

Oral Immunotherapy with a Phosphorylated Hypoallergenic Allergen Ameliorates Allergic Responses More Effectively Than Intact Allergen in a Murine Model of Buckwheat Allergy

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Scope: Buckwheat is a common food allergen frequently consumed in Asian countries, with Fag e 1 and Fag e 2 being the major buckwheat allergens. The purpose of this study is to prepare an oral immunotherapy agent by attenuating these allergens via phosphorylation. The immunomodulatory effects of phosphorylated Fag e 2 (P-Fag e 2) in a mouse model of buckwheat allergy are evaluated.

Methods and results: Phosphorylated Fag e 1 (P-Fag e 1) and P-Fag e 2 are prepared by dry-heating in the presence of pyrophosphate. Subsequent dot-blot analysis using serum from food-allergic patient indicates that both proteins exhibit reduced allergenicity upon phosphorylation. Mice subjected to oral administration of P-Fag e 2 for 6 weeks exhibit decreased specific serum IgE and increased specific IgA after Fag e 2 sensitization compared to the sham-treated mice. Moreover, the Peyer's patches (PP) of phosphorylated antigen-fed mice show decreased IL-4 production and induction of T follicular helper (T_{fh}) cells. Increased production of IL-6 is observed in the CD11c⁺ cells isolated from the PPs of P-Fag e 2-fed mice.

Conclusion: These results indicate that attenuated allergens can suppress Th2-induced allergic responses via induction of T_{fh} cells, which are regulated by IL-6 secreted from dendritic cells.

China, and Korea, and has recently become more popular in Europe.^[1] Notably, consumption of even small amounts of flour or food products made from this grain can result in severe anaphylactic reactions. Indeed, approximately 3% of all cases of anaphylaxis in Japan were found to be caused by buckwheat allergy.^[2] A previous study identified four major allergenic proteins in buckwheat, with molecular weights of 9, 16, 19, and 24 kDa. Of these, the 24- and 16-kDa proteins are referred to as Fag e 1 and Fag e 2, respectively.^[3] Fag e 1, the β subunit of a 13 S globulin, was reported to be the predominant allergen with a high IgE binding frequency in patients with buckwheat allergy.^[4] On the other hand, Fag e 2, a member of 2S albumin family, is reported as being highly resistant to pepsin digestion and appears to be responsible for an immediate hypersensitivity reaction of buckwheat allergy including anaphylaxis.^[5]

Allergen-specific immunotherapy, which entails gradual exposure to increasing amounts of an allergen in the hopes of desensitization and promoting tolerance, is one of the most researched approaches for allergy treatment.^[6,7] Currently, oral immunotherapy appears to be the most promising therapeutic approach for

1. Introduction

Buckwheat (*Fagopyrum esculentum*) is a common food allergen that is frequently consumed in Asian countries such as Japan,

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food allergies, as subcutaneous immunotherapy is found to result in unacceptably high rates of severe adverse reactions.^[7–9] However, oral administration is still associated with a high risk for IgE-mediated side effects;^[10] thus, the development of effective and safer tolerogens is urgently needed.

Multiple studies have evaluated the modification of allergenic proteins for use in specific immunotherapies. In particular, Maillard-type glycosylation comprises an effective method for the surface modification of a target protein.^[11] Previously, the attachment of polysaccharides such as arabinogalactan, glucomannan, and xyloglucan via this reaction was shown to mask the IgE epitopes of allergenic proteins, resulting in reduced IgE-binding capacity in the presence of sera from allergic patients.^[12,13] In addition, a Fag e 1-glucomannan conjugate prepared through Maillard-type linkage was shown to shift the Th1/Th2 balance in spleens toward a Th1-dominant immune response, and to suppress buckwheat allergy in mice.^[13] While these hypoallergenic proteins, therefore, comprise promising tolerogens associated with low risks for IgE-mediated side effects, polysaccharide moieties are not suitable for masking epitopes located at the interior of a protein owing to their long side chains. Thus, we hypothesized that phosphorylation might serve as a more effective method for masking the epitope sites of allergenic proteins. Phosphate groups play an important role in immunomodulation, for example by promoting type-I IFN secretion.^[14] Notably, in a previous study, casein phosphopeptide was shown to yield reduced allergy symptoms by promoting intestinal IgA production and reducing serum IgE levels.^[15] The introduction of a phosphate group to allergic proteins might therefore contribute not only to the masking of epitope sites, but also to enhanced immunomodulation. The purpose of this study was to attenuate buckwheat allergens, Fag e 1 and Fag e 2, via phosphorylation and to investigate the effects of hypoallergenic protein on induction of desensitization in a mouse model of buckwheat allergy.

2. Experimental Section

2.1. Materials

Antibodies and reagents used for flow cytometry analyses were purchased from BD Biosciences Co., Ltd. (San Jose, CA, USA). Human serum was obtained from a food-allergy patient (male, 20 years old; female, 18 years old) exhibiting generalized urticaria or angioedema. All reagents were of reagent grade.

2.2. Mice

BALB/c mice (female, 5 weeks old) were purchased from Charles River, Ltd. (Tokyo, Japan) and acclimatized for 1 week prior to the start of the study. Mice were provided free access to a commercial pellet diet (MF; Oriental Yeast Co., Tokyo, Japan) and water. The animal room was maintained at a constant temperature (22 ± 2 °C) and humidity ($55 \pm 10\%$), with a 12 h light/dark cycle. All experiments conformed to the guidelines for Animal Experimentation at Shinshu University (permission number: 270075).

2.3. Preparation of P-Fag e 1 and P-Fag e 2 and Dry-Heated Fag e 1 and Fag e 2

For in vitro allergen production, Fag e 1 and Fag e 2 cDNA molecules were cloned from buckwheat seeds and recombinant proteins were prepared using the *Pichia* expression system (Invitrogen, Waltham, MA, USA). Secreted proteins were purified by size exclusion chromatography on a Superdex S200 HR column (GE Healthcare, Little Chalfont, UK) and lyophilized to obtain powder samples. Phosphorylated Fag e 1 (P-Fag e 1) and Fag e 2 (P-Fag e 2) were then prepared according to the method described by Enomoto et al.^[16] Briefly, the allergens were dissolved in 0.1 M sodium pyrophosphate at a protein concentration of 1 mg mL⁻¹ and lyophilized. The samples were incubated at 85 °C for 7 days, and then dissolved in water. The resulting solution was dialyzed for 3 days against distilled water, using 6–8 kDa molecular weight cutoff tubes (Spectrumlabs, Rancho Dominguez, CA, USA), to remove free pyrophosphate and then lyophilized. For the preparation of dry-heated Fag e 1 (DH-Fag e 1) or Fag e 2 (DH-Fag e 2), freeze-dried Fag e 1 or Fag e 2 was dissolved in distilled water at a protein concentration of 1 mg mL⁻¹, and the solution pH was adjusted to 4.0 with 1 M HCl, followed by lyophilization. Lyophilized samples were then incubated at 85 °C for 7 days, after which the solution was again lyophilized.

2.4. Determination of Phosphorus Content

Phosphorus contents were determined according to the method of Chen et al.^[17] Briefly, samples were mixed with potassium persulfate in test tubes, digested for 20 min at 120 °C, and cooled to room temperature. Samples were then mixed with ascorbic acid and ammonium molybdate-antimony potassium tartrate, and the absorbance at 880 nm was measured using a UV spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was generated using stock solutions of potassium dihydrogen phosphate.

2.5. Immuno Dot-Blotting

Nitrocellulose membranes (Hybond-ECL; GE Healthcare) were incubated in distilled water overnight, and then dried before use. For dot-blotting analysis, 5- μ L sample solutions (2.5 mg mL⁻¹) of Fag e 1, Fag e 2, P-Fag e 1, P-Fag e 2, DH-Fag e 1, or DH-Fag e 2 were spotted onto a washed nitrocellulose membrane. Membranes were blocked by incubation with Tris-buffered saline with 0.05% Tween-20 containing 1% (w/v) BSA at room temperature for 1 h, then incubated with human sera (diluted 1:100) overnight at 37 °C followed by goat anti-human IgE labeled with horseradish peroxidase (HRP; diluted 1:1,000) for 1 h at room temperature. Immunoreactive signals were detected using Pierce Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA). The membrane was washed well using Tris-buffered saline with 0.05% Tween-20 after each step.

2.6. Mouse Allergy Model

In the in vivo allergy experiments, the mice were divided into four groups (six mice per group). For the sham-treated group, Fag e 2

group, and P-Fag e 2 group, naive mice were immunized with 50 μg Fag e 2 dissolved in 200 μL of PBS and alum adjuvant by intraperitoneal injection, respectively. Mice were then boosted by intraperitoneal injection with 50 μg Fag e 2 and alum adjuvant on 14 days after the initial immunization. On day 21, after confirmation of elevated serum levels of specific IgE antibodies via ELISA analysis, mice were provided ad libitum access to home-made feed containing 30 mg Fag e 2 or P-Fag e 2 per 100 g MF pellet for 6 weeks. Mice were then orally challenged with 30 mg of Fag e 2 on day 65. For the naive control group, mice were provided the commercial pellet diet on the same schedule. All mice were euthanized by asphyxiation with CO_2 , and their sera and small intestines were harvested. For cytokine analyses, Peyer's patch (PP) cells harvested from mice in the naive, sham-treated, Fag e 2, and P-Fag e 2 groups were incubated with 50 $\mu\text{g mL}^{-1}$ (final concentration) Fag e 2, respectively, at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. For the naive control group, cells were incubated with PBS instead of allergens.

2.7. Assessment of Hypersensitivity Reactions

Anaphylactic symptoms were evaluated 40 min post challenge using a scoring system as described by Li et al.^[18] and were scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; and 4, no activity after prodding or tremor and convulsion.

2.8. Measurement of Serum Levels of Total and Fag e 2-Specific IgE and IgA

Serum levels of total IgE and IgA were measured via sandwich ELISA, using HRP-labeled antibodies and hydrogen peroxide with *o*-phenylenediamine as the substrate. The anti-mouse IgE and IgA antibodies and HRP-conjugated anti-mouse IgE and IgA antibodies used in these experiments were purchased from Pierce (Rockford, IL, USA). Fag e 2-specific IgE and IgA were detected as described for the total IgE and IgA, except the coating antigen was replaced with Fag e 2 (100 $\mu\text{g mL}^{-1}$).

2.9. Flow Cytometric Analysis

Flow cytometry was utilized to evaluate changes in the numbers of B cells and T follicular helper (Tfh) cells. After oral administration of P-Fag e 2 to Fag e 2-sensitized mice for 6 weeks, PP cells were collected. For analysis of B cells, the cells were stained with PE Anti-mouse CD45R/B220 (1:100) (BD Biosciences) and FITC-labeled anti-CD19 (1:100) (BD Bioscience) antibodies for 30 min at 4 °C. Tfh cells were detected by staining with FITC-labeled anti-CD4, PE-labeled anti-CXCR5, and APC Anti-mouse CD279 (PD-1) antibodies (1:100) (BD Biosciences). The resulting cell samples were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

2.10. Analysis of Cytokine Production by CD11c⁺ Cells

For separation of CD11c⁺ cells from the PP cells of the naive, sham-treated, Fag e 2, and P-Fag e 2 groups, cell suspensions from each respective group were stained with PE-labeled anti-CD11c (1:30) (BD Biosciences), incubated for 30 min in the dark at 4 °C. CD11c⁺ cells were sorted using a Cell Sorter SH-800 (Sony, Tokyo, Japan). The resulting cells, regarded as CD11c⁺ DCs isolated from PP cells, were suspended (1×10^5 cells per well) in RPMI 1640 medium supplemented with 10% FBS, 50 U mL^{-1} penicillin, and 50 $\mu\text{g mL}^{-1}$ streptomycin, and further incubated for 72 h in the presence of 50 $\mu\text{g mL}^{-1}$ Fag e 2 (sham-treated, Fag e 2, and P-Fag e 2 groups) or PBS (naive unsensitized group). The cytokine concentrations in the resulting culture supernatants were measured by ELISA analysis.

2.11. Measurement of Cytokine Levels in Culture Supernatants

Cytokine levels in culture supernatants were determined by sandwich ELISA analysis, using the following HRP-conjugated antibodies and hydrogen peroxide with *o*-phenylenediamine as the substrate: rat anti-mouse IL-4, IFN- γ , IL-21, and IL-6 antibodies, biotinylated rat anti-mouse IL-4, IFN- γ , IL-21, and IL-6 antibodies, and streptavidin-HRP conjugate (all purchased from Peprotech, Rocky Hill, NJ, USA).

2.12. Statistical Analysis

Results are expressed as mean $\pm$ SEM. To evaluate differences between the groups, unpaired Student's *t*-tests were carried out using Statcel software ver. 2.0 (OMS-Publishing, Saitama, Japan); $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Effects of Phosphorylation on the Reactivity of Serum from a Food-Allergic Patient to Fag e 1 and Fag e 2

First, we prepared P-Fag e 1 and P-Fag e 2 by dry-heating in the presence of pyrophosphate. As shown in **Figure 1A**, the phosphorus content of P-Fag e 1 and P-Fag e 2 when dry-heated at 85 °C for 7 days was 0.38% and 0.42%, respectively, which was significantly higher than that of the control. The allergenicity of the resulting proteins was then measured by dot-blotting immunoassay analysis, using serum from a food-allergic patient. As a result, P-Fag e 1 and P-Fag e 2 exhibited drastically reduced allergenicity against sera from two allergenic patients when compared to intact Fag e 1 and Fag e 2 (**Figure 1B**). The serum showed similar levels of reactivity to the samples dry-heated in the absence of pyrophosphate and the untreated control (**Figure S1**, Supporting Information), suggesting that reduced allergenicity was not induced by dry-heating without phosphorylation. Serine, threonine, tyrosine, arginine, and lysine are the primary amino acids that are susceptible to phosphorylation. Previously, Li et al. revealed that the serine, threonine, and tyrosine residues

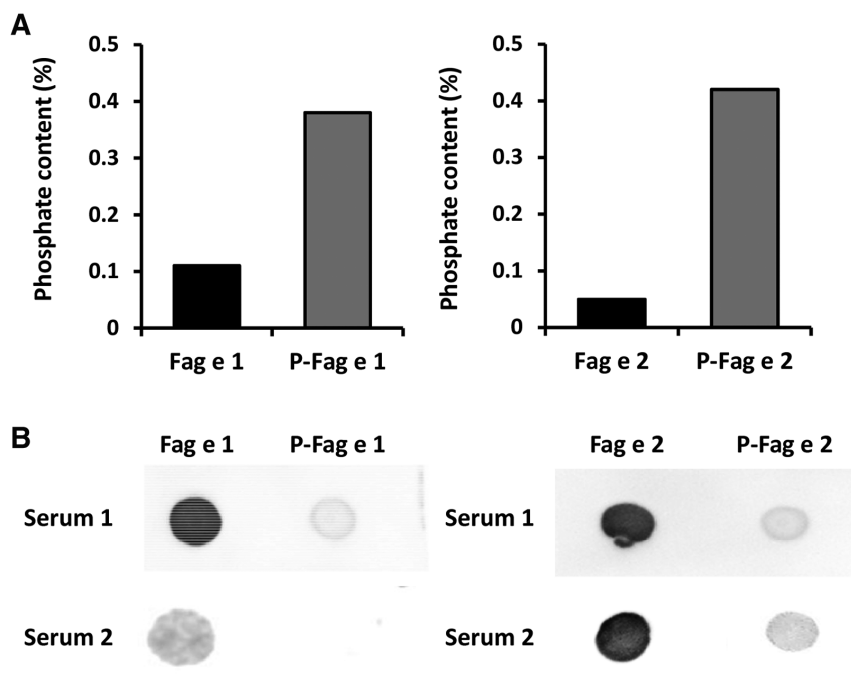


Figure 1. Phosphorylation of Fag e 1 and Fag e 2 results in reduced allergenicity. Graphic depictions of changes in A) phosphate content and B) allergenicity of Fag e 1 and Fag e 2 after dry-heating in the presence of pyrophosphate, as determined by spectrometry and immune dot-blot analysis, respectively. P-Fag e 1, phosphorylated Fag e 1; P-Fag e 2, phosphorylated Fag e 2.

of lysozyme and ovalbumin were phosphorylated by dry-heating with pyrophosphate.^[19] Notably, six of the eight IgE-binding regions in Fag e 1^[20] contain serine, tyrosine, arginine, and lysine residues. Likewise, the peptide EGVRDLKE, which was identified as the epitope region of Fag e 2,^[21] also contains arginine and lysine residues. These findings suggest that the masking effect of phosphorylation could be attributed to reduced patient-specific IgE recognition of Fag e 1 and Fag e 2.

3.2. Effects of Phosphorylation of Fag e 2 on Buckwheat Allergy in Mice

Since Fag e 2 is mainly considered to contribute to the induction of an immediate hypersensitivity reaction of buckwheat allergy, including anaphylaxis, we next investigated the effectiveness of hypoallergenic P-Fag e 2 as a desensitization inducing agent using an animal model of Fag e 2-sensitized mice. Since intact allergen is usually used in immunotherapy, we compared the effects of intact Fag e 2 and P-Fag e 2 in their respective treatment groups. After immunization with Fag e 2 twice, the effects of oral administration of P-Fag e 2 for 6 weeks was investigated (Figure 2). As shown in Figure 3A and B, respectively, the mice provided phosphorylated proteins exhibited a lower allergic score and lower levels of total IgE compared with the control and Fag e 2 groups of mice. A similar decrease was also observed in the levels of Fag e 2-specific IgE in serum (Figure 3C). In contrast, while the mice in the sham-treated groups exhibited lower levels of total IgA than the naive control mice, the mice in the P-Fag e 2 group exhibited higher total and Fag e 2-specific IgA levels than the

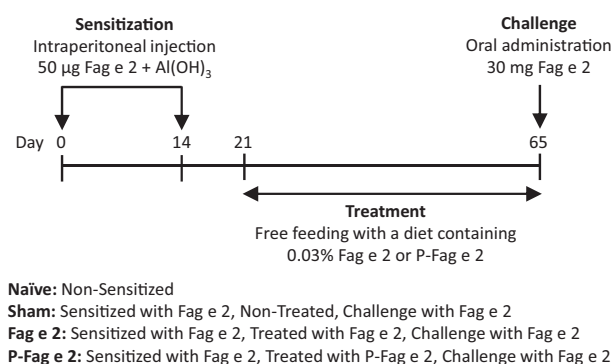


Figure 2. Schematic depicting the design of the in vivo experiments employed in this study. Mice were divided into five groups ($n = 6$ mice/group): Naïve, no sensitization and normal diet; sham-treated, Fag e 2 sensitization and normal diet; Fag e 2, Fag e 2 sensitization and diet containing 0.03% (w/w) P-Fag e 2; and P-Fag e 2, Fag e 2 sensitization and diet containing 0.03% (w/w) P-Fag e 2.

mice in the sham and Fag e 2 groups (Figure 3D and E). Moreover, the B220⁺CD19⁺ B-cell population of the PP cells isolated from each mouse was significantly decreased by oral administration of P-Fag e 2 (Figure 3F). These results suggest that phosphorylation might attenuate the allergic reaction against buckwheat allergens more safely and effectively than intact allergen.

We then examined the effects of Fag e 2 phosphorylation on cytokine production levels to assess whether modification of these proteins affected the differentiation of Th cell subsets in the PP of allergic mice. The production of IL-4 (Th2-associated cytokine) in PP cells was decreased in the P-Fag e 2 group, compared to that in

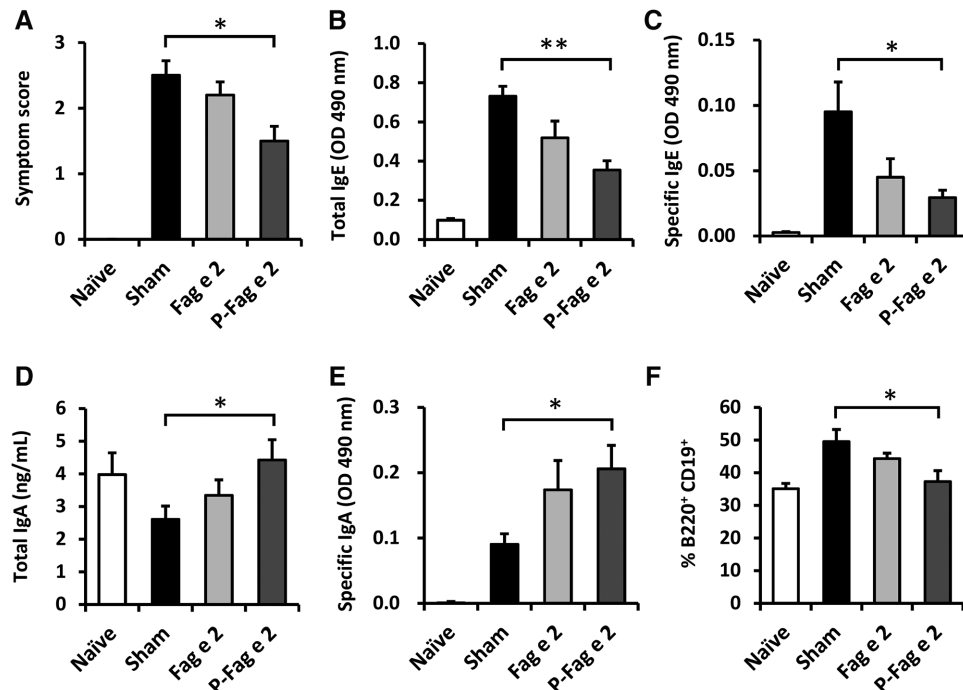


Figure 3. Effects of oral administration of P-Fag e 2 on the immune response of Fag e 2-sensitized mice. A) Allergic score and the serum levels of B) total IgE, C) Fag e 2-specific IgE, D) total IgA, E) Fag e 2-specific IgA, and F) the population of B220⁺CD19⁺ cells in cultured Peyer's patch (PP). IgE and IgA levels were measured by ELISA, while the cell population was measured by flow cytometry. Bars represent the mean values $\pm$ SEM; * p < 0.05 and ** p < 0.01 as compared to the sham.

the sham-treated group (Figure 4A). Conversely, no differences in PP cell IFN- γ levels were observed between groups (Figure 4B). Furthermore, increased production of IL-21 was observed in the PP cells of mice in the P-Fag e 2 group, compared to that in the sham and Fag e 2 groups (Figure 4C). In general, food allergies are characterized as IgE-mediated hypersensitivity reactions. In such reactions, IL-4 induces the differentiation of native CD4⁺ T cells into helper Th2 cells, which in turn promote the production of IgE via cytokines such as IL-4 and IL-5. On the other hand, IL-21 is one of the predominant cytokines produced by Tfh cells, and has been shown to play a compensatory role for IL-4 in negatively impacting IgE switching.^[22] Thus, our data suggest that IL-21 produced by Tfh cells attenuated the Th2-type allergic response, including IgE production, following Fag e 2-sensitization without drastically affecting the Th1 response.

Subsequently, we further investigated the effects of orally administered P-Fag e 2 on the differentiation of precursor cells into Tfh cells. We hypothesized that the induction of Tfh cells could contribute to the enhancement of IgA production in the serum of P-Fag e 2 administered mice because Tfh cells promote IgA⁺ B cell differentiation and IgA production.^[23] PP cells were stained with antibodies specific to Tfh lineage markers and subjected to flow cytometric analysis. The PP cells of mice fed phosphorylated proteins exhibited higher percentages of CD4⁺CXCR5⁺ PD-1⁺ cells than those of the sham-treated and Fag e 2 groups (Figure 4D and E). Tfh cells have been demonstrated to suppress the Th2 type immune response and to enrich particularly in the intestinal lamina propria.^[22,24] These results therefore suggest that the Th2-induced allergic response to P-Fag e 2 was suppressed via the induction of Tfh.

3.3. Effects of Phosphorylation of Fag e 2 on Dendritic Cell Production of IL-6

IL-6 produced by dendritic cells is widely accepted as being an inducer of Tfh cells.^[22,25] Increased production of IL-6 was observed in the PP cells of mice in the P-Fag e 2 group, compared to that in the sham-treated and Fag e 2 groups (Figure 5A). Moreover, increased IL-6 production was observed in CD11c⁺ cells isolated from the PPs of P-Fag e 2-fed mice (Figure 5B). These results suggest that CD11c⁺ cells, which are mainly dendritic cells, were able to produce Tfh-inducing cytokines.

We then investigated whether P-Fag e 2 directly enhanced the IL-6 production, which subsequently regulated the differentiation of Th cells. The PP cells collected after Fag e 2-sensitization on day 21 (Figure 2) were incubated with Fag e 2 or P-Fag e 2 for 3 days. For the cells incubated with P-Fag e 2, IL-4 production was lower, and IL-6 and IL-21 production were higher compared to the PP cells incubated with Fag e 2 (Figure 6A–C), which was consistent with the results from the experiments using oral administration of the allergen challenge (Figures 4A, C, and 5A). Furthermore, increase in IL-6 level was observed in CD11c⁺ cells of the P-Fag e 2 group (Figure 6D). These results suggest that dendritic cells were capable of recognizing P-Fag e 2 regardless of oral administration of P-Fag e 2. Thus, dendritic cells within the PP might directly recognize active sites, such as the phosphorus moiety, of P-Fag e 2. The oral administration of intact Fag e 2 resulted in no detectable induction of IL-6 production or differentiation of precursor cells into Tfh cells, while the phosphorylation of Fag e 2 resulted in the Tfh-mediated attenuation of the allergic reaction. Increased IL-21 levels in PP cells appear to be caused by

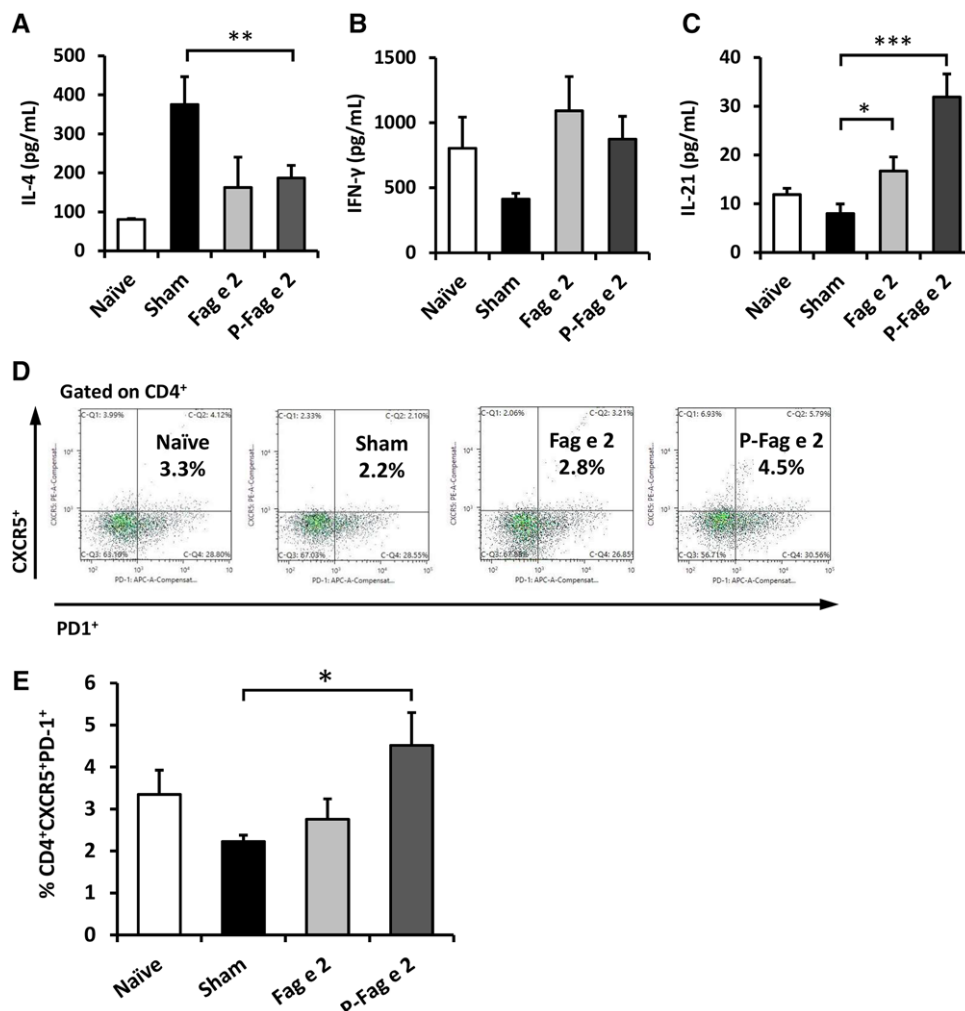


Figure 4. Effects of oral administration of P-Fag e 2 on cytokine production and the population of T follicular helper (Tfh) cells in the PP cells of Fag e 2-sensitized mice. Fag e 2-sensitized mice were fed P-Fag e 2 for 6 weeks. Peyer's patch (PP) cells were then harvested and incubated with 50 $\mu\text{g mL}^{-1}$ (final concentration) Fag e 2 (sham-treated, Fag e 2, and P-Fag e 2 groups) or PBS (naive nonsensitized group) at 37 °C in a humidified atmosphere with 5% CO₂ for 72 h for the determination of cytokine levels. The levels of A) IL-4, B) IFN- γ , and C) IL-21 within the culture supernatants of the PP cells were measured by ELISA. For the measurement of the Tfh population, PP cells harvested from mice were analyzed by flow cytometry without incubation. D) Dots represent cells proliferation. E) The percentage of Tfh cells in the PP cells collected from mice. Bars represent the mean values $\pm$ SEM; * p < 0.05, ** p < 0.01, and *** p < 0.005 as compared to the sham.

Tfh cells; however, it remains unclear whether P-Fag e 2 may also be able to directly affect the Tfh cells. Future investigation of the IL-21 signaling pathways in Tfh cells will be necessary to reveal the detailed mechanism by which P-Fag e 2 induces Tfh.

IgA is an important component of mucosal immune homeostasis and plays a critical role in the maintenance of a vast community of commensal bacteria in the intestinal lumen, while minimizing penetration of the gut epithelium.^[26] Frossard et al. reported that food allergy-tolerant mice show increased numbers of antigen-specific IgA-secreting cells within the PP.^[27] The increases in Tfh cells within the PP, as well as the increased serum levels of IgA, observed in P-Fag e 2-fed mice, compared to that in mice provided normal feed, suggest that IgA-producing B cells are induced by modulation of Tfh cells. Generally, regulatory T (Treg) cells regulate immune responses and maintain self-tolerance by inhibiting B cells responses,^[28,29] whereas Tfh cells help B cells to differentiate into IgA-producing plasma cells.

Thus, the balance between Tregs, Tfh cells, and effector T cells might contribute to the homeostasis of immune response. In this study, the mechanism of changes in the population of Tregs, Tfh, and effector T cells over time still remains unclear, and it should be deeply studied.

In a recent study, Rupa et al. reported that mannosylation effectively suppressed orally induced allergic immune responses to egg white proteins, but not whey or peanut proteins, in mice.^[30] Specifically, this mannosylation resulted in increased populations of Treg cells, but no changes in IgA levels. Conversely, in the current study, phosphorylation promoted IgA production, followed by increased populations of Tfh cells. Thus, the mechanisms underlying the immunosuppressive effects of mannosylation and phosphorylation appear to be different. In any case, further studies are needed to investigate whether phosphorylation is applicable for the development of hypoallergenic agents for other food allergens, including whey and peanut proteins.

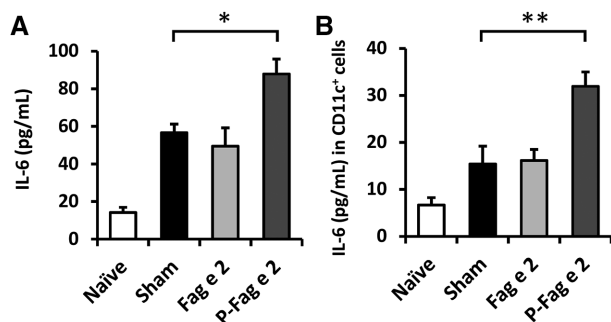


Figure 5. Effects of oral administration of P-Fag e 2 on IL-6 production by PP cells of Fag e 2-sensitized mice. Fag e 2-sensitized mice were fed P-Fag e 2 for 6 weeks. Peyer's patch (PP) cells were then harvested and the CD11c⁺ cells isolated. PP and CD11c⁺ cells were then incubated with 50 $\mu\text{g mL}^{-1}$ (final concentration) Fag e 2 (sham-treated, Fag e 2, and P-Fag e 2 groups) or PBS (naïve nonsensitized group) at 37 °C in a humidified atmosphere with 5% CO₂ for 72 h. A) The level of IL-6 within the culture supernatants of the PP cells was measured by ELISA. B) CD11c⁺ cells were isolated from PP cells, and the IL-6 level within the culture supernatants was measured by ELISA. Bars represent the mean values $\pm$ SEM; * p < 0.05 and ** p < 0.01 as compared to the sham.

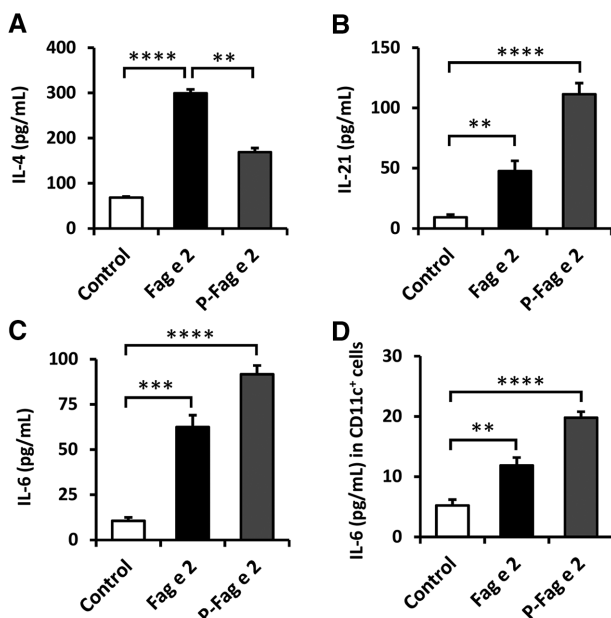


Figure 6. Effects of oral administration of P-Fag e 2 on cytokine production by PP cells and CD11c⁺ cells of Fag e 2-sensitized mice. Mice were sensitized with Fag e 2. Peyer's patch (PP) cells were then harvested and CD11c⁺ cells isolated from the PP cells. PP and CD11c⁺ cells were then incubated with 50 $\mu\text{g mL}^{-1}$ (final concentration) Fag e 2 or P-Fag e 2 at 37 °C in a humidified atmosphere with 5% CO₂ for 72 h. As a control, cells were incubated with PBS instead of an allergen. The levels of (A) IL-4, (B) IL-21, and (C) IL-6 within the culture supernatants of the PP cells were determined by ELISA analysis. D) CD11c⁺ cells were isolated from PP cells, and the IL-6 level within the culture supernatants was measured by ELISA analysis. Bars represent the mean values $\pm$ SEM; ** p < 0.01, *** p < 0.005, and **** p < 0.001 as compared to the sham.

4. Concluding Remarks

In conclusion, we demonstrated that oral administration of P-Fag e 2 attenuated the Th2-type allergic response in Fag e 2-sensitized mice. Suppression of the allergic response was primarily due to induction of Tfh cells, which was regulated by IL-6 secreted from dendritic cells. The orally administered Fag e 2 also induced desensitization of the allergic response; however, the phosphorylation of Fag e 2 enabled a stronger and safer induction of desensitization than the intact Fag e 2. These findings could facilitate the development of a hypoallergenic agent for buckwheat allergy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

allergenicity, buckwheat, oral immunotherapy, phosphorylation, T follicular helper cells

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Supporting Information

Oral immunotherapy with a phosphorylated hypoallergenic allergen ameliorates allergic responses more effectively than intact allergen in a murine model of buckwheat allergy

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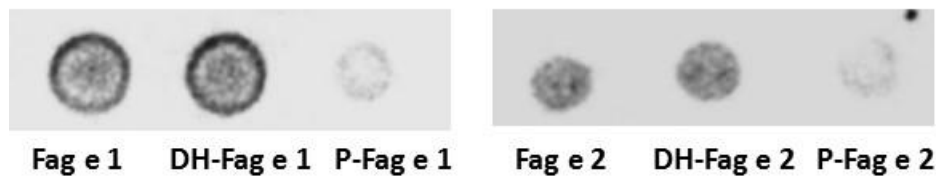


Figure S1. Allergenicity of Fag e 1 and Fag e 2 after dry-heating in the absence or presence of pyrophosphate, as determined by immune dot-blot analysis. P-Fag e 1, phosphorylated Fag e 1; P-Fag e 2, phosphorylated Fag e 2; DH-Fag e 1, dry-heated Fag e 1; DH-Fag e 2, dry-heated Fag e 2..