The role of Chitotriosidase in Regulation of Allergen-induced Airway Inflammation

Jung Yeon Hong

Department of Medical Science

The Graduate School, Yonsei University

The role of Chitotriosidase in Regulation of Allergen-induced Airway Inflammation

Directed by Professor Myung Hyun Sohn

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This certifies that the Doctoral Dissertation of Jung Yeon Hong is approved.

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Thesis Supervisor: Myung Hyun Sohn

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Thesis Committee Member#1: Jae Myun Lee

_____

Thesis Committee Member#2: Young Sam Kim

_____

Thesis Committee Member#3: Ho-Geun Yoon

_____

Thesis Committee Member#4: Chul Hoon Kim

The Graduate School

Yonsei University

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ABSTRACT

The role of Chitotriosidase in Regulation of Allergen-induced Airway Inflammation

Jung Yeon Hong

Department of Medical Science

The Graduate School, Yonsei University

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The mammalian true chitinase, Acidic mammalian chitinase (AMCase) and chitotriosidase (Chitnase 1, Chit1), were known to be important regulators of inflammation and remodeling. Allergic asthma is the immunologic hypersensitive disease which is characterized by eosinophil recruitment, increase of mucus

secretion and airway hyperresponsiveness. During asthma development, Th2 cells modulate pathological symptoms of asthma by secretion of IL-4, IL-5 and IL-13. However, the specific role and mechanism by which Chit1 regulates Th2 responses has not been fully defined.

To explore the contribution of Chit1 during allergic inflammation, wild type (WT) and chitotriosidase deficiency ($Chit1^{-/-}$) mice were subjected to ovalbumin (OVA) or house dust mite (HDM) sensitization and challenge. Bronchoalveolar lavage fluid (BALF) cell number and differential were assessed, cytokines and total and OVA-specific IgE were evaluated using enzyme-linked immunosorbent assay (ELISA) and airway hyperresponsiveness (AHR) to metacholine was measured by with a Flexivent apparatus. The proportion of $CD4^{+}Foxp3^{+}$ cells and $CD4^{+}GATA3^{+}$ cells were determined by flow cytometry.

In OVA-challenged $Chit1^{-/-}$ mice, Th2 cytokine production, eosinophil infiltration, IgE production and AHR were increased compared to WT mice. The levels of TGF-β1 and IL-10 expression were also significantly decreased in the lungs of $Chit1^{-/-}$

mice. The ratio of $CD4^+Foxp3^+/CD4^+GATA3^+$cells and their mRNA level were significantly diminished in $Chit1^{-/-}$ mice compared to WT mice. In naïve mice, the percentage and proliferation of Treg cells were comparable to WT cells. In vitro studies, $Chit1^{-/-}$ $CD4^+CD25^-$T cells upregulated Foxp3 and TGF-β receptors following TGF-β1 and rChit1 stimulation as much as WT $CD4^+CD25^-$ T cells. In addition, $Chit1^{-/-}$ $CD4^+CD25^+$ Treg cell displayed similar suppression ability in APC–stimulated $CD4^+CD25^-$ T cells, as compared with WT $CD4^+CD25^+$ Treg cells.

Collectively, these results indicate that Chit1 play a protective effect against allergen-induced allergic airway inflammation and airway responses potentially through the regulation of $Foxp3^+$Treg cells.

Key words: chitotriosidase, asthma mouse model, $Foxp3^+$ regulatory T cell, TGF-β

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I. INTRODUCTION

Asthma is one of the most common chronic diseases in the world, affecting people of all ages and ethnic backgrounds.[1] It is characterized by airway hyperresponsiveness, chronic pulmonary eosinophilia, elevated serum IgE, excessive mucus production, and airway remodeling.[2] In asthmatics, $CD4^+$ Tcells producing IL-4, IL-5 and IL-13 have been identified in BALF and airway biopsies.

After antigen challenge in allergic asthmatics, these cytokines and Th2 lymphocytes are increase in the airway. Because Th2 cytokines are required for the development of airway eosinophilia and IgE, it has been proposed that Th2-cells stimulate an inflammatory response that results in asthma.[3,4] However, mechanisms underlying the pathogenesis of asthma are not fully understood.

Th2-cell driven inflammation is likely to represent an abnormal response to allergens. These reactions are normally suppressed by regulatory T cells (Treg), which are essential in the maintenance of immunological tolerance and represent a major pathway proposed to contribute to the maintenance of immune homeostasis in the airways. The forkhead box P3 (Foxp3), which is encodes a transcription factor, has been shown to be not only specifically expressed in professional Treg, but also required for their development.[5] $CD4^{+}Foxp3^{+}$ Treg consist of two indistinguishable subsets induced in either the thymus (called natural Tregs or nTregs) or the periphery (called induced Tregs or iTregs).[6] In mouse, $CD4^{+}CD25^{-}Foxp3^{-}$ cells can be induced to express Foxp3 when activated in vitro

by TCR stimulation in the presence of TGFβ appear to resemble nTregs in all their phenotypic and functional properties. They exert potent suppressor function in vitro and can prevent or control disease in vivo.[7]

Chitinases are a pivotal component of the host response to chitinous organism.[8] Mammals encode two functional chitinases, chitotriosidase (Chit1, chitinase 1) and acidic mammalian chitinase (AMCase).[9] In recent years, there are mounting evidences demonstrating that mammalian chitinases may play a key role in mediating the T-helper 2 cell-driven inflammatory response.[10,11] Chit1 is the most readily appreciated true chitinase in mammals and man.[1,12,13] Recently, Vicencio et al[14] studied that severe asthma children with fungal sensitization were carried a mutation in CHIT1 and CHIT1 mutations may increase the risk of pulmonary fungal infection or colonization, thereby facilitating sensitization and complicating lung disease. Animal experimentation has also demonstrated significant changes in Chit1 expression at sites of inflammation, remodeling, and fibrosis.[15,16] However, the roles of Chit1 in the pathogenesis of tissue inflammation in asthma are

unknown. Here, we showed that chit1 is up regulated in the lungs and $GATA3^+$ T cells of allergen-induced mice compared to control animals.

In this present study, $Chit1^{-/-}$ leads to increased Th2 polarization shown by elevated concentrations of IL-4, IL-5 and IL-13 and increased the ratio of $GATA3^+/Foxp3^+$ T cells in the lung in a murine model of allergic asthma. Furthermore, the addition of recombinant TGF-β1 and/or Chit1 induced the $Foxp3^+$ T cells in vitro, providing insight into the function of Chit1 in allergic asthma. Our studies also demonstrate that Chit1 concentration is increased in the pediatric patients with asthma.

II. MATERIALS AND MEHTODS

1. Animals

All experiments used 7-13-wk-old, sex- and age-matched mice that were housed under specific pathogen-free conditions. Chitotriosidase null mutant (Chit1$^{-/-}$) mice were a generous gift from Jack Elias (Brown University, RI, USA) and generated on C57BL/6 background as previously described.[17] OT-II mice were purchased from Jackson Laboratories (Ba Harbor, ME, USA). All animal experiments were performed in compliance with Korea Research Institute of Bioscience and Biotechnology and approved by the institutional review boards of Yonsei University College of Medicine Council of Science and Technology.

2. Ovalbumin and house dust mite challenge

Specific-pathogen-free female wild type (WT) and Chit1$^{-/-}$ mice were sensitized by intraperitoneal injection (i.p) of 20 μg OVA (Sigma-Aldrich, St. Louis, MO, USA), with 2 mg aluminum hydroxide (Pierce, Thermo scientific, Rockford, IL) in 200 μl,

on days 1 and 14. The mice underwent intranasal (i.n) challenges with 1% OVA in PBS or PBS alone (control mice) on days 28-30. They were sacrificed 1 day after the final challenge and their lungs were evaluated.

Mice were immunized with two i.p of 10 μg house dust mite (HDM) (Greer Laboratories, Lenoir, NC) with 2 mg aluminum hydroxide in 200 μl, on days 1 and 5. Starting on day 12, mice were challenged by i.n with 50 μg HDM in PBS or PBS alone (control mice) daily for 3 day.

3. Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid

After last challenge, lung was lavaged with 2 X 0.9 mL PBS. The lavage fluids were centrifuged, bronchoalveolar lavage (BAL) fluid cells isolated, and the supernatant frozen for later analysis. BAL cell pellets were resuspended in PBS for total and differential cell counts. Total BAL leukocyte counts were determined for each mouse via light microscopy using trypan blue exclusion. BAL cells were centrifuged onto slides using a Cytospin centrifuge (Thermo scientifics).

Differential cell counts were determined by Diff-Quick® staining reagent (Merck, Germany) of air-dried and fixed cytospin slides.

4. Measurement of released cytokines

The concentrations of interleukin (IL) - 4, 5 and 13 in BAL fluid were measured by ELISA (R&D Systems, Minneapolis, MN) as manufacturer's instructions. Briefly, ELISA plates were coated with purified anti-cytokine antibodies and blocked with 1% BSA-PBS for 1h. Samples and dilution standards were loaded and incubated for 2 hr at room temperature. The bounded cytokine was detected with anti-mouse-cytokine antibodies for 2 hr, followed by streptavidin-HRP for 20 min. The plates were developed by addition of the substrate a tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) and stopped with 2N Sulfuric acid. Plates were read at 450nm.

5. Measurement of total IgE and OVA-specific IgE

Blood was collected by cardiac puncture. Serum total IgE level was measured using mouse IgE ELISA assay kit (BD bioscience, San Diego, CA) according to the manufacturer's instructions. Microplates were coated with anti–IgE antibody in coating buffer. Serum samples and diluted standards were added and incubated for 2 hr at room temperature. After washing, detection antibody and SAv-HRP regent were applied on to plate for 2 hr at room temperature. The reaction was developed with a TMB (KPL) and stopped with 2N Sulfuric acid. Plates were read at 450nm.

6. Lung fixation and histology

Lung sections were stained with hematoxyline and eosin (H&E). Birefly, left lung was fixed with 10% buffered formalin, embedded in paraffin and cut into 5-μm sections for H&E staining to assess airway inflammation respectively. Microphotographs were taken by Nikon microscope (Elipes 90i) with camera.

7. Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) to methacholine (Mch; Sigma-Aldrich) was measured by animal ventilator (Flexivent; SCIREQ, Montreal, QC, Canada). Anaesthetized and tracheotomized mice were challenged with aerosolized saline or Mch in increasing concentrations (3.125 - 50 mg/ml). Lung resistance (Rn) was calculated from measures of pressure and flow and expressed as cm/H_20/ml/s.

8. Preparation of Leukocyte

Spleen, lymph nodes (LN), thymus were removed from mice, and single-cell suspensions were prepared.

Lungs were minced finely. The tissues were then transferred into digestion solution (PBS, containing 0.75 mg/ml type I collagenase and 2 mg/ml DNase I; roche, Freehold, NJ) and were incubated for 1 hr at 37 °C with agitation. Digested tissue were transferred to and ground through a cell strainer using a syringe plunger. Red blood cells were lysed using ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$,

0.1mM Na_2EDTA, pH 7.2-7.4). Cells were centrifuged (5 min, 4℃, 300g) and the cell pellets were washed with RPMI-1640-10% FBS medium and counted.

The spleens (SPL) and mediastinal lymph nodes (mLN) were transferred into cell strainers; the cells were passed through the strainer using plungers, then the cells were washed and resuspended in fresh medium. The red blood cells were lysed using ACK lysis buffer for 5 min. The cells were washed again and the cell numbers were determined as above.

9. Isolation of CD4+ T cell

A. Isolation of $CD3^+$ T cells using AutoMACS

The single cells were washed and then resuspended in MACS buffer (PBS, pH=7.2, 5 % BSA, 2 mM EDTA) per 10^7 total cells, then CD3ε-specific antibody (Miltenyi Biotec, Auburn, CA) per were added to the suspension, which was then mixed and incubated for 15 min at 4°C. The cells were then washed with 1 ml of MACS buffer and centrifuged at 300 x g for 10 min and the cells resuspended in MACS buffer,

and then anti- microbeads (Miltenyi Biotec) were added to the suspension for 10 min at 4°C. The cells were then washed with MACS buffer and centrifuged and the cells resuspended in 500 μl of MACS buffer. The cell suspension was applied onto the AutoMACS (Miltenyi Biotec). The marker negative- and positive- cells were collected.

B. Isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells using FACS Aria II

To sort $CD4^+CD25^+$ T cells, $CD3^+$cells were resuspened in FACS buffer and stained with anti-CD25-APC (eBioscience), anti-CD4-FITC (eBioscience), then incubated for 30 min at 4 ℃. After washing cells with FACS buffer (5% FBS in PBS) and sorted on a BD Biosciences FACSAria II (BD Biosciences, San Jose, CA).

10. Flow cytometry

Single-cell suspensions (1 X 10^6 to 2 X 10^6 cells) were resuspended in 100 μl of FACS buffer (0.5% FBS in PBS) and blocked with anti-CD16/32 (San Diego, CA,

BioLegend, USA) and simultaneously stained with optimal concentrations of mAbs specific for CD4 (clone RM4.5), CD8 (clone 53-6.7), CD25 (clone PC61.5), CD44 (clone IM7), CD62L (clone MEL-14), and CD103 (clone M290). All mAbs were obtained from eBioscience (San Diego, CA, USA). Cells were stained for 30 min at 4˚C, washed twice with cold staining buffer (PBS, 2% FCS, and 0.02% sodium azide). Samples were run on a FACS LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

For intracellular staining, cells were washed twice with PBS and centrifuged to remove the supernatant, then treated with RPMI-1640 medium containing 10% FBS and 50 ng/mL PMA (Sigma-Aldrich) and 500 ng/ mL ionomycin (Sigma-Aldrich), and incubated in a 5% CO^2 incubator at 37°C for 4 hr. Incubated cells were then collected, centrifuged and incubated in the dark at 4℃ for 30 min with anti-mouse surface antibodies. 1 ml of freshly prepared Foxp3 Staining Kit (eBioscience) was added to each sample, pulse vortexed, and incubated at 4℃ for 1 hr in the dark. Cells were washed twice with 2 ml of permeabilization buffer. Foxp3 (clone FJK-

16s) and GATA3 (clone L50-823) were added in 100 μl of permeabilization buffer, incubated at 4℃ for 45 min in the dark, and then washed twice with 2 ml of permeabilization buffer. Samples were run on a FACS LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

11. *In vitro* Induction of Foxp3+ Treg

Splenic $CD4^{+}CD25^{-}CD62L^{hi}CD44^{low}$ T cells were cultured at density of $2X10^{5}$ cell/well in flat bottom 96-well plates precoated with anti-CD3 (2 μl/ml). Cells were cultured for 72 hr with rIL-2 (100 U/ml) in the presence or absence of different concentrations of recombinant human TGF-β1 (R &D systems; 1–10 ng/ml). Foxp3 expression was assessed by flow cytometry and real-time PCR.

For Treg suppression assay, OTII or $Chit1^{-/-}$ OTII Tresps ($5X10^{4}$/well) were cocultured separately with OTII or $Chit1^{-/-}$ OTII Tregs at different ratios, as

indicated, along with mitomycon C (20 μg/ml)-treated APCs ($5X10^4$/well) plus anti-CD3 (2 μg/ml) or 10 μg/ml OVA for 72 hr. Plates were pulsed with CCK-8, and cell proliferation in triplicates was measured at 405 nm using ELISA reader.

12. T cell proliferation

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were labeled with CFSE (2 μM; Invitrogen) for 20 min at 37°C, washed, and then cultured for 72 hr in the presence of 2 μ g anti-CD28, 4 μg anti-CD3 antibody and recombinant IL-2 (10 U/ml; R&D systems). After 5 days, cells were fixed, permeabilized and stained with anti-mouse Foxp3 antibody (eBioscience) and anti-mouse CD4 antibody (eBioscience). T cell proliferation was determined using the CCK-8 (Roche, Heidelberg, Germany).

13. Real-time RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen). First strand cDNA was synthesized using superscript with random hexamer primers (Invitrogen). Real-time

PCR was performed with a Exicycler 96 (Bioneer, Korea), and message levels were quantified using the AccuPower® GreenStar qPCR Master Mix (Bioneer), according to the manufacturer's instructions. Amplification was conducted for 45 cycles. The recovered PCR product and amplicon were checked by agarose gel electrophoresis for a single band of the expected size. The relative expression of Foxp3 was determined by normalizing expression of each target to GAPDH.

14. Cell culture

Splenic $CD4^+CD25^-$ T cells sorted by flow cytometry were used at a density of $1X10^6$ cell/ml in flate bottom 96-well plates precoated with anti-CD3 (2 μg/ml). Cells were cultured for 72 hr with IL-2 (100U/ml) in the presence or absence of different concentrations of recombinant human TGF-β1 and/or mouse chitotriosiase (R&D systems). Foxp3 expression was assessed by flow cytometry and real-time PCR.

15. Subject

A total of 80 children were enrolled in this study. The children were divided into 2 groups, atopic asthma and controls. Asthma was defined as bronchial hyperresponsiveness (BHR) in the methacholine challenge (PC_{20} ≤16 mg/ml) or at least 12% reversibility of forced expiratory volume in 1 s (FEV_1) after inhalation of β_2 agonist accompanied by any typical asthma symptoms. Among the 40 asthma patients, 17 were mild persistent asthma and 23 were moderate-to-severe persistent asthma [18]. Children treated with systemic corticosteroids due to asthma exacerbation in the preceding month were excluded from the study. The control group visited the hospital for general health workup or vaccination. And they had no history of chronic diseases, recurrent wheezing, or respiratory infection for at least 4 weeks prior to the evaluation. Total serum immunoglobulin E (IgE) levels, peripheral blood eosinophil count, and eosinophil cationic protein (ECP) levels were determined at the initiation of the evaluations. A specific IgE test was performed

with 6 allergens common in Korea: Dermatophagoides pteronyssinus, Dermatophagoides farina, egg whites, cow milk, German cockroach, and Alternaria alternata. Atopy was defined as more than 0.7 KUa/L specific IgE to more than one allergen, or 150 IU/ml total IgE. Atopy was defined as more than one positive skin test result of 12 common aeroallergens, including 2 types of dust mites, cat and dog epithelium, as well as mold and pollen allergens. This study was approved by the Institutional Review Board of Severance Hospital (Seoul, Korea) (4-2004-0036).

16. Statistical analyses

Data are expressed as mean±SD. significant differences between two groups were estimated using unpaired Stud ent's t-test. Statistical significant was set at $p \leq 0.05$.

III. RESULTS

1. Chit1 regulates OVA-induced airway inflammation and airway hyperresponsiveness

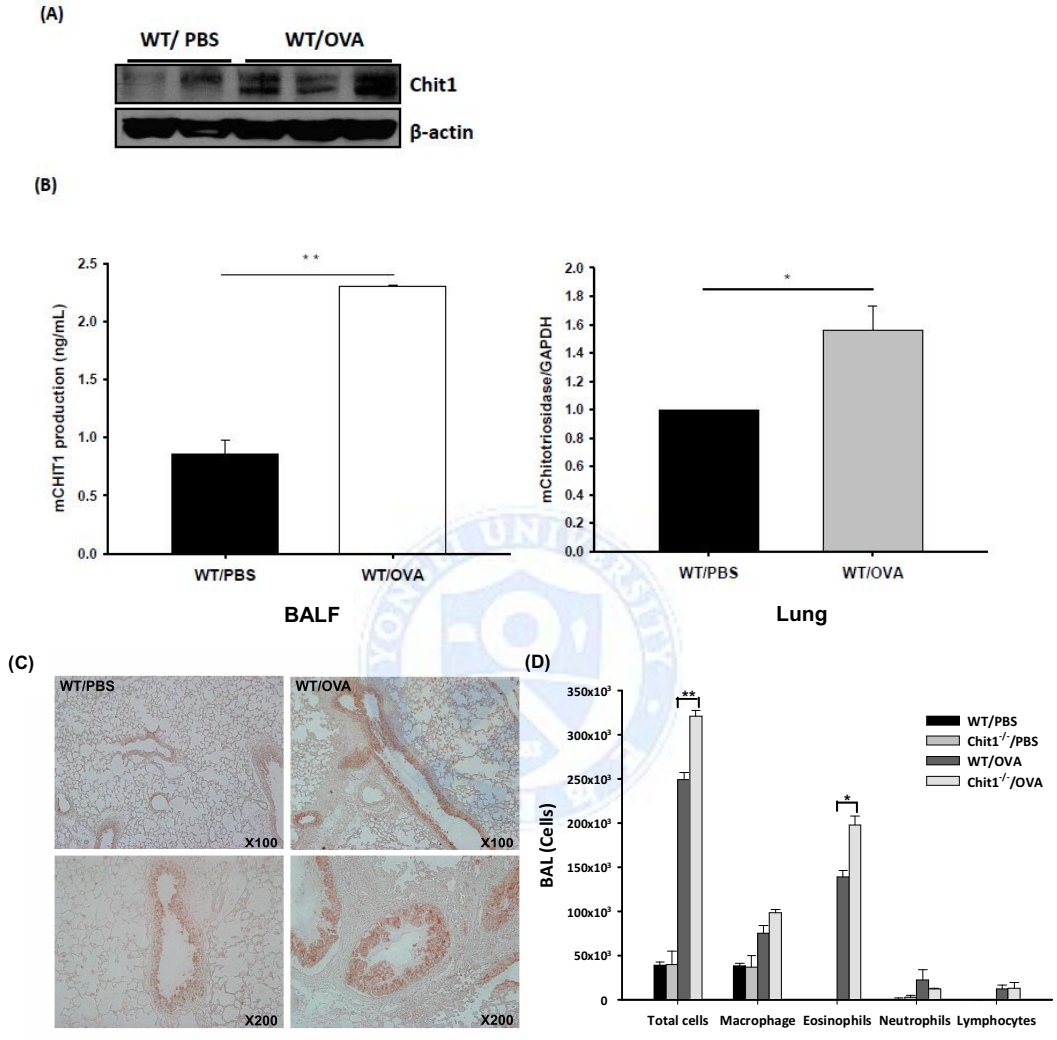
To determine the contribution of the Chit1 in Th2 inflammation in asthma, we compared the expression of Chit1 in lungs from control mice and that had been sensitized and challenged with the chitin-free antigen OVA. These studies demonstrated that the levels of Chit1 mRNA and protein were significantly increased after OVA sensitization and challenge (Fig. 1A and B).

In immunohistochemistry, Chit1 expressed localized predominantly within airway epithelial cells and alveolar macrophages (Fig. 1C). Thus, Chit1 is prominently induced during the course of chitin-free antigen-induced Th2 inflammation.

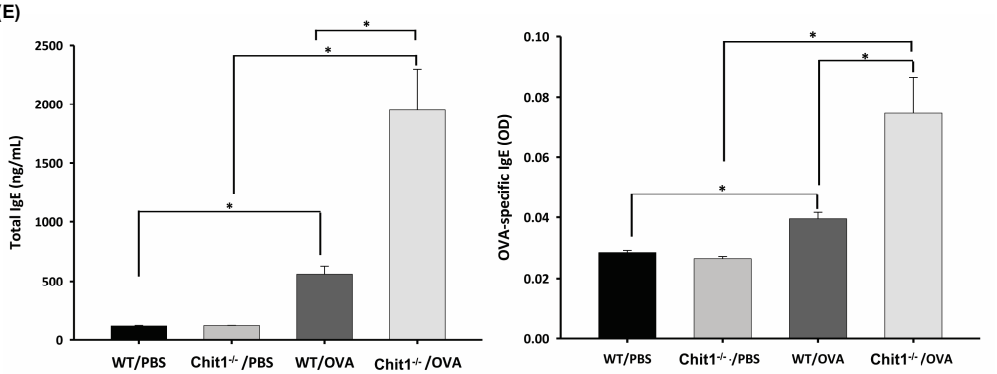
To begin to assess the mechanisms that underlie the Th2 response in Chit null mutant (Chit1 $^{-/-}$) mice, studies were undertaken to determine if Chit1 played a role

in OVA-induced allergen sensitization. In these experiments, WT and Chit1 $^{-/-}$ mice received i.p. OVA plus alum. They were then boosted with antigen and antigen-induced inflammatory cell proliferation, the levels of total and antigen-specific IgE and AHR were assessed.

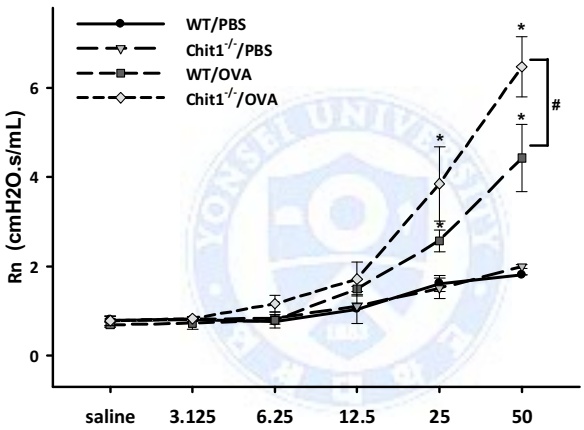
In WT mice, OVA-plus-alum sensitization and challenge caused a significant increase in tissue inflammation and BAL total cell, eosinophil, and lymphocyte recovery, increases in the levels of total and OVA-specific IgE and AHR (Fig. 1D-F) and exhibited increased histological changes, as demonstrated by perivascular and peribronchiolar inflammatory cell infiltration (Fig. 1G) In these experiment systems, null mutations of Chit1 showed a significant increase in each of these parameters compare to WT (Fig. 1D-G).


(A)
WT/ PBS
WT/OVA
Chit1
β-actin
(B)
mCHIT1 production (ng/mL)
WT/PBS
WT/OVA
BALF
mChitotriosidase/GAPDH
WT/PBS
WT/OVA
Lung
(C)
WT/PBS
WT/OVA
X100
X100
X200
X200
(D)
BAL (Cells)
WT/PBS
Chit1-/-/PBS
WT/OVA
Chit1-/-/OVA
Total cells
Macrophage
Eosinophils
Neutrophils
Lymphocytes

(E)


Total IgE (ng/mL)
2500
2000
1500
1000
500
0
WT/PBS
Chit1-/- /PBS
WT/OVA
Chit1-/- /OVA
OVA-specific IgE (OD)
0.10
0.08
0.06
0.04
0.02
0.00
WT/PBS
Chit1-/- /PBS
WT/OVA
Chit1-/- /OVA

(F)


WT/PBS
Chit1-/-/PBS
WT/OVA
Chit1-/-/OVA
Rn (cmH2O.s/mL)
6
4
2
0
saline
3.125
6.25
12.5
25
50
#

(G)

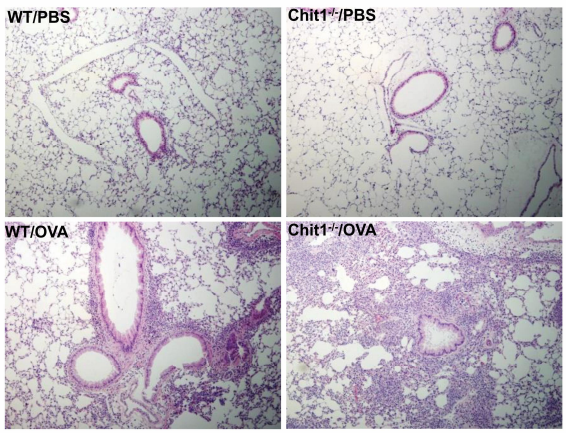
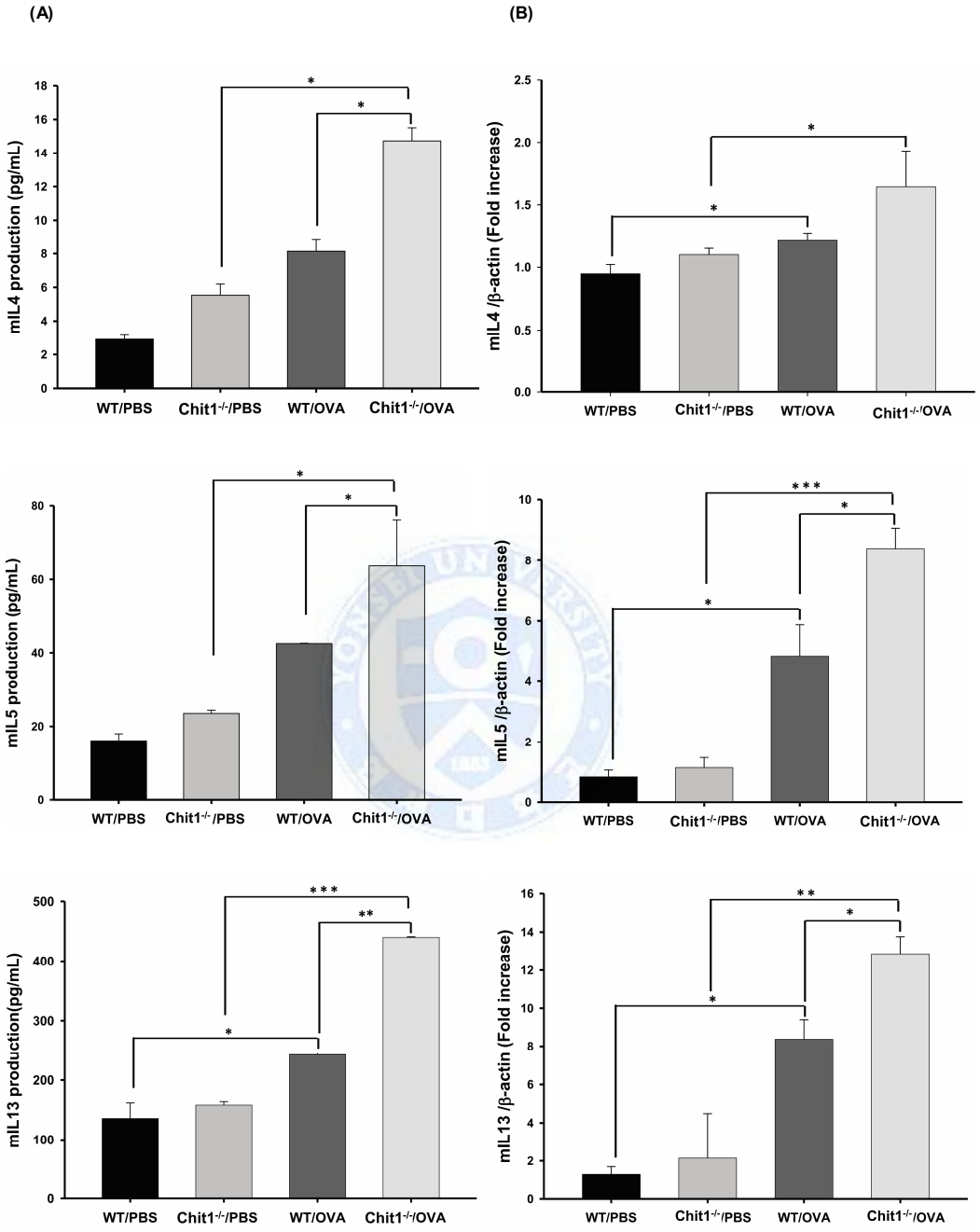

WT/PBS
Chit1-/-/PBS
WT/OVA
Chit1-/-/OVA

Figure 1. Chit1 regulates OVA-induced airway inflammation and airway hperresponsiveness. WT and Chit1$^{-/-}$ mice were sensitized OVA/Alum and challenged with OVA. The level of Chit1 was assessed via western blot (A), ELISA and real-time PCR (B). Immunohistochemistry was used to localize the Chit1 (C). Total cells and inflammatory cell were enumerated in BAL fluid (D). Total and OVA-specific IgE (E) and airway hyperreponsiveness were measured (F), and H&E stain of lung sections (G). Mean data was estimated from triplicated experiments. * $p<0.05$ (control vs. OVA), # $p<0.05$ (WT/OVA vs. Chit1$^{-/-}$/OVA).

2. Chit1 regulates the expression of Th2 cytokines, TGF-β and IL-10 in asthma mouse model

In Chit1$^{-/-}$ mice, OVA-plus-alum sensitization and challenge caused significant increases in inflammatory parameters compare to WT mice. Next, we identified inflammation related cytokines. Higher Th2 cytokine induction was noted in mice with null mutations of Chit1, with lung IL-13 and IL-5 being increased by 1.5~ 1.9 fold (Fig. 2A and B). Importantly, Treg related cytokine TGF-β1 and IL-10 were decreased (Fig. 2C) compare to WT/OVA. When viewed in combination, these studies demonstrate that Chit1 plays a critical role in Th2 inflammation, physiological dysregulation, and Treg related cytokine production.


(A)
(B)
mIL4 production (pg/mL)
mIL4 /β-actin (Fold increase)
mIL5 production (pg/mL)
mIL5 /β-actin (Fold increase)
mIL13 production(pg/mL)
mIL13 /β-actin (Fold increase)
WT/PBS
Chit1-/-/PBS
WT/OVA
Chit1-/-/OVA

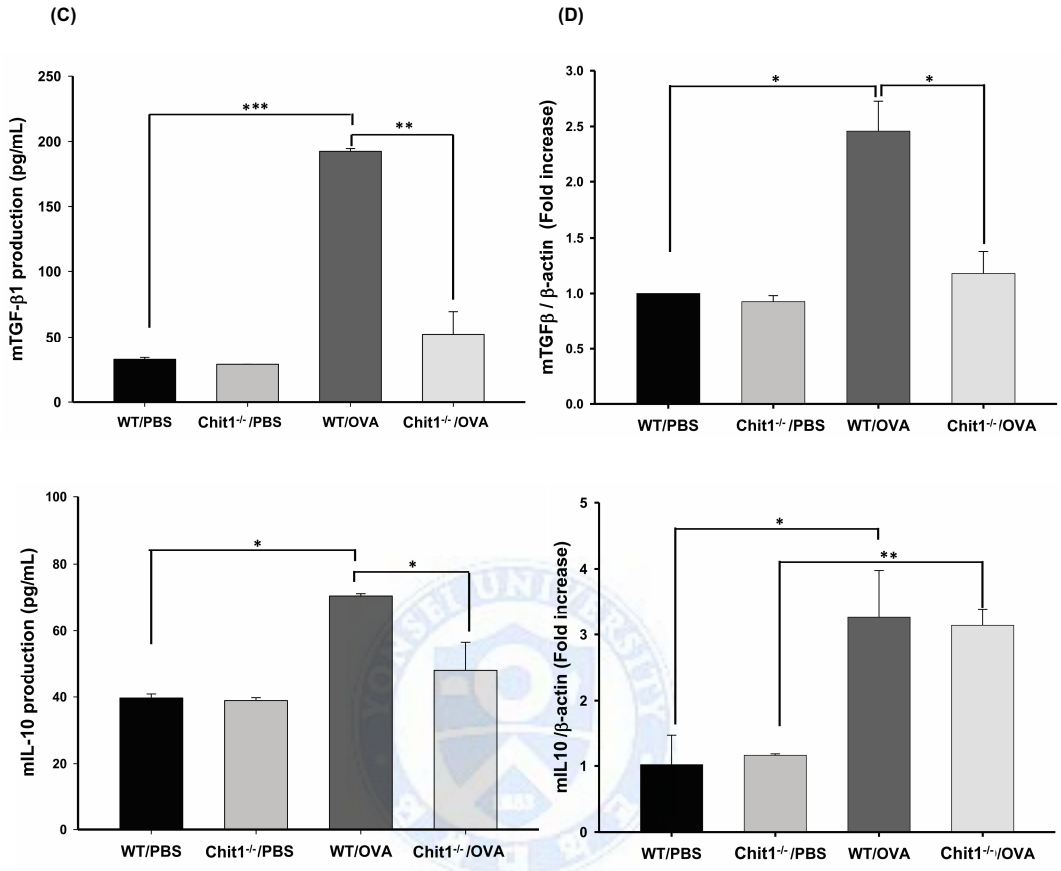

(C)
(D)
mTGF-β1 production (pg/mL)
250
200
150
100
50
0
***
**
WT/PBS
Chit1-/- /PBS
WT/OVA
Chit1-/- /OVA
mTGFβ / β-actin (Fold increase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT/PBS
Chit1-/- /PBS
WT/OVA
Chit1-/- /OVA
mIL-10 production (pg/mL)
100
80
60
40
20
0
*
*
WT/PBS
Chit1-/-/PBS
WT/OVA
Chit1-/- /OVA
mIL10 /β-actin (Fold increase)
5
4
3
2
1
0
*
**
WT/PBS
Chit1-/- /PBS
WT/OVA
Chit1-/-/OVA

Figure 2. Chit1 regulates the expression of Th2 cytokines, TGF-β and IL-10 in asthma mouse model. WT and Chit1$^{-/-}$ mice were sensitized OVA/Alum and challenged with OVA. The level of Chit1 in BAL fluid from PBS- or OVA-challenged mice, were measured by ELISA (A and C). RNA levels of cytokines in the lung quantified by real-time RT-PCR (B and D). Mean data and fold increases were estimated from triplicated experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

3. The absence of Chit1 increased the number of GATA3^{+}Foxp3^{-} T cells in asthma mouse model

To identify whether the exaggerated Th2 response after OVA exposure in Chit1$^{-/-}$ mice was driven an augmented Th2 response by depressed regulatory T (Treg) cell related cytokines that we noted. Th2 differentiation was measured by the induction of GATA3, one of the main transcription factors, which potentiates Th2 responses, whereas Treg differentiation was measured by the induction of FOXP3[6,19]. We next compared the GATA3^{+} effector T cell (Teff) and Foxp3^{+} Treg in madiastinal lymph nodes, spleens and lungs from WT and Chit1$^{-/-}$ mice. In these experiments, the numbers of Teff (CD3^{+}CD4^{+}GATA3^{+}) and Treg (CD3^{+}CD4^{+}Foxp3^{+}) were increased from WT and Chit1$^{-/-}$ mice that had been sensitized and challenged with OVA (Fig. 3A and B). Importantly, the dominant expression of GATA3^{+} occurred in the lung of OVA challenged mice from both WT and Chit1$^{-/-}$. However, in WT mice, the ration of GATA3/Foxp3 was significantly lower than Chit1$^{-/-}$ mice, and that the GATA3^{+}Foxp3^{-} T cell was predominantly expressed in lungs of Chit1$^{-/-}$ OVA

challenged mice. These results indicate that Chit1 is one of the pathways for OVA induced airway inflammation and is closely related to development, expansion and occurrence of $GATA3^{+}$ T cell and Th2 type inflammation.

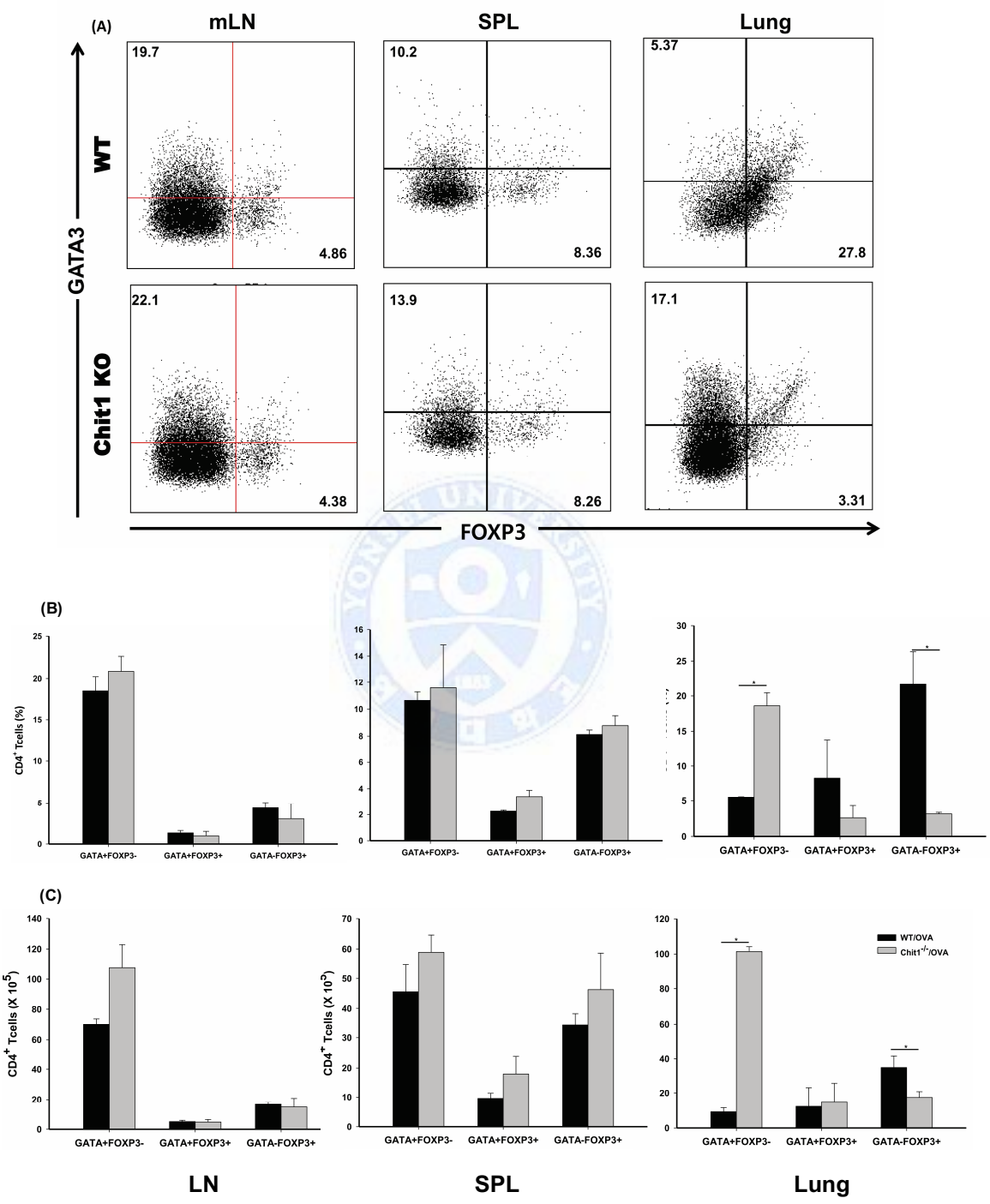

(A)
mLN
SPL
Lung
WT
Chit1 KO
GATA3
FOXP3
19.7
4.86
10.2
8.36
5.37
27.8
22.1
4.38
13.9
8.26
17.1
3.31
(B)
CD4+ Tcells (%)
GATA+FOXP3-
GATA+FOXP3+
GATA-FOXP3+
(C)
CD4+ Tcells (X 10^5)
CD4+ Tcells (X 10^5)
WT/OVA
Chit1-/-/OVA
LN
SPL
Lung

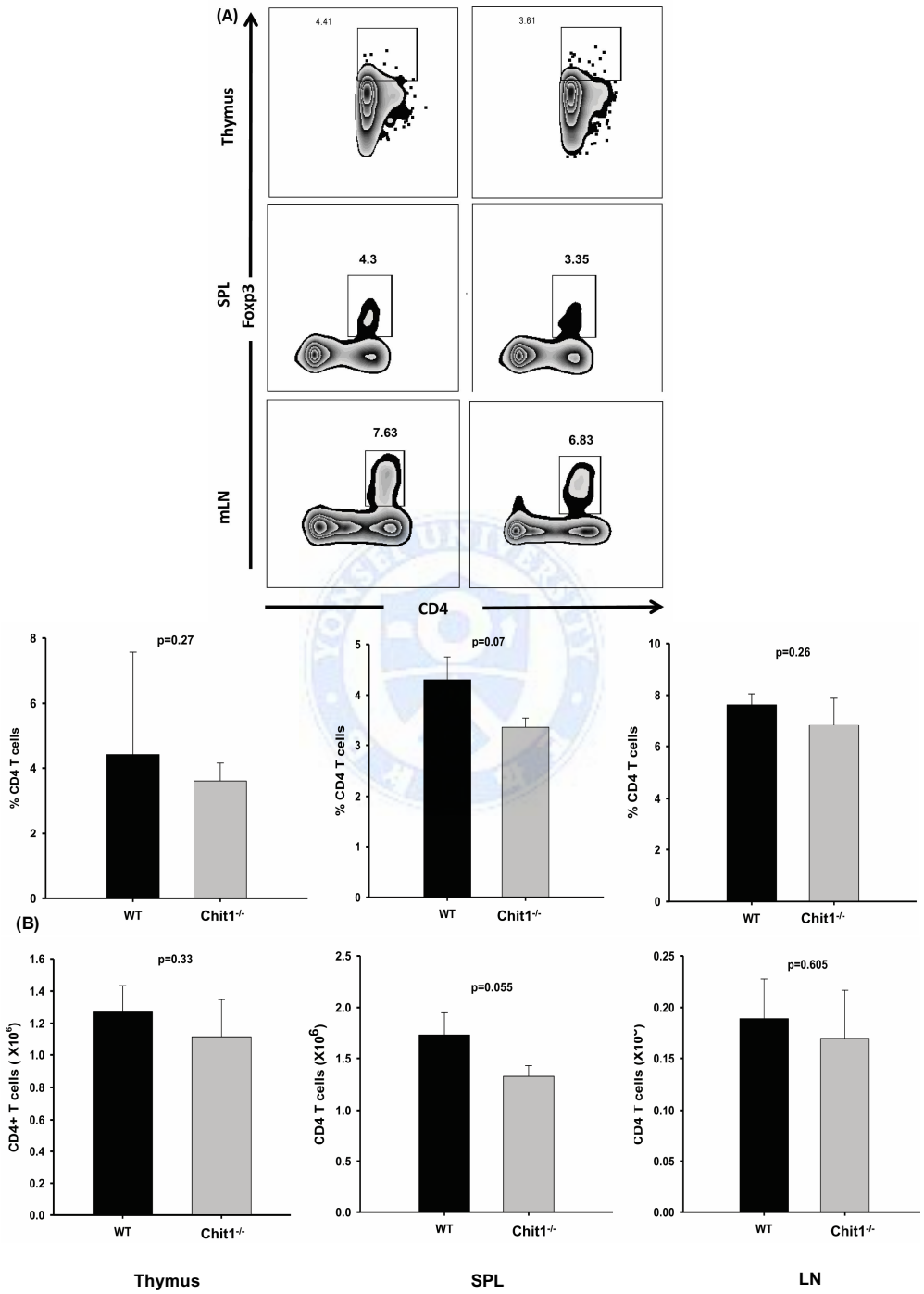
Figure 3. The absence of Chit1 increased the number of GATA3^{+}/Foxp3^{-} T cells in asthma mouse model. (A) After 24 hr of last OVA challenge, cells were isolated from mLN, spleen and lung from WT and Chit1$^{-/-}$ mice, stained with anti-CD4, Foxp3 and GATA3 and analyzed by flow cytometry. (B) Graphs are representing absolute numbers of CD4^{+}GATA3^{+}, CD4^{+}Foxp3^{+} and CD4^{+}GATA3^{+}Foxp3^{+} cells in two groups. Mean frequency was estimated from triplicated experiments. * $p<0.05$.

4. The absence of Chit1 does not affect the cell numbers, Foxp3 expression and proliferation without allergen challenge

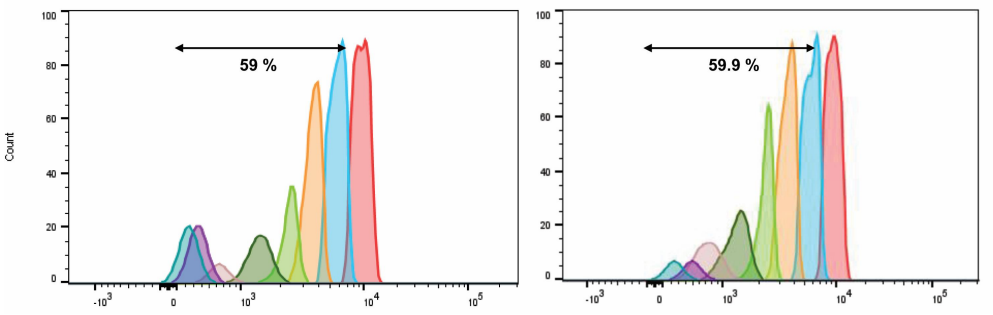
Chit1$^{-/-}$ mice with OVA challenged present with lower CD4^{+}GATA^{-}Foxp3^{+} and CD4^{+}GATA^{+}Foxp3^{+} expression. We therefore investigated whether Chit1 deficiency would affect Foxp+ Treg development in vivo. However, following examination of the thymus, spleen, mLN and other pheripheral LN (axillary and brachial), similar frequencies (Fig. 4A) and absolute numbers (Fig. 4B) of CD4^{+}Foxp3^{+} T cells showed in WT and Chit1$^{-/-}$ mice. This result suggests that Chit1 does not regulate Foxp3^{+} Treg development under steady-state conditions.

Next, we examined whether Chit1 deficiency would affect Treg cell proliferation. Peripheral Tregs were purified by magnetic selection and FACS from WT and Chit1$^{-/-}$ mice, and their ability to proliferate following in vitro TCR stimulation in the presence of exogenous IL-2 was evaluated by CFSE dilution and CCK-8 detection. Both WT and Chit1$^{-/-}$ CD4^{+}CD25^{+} T cells were strongly proliferated (Fig 4C). We then tested the functionality of Chit1$^{-/-}$ Tregs in vitro

suppression of T cell proliferation. Using highly purified $CD4^+CD25^+$ T cells, we found that $Chit1^{-/-}$ Tregs were also effective at suppressing the increased proliferation of WT $CD4^+CD25^-$ (Tresps) as WT Tregs (Fig. 4D). Therefore, Chit1-deficient $Foxp3^+$ Tregs are presented with normal proliferation and suppression ability.


(A)
Thymus
SPL
Foxp3
mLN
4.41
3.61
4.3
3.35
7.63
6.83
CD4
(B)
% CD4 T cells
p=0.27
p=0.07
p=0.26
WT
Chit1-/-
CD4+ T cells (X10^6)
CD4 T cells (X10^6)
CD4 T cells (X10^7)
p=0.33
p=0.055
p=0.605
Thymus
SPL
LN

(C)


Count
59 %
59.9 %

(D)

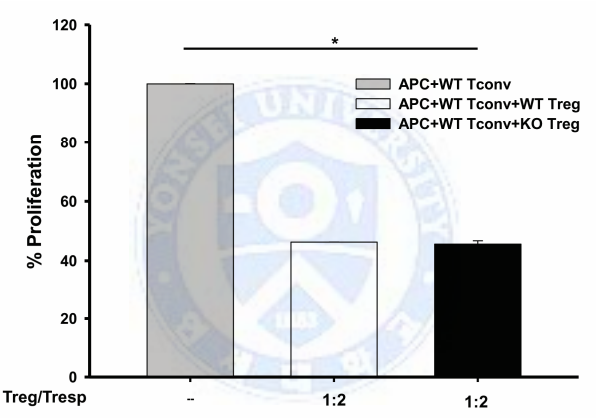

% Proliferation
APC+WT Tconv
APC+WT Tconv+WT Treg
APC+WT Tconv+KO Treg
Treg/Tresp
--
1:2
1:2
*

Figure 4. The absence of Chit1 does not affect the cell numbers, Foxp3 expression and proliferation without allergen challenge. (A) Cells were isolated from thymus, spleen and LN from WT and Chit1$^{-/-}$ mice, stained with CD4 and Foxp3 and analyzed. (B) Graphs representing absolute numbers of total cells in age- and sex- matched mice WT and Chit1$^{-/-}$ mice. (C) Purified CD4^{+} CD25^{+} cells were labeled with CFSE and stimulated with plate bounded anti-CD3/CD28 in the presence of IL-2. After 72 hr, Treg proliferation was evaluated by CFSE dilution. (D) Suppression of T cell-proliferative response by CD4^{+}CD25^{+} T cells sorted from WT and Chit1$^{-/-}$ mice following co-culture with WT CD4^{+}CD25^{-} (Tresps) cells. Proliferation was assessed as CCK-8 incorporation. Proliferations are representative of at least three independent experiments. * $p<0.05$.

5. TGF-β or Chit1 stimulation enhance the differentiation of Foxp3^{+} iTreg cells in a dose dependent way.

Because OVA-challenged Chit1deficiency mice impaired in Foxp3 induction, we hypothesized that Chit1 might be important for Treg induction, and we therefore examined whether Chit1 deficiency would affect Foxp3 induction in CD4^{+}T cells. Purified naïve reponse (CD4^{+}CD25^{-}CD62L^{hi}CD44^{low}) T cells were stimulated with TGF-β1 and Foxp3 expression levels examined after 4 day in culture. It was interest to investigate whether Chit1 regulated the development and/or Foxp3^{+} T cells. Following 3 day of culture with TGF-β1 only, the frequency of CD4^{+}Foxp3^{+} Treg cells were significantly lower in the absence of Chit1 when compared with WT (Fig. 5). We consequently examined the Chit1 effect on Foxp3^{+} Treg of WT and Chit1$^{-/-}$ CD4^{+}CD25^{-}T cells stimulated with TGF-β1 and/or Chit1 following 4 day of culture. Stimulation of CD4^{+}CD25^{-}T with Chit1 led to Foxp3^{+} Treg cells. However, significantly higher frequency of Foxp3+ Treg cell was detected in the presence of both TGF-β1 and Chit1, as compared with TGF-β1 stimulation only. Otherwise

stimulation of Chit1 only did not induced Foxp3^{+} Treg and did not showed synergistic effect on iTreg induction in WT (Fig. 5A and B). TGF-β1 or Chit1 treatment induced TGF-β II receptor expression but both TGF-β1 and Chit1 treatment were showed highest mRNA expression (Fig. 5D). These data suggested that Chit1 deficiency impaired that development of CD4^{+}Foxp3^{+} Treg cells in the periphery.

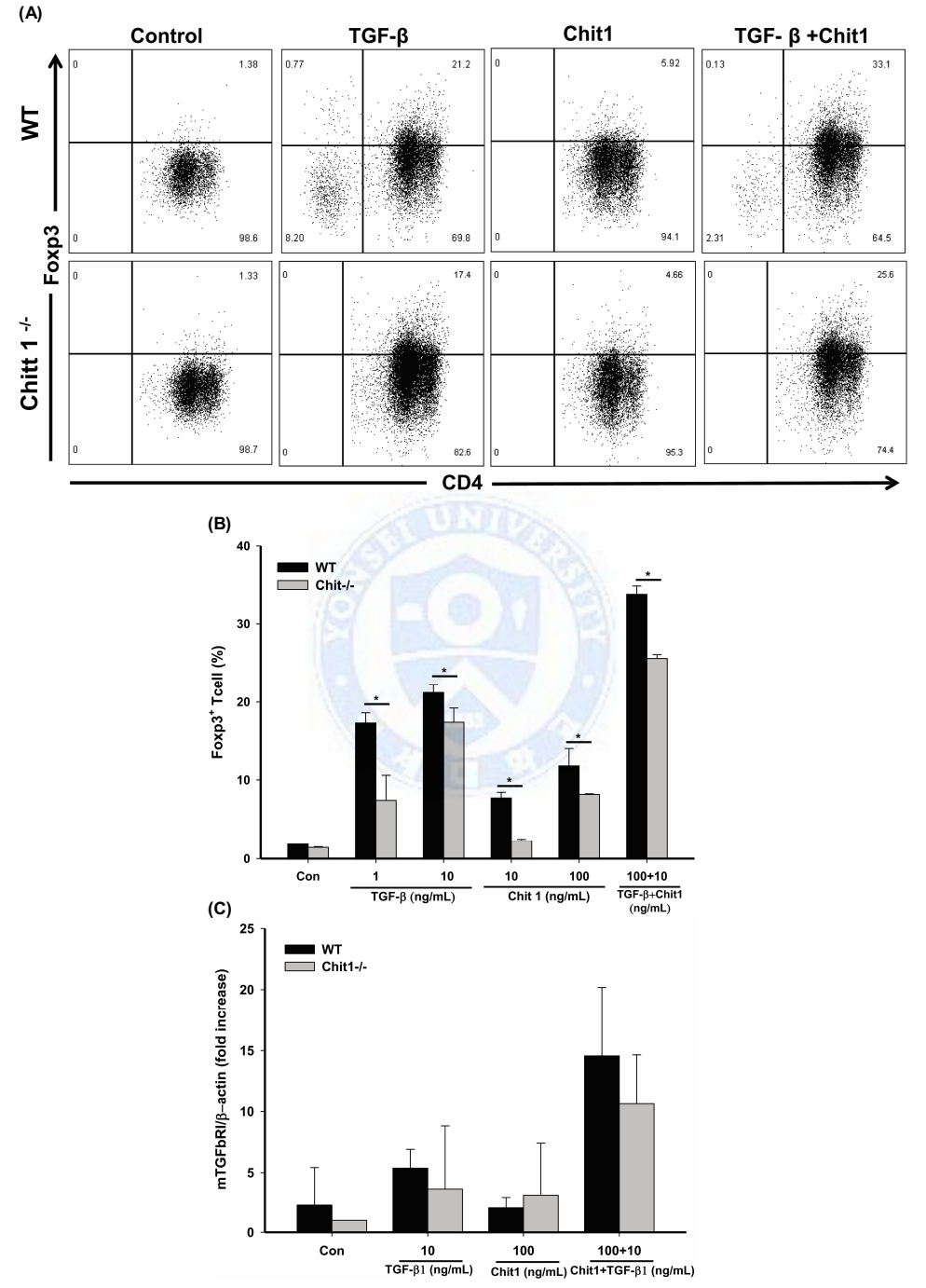
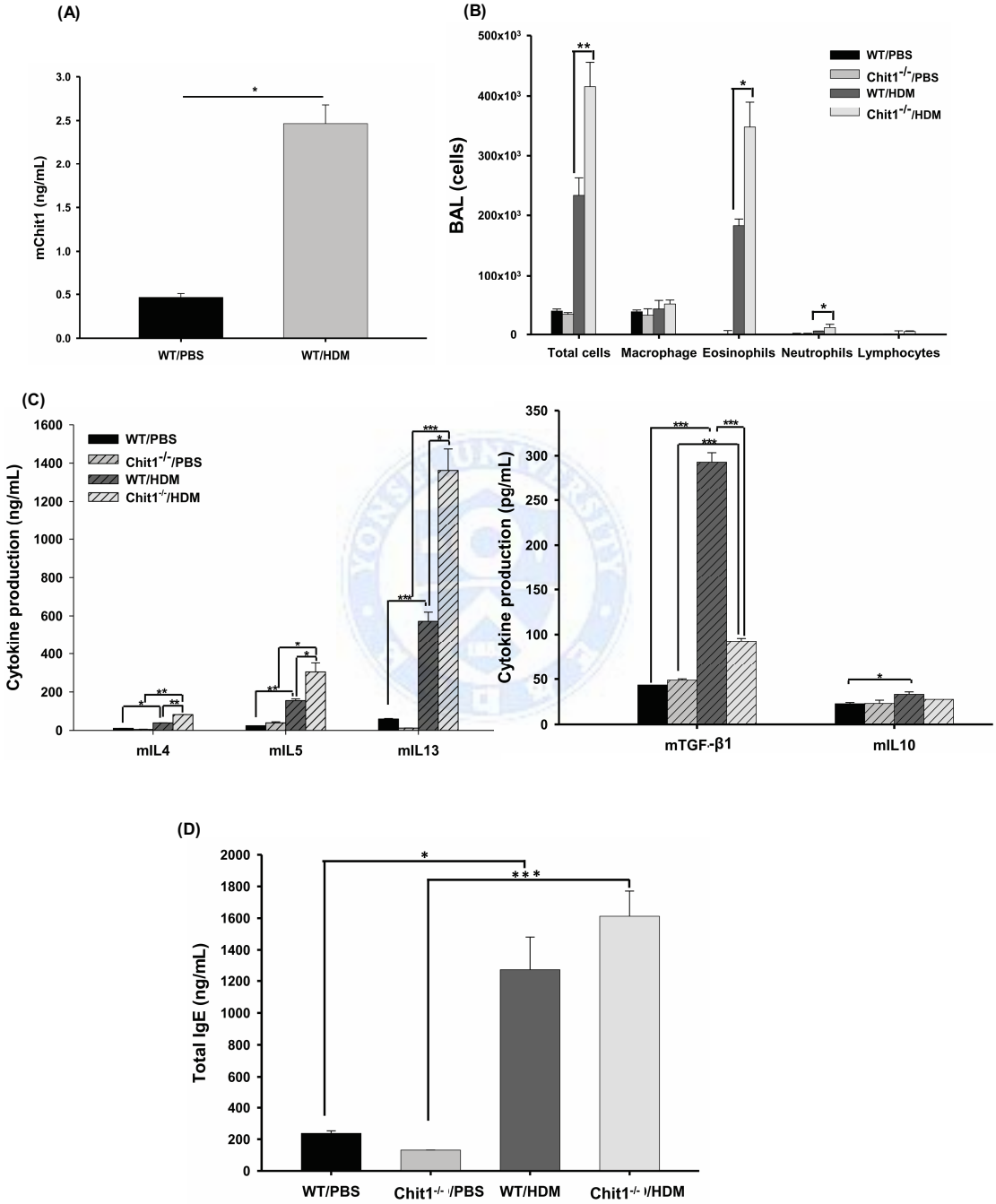

(A)
Control
TGF-β
Chit1
TGF- β +Chit1
WT
Chitt 1 -/-
Foxp3
CD4
1.38
98.6
21.2
69.8
5.92
94.1
33.1
64.5
1.33
98.7
17.4
82.6
4.66
95.3
25.6
74.4
(B)
WT
Chit-/-
Foxp3+ Tcell (%)
Con
TGF-β (ng/mL)
Chit 1 (ng/mL)
TGF-β+Chit1 (ng/mL)
(C)
WT
Chit1-/-
mTGFbRI/β−actin (fold increase)
Con
TGF-β1 (ng/mL)
Chit1 (ng/mL)
Chit1+TGF-β1 (ng/mL)

Figure 5. TGF-β1 or Chit1 stimulation enhance the differentiation of Foxp3^{+} iTreg cells in a dose dependent way. CD4^{+}CD25^{-} cells were isolated from spleen from WT and Chit1$^{-/-}$ mice. The cells were then stimulated with plate bound-anti CD3 and anti-CD28 in the presence of IL-2 and different concentrations of TGF-β1 or Chit1. After 72 hr, cells were harvested and stained with anti-CD4/Foxp3 and were subjected to FACS (A and B) and TGF- β receptor II mRNA expression were real-time PCR (C) evaluation. * $p<0.05$.

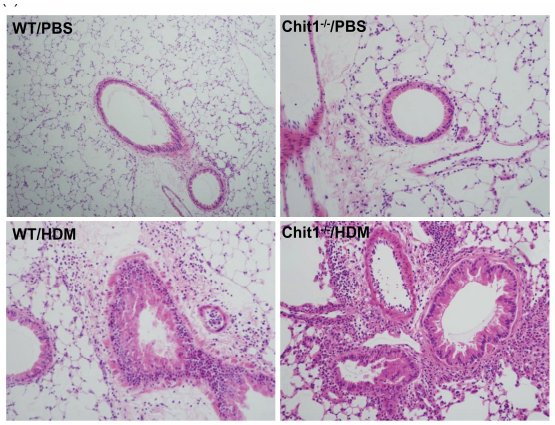
6. Chit1 regulates House dust mite-induced airway inflammation and airway hperresponsiveness

Hallmark indicators of allergic asthma include airway eosinophilia and the production of mucin, Th2-patterned cytokines, and IgE Abs. We also tested the role of Chit1 in the chitin-containing antigen-driven systems. To assess the influence of Chit1 on these parameters, Chit1$^{-/-}$ and WT mice were sensitized and challenged with house dust mice (HDM), and the lungs were lavaged, excised, and prepared for analysis. As expected, HDM-treated mice was increased the level of Chit in BAL fluid (Fig. 6A). The Chit1$^{-/-}$ HDM mice exhibited increased total cell and eosinophil counts (Fig. 6B) in the BAL fluid compared with their WT counterparts. Chit1$^{-/-}$/OVA mice had greater BAL fluid IL-4, IL-5 and IL-13 protein levels but not TGF-β1 and IL-10 (Fig. 6C) and serum IgE levels (Fig. 6D) compared with WT HDM mice. WT and Chit1$^{-/-}$ HDM mice exhibited the expected HDM mediated histological changes, as demonstrated by perivascular and peribronchiolar inflammatory cell infiltration (Fig. 6E), suggesting that Chit1 modulates the extent

of the Th2 response and subsequent humoral response in allergen mediated lung inflammation.


(A)
mChit1 (ng/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
WT/PBS
WT/HDM
(B)
BAL (cells)
500x10³
400x10³
300x10³
200x10³
100x10³
0
**
*
*
WT/PBS
Chit1-/-/PBS
WT/HDM
Chit1-/-/HDM
Total cells
Macrophage
Eosinophils
Neutrophils
Lymphocytes
(C)
Cytokine production (ng/mL)
1600
1400
1200
1000
800
600
400
200
0
WT/PBS
Chit1-/-/PBS
WT/HDM
Chit1-/-/HDM
mIL4
mIL5
mIL13
Cytokine production (pg/mL)
350
300
250
200
150
100
50
0
mTGF-β1
mIL10
(D)
Total IgE (ng/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
***
WT/PBS
Chit1-/-/PBS
WT/HDM
Chit1-/-/HDM

(E)


WT/PBS
Chit1-/-/PBS
WT/HDM
Chit1-/-/HDM

(F)

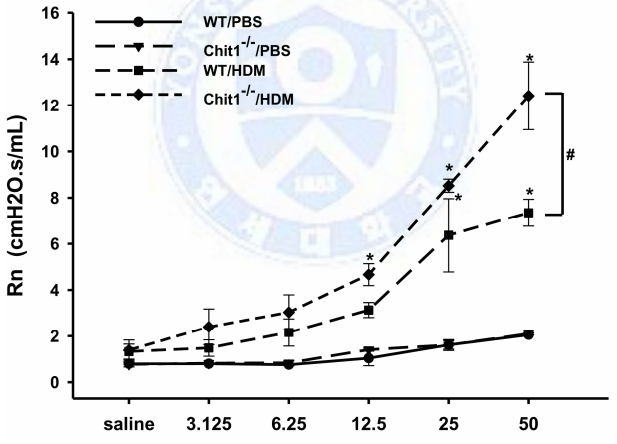

WT/PBS
Chit1-/-/PBS
WT/HDM
Chit1-/-/HDM
Rn (cmH2O.s/mL)
0
2
4
6
8
10
12
14
16
saline
3.125
6.25
12.5
25
50
*
#

Figure 6. Chit1 regulates House dust mite-induced airway inflammation and airway hperresponsiveness. WT and Chit1$^{-/-}$ mice were sensitized house dust mite (HDM)/Alum and challenged with HDM. The level of Chit1 was assessed via ELISA (A) using BAL fluid. BAL total and inflammatory cell recovery were quantitated (B), cytokine (C), total IgE (D) and airway hyperreponsiveness were measured (F), and H&E stain of lung sections (E) Mean frequency and fold increases were estimated from triplicated experiments. * $p<0.05$, **$p<0.01$, ***$p<0.005$ (control vs. OVA), # $p<0.05$ (WT/OVA vs. Chit1$^{-/-}$/OVA).

7. **Chit1 expression is increased in pediatric asthma patients**

To determine whether Chit1 expression is increase in the circulation and sputum of patients with asthma we applied ELISA approaches to the serum and sputum samples. The clinical characteristics of the study subjects are summarized in Table 1. There were no significant differences in age and gender between the groups. The percentage of children with atopy was significantly higher in asthma group than those in control group ($p < 0.001$). Pulmonary function parameters, including FEV_1 ($p < 0.001$), percentage change in FEV_1 after BD therapy ($p < 0.001$), FEV_1/FVC ($p < 0.001$), and forced expiratory flow midexpiratory phase ($FEF_{25–75}$) [$p < 0.001$], showed significantly lower levels in children with asthma than in control subjects. Asthma subjects showed lower levels in FVC, but there was no statistical difference ($p=0.054$). The percentage of eosinophils in induced sputum was significantly higher in children with asthma than in control subjects ($p < 0.001$). The blood eosinophil count, serum ECP, and serum total IgE levels were increased in children with asthma compared with those in control subjects ($P <$

0.001).

Compared with control samples, which showed only modest levels of chit1, the levels of sputum and serum Chit1 were higher in patients with asthma (41.1±23.288 versus 53.65±4.6, 48.43±1.712 versus 53.58±1.945; Fig. 7A and B). This suggests that Chit1 may be a useful biomarker of disease in this patient population.

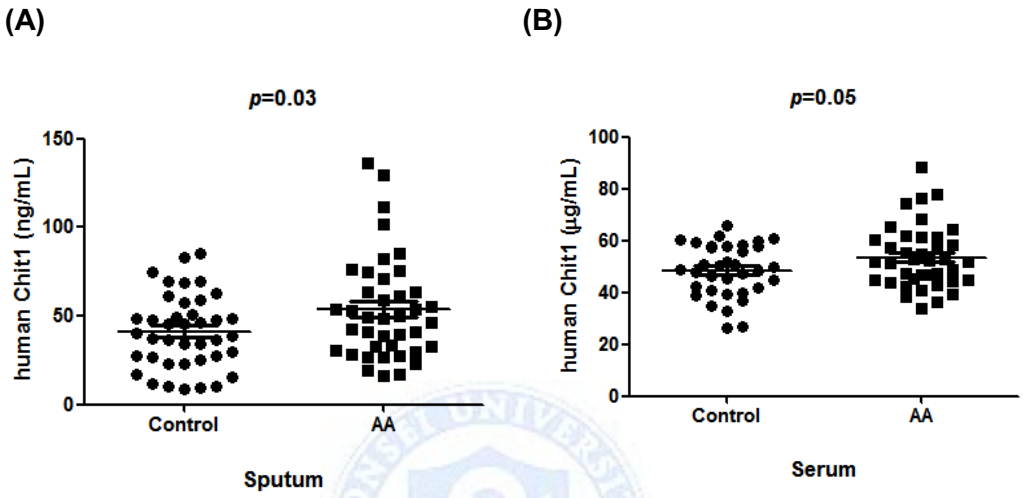


Figure 7. Chit1 expression is increase from pediatric patients with asthma. Comparison of normal controls (left, n=40) versus asthma subjects (right, n=40) reveals significantly increased Chit1 concentration in (A) sputum and (B) serum in asthma. (A) The level of Chit1 is enhanced in patients with asthma (right, n=40) compare to normal control (left, n=40) in suptum.

Table 1. Subject characteristics

Characteristics	Asthma (n=40)	Control (n=40)
Age, yr	9.2 ± 2.1	9.1 ± 2.6
Sex, M (%)	26 (65)	18 (45)
Atopy, with (%)	40 (100)*	0 (0)
FVC, % pred	88.9 (76.1-95.8)	90.1 (83.9-104.3)
FEV_1, % pred	94.5 (85.0-103.1)*	98.5 (92.5-106.8)
Change in FEV_1, %	5.8 (3.3-14.3)*	4.25 (3.3 -6.0)
FEV_1/FVC, %	94.3 (86.1-98.7)*	97.3 (91.5-99.0)
FEF_{25-75}, % pred	91 (76.4-108.9)*	101.1 (89.3-118.0)
Total IgE, IU/mL	79.8 (10.3-708)	10.4 (4.8-21.0)
Blood eosinophil, uL	230 (120-560)*	120 (70-180)
Serum ECP, ug/L	14.4 (6.8-28.1)*	7.5 (4.9-17.3)

Values are expressed as number (percentage), mean ± SD, or median (Interquatile range) *p < 0.001 compared to control subjects. FVC, forced expiratory vital capacity; FEV1, forced expiratory volume in 1 sec; FEF25-75, forced expiratory flow between 25% and 75%; IgE, immunoglobulin E; ECP, eosinophil cationic protein.

IV. DISCUSSION

The present studies demonstrate that the regulation of Chit1 in a murine model of allergen allergic airway disease amplifies the inflammatory response and leads to a higher percentage of $CD4^{+}GATA3^{+}$ T cells within the lungs of $Chit1^{-/-}$ mice. Moreover, allergen-challenged $Chit1^{-/-}$ lung produced increased IL-4, 5 and 13 cytokine levels upon Th2 polarization compared to wild type mice. Whereas lower level of TGF-β1 and IL-10 were exhibited in BAL and lung from $Chit1^{-/-}$ mice. Adding exogenous recombinant TGF-β1 and Chit1 in vitro to replicate its function correspondingly increase the proportion of $Foxp3^{+}$ T cells. These studies demonstrate that Chit1 mediates these effects, at least in part, by synergizing with TGF-β1. Lastly, we compared the level of Chit1 in sputum and serum was increased in asthma patients compare to healthy control.

The present studies have revealed a regulatory profile with Chit1 contributing to the pathogenesis of adaptive Th2 inflammation in chitin-free experimental systems. However, our data also demonstrates that deficiency of Chit1 has similar effects in

chitin-free and chitin-containing antigen-driven systems. This finding suggests that the contributions of Chit1 in this setting may also be independent of its chitinase enzyme activity.

Our data showed that Chit1 is required for TGF-β1 mediated Foxp3 induction in allergen stimulated T cell development. The fate of $CD4^+$T cells is governed by the cytokines they encounter, leading to the increase expression of master regulators, determining in particular their cytokine secretion profile. As reported by many studied, $CD4^+$ lineages have some degree of plasticity, allowing coexpression of master transcriptional regulators and cytokines specific for other lineages. Indeed, Chit1 involve in TGF- β1- induced receptor expression and signaling by activation of TGF-β receptor (TGFβR) at that expense of Treg induction. These observations are well supported by several recent studies showing that in the presence of both TGF- β1 and Chit1, enhance to induce TGFβR expression and TGF- β1 signaling compare to TGF- β1 alone.[16,20]

Chit1 bioactivity and protein levels were significantly increased in the circulation

and lung of patients compared to controlls.[21] Using animal models, they further demonstrated that Chit1 enhances TGFβR expression and its signaling in fibroblasts. And Chit1 augmented TGF- β1–induced Smad2 phosphorylation, Smad2 reporter activation and MAPK ERK1/2 phosphorylation. Thus they identified that Chit1 interacts with TGF- β1 to augment TGF- β1 receptor expression and TGF- β1 signaling.[16,22,23] In this regard, our results show that in the absence of Chit1, failure to induction Foxp3+ Tregs is associated with the loss of activation of TGFβR expression and TGF- β1 signaling and consequently acquisition of Th2 phenotype, likely due to increased GATA3 expression levels. Therefore, TGFβR and TGF- β1 signaling activation levels seems to be required for an acquisition of Foxp3+ phenotype, and Chit1 may play a synergetic role in this process.

Previous study demonstrated that Foxp3 induction is inhibited by GATA3, which is the key regulator for polarization toward Th2 cells. After differentiation, the effector Th2 cells become refractory to conversion into a $Foxp3^{+}$ phenotype.[24] GATA3 repressed Foxp3 expression directly by binding to the Foxp3 promoter

region. Naïve T cells could efficiently up-regulate Foxp3 when treated with TGF-β to iTreg cells, but already-committed cells such as memory T cells and Th1 cells showed not sufficient to change the phenotype toward a regulatory T cell profile. And the Th2 cytokine IL-4 but not IL-13 were able to inhibit TGF- β -mediated Foxp3 induction and therefore prevented conversion into the regulatory phenotype.[25] Similar effects have been recently described for IL-6, which combined with TGF- β, inhibits the generation of iTreg cells and induces differentiation into the Th17 cells by an unknown mechanism.[26] In this sense, GATA3 restrains the development of Treg subsets of the inducible and peripheral population. We have shown that the level of TGF- β was significantly low in allergen-induced Chit1$^{-/-}$ mice. In addition to, the deficiency of Chit1 leads to the enhanced frequency of GATA3^{+} T cells and level of IL-4 in allergen challenged mice, which may be responsible for the decreased Foxp3^{+} T cell differentiation in Chit1$^{-/-}$. Thus Chit1 could function to promote TGF- β -mediated Treg pathways and dampen T cell activation in the site of inflammation.

V. CONCLUSION

Based on our findings, the presence of adequate amounts of Chit1 in the lung milieu positively regulates the prevalence of $Foxp3^+$ Treg cells in the lung tissue during allergen-induced inflammation. Because of a deficiency of Chit1, there is a concomitant decrease in the production of Treg-associated cytokines, which, in turn, affects the induction of Tregs and increased the Th2-associated inflammation response. Thus, Chit1 regulate Treg induction synergically from acquiring Th2 effector characteristics by cooperating with TGF-β1-induced Treg induction.

REFERENCES

1. Shuhui L, Mok YK, Wong WS. Role of mammalian chitinases in asthma. International archives of allergy and immunology 2009;149:369-77.

2. Venuprasad K, Poojary KV, Kong YC, Farrar MA. Control of Th2-mediated inflammation by regulatory T cells. The American journal of pathology 2010;177:525-31.

3. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nature medicine 2012;18:716-25.

4. Ray A, Cohn L. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. The Journal of clinical investigation 1999;104:985-93.

5. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.

6. Zeng H, Zhang R, Jin B, Chen L. Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. Cellular & Molecular

Immunology 2015;12:566-71.

7. Palomares O, Martín Fontecha M, Lauener R, Traidl Hoffmann C, Cavkaytar O, Akdis M, et al. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes and Immunity 2014;15:511-20.

8. Wiesner DL, Specht CA, Lee CK, Smith KD, Mukaremera L, Elias JA, et al. Chitin recognition via chitotriosidase promotes pathologic type-2 helper T cell responses to cryptococcal infection. PLOS pathogens 2015;11:e1004701.

9. Lee CG, Da Silva CA, Lee J, Hartl D, Elias JA. Chitin regulation of immune responses: an old molecule with new roles. Current opinion in immunology 2008;20:684-9.

10. Zhu Z, Zheng T, Homer RJ, Kim Y, Chen NY, Cohn L, et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 2004;304:1678-82.

11. He CH, Lee CG, Dela Cruz CS, Zhou Y, Ahangari F, Ma B, et al. Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor α2. Cell Reports 2013;4:830-41.

12. Di Rosa M, Malaguarnera G, De Gregorio C, D'Amico F, Mazzarino MC, Malaguarnera L. Modulation of chitotriosidase during macrophage differentiation. Cell biochemistry and biophysics 2013;66:239-47.

13. Di Rosa M, De Gregorio C, Malaguarnera G, Tuttobene M, Biazzo F, Malaguarnera L. Evaluation of AMCase and CHIT-1 expression in monocyte macrophages lineage. Molecular and cellular biochemistry 2013;374:73-80.

14. Vicencio AG, Chupp GL, Tsirilakis K, He X, Kessel A, Nandalike K, et al. CHIT1 mutations: genetic risk factor for severe asthma with fungal sensitization? Pediatrics 2010;126:e982-5.

15. Seibold MA, Donnelly S, Solon M, Innes A, Woodruff PG, Boot RG, et al. Chitotriosidase is the primary active chitinase in the human lung and is

modulated by genotype and smoking habit. Journal of Allergy and Clinical Immunology 2008;122:944-50.e3.

16. Lee CG, Herzog EL, Ahangari F, Zhou Y, Gulati M, Peng X, et al. Chitinase 1 is a biomarker for and therapeutic target in scleroderma-associated interstitial lung disease that augments TGF-β1 signaling. The journal of immunology 2012;189:2635-44.

17. Lee CG, Herzog EL, Ahangari F, Zhou Y, Gulati M, Peng X, et al. The journal of immunology 2012;189:2635-44.

18. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. Journal of allergy and clinical immunology 2007;120:S94-138.

19. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. Journal of biological chemistry 1997;272:21597-603.

20. Cho SJ, Weiden MD, Lee CG. Chitotriosidase in the Pathogenesis of Inflammation, Interstitial Lung Diseases and COPD. Allergy, asthma & immunology research 2015;7:14-21.

21. Létuvé S, Kozhich A, Humbles A, Brewah Y, Dombret M, Grandsaigne M, et al. Lung chitinolytic activity and chitotriosidase are elevated in chronic obstructive pulmonary disease and contribute to lung inflammation. The American journal of pathology 2010;176:638-49.

22. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. The journal of immunology 2004;172:5149-53.

23. Venuprasad K, Poojary KV, Kong YM, Farrar MA. Control of Th2-mediated inflammation by regulatory T cells. The American journal of pathology 2010;177:525-31.

24. Mantel PY, Kuipers H, Boyman O, Rhyner C, Ouaked N, Rückert B, et al.

GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS Biology 2007;5:e329.

25. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nature Immunology 2008;9:1347-55.

26. Kastner L, Dwyer D, Qin FX. Synergistic effect of IL-6 and IL-4 in driving fate revision of natural Foxp3+ regulatory T cells. The journal of immunology 2010;185:5778-86.

ABSTRACT (IN KOREAN)

알러젠으로 유도된 천식 마우스에서 Chitotriosidase의 알레르기 면역 반응 조절 연구

<지도교수 손 명 현>

연세대학교 대학원 의과학과

홍 정 연

인간에 존재하는 true chitinase 중 Acidic mammalian chitinase (AMCase) 와 Chitotriosidase (Chitinase 1, chit1)는 기관지 염증 또는 변형에 중요한 조절자로 알려져 있다. 알러지성 천식은 Th2 세포들이 생성하는 사이토카인, 호산구, 기도 과민성의 증가를 특징으로 한다. 그러나 이러한 Th2 반응에서의 Chit1의 역할과 메커니즘에 대한 연구는 밝혀진 것이 없다.

6주령 된 C57BL/6 를 background로 갖는 wild type (WT)과 Chit1 Knock out (Chit1$^{-/-}$) 마우스에 ovalbumin (OVA)과 house dust mite (HDM)을 복강을 통해 감작시키고 기도에 처리하여 알러지성 기도 염증을 유도하였다. 기관지 폐포 세척액 (Bronchoalveolar lavage fluid; BALF) 에서 총 세포와 염증 세포수를 측정하고 사이토카인과 IgE 농도는 ELSIA로 측정하였다. 메타콜린 흡입에 따른 기도과민성 (airway hyperresponsiveness; AHR) 은 Flexivent apparatus를 이용하여 측정한다. 마우스의 비장, 림프절, 폐에서 분리한 세포는 OVA로 재감작을 하거나 분리한 $CD4^{+}CD25^{-}$ T 세포에 TGF-β 와 Chit1를 처리하여 Treg을 유도한 뒤 유세포 분석기를 이용해 세포를 분석한다.

OVA에 의해 유도된 Chit1$^{-/-}$ 군은 WT군에 비해 저항값, 기관지폐포세척액 내의 염증세포 수, IL-5 및 IL-13 농도, 혈청 내의 총 또는 OVA 특이 IgE 농도, 폐조직 내 염증세포의 과다형성 정도가 유의하게 높게 측정되었으나 TGF-β1 과 IL-10 의 농도는 유의하게 낮게 측정되었다. 특히 천식 마우스군 폐에서 측정한 $CD4^{+}Foxp3^{+}$/$CD4^{+}GATA3^{+}$ 비율과 절대 세포수도

Chit1$^{-/-}$ 군에서 유의하게 낮은 수치를 나타냈다. 또한 분리한 $CD4^{+}CD25^{-}$ T 세포에 TGF-β 와 Chit1을 처리하여 Treg 세포 분화 유도시 TGF-β1 과 Chit1를 함께 처리해준 Chit1$^{-/-}$ 군에서 가장 높은 분화효과를 확인할 수 있었다.

또한 chitin이 포함된 HDM를 이용한 천식마우스 모델에서도 Chit1$^{-/-}$ 마우스는 WT에 비해 염증관련 parameter들이 모두 증가되어 있었으나 TGF-β1 과 IL-10 의 농도는 유의하게 낮게 측정되었다.

마지막으로 천식환자의 가래와 혈청에서 Chit1의 농도를 측정한 결과 정상군에 비해 유의하게 높은 Chit1 농도를 가래에서 확인 할 수 있었다.

따라서 위 결과를 통해 알러젠 유도 천식 마우스 모델에서 Chit1이 염증 부위에 증가되고 $CD4^{+}$ T 세포 분화에 관여하여 염증 부위의 T cell population 을 조절하여 염증반응을 감소시키는 것을 확인하였다.

핵심 되는 말: chitotriosidase, 천식 마우스 모델, Foxp3^{+} regulatory T cell, TGF-β