The role of chitinase 3-like 1 in atopic dermatitis

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Directed by Professor Myung Hyun Sohn

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졸업과 동시에 인생이 퍽퍽해진다는 명언 및 제가 올바른 길로 나아갈 수 있도록 조언을 해주신 강상욱 교수님, 중학교부터 고등학교까지 제 학창기 시절을 같이 보낸 이제는 편하게 고민을 털어놓을

수 있는 친구 같은 신지은 선생님께 감사 드립니다.

학위 생활을 같은 곳에서 하지는 않았지만 마치 옆에 있는 듯 나오라고 부르면 언제든지 나와서 같이 맛있는 음식 먹으며 스스럼없이 모든 이야기를 나누고 공감해준 민혜, 항상 제 입장에서 생각해주고 이해해주며 명쾌한 해결책을 제시해 준 늘 실패하지 않는 태희 스티로드 태희언니, 어엿한 직장인이 되어 이제는 대학생 때처럼 자주 만나지는 못해도 제가 힘들어 할 때면 늘 응원해주고 위로해준 안주와 다혜, 언제나 제 이야기를 잘 들어주고 겉모습은 그렇지 않지만 누구보다 따듯한 마음을 가진 현민오빠, 늘 소소한 재미를 안겨준 영덕이, 바빠서 자주 만나지는 못하지만 만나면 늘 수다삼매경에 빠지는 도연언니와 희경언니에게도 고마운 마음을 전하고 싶습니다.

제가 잘될 수 있도록 항상 잊지 않고 기도해주시고 친할머니와 같은 마음으로 챙겨주신 박명자 권사님과 이은경 집사님께도 감사 드립니다.

끝으로 학위과정 중 한결같이 제 옆을 든든하게 지켜주며 다독여준 태문오빠, 인생의 선배로서 늘 조언해주시고 제가 잘 마무리 하기까지 북돋아 주시며 믿고 기다려 주신 부모님과 은종이 오빠에게도 감사한 마음을 전하고 싶습니다.

마지막으로 다시 한번 모든 분들께 진심으로 감사 드립니다.

2019년 6월

곽은지

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ABSTRACT

The role of chitinase 3-like 1 in atopic dermatitis

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Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by defective skin barrier and Th2 immune responses. Chitinase 3-like 1 (CHI3L1), also known as breast regression protein 39 (BRP-39) in mice and human homologue YKL-40, plays important roles in Th2 inflammation and allergen sensitization. CHI3L1 has been implicated in a variety of diseases including asthma characterized by inflammation, apoptosis, and tissue remodeling, but its role in AD remains elusive. The aim of this study was to investigate the role of CHI3L1 in the development and progression of AD.

We investigated YKL-40 level in the serum and skin of AD patients by ELISA and immunofluorescence, respectively. Using a murine model of AD induced by ovalbumin (OVA), we investigated Th2 immune responses, M2 macrophage activation, and skin barrier gene expression using wild-type (WT) and BRP-39 null mutant (BRP-39$^{-/-}$) mice.

YKL-40 level was significantly increased in the serum of AD patients. In addition, both mRNA and protein expression levels of BRP-39 were higher in OVA-sensitized WT mice than in control mice. OVA-sensitized BRP-39$^{-/-}$ mice showed decreased epidermal thickness, lower total serum IgE, Th2 cytokine levels, and CD4^{+} effector T cell populations than OVA-sensitized WT mice. Induction of BRP-39 was dominant in dermal macrophages and BRP-39 promoted M2 macrophage activation. Consistently, the YKL-40 level in the skin of AD patients was higher than in normal subjects and it was expressed in dermal macrophages. BRP-39 deficiency attenuated dysregulation of skin barrier and tight junction genes.

These findings demonstrate that CHI3L1 mediates the development of AD induced by OVA, affecting Th2 inflammation, M2 macrophage activation and skin barrier function.

Key words: atopic dermatitis, chitinase 3-like 1, skin barrier, type 2 immunity

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I. INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disorder, with a prevalence rate of 2% to 10% in adults and up to 30% in pediatric population worldwide.[1] It is characterized by complex interactions between environmental and genetic factors, including a skin barrier defect, Th2 inflammation with elevated serum IgE and eosinophilia.[2,3] Classically, AD is associated with the Th1/ Th2 immune response, however, there is growing evidence that Th17 and Th22 inflammatory responses are also involved.[4,5] One of the most well-known risk factors for AD is a mutation of *filaggrin (Flg)* gene which is important for skin barrier.[6] During epidermal

differentiation, approximately over 400kDa of profilaggrin is dephosphorylated and cleaved into monomeric filaggrin (37kDa). These free filaggirn monomers functions to aggregate and collapse of keratin filament to form impermeable skin barrier.[7] Flaky tail mice (*Flg*^{ft} mice), analogous to *Flg* gene mutation in human AD patients, exhibit spontaneous skin inflammation.[8] These mice have genetic mutations of *Flg* and *matted* gene. *Tmem79 (ma/ma)* mutation causes spontaneous dermatitis and deficiency of *Flg* express an abnormal profilaggrin protein which is incapable of being proteolytic processed to filaggrin monomers.[9]

Chitin, a component of fungal cell walls and the exoskeletons of insects, protects organisms from the harsh environment.[10] Chitinase 3-like 1 (CHI3L1) belongs to chitinase-like proteins (CLPs) which do not have enzymatic activity to directly degrade chitin.[11] CHI3L1, also known as breast regression protein 39 (BRP-39) in mice and human homologue YKL-40, are expressed in a variety of immune cells (e.g., macrophages and neutrophils) and structural cells (e.g., endothelial cells and smooth muscle cells).[12,13] Previously, CHI3L1 has been shown to enhance Th2 allergic inflammation in a point of dendritic cell (DC) accumulation and alternative macrophage (M2) activation in a murine model of asthma induced by ovalbumin (OVA).[14] Elevated serum levels of YKL-40 have been correlated with various diseases characterized by inflammation, infection, apoptosis and tissue remodeling such as bacterial infections, rheumatoid arthritis, inflammatory bowel disease and asthma.[10,15] YKL-40 has been shown to play a pivotal role in asthma, and has been indicated as a biomarker of disease

severity, as well as a therapeutic target.[16] However, the biological functions of CHI3L1 in AD has not been addressed yet.

As noted above, AD is characterized not only by Th1/2 imbalance, but also by dysregulation of various immune cells, including macrophages, which play roles in phagocytosis, tissue healing, and the regulation of adaptive immunity. Although the roles of M2 macrophages induced by Th2 cytokines are not yet fully understood, it is known that they contribute to protection from parasitic infections, promote Th2 immune responses, and dampen excessive inflammation.[17-19] A previous study reported that excessive activation of macrophages can lead to the development of chronic inflammation, including psoriasis and AD.[20] However, the in vivo roles of M2 macrophages in AD remain elusive.

In the present study, we hypothesized that CHI3L1 is involved in AD. To clarify this issue, we examined the YKL-40 level in serum and skin of AD patients and compared the immune responses of OVA-sensitized wild-type (WT) and BRP-39 null mutant (BRP-39$^{-/-}$) mice. We found that CHI3L1 affects the Th2 immune response, M2 macrophage activation and skin barrier function during the development of AD.

II. MATERIALS AND METHODS

1. Human subjects

A total of 68 children who visited the allergy clinic at Severance Children's hospital for treatment of AD or for a general health check-up between May, 2013 and December, 2017 were enrolled in this study. Children with AD met the revised Hanifin and Rajka criteria[21], and reported no previous symptoms of other allergic diseases such as asthma or allergic rhinitis. Healthy controls had no history of any allergic or inflammatory disease. After obtaining consent, blood samples were drawn from all children during their first visit. Total eosinophil numbers were determined using a hematology analyzer (NE-8000; Sysmex, Kobe, Japan), and IgE level was measured using the Pharmacia CAP assay (Uppsala, Sweden). This study was approved by the institutional review board of Severance Hospital, and written informed consent was obtained from participants or their parents (protocol no. 4-2004-0036).

Skin biopsy samples were obtained from 2 healthy controls and 2 AD patients who were diagnosed according to the criteria of Hanifin and Rajka.[21] This study was approved by the Institutional Review Board of Yonsei University Health System, Severance Hospital (No. 4-2013-0624), and informed consent was obtained from all subjects before they participated in the study. This study protocol was performed in accordance with the ethical guidelines of the Korean Bioethics and Safety Act.

2. Animals

Female C57BL/6 mice (7 to 10 weeks old) were purchased from Orient Bio (Sungnam, Korea). BRP-39$^{-/-}$ mice were generated on a C57BL/6 background and maintained as previously described.[14] *Flg*^{ft} mice, which carry double-homozygous *Flg* and *matted (ma)* gene mutations,[8] with a BALB/c background were kindly provided by Kenji Kabashima (Kyoto University Graduate School of Medicine, Kyoto, Japan) and we backcrossed these with C57BL/6 mice over seven generations. For the OVA-induced AD model, *Flg*^{ft} mice were backcrossed with BRP-39$^{-/-}$ mice. All experiments in this study were conducted according to the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Experiment provided by the Institutional Animal Care and Use Committee and were approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System.

3. Epicutaneous sensitization

An experimental murine model of atopic dermatitis was induced using ovalbumin (OVA) (grade V; Sigma-Aldrich, St. Louis, MO). Mice were anesthetized with isoflurane (Hana Pharm, Sungnam, Korea) and shaved with an electric shaver. The shaved dorsal skin was tape-stripped with cellophane tape (Nichiban, Tokyo, Japan) eight times. After tape-stripping, mice were epicutaneously sensitized with 100 μg OVA in 100 μl normal saline. Mice had a total of three 1-week exposures to the patch,

separated by 2-week intervals as previously described.[22] Mice were sacrificed after the third sensitization (day 50).

4. Clinical score and Transepidermal water loss (TEWL)

TEWL was measured using a vapometer (Delfin, Kuopio, Finland) 1 week after each sensitization period, and clinical scores were recorded. The severity of lesional skin was clinically scored based on macroscopic criteria, as described previously.[23] In brief, the criteria of scoring were erythema, edema, erosion, and scaling (0, no symptoms; 1, mild; 2, moderate; 3, severe). The total clinical score was calculated as the sum of individual scores for each parameter.

5. Histology and Immunohistochemistry

The dorsal skin of mice was fixed in 10% formaldehyde, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described,[14] using a polyclonal anti-CHI3l1 antibody (Invitrogen, Carlsbad, CA) or normal rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) diluted 1:100 in antibody diluent (Dako, Carpinteria, CA).

6. Quantitative real-time PCR

Skin total mRNA was extracted with TRIzol reagent (Invitrogen) and cDNA was synthesized as previously described.[24] Quantitative real-time PCR was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with

AccuPower 2X Greenstar qPCR Master Mix (Bioneer, Oakland, CA) according to the manufacturer's instructions. Target gene mRNA levels were calculated relative to β-actin.

7. ELISA

Serum total and OVA-specific IgE levels were determined, as previously described.[25] Mouse chitinase 3-like 1 DuoSet ELISA (R&D Systems, Minneapolis, MN) was used to assess mouse BRP-39 level and Human Chitinase 3-like 1 DuoSet ELISA (R&D Systems) was used for human YKL-40 level.

8. Western blotting

The epidermis and dermis were separated by incubating the skin at 4°C overnight in 4mg/mL dispase solution. Then, proteins in epidermis were extracted in radioimmunoprecipitation assay (RIPA) buffer with HALT protease inhibitor cocktail (both from Thermo Fisher Scientific, Waltham, MA). Subsequently, 30 μg of proteins was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Western blotting was carried out as previously described,[26] using rabbit antibodies to filaggrin (1: 1,000; Biolegend, San Diego, CA), β-actin (1: 1,000; Cell Signaling Technology, Danvers, MA), HRP conjugated anti-rabbit secondary antibody (1: 1,000; Santa Cruz Biotechnology).

9. Immunofluorescence

Paraffin-embedded mouse and human skin sections were deparaffinized and rehydrated, and heat retrieval was performed for 20 min at 95°C, followed by blocking with 5% bovine serum albumin (Sigma-Aldrich) for 30 min at room temperature. Next, mouse skin samples were incubated with anti-CHI3l1 antibody (Invitrogen), and anti-F4/80 antibody (Abcam, Cambridge, UK) overnight at 4°C. After washing, this was followed by a 1 hr incubation at room temperature with matched secondary antibodies: Alexa Fluor 555-conjugated goat anti-rabbit IgG (Abcam) and Alexa Fluor 488-conjugated donkey anti-rat IgG (Abcam). Cell nuclei were stained with DAPI (Thermo Fisher Scientific) for 10 min.

For triple immunofluorescence staining, human skin sections were incubated for a 1 hr with anti-CHI3l1 (Invitrogen) overnight at 4 °C. After washing, sections were stained with Alexa Fluor 555-conjugated goat anti-rabbit IgG (Abcam). Then, sections were washed and stained with a mixture of anti-CD163 (Abcam), and anti-Factor FXIIIa (Abcam) antibodies. After washing, sections were incubated with Alexa Flour 647-conjugated donkey anti-mouse IgG (Abcam), and Alexa Flour 488-conjuagted anti-goat IgG (Abcam) antibodies. Sections were mounted in VECTASHIELD mounting media with DAPI (Vector Laboratories, Burlingame). Images were captured with confocal microscopes (LSM 700, and LSM 780; Zeiss, Oberkochen, Germany) using Zen software.

10. Flow cytometry

Single-cells (1×10^6 of cells) from mouse spleens and lymph nodes were isolated and analyzed by a FACSVerse flow cytometer (BD Biosciences) as previously described,[26] using monoclonal antibodies for CD3, CD4, CD44 and CD62 ligand (CD62L) (all from eBioscience, San Diego, CA). Dead cells were excluded with viability dye eFluor780 (eBioscience) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

11. Microarray gene expression analysis

Microarray gene expression analysis was performed using Affymetrix Mouse Gene 2.0 ST Array chips (Affymetrix, Santa Clara, CA). The data were summarized and normalized using the robust multi-average (RMA) method implemented in Affymetrix® Power Tools (APT). Gene-enrichment and functional annotation analysis for the significant probe list was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Statistical tests and visualization of differentially expressed genes were conducted using R 3.3.2.

12. Statistical analysis

Statistical analyses for human data were conducted with R (version 3.3.2; R Foundation for Statistical Computing, Vienna, Austria). Continuous data were reported as the mean (± standard deviations) or median (interquartile range). Comparisons of two groups were performed by using the Student's *t*-test or Mann-Whitney U test for

continuous variables, and the chi-square test or Fisher's exact test was used to analyze categorical variables. Correlations between YKL-40 levels and total serum eosinophil counts, or total IgE levels were analyzed using Spearman's rank correlation test.

Data are presented as the mean ± standard error of the mean (SEM). Analysis of variance was evaluated using the Student's *t*-test for two groups, and one-way ANOVA followed by the Turkey test for 3 or more groups. P-value <0.05 was considered statistically significant.

III. RESULTS

1. YKL-40 expression is increased in the serum of AD patients

To investigate the relationship between CHI3L1 and AD, we measured YKL-40 levels in the serum of pediatric AD patients and healthy controls. The clinical characteristics of the study subjects are summarized in Table 1. The two groups did not differ in age and sex. Total serum eosinophil counts and IgE levels were significantly higher in the AD group than in the healthy controls. Serum levels of YKL-40 were also significantly elevated in children with AD, compared to healthy controls (63.1 ± 11.9 ng/mL vs. 52.7 ± 11.2 ng/mL, $P < 0.001$; Figure 1. A). Moreover, YKL-40 levels were positively correlated with both total eosinophil count ($r = 0.390$, $P = 0.002$) and total IgE levels ($r = 0.312$, $P = 0.015$).

2. BRP-39 expression levels are altered in a murine model of AD induced by OVA

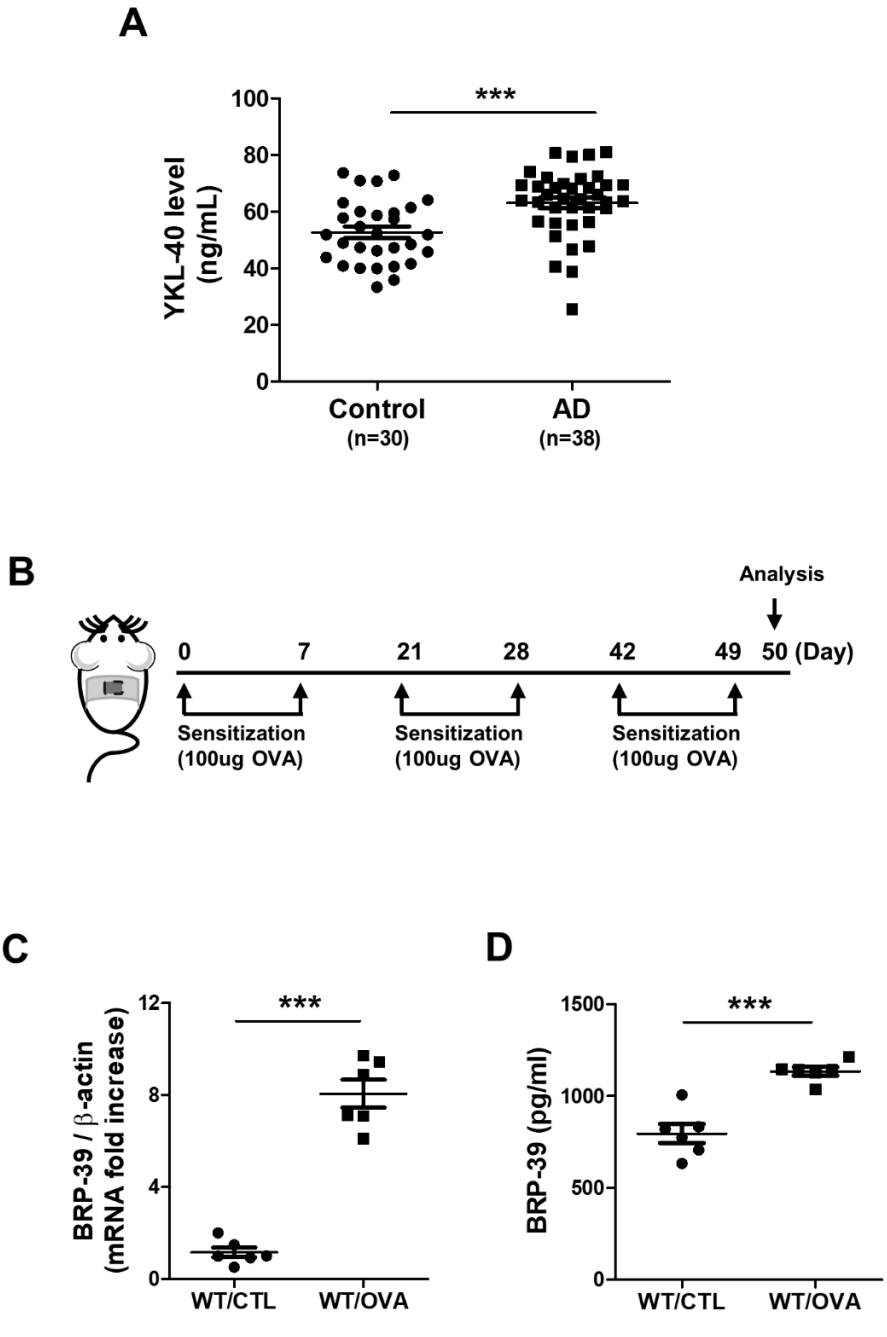
To define the role of BRP-39, we used an OVA-induced AD mouse model, mimicking acute lesions of human AD characterized by Th2-dominated immune responses.[22] Mice were treated a total of three times with the OVA patch, for 1 week each time at 2-week intervals. (Figure 1. B). After the final sensitization, both mRNA and protein levels of BRP-39 were found to be higher in OVA-sensitized WT mice than in control mice (Figure 1. C, D). In addition, infiltrated inflammatory cells expressing BRP-39 were predominantly observed in the dermis of OVA- sensitized skin, as detected by immunohistochemistry (Figure 1. E, F). These findings suggest that BRP-39 is involved in AD induced by OVA.

Table 1. Characteristics of subjects

	Control (n = 30)	AD (n = 38)	*P*-value
Age (years)	6.8 (1.7-10.7)	3.3 (1.4-7.3)	0.099
Sex, M (%)	17 (56.7)	22 (57.9)	> 0.099
Blood eosinophil (/μL)	190.0 (130.0-250.0)	760.0 (465.0-1385.0)	< 0.001
Serum total IgE (IU/mL)	24.9 (17.2-33.1)	875.0 (394.0-2072.0)	< 0.001

AD, atopic dermatitis; IgE, immunoglobulin E

Data are given as number (%), mean (± standard deviation), or median (interquartile range), as appropriate.



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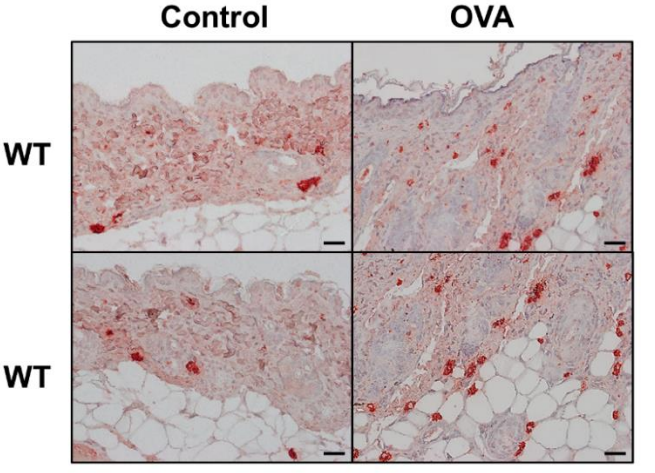

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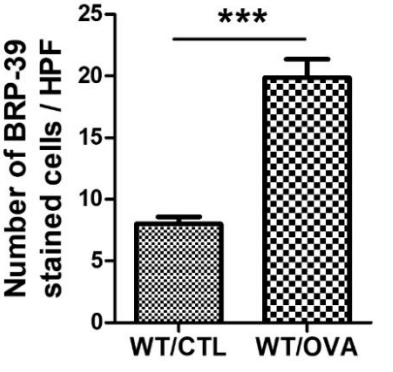
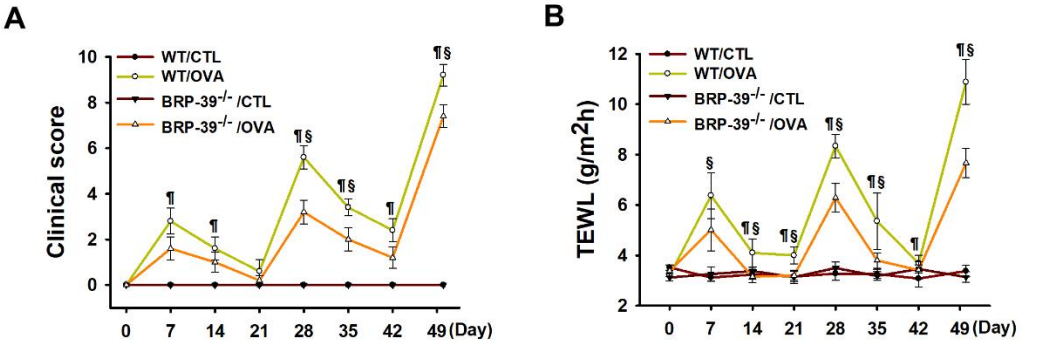
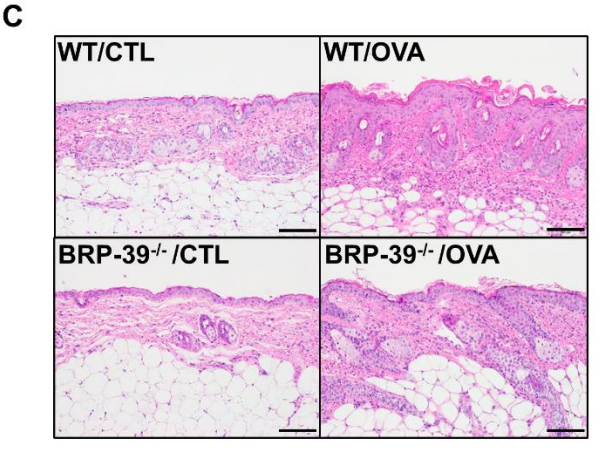


Figure 1. Increased YKL-40 expression level in pediatric atopic dermatitis patients and a murine model of AD induced by OVA. (A) YKL-40 levels were measured in children with atopic dermatitis (n=38) and healthy controls (n=30) by ELISA. (B) Mice were epicutaneously sensitized with 100ug of OVA a total of three 1 week exposures. (C) Increased BRP-39 mRNA and (D) protein expression level in OVA-sensitized WT mice. (E) Representative IHC of BRP-39 and (F) quantification of positive cells per HPF (400× magnification). Results represent the mean ± SEM of two independent experiments. Scale bars;100μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AD; atopic dermatitis, CTL; control, OVA; ovalbumin, WT; wild-type, ELISA; enzyme-linked immunosorbent assay, IHC; immunohistochemistry, HPF; high-power field.

3. BRP-39 alters Th2 immune responses in OVA-induced AD

As previously mentioned, BRP-39 plays an important role in Th2 inflammation.[14] BRP-39$^{-/-}$ mice sensitized with OVA showed less severe characteristics of AD as demonstrated by a decreased clinical score, TEWL, and epidermal thickness compared to OVA-sensitized WT mice (Figure 2. A-C). Total and OVA-specific IgE serum levels, as well as the skin mRNA levels of Th2 cytokines such as IL-4, IL-5, and IL-13 were diminished in BRP-39$^{-/-}$ mice treated with OVA (Figure 2. D, E). Additionally, OVA-sensitized BRP-39$^{-/-}$ mice had reduced CD4^{+} effector T cell populations ($CD3^{+}CD4^{+}CD44^{high}CD62L^{low}$) in the skin-draining lymph node (s-dLN) and spleen compared to OVA-sensitized WT mice, as analyzed by flow cytometry (Figure 3. A-C). These findings demonstrate that BRP-39 mediated the development of OVA-induced AD, partly via the upregulation of the Th2 inflammatory responses.





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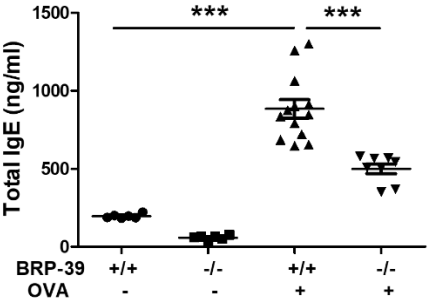

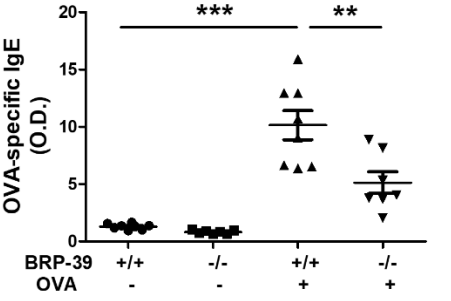

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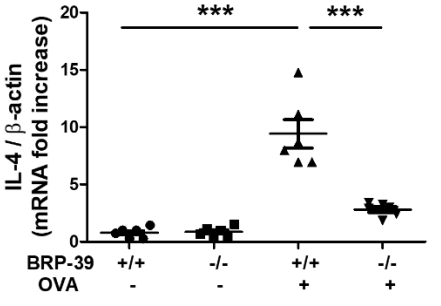
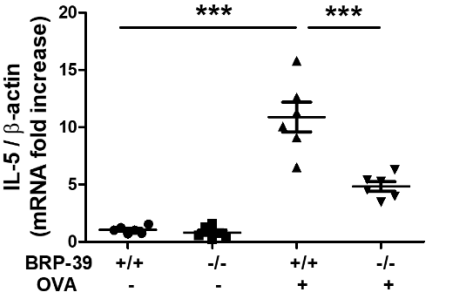




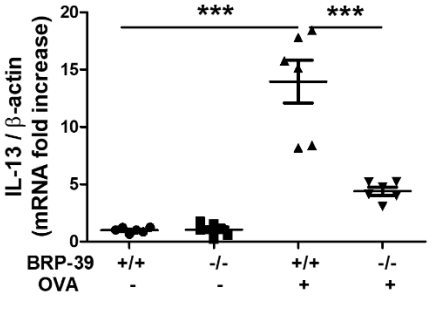
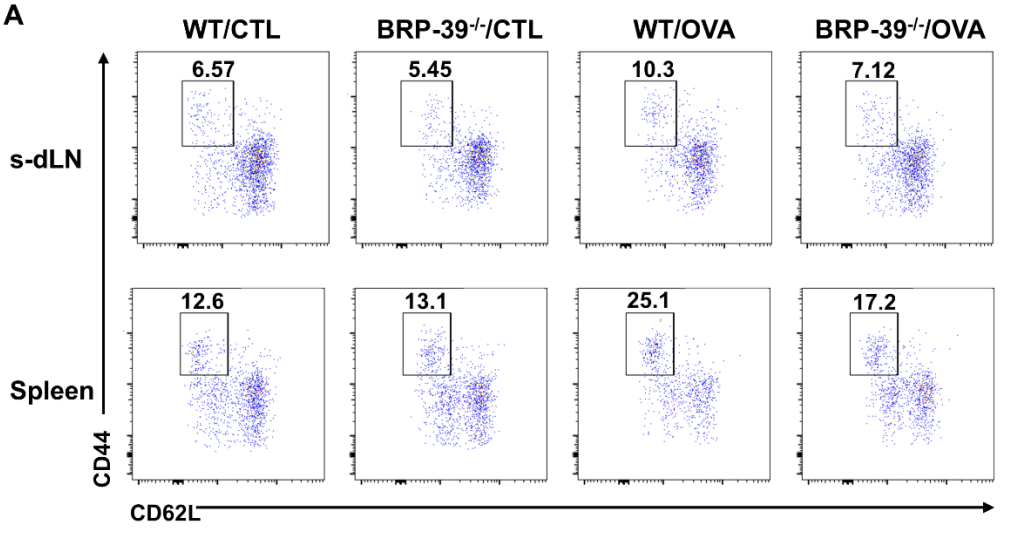
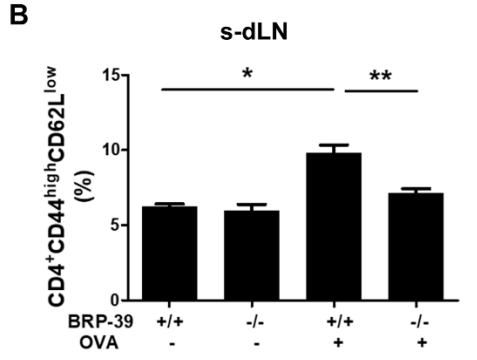


Figure 2. Attenuated Th2 inflammatory responses to OVA-induced AD in BRP-39$^{-/-}$ mice. (A) Clinical score, (B) TEWL level were measured in OVA-sensitized WT and BRP-39$^{-/-}$ mice. (C) H&E staining (200× magnification), (D) Levels of total IgE and O.D values of OVA-specific IgE in mouse serum measured by ELISA. (E) The mRNA expression level of Th2 cytokines in lesional skin of the mice. Results represent the mean ± SEM of three independent experiments in a minimum of 5 mice in each group. Scale bars;100μm. ¶$P < 0.05$ (WT/CTL vs WT /OVA). §$P < 0.05$ (WT/OVA vs BRP-39$^{-/-}$ /OVA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. TEWL; transepidermal water loss, O.D; optical density, OVA; ovalbumin, AD; atopic dermatitis, H&E; hematoxylin and eosin, IgE; immunoglobulin E, Th2; T helper 2.





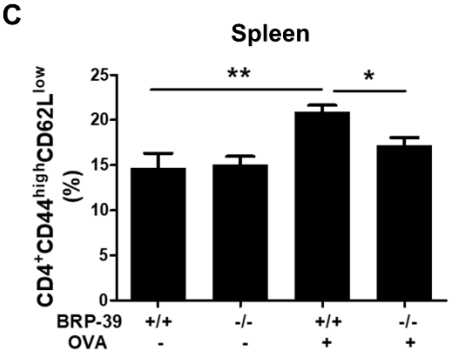


Figure 3. Decreased $CD4^+$ effector T cell population in OVA-sensitized BRP-$39^{-/-}$ mice. (A) A representative dot plot of effector T cell populations ($CD4^+CD44^{high}CD62L^{low}$) in s-dLN and spleen of OVA-sensitized WT and BRP-$39^{-/-}$ mice. (B) Data of flow cytometry in s-dLN showed as a graph. (C) Data of flow cytometry in spleen showed as a graph. Results represent the mean ± SEM of three independent experiments in a minimum of 4 mice in each group. * $P < 0.05$, ** $P < 0.01$. OVA; ovalbumin, s-dLN; skin-draining lymph node, OVA; ovalbumin, WT; wild-type.

4. BRP-39/YKL-40 is expressed in dermal M2 macrophages

We next examined the expression of YKL-40 in the skin of AD patients and normal subjects. Strikingly, the abundance of YKL-40^{+} dermal cells was higher in AD patients than in healthy controls. Transglutaminase factor XIIIa (FXIIIa) is known to identify tissue resident macrophages in situ,[27] we found YKL-40 was expressed in FXIIIa^{+} human dermal macrophages (Figure 4. A).

M2 macrophages are known to drive Th2 inflammation in allergic airway inflammation.[18,19] Given that BRP-39 from macrophages has been intensively studied with regard to lung diseases, we sought to clarify the main source of BRP-39 in the skin. Notably, BRP-39 was produced by dermal F4/80^{+} macrophages in the skin (Figure 4. B). These macrophages were found to be M2 macrophages marked by CD206 (Figure 4. C). We next compared the mRNA levels of iNOS for M1 macrophages, and CD206, Ym1/2, and Fizz1 for M2 macrophages, in OVA-sensitized WT and OVA-sensitized BRP-39$^{-/-}$ mice. While the mRNA levels of iNOS were not significantly different, the levels of CD206, Ym1/2, and Fizz1 were significantly decreased in BRP-39$^{-/-}$ mice (Figure 4. D-F).

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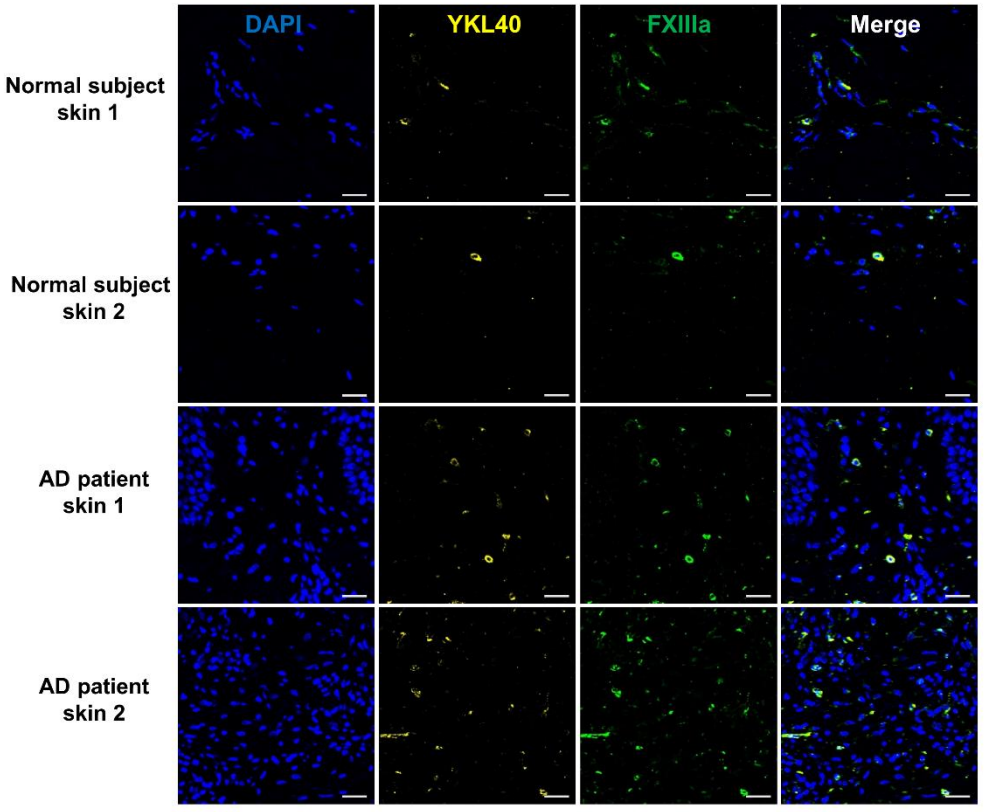

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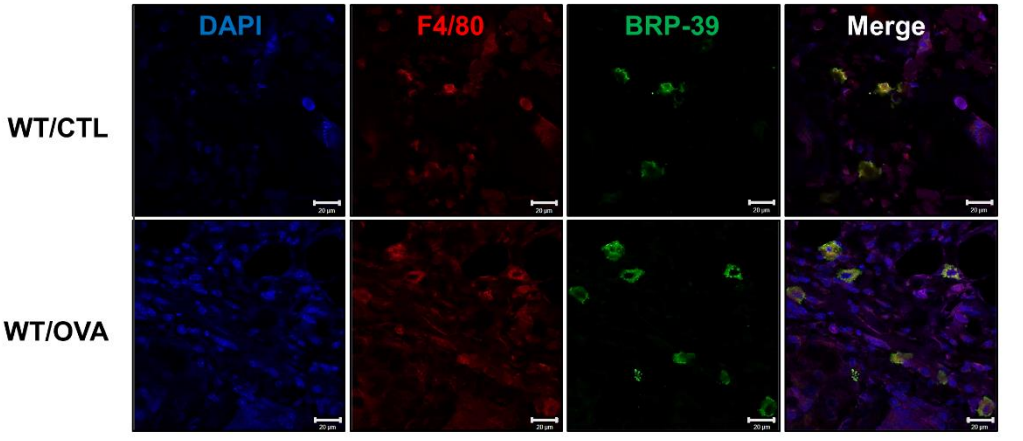

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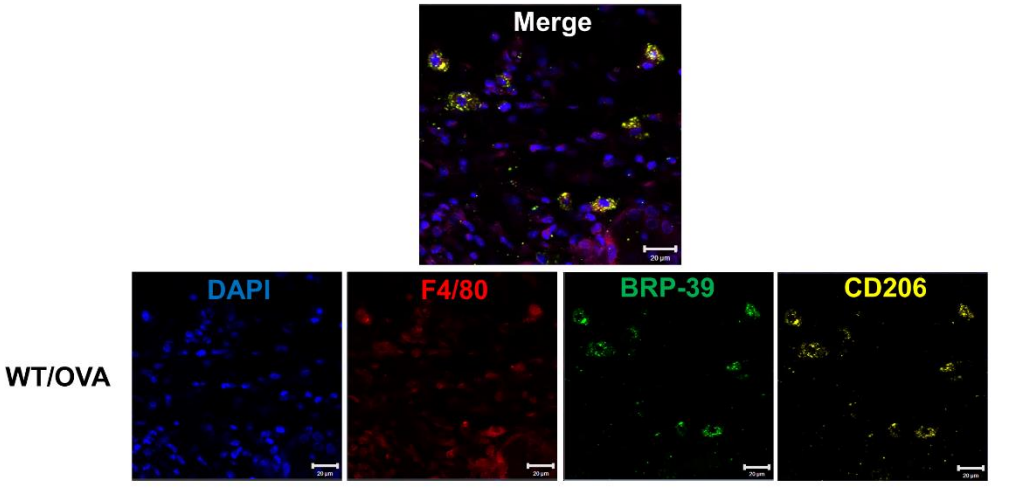


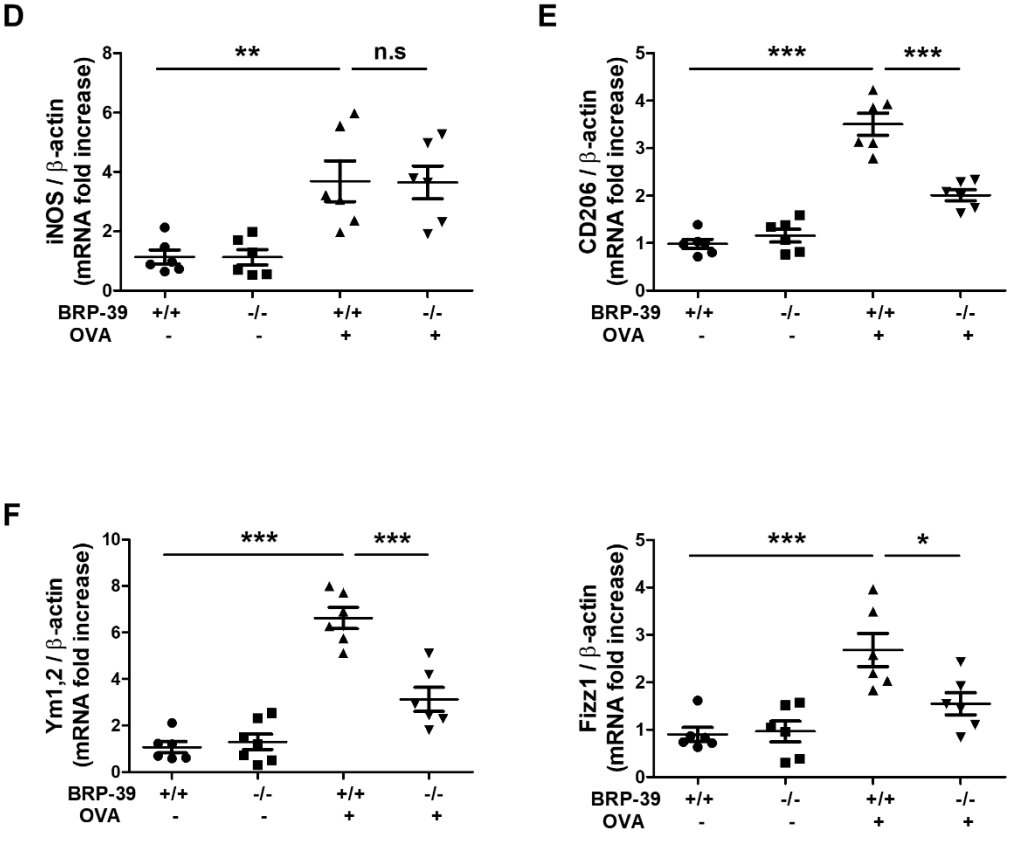
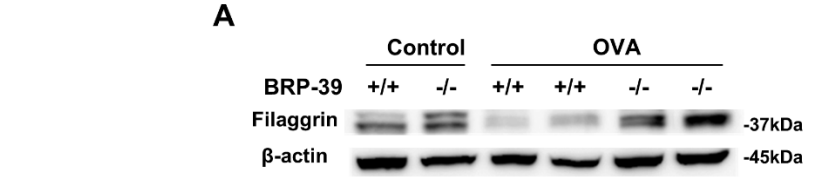
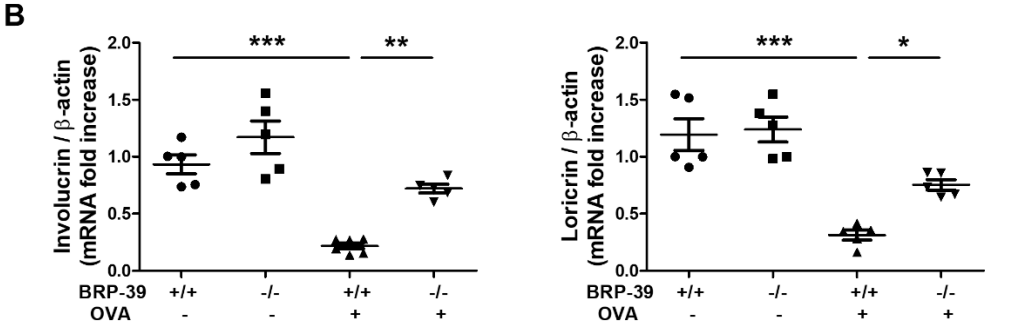


Figure 4. Expression of BRP-39/YKL-40 in dermal macrophages in the skin of mouse and human AD patients. (A) Immunofluorescence staining of $FXIIIa^+$ macrophages expressing YKL-40 in the skin of AD patients and normal subjects (400× magnification). (B) Immunofluorescence staining to identify dermal $F4/80^+$ macrophages expressing BRP-39, and (C) images of $CD206^+$ $F4/80^+$ macrophages expressing BRP-39 in OVA-sensitized WT mice (400× magnification). (D) The mRNA expression level of iNOS in lesional skin of the mice. (E) The mRNA expression level of CD206, and (F) Ym1,2, and Fizz1 in lesional skin of the mice. Results represent the mean ± SEM of three independent experiments in a minimum of 4 mice in each group. Scale bars;20μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.s = not significant. OVA; ovalbumin

5. Dysfunction of genes encoding epidermal differentiation complex and tight junction is attenuated in BRP-39$^{-/-}$ mice

We next evaluated the expression of skin barrier genes, including those of the epidermal differentiation complex and tight junctions. Tight junction proteins such as claudin (CLDN)-1 and zonula occludens (ZO)-1 have a complex structure which enables the passage of water, ions, and solutes in the epithelium.[28] We assessed the level of FLG protein expression, which plays a critical role in maintaining the skin barrier. The FLG protein levels were higher in BRP-39$^{-/-}$ mice treated with OVA than in WT mice treated with OVA (Figure 5. A). We assessed the mRNA expression levels of loricrin (LOR), and involucrin (INV) for epidermal barrier function, and of ZO-1, and CLDN-1 for tight junctions in lesional skin of WT and BRP-39$^{-/-}$ mice. Interestingly, expressions of LOR, INV, CLDN-1, and ZO-1 was higher in OVA-sensitized BRP-39$^{-/-}$ mice than in OVA-sensitized WT mice (Figure 5. B, C). Taken together, these findings suggest that BRP-39 affects not only Th2 immune responses, but also skin barrier integrity.





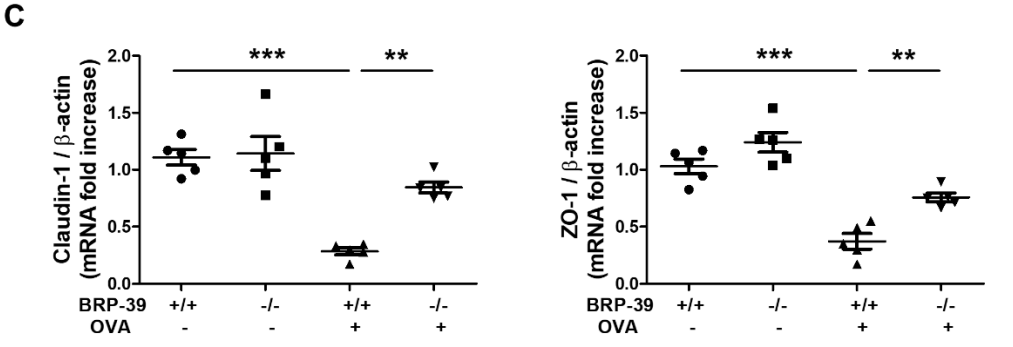
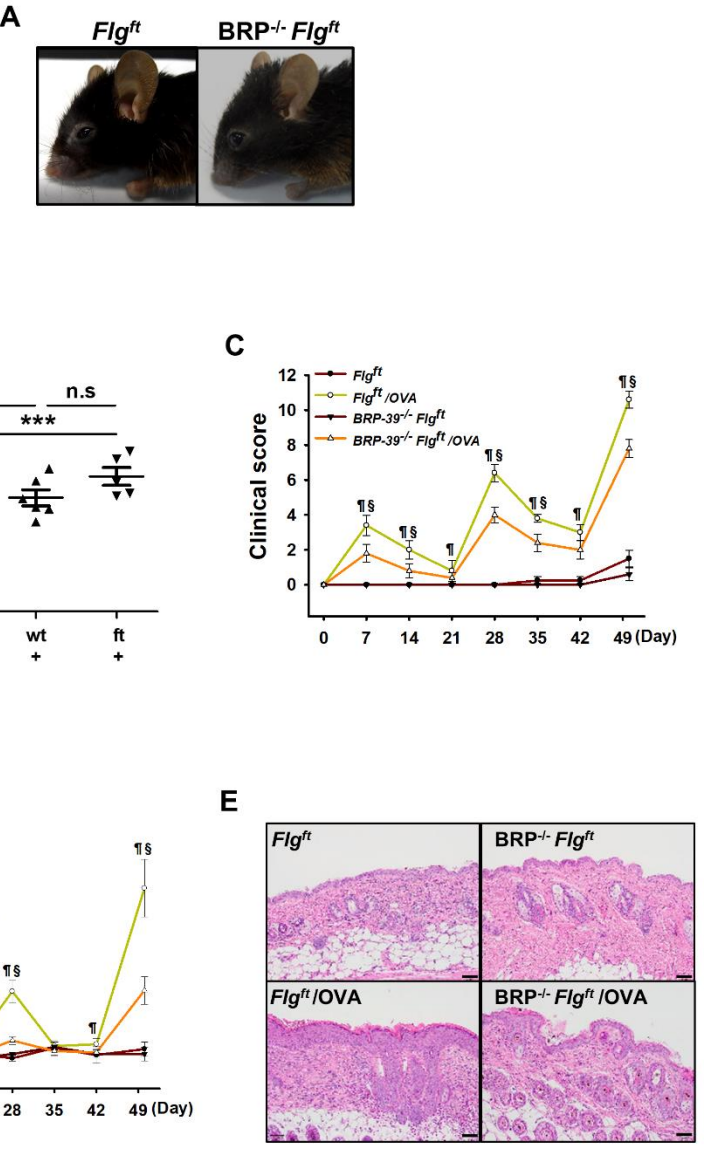


Figure 5. Regulation of skin barrier dysfunction in OVA-sensitized BRP-39$^{-/-}$ mice. (A) Western blot analysis of FLG in epidermal extract of WT and BRP-39$^{-/-}$ mice. (B) The mRNA expression level of INV and LOR, (C) CLDN-1 and ZO-1 in lesional skin of WT and BRP-39$^{-/-}$ mice measured by real-time PCR. Results represent the mean ± SEM of three independent experiments in a minimum of 4 mice in each group. Scale bars; 50μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OVA; ovalbumin, FLG; filaggrin, CLDN-1; claudin-1, ZO-1; zonulae occludens-1, LOR; loricrin, INV; involucrin WT; wild-type.

6. BRP-39 deficiency leads to diminished Th2 inflammatory responses in *Flg*^{ft} mice

To further investigate whether BRP-39 affects FLG expression, we crossed *Flg*^{ft} mice with BRP-39$^{-/-}$ mice with a B6 background. *Flg*^{ft} mice are known to exhibit increased TEWL, serum IgE and IgG1 levels, as well as IL-4 and Th17 expression in the skin.[29,30] The representative clinical phenotype of *Flg*^{ft} mice exhibited marked eczematous inflammation of the eyelid skin compared to BRP-39$^{-/-}$ *Flg*^{ft} (Figure 6. A). The mRNA level of BRP-39 was not significantly different between untreated *Flg*^{ft} mice and WT control mice, however, it was increased in both WT and *Flg*^{ft} mice treated with OVA (Figure 6. B). To clarify the effects of BRP-39 deficiency in *Flg*^{ft} mice, we epicutaneously sensitized *Flg*^{ft} and BRP-39$^{-/-}$ *Flg*^{ft} mice using OVA. *Flg*^{ft} mice treated with OVA showed significant increases of clinical score, TEWL and epidermal thickness compared to BRP-39$^{-/-}$ *Flg*^{ft} mice treated with OVA (Figure 6. C-E). Total serum and OVA-specific IgE levels, and Th2 type cytokine mRNA levels were also higher in *Flg*^{ft} mice treated with OVA than those in BRP-39$^{-/-}$ *Flg*^{ft} mice treated with OVA (Figure 6. F, G). These findings demonstrate that BRP-39 deficiency attenuated allergic skin inflammation in the *Flg*^{ft} AD mouse model.



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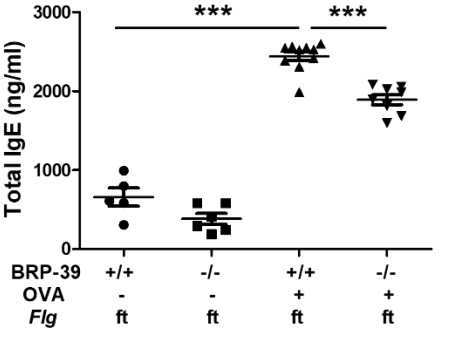

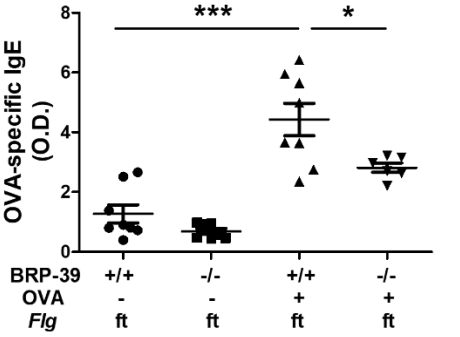

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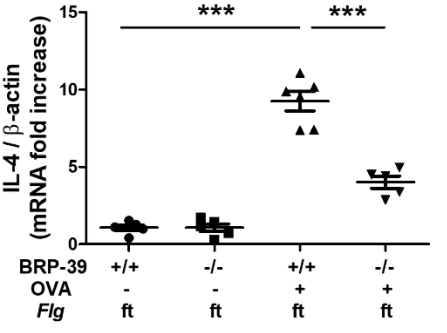
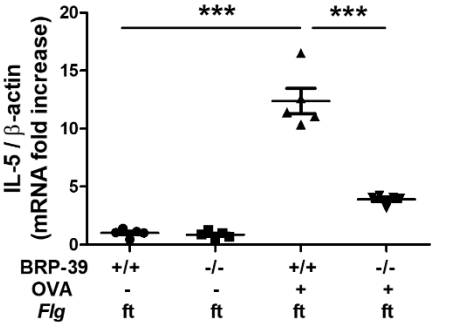




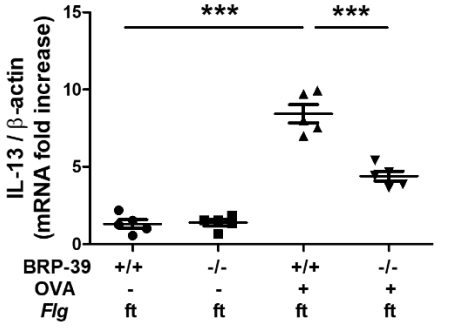


Figure 6. BRP-39 deficiency attenuated skin inflammation and Th2 responses in *Flg^{ft}* mice. (A) Gross phenotype of a representative *Flg^{ft}* and BRP-39$^{-/-}$ *Flg^{ft}* mice at 18 weeks. (B) The mRNA expression of BRP-39. (C) Clinical score. (D) TEWL level. (E) H&E staining (× 200). (F) Levels of total IgE and O.D values of OVA-specific IgE in mouse serum measured by ELISA. (G) The mRNA expression levels of Th2 cytokines in lesional skin of the mice. Results represent the mean ± SEM of two independent experiments in a minimum of 5 mice in each group. Scale bars; 50 μm. ¶P < 0.05 (*Flg^{ft}* vs *Flg^{ft}* /OVA). §P < 0.05 (*Flg^{ft}* /OVA vs BRP-39$^{-/-}$ *Flg^{ft}* /OVA). * P < 0.05, ** P < 0.01, *** P < 0.001. n.s. = not significant. WT; wild-type, TEWL; transepidermal water loss, H&E; hematoxylin and eosin, IgE; immunoglobulin E, O.D; optical density, OVA; ovalbumin, ELISA; enzyme-linked immunosorbent assay, Th2; T helper 2.

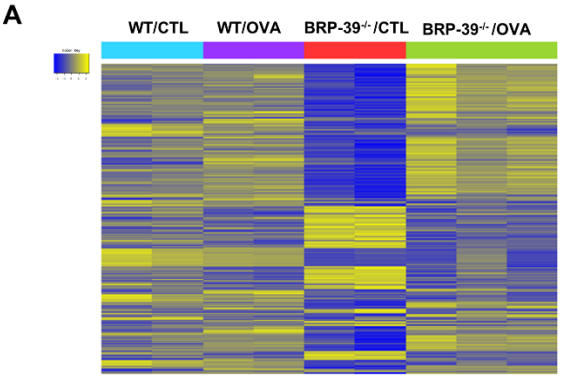
7. BRP-39 deficiency alters the transcriptome

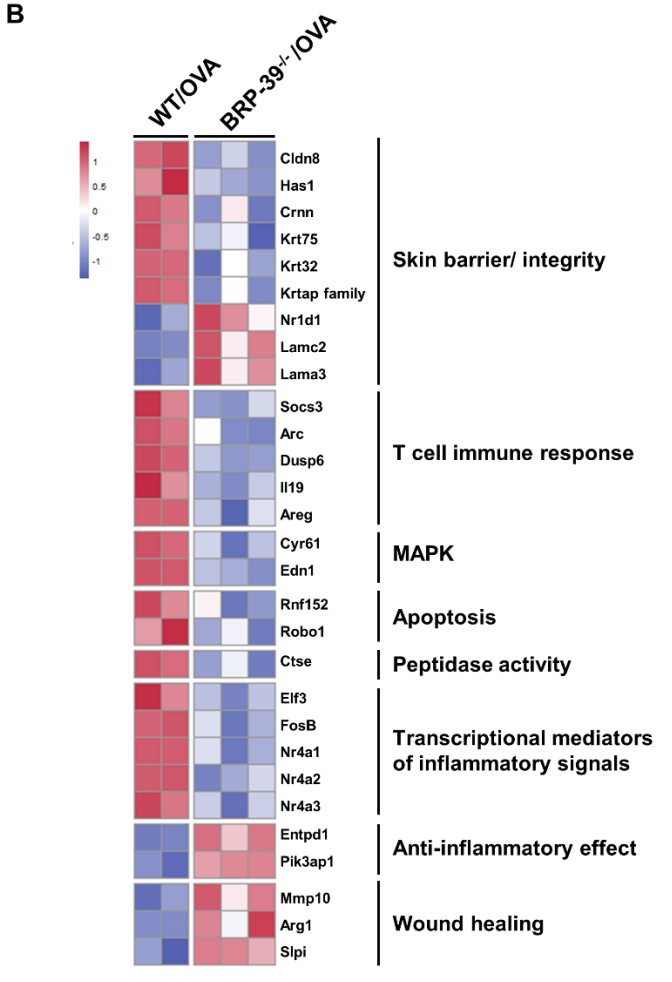
To further elucidate the inflammatory pathways influenced by BRP-39 in OVA-induced AD mouse model, we performed a microarray transcriptomic analysis. Hierarchical clustering of 1,671 altered genes (fold change >2, P-value <0.05) was shown in WT/CTL, WT/OVA, BRP-39$^{-/-}$ /CTL, BRP-39$^{-/-}$ /OVA (Figure 7. A).

To identify differentially expressed genes (DEGs), we excluded significant probes between WT and BRP-39$^{-/-}$ control mice, and then selected transcripts whose expression levels were significantly different (fold change >2, P-value <0.05) in OVA-sensitized WT and BRP-39$^{-/-}$ mice. BRP-39$^{-/-}$ mice treated with OVA showed reduced expression of genes related to skin barrier/ integrity (Cldn8, Has1, Crnn, Krt75, Krt32, Krtap family [including krtap27-1, krtap4-9, krtap1-3, krtap4-8, krtap26-1, krtap24-1, krtap13-1, krtap1-4, krtap5-5]), T cell immune response (Socs3, Arc, Dusp6, Il19, Areg), MAPK (Cyr61, Edn1), apoptosis (Rnf152, Robo1), peptidase activity (Ctse), and transcriptional mediators of inflammatory signals (Elf3, FosB, Nr4a1, Nr4a2, Nr4a3). However, skin barrier/ integrity (Nr1d1, Lamc2, Lama3), anti-inflammatory effect (Entpd1, Pik3ap1) and wound healing related (Mmp10, Arg1, Slpi) genes were increased in BRP-39$^{-/-}$ mice treated with OVA compared to WT mice treated with OVA (Figure 7. B).

Next, KEGG pathway analysis, was used to identify immune pathways involved. Of note, IL-17, TNF, C-type lectin receptor, cytokine-cytokine receptor interaction, Toll-like receptor (TLR), PI3K-Akt, NF-κB, chemokine signaling, mitogen-activated

protein kinase (MAPK), JAK-STAT, and NOD-like receptor (NLR) signaling pathways were significantly differed between OVA-sensitized WT and BRP-39$^{-/-}$ mice (Figure 7. C). The statistically significant genes in each category are described in Table 2.





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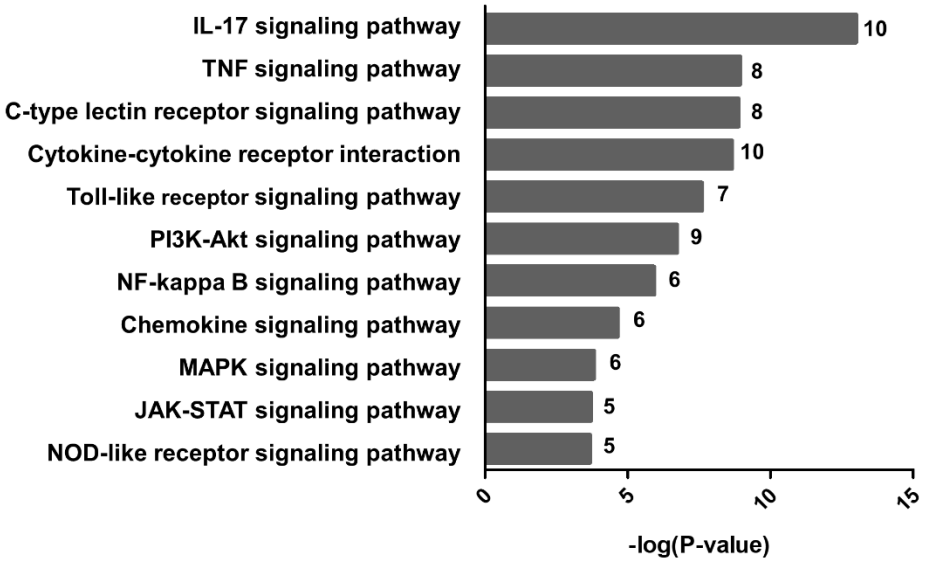

Figure 7. Microarray and KEGG pathway analysis of RNA from OVA-sensitized dorsal skins. (A) Hierarchical clustering of 1,671 genes altered (fold change>2, P-value <0.05) in OVA-induced AD mice model. Horizontal stripes represent genes, and columns show experimental samples. The average normalized value are shown in the heat map using yellow and blue color codes for up- and down-regulation, respectively. (B) Heatmap representing differences in expression for various genes related to immune and skin barrier integrity, as indicated (WT/OVA n=2, BRP-39$^{-/-}$ /OVA n=3 animals per group). (C) KEGG analysis of immune system and signal transduction. Bars represent –log (P-value), number of statistically significant gene is indicated in each category. *** $P < 0.001$. OVA; ovalbumin, KEGG; Kyoto Encyclopedia of Genes and Genomes.

Table. 2. Detailed analysis of KEGG pathways

Term	*P*-value	Number of DEGs	DEGs
IL-17 signaling pathway	9.94054E-14	10	Fosb, Fosl1, Il1b, Mmp9, S100a8, S100a9, Cxcl2, Tnf Cxcl3, Mmp1b
TNF signaling pathway	1.15681E-09	8	Socs3, Edn1, Il1b, Mmp9, Pik3r3, Cxcl2, Tnf, Cxcl3
C-type lectin receptor signaling pathway	1.30321E-09	8	Il1b, Clec4d, Pik3r3, Nlrp3, Tnf, Clec4e, Clec4n, Clec7a
Cytokine-cytokine receptor interaction	2.23832E-09	10	Ccr1, Csf3r, Il1b, Osm, Ccl3, Ccl4, Cxcl2, Tnf, Il19, Cxcl3
Toll-like receptor signaling pathway	2.50793E-08	7	Cd14, Il1b, Pik3r3, Ccl3, Ccl4, Spp1, Tnf
PI3K-Akt signaling pathway	1.86191E-07	9	Areg, Csf3r, Nr4a1, Lama3, Lamc2, Osm, Pik3r3, Spp1, Pik3ap1
NF-kappa B signaling pathway	1.17126E-06	6	Cd14, Il1b, Plau, Ccl4, Cxcl2, Tnf
Chemokine signaling pathway	2.26119E-05	6	Ccr1, Pik3r3, Ccl3, Ccl4, Cxcl2, Cxcl3
MAPK signaling pathway	0.00015121	6	Areg, Cd14, Nr4a1, Il1b, Tnf, Dusp6
Jak-STAT signaling pathway	0.000194097	5	Socs3, Csf3r, Osm, Pik3r3, Il19
NOD-like receptor signaling pathway	0.000207599	5	Il1b, Cxcl2, Nlrp3, Tnf, Cxcl3

DEGs, differentially expressed genes

IV. DISCUSSION

CHI3L1 belongs to CLPs which do not have enzymatic activity to directly degrade chitin, however, chitinase functions as a defensive enzyme against chitin-containing pathogen in lower organisms.[31,32] The biological activities of CHI3L1 have previously been studied in relation to regulation of cell proliferation, activation, migration and adhesion.[33]

In the present study, we demonstrated that CHI3L1 stimulates Th2 responses and M2 macrophage activation, thereby affecting the development of AD. We found YKL-40 expression was markedly higher in the serum and skin of AD patients compared to healthy controls, and that dermal macrophages express YKL-40 in the skin of AD patients. In line with these findings, both mRNA and protein expression levels of CHI3L1 were significantly increased in OVA-sensitized WT mice compared to control WT mice. Numerous studies have shown elevated serum levels of YKL-40 to be correlated with disease severity, and with poorer outcomes for many diseases, including cancer, psoriasis, rheumatoid arthritis, type 2 diabetes, liver fibrosis, atherosclerosis, and coronary artery disease.[11,15,34,35] CHI3L1 is considered a risk factor gene for allergic diseases, and has polymorphisms which correlate with asthma prevalence, and abnormal lung function.[36] Taken together, CHI3L1, promoting the expression of inflammatory mediators (e.g. CCL2, CXCL2, MMP-9), has been suggested as a pro-inflammatory biomarker and therapeutic target.[33]

In our murine model of AD induced by OVA, CHI3L1 deficiency resulted in the attenuation of allergic skin inflammation, and was accompanied by low Th2 cytokine levels and effector $CD4^+$ T cell populations, suggesting that CHI3L1 promotes Th2 immune responses. Consistent with this finding, a recent study demonstrated that BRP-$39^{-/-}$ $CD4^+$ T cells are prone to Th1 differentiation, and that they show reduced Th2 differentiation via an IFNγ signaling pathway.[37] Additionally, in the absence of CHI3L1, IL-18-induced pulmonary inflammatory responses were significantly ameliorated by down regulation of Th2 and Th17 responses.[38,39]

CHI3L1 is widely recognized as an important regulator of both M2 macrophage activation and Th2 inflammation. While M2 macrophages are known to stimulate Th2 inflammation in mouse models of allergic airway inflammation, they also play a role in inflammatory skin diseases such as psoriasis and AD.[40] A previous study reported elevated macrophage populations in acutely and chronically inflamed human AD skin.[41] In addition, M2 macrophages accumulation was reported in a murine model of contact hypersensitivity.[42]

Regarding the relationship between CHI3L1 and M2 macrophages, CHI3L1 has been found to stimulate respiratory syncytial virus (RSV)-induced airway inflammation, partly via M2 macrophage activation.[43] Our data suggests that dermal M2 macrophages expressing CHI3L1 synergistically promote Th2 immune responses in OVA-induced AD. Based on the findings of several in vitro studies, M2 macrophages have recently been subdivided into four sub-groups: M2a, M2b, M2c, and M2d.[42] Further studies

should be conducted to determine the functions of these different M2 macrophage subsets in atopic skin inflammation.

With respect to skin barrier defects, CHI3L1 deficiency attenuated dysregulation of FLG, LOR, INV, CLDN-1 and ZO-1. *Flg*^{ft} mice have genetic mutations of *Flg* and *ma* gene. The *Tmem79*$^{ma/ma}$ mutation causes spontaneous dermatitis, while the *Flg* mutation results in expression of an abnormal profilaggrin protein, which is incapable of being proteolytically processed to FLG monomers.[7,9] Macroscopic findings such as marked hair loss on the neck, matted hair and eczematous inflammation in the eyelid skin were ameliorated in a steady state of BRP-39$^{-/-}$ *Flg*^{ft} mice compared to *Flg*^{ft} mice. We confirmed that CHI3L1 deficiency attenuated skin inflammation in both OVA-induced WT AD mice and the *Flg*^{ft} AD mouse model. However, we observed that FLG deficiency did not affect the expression of CHI3L1, and vice versa. These results suggest that the expression of both proteins is independent, indicating that skin inflammation attenuated by CHI3L1 deficiency is mainly associated with the reduction of Th2 inflammation and M2 macrophage activation, rather than with the skin barrier function influenced by FLG deficiency.

A number of interacting molecules and signaling pathways involving CHI3L1 have been identified so far. A recent study reported that CHI3L1 binds to IL-13Rα2, composing a multimeric complex with IL-13.[44] Furthermore, CHI3L1 has been found to regulate hyperoxia-induced airway epithelial cell death via the MAPK and PI3K pathways.[45] In our murine model of OVA-induced AD, KEGG analysis showed the

TNF, TLR, PI3K-Akt, NF-κB, MAPK, JAK-STAT and NLR signaling pathways to be significant. The inflammatory mediators interacting with CHI3L1 in skin diseases should be further studied.

It is known that galectin-3 (gal-3), a member of β-galactosidase-binding lectins, expressed in many cell types such as epithelial cells, can directly interact with complex of IL-13Rα2 and CHI3L1, and competes with TMEM219 for IL-13Rα2 binding.[46] It leads to decreased CHI3L1-induced anti-apoptotic signaling in epithelial cells while promoting Wnt/β-catenin signaling in the lungs of *pale ear* mice (*HPS-1*$^{-/-}$). With regarding to gal-3, one study showed that OVA-treated gal-3$^{-/-}$ mice exhibited reduced epidermal thickness, lower eosinophil infiltration and lower serum IgE levels compared to WT mice.[47] These results suggest that gal-3 is important for the development of OVA-induced AD mice model, therefore, further studies needs to be done with respect to the interaction of gal-3 and CHI3L1 in AD.

In conclusion, we found the expression of YKL-40 to be significantly elevated in the serum and skin of AD patients. YKL-40 was expressed in dermal macrophages in the skin of AD patients. In a murine model of OVA-induced AD, CHI3L1 was found to be highly expressed in dermal macrophages, with its deficiency leading to attenuated allergic skin inflammation. Our findings reveal a critical role for CHI3L1 in the development of AD via Th2 inflammatory responses, M2 macrophage activation and skin barrier function.

V. CONCLUSION

In the present study, expression level of YKL-40 was highly increased in the serum and skin of AD patients and it was produced by dermal macrophages in the lesional skin. In a murine model of OVA-induced AD, CHI3L1 was found to be highly expressed is in dermal macrophages and its deficiency leads to attenuated allergic skin inflammation measured by clinical score, TEWL, Th2 cytokine levels, $CD4^+$ effector T cell populations, M2 macrophage activation and skin barrier gene expression. Consistently, OVA-sensitized BRP-$39^{-/-}$ Flg^{ft} mice showed attenuated skin inflammation compared to Flg^{ft} mice. Our results suggest that CHI3L1 plays critical roles in the development of AD via Th2 inflammatory responses, M2 macrophage activation and skin barrier function.

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ABSTRACT(in korean)

아토피피부염에서 Chitinase 3-like 1의 역할 규명

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곽 은 지

18-glycosyl-hydrolase family에 속하는 Chitinase 3-like 1 (CHI3L1)은 chitinase 활성이 결여된 39 kDa의 chitinase-like protein (CLP)을 암호화하고 있는 유전자로서 chitin을 합성하거나 대사작용을 할 수 없는 포유류에서도 진화적으로 보존되어왔다. CHI3L1은 마우스에서는 breast regression protein 39 (BRP-39), 인간에서는 YKL-40로 불리며 단핵구, 대식세포, 내피세포와 같은 다양한 세포에서 분비되며 염증, 세포자살 등을 특징으로 보이는 류마티스 관절염, 염증성 장 질환, 간 섬유증 그리고 천식과 같은 다양한 질병과 연관이 있다는 사실이 연구되었다. 이처럼 다양

한 질병과 상관관계를 보이는 CHI3L1은 천식에서 미치는 영향에 대해서는 많은 연구가 이루어졌지만 또 다른 알레르기 질환인 아토피 피부염에서의 그 역할은 아직 알려져 있지 않다. 따라서 본 연구에서는 아토피피부염 환자의 혈청과 피부 병변 조직에서 YKL-40의 발현 변화를 확인한 뒤 난백알부민으로 유도한 아토피피부염 마우스 모델을 통해 아토피 피부염에서 CHI3L1의 역할에 대해 규명하고자 하였다.

정상대조군과 비교하여 아토피피부염 환자의 혈청에서 YKL-40의 발현이 높게 증가되어 있었다. 난백알부민을 이용해 아토피피부염이 유도된 야생형 마우스의 피부 조직에서 BRP-39의 발현이 증가하였고, 야생형 마우스보다 BRP-39 결핍 마우스에서 홍반, 가려움, 부종, 표피의 두께와 경피 수분 손실도가 낮았다. 또한 야생형 마우스와 비교했을 때, BRP-39 결핍 마우스에서 혈청 면역글로불린 E와 피부조직에서의 Th2 사이토카인 (인터류킨-4, 인터류킨-5, 인터류킨-13) 의 발현량이 낮았으며, 더 나아가, 림프절과 비장에서 활성화 되어있는 T 림프구($CD3^{+}CD4^{+}CD44^{high}CD62L^{low}$) 의 수가 낮은 것을 통해서 아토피피부염 염증반응이 완화되어 있는 것을 확인하였다. 아토피피부염이 유발된 야생형 마우스의 피부 조직에서 대식세포에서 BRP-39이 분비되고, M2 대식세포 활성이 높은 반면 BRP-39 결핍 마우스에서는 상대적으로 낮았다. 뿐만 아니라 정상대조군과 비교하여 아토피피부염 환자의 피부 병변 조직에서 YKL-40의 발현이 증가되어 있고, 대식세포에서 분

비되는 것을 확인하였다. 마지막으로 피부 장벽 관련 유전자들의 발현을 비교한 결과, 아토피피부염이 유발된 야생형 마우스에서는 발현이 매우 낮은 반면 BRP-39 결핍 마우스에서는 상대적으로 발현이 높은 것을 확인하였다.

결론적으로, 본 연구에서는 대식세포에서 분비되는 CHI3L1이 제 2형 T 도움 세포의 면역 반응과 피부 장벽 기능에 영향을 미침으로써 아토피피부염에 연관되어 있다는 것을 확인하였다.

핵심되는 말: 아토피피부염, Chitinase 3-like 1, 피부 장벽, 제 2 형 도움 T 세포