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B-FAHF-2 plus oral immunotherapy (OIT) is safer and more effective than OIT alone in a murine model of concurrent peanut/tree nut allergy

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Abstract

Background—Concurrent sensitization to peanut (PN) and tree nuts (TN), the most dangerous food allergies, is common. Current oral immunotherapy (OIT) is not fully satisfactory.

Objective—To determine if the herbal formula B-FAHF-2 (BF2) ameliorates PN/TN OIT adverse reactions and enhances persistence of a tolerant state.

Methods—Concurrently sensitized peanut, walnut (WN) and cashew (CSH) allergic mice received 1 day PN/WN/CSH rush OIT plus 3 weeks of maintenance dosing, with or without 3-weeks prior and 3-weeks BF2 co-treatment. Anaphylactic symptom scores, core body temperatures, plasma histamine levels, basophil numbers, antigen-specific IgE, cytokine levels, and IL-4, INF- γ and Foxp3 gene promoter DNA methylation status, and their correlation with final challenge symptom scores were determined.

Results—BF2+OIT treated mice experienced significantly fewer and less severe adverse reactions than OIT only treated mice ($p < 0.01$) during the one-day rush OIT buildup dose phase. Both OIT only and BF2+OIT mice showed significant desensitization ($p < 0.01$ and 0.001 respectively) at one-week post therapy challenge; being greater in BF2+OIT mice. All sham treated and 91% of OIT treated mice experienced anaphylaxis whereas only 21% of BF2+OIT treated mice exhibited reactions during 5–6 weeks of dose escalation single PN and TN challenges. Greater and more persistent protection in BF2+OIT mice was associated with significantly lower plasma histamine and IgE levels, increased IFN- γ /IL-4 and IL-10/IL-4 ratios, DNA re-methylation at the IL-4 promoter and de-methylation at IFN- γ and Foxp3 promoters. Final challenge symptom scores were inversely correlated with IL-4 DNA methylation levels ($p < 0.0002$), and positively correlated with IFN- γ and Foxp3 gene promoter methylation levels ($p < 0.0011$) ($p < 0.0165$).

Conclusions and clinical relevance—Combined BF2/OIT therapy was safer and produced longer post treatment protection, and more tolerance-prone immunological and epigenetic

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modifications than OIT alone. BF2/OIT may provide an additional OIT option for patients with concurrent peanut/ tree nut and other food allergies.

Keywords

Food allergies; murine model of peanut allergies and tree nut allergies; oral immunotherapy; Chinese herbal medicine; B-FAHF-2; cytokines and epigenetic regulation

Introduction

Food allergy (FA), a major public health issue in Western countries, which affects 1 in 13 American children,[1] and accounts for 1/3 of anaphylaxis cases, is potentially life threatening.[2] FA has a drastic negative impact on the quality of life of affected individuals and families,[3] and there is no established treatment for use in routine clinical practice. Management involves avoiding offending foods and rescue medications in the event of accidental reactions.[4, 5] FA is characterized by a Th2-skewed immune response presenting as elevated IL-4 and food protein IgE levels.[6] IL-4 is required for B cells switching to IgE production, mast cell activation, and Th2 cell differentiation.[7, 8] IL-4 levels are associated with clinical FA [9, 10] and FA animal models.[11, 12] Turcanu et al. found that peanut-specific lymphocytes from patients with persistent peanut allergy showed Th2 skewed cytokine production (low IFN- γ /IL-4 ratio) whereas lymphocytes from children who outgrew their allergy showed a Th1 skewed response to peanut antigens (high IFN- γ /IL-4 ratio).[13] In addition, Krogulska et al. found that peripheral blood mononuclear cells from FA children exhibited statistically lower Foxp3 and IL10 gene expression than cells from healthy children, whereas children acquiring tolerance to the food showed significantly higher levels of the Foxp3 gene expression than children with active FA. An inverse correlation between Foxp3 expression and total IgE levels was also found.[14]

Peanut (PN) and tree nut (TN) allergies are the most dangerous food allergies; and concurrent sensitization to both peanut and tree nut further increases the risk of severe reactions. Although food allergen OIT has desensitized some patients, many regained reactivity shortly discontinuing OIT. [15–20] The mechanisms underlying these clinical observations are not clear, but it appears that current OIT protocols do not generate sustained alteration of Th2 allergic immune status in the majority of patients. A recent study also found that reduced expression of allergy biomarkers, including IL-4 expression and basophil activation, during PN OIT was transient. Initially increased IL-10 expression was also transient in this phase II study in which increased IL-10 levels declined toward baseline during the second half of OIT. [21]

DNA methylation status is an important epigenetic mechanism of IL-4, IFN- γ and Foxp3 gene regulation. Increased DNA methylation (or remethylation) of cytokine promoters suppresses, whereas reduced DNA methylation (demethylation) activates cytokine gene expression. This provides a potential target for induction of immune tolerance. A recent phase I study suggested that Foxp3 locus demethylation was associated with peanut OIT induced sustained unresponsiveness when compared to individuals who regained sensitivity to peanut 3 months off OIT. Subsequent remethylation was associated with regained

sensitivity, [22] suggesting that DNA methylation status of the Foxp3 locus might be a biomarker predicting clinical tolerance. OIT modulation of DNA methylation at the IL-4 and IFN- γ promoters has not been reported.

Because of significant safety concerns regarding routine use of OIT, [16–20, 23] several combined treatment approaches have been investigated. OIT plus anti-IgE antibody administration produced more rapid desensitization to milk and egg proteins with fewer and less severe adverse reactions than OIT only.[24] [25, 26] Administration of a probiotic during 18 months of peanut OIT did not reduce the incidence of adverse reactions. [27] A safe and effective peanut and tree nut allergy therapy that both desensitizes effector cells and induces tolerogenic immunity by reducing Th2 status and increasing IFN- γ and IL-10 has not yet been developed.

Murine models of food allergy are useful tools to investigate new treatments, including OIT. [28] Leonard et al reported that 3 weeks of daily ovomucoid OIT significantly reduced oral egg challenge induced gastrointestinal reactions (diarrhea). However reactions returned 2 weeks post therapy, and OIT did not result in desensitization of systemic effector cells.[29] Maeta et al [30] reported that 10 day rush ovomucoid OIT treatment of mildly ovomucoid allergic mice failed to reduce allergic symptoms and increased vascular permeability, it further increased total plasma IgE levels and reduced IFN- γ /IL4 and IL-10/IL-4 ratios compared to sham OIT mice. No animal model study of OIT for peanut, tree nut, or multiple allergies has been reported.

Food Allergy Herbal Formula 2 (FAHF-2) protects against peanut anaphylaxis in murine models, [31] suppresses basophil activation and Th2 cytokine production, and has high safety profile in clinical studies.[32, 33] BF-2 is butanol purified version of (FAHF-2). This formulation addresses the need to reduce the treatment dose burden, which hindered compliance in FAHF-2 human trials [34]. BF2 suppressed anaphylactic reactions, IgE production, mast cell degranulation, and modulated Th1 and Th2 cytokine production in a murine peanut allergy model at only 20% of the FAHF-2 dose, and the effect was sustainable.[35] BF2 inhibited human B cell IgE production in vitro 10 times more potently than FAHF-2.[35] Some major active compounds including alkaloids and triterpenoids from BF2 have been identified and found to inhibit IgE production[36], mast cell activation, [37] and inhibited of pro-inflammatory cytokines in allergy [38] and other inflammatory conditions.[39] We, therefore, hypothesized that combining BF2 and multiple PN/ TN OIT would be more beneficial than OIT alone. The objectives of this study were to first generate a murine model of concurrent PN and tree nut allergies for which there was no previous report and test our hypothesis in this model.

Methods

Mice

Five-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free facilities at the Mount Sinai vivarium on PN-free chow according to standard guidelines for the care and use of animals.[40] Animal experiments were approved by Mount Sinai IACUC (Institutional Animal Care and Use

Committee). Its institutional Public Health Service (PHS) animal welfare assurance number is A3111-01. The program is licensed under the United States Department of Agriculture (USDA) as a Research Facility, No. 21-R-040

In vivo experimental protocol

Pre-OIT phase—To generate a murine model concurrently sensitized to PN and TN, mice in groups (G) 1–3 (described in Figure 1) were sensitized intragastrically (i.g) with a PN, CSH and WN cocktail (equivalent 2.5 mg protein /mouse), and cholera toxin (10 µg/mouse) as mucosal adjuvant in a vehicle solution of 1.5 % bicarbonate-PBS and 16.5 µl of 40% alcohol (vodka - Stolichnaya Vodka®) added to enhance gastrointestinal absorption.[41] Protein content for PN, CSH and WN was adjusted using values listed in the USDA Nutrient Data Laboratory database (www.nal.usda.gov/fnic/foodcomp/search). Protein concentration of each nut was 2.5mg/dose at weeks 0–4 and 12.5 mg/dose at week 5 for boosting. Mice were fasted for 2 hours prior to sensitization or boosting. Unsensitized naïve mice (G4) served as controls. To assess the hypersensitivity reactions to each of PN/TN challenge, these polysensitized mice were i.g. challenged with PN followed by CSH and WN (50 mg/mouse) on weeks 6, 7 and 8 respectively. Challenge solution contained homogenized PN or WN or CSH without cholera toxin and was dissolved in sensitization-vehicle solution described above. Identifying cage cards were concealed throughout the challenge procedure. Symptom scores were visually assessed 30 minutes after challenge and scores were assigned according to criteria described below. Body temperatures were recorded using rectal thermometer. These mice, concurrently allergic to PN and the TN (CSH and WN) were designated as PN and TN allergic mice (PTNA mice). IgE levels and severity of reactions served as baseline prior to OIT.

Treatment protocols—Two days following the baseline pre-OIT challenge (at week 8), G1 PTNA mice received water sham treatment (Sham group), G2 PTNA mice received OIT alone treatment (OIT group). G3 PTNA mice received BF2 (12 mg/day dissolved in drinking water given in two daily gavages) for 3 weeks prior to and during 3 weeks of OIT (BF2+OIT group). The OIT protocol included one-day rush OIT protocol and 3 weeks maintenance OIT protocol. Rush OIT protocol consisted of a one-day rush OIT build up phase using three i.g. administrations at 30 min intervals of a PN/CSH/WN cocktail containing either 2.5, 6.25 or 12.5 mg of each PN/TN protein. Because reactions were observed at 12.5 mg, 6.25 mg maintenance doses were administered daily for 3 weeks. BF2 formula product preparation and quality control methods and results are described in supplemental methods (E Method 2 and E Figure 2). The FDA guidance for industry (Q3C tables and list) classifies butanol, ethanol and ethyl acetate as class 3 (less toxic) solvents. [42] The daily amount of butanol consumed should be less than 50 mg/day. [42] The amount of butanol in the daily dose of BF2 tablets is only 0.228mg, which is far below the allowable limits. BF2 has received US FDA approval to replace FAHF-2 in clinical studies. Naïve mice (G4) served as normal controls.

Post-OIT challenges—To determine the desensitization effect all treated PTNA mice (G1–3) were orally challenged with a PN, WN and CSH cocktail (25 mg of each protein) 1 week (week 14) post-therapy, and re-challenged 2 weeks later. Naïve mice received PBS

challenge. To determine the effect on induction of tolerance, at 5 weeks post-therapy (week19), mice were given dose escalation challenges with 12.5, 25 and 50 mg of PN and individual nut proteins at 3 day intervals beginning with PN followed by WN and finally CSH at 6 weeks post-therapy (final challenges at week 20). Mice reacting to the lower doses did not receive additional challenges. This protocol mimics the standard clinical challenge protocol. Mice were sacrificed immediately after completing symptom evaluation following the final CSH challenge at week 6 post OIT.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were observed over 30 minutes and scored as previously described with slight modifications because multiple-antigen allergic mice exhibited more severe anaphylactic reactions than PN or single TN allergic mice. Symptoms included a sustained lack of voluntary motility and splaying of limbs with belly resting on cage floor. Therefore, in this study we used grades 0–6 to score the reactions. :0 - no signs; 1 - scratching and rubbing around the snout and head, diarrhea without other systemic symptoms; 2 - puffiness around the eyes and snout, redness around snout, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 - Labored respiration, diarrhea accompanied by drop in body temperature, cyanosis around the mouth and the tail; 4 – Labored respiration accompanied by drop in body temperature and sustained lack of voluntary motility, but activity after prodding, 5- Labored respiration, drop in body temperature, splaying of limbs with belly resting on cage floor, minimal or no activity after prodding, tremor and convulsions. 6- Death. Reactions with score 1 were defined as mild; scores 2–3 as moderate-to severe; score 4 as severe, Score 5–6 very severe.

Plasma histamine, serum antibody and cytokine measurements

Plasma histamine, food-specific immunoglobulins and cytokine levels were determined by ELISA as described previously, [35] (Supplementary information E methods 3, 4 and 5)

CpG methylation analysis

Genomic DNA isolated from intestinal tissue was preserved in AllProtect (Qiagen, Valencia, CA.) and bisulfite converted prior to pyrosequencing using a Pyromark Q24 system (Qiagen, Valencia, CA). Methylation status of CpG residual in promoter regions of IL-4, IFN- γ and Foxp3 described previously were examined.[43] Detailed information including PCR and sequencing primers is provided in supporting information (E Methods 6)

Statistical analysis

Data were analyzed using GraphPad Prism. One Way ANOVA with Bonferroni post-test was applied to determine significant differences across treatment groups. Student's t test was used to analyze differences between selected groups. For data that were not normally distributed, Kruskal-Wallis test was used. Spearman correlation was used for regression analysis. P values less than P 0.05 were considered significant.

Results

BF2 reduced rush OIT buildup dose phase-associated adverse reactions

One-day dose escalating rush OIT with PN/TN cocktail was initiated the day after completing 3 weeks of BF2 or sham treatment. OIT and BF2-OIT PTNA mice received the first dose of PN, CSH and WN mixture containing 2.5 mg each food protein. Sham-treated PTNA mice received an equal volume of water. After 30 minutes of observation during which no mice reacted, a mixture containing 6.5 mg of each protein was administered. This dose also induced no reactions. After administration of a mixture containing 12.5 mg of each protein, 7 of 16 (43%) of the OIT-treated only mice exhibited moderate-to severe reactions (symptom scores 2–3). In contrast only 2 of 16 (12 %) BF2+ OIT mice showed reactions, which were mild (symptom score 1) (Figure 1A). Median symptom scores of OIT-treated only PTNA mice were also significantly higher than those of the BF2+ OIT-treated group of mice ($P<0.001$, Figure 2A). Six of 7 OIT reactor mice, but only 1 of 2 BF2+ OIT reactor mice exhibited hypothermia (core body temperature below 36 °C), Figure 1B). Consistent with symptom scores, plasma histamine levels were significantly elevated in the OIT-treated only mice but not the BF2+OIT-treated mice ($P<0.05$, Figure 1C). Sham OIT PTNA mice did not show any sign of reactions. These findings demonstrated that BF2 pretreatment significantly increased the safety of OIT during the rush OIT build-up phase. Because a 6.5 mg protein dose was the highest safe dose for all mice, this dose was used for daily OIT throughout the remainder of the three weeks treatment regimen.

Complementary BF2 treatment generated greater OIT desensitization at one week post-OIT challenge

One week after completing therapy all mice, except naïve, were orally challenged with a PN/CSH/WN cocktail (12.5mg protein each). Naïve mice received PBS and served as normal controls. Naïve mice were not challenged at this time and only received the final challenge with these foods. All Sham treated PTNA mice reacted to challenge (Figure 2A). 6 of 16 (37.5%) challenged mice in the OIT group exhibited reactions. Only 2 of 11 (18%) mice in the BF2+OIT group showed reactions, and these reactions were mild (scores 1 and 2). Median scores were significantly lower in OIT alone and BF2+OIT than in the Sham group of mice ($p<0.01$, OIT vs Sham; $p<0.001$ BF2 vs Sham). In addition, 90% of reactors in the sham-treated group and 50% in the OIT only group developed diarrhea, indicating both GI reactions and systemic reactions occurred. In contrast diarrhea was absent in BF2+OIT reactor mice.

Mean core body temperatures of OIT and BF2+OIT mice were essentially similar those of naïve mice, whereas those of Sham treated and OIT only mice were decreased (Figure 2B, $P<0.05$ for OIT and $P<0.001$ for BF2+OIT).). Plasma histamine levels were significantly lower in BF2+OIT, but not OIT mice, compared to Sham-treated mice (Figure 2C, $p<0.05$ vs Sham). A similar pattern was found following mixed PN/TN challenge 2 weeks later (data not shown). These findings show that BF2 treatment significantly enhanced OIT desensitization. To further evaluate desensitization, we determined peripheral blood basophil numbers. Basophil numbers in both OIT alone and BF2+OIT mice were reduced when compared to Sham treated mice, but the reduction reached statistical significance only in

BF2+OIT mice (E Figure 2, $p<0.05$). Therefore, basophil numbers may not be a key biomarker to distinguish the beneficial influence of BF2 in OIT therapy. We next conducted correlation analysis, and data showed that symptoms scores were significantly negatively correlated with body temperature ($r=-0.801$, $p<0.000$, Fig 3E) and positively correlated with plasma histamine levels ($r=0.733$, $p<0.0001$, Fig 3D), suggesting plasma histamine levels may be a biomarker to distinguish the superior of protection of BF2 +OIT therapy over OIT alone.

BF2+OIT produced more sustained protection than OIT alone at 5–6 weeks post-OIT challenge

Prior to treatment, we challenged the 3 groups of concurrently PN/TN poly sensitized mice with individual PN, WN and CSH to generate a baseline, mimicking human clinical studies. We found that all three groups of PN/TN poly-sensitized mice showed anaphylactic reactions to PN, WN and CSH following i.g. challenges (Figure 4A). For comparison and to evaluate tolerance effect of treatment, 5–6 weeks post-OIT, mice received single PN, WN and CSH dose escalation challenges at 3 day intervals. The final CSH challenge occurred 9 days later at week 6 post OIT. No reactions occurred following the 12.5 mg challenges (data not shown), whereas 25 mg PN protein challenges induced reactions in 3/10 sham, 1/10 OIT and 1/11 BF2-OIT mice. Median scores did not differ between groups (data not shown). Following 50 mg PN protein challenge, all Sham treated and 8/9 OIT-treated only mice reacted. Median scores were not significantly different between Sham and OIT groups (Figure 4A, PN challenge). In contrast, only 2 of 10 BF2+OIT-treated mice reacted, and median scores were significantly lower than in Sham-treated mice ($p<0.05$). Median score in BF2+OIT mice was lower than in OIT mice although differences did not reach statistical significance (Figure 4B). Similar results were found following WN and CSH dose escalation challenges, and the symptom scores following 50 mg challenges, except that significant differences between BF2+OIT and OIT alone group were observed for WN (Figure 4B). These data showed the protection in OIT group to PN, WN and CSH had markedly declined whereas majority mice in BF2+OIT group were still well protected. Overall, challenges with 50 mg of all three foods induced reactions in 100% of Sham-treated mice (22/22), 91% (20/22) of OIT-treated mice, but only 23% (7/30) of BF2+OIT-treated mice (Figure 4B). Symptom scores from PN, WN and CSH challenges were combined to yield a cumulative symptom score for each mouse. Cumulative symptom score following all three foods was 9.3 ± 0.78 in the Sham group, 9.1 ± 1.19 in the OIT only group and 2.6 ± 1.00 in the BF2+OIT group (Fig 4C). Rate of reactions and cumulative symptom scores were not different between Sham and OIT only groups; however they were significantly lower in BF2 than in Sham and OIT alone groups (Fig 4B and C, $p<0.001$ and $p<0.01$ respectively). Core body temperatures were consistent with symptom scores (data not shown). Plasma histamine levels were significantly lower in BF2 +OIT mice than in OIT-treated only and sham mice following the final CSH challenge at week 6 (Figure 4D, $p<0.01-0.05$). These findings demonstrate that BF2+OIT produced significantly greater sustained protection than OIT alone. We also found significant positive correlations between accumulative symptom scores and histamine levels at 5–6 weeks post-therapy as Fig 4E ($r=0.874$, $p<0.0001$).

BF2+OIT but not OIT-treated mice exhibited reduced antigen specific IgE levels

Prior to treatment, all three PN/TN poly sensitized mice showed elevated IgE production against PN, NW and CSH (Figure 5A) compared to non-sensitized mice (naïve mice). One-week post-treatment mean plasma levels of PN- WN- and CSH-specific IgE of OIT mice were essentially the same as that of Sham-treated P mice. However, PN-specific IgE levels in two OIT mice spiked (one of which experienced a fatal reaction) (Figure 5B). In contrast, BF2+OIT-treated mouse plasma PN, WN and CSH IgE levels were significantly below those of Sham-treated mice ($P<0.01$ for all, Figure 5A–C), and the reductions of WN and CSH IgE reached statistical significance when compared to OIT only mouse levels ($P<0.05$ – 0.01). BF2+OIT reduction of PN and CHS IgE remained statistically significant and WN trended towards significance when compared to Sham and OIT only treated mice at 5–6 weeks post therapy (Figure 5B5C, PN, $p<0.05$; CSH, $p<0.001$ and WN $p=0.08$). In contrast, PN, CHS- and WN-specific IgE levels in the OIT only group were not significantly different from Sham-treated mice. Figure 5D shows the means of cumulative PN, CHS- and WN-specific IgE levels in each group at pre-, one week post- and 5–6 weeks post- therapy. These results demonstrated that BF2+OIT was superior to OIT alone treatment in suppressing antigen specific IgE responses.

BF2+OIT therapy beneficial effects were associated with increased IFN- γ /IL-4 and IL10/IL-4 ratios

Outgrowth of peanut allergy in patients has been found to be associated with an increased ratio of IFN- γ to IL-4;[13] and failure of egg OIT to induce tolerance was associated with reduced the IFN- γ /IL-4 and IL-10/IL-4 ratios.[30] To investigate BF2+OIT treatment induction of more sustained tolerance, we determined IFN- γ /IL-4 and IL-10/IL-4 ratios in individual PN, CSH and WN antigen re-stimulated splenocyte (SP) cultures. IFN- γ /IL-4 ratios and IL-10/IL4 ratios in splenocyte cultures of OIT-treated only mice were similar to Sham group SP cultures stimulated with all three food proteins individually (Table I). In contrast, SP cultures from BF2+OIT mice stimulated with all three food proteins exhibited a significantly elevated IFN γ /IL-4 ratio when compared to Sham ($P<0.05$ – 0.01) and OIT only cultures ($P<0.05$ – 0.01). IL-10/IL-4 ratios were also higher in SP cultures from BF2+OIT treated mice than in cultures from Sham group mice and the increases reached statistical significance following PN and WN stimulation ($p<0.05$ and 0.01) and approached significance following CSH stimulation ($p<0.06$) when compared to sham treated cultures, but only a trend of increases when compared to OIT only SP cultures. Similar alterations of cytokine ratios in BF2+OIT mice were found in MLN cultures but to a lesser extent than in SP cultures (Table 1). These results demonstrated that BF2+OIT therapy is associated with greater induction of a tolerogenic cytokine profile.

BF2+OIT induced pro-tolerance modulation of DNA methylation levels at IL-4, INF- γ and Foxp3 gene promoters

We wished to determine whether BF2+OIT-induced tolerogenic cytokine profiles, which persisted for or were present at 5–6 weeks post-therapy, were associated with epigenetic alterations. Based on our and other previous publications, [43–45] we analyzed IL-4, IFN- γ , Fox p3 gene promoter methylation levels at selected CpG sites in genomic DNA of intestinal

tissues. We found that the percent methylation of CpG⁻³⁹³ in the IL-4 promoter from mice receiving BF2+OIT was significantly greater when compared to CpG⁻³⁹³ from Sham treated- and OIT-treated only mice (Figure 6A), indicating IL-4 gene suppression. Conversely, CpG⁻⁴⁵ in the IFN- γ gene promoter and CpG⁻⁷¹ in the Foxp3 gene promoter from BF2+OIT group mice was less methylated when compared to DNA from the Sham- and OIT-treated only groups (Figure 6B and C) indicating IFN- γ and Foxp3 gene activation. This finding demonstrated that BF2+OIT induced beneficial modulation of DNA methylation at the IL-4, INF- γ and Foxp3 gene promoters.

DNA methylation levels at IL-4, IFN- γ and Foxp3 promoters were correlated with anaphylaxis symptom scores

We next investigated potential correlations between methylation levels of IL-4 CpG⁻³⁹³, INF- γ promoter CpG⁻⁴⁵ and Foxp3 promoter CpG⁻⁷¹ and the cumulative symptom scores provoked by individual PN, CSH and WN challenges following 5–6 weeks off therapy. IL-4 CpG⁻³⁹³ methylation levels and severity of symptom scores were inversely and significantly correlated ($r=-0.59$, $p<0.0002$, Figure 7A). Methylation levels of INF- γ promoter CpG⁻⁴⁵ and symptom scores as well as Foxp3 promoter CpG⁻⁷¹ methylation levels and cumulative symptom scores ($r=0.52$; $p<0.0011$ and $r=0.4$; $p<0.0165$, Figures 7B and C) were positively correlated. Correlation between DNA methylation levels and symptom scores was stronger for the IL-4 promoter CpGs than those in the INF- γ and Foxp3 promoters.

Discussion

Concurrent PN and TN allergies are the most severe food allergies. Multiple OIT may prove to be an effective approach for this population of patients, [25, 46] but OIT safety and persistence of protection need to be improved. In this study, we for the first time generated a murine model that displayed concurrent PN and TN allergies as evidenced by elevation of IgEs against PN, CSH and WN following concurrent sensitization, and anaphylactic reactions following individual PN and CSH and WN challenges. We previously showed that BF2 provided protection in a peanut allergy model.[30] As seen in the peanut allergy model, our preliminary data showed BF2 also provided significant protection and reduction of specific IgE levels in concurrent PN and TN allergic mice (Srivastava et al. unpublished data). The present study shows that combining BF2 with PN and TN OIT therapy significantly improved safety, efficacy and persistence of protection in a novel murine model of concurrent PN and TN allergies. The OIT alone desensitization effect in the present study is consistent with the findings of human trials, as well as murine models as reported by Leonard et al,[29] in which desensitization was achieved, but reactivity returned as soon as 2 weeks post- therapy. In our study, approximately 63% of OIT treated mice were desensitized at one week post-therapy. Although median symptom scores were significantly lower than in Sham-treated PTNA mice, some mice developed severe, including one fatal, reactions. Our study differs from a previous OIT study by Maeta [30] in which ovomucoid OIT did not induce protection in a ‘mild food allergy’ model. This might be because a 10 day OIT protocol was used. We, as did Leonard et al, [47] used a 21 day protocol and also found significant protection in the OIT alone group. Importantly, we found that BF2+OIT

produced greater and more persistent protection than OIT alone. This combined therapy may provide an option for developing an improved OIT regimen for multiple food allergies, in particular concomitant peanut and tree nut allergies.

Acute anaphylactic symptoms upon exposure to Ag in sensitized individuals are due to release of histamine and other mast cell/basophil mediators. It was found previously that OIT also induces transient effector cell (mast cell/basophil) desensitization. [21] In the present study, combining BF-2 with OIT produced greater reduction of plasma histamine levels than OIT and symptoms scores were significantly and positively correlated with plasma histamine levels following challenges immediately and 5–6 weeks post-therapy. The mechanisms underlying the superior protection against anaphylaxis upon PN, CSH and WN exposure by BF2+OIT compared to OIT alone in this model may have been achieved by preventing mast cell/basophil histamine release. Our previous study by Song et al. [37] showed that the BF2 active compounds berberine, palmatine and jatrorrhizine directly inhibit mast cell activation by suppressing phosphorylation of Syk, the key signaling event in mast cell/basophil degranulation. In this study we showed that basophil number reduction was observed in both treated groups, being greater in the BF2+OIT group immediately post OIT challenge, but the differences between the groups was not statistically significant. Basophil number alone does not determine desensitization status.

Many previous studies found that OIT causes transient elevation of IgE in patients [48, 49] and murine models.[30] Our study also found that OIT alone induced higher levels of IgE immediately after completing therapy. In contrast, combined BF2+OIT prevented OIT increase of antigen specific IgE. Importantly BF2+OIT suppression persisted. The mechanisms underlying the superior effect of BF2+OIT on IgE response are not fully understood, but might be attributed to the effect of active compounds in BF2. We have reported that berberine decreased IgE production by the human B cell line U266, and IgE production by IL-4 and CD40-activated human PBMCs from children with food allergies. These effects on PBMCs were associated elevation of STAT3, TBX21 and T bet mRNA levels, as well as suppression of ϵ germline transcript. Therefore BF2 prevention of OIT elevation of IgE may be attributed to the anti-IgE effect of active compounds in BF2.

Th2 cytokine IL-4 is required for B cell switching to IgE production, and mast cell activation, whereas IFN- γ and IL-10 negatively regulate IgE production and mast cell activation. IFN- γ /IL-4, and IL10/IL-4 ratios have been associated with food allergy status, and a higher ratio was associated with food tolerance restoration in patients. BF2+OIT therapy resulted in pro-tolerogenic IFN- γ /IL-4 and IL10 /IL4 ratios at 6 weeks post-therapy, which were associated with more persistent protection.[35] Sustained favorable cytokine profiles i.e. higher ratio of IFN- γ /IL-4 and IL10 /IL4 have not been previously established in human or animal studies using OIT alone or other combined therapies.[21][24, 25, 27] In OIT patients, reduced Th2 status was lost 4–6 weeks post-OIT, and even during OIT in some individuals. In a murine study, failure of egg OIT to produce protection against anaphylaxis was associated with reduced IFN- γ /IL-4 and IL-10/IL-4 ratios. [30] The pro-tolerogenic effect of BF2+OIT on IFN- γ , IL-10 and IL-4 production may, at least in part, explain the sustained reduction of IgE, mast activation and the consequent clinical protection in the present study.

Alteration to DNA methylation status of CpG residues in cytokine gene promoters has been shown to be key molecular mechanism of IL-4, IFN- γ and FoxP3 gene regulation.[50, 51] Our previous study showed that demethylation of the IL-4 promoter was associated with increased IL-4 and IgE production and the risk of offspring of peanut allergic mothers to develop peanut allergy.[43] Demethylation of the FoxP3 gene promoter was found in a small number of individuals, who showed persistent protection following PN OIT in a clinical study.[22] The current study demonstrates for the first time significant and simultaneous modulation of IL-4, IFN- γ and Fox-p3 promoters, i.e. re-methylation of IL-4 promoter and demethylation of IFN- γ and Fox-3 promoters. We further found that cumulative symptom scores following 5–6 weeks post-therapy PN, CSH and WN individual challenges were inversely correlated with DNA methylation levels at the IL-4 promoter and positively correlated with DNA methylation levels at the IFN- γ and Foxp3 promoters, suggesting that these alterations in DNA methylation may also be a biomarker of clinical tolerance following combined OIT. While we observed decreased methylation of the FoxP3 promoter, we did not find an association between DNA methylation at the Foxp3 gene promoter and number of SPC CD4+CD25+FoxP3+Tregs (Data not shown). Thus the functional contribution of Tregs to BF2+ OIT effects requires further study. Smaldini et al reported induction of CD4+CD25+FoxP3 Tregs localized to gut lamina propria after prolonged antigen desensitization. [52] Additionally, in a recent study by Tordesillas et al, LAP+ CD25-FoxP3- Tregs generated via epicutaneous desensitization localized to the gut but not spleen and inhibited mast cell activity and anaphylaxis in a murine food allergy model.[53] Treg activity in the Smaldini study was associated with tissue elevation of IL-10 and TGF- β and the Tordesillas study implicated a role for TGF- β . While we did not observe differences in TGF- β levels in splenocyte or MLN cultures (Data not shown), consistent with our previous finding that BF2 had no effect on TGF- β production[35], IL-10:IL-4 ratios were significantly higher in both types of cultures. Additional studies specifically targeting intestinal tissue-resident Tregs, including non-classical Treg types and related cytokines are needed to determine functional role of such cells to BF2+OIT effects. That the inverse correlation between DNA methylation at the IL-4 promoter was stronger than for IFN- γ and Foxp3 promoters suggests that BF2+OIT may directly inhibit IL4 production. A recent novel finding showed that Tregs can be reprogrammed to produce Th2 cytokines in the presence of antigen in sensitized individuals, which impairs oral tolerance and promotes food allergy.[54] It is possible that BF2 remethylation of the IL-4 gene promoter prevents OIT antigen reprogramming of Treg toward a Th2-cell-like lineage regardless of the number of Tregs. Future studies will test these possibilities.

In conclusion, our data show that BF2+OIT has a superior safety profile and produces more sustained protection than OIT alone in PN and TN allergic mice. These effects were associated with suppression of IgE and histamine release, and favorable immunomodulation of IL-4, IL-10 and IFN- γ ratios. Also, BF2+OIT therapy but not OIT alone resulted in beneficial epigenetic modulation of IL-4, IFN- and FoxP3 gene promoters, which were correlated with sustained protection. This combined approach may be an additional option for developing safe and effective immunotherapy for concurrent PN/TN allergies, particularly for the most sensitive individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experiment Protocol

Pre-OIT phase: mice in groups (G) 1–3 were orally sensitized and boosted weekly with a peanut (PN), cashew (CSH) and walnut (WN) mixture and cholera toxin for 5 weeks as indicated. Individual PN, CSH and WN oral challenges were administered at week (W) 6, 7, and 8. They were designated PTNA mice. Naïve mice were not sensitized or challenged. **Treatment phase:** B-FAHF-2 therapy began on w8 (2 days after walnut challenge) and continued throughout OIT. PN/TN OIT was started following 3 weeks of B-FAHF-2 therapy. Day 1 of OIT was the build-up phase. The highest dose safe for all mice (6.25mg) was chosen as maintenance dose and administered daily for the remainder of 3 weeks. G1, 2 and 3 PTNA mice received water (Sham); G2 OIT alone, or BF2+OIT respectively. **Post-OIT phase:** mice were challenged with a PN/TN cocktail at 1 and 3 weeks post therapy to evaluate the effect on desensitization, and individual PN, WN and CSH dose escalating challenges were administered 5–6 weeks post –OIT) and given at 3 day intervals to evaluate induction of tolerance. Mice were sacrificed 48 hours after the last challenge. Data are from 2 sets of experiments. PTNA, peanut and tree nut allergic mice. * indicating that PTNA mice received one day rush OIT prior to the 6.5mg mixture of each peanut/TN maintenance treatment.

(Day one rush OIT build-up phase)

Figure 2. Reactions during the OIT build-up phase

Dose escalating OIT with a PN, CHS and WN cocktail was administered at 30 minutes intervals. Reactions were observed following a 12.5mg dose. Symptom scores were determined using criteria described in methods (A). Core body temperatures were measured using a rectal probe (B). Plasma histamine levels were measured using a commercial ELISA kit (C). Data shown are from two sets of experiments (N=10–16). Bars are group medians in A and group means in B and C. *P<0.05; **P<0.01. For statistical analysis, One Way ANOVA with Bonferroni post-test was applied to determine significant differences across treatment groups. P values less than P 0.05 were considered significant.

(One week post OIT challenge)

Figure 3. Reactions following one week post-OIT challenge with a peanut, cashew and walnut mixture

Symptom scores were determined using the scoring system described in methods (A). Core body temperatures were measured using a rectal probe. Plasma histamine levels were measured using a commercial ELISA kit. Bars in (A) are group medians. Bars in (B) and (C) are group means. Data shown are from two sets of experiments (N=10–16). *P<0.05; ***P<0.001. For statistical analysis, For (A) Kruskal-Wallis test was used. For (B) and (C) One-Way ANOVA followed by Bonferroni's post-test was used. Pearson correlation was used for regression analysis (D&E). P values less than P 0.05 were considered significant.

(5-6 weeks post-OIT challenges)

Figure 4. Reactions to individual peanut, cashew and walnut challenges during 5–6 weeks post-OIT challenges

A. Symptom scores of reactions in response to dose escalating individual 12.5g (not shown), 25 and then 50 mg PN, WN and CSH protein challenges. Reactions were scored using criteria described in methods. Bars are group medians. B. Rate of reactions following each 50 mg of PN, WN and CSH challenges. Data are expressed as percent of reactions. C. Anaphylactic symptom scores at each of the 5–6 week post-therapy challenges with PN, WN and CSH were added to compute cumulative symptom score for each mouse. Data shown as group means $\pm$ SEM. D plasma histamine levels, bars represent group means. E Correlation between plasma histamine levels and cumulative symptom scores. For statistical analysis, Kruskal-Wallis test was used for (A), Fisher's exact test was used for (B), One-way ANOVA followed by Bonferroni post test was used for C and D and Spearman correlation was used for (E). * $P<0.05$; ** $P<0.01$; *** $P<0.001$. N=10–11 mice/group from two sets of experiments.

Figure 5. Peanut, cashew and walnut-specific IgE levels in serum

Specific IgE for PN, WN and CSH at 1 week post-therapy (panel A) and 5–6 weeks post-therapy (panel B) was measured by ELISA as described in methods. Data shown as Mean $\pm$ SEM. One Way ANOVA with Bonferroni post-test was applied to determine significant differences across treatment groups * $P < 0.05$; ** $P < 0.01$. N=10–11 mice/group.

Figure 6. DNA methylation profiles of IL-4, IFN- γ and Foxp3 promoter CpGs

Percent methylation of selected CpG residues in the IL-4, IFN- γ and Foxp3 promoter regions were evaluated by pyrosequencing of bisulfate converted intestinal genomic DNA harvested following the final challenge. Bars represent group means. One Way ANOVA with Bonferroni post-test was applied to determine significant differences across treatment groups * $P < 0.05$; *** $P < 0.001$. N=10–11 mice/group.

Figure 7. Correlation between DNA methylation and symptom severity

Correlation between DNA methylation at IL-4, IFN- γ and Foxp3 gene promoters (shown in Figure 6) and cumulative symptom scores in (shown in Figure 4C) following three 50mg PN, WN and CHS challenges were analyzed using Spearman correlation. * $P < 0.05$ and ** $P < 0.01$ (N = 10–11 mice/group).

Table 1

Splenocyte and mesenteric lymphnode culture cytokine ratios

Cell suspensions from spleen and MLN were prepared following the final CSH challenge 6 weeks post OIT. IL-4, IFN- γ and IL-10 levels in splenocyte cultures re- stimulated separately with PN, WN and CSH proteins were measured by commercial ELISA. IFN- γ /IL-4 and IL-10/IL-4 ratios were calculated. Data are shown as ratio means $\pm$ SEM. Students T-test was used to determine significance.

Allergen	Experimental Group	Splenocytes		Mesenteric lymph node cells	
		IFN- γ /IL-4 ratio	IL10/IL-4 ratio	IFN- γ /IL-4 ratio	IL10/IL-4 ratio
Peanut	Sham	16.47 $\pm$ 2.04	6.90 $\pm$ 1.72	2.24 $\pm$ 0.69	5.52 $\pm$ 10.55
	OIT	13.72 $\pm$ 2.70	12.01 $\pm$ 3.86	0.84 $\pm$ 0.36	5.03 $\pm$ 4.28
	BF2+OIT	456.91$\pm$163.14^{*,#}	144.61$\pm$55.93[*]	32.34$\pm$10.55^{*,#}	5.38 $\pm$ 2.01
	Naive	ND	ND	ND	ND
CSH	Sham	25.41 $\pm$ 3.79	23.71 $\pm$ 6.36	1.76 $\pm$ 1.01	3.41 $\pm$ 1.43
	OIT	16.52 $\pm$ 5.36	12.9 $\pm$ 3.74	5.04 $\pm$ 4.40	1.46 $\pm$ 1.23
	BF2+OIT	296.5$\pm$157.10^{*,##}	154.8 $\pm$ 77.92	25.91$\pm$3.0^{*,##}	6.45 $\pm$ 2.10
	Naive	ND	ND	ND	ND
WN	Sham	6.519 $\pm$ 0.91	5.98 $\pm$ 0.65	4.29 $\pm$ 1.07	4.46 $\pm$ 0.52
	OIT	12.13 $\pm$ 1.80	11.38 $\pm$ 3.26	12.33 $\pm$ 11.52	5.00 $\pm$ 4.60
	BF2+OIT	54.90$\pm$13.80^{*,#}	77.70$\pm$37.96^{*,#}	9.56 $\pm$ 2.04	13.31$\pm$1.80^{*,#}
	Naive	ND	ND	ND	ND

* P<0.05;

** P<0.01.

N=10–11 mice/group.