의학박사 학위논문

The Role of House Dust Mite Derived Chitin as an Adjuvant in Airway Inflammation Induced by Ovalbumin

난알부민으로 유도한 기도염증에서 집먼지진드기로부터 추출한 키틴의 면역보강제로서의 역할

2014년 7월

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의학과 협동과정 임상약리학 전공

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The Role of House Dust Mite Derived Chitin as an Adjuvant in Airway Inflammation Induced by Ovalbumin

By

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Medicine

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Abstract

Introduction: House dust mite (HDM), the most common inhalant allergen, sometimes promotes allergic response to other inhalant allergens. The exoskeleton of HDM mainly consists of chitin which is the second-most abundant polysaccharide in nature. Recently, chitin was reported to be a potent adjuvant in Th1-, Th2- and Th17- type adaptive immune responses through toll-like receptor 2 (TLR2). In previous study, tumor necrosis factor – alpha (TNF-α) and natural killer T (NKT) cell were observed to be important in the development of Th2 cell response to inhaled allergens.

The objective of this study **is to** verify our hypothesis that HDM promotes adaptive immune response to other inhalant allergen through its component, chitin, a multifaceted adjuvant, administrating intranasally ovalbumin (OVA) with HDM or HDM-derived chitin to mouse during sensitization. We also evaluated the role of TLR2, TNF-α and NKT cell in this murine model, using TLR2-, TNF-α-, and CD1d- deficient mice, respectively.

Methods: Wild-type (WT) mice (C57BL/6 background), TLR2-, TNF-α-, and CD1d-deficient mice were sensitized intranasally with 75 μg of OVA and 100 μg of HDM or HDM-derived chitin, then challenged intranasally with 50 μg of OVA. WT mice were also sensitized intranasally with 75 μg of OVA and 100 μg of HDM or HDM-derived chitin in the presence of chitinase.

Results: HDM and HDM-derived chitin promoted airway inflammation and all of Th1, Th2, and Th17 immune response to OVA as well as the serum level

of OVA-specific IgE, IgG1, and IgG2a. In the absence of TLR2, all types of immune responses and serum level of OVA-specific IgE, IgG1, and IgG2a were diminished. In the absence of TNF-α or in the presence of chitinase, the expression of Th2 cytokines and the serum level of OVA-specific IgE were alleviated, while the expression of Th1 and Th17 cytokines and the serum level of OVA-specific IgG1 and IgG2a were preserved or more enhanced. In the absence of CD1d, all types of immune responses and serum level of OVA-specific antibodies were diminished, except IL-10 and serum OVA-specific IgG2a which are known to relate to regulator T cell response.

Conclusion HDM promotes adaptive immune response to other inhalant allergen through its component, chitin which is a multifaceted adjuvant. Chitin stimulates all of Th1, Th2, and Th17, but mainly Th2 immune response to concomitantly inhaled other aeroallergen through TLR2, TNF-α and NKT cell.

Key word: house dust mite, chitin, adjuvant, toll-like receptor 2, tumor necrosis factor–alpha, natural killer T cell

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Abbreviation

ANOVA:	Analysis of variance
Alum:	Aluminum hydroxide
BAL:	Bronchoalveolar lavage
CD1d:	Cluster of differentiation 1d
CD3:	Cluster of differentiation 3
CD4:	Cluster of differentiation 4
CD11b-APC:	Anti-CD11b monoclonal antibody labeled with allophycocyanin
CD11c-APC:	Anti-CD11c monoclonal antibody labeled with allophycocyanin
CD28:	Cluster of differentiation 28
CD40-PE:	Anti-CD40 monoclonal antibody labeled with R-phycoerythrin
CD80-PE:	Anti-CD80 monoclonal antibody labeled with R-phycoerythrin
CD86-PE:	Anti-CD86 monoclonal antibody labeled with R-phycoerythrin
F4/80-FITC:	Anti-F4/80 monoclonal antibody labeled with fluorescein isothiocyanate
CFSE:	Carboxyfluorescein succinimidyl ester
Der f:	*Dermatophagoides farinae*

Der p:	*Dermatophagoides pteronyssinus*
ELISA:	Enzyme-linked immunosorbent assays
FACS:	Fluorescence-activated cell sorting
FT-IR:	Fourier transform infrared
H&E:	hematoxylin and eosin
HDM:	House dust mite
IFN-γ:	Interferon gamma
IgE:	Immunoglobulin E
IgG1:	Immunoglobulin G1
IgG2a:	Immunoglobulin G2a
IL-1β:	Interleukin 1 beta
IL-4:	Interleukin 4
IL-5:	Interleukin 5
IL-6:	Interleukin 6
IL-10:	Interleukin 10
IL-12:	Interleukin 12
IL-17:	Interleukin 17
IL-23:	Interleukin 23
LN:	Lymph node
MHC:	Major histocompatibility complex
MCT:	Mercury cadmium telluride
Myd88:	Myeloid Differentiation Primary 88
NKT cell:	Natural killer T cell

OVA:	Ovalbumin
PAMP receptor:	Pathogen associated molecular pattern receptor
TCR:	T cell receptor
Th1 cell:	Type 1 T helper cell
Th2 cell:	Type 2 T helper cell
Th17 cell:	Type 17 T helper cell
TGF-β:	Transforming growth factor beta
TLR2:	Toll-like receptor 2
TNF-α:	Tumor necrosis factor – alpha
TNFR:	Tumor necrosis factor receptor
TSLP:	Thymic stromal lymphopoietin
VEGF:	Vascular endothelial growth factor
WT:	Wild-type

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allergen, however chitinase treatment alleviates these airway inflammation and Th2 immune response, but not Th1 or Th17 immune response.

Fig. 6 Airway inflammation and all types of immune response to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is suppressed in the absence of toll-like receptor 2 (TLR2).

Fig. 7 Airway inflammation and Th2 immune responses to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is diminished in the absence of TNF-α, whereas the Th1 and Th17 responses are more enhanced.

Fig. 8 Airway inflammation and all types of immune response to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is suppressed in the absence of natural killer T (NKT) cell.

Suppl. Fig. 1 Airway inflammation and Th1, Th2, and Th17 immune responses to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin are not mediated by toll-like receptor (TLR) 1, TLR 4, or TLR 6.

Suppl. Fig. 2 Airway inflammation induced by house dust mite derived (HDM-derived) chitin is mediated by Th2 immune response dominantly

rather than Th1 or Th17 immune responses.

Suppl. Fig. 3 Levels of IL-6 and TNF-α released by murine lung epithelial (MLE) cells and murine alveolar macrophage (MH-S) cells which were cultured with phosphate-buffered saline (PBS), chitinase, house dust mite derived (HDM-derived) chitin, or chitinase-treated HDM-derived chitin for 24 h.

Suppl. Fig. 4 The distribution of chitin according to its size

Suppl. Fig. 5 Scheme of TLR2-TNF-α-NKT axis in adaptive immune response to ovalbumin (OVA) allergen induced by house dust mite (HDM)-derived chitin

Contents

Introduction

House dust mite (HDM) including *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* (Der p) is the major inhalant allergen which is most frequently sensitized in general population and causes Th2-type airway inflammatory diseases that mainly consists of allergic rhinitis and asthma (1-3). We can frequently find this fact when we perform skin prick test or measure the level of serum specific IgE in general population or patients who suffer from allergic rhinitis or asthma, and when we perform allergen specific nasal or bronchial provocation test in these patients (4-6). HDM was also known to promote sensitization to other antigen such as ovalbumin (OVA) in addition to its own allergenic properties (7). However, the exact mechanism remained to be investigated.

Chitin, a biopolymer of N-acetyl-b-D-glucosamine, is the second-most abundant polysaccharide in nature after cellulose, and it is found in the exoskeleton and the lining of the digestive tracts of arthropods including HDM; the walls of fungi; and the microfilarial sheath of parasitic nematodes (8-11). With regard to HDM, many allergens from mite group 5, 12, 15, 18, 21, and 23 are thought to bind to chitin, some of which are frequently found in the midgut epithelium and fecal pellets (12, 13).

Some studies demonstrated that oral or intranasal administration of chitin down regulates allergen-induced IgE production and lung inflammation with IL-4, IL-5, and IL-10 of Th2 cytokines inhibited (14, 15). They further

demonstrated that the production of Th1 cytokines including IL-12, IFN-γ, and TNF-α was responsible for the inhibition of allergen-induced Th2 cytokine production. However, those studies could not explain why chitin-rich inhalant allergen such as HDM produces IgE production and Th2 airway inflammation.

A recent study reported that chitin can be a potent multifaceted adjuvant that induces adaptive Th2, Th1, and Th17 immune responses via toll-like receptor 2 (TLR2), MyD88, and IL-17A (16). In this study, mice were sensitized intraperitoneally with OVA plus chitin or OVA plus alum, and received aerosol challenge with OVA. However, the intraperitoneal administration of chitin in this study as well as oral administration of chitin in a previous study is not pathophysiological, and it might not sufficiently represent the mechanism of allergic sensitization and inflammation induced by airborne chitin-associated allergen including HDM and fungi. In addition to route of chitin administration, the chitin preparation (size difference), doses, and duration could differently affect inflammation and tissue responses (17).

Meanwhile, tumor necrosis factor-alpha (TNF-α), an important mediator of inflammatory response, is produced by innate immune cells, including macrophages, mast cells, and lung epithelial cells. TNF-α induces the infiltration of inflammatory cells to the inflamed site through up-regulation of adhesion molecules and increased cytokine production (18).

Regarding adaptive immune responses, TNF-α acts like Th1 cytokine in lung inflammation in some studies; however, others have reported that Th2

inflammation is induced by recombinant TNF-α (rTNF-α) administration and diminished through TNF-a signaling inhibition (19-22).

In previous study, Th2 airway inflammation was successfully induced when mice were intranasally sensitized and challenged with allergen, and it is found that TNF-α has a key role in the induction of Th2 airway inflammation (23).Furthermore, it was also demonstrated that TNF-α, produced by allergen sensitization with dsRNA, mediates the development of adaptive immunes response to allergen through TNF receptor (TNFR) on NKT cells. TNFR on NKT cells was also suggested to have important role in inducing allergic airway inflammation and Th1 and Th2 immune response in other recent study (24).

In present study, we verified our hypothesis that HDM promotes adaptive immune response to other inhalant allergen through its component, chitin, a multifaceted adjuvant, administrating intranasally OVA with HDM or HDM-derived chitin to mouse during sensitization. We also evaluated the role of TLR2, TNF-α, and NKT cell in this murine model, using TLR-2, TNF-α-, and CD1d-deficient mice, respectively.

Materials and Methods

Mice

TLR2-deficient, TNF-α-deficient, CD1d-deficient, and wild-type (WT) mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred in a pathogen-free facility, and 5 ea were used for each group. All live animal experiments were approved by Ethics Committee.

Reagents

OVA, HDM, and Brugua malayi chitinase were obtained from Sigma-Aldrich (St. Louis, MO, USA), Korean National Arthropods of Medical Importance Resource Bank (Seoul, Korea), and New England Biolabs (Ipswich, MA, USA), respectively.

Purification of chitin from HDM and FTIR analysis

For purification of chitin from HDM, 100 mg of house dust mite was washed in phosphate buffered saline (PBS) for 24h and then dried in 55℃ for 10h. Next, 2M NaOH was added and incubated at room temperature for 15h. Then, NaOH added HDM was washed with distilled water three times and then incubated with 0.5M H_2SO_4 at 50℃ for 16h. After incubation, it was washed again and incubated in 2M NaOH for 30 min. Finally, chitin was washed with

distilled water three times and lyophilized to remove moisture.

Fourier transform infrared (FT-IR) spectra were acquired at a spectral resolution of 4 cm^{-1} with a Bomem DA8 FTIR spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector which is in the range 800~4000 cm^{-1} under vacuum. The diffuse reflectance attachment (Harrick Scientific Corporation, Pleasantville, NY, USA) was used in this paper. Sample was prepared using the mortar to make properly fine powder to get FTIR spectra. All diffuse-reflectance FTIR spectra were measured by co-adding 256 scans.

Acquired chitin amount was 700 μg per 1 mg of mite, and endotoxin level was less than 0.6 endotoxin unit per 100 μg of mite. Protein was not detected when we evaluated using BCA methods.

Generation of murine model

Six week-old mice were administrated with 75 μg of OVA plus 100 μg of HDM or HDM-derived chitin with or without chitinase and sensitized intranasally on day 0, 1, 2, and 7. We administered HDM or HDM-derived chitin with the maximally tolerable dose of 100 μg according to previous studies in which 10-100 μg of HDM was administrated intranasally (25-32). After sensitization, 50 μg of OVA was challenged intranasally on day 14, 15, 21 and 22, and 24h after last OVA challenge, mice were sacrificed for evaluation. In the case of chitinase treated group; 1 unit of chitinase was co-incubated with 100 μg of HDM or HDM-derived chitin (reaction volume-in

30 μl PBS) in 37 °C for 1 hr, and after enzyme digestion, heat inactivation was done for stopping the reaction. Thereafter, it was intranasally administrated with OVA.

Cellularity in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) cellularity was analyzed as described previously (33). Briefly, cell pellets were diluted in 50 μl PBS, and 300 inflammatory cells were counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Macrophage preparation from peritoneum and alveolus

To isolate the macrophage in the alveolus, lung was washed with 0.22 μm filtered PBS more than 3 times. Cells were isolated by centrifugation and seeded in culture plate as 1×10^6 cell/ml. Six hour after seeding, cells were stimulated with HDM-derived chitin. For isolation of peritoneal macrophage, 3% thioglycollate was injected to mouse peritoneum, and cells were harvest from peritoneal fluid. Seeding and stimulation were done as above mentioned.

Single-cell preparation from lung and lung-draining lymph nodes

Briefly, for single-cell isolation from the lung tissue, tissue was chopped and incubated in 37°C with 0.05% trypsin (GIBCO, Grand Island, NY, USA) and

200 unit/ml of collagenase (GIBCO, Grand Island, NY, USA). After digestion for 10 min, tissue was ground using the cell strainer (BD Falcon, Bedford, MA, USA) and incubated in 4°C with RBC lysis buffer (StemCell Technologies, Vancouver, Canada). For isolating lymph node (LN), tissue was ground using the cell strainer and incubated with RBC lysis buffer as in single-cell preparation from the lung tissue.

Lung tissue histology

Lung sections were stained with hematoxylin and eosin (H&E) after pressure fixation with Streck solution (Streck Laboratories, La Vista, NE, USA). All slides were compared at the same magnification. Lung inflammation was measured by assessing the degree of peribronchiolar and perivascular inflammation as described previously (34).

Immune response in the lung and lung-draining lymph nodes

After harvest, lung-draining LNs were cultured (2.0 x 10^6/ml) in 24-well plates at 37°C in RPMI 1640 (Hyclone, UT, USA) in the presence or absence of CD3 and CD28 antibodies (1 μg/ml each; eBioscience, San Diego, CA, USA). The level of cytokines including IL-4, IL-17, and IFN-γ produced by the restimulated T cells were determined from culture supernatant fractions collected 12 h after CD3/CD28 antibody stimulation.

Cytokine measurement

The level of cytokines including IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17 IL-23, TNF-α, eotaxin, TGF-β, TSLP, IFN-γ, and IP-10 in BAL fluid and culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

OVA specific antibody level evaluation

The level of OVA specific antibodies including IgG1, IgG2a and IgE in serum was evaluated by using enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer's instructions (Bethyl laboratories INC, Montgomery, TX, USA).

Fluorescence-activated cell sorting analyses

For intracellular cytokine staining, isolated cells from lung-draining LN (4.0×10^6 cells/ml) were incubated at 37°C for 6 h in 48-well plates coated with the CD3 and CD28 antibodies (1 μg/ml each; ebioscience, San Diego, CA, USA). Two hours before harvest, Brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added. After harvest, cells were stained with surface antibodies (CD3-APC and CD4-FITC; BD Biosciences, San Jose, CA, USA) for 30 min at 4°C and then fixed for 10 min in 4% paraformaldehyde at room

temperature. Cells were incubated with antibodies (anti-IL-4-PE, anti-IL-17-PE, and anti-IFN-γ-PE; BD Biosciences, San Jose, CA, USA) for 30 min at room temperature and then analyzed on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest Pro software. For evaluating the T cell proliferation, Crystal field stabilization energy (Invitrogen, Carlsbad, CA, USA) was labeled as a manufacturer's instructions. After labeling, cells were re-stimulated with 100 μg/ml of OVA for 72 h, and stained with anti-CD3 antibody as above mentioned. Surface staining, for evaluating the levels of co-stimulatory molecules, was done by incubating with antibodies (F4/80-FITC, CD11b-, CD11c-APC, CD40-PE, CD80-PE and CD86-PE) and then, cells were analyzed as intracellular cytokine staining.

Statistical analyses

Analysis of variance (ANOVA) was used to determine the statistical significance of differences between all groups. Significant differences between treatments were assessed using ANOVA, or Wilcoxon's rank sum test. For multiple comparisons, ANOVA was used first, and if significant differences were found, individual Wilcoxon's rank sum tests were performed between pairs of groups. Differences were considered statistically significant if $P < 0.05$.

Results

House dust mite promotes innate and adaptive immune response to other inhalant allergens through their component, chitin, a multifaceted adjuvant.

HDMs and their body crust are usually founded in house dust. Recent evidence indicated that chitin particle is one of candidate to induce the immune responses. In this context, to evaluate the role of HDM-derived chitin in allergic sensitization, we adjusted above scheme *in vivo*. We sensitized the mice intranasally with 75 μg of OVA + 100 μg of HDM with or without chitinase treatment, and evaluated 12 h after the sensitization. BAL cellularity showed that inflammatory cell infiltration such as macrophage, neutrophil and eosinophil was increased in mice sensitized with OVA and HDM compared to control groups, meanwhile it was reversed and became comparable to negative control group in chitinase-treated group (Fig. 1A). In terms of the production of pro-inflammatory cytokines, IL-1β, TNF-α IL-4 and TSLP (mainly related with Th2 responses) were elevated in OVA and HDM sensitized mice, however the result was reversed in chitinase treatment (Fig. 1B, left). In the case of IL-6, IL-10, IL-12p70 and IL-23 (for Th1 and Th17 immune responses), the production were enhanced in the mice sensitized with OVA and HDM, and the chitinase treatment to HDM induced up-regulation of cytokines (Fig. 1B, right). These findings indicated that chitin in HDM is

possible to promote all type of innate immune response, mainly Th2 related responses, in airway.

Then, to test the effect of HDM-derived chitin on the development of adaptive immune response to inhaled allergen, we sensitized 75 μg of OVA and 100 μg of HDM with or without chitinase treatment on day 0, 1, 2, and 7, then 50 μg of OVA was challenged intranasally on day 14, 15, 21 and 22. The evaluation was performed 24 h after the last OVA challenge. Lung inflammatory cell infiltration showed similar patterns with the result after sensitization (Fig. 2A). Histological findings showed that, inflammatory cell infiltration into lung was observed in the mice sensitized with OVA and HDM compared to its control group, but the result reversed by chitinase treatment (Fig. 2B). When we measured the production of allergen specific antibodies, IgE induction by HDM stimulation was down-regulated by chitinase treatment, while IgG1 and IgG2a production was more enhanced (Fig. 2C). In regard to the production of cytokines, Th2 type cytokines including IL-4, IL-5, eotaxin and TGF-β were induced in OVA and HDM administrated group, but this effect is alleviated by chitinase treatment (Fig. 2D, upper). On the contrary, Th1 and Th17 related cytokines including IL-10, IL-17, IP-10 and IFN-γ were more increased (Fig. 2D, lower). Next, to clarify the T cell response, we isolated and re-stimulated T cells from lung and draining lymph nodes. The results revealed that IL-4 production in lung and lymph node T cell was increased in OVA and HDM sensitized group and this increased production was suppressed by chitinase treatment, meanwhile the production

of IL-17 and IFN-γ was more enhanced as Th1 and Th17 related cytokine in BAL fluids (Fig. 2E). These findings suggested that HDM promotes all of Th1, Th2 and Th17 immune responses (especially Th2) to inhaled OVA allergens though its component, chitin.

The isolation of chitin from house dust mite and the determination of adequate chitin's dose for inducing airway inflammation

Based on above findings, we could indirectly suggest that chitin has a great role in inflammation induced HDM inhalation. In this time, to elucidate more directly the role of chitin in the airway inflammation induced by inhalation of HDM, we isolated chitin from HDM. We could extract at least 700 μg of chitin from 1mg of HDM without protein contamination. When compared with commercial chitin to check the isolation of chitin using FT-IR, we confirmed that isolated chitin from HDM was not different compared with commercial crab chitin (Fig. 3A). Next, to evaluate the role of HDM-derived chitin in inducement of innate immune response, we stimulated chitin to naïve mice and evaluate the production of pro-inflammatory cytokines, IL-6 and TNF-α, on 12 h after sensitization. With 1 μg or 100 μg of HDM-derived chitin, production of inflammatory cytokines was not induced significantly and comparable to control group with phosphate buffered saline (PBS), while both cytokine were markedly induced with 100 μg of HDM-derived chitin

(Fig. 3B). Following, to evaluate adjuvant role of HDM-derived chitin, we administrated OVA and diverse dose of HDM-derived chitin to mice, and evaluation was performed 24h after last OVA challenge. The infiltration of inflammatory cell into lung was observed in 10 and 100 μg sensitized group, and the pattern of infiltrated cells in both groups was similar (Fig. 3C). However, in the case of OVA specific antibody production, only 100 μg of HDM-derived chitin during sensitization induced up-regulation of specific antibody (Fig. 3D). In addition, all of cytokines in BAL fluids (related with Th1, Th2 and Th17 immune responses) were significantly increased only with 100 μg of chitin compared with control group with OVA (Fig. 3E). These data indicated that 100 μg of HDM-derived chitin is sufficient to induce the adaptive immune responses.

House dust mite derived (HDM-derived) chitin induces airway inflammation and innate immune responses to inhalant allergen.

Previous experiments showed that HDM-derived chitin induced pro-inflammatory cytokine effectively in airways. In this time, to evaluate the innate immune response induced by HDM-derived chitin, we administrated 100 μg of chitin to mice, referring to previous results, and evaluated in each time point. We observed inflammatory cell infiltration from 3 h after airway stimulation (Fig. 4A). In terms of the inflammatory cytokine production in

airways, Th1, Th2 and Th17 related cytokine production was observed (Fig. 4B). The level of IL-4 and TSLP were slightly increased from 3 h after stimulation, and peaked in 12 h after. TNF-α, a famous pro-inflammatory cytokine and Th2 cytokine inducer according to previous study (23), was up-regulated more rapidly, and peaked 6 h after stimulation. In the case of Th1 and Th17 related cytokines, IL-12p70 and IL-6 were highest 6 h and 12 h after stimulation of HDM-derived chitin, respectively. Vascular endothelial growth factor (VEGF), IL-6 inducer as previous study (23), showed fate elevation for early 3 h after stimulation and plateau for 9 h thereafter (Fig 4B).

Then, to test the effect of HDM-derived chitin *in vivo* during the sensitization period, we sensitized mice with HDM-derived chitin with or without chitinase, and evaluated immunological parameter 12 h after the sensitization. HDM-derived chitin induced the infiltration of inflammatory cells including macrophage, neutrophil and eosinophil (Fig. 3C). However, this infiltration was alleviated when chitinase administrated during sensitization (Fig. 4C). In the case of cytokine expression, the expression of Th2 polarization related cytokines were increased in mice treated with HDM-derived chitin during sensitization, however this effect was alleviated when chitinase was added. On the contrary, the production of Th1 and Th17 related cytokines including IL-6, IL-10, IL-12p70 and IL-23 were more up-regulated (Fig. 4D). Next, we evaluated the role of HDM-derived chitin in the expression of co-stimulatory molecules in $F4/80^{-}CD11c^{+}$ cells, important for T cell differentiation in addition to cytokine environment. The expression levels

of CD40, CD80 and CD86 were increased in mice treated with HDM-derived chitin during sensitization compared to control group, however this effects was alleviated when chitinase was added during sensitization (Fig 4E). Taken together, these findings indicated that HDM-derived chitin induces the environment inducing adaptive immune response to inhaled allergen through the up-regulation of pro-inflammatory cytokines and co-stimulatory molecules.

House dust mite derived (HDM-derived) chitin induces Th1, Th2, and Th17 adaptive immune responses to inhalant allergen, which was reversed by chitinase.

Previously, we showed that HDM-derived chitin induces pro-inflammatory response in lung tissue during the sensitization period. Particularly, chitin up-regulated all of Th1, Th2 and Th17 related cytokines. Interestingly, when we treated chitinase, we observed down-regulation of Th2 related cytokines and up-regulation of Th1 and 17 related cytokines. Next, we extended to the model of acute OVA challenge to confirm the role of HDM-derived chitin in the development of OVA specific adaptive immune responses. To test this, mice were sensitized with 75 μg of OVA and 100 μg of HDM-derived chitin with or without chitinase treatment, then challenged with OVA (50 μg) alone, and all evaluation was performed 24 h after the last OVA challenge. Similar to the result of sensitization, lung inflammation was induced in group treated

with HDM-derived chitin during sensitization. In addition, infiltration of neutrophil and eosinophil were also significantly induced, while it was reversed significantly by chitinase treatment. Histological findings showed similar manner of result of lung inflammatory cell infiltration (Fig. 5A and 5B). In the case of OVA specific antibody production, the production of IgE was induced in group treated with HDM-derived chitin during sensitization and this production was suppressed by chitinase treatment. On the contrary, OVA specific-IgG1 and -IgG2a were more up-regulated by chitinase treatment (Fig. 5C). In terms of the production levels of cytokines, Th2 related cytokines such as IL-4, IL-5, eotaxin and TGF-β were enhanced in group treated with HDM-derived chitin during sensitization, while it was reversed by chitinase treatment (Fig. 5D; upper). On the contrary, Th1 and 17 related cytokines such as IL-10, IL-17, IL-12p70 and IFN-γ were much more enhanced (Fig 4D; lower). Next, to evaluate the T cell immune response, we re-stimulated lung and draining lymph node T cell with anti CD3/CD28 antibodies and evaluated the expression of cytokine including IL-4, IL-17, and IFN-γ. The result revealed that, as the result of BAL cytokines, we observed the expression of IL-4 was enhanced in group treated with HDM-derived chitin during sensitization; however it was reversed by chitinase treatment (Fig. 5E). Meanwhile, IL-17 and IFN-γ (Th17 and Th1 cytokines, respectively) were much more increased. These findings indicated that HDM-derived chitin acts as an adjuvant and induce the mixed Th1, Th2 and Th17 immune responses. In addition, airway of inflammation (mainly Th2 immune response

in terms of adaptive immunity) was suppressed when chitin structure was modified by chitinase.

House dust mite derived (HDM-derived) chitin induces Th1, Th2, and Th17 adaptive immune responses to inhalant allergen via toll-like receptor 2 (TLR2).

In the above experiments, we used chitinase to exclude the effect of chitin in the inflammation by destruction of its structure. However, airway inflammation was not fully suppressed, and we observed immune-modulation by enzyme digestion. According to previous reports, chitin is known to be recognized by TLR2. It is possible that TLR2 exist forming homo-dimer or hetero-dimer with TLR1 or TLR6. Considering it, we sensitized HDM-derived chitin in TLR1- and TLR6-deficient mice. In addition, to exclude the possible effect of contaminated LPS in HDM-derived chitin, we repeated test in TLR4-deficient mice. As a result, all of TLR deficiencies did not affect on the development of airway inflammation by intranasal administration of HDM-derived chitin (Suppl. Fig. 1). So in this context, we adjusted previous experiments to TLR2-deficient mice. To evaluate the effect of HDM-derived chitin during sensitization period, we administrated OVA and HDM-derived chitin in wild type (WT) and TLR2-deficient mice. When we evaluate the airway inflammation 12h after sensitization, the airway infiltration of macrophage and eosinophil were significantly down-regulated as the level of

negative control, and that of neutrophil was also significantly but not fully reduced (Fig. 6A). In the case of inflammatory cytokines, not only Th2 related cytokines, but also Th1/17 related cytokines were down-regulated in the absence of chitin recognition by TLR2 (Fig. 6B). Next, we extended to OVA challenge model to evaluate the effect of HDM-derived chitin on adaptive immune response. In regard to airway inflammation, airway infiltration of all inflammatory cells including macrophage, neutrophil and eosinophil were markedly reduced in TLR2-deficient mice (Fig. 6C). Lung histological findings also demonstrated similar manner (Fig. 6D). In addition, when we evaluated the level of OVA-specific antibody and cytokines, without TLR2, all of antibodies and cytokines were down-regulated and comparable to negative control (Fig. 6E & 6F). In terms of T cell immune response, proliferation of T cell from draining lymph node by re-stimulation with OVA was enhanced in WT mice treated with HDM-derived chitin during sensitization, however it was reversed in TLR2-deficient mice (Fig. 6G). In addition, T cells of lung tissue and draining lymph node from TLR2-deficient mice treated with OVA and HDM-derived chitin during sensitization showed diminished production of IL-4, IL-17, and IFN-γ compared to its positive control in WT mice (Fig. 6H). Taken together, these data indicated that TLR2, which is essential in the recognition of chitin, plays a key role in the development of all types of adaptive immune response to HDM-derived chitin containing inhalant allergens.

The main adaptive immune response to inhalant allergen induced by house dust mite derived (HDM-derived) chitin is Th2 rather than Th1 or Th17, and it is mediated by TNF-α signaling.

To sum up the result of HDM-derived chitin, it induced variable adaptive immune responses, including Th1, Th2 and Th17. However, it was not revealed that which immune response is the main event in the airway inflammation induced by HDM-derived chitin. To elucidate this, we applied previous animal model in the IL-4 receptor 1-, IL-17-, and IFN-γ- deficient mice, and airway inflammation was evaluated 24 after last OVA challenge. Absence of IL-4 signaling showed total inflammatory cells in BAL fluid decreased more than 60 percent (Suppl. Fig. 2). In addition, all kinds of inflammatory cells except lymphocyte, whose level was too low in all groups to compare, were decreased significantly. On the contrary, the absence of IL-17- and IFN-γ reduced total inflammatory cells in BAL fluid just less than 30 percent. This means that HDM-derived chitin induces airway inflammation mainly through Th2 immune response.

According to previous study, TNF-α plays a key role in the development of Th2 immune response to inhaled allergens. In the present study, we confirmed that TNF-α is produced by stimulation with HDM-derived chitin *in vivo*, and that its expression time was faster than other Th2 polarizing cytokine, such as IL-4 and TSLP, as previous studies. In this regard, we hypothesized that TNF-

α induced by HDM-derived chitin plays a key role in the development of Th2 type immune response as previous study regarding the role of TNF-α in dsRNA-treated animal model. To evaluate the role of TNF-α in sensitization to inhalant allergen induced by HDM-derived chitin, we performed *in vitro* test at first. We cultured lung epithelial cells and alveolar macrophages, which are the first-line cells responding to inhaled agents, with PBS, chitinase, HDM-derived chitin or chitinase-treated HDM-derived chitin for 24 h, and measured the expression of TNF-α as well as IL-6 which is known to induce Th1 and Th17 immune response. As a result, the production of TNF-α, a key mediator of Th2 inflammation in previous study, was markedly enhanced in the presence of chitin; however the production was significantly alleviated when chitin was treated with chitinase; meanwhile, the production of IL-6 was more enhanced (Suppl. Fig. 3). Next, we further extended to mouse model. WT and TNF-α-deficient mice were sensitized with OVA alone or OVA and HDM-derived chitin as previous experiment. As a result, infiltration of inflammatory cells in airway observed 24h after sensitization were significantly suppressed by the absence of TNF-α signaling as previous results (Fig. 7A). In the case of BAL cytokine expression, however, only Th2 polarizing cytokine including IL-4 and TSLP were decreased to control level, while Th1/17 polarizing cytokines were more enhanced (Fig. 7B). Next, as previous results, we extended to OVA challenge model, and evaluated adaptive immune response including T cell responses. BAL cellularity and histological findings showed that airway inflammation was reduced in TNF-α-

deficient mice like the result of sensitization (Fig. 7C and D). In terms of OVA specific antibodies, up-regulated level of IgE in WT mice was decreased in the absence of TNF-α signaling. While, IgG1 and IgG2a were more enhanced in TNF-α deficiency compared with WT control mice (Fig. 7E). Similarly, in regard to the level of cytokine production in BAL fluid, Th2 immune response related cytokines and chemokine including IL-4, IL-5, eotaxin and TGF-β were decreased in TNF-α-deficient mice compared to its WT control. Otherwise, Th1/17 immune response related cytokines, such as IL-10, IL-17, IP-10 and IFN-γ, showed opposite manner (Fig. 7F). Next, to evaluate the effect of TNF-α on the adaptive immune response induced by HDM-derived chitin, T cell proliferation and cytokine production by T cell re-stimulated with OVA were assessed. When T cells from lung regional lymph node were stimulated with OVA, the proliferation was observed to elevate, however, it was down-regulated in TNF-α deficiency (Fig. 7G). In terms of cytokine production of T cell from lung and draining lymph node, the result showed that reduced production of IL-4 and increased production of IL-17 and IFN-γ in TNF-α deficiency, like the results of cytokines in BAL fluid and OVA specific antibodies in serum (Fig. 7H). Taken together, TNF-α is an important mediator for the development of Th2 immune response to inhaled HDM-derived chitin containing allergen.

House dust mite derived (HDM-derived) chitin induces Th1, Th2, and Th17 adaptive immune responses to

inhalant allergen though natural killer T (NKT) cell.

In previous study, it was demonstrated that TNF- α, produced in mice treated with inhalant allergen and dsRNA during sensitization, mediates the development of adaptive immunes response to the allergen through TNFR on NKT cells. In this context, we hypothesized that NKT cell is also a key player for the establishment of adaptive immune responses to inhaled allergen in mice treated with HDM-derived chitin during sensitization. To test this, WT and CD1d-deficient mice were sensitized with OVA alone or OVA and HDM-derived chitin. The number of infiltrated cells in lung tissue was robustly decreased in CD1d-deficient mice compared to WT mice when sensitized with OVA and HDM-derived chitin (Fig 8A). Histological aspects also indicated that CD1d-deficient mice showed reduced lung inflammation compared to WT mice (Fig 8B). Allergen specific IgE and IgG1 but not IgG2a showed similar manner. In regard to BAL fluid cytokine production, all of cytokines related to Th1, Th2 and Th17 immune responses except IL-10 were diminished in CD1d-deficient mice compared to WT mice (Fig 8D). Next, to evaluate the role of NKT cells on the development of T cell immune response induced by HDM-derived chitin, we assessed T cell proliferation and cytokine production by T cell which was re-stimulated with OVA. In terms of T cell proliferation, T cells from CD1d-deficient mice proliferated less than those from WT mice when stimulated with OVA and chitin during sensitization (Fig 8E). In addition, all of cytokine produced by T cells, from lung tissue and lung regional lymph node were down-regulated in CD1d-deficient mice (Fig 8F).

These findings suggest that NKT cell play an important role for the establishment of adaptive immune responses to HDM-derived chitin containing inhalant allergen as in previous dsRNA-treated animal model.

Discussion

Many kinds of mouse models of allergic airway diseases have been tried, which actually improved our understanding of the pathogenesis of those diseases. Most of these models evaluated responses to a single antigen or allergen, especially OVA which is a prototypic innocuous antigen (35, 36). In many studies, many kinds of adjuvants including aluminum hydroxide, lipopolysaccharide (LPS), and double stranded RNA were added to OVA in order to induce certain type of airway inflammation (23, 34, 35, 37). Meanwhile humans are, in their real life, frequently exposed to various allergens simultaneously, each with distinct antigenic potential. In previous study, HDM, a major inhalant allergen in asthma and allergic rhinitis, was reported to promote sensitization to other antigen such as OVA in addition to its own allergenic properties (7). However, the exact mechanism remained to be investigated.

Chitin is frequently found in exoskeleton, mid gut epithelium and fecal pellets of HDM (13). Around twenty years ago, chitin and chitin derivatives were demonstrated to stimulate macrophages to produce cytokines that induced nonspecific innate immune response to bacterial or viral infections and neoplasm in many studies (38-42). According to recent studies, in the context of chitin's size, various immune responses are induced, determined by its size. Shibata et al. reported that phagocytosable chitin (1-10 μm) induced IFN-γ production in alveolar macrophage (43). Next, Reese et al. showed that

chitin (unknown, diver size) induced Th2 immune response (44). Da Silva et al. reported that intermediate size chitin (IC, 40-70 μm) induced mixed immune responses (Th1, Th2 and Th17) via TNF-α signaling, meanwhile small size chitin (SC, <40 μm) induced production of mainly IL-10 rather than TNF-α in alveolar macrophage (16, 45).

Chitinases (*chitodextrinase*, *1,4-beta-poly-N-acetylglucosaminidase*, *poly-beta-glucosaminidase*, *beta-1,4-poly-N-acetyl glucosamidinase*, *poly[1,4-(N-acetyl-beta-D-glucosaminide)] glycanohydrolase*, *(1->4)-2-acetamido-2-deoxy-beta-D-glucan glycanohydrolase*) are hydrolytic enzymes that break down glycosidic bonds in chitin(17). They are generally found in organisms that either need to reshape their own chitin (parasites, arthropods, and fungi) or dissolve and digest the chitin of others (human and other mammals)(17).

In our study, the amount of small size chitin less than 10 μm was increased (Suppl. Fig. 4A), while that of larger chitin (10 ~ 500 μm) including IC was decreased (Suppl. Fig. 4B) when chitin was treated with chitinase. As a result, increased amount of small size chitin might stimulate macrophage to produce more IFN-γ and IL-10 which could consequently up-regulate Th1 and Th17 immune response. In addition to IFN-γ and IL-10, IL-6 was recently reported to relate with Th1 and Th7 immune response (46-49). In our *in vitro* study, production of IL-6 by macrophage was increased when chitin was treated with chitinase (Suppl. Fig. 3), which might contribute to enhanced Th1 and Th17 immune response. On the contrary, decreased IC might lead to reduced production of TNF-α which could subsequently result in suppression of Th2

immune response, seeing that TNF-α was observed to have a key role in inducing Th2 immune response to inhalant allergen in this study as well as the previous report (23). Especially, TNF-α produced by macrophage was reduced when chitin was treated with chitinase in our *in vitro* study (Suppl. Fig. 3).

Da Silva et al. also reported the kinds of chitin's receptor according to its size (IC: TLR2 mainly and Dectin-1, SC: Dectin-1 mainly, TLR2, and mannose receptor) (45). Although other receptors also react to chitin, TLR2 seemed to play a more important role in the development of airway inflammation than others, for airway inflammation and all type of Th1, Th2, and Th17 immune response were suppressed in TLR2-deficient mice in our study.

From the point of immune responses, previous studies with intraperitoneal sensitization showed that OVA specific mixed Th1, Th2 and Th17 adaptive immune responses were induced by crab chitin which has intermediate size (40-70 μm) (16). In addition, the immune responses were mediated by TLR2 and IL-17, mainly (16, 50). In this study, we also observed the similar mixed and TLR2-mediated adaptive immune response to OVA when HDM-derived chitin was intranasally administrated during sensitization. However, unlike the previous studies, we proved that OVA specific adaptive immune responses induced by inhaled HDM-derived chitin are mainly mediated by Th2 immune responses via IL-4 signaling, suggesting that airway inflammation was much more decreased in IL-4R1-deficient mice than IL-17- or IFN-γ-deficient mice. To exclude the possible effect of low dose LPS on inducing the Th2 immune

response (51), we measured the level of LPS in HDM-derived chitin, and found that LPS was not detected (data not shown). In addition, there was no difference between wild type and TLR4-deficient mice when exposed to HDM-derived chitin. Our experiment and previous studies are different in term of route of immunization, size, and composition of chitin; therefore they might show different immune responses consequently. However, considering that mice in our study were sensitized to HDM or other inhalant allergen not through peritoneal route but through airway, our result may be closer to HDM induced airway inflammation in real life than previous reports. Collectively, diverse size of inhaled HDM-derived chitin was recognized by TLR2 and induced mixed adaptive immune response to allergen, which was mainly Th2-type.

In regard to TNF-α, a proinflammatory cytokine inducing Th2 response in murine model of intranasal sensitization and challenge [23], intermediate and small size of chitin was recognized by macrophage which consequently produced TNF-α in a previous study (45). In this study, we also confirmed that the production of TNF-α was enhanced in alveolar macrophage and airway epithelial cell line after stimulation with chitin, and that it was reversed when chitin was treated with chitinase; meanwhile the expression of IL-6 which is known to induce Th1 and Th17 immune response [46-49] was more enhanced. In addition to *in vitro* assay, we observed the up-regulation and rapid production of TNF-α which is faster than that of other Th2 related cytokines in early time *in vivo*. According to previous study, TNF-α can

induce antigen specific Th2 immune responses in the lung (23), we postulated that TNF-α induced by HDM-derived chitin produces OVA specific Th2 immune response. To clarify this hypothesis, we administrated HDM-derived chitin in TNF-α-deficient mice, and confirmed the down-regulation of airway inflammation and Th2 related cytokines' production. In contrast to previous reports in which Th1 and/or Th17 immune responses induced by the usage of limited size or diverse size of chitin, our data indicated that diverse-sized HDM-derived chitin is recognized by TLR2 and induce the Th2 immune response dominantly through TNF-α signaling.

When it comes to NKT cells and TNFR on these cells, previous reports showed that chitin induced Th2 cytokine, IL-4 and IL-13 by the activation of eosinophil, basophil, and macrophage (44). Recently, it was reported that macrophage-derived TNF-α induces Th2 cell response to inhaled allergen through TNF-α receptor on the surface of NKT cells (23). In this study, to confirm the exact mechanism, we applied the animal model using HDM-derived chitin in CD1d-deficient mice. As a result, we observed that all of adaptive airway inflammation and cytokine production were down-regulated, except IL-10 and serum OVA-specific IgG2a antibody which are known to relate to regulatory T cell (Treg). In addition, OVA specific T cell proliferation was also showed similar manner. Seeing this result, the role of NKT cells seemed to be crucial in Th2 immune response as well as Th1 and Th17 immune response. To sum up our data, we suggest that HDM-derived chitin induces Th2 immune response through TLR2-TNF-α-NKT axis (Suppl. Fig.

5).

In this study, albeit both Th1 and Th17 immune responses are not the main aspect in airway inflammation, and they showed somewhat different results compared to Th2 immune response. Under the TLR2-deficient state, all type of responses disappeared. This phenomenon might be caused by the absence of chitin recognition. In the absence of NKT cells, all of adaptive immune responses were also down-regulated, with the exception of IL-10 and serum OVA-specific IgG2a which are known to relate to Treg. NKT cell is well known as Th1, Th2 and Th17 cytokine producer and TLR2 is also expressed on NKT cells (52-55). Although further investigation is required to clarify the exact mechanism of Th1/17 response in NKT cells, NKT cells might also play an important role in Th1 and Th17 responses induced by HDM-derived chitin. On the contrary, in the absence of TNF-α, as opposed to above results, Th1 and Th17 immune responses were enhanced during both sensitization and challenge. As suggested in a previous study, both responses might be enhanced by the absence of suppression by TNF-α or IL-4 (23). In addition, the production of IL-6 which was recently known to induce Th1 and Th17 immune response (46, 48, 49), was increased when alveolar macrophage and lung epithelial cell were cultured with chitinase-treated chitin, which might contribute to enhanced Th1 and Th17 immune response.

With regard to the recruitment of inflammatory cells to airway, we observed down-regulation of airway inflammation in wild-type mice in which HDM or HDM-derived chitin was treated with chitinase and in all of knock-out mice.

When HDM-derived chitin was treated with chitinase, the level of dendritic maturation was down regulated. Previous experimental data showed that the absence of TNF-α induced down-regulation of dendritic cell maturation, and adaptive immune response to OVA (23). In addition to TNF-α, IL-1, a pro-inflammatory cytokine related with dendritic cell maturation, was down-regulated by chitinase treatment in this experiment. Based on above mechanism, we can postulate that down-regulation of airway inflammation was caused by highly suppressed production of pro-inflammatory cytokines including TNF-α and IL-1 in wild-type mice in which HDM or HDM-derived chitin was treated with chitinase and in all of knock-out mice.

In conclusion, HDM promotes adaptive immune response to other inhalant allergen through its component, chitin which is a multifaceted adjuvant. Chitin stimulates all of Th1, Th2, and Th17, but mainly Th2 immune response to concomitantly inhaled other aeroallergen through TLR2, TNF-α and NKT cell.

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Figure legends

Fig. 1 The infiltration of inflammatory cells and the expression of Th2 related cytokines in bronchoalveolar lavage (BAL) fluid were increased in mice treated with house dust mite (HDM) in addition to ovalbumin (OVA) during sensitization, and it was alleviated by chitinase treatment. Meanwhile Th1 and Th17 related cytokines were more enhanced. For all panels, wild-type mice (C57BL/6 background) were sensitized with 75 μg of OVA alone, 75 μg of OVA + 100 μg of HDM with or without chitinase treatment, and evaluated 12 h after sensitization. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$ compared to each control groups, OVA or OVA + chitinase, respectively; # $P< 0.05$; ## $P< 0.01$; ### $P< 0.001$; n.s.: not significant; (A) Bronchoalveolar lavage (BAL) cellularity level, (B) Production of IL-1β, TNF-α, IL-4, TSLP, IL-6, IL-10, IL-12p70, and IL-23 in BAL fluid

Fig. 2 Airway inflammation and Th2 immune response promoted by addition of house dust mite (HDM) to ovalbumin (OVA) during sensitization was down-regulated by chitinase treatment, meanwhile Th1 and Th17 immune responses were more enhanced. For all panels, wild-type mice (C57BL/6 background) were sensitized with 75 μg of OVA+100 μg of HDM with or without chitinase treatment, then challenged with OVA (50 μg) alone. The evaluations were performed 24 h after the last OVA challenge. * $P< 0.05$; **

P< 0.01; *** *P*< 0.001 compared to each control groups, OVA or OVA+chitinase, respectively; # *P*< 0.05; ## *P*< 0.01; ### *P*< 0.001; n.s.: not significant; (A) Bronchoalveolar lavage (BAL) cellularity, (B) Lung histologic findings (a, OVA; b, OVA+chitinase; c, OVA+HDM; d, OVA+HDM+chitinase), (C) The production of OVA-specific IgE, IgG1 and IgG2a in serum, (D) Cytokine productions of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IP-10, and IFN-γ in BAL fluid; For (E) panel, cells were isolated from lung tissues and lung-draining lymph nodes (LN), and incubated with PBS or anti-CD3 and CD28 antibodies for 12h. Levels of each cytokine were evaluated in supernatant fraction. (E) Levels of IL-4, IL-17, and IFN-γ from lung cells, and lung-draining LN cells.

Fig. 3 House dust mite derived (HDM-derived) chitin as an adjuvant in immune response to inhaled ovalbumin (OVA) allergen and its extraction (A) Fourier transform infrared (FTIR) spectroscopy of commercial crab chitin and HDM-derived chitin; For (B) panel, phosphate buffered saline (PBS) or 1, 10, 100 μg of HDM-derived chitin was intranasally administrated to wild-type mice (C57BL/6 background), and evaluated 12 h later. * *P*< 0.05; ** *P*< 0.01; *** *P*< 0.001 compared to PBS group; (B) Levels of IL-6 and TNF-α in BAL fluid; For (C)-(E) panels, wild-type mice (C57BL/6 background) were sensitized with 75 μg of OVA, or 75 μg of OVA plus 1, 10, or 100 μg of HDM-derived chitin. Then, mice were challenged with OVA (50 μg) alone, and evaluated 24 h after the last OVA challenge. * *P*< 0.05; ** *P*< 0.01; ***

P< 0.001 compared to OVA group; (C) Bronchoalveolar lavage (BAL) cellularity, (D) Levels of OVA-specific IgE, IgG1 and IgG2a in serum, (E) Levels of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IL-12p70, and IP-10 in BAL fluid

Fig. 4 House dust mite (HDM) derived chitin induces airway inflammation and all types of Th1, Th2, and Th17 related cytokines during sensitization, and it was reversed by chitinase treatment except Th1 and Th17 related cytokines. * *P*< 0.05; ** *P*< 0.01; *** *P*< 0.001; n.s.: not significant compared to basal or OVA group; For panel (A) and (B), each dose of HDM-derived chitin was administrated to wild type (WT) mouse airways and then evaluated at various time points. (A) Inflammatory cell infiltration of airway after HDM-derived chitin administration; (B) The levels of TNF-α, IL-4, TSLP, VEGF, IL-6 and IL-12p70 in bronchoalveolar lavage (BAL) fluids; For (C)-(E), wild-type mice (C57BL/6 background) were sensitized with 75 μg of OVA, or 75 μg of OVA plus 100 μg of HDM-derived chitin with or without chitinase treatment. The evaluation was performed 12h later. (C) BAL cellularity; (D) Levels of IL-1β, TNF-α, IL-4, TSLP, IL-6, IL-10, IL-12p70, and IL-23 in BAL fluid; (E) Expression levels of co-stimulatory molecules, such as CD40, CD80 and CD86, on lung $F4/80^{-}CD11c^{+}$ dendritic cells.

Fig. 5 House dust mite (HDM) derived chitin induces airway inflammation and Th1, Th2, and Th17 immune response to inhaled ovalbumin (OVA)

allergen, however chitinase treatment alleviates these airway inflammation and Th2 immune response, but not Th1 or Th17 immune response. For all panels, wild-type (WT) mice (C57BL/6 background) were sensitized with 75 μg of OVA, 75 μg of OVA + chitinase, 75 μg of OVA + 100 μg of HDM-derived chitin with or without chitinase treatment, then challenged with OVA (50 μg) alone, and evaluated 24 h after the last OVA challenge. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$ compared to each control groups, OVA or OVA + chitinase, respectively; # $P< 0.05$; ## $P< 0.01$; ### $P< 0.001$; (A) Bronchoalveolar lavage (BAL) cellularity; (B) Lung histologic findings (a, OVA; b, OVA + chitinase; c, OVA + HDM-derived chitin; d, OVA + HDM-derived chitin + chitinase); (C) Levels of OVA-specific IgE, IgG1, IgG2a in serum; (D) Levels of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IL-12p70, and IFN-γ in BAL fluid; For panels (E), cells were isolated from lung tissues and lung-draining lymph nodes (LN), and incubated with PBS or CD3 and CD28 antibodies for 12h. Levels of each cytokine were evaluated in supernatant fraction. (E) Levels of IL-4, IL-17, and IFN-γ from lung and lung-draining lymph node cells.

Fig. 6 Airway inflammation and all types of immune response to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is suppressed in the absence of toll-like receptor 2 (TLR2). For all panels, TLR2-deficient mice and wild-type (WT) mice (C57BL/6 background) were sensitized with 75 μg of OVA, or 75 μg of OVA + 100 μg of HDM-

derived chitin. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$ compared to wild-type OVA group; # $P< 0.05$; ## $P< 0.01$; ### $P< 0.001$; For (A)-(B) panels, evaluations were performed after 12 h sensitization. (A) Bronchoalveolar lavage (BAL) cellularity; (B) Levels of IL-1β, TNF-α, IL-4, TSLP, IL-6, IL-10, IL-12p70, and IL-23 in BAL fluid; For panels (C)-(H), mice were further challenged with OVA (50 μg) alone, and evaluated 24 h after the last OVA challenge. (C) BAL cellularity; (D) Lung histologic findings (a, WT mice treated with OVA; b, TLR2-deficient mice treated with OVA; c, WT mice treated with OVA + chitin; d, TLR2-deficient mice treated with OVA + chitin); (E) Levels of OVA-specific IgE, IgG1, IgG2a in serum; (F) Levels of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IFN-γ, and IP-10 in BAL fluid; For panels (G)-(H), cells were isolated from lung tissues and lung-draining lymph nodes (LN). (G) The levels of memory T cell proliferation by stimulation of OVA in LN cells from each mouse. For (H), isolated cells were incubated with PBS or CD3 and CD28 antibodies for 12h. Levels of each cytokine were evaluated in supernatant fraction. (H) Levels of IL-4, IL-17, and IFN-γ from lung and lung-draining LN cells.

Fig. 7 Airway inflammation and Th2 immune responses to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is diminished in the absence of TNF-α, whereas the Th1 and Th17 responses are more enhanced. For all panels, TNF-α-deficient (TNF KO) mice and wild-type (WT) mice (C57BL/6 background) were sensitized with 75 μg of OVA,

or 75 μg of OVA + 100 μg of HDM-derived chitin. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$ compared to wild-type OVA group; # $P< 0.05$; ## $P< 0.01$; ### $P< 0.001$; n.s.: not significant. For panel (A) and (B), evaluations were performed 12 h after sensitization. (A) Bronchoalveolar lavage (BAL) cellularity; (B) Levels of IL-1β, TNF-α, IL-4, TSLP, IL-6, IL-10, IL-12p70, and IL-23 in BAL fluid; For (C)-(H), mice were further challenged with OVA (50 μg) alone, and evaluated 24 h after the last OVA challenge. (C) BAL cellularity; (D) Lung histologic findings (a, WT mice treated with OVA; b, TNF-α-deficient mice treated with OVA; c, WT mice treated with OVA + chitin; d, TNF-α-deficient mice treated with OVA + chitin); (E) Levels of OVA-specific IgE, IgG1 and IgG2a in serum ; (F) Levels of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IP-10, and IFN-γ in BAL fluid; For panels (G)-(H), cells were isolated from lung tissues and lung-draining lymph nodes (LN). (G) The levels of memory T cell proliferation by stimulation of OVA in LN cells from each mouse. For (H), isolated cells were incubated with PBS or CD3 and CD28 antibodies for 12 h. Levels of each cytokine were evaluated in supernatant fraction. (H) Levels of IL-4, IL-17, and IFN-γ from lung and lung-draining LN cells.

Fig. 8 Airway inflammation and all types of immune response to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin is suppressed in the absence of natural killer T (NKT) cell. For all panels, CD1d-deficient mice and wild-type (WT) mice (C57BL/6 background)

were sensitized with 75 μg of OVA, or 75 μg of OVA + 100 μg of HDM-derived chitin, then challenged with OVA (50 μg) alone, and evaluated 24 h after the last OVA challenge. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$ compared to wild-type OVA group; # $P< 0.05$; ## $P< 0.01$; ### $P< 0.001$; n.s.: not significant; For (A)-(F) panels, evaluations were performed 24h after last challenge. (A) Bronchoalveolar lavage (BAL) cellularity; (B) Lung histologic findings (a, WT mice treated with OVA; b, CD1d-deficient mice treated with OVA; c, WT mice treated with OVA + chitin; d, CD1d-deficient mice treated with OVA + chitin); (C) Levels of OVA-specific IgE, IgG1, IgG2a in serum; (D) Levels of IL-4, IL-5, eotaxin, TGF-β, IL-10, IL-17, IP-10, and IFN-γ in BAL fluid; For panels (E)-(F), cells were isolated from lung tissues and lung-draining lymph nodes (LN). (E) The levels of memory T cell proliferation by stimulation of OVA in LN cells from each mouse. For (F), isolated cells were incubated with PBS or CD3 and CD28 antibodies for 12h. Levels of each cytokine were evaluated in supernatant fraction. (F) Levels of IL-4, IL-17, and IFN-γ from lung and lung-draining LN cells.

Suppl. Fig. 1 Airway inflammation and Th1, Th2, and Th17 immune responses to inhaled ovalbumin (OVA) allergen induced by house dust mite derived (HDM-derived) chitin are not mediated by toll-like receptor (TLR) 1, TLR 4, or TLR 6. For all panels, TLR-deficient mice and wild-type mice (C57BL/6 background) were sensitized with 75 μg of OVA, or 75 μg of OVA + 100 μg of HDM-derived chitin, and then evaluated 12 h after sensitization.

(A) Bronchoalveolar lavage (BAL) cellularity; (B) Levels of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-23, TNF-α, and TSLP in BAL fluid; +: sensitized, -: not sensitized

Suppl. Fig. 2 Airway inflammation induced by house dust mite derived (HDM-derived) chitin is mediated by Th2 immune response dominantly rather than Th1 or Th17 immune responses. Wild-type (WT) mice (C57BL/6 background) and IL-17-, IL-4R1-, and IFN-γ- deficient mice were sensitized with 75 μg of OVA, or 75 μg of OVA + 100 μg of HDM-derived chitin, then challenged with OVA (50 μg) alone. The evaluation of cellularity was performed in bronchoalveolar lavage fluids 24h after last OVA challenge. # $P< 0.05$; ### $P< 0.001$ compared to wild-type group

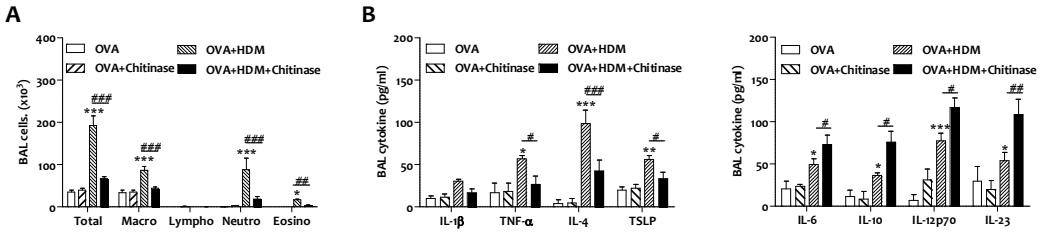
Suppl. Fig. 3 Levels of IL-6 and TNF-α released by murine lung epithelial (MLE) cells and murine alveolar macrophage (MH-S) cells which were cultured with phosphate-buffered saline (PBS), chitinase, house dust mites derived (HDM-derived chitin), or chitinase-treated HDM-derived chitin for 24 h.

Suppl. Fig. 4 The distribution of chitin according to its size; (A) Chitin sized less than 10 μm, (B) Whole chitin (0-1000 μm)

Suppl. Fig. 5 Scheme of TLR2-TNF-α-NKT axis in adaptive immune

response to ovalbumin (OVA) allergen induced by house dust mite (HDM)-derived chitin; PAMP receptor: pathogen associated molecular pattern receptor: TLR2: toll-like receptor 2, TNF-α: tumor necrosis factor alpha, TNFR: tumor necrosis factor receptor, NKT cell: natural killer T cell, MHC: major histocompatibility complex, TCR: T cell receptor, Th2 cell: type 2 T helper cell, Th1 cell: type 1 T helper cell, Th17 cell: type 17 T helper cell

Fig. 1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Lympho
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Eosino
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IL-10
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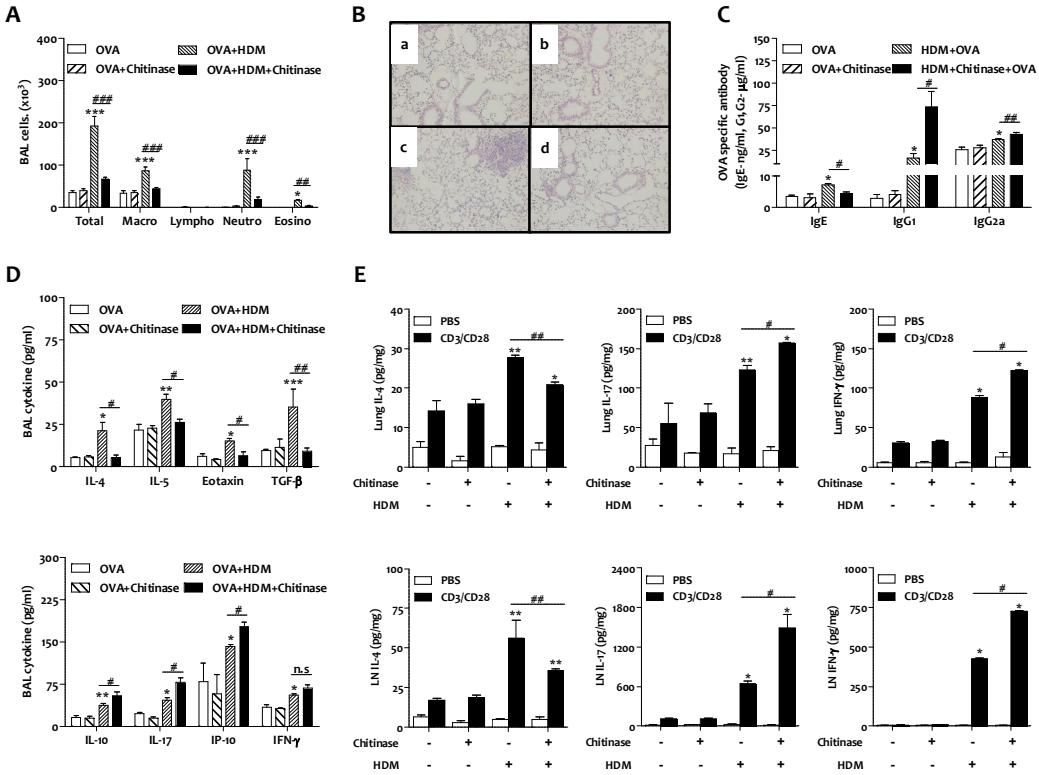
IL-23

Fig. 2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Lympho
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Eosino
B
a
b
c
d
C
OVA
OVA+Chitinase
HDM+OVA
HDM+Chitinase+OVA
OVA specific antibody (IgE- ng/ml, G1,G2- µg/ml)
IgE
IgG1
IgG2a
D
BAL cytokine (pg/ml)
IL-4
IL-5
Eotaxin
TGF-β
IL-10
IL-17
IP-10
IFN-γ
n.s
E
PBS
CD3/CD28
Lung IL-4 (pg/mg)
Lung IL-17 (pg/mg)
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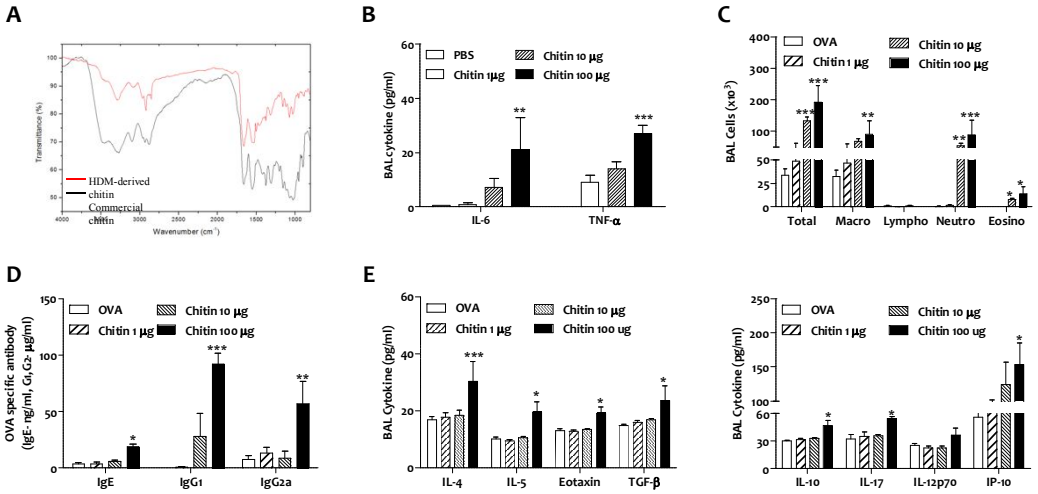
LN IL-17 (pg/mg)
LN IFN-γ (pg/mg)
Chitinase
HDM

Fig. 3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B
C
D
E
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Wavenumber (cm⁻¹)
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Chitin 10 μg
Chitin 100 μg
BAL cytokine (pg/ml)
IL-6
TNF-α
OVA
Chitin 1 μg
Chitin 10 μg
Chitin 100 μg
BAL Cells (x10³)
Total
Macro
Lympho
Neutro
Eosino
OVA specific antibody (IgE- ng/ml), G1,G2- μg/ml)
IgE
IgG1
IgG2a
Chitin 100 ug
BAL Cytokine (pg/ml)
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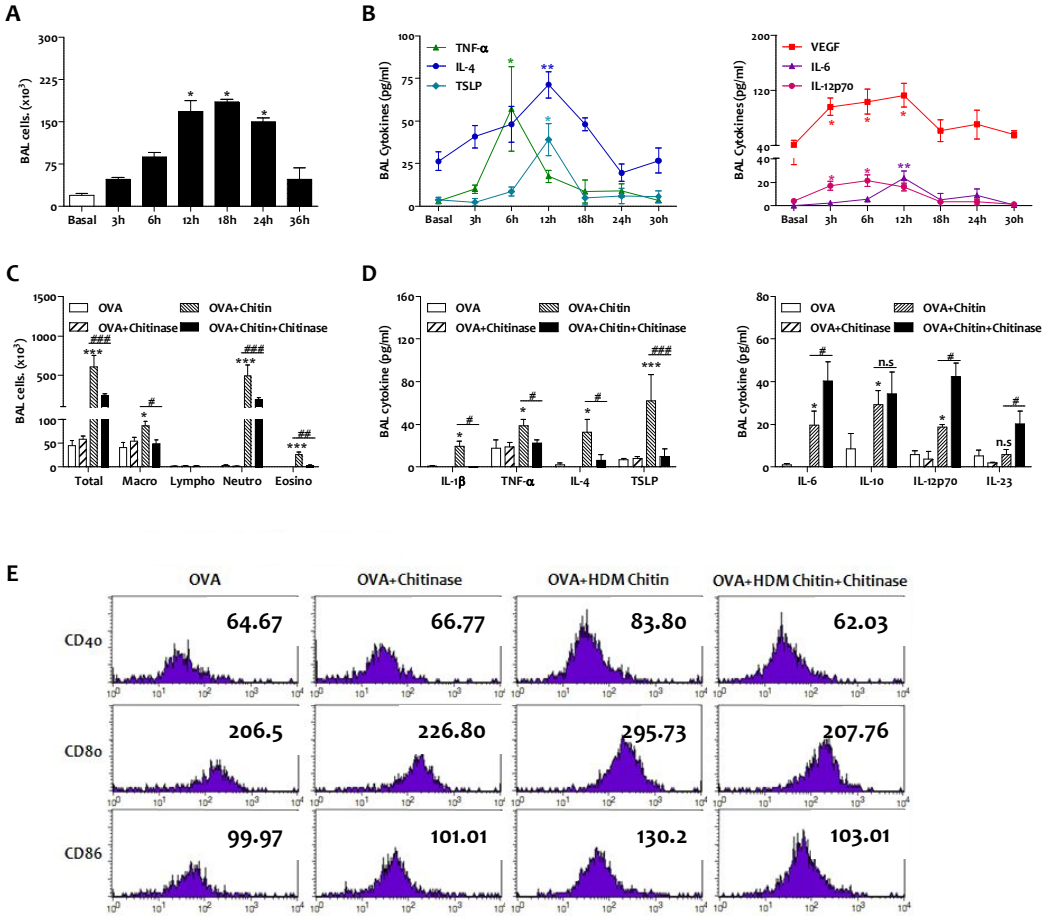
IL-5
Eotaxin
TGF-β
IL-10
IL-17
IL-12p70
IP-10

Fig. 4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6h
12h
18h
24h
36h
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BAL Cytokines (pg/ml)
VEGF
IL-6
IL-12p70
30h
C
OVA
OVA+Chitin
OVA+Chitinase
OVA+Chitin+Chitinase
Total
Macro
Lympho
Neutro
Eosino
D
IL-1β
TNF-α
IL-4
TSLP
BAL cytokine (pg/ml)
IL-6
IL-10
IL-12p70
IL-23
n.s
E
OVA
OVA+Chitinase
OVA+HDM Chitin
OVA+HDM Chitin+Chitinase
CD40
64.67
66.77
83.80
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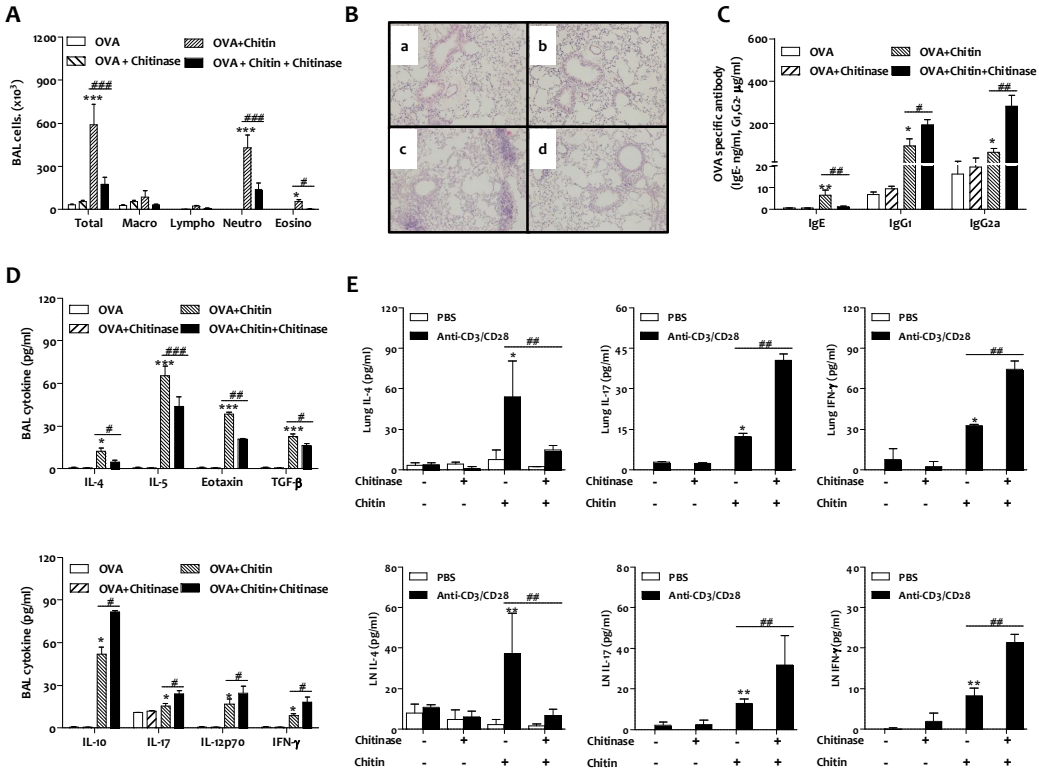
103.01

Fig. 5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OVA + Chitinase
OVA + Chitin + Chitinase
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Total
Macro
Lympho
Neutro
Eosino
B
a
b
c
d
C
OVA
OVA+Chitin
OVA+Chitinase
OVA+Chitin+Chitinase
OVA specific antibody (IgE- ng/ml, G1,G2- µg/ml)
IgE
IgG1
IgG2a
D
OVA
OVA+Chitin
OVA+Chitinase
OVA+Chitin+Chitinase
BAL cytokine (pg/ml)
IL-4
IL-5
Eotaxin
TGF-β
BAL cytokine (pg/ml)
IL-10
IL-17
IL-12p70
IFN-γ
E
PBS
Anti-CD3/CD28
Lung IL-4 (pg/ml)
Lung IL-17 (pg/ml)
Lung IFN-γ (pg/ml)
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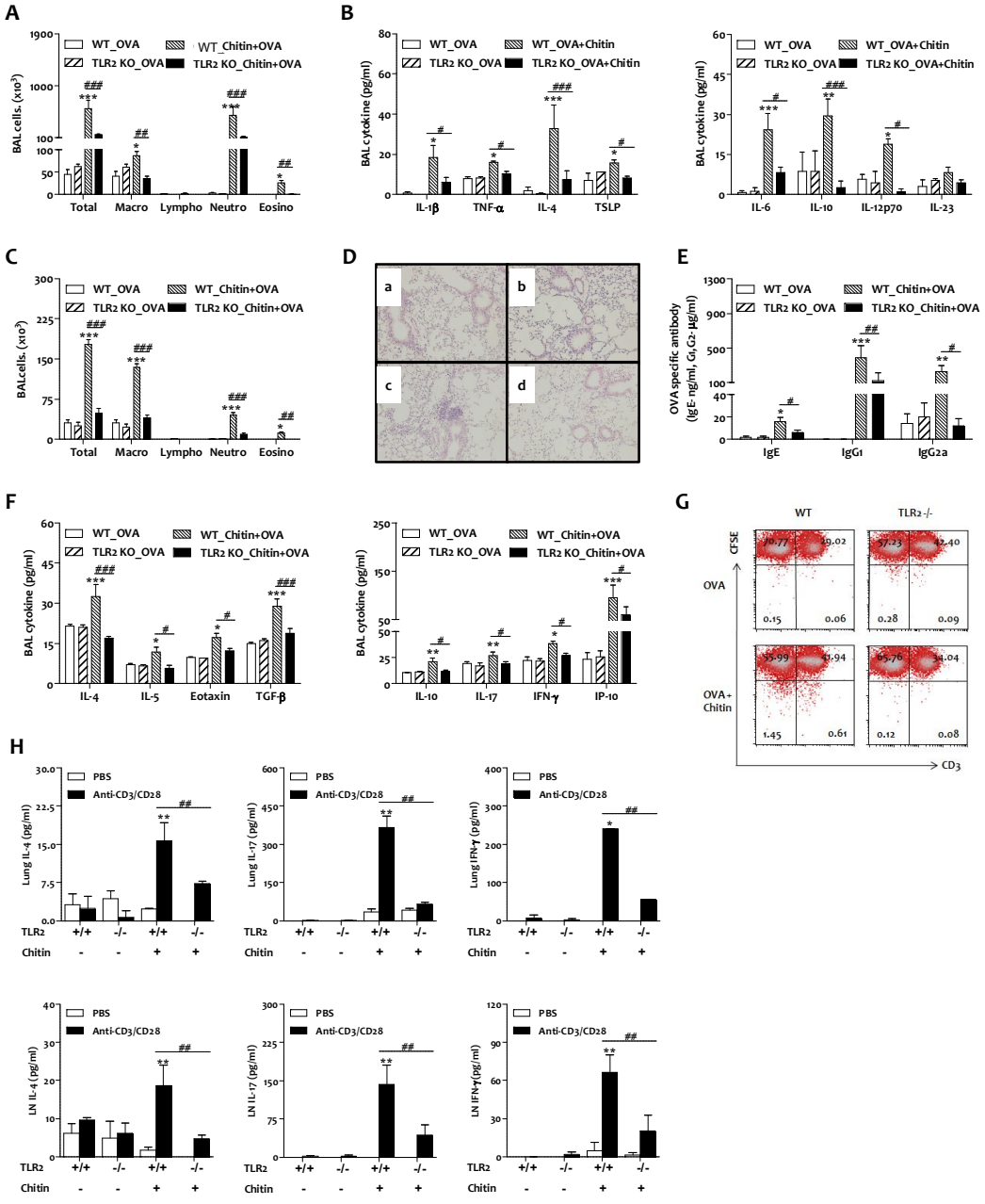
LN IFN-γ(pg/ml)
Chitinase
Chitin

Fig. 6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WT_Chitin+OVA
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Macro
Lympho
Neutro
Eosino
B
WT_OVA+Chitin
TLR2 KO_OVA+Chitin
BAL cytokine (pg/ml)
IL-1β
TNF-α
IL-4
TSLP
IL-6
IL-10
IL-12p70
IL-23
C
D
a
b
c
d
E
OVA specific antibody (IgE- ng/ml, G1,G2- µg/ml)
IgE
IgG1
IgG2a
F
IL-4
IL-5
Eotaxin
TGF-β
IL-10
IL-17
IFN-γ
IP-10
G
WT
TLR2 -/-
CFSE
OVA
OVA+ Chitin
CD3
H
PBS
Anti-CD3/CD28
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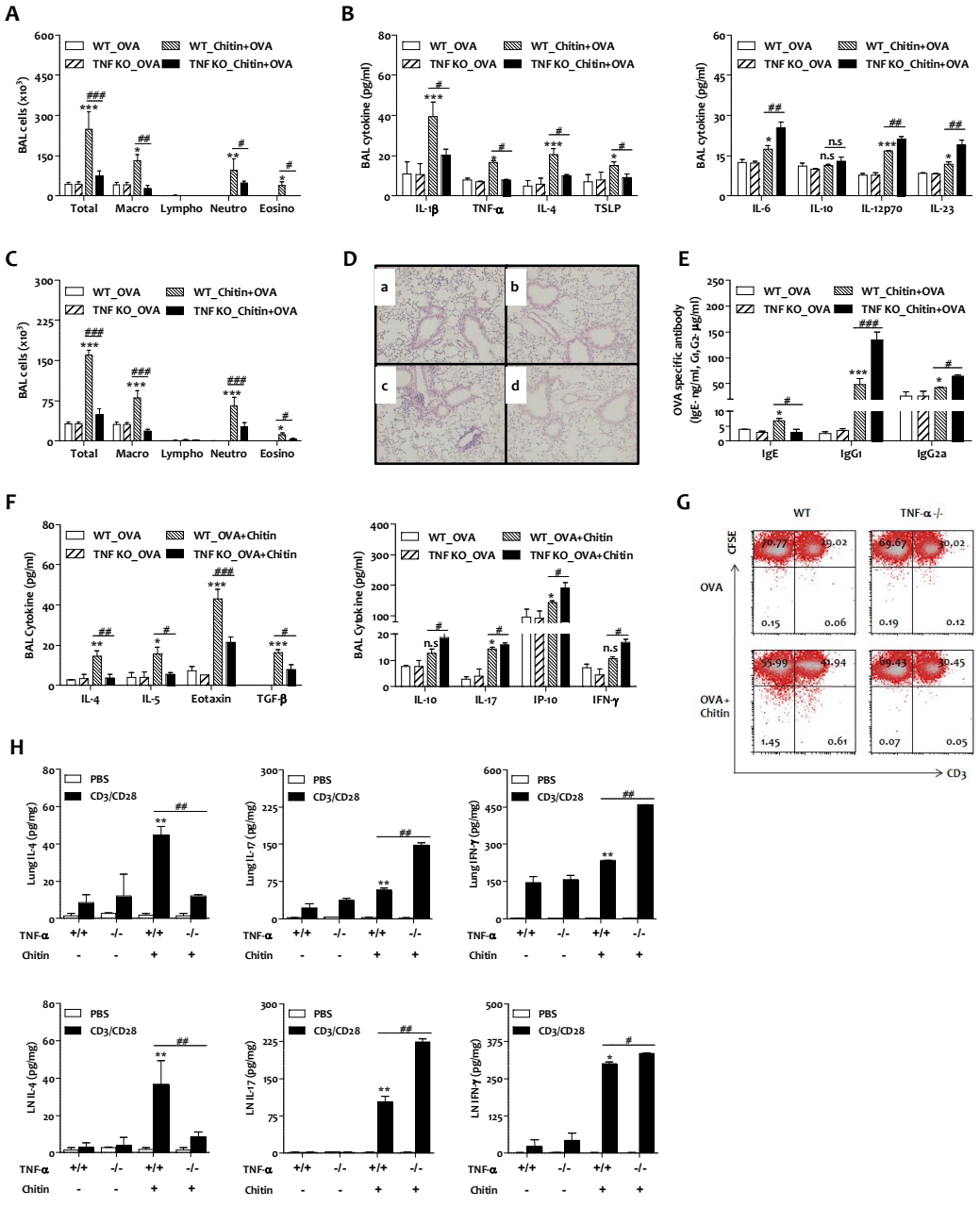
LN IL-17 (pg/ml)
LN IFN-γ(pg/ml)
TLR2
Chitin

Fig. 7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WT_Chitin+OVA
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Macro
Lympho
Neutro
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TNF-α
IL-4
TSLP
IL-6
IL-10
IL-12p70
IL-23
n.s
C
BAL cells (x10³)
D
a
b
c
d
E
OVA specific antibody
(IgE - ng/ml, G1,G2- µg/ml)
IgE
IgG1
IgG2a
F
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BAL Cytokine (pg/ml)
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IL-10
IL-17
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IFN-γ
G
WT
TNF-α -/-
CFSE
OVA
OVA+ Chitin
CD3
70.77
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0.15
0.06
69.67
30.02
0.19
0.12
55.99
43.94
1.45
0.61
69.43
30.45
0.07
0.05
H
PBS
CD3/CD28
Lung IL-4 (pg/mg)
Lung IL-17 (pg/mg)
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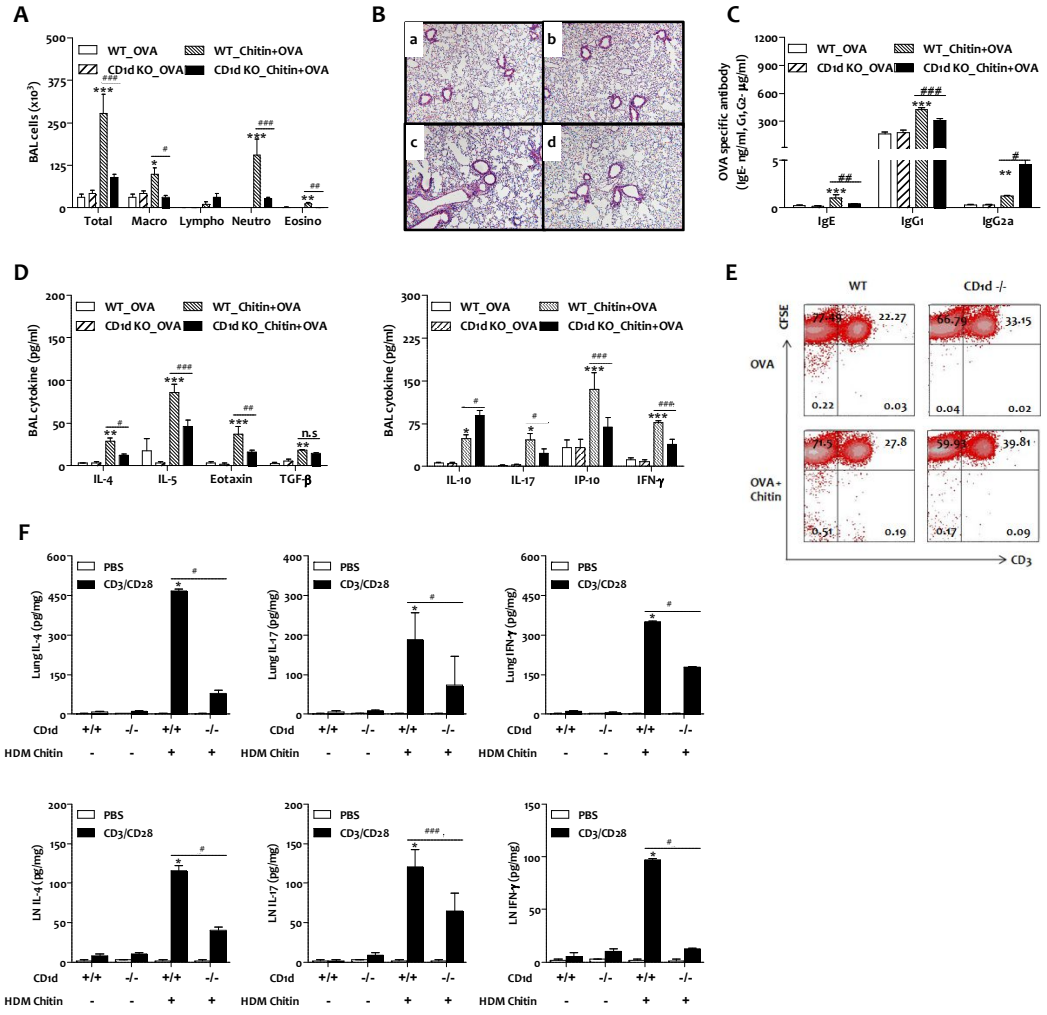
LN IFN-γ (pg/mg)
TNF-α
Chitin

Fig. 8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CD1d KO_OVA
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BAL cells (x10³)
Total
Macro
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b
c
d
C
OVA specific antibody (IgE- ng/ml, G1, G2- µg/ml)
IgE
IgG1
IgG2a
D
BAL cytokine (pg/ml)
IL-4
IL-5
Eotaxin
TGF-β
IL-10
IL-17
IP-10
IFN-γ
n.s
E
WT
CD1d -/-
CFSE
OVA
OVA+ Chitin
CD3
77.48
22.27
0.22
0.03
66.76
33.15
0.04
0.02
71.5
27.8
0.51
0.19
59.93
39.81
0.17
0.09
F
PBS
CD3/CD28
Lung IL-4 (pg/mg)
Lung IL-17 (pg/mg)
Lung IFN-γ (pg/mg)
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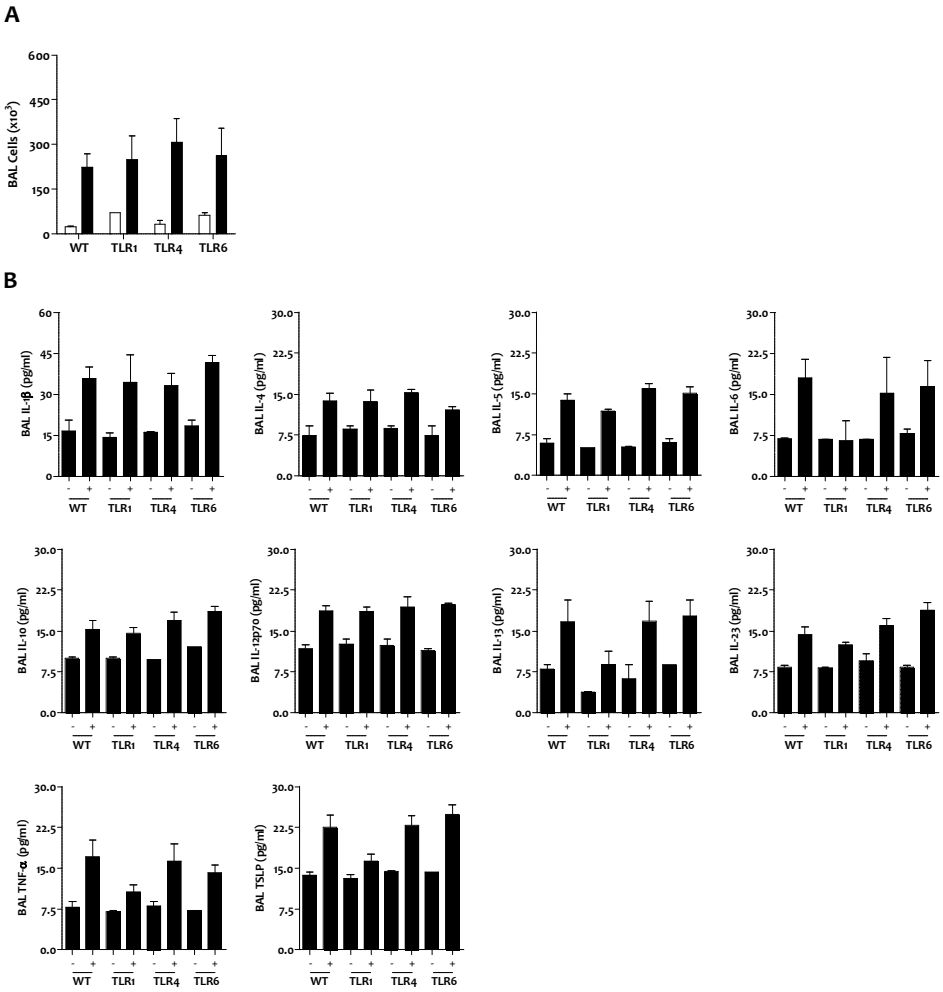
LN IL-17 (pg/mg)
LN IFN-γ (pg/mg)
CD1d
HDM Chitin

Suppl. Fig. 1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600
450
300
150
0
WT
TLR1
TLR4
TLR6
B
BAL IL-1β (pg/ml)
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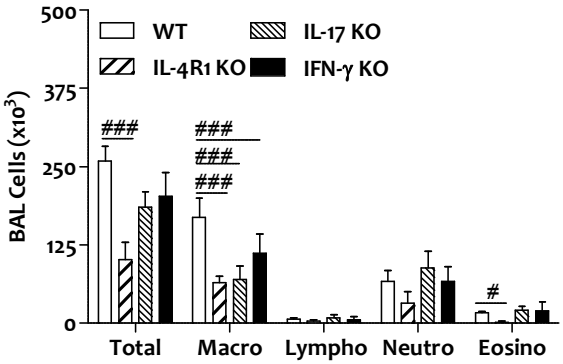
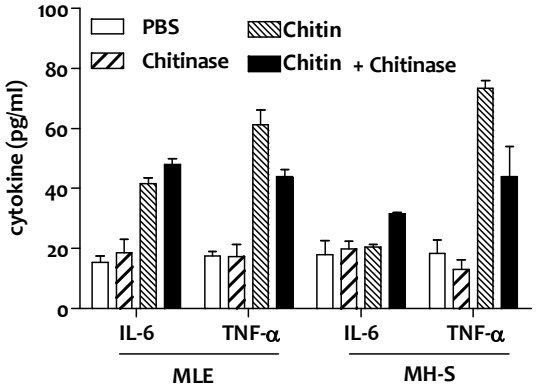
BAL IL-5 (pg/ml)
BAL IL-6 (pg/ml)
BAL IL-10 (pg/ml)
BAL IL-12p70 (pg/ml)
BAL IL-13 (pg/ml)
BAL IL-23 (pg/ml)
BAL TNF-α (pg/ml)
BAL TSLP (pg/ml)

Suppl. Fig. 2

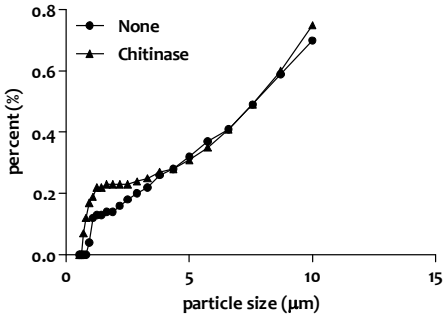

500
375
250
125
0
BAL Cells (x10³)
WT
IL-17 KO
IL-4R1 KO
IFN-γ KO
###
###
###
###
#
Total
Macro
Lympho
Neutro
Eosino

Suppl. Fig. 3

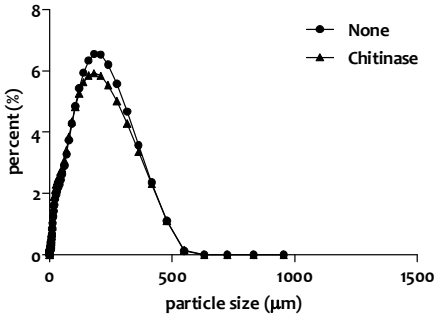

100
80
60
40
20
0
cytokine (pg/ml)
PBS
Chitinase
Chitin
Chitin + Chitinase
IL-6
TNF-α
IL-6
TNF-α
MLE
MH-S

Suppl. Fig. 4

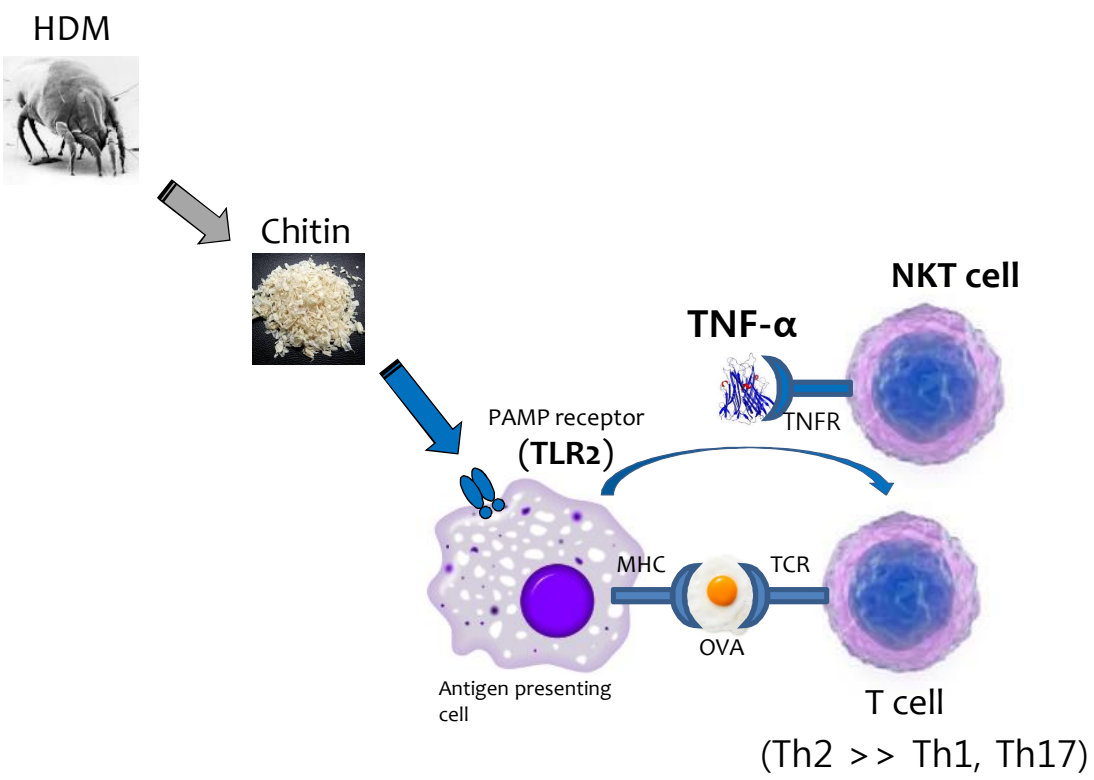
A



B



Suppl. Fig. 5


HDM
Chitin
NKT cell
TNF-α
TNFR
PAMP receptor
(TLR2)
MHC
TCR
OVA
Antigen presenting cell
T cell
(Th2 >> Th1, Th17)

국문 초록

서론: 집먼지진드기는 가장 흔한 흡입항원으로서 종종 다른 흡입항원에 대한 알레르기 반응을 촉진시키기도 한다. 집먼지진드기 외골격의 주성분은 키틴(chitin)으로서, 자연계에서 2번째로 흔한 다당질(polysaccharide)이다. 최근 키틴은 톨유사수용체2(toll-like receptor 2: TLR2)를 통해 Th1, Th2, Th17 적응면역반응을 일으키는 것으로 알려졌다. 또한 이전 연구에서 종양괴사인자알파(tumor necrosis factor-alpha: TNF-α)와 자연살해T세포(natural killer T cell: NKT cell)는 흡입항원에 대한 Th2 면역반응에서 중요한 역할을 하는 것으로 보고되었다. 이번 연구에서는 집먼지진드기가 다른 흡입항원에 대한 적응면역반응을 촉진시키는 것은 그 구성성분이면서 다양한 면역반응을 일으키는 면역보강제인 키틴 때문이라는 가설을 검증하고자 하였으며, 이를 위해 감작 단계에서 난알부민과 함께 집먼지진드기 혹은 집먼지진드기유래키틴(HDM-derived chitin)을 마우스에게 투여하였다. 또한 톨유사수용체2, 종양괴사인자알파, CD1d가 결여된 마우스를 이용하여 톨유사수용체2, 종양괴사인자알파, 자연살해T세포의 역할을 이러한 마우스 모델에서 각각 평가하고자 하였다.

방법: C57BL/6 배경의 야생형 마우스와 톨유사수용체2, 종양괴사인

자알파, CD1d가 각각 결여된 마우스를 난알부민 75 ㎍과 함께 집먼지진드기 혹은 집먼지진드기유래키틴(HDM-derived chitin) 100 ㎍으로 감작시킨 후 난알부민 50 ㎍으로 유발시켰다. 또한, 야생형 마우스에서는 난알부민 75 ㎍과 함께 키틴분해효소(chitinase)로 처리한 집먼지진드기 혹은 집먼지진드기유래키틴 100 ㎍으로 감작을 유도하는 실험을 추가적으로 시행하였다.

결과: 집먼지진드기와 집먼지진드기유래키틴은 기도염증과 함께 Th1, Th2, Th17 면역반응의 모든 싸이토카인의 발현과 혈청내 난알부민 특이 IgE, IgG1, IgG2a 항체의 농도를 증가시켰다. TLR2가 결여된 마우스에서는 모든 면역반응과 함께 혈청내 난알부민 특이 IgE, IgG1, IgG2a 항체의 농도가 감소하였다. 종양괴사인자알파가 결여되거나 키틴분해효소를 처리한 경우에는 Th2 싸이토카인의 발현과 혈청내 난알부민 특이 IgE 항체의 농도는 감소하는 반면에 Th1, Th17 싸이토카인의 발현과 혈청내 난알부민 특이 IgG, IgG2a 항체의 농도는 유지되거나 더욱 증가하였다. CD1d가 결여된 마우스에서는 면역조절세포 반응과 관련된 것으로 알려진 IL-10와 난알부민 특이 IgG2a 항체를 제외하고, 모든 면역반응과 함께 혈청내 난알부민 특이 항체의 농도가 감소하였다.

결론: 집먼지진드기가 다른 흡입항원에 대한 적응면역반응을 촉진시

키는 것은 그 구성성분이면서 광범위한 면역보강제인 키틴을 통해서 이루어진다. 키틴은 톨유사수용체2, 종양괴사인자알파, 그리고 자연살해T세포를 통해 집먼지진드기와 함께 흡입하는 다른 항원에 대한 Th1, Th2, Th17 면역반응을 모두 일으킨다.

주요어: 집먼지진드기, 키틴, 면역보강제, 톨유사수용체2, 종양괴사인자알파, 자연살해T세포

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