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The role of activated leukocyte cell
adhesion molecule (ALCAM/CD166)
in the pathogenesis of atopic dermatitis

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The role of activated leukocyte cell
adhesion molecule (ALCAM/CD166)
in the pathogenesis of atopic dermatitis

Directed by Professor Myung Hyun Sohn

The Master's Thesis submitted to
The Department of Medical Science,
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This certifies that the Master's Thesis of
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감사의 글

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저의 학문적인 부족함을 늘 세심한 조언으로 채워주시고, 제가 연구에 능동적으로 임할 수 있게 옆에서 지도해주신 손명현 교수님께 깊은 감사드립니다. 그리고 반복되는 실험으로 심신이 지쳤을 때, 따듯한 조언과 배려로 응원해주셨던 김규언 교수님께 감사 드립니다. 제가 부진할 때마다 안타까워하시며 제가 큰 성과를 이룰 수 있도록 옆에서 다독여주셨던 김경원 교수님, 제 논문을 더 풍성하게 채워갈 수 있도록 귀한 조언을 주셨던 이재면 교수님께도 감사 드립니다.

영똥한 저에게 늘 귀 기울여주시고 제가 이 곳에서도 소중한 존재라는 것을 느끼게 해주신 이정은 선생님 정말 감사합니다. 제 연구의 시작과 마무리까지 늘 관심 가져주시고 저의 많은 질문에도 항상 자상하게 알려주셨던 홍정연 선생님, 연구자로서의 제 단점도 장점으로 승화시켜주시며 긍정적으로 연구실 생활을 할 수 있게 도와주신 김미나 선생님께 감사드립니다. 더 좋은 연구 결과를 낼 수 있도록 바쁘신 상황에서도

저를 위해 시간을 내어주신 설인숙 선생님, 김수연 선생님께도 감사 드립니다. 그리고 힘든 석사 과정을 함께 하면서 저의 부족함을 채워주던 친구, 윤선이에게 미안함과 고마움을 함께 전합니다.

지루한 삶에서 활력소가 되어준 유진, 진아, 민지. 나의 짜증도 유쾌하게 받아주었던 희재, 영재. 연구실에서 깜짝선물과 같았던 보라, 호균 오빠, 지수오빠. 그리고 나의 어떤 모습도 이해해주고, 잘 될 거라며 자신감을 북돋아주었던 상만이. 변함없는 마음으로 제게 큰 힘과 사랑을 주고, 끝까지 노력할 수 있도록 응원해준 친구들에게도 감사합니다.

마지막으로 오랫동안 공부하는 막내딸을 한결같이 응원해주시고 지금껏 뒷바라지 하시느라 고생하신 부모님, 학업으로 가족에 소홀해진 저를 대신해 집을 항상 화기애애하게 해주고 저를 자랑스러운 동생으로 생각 해준 언니, 나에게 웃음을 선사하는 폭스, 그리고 나의 곁에 오랜 시간 함께 함으로 마음의 안정을 주었던 소중한 비다. 사랑하는 가족들이 있었기에 포기하지 않을 수 있었습니다. 정말 감사합니다.

다시 한번, 제 인생에 이처럼 영광스러운 순간을 마련해주신 모든 분들께 깊은 감사의 마음을 전합니다.

2016년 12월

오미선

TABLE OF CONTENTS

| | |
|--|----|
| ABSTRACT | 1 |
| I. INTRODUCTION | 3 |
| II. MATERIALS AND METHODS | 6 |
| 1. Mice and Induction of Atopic Dermatitis | 6 |
| 2. Hematoxylin and Eosin Staining | 7 |
| 3. Clinical Score | 7 |
| 4. Histological Score | 7 |
| 5. Enzyme-linked Immunosorbent Assay | 8 |
| 6. Real-time Polymerase Chain Reaction | 8 |
| 7. Electron Microscopy | 9 |
| 8. Flow Cytometry | 10 |
| 9. Subjects | 11 |
| 10. Statistical Analysis of Data | 12 |
| III. RESULTS | 13 |
| 1. ALCAM is increased in OVA-induced AD mice | 13 |
| 2. ALCAM deficiency attenuates Th2 inflammatory responses | 15 |
| 3. ALCAM deficiency suppresses the activation of T cell | 18 |
| 4. ALCAM deficiency diminishes the skin barrier dysfunction | 21 |
| 5. ALCAM deficiency attenuates Th2 inflammatory responses in OXA-induced AD mice | 24 |
| 6. ALCAM is increased in the serum of pediatric AD patients | 27 |

| | |
|----------------------------|----|
| IV. DISCUSSION | 30 |
| V. CONCLUSION | 33 |
| REFERENCES | 34 |
| ABSTRACT (IN KOREAN) | 40 |

LISTS OF FIGURES

| | |
|---|----|
| Figure 1. Increase of ALCAM in wild type mice induced to atopic dermatitis by ovalbumin | 14 |
| Figure 2. Attenuation of ovalbumin-induced atopic dermatitis in ALCAM deficient mice | 16 |
| Figure 3. Suppression of T cell activation in ALCAM deficient mice | 19 |
| Figure 4. Inhibition of skin barrier dysfunction in ALCAM deficient mice | 22 |
| Figure 5. Attenuation of oxazolone-induced atopic dermatitis in ALCAM deficient mice | 25 |
| Figure 6. Increase of ALCAM in pediatric atopic dermatitis patients | 28 |

LISTS OF TABLES

| | |
|--|----|
| Table 1. Characteristics of the study population | 29 |
|--|----|

ABSTRACT

The role of activated leukocyte cell adhesion molecule (ALCAM/CD166)
in the pathogenesis of atopic dermatitis

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(Directed by Professor Myung Hyun Sohn)

Activated leukocyte cell adhesion molecule (ALCAM/CD166) is a transmembrane protein expressed highly on dendritic cells (DC). ALCAM binds heterophilically to CD6 expressed on resting T cells. It has been reported that ALCAM-CD6 interaction is involved in the formation of immunological synapse. The interaction mediates the stable contact between DC and T cells. Moreover, it is associated with T cell activation, proliferation and maturation. Atopic dermatitis (AD) is known to T cell-mediated allergic skin diseases. The disease is characterized by skin barrier dysfunctions and Th2 inflammatory immune reactions. However, the role of ALCAM has not been fully identified in the allergic diseases. Therefore, this study determined the possibilities that ALCAM mediates the immunologic responses and affects to the function of skin barrier in the pathogenesis of AD.

To explore the contribution of ALCAM in allergic skin inflammation, wild-type (WT) mice were induced to AD, using ovalbumin (OVA). As a different allergen, oxazolone (OXA) was applied to induce AD murine model. ALCAM deficient (ALCAM^{-/-}) mice were used to analyze the effects of the immunologic

response and barrier dysfunction in the pathogenesis of AD.

In this study, ALCAM mRNA expression was up-regulated in the skin of WT AD mice, comparing with a sham group. Although mice were induced to AD with OVA or OXA respectively, ALCAM deficiency was less worsened the skin barrier dysfunction than WT AD mice. In addition, T cell activation was inhibited by ALCAM deficiency. Serum IgE and Th2 type cytokines were down-regulated in ALCAM^{-/-} AD mice

Therefore, these findings indicate that ALCAM mediates not only immunologic dysregulation but also impaired barrier function in the pathogenesis of AD.

KEY WORDS: atopic dermatitis, ALCAM, CD166, ovalbumin, oxazolone, Th2 dominant immune response, skin barrier

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I. INTRODUCTION

Atopic dermatitis (AD) is associated with skin barrier defects and T cell-mediated chronic inflammation. AD symptoms are accompanied by dry, itchy skin and relapsing eczema.¹⁻³ Dendritic cell (DC) and type 2 helper T cells (Th2) contribute to development of allergic skin inflammation. Th2 cells are classically dominant to elicit AD, producing the cytokines such as interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13). DC is found in the stratified epithelium of the skin and mucosa.³⁻⁶ An antigen penetrated through the impaired epithelial barrier, is uptaken by DC. DC is acquired to initiate atopic dermatitis after contact with an antigen. The activated DC migrates to the T cell zone of skin draining lymph node and promotes Th2 differentiation.⁷ As an antigen presenting cell (APC), DC gains the capacity to differentiate naïve CD4⁺ T cells into effector Th2 cells.⁸ T cell activation requires two receptor signals expressed on the T cells and DC respectively to initiate an immune response. The first signal is an interaction of T cell receptor (TCR) and MHC-peptide complexes of dendritic cell (DC), forming an immune synapse. The second signal is an interaction of co-

stimulatory molecules. The co-stimulatory molecules are expressed typically on T cell (CD28 and CTLA-4) and DC (CD80/B7.1 and CD86/B7.2).⁸⁻¹⁰ As an another co-stimulatory molecule, CD6 is a surface glycoprotein found on mature T cells, immature B cells. It binds to ALCAM (Activated leucocyte cell adhesion molecule / CD166) through their extracellular domains.¹¹

ALCAM is a transmembrane protein of immunoglobulin superfamily.¹² The molecule is structurally characterized by the presence of five extracellular immunoglobulin (Ig) domains including two membrane-distal variable (V) type and three membrane-proximal constant (C₂) type Ig folds.¹³ ALCAM is expressed on diverse cell types, such as monocytes, keratinocytes, thymic epithelium, nervous ends, langerhans cells. Its expression is particularly high on DC. ALCAM has been reported to a critical role in tumor progression, metastasis, cell migration and immune response.¹⁴⁻¹⁶ ALCAM-CD6 interaction is important to mediate immunologic process, including initiation and sustaining of DC-T cell contact called 'immune synapse'. In addition ALCAM-CD6 has a pivotal role in T cell differentiation, proliferation and maturation through enhancement of CD3 proliferation and increase of CD25 molecules.^{11,16}

Meanwhile, an abnormal skin barrier is also a hallmark of AD. Epidermis is the outer layer of the skin and provides a barrier against the outside. It is composed of four layers, stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS) and stratum basale (SB). SC normally is composed with proteins, lipid rich peripheral envelope and lamellar body (LB) located in SG. The main function of the proteins and envelope is to prevent transepidermal water loss (TEWL) and to provide a cutaneous permeability barrier against environmental allergen. In AD patients, however, the proteins related with skin barrier and the lipids are deficient significantly. Moreover, allergic skin inflammatory response demonstrates Th2 dominant cytokine profile through the suppressed Th1 and enhanced Th2 cytokines.^{3,5,17-19} Impaired skin barrier might also provide an initial entry point for food allergens, potentially leading to development of eosinophilic esophagitis (EoE)

and food anaphylaxis by modulation of IL-33.^{20,21} Thus, compromised epithelial barrier can be a risk factor for AD as well as other allergic diseases.

Taken together, it is suggested that ALCAM is needed for T cell-mediated allergic inflammatory diseases. However, ALCAM is still unclear in pathogenesis of AD. Thus, the present study identified the role of ALCAM in immune response and skin barrier function, using ALCAM deficient and wild type mice.

II. MATERIALS AND METHODS

1. Mice and Induction of Atopic Dermatitis

WT C57BL/6 mice, aged 7 - 10 wk old, were purchased from Orient Bio (Sungnam, Korea). ALCAM^{-/-} mice with C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were kept under controlled humidity, temperature and pathogen-free conditions.

To induce atopic dermatitis on the dorsal skin of mice by egg protein ovalbumin (OVA / Grade V; Sigma-Aldrich, St. Louis, MO, USA), Mice were anesthetized with isoflurane (Hana pharm, Sungnam, Korea) inhalation. The dorsal skin of mice was also shaved with an electric clipper and then tape stripped five times by tegaderm (1624W; 3M Health Care, St Paul, MN, USA). Then 100 µg of OVA in 100 µl of PBS was placed on a patch of sterile gauze (1 × 1 cm²). It was secured to the dorsal skin with tegaderm. The patch was kept on the skin for 1 wk. Thus, mice have a total of three 1 wk OVA exposures with 2 wk intervals between each exposure week. During the experiment periods, transepidermal water loss (TEWL) was assessed on the skin by Vapometer (Delfin, Kuopio, Finland) at controlled humidity and temperature conditions. On day 50, the dorsal skin and blood were collected after mice were sacrificed.²²

To induce atopic dermatitis on the ear of mice by oxazolone (OXA / 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, St. Louis, MO, USA), Mice were sensitized with 50 µl of 3 % OXA in the vehicle mixed acetone and olive oil in the ratio of 4:1 at day 0. After 5 days, mice were challenged with 20 µl of 0.6 % OXA in vehicles every other day until day 13. Mice were measured to TEWL and ear thickness with a micrometer (Mitutoyo, Kanagawa, Japan) during the experiment. On the day 14, the ear and blood were collected after mice were sacrificed.⁴

2. Hematoxylin and Eosin Staining

Skin samples of mice were fixed in 10% buffered formalin at room temperature for 24 hr. And then, the samples were embedded in paraffin. The sections were cut at 4 μm for hematoxylin and eosin staining. Briefly, the sections were deparaffinized by xylene (Sigma-Aldrich, St. Louis, MO, USA) and rehydrated by ethanol (Duksan, Ansan, Korea). Deparaffinized sections were stained with hematoxylin and eosin.²³ Stained sections were analyzed by light microscopy at $\times 100$ or $\times 200$.

3. Clinical Score

During the experimental periods, mice were assessed to AD symptoms by clinical observation. Clinical score was graded as follows: 0 (none), 1 (mild), 2 (moderate), 3 (severe) for the symptoms of erythema, edema, erosion, dryness in the skin lesions of mice.^{24,25} Clinical score was expressed by the average of each symptom in each group. Thus maximum score of each group was 3 points.

4. Histological Score

The hematoxylin and eosin stained sections were analyzed to histological characteristics. The sections were graded as follows: 0 (none), 1 (minimal), 2 (mild), 3 (moderate), 4 (marked) representing the histological responses of inflammation, edema, epithelial hyperplasia and immune cell infiltration under light microscopy at $\times 200$.²⁴ Histological score was expressed by the sum of each response in each group. Thus maximum score of each group was 16 points.

5. Enzyme-linked Immunosorbent Assay (ELISA)

After mice were sacrificed, blood was collected by cardiac puncture. Then, blood was centrifuged (20 min, 4 °C, 4,000 rpm) to obtain the serum. IgE level was measured from the serum using mouse IgE ELISA assay kit (BD bioscience, San Diego, CA, USA). Briefly, 96-well plates were coated and incubated overnight at 4 °C with anti-IgE antibody in coating buffer (0.1 M sodium carbonate in PBS, pH 9.5). The plates were blocked with assay diluent (10 % FBS in PBS) and incubated for 1 hr at room temperature. The samples and diluted standards were added and incubated for 2 hr at room temperature. Then, working detector (detection antibody and SA_v-HRP reagent) was added to the plate for 1 hr at room temperature. After each step was finished, the plates were washed with PBS containing 0.05% tween (Sigma-Aldrich, St. Louis, MO, USA). Substrate solution (TMB / KPL, Gaithersburg, MD, USA) was used to develop the reaction of serum and standards and incubated for 30 min at room temperature in the dark. The reaction of the samples was stopped by 1 % of sulfuric acid. The plates were read at absorbance 570 nm from absorbance 450 nm.

ALCAM levels of human serum were also measured by ELISA using human ALCAM/CD166 ELISA assay kit (BD bioscience, San Diego, CA, USA). The protocol was generally same with mouse IgE. However, ALCAM/CD166 ELISA assay kit was required to use PBS as coating buffer. The assay diluent was consisted of 1 % BSA in PBS. The concentration of standards was applied half as much again in the plates. Moreover, steps to detect and to use streptavidin-HRP were conducted respectively unlike mouse IgE ELISA assay kit.

6. Real-time Polymerase Chain Reaction (Real-time PCR)

Total RNA was isolated from the skin with TRIzol reagent (Invitrogen, Charlsabad, CA, USA). The isolated RNA was dissolved in RNase-free water and quantified. 2 µg of total RNA was used to synthesize complementary DNA (cDNA)

using 3 μg of random hexamer, 1 μl of 10 mM dNTP Mix (2'-deoxynucleoside 5'-triphosphate) and 200 U of Superscript II reverse transcriptase (Invitrogen, Charlsabad, CA, USA) in 20 μl volumes at 42 $^{\circ}\text{C}$ for 60 min and subsequently at 70 $^{\circ}\text{C}$ for 15 min. Real-time PCR was performed using an Exicycler96 (Bioneer, Daejeon, Korea) and target gene levels were quantified with an AccuPower® Greenstar qPCR PreMix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Primers of target genes were synthesized by integrated DNA technologies (Coralville, IA, USA) and were followed: ALCAM: 5'-TGG TAC ACT GTC AAC TCA GCA-3' (forward primer) and 5'-ACC CAT CGG GCT TTT CAT ATT TC-3' (reverse primer); IL-4: 5'-GGT CTC AAC CCC CAG CTA GT-3' (forward primer) and 5'-GCC GAT GAT CTC TCT CAA GTG AT-3' (reverse primer); IL-5: 5'-CTC TGT TGA CAA GCA ATG AGA CG-3' (forward primer) and 5'-TCT TCA GTA TGT CTA GCC CCT G-3' (reverse primer); IL-13: 5'-CCT GGC TCT TGC TTG CCT T-3' (forward primer) and 5'-GGT CTT GTG TGA TGT TGC TCA-3' (reverse primer); Filaggrin: 5'-CAC TGA GCA AAG AAG AGC TGA A-3' (forward primer) and 5'-CGA TGT CTT GGT CAT CTG GA-3' (reverse primer); Loricrin: 5'-TCC TTC CCT CAC TCA TCT TCC-3' (forward primer) and 5'-CTC CTC CAC CAG AGG TCT TT-3' (reverse primer); Involucrin: 5'-CTC CTG TGA GTT TGT TTG GTC T-3' (forward primer) and 5'-GGA TGT GGA GTT GGT TGC TT-3' (reverse primer); β -actin: 5'-GGC TGT ATT CCC CTC CAT CG-3' (forward primer) and 5'-CCA GTT GGT AAC AAT GCC ATG T-3' (reverse primer). The results of mRNA expression were quantified relative to the housekeeping gene, β -actin.

7. Electron Microscopy (EM)

Skin samples were pre-fixed in Karnovsky's fixative solutions (2 % of glutaraldehyde-paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 wk. After washing the samples with phosphate buffer saline (PBS), 1 % of osmium

tetroxide (OsO_4) was added for post-fixation and then ethanol was used to dehydrate. To analyze the samples with transmission electron microscope (TEM), the samples were infiltrated with propylene oxide and embedded by Ply/Bed 812 kit (Polysciences Inc., Warrington, PA, USA). 70 nm of thin sections were cut from the embedded samples and stained with 7 % of uranyl acetate and lead citrate for contrast staining. Then, the thin section was observed by JEM-1011 TEM (JEOL, Tokyo, Japan). Images of the section were obtained by Mega View III camera (Olympus, Tokyo, Japan).

8. Flow Cytometry

Cell suspensions were isolated from skin and skin draining (dLN / axillary, brachial) lymph nodes.

Skin was detached from the body or ears and floated on RPMI 1640 (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) containing 1 mg/ml of dispase II (Sigma-Aldrich, St. Louis, MO, USA) for 60 min. Whole skin was cut into small pieces and incubated RPMI containing 10 % inactivated fetal bovine serum (FBS / Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 0.8 mg/ml of collagenase type II (Worthington Biochemical Corp. Lakewood, NJ, USA) and 50 $\mu\text{g}/\text{ml}$ of DNaseI (Roche, Freehold, NJ, USA) at 37 $^\circ\text{C}$ for 90 min. Digested skin was filtered through a 40 μm cell strainer (BD Falcon, Corning, NY, USA), using a syringe plunger to obtain single cell suspension.^{26,27} On the other hands, skin draining lymph nodes were floated on PBS and passed through the 40 μm cell strainer, using the syringe plunger without the process of digestion. Red blood cells were lysed using ACK lysis buffer (150 mM of NH_4Cl , 10 mM of KHCO_3 , 0.1 mM of Na_2EDTA , pH 7.2-7.4) at room temperature for 5 min. Then, cells were centrifuged (10 min, 4 $^\circ\text{C}$, 400 g). Cell pellets were washed with PBS and counted by a light microscopy.

Single cell suspensions (4×10^5 to 1×10^6 cells) were re-suspended in 100 μl

of FACS buffer (0.5 % FBS in PBS) and simultaneously stained with optimal concentration (1/100) of mouse antibodies (mAbs) specific for CD3 (clone: 17A2), CD4 (clone: GK1.5), CD44 (clone: IM7), CD62L (clone: MEL-14). mAbs of CD3 and CD4 were purchased from eBioscience Inc. (San Diego, CA, USA). mAbs of CD44 and CD62L were obtained from BD Biosciences (San Jose, CA, USA). The cells were stained for 1 hr and tapped slightly for blend every 15 min. After the process of staining, the suspensions were washed with FACS buffer. The samples were run on a FACS LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed by Flow Jo Software (Tree Star, Ashland, OR, USA).

9. Subjects

ALCAM level was evaluated from serum of children who visited the allergy clinic for AD or for a general health workup at Severance Children's Hospital between June 2010 and November 2014. Children who were enrolled had no symptoms of other allergic disease such as asthma or allergic rhinitis besides AD. Children with AD fulfilled the revised Hanifin and Rajka criteria.²⁸ Children with AD were subdivided into three groups, mild AD (SCORAD score ≤ 25), moderate AD ($25 < \text{SCORAD score} \leq 50$) or severe AD (SCORAD score > 50) according to disease severity based on the SCORing Atopic Dermatitis (SCORAD) index.^{29,30} Healthy controls had no history of AD or other allergic diseases such as asthma, allergic rhinitis or inflammatory disease. At first visit, blood samples were obtained from all the children. The number of eosinophil was determined by using a hematology analyzer (NE-8000; Sysmex, Kobe, Japan). IgE was measured by 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 (IRB No. 4-2004-0036).

10. Statistical Analysis of Data

The results of allergen-induced AD mice were expressed as mean \pm standard deviations (SDs). Significant differences between each group were estimated with unpaired Student's *t*-test.

In human serum analysis, continuous data were also reported as mean \pm SDs. The Student's *t*-test and one-way analysis of variance were used for analyzing continuous variables. Categorical data were presented as counts and percentages, and the Chi-square test was used for comparisons. Correlations between ALCAM levels and SCORAD scores were evaluated by using the Pearson correlation analysis.

All statistical analysis was performed by using SPSS 20 (SPSS Inc., Chicago, IL, USA). A result was deemed significant at *P*-value < 0.05 .

III. RESULTS

1. ALCAM is increased in OVA-induced AD mice

To identify the effect of ALCAM in the pathogenesis of AD, WT mice were sensitized by OVA, conducting the protocol (Fig. 1. A). This study investigated the level of ALCAM at skin lesion and serum respectively. OVA-induced AD WT mice were significantly increased to mRNA expression of ALCAM on the AD skin lesion, comparing with a sham (Fig. 1. B). ALCAM level of serum also was significantly increased in WT mice induced to AD (Fig. 1. C). Thus, the study supports a hypothesis that ALCAM is involved in AD.

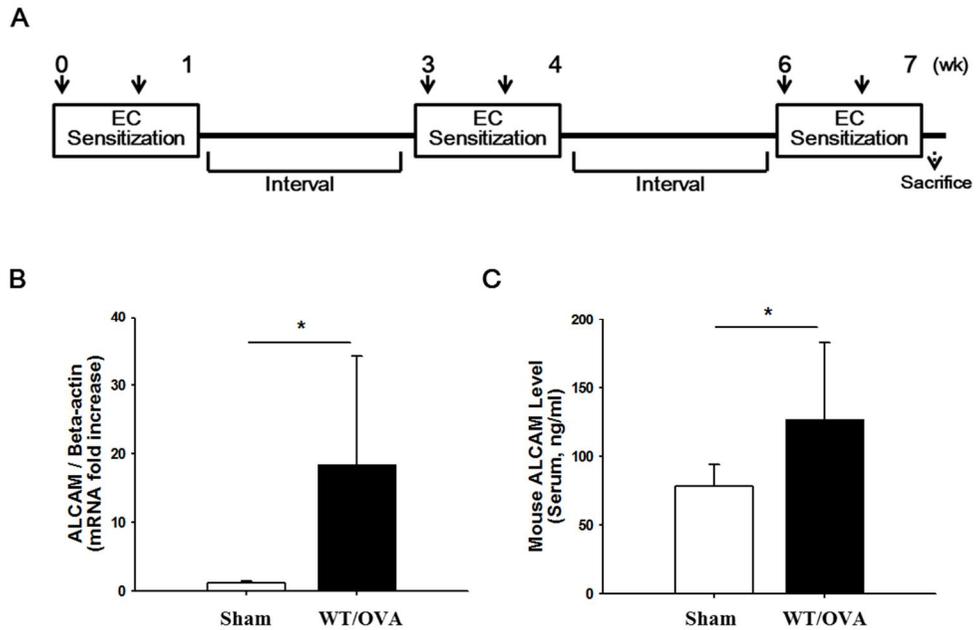
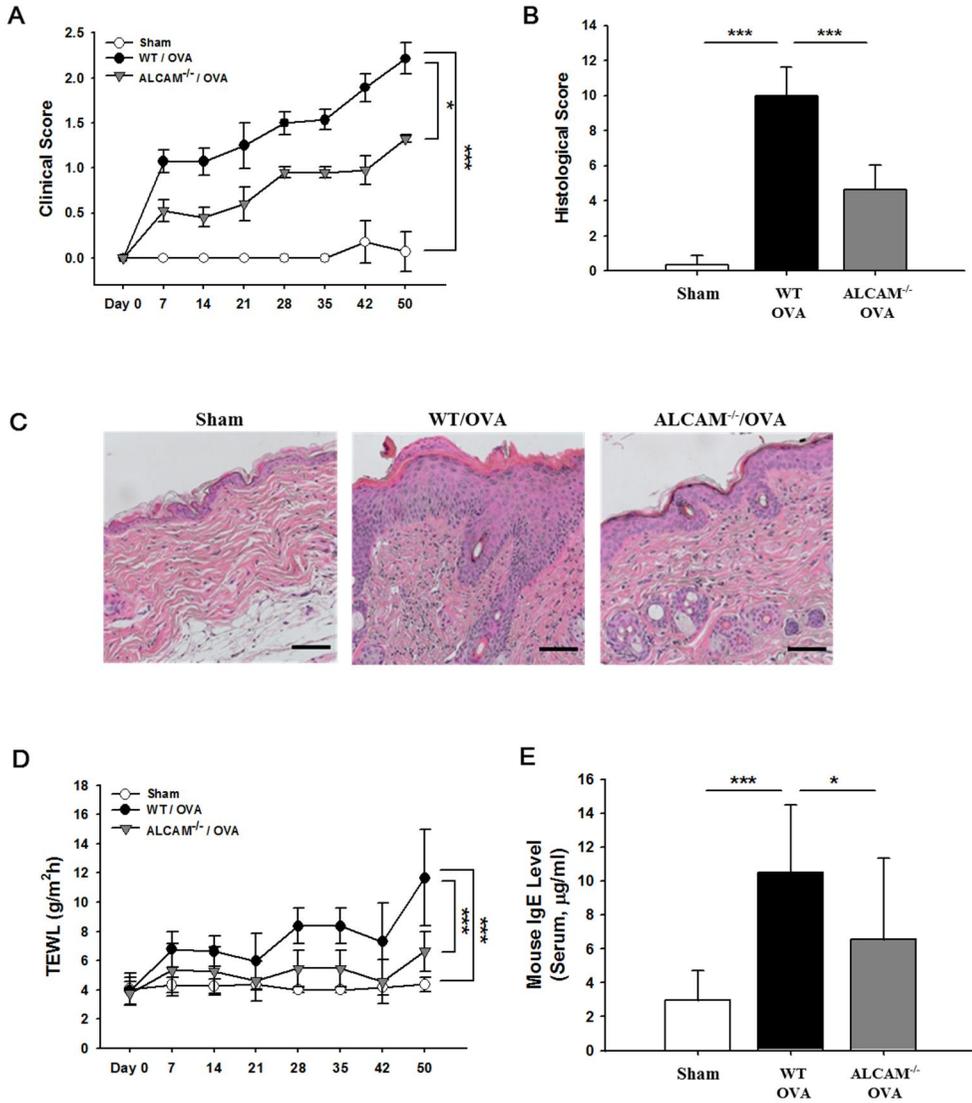


Figure 1. Increase of ALCAM in wild type mice induced to atopic dermatitis by ovalbumin (A) Experimental protocol for ovalbumin-induced atopic dermatitis model. (B) The mRNA expression of ALCAM in the skin lesion. (C) The level of ALCAM in the serum. The data represent means \pm SD. * $P < 0.05$, $n = 3 - 7$ mice / group.

2. ALCAM deficiency attenuates Th2 inflammatory responses

To determine the role of ALCAM in Th2 dominant AD, the study used with ALCAM^{-/-} mice, comparing WT mice. AD symptom was measured by clinical score and histological score. Clinical score was included some symptoms such as erythema, edema, erosion, dryness. Histological score was assessed by inflammation degree, edema, epithelial hyperplasia, immune cell infiltration. After induction of AD, ALCAM^{-/-} mice showed that attenuated clinical score (Fig. 2. A) and alleviated skin lesion histologically (Fig. 2. B, C). TEWL was also a representative AD symptom. TEWL was lower in ALCAM^{-/-} mice than WT mice (Fig. 2. D). AD was regulated by Th2 dominant immune response. The level of IgE obtained from serum and mRNA levels of IL-4, IL-5 and IL-13 gained from the skin were quantified to investigate the effect of ALCAM in Th2 dominant condition. ALCAM^{-/-} mice significantly decreased serum IgE level (Fig. 2. E) and Th2 type cytokines (Fig. 2. F).

These findings suggest that ALCAM affects to the development of Th2 dominant AD induced by OVA in mice.



F

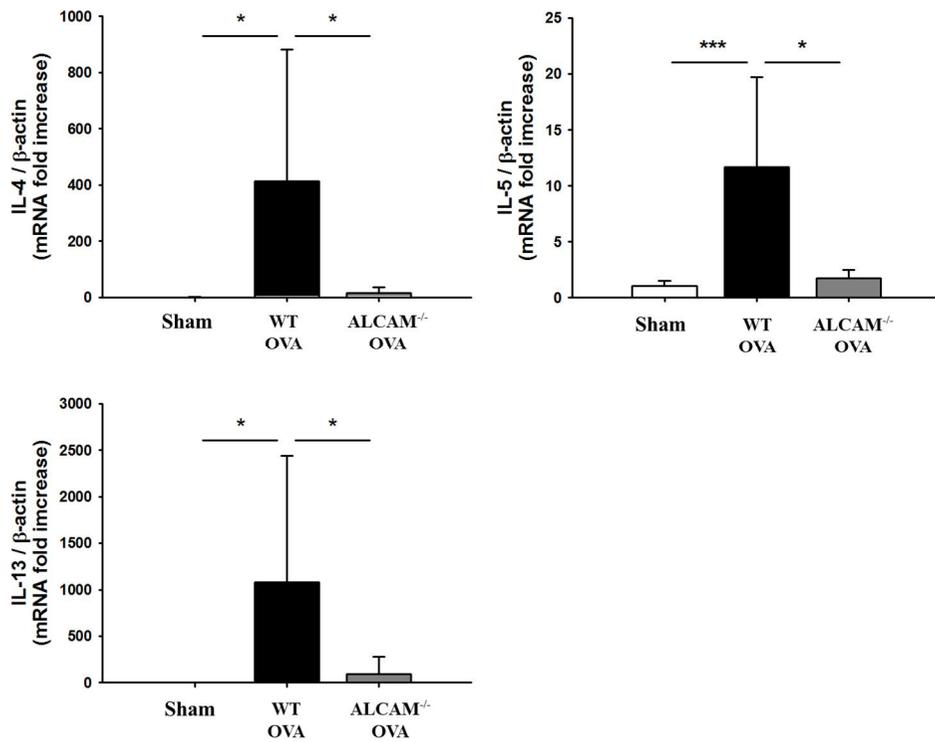


Figure 2. Attenuation of ovalbumin-induced atopic dermatitis in ALCAM deficient mice (A) Clinical score. (B) Histological score. (C) Hematoxylin and eosin (H&E) staining ($\times 200$, Bar = 500 μm). (D) Transepidermal water loss (TEWL). (E) The level of IgE in serum. (F) The levels of Th2 type cytokines in the skin. The data represent means \pm SD. * $P < 0.05$, *** $P < 0.001$, $n = 4 - 10$ mice / group.

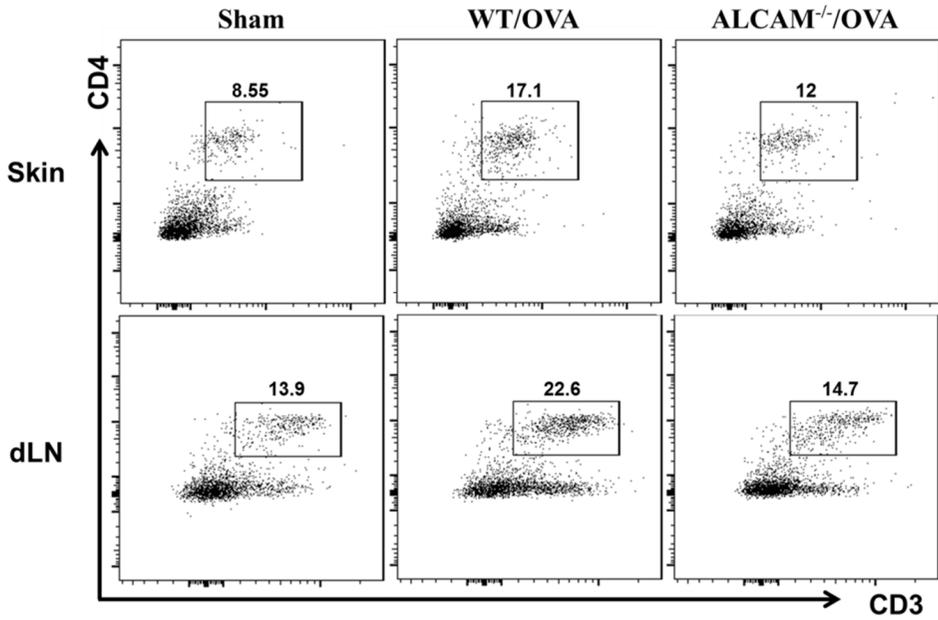
3. ALCAM deficiency suppresses the activation of T cell

To determine that ALCAM mediates the activation of T cell to develop an immune response in the skin and secondary lymphatic organ, flow cytometry was used to analyze the population of CD4⁺ T cells and the activation of CD4⁺ T cells in the skin lesion and skin draining lymph nodes (dLN) obtained from axillary and brachial sites.

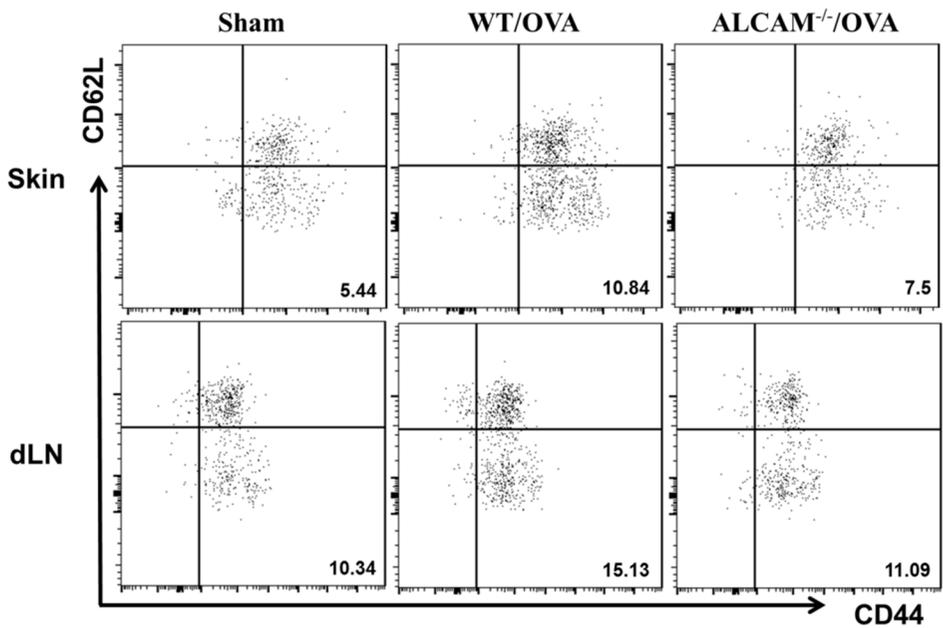
CD4⁺ T cells were detected by CD3⁺CD4⁺ surface marker on the cells. The population of CD4⁺ T cells located in the skin and dLNs respectively was significantly increased in WT AD mice, comparing with a sham. Although mice were induced to AD, CD4⁺ T cells of ALCAM deficient mice were significantly lower than WT AD mice in both of the tissues (Fig. 3. A, C). Activated CD4⁺ T cells were called to effector CD4⁺ T cells. They were detected by CD4⁺CD44^{high}CD62L^{low} surface marker on the cells. Importantly, the dominant expression of CD4⁺CD44^{high}CD62L^{low} was observed in the skin and dLNs of WT AD mice, comparing ALCAM deficient AD mice (Fig. 3. B, D).

These findings suggest that ALCAM has an ability to activate CD4⁺ T cells in the skin and dLNs.

A



B



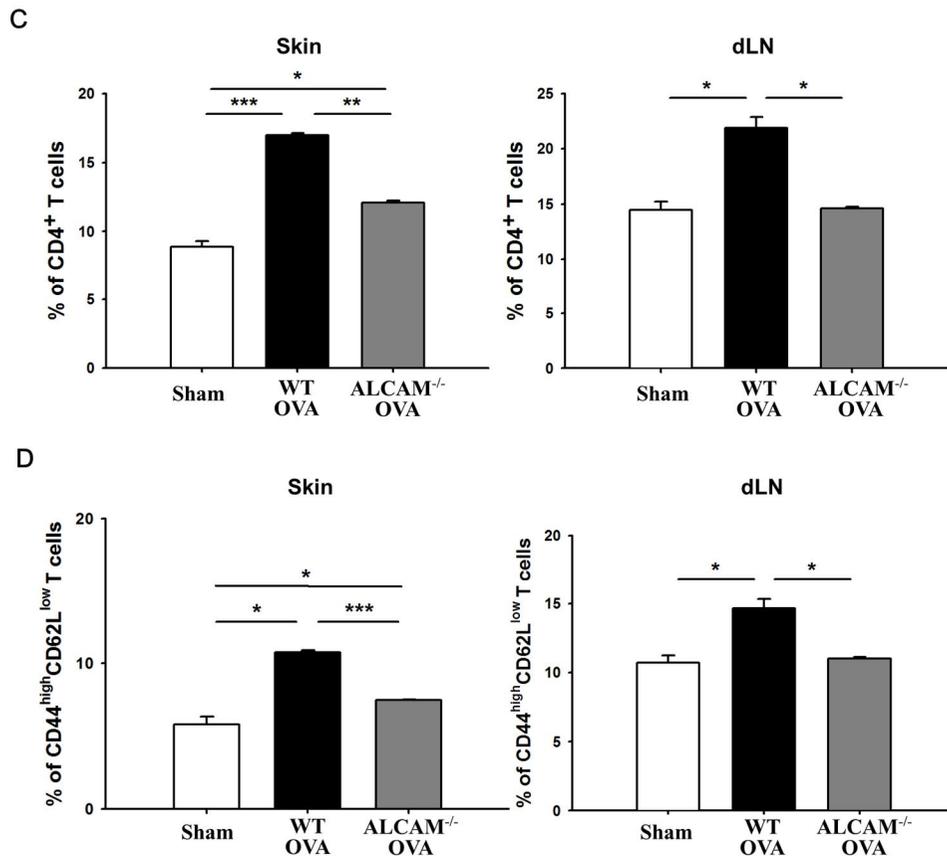


Figure 3. Suppression of T cell activation in ALCAM deficient mice (A) The population of CD4⁺ T cells in the skin and dLNs. Cells were stained with anti-CD3, anti-CD4. (B) The activation of CD4⁺ T cells in the skin and dLNs. Cells were stained with anti-CD4, anti-CD44, anti-CD62L. The figure represented CD4⁺ cells stained with anti-CD44 and anti-CD62L. (C) The percentage of CD4⁺ T cells. (D) The percentage of CD4⁺CD44^{high}CD62L^{low} (effector) T cells. The cells were analyzed by flow cytometry. The experiments were representative of at least two independent experiments. The data represent means \pm SD. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, $n = 3$ mice / group.

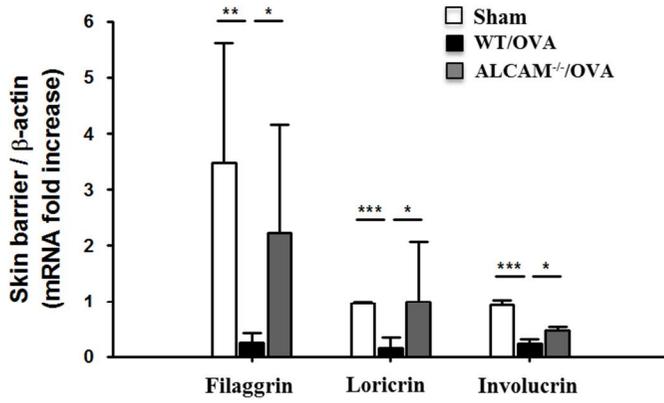
(Abbreviation; dLN, Skin draining lymph nodes)

4. ALCAM deficiency diminishes the skin barrier dysfunction

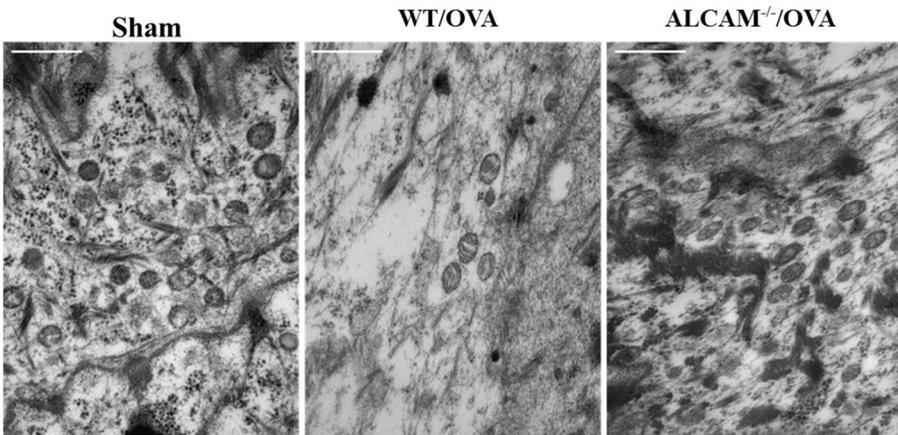
To determine the effect of ALCAM in the skin barrier dysfunction, this study investigated that the changes of skin barrier genes and lamellar body in the skin lesion after mice were induced to AD by OVA. The mRNA levels of skin barrier related proteins (filaggrin, loricrin, involucrin) were quantified by real-time PCR. ALCAM deficient mice showed lower reduction of skin barrier genes expression than WT AD mice (Fig. 3. A). Moreover, ALCAM^{-/-} AD mice showed lower decrease of the number (Fig. 3. B, C) of lamellar bodies located in SG than WT mice induced to AD. The aggregated shape was also decreased in the epidermis of ALCAM deficiency (Fig. 3. D). The number of lamellar bodies located in SG was expressed by the average of sum. The sum was gained from three sites chosen randomly of a mouse.

These findings suggest that ALCAM is involved in the regulation of skin barrier.

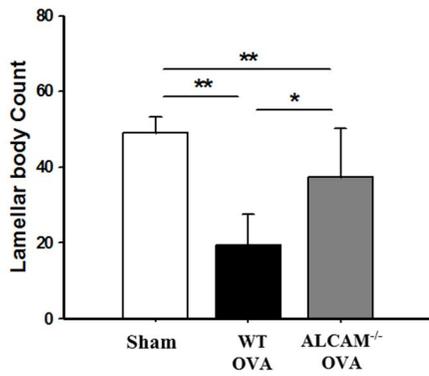
A



B



C



D

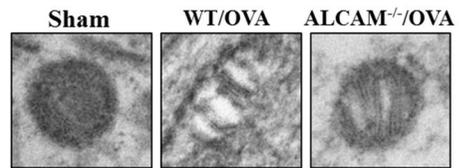


Figure 4. Inhibition of skin barrier dysfunction in ALCAM deficient mice (A)

The mRNA levels of genes associated with skin barrier. (B) The population of lamellar body in the stratum granulosum ($\times 50,000$, Bar = 500 nm). (C) The number of lamellar bodies located in stratum granulosum. (D) The aggregation of lamellar body in epidermis ($\times 120,000$). The data represent means \pm SD. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, n = 2 - 6 mice / group.

5. ALCAM deficiency attenuates Th2 inflammatory responses in OXA-induced AD mice

This study also performed in OXA-induced AD mice. WT and ALCAM^{-/-} mice were induced to AD by OXA using the protocol (Fig. 4. A). OXA-induced AD ALCAM^{-/-} mice showed same effects compared with OVA-induced AD murine models. ALCAM^{-/-} mice lower increased clinical score (Fig. 4. B) and TEWL (Fig. 4. C) than WT AD mice. Ear thickness also was reduced in ALCAM^{-/-} AD mice (Fig. 4. D). It could be observed by histological results (Fig. 4. E, F). Like OVA-induced AD, mRNA ALCAM expression was increased in skin lesion of WT AD mice induced by OXA (Fig. 4. G). Moreover, IgE level and IL-4, IL-13 mRNA level were quantified to investigate the effect of ALCAM in Th2 dominant condition. ALCAM^{-/-} mice significantly decreased serum IgE level (Fig. 4. H) and Th2 type cytokines (Fig. 4. I).

These findings suggest that ALCAM affects to the development of Th2 dominant AD induced by OXA in mice.

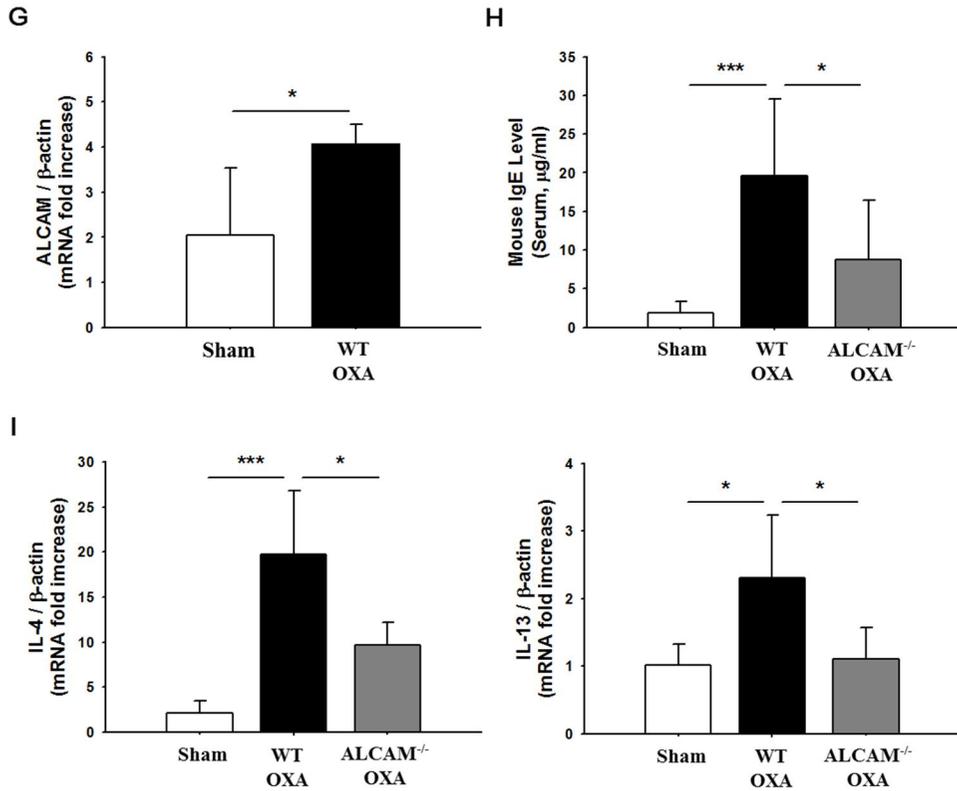


Figure 5. Attenuation of oxazolone-induced atopic dermatitis in ALCAM deficient mice (A) Experimental protocol for oxazolone-induced atopic dermatitis model. (B) Clinical score. (C) Transepidermal water loss (TEWL). (D) Ear thickness. (E) Histological score. (F) Hematoxylin and eosin (H&E) staining ($\times 100$, Bar = 200 μ m). (G) The mRNA expression of ALCAM in the skin lesions. (H) The level of IgE in the serum. (I) The level of Th2 type cytokines. The data represent means \pm SD. * $P < 0.05$, *** $P < 0.001$, n = 2 - 8 mice / group.

6. ALCAM is increased in the serum of pediatric AD patients

In addition to mice, identification of ALCAM level in human serum was performed as well. The clinical characteristics of the study subjects are summarized in Table 1. AD group was significantly younger in age and male dominant compared to control group. Serum total eosinophil count and IgE levels were significantly higher in AD group than healthy controls. Serum ALCAM level was also significantly elevated in AD group (26 ± 3.4 ng/ml vs. 30.8 ± 3.5 ng/ml, $P < 0.001$) (Fig. 5. A). After adjusting age and gender, serum ALCAM level was still statistically significantly increased in AD group ($\beta = 3.68$, $P < 0.001$). Moreover, ALCAM levels showed tendency of increase in children with AD as the severity increases, and revealed significantly positive correlations with SCORAD score ($r = 0.392$, $P < 0.001$) (Fig. 5. B, C).

These findings also suggest that ALCAM may be involved in the pathogenesis of human AD.

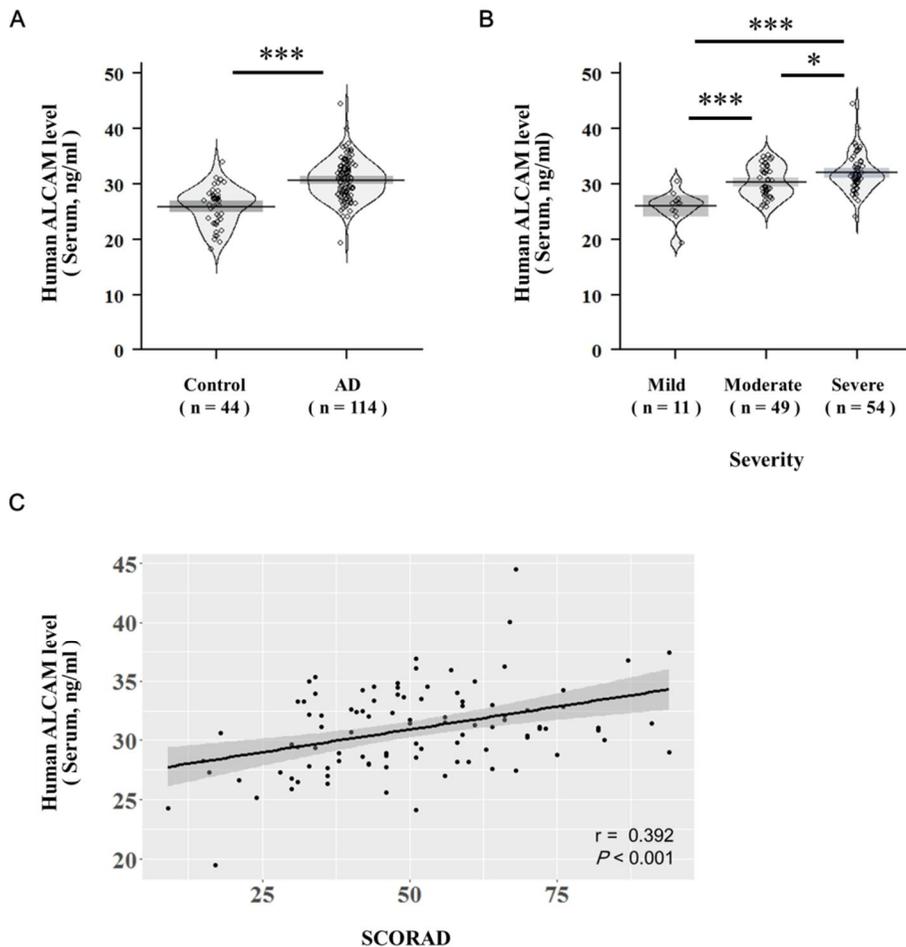


Figure 6. Increase of ALCAM in pediatric atopic dermatitis patients The level of ALCAM was measured by ELISA in the human serum of healthy controls (n = 44) and atopic dermatitis patients (n = 114). Pirteplots represent the distribution of ALCAM levels of each sample, with the horizontal mean line and boxes representing the 95% confidence intervals. (A) The level of ALCAM in human serum. (B) Increment of serum ALCAM level as atopic dermatitis severity increases. (C) The correlation of serum ALCAM level with atopic dermatitis SCORAD index. The data represent means \pm SD. * $P < 0.05$, *** $P < 0.001$.

Table 1. Characteristics of the study population

| | Control (n = 44) | Atopic Dermatitis (n = 114) |
|-------------------------|---------------------|--------------------------------|
| Age, years | 8.9 ± 2.7 | 4.0 ± 4.0 *** |
| Sex, male (%) | 24 (54.5) | 89 (78.1) * |
| Blood eosinophils, / μl | 183.7 ± 148.5 | 870.6 ± 767 *** |
| Total IgE, IU / ml | 45.7 ± 33.2 | 1036.1 ± 1262.2 *** |

The study population is included control (n = 44) and atopic dermatitis (n = 114).

Values are presented as mean ± standard deviations, or number (%).

Abbreviation; IgE, immunoglobulin E

* $P < 0.05$, *** $P < 0.001$, as compared with healthy control using the Student's *t*-test.

IV. DISCUSSION

AD skin lesion contains a number of resident and infiltrated immune cells, such as dendritic cells, macrophages, mast cells, neutrophils, basophils, eosinophils, innate lymphoid cells (ILCs), natural killer cells, fibroblast and various T cell subsets. Functional interactions of these skin immune cells are important for the pathogenesis of AD.³¹ Leukocytes coordinately use adhesion molecules in a highly regulated immune process to be trafficked from the blood into target peripheral tissues. Adhesion molecules are consisted of selectins, integrins and chemoattractant receptors. These molecules are up-regulated in tissue and vascular beds and provide directional cues for inflammatory T cells. The altered expression of trafficking receptors is involved in the states (activation, polarization and differentiation) of T cells.³²

As an adhesion molecule, ALCAM is detectable in a wide range of vertebrate tissues of fetal, neonatal, adult stages in all three embryonic lineages. Although it is associated with T cell activation, hematopoiesis, growth of primary tumor, inflammation and migration of neutrophils, ALCAM does not appear abundant in the body. Its expression is also usually restricted to subsets of cells and selected time frames. ALCAM also mediates homophilic avidity as well as heterophilic avidity with CD6, forming the clustering at the cell surface. Meanwhile, CD6 is surface receptors expressed on T cells, thymocytes and a subset of B cells. ALCAM binds to the membrane proximal scavenger-receptor cysteine-rich (SRCR) domain of CD6. In several studies, CD6 has been reported as a co-stimulatory molecule to activate T-cell function. ALCAM-CD6 interaction recruits T cell and APC (antigen presenting cell) and contributes for the stabilization of the immune synapse. The interaction also mediates a long term engagement of CD6 and ALCAM to induce T-cell activation and proliferation. In addition, ALCAM-CD6 can result in activation of three signals, mitogen activated protein kinase (MAPK) cascades, extracellular signal-regulate kinases 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) to develop the adaptive immune response.^{16,33-35} Interestingly, ALCAM positive

transcription profile shows overexpression of protein kinase C α (PKC α) and suppression of PKC ϵ in metastatic melanoma cells. PKC θ , other PKC subfamily, is a kinase in mediating TCR signals and regulates the activation of transcriptional factors (NF κ B, AP-1) in autoimmunity. ALCAM activation and PKC signaling can be a consecutive signaling event in immune response.³⁶⁻³⁸ On the other hand, the receptor for advanced glycation end products (RAGE) is a single transmembrane receptor of immunoglobulin superfamily mainly expressed on immune cells, neurons and a variety of cancer cells. RAGE binds with high mobility group box1 (HMGB1) or S100B and activates NF κ B pathway to mediate inflammation in house dust mite induced AD NC/Nga transgenic mice. ALCAM and RAGE have a structural similarity and express complementarily. Other reports demonstrated that possible time- and spatial-dependent contributions of ALCAM and RAGE in the context of S100B-driven initiation, perpetuation and possibly termination of inflammation.³⁹⁻⁴³

The role of ALCAM in the pathogenesis of AD related with intra-cutaneous T-cell activation and impaired skin barrier function has not been well resolved.^{4,44-46} Classically AD is defined by Th2 type dominant condition. Th2 type cytokines are consisted of IL-4, IL-5, IL-13. IL-4 is a critical key for Th2 cell differentiation, IgE production and eosinophil recruitment. IL-5 is an essential cytokine for eosinophil development, survival and proliferation. It is related in epidermal thickening in AD. IL-13 is a mediator of allergic inflammation and it is expressed in both acute and chronic lesions of AD.⁵ In this study, WT AD-like mice induced by repeated exposure of OVA or OXA in the skin showed immune cell infiltration, altered the levels of IgE and IL-4, IL-5, IL-13, comparing with a sham group. However, ALCAM deficient AD-like mice showed attenuation of AD symptoms and lower levels of IgE and Th2 type cytokine, comparing with WT AD-like mice. The study also demonstrated that CD4⁺T cell activation and population were decreased in the skin and dLNs of ALCAM deficient AD-like mice. The results suggest that ALCAM affects to Th2 dominant immune response on the skin lesion by co-

stimulatory interaction of CD6-ALCAM in immune synapse.

This study also showed that ALCAM deficient AD-like mice were lower reduced skin barrier related gene expressions (Filaggrin, Loricrin, Involucrin) than WT AD-like mice. ALCAM deficiency also attenuates the damage of lamellar body located in the stratum granulosum after induction of AD. Cytokine signaling has a pivotal role in multiple consequences for the barrier function of the skin. The signaling affects to keratinocyte proliferation and differentiation. Thus, altered cytokine expression may contribute to dysregulation of the epidermal barrier and elicit to skin diseases, including AD and psoriasis.^{17,47} Although the correlation of Th2 type cytokine and skin barrier related gene expression still remain to be elucidated, IL-4 and IL-13 were down regulated to loricrin and involucrin using primary human keratinocyte differentiated with CaCl₂ or filaggrin deficient skin equivalent in vitro.^{3,48,49} An another study also demonstrated that the absence of IL-4 increased epidermal differentiation complex gene expression such as filaggrin in the presence of high Th2 environment, using OVA-induced mice only lacked to IL-4 but expressed active Stat6 on T cell specifically.⁵⁰ With recent reports, the findings may be suggested that ALCAM indirectly regulates to the function of skin barrier in Th2 dominant immune response.

However, there are some limitations in this study. First, ALCAM can be expressed in diverse type of cell. Further study is needed to determine the effect of ALCAM expressed on specific tissue or cell in the pathogenesis of AD. Second, T-cell can be divided into two major subsets, $\alpha\beta$ and $\gamma\delta$ T-cells which can differentiate to Th2 type. Human and mice have different populations of $\alpha\beta$ and $\gamma\delta$ T-cell on the skin.⁵¹ Other reports indicated that ALCAM is recruited for the activation of $\gamma\delta$ T-cells but also $\alpha\beta$ T-cell interacting with CD6 in the tumor cells sensitized with non-peptide antigen.⁵² Taken together, this study demonstrated that ALCAM regulates the expression of Th2 type cytokines and indirectly affects the skin barrier functions in OVA or OXA-induced AD mice. Therefore, ALCAM contributes to elicit an immune response in the pathogenesis of AD.

V. CONCLUSION

ALCAM is expressed on a variety cell types, including in neurons, fibroblasts, endothelial cells and dendritic cells. ALCAM also has been reported to have important roles in cell trafficking, tumor metastasis and a ligand for CD6 to stabilize the immunological synapse and activate T cell. Although ALCAM has been demonstrated to an effect to mediate immune response, the role of ALCAM has not been identified in the pathogenesis of AD.

In the study demonstrated that **1)** ALCAM level was increased in the serum and skin lesion of OVA-induced WT AD mice. **2)** ALCAM deficiency showed attenuation of Th2 dominant AD symptoms. **3)** ALCAM deficiency also alleviated to activate T cell. **4)** ALCAM was related to impaired function of skin barrier in AD. **5)** ALCAM showed attenuation of AD symptoms in OXA-induced WT AD mice. Moreover, **6)** ALCAM level was increased in the serum of pediatric AD patients.

Thus, the study has a hypothesis that ALCAM is involved in the expression of Th2 type cytokines in AD development. ALCAM also mediates to T cell activation and impaired skin barrier function in the AD skin lesion.

Taken together, ALCAM might play a potential role to develop inflammatory responses in the pathogenesis of Th2-dominant AD.

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ABSTRACT (IN KOREAN)

아토피피부염 병인기전에서 Activated leukocyte cell adhesion molecule
(ALCAM/CD166)의 역할 규명

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Activated leukocyte cell adhesion molecule의 약자인 ALCAM은 5개의 면역글로불린 도메인의 구조로 이루어진 막관통단백질이다. ALCAM은 체내의 신경세포, 내피세포, 섬유아세포 등 다양한 세포에서 발현한다고 알려져 있으며, 특히 항원제시세포인 수지상세포에서 가장 높게 발현된다. 또한, T 림프구 표면에서 발현하는 CD6의 리간드로 알려진 ALCAM은 T 림프구와 수지상세포의 접촉면에서 형성되는 면역시냅스를 안정적으로 유지하는데 기여하고, T 림프구의 활성화와 분화를 매개하여 면역반응을 조절하는 보조인자로서 보고된 바가 있다.

따라서 ALCAM이 면역시냅스에서 T 림프구의 활성화에 기여한다는 점과 아토피피부염이 T 림프구 매개의 만성적인 알레르기성 피부 질환이라는 점을 바탕으로, 본 연구에서는 아토피피부염 병인기전에서 ALCAM의 역할을 규명하고자 하였다.

아토피피부염 병인기전에서 ALCAM의 역할을 확인하기 위해, C57BL/6를 유전적 배경으로 갖는 7-10주령의 야생형 마우스와 ALCAM

결핍마우스를 사용하였다. 마우스의 등 피부 털을 제거한 후, 피부에 테이프를 붙였다가 떼어냄을 5회 반복하는 것과 거즈에 분주한 100 µg의 난백 알부민을 반창고를 사용하여 등 피부에 노출시키는 것을 통해 아토피피부염을 유도하였다. 그 결과, 아토피피부염을 유도한 야생형 마우스의 피부와 혈청에서 ALCAM의 수치가 높게 측정됨을 확인했다. ALCAM 결핍마우스에서는 아토피피부염을 유도했음에도 불구하고, 야생형 마우스보다 T 림프구의 활성화가 적게 일어나며 혈청에서의 면역글로불린 E와 제 2형 T 도움세포에서 생성되는 사이토카인의 수치가 낮았다. 또한 ALCAM이 결핍된 마우스에서 난백알부민을 노출시켜도 피부장벽을 조절하는 단백질의 발현의 감소가 적었다. 합텐인 옥사졸론으로 마우스의 귀에 아토피피부염을 유도한 연구에서도 난백알부민으로 유도한 아토피피부염 마우스와 같은 결과를 보였다. 더 나아가, 아토피피부염이 없는 소아의 혈청과 비교하여 아토피 피부염이 있는 소아의 혈청에서 ALCAM의 수치가 증가되어 있음을 확인했다.

위의 결과를 통해서, 본 연구에서는 ALCAM이 T 림프구의 활성화와, 피부에서 발생하는 제 2형 T 도움 세포의 면역반응 및 피부장벽의 기능조절장애를 매개할 수 있음을 확인하였다.

핵심되는 말: 아토피피부염, ALCAM, CD166, 난백알부민, 제 2형 도움 T 세포, 면역반응, 피부장벽