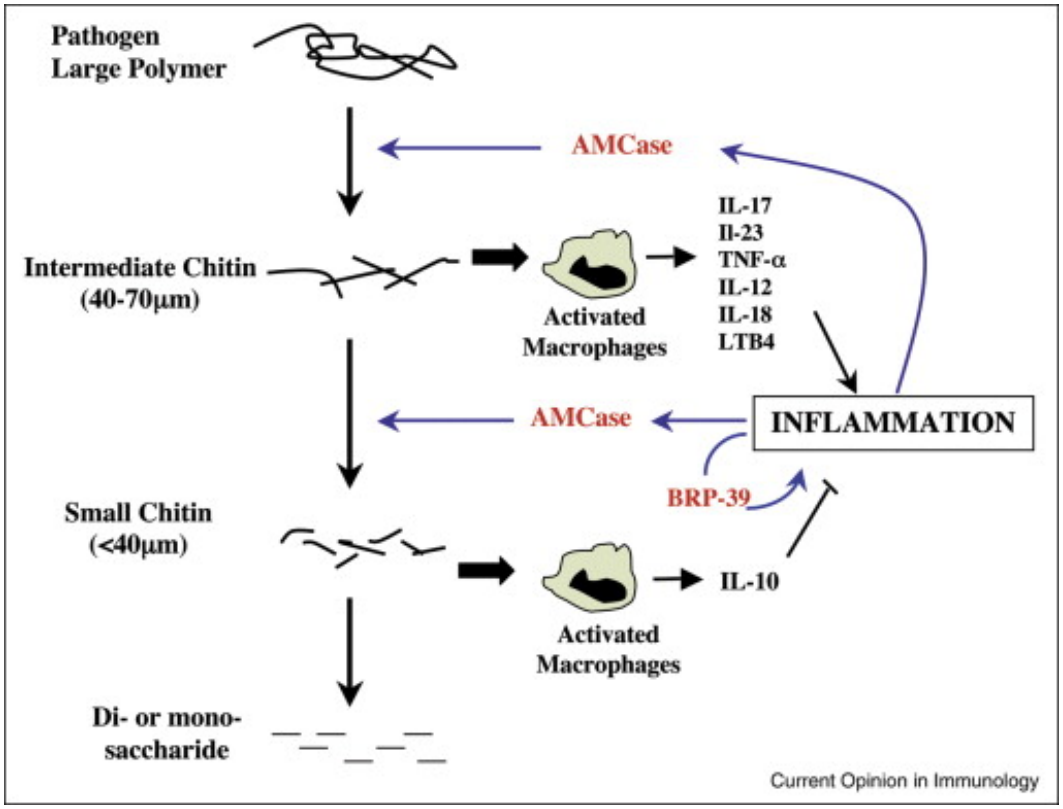

Figure 1-2. The proposed effects of chitin fragment size on immune cell function and how does AMCase contribute to this process during infection (Chun Geun Lee et al., 2008).

Allergic diseases including asthma, chronic rhinosinusitis, and allergic rhinitis are among the most common diseases seen in clinical practices today. Cases have increased over

the last 50 years such that allergic inflammatory disorders are now the most prevalent chronic childhood illnesses in developed countries (Locksley, 2010). Allergic responses have been linked with specific environmental triggers and are thought to result from airway inflammatory responses to ubiquitous pathogen-associated molecules (T Umetsu, Jones, Akbari, Macaubas, & H DeKruyff, 2002). Human fungal pathogens such as the allergenic species *Alternaria alternata* and *Aspergillus fumigatus*, have protective cell walls containing layers of rigid structural polymers that fall into two distinct groups depending on their solubility in alkaline solutions (Fontaine et al., 2000). While soluble components such as galactomannan play important roles in cell wall integrity and host immune system elicitation, alkali-insoluble complexes of chitin and ß-glucan are more abundant (Cantu, Greve, Labavitch, & Powell, 2009; Latge et al., 1994). Host immune recognition by Dectin-1 and the subsequent responses to challenge with fungal ß-glucan have been worked out in rich detail (Brown et al., 2007; Rosas et al., 2008; Taylor et al., 2007). In contrast, the body of evidence for the role of chitin in driving inflammatory processes and associated mechanisms remains sparse.

Chitin exposure has long been associated with allergic pathology in susceptible populations, prompting further study of chitin as an elicitor of Th2 immunity in models of airborne respiratory allergies (Cartier et al., 2004; Van Dyken et al., 2011). While early work suggested that chitin may induce Th1 responses such as IFN-γ secretion in natural killer (NK) cells, it has since been shown that chitin particles induce the accumulation of IL-4-producing innate immune cells in mouse models. Such accumulation is abolished when mice are challenged with chitin pretreated with copious amounts of recombinant

acidic mammalian chitinase (AMCase) (Reese et al., 2007; Semenuk et al., 2001). The accumulation of IL-4 producing cells is also lost in chitin-challenged mice overexpressing AMCase, strongly establishing chitin molecules as elicitors of Th2 immunity. In addition, chitin induces alternative activation of mouse macrophages in vivo, which appears to be mediated through the CCL2 chemokine receptor CCR2. CCR2 knockout mice exhibited decreased reactive oxygen species (ROS) production in the lung, attenuated recruitment of eosinophil and monocyte, and decreased eosinophil function in response to chitin challenge versus unchallenged controls (R. M. Roy, Wuthrich, & Klein, 2012).

Airway epithelial cells represent the first line of defense against inflammatory stimuli and antigens. Activation of epithelial cells is one of characteristics of asthma and allergic rhinitis and is significantly associated with allergic sensitization (Wang, Bai, Li, Adler, & Wang, 2008). Cytokines produced by epithelial cells at the barrier surface, including IL-25, IL33 and thymic stromal lymphopoietin (TSLP), play an important role in regulating type 2 immunity (Uchida et al., 2017). Direct effects of chitin on airway epithelial cells are considered to support the development of allergic airway diseases such as asthma (Khosravi & Erle, 2016). It was shown that inhaled chitin stimulated epithelial cells to release the Th2 cytokines IL-25, IL-33, and TLSP, which in turn, induced secretion of IL-13 and IL-5 in innate lymphoid type 2 cells (Van Dyken et al., 2014). However, IL-17A-producing γδ T cell activation was also increased, as was prolonged neutrophil influx to the site of challenge. These results suggest that complex interacting immune system pathways are activated during the chitin inflammatory response, making elucidation of the mechanism of chitin recognition critical. While it is well supported that fragments of chitin

elicit immediate innate and prolonged Th2-driven inflammatory responses both in humans and in animal models, it is currently unclear how host cells recognize these fragments and propagate the signaling events required for immune cell recruitment. Little has been elucidated regarding the pattern recognition receptors (PRRs) in epithelial cells that mediate these responses. A chitin specific receptor in human airway epithelial cells has not been identified to this point.

1.3 How is chitin recognized by plants and mammals?

PRR play an important role in sensing the extracellular environment and shaping the host response to pathogens. Innate immune signaling pathways in plants and animals are evolutionarily conserved (Ausubel, 2005). The lysin motif (LysM) is a carbohydrate binding module conserved across all kingdoms, including viruses, bacteria, fungi, plants and mammals. In a survey of both prokaryotic and eukaryotic proteins, more than 4000 proteins have been found to contain Lysin motifs, including secreted proteins, outer-membrane proteins, and lipoproteins (Buist, Steen, Kok, & Kuipers, 2008). LysM domains have been shown to bind to chitin and peptidoglycan (Mesnage et al., 2014). In plants, LysM domain containing cell surface receptors such as OsCEBiP, AtCERK1, AtLYK4 and AtLYK5 are involved in the recognition of chitin and trigger an innate immune response against chitin containing pathogens such as fungi (Cao et al., 2014; Wan et al., 2008).

Some innate immune receptors in mammals, including TLR-2, Dectin-1, mannose receptor and NOD2, have been shown to be involved in mediating a variety of immunes induced by chitin (Da Silva, Hartl, Liu, Lee, & Elias, 2008; Fuchs et al., 2018; Koller, Muller-Wiefel, Rupec, Korting, & Ruzicka, 2011; Louis et al., 2019; Mora-Montes et al., 2011; Wagener et al., 2014). Chitin particles with certain sizes (1-10 μm) can be phagocytosed. Fc-γ receptor on human peripheral blood mononuclear cells (PBMCs) that recognizes IgG-opsonized *Aspergillus* chitin and induces phagocytosis has been demonstrated (Becker et al., 2016). Another study showed that the acetyl group on chitin is the molecular pattern recognized by TLR2, resulting in phagocytosis of chitin and formation of phagosomal chitin-TLR2 cluster (Davis et al., 2018). Although a large number

of reports show that TLR2 contributes to sensing of chitin, only one study demonstrated that Alexa Fluor 647 labeled chitin oligomer directly bound TLR2 using Microscale Thermophoresis (MST) (Fuchs et al., 2018). However, the possibility that TLR2 bound to the hydrophobic fluorescence tag has not been excluded. Fibrinogen C domain-containing protein 1 (FIBCD1), a transmembrane protein expressed in the gastrointestinal tract (Schlosser et al., 2009) and human lung epithelium, directly binds chitin through a conserved S1 binding site and mediates suppression of IL-8 secretion in A549 cells in response to *A. fumigatus* cell walls. However, recognition of the *A. fumigatus* cell wall by FIBCD1 is S1 site-independent and there is no clear evidence that FIBCD1 recognizes chitin itself in the fungal cell wall. Although co-localization was found between the chitin-binding fluorescent-labeled Wheat Germ Agglutinin (WGA) and purified FIBCD1 on *A. fumigatus* (Jepsen et al., 2018), the possibility that WGA, which has high affinity for Sialic acid and N-acetylglucosaminyl residues, bound directly to FIBCD1 has not been excluded. More conflicting results have been reported on the role of Dectin-1 in chitin signaling. Dectin-1 reconstitution in HEK293T did not result in NF-κB activation in response to chitin and macrophages from Dectin-1-deficient mice respond as efficiently to chitin as WT macrophages in terms of TNF-α production (Fuchs et al., 2018). Dectin-1 does not directly bind chitin as shown in multiple studies (Gantner, Simmons, & Underhill, 2005; Gour et al., 2018; Mora-Montes et al., 2011).

CHAPTER II: MATERIALS AND METHODS

2.1 Protein, plasmid and reagents

The ectodomain of human LYSMD3 was synthesized and subcloned into His-Trx fusion vector pET32a and further recombinantly produced in *Escherichia coli* by Genscript. Purification and tag removal was performed to get the tag free ectodomain of rLYSMD3 with a purity >75%. Endotoxin was removed from rLYSMD3. The recombinant human LYSMD3 (Ag19496) was from Proteintech. The expression plasmid pcDNA3-RFP-LYSMD3 was a gift from Dr. Craig M. Crews (Yale University). The cDNA encoding LYSMD3-Flag was chemically synthesized and cloned into the vector pcDNA3.1 by GeneArt (Invitrogen). The recombinant human TLR2 (2616-TR), TLR4 (1478-TR) and Dectin-1 (1859-DC) were obtained from R&D Systems. The recombinant GFP protein (13105-S07E) was obtained from Sino Biological. LYSMD3 (24313-1-AP) and HSP90 (13171-1-AP) antibodies were obtained from Proteintech. For neutralizing assays, LYSMD3 antibody in a carrier-free format was provided by Proteintech. Endotoxin was removed with ToxOut rapid endotoxin removal kit (BioVision). The antibody was 0.22 μm sterile-filtered using Spin-X centrifuge tube filter (Corning). Anti-β-Actin (sc-47778) was purchased from Santa Cruz. Anti-sodium potassium ATPase antibody (ab185065) was from Abcam. Mouse IgG_{2B} isotype control (MAB004), human TLR2 (AF2616), TLR4 (MAB1478) and Dectin-1 (MAB1859) antibodies were from R&D Systems. Rabbit IgG isotype control (31235) and 6×-His tag antibody (MA1-21315-HRP) were from ThermoFisher Scientific. Human IgA2 control (maba2-ctrl) and anti-hTLR2-IgA (maba2-htlr2) were from invivoGen. Anti-Syk (13198), anti-Phospho-Syk (2710) were from Cell Signaling Technology. Anti-Phosphotyrosine (16-105) was from EMD Millipore. Anti-Flag (F1804) antibody was from Sigma. Chitin oligosaccharides DP2, DP3, DP4, DP5 and DP6

were obtained from Elicityl. Chitin DP7 was from Elicityl and Isosep. Chitin from crab shells (Sigma, C9752) was placed in endotoxin-free sterile PBS and sonicated with a Branson ultrasonics sonicator at 25% output power for 5 min. Sonication was repeated two more times for a total of three 5-minute intervals. Afterwards, the suspensions were passed through a 10 μm cell strainer. All chitin preparations were endotoxin-free. LPS-RS, Pam3CSK4, FLA-ST Ultrapure, Poly(I:C) HMW, laminarin, WGP Soluble and curdlan were obtained from InvivoGen. Extracts of *Alternaria* were purchased from Greer Laboratories. Piceatannol was obtained from EMD Millipore. QUANTI-Blue was from InvivoGen.

2.2 Fungal Strain and Growth Conditions

Aspergillus fumigatus strain 293 expressing GFP was cultured on Sabouraud dextrose agar plates for 4-5 days at 37°C. *Alternaria alternata* (ATCC 66981) was cultured on PDA (0.4% potato starch, 2% dextrose, 1.5% agar) and incubated at 25°C in the absence of light. Spores or conidium were collected using gentle agitation in PBS and counted on a hemacytometer. Media was purchased from Sigma-Aldrich.

2.3 Cell culture and transfection

BEAS-2B, A549, LA-4 and RAW 264.7 cells were obtained from ATCC. NHBE cells were obtained from Lonza. A549-Dual cells were purchased from InvivoGen. hBE33 cells containing the human IL-33 gene were developed as previously described (Uchida et al., 2017). A549 and RAW 264.7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). BEAS-2B, NHBE, DHBE and hBE33 cells were maintained in bronchial epithelial growth media (BEGM) supplemented with defined growth factors and retinoic acid (Lonza). LA-4 cells were cultured in F-12 Ham's media supplemented with 15% FBS. The siRNA pools (sc-91992 and sc-149191) targeting human or mouse LYSMD3 and the negative control siRNA (sc-37007) were obtained from Santa Cruz. The single siRNA duplex (J-024521-09) against human LYSMD3 was obtained from Dharmacon. BEAS-2B and LA-4 were transfected with siRNA duplexes through the use of Lipofectamine RNAiMAX transfection reagent (Invitrogen). Transfecting siRNA into NHBE, hBE33 or RAW 264.7 cells was performed using the HiPerFect transfection reagent (QIAGEN). X-tremeGENE HP DNA transfection reagent (Roche) was used for plasmid transfection.

2.4 CRISPR–Cas9-mediated LYSMD3 gene knockout

BEAS-2B cells were infected with non-targeting control or LYSMD3-sgRNAs Edit-R All-in-one Lentiviral particles (Dharmacon, VSGC11964, VSGH11936-247547638 and VSGH11936-247618492) to establish stable LYSMD3 KO cells. Cells were selected with 1 μg/mL puromycin for 4 days.

2.5 Chitin binding assay

Chitin-binding assay was performed according to the previously described method with modifications (Cadoret, Ball, Douzi, & Voulhoux, 2014). Briefly, 100 µl of chitin magnetic beads (New England BioLabs) in chitin-binding buffer (NaCl 500 mM, Tris-HCl 20 mM, EDTA 1 mM, Tween-20 0.1%, pH 8) was incubated with a 200 µl solution containing LYSMD3 and BSA proteins at 200 µg/ml each in chitin-binding buffer. The sample was incubated at 4°C with agitation for 1 hour. Separation of the supernatant (unbound fraction) from the beads (bound fraction) was performed by applying a magnet. After collection of the supernatant, the beads were washed three times with 200 µl of chitin-binding buffer. 15 µl of samples from Total, Unbound and Bound fractions were analyzed using SDS-PAGE and subsequent Coomassie blue staining.

2.6 LYSMD3-binding ELISA

Nunc MaxiSorp 96-well ELISA plates (ThermoFisher Scientific) were coated with chitin 1 µg chitin oligosaccharides, 20 µg LPS, 4 µg curdlan, 4 µg laminarin or 4 µg WGP soluble in carbonate buffer, pH 9.5 or 5 µg BSA in PBS overnight at 4 °C. Wells were blocked with StartingBlock T20 (TBS) Blocking Buffer (ThermoFisher Scientific) and then incubated with recombinant human LYSMD3 ectodomain at increasing concentration for 1 h at room temperature. After washing, LYSMD3 was detected by LYSMD3 antibody, 1/3000 dilution, followed by mouse anti-rabbit IgG monoclonal antibody conjugated to HRP (GenScript, A01827), 1/20000 dilution, and addition of tetramethylbenzidine (TMB).

Alternatively, chitin or WGP-coated wells were incubated with recombinant human LYSMD3, Dectin-1 or BSA at 4 µg/ml, followed by rinsing and addition of anti-His-HRP monoclonal antibody and processed as described above.

2.7 Fungal binding assay

The assay was adapted from a previously described method (Bessa Pereira et al., 2016). In order to expose cell wall PAMPs, fungal spores were suspended in complete RPMI 1640 medium and incubated for 2 h at 30 °C to induce germination. Recombinant proteins LYSMD3, TLR2, TLR4 and GFP (2.5 μg per assay) were incubated for 1 h at 4°C under rotation with suspension of *Alternaria alternata* spore (5×10^6) in binding buffer (TBS, 1% BSA, 5 mM $CaCl_2$) to a final volume of 0.5 ml. Following incubation, cells were collected using Spin-X centrifuge tube filter (Corning) by centrifugation and washed thoroughly with TBS plus 5 mM $CaCl_2$ to remove nonspecifically bound proteins. Cells were then resuspended in 60 μl Laemmli's sample buffer and denatured by heating at 100°C for 15 min. Next, 30 μl of the lysate and pure recombinant proteins (25 or 100 ng) were separated by 10% SDS-PAGE followed by western blot analysis.

2.8 Confocal microscopy

For microscopy experiments epithelial cells were grown on Falcon 8-well culture slides (Corning) and, after the indicated treatment, the cells were fixed in 3% paraformaldehyde in PBS. Images were obtained by stacking optical sections along the z axis on a Zeiss LSM 880.

2.9 ELISA

BEAS-2B, NHBE, A549, LA-4 or RAW 264.7 cells were stimulated for 24 h with chitin, *Alternaria* spores, Pam3CSK4, FLA-ST or Poly(I:C). hBE33s were stimulated for 1 h with *Alternaria* extract. Supernatants were collected and the amount of IL-6, IL-8, IL-33, CCL2 or RANTES was measured with ELISA kits according to the manufacturer's recommendations (R&D Systems or BioLegend).

2.10 Immunoprecipitation

Cells were lysed in Pierce IP lysis buffer (ThermoFisher Scientific) supplemented with Halt protease inhibitor cocktail (ThermoFisher Scientific) and Halt phosphatase inhibitor cocktail (ThermoFisher Scientific). Proteins were immunoprecipitated from cell lysates by incubation overnight with Anti-FLAG M2 magnetic beads (Sigma) and subjected to western blotting analysis.

2.11 Protein Preparation

Total cellular protein was isolated with RIPA Buffer. Fractions of membrane-and-cytoplasmic proteins from cultured cells were obtained by using Mem-PER Plus Membrane Protein Extraction Kit (ThermoFisher Scientific). Biotinylation of the cell surface proteins of BEAS-2B cells was performed with the Cell Surface Protein Isolation Kit (BioVision) according to the manufacturer's protocol. Briefly, cells were grown in a T75 flask until they reached confluency. The cells were then washed with PBS and incubated with Sulfo-NHS-SS-biotin for 30 min at 4 °C. A quenching solution was added, and cells were lysed with lysis buffer (500 µL) containing the Halt protease inhibitor cocktail. The biotinylated cell surface proteins were isolated with Streptavidin beads, eluted by the elution buffer (100 µL) with DTT, and analyzed by Western blotting.

2.12 RNA preparation and real-time PCR

RNA was extracted from BEAS-2B cells with TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was generated from 1 μg total RNA using a SuperScrip IV VILO Master Mix (Invitrogen). Real-time PCR was performed with a Premix Ex Taq (Probe qPCR) Master Mix (Takara) on a StepOnePlus Real-Time PCR System (Life Technologies). The following primer–probe sets were used: LYSMD1, Hs.PT.58.21016257; LYSMD2, Hs.PT.58.40064258; LYSMD4, Hs.PT.58.2438397; β-Actin (ACTB), Hs.PT.39a.22214847 (Integrated DNA Technologies). The expression of genes encoding LYSMD1, LYSMD2 and LYSMD4 were normalized to the expression of β-actin.

2.13 MTT assay

MTT assays were carried out using MTT Cell Proliferation Assay Kit following the manufacturer's instructions (Cayman Chemical).

2.14 LDH assay

Lactate dehydrogenase (LDH) assays were performed with Pierce LDH Cytotoxicity Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions.

2.15 Electronic Medical Records

BioVU is the Vanderbilt University Medical Center'sbiobank of DNA extracted from discarded clinical blood samples combined with a de-identified copy of the electronic medical record. For this study we analyzed the records from 20,003 adult Caucasian patients who had genotyping available from the Illumina Infinium Human-Exome BeadChip (Illumina, San Diego, CA, USA) E4. Phenotypes were defined following the PheWAS method E5 for 29 targeted phenotypes. Briefly, each PheWAS phenotype code is defined by a curated list of International Classification of Disease (ICD.9) codes. Two or more instances of the relevant ICD.9 codes are necessary to define a patient as a case for that PheWAS phenotype. Targeted phenotypes (**Supplementary file 1A**) were chosen for involvement in the airway, fungal or immune system, with > 250 cases in our cohort. The genetic variants were tested for association with each targeted phenotype by logistic regression, adjusting for sex and age at last record. The Exome BeadChip contained 6 rare nonsynonymous variants in LYSMD3 15 in LYSMD4. For rare variant analysis, patients were classified as carriers or non-carriers of rare variants (< 1% minor allele frequency in this cohort), and then tested for phenotype association by logistic regression, adjusting for sex and age (data analysis courtesy of Dr. David Samuels, Vanderbilt University).

2.16 Statistical analysis

Data are expressed as mean ± SD. Data were statistically analyzed by unpaired 2 sample t-test with $p<0.05$ indicating of statistical significance.

CHAPTER III: RESULTS

3.1 LYSMD3 is localized in plasma membrane of airway epithelial cells and in sites of fungal-epithelial interactions

We first sought to define the subcellular localization of LYSMD3 in epithelial cells. In silico analysis predicts that LYSMD3 contains a transmembrane domain (Fig. 3-1). It was demonstrated previously that exogenous RFP-tagged LYSMD3 was found at both the Golgi apparatus and plasma membrane of HeLa cells (Serebrenik et al., 2018). By biochemical fractionation experiments, we observed LYSMD3 expression in the membrane compartments of both human bronchial epithelial cells (BEAS-2B) and alveolar type II epithelial cells (A549). No expression was detectable by immunoblot analysis in the cytosolic fractions (Fig. 3-2).

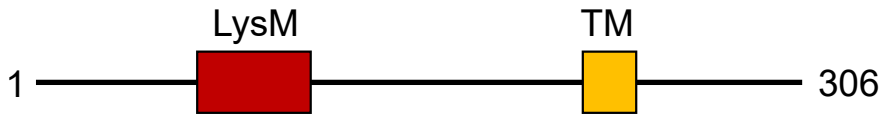

Figure 3-1. Schematic illustration of the predicted domains of human LYSMD3. The LysM domain is located at amino acid residues 65-109. The transmembrane domain is located between amino acid residues 218-238.

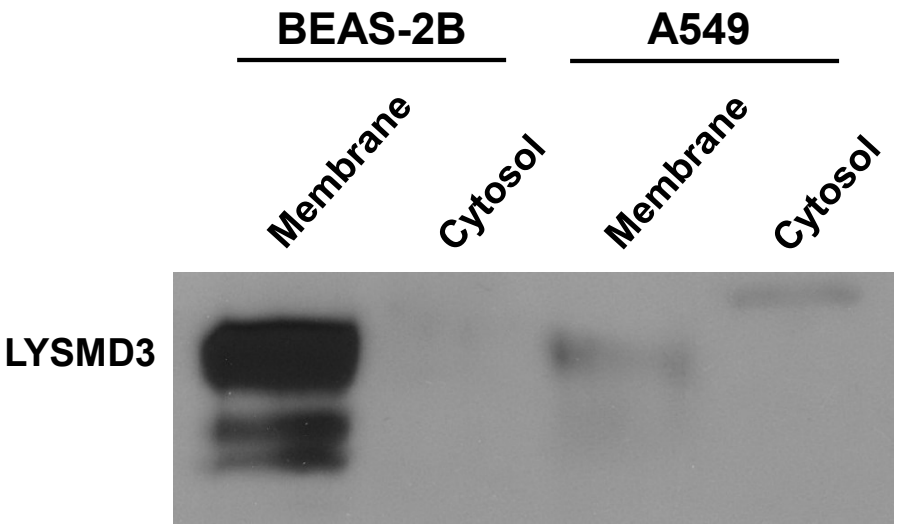

Figure 3-2. LYSMD3 is associated with the membrane fractions of lung epithelial cells. Immunoblot analysis of membrane and cytosolic fractions (10 μg) from BEAS-2B and A549 cells probed with anti-LYSMD3.

Next, we performed biotinylation of surface membrane and subsequent isolation of cell surface proteins. Using this approach, we observed that LYSMD3 is expressed on the surface of unstimulated BEAS-2B cells (Fig. 3-3).

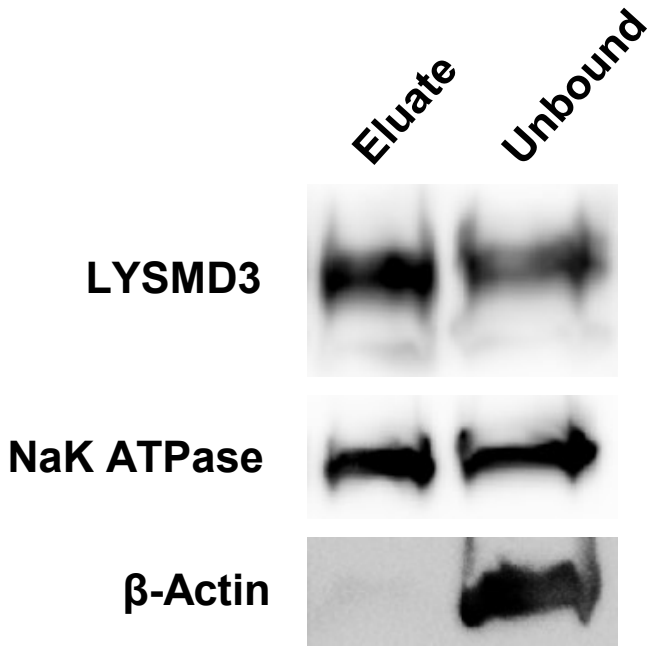

Figure 3-3. LYSMD3 is expressed on the surface of BEAS-2B cells. Immunoblot analysis of biotinylated cell surface proteins (eluate, 25 µL) and unbound lysate (25 µL) from BEAS-2B cells.

We next investigated whether the localization of LYSMD3 is associated with interaction between epithelial cells and fungus. We expressed red fluorescent protein (RFP) fusion LYSMD3 in BEAS-2B cells and then treated cells with *Aspergillus* conidium or *Alternaria* spores. By confocal microscopic imaging, we observed that LYSMD3 accumulated around *Aspergillus* conidium after 15 min of infection and Alternaria spores and hyphae after 90 min of incubation (Fig. 3-4, Fig. 3-5 and Fig. 3-6).

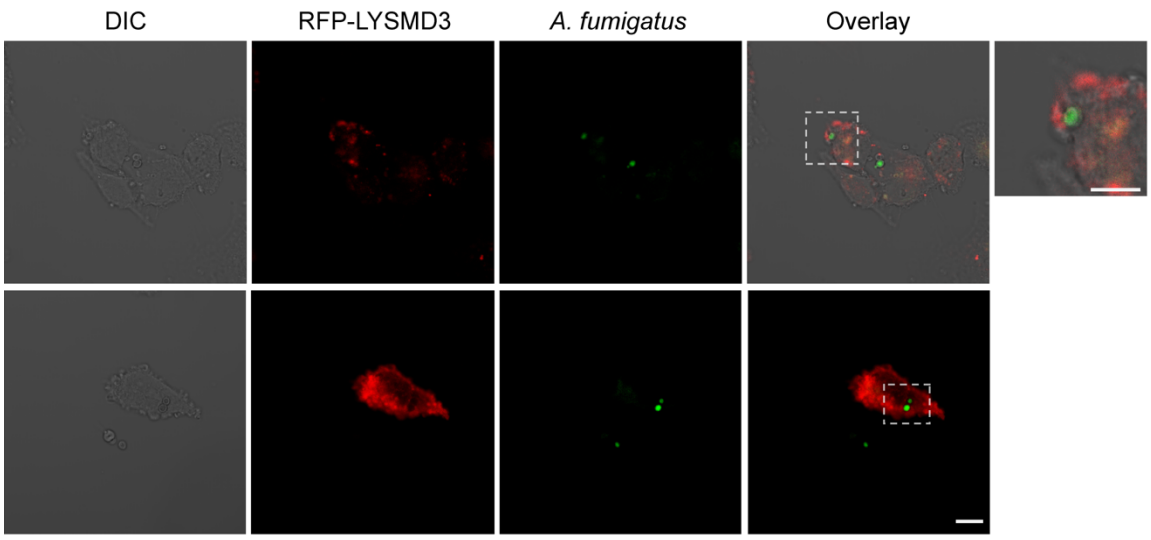

Figure 3-4. LYSMD3 accumulation around *Aspergillus* conidia. Confocal microscopic images of BEAS-2B cells transfected with RFP-LYSMD3 and infected with GFP-expressing *Aspergillus* conidium. Scale bars, 5 μm.

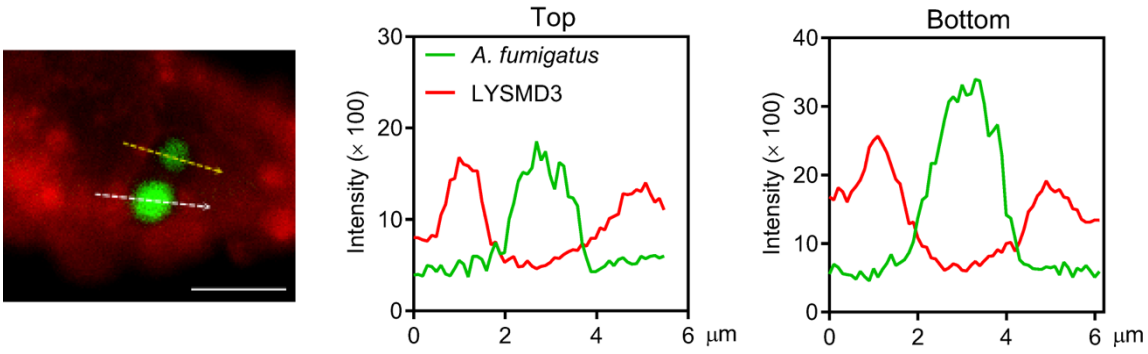

Figure 3-5. LYSMD3 accumulation around *Aspergillus* conidia. Magnified image of *A. fumigatus* conidium in d with plots of fluorescent intensity at the regions indicated by the dashed lines. Scale bars, 5 μm.

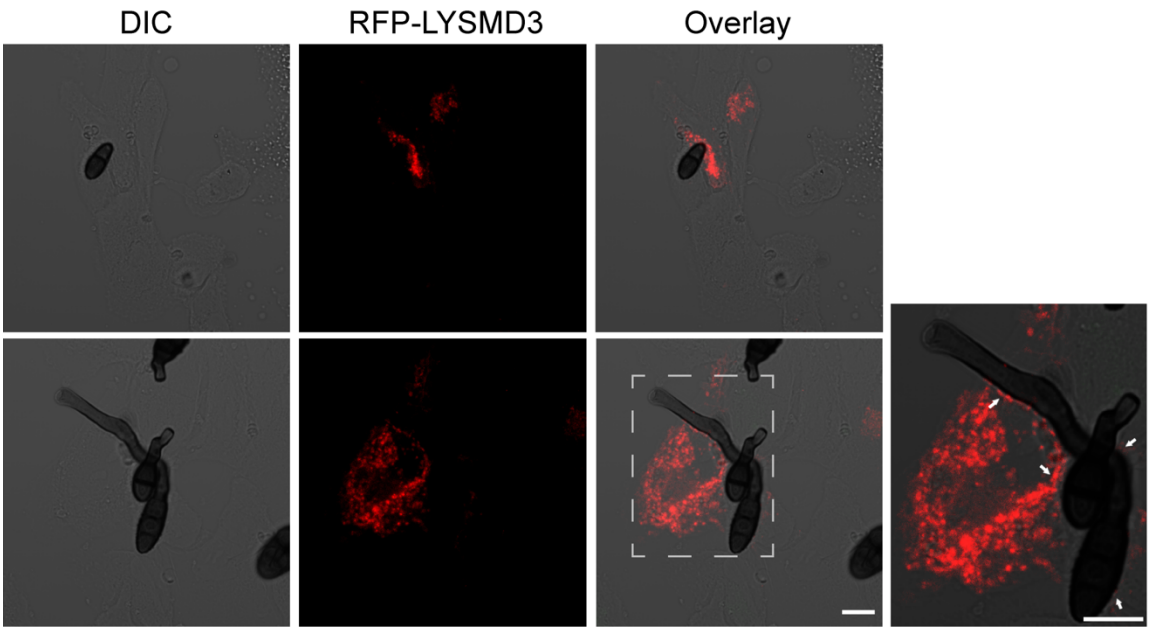

Figure 3-6. LYSMD3 accumulation around *Alternaria* spores and hyphae. Confocal microscopy images of BEAS-2B cells transfected with RFP-LYSMD3 and infected with *Alternaria* spores. Arrows pointing to the accumulation of LYSMD3 around the *A. alternata* spores and hyphae. Scale bars, 5 μm.

3.2 LYSMD3 is a chitin and β-glucan binding protein

Since some lysin motif (LysM)-containing receptors in plants bind to chitin oligosaccharides (Liu et al., 2012; Miya et al., 2007), we first determined whether human LYSMD3 could bind soluble chitin oligosaccharides. Using an ELISA assay for protein binding to immobilized chitin, we found that purified ectodomain (ECD) of human LYSMD3 was able to directly bind to chitin oligosaccharides with degrees of polymerization of 5-7 (DP5-7) but not to bovine serum albumin (BSA) or LPS (Fig. 3-7 and data not shown).

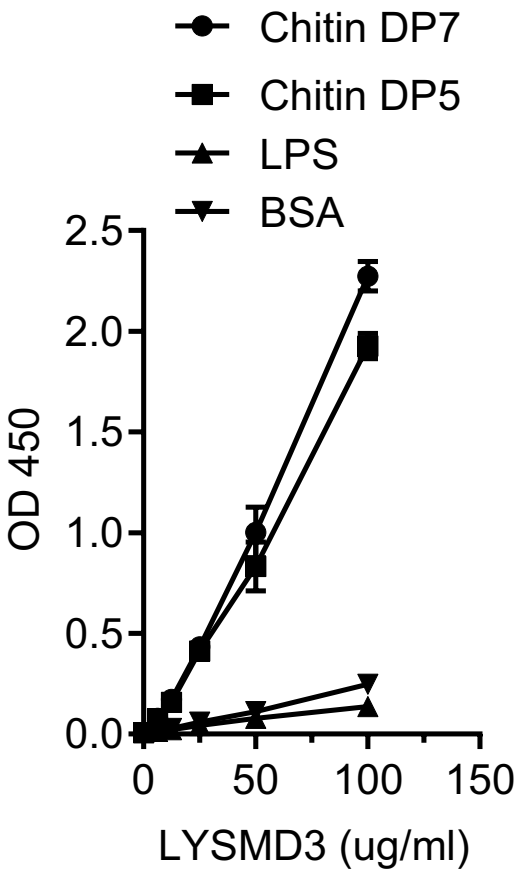

Figure 3-7. Direct binding of recombinant LYSMD3 to immobilized carbohydrates or bovine serum albumin (BSA), as determined by ELISA. The ELISA plate was coated with chitin DP5, DP7, LPS or BSA. Increasing concentrations of the ectodomain of LYSMD3 were added, and the amount of bound LYSMD3 was detected using LYSMD3 antibody, horseradish peroxidase conjugated secondary antibody and TMB (mean and SD of three samples per group).

In line with the ELISA results, pull-down assays demonstrated that LYSMD3 ectodomain co-immunoprecipitated with chitin beads (20-180 μm) (Fig. 3-8), indicating that LYSMD3 can also interact with a large, insoluble particulate form of chitin.

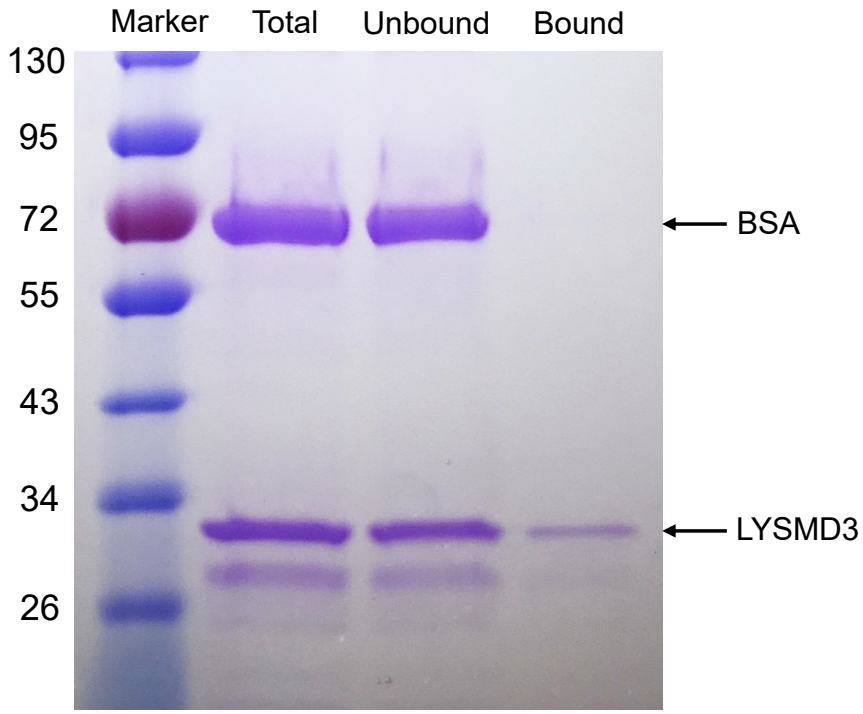

Figure 3-8. LYSMD3 bind directly to chitin beads. Chitin magnetic beads was incubated with a mixture of purified ectodomain of LYSMD3 and BSA. LYSMD3 bound to chitin, while the BSA was found only in the soluble fraction (Unbound). The samples were analyzed by SDS-PAGE and subsequent Coomassie blue staining.

Therefore, we next examined if LYSMD3 can recognize live fungal spores. We found that LYSMD3 ECD bound intensely to *Alternaria* spores (Fig. 3-9).

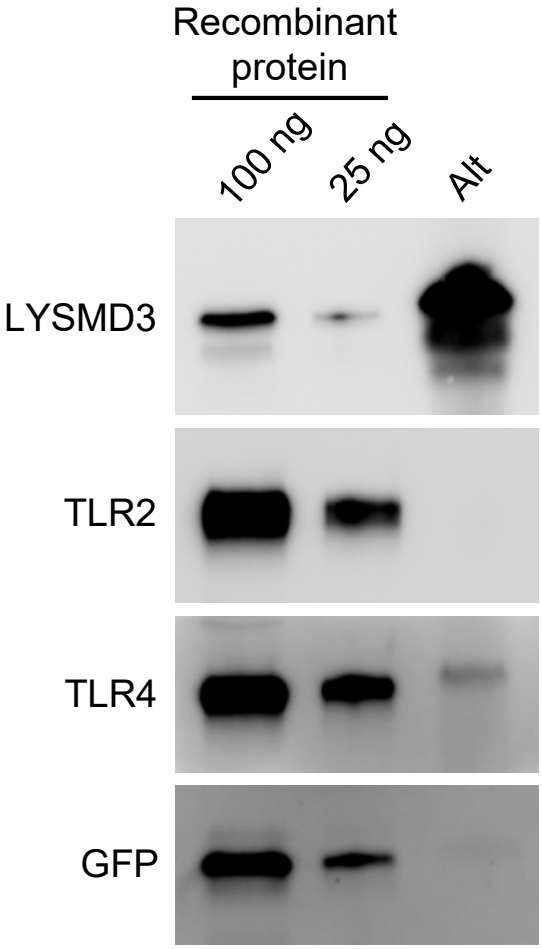

Figure 3-9. LYSMD3 binds to *Alternaria* spore cell wall. Recombinant LYSMD3, TLR2, TLR4 and GFP were incubated (2.5 μg each sample) with suspensions of 5×10^6 live *Alternaria* (Alt) spores. Proteins bound to fungal cell wall were detected by immunoblotting using anti-LYSMD3, anti-TLR2, anti-TLR4 or anti-His tag (GFP) antibody. Pure recombinant proteins were used to determine the sensitivity of the assay.

Given that chitin only constitutes a small portion (~3-4%) of the fungal cell wall and is mainly present in its inner layer (Gow, Latge, & Munro, 2017), we wondered whether LYSMD3 can also bind other fungal cell wall components. Eukaryotic LysM domains have been shown to bind mainly to chitin and peptidoglycan (Mesnage et al., 2014). However, in addition to chitin, plant LysM receptor AtCERK1 has recently been demonstrated to be able to recognize non-branched β-1,3-glucan oligosaccharides (Melida et al., 2018). Thus, we next assayed the binding of LYSMD3 ECD to immobilized β-glucan by ELISA as above. Surprisingly, LYSMD3 ECD also bound curdlan, a particulate linear β(1,3)-glucan without branches (Fig. 3-10), as well as laminarin, a linear soluble β(1-3)-glucan with β(1-6)-linkages (Fig. 3-11) (Bashir & Choi, 2017), in a dose-dependent manner (Fig. 3-12). Notably, LYSMD3 showed no affinity for a soluble yeast whole glucan particles (WGP) preparation (Fig. 3-12). These data are further supported by similar binding results with a commercially available purified ECD of LYSMD3 (Fig. 3-13 and Fig. 3-14). This suggests that the interaction of the LYSMD3 with fungi is likely mediated through recognition of either chitin or β-glucan, or both. Together, our findings indicate that both chitin and β-glucan are ligands for LYSMD3.

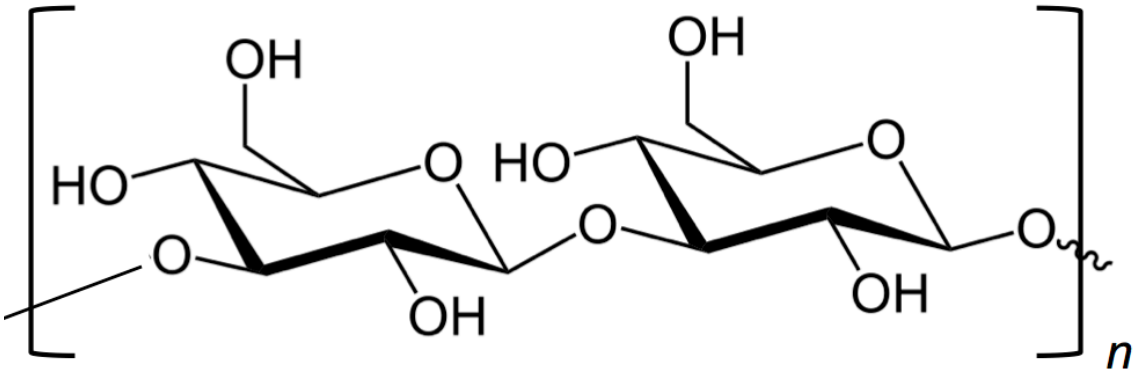

Figure 3-10. Structure of curdlan.

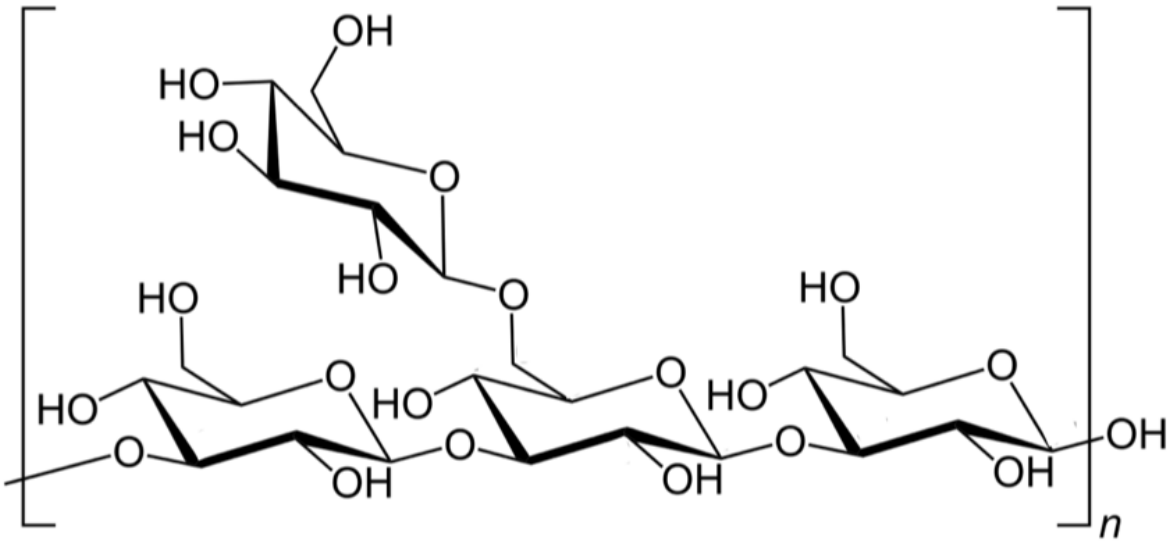

Figure 3-11. Structure of laminarin.

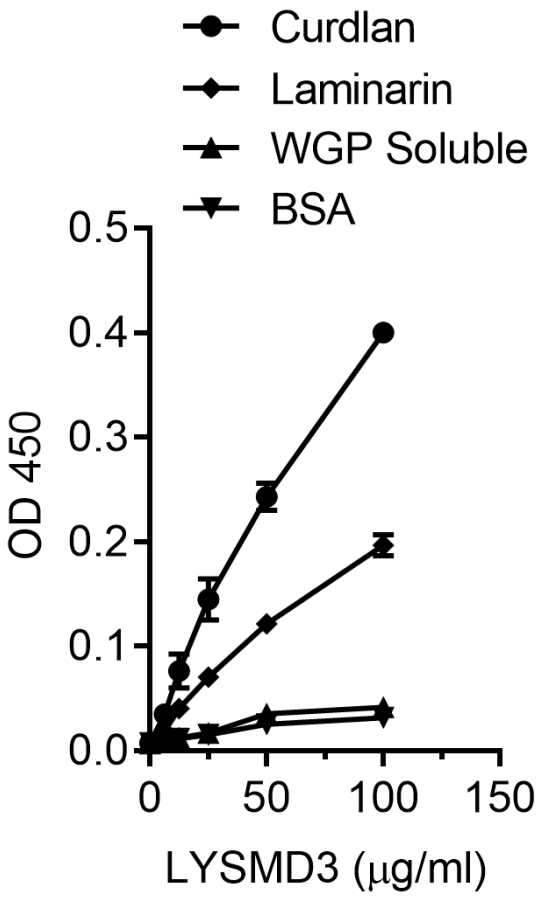

Figure 3-12. Direct binding of recombinant LYSMD3 to immobilized β-glucans, as determined by ELISA. The ELISA plate was coated with curdlan, laminarin, WGP soluble or BSA. Increasing concentrations of ectodomain of LYSMD3 was added, and the amount of bound LYSMD3 was detected using LYSMD3 antibody, HRP conjugated secondary antibody and TMB (mean and SD of three samples per group).

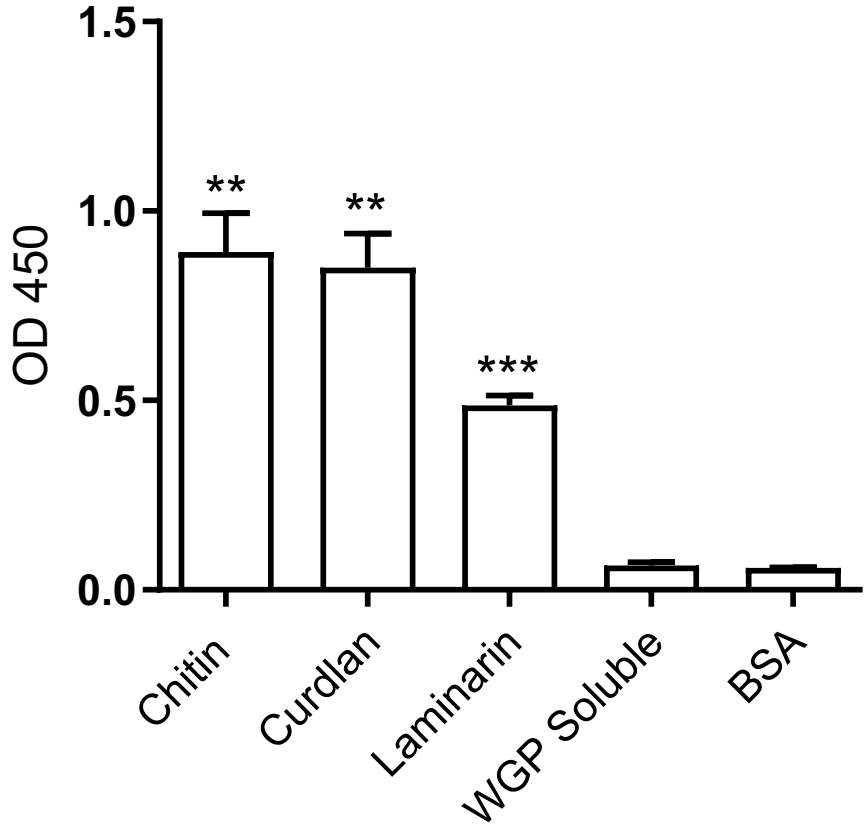

Figure 3-13. A commercially available recombinant human LYSMD3 bind to chitin, curdlan and laminarin but not WGP soluble. Direct binding of recombinant LYSMD3 (6xHis-Met 1-Gln 142) to immobilized various carbohydrates or bovine serum albumin (BSA), as determined by ELISA (mean and SD of three samples per group). **P < 0.01 and ***P < 0.001 (Student's t-test).

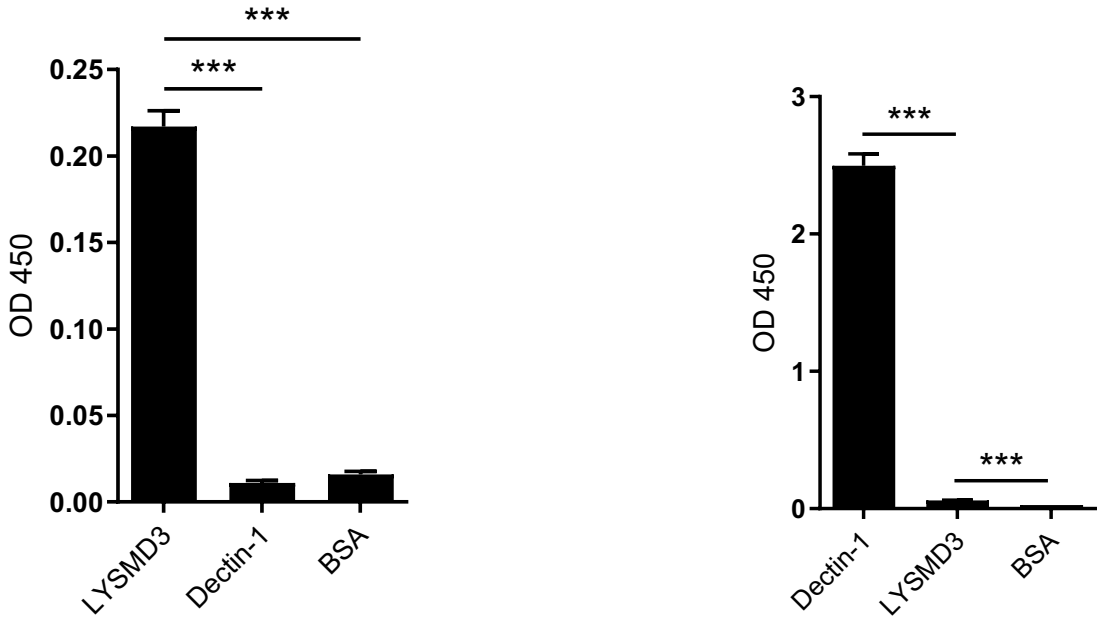

Figure 3-14. A commercially available recombinant human LYSMD3 binds to chitin, but not WGP soluble. Binding of recombinant LYSMD3 (6xHis-Met 1-Gln 142), Dectin-1 and BSA to immobilized chitin or WGP soluble, as determined by ELISA. The ELISA plate was coated with chitin DP7 (left) or WGP soluble (right). Recombinant human LYSMD3, Dectin-1 or BSA (all at 4 µg/ml) were added, and the amount of bound protein was detected using anti-His-HRP monoclonal antibody and TMB (mean and SD of three samples per group). ***P < 0.001 (Student's t-test).

3.3 LYSMD3 mediates the expression of genes encoding pro-inflammatory cytokines or chemokines upon stimulation with chitin or chitin containing fungi

We next assessed the importance of the LYSMD3-chitin interaction by studying its role in chitin-induced innate immune responses in mammalian lung epithelial cells. In plants, recognition by LysM receptors of chitin oligosaccharides of at least seven NAG repeats triggers strong immune responses (Hamel & Beaudoin, 2010). However, little is known about the chitin structure that is recognized in mammals. The chitin oligosaccharides were recently reported to induce “size-dependent” immunostimulatory effects in human and mouse immune cells as well as in mice (Fuchs et al., 2018). the immunological effects of oligomeric chitin fragments in epithelial cells have not yet been assessed. The findings that plant LysM receptors recognize chitin oligomers and that AMCase is secreted constitutively in airways (Van Dyken et al., 2017), prompted us to evaluate the immunological effects of chitin oligosaccharides in airway epithelial cells. We first tested the chitin oligomers with defined length (DP 2-7) and found that only chitin heptamers (DP7) elicited substantial IL-6 and IL-8 secretion in BEAS-2B cells and little or no activity for smaller oligomers (DP < 6) (Fig. 3-15 and Fig. 3-16). This was in line with the size-dependent chitin sensing and downstream activation of defense mechanisms via conserved LysM receptors in plants (Hamel & Beaudoin, 2010). These results also suggest that the production of IL-6 and IL-8 in response to larger chitin oligomers was not due to contamination in the chitin preparations.

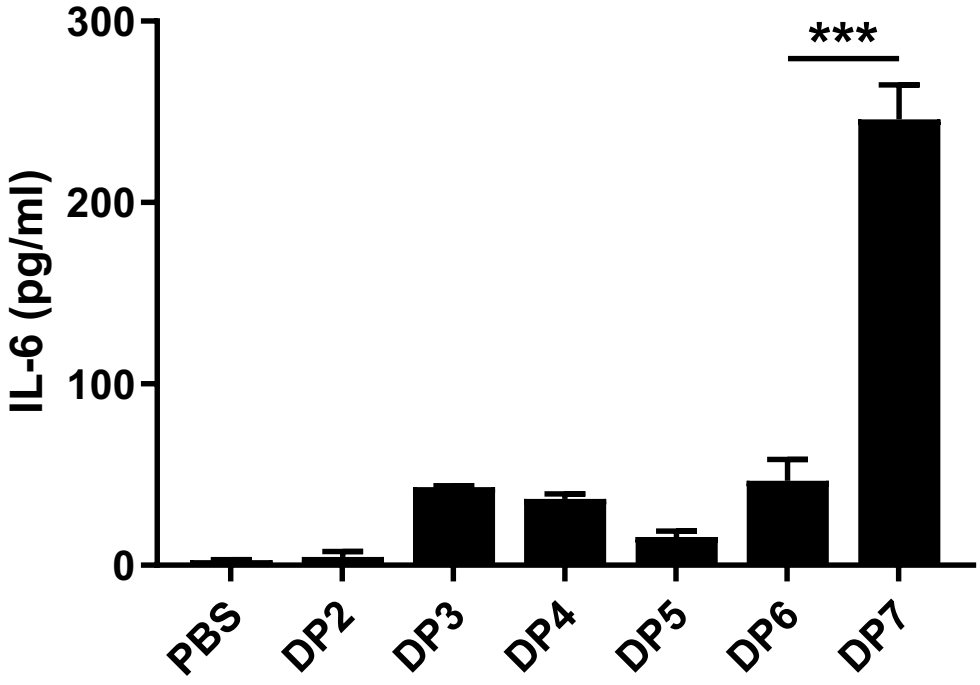

Figure 3-15. Size-dependent effects of chitin oligomers on IL-6 production in BEAS-2B cells. ELISA of IL-6 in supernatants of BEAS-2B cells stimulated for 24 h with 500 μM chitin oligomers with varied degrees of polymerization (mean and SD of three samples per group). ***P < 0.001 (Student's t-test). Data courtesy of Brad Howard, Lawrence lab.

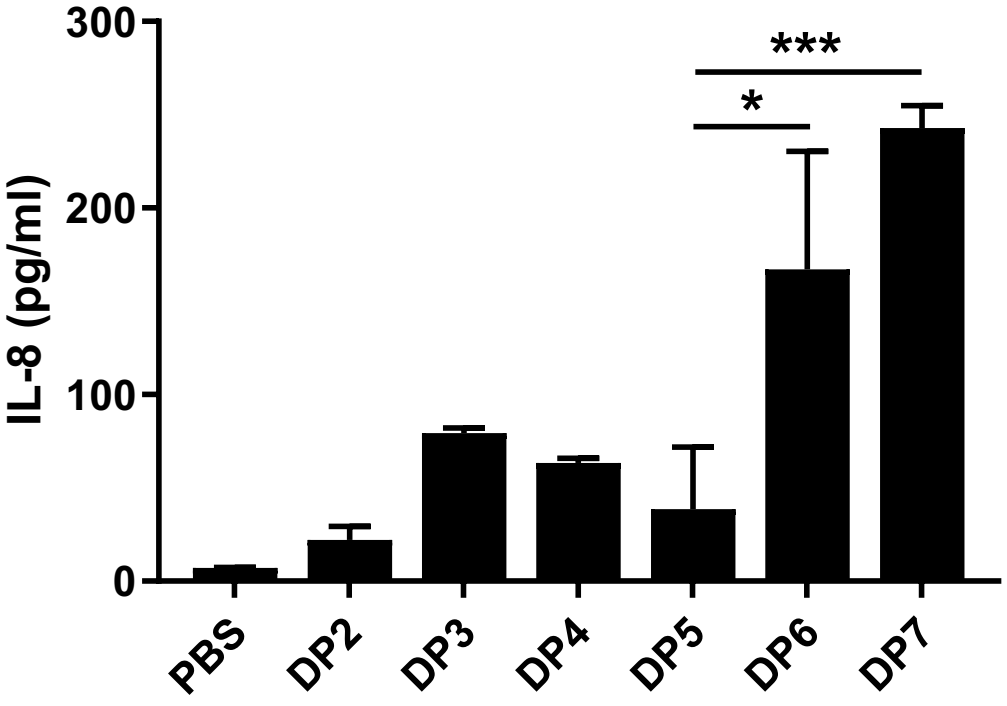

Figure 3-16. Size-dependent effects of chitin oligomers on IL-8 production in BEAS-2B cells. ELISA of IL-8 in supernatants of BEAS-2B cells stimulated for 24 h with 500 μM chitin oligomers with varied degrees of polymerization (mean and SD of three samples per group). *P < 0.05 and ***P < 0.001 (Student's t-test). Data courtesy of Brad Howard, Lawrence lab.

Notably, it appears that the production of CCL2 by chitin oligomers was independent of the size of chitin (Fig. 3-17).

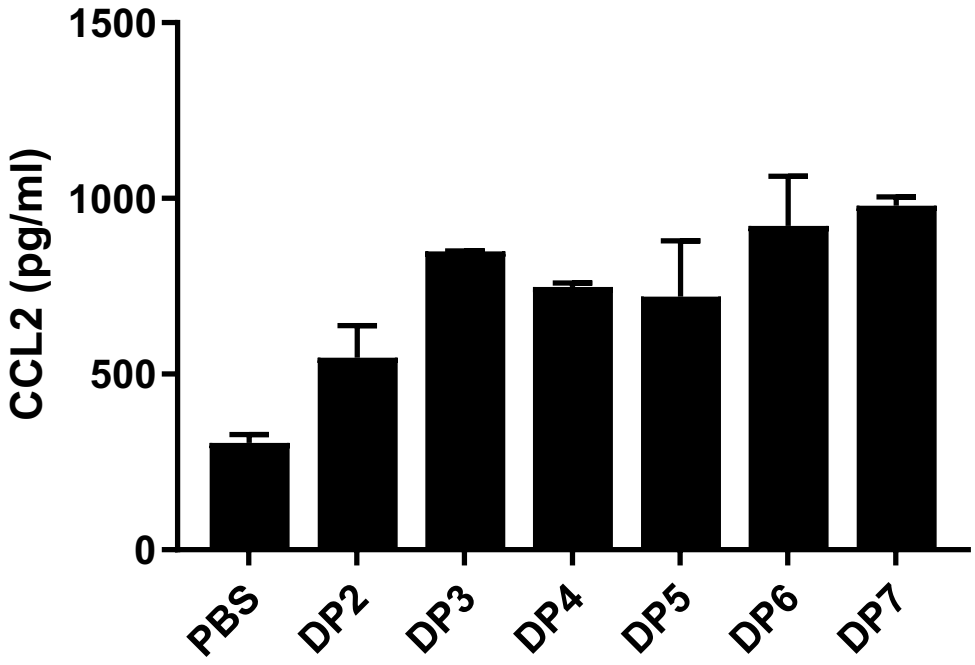

Figure 3-17. Size-dependent effects of chitin oligomers on IL-8 production in BEAS-2B cells. ELISA of CCL2 in supernatants of BEAS-2B cells stimulated for 24 h with 500 μM chitin oligomers with varied degrees of polymerization (mean and SD of three samples per group). Data courtesy of Brad Howard, Lawrence lab.

We then investigated the effect of LYSMD3 knockdown on the expression of cytokines in lung epithelial cells when stimulated with chitin or chitin-containing fungi. Knockdown of LYSMD3 in BEAS-2B cells using a siRNA pool (Fig. 3-18) nearly abolished the production of IL-8 in response to stimulation with chitin oligomers (DP7) or chitin particles (Fig. 3-19). LYSMD3 knockdown also reduced IL-8 production induced by the spores of the allergenic, fungus *A. alternata* (Fig. 3-19). Similar results were obtained using primary human bronchial epithelial (NHBE) cells (Fig. 3-20 and Fig. 3-21) and human alveolar type II epithelial cells (A549) (Fig. 3-22 and Fig. 3-23). To confirm those results, we used a second, non-overlapping single siRNA to silence LYSMD3 (Fig. 3-24). LYSMD3 knockdown by the single siRNA duplex also decreased chitin-induced IL-8 production in BEAS-2B cells (Fig. 3-25). To determine the specificity of LYSMD3 signaling, we investigated whether knockdown of LYSMD3 could also affect cytokine production induced by other PRR ligands. In contrast, LYSMD3 knockdown had no effect on cytokines production induced by the TLR3 ligand Poly (I:C) and TLR5 agonist FLA-ST (Fig. 3-19 and Fig. 3-26).

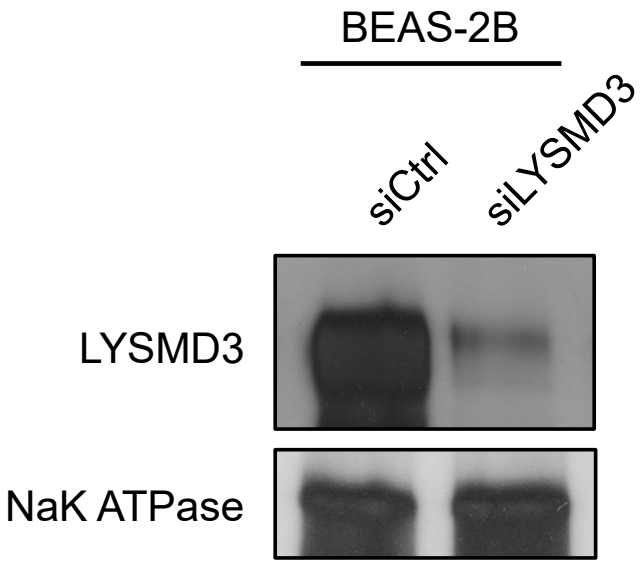

Figure 3-18. Immunoblot analysis of LYSMD3 in membrane protein fraction of BEAS-2B cells 48 h after transfection with control siRNA or a siRNA pool specific for LYSMD3. NaK ATPase serves as a loading control.

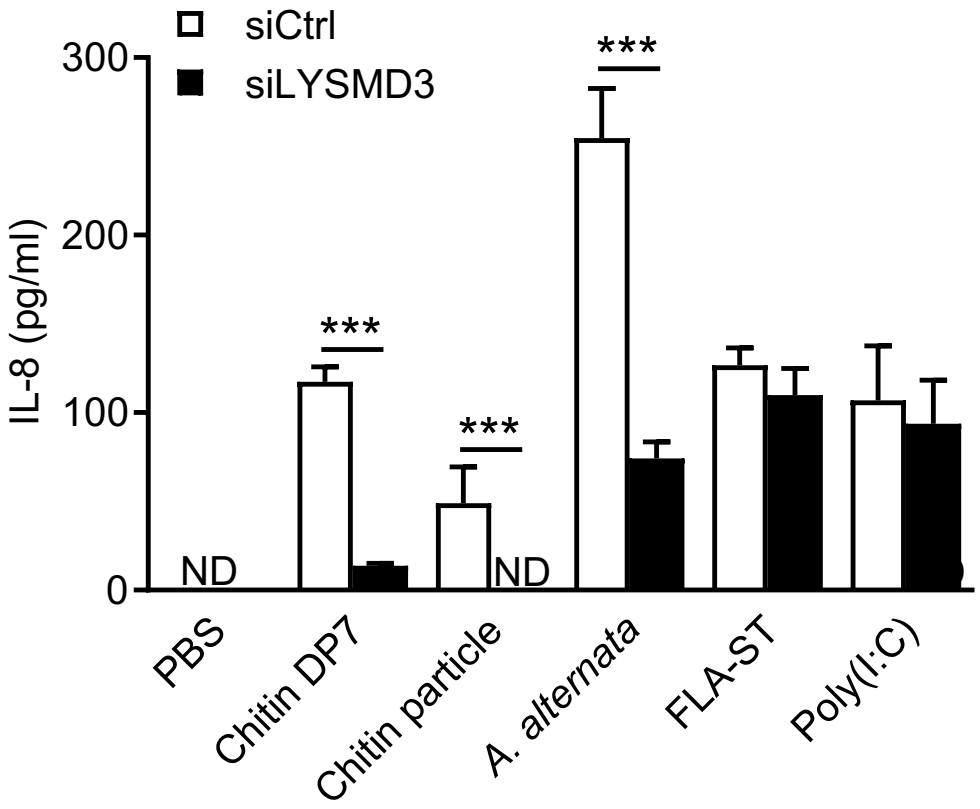

Figure 3-19. Knockdown of LYSMD3 impaired chitin and *Alternaria* spore induced IL-8 production, but had no effect on had no effect on IL-8 production induced by Poly (I:C) and FLA-ST in BEAS-2B cells. ELISA of IL-8 in supernatants of BEAS-2B cells transected with siRNAs, then stimulated for 24 h with chitin DP7 (500μg/ml), chitin particles (500 μg/ml), *Alternaria alternata* spores (2×10^5/ml), FLA-ST (300 ng/ml) or Poly(I:C) (3 μg/ml) (mean and SD of three samples per group). ***P < 0.001 (Student's t-test).

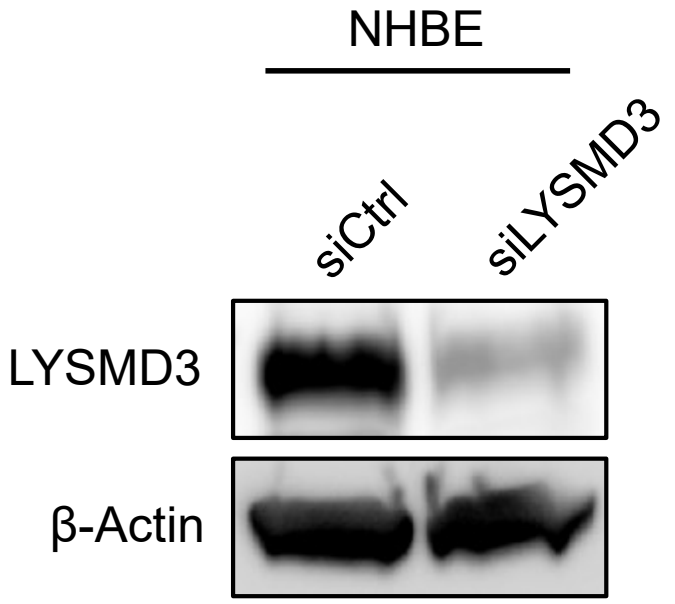

Figure 3-20. Immunoblot analysis of LYSMD3 in whole-cell lysates of NHBE cells 48 h after transfection with control siRNA or siRNA specific for LYSMD3. β-Actin serves as a loading control.

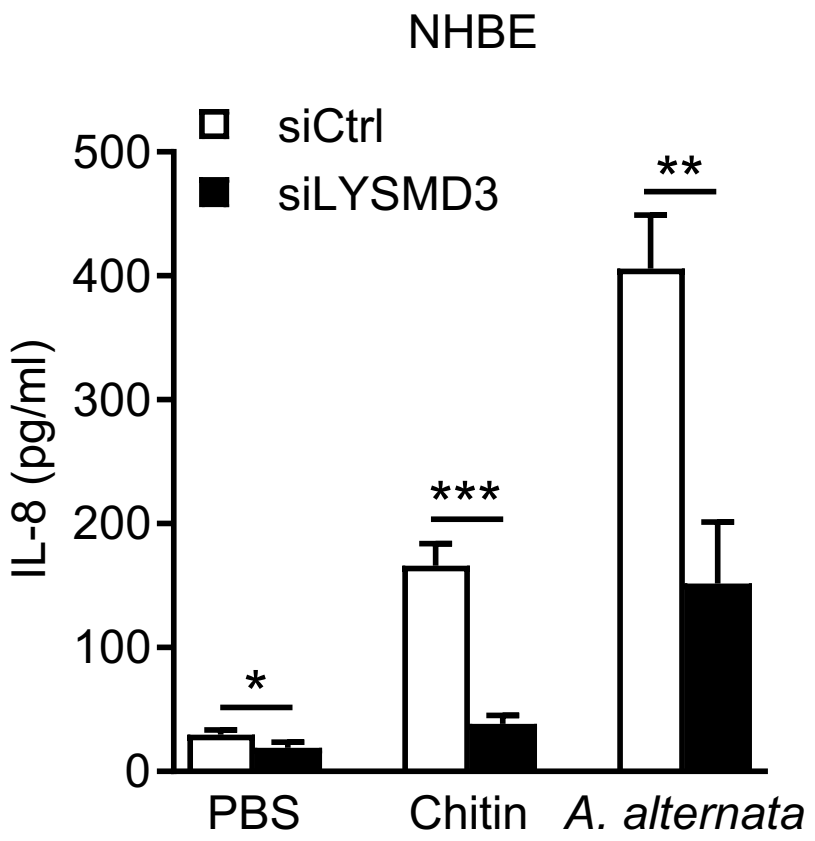

Figure 3-21. Knockdown of LYSMD3 reduced chitin and *Alternaria* spore induced IL-8 production in NHBE cells. ELISA of IL-8 in supernatants of NHBE cells transected with siRNA, then stimulated for 24 h with chitin DP7 (500 μg/ml) or *Alternaria alternata* spores (2×10^5/ml). (mean and SD of three samples per group). *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

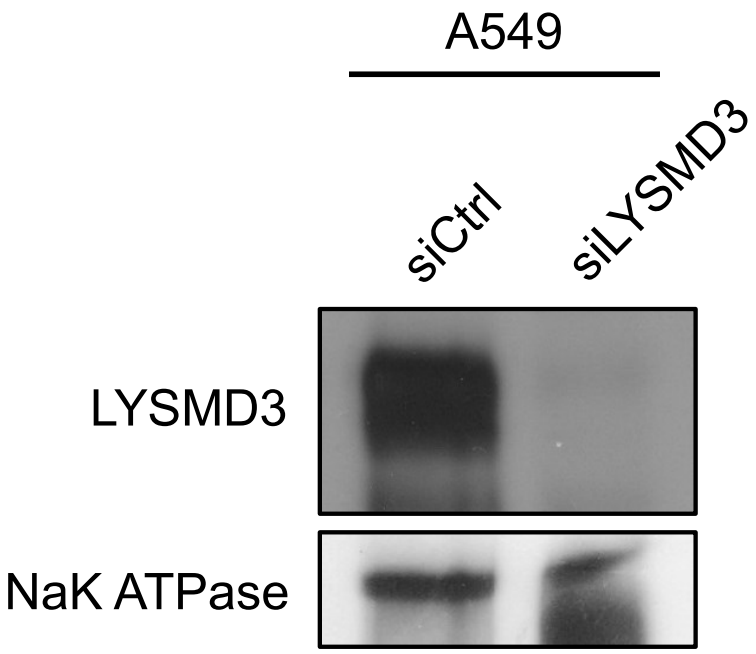

Figure 3-22. Immunoblot analysis of LYSMD3 in membrane protein fraction of A549 cells 48 h after transfection with control siRNA or siRNA specific for LYSMD3. NaK ATPase serves as a loading control.

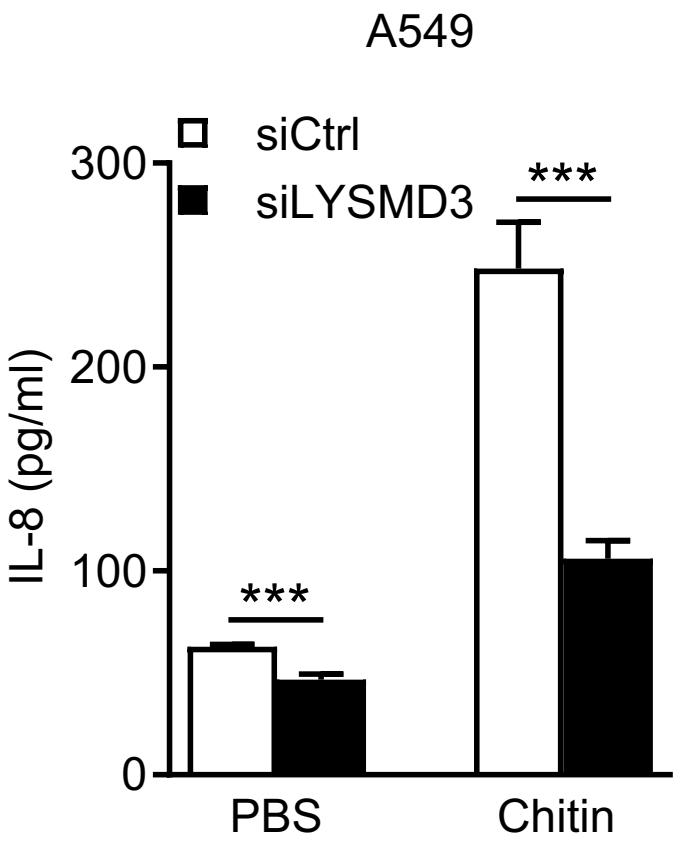

Figure 3-23. Knockdown of LYSMD3 reduced chitin induced IL-8 production in A549 cells. ELISA of IL-8 in supernatants of A549 cells transected with siRNA, then stimulated for 24 h with chitin DP7 (500 µg/ml) (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

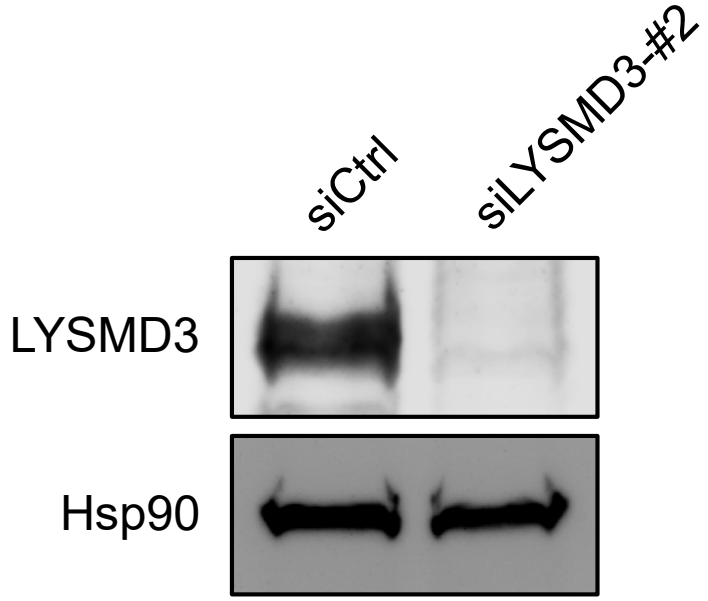

Figure 3-24. Immunoblot analysis of LYSMD3 in whole-cell lysates of BEAS-2B cells 48 h after transfection with control siRNA or siRNA individual for LYSMD3. Hsp90 serves as a loading control.

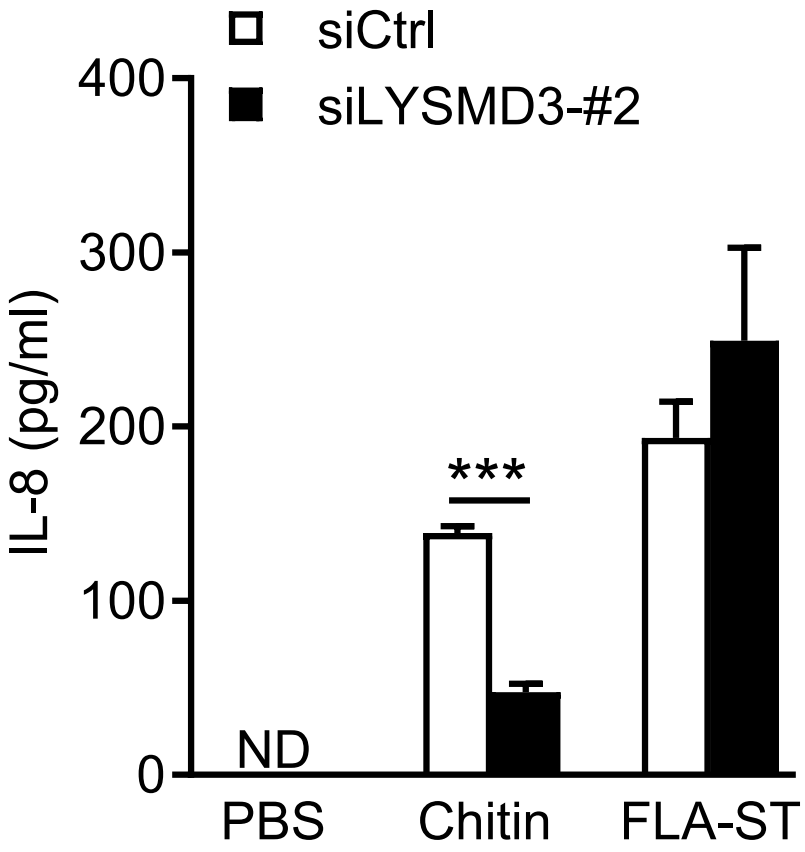

Figure 3-25. LYSMD3 depletion with single siRNA duplex reduces epithelial cell production of IL-8 in response to chitin. ELISA of IL-8 in supernatants of BEAS-2B cells transected with siRNA, then stimulated for 24 h with chitin DP7 (500 µg/ml) or FLA-ST (300 ng/ml) (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

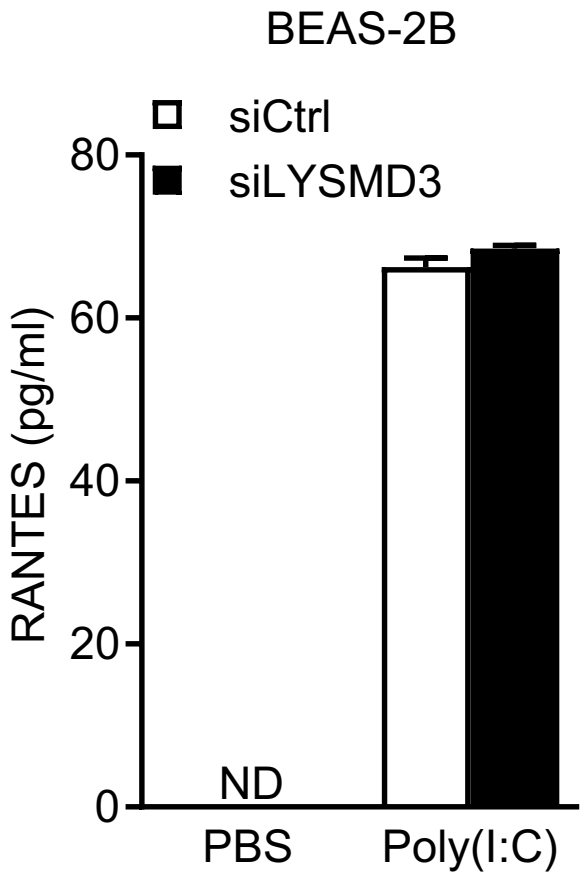

Figure 3-26. LYSMD3 depletion had no effect on RANTES production in BEAS-2B cells in response to Poly(I:C). ELISA of RANTES in supernatants of BEAS-2B cells transected with siRNA, then stimulated for 24 h with Poly(I:C) (3 μg/ml) (mean and SD of three samples per group).

Similarly, in immune cells, silencing of LYSMD3 resulted in lower production of IL-6 in mouse macrophages stimulated with chitin but had no effect on IL-6 production in response to Pam3CSK4 (Fig. 3-27 and Fig. 3-28).

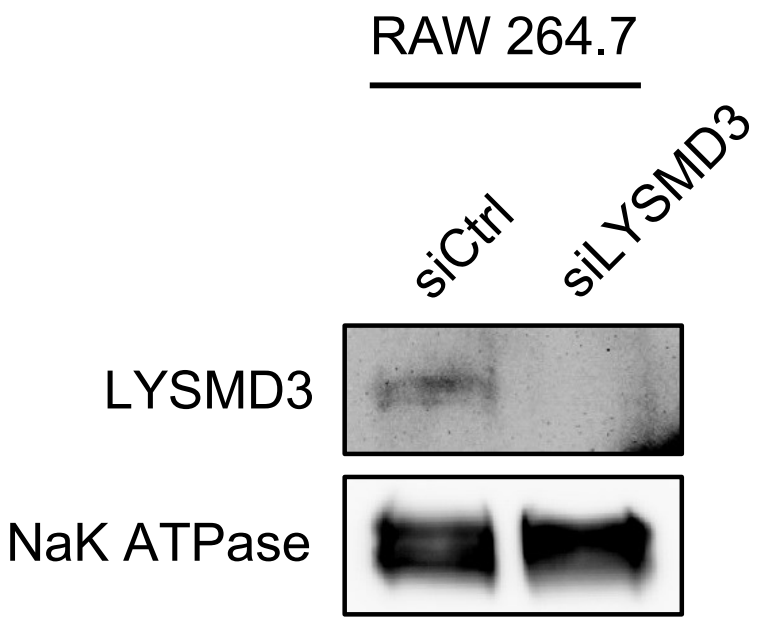

Figure 3-27. Immunoblot analysis of LYSMD3 in the membrane protein fraction of RAW 264.7 cells 48 h after transfection with control siRNA or siRNA specific for LYSMD3. NaK ATPase serves as a loading control.

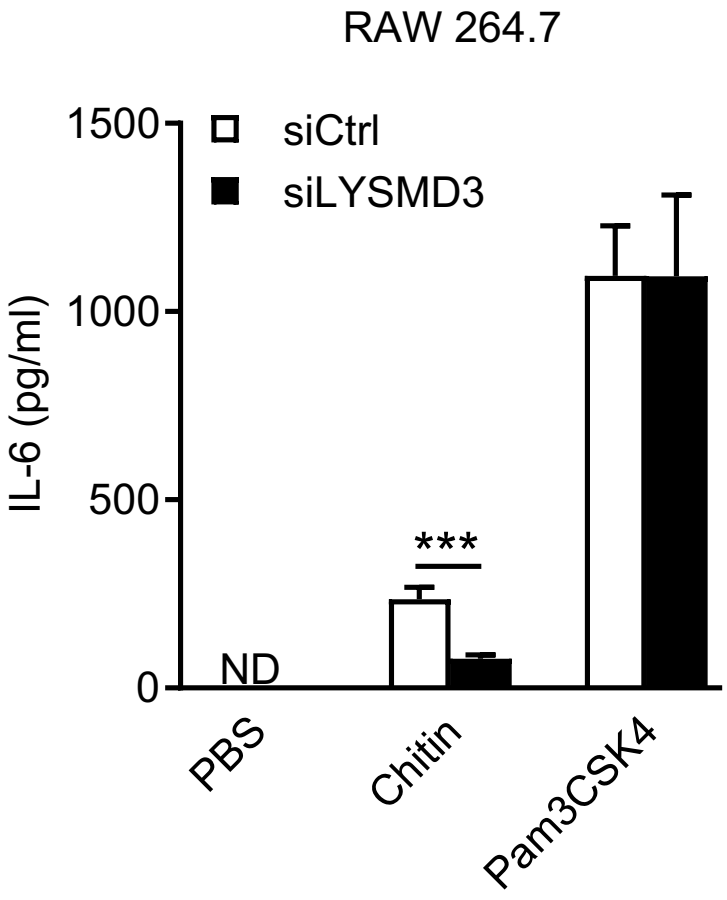

Figure 3-28. Knockdown of LYSMD3 reduced chitin but not Pam3CSK4 induced IL-8 production in RAW 264.7 cells. ELISA of IL-6 in supernatants of RAW 264.7 cells transected with siRNA, then stimulated for 24 h with chitin DP7 (100 µg/ml) or Pam3CSK4 (100 ng/ml) (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

To further exclude the possibility that LYSMD3 knockdown had a more global effect, such as causing loss of cell viability or fitness and therefore hyporesonsiveness to certain innate immune stimuli, we performed MTT and LDH cytotoxicity assays. LYSMD3 knockdown had no effect on bronchial epithelial cell viability or proliferation (Fig. 3-29 and Fig. 3-30), indicating LYSMD3 is not essential for bronchial epithelial cell viability, cytotoxicity, or membrane integrity.

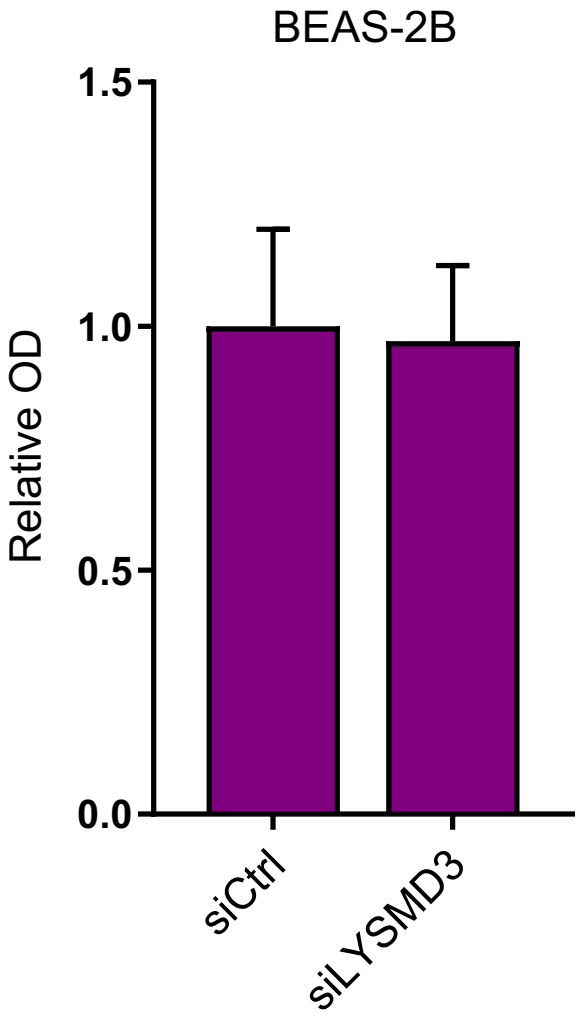

Figure 3-29. LYSMD3 depletion did not affect bronchial epithelial cell viability. MTT assay 48 h after transient transfection of siRNA in BEAS-2B cells. Cell growth rate was expressed as absorbance at 570 (mean and SD of eight samples per group).

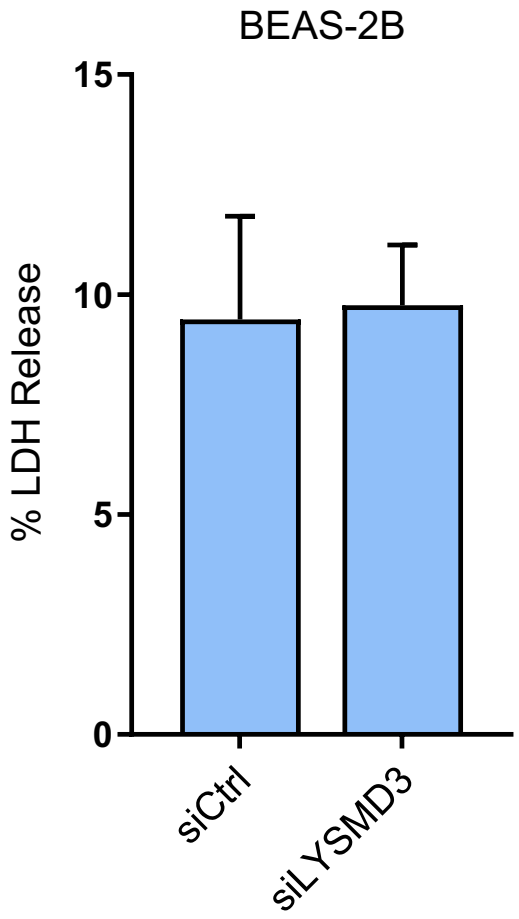

Figure 3-30. LYSMD3 depletion had no effect on LDH release by bronchial epithelial cells. LDH cytotoxicity assay 48 h after transient transfection of siRNAs in BEAS-2B cells (mean and SD of eight samples per group).

Next, we used CRISPR–Cas9 to further validate the results of LYSMD3 knockdown. We used two single guide RNAs (sgRNAs) to knock out LYSMD3 in BEAS-2B cells (Fig. 3-31). Consistent with the siRNA results, both the two LYSMD3 sgRNAs significantly decreased chitin-induced IL-8 production, but had no effect on IL-8 secretion when stimulated with FLA-ST, as compared with a non-targeting sgRNA control (Fig. 3-32). Together, these data suggest that LYSMD3 was specifically involved in chitin induced innate immune responses in epithelial cells.

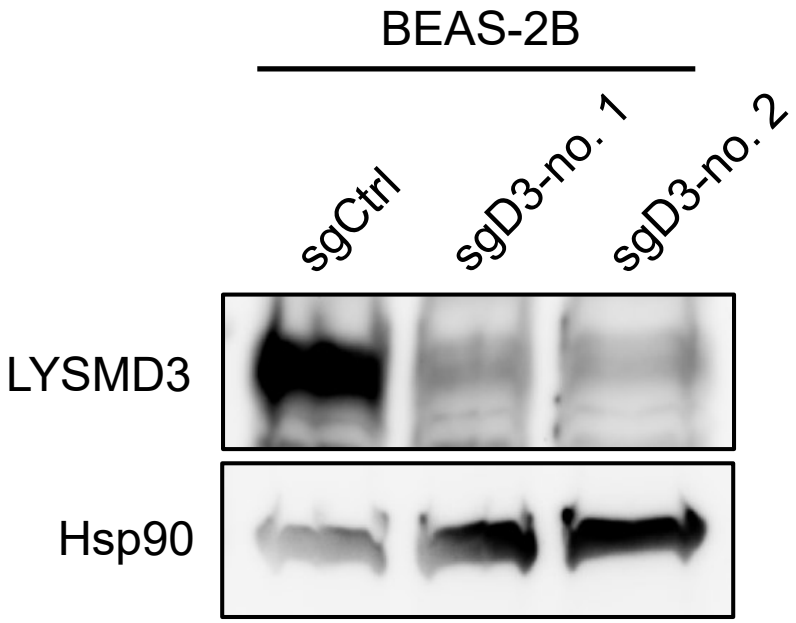

Figure 3-31. The protein expression of LYSMD3 in BEAS-2B cell knockout of LYSMD3 (sgLYSMD3) by the CRISPR–Cas9 system. Hsp90 was used as the loading control.

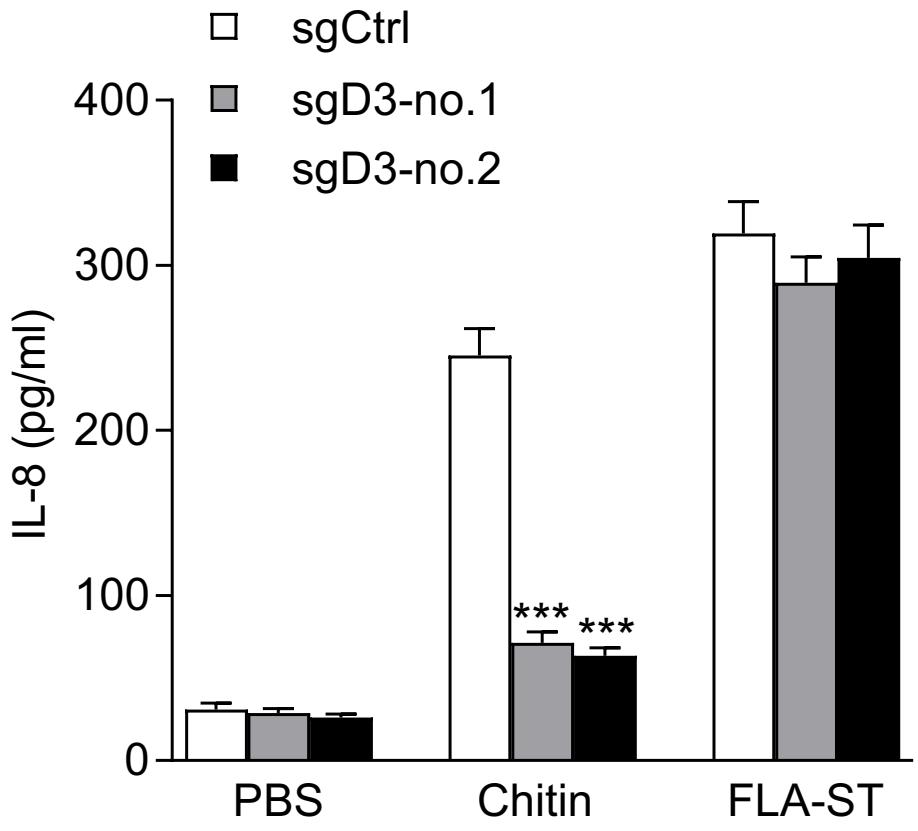

Figure 3-32. CRISPR knockout of LYSMD3 reduces chitin induced IL-8 production in BEAS-2B cells. ELISA of IL-8 in supernatants of BEAS-2B cell knockout of LYSMD3, and left unstimulated or stimulated for 24 h with chitin DP7 (500 μg/ml) or FLA-ST (300 ng/ml) (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

Chitin particles induce secretion of CCL2 in mouse airway epithelial cells and CCL2 triggers alternative activation of macrophages and allergic inflammation in vivo (R. M. Roy et al., 2012). We assessed whether LYSMD3 is involved in chitin-induced CCL2 secretion in Mouse airway epithelial cells. We determined that LYSMD3 knockdown significantly impaired CCL2 production in LA-4 cells in response to chitin particles (Fig. 3-33 and Fig. 3-34).

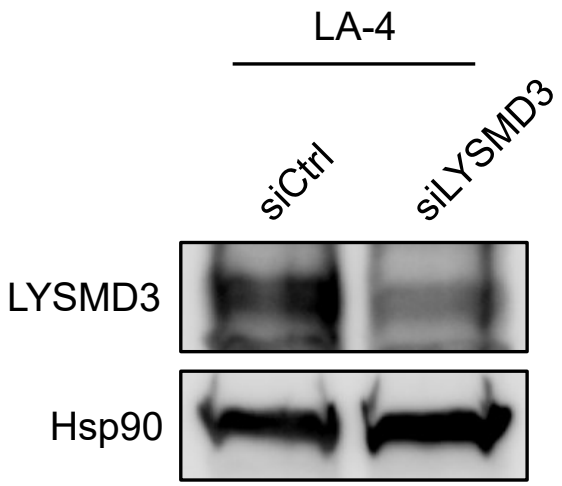

Figure 3-33. Immunoblot analysis of LYSMD3 in whole-cell lysates of LA-4 cells 48 h after transfection with control siRNA or siRNA for LYSMD3. Hsp90 serves as a loading control.

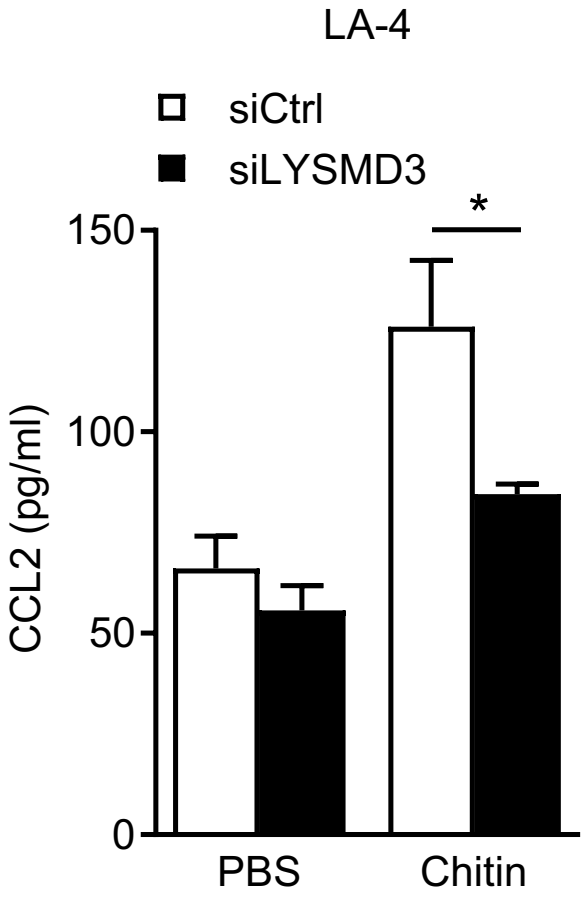

Figure 3-34. Knockdown of LYSMD3 reduced chitin induced CCL2 production in LA-4 cells. ELISA of CCL2 in supernatants of LA-4 cells transected with siRNA, then stimulated for 24 h with chitin particles (500 μg/ml) (mean and SD of three samples per group). *P < 0.05 (Student's t-test)

Interestingly, IL-33, which is stored in the nuclei of airway epithelial cells, was released quickly into the airway lumen when exposed to *Alternaria* antigen extract but not to other common aeroallergens (Bartemes & Kita, 2018). The molecular mechanisms that mediate IL-33 release are a subject of intense research. We found that knockdown of LYSMD3 significantly reduced *Alternaria* extract induced IL-33 release in IL-33-producing human bronchial epithelial cells (HBE33) (Uchida et al., 2017) (Fig. 3-35). Together, our data support the hypothesis that LYSMD3 has bona fide pathological relevance in the context of fungal innate immunity and allergic inflammation.

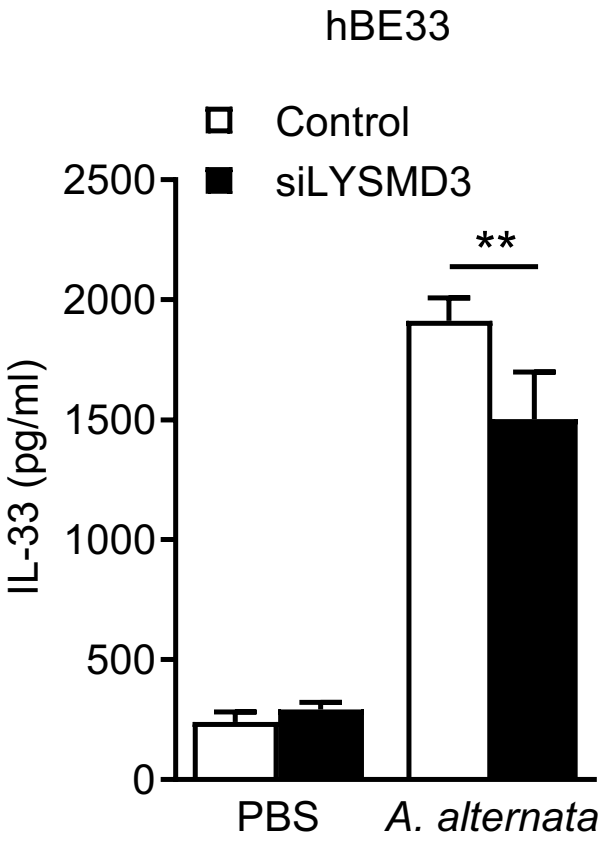

Figure 3-35. Knockdown of LYSMD3 reduced *A. alternata* induced IL-33 release in hBE-33 cells. ELISA of IL-33 in supernatants of hBE33 cells transected with siRNA as in Fig 3d, then stimulated for 1 h with Alternaria extract (200 µg/ml) (mean and SD of four samples per group). **P < 0.01 (Student's t-test).

3.4 Antagonizing LYSMD3 ectodomain function impairs epithelial cell inflammatory responses induced by chitin

We next examined whether antagonization of LYSMD3 ectodomain can block chitin-induced innate immune responses. First, we found that the addition of soluble LYSMD3 ectodomain to primary human bronchial epithelial cells negatively regulated chitin-induced IL-8 production in a dose-dependent manner (Fig. 3-36), but had no effect on secretion of IL-8 induced by flagellin (Fig. 3-37).

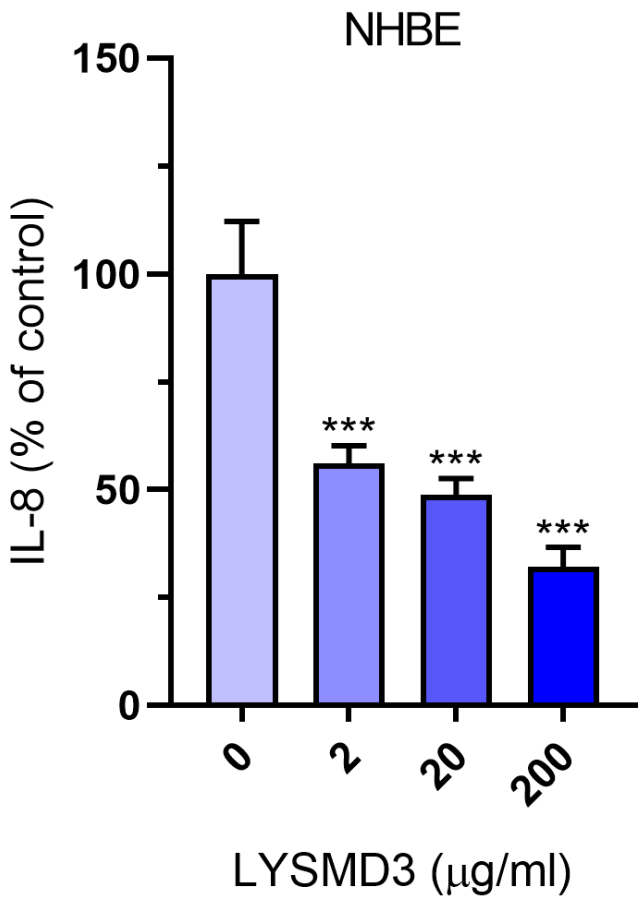

Figure 3-36. Antagonizing LYSMD3 ectodomain function by soluble LYSMD3 ECD inhibited epithelial cell IL-8 production induced by chitin. ELISA of IL-8 in supernatants of NHBEs in the presence of indicated concentrations of soluble ectodomain of LYSMD3, then stimulated for 24 h with chitin DP7 (500 μg/ml). IL-8 concentrations in cell-free supernatants were normalized to the values without ectodomain of LYSMD3 as 100% (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

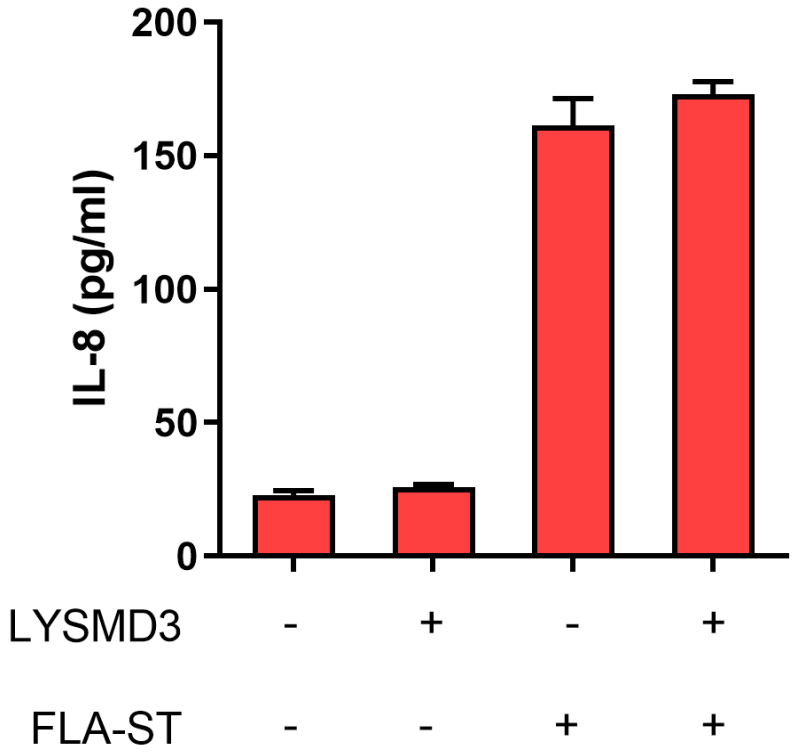

Figure 3-37. Soluble LYSMD3 ECD had no effect on secretion of IL-8 induced by flagellin. ELISA of IL-8 in supernatants of NHBEs in the presence of soluble ectodomain of LYSMD3 (200 µg/mL), then stimulated for 24 h with FLA-ST (300 ng/mL) (mean and SD of four samples per group).

More importantly, treatment with a polyclonal antibody against LYSMD3 ectodomain significantly inhibited chitin-induced IL-8 production in both BEAS-2B and A549 cells in a dose-dependent manner, as compared with a polyclonal control antibody (Fig. 3-38).

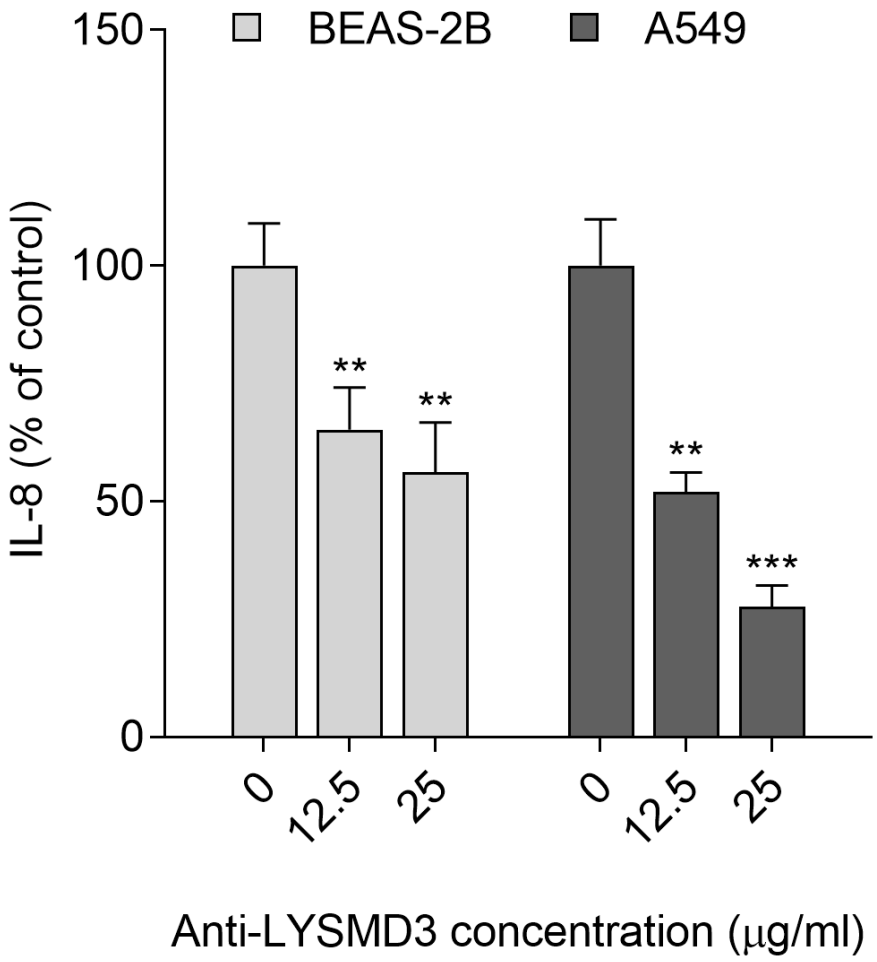

Figure 3-38. **Antagonization of LYSMD3 ectodomain by LYSMD3 antibody reduced epithelial cell IL-8 production induced by chitin**. ELISA of IL-8 in supernatants of BEAS-2B or A549 cells pre-incubation with various concentrations of LYSMD3 antibody or isotype control antibody for 30 min, then stimulated for 24 h with chitin DP7 (500 μg/ml). IL-8 concentrations in supernatants were normalized to the values with isotype control antibody as 100% (mean and SD of three samples per group). **P < 0.01 and ***P < 0.001 (Student's t-test).

In addition to chitin, we found that LYSMD3 also bound to β-glucan. It is unclear whether LYSMD3 plays a role in sensing β-glucan on epithelial cells. However, airway epithelial cells are unresponsive or respond poorly to β-glucan that is consistent with the low or no expression of Dectin-1 (Heyl et al., 2014; H. M. Lee, Yuk, Shin, & Jo, 2009; Mayer et al., 2007). A549 cells do not express Dectin-1 (Heyl et al., 2014; H. M. Lee et al., 2009). Although Dectin-1 is inducible in A549 cells by zymosan, *Staphylococcus aureus*, or *Mycobacterium tuberculosis*, curdlan (pure β-glucan) alone does not induce the expression of Dectin-1 (H. M. Lee et al., 2009). We found that the activation of NF-κB in A549-Dual reporter cells was not induced by even high dose (1 mg/ml) of various pure β-glucans (laminarin, whole glucan particles (WGP) soluble, and curdlan) or chitin DP5 at 200 μM (data not shown). However, preincubation of A549 cells with laminarin, curdlan or chitin DP5 led to a significant reduction in the level of NF-κB activation subsequently induced by chitin (Fig. 3-39). Our results and other reports show that Dectin-1 does not bind chitin (Fig. 3-14) (Gantner et al., 2005; Gour et al., 2018; Mora-Montes et al., 2011). The addition of either Dectin-1 antagonist WGP soluble, or Dectin-1 blocking antibody had no effect on NF-κB activation in response to chitin (Fig. 3-39), indicating that Dectin-1 was not involved in the recognition of chitin by A549 cells and the inhibition of chitin-induced NF-κB activation by β-glucans was independent of blockage of Dectin-1. Furthermore, preincubation of A549 cells with laminarin, curdlan, or chitin DP5 did not affect the induction of NF-κB activation stimulated by FLA-ST (Fig. 3-39), suggesting that blocking was not due to steric masking of specific cell surface receptors.

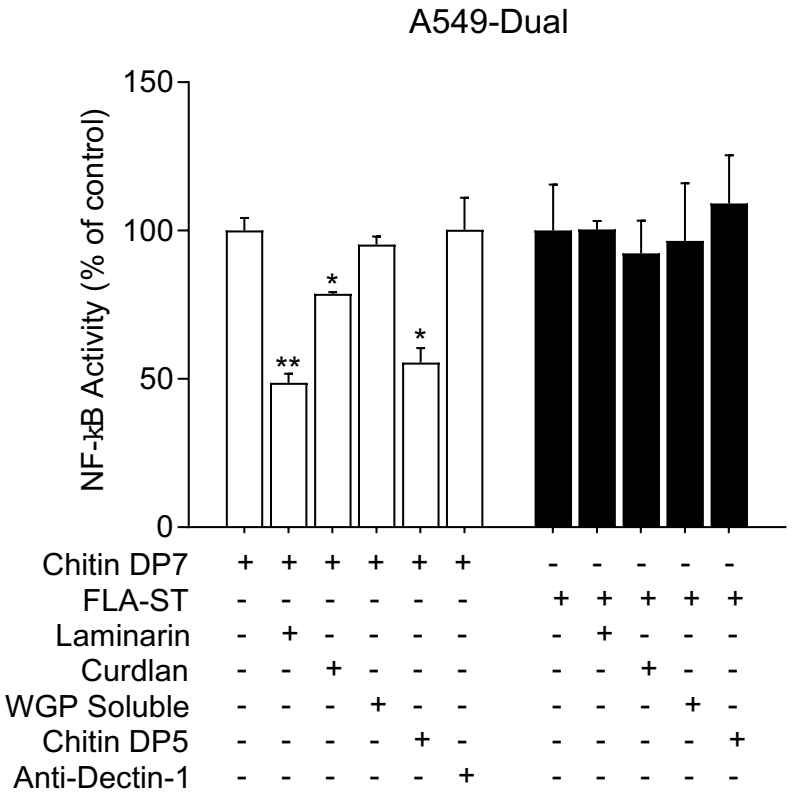

Figure 3-39. **Blockage of LYSMD3 by various LYSMD3 ligands inhibited NF-κB activation induced by chitin**. A549-Dual cells were preincubated with laminarin (1 mg/ml), curdlan (1 mg/ml), WGP soluble (1 mg/ml), chitin DP5 (200 μM), Dectin-1 blocking antibody (3 μg/ml), or corresponding isotype control antibody and then stimulated with chitin DP7 (500 μg/ml) or FLA-ST (300 ng/ml) for 24 h. NF-κB activation was determined using QUANTI-Blue, and optical density (OD) at 655 nm was measured. NF-κB activities were normalized to the values in cells stimulated with chitin or FLA-ST in the absence of receptors antagonists or in the presence of isotype control antibodies (not shown) (mean and SD of three samples per group). *P < 0.05 and **P < 0.01 (Student's t-test).

Finally, laminarin, curdlan and chitin DP5, but not WGP soluble, were able to bind LYSMD3 ECD and presumably block binding to chitin of purified LYSMD3 (Fig. 3-40), suggesting that the inhibition of chitin signaling by laminarin, curdlan and chitin DP5 was due to blocking of LYSMD3.

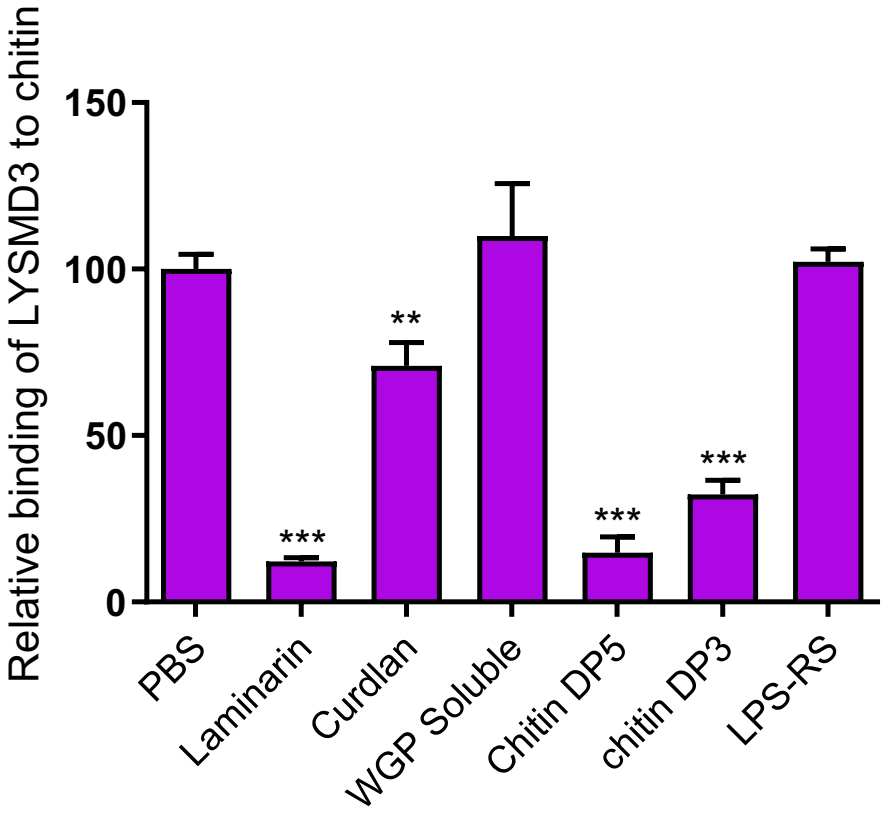

Figure 3-40. **Laminarin, curdlan, chitin DP5 and chitin DP3 blocked binding of LYSMD3 to immobilized chitin DP7.** Relative binding of recombinant LYSMD3 (50 μg/ml) to immobilized chitin DP7 in the presence of laminarin (100 μg/ml), curdlan (500 μg/mL), WGP soluble (500 μg/ml), chitin DP5 (200 μM), chitin DP3 (200 μM), or LPS-RS (500 μg/ml), as determined by ELISA. The binding abilities were normalized to the values without carbohydrates as 100% (mean and SD of three samples per group). **P < 0.01 and ***P < 0.001 (Student's t-test).

3.5 Syk mediates LYSMD3-induced immune responses to chitin

Next we investigated how LYSMD3 mediates chitin signal transduction. We noticed that LYSMD3 contains a single cytoplasmic YxxL sequence which is known as a hemi-immunoreceptor tyrosine-based activation motif (hemi-ITAM). The tyrosine residue of hemi-ITAM is often phosphorylated by Src family kinases and binds and activatestyrosine kinase Syk through its two SH2 domains (Manne et al., 2015). We detected the tyrosine phosphorylation of exogenous LYSMD3 protein and the association between LYSMD3 and Syk in resting HEK 293 cells (Fig. 3-41). Whether the tyrosine phosphorylation of LYSMD3 and the interaction of LYSMD3 and Syk will be enhanced by stimulation with chitin needs further investigation.

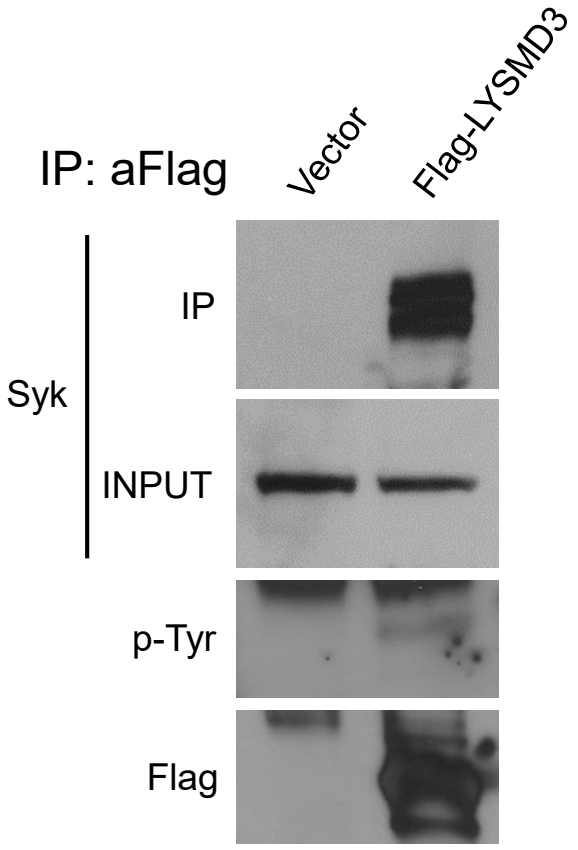

Figure 3-41. **LYSMD3 interacts with Syk.** Immunoblot analysis of proteins immunoprecipitated (with anti-Flag antibody) from lysates of HEK 293 cells transiently transfected with plasmid expressing Flag-tagged LYSMD3.

Remarkably, we also found that phosphorylation of Syk at $Tyr^{525/526}$ was highly induced in primary normal human bronchial epithelial (NHBE) cells stimulated with chitin and impaired in LYSMD3 knockdown cells (Fig. 3-42), which indicated that chitin-induced activation of Syk was LYSMD3 dependent.

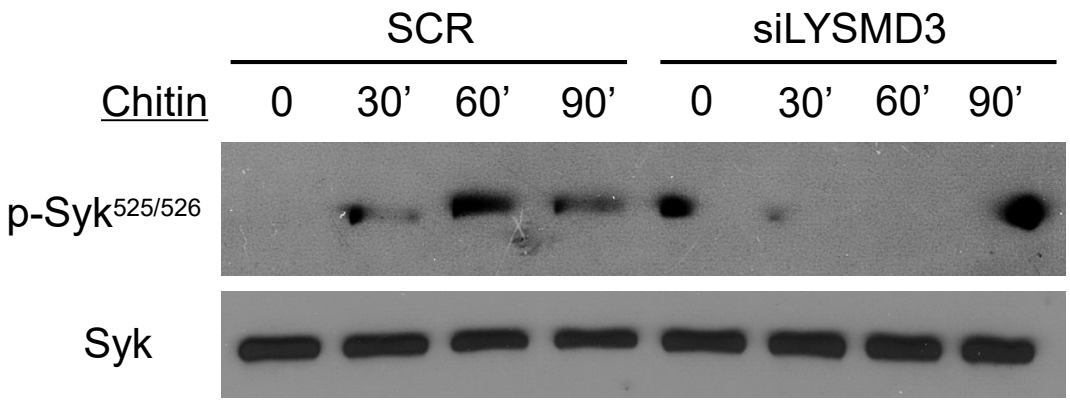

Figure 3-42. **LYSMD3 knockdown impaired Syk activation induced by chitin.** Immunoblot analysis of whole-cell lysates of NHBE transected with control siRNA or siRNA specific for LYSMD3 and left unstimulated (0 min) or stimulated for 30, 60, or 90 min with chitin DP7 (500 μg/mL).

Next, using a Syk-specific inhibitor, piceatannol, it was revealed that functional Syk is essential for chitin-Induced IL-8 production by NHBEs (Fig. 3-43). These results collectively suggested that LYSMD3 mediated chitin signaling through interacting and triggering activation of Syk.

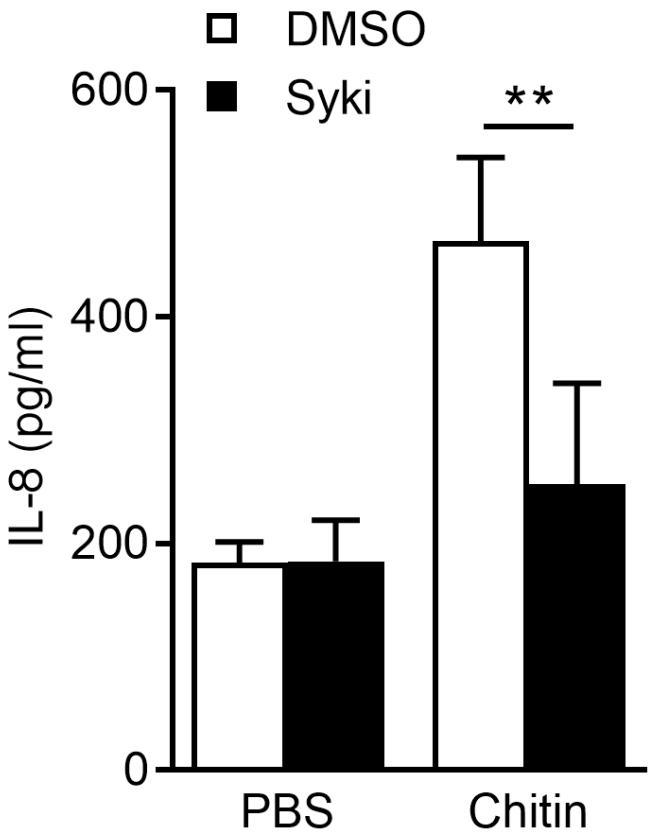

Figure 3-43. **Inhibition of Syk impaired production of IL-8 induced by chitin.** ELISA of IL-8 in supernatants of NHBE cells pre-treated with Syk inhibitor piceatannol (25 μM), then stimulated for 24 h with chitin DP7 (500 μg/mL) (mean and SD of four samples per group). **P < 0.01 (Student's t-test).

3.6 Human LysM-proteins in bronchial epithelial Cells differentially regulate innate immune responses to various PAMPs and Fungi

We also investigated the putative role of other human LysM-proteins (D1, D2, and D4) in the context of PAMP and pathogen-induced immunity in BEAS-2B cells. LYSMD1 and LYSMD2 are not predicted to be membrane-bound proteins except LYSMD4 that also contains a predicted transmembrane domain (Fig. 3-44).

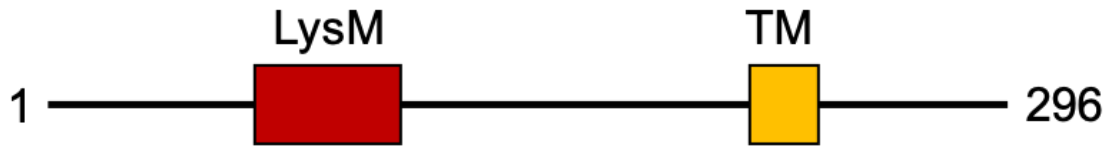

Figure 3-44. Schematic illustration of the predicted domains of human LYSMD4. The LysM domain is located at amino acid residues 74-118. The transmembrane domain is located between amino acid residues 218-238.

Interestingly, we found that knockdown of LYSMD1 or LYSMD2 using gene specific siRNAs resulted in a lower immune response following stimulation with chitin, other PAMPs, or fungal spores stimulation compared to scrambled siRNA control treated cells (Fig. 3-45, Fig. 3-46, Fig. 3-47 and Fig. 3-48).

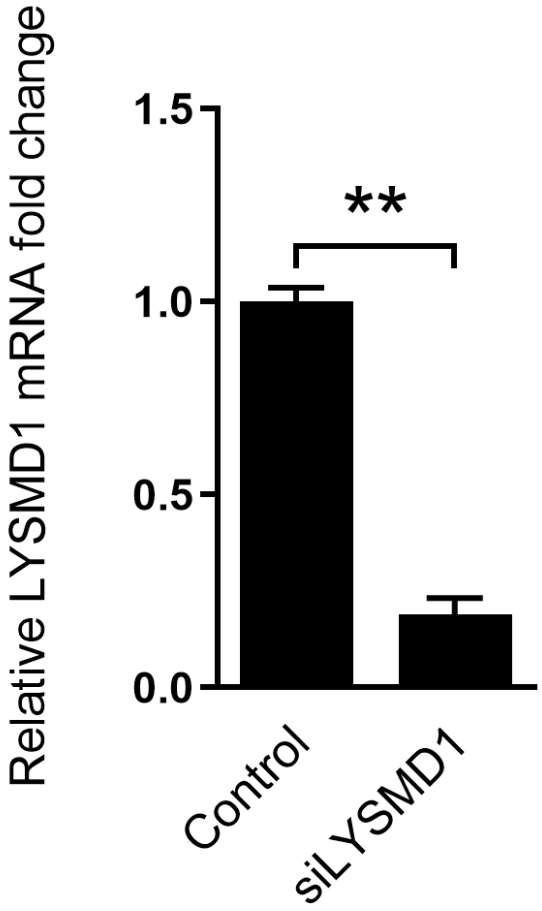

Figure 3-45. **The relative mRNAs expression level of human LYSMD1 in BEAS-2B cells 48 h after transfection with control siRNA or siRNAs specific for LYSMD1 (mean and SD of two samples per group).** **P < 0.01 (Student's t-test).

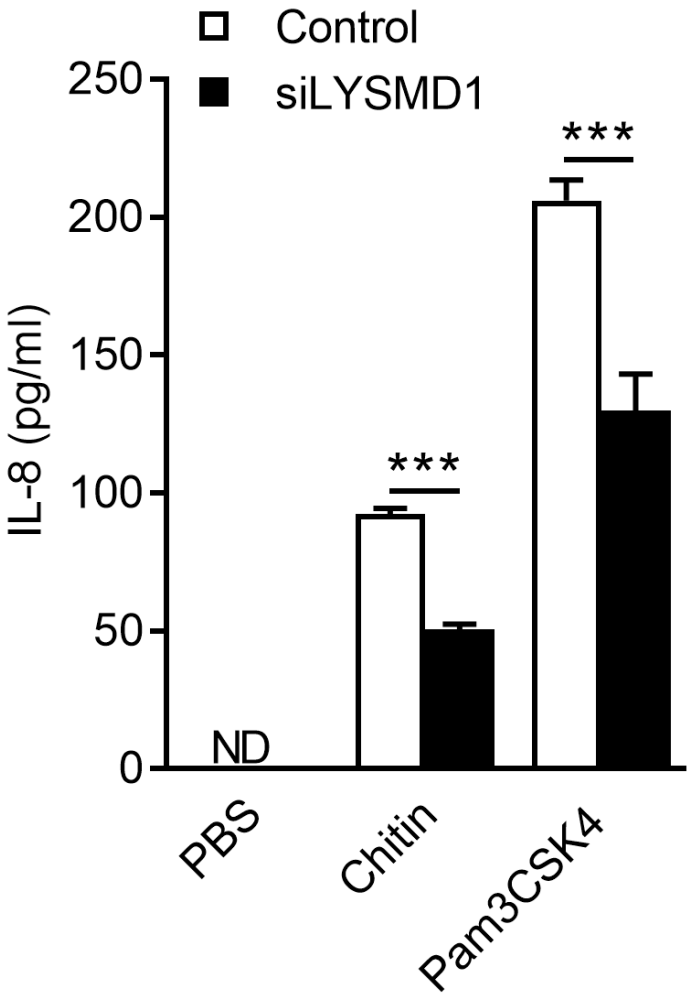

Figure 3-46. **Knockdown of LYSMD1 reduced production of IL-8 induced by chitin and Pam3CSK4.** ELISA of IL-8 in supernatants of BEAS-2B cells transected with siRNAs as in a, then stimulated for 24 h with chitin DP7 (500 µg/ml) or Pam3CSK4 (100 ng/mL) (mean and SD of four samples per group). ***P < 0.001 (Student's t-test).

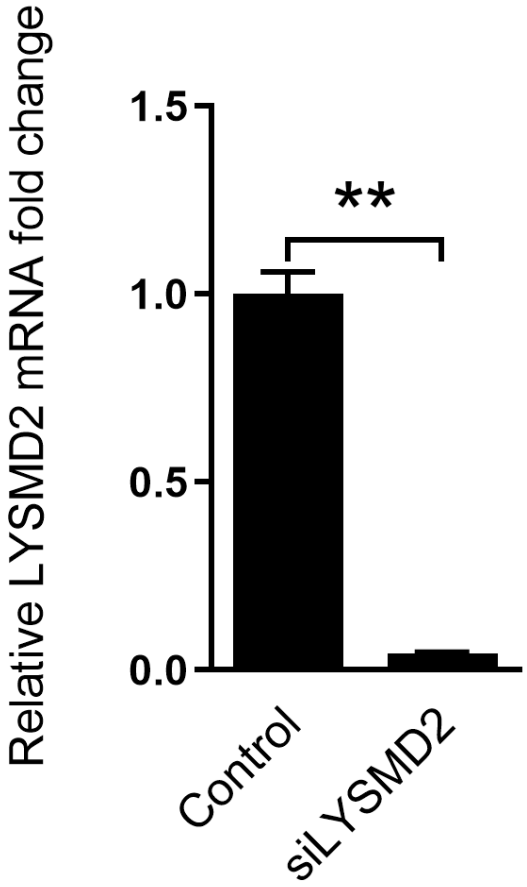

Figure 3-47. **The relative mRNAs expression level of human LYSMD2 in BEAS-2B cells 48 h after transfection with control siRNA or siRNAs specific for LYSMD2 (mean and SD of two samples per group).** **P < 0.01 (Student's t-test).

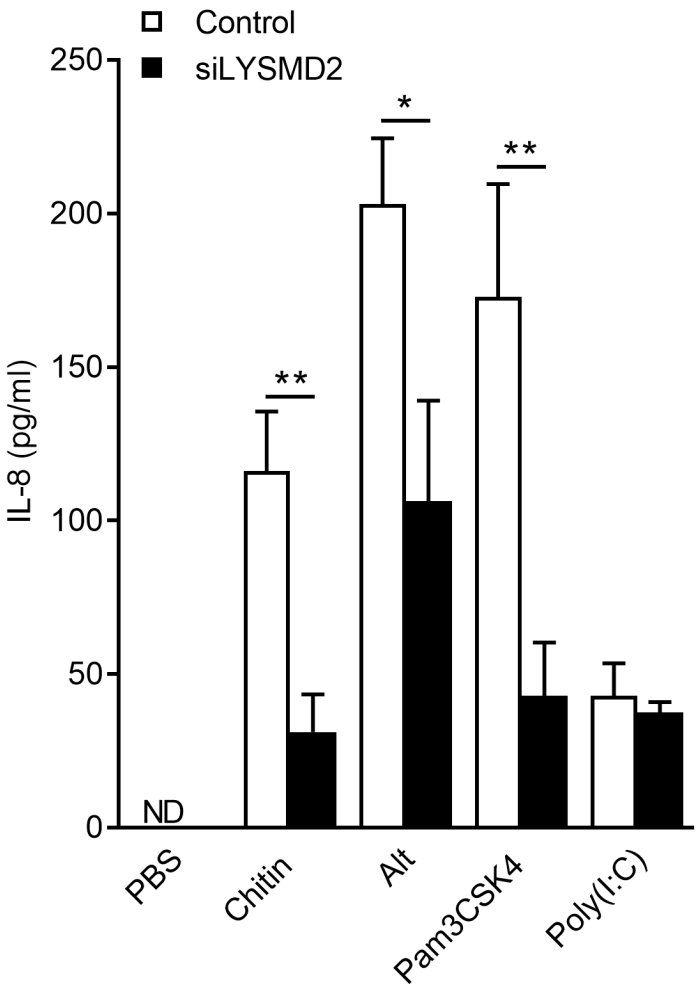

Figure 3-48. **Knockdown of LYSMD2 reduced production of IL-8 induced by chitin, Pam3CSK4 and *Alternaria* spores.** ELISA of IL-8 in supernatants of BEAS-2B cells transected with siRNAs as in a, then stimulated for 24 h with chitin DP7 (500 μg/ml), *Alternaria alternata* spores (2 x 10^5/mL), Pam3CSK4 (100 ng/mL) or Poly(I:C) (3 μg/mL) (mean and SD of three samples per group). *P < 0.05 and **P < 0.01 (Student's t-test).

However, knockdown of LYSMD4 resulted in an enhanced immune response following chitin or other PAMPs treatment, suggesting it may play an anti-inflammatory role (Fig. 3-49 and Fig. 3-50).

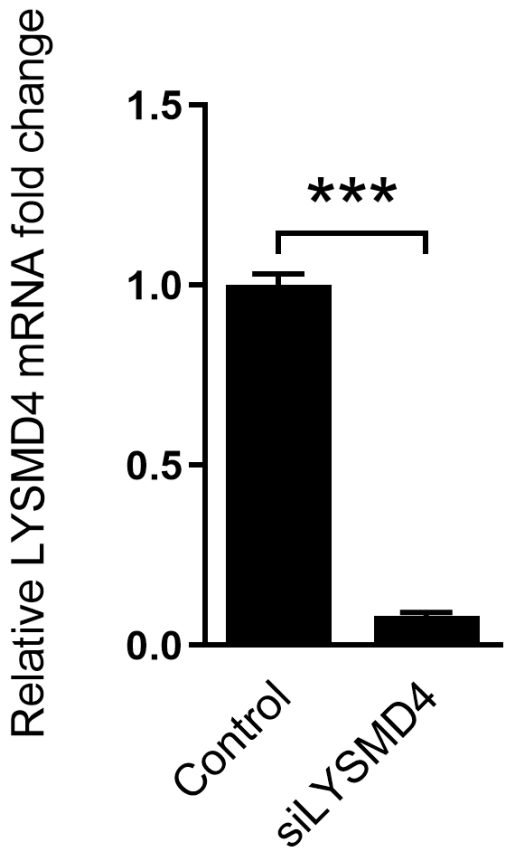

Figure 3-49. **The relative mRNAs expression level of human LYSMD4 in BEAS-2B cells 48 h after transfection with control siRNA or siRNAs specific for LYSMD4 (mean and SD of two samples per group).** ***P < 0.001 (Student's t-test).

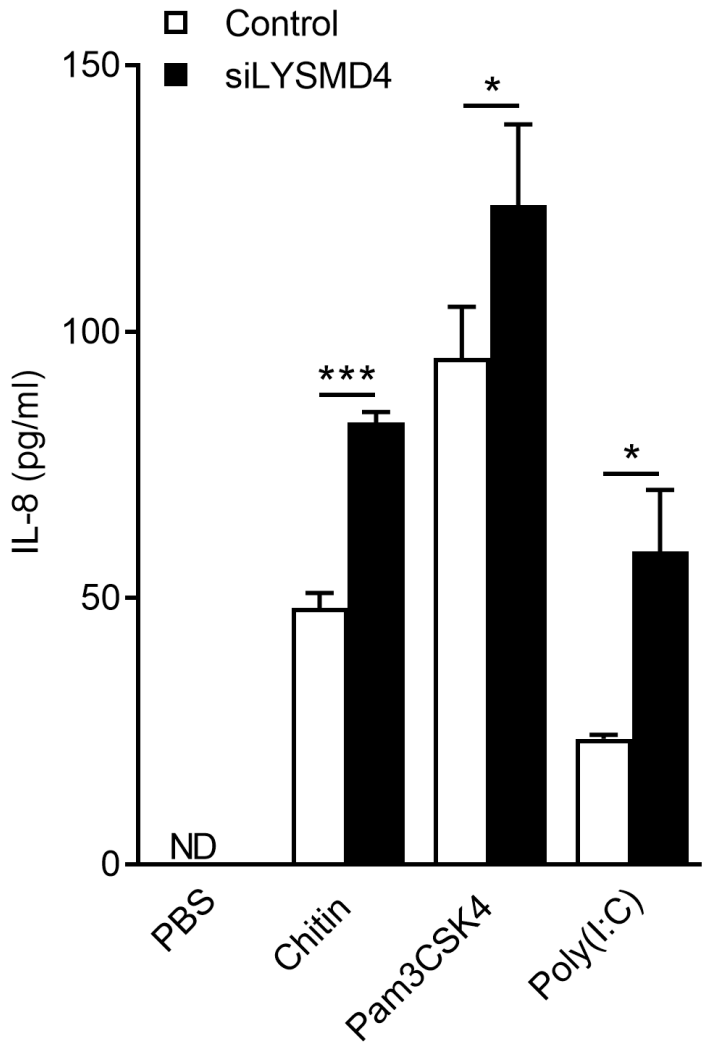

Figure 3-50. **Knockdown of LYSMD4 enhanced production of IL-8 induced by chitin, Pam3CSK4 and Poly(I:C).** ELISA of IL-8 in supernatants of BEAS-2B cells transected with siRNAs as in a, then stimulated for 24 h with chitin DP7 (500 μg/ml), Pam3CSK4 (100 ng/mL) or Poly(I:C) (3 μg/mL) (mean and SD of three samples per group). *P < 0.05 and ***P < 0.001 (Student's t-test).

In addition, MTT assays, LDH assays and cell numbers were comparable in control cells and LYSMD1-4 knockdown cells (Fig. 3-51), indicating that these human LysM-proteins are not essential for bronchial epithelial cell viability and proliferation.

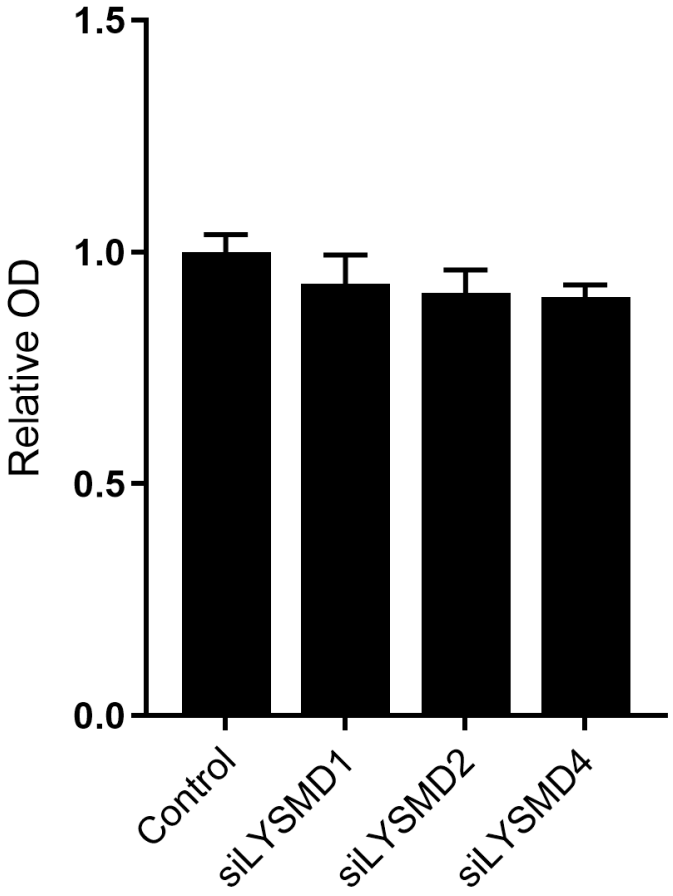

Figure 3-51. **Knockdown of human LysM-proteins had no effect on epithelial cell viability.** MTT assay 48 h after transient transfection of siRNAs for LYSMD1, LYSMD2, and LYSMD4 in BEAS-2Bs. Cell growth rate was expressed as absorbance at 570 (mean and SD of three samples per group).

Interestingly, LDH release was significantly decreased in cells treated with LYSMD1, LYSMD2, or LYSMD4 siRNA when compared with the control) and warrants further investigation (Fig. 3-52).

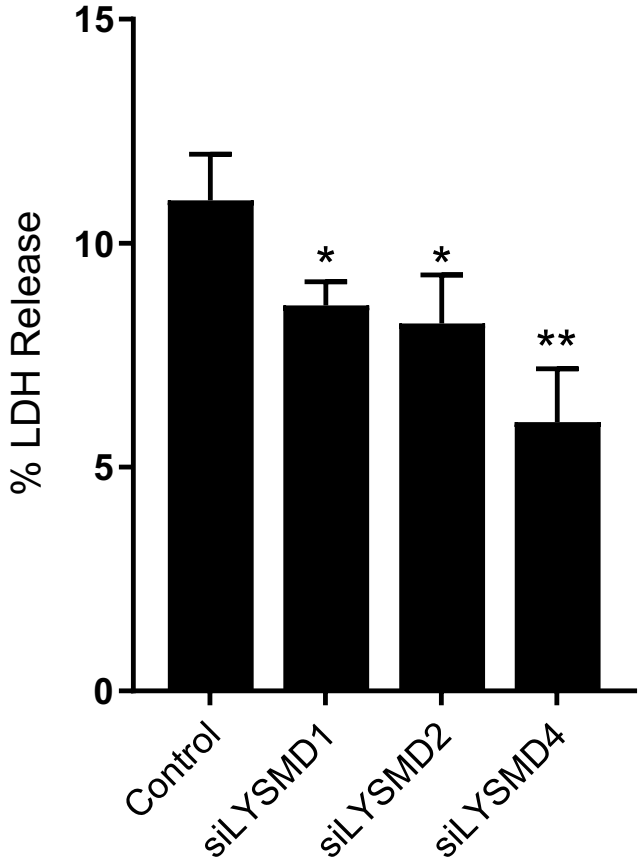

Figure 3-52. **Knockdown of human LysM-proteins decreased LDH release by bronchial epithelial cells.** LDH cytotoxicity assay 48 h after transient transfection of siRNAs for LYSMD1, LYSMD2 or LYSMD4 in BEAS-2B (mean and SD of three samples per group). *P < 0.05 and **P < 0.01 (Student's t-test).

3.7 LYSMD3 and LYSMD4 are associated respectively with systemic lupus erythematosus (SLE) and psoriasis (data analysis courtesy of Dr. David Samuels, Vanderbilt University)

Finally, using our BioVU Electronic Medical Records cohort, we tested for the association of nonsynonymous variants in LYSMD3 and LYSMD4 with selected airway, fungal and immune system phenotypes in 20,003 Caucasian adults using a PheWAS approach (Denny et al., 2010) (Table. 3-1). LYSMD3 has no common nonsynonymous variants and one low-frequency nonsynonymous variant (rs62375061, S150Y), which had 2.3% frequency in our cohort (Table. 3-2). The LYSMD3 S150Y variant was significantly associated with systemic lupus erythematosus (odds ratio [95% confidence interval] = 2.2 [1.4-3.3], p=0.000173). Carriers of the six genotyped rare nonsynonymous LYSMD3 variants (only 0.2% of our cohort) were not significantly associated with any targeted phenotype. LYSMD4 has four common nonsynonymous variants (Table. 3-3), but none of these variants were significantly associated with the targeted phenotypes. However, carriers of the genotyped LYSMD4 rare variants (0.5% of the cohort) were strongly significantly associated with psoriasis (OR = 4.2 [2.1-7.5], p=5E-6). These results suggested that human LysM-proteins are associated with autoimmune diseases.

Table 3-1. Targeted Phenotypes

Group	Phenotype	Number of Cases	ICD9 code ranges
Airway	Chronic sinusitis	972	473-473.9
Airway	Allergic rhinitis	1994	477, 477.0, 477.2-477.9
Airway	Other upper respiratory disease	558	478, 478.1-478.29, 478.8, 478.9, 519.1, 519.19, 519.2, 519.3, 786.1
Airway	Acute bronchitis and bronchiolitis	803	464.4, 466, 466.0, 466.1, 466.11, 466.19
Airway	Asthma	1071	493-493.00, 493.1, 493.10, 493.8, 493.82-493.9
Airway	Chronic airway obstruction	1830	496
Airway	Emphysema	426	492-492.8
Airway	Chronic bronchitis	553	491-491.1, 491.8, 491.9
Airway	Other pulmonary inflammation or edema	260	518.3-518.4
Airway	Empyema and pneumothorax	713	510-510.9, 512-512.89
Airway	Pleurisy; pleural effusion	2186	511-511.8, 511.89, 511.9
Airway	Pulmonary collapse; interstitial/compensatory emphysema	2914	518.0-518.2
Airway	Respiratory failure; insufficiency; arrest	2017	517.3
Airway	Respiratory failure	1302	518.81, 518.83, 518.84
Airway	Respiratory insufficiency	324	518.82
Airway	Other diseases of lung	1612	517, 517.8, 518, 518.8, 518.89
Airway	Wheezing and painful respiration	611	519
Airway	Painful respiration	281	786.52
Airway	Respiratory abnormalities	548	786.0, 786.00
fungal	Dermatophytosis / Dermatomycosis	705	110
fungal	Dermatophytosis	674	110.0, 110.2, 110.6-110.9
fungal	Dermatophytosis of nail	498	110.1
fungal	Candidiasis	607	112-112.2, 112.5, 112.8, 112.82, 112.84-112.89, 112.9
immune	Immune disorders	412	279, 279.9
immune	Immunity deficiency	354	279.1-279.3
immune	Discoid lupus erythematosus	298	373.34
immune	Systemic lupus erythematosus	275	710.0
immune	Psoriasis & related disorders	319	696
immune	Psoriasis	308	696.8

Table 3-2. LYSMD3 variants tested

SNP	Category	AA change
rs62375061	Low frequency	S150Y
rs78764058	Rare	M194L
rs200384766	Rare	P198T
rs139750200	Rare	R131H
rs192126274	Rare	S257T
rs148338096	Rare	P265S
rs201890408	Rare	R4G

Table 3-3. LYSMD4 variants tested

SNP	Category	AA change
rs2061007	Common	A181G
rs8041078	Common	A162V
rs8041089	Common	A158V
rs72760587	Common	T196S
rs78073940	Rare	K118M
rs143085338	Rare	D286A
rs150937647	Rare	H124L
rs145468893	Rare	T77I
rs147293942	Rare	P209L
rs140893433	Rare	V3A
rs145292960	Rare	T32I
rs149471133	Rare	Q294R
rs200602575	Rare	E190Q
rs201005514	Rare	S57R
rs148658487	Rare	P203L
rs139937601	Rare	K95T
rs148348952	Rare	R274I
rs139777997	Rare	N260I
rs201793909	Rare	R180H

CHAPTER IV: DISCUSSION

β-glucan and chitin are PAMPs in the fungal cell wall that are covalently cross-linked to one another. *Aspergillus fumigatus* alkali-insoluble cell wall fragments (AIF), which consist of chitin linked to β-glucan, induced enhanced immune responses compared with single cell wall polysaccharides. These fungal chitin-β-glucan particles induced recruitment of eosinophil and neutrophil, chitinase activity, and production of TNF-a and TSLP in mouse lungs (Dubey, Moeller, Schlosser, Sorensen, & Holmskov, 2014), It is possible that composite PAMPs induce synergy between individual receptors. Alternatively, a single receptor can recognize composite PAMPs more efficiently than non-composite PAMPs. Our data revealed that LYSMD3 is the only receptor thus far found in humans that can interact with both chitin and β-glucan. Thus, it is possible that LYSMD3 can recognize composite fungal chitin-β-glucan more efficiently than chitin or β-glucan alone, and may warrant further investigation in the future.

TLR2 has long been implicated in mediating immune responses to chitin. However, direct binding of TLR2 to chitin has not been fully demonstrated. Moreover, the possibility that contaminants (Zhang et al., 2010) might contribute to the activation of TLR2 cannot be excluded. Our data suggested that TLR2 did not bind the cell wall of *Alternaria alternata*. Similarly, TLR2 ECD bind moderately to cell wall of *Candida albicans* (Fuchs et al., 2018). TLR2, which traditionally has shown a preference for soluble ligands (Goodridge et al., 2011), such as components shed from the surface of microbes located at a distance, may not be able to directly bind or recognize large or insoluble particulate chitin and fungal cell wall. Indeed, although TLR2 plays a critical role in cytokines production in response to *C. albicans*, it is not required for phagocytosis of *C. albicans* by mouse macrophage

(Villamón et al., 2004). Chitin particles are readily phagocytosed. Receptors that mediate the phagocytic response of chitin particles have yet to be definitively identified (Bueter et al., 2013). Chitin elicits distinct patterns of innate cytokines and other immunologic factors compared to known mycobacterial TLR2 agonists (Fuchs et al., 2018), suggesting the existence of other chitin receptors. Chitin, chitinase, and chitinase-like proteins have been associated with an increasing number of human pathologies including a number of immune-associated disorders, though the mechanisms by which these function are unclear (Ziatabar et al., 2018). Large chitin particles have been reported to induce expression of chitinase in epithelial cells (Lalaker, Nkrumah, Lee, Ramanathan, & Lane, 2009) and immune cells (Fuchs et al., 2018) and in vivo (Kim et al., 2015). However, the receptor that recognizes chitin particles and mediates chitinase activity remains unknown. In bronchial epithelial cells, the expression of TLR2 is low and the expression of co-receptor CD36 is missing (Mayer et al., 2007). Our data suggested that TLR2 is not required for chitin-induced NF-κB activation and cytokine production by airway epithelial cells.

Dectin-1 has been implicated in mediating immune responses to chitin. It was demonstrated previously that treatment with the Dectin-1 blocker laminarin (1 mg/ml) completely abrogates the production of TNF-α and IL-10 by chitin stimulated mouse peritoneal macrophages (Da Silva et al., 2008). However, there is no other evidence that Dectin-1 is involved in this process and Dectin-1 does not bind chitin. Dectin-1 reconstitution in HEK293T does not result in NF-κB activation in response to chitin. Immortalized macrophages from Dectin-1-deficient mice respond as efficiently to chitin

as WT macrophages in terms of TNF-α production (Fuchs et al., 2018). Of note, the use of low concentration of laminarin (100 μg/ml), which is sufficient to block Dectin-1 activity, has no effect on chitin induced IL-10 production in mouse macrophages (Wagener et al., 2014). These data suggest that blockage of a receptor with a relative low affinity for laminarin is actually responsible for the abolished chitin responsiveness in immune cells. We have now shown that laminarin directly binds LYSMD3 and was able to block LYSMD3 activity. Furthermore, knockdown of LYSMD3 specifically inhibited chitin-induced IL-6 production in mouse macrophages. Similarly in bronchial epithelial cells, curdlan and laminarin treatment reduced IL-8 release induced by allergenic fungi *Fusarium proliferatum* to 20% and 53% of the controls, respectively, while Dectin-1 blocking antibody showed less inhibitory effect (76% of the controls) compared with β-glucans (Yeh et al., 2017). These results suggest that other receptors blocked by curdlan and laminarin may be also involved in recognition of *F. proliferatum* by airway epithelial cells. Collectively, our results provide an explanation of previous conflicting reports about the role of Dectin-1 in chitin signaling and suggest that blocking of LYSMD3 by laminarin reduced chitin or chitin-containing fungi triggered innate immune responses in immune cells and epithelial cells.

Syk was recently reported to be a critical kinase that phosphorylates MyD88, promotes MyD88-dependent signaling (Gurung et al., 2017), and plays a critical role in the expression and activation of IRAK1 in macrophages. We used the *Arabidopsis* chitin receptor AtCERK1 protein sequence and performed a BLASTP analysis against human and mouse proteomes. Interestingly, we found no homology with the LysM domain

containing extracellular region. However, we found high homology of the kinase portion of the plant protein with human IRAK1 (E-value: 7.5e-38, 30% identity) and IRAK4 (5e-44, 36% identity). Similar homology was also found with corresponding mouse orthologs of these kinases. These results were intriguing since most mammalian receptors in the innate immune system do not typically have kinases attached but are recruited along with adaptors during signaling. It was recently found that chitin oligomers (DP10-15) induce a novel phosphorylation site at Ser 186 in IRAK4 and is 8-fold greater in abundance than in TLR2 ligand Pam2CSK4-stimulated THP-1 cells (Fuchs et al., 2018). Our RNAi data also suggested that IRAK1 is another key molecule in mediating the response to chitin in human lung epithelial cells (data not shown). MyD88 has also been linked to chitin signaling in macrophages (Da Silva et al., 2008). Taken together, these results suggest a Syk-MyD88-IRAK1 pathway downstream of LYSMD3 and we also speculate that LYSMD3 and TLR pathways converge downstream at MyD88.

Interestingly, human LYSMD3 has three alternatively spliced isoforms while mouse LYSMD3 does not have such predicted isoforms. Therefore, elucidation of the role of the LYSMD3 isoforms in human may reveal differences in mechanisms and pathways of innate immune activation and regulation between human and mouse, and thus contribute to the evaluation of rodents as models of human diseases, particularly infection and inflammation (Seok et al., 2013).

Our PheWAS study revealed rare nonsynonymous mutations in coding regions of LYSMD3 and LYSMD4 that are associated with increased odds of having autoimmune

disorders like systemic lupus erythematosus and psoriasis. These results suggested that LysM-domain containing proteins may function as a critical link between the innate immune responses and the adaptive immune responses. Continuous activation or dysregulation of LYSMD3 signaling might contribute to the pathogenesis of autoimmunity. Growing evidence suggests that genetic variations that alter the functions of pattern recognition receptors are not only associated with increased susceptibility to infectious diseases, but also autoinflammatory and autoimmune disorders as well (Seibl, Kyburz, Lauener, & Gay, 2004). There has been increasing interest in targeting these receptors to develop new therapeutic approaches for autoimmune disorders in the clinic (Mullen, Chamberlain, & Sacre, 2015).

It is interesting in our study that the presence of LPS was associated with chitin binding to LYSMD3 and was dependent on the type and purity of LPS (data not shown). LPS exposure is found to be associated with asthma and respiratory infections, but different types of endotoxin could have different effects, and the mechanisms remain unclear (Norback et al., 2014). Bacterial-fungal interactions are of great significance in human disease. Studies have shown increased fungal-related mortality in the presence of *E.coli*, and endotoxin was thought to be important for this enhanced virulence (Peleg, Hogan, & Mylonakis, 2010). Both LPS and *Pseudomonas aeruginosa* can modify the immune response against *C. albicans* by shifting from Th2 to Th1 immune response when they are concomitantly administered. Although *P. aeruginosa* contains LPS, the immunomodulatory effect that *P. aeruginosa* has on the response to fungi is TLR4-independent. However, the immunodeviation of the adaptive response to *C. albicans* by

LPS was considered TLR4-dependent by default and therefore has not been tested (Allard et al., 2009). Our finding may provide new insight into fungal-bacterial interactions in that the presence of LPS or bacteria may promote fungal-host Interaction by facilitating adhesion, recognition, or endocytosis of fungi or fungal products by host epithelial cells. Intriguingly, our findings also suggested that LPS may modulate host immune responses to fungi independent of TLR4.

CHAPTER V: CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusion

In conclusion, direct interaction between Inhaled fungi and airway epithelial cells is an early event, which can shape the eventual immunological outcome (René M. Roy & Klein, 2013). In contrast to innate immune cells, the recognition of chitin or fungal cell wall by epithelial cells is less defined. We have discovered a novel chitin receptor, LYSMD3, expressed in human lung epithelial cells. It contains an extracellular chitin (LysM) binding domain and a hemi-ITAM-like motif in the cytoplasmic tail. Our identification and characterization of LYSMD3 as the elusive human epithelial cell receptor for chitin, resolves a long-standing mystery and provides a new insight into the context of innate immunity in mammals against chitin-containing organisms and allergic inflammation.

5.2 Further characterization of LYSMD3 as a PRR

One experiment will be performed is the fluorescent labeling of LYSMD3 and then probing killed resting *A. fumigatus* conidia, killed swollen conidia, germlings, and hyphae. We expected that we can only see labeling for the killed swollen conidia and hyphae. This type of assay was instrumental in dissecting Dectin-1, Dectin-2 and several other PRR specificity to specific morphologies of *A. fumigatus*. The assay is becoming a gold standard.

We were also interested in how LYSMD3 mediates chitin signal transduction. We have detected the tyrosine phosphorylation status of exogenous LYSMD3 protein and an association between LYSMD3 and Syk in resting HEK 293 cells. We will next determine whether the phosphorylation of Tyr and the interaction of LYSMD3 and Syk will be enhanced by stimulation with chitin. Co-localization of LYSMD3 and Syk after chitin stimulation will also be examined by confocal microscopy. A pull down assay will be performed to determine direct/indirect interaction of proteins if necessary. A plasmid expressing Flag-tagged LYSMD3 with mutation of Tyr will be constructed to confirm the site of phosphorylation and the role of hemi-ITAM in chitin signaling and the interaction of LYSMD3 and Syk upon chitin stimulation. We will also investigate whether the function of Syk is critical for chitin induced cytokines production by combining knockdown, knockout and inhibition of Syk.

We will also investigate whether LYSMD3 interacts with other molecules involved in chitin signaling by using immunoprecipitation combined with mass spectrometry and then

confirm the interaction with specific antibodies. Some proteins will be given special attention such as other Src family kinases since phosphorylation of key tyrosine residues within ITAMs is considered as a primary function of the Src family (Lowell, 2011). Other potential interacting proteins include TLR2, MYD88 and IRAK-1.

5.3 Identify the role of LYSMD3 in chitin immunity

The link between IL-8, CCL2 as well as IL-33 with LYSMD3 in our in vitro studies is exciting. However, we didn't get any significant induction of type 2-promoting epithelial cytokines IL-25, IL-33 or TSLP in vitro after stimulation with either chitin (NAG)7 or shellfish chitin (<10μm). It is possible that the chitin we used was too small or chitin alone does not induce the production of type 2-promoting epithelial cytokines. This may suggest that LYSMD3 only contribute the production of a limited number of cytokines that support the development of Th2-associated inflammation. Alternatively, costimulation of other receptors is required for LYSMD3 mediated expression of these genes (Lalaker et al., 2009). Thus, LYSMD3 obviously plays a critical role as a mediator in chitin immunity, but the exact role of LYSMD3 in airway inflammation and allergic response needs to be validated in murine knock-out models. Therefore, we will next generate LYSMD3 knockout mice. To establish airway inflammation model in mice, wildtype mice (C57/BL6) or LYSMD3 KO mice will be given with 50 μL of 10^5 beads/ml pure chitin beads (NEB) or Alternaria spores by intranasal administration. Mice will be sacrificed at 3, 6, 12, and 24 hours following exposure to the various treatments. Lung BALF will then be collected and analyzed for various cytokines/chemokines including TNF-α, KC, CCL2, IL6, -4, -5, -13, 25, -33, Eotaxin-1, 2, 3, and TSLP. Immune cells in BALF will be analyzed (macrophage, eosinophils, neutrophils, and lymphocytes). We will also excise and section lungs and perform histopathology studies in order to visualize inflammation, cell infiltration, morphological changes, and potential fungal growth in the case of *Alternaria*.

Since the size of the chitin is important for the type of response elicited by the immune system, we will determine what size of chitin shows the strongest affinity to LYSMD3

using ITC or SPR technique. Combine with results from mouse model, we will therefore define the role of LYSMD3 in the size effect of chitin and further explain the controversial immunological properties of chitin from LYSMD3 perspective.

We expect that LYSMD3 deficiency will impede Th2 programs in the mouse allergic inflammation model. The loss of LYSMD3 in no-HSC such as airway epithelial cells will mediate decreased neutrophil influx in response to *Alternaria*. Loss of IL-33 should mitigate Th2 mediated responses to looking at T cell and DC populations will be important. Otherwise, using LYSMD3 blocking antibody or ectodomain of LYSMD3 to block the transmission of chitin in mouse airway will be the alternative therapeutic approach to respiratory allergies or asthma.

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