



Characterization of the potential allergenicity of irradiated bovine α -lactalbumin in a BALB/c mouse model



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ABSTRACT

Bovine α -lactalbumin (ALA) is a known food allergen present in milk to induce anaphylaxis. A previous study demonstrated that irradiated ALA (iALA) decreased the IgE-binding properties and weakened the degranulation capacity of basophils *in vitro*. The present study aimed to further assess the potential allergenicity of iALA *in vivo* in a BALB/c mouse model. The mice ($n = 10/\text{group}$) were intragastrically sensitized and orally challenged with either iALA or ALA using cholera toxin as adjuvant. In contrast to the ALA group, the iALA group did not show anaphylactic shock symptoms. A tendency toward decreased serum allergen-specific IgG/IgG₁/IgE levels, plasma histamine levels and mast cell protease-1 (mMCP-1) concentrations in the iALA group were also observed, accompanied by a decrease in Th2-related cytokine levels and an increase of IFN- γ production in spleen cell cultures. Moreover, the peritoneal mast cell surface expression of Fc ϵ RI and peripheral blood basophil CD200R⁺ expression were decreased by 64.3% and 35.19%, respectively. Conversely, the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells increased in the iALA group. All of these findings indicated that iALA induces a shift toward the Th1 response, which ultimately reduces its potential allergenicity.

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1. Introduction

Food allergy is an important public health concern, affecting nearly 8% of children and 5% of adults (Sicherer and Sampson, 2014). Among the eight most common food allergens, cow milk is the chief food allergen that can induce severe or fatal allergic reactions, such as asthma, atopic dermatitis, and allergic rhinitis (Kim et al., 2011; Schichter-Konfino et al., 2015). The allergenicity of cow milk is related to protein constituents, and the most prominent allergens are caseins, β -lactoglobulin (BLG), and α -lactalbumin (ALA) (El-Hatmi et al., 2015; Host and Halken, 2014). ALA, as a potent allergen, accounts for 25% of the whey proteins (Caira et al., 2012). Over 75% of milk-allergic patients were reported to be allergic to this protein (Meulenbroek et al., 2014). On the other hand, given its high nutritional value and desirable techno-functional properties, ALA is frequently used as a food additive in many food products (Matsumoto et al., 2001). Hence, this milk

component is difficult to eliminate from the diet. Accordingly, many studies have explored possible available methods for reducing milk protein allergenicity.

Currently, some strategies to reduce food protein allergenicity are available, such as thermal and nonthermal treatments (Rahaman et al., 2016; Verhoeckx et al., 2015). The latter has been paid more attention because such strategy allows the retention of nutritional and sensory properties to the maximum extent (Shriver and Yang, 2011). The most popular nonthermal treatments include enzymatic hydrolysis (Kasera et al., 2015), high-pressure treatment (Jin et al., 2015), gamma irradiation (Vaz et al., 2012), and so on. According to the previous research, we found that gamma irradiation is a most useful physical technique for reducing or abolishing the allergenicity of certain milk allergens (Kuan et al., 2013; Oduke et al., 2016). For instance, Byun suggested that the irradiation of BLG at 10 kGy led to a significant reduction of IgE binding in inhibition enzyme linked immunosorbent assay (ELISA) using pooled sera from 20 milk allergy patients (Byun et al., 2002). Similarly, lower antigenicity and allergenicity of the irradiated bovine α -casein (ACA) and BLG were also observed through inhibition ELISA (Lee et al., 2001). However, all of these findings were based on *in vitro* IgE and IgG binding reactivity. Information on the change of

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allergenicity after gastrointestinal digestion is extremely limited, even more so on bioactivity. Consequently, the studies highlighted that further assessment of irradiated milk proteins *in vivo* is an important investigation.

In vivo animal models hold great potential as powerful tools for predicting possible allergy triggers, identifying possible mechanisms involved in the allergic pathway, and reflecting the relevant clinical read-out parameters of allergic reactions (Aldemir et al., 2009; Bock et al., 2001; Chen et al., 2013; Ladics, 2008; Van Gramberg et al., 2013). More recently, different animal models have been adopted for these purposes. Such animal models include the mouse, rat, guinea pig, swine, and dog (Dearman and Kimber, 2009). In particular, in the study of cow's milk allergy, BALB/c mice is the most commonly used among the mouse models to assess allergenicity. This model is used mainly because intragastric sensitization generates specific IgE antibodies to milk proteins that are of similar specificity to those produced by humans (Li et al., 1999). Another advantage is that a predominantly IgE-mediated mouse model for orally induced cow milk protein allergy (CMPA) for certain milk allergens was introduced in recent years, indicating that this model can be widely used to evaluate the allergenicity of products based on cow milk (Adel-Patient et al., 2012; Gomes-Santos et al., 2015; Stojadinovic et al., 2014).

Food allergy is well known to be usually related to the gastrointestinal tract (GIT). Our previous work showed that IgE-binding properties and mediator release capacity of irradiated ALA (iALA) gradually decreased with increasing irradiation doses *in vitro* (1–10 kGy; Meng et al., 2016). Obviously, the support for the allergenicity assessment of the iALA provided by the study is insufficient. Accordingly, this work aims to further evaluate the allergenicity of iALA (10 kGy-treated) after gastrointestinal digestion in a BALB/c mouse model *in vivo*. This goal is achieved by measuring serum allergen-specific antibody levels, splenic cytokine levels, plasma histamine levels, peritoneal mast cell counts, peripheral blood basophil expression, and regulation T cells activation.

2. Materials and methods

2.1. Preparation of ALA and iALA

Since bovine ALA is a very common used protein in many relevant works, it was easily purified from fresh cow milk (SUNSHINE DAIRY, Nanchang, China) by the method as described previously (Li et al., 2008; Neyestani et al., 2003) with some modifications. Briefly, cow milk (40 L, 3.6% fat) was defatted by centrifugation at 1000g for 30 min at 4 °C. To further remove the casein, the skimmed milk was acidified to pH 4.6 by adding 5 M HCl slowly. After centrifugation at 8000g for 30 min at 4 °C, the supernatant (whey) was concentrated by hollow-fiber membrane with 10 kDa cut-off. The obtained whey (final protein concentration at 40 mg/mL) was stored at –20 °C until used. To purify ALA from whey, 30 mL whey was applied to a gel chromatography column (3.5 × 100 cm) filled with about 800 mL Sephadex G-75 (General Electric Company, USA) and eluted with phosphate buffer (0.02 M, pH6.8) with the flow rate of 2 mL/min (Supplementary Fig. 1). The obtained crude ALA solution was collected for further purification using DEAE-Sepharose Fast Flow (500 mL; General Electric Company, USA) anion-exchange chromatography column (3.5 × 100 cm), and eluted with a linear gradient of NaCl (0–0.5 M) in phosphate buffer (0.02 M, pH6.8) (Supplementary Fig. 2A). Afterward, the target eluent was collected and dialyzed against distilled water for 48 h at 4 °C, followed by freeze-drying and stored at 4 °C until used. The purity and identity of the lyophilized sample were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass

spectrometry (MS) (Supplementary Fig. 2B and Fig. 3), and the lyophilized protein was identified as ALA with purity of above 96%.

Before the beginning of the study, ALA solutions were produced in two concentrations. An amount of 500 mg lyophilized ALA was dissolved in 50 mL PBS (0.05 M, pH6.8) to get 10 mg/mL ALA solution, and 360 mg lyophilized ALA was dissolved in 12 mL PBS (0.05 M, pH6.8) to make 30 mg/mL ALA solution. To prepare iALA sample for the oral sensitization and challenge of mice, an amount of 25 mL (10 mg/mL) and 6 mL (30 mg/mL) ALA solutions were irradiated by a cobalt-60 gamma irradiator (Jiangxi Academy of Agricultural Sciences, Nanchang, China) using a dose of 10 kGy at a rate of 0.32 kGy/h. The same volume of untreated ALA solution was stored at –20 °C until used. ALA and iALA solutions were vortexed thoroughly before use to ensure all solutions were homogenous.

2.2. Animals

Thirty female BALB/c mice with the body weight of 18.0 ± 2.0 g (4–5 weeks) were purchased from the Laboratory Animal Center of Nanchang University (Nanchang, China; permission number “SYXK (Gan) 2015-0001”). The mice were housed in an animal room with controlled temperature of 23 ± 1 °C, relative humidity of $50 \pm 5\%$, and with the altering of 12/12 h light-dark cycle. The mice were housed in polypropylene cages (32 cm × 20 cm × 14 cm) in ten mice/group and provided *ad libitum* access to a commercially available rodent diet and water. Water was made available continuously through automatic ports, and the diet did not contain any milk allergens which could influence the outputs of the experiments. Moreover, the bedding (wood shaving) of mice were replaced every two days. Animals were acclimatized to the laboratory condition for 1 week before commencement of the animal experiment. All mice used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, 1996), and all experimental procedures were approved by the Animal Care Review Committee, Nanchang University.

2.3. Oral sensitization and challenge of mice

As illustrated in Fig. 1A, mice were randomly divided into three groups ($n = 10$ /group). All the mice were sensitized by the intragastrically (i.g.) on days 0, 7, 14, and 21, and orally challenged on day 28. More specifically, the first group (CT) is the control group which was sensitized with cholera toxin (CT; Sigma-Aldrich, St. Louis, MO, USA) adjuvant (15 µg) in 500 µL PBS, and challenged with PBS (500 µL) alone. The second group (ALA) was sensitized with 500 µL ALA solution (10 mg/mL; 5 mg ALA) containing 15 µg CT, and challenged with 500 µL ALA solution (30 mg/mL; 15 mg ALA). The third group (iALA) was sensitized with 500 µL iALA solution (10 mg/mL; 5 mg iALA) containing 15 µg CT, and challenged with 500 µL iALA solution (30 mg/mL; 15 mg iALA). Then, 40 min after final challenge, the allergic or anaphylactic symptoms were monitored using a validated anaphylactic scoring (Table 1). Thereafter, blood samples, peritoneal cells, and mouse spleens were collected to measure the following different biomarkers.

2.4. Quantification of ALA-specific immunoglobulins and mouse mast cell protease-1 (mMCP-1) in blood

Serum allergen-specific IgG/IgG₁/IgE levels were determined by an indirect ELISA ($n = 6$ /group). Briefly, 100 µL of 5 µg/mL ALA or iALA in 50 mM sodium carbonate buffer (pH 9.6) was added to a 96-well microtiter plate, and the plate was allowed to incubate overnight at 4 °C. Each diluted serum were added (1:100 for specific IgG,

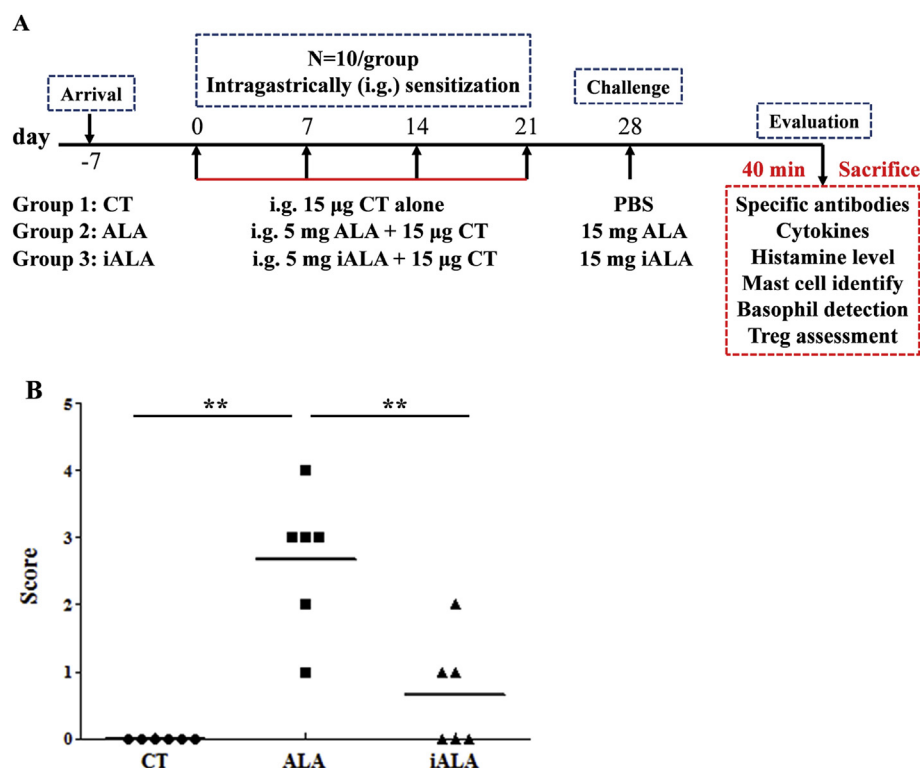


Fig. 1. Oral immunization of BALB/c mice with ALA or irradiated ALA. (A) Experimental scheme. BALB/c mice were sensitized weekly by intragastric gavage with ALA or iALA using CT as adjuvant. Control group received CT only. After challenge, blood and spleens were taken for specific antibodies, cytokine and histamine level assays, and identify mast cell, basophil, and Treg cell. (B) Score of hypersensitivity symptoms. The symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 5 (death) as described in Table 1. Student's t-test was used to determine statistical significance. **P < 0.01.

Table 1
Anaphylactic symptom scoring.

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erection; reduced activity; high breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

1:50 for specific IgG₁, and 1:20 for specific IgE), followed by 100 µL of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG₁, and IgE antibodies (1:6,000, SouthernBiotech, Birmingham, USA). Plates were allowed to develop with *o*-phenylenediamine substrate, and the reaction was terminated with 2 M H₂SO₄. The optical density was measured at 490 nm by a microplate reader (Model 1860, Bio-Rad, USA). Serum concentrations of mMCP-1 were quantified following the manufacturer's instructions using a commercially available ELISA kit (eBioscience, San Diego, USA).

2.5. Splenic cells culture and cytokine evaluation

Individual spleen was aseptically collected and minced (n = 6/group). After erythrocyte lysis, spleen cells were suspended in complete medium (RPMI-1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES buffer, 100 IU/mL penicillin, and 100 mg/mL streptomycin). Cells (2 × 10⁶/well) were seeded in a 48-well plate. These cells were then stimulated with plain medium or in the presence of either ALA or iALA (50 µg/mL) for 72 h (37 °C and 5% CO₂). Subsequently, supernatants were collected and stored

at –80 °C until further analysis. The levels of interleukin (IL)-4, IL-5, IL-13, IL-17E, IL-21, and interferon (IFN)-γ were determined by commercial ELISA kits (eBioscience, San Diego, USA).

2.6. Determination of plasma histamine levels

To measure the level of histamine in the plasma, 500 µL of a mouse blood sample (n = 6/group) was collected into anticoagulant tubes, followed by centrifugation for 10 min at 4000g. The supernatant was collected and stored at –80 °C until further analysis. Plasma histamine levels were measured using a histamine ELISA kit (eBioscience, San Diego, USA) in accordance with the manufacturer's protocol.

2.7. Identification of peritoneal mast cells in mice

The remaining four mice in each group (n = 4/group) were used for the detection of mast cells, basophils, and regulatory T cells. In this experiment, to collect peritoneal cells, we injected 5 mL RPMI-1640 containing 10% fetal bovine serum into the mouse's peritoneal cavity. Afterward, the abdomen was massaged gently for 1 min. The peritoneal cell-containing fluid was collected, followed by centrifugation at 150g for 10 min to precipitate the peritoneal cell population, and the cells were suspended in flow cytometry staining buffer.

Mast cells were detected by their expression of the high-affinity IgE receptor (FcεRI) and c-kit. Cells (1 × 10⁷ cells/mL) were stained with anti-FcεRI antibody (0.5 µg/test, eBioscience, San Diego, USA; clone MAR-1) and anti-c-kit antibody (0.5 µg/test, eBioscience, San Diego, USA; clone 2B8) for 30 min in the dark at 4 °C for 30 min. Then, the cells were washed twice with flow cytometry staining buffer and subjected to flow cytometry. Then, data were analyzed

with BD FACSDiva software (BD Biosciences, CA, USA).

2.8. Detection of circulating basophils after challenge

Forty minutes after the challenge, 100 μ L blood was collected into the anticoagulant tubes and diluted to 1:1 with flow cytometry staining buffer. Regarding the detection of basophils, antibodies against CD4 (0.125 μ g/test, clone GK1.5, for negative gating), CD45R (0.25 μ g/test, clone RA3-6B2, for negative gating), IgE (1 μ g/test, clone 23G3), and CD200R (0.25 μ g/test, clone OX110, all from eBioscience, San Diego, USA) were stained with cells for 30 min in the dark at 4 °C. Afterward, the lysed erythrocyte was washed twice with flow cytometry staining buffer, and the amount of dyed cells was determined by flow cytometry.

2.9. Assessment of regulatory T cells (Tregs) in mice

CD4⁺CD25⁺Foxp3⁺ cells from Mouse Tregs were identified by Foxp3 Staining Kit (eBioscience, San Diego, USA). Single-cell suspensions were obtained from spleens. Cells (2×10^7 cells/mL) were stained with anti-CD4 (0.375 μ g/test), anti-CD25 (0.18 μ g/test), and anti-Foxp3 (1.5 μ g/test, all from eBioscience, San Diego, USA; clone FJK-16s) in accordance with the manufacturer's protocol. The cells were then measured and analyzed by flow cytometry.

2.10. Statistical analysis

Statistical evaluation was performed with the student's *t*-test, using the GraphPad Prism 5 programmer (GraphPad Software Inc., San Diego, CA, USA). A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Anaphylactic shock score

The system anaphylactic reactions of iALA were assessed in BALB/c mouse model. All the mice in the ALA group developed anaphylactic reactions (median score 3; $P < 0.05$ compared with CT mice), and one of the mice achieved a severe condition in which shivering occurred, thereby resulting in a score of 4 (Fig. 1B). By contrast, in the iALA group, no anaphylactic reaction ensued (median score 1), and no significant difference existed between the iALA and CT groups.

3.2. Reduction in serum-specific IgG, IgG₁, and IgE levels

The immune response to iALA was evaluated by testing the serum-specific antibody levels *in vivo*. As shown in Fig. 2, the mice challenged with iALA generated a significantly lower IgG ($P < 0.05$), IgG₁ ($P < 0.01$), and IgE ($P < 0.01$) response when compared with that of mice challenged with ALA (Fig. 1C). In particular, decreases in IgE and IgG₁ serum levels were not as apparent as the decrease in IgG levels.

3.3. Cytokine production in splenic cells

To further determine whether iALA could change cytokine production in the immune response, Th1- (IFN- γ), Th2- (IL-4, IL-5, and IL-13), Th17- (IL-17E), and Tfh- (IL-21) related cytokines were analyzed in the spleen. None or minimal Th2-related cytokine (except IL-5) was detected in the ALA and iALA groups (Fig. 3A). However, both ALA and iALA sensitization led to an increase in IFN- γ production in splenic cultures re-stimulated by ALA and iALA, respectively. By contrast, the cytokine levels from the iALA group

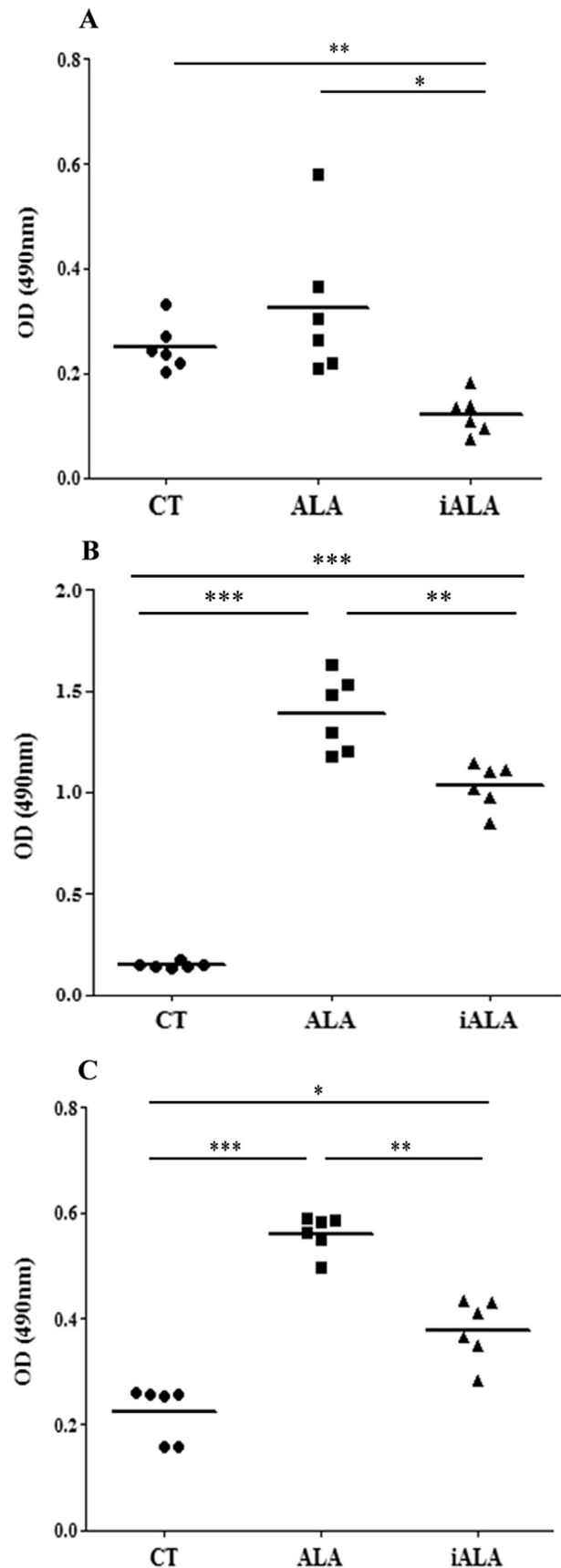


Fig. 2. Levels of specific IgG (A), IgG₁ (B), and IgE (C) in sera from mice exposed to CT, ALA or iALA were detected by ELISA. Student's *t*-test was used to determine statistical significance. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

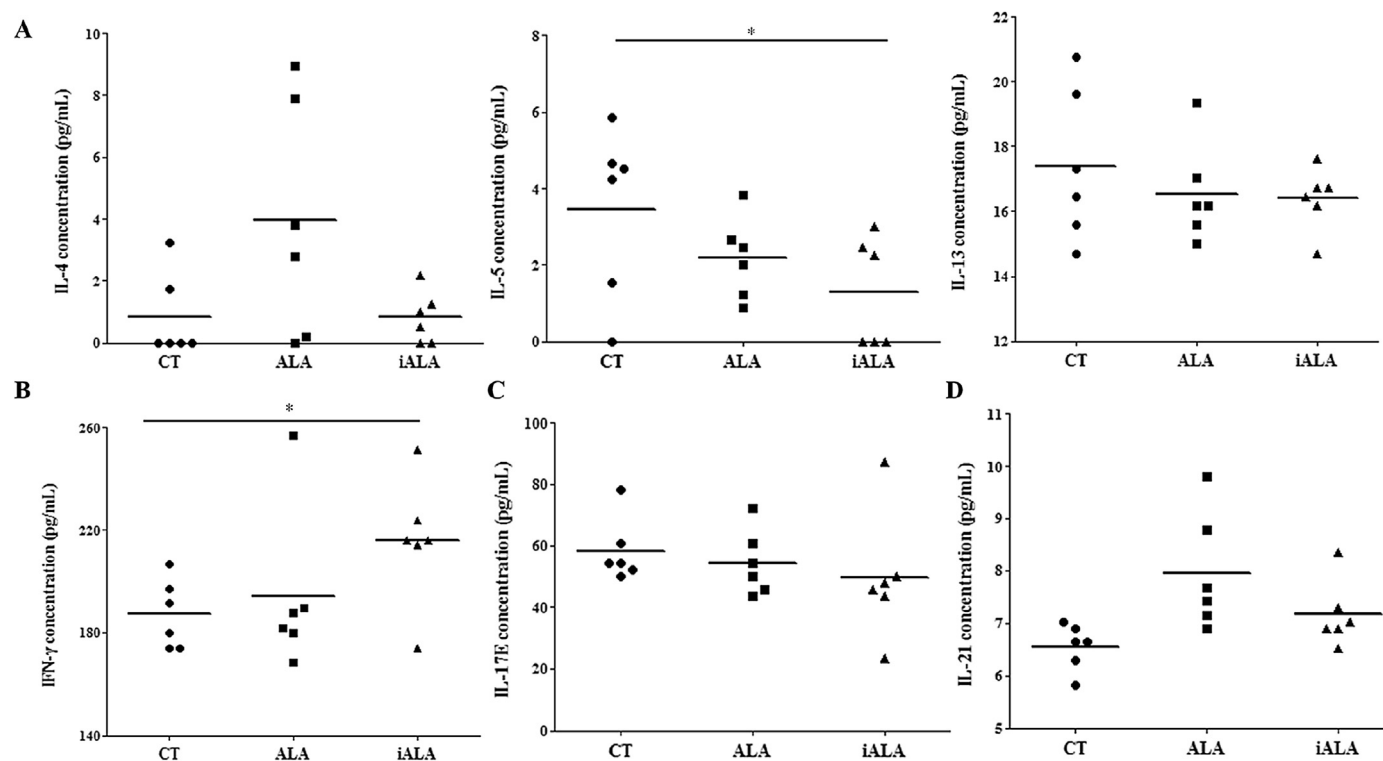


Fig. 3. Cytokine concentration. Spleen was collected from each mice, and splenocytes were isolated and cultured at 2×10^6 /well as stimulated with 50 μ g/mL of ALA or iALA. After 72 h, the supernatants were assessed for Th2-related cytokines IL-4, IL-5, and IL-13 (A), Th1-related cytokine IFN- γ (B), Th17-related cytokine IL-17E (C), and Tfh-related cytokine IL-21 (D) by ELISA kits. Significance was taken at * $P < 0.05$.

was significantly different from those in the control group ($P < 0.05$) (Fig. 3B). The expression of IL-17E and IL-21 did not change significantly between the experimental and control groups (Fig. 3C and D).

3.4. Reduction in plasma histamine level

The plasma histamine level in the iALA group was slightly higher than the CT group, whereas no significant difference between the two groups was found (Fig. 4). This finding indicates that the two groups exerted similar effects on the induction of plasma histamine level. However, plasma histamine level was significantly decreased ($P < 0.001$) in the iALA-challenged mice, which was reduced by 66.67% compared with the ALA group. These results suggested that iALA exerts a suppressive effect on the development of allergic clinical manifestations, which was consistent with the observed anaphylactic shock symptoms described above (Fig. 1B).

3.5. Attenuation of mast cell activation

To evaluate if subclinical mast cell activation occurred after the challenge with iALA, peritoneal mast cells were collected and Fc ϵ RI $^+$ and c-kit $^+$ cells were tested by flow cytometry. We found that the percentage of Fc ϵ RI $^+$ and c-kit $^+$ cells in the ALA-challenged mice (~8.0% of the total cells) was much higher than those in any of the other groups (Fig. 5A). The percentage of Fc ϵ RI $^+$ and c-kit $^+$ cells obtained from the iALA-challenged mice was only about 2.82%, which was reduced by 64.30% compared with that of the ALA group.

Serum mMCP-1, another marker of allergen-induced mast cell activation, was also measured and shown in Fig. 5B. Similarly, ALA-challenged mice enhanced mMCP-1 concentration in the serum than that of the CT group ($P < 0.01$). Upon oral challenge with iALA,

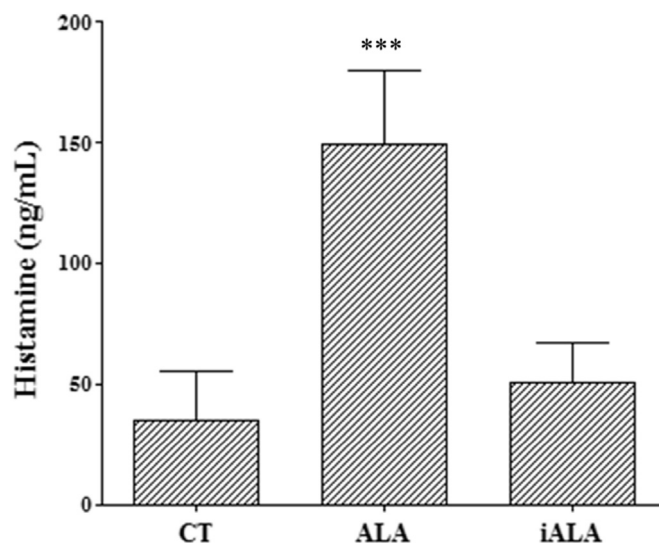


Fig. 4. Plasma histamine level was determined by enzyme immunoassay kit. Student's t-test was used to determine statistical significance. *** $p < 0.001$ compared with iALA group.

the serum levels of mMCP-1 was lower than that of the mice orally challenged by ALA, and a remarkable attenuation was observed in the iALA group ($P < 0.05$).

3.6. Identification of basophils from blood

The peripheral blood basophils as another marker of effector cell was identified by the percentage of IgE $^+$ CD200R $^+$ cells (Fig. 6). A representative image of the IgE $^+$ CD200R $^+$ cells for all treatment

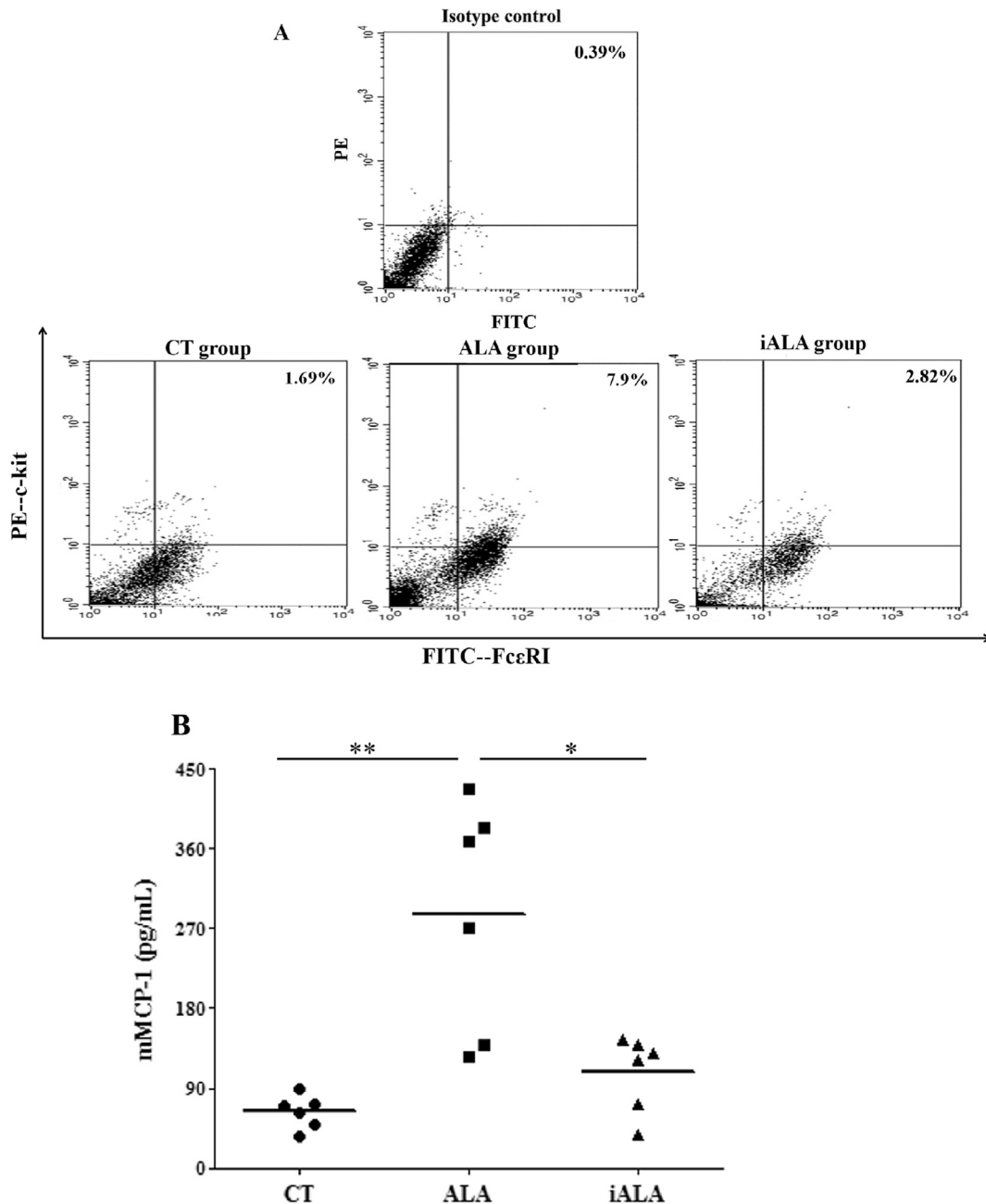


Fig. 5. Identification of mast cells. (A) FcεRI and c-kit expression on peritoneal mast cells. (B) The level of mMCP-1 in the serum after challenged with ALA or iALA. * $P < 0.05$, ** $P < 0.01$.

groups is shown in Fig. 6A. In the CT group, the percentage of IgE⁺CD200R⁺ cells was only 7.93%, and a significant increase was observed in the ALA (28.36% and $P < 0.01$) and iALA (18.38% and $P < 0.05$) groups. For the iALA-challenged mice, the percentage of IgE⁺CD200R⁺ cells was reduced by 35.19% compared with the ALA group.

3.7. Proportion of CD4⁺CD25⁺Foxp3⁺ T cells

Regulatory T cells (Tregs) are a crucial factor in inducing oral tolerance in immune response. Thus, the proportions of Tregs in the spleens of the ALA-, iALA-, and CT-treated mice were monitored (Fig. 7). Only a slight higher level of Tregs in the ALA-treated mice was observed relative to the CT group. By contrast, a remarkable increase was found in the iALA-treated mice than that of the control ($P < 0.01$).

4. Discussion

Although fundamental work on the protein structural changes induced by gamma irradiation has been ongoing for more than 40 years, clinical research on the effect of structural changes on food allergens remain unclear (Kuan et al., 2013). Particularly, information is unknown on whether irradiated bovine ALA differs from the native ALA in terms of their ability to stimulate a systemic immune response. Fortunately, this work has successfully evaluated the effect of iALA on sensitization and allergic response in a BALB/c mouse model with relevant clinical read-out parameters.

In our study, ALA and iALA triggered different levels of allergic symptoms in the BALB/c mice. In particular, iALA attenuated the anaphylactic scores relative to that of ALA group. These results

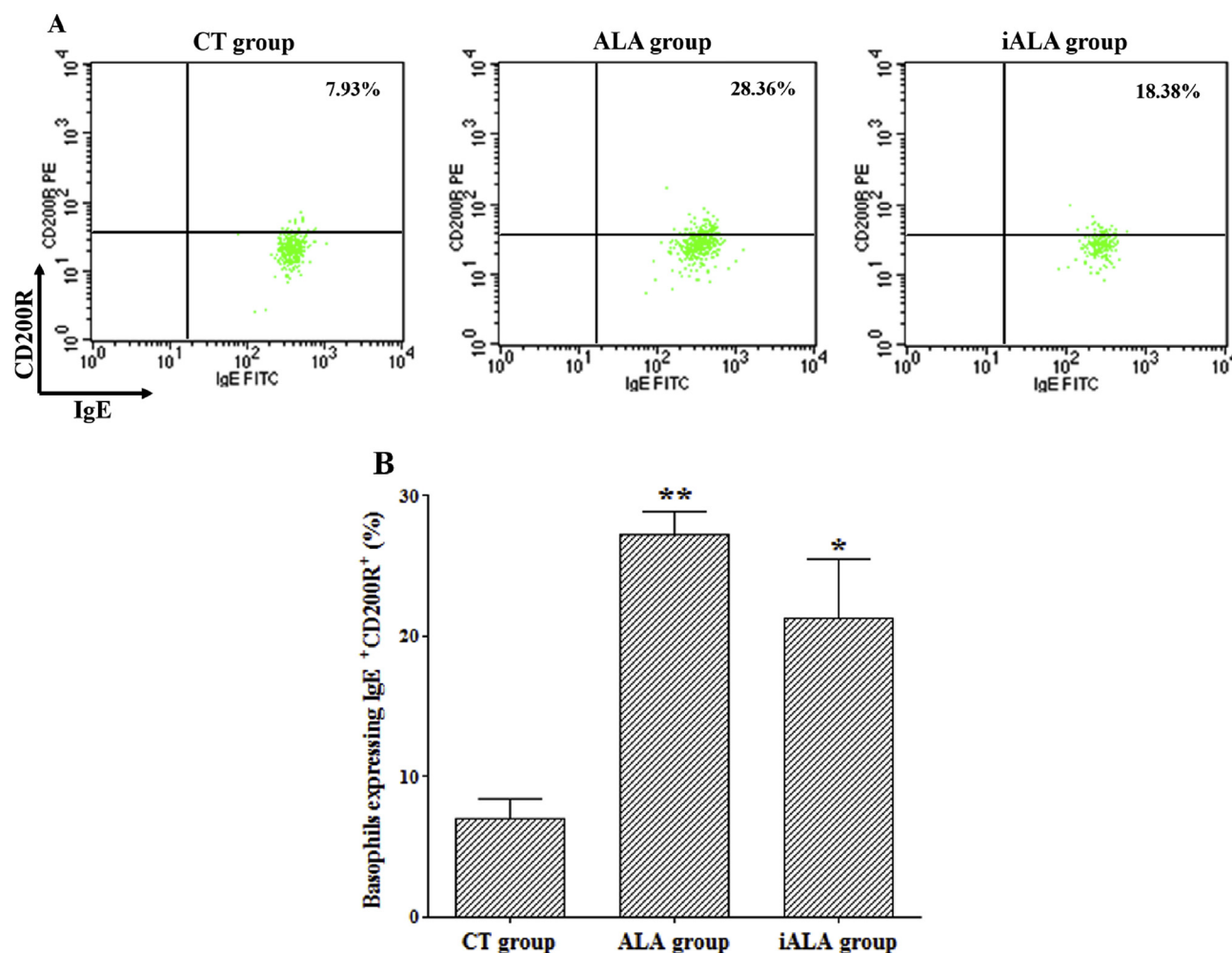


Fig. 6. Blood basophil detection by flow cytometry. (A) Typical plots depicting numbers of basophils in mouse blood after treated with ALA, iALA or CT, respectively. Numbers in upper right quadrants shows proportions of IgE⁺CD200R⁺ cells out of all cells. (B) The percentage of IgE⁺CD200R⁺ cells were quantified of blood basophils. **P* < 0.05, ***P* < 0.01 compared with CT group.

generally suggested a lower allergenicity of iALA compared with ALA. Actually, anaphylaxis in mice may be triggered by multiple mechanisms, and serum-specific IgE antibodies are particularly important because these antibodies play a crucial role in mediating type I hypersensitivity responses in CMPA (Duan et al., 2014; Li et al., 1999). Furthermore, IgE also play a key role in the cross-linking of high-affinity IgE receptors FcεRI to activated effector cells, such as mast cells or basophils (Levy et al., 2012; Van Esch et al., 2013; Yamaguchi et al., 1999). Hence, the serum-specific IgE levels were evaluated as the hallmark of the Th2-type cell immune response. Our finding revealed that iALA induced significantly lower levels of iALA-specific IgE compared with those induced by ALA. The ability of irradiation-denatured allergens to reduce Th2-associated IgE is also exhibited by other allergens, such as ovalbumin (OVA) (Seo et al., 2007). Moreover, previous studies demonstrated that the serum IgG₁ and IgE levels of the Th2 immune response are positively correlated in mice (El Mecherfi et al., 2015). Accordingly, the IgG₁ level in the sera was also significantly suppressed in the iALA-induced mice than in the ALA group, thereby indicating that iALA led to a specific immune hyporesponsiveness compared with ALA.

To further explain the allergic response mechanism, evaluation of the changes of the effector cells was essential. As a primary effector cell, mast cells were identified as FcεRI⁺ and c-kit⁺ cells by

flow cytometry. The percentages of FcεRI⁺ and c-kit⁺ cells were found to be markedly decreased in iALA-challenged mice than in ALA-challenged mice. In parallel with a diminished number of mucosal mast cells, serum mMCP-1 concentrations did not increase when the mice were challenged with iALA. Hence, the iALA may have altered mast cell activation and function. Additionally, as another marker of effector cell, the basophils in the blood exhibited similar changes as those exhibited by the mast cells. The result showed that iALA-challenged mice led to a sharp decline of IgE⁺CD200R⁺ cell number in circulation. Theoretically, basophil activation after anaphylaxis can lead to a redistribution and homing of basophils to the peripheral tissues or lymph nodes, where they can influence the adaptive immune response (López-Expósito et al., 2012; Sokol and Medzhitov, 2010; Torrero et al., 2009). Therefore, the results indicated that in addition to mast cells, iALA could also inhibit basophil activation. Mast cell and basophil degranulation will release histamine and thereby induce allergic reaction (Levy et al., 2012); the plasma histamine level was then examined in the following step. We noted that iALA induced significantly a lower level of histamine than that of ALA, suggesting that the histamine released from the activated mast cell and basophil is related to the weak systemic allergic symptoms.

A large variety of cytokines comprise another important bioactive mediator besides histamine in allergic reaction. This work

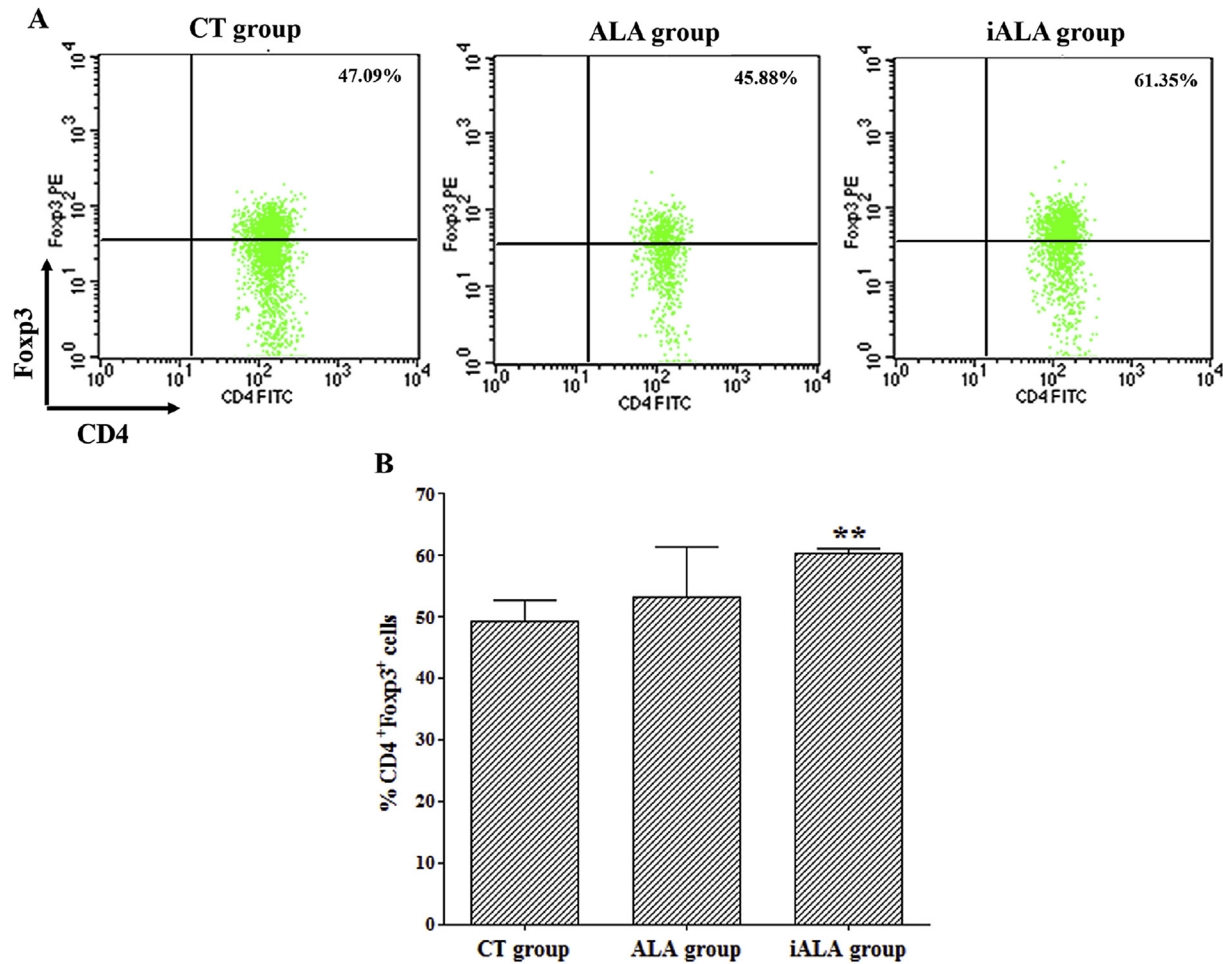


Fig. 7. Percentage of Tregs in splenocytes of ALA or iALA treated mice was determined by Flow cytometry. (A) Typical plots depicting numbers of Tregs in mouse splenocytes in gated CD4⁺CD25⁺ T helper cells after treated with ALA, iALA or CT, respectively. Numbers in upper right quadrants shows proportions of CD4⁺Foxp3⁺ cells out of all cells. (B) The percentage of CD4⁺Foxp3⁺ cells were quantified from splenocytes. **P < 0.01 compared with CT group.

focused on the cytokine expression profiles of ALA- and iALA-specific immune responses. To our knowledge, IL-4, IL-5, and IL-13 are Th2-related cytokines and important in inducing B-cell growth and differentiation, as well as IgE and IgG₁ secretion (Dearman and Kimber, 2009; Ngoc et al., 2005). On the contrary, IFN- γ is a major cytokine in Th1-type cells and can inhibit the production of IgE. This observation suggests that IFN- γ could suppress allergic reactions by resting the Th2 cells (Adel-Patient et al., 2012). We found a slightly lower production of Th2-type cytokines in the spleen from the iALA-treated mice, accompanied by a significantly higher IFN- γ production after the splenocytes were restimulated by iALA. A similar result was concluded by Oh et al. (Oh et al., 2009), who showed an increased IFN- γ production as well as decreased IL-4 secretion by splenocytes from the challenged mice by irradiated peanut extract. Therefore, our findings suggest that iALA could lead to a shift from the Th2- to Th1-type response. Ultimately, this effect decrease the level of specific IgE and IgG₁ antibodies. This finding is notable, as several reports have shown that the Th17 and Tfh lineages, which are associated with the production of IL-17E and IL-21, might contribute to allergic reactions by inhibiting IFN- γ expression and consequently modulating the balance between Th1 and Th2 cells (Herberth et al., 2010; Zaretsky et al., 2009). This conclusion was confirmed by our work, which showed that the levels of IL-17E and IL-21 in the iALA-treated mice showed a similar decrease with the Th2 cytokines.

Simply exploring the Th1/Th2 dichotomy was insufficient to effectively explain the dysregulated immune system involved in allergy and asthma, and another mechanism may involve T-regulatory cells (Tregs) (Akdis and Akdis, 2009; Kanjarawi et al., 2011; Ngoc et al., 2005). Therefore, the proportion of CD4⁺CD25⁺Foxp3⁺ T cells in mice was measured in this study. We found that Tregs levels remarkably increased in the iALA-treated mice, thereby indicating that the increase in Tregs may have been controlled by the modulation of the Th2, IL-17E, and IL-21 response and the increase in Th1-type response (IFN- γ). Therefore, all the findings suggested that iALA could induce a shift toward the Th1 response and ultimately suppress immune reaction.

In conclusion, the data presented in this study demonstrated that the iALA exhibited a low potential allergenicity *in vivo*, suppressing the levels of serum-specific IgG/IgG₁/IgE antibodies, mast cell surface expression of Fc ϵ RI⁺c-kit⁺, peripheral blood basophil expression of IgE⁺CD200R⁺, and plasma histamine levels. Moreover, the iALA displayed a shift from the Th2- to Th1-type response relative to the response against native ALA. These findings suggested that iALA is not allergenic under irradiation processing using safe doses.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fct.2016.10.010>.

Transparency document

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