

Effects of Egg White Consumption on Immune Modulation in a Mouse Model of Trimellitic Anhydride-induced Allergy

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Abstract

Egg allergy has been shown to be the most common food allergy in children with atopic dermatitis. Allergic reactions to proteins derived from egg white (EW) are more common than those derived from egg yolk. Ovomuroid, ovalbumin, ovotransferrin, and lysozyme have been identified as major allergens in EW. This study was conducted to evaluate the effect of EW on immune modulation in an induced allergy mouse model. A total of 50 five-week-old BALB/c male mice were treated with trimellitic anhydride (TMA) for three weeks to induce allergy-like symptoms. The TMA-treated mice were rested for one week and then divided into five groups and fed 0, 10, 50, and 100 mg/d EW for four weeks. All EW consumption groups showed no significant increase or decrease in the populations of white blood cells; however, a significant increase in B-lymphocyte activity was observed in the fourth week. Furthermore, EW consumption did not influence serum immunoglobulin G and immunoglobulin E levels. Taken together, these data demonstrate that the consumption of EW by TMA-treated mice did not increase allergic parameters such as serum IgE level, but enhanced the lymphocyte activities against pathogens. Therefore, this study suggests that the consumption of EW promotes Th2 immune modulation, and EW could be an excellent candidate for maintaining health.

Keywords: egg white, allergen, immunoglobulin, lymphocyte activation

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Introduction

Food allergies are adverse immune reactions to food proteins that induce atopic dermatitis and allergic gastrointestinal disorders. After cow's milk, chicken egg allergy is the second most common food allergy in infants and young children (Sampson, 2004). A recent meta-analysis of the prevalence of food allergies estimated that egg allergy affects 0.5-2.5% of young children (Rona *et al.*, 2007).

Compared to allergic reactions against egg yolk, reactions against egg white (EW) proteins are more frequent

(Anet *et al.*, 1985). Five major allergens have been characterized in hen's egg, which are designated Gal d 1-5 (Heine *et al.*, 2006). EW contains several allergens, including ovomucoid (Gal d 1), ovalbumin (Gal d 2, conalbumin), ovotransferrin (Gal d 3), lysozyme (Gal d 4), and ovomucin (Bernhisel-Broadbent *et al.*, 1994; Heine *et al.*, 2006). Although ovalbumin is the most abundant protein comprising hen's EW, ovomucoid has been shown to be the predominant allergen found in egg (Cooke and Sampson, 1997).

Egg allergies are currently diagnosed based on a patient's clinical history, a physical examination, a skin prick test, and the presence of immunoglobulin (Ig) E antibodies specific for EW. Egg allergies include IgE antibody-mediated allergy as well as other allergic syndromes such as atopic dermatitis and eosinophilic esophagitis, which are mixed IgE- and cell-mediated disorders (Hill *et al.*, 2008). IgE-mediated reactions, however, are the most common type of allergic reactions to egg. One research

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group has demonstrated that children with persistent egg allergies have significantly higher levels of serum IgE antibodies to ovomucoid and ovalbumin than those with a transient egg allergy (Järvinen *et al.*, 2007).

Trimellitic anhydride (TMA), a known respiratory sensitizer, is often used to trigger T cell-dependent contact hypersensitivity reactions in mice and to elicit eosinophil and T cell infiltration, Th2 cytokine production, and an increase in IgE (Bernstein *et al.*, 1982). The effects of TMA-induced inflammation on the cutaneous cytokine profile, immune cell infiltration, and serum IgE levels have been intensively studied (Dearman *et al.*, 2002; Sailstad *et al.*, 2003). Lymph node cells derived from TMA-treated BALB/c mice expressed high levels of the Th2 cytokines interleukin (IL)-4 and IL-10, but little of the Th1 cytokine interferon (INF)- γ . Exposure to 2,4-dinitrochlorobenzene (DNCB) provoked the inverse pattern of cytokine secretion (Dearman *et al.*, 1996).

Although eggs have both beneficial and detrimental biological effects, the effects of EW are yet to be demonstrated in animal models of allergy. In the present study, various concentrations of EW were fed to TMA-sensitized mice to determine the correlation between EW consumption and health parameters by measuring changes in allergy sensitization and immune modulatory activity. Furthermore, we discuss the advantages and disadvantages of analyzing EW consumption in this mouse model of allergy.

Materials and Methods

Preparation of EW

Normal, commercially available, fresh chicken eggs were obtained from a local egg farm. The EW was separated from the yolk, freeze-dried using a freeze dryer (Ilshintech, Korea), and resuspended in distilled water (DW).

Animals

All animal care protocols were approved by the Konkuk University Institutional Animal Care and Use Committee (Approval No.: KU13144-1). A total of 50 male BALB/c mice (five weeks old; Nara Biotech Co., Korea) were housed in a conventional temperature- and humidity-controlled room and provided with standard laboratory food and water.

Preparation of the TMA-sensitized and IgE-elevated mouse model

Five-week-old male BALB/c mice were used to pro-

duce a serum IgE-elevated model animal. Briefly, the fur on the backs of the mice was removed one day prior to the treatment with TMA. The treatment to the skin consisted of 40 mg of TMA dissolved in 200 μ L of a 3:1 solution of acetone in olive oil (0.2 mg/ μ L). Additional treatments with 40 mg TMA were then administered once every three days for three weeks. The TMA-sensitized mice were rested for one week before EW treatment.

Experimental design

Five groups of mice (10 mice per group) were used in the study. Group 1 animals were treated for four weeks with an oral dose of 0 mg/d EW. Group 2, 3, and 4 animals were treated for four weeks with an oral dose of 10, 50, or 100 mg/d EW, respectively. Group 5 animals were a model for studying the production of elevated serum IgE using DNCB as described in a previous study (Song *et al.*, 2014). The starting group and the control of TMA treated only groups were used by randomly selection.

Lymphocyte and serum preparation

All ten mice from each experimental group were sequentially anesthetized using 2.5% (v/v) avertin, and blood samples were collected by direct heart puncture. Approximately 0.2 mL of blood from each animal was collected into a tube containing ethylenediaminetetraacetic acid (EDTA; Becton Dickinson, USA), and 0.1 mL of whole blood was mixed with 0.1 mL of Histopaque-1077 (Sigma) and centrifuged at 1,200 g for 20 min. The lymphocytes were carefully collected from the middle phase of the gradient. To separate the serum, 0.5 mL of whole blood was incubated for 1 h at room temperature and then centrifuged at 1,200 g for 15 min. The supernatant was carefully collected and stored at -70°C .

White blood cell analysis

The collected whole blood samples were inverted several times to prevent coagulation in the EDTA-coated tube. The concentration of white blood cells and the percentages of lymphocytes, neutrophils, eosinophils, basophils, and monocytes were compared among the four treatment groups. Samples were analyzed immediately by an ADVIA 2120 hematology system (Siemens, Germany), according to the manufacturer's instructions.

Measurement of lymphocyte activation during EW treatment

Isolated lymphocytes (1×10^5 cells) from each treatment group were seeded into a 96-well plate and incubated for

1 h at 37°C. Lipopolysaccharide (2.5 µg/mL) was added to the cultured cells to detect B lymphocyte activity, and 2.5 µg/mL concanavalin A was added to assess T lymphocyte activity. The cells were then incubated for 48 h. Cell viability and proliferation were analyzed using an EZ-Cytotox kit (Daeil Lab Service, Korea) according to the manufacturer's instructions. The cell proliferation rate was determined using a microplate reader at an absorbance of 450 nm.

Analysis of immunoglobulin

The IgG and IgE concentrations in the whole blood samples were measured by a mouse IgG and IgE enzyme-linked immunosorbent assay, respectively, using a quantitation kit (Bethyl Laboratory Inc., USA) in accordance with the manufacturer's instructions. Serum IgG and IgE concentrations were evaluated by comparison with serial dilutions of IgG and IgE standards provided in the kit.

Analysis of interleukin 4 (IL-4)

The IL-4 concentration in serum samples was measured by a mouse IL-4 quantification assay kit (R&D systems, USA) according to the manufacturer's instructions. Serum IL-4 concentrations were evaluated by comparison with serial dilutions of IL-4 standards provided in the kit.

RNA extraction, Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA was extracted from spleen tissues using TRIzol (Life Technologies, USA). The extracted RNA was treated with DNaseI (New England BioLabs, USA) for 30 min, and enzymes were inactivated using a 0.2 M EDTA solution (Sigma-Aldrich; E6635) for 10 min. cDNA was synthesized from 1 µg of total RNA using an RT-PCR premix kit (iNtRON, Korea). Relative levels of GATA3, IL-4, IL-13, INF-γ, and TNF-α mRNA expression were estimated in duplicate samples by fluorescence and quantified using a Rotor-gene Q Real-Time PCR Detection System (Qiagen, USA). The reaction was initiated in a total volume of 20 µL containing 10 ng of cDNA and 1

pM of each primer in a reaction buffer containing iQ SYBR Green Supermix (Bio-Rad Laboratories, USA). All cycle threshold (Ct) values were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The results are presented as target gene expression relative to control gene expression. PCR amplification was performed using 40 cycles for 20 sec at 95°C, 20 sec at 55°C, and 20 sec at 72°C. All primer sets are listed in Table 1.

Statistical analysis

Data were analyzed by one-way analysis of variance using SPSS statistical package ver. 21.0 for Windows. A *t*-test was performed for comparisons between control and experimental groups. All data are expressed as the mean± standard deviation. The null hypothesis was rejected when the probability was $p < 0.05$.

Results and Discussion

Effect of EW consumption on immunoglobulin levels

To investigate the ability of EW to modulate blood Ig levels in allergy-sensitized mice, serum IgG and IgE concentrations in the blood were analyzed after four weeks of EW treatment. Serum IgG levels neither significantly decreased nor increased compared with those of the negative control (normal mice; Fig. 1A). In contrast, the serum IgE concentration significantly increased in the TMA-treated only group compared to the negative control group (Fig. 1B). However, after four weeks, the serum IgE levels of the EW consumption groups significantly decreased, while the serum IgE level of the DNCB-treated group significantly increased compared with that of the control group (Fig. 1B).

Food allergies are frequently the result of an IgE-mediated hypersensitivity reaction, and the serum IgE level has a strong association with an allergic response (Burrows *et al.*, 1989). In our study, however, serum IgE levels were unchanged in EW-treated groups compared with levels in the TMA-treated, 0 mg/d EW group (Fig. 1B).

Table 1. Primers used for the reverse transcription-polymerase chain reaction (RT-PCR) of cDNA from mouse spleen

Gene	Forward Primer	Reverse primer
GATA3	5'- TTTACCCTCCGGCTTCATCCTC-3'	5'- TGCACCTGATACTTGAGGCACTCT-3'
IL4	5'- AACGAGGTCACAGGAGAAGG -3'	5'- TCTGCAGCTCCATGAGAACA -3'
IL13	5'- TGAGGAGCTGAGCAACATCACACA-3'	5'- TGCGGTTACAGAGGCCATGCAATA -3'
INF-γ	5'- GGCCATCAGCAACAACATAAGCGT -3'	5'- GCTACGACGTGGGCTACAG-3'
TNF-α	5'- CCCTCACACTCAGATCATCTTCT-3'	5'-CATCTGCTGGAGGCTGAGGT-3'
GAPDH	5'- GTTGTCTCCTGCGACTTCA-3'	5'- TGGTCCAGGGTTTCTTACTC-3'

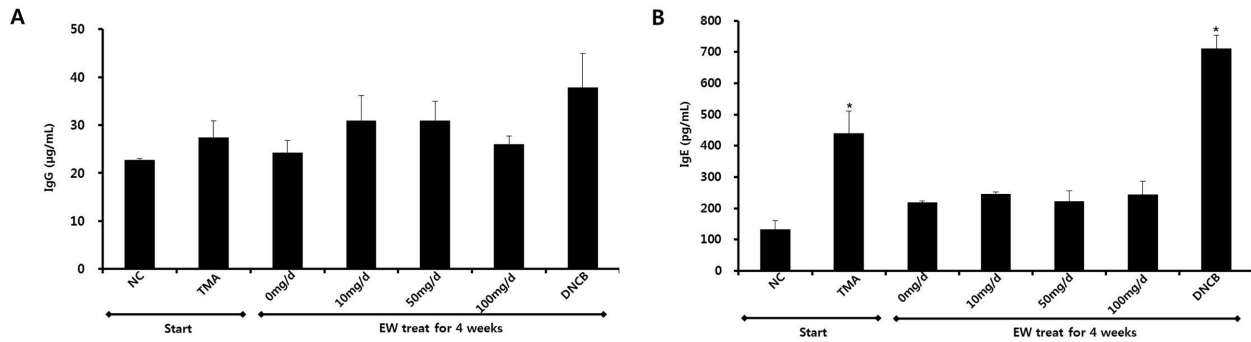


Fig. 1. Effect of egg white (EW) treatment on mouse immunoglobulin production. Blood samples were collected from start group (negative control, NC), control of TMA treated only group (TMA) and EW-treated (0, 10, 50, and 100 mg/d) mice at wk 4. The samples were incubated with anti-mouse IgG (A) and IgE (B) antibodies. A horseradish peroxidase-conjugated secondary antibody was used to detect the absorbance of each Ig. *P*-values were calculated for the differences between immunoglobulin levels in the 0 mg/d (control) group and each EW treatment group at week 5 ($*p < 0.05$, $n = 10$). EW: egg white; DNCB: 1-chloro-2,4-dinitrobenzene.

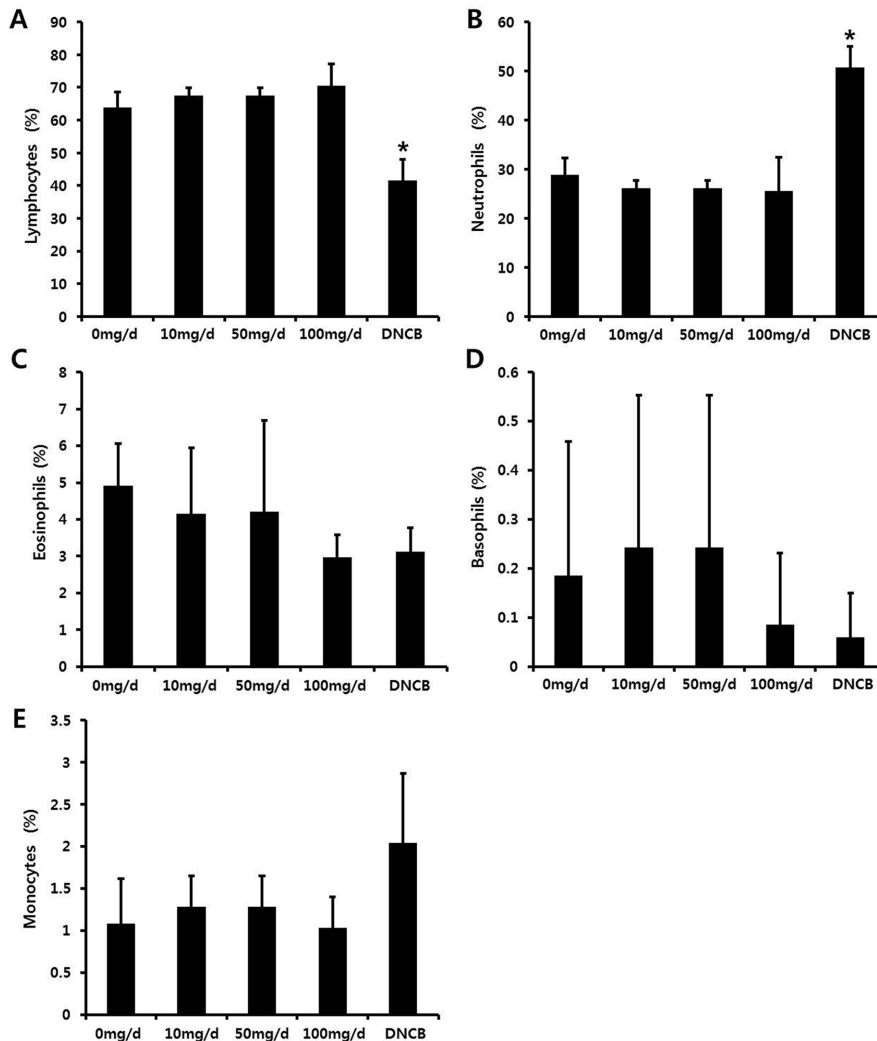


Fig. 2. Populations of lymphocytes, granulocytes, and monocytes. (A) Lymphocytes, (B) neutrophils, (C) eosinophils, (D) basophils, and (E) monocytes are presented as the percentages of total white blood cells. A statistical analysis was conducted to determine differences in cell populations between the 0 mg/d (control) group and each EW treatment group in the same week ($*p < 0.05$, $n = 10$). EW: egg white; DNCB: 1-chloro-2,4-dinitrobenzene.

However, IgE levels alone are not sufficient to explain food allergies. Allergy to cow's milk affects about 2.5% of infants in the first year of life (Host and Halcken, 1990), with IgE reactions accounting for about 60% of milk allergy-related disorders in 1.5% of all infants. Although most infants with a cow's milk allergy will outgrow their sensitivity by their third year of life, 15% of infants with IgE-mediated cow's milk allergy retain this sensitivity into their second decade, and 35% of infants have allergic reactions to other foods (Host *et al.*, 1997). IgE-mediated allergic reactions to egg occur in about 1.3% of young children (Nickel *et al.*, 1997). In this study, we observed total IgE levels, not IgE levels specifically reacting to EW. The total IgE levels were not sufficient for explain allergy reaction in this study.

Effect of EW consumption on immune cell populations

To determine whether EW influences populations of blood immune cells, we analyzed changes in the number of lymphocytes, neutrophils, eosinophils, basophils, and monocytes. There were no significant differences in the populations of these cells among the EW treatment groups (Fig. 2). The percentage of lymphocytes among total white blood cells significantly decreased, and the percentage of neutrophils significantly increased in the DNCB-treated group after four weeks of treatment (Fig. 2A and B). These results were consistent with our previous study (Song *et al.*, 2014). Neutrophils primarily play a role in acute inflammation, and the neutrophil-to-lymphocyte ratio (NLR) is a measure of the balance between neutrophil and lymphocyte levels in the body, with a high NLR indicating systemic inflammation (Zahorec, 2001). In this study, DNCB was used to induce an allergic reaction and inflammation. EW, in contrast, did not promote inflammation.

Effect of EW consumption on lymphocyte activation

The activation of B and T lymphocytes was compared among the control and EW-treated groups. In the present study, we found that B lymphocyte activation was significantly higher in the 10 and 50 mg/d EW groups than the control group (Fig. 3A). Additionally, T lymphocyte activation was not significantly increased in all TMA-treated EW groups compared to that of the control group (Fig. 3B). Taken together, our data showed that EW consumption regulates B lymphocyte activation, suggesting that EW may be an immune activator in the TMA-induced allergy model. A recent study showed that water-soluble egg

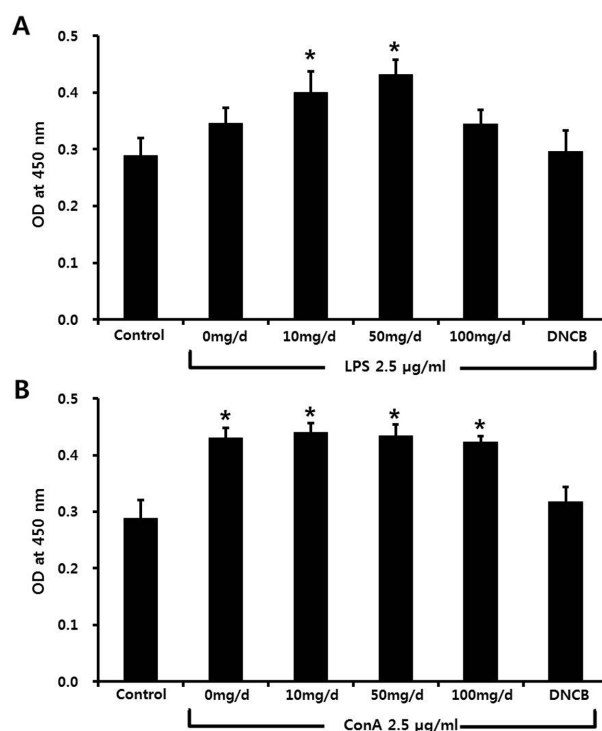


Fig. 3. B and T lymphocyte activation by egg white. Lymphocytes were isolated from EW-treated (0, 10, 50, and 100 mg/d) mice at wk 4. Isolated lymphocytes (1×10^5 cells) were treated with (A) 2.5 µg/mL lipopolysaccharide (LPS) for B lymphocyte activation or (B) 2.5 µg/mL concanavalin A (ConA) for T lymphocyte activation. *P*-values were calculated for the differences in lymphocyte activation between the control (normal mice) group and each EW treatment group at wk 4 ($*p < 0.05$, $n = 10$). EW: egg white; OD: optical density; DNCB: 1-chloro-2,4-dinitrobenzene.

yolk extract significantly increased monocyte and B lymphocyte activity (Lee *et al.*, 2013). In this study, the TMA-stimulated B lymphocytes were re-stimulated by EW intake, but T lymphocyte activation was not promoted by EW consumption.

Effect of EW consumption on allergy-related gene expression

To determine whether EW influences allergy-related gene expression, we analyzed IL-4 levels in serum. Serum IL-4 levels significantly increased in all EW consumption groups (10, 50 and 100 mg/d) compared with the control group (0 mg/d; Fig. 4). In addition, TNF- α levels significantly increased in the 50 and 100 mg/d EW consumption groups compared with the control group (0 mg/d), but INF- γ expression was significantly reduced in the 100 mg/d EW consumption group and the DNCB-treated group (Fig. 5D and E).

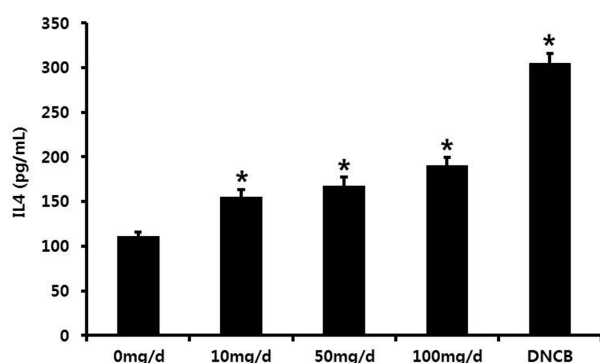


Fig. 4. IL-4 levels after egg white treatment. Blood samples were collected from EW-treated (0, 10, 50, and 100 mg/d) mice at wk 4. The samples were incubated with anti-mouse IL-4 antibody. A horseradish peroxidase-conjugated secondary antibody was used to detect the absorbance of each cytokine. *P*-values were calculated for the differences in IL-4 levels between the 0 mg/d (control) group and each EW treatment group at wk 5 ($*p < 0.05$, $n = 10$). EW: egg white; DNCB: 1-chloro-2,4-dinitrobenzene; IL-4: interleukin 4.

IL-4 is a key cytokine in the development of allergic inflammation. It is associated with the induction of the ϵ isotype switch and secretion of IgE by B lymphocytes (Coffman *et al.*, 1986). An essential role of IL-4 in the development of allergic inflammation is driving the differentiation of naive T helper type 0 (Th0) lymphocytes

into Th2 lymphocytes (Hsieh *et al.*, 1992; Seder *et al.*, 1995). These Th2 cells can secrete IL-4, IL-5, IL-9, and IL-13, but lose the ability to produce INF- γ (Jutel *et al.*, 1995). Conversely, Th1 cells, another outcome of Th0 differentiation, secrete INF- γ and TNF- β and are efficient in eliminating intracellular pathogens (Abbas *et al.*, 1996). The Th1/Th2 balance is extremely important and may determine whether the immune response is appropriate or leads to severe immunopathology. Overproduction of Th1 cytokines has been implicated in delayed-type hypersensitivity reactions and autoimmune disease. Interestingly, increased TNF- α has been detected in patients with asthma (Kips *et al.*, 1993), raising the possibility that TNF- α and IL-4 mediate selective recruitment of lymphocytes and eosinophils through the synergistic upregulation of vascular cell adhesion molecule-1 expression. In this study, both IL-4 and TNF- α levels increased, but INF- γ levels decreased after EW and DNCB treatment (Figs. 4 and 5). The Th2 cytokine IL-4 is necessary for IgE synthesis (Böhm and Bauer, 1997). We found that EW consumption increased IL-4 and TNF- α levels, but it did not affect IgE expression. Previously, it was shown that for calculating the IgE/IgG₄ ratio, EW is more useful a parameter for predicting the outcome of oral challenge with eggs than is IgE alone (Okamoto *et al.*, 2012). We compared the EW consumption groups with the DNCB-treated group. The DNCB treatment increased atopic dermatitis, but EW

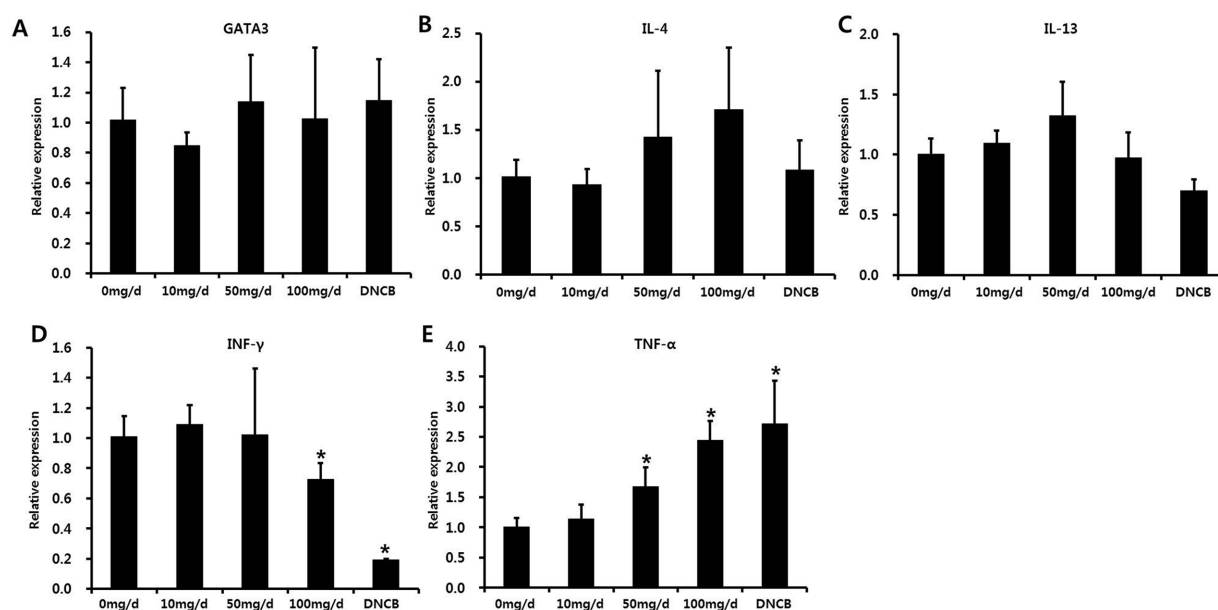


Fig. 5. Allergy-related gene expression after egg white treatment. (A) GATA3, (B) IL-4, (C) IL-13, (D) INF- γ , and (E) TNF- α are presented as the relative mRNA expression in the spleen. A statistical analysis was conducted to determine differences in expression between the 0 mg/d (control) group and each EW treatment group in the same week ($*p < 0.05$, $n = 10$). EW: egg white; DNCB: 1-chloro-2,4-dinitrobenzene; IL-4: interleukin 4; INF- γ : interferon- γ ; TNF- α : tumor necrosis factor α .

consumption was not sufficient to affect atopic dermatitis. Taken together, these results indicate that EW intake stimulates Th2 immune response in the induced allergy mouse model.

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