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2 **Lactalbumin and β -Lactoglobulin through NF- κ B Pathway in Immune Cells**

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ABSTRACT

α -Lactalbumin and β -lactoglobulin are two major whey proteins which specifically bind immunoglobulin E, and are suspected as major allergens causing cow milk allergy (CMA). Recent reports have shown that thymic stromal lymphopoietin is a critical factor linking at the interface of the body and environment to the T-helper 2 response. However, it is not known whether or not thymic stromal lymphopoietin expression is changed by α -lactalbumin and β -lactoglobulin in immune cells. Using RT-PCR and ELISA, the present study was conducted to examine if intravenous injection of α -lactalbumin and β -lactoglobulin increase proinflammatory cytokines, T-helper 2 cytokines, and thymic stromal lymphopoietin expression in several immune cells, including macrophages, mast cells and keratinocytes. Results showed that α -lactalbumin and β -lactoglobulin induced thymic stromal lymphopoietin, interleukin-6 and tumor necrosis factor- α expression. It was concluded that the allergenicity of α -lactalbumin and β -lactoglobulin may be attributed to thymic stromal lymphopoietin induction, T-helper 2 cytokines and proinflammatory cytokines.

KEYWORDS: *α -lactalbumin, β -lactoglobulin, thymic stromal lymphopoietin, T-helper 2 cytokine, proinflammatory cytokine*

INTRODUCTION

It is generally known that cow milk allergy (CMA) is the most common food allergy in infants and children,¹ with symptoms ranging from mild to severe and life-threatening.² Symptoms associated with immunoglobulin E (IgE)-mediated cow milk allergy include: gastrointestinal, cutaneous, respiratory and generalized allergy (anaphylactic shock).³⁻⁴ In addition, patients with CMA develop an IgE response against cow milk proteins such as α_{S1} -casein, α -lactalbumin (α -LA), and β -lactoglobulin (β -LG).⁵ Especially, α -LA and β -LG among whey proteins bind specific IgE⁶ and are suspected to be significant allergens in cowmilk.⁷⁻⁸ These milk proteins activate T-helper 2 (Th2) cells, as demonstrated by interleukin-4 (IL-4) and interleukin-5 (IL-5) secretions.⁹ IL-4 is important in driving Th2 cell differentiation and induces production of allergen-specific IgE by B cells, which bind to the high affinity immunoglobulin E receptor (Fc ϵ RI) on mast cells and basophils.¹⁰

Thymic stromal lymphopoietin (TSLP) is a key mediator of atopic dermatitis¹¹⁻¹² and a critical factor linking responses at the interface of the body and environment to the Th2 response.¹³⁻¹⁴ TSLP is an interleukin-7 (IL-7)-like cytokine expressed mainly by epithelial cells,¹⁵ and recent studies have revealed that epidermal keratinocytes (such as mast cells, airway smooth muscle cells, fibroblasts, dendritic cells, and cancer or cancer associated cells) also express TSLP.¹⁶ Generally, at barrier interfaces, environmental stimuli such as allergen exposure, viral infection, microflora, helminth infections, cigarette smoke, and chemicals trigger TSLP production, result in initiation of the sensitization process and endogenous triggers and regulators.¹⁶ In addition to environmental triggers, proinflammatory cytokines, Th2-related cytokines, and IgE contribute to TSLP production, suggesting endogenous amplification cycles.¹⁷⁻¹⁸

Numerous studies have shown that TSLP acts as a master switch that triggers both the

initiation and maintenance of atopic dermatitis and the atopic march.¹⁹⁻²¹ However, to date, it has not been known whether or not allergens such as whey proteins (α -LA and β -LG) can induce TSLP, nor has been demonstrated if TSLP expression is changed by α -LA and β -LG in immune cells. Therefore, the objectives of our study were to: (1) investigate whether α -LA and β -LG increase TSLP expression in immune cells, (2) confirm the modulation of IL-6 and TNF- α expression, (3) evaluate if α -LA and β -LG cause the elevation of IL-6 and TSLP levels in mice *in vivo* through intravenous injections, and (4) determine if α -LA and β -LG induce TSLP, IL-6 and TNF- α expression, which are involved in the nuclear factor-kappa B (NF-kB) pathway in RAW264.7 cells.

■ MATERIALS AND METHOD

Cell Culture

The murine macrophage-like RAW264.7 cell line and human keratinocyte cell line, or HaCaT, were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 and HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA). The human mast cell line (HMC-1) cell was cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco BRL, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA). The cells were cultured at 37 °C in a 5% CO₂ humidified incubator.

RNA Isolation and RT-PCR Analysis

94 RAW264.7, HaCaT and HMC-1 cells were plated at 5×10^5 cells/mL into a 12-well plate.
95 The cells were treated with 0.5, 5, 50 $\mu\text{g/mL}$ α -LA and β -LG, respectively, and with 1 $\mu\text{g/mL}$
96 lipopolysaccharide (LPS) or 1 $\mu\text{g/mL}$ phorbol myristate acetate (PMA) together. After 4.5 hrs,
97 total RNA was isolated from these cells using Trizol reagent (Invitrogen, Carlsbad, CA,
98 USA). One microgram of total RNA was converted to cDNA with 25 units/ μL Oligo (dT)
99 primer, 1 mM dNTPs, 1.75 units/ μL ribonuclease inhibitor, 2.5 units/ μL M-MLV Reverse
100 Transcriptase, M-MLV RT $5 \times$ buffer (Promega, Madison, WI, USA). To detect TSLP, IL-6
101 and TNF- α mRNA, the synthesized cDNA was used per 20 μL reaction, which comprised of
102 PCR $2 \times$ dye mix (Enzynomics, Daejeon, South Korea) with specific primer pairs.

103 Sequences of the PCR primer pairs used for the amplification of mouse TSLP are as follows:

104 forward 5' CGACAGCATGGTTCTTCTCA-3' and reverse 5'-
105 GGACTTCTTGTGCCATTTC-3'; mouse IL-6, forward 5'-
106 GATGCTACCAAAGTGGATATAATC-3' and reverse 5'-
107 GGTCCTTAGCCACTCCTTCTGTG-3'; mouse TNF- α , forward 5'-
108 ATGAGCACAGAAAGCATGATCCGC-3' and reverse 5'-
109 CCAAAGTAGACCTGCCCCGGACTC-3', glutaldehyde-3-phosphate dehydrogenase
110 (GAPDH), forward 5'-CCATCACCATCTTCCAGGAG-3' and reverse 5'-
111 ACAGTCTTCTGGGTGGCAGT-3'; human TSLP, forward 5'-
112 GCCCAATTCCCCTAAAAGAA-3' and reverse 5'-AGTCCACCAAGAAAGCCTCA-3'.

113 PCR was carried out using the GeneAmp PCR System 2700 (Applied Biosystems, Foster
114 City, CA, USA). The amplified products were separated by electrophoresis on a 2% agarose
115 gel, stained with ethidium bromide, and photographed under UV illumination with Gel-Doc
116 2000 UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA).

117

Cytokine Assays

RAW264.7 cells were seeded and cultured in a 48-well plate at 4×10^5 cells/mL. The cells were stimulated with 1 μ g/mL LPS (Sigma Aldrich, St. Louis, MO, USA) and treated with 0.5, 5, 50 μ g/mL α -LA (Sigma Aldrich, St. Louis, MO, USA) and β -LG (Sigma Aldrich, St. Louis, MO, USA), respectively. After 14hrs, the supernatant was collected and assayed by enzyme-linked immunosorbent assay (ELISA). Secretion levels of IL-6 and TNF- α were detected using an ELISA kit (BD Bioscience, San Diego, CA, USA) according to the manufacturer's instructions.

TSLP expression was detected in the supernatant, which was collected from the treated cell. The cells were treated with α -LA and β -LG and incubated for 20 hrs using an ELISA kit (Biolegend, San Diego, USA). The secretion levels of TSLP, IL-6 and TNF- α were quantified according to standard curves using the SOFT max curve-fitting software program (Molecular Devices, Corporation, Ramsey, MN, USA).

In order to evaluate whether or not the nuclear factor (NF)- κ B pathway is involved in TSLP induction by α -LA and β -LG treatments, Bay11 (Sigma Aldrich, St. Louis, MO, USA), NF- κ B inhibitor, was used to treat RAW264.7 cells with three different (0.1, 1, 10 μ M) concentrations. Subsequently, the supernatant was analyzed by ELISA to determine the reduction of TSLP, IL-6 or TNF- α release.

Transient Transfection and Luciferase Assay

RAW264.7 cells were plated at a density of 1×10^5 cells/mL and transfected each with 1 μ g of pAP-1-Luc (Agilent Technologies, Santa Clara, CA, USA), pNFAT-Luc (Agilent Technologies, Santa Clara, CA, USA) and pNF- κ B-Luc (Agilent Technologies, Santa Clara, CA, USA) plasmid vectors with Attractene Transfection Reagent (Qiagen, Chatsworth, CA,

USA) according to manufacturer's instructions. The transfected RAW264.7 cells were cultured in a 96-well plate (1×10^4 cells/well), and then LPS, α -LA and β -LG were treated and each treated cells were incubated. After 6 hrs, ONE-gluciferase assay (Promega, Madison, WI, USA) was used according to manufacturer instructions. The detection was performed using a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Western Blot Analysis

RAW264.7 cells were seeded at 4×10^5 cells/mL in a six-well plate, and then the cells were incubated overnight. The cells were treated with α -LA (50 μ g/mL) and β -LG (50 μ g/mL) for 1 hr. Subsequently, 1 μ g/mL LPS was placed into each well and incubated for 1 hr. Next, soluble lysates from the cells were extracted in a lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease (Roche, Nutley, NJ, USA) and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) for 30min on ice. Equal amounts of protein were subjected to electrophoresis in 8-12% SDS-polyacrylamide gel for 2 hrs at 100V and then transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA). The membranes were incubated for 1hour in 5% (w/v) skim milk in PBS containing 0.05% (v/v) Tween 20 (PBST). After washing with PBST, the membrane was reacted with anti-Inhibitor κ B (IkB) (Cell Signaling Technology, Beverly, MA, USA) and anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4 °C. Immunoreactive proteins were detected with secondary antibody and visualized using ECL reagent.

Immunofluorescence and Confocal Microscopy

RAW264.7 cells (4×10^5 cells/mL DMEM) were plated in a six-well plate, and the plate was

incubated overnight. The cells were treated with α -LA (50 μ g/mL), β -LG (50 μ g/mL) and LPS (1 μ g/mL) in each well, then incubated for 1 hr. The supernatants were removed and the cells were fixed to the plate with a fixation/permeabilization solution for 20 min at 4 °C. After 20 min, the fixer was removed, followed by washing the cells with BD Perm/Wash buffer. Fixer and washing buffer used were the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscience, San Diego, CA, USA), and the procedure was performed according to the manufacturer's instructions. Cells were incubated with anti-NF- κ B/p65 antibody (Invitrogen, Carlsbad, CA, USA) diluted at 1:200 in blocking buffer overnight at 4°C. The cells were then washed, and incubated with FITC-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), which was diluted at 1:1000 in blocking buffer for 1 hr. Nuclear staining was carried out with Hoechst for 2 min. Translocation of p65 was observed using a Zeiss confocal microscope (Carl Zeiss, Jena, Germany).

Mice *in vivo* Experiment

Eight week-old male Balb/c mice were purchased from Koatech Corporation (Pyeongtaek, South Korea), and housed according to a 12-12 hour light-dark cycle. The animals were maintained under specific pathogen-free conditions, and managed according to the guidelines for the care and use of laboratory animals (KRIBB, Daejeon, Korea). All animal experiments were approved by the institutional review board (KRIBB Institutional Animal Care and Use Committee/KRIBB-IACUC, approval number: KRIBB-AEC-13107), and the study was conducted in accordance with institutional guidelines.

Experimental mice (20 g/animal) were divided into four treatment groups with five animals each. The experimental animals were received four different treatments of intravenous injections as control (PBS-injected, n=5), LPS (0.4 μ g/mouse)-treated (n=5), α -

LA (200 $\mu\text{g}/\text{mouse}$)-treated ($n=5$) and β -LG (200 $\mu\text{g}/\text{mouse}$)-treated ($n=5$). Four hours later, the entire blood of each mouse was collected using a 1 mL syringe with 28G needle. Plasma were obtained by centrifugation at $16,000\times g$ (Hanil Co. Mega 17R, Incheon, Korea) for 5 min at 4°C , and analyzed for detection of TSLP and IL-6 expression using ELISA.

Statistical Analysis

Statistical significance was evaluated using the Student's unpaired t -test. A confidence level ($p<0.05$) was considered significant. Values are reported as the mean $\pm$ SD except for the values from the *in vivo* study, which are computed as the mean $\pm$ Standard Error of the mean (SEM).

■ RESULTS

Effects of α -LA and β -LG on Induction of mRNA Expression of Allergy-Mediated Cytokines and Immune Factor Secretion

As aforementioned, TSLP has recently been identified as a major contributing factor in allergic reactions¹¹⁻¹² and in activity of dendritic cells (DCs), which resulted in their activation and production of Th2 cytokines such as IL-4, IL-5 and IL-13, and a proinflammatory cytokine similar to TNF- α .²² α -LA and β -LG increased TSLP mRNA expression in dose- (Fig. 1A and 1C) and time-dependent manners (Fig. 1B and 1D). Proper dose for both α -LA and β -LG turned out to be 50 $\mu\text{g}/\text{mL}$ for the experiment. In cases of TSLP and IL-6 expression, the increases of mRNA expression by α -LA and β -LG were observed after 3 hrs of the treatment of the cells and persisted 9 hrs later. TNF- α mRNA expression following α -LA and β -LG treatments increased more rapidly than TSLP and IL-6 expression in RAW264.7 cells. In addition to RAW264.7 cells, HeCaT and HMC-1 cells responded to α -LA and β -LG, which

resulted in TSLP mRNA induction (Fig. 1E and 1F).

The LPS and PMA were used as positive controls as stimulants in expressions of cytokines (TNF- α , IL-6) and related factors, which are associated with inflammation response in the immune cells in vitro system. Thus, PMA (1 μ g/mL) and LPS (1 μ g/mL) were used as stimulating factors for TSLP mRNA expression. The effects of α -LA and β -LG were compared with those of positive controls on the expression of inflammation related factors in immune cells. HMC-1 and RBL-2H3 cells are the mast cell line which are directly associated with the secretion of allergy related cytokines. Our experiment was to confirm whether the expression of the allergy eliciting factor, TSLP, is increased by α -LA or β -LG treatments or not.

As expected, α -LA and β -LG applied to RAW264.7 cells exhibited an increase in TSLP (Fig. 2A and 2B), TNF- α (Fig. 2C and 2D) and IL-6 (Fig. 2E and 2F) secretion. Moreover, these cytokines were significantly increased with increased dosage (Fig. 2). However, β -LG showed less increase in the secretion of these cytokines than that of α -LA. These results indicated that α -LA and β -LG increased the secretion of TSLP as well as proinflammatory cytokines. The outcome of this experiment indicated that α -LA and β -LG could increase of TSLP mRNA expression in RAW264.7 cells, as well as increase in TNF- α and IL-6 mRNA expression.

α -LA and β -LG Increased TSLP, TNF- α and IL-6 Expression Mediated by the NF- κ B Pathway

TSLP is expressed and produced by the NF- κ B pathway in various cells.²³ In addition, it is known that NFAT or AP-1 involved with TSLP expression in transcription or post-transcription.¹⁶ To verify the effects of α -LA and β -LG on NF- κ B activation in RAW264.7

cells, an assay was performed using the reporter gene after α -LA and β -LG treatment and analyzed NF- κ B-luciferase activity. As shown in Fig. 3A and 3B, α -LA and β -LG stimulated and increased NF- κ B-luciferase activity in a dose-dependent manner. However, AP-1 and NFAT luciferase activities were weakly activated by α -LA and β -LG (Fig. 3C and 3D). These results suggest that α -LA and β -LG activate NF- κ B, which might be involved in TSLP production in RAW264.7 cells. For further verification of this involvement, we have used Bay11, an NF- κ B inhibitor, on α -LA- and β -LG-treated RAW264.7 cells, and then the secreted TSLP, TNF- α and IL-6 levels were detected by ELISA. As shown in Fig. 4, the induced secretion of TSLP (Fig. 4A and 4B), TNF- α (Fig. 4C and 4D) and IL-6 (Fig. 4E and 4F) by α -LA and β -LG is inhibited by Bay11. Bay11 decreased the secretion of cytokines dose-dependently. In most cases, 10 μ M Bay11 significantly blocked the induction of TSLP, TNF- α and IL-6 secretion by α -LA and β -LG. LPS was used as a positive control. These data collectively revealed that α -LA and β -LG among whey proteins stimulated NF- κ B activation and TSLP induction, where these two processes were mediated by the NF- κ B pathway.

Effects of α -LA and β -LG on Degradation of I κ B Protein and p65 Translocation into Nucleus

In order to have further confirmation on the fact that α -LA and β -LG stimulated NF- κ B activation, we have evaluated I κ B degradation in the cytosol (Fig. 5A) and the translocation of the NF- κ B p65 subunit into the nucleus (Fig. 5B) in α -LA- and β -LG-treated RAW264.7 cells. Generally, it is known that NF- κ B activation is not only based on degradation of I κ B, but also depends on I κ B kinase.²⁴ When the cell is treated with the medium containing α -LA or β -LG, the signal transduction was achieved across the cell membrane, and the upstream signal transduction which induced the dissolution of I κ B in cytoplasm, was activated, and

subsequently NF- κ B was activated and the expression of TSLP was increased. This phenomenon may indicate that α -LA and β -LG at high dosage (50 μ g/mL; compared to low dosage 5 μ g/mL) levels activate the induction of dissolution of I κ B in cytoplasm which in turn causes disruption of the cellular integrity resulting in the allergenicity of the whey proteins, as shown in Fig. 5A. To demonstrate the effect of α -LA and β -LG on the NF- κ B pathway, the nuclear translocation of NF- κ B/p65 was examined under confocal microscopy (Fig. 5B). RAW264.7 cells stimulated with LPS showed a marked increase in the translocation of NF- κ B into the nucleus. Similarly, α -LA and β -LG induced NF- κ B activation and translocation of a p65 subunit into the nucleus.

Effects of α -LA and β -LG on the Increase of Serum TSLP and IL-6 in Mice

To investigate if whey proteins facilitate allergic cytokine induction in mice, α -LA (10 mg/kg) and β -LG (10 mg/kg) were intravenously injected into mice, and their sera were collected 4 hrs after the whey proteins treatment. Simultaneously, LPS (0.1 mg/kg) was also injected as a positive control for inducing TSLP and IL-6 secretion. The data demonstrated that α -LA and β -LG induced TSLP expression based on serum levels (Fig. 6A), although there was a noticeable difference between the subjects. The serum IL-6 level of the mouse was markedly increased after LPS injection, and α -LA and β -LG also significantly increased the levels of serum IL-6 (Fig. 6B). Our results confirmed that among whey proteins increased TSLP production *in vivo* mouse study.

■ Discussion

As aforementioned, we have observed that TNF- α and IL-6 were induced by α -LA and β -LG in RAW264.7 cells (Fig. 1 and 2), where TNF- α and IL-6 appeared to be significant

mediators in inflammatory response, which may cause CMA reactions by the allergens in cow milk, such as the major whey proteins (α -LA and β -LG). Our observations from our study are in agreement with the previous report of Cho et al.²⁵, where they observed that TNF- α , an important mediator of the inflammatory response, is produced by innate immune cells, including macrophages, mast cells, and lung epithelial cells. Among whey proteins, α -LA and β -LG bind specific IgE, which is induced by Th2 cytokines such as IL-4 or IL-13²⁶, and are suspected to be the primary allergens in CMA.⁶⁻⁸

Our experimental data revealed that the whey proteins (α -LA and β -LG) stimulated and proportionally increased NF- κ B-luciferase activity by increased dosage level, as demonstrated in Fig. 3A and 3B. On the other hand, α -LA and β -LG minimally activated AP-1 and NFAT luciferase activities (Fig. 3C and 3D). These results may imply that α -LA and β -LG activate NF- κ B, which might be involved in TSLP production in RAW264.7 cells.

For further verification of this involvement, we have used Bay11, an NF- κ B inhibitor, on α -LA- and β -LG-treated RAW264.7 cells, and then the secreted TSLP, TNF- α and IL-6 levels were detected by ELISA. As shown in Fig. 4, the induced secretion of TSLP (Fig. 4A and 4B), TNF- α (Fig. 4C and 4D) and IL-6 (Fig. 4E and 4F) by α -LA and β -LG is inhibited by Bay11.

We have evaluated I κ B degradation in the cytosol (Fig. 5A) and the translocation of the NF- κ B p65 subunit into the nucleus (Fig. 5B) in α -LA- and β -LG-treated RAW264.7 cells. Generally, it is known that NF- κ B activation is not only based on degradation of I κ B, but also depends on I κ B kinase.²⁴ When the cell is treated with the medium containing α -LA or β -LG, the signal transduction is carried out across the cell membrane, and the upstream signal transduction which induces the dissolution of I κ B in cytoplasm, is activated, and subsequently NF- κ B is activated and the expression of TSLP is increased. This phenomenon may indicate that α -LA and β -LG at high dosage (50 μ g/mL; compared to low dosage 5

310 $\mu\text{g/mL}$) levels activate the induction of dissolution of I κ B in cytoplasm, which in turn causes
311 disruption of the cellular integrity, resulting in the allergenicity of the whey proteins, as
312 shown in Fig. 5A.

313 Zaph et al.²⁷ reported that NF- κ B activation is considered essential for TSLP production,
314 whereby our data have also shown that the NF- κ B signaling pathway is mainly related to
315 TSLP production in RAW264.7 cells (Fig. 3-5). The presence of α -LA and β -LG lead to TSLP,
316 TNF- α and IL-6 induction. TSLP, TNF- α and IL-6 are modulated by the activation of NF- κ B
317 in RAW264.7 cells. The activation of NF- κ B can be inhibited by blocking the activation of
318 IKK, leading to the phosphorylation, ubiquitination, and degradation of I κ B in the cytoplasm
319 by Bay11. Therefore, we confirmed that induction of TSLP, TNF- α and IL-6 by α -LA and β -
320 LG was dramatically blocked by Bay11 (Fig. 4). These results suggest that TSLP induction
321 by α -LA and β -LG is mainly dependent on NF- κ B activation. Generally, activation protein-1
322 (AP-1) and nuclear factor of activated T cells (NFAT) levels increased due to LPS
323 stimulation.^{25, 28} However, AP-1 and NFAT activities slightly increased due to presence of α -
324 LA and β -LG in our experiment. Further investigations are necessary to determine if AP-1
325 and NFAT activities are stimulated by α -LA and β -LG.

326 In order to explore any difference in *in vitro* and *in vivo* data, we have intravenously
327 administered α -LA and β -LG to Balb/c mice and evaluated the serum levels of TSLP and IL-6
328 using ELISA (Fig. 6). In a previous study⁹, mice were sensitized intragastrically to milk
329 proteins by intraperitoneal injection. In addition, mice were sensitized to milk protein plus
330 cholera toxin to enhance the immune response, where the immune response showed weak
331 oral sensitization. Takai¹⁶ reported that the TSLP expressed in intestinal epithelial cells may
332 be involved in tolerance to commensal microflora through modulation of dendritic cell
333 function. Cultured human intestinal epithelial cells produced TSLP without stimuli or in

response to stimulation *in vitro*. However, the potential roles of breast milk TSLP in the development of the neonatal gastrointestinal tract and of allergies in childhood may need further investigations.

Other studies²⁹⁻³⁰ have been conducted on lactoferrin injection into the tail vein of mice, and showed a rapid activation of immune response, indicating that the immune system activation may have been caused by intravenous administration of the whey proteins. Hence, it was expected that the administration of α -LA and β -LG into the tail vein in our study would exhibit the similar results.

The results of our study suggest that a cause of allergenicity of α -LA and β -LG is probably attributed to the TSLP induction as well as proinflammatory cytokines. Therefore, the induction of TSLP as well as TNF- α and IL-6 by α -LA and β -LG may contribute to cow milk allergy.

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Notes

The authors declare no competing financial interest.

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Figure Legends

Fig. 1. Effects of α -lactalbumin (α -LA) and β -lactoglobulin (β -LG) on thymic stromal lymphopoietin (TSLP), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) mRNA expression in immune cells. RAW264.7 cells were treated with the indicated concentrations of α -LA (A) and β -LG (C) for 4.5 hrs and treated with 50 μ g/mL α -LA (A) and β -LG in a time-dependent manner (0 to 9 hrs, B and D), and mRNA expression of TSLP, IL-6, and TNF- α was evaluated with RT-PCR. (E) HaCaT cells were treated with 50 μ g/mL α -LA and β -LG for 3 hrs. (F) HMC-1 cells were also treated with 50 μ g/mL α -LA for 3 hrs. Phorbol myristate acetate (PMA) (1 μ g/mL) and lipopolysaccharide (LPS) (1 μ g/mL) were used as the stimulating factors in TSLP induction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of the transcript.

Fig. 2. Effects of α -LA and β -LG on the increase in TSLP, TNF- α , and IL-6 production. RAW264.7 cells were treated with the indicated concentrations of α -LA and β -LG for 20 hrs (A and B), and TNF- α (C and D) and IL-6 (E and F) were activated for 14 hrs. LPS was used as a positive control to compare with α -LA and β -LG.

Fig. 3. Luciferase assay of nuclear factor-kappaB (NF- κ B), nuclear factor of activated T-cells (NFAT), and activator protein-1 (AP-1) activation induced by α -LA and β -LG in RAW264.7 cells. The NF- κ B reporter assay was performed on RAW264.7 cells treated with the indicated concentrations of α -LA and β -LG for 6 hrs (A and B). (C): The AP-1 reporter assay was performed on RAW264.7 cells treated with the indicated concentrations of LPS, BSA, α -LA, and β -LG for 6 hrs. (D): The NFAT reporter assay was performed on RAW264.7 cells

501 treated with the indicated concentrations of LPS, BSA, α -LA, and β -LG for 6 hrs.

502
503 **Fig. 4.** Inhibitory effect of Bay11 on α -LA- and β -LG-induced TSLP, TNF- α , and IL-6
504 production. RAW264.7 cells were pretreated with the indicated concentrations of Bay11 for 1
505 h followed by stimulation with α -LA and β -LG for 20 hrs and then analyzed using the TSLP
506 ELISA kit (A and B). RAW264.7 cells were pretreated with the indicated concentrations of
507 Bay11 for 1 h followed by stimulation with α -LA and β -LG for 14 hrs and then analyzed
508 using the TNF- α ELISA kit (C and D). RAW264.7 cells were pretreated with the indicated
509 concentrations of Bay11 for 1 h followed by stimulation with α -LA and β -LG for 14 hrs and
510 then analyzed using the IL-6 ELISA kit (E and F).

511
512 **Fig. 5.** Effects of α -LA and β -LG on I κ B degradation and NF- κ B translocation into the
513 nucleus. RAW264.7 cells were treated with the indicated concentrations of α -LA and β -LG
514 for 1 h, and then analyzed with western blot (A). RAW264.7 cells were treated with α -LA and
515 β -LG for 1 h. The samples were prepared for confocal microscopy analysis (B).

516
517 **Fig. 6.** Increase in TSLP and IL-6 cytokine expression in mice sera by intravenous injection.
518 TSLP in mice sera was assayed following LPS, α -LA, and β -LG injection intravenously (A).
519 IL-6 in mice sera was assayed following LPS, α -LA, and β -LG injection intravenously (B).

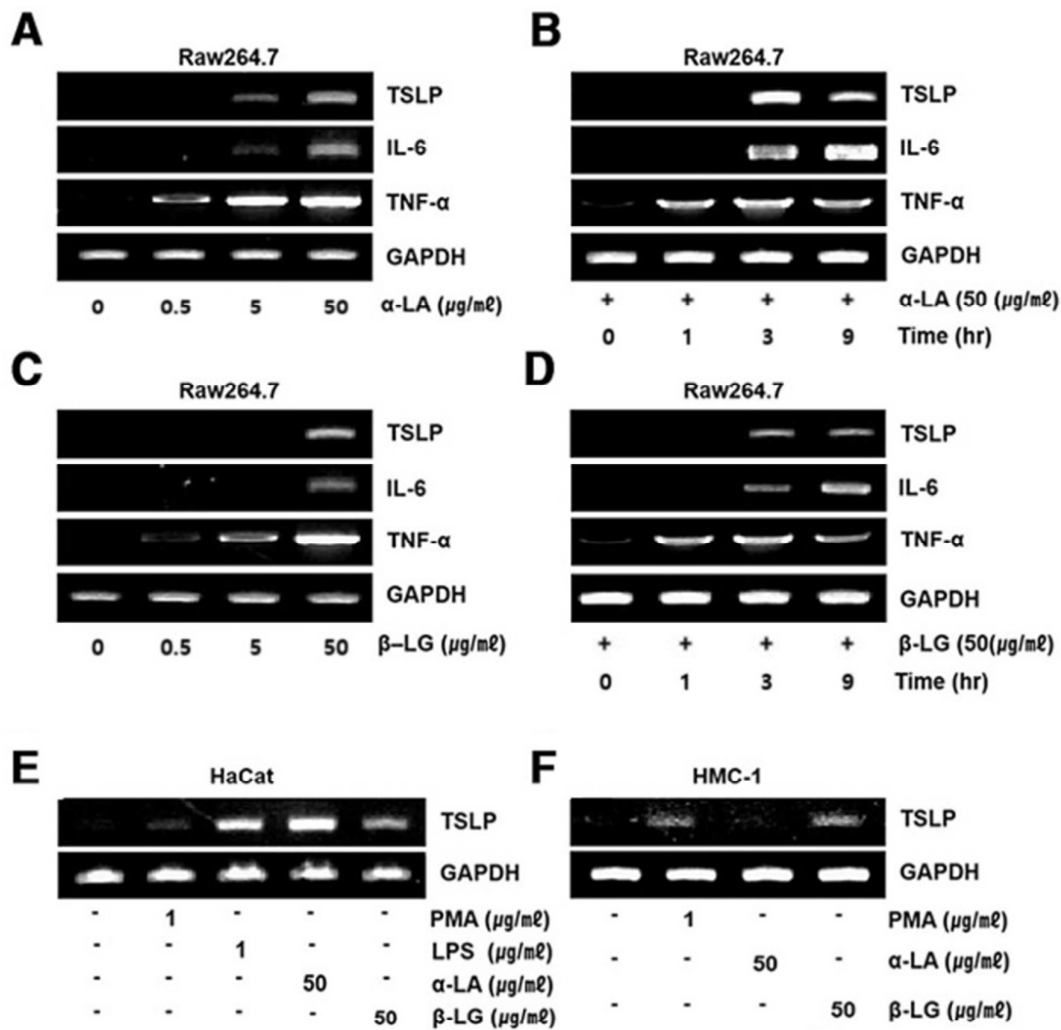


Figure 1

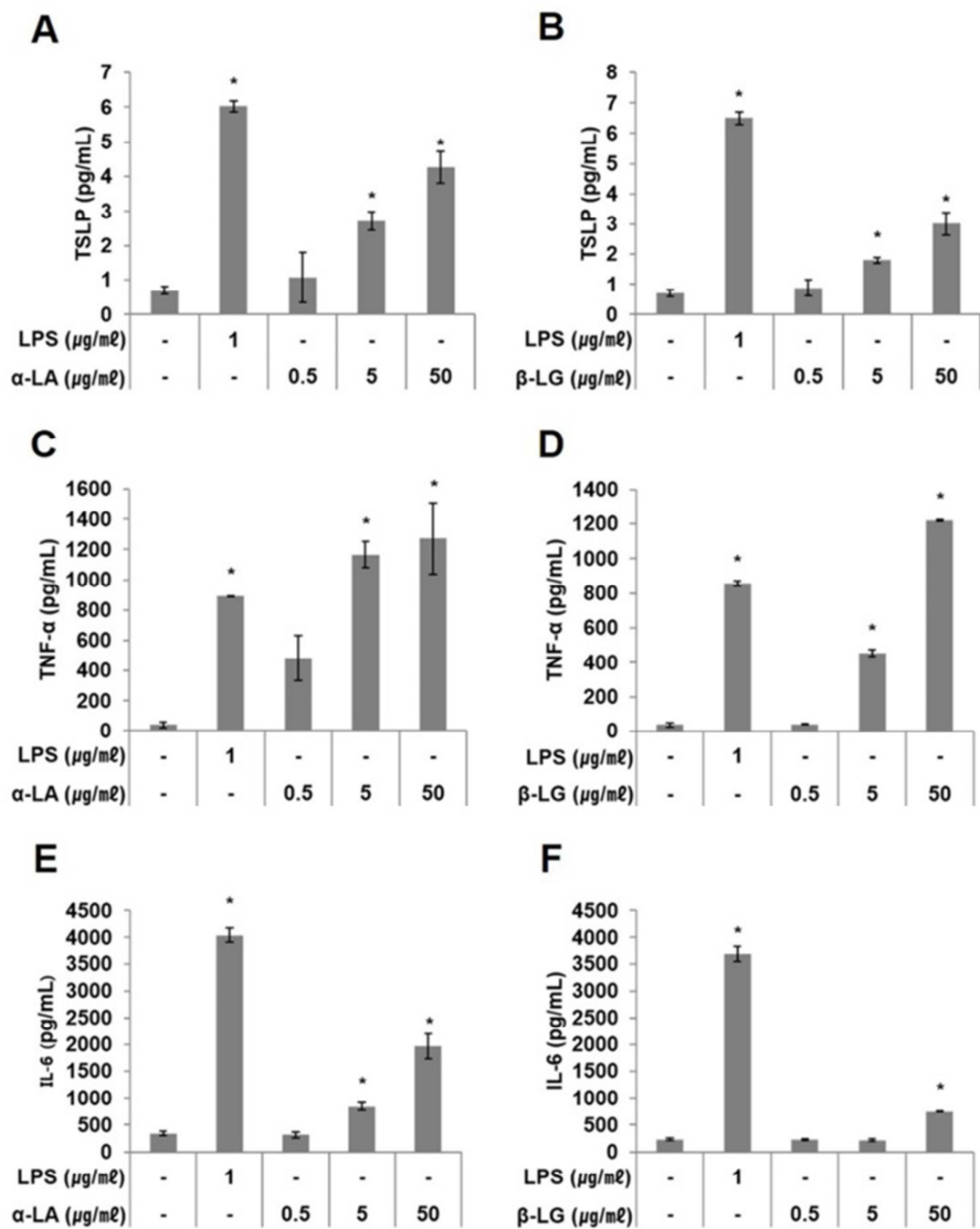


Figure 2

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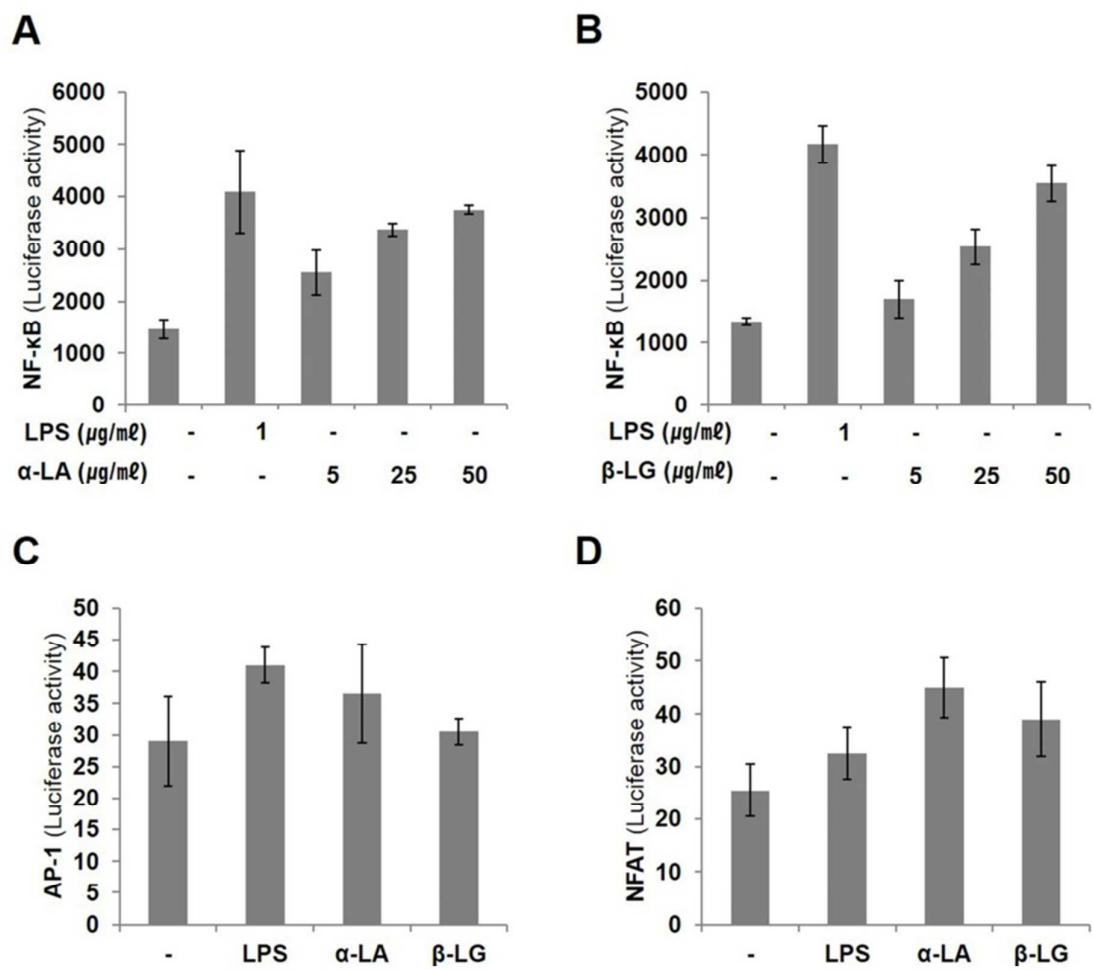


Figure 3

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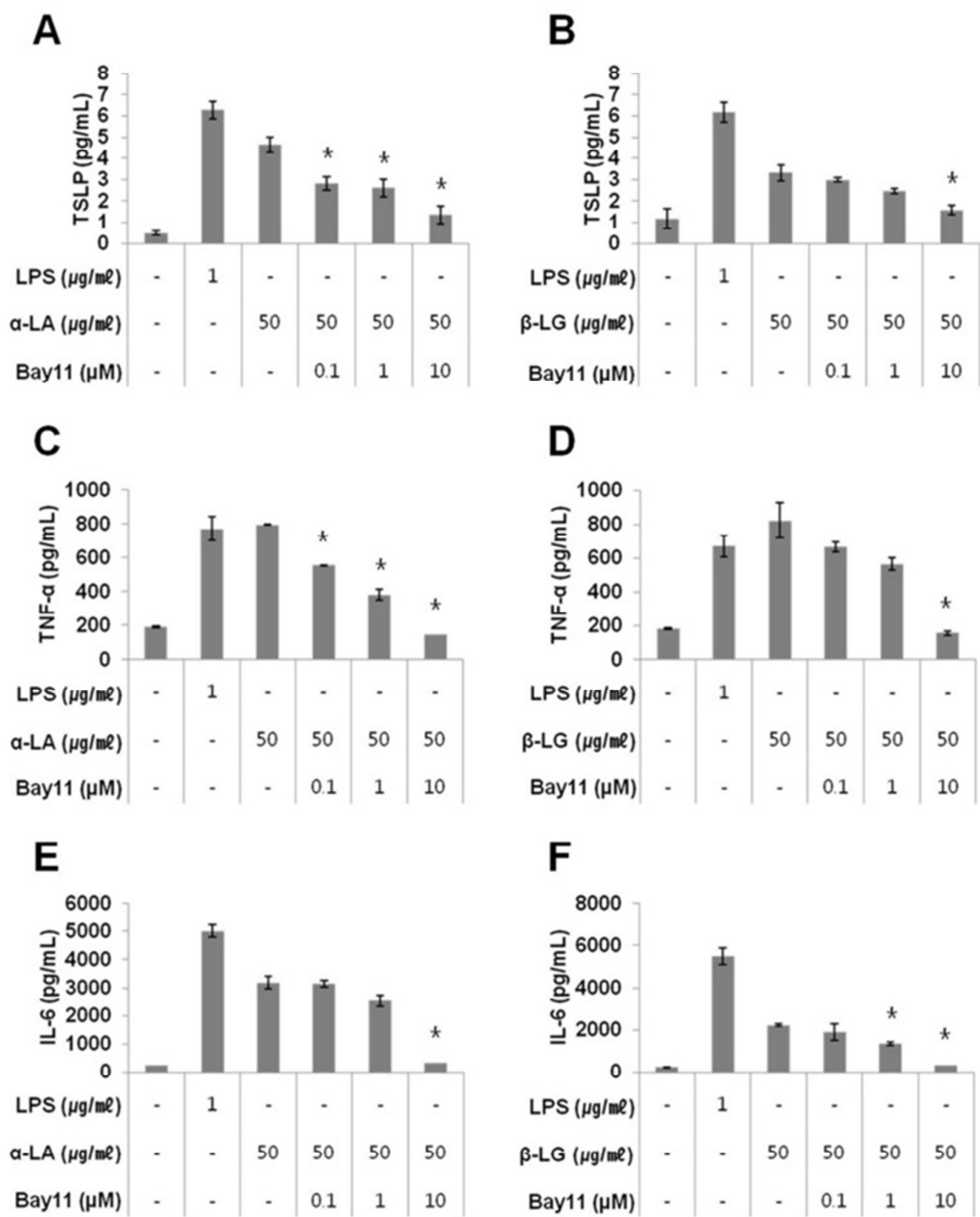


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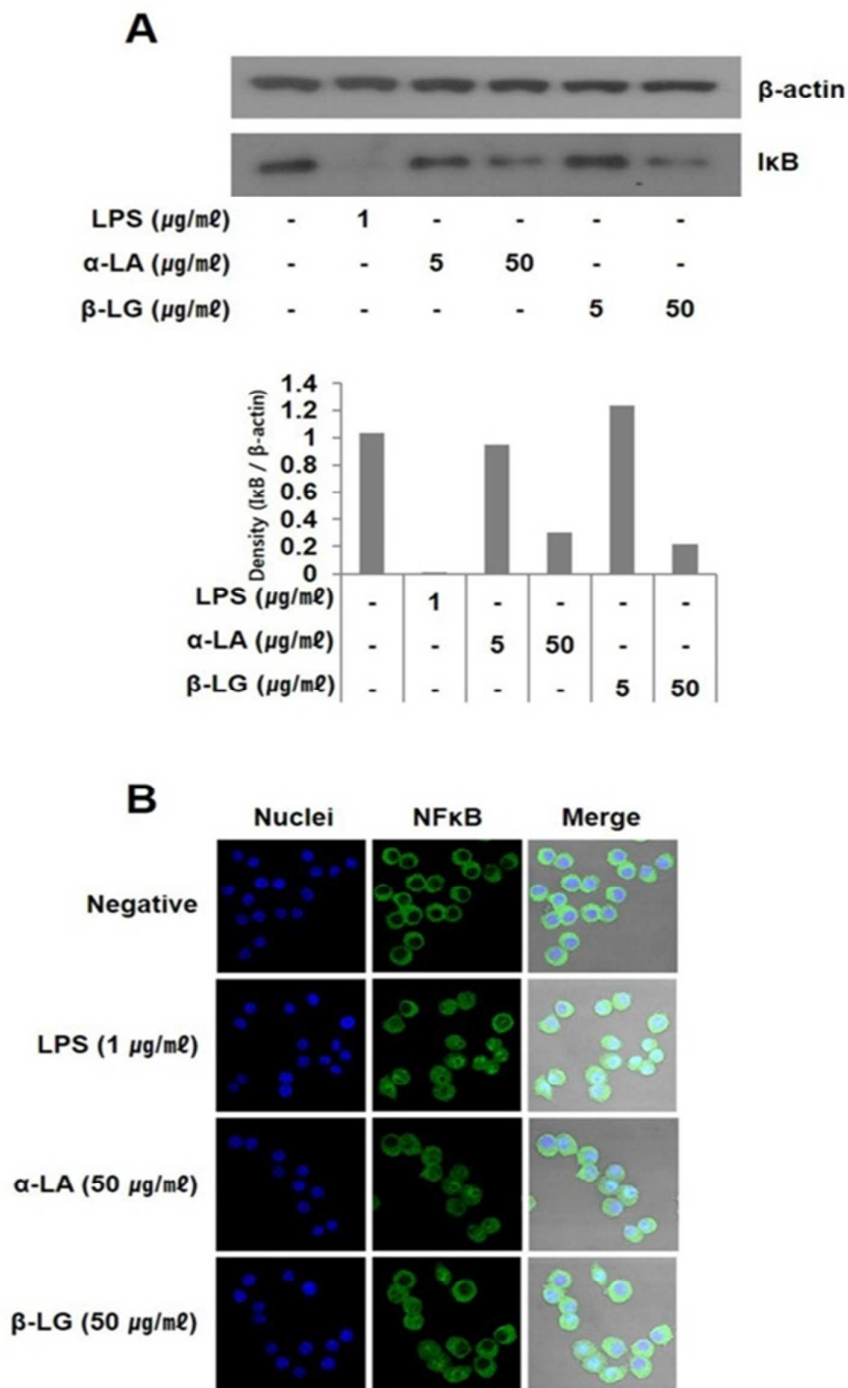


Figure 5

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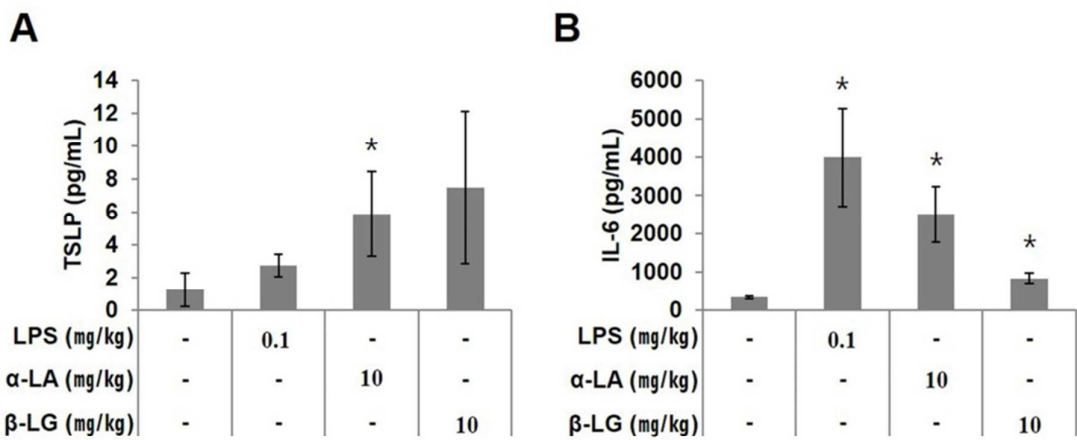
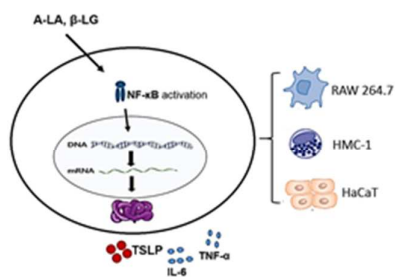


Figure 6

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