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Chitinase 3-like 1 drives allergic skin inflammation via Th2 immunity and M2 macrophage activation

Running title: The role of Chitinase 3-like 1 in atopic dermatitis

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Authorship

EJK, performed the experiments and data analysis and wrote the manuscript; JYH, MNK helped to design the experiments; SYK and KWK analyzed data of the human YKL-40 serum; SHK and COP performed human YKL-40 immunofluorescence; CGL and JAE generated BRP-39 null mutant mice; HMJ and MHS provided financial support for the study, contributed to the conception of the study and supervised the study.

Abstract

Background: 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.

Objective: The aim of this study was to investigate the role of CHI3L1 in the development and progression of AD.

Methods: We investigated YKL-40 levels 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.

Results: YKL-40 level was significantly increased in 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. BRP-39 deficiency was found to be involved in 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.

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

Keywords: Atopic dermatitis, Chitinase 3-like 1, Skin barrier, Type 2 immunity

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 Flaky tail mice (*Flg^{ft}* mice), analogous to *Flg* gene mutation in human AD patients, exhibit spontaneous skin inflammation.⁷

Chitin, a component of fungal cell walls and the exoskeletons of insects, protects organisms from the harsh environment.⁸ Chitinase 3-like 1 (CHI3L1) belongs to chitinase-like proteins (CLPs) which do not have enzymatic activity to directly degrade chitin.⁹ 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).^{10, 11} 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).¹² Elevated serum levels of YKL-40 have been correlated with various

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diseases characterized by inflammation, infection, apoptosis and tissue remodeling such as bacterial infections, rheumatoid arthritis, inflammatory bowel disease and asthma.^{8, 13} 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.¹⁴ 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.¹⁵⁻¹⁷ A previous study reported that excessive activation of macrophages can lead to the development of chronic inflammation, including psoriasis and AD.¹⁸ 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.

Methods

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 criteria¹⁹, 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.¹⁹ 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.

Animals

Female C57BL/6 mice (7 to 10 weeks old) were purchased from Orient Bio (Sunngam, Korea). BRP-39^{-/-} mice were generated on a C57BL/6 background and maintained as previously described.¹² *Flg^{fl}* mice, which carry double-homozygous *Flg* and *matted* (*ma*) gene mutations,²⁰ 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^{fl}* 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.

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 treated a total of three times with 100 µg OVA in 100 µl normal saline, for 1 week each time at 2-week intervals as previously described.²¹ Mice were sacrificed after the third sensitization (day 50).

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, San Jose, USA) as previously described,²² 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).

Skin cell sorting

Mouse skin cells were isolated, as previously described,²³ using monoclonal antibodies for MHCII, CD11b, and F4/80 (all from eBioscience). Dead cells were excluded with viability dye eFluor780 (eBioscience) and skin macrophages were sorted using FACSARIAII (BD Biosciences).

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.

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 ($\pm$ 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 $\pm$ 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.

Results

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 ($r = 0.312$, $P = 0.015$).

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.²¹ 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.

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

As previously mentioned, BRP-39 plays an important role in Th2 inflammation.¹² 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-D). We examined the number of CD4⁺ T cells and MBP⁺ eosinophils, the major infiltrating inflammatory cells in AD.² The number was significantly lower in OVA-sensitized BRP-39^{-/-} mice than in OVA-sensitized WT mice, as seen by immunofluorescence (Figure 2. E, F). 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. G, H).

Additionally, OVA-sensitized BRP-39^{-/-} mice had reduced CD4⁺ effector T cell populations (CD3⁺CD4⁺CD44^{high}CD62L^{low}) in the skin-draining lymph nodes (s-dLN) and spleens 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.

BRP-39/YKL-40 is expressed in skin macrophages of mouse and human AD patients and is involved in M2 macrophage activation

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. A). These macrophages were found to be M2 macrophages marked by CD206 (Figure S1). M2a macrophages are known to drive Th2-mediated inflammation by producing CCL17 and CCL22 in allergic airway inflammation.²⁴ The mRNA levels of M1 macrophage markers such as iNOS, CD16, and CD80 were not significantly different, however, the levels of M2 macrophage markers such

as CD206, Ym1/2, and Fizz-1 were significantly decreased in the skin of OVA-sensitized BRP-39^{-/-} mice compared to WT mice (Figure 4. B, C). To further explore the role of BRP-39, we sorted MHCII⁺ CD11b⁺ F4/80⁺ skin macrophages in OVA-induced AD to examine cytokine expression altered by BRP-39 deficiency. mRNA levels of CD206, Ym1/2, Fizz-1, CCL17, and CCL22 were higher in BRP-39^{+/+} skin macrophages than in BRP-39^{-/-} macrophages, which is characterized as M2a-like phenotype (Figure 4. D, E). However, the mRNA levels of iNOS, CD80, and CD16 were not significantly different between BRP-39^{+/+} and BRP-39^{-/-} skin macrophages (Figure 4. F).

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 normal subjects. Transglutaminase factor XIIIa (FXIIIa) is known to identify tissue resident macrophages in situ,²⁵ we found YKL-40 was expressed in FXIIIa⁺ human dermal macrophages (Figure 4. G).

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.²⁶ 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 were higher in OVA-sensitized BRP-39^{-/-} mice than in OVA-sensitized WT mice (Figure 5. A, B). Taken together, these findings suggest that BRP-39 affects not only Th2 immune responses, but also skin barrier integrity.

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

Next, 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 6. A, B). To further investigate whether BRP-39 affects FLG expression, we crossed *Flg^{fl}* mice with BRP-39^{-/-} mice with a B6 background. *Flg^{fl}* mice are known to exhibit increased TEWL, serum IgE and IgG1 levels, as well as IL-4 and Th17 expression in the skin.^{27, 28} The representative clinical phenotype of *Flg^{fl}* mice exhibited marked eczematous inflammation of the eyelid skin compared to BRP-39^{-/-} *Flg^{fl}* (Figure 6. C). The mRNA level of BRP-39 was not significantly different between untreated *Flg^{fl}* mice and WT control mice, however, it was increased in both WT and *Flg^{fl}* mice treated with OVA (Figure 6. D). To clarify the effects of BRP-39 deficiency in *Flg^{fl}* mice, we epicutaneously sensitized *Flg^{fl}* and BRP-39^{-/-} *Flg^{fl}* mice using OVA. *Flg^{fl}* mice treated with OVA showed significant increases of clinical score, TEWL and epidermal thickness compared to BRP-39^{-/-} *Flg^{fl}* mice treated with OVA (Figure 6. E-H). Total serum and OVA-specific IgE levels, and Th2 type cytokine mRNA levels were also higher in *Flg^{fl}* mice treated with OVA than those in BRP-39^{-/-} *Flg^{fl}* mice treated with OVA (Figure 6. I, J). Macrophages are known to be activated in the skin of *Flg^{fl}* mice compared to that in WT mice.²⁹ The number of F4/80⁺ macrophages and the expression levels of M2 macrophage markers, including CD206 and Ym1/2, were lower in OVA-sensitized BRP-39^{-/-} *Flg^{fl}* mice than in OVA-sensitized *Flg^{fl}*; however, the levels of Fizz-1, iNOS, CD16, and CD80 were not significantly different (Figure S2). These findings demonstrate that BRP-39 deficiency attenuated allergic skin inflammation in the *Flg^{fl}* AD mouse model.

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 S3).

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. A).

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. B). The statistically significant genes in each category are described in Table. S1.

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.^{30, 31} The biological activities of CHI3L1 have previously been studied in relation to regulation of cell proliferation, activation, migration and adhesion.³²

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.^{9, 13, 33, 34} CHI3L1 is considered a risk factor gene for allergic diseases, and has polymorphisms which correlate with asthma prevalence, and abnormal lung function.³⁵ 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.³²

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 IFN γ

signaling pathway.³⁶ Additionally, in the absence of CHI3L1, IL-18-induced pulmonary inflammatory responses were significantly ameliorated by down regulation of Th2 and Th17 responses.^{37, 38}

CHI3L1 is widely recognized as an important regulator of both M2 macrophage activation and Th2 inflammation. M2 macrophages also play a role in inflammatory skin diseases such as psoriasis and AD.³⁹ A previous study reported elevated macrophage populations in acutely and chronically inflamed human AD skin.⁴⁰ In addition, M2 macrophages accumulation was reported in a murine model of contact hypersensitivity.⁴¹

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.⁴² Based on the findings of several in vitro studies, M2 macrophages have recently been subdivided into four sub-groups: M2a, M2b, M2c, and M2d.⁴¹ As previously mentioned, M2a macrophages are known to enhance Th2 inflammation by highly expressing CCL17 and CCL22 in allergic airway inflammation.²⁴ Our data suggested that BRP-39^{+/+} skin macrophages, characterized as M2a-like phenotype, synergistically promote Th2 immune responses by producing CCL17 and CCL22 in OVA-induced AD. However, further studies should be conducted to determine the functions of other 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*^{fl} 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.^{43, 44} 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*^{fl} mice compared to *Flg*^{fl} mice. We confirmed that CHI3L1 deficiency

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attenuated skin inflammation in both OVA-induced WT AD mice and the *Flg^{fl}* 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-13R α 2, composing a multimeric complex with IL-13.⁴⁵ Furthermore, CHI3L1 has been found to regulate hyperoxia-induced airway epithelial cell death via the MAPK and PI3K pathways.⁴⁶ In our murine model of OVA-induced AD, KEGG analysis showed the TNF, TLR, PI3K-Akt, NF- κ B, MAPK, JAK-STAT and NLR signaling pathways to be significant. The inflammatory mediators interacting with CHI3L1 in skin diseases should be further studied.

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.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table

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 ($\pm$ standard deviation), or median (interquartile range), as appropriate.

Figure legends

Figure 1. Increased YKL-40 expression level in pediatric atopic dermatitis patients and

in a murine model of AD induced by OVA. (A) Serum levels of YKL-40 were measured by ELISA in children with atopic dermatitis (n=38) and in healthy controls (n = 30). (B) Mice were treated a total of three times with 100 µg of OVA, for 1 week each time at 2-week intervals. (C) Increased BRP-39 mRNA and (D) protein expression levels in the skin of OVA-sensitized WT mice. (E) Representative IHC of BRP-39 and (F) quantification of BRP-39⁺ cells per HPF (400× magnification). Results represent the mean ± SEM of three 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.

Figure 2. Attenuated Th2 inflammatory responses to OVA-induced AD in BRP-39^{-/-} mice.

(A) Clinical score, and (B) TEWL levels were measured in OVA-sensitized WT and BRP-39^{-/-} mice. (C) H&E staining (200× magnification), (D) Epidermal thickness, (E) Representative immunofluorescence of MBP⁺ eosinophils and CD4⁺ T cells and (F) quantification of eosinophils and CD4 T cells per HPF (400× magnification). (G) Total IgE levels and O.D values of OVA-specific IgE in mouse serum measured by ELISA. (H) Th2 cytokines mRNA expression levels in lesional skin of mice. Results represent the mean ± SEM of three independent experiments including 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, HPF; high-power field.

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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 spleens of OVA-sensitized WT and BRP-39^{-/-} mice. (B) Graph of s-dLN flow cytometry. (C) Graph of spleen flow cytometry. 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.

Figure 4. BRP-39/YKL-40 is expressed in skin macrophages of mouse and human AD patients and is involved in M2-like phenotype. (A) Dermal F4/80⁺ macrophages expressing BRP-39 identified by immunofluorescence staining (400× magnification).

(B) mRNA expression levels of CD206, Ym1/2, and Fizz-1 in lesional skin of mice. (C) mRNA expression levels of iNOS, CD80, and CD16 in lesional skin of mice. mRNA expression levels of (D) CD206, Ym1/2, and Fizz1, (E) CCL17 and CCL22, and (F) iNOS, CD80, and CD16 in skin macrophages of OVA-induced AD. (G) Immunofluorescence staining of FXIIIa⁺ macrophages expressing YKL-40 in the skin of AD patients and healthy controls (400× magnification). Results represent the mean ± SEM of three independent experiments, with a minimum of 4 mice included in each group. Scale bars; 20μm. * P < 0.05, ** P < 0.01, *** P < 0.001. n.s. = not significant. OVA; ovalbumin.

Figure 5. Regulation of skin barrier dysfunction in OVA-sensitized BRP-39^{-/-} mice. (A)

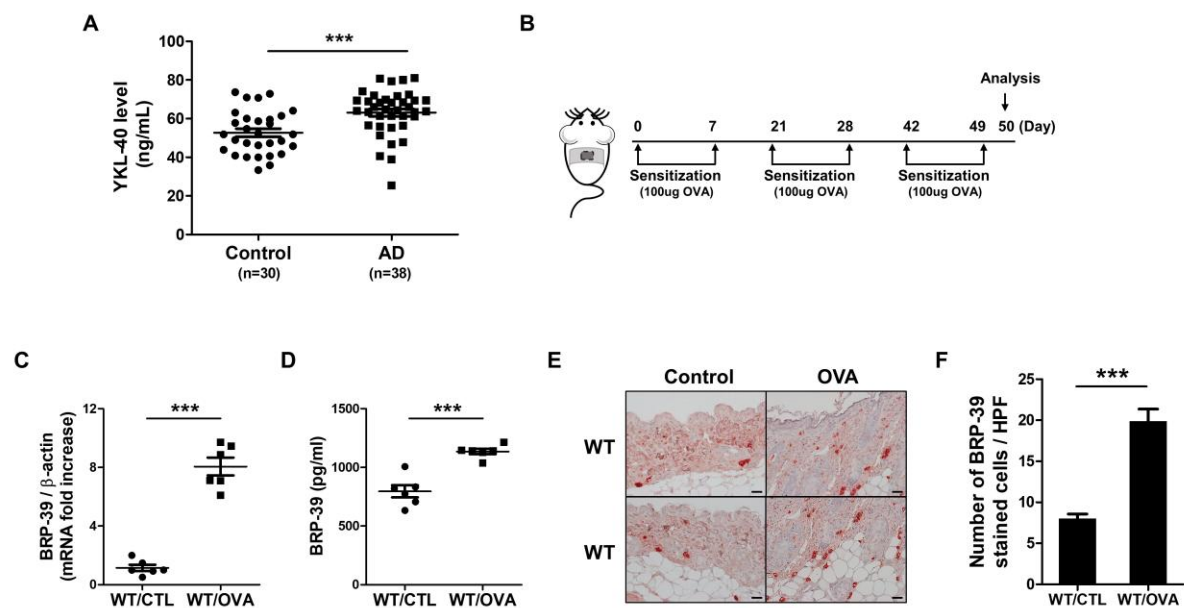
The mRNA expression level of LOR and INV, (B) CLDN-1 and ZO-1 in lesional skin of WT and BRP-39^{-/-} mice measured by real-time PCR. Results represent the mean ± SEM of three independent experiments, with a minimum of 4 mice included in each group. Scale bars;

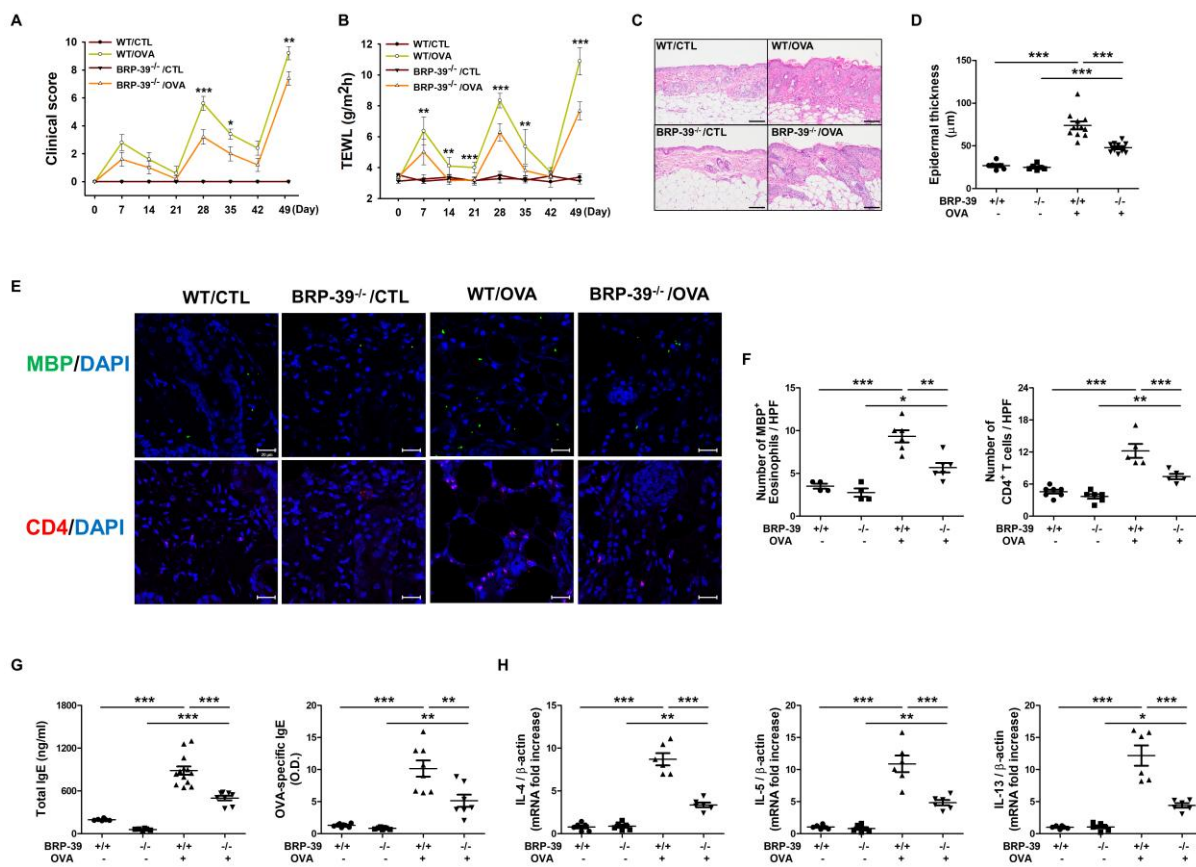
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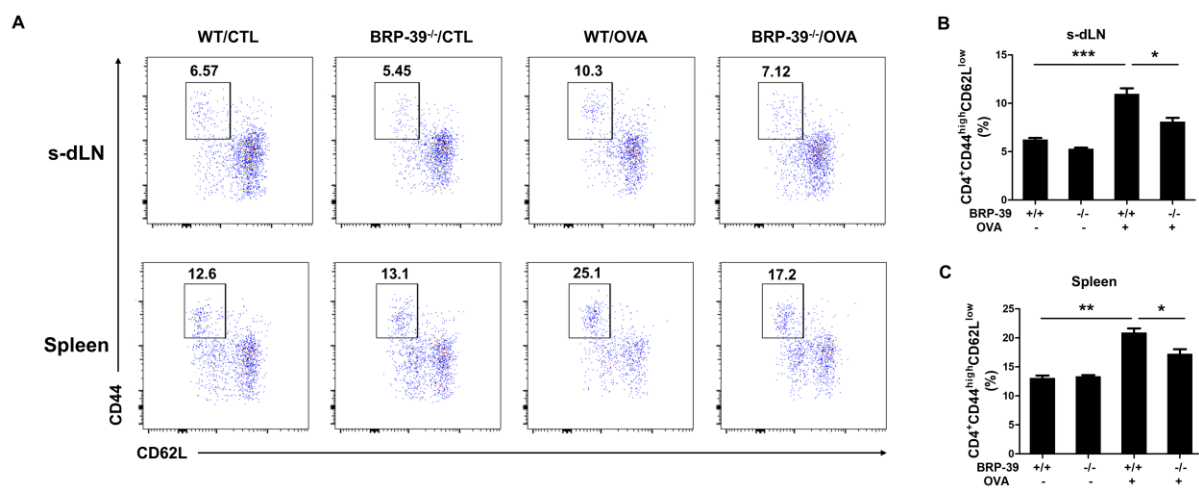
50μm. * P < 0.05, ** P < 0.01, *** P < 0.001. OVA; ovalbumin, CLDN-1; claudin-1, ZO-1; zonula occludens-1, LOR; loricrin, INV; involucrin WT; wild-type.

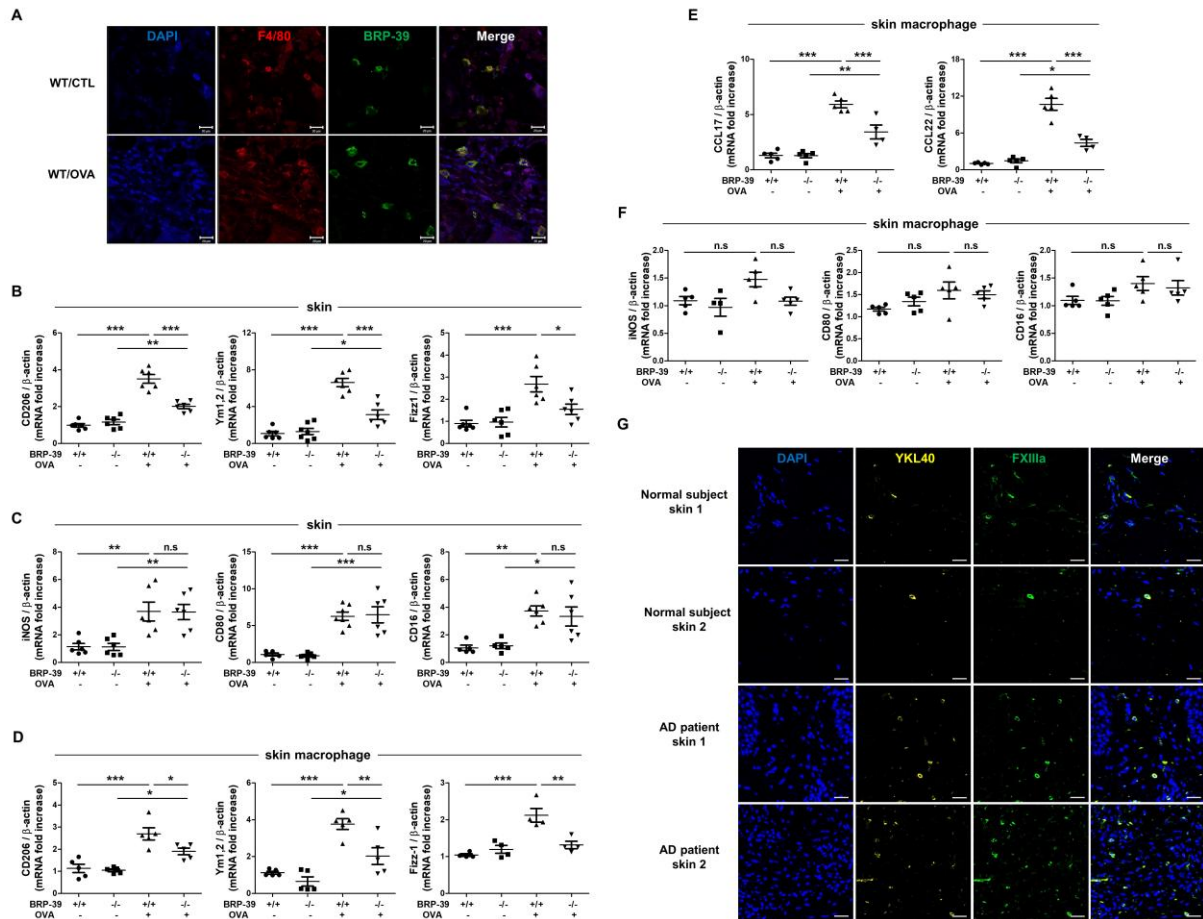
Figure 6. BRP-39 deficiency attenuated skin inflammation and Th2 response in *Flg^{fl}* mice. (A) Western blot analysis of FLG in epidermal extract of WT and BRP-39^{-/-} mice. (B) Densitometric analysis of FLG. (C) Gross phenotype of a representative *Flg^{fl}* and BRP-39^{-/-} *Flg^{fl}* mice at 18 weeks. (D) mRNA expression of BRP-39. (E) Clinical score. (F) TEWL level. (G) H&E staining (× 200). (H) Epidermal thickness. (I) Total IgE levels and O.D values of OVA-specific IgE in mouse serum measured by ELISA. (J) Th2 cytokine mRNA expression levels 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; 50 μm. [†]P < 0.05 (*Flg^{fl}* vs *Flg^{fl}* /OVA). [§]P < 0.05 (*Flg^{fl}* /OVA vs BRP-39^{-/-} *Flg^{fl}* /OVA). * P < 0.05, ** P < 0.01, *** P < 0.001. n.s. = not significant. FLG; filaggrin, 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.

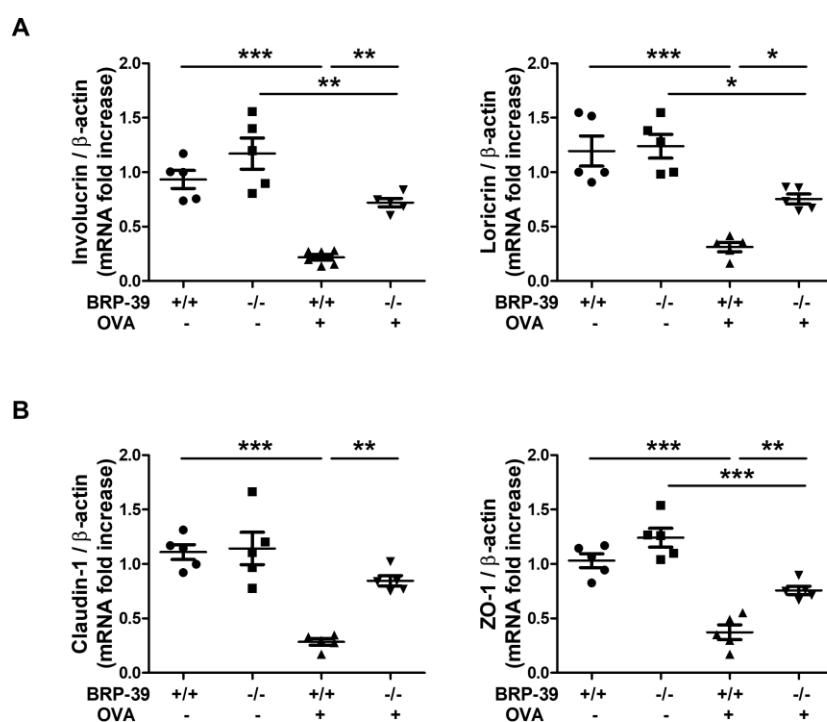
Figure 7. Microarray and KEGG pathway analysis of RNA from OVA-sensitized dorsal skins. (A) Heatmap representing differences in expression of genes related to immunity and skin barrier integrity, as indicated (WT/OVA n = 2, BRP-39^{-/-} /OVA n = 3 animals per group). (B) KEGG analysis of immune system and signal transduction. Bars represent -log(P-value), the number of statistically significant genes is indicated in each category. *** P < 0.001. OVA; ovalbumin, KEGG; Kyoto Encyclopedia of Genes and Genomes.

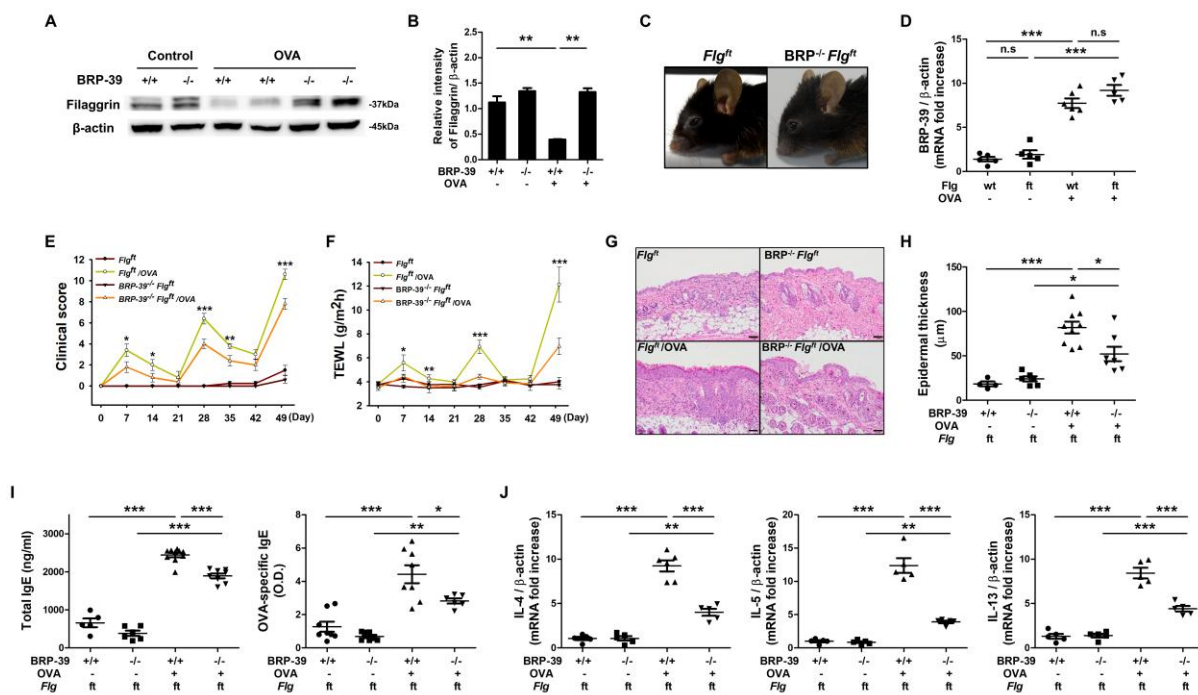


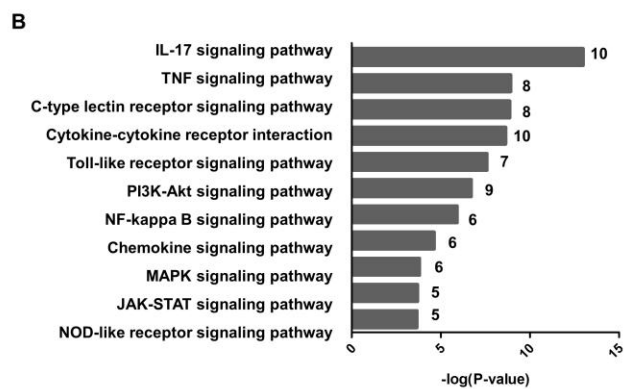
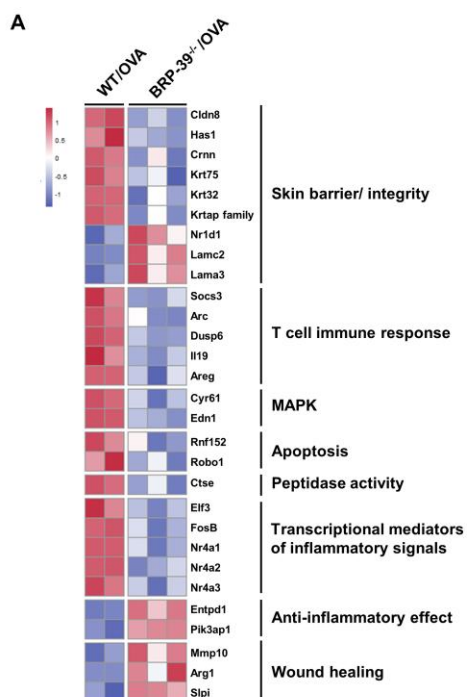












Supplementary Methods

Clinical score and Transepidermal water loss (TEWL)

TEWL was measured using a vapometer (Delfin, Kuopio, Finland) one 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.¹ 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.

Histology and Immunohistochemistry

The dorsal skin of mice was fixed in 10% formaldehyde, embedded in paraffin, cut into 4- μ m sections, and stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described,² 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).

Quantitative real-time PCR

Skin total mRNA was extracted with TRIzol reagent (Invitrogen) and cDNA was synthesized as previously described.³ 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.

ELISA

Total serum and OVA-specific IgE levels were determined, as previously described.⁴ 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.

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,⁵ 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). Image J software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis.

Immunofluorescence staining

Paraffin-embedded mouse and human skin sections were deparaffinized and rehydrated, and heat retrieval was performed for 20 minutes 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-CD4 antibody (Abcam, Cambridge, UK), anti-major basic protein (MBP) antibody (Abcam), anti-CHI3l1 antibody (Invitrogen), and anti-F4/80 antibody (Abcam) overnight at 4°C. After washing, this was followed by a 1 h 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 h 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 Fluor 647-conjugated donkey anti-mouse IgG (Abcam), and Alexa Fluor 488-conjugated 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.

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Supporting information

Table. S1. Detailed analysis of KEGG pathways

Term	<i>P</i> -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

Supplementary Figures

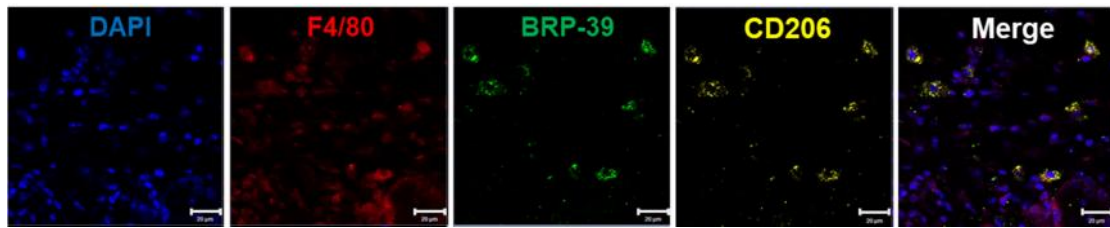


Figure S1. Immunofluorescence images of CD206⁺ macrophages expressing BRP-39 in OVA-sensitized WT mice. (400× magnification). Scale bars; 20μm.

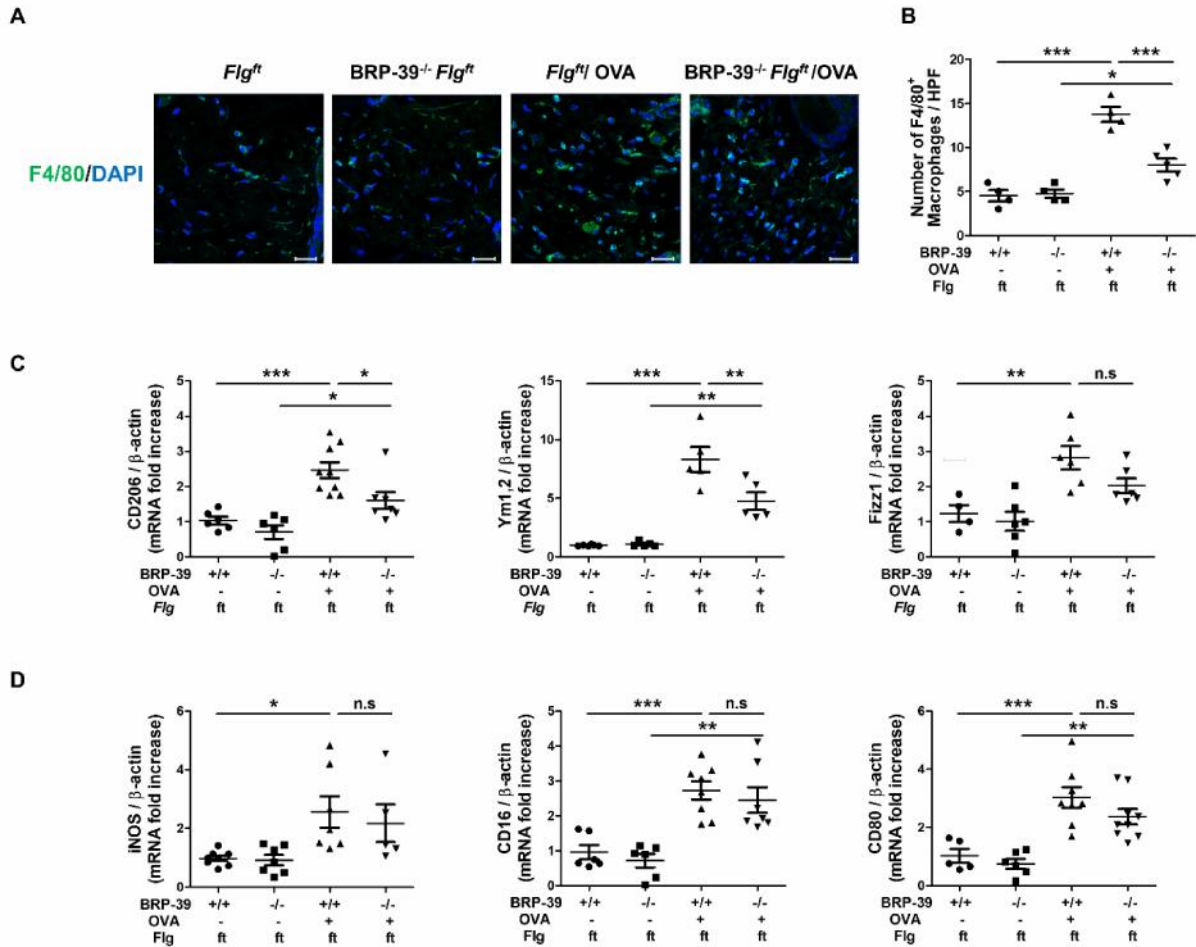


Figure S2. M2 macrophage activation in the *Flg^{fl}* AD mouse model. (A) Representative immunofluorescence images of F4/80⁺ macrophages (400× magnification). (B) Quantification of F4/80⁺ macrophages per HPF. (C) mRNA expression levels of CD206, Ym1/2, and Fizz1 in lesional skin of mice. (D) mRNA expression levels of iNOS, CD16, and CD80 in lesional skin of mice. Results are represented as the mean ± SEM of three independent experiments, with a minimum of 5 mice included per group. Scale bar; 20 μm. * P < 0.05, ** P < 0.01, *** P < 0.001; n.s. = not significant. HPF; high-power field.

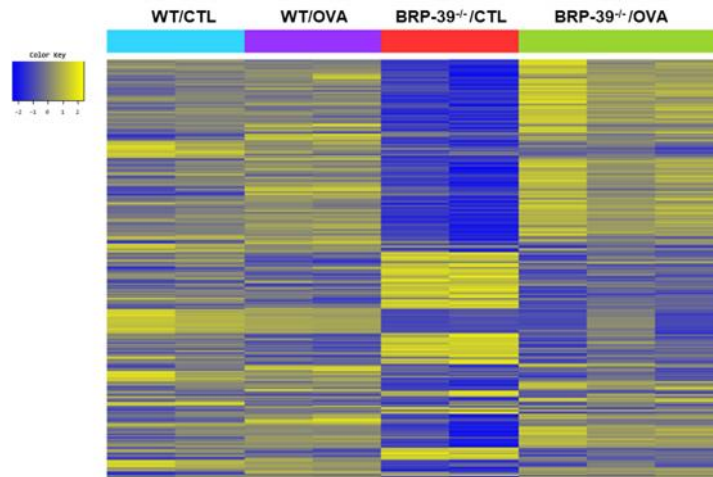


Figure S3. Hierarchical clustering of altered 1,671 genes (fold change>2, P-value <0.05) in OVA-induced AD mice model. Horizontal stripes represent genes, and columns show experimental samples. The average normalized value are shown in the heat map using yellow and blue color codes for up- and down-regulation, respectively.