Activated leukocyte cell adhesion molecule (ALCAM/CD166) affects immune responses in ovalbumin-induced food allergy

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Activated leukocyte cell adhesion molecule (ALCAM/CD166) affects immune responses in ovalbumin-induced food allergy

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김 윤선

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ABSTRACT

Activated leukocyte cell adhesion molecule (ALCAM/CD166) affects immune responses in ovalbumin-induced food allergy

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Food allergy is an abnormal immune response resulting from failing to establish oral tolerance and considered one of the worldwide major health problems. Activated leukocyte cell adhesion molecule (ALCAM/CD166) is a member of immunoglobulin superfamily transmembrane glycoproteins and express on surface of antigen presenting cells (APCs), especially dendritic cells (DCs). CD6 is known for co-stimulatory molecule and binds with ALCAM. There are many studies that long term interaction of CD6 and ALCAM affects immune response. However, there are currently no studies identified functions of ALCAM in food allergy. So,

this study aimed to identify the functions of ALCAM in OVA-induced food allergy.

Female BALB/c wild type (WT) mice and ALCAM mutant ($ALCAM^{-/-}$) mice were intraperitoneally sensitized and orally fed with ovalbumin (OVA). After last challenge, mice were assessed parameters of experimental food allergy, including clinical and diarrhea scores, and core temperature. Mice were sacrificed and analyzed systemic immune responses such as expression of T_H2 cytokines, levels of serum total IgE and OVA-specific IgE, injury scores of H&E stained intestine, and T cell responses.

Food allergy-induced WT mice showed decreased expression of ALCAM mRNA in small intestine and increased levels of ALCAM in serum. Clinical and diarrhea scores, serum total IgE and OVA-specific IgE levels, mRNA expressions of T_H2 cytokines (IL-4, IL-5, IL-13), and injury scores of H&E stained small intestine were enhanced in OVA-challenged WT mice against control mice. In contrast, these parameters and immune responses of food allergy were attenuated in OVA-challenged $ALCAM^{-/-}$ mice. T cell proliferation of total cells from spleen and mesenteric lymph node (mLN), and T_H2 cytokine levels of splenocytes cultured media were diminished in OVA-challenged $ALCAM^{-/-}$ mice. Furthermore, proportion of $CD3^+CD4^+$ T cell population and activated T cell of spleen, mLN, small intestine were dropped in OVA-challenged $ALCAM^{-/-}$ mice. Lastly, levels of serum ALCAM increased in children with food allergy against healthy control subjects.

In conclusion, this study suggests that ALCAM affects immune response in experimental food allergy. That is, ALCAM regulates allergic disease and affects immune responses by alternation of T cell activation and T_H2 response in OVA-induced food allergy.

Key words : activated leukocyte cell adhesion molecule, ALCAM, CD166, food allergy, food allergy murine model

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Ⅰ. INTRODUCTION

The prevalence of food allergy has significantly increased and considered as a lethal disease.[1-3] Although food allergy is a serious public health problem, mechanism that systemic allergic immune response to food allergens in the gastrointestinal tract remains limited.[4,5] Gastrointestinal tract, the main organ of food allergy, is the largest immunologic barrier in the body; it constantly encounters microbiota antigens and dietary proteins. Therefore, the balance between tolerance and allergic sensitization is crucial for induction of food allergy.[6-10] When maintenance of the balance is failed, orally ingested food allergens are permeated

and processed as small fragment and then presented to T cells by antigen presenting cells (APCs).[11] These interactions lead to T cell proliferation and T_H2 cytokine secretion such as interleukin (IL)-4, IL-5, and IL-13. These T_H2 cytokines can promote the production of food allergen-specific immunoglobulin (Ig) E from B cells. IgE antibodies bind to FcεR Ⅰ on the surface of mast cells and induce degranulation of mast cells. Finally, inflammatory mediators from mast cells, such as histamine, prostaglandin, and leukotrienes and immune cells present symptoms of food allergy.[12,13]

It is important that APCs present allergens to T cells in the immune response. A synapse is a specialized structure that forms when the plasma membranes of two cells come into close apposition to transmit signals. Formation of synapse between T cells and APCs called immunological synapses and maintenance of this interaction is crucial for T cell activation. And there are many co-stimulatory molecules on immune cells that engage in prolonged maintenance of immunological synapse.[14]

Activated leukocyte cell adhesion molecule (ALCAM/CD166) is a member of the immunoglobulin superfamily (IgSF).[15] There are many studies that ALCAM is presented on APCs, especially dendritic cells (DCs) and has five immunoglobulin (Ig) domains, include two membrane-distal variable(V)-type and three membrane-proximal constant(C_2)-type Ig folds.[15,16] And ALCAM is involved in maintenance of tissue architecture, immune response and tumor progression.[17]

Previous studies demonstrated that expression of ALCAM is correlated with aggressive disease in a variety of cancers, including melanoma, prostate, breast, ovarian, esophageal, bladder, and intestinal cancers and used as prognostic marker in human.[18-24] ALCAM engages in homophilic interactions with ALCAM or heterophilic interactions with CD6 on T cells in immunological synapse.[25] The heterophilic interactions are stronger than homophilic interactions. CD6, ligand for ALCAM, belongs to the scavenger receptor cysteine-rich protein superfamily (SRCRSF) and plays a role in T cell activation as an accessory molecule in immunological synapse.[26-28] Interaction of ALCAM and CD6 is the first characterized the interaction of IgSF and SRCRSF and they play a role during T cell activation as co-stimulatory molecules.[15,25,29]

ALCAM mediates immune responses as co-stimulatory molecule by the long-term interactions with CD6,[29] and plays a role in pathogenesis of disease, such as cancer. However, there are few studies that effects of ALCAM in allergic disease, which is related to immune response. Therefore, this study aimed to identify the effects of ALCAM in allergic disease, especially food allergy.

Ⅱ. MATERIALS AND METHODS

1. Subjects

A total of 143 children among who visited the Severance Children's hospital for work-up or treatment of food allergy or routine health check-up between April, 2012 and September, 2014 were enrolled in this study. Food allergy was defined according to the guidelines of the National Institute of Allergy and Infectious Diseases' Expert panel report on 2010.[30] Thorough medical history including previous adverse food reactions and physical examination were performed at the first visit. Patients who diagnosed as other allergic diseases such as atopic dermatitis, chronic urticaria, allergic rhinitis or asthma were excluded considering potential effect on allergic sensitization. Blood sampling were done on the same day after obtaining consent. After blood sample was taken, serum samples were stored at -20 ℃ and we measured the serum levels of total IgE and specific IgE levels by the Pharmacia CAP assay (Uppsala, Sweden). A specific IgE test was performed for individual suspicious food allergens and if not specified, for five most common food allergens in Korea, including cow milk, egg white, wheat, peanut, soybean, and peanut. Serum ALCAM levels were assessed with a commercially available enzyme-linked immunosorbent assay kit (R&D systems, Minneapolis, USA), according to the manufacturer's instructions. This study was approved by the Institutional Review Board of Severance Hospital (protocol no. 4-2004-0036). Written informed consent was obtained from the participants and their parents.

2. Animals

Four- to six-wk-old female BALB/c mice were purchased from OrientBio Inc. (Kyeonggi, Korea). ALCAM mutant ($ALCAM^{-/-}$) mice with C57BL/6 background were purchased from JAX Laboratories (Bar Harbor, ME, USA) and backcrossed to BALB/c background for more than seven generations. All animal experiments were performed in compliance with Korea Research Institute of Bioscience and Biotechnology and approved by the institutional review boards of Yonsei University College of Medicine Council of Science and Technology.

3. Antibodies and reagents

For Flow cytometric analysis, cells were stained with allophycocyanin (APC) - conjugated anti-CD3, fluorescein isothiocyanate (FITC) - conjugated anti-CD4, phycoerythrin (PE) - conjugated anti-CD44, and APC – conjugated anti-CD62 ligand (CD62L) antibodies. These antibodies were obtained from eBioscience (San Diego, CA, USA). Ovalbumin (grade Ⅴ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholera toxin was purchased from List Biologicals (Campbell, CA, USA).

4. Experimental food allergy

Wild type mice and ALCAM mutant mice were sensitized twice within a 2-wk interval by intraperitoneal (i.p.) injection with 50 μg ovalbumin (OVA) and 10 μg cholera toxin (CT) as adjuvants. Then all mice were orally challenged with OVA

(50 ㎎ in 200 ㎕ saline) six times within a one-day interval. Control mice were sensitized and challenged with PBS alone. They were sacrificed one day after the final challenge and subjected to analysis of allergic responses in the small intestine. (Figure1. A)

5. Measurement of parameters of food allergy

At last challenge, mice were measured core temperature before and 15, 30, and 60 min after challenge in the rectum with a digital thermometer. (Testo, Lenzkirch, Germany) Mice were also observed for 2 hrs to record diarrhea and clinical sores after the final challenge as previously described.[12,31] Briefly, Diarrhea was scored as follows: 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; 3, severe and watery stool with severe perianal staining of the coat. The clinical response was also scored as follows: 0, no symptom; 1, scratching and rubbing around the nose, ear and head; 2, puffiness around the eyes and mouth, reduced activity, and/or increased respiratory rate; 3, labored respiration; 4, no activity after prodding, or convulsion; 5, death.

6. Isolation and culture of immune cells

Spleen cells, mesenteric lymph node (mLN) cells, and lamina propria mononuclear cells were isolated from mice. Spleen and mLN were homogenized using a syringe plunger in a cell strainer (BD Falcon; 40 ㎛). The cells were centrifuged and

washed with Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal bovine serum (FBS) and then red blood cells (RBCs) were lysed with ACK lysis buffer (0.15 M NH_4Cl, 0.1 mM $KHCO_3$, 0.1 mM Na_2-EDTA in distilled water; pH 7.2). Cells were washed with RPMI containing 5% FBS. The cells were cultured 96-well flat bottom plate in the absence or presence of 100 ㎕ ovalbumin(1 ㎎/㎖). Small intestine (jejunum) lamina propria mononuclear cells were isolated as previously described.[32] Briefly, jejunum was removed from mice and placed into cold RPMI containing 10 % FBS. Jejunum was gently flushed with PBS, cut into 3-4 inch segments, removed residual fat by rolling, and inverted inside out by curved forceps. Place the tissue segments in extraction media (500 rpm, 15 min, 37 ℃), and digestion media (500 rpm, 30 min, 37 ℃) after mince the tissue. After digestion, filter the digested tissue and centrifuge that solution with RPMI containing 10 % FBS twice. After 72 hrs of culture, cells with medium were collected and centrifuged. The supernatant and centrifuged cells were frozen separately.

7. Proliferation assay

Isolated cells from spleen and mLN were cultured 96-well flat bottom plate in the absence or presence of 50 ㎕ ovalbumin (10 ㎎/㎖) for 5 days. At the fifth day, 10 ㎕ of cell counting kit-8 (CCK-8) solution was added per well with cultured cells and incubated two-to-four hrs in incubator. And then, 96-well flat bottom culture plates were read at 450 nm with ELISA reader.

8. Determination of the concentrations of cytokines in splenocyte cultured media

The concentrations of IL-4, IL-5 and IL-13 in cell-culture supernatants were determined by ELISA (R&D Systems, Minneapolis, MN) as manufacturer's instructions. Briefly, ELISA plates were coated with purified anti-cytokine antibodies and blocked with 1 % BSA/PBS for 1 hr. Samples and dilution standards were loaded and incubated for 2 hrs at room temperature. The bounded cytokine was detected with anti-mouse-cytokine antibodies for 2 hrs, followed by streptavidin-HRP for 20 min. The plates were developed by addition of the substrate a tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) and stopped with 2 N sulfuric acid. Plates were read at 250 nm.

9. Flow cytometric analysis

Single-cell suspensions (1×10^6 to 2×10^6 cells) were resuspended in 100 ㎕ of FACS buffer (0.5 % FBS in PBS) and dead cells excluded by Viability dye eFluor780 (eBioscience, San Diego, CA, USA). Cells were simultaneously stained with optimal concentrations of monoclonal antibodies specific for CD3, CD4, CD44, and CD62 ligand (CD62L). Therefore, population of T cell was selected by CD3, and CD4 and activation of T cell was selected by CD4, CD44, and CD62L. Cells were stained for 30 min at 4 ℃, washed twice with cold staining buffer (PBS, 2 % FCS, and 0.02 % sodium azide). Samples were run on a FACSVerse flow cytometer (BD Biosciences), and data were analyzed using FlowJo (Tree Star, Ashlnd, OR).

10. Measurement of cytokines by real-time PCR

Total RNA was prepared with small intestine (jejunum) by TRIzol reagent (Invitirogen). First strand cDNA was synthesized using superscript with random hexamer primers (Invitrogen). Real-time PCR was performed with Exicycler 96 (Bioneer, Korea), and mRNA levels were quantified using the AccuPower® GreenStar qPCR Master Mix (Bioneer), according to the manufacturer's instructions. Amplification was conducted for 45 cycles. Quantification values were normalized to the mean value of housekeeping gene; β-actin. Based on the cycle threshold (C_t) values obtained, a relative and normalized mRNA expression level was determined for each gene using ΔC_t method. The C_t value for each gene was corrected by the mean C_t value of the two housekeeping genes. The results were calculated as a relative expression using the following formula: $2^{-\Delta Ct} \times K$, where K is a 10^6 factor, and expressed as arbitrary units. Fold increase in the expression levels of the experimental groups was normalized to those of the negative control group.

11. Histologic evaluation

Small intestine (jejunum) sections were stained with hematoxyline and eosin (H&E). Briefly, jejunum was fixed with 10 % buffered formalin, embedded in paraffin and cut into 5 ㎛ sections for H&E staining to assess gut inflammation respectively. Microphotographs were taken by Nikon microscope (Elipes 90i) with camera. Histological damaged score was measured as follow;[33] 0, no damage; 1, low amount of damage is seen with distinct structural components (epithelial layer

and lamina propria); 2, structural components can still be differentiated but the epithelial layer is noticeably separating from the lamina propria; 3, disorganization of the villi is beginning and differentiating between structural components is difficult; 4, organization of the villi is chaotic; 5, structure of villi is chaotic and many villi are completely destroyed down to the basal layers of tissue.

12. Electron microscopy

Small intestine (jejunum) specimens were pre-fixed in Karnovsky's fixative solutions (2 % glutaraldehyde – paraformaldehyde in 0.1 M phosphate buffer for 30 min, they were post fixed with 1 % osmium teroxide (OsO_4) dissolved in 0.1 M phosphate buffer for 2 hrs and dehydrated in ascending gradual series (50 ~ 100 %) of ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences Inc., Warrington, PA, USA). Pure resin embedding and polymerization was carried out at 60 ℃ electron microscope oven (TD-700, DOSAKA, Tokyo, Japan) for 24 hrs. Three hundred fifty ㎚ thick section were initially cut and stained with toluidine blue for light microscope. Seventy ㎚ thin sections were double stained with 7 % uranyl acetate and lead citrate for contrast staining. These sections were cut by LEICA Ultracut UCT Ultra-microtome (Leica Microsystems, Vienna, Austria). All of the thin sections were observed by JEM-1011 transmission electron microscopy (JEOL, Tokyo, Japan) at the acceleration voltage of 80 ㎸. The images were obtained with a MegaView Ⅲ camera (Olympus, Tokyo, Japan).

13. Quantification of the serum concentrations of total IgE and specific IgE (ELISA)

Blood was collected by cardiac puncture. Serum total immunoglobulin E (IgE) and ovalbumin-specific IgE level was measured using mouse IgE ELISA assay kit (BD Bioscience, San Diego, CA) according to the manufacturer's instructions. Microplates were coated with anti-IgE antibody in coating buffer. Serum samples and diluted standards were added and incubated for 2 hrs at room temperature. After washing, detection antibody and SAv-HRP reagent were applied onto plate for 2 hrs at room temperature. The reaction was developed with a TMB (KPL) and stopped with 2 N sulfuric acid. Plates were read at 450 nm. For measurement of ovalbumin-specific IgE, microplates were coated with anti-IgE antibody and ovalbumin in coating buffer.

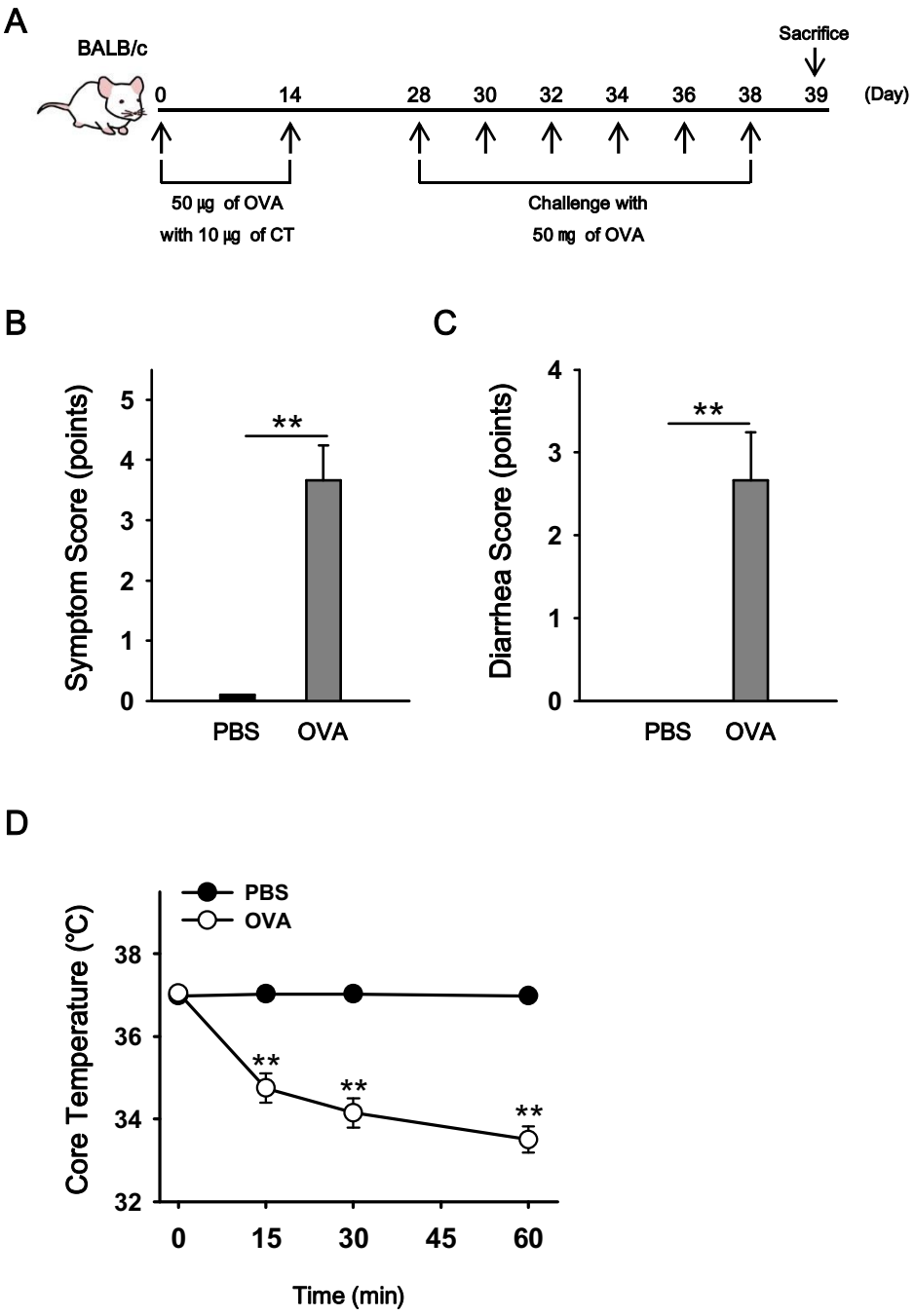
14. Statistical analysis

Data were expressed as mean ±SD. Significant differences between two groups were estimated using unpaired Student's t-test. Statistical significance was set at $P \leq 0.05$.

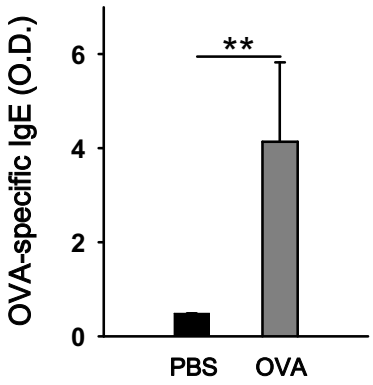
Ⅲ. RESULTS

1. Increased ALCAM levels in serum of OVA-challenged food allergy model

To address the effects of ALCAM in food allergy, we investigated ALCAM level in OVA-challenged wild type (WT) mouse model. WT mice was intraperitoneally sensitized, and orally challenged with OVA, (Figure 1. A) and evaluated with parameters of food allergy. Clinical and diarrhea scores were increased, and core temperature was decreased in OVA-challenged WT mice. (Figure 1. B-D) Levels of serum OVA-specific IgE were also enhanced in OVA-challenged WT mice against to control mice. (Figure 1. E) Unlike increased levels of ALCAM in serum, mRNA expression of ALCAM decreased in small intestine (jejunum) of food allergy-induced mice. (Figure 1. F)



E



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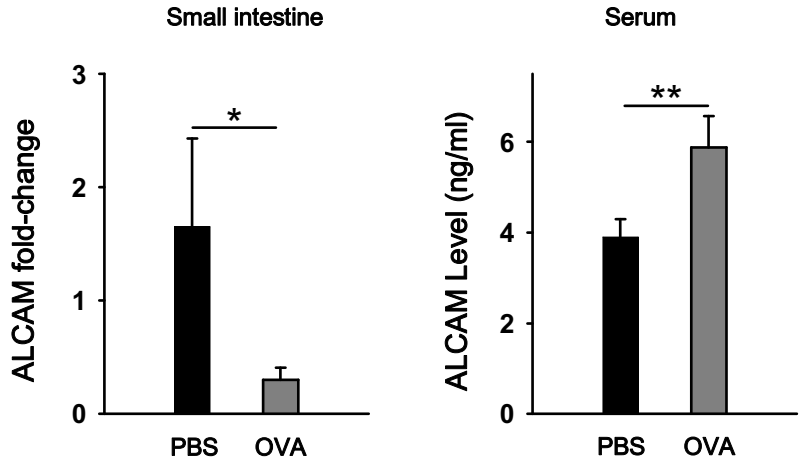
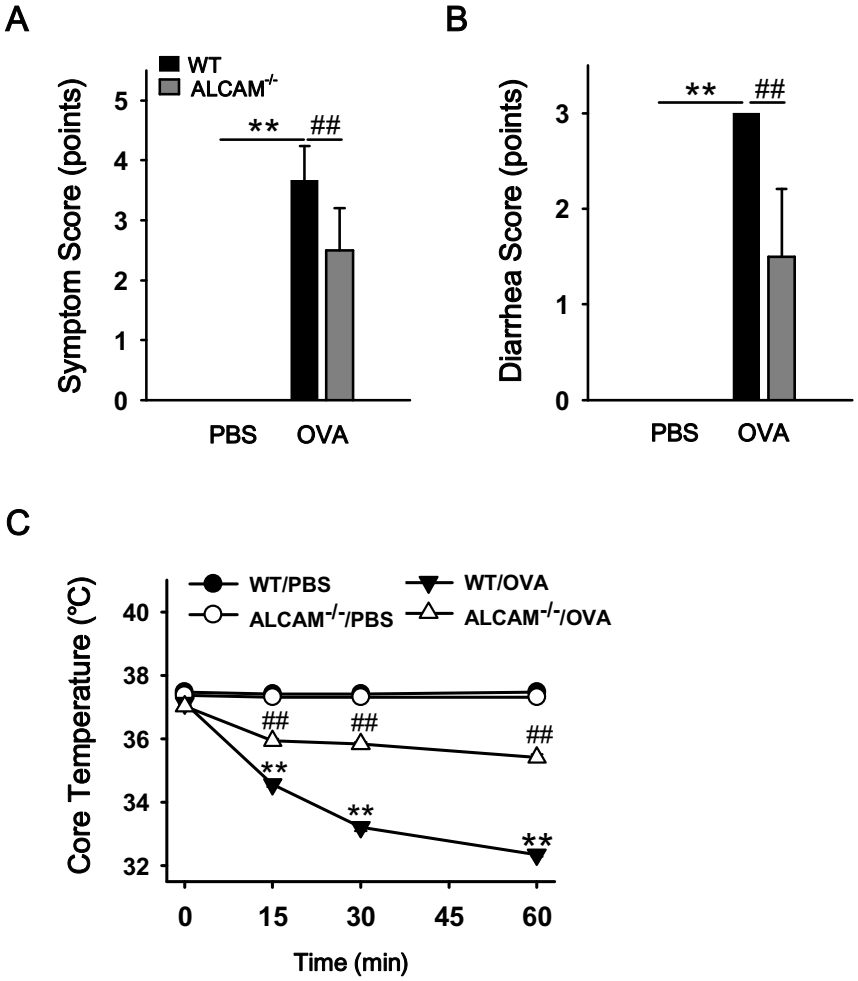
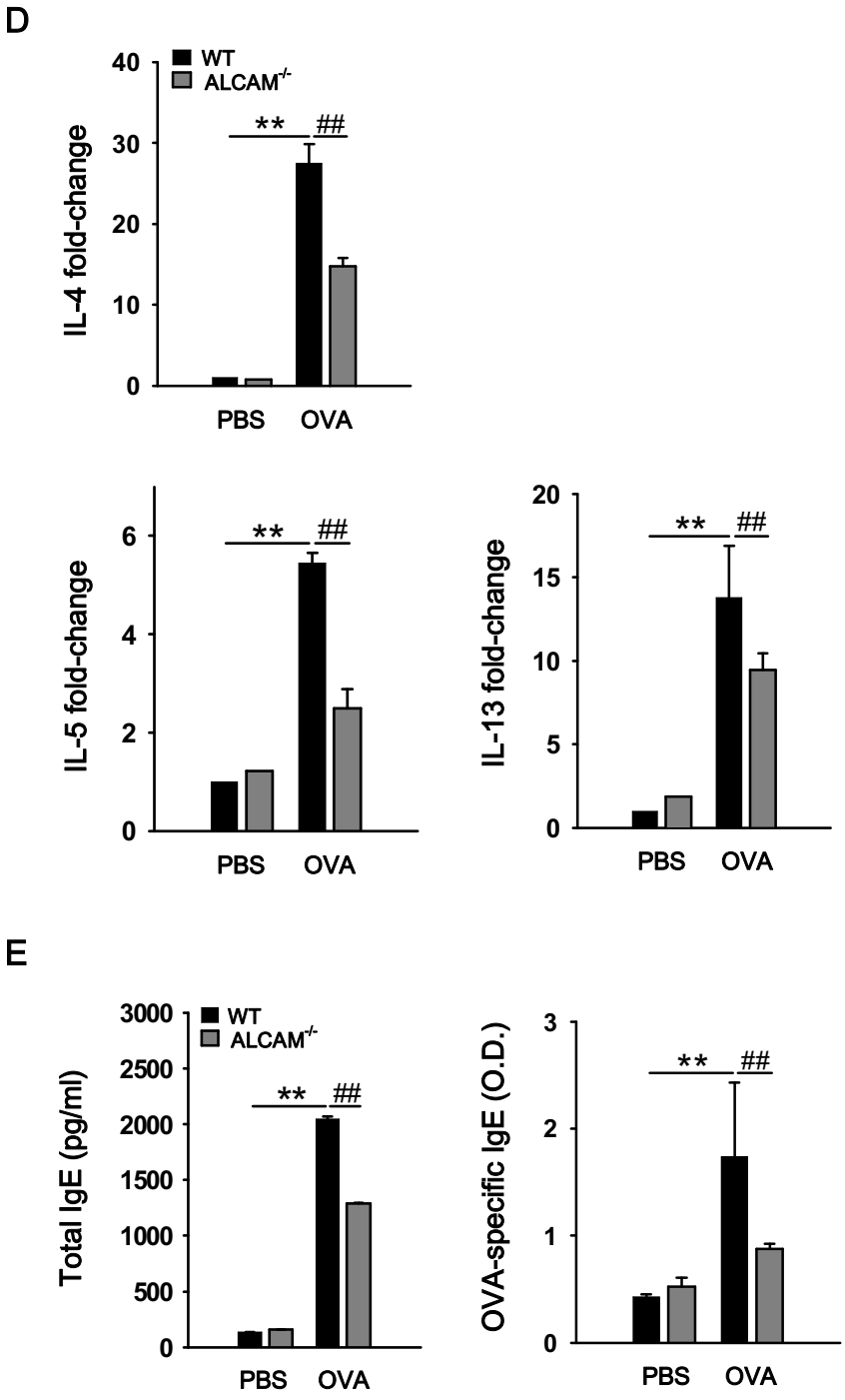


Figure 1. Increased levels of ALCAM in serum of food allergy- induced wild type mice with ovalbumin. (A) Experimental protocol for (OVA)-induced food allergy model. Mice were sensitized with OVA/CT and challenged with OVA. Clinical score (B), diarrhea score (C), and core temperature (D) were measured after the last challenge. (E) Level of OVA-specific IgE in mice serum. (F) ALCAM levels were measured in small intestine, and serum. Mean data were estimated from triplicated experiments. * $P < 0.05$, ** $P < 0.01$.

2. Attenuated immune responses in ALCAM$^{-/-}$ mice model

To identify contributions of ALCAM to OVA-induced food allergy, we induced food allergy to WT and ALCAM mutant (ALCAM$^{-/-}$) mice with OVA. Levels of the parameters of food allergy, T_H2 cytokines, total IgE, OVA-specific IgE, and histological injury of small intestine were increased in OVA-challenged WT mice against to control mice. (Figure 2.) In OVA-challenged ALCAM$^{-/-}$ mice, clinical and diarrhea scores were decreased against OVA-challenged WT mice. (Figure 2. A, B) Level of core temperature was less dropped in OVA-challenged ALCAM$^{-/-}$ mice than OVA-challenged WT mice. (Figure 2. C) In addition, mRNA expressions of T_H2 cytokines such as IL-4, IL-5, and IL-13 were diminished in small intestine of OVA-challenged ALCAM$^{-/-}$ mice. (Figure 2. D) Levels of serum total IgE, and OVA-specific IgE were also attenuated in OVA-challenged ALCAM$^{-/-}$ mice. (Figure 2. E) In OVA-challenged ALCAM$^{-/-}$ mice, the characteristics of injured small intestine, such as damaged villi and infiltrated cells were milder than OVA-challenged WT mice. (Figure 2. F, G) Chen T. et al. identified that enlarged intestinal permeability and damaged intestinal tight junction in sensitized juvenile rat with OVA.[34] So, we observed intestinal tight junction through electron microscopy (EM). The intestinal tight junction and desmosomes of OVA-challenged WT mice smeared and spread against to control mice. However, these phenomena were weakened in OVA-challenged ALCAM$^{-/-}$ mice. (Figure 2. H) Put together, ALCAM$^{-/-}$ mice showed diminished immune response.





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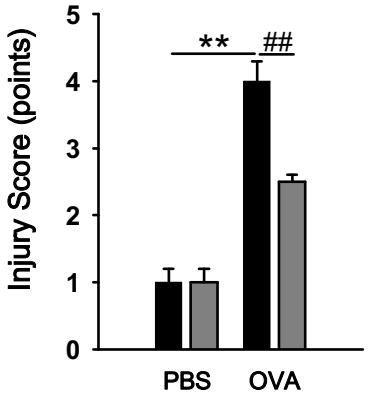

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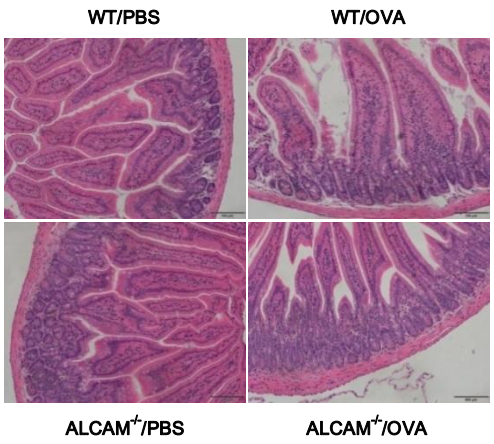

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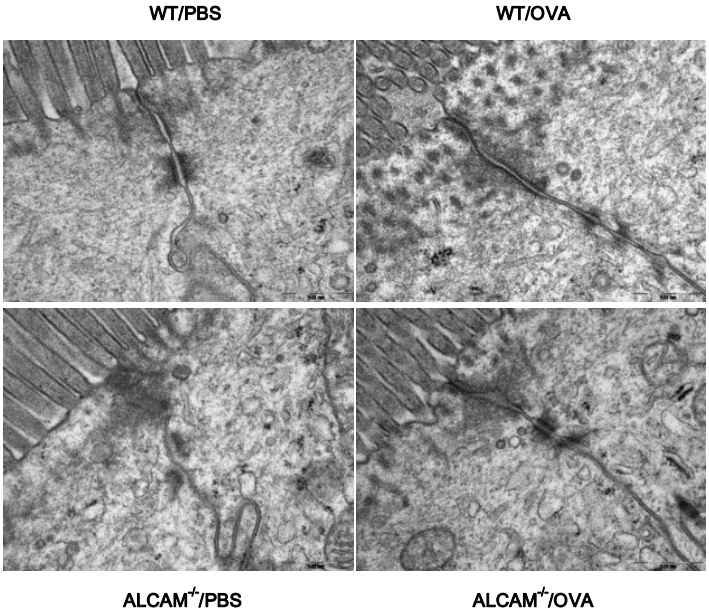


Figure 2. Attenuated immune responses in food allergy-induced ALCAM deficient mice with ovalbumin. After last challenge, clinical score (A), diarrhea score (B), and core temperature (C) were measured in OVA-challenged WT and ALCAM$^{-/-}$ mice. (D) Expression of T_H2 cytokines mRNA (IL-4, IL-5, and IL-13) in small intestine of mice. (E) Levels of total IgE and O.D. values of OVA-specific IgE were measured in mice serum by ELISA. Histological observations of small intestine (G) and its injury score (F). (H) Morphological observation of intestinal tight junction using electron microscopy (EM). Mean data were estimated from triplicated experiments. ** means $P < 0.01$ (WT/PBS vs. WT/OVA). ## means $P < 0.01$ (WT/OVA vs. ALCAM$^{-/-}$/OVA). Magnification; (F, x200); (I, x50K).

3. Diminished T cell responses in ALCAM$^{-/-}$ mice

It is well known that ALCAM on APCs and CD6 on T cells interact in immunological synapse and play roles as co-stimulatory molecule.[35] Therefore, we figured out the role of ALCAM in T cell responses through identifying T cell responses. Splenocytes and mesenteric mLN cells were isolated from WT mice and ALCAM$^{-/-}$ mice, and cultured with OVA. And then T cell proliferation was measured with CCK-8. The rate of total T cell was diminished in both cell types from OVA-challenged ALCAM$^{-/-}$ mice compared with those from OVA-challenged WT mice. (Figure 3. A) Levels of T_H2 cytokines (IL-4, IL-5, IL-13) were decreased in splenocytes cultured media of OVA-challenged ALCAM$^{-/-}$ mice against to OVA-challenged WT mice. (Figure 3. B)

The change of T cell proliferation in whole cell from spleen and mLN was identified. (Figure 3.) To address more specific changes of T cell responses in immune tissues, splenocytes, mesenteric lymph nodes (mLN) cells, and lamina propria mononuclear cells were isolated and cultured from spleen, mLN, and small intestine. For identify T cell population, CD3, and CD4 were used as markers. Number of $CD3^+CD4^+$ T cells enhanced in immune systems, especially in small intestine, of OVA-challenged WT mice against that of control mice. However, the number of $CD3^+CD4^+$ T cells decreased in OVA-challenged ALCAM$^{-/-}$ mice than OVA-challenged WT mice. We obtained similar data in T cell activation with markers, CD4, CD44, and CD62 ligand (CD62L). Number of activated cells

($CD4^{+}CD44^{high}CD62L^{low}$) decreased in OVA-challenged $ALCAM^{-/-}$ mice than OVA-challenged WT mice. (Figure 4. A, B) Graphs represent populations of cells. (Figure 4. C, D) These results suggest that ALCAM has an effect on T cell population and activation in the immune systems by interaction with CD6 on T cells.

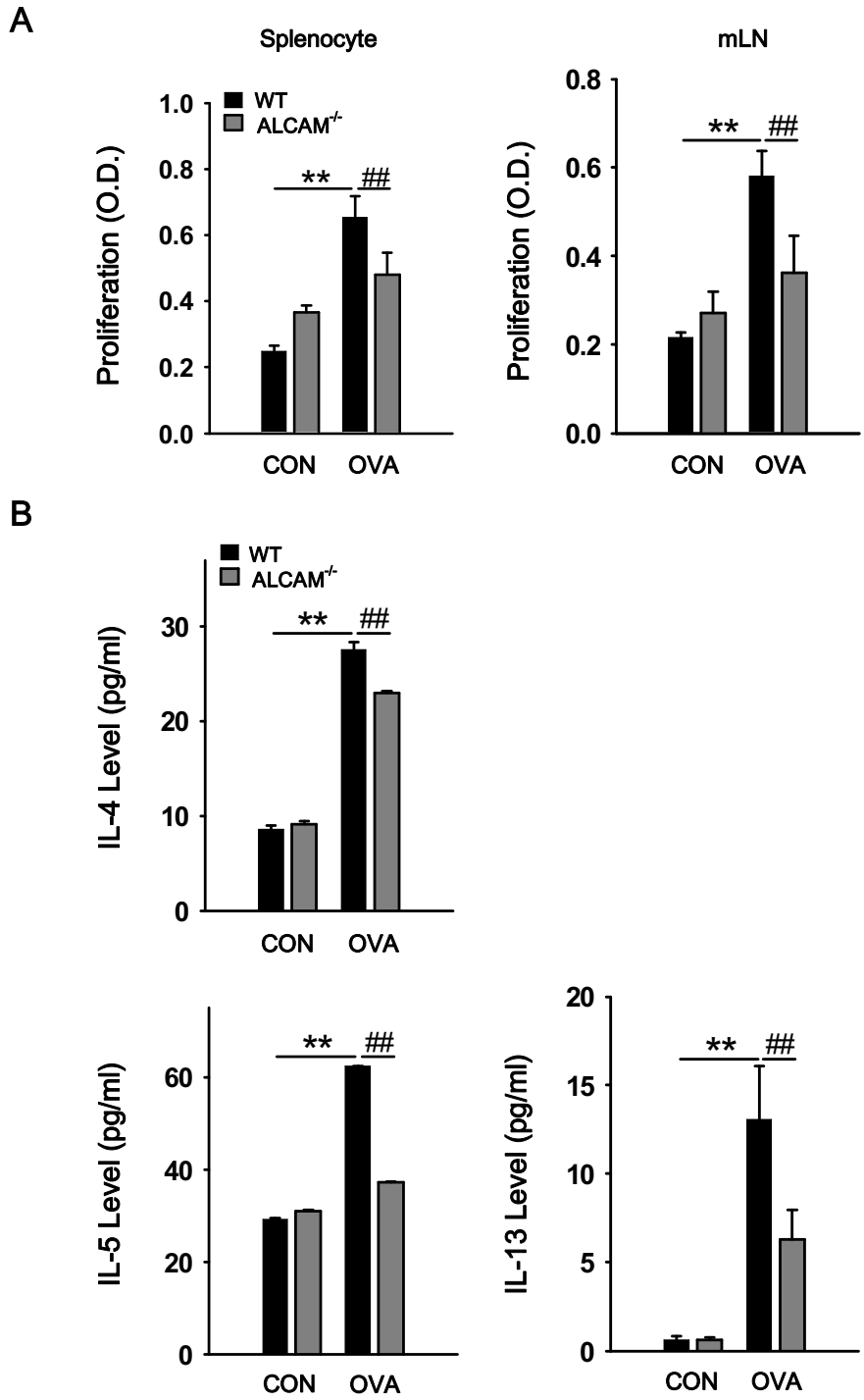
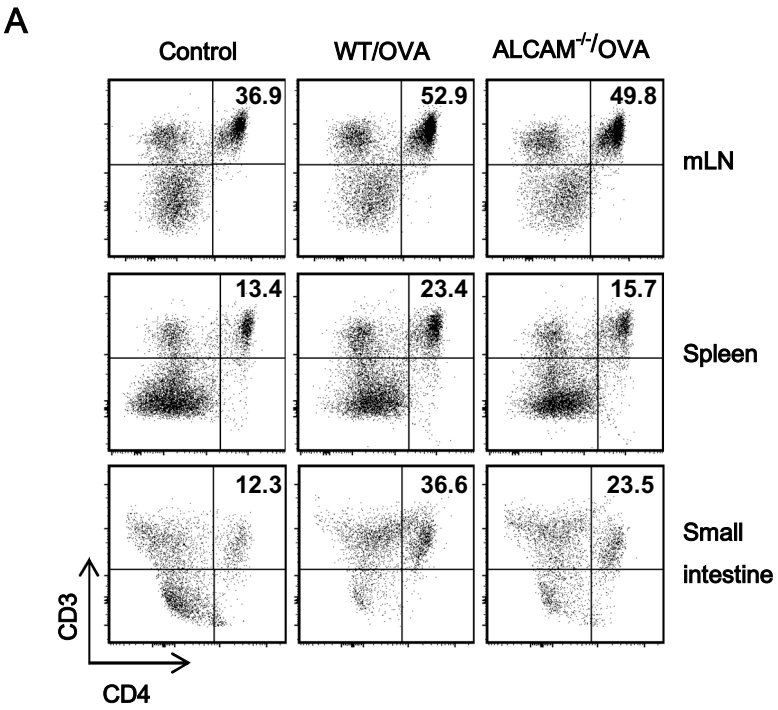
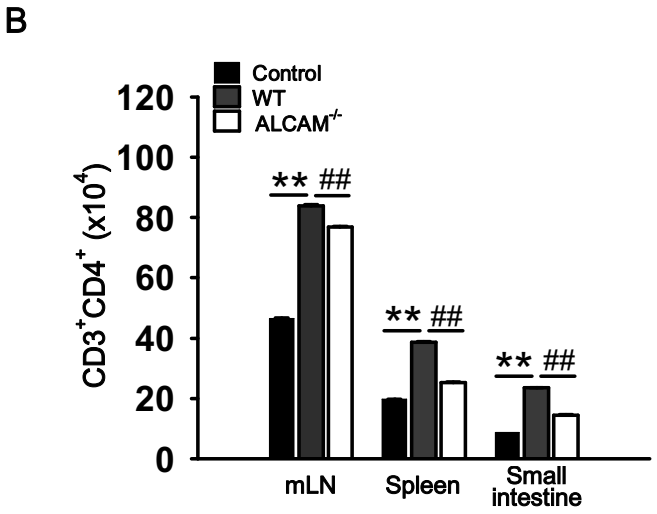
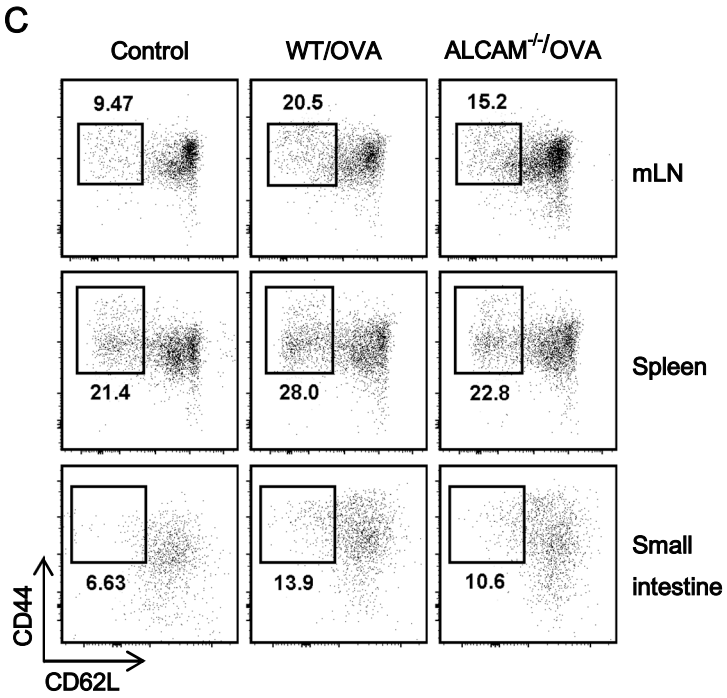


Figure 3. Decreased T cell proliferation in food allergy-induced ALCAM deficient mice with ovalbumin. Spleen and mLN were removed and cells were isolated from these tissues. Total cells of spleen and mLN were cultured in the absence (Control; CON) or presence of OVA for 5days. T cell proliferation was assessed with CCK-8. The supernatant of splenocyte was used for measurement of released T_H2 cytokine level. (A) Values of total cell proliferation in spleen and mLN. (B) T_H2 cytokine levels in supernatant of splenocytes cultured media by ELISA. ** means $P < 0.01$ (WT/CON vs. WT/OVA). ## means $P < 0.01$ (WT/OVA vs. $ALCAM^{-/-}$/OVA).







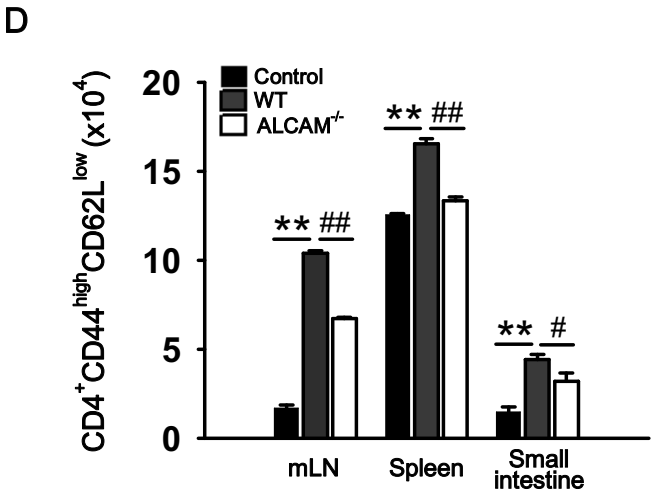


Figure 4. Diminished T cell population and T cell activation in food allergy-induced ALCAM deficient mice with ovalbumin. Single cells of spleen, mesenteric lymph node (mLN), and small intestine (jejunum) were gated with CD3, and CD4 or CD4, CD44, and CD62 ligand (CD62L) by flow cytometry. (A) $CD3^{+}CD4^{+}$ T cell populations of splenocytes, mLN cells and small intestine lamina propria mononuclear cells of OVA-challenged WT and $ALCAM^{-/-}$ mice. (B) Data of flow cytometry (A) showed as a graph. (C) Activated T cells were measuredand gated with $CD4^{+}CD44^{high}CD62L^{low}$ in lymphatic tissues of OVA-challenged WT and $ALCAM^{-/-}$ mice. (D) Data of flow cytometry (C) showed as a graph. Mean data were estimated from triplicated experiments. ** means $P < 0.01$ (WT/Control vs. WT/OVA). # means $P < 0.05$, ## means $P < 0.01$ (WT/OVA vs. $ALCAM^{-/-}$/OVA).

4. Increased levels of ALCAM in serum of children with food allergy

To determine the level of ALCAM in children with food allergy, we evaluated 143 children aged 0.5 – 10.8 years, with 53 and 90 in the food allergy and control groups, respectively. The clinical characteristics of the subjects are summarized in Table 1. There was no significant difference in gender, while mean age was higher in the control group than that in the food allergy group (7.4 ± 1.44 vs. 2.24 ± 1.9, $P < 0.0001$). Serum total IgE levels were significantly increased in children with food allergy compared to healthy controls (260.8 ± 294.6 vs. 40.4 ± 30.5, $P < 0.0001$). ALCAM levels in serum were significantly higher in those with food allergy than in healthy controls (35.4 ± 4.7 vs. 28.1 ± 4.2, $P < 0.0001$). (Figure 5.) A multiple regression analysis performed to evaluate whether ALCAM level relates to food allergy after controlling for age demonstrated similar results that ALCAM level was significantly increased in the food allergy group. ($\beta = 5.75$, $P < 0.0001$)

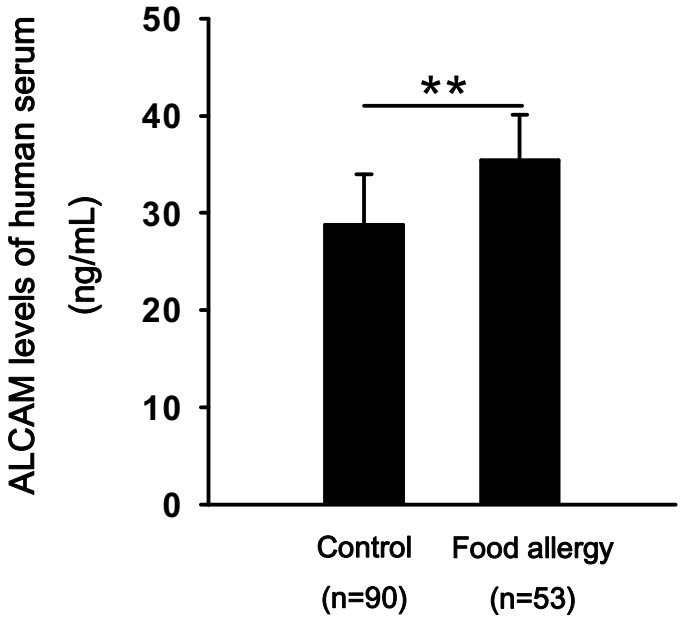


Figure 5. Enhanced levels of ALCAM in serum of children with food allergy. The ALCAM level of human serum was measured in children with food allergy (n=53) and healthy control subjects (n=90) by ELISA. ** $P < 0.01$.

Table 1. Subject characteristics

Characteristics	Control (n=90)	Food allergy (n=53)	*P* value
Age, yr	7.4 ± 1.44	2.24 ± 1.9	*P* < 0.0001
Sex, M (%)	42 (47)	32 (60)	0.158
Serum total IgE, kU/L	40.4 ± 30.5	260.8 ± 294.6	*P* < 0.0001

Values are expressed as number (percentage), mean ± SD. IgE, immunoglobulin E.

Ⅳ. DISCUSSION

Allergenic food proteins may induce not production of IgE but activation of special subsets of T cells to establish food allergy.[36] It means that the most common form of food allergy is mediated by IgE antibodies, but food allergy is also induced by T cells.[37] In small intestine, the main tissue of food allergy, lymphocytes are found at various locations in the gut mucosa. T cells especially resident in the epithelium.[38] When APCs, such as DCs, are present food allergens to T cells, the immunological synapse is formed. And there are many co-stimulatory molecules on immune cells engage in prolonged maintenance of immunological synapse.[14] ALCAM, one of co-stimulatory molecules, is expressed by DCs and interacts with CD6 on T cells. Many studies demonstrated that ALCAM mediates immune responses by interaction with CD6[29] and affects pathogenesis of cancers. One of the studies, they characterized ALCAM as intestinal cancer stem cell marker in the human and mouse gastrointestinal tract.[39]

This study identified that enhanced ALCAM levels in serum of OVA-challenged WT mice and children with food allergy against to control. These findings indicate that ALCAM has an effect on OVA-induced food allergy and correlate to studies that addressed the role of ALCAM in murine model or human disease. Hansen AG. et al. identified that ALCAM is overexpressed in murine model of metastatic prostate cancer and patient with prostate cancer.[40] Moreover, levels of ALCAM increased in patients with a variety of cancers, including melanoma, prostate, breast,

ovarian, esophageal, bladder, and intestinal cancers.[18-24,41] Here, this study show that the attenuated symptoms of food allergy in OVA-challenged ALCAM$^{-/-}$ mice compared with WT mice, such as decreased levels of T_H2 cytokines, injury of small intestine, and scores of parameters. It means ALCAM promotes immune response in food allergy and these data correlate with other studies that demonstrated the role of ALCAM in disease.[42-44] von Bauer R. et al. demonstrated that ALCAM similar to structure of receptor for advanced glycation end products (RAGE) and plays a role in delayed type hypersensitivity (DTH) as a pattern recognition receptor.[45] They induced DTH to mice and identified that decreased clinical score of allergic disease in DTH-induced ALCAM$^{-/-}$ mice against to DTH-induced WT mice.

Li XM et al. characterized T cell response to peanut allergen in a murine model of peanut allergy.[46] Authors identified that levels of allergen-specific IgE and systemic anaphylaxis score are significantly enlarged in peanut-induced mice compared to control mice. Moreover, T cell proliferation of splenocytes which were stimulated by peanut increased in peanut-induced mice. In another study, authors induce food allergy to BALB/c mice by sensitization with OVA.[47] OVA-challenged mice exhibited symptoms of food allergy such as incidence of diarrhea, decreased body temperature, increased levels of OVA-specific IgE, and expanded the number of mast cells. In addition, levels of T_H2 cytokines, and T cell proliferation of mLN and splenocytes were enhanced in OVA-challenged mice. Another previous studies identified that CD6, ligand for ALCAM, is a co-stimulatory molecule in T cell

activation.[28] Both ALCAM and CD6 are actively recruited and contribute to stabilization of the immunological synapse. Moreover, it is already known that ALCAM-CD6-mediated adhesion contributes to both early and later stages of DC-induced T cell activation and proliferation.[29,35] Aukje W. Zimmerman demonstrated that CD6 and ALCAM form a key adhesive receptor-ligand pair that is not only involved in early DC-T-cell binding but also in sustaining DC-induced T-cell proliferation long after the initial contact has been established.[29] These studies mean that study of T cell biology is the most widely used to investigate the role of ALCAM. For these reasons, we measured total T cell proliferation, CD4+T cell population, and activated T cells of systemic immune tissues to identify the reasons of attenuated symptoms in OVA-challenged ALCAM$^{-/-}$ mice in this study. As a result, total T cell proliferation of cultured mLN and splenocytes was diminished in OVA-challenged ALCAM$^{-/-}$ mice. More specifically, $CD3^{+}CD4^{+}$ T cell population and T cell activation were decreased in mLN, spleen, and small intestine of OVA-challenged ALCAM$^{-/-}$ mice than OVA-challenged WT mice. These results suggest that defected ALCAM draw attenuated T cell activation by diminished interactions with CD6 on T cells.

In this study, ALCAM levels of serum enlarged in OVA-challenged WT mice and children with food allergy, and OVA-challenged ALCAM$^{-/-}$ mice showed attenuated symptoms of food allergy. Diminished T cell responses in OVA-challenged ALCAM$^{-/-}$ mice indicate that ALCAM affects immune responses in food allergy

through change T cell activation and T_H2 response. Although more mechanism studies are required to specifically address the role of ALCAM in food allergy, this study suggests that ALCAM affects immune response in OVA-induced food allergy.

Ⅴ. CONCLUSION

In this study, expression of ALCAM in small intestine and levels of ALCAM in serum were changed in OVA-challenged WT mice compared to control mice. ALCAM levels in serum of children with food allergy were also enhanced compared to healthy control subjects. In OVA-challenged ALCAM$^{-/-}$ mice, immune responses were attenuated against to OVA-challenged WT mice. Total T cell proliferation of spleen and mLN, CD3^{+}CD4^{+} T cell population, and CD4^{+}CD44^{high}CD62L^{low} activated T cell of spleen, mLN, small intestine were also diminished in OVA-challenged ALCAM$^{-/-}$ mice compared to OVA-challenged WT mice.

In conclusion, this study suggests that ALCAM affects immune response in experimental food allergy. That is, ALCAM regulates allergic disease and affects immune responses by alternation of T cell activation and T_H2 response in OVA-induced food allergy.

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ABSTRACT (in Korean)

난백알부민에 의해 유도된 식품알레르기의 면역반응에서 활성백혈구부착분자 (ALCAM/CD166)의 역할

<지도교수 손 명 현>

연세대학교 대학원 의과학과

김 윤 선

식품알레르기는 경구 면역관용에 실패함으로써 나타나는 비정상적인 면역반응으로, 전세계적으로 주요하게 다뤄지는 건강 문제 중 하나의 질병으로 손꼽힌다. 활성백혈구부착분자 (ALCAM/CD166)는 면역글로불린상과 (IgSF) 에 속하는 막관통 당단백질로, T 세포의 표면에 존재하는 CD6와 상호작용하여 면역반응에서 역할을 하는 것으로 알려져 있다. 또한 ALCAM은 여러 가지 기관에서 발병되는 암의 발생에서 역할을 한다고 알려져 있다. 그러나, 현재 이러한 ALCAM이 알레르기 질병의 면역반응에 미치는 영향이나 기능을 확인한 논문은 없는 것으로 알려져 있다. 따라서 본 연구는 알레르기 질병 중 하나인 식품알레르기 모델에서 ALCAM의 기능을 확인하기 위하여 진행되었다.

BALB/c 야생형 마우스와 ALCAM 결핍 마우스에 난백알부민을 복강주사하여 감작시키고 경구투여했다. 그 후 마우스에서 증상에 대한 정도, 체온, 소장의 형태, 혈청 면역글로불린 E, T_H2 면역 반응 등을 포함한 식품알레르기 모델의 척도를 확인했다.

식품알레르기를 유발한 야생형 마우스의 소장에서는 ALCAM의 발현량이 감소하였고 혈청에 존재하는 ALCAM의 양은 증가한다는 것을 확인했다. 또한 대조군 보다 난백알부민으로 식품알레르기를 유발한 야생형 마우스에서 증상에 대한 정도, 혈청에 존재하는 총 면역글로불린 E와 난백알부민에 특이적인 면역글로불린 E의 양, T_H2 사이토카인(인터류킨-4, 인터류킨-5, 인터류킨-13)의 발현량, 헤마톡실린&에오신 염색을 한 소장의 손상 정도 등이 증가한 것을 확인하였다. 그에 반해서, 이러한 식품알레르기에 대한 척도가 ALCAM 결핍 마우스에서 억제되어 나타나는 것을 확인했다. T 세포의 보조자극신호 분자로 알려진 CD6가 그의 리간드인 ALCAM에 어떠한 영향을 미치는 지 알아보기 위해 마우스의 비장과 림프절에서 T 세포의 급증을 확인하였을 때, 식품알레르기가 일어난 야생형 마우스보다 ALCAM 결핍 마우스에서 더 감소하는 현상을 확인하였다. ALCAM이 관여하는 T 세포의 변화를 좀 더 특이적으로 확인하기 위하여 마우스의 비장과 림프절, 소장에서 세포를 분리하고 염색하여 $CD3^+CD4^+$ T 세포의 분포와 $CD4^+CD44^{high}CD62L^{low}$에 해당하는 활성된 T 세포의 수를 유세포분석으로 측정하였다. 그 결과 T 세포의 분포와 활성 정도 역시 ALCAM 결핍

마우스의 비장, 림프절, 소장에서 감소하는 것을 확인했다. 마지막으로, 마우스에서 확인한 결과와 같이 건강한 대조군 보다 식품알레르기 증상을 보이는 환아의 혈청에서도 ALCAM의 양이 증가하는 것을 확인하였다.

결론적으로, 본 연구에서 진행한 실험 결과를 통해 ALCAM이 T 세포의 표면에 존재하는 CD6와 상호작용하여 T 세포의 급증, 분포, 활성 등을 유도하고, T_H2 면역반응에 영향을 미침으로서 식품알레르기 모델에 연관되어 있다는 것을 확인하였다.

핵심 되는 말 : 식품알레르기, 식품알레르기 마우스 모델, 활성백혈구부착분자, ALCAM, CD166