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Chitinase 3-Like 1 Contributes to Food Allergy via M2 Macrophage Polarization

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ABSTRACT

Purpose: Food allergy is a hypersensitive immune response to specific food proteins. Chitinase 3-like 1 (CHI3L1, also known as YKL-40 in humans or BRP-39 in mice) is associated with various chronic diseases, such as cancer, rheumatoid arthritis, and allergic disease. CHI3L1 is involved in allergen sensitization and type 2 helper T (Th2) inflammation, but the role of CHI3L1 in food allergy remains unclear. In this study, we sought to investigate the role of CHI3L1 in the development of food allergy.
Methods: We measured serum levels of CHI3L1 in food allergic patients. Food allergy was induced in wild-type (WT) and CHI3L1 null mutant (CHI3L1$^{-/-}$) BALB/c mice with ovalbumin (OVA). We investigated Th2 immune responses, M2 macrophage polarization, and mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) signaling pathways, and also performed transcriptome analysis.
Results: Serum levels of CHI3L1 were significantly higher in children with food allergy compared with those in healthy controls. Furthermore, CHI3L1 expression levels were elevated in WT mice after OVA treatment. Food allergy symptoms, immunoglobulin E levels, Th2 cytokine production, and histological injury were attenuated in food allergy-induced CHI3L1$^{-/-}$ mice compared with those in food allergy-induced WT mice. CHI3L1 expression was increased in OVA-treated WT intestinal macrophages and caused M2 macrophage polarization. Furthermore, CHI3L1 was involved in the extracellular signal-regulated kinases (ERK) and AKT signaling pathways and was associated with immune response and lipid metabolism as determined through transcriptome analysis.
Conclusions: CHI3L1 plays a pivotal role in Th2 inflammation and M2 macrophage polarization through MAPK/ERK and PI3K/AKT phosphorylation in food allergy.

Keywords: Chitinase; food allergy; immunoglobulin E; macrophages; M2 macrophage polarization; mitogen-activated protein kinase; phosphoinositide 3-kinase; signaling pathway; type 2 helper T cells

INTRODUCTION

Immunoglobulin E (IgE)-mediated food allergy is a global health concern that affects millions of people, with increasing prevalence over the last decade.[1,2] Symptoms of food

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There are no financial or other issues that might lead to conflict of interest.

allergy include skin rash, urticaria, vomiting, diarrhea, dyspnea, and even life-threatening anaphylaxis after food intake.[3] Food allergy is associated with allergen-specific type 2 helper T (Th2) cells that produce cytokines such as interleukin (IL)-4, IL-5, and IL-13. These cytokines induce class switching accompanied by the production of allergen-specific IgE.[4] When normal defenses against food antigens are compromised, Th2 and IgE responses are strengthened, while IgG and IgA responses are weakened, leading to an allergic inflammatory reaction.[4,5] Macrophages also modulate the food allergy response via secretion of inflammatory cytokines. During an inflammatory response, macrophages are activated by several factors and stimulate the proliferation and activation of T cells.[6,7] However, the role of macrophages in food allergy is not thoroughly understood, and an incomplete understanding of immunologic responses during food allergy results in limited treatment strategies.[8,9]

Chitinase 3-like 1 (CHI3L1, also known as YKL-40 in humans or BRP-39 in mice), a member of evolutionarily conserved glycosyl hydrolase family 18, is characterized by a strong binding affinity for chitin, which it lacks the enzymatic activity to directly degrade.[10,11] CHI3L1 is synthesized as a 39-kDa protein encoded by chromosome 1 in humans and mice.[12,13] Numerous studies have shown that CHI3L1 is expressed in a variety of cells, including macrophages, neutrophils, fibroblasts, tumor cells, and epithelial cells, and is associated with various diseases, such as cancer, rheumatoid arthritis, and inflammatory bowel disease.[14-16] Previous studies have also demonstrated that serum levels of CHI3L1 were significantly elevated in patients with asthma or atopic dermatitis, suggesting that CHI3L1 is associated with allergic disease.[17-19] CHI3L1 is reportedly expressed in epithelial cells and macrophages at the sites of Th2 inflammation and contributes to M2 macrophage differentiation by activating local dendritic cells.[20] CHI3L1 also contributes to tissue remodeling and apoptosis as well as Th2 inflammation. These studies suggest that CHI3L1 could be a potential therapeutic target for various immunological disorders.[21] Although CHI3L1 is known to be closely associated with various human diseases, the molecular and cellular functions of CHI3L1 in food allergy have not yet been elucidated.

In this study, we tested the hypothesis that CHI3L1 is involved in food allergy. We examined serum levels of CHI3L1 in food allergic patients and investigated CHI3L1 expression levels in an ovalbumin (OVA)-induced mouse model of food allergy. We also compared inflammatory responses in food allergy-induced wild-type (WT) and CHI3L1 null mutant (CHI3L1$^{-/-}$) BALB/c mice.

MATERIALS AND METHODS

Human subjects

A total of 68 children who visited a university hospital located in Seoul, Republic of Korea for diagnostic workup, and treatment for egg allergy or routine health check-up between July 2013 and July 2017 were enrolled in this study. Egg allergy was defined according to the guidelines of the National Institute of Allergy and Infectious Diseases-sponsored expert panel report.[1] A thorough medical history was taken and physical examination was performed at the first visit. Children reporting symptoms of other allergic diseases, such as allergic rhinitis and asthma, were excluded from the study. Healthy controls had no history of any allergic or inflammatory disease. Blood samples were drawn and subsequently stored at −20°C. Total white blood cell and eosinophil counts were determined using a NE-8000 hematology analyzer (Sysmex Corporation, Kobe, Japan) and levels of total and egg white-specific IgE were measured using the Pharmacia CAP assay (Uppsala, Sweden). Serum levels of CHI3L1 were determined using

an enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. This study was approved by the Institutional Review Board of the affiliated hospital and written informed consent was obtained from participants or their parents (protocol No. 4-2004-0036).

Animals

BALB/c mice were obtained from Orient Bio Inc. (Seongnam, Korea). CHI3L1$^{-/-}$ mice were generated in a C57BL/6 background and maintained as previously described.[21] CHI3L1$^{-/-}$ mice in the C57BL/6 background were backcrossed for more than 6 generations with BALB/c mice. Four- to 6-week-old female mice were used in all experiments and were housed in specific pathogen- free conditions in an air-conditioned room (23°C±1°C) under a 12-hour light/dark cycle. The animals were allowed free access to standard rodent food and tap water. All animal experiments were approved by the Institutional Animal Care and Use Committee of the affiliated university (protocol No. 2017-0195; Seoul, Korea).

Murine models of food allergy

Mice were intraperitoneally sensitized with 50 μg of OVA (grade V; Sigma-Aldrich, Munich, Germany) and 10 μg of cholera toxin (CT; List Biological Laboratories, Los Angeles, CA, USA) as an adjuvant on Days 0 and 14. From Day 28 onward, mice were intragastrically challenged with 50 mg of OVA 6 times at 1-day intervals. Control mice were sensitized and challenged with phosphate-buffered saline (PBS). Core temperature, clinical and diarrhea scores were measured after the final intragastric challenge, as previously described.[22] Blood and tissue samples were collected from mice 1 day after rectal temperature and symptom score measurement.

Isolation and culture of immune cells

Mouse spleen was harvested and passed through a 40-μm cell strainer in RPMI 1640 medium (Hyclone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer. The cells were washed twice and 1×10^6 cells were cultured in wells of a 96-well plate for 3 days at 37°C in a 5% CO_2 incubator. The cells were then collected, and the supernatants were frozen at -80°C separately.

Lamina propria mononuclear cells were isolated from mice as previously described, with a slight modification.[23,24] In brief, the jejunum was isolated, and fecal content was washed out with PBS after removal of Peyer's patches. The jejunum was cut into 1-cm pieces and shaken for 20 minutes in HBSS medium (Thermo Fisher Scientific, Waltham, MA, USA) with 5% FBS and 2 mM EDTA. This process was repeated 2 additional times. The remaining tissue was cut into small pieces and digested with 1.5 mg/mL collagenase and 40 μg/mL DNase 1 (both from Sigma-Aldrich). The digested tissue and supernatant were passed through a 100-μm cell strainer, before centrifugation at 1,500 rpm for 5 minutes at 4°C and before washing cells to isolate lamina propria mononuclear cells.

Peritoneal macrophages were isolated from mice as previously described, with a slight modification.[25,26] In brief, the peritoneal cavity was injected with 5 mL of PBS containing 5% FBS, and the peritoneal fluid was collected. This process was repeated 2 additional times. The peritoneal fluid was centrifuged at 1,500 rpm for 8 minutes at 4°C and isolated cells were washed. These cells were then cultured in RPMI 1640 medium (Hyclone Laboratories) containing 10% FBS and 1% penicillin-streptomycin at a density of 2×10^6 cells per well on

the 6-well plates. The cells were allowed to adhere for 2 hours at 37°C in a 5% CO_2 incubator. The cultures were then washed 3× with PBS to remove non-adherent cells, and peritoneal macrophages were harvested using a cell scraper.

Flow cytometric analysis

Cells were isolated from the jejunum as described above. Cellular debris was removed from lamina propria mononuclear cells using Percoll gradient (Sigma-Aldrich). Antibodies for flow cytometry were purchased from eBioscience (Waltham, MA, USA) unless otherwise stated. Dead cells were excluded from analysis with a viability dye eFluor 780. Macrophages in the lamina propria were identified through staining with anti-major histocompatibility complex (MHC) class II (eFluor 450), anti-CD11b (PerCP/Cy5.5), and anti-F4/80 (BV605; BD Biosciences, San Diego, CA, USA) antibody. We also performed staining with anti-CHI3L1 (PE; Biorbyt, Cambridge, UK) antibody to determine if CHI3L1 is expressed in lamina propria macrophages. Stained cells were detected by flow cytometry using an LSR Fortessa™ X-20 cell analyzer (BD Biosciences), and data were analyzed using FlowJo software (version 10.6.0; Tree Star, Inc., Ashland, OR, USA). The gating strategy is shown in **Supplementary Fig. S1**.

Cell sorting

Cells were isolated from the jejunum as described above. Dead cells were removed using a Dead Cell Removal Kit (Miltenyi Biotec, Bergisch, Germany), according to the manufacturer's protocol for magnetic cell sorting (auto-MACS; Miltenyi Biotec). Intestinal macrophages were identified by staining with anti-MHC class II (PE), anti-CD11b (PerCP/Cy5.5), and anti-F4/80 (FITC) antibody. Stained cells were sorted using a FACS Aria™ II flow cytometer (BD Biosciences).

Microarray transcriptome analysis

Microarray transcriptome analysis was performed using GeneChip® Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA). Total RNA in the jejunum was extracted using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cDNA was synthesized using the GeneChip® Whole Transcript Amplification Kit (Affymetrix) according to the manufacturer's instructions. The cDNA was then fragmented and biotin-labeled using the GeneChip® Whole Transcript Terminal Labeling Kit (Affymetrix), and the labeled DNA target was hybridized to GeneChip® array. Hybridized arrays were stained on a GeneChip® Fluidics Station 450 and scanned using a GeneChip® Scanner 3000. Array data export processing and analysis were performed using the GeneChip® Command Console® Software (version 4.0.0). The data were summarized and normalized using a robust multi-average method implemented in Affymetrix® Power Tools, and differentially expressed gene (DEG) analysis was performed. Gene-enrichment and functional annotation analysis for the significant probe list was performed using Gene Ontology (GO)[27,28] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[29] databases.

Statistical analysis

Statistical analyses of human data were performed using R Statistical Software (version 3.3.3; R Foundation for Statistical Computing, Vienna, Austria). Categorical data are presented as counts and percentages. Continuous data were tested for normality using the Kolmogorov-Smirnov test and reported accordingly as the mean (± standard deviation) or median (interquartile range). Two-group comparisons were performed using Student's *t* test or the Mann-Whitney *U* test for continuous variables, or the χ^2 test for categorical variables. Correlations between levels of CHI3L1 and total IgE were analyzed using the Spearman's

rank correlation test. A *P*-value < 0.05 was considered statistically significant. Statistical analyses of mouse data are presented as mean ± standard error of the mean (SEM). Statistical differences were analyzed using Student's *t* test for 2-group comparisons, or the 1-way analysis of variance (ANOVA) test, followed by Tukey's test for multiple-group comparisons. A *P*-value of < 0.05 was considered statistically significant.

Additional details of the materials and methods are provided in the **Supplementary Data S1**.

RESULTS

Serum levels of CHI3L1 are increased in children with food allergy.

To investigate whether CHI3L1 is involved in food allergy, we measured serum levels of CHI3L1 in children with food allergy or healthy controls. The clinical characteristics of the subjects are summarized in **Table**. The 2 groups did not differ significantly in age, sex and serum white blood cell count. Blood eosinophil and total IgE levels were significantly elevated in the food allergic patient group compared with those in the healthy control group. Serum levels of CHI3L1 were also significantly higher in children with food allergy compared than in healthy controls (21.819 ± 6.979 ng/mL *vs.* 16.16 ± 5.619 ng/mL; $P < 0.001$; **Fig. 1A**). In addition, CHI3L1 serum levels were positively correlated with total IgE levels ($r = 0.261$, $P = 0.031$; **Supplementary Fig. S2**). Among children with food allergy, the level of CHI3L1 presented a positive correlation with the class of serum egg white-specific IgE ($r = 0.355$, $P = 0.039$).

CHI3L1 expression levels are elevated in murine models of food allergy.

We established a food allergy mouse model and confirmed CHI3L1 expression levels to identify the contribution of CHI3L1 to food allergy in mice. To induce food allergy, mice were intraperitoneally sensitized with OVA plus CT and intragastrically challenged with OVA (**Fig. 1B**). OVA treatment resulted in decreased core temperature (**Fig. 1C**) and increased clinical and diarrhea scores (**Fig. 1D, E**). Total IgE serum levels were also elevated after OVA treatment (**Fig. 1F**). Next, we measured CHI3L1 expression levels in mouse jejunum. We confirmed that CHI3L1 mRNA (**Fig. 1G**) and protein (**Fig. 1H and I**) expression levels were significantly elevated in the OVA-treated WT mice compared with those in the control mice. Consequently, these results indicate that CHI3L1 is associated with food allergy.

CHI3L1 regulates Th2 immune responses.

To clarify the relationship between CHI3L1 and food allergy, we induced food allergy with OVA in the WT and CHI3L1$^{-/-}$ mice. Core temperature, which was decreased in OVA-treated WT mice, was restored in OVA-treated CHI3L1$^{-/-}$ mice (**Fig. 2A**). Clinical and diarrhea scores, which were increased in OVA-treated WT mice, were reduced in OVA-treated CHI3L1$^{-/-}$ mice (**Fig. 2B and C**). Food allergy is well known as a Th2-mediated immune response, and Th2

Table. Characteristics of study subjects

Characteristics	Control (n = 34)	Food allergy (n = 34)	*P*-value
Age (year)	2.4 (1.0–7.5)	2.1 (1.2–4.4)	0.342
Sex, male (%)	14 (41.2)	19 (55.9)	0.332
White blood cell (/μL)	7,545.0 (6,750.0–9,510.0)	8,185.0 (6,870.0–9,390.0)	0.946
Blood eosinophil (/μL)	220.0 (140.0–300.0)	395.0 (290.0–500.0)	0.001
Serum total IgE (IU/mL)	15.4 (8.6–30.0)	398.0 (90.5–847.0)	< 0.001
Serum egg white-specific IgE (IU/mL)		20.6 (8.0–51.8)	N/A

Data are shown as number (%) or median (interquartile range), as appropriate.
IgE, immunoglobulin E; N/A, not applicable.

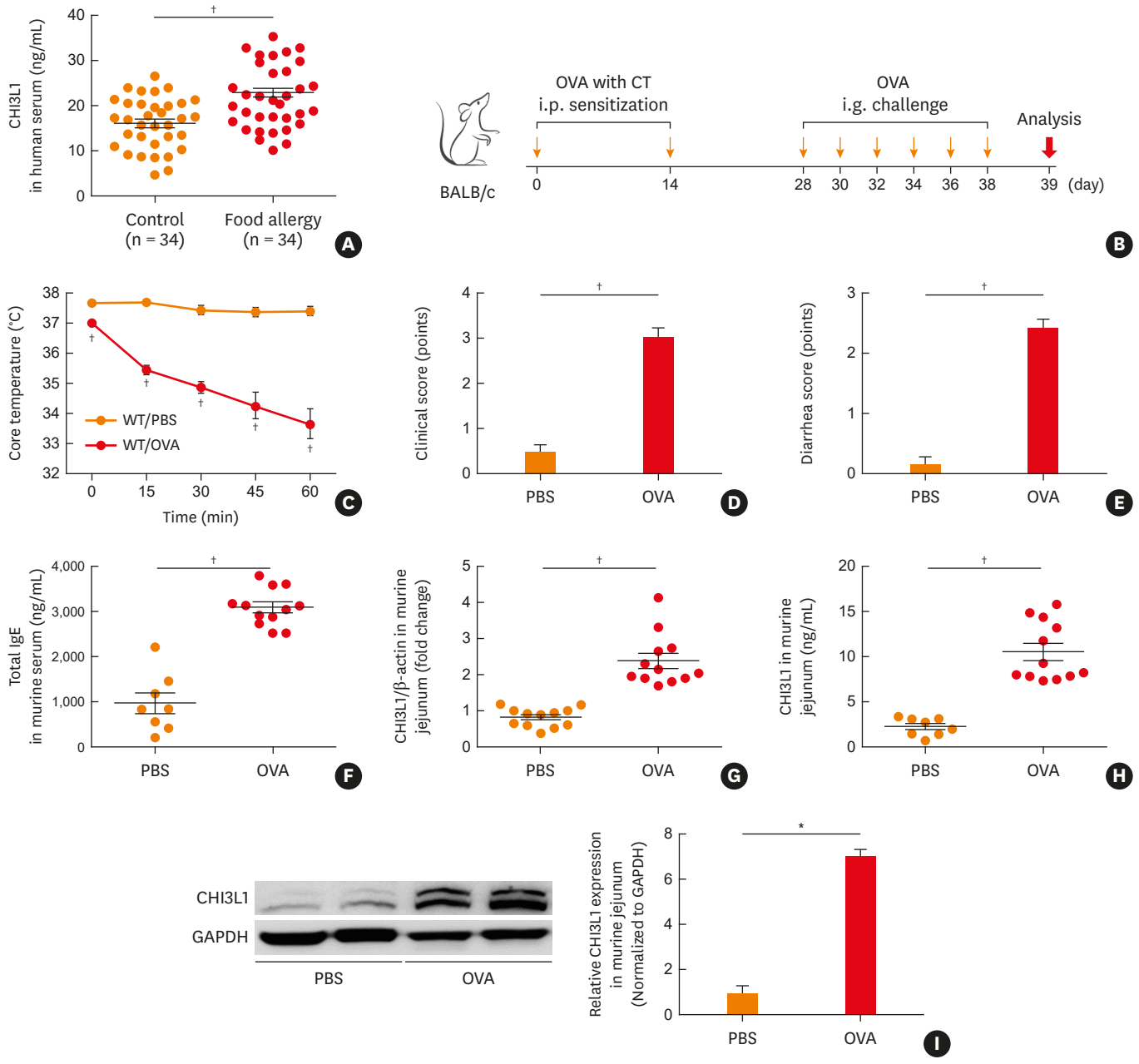

Fig. 1. CHI3L1 expression is increased in children with food allergy and in a mouse model of OVA-induced food allergy. (A) Serum levels of CHI3L1 measured in children with food allergy (n = 34) and healthy control subjects (n = 34) by ELISA. (B) Mouse model of experimental food allergy protocol. (C) Core temperature, (D) clinical score and (E) diarrhea score measured in mouse model of OVA-induced food allergy. (F) Total IgE levels in the serum analyzed by ELISA. (G) mRNA expression levels of CHI3L1 in the jejunum measured by real-time PCR. (H) CHI3L1 levels in jejunum lysate assessed by ELISA. (I) Protein levels of CHI3L1 in the jejunum measured by western blot analysis and quantified signal intensity. Data are presented as mean ± SEM and are representative of at least 3 independent experiments (n = 7–12 mice per group).
CHI3L1, chitinase 3-like 1; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; PCR, polymerase chain reaction; SEM, standard error of the mean; CT, cholera toxin; i.p., intraperitoneal; i.g., intragastric; WT, wild-type; PBS, phosphate-buffered saline.
*$P < 0.01$, †$P < 0.001$.

cells and their cytokines play important roles in the development of food allergy.[4] Therefore, we evaluated Th2 cytokine levels in mouse jejunum and splenocytes. mRNA expression levels of IL-4, IL-5, and IL-13 in the jejunum were significantly elevated in the OVA-treated WT mice compared with those in the control mice, but were attenuated in OVA-treated CHI3L1$^{-/-}$ mice

(**Fig. 2D**). IL-4, IL-5, and IL-13 concentrations in the supernatant of cultured splenocytes were also diminished in the OVA-treated CHI3L1$^{-/-}$ mice compared with those in the OVA-treated WT mice (**Fig. 2E**). Additionally, total IgE and OVA-specific IgE levels in the serum were inhibited in the OVA-treated CHI3L1$^{-/-}$ mice compared with those in the OVA-treated WT mice (**Fig. 2F**). Histological studies performed via hematoxylin-eosin (H&E) staining of jejunal sections showed that intestinal damage, such as villus injury and inflammatory cell accumulation, was reduced in the OVA-treated CHI3L1$^{-/-}$ mice compared with that in the OVA-treated WT mice (**Fig. 2G and H**). Next, we examined junctional complexes in the jejunum by transmission electron microscopy (TEM), including the tight junction, adherens junction, and desmosome. TEM analysis revealed that the OVA-treated WT mice had severe structural alterations in the junctional complexes, whereas the OVA-treated CHI3L1$^{-/-}$ mice had fewer intercellular junction abnormalities than the OVA-treated WT mice (**Fig. 2I**). These studies demonstrate that CHI3L1 plays a critical role in food allergy phenotypes and Th2 responses.

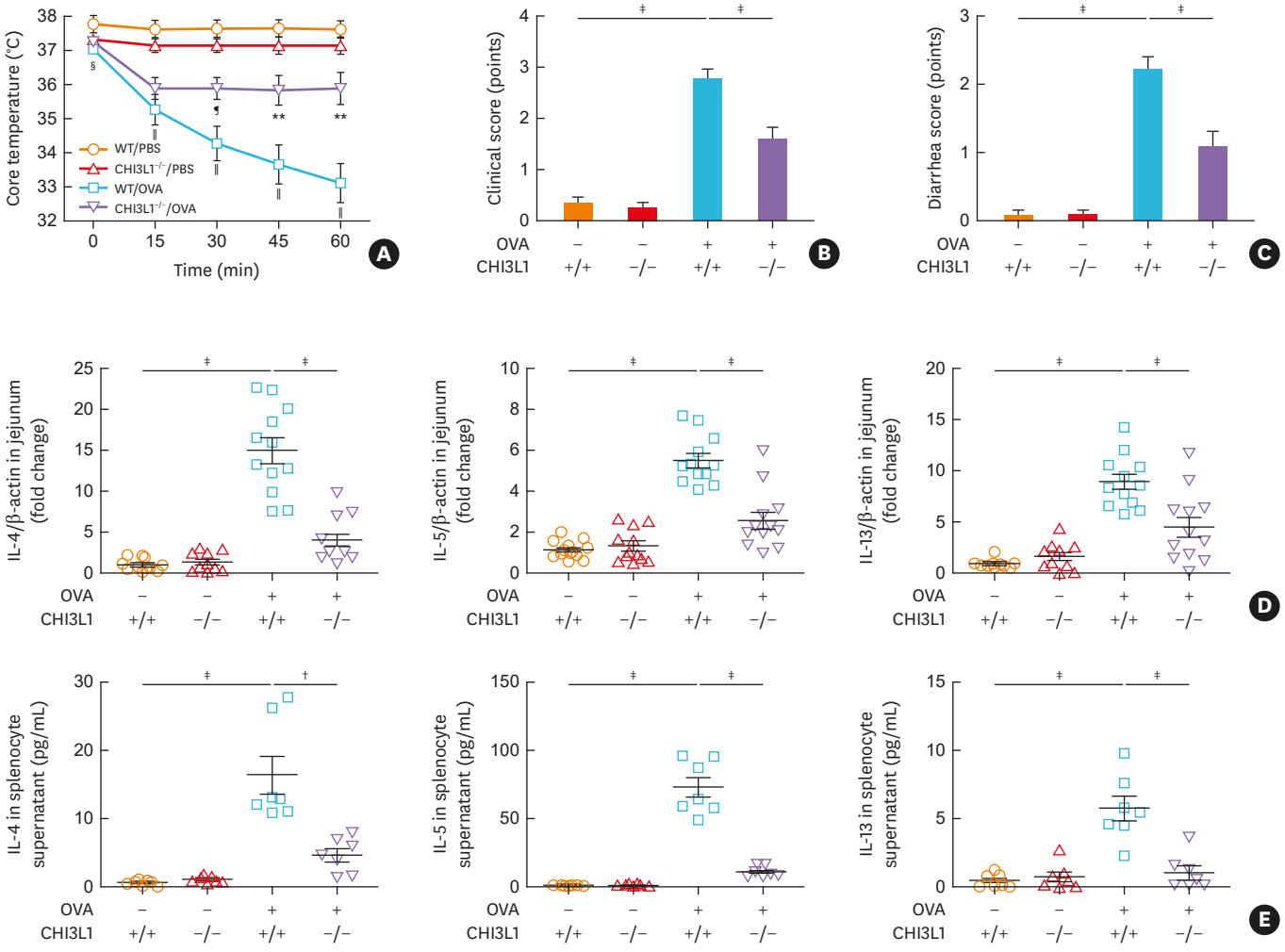

Fig. 2. Th2 immune responses are diminished in OVA-treated CHI3L1$^{-/-}$ mice. (A) Core temperature, (B) clinical score and (C) diarrhea score measured in mouse model of OVA-induced food allergy. (D) mRNA expression levels of IL-4, IL-5 and IL-13 in the jejunum assessed by real-time PCR. (E) Concentrations of IL-4, IL-5 and IL-13 in the splenocyte supernatant measured by ELISA.

Th2, type 2 helper T; OVA, ovalbumin; CHI3L1, chitinase 3-like 1; IL, interleukin; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; O.D., optical density; H&E, hematoxylin-eosin; TEM, transmission electron microscopy; SEM, standard error of the mean; WT, wild-type; PBS, phosphate-buffered saline.

$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. In panel (A), $^{\S}P < 0.05$, $^{\|}P < 0.001$ for WT/PBS *vs.* WT/OVA and $^{\P}P < 0.05$, $^{**}P < 0.01$ for WT/OVA *vs.* CHI3L1$^{-/-}$/OVA.

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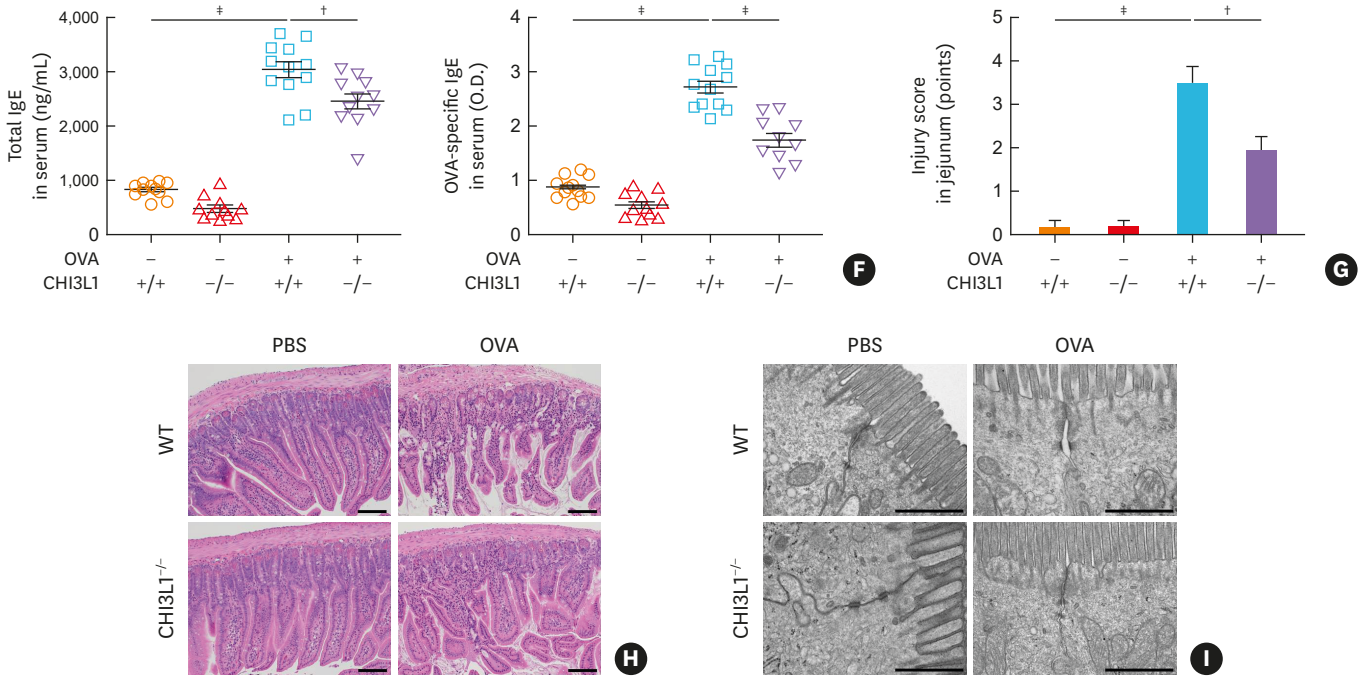

Fig. 2. (Continued) Th2 immune responses are diminished in OVA-treated CHI3L1$^{-/-}$ mice. (F) Total IgE levels and O.D. values of OVA-specific IgE in the serum analyzed by ELISA. (G) Injury score and (H) histological analysis of jejunum observed by H&E staining. (I) Morphological observations of the junctional complexes in the jejunum conducted by TEM. Data are presented as mean ± SEM and are representative of at least 3 independent experiments (n = 7–12 mice per group). Scale bars, (H) 100 μm and (I) 1,000 nm.

Th2, type 2 helper T; OVA, ovalbumin; CHI3L1, chitinase 3-like 1; IL, interleukin; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; O.D., optical density; H&E, hematoxylin-eosin; TEM, transmission electron microscopy; SEM, standard error of the mean; WT, wild-type; PBS, phosphate-buffered saline.

$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. In panel (A), $^{\S}P < 0.05$, $^{\|}P < 0.001$ for WT/PBS *vs.* WT/OVA and $^{\P}P < 0.05$, $^{**}P < 0.01$ for WT/OVA *vs.* CHI3L1$^{-/-}$/OVA.

CHI3L1 is expressed in macrophages and activates M2 macrophage polarization. Macrophages are known to affect Th2 inflammation, and our previous study demonstrated that CHI3L1 is predominantly induced in macrophages during Th2-dependent skin inflammation.[19] We therefore used flow cytometric analysis to determine whether CHI3L1 is expressed in intestinal macrophages during food allergy. Strikingly, the population of CHI3L1-positive cells in intestinal macrophages was higher in the OVA-treated WT mice than in the control mice (**Fig. 3A**). Additionally, the percentage of CHI3L1-expressing intestinal macrophages was increased in the OVA-treated WT mice compared with that in the control mice (**Fig. 3B**). We further investigated mRNA expression levels of representative M1 and M2 macrophage markers in the mouse jejunum, revealing higher expression levels of M2 markers, such as arginase 1, YM1/2, and CD206, in the OVA-treated WT mice compared with those in control mice. However, these levels were significantly lower in the OVA-treated CHI3L1$^{-/-}$ mice than in the OVA-treated WT mice (**Fig. 3C**). Expression levels of M1 markers, such as iNOS, CD16, and CD86, did not significantly differ among the 4 groups (**Fig. 3D**). To further explore whether CHI3L1 activates M2 macrophage polarization during food allergy, we examined mRNA expression levels of IL-10, known as a Th2-differentiating cytokine or M2 macrophage cytokine, and IL-12, known as a Th1-differentiating cytokine or M1 macrophage cytokine, in sorted intestinal macrophages (MHC class II^{+}, CD11b^{+} and F4/80^{+} cells). mRNA expression levels of IL-10 were significantly up-regulated in the OVA-treated CHI3L1$^{+/+}$ intestinal macrophages compared with those in the control CHI3L1$^{+/+}$ intestinal macrophages, but were down-regulated in the OVA-treated CHI3L1$^{-/-}$ intestinal macrophages (**Supplementary Fig. S3A**). Expression levels of IL-12p35 or IL-12p40 did not significantly differ among the 4 groups (**Supplementary Fig. S3B**). Similarly, mRNA expression levels of M2 markers,

such as arginase 1 and YM1/2, in addition to M2 chemokines, such as CCL22, were significantly diminished in the OVA-treated CHI3L1$^{-/-}$ intestinal macrophages compared with those in the OVA-treated CHI3L1$^{+/+}$ intestinal macrophages (**Fig. 3E**). Taken together, these results suggest that CHI3L1 can polarize intestinal macrophages to the M2 phenotype during food allergy.

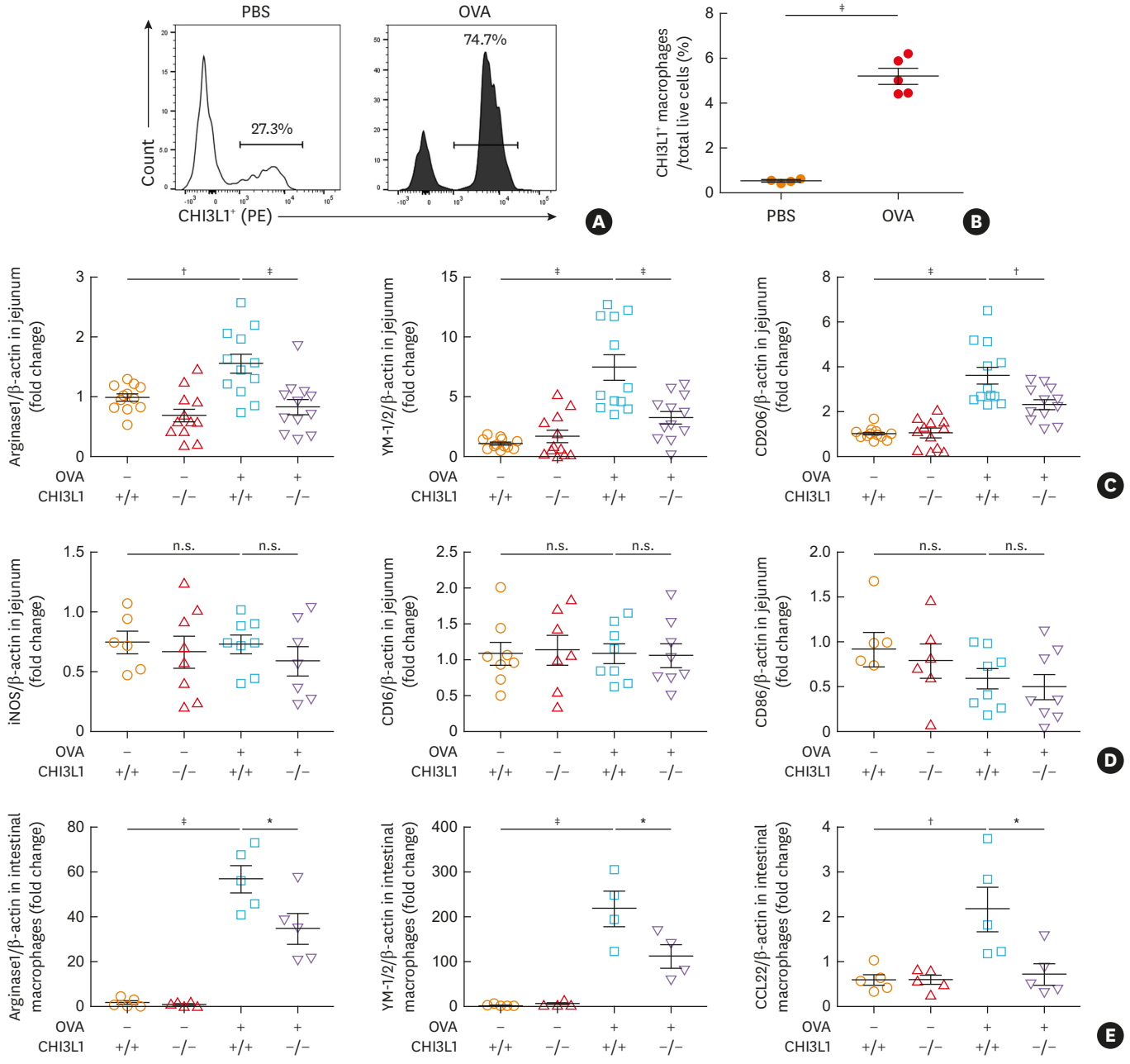

Fig. 3. CHI3L1 is expressed in intestinal macrophages and M2 macrophage polarization is suppressed in OVA-treated CHI3L1$^{-/-}$ mice. (A) Expression of CHI3L1 in intestinal macrophages from WT mice quantified by flow cytometric analysis. (B) Percentage of CHI3L1-expressing macrophages normalized to live cells from WT mice quantified by flow cytometric analysis. (C) mRNA expression levels of M2 macrophage markers (arginase 1, YM 1/2 and CD 206) in the jejunum from WT and CHI3L1$^{-/-}$ mice measured by real-time PCR. (D) mRNA expression levels of M1 macrophage markers (iNOS, CD16, and CD86) in the jejunum from WT and CHI3L1$^{-/-}$ mice measured by real-time PCR. (E) mRNA expression levels for M2 macrophage markers (arginase 1 and YM 1/2) and chemokines (CCL22) in the intestinal macrophages from WT and CHI3L1$^{-/-}$ mice assessed using real-time PCR. Data are presented as mean ± SEM and representative of at least 3 independent experiments (n = 4–12 mice per group).
CHI3L1, chitinase 3-like 1; OVA, ovalbumin; WT, wild-type; PCR, polymerase chain reaction; SEM, standard error of the mean; PBS, phosphate-buffered saline; n.s., not significant.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

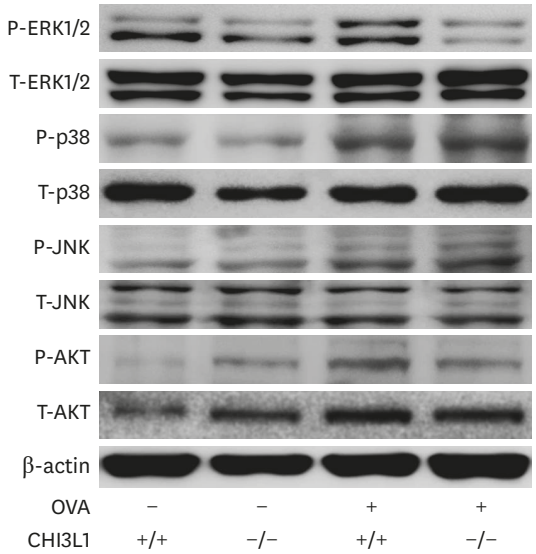

Fig. 4. ERK1/2 and AKT phosphorylation are reduced in OVA-treated CHI3L1$^{-/-}$ mice. Peritoneal macrophages from WT and CHI3L1$^{-/-}$ mice were harvested and western blot analysis was used to evaluate P-ERK1/2 and T-ERK1/2, P-p38 and T-p38, P-JNK and T-JNK, P-AKT and T-AKT. Representative panel of at least 3 independent experiments. ERK, extracellular signal-regulated kinases; OVA, ovalbumin; CHI3L1, chitinase 3-like 1; WT, wild-type; P, phosphorylation; T, total; JNK, c-Jun N-terminal kinase.

CHI3L1 participates in mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) and phosphatidylinositol 3 kinase (PI3K)/AKT signaling pathways.

To further evaluate the signaling pathways influenced by CHI3L1 in food allergy, we performed western blot analysis on murine peritoneal macrophages. Peritoneal macrophages constitute a critical element in the effector phase of immune reactions, and subpopulations of peritoneal macrophages exacerbate allergic reactions.[7,30] CHI3L1 is thought to be associated with various MAPK and PI3K signaling pathways in mice and humans.[31-34] Thus, we examined the phosphorylation levels of ERK1/2, p38, c-Jun N-terminal kinase (JNK) and AKT in peritoneal macrophages (**Fig. 4**). In MAPK signaling pathways, only ERK1/2 phosphorylation was enhanced in the OVA-treated WT mice and reduced in the OVA-treated CHI3L1$^{-/-}$ mice. The p38 and JNK phosphorylation levels were enhanced in both OVA-treated WT and OVA-treated CHI3L1$^{-/-}$ mice, without significant difference between the 2 groups. In PI3K signaling pathways, AKT phosphorylation was enhanced in the OVA-treated WT mice and reduced in the OVA-treated CHI3L1$^{-/-}$ mice. These results demonstrate that CHI3L1 is involved with MAPK/ERK and PI3K/AKT signaling pathways in food allergy.

CHI3L1 alters expression of immune response-related genes.

To further elucidate the biological and molecular functions of CHI3L1 in food allergy, we performed microarray transcriptome analysis of the mouse jejunum. A total of 157 genes were differentially expressed (51 up-regulated and 106 down-regulated) in the OVA-treated CHI3L1$^{-/-}$ mice compared with the OVA-treated WT mice (**Fig. 5A**). A list of DEGs in the whole transcriptome analysis of the OVA-treated CHI3L1$^{-/-}$ mice is provided in **Supplementary Table S1**. Statistically significant genes in the GO and KEGG pathway categories are summarized in **Supplementary Table S2**. GO analysis revealed that immune response-related genes were highly down-regulated in the OVA-treated CHI3L1$^{-/-}$ mice compared with those in the OVA-treated WT mice (**Fig. 5B**). These GO terms are associated with specific immune responses mediated by lymphocytes, and with activation or perpetuation of immune responses. KEGG

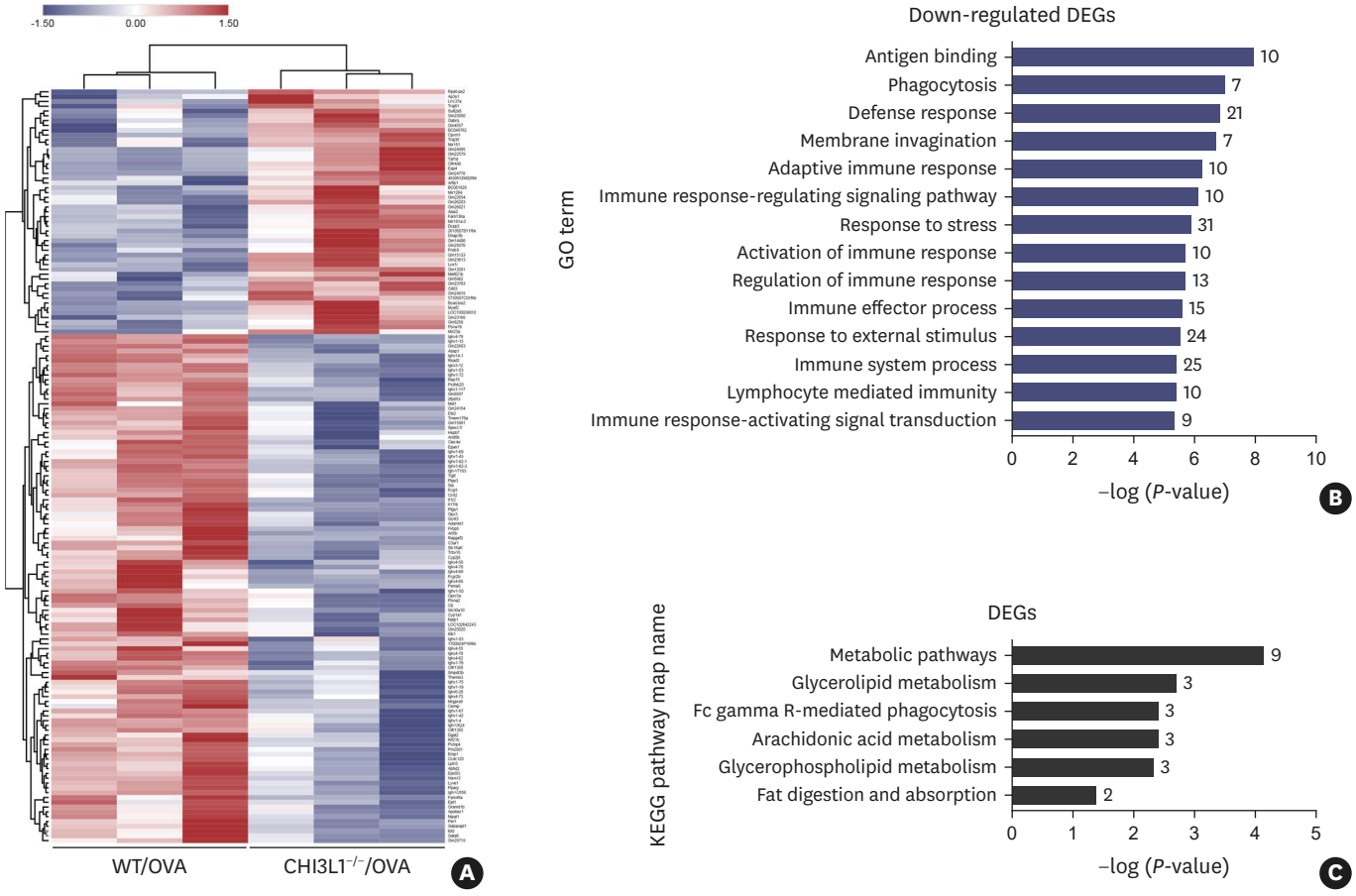

Fig. 5. Immune response-related genes are down-regulated in OVA-treated CHI3L1$^{-/-}$ mice. (A) Transcriptome analysis showing DEGs in the jejunum from OVA-treated WT and OVA-treated CHI3L1$^{-/-}$ mice by heat map. (B) GO terms and (C) KEGG pathway map names of DEGs identified as statistically significant in the OVA-treated CHI3L1$^{-/-}$ mice compared with the OVA-treated WT mice. n = 3 mice per group.
OVA, ovalbumin; CHI3L1, chitinase 3-like 1; DEG, differentially expressed gene; WT, wild-type; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

pathway analysis indicated that several pathways were involved in the OVA-treated CHI3L1$^{-/-}$ mice compared with those in the OVA-treated WT mice (**Fig. 5C**). Fc gamma R-mediated phagocytosis observed in our KEGG pathway analysis plays a key role in the uptake and degradation of foreign particles mediated by macrophages.[35,36] Pathways associated with lipid metabolism, such as glycerolipid metabolism, arachidonic acid metabolism, glycerophospholipid metabolism, and fat digestion and absorption, were also discovered in our KEGG pathway analysis. Previously, macrophages were found to play an important role in lipid metabolism in addition to the phagocytosis of pathogens.[37] Taken together, these results suggest that CHI3L1 is associated with macrophage-related responses and lipid metabolism, and a lack thereof weakens the immune response.

DISCUSSION

In this study, we demonstrated that CHI3L1 promotes food allergy through Th2 immune responses and M2 macrophage polarization in combination with MAPK/ERK and PI3K/AKT signaling pathways (**Fig. 6**).

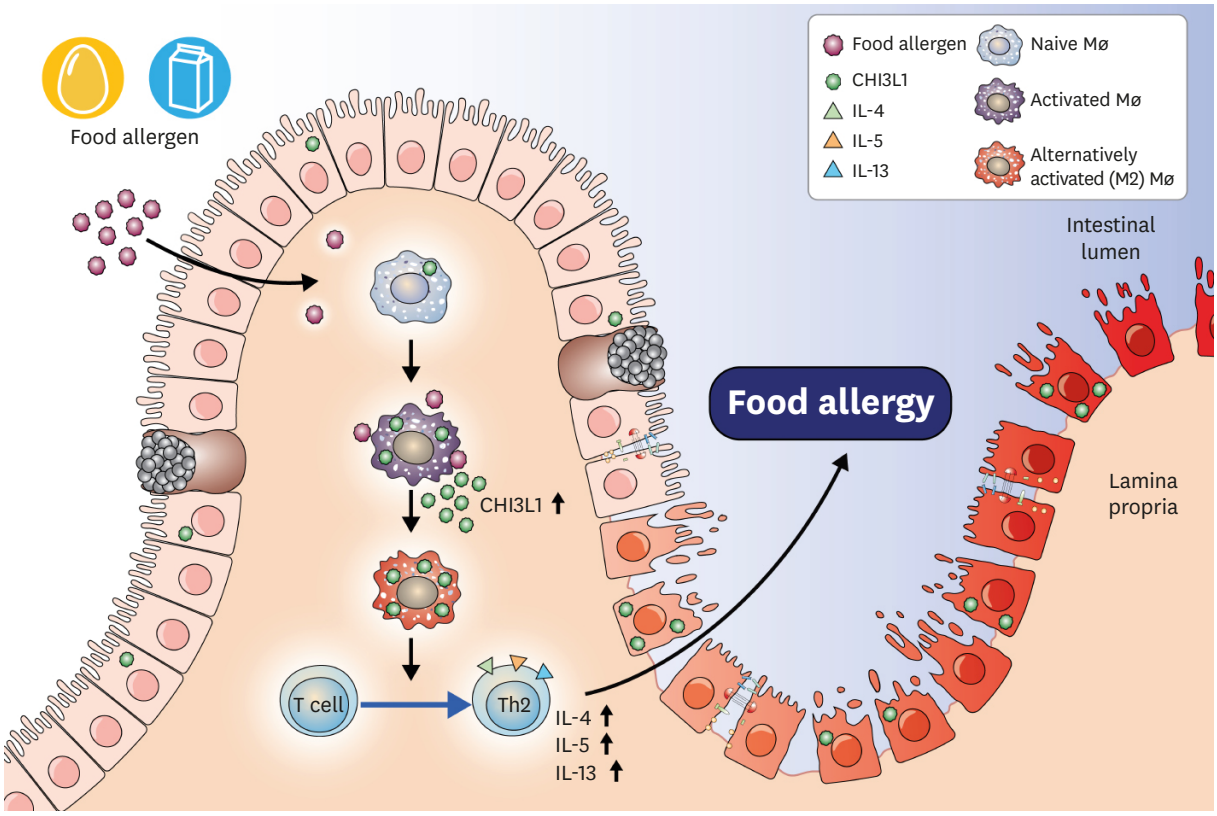

Fig. 6. CHI3L1 contributes to food allergy through M2 macrophage polarization and Th2-associated inflammation. CHI3L1 was predominantly expressed in intestinal macrophages and caused M2 macrophage polarization. Furthermore, CHI3L1 induced Th2 immune responses that could influence the development of food allergy. CHI3L1, chitinase 3-like 1; Th2, type 2 helper T cell; IL, interleukin; Mø, macrophage.

Previous studies have revealed that CHI3L1 serum levels were elevated in several chronic disease patients, such as rheumatoid arthritis, atherosclerosis, osteoarthritis, cancer, and asthma.[18,38-40] CHI3L1 levels have also been correlated with disease severity.[17,41] In this study, we showed that CHI3L1 serum levels were significantly higher in children with food allergy than in healthy controls. Consistent with this finding, CHI3L1 levels were reportedly up-regulated in adults with food allergy and were associated with airway allergic responses.[42] Additionally, we observed that CHI3L1 mRNA and protein expression levels were considerably increased in the OVA-treated WT mice compared with those in the control mice. Therefore, these results suggest that CHI3L1 may be a useful prognostic biomarker and potential therapeutic target in allergic inflammatory disorders.

Using CHI3L1$^{-/-}$ mice, we discovered that food allergy phenotypes and expression of Th2 cytokines were attenuated in the OVA-treated CHI3L1$^{-/-}$ mice compared with that in the OVA-treated WT mice, which is consistent with results of recent reports. Th2 cytokine levels and airway inflammation are known to be elevated in respiratory syncytial virus (RSV) infection but were reportedly diminished in the absence of CHI3L1.[43] In lung metastasis, CHI3L1 also plays a significant role in the pathogenesis of Th2 inflammation. CHI3L1 expression levels were found to be elevated in Th2 cells and CHI3L1-deficient T cells were prone to differentiate into Th1 cells as a result of increased interferon-gamma signaling.[44] These results suggest that CHI3L1 is involved in Th2 cell-related immune responses.

With respect to macrophage polarization, we found that CHI3L1 expression was increased in the OVA-treated intestinal macrophages. Furthermore, sorted OVA-treated CHI3L1$^{+/+}$ intestinal macrophages were significantly polarized to the M2 phenotype compared with the OVA-treated CHI3L1$^{-/-}$ intestinal macrophages. Recent studies have also indicated that CHI3L1 is involved in M2 macrophage polarization in atopic dermatitis and RSV infection.[19,43] This effect could be due to Th2 cytokines, such as IL-4 and IL-13, which are M2 polarization drivers.[45] Therefore, we suspect that increased expression of Th2 cytokines is involved in the development of the M2 phenotype, and that these synergistic effects may promote food allergy.

CHI3L1 binds to the IL-13 receptor α2 and participates in a multimeric complex with IL-13, IL-13 receptor α2, and transmembrane protein 219 that mediates various CH13L1 signaling responses. Treatment with recombinant CHI3L1 was shown to enhance ERK/AKT phosphorylation and β-catenin nuclear translocation in macrophages in a time-dependent and dose-dependent manner.[31,32] Previous research has also indicated that CHI3L1 is involved in MAPK and PI3K pathways during oxidative stress in human airway epithelial cells.[33] CHI3L1 is also critical for activating AKT signaling in colonic epithelial cells, which can contribute to the development of chronic colitis.[34] Here, we demonstrated that CHI3L1 in food allergy is associated with activation of the MAPK and PI3K signaling pathways, particularly the ERK and AKT signaling pathways. These pathways are presumably important for maintaining allergic inflammation according to the development of food allergy.

GO analysis of our transciptomics data indicated that immune response genes were down-regulated in the OVA-treated CHI3L1$^{-/-}$ mice. These results correlated with the data in **Fig. 2**, which shows decreased Th2 immune responses in the OVA-treated CHI3L1$^{-/-}$ mice. Our KEGG pathway analysis revealed that phagocytosis and lipid metabolism-related pathways were affected in the OVA-treated CHI3L1$^{-/-}$ mice. Macrophages are important for phagocytosis and lipid metabolism that orchestrates inflammation.[46,47] Additionally, lipid mediators are also known to participate in the crosstalk between metabolism and inflammation.[47] Glycerolipid metabolism, which was found in our KEGG pathway analysis, is known to participate in T cell activation and proliferation.[48,49] Thus, our findings indicate that the immune response and lipid metabolism are involved in CHI3L1-mediated food allergy. However, the interaction between lipid metabolism and CHI3L1 in food allergy warrants further studies.

In conclusion, our data demonstrates that CHI3L1 expression is elevated in children with food allergy and in an IgE-mediated food allergy mouse model. CHI3L1-deficient mice had suppressed Th2 immune responses and exhibited M2 macrophage polarization. Additionally, we confirmed that CHI3L1 is associated with the ERK and AKT signaling pathways, and transcriptome analysis revealed that CHI3L1 is also associated with the immune response and lipid metabolism in food allergy. Taken together, our work highlights a novel role for CHI3L1 in promoting Th2-associated inflammation and M2 macrophage polarization through MAPK/ERK and PI3K/AKT signaling pathways in food allergy, which may influence the development of the food allergy. CH13L1 may be a useful biomarker and potential target for developing new treatments for patients with food allergy.

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SUPPLEMENTARY MATERIALS

Supplementary Data S1

Materials and methods

Click here to view

Supplementary Table S1

List of DEGs in OVA-treated CHI3L1$^{-/-}$ mice compared with those in OVA-treated WT mice, excluding DEGs between the WT and CHI3L1$^{-/-}$ control mice

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Supplementary Table S2

Detailed analysis of GO (up) and KEGG pathway (down)

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Supplementary Fig. S1

Flow cytometric gating strategy for intestinal macrophages. Small intestinal cells were first gated according to a FSC/SSC plot, and then doublets and dead cells were excluded from the analysis. Macrophages were defined by the expression of MHC class II, CD11b and F4/80.

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Supplementary Fig. S2

Association between serum CHI3L1 and total IgE levels. Serum CHI3L1 levels showed a significant correlation with the total IgE levels ($r = 0.261$, $P = 0.031$).

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Supplementary Fig. S3

Th2 inflammation and M2 macrophage polarization are attenuated in OVA-treated CHI3L1$^{-/-}$ mice. mRNA expression levels of (A) IL-10 (Th2-differentiating cytokine or M2 macrophage cytokine) and (B) IL-12p35 and IL-12p40 (Th1-differentiating cytokine or M1 macrophage cytokine) in the intestinal macrophages from WT and CHI3L1$^{-/-}$ mice assessed by real-time PCR. Data are presented as mean ± SEM and representative of at least three independent experiments (n = 4-5 mice per group).

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Supplementary Data S1. Materials and methods

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of total immunoglobulin E (IgE) and ovalbumin (OVA)-specific IgE were measured using an ELISA Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. To measure OVA-specific IgE, a 96-well plate was coated with OVA (grade V; Sigma-Aldrich, Munich, Germany). Chitinase 3-like 1 (CHI3L1) levels in the jejunum and interleukin (IL)-4, IL-5, and IL-13 levels in the *splenocyte supernatant* were determined using an ELISA Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

mRNA expression analysis by real-time polymerase chain reaction (PCR)

Total RNA was isolated from jejunum and intestinal macrophages using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Jejunum was homogenized using the T10 Basic Ultra-Turrax® homogenizer (IKA Labortechnik, Staufen, Germany). RNA was extracted and cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. Quantitative PCR was performed using Power SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with the *StepOnePlus™ Real-Time PCR System* (Applied Biosystems) according to the manufacturer's protocol. β-actin was used as a housekeeping gene and the results were analyzed using the $2^{-\Delta\Delta CT}$ method.[1]

Western blotting

Total jejunum protein was extracted by homogenizing mouse jejunum using radioimmunoprecipitation assay (RIPA) buffer containing a Halt™ Protease Inhibitor Cocktail (both from Thermo Fisher Scientific, Waltham, MA, USA), and peritoneal macrophage protein was extracted using mammalian protein extraction reagent (MPER) buffer (Thermo Fisher Scientific), according to the manufacturer’s instructions. Protein quantification was performed using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Quantified protein was electrophoresed in 10%

SDS polyacrylamide gels and electrotransferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skimmed milk for 1 hour, the membrane was incubated overnight with specific primary antibodies against CHI3L1 (Invitrogen) and GAPDH, phosphorylation (P)-extracellular signal-regulated kinases (ERK)1/2, total (T)-ERK1/2, P-p38, T-p38, P-c-Jun N-terminal kinase (JNK), T-JNK, P-AKT, T-AKT, and β-actin (all from Cell Signaling Technology, Beverly, MA, USA) at 4°C. The next day, the membrane was incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). ImageQuant™ LAS 4000 Mini Biomolecular Imager (GE Healthcare, Buckinghamshire, UK) and Chemiluminescent Substrate (Thermo Fisher Scientific) were used to visualize the protein bands. Image J software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis.[2]

Hematoxylin-eosin (H&E) staining and histological evaluation

Specimens of jejunum were fixed in 4% paraformaldehyde for 3 days and then embedded in paraffin. The paraffin block was cut and 4-μm sections were stained with H&E. Tissue structure and inflammation of intestinal mucosa were observed using a BX43 Upright Microscope (Olympus, Tokyo, Japan) at 200× magnification. The histological damage score was determined, as previously described.[3]

Electron microscopy analysis

Specimens of jejunum were prefixed with 2% glutaraldehyde–paraformaldehyde in 0.1 M phosphate buffer at 12 hours and postfixed with 1% OsO_4 dissolved in 0.1 M phosphate buffer for 2 hours. Postfixed jejunum was dehydrated with ethanol and infiltrated with propylene oxide. The jejunum was then embedded using the Poly/Bed® 812 Embedding Kit (Polysciences, Warrington, FL, USA) and polymerized at 65°C in an Electron Microscope Oven (TD-700; Dosaka Em Co., Ltd., Kyoto, Japan) for 24 hours. The jejunum was then cut and transferred to copper and nickel grids, and a 70-nm thin jejunum section was double stained with 6% uranyl acetate and lead citrate. The section was

observed using JEM-1011 transmission electron microscopy (Jeol Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV.

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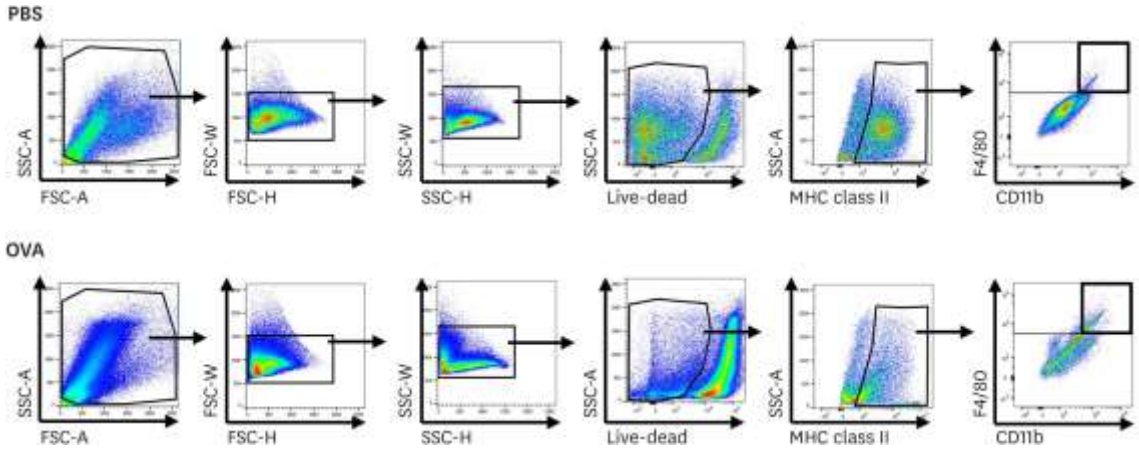

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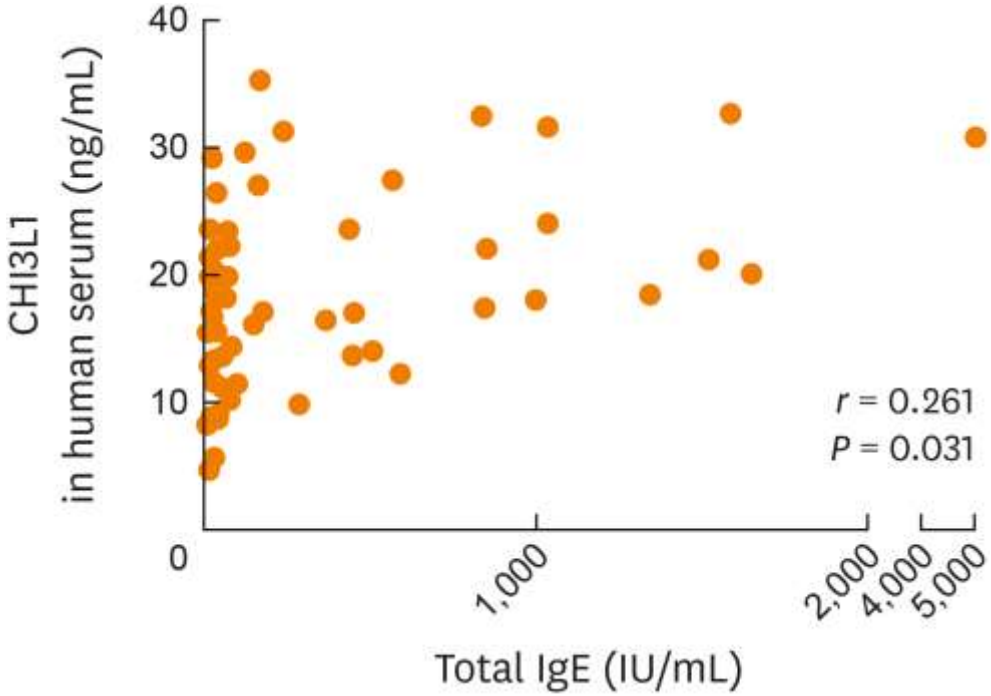

Supplementary Fig. S2. Association between serum CHI3L1 and total IgE levels. Serum CHI3L1 levels showed a significant correlation with the total IgE levels (r = 0.261, P = 0.031).

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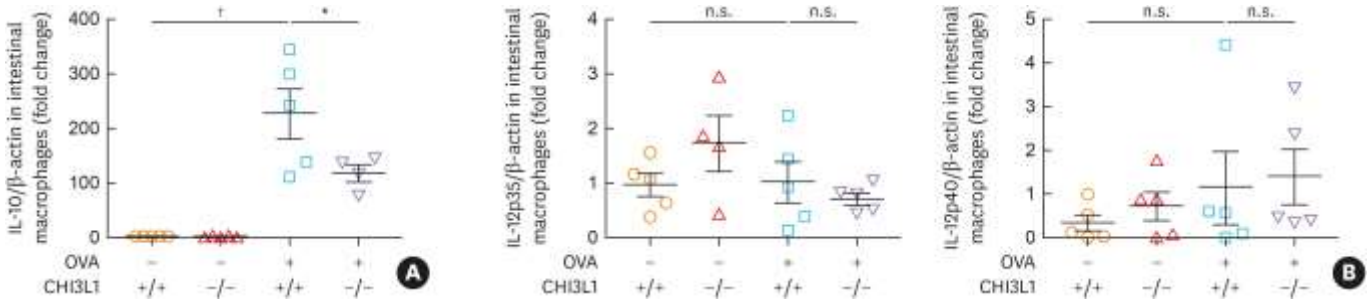

Supplementary Fig. S3. Th2 inflammation and M2 macrophage polarization are attenuated in OVA-treated CHI3L1$^{-/-}$ mice. mRNA expression levels of (A) IL-10 (Th2-differentiating cytokine or M2 macrophage cytokine) and (B) IL-12p35 and IL-12p40 (Th1-differentiating cytokine or M1 macrophage cytokine) in the intestinal macrophages from WT and CHI3L1$^{-/-}$ mice assessed by real-time PCR. Data are presented as mean ± SEM and representative of at least three independent experiments (n = 4-5 mice per group).

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