

Food Allergy Herbal Formula-2 silences peanut-induced anaphylaxis for a prolonged posttreatment period via IFN- γ -producing CD8⁺ T cells

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Background: Food allergy is a serious and sometimes fatal condition for which there is no cure. We previously reported that Food Allergy Herbal Formula (FAHF)-2 protected peanut-allergic mice against anaphylactic reactions as long as 4 weeks posttherapy. This formula is now in clinical trials in the United States.

Objective: We sought to determine whether FAHF-2-mediated protection could be extended long-term and explored the mechanisms underlying its persistent immunomodulatory effects.

Methods: Peanut-allergic mice received FAHF-2 daily orally by gavage for 7 weeks, and then received 7 oral peanut challenges at intervals of 4 to 10 weeks over a period of 36 weeks. For mechanistic studies, some mice received CD4⁺ or CD8⁺ T-cell-depleting antibodies or IFN- γ -neutralizing antibodies. Anaphylactic symptoms, body temperatures, and plasma histamine levels were recorded after each challenge, and peanut-specific immunoglobulin levels and cytokine profiles of splenocytes, mesenteric lymph node cells, and purified CD4⁺ and CD8⁺ T cells were determined.

Results: Food Allergy Herbal Formula-2 treatment protected mice from anaphylaxis for more than 36 weeks after discontinuing treatment. Peanut-specific IgE levels were reduced as much as 50%, whereas IgG_{2a} levels were increased as much as 60%, and these effects persisted over time. T_H2 cytokine production by CD4⁺ T cells from FAHF-2-treated mice was reduced as much as 75%, whereas CD8⁺ T-cell IFN- γ production was markedly increased by as much as 85% at the

final challenge. Neutralization of IFN- γ and depletion of CD8⁺ T cells markedly attenuated FAHF-2 efficacy.

Conclusions: Food Allergy Herbal Formula-2 provides long-term protection from anaphylaxis by inducing a beneficial shift in allergen-specific immune responses mediated largely by elevated CD8⁺ T-cell IFN- γ production. (J Allergy Clin Immunol 2009;123:443-51.)

Key words: Peanut allergy, murine model, anaphylaxis, IgE, T_H2 cytokines, CD8, T cells, traditional Chinese herbal medicine

Allergic diseases are a significant health problem in Westernized countries and represent a tremendous burden with respect to both quality of life and health care expenditures.¹⁻³ Food allergy reactions account for 30% to 50% of anaphylaxis cases in emergency departments in North America, Europe, Asia, and Australia (see review⁴). Peanut allergy (PNA) specifically accounts for approximately 80% of fatal and near-fatal anaphylactic reactions in the United States.^{5,6} The prevalence of childhood PNA doubled between 1997 and 2002⁷ and affects ~1% of the American population.⁸ Although tremendous strides have been made in food allergy awareness, there is no satisfactory therapy to prevent or reverse this disease. Other than immediate access to postanaphylactic rescue medications, strict avoidance is the only way to manage this condition. Unfortunately, accidental ingestion is common. Conventional allergen-specific immunotherapy is not satisfactory because of the unacceptable number of adverse reactions and poor maintenance of tolerance afterward.^{9,10} Anti-IgE therapy for food allergy has shown only modest and transient benefits.¹¹ There is a need for safe, effective, and long-lasting therapy.

We previously reported that food allergy herbal formula (FAHF)-1 protected mice against peanut anaphylaxis.¹² To increase the ease of quality control and safety profile, we generated FAHF-2 by eliminating 2 herbs from FAHF-1 and demonstrated that FAHF-2 completely prevented peanut induced anaphylaxis.¹³ We also tested the pharmacologic actions of individual herbs in FAHF-2 and found that no individual herbs or further simplified formulas were as effective as FAHF-2,¹⁴ suggesting a potential synergistic or additive effect of individual herbs in FAHF-2. FAHF-2 may be the optimal candidate for developing an anti-food allergy botanical drug. A subsequent study demonstrated that FAHF-2 treatment re-established tolerance to peanut after peanut hypersensitivity was fully established in mice.¹⁵ This effect was shown to persist for 4 weeks posttherapy, and was associated with upregulation of CD8⁺ T-cell IFN- γ production. There was no effect on TGF- β and a slight but significant reduction of IL-10 production. These findings suggested that FAHF-2 might have a long-lasting effect

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Abbreviations used

CPE: Crude peanut extract
FAHF: Food Allergy Herbal Formula
MLN: Mesenteric lymph node
PNA: Peanut allergy

and that enhancement of IFN- γ production by CD8⁺ T cells may be responsible for the therapeutic effect. However, how long the treatment effects might persist and whether CD8⁺ T-cell IFN- γ production is required in FAHF-2-mediated long-term protection have not been previously investigated.

In this study, we used a chronic peanut hypersensitivity model and challenged at intervals of 4 to 10 weeks for a total of 7 challenges over a period of 36 weeks after discontinuation of FAHF-2 treatment. Treated mice were protected against anaphylaxis for as long as 36 weeks compared with sham-treated mice. We then determined cytokine profiles of splenocytes and mesenteric lymph node (MLN) cells and found that, even 36 weeks posttherapy, the cytokine profiles were similar to those immediately after treatment, which is increased IFN- γ and reduced T_H2 cytokine production. CD8⁺ but not CD4⁺ T cells from FAHF-2-treated mice showed enhanced IFN- γ production. By using *in vivo* IFN- γ neutralization and CD8⁺ T-cell depletion methods, we demonstrated that increased IFN- γ production by CD8⁺ T cells is a crucial component of FAHF-2-mediated protection.

METHODS

Mice

Five-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, Me) were maintained on peanut-free chow under specific pathogen-free conditions according to standard guidelines for the care and use of animals. There were 13 mice in the sham group, 8 mice in the FAHF-2 treatment group, and 20 naive controls.

Peanut sensitization, boosting, and challenge

Whole roasted peanuts (White Rose, Carteret, NJ) were shelled, and skins were left on. Peanuts were homogenized in sterile PBS (Mediatech, Va) until a smooth suspension was obtained. As shown in Fig 1, mice were sensitized intragastrically with peanut (10 mg) and cholera toxin (20 μ g; List Laboratories, Campbell, Calif) in a total volume of 500 μ L PBS on 3 consecutive days of week 0, and once a week from weeks 1 to 5. Mice were boosted at weeks 6 and 8 with peanut (50 mg) and cholera toxin (20 μ g). All animals were challenged intragastrically with 200 mg peanut at week 14 and subsequently at the times indicated in Fig 1. Age-matched naive mice were also challenged at weeks 14 and 18. As expected, naive mice did not respond to peanut challenge. Therefore, the remaining naive mice were not challenged until week 50. Plasma and serum samples were collected at the time of each challenge, and body temperatures were recorded to obtain baseline data.

FAHF-2 formula and treatment

Dried aqueous extract of FAHF-2, produced in a good manufacturing practice-certified facility, was obtained from Beijing Shen Hua Shi Di Medical Technology, Beijing, China, and stored at room temperature.^{13,14} The raw herb quality was ascertained according to the standards required by the *Pharmacopoeia of the People's Republic of China*.¹⁶ On the basis of organoleptic and microscopic examination, the raw herbs used in FAHF-2 were identified as *Prunus mume*, *Zanthoxylum schinifolium*, *Angelica sinensis*, *Zingiber officinalis*, *Cinnamomum cassia*, *Phellodendron chinense*, *Coptis chinensis*, *Panax ginseng*, and *Ganoderma lucidum*. The quality and ratio of herbs used has been described previously.¹⁴ Product quality was monitored

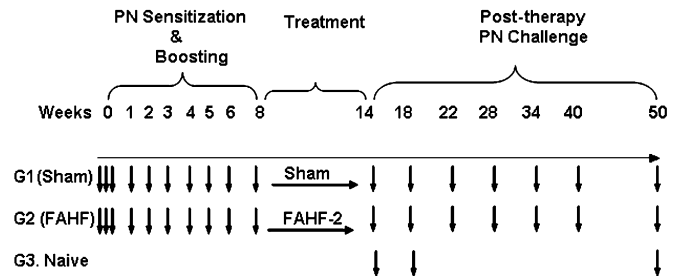


FIG 1. Experimental design. C3H/HeJ mice were subjected to weekly intragastric peanut (PN) sensitization from week 0 through week 5 and boosted thereafter at weeks 6 and 8. Peanut-allergic mice were treated with FAHF-2 (32 mg, twice a day), or water beginning at week 8 (at which time peanut hypersensitivity is fully established¹⁵) and continued daily for 7 weeks. Mice were challenged at week 14 (first challenge) and 6 subsequent peanut challenges at the intervals indicated. Sham: peanut-sensitized and boosted mice receiving water treatment (n = 13 at first through sixth challenges and n = 12 at the seventh challenge); FAHF-2: peanut-sensitized and boosted mice receiving FAHF-2 treatment (n = 8); naive: neither sensitized nor treated. Twenty naive mice served as controls. Because naive mice do not respond to peanut challenge,¹⁵ only 5 naive mice at week 14 and 5 at week 18 received peanut challenge. The remaining 10 mice were not challenged until week 50 to maintain naive status; however, plasma and serum samples were collected at the time of each challenge (weeks 22-40) and body temperatures were recorded to generate normal control data.

by HPLC fingerprinting according to the US Food and Drug Administration's Guidance for Industry Botanical Drug Products.¹⁷ The dose of FAHF-2 (64 mg/day) used in this study was calculated by normalizing the daily human dose to mouse body weight.¹⁵ Treatment with FAHF-2 commenced at week 8, by which time peanut-induced hypersensitivity was fully established.¹⁵ The formula, dissolved in 0.5 mL drinking water, was given by oral gavage twice daily for 7 weeks. Sham treated mice received 0.5 mL drinking water.

Endotoxin testing of FAHF-2 product

Endotoxin levels in FAHF-2 and peanut preparations were measured using the Pyrogen Plus assay kit (Lonza, Hopkington, Mass). Briefly, 250 μ L test solutions of FAHF-2 or peanut were added to tubes containing reconstituted *Limulus* amoebocyte lysate and incubated for 30 minutes in a 37°C water bath. Endotoxin at concentrations from 0.5 to 0.01 EU/mL was used as control. No FAHF-2 or peanut preparation clot formation was found, indicating endotoxin levels below 0.03 EU/mL or 0.02 ng/mL, which is the limit of sensitivity for this kit. Clot formation was observed in endotoxin control tubes at and above 0.0325 EU/mL.

Assessment of hypersensitivity reactions, measurement of plasma histamine levels, and histology

Anaphylactic responses were evaluated 25 to 30 minutes after intragastric challenge. Visually observed symptoms were scored by using a score key described previously¹⁵ and as follows: 0, no symptoms; 1, scratching and rubbing around the snout and head (mild reaction); 2, puffiness around the eyes and snout, diarrhea, pilar erection, reduced activity, and/or decreased activity with increased respiratory rate (moderate reactions); 3, wheezing, labored respiration, cyanosis around the mouth and the tail (severe reactions); 4, no activity after prodding, or tremor and convulsion (near fatal); and 5, death. Rectal temperatures were measured immediately after scoring using a rectal probe (Harvard Apparatus, Holliston, Mass).

Blood was collected into chilled EDTA-coated tubes (Fisher Scientific, Pittsburgh, Pa) by retro-orbital bleeding immediately after symptom scores and temperatures were recorded. Plasma was harvested and stored at -80°C until used. Histamine was measured by using an enzyme immunoassay kit (ImmunoTECH Inc, Marseille, France) as described by the manufacturer.

Ear tissue was collected in buffered formalin after completion of challenge. Tissue sections were stained with toluidine blue and counterstained with

TABLE I. FAHF-2 treatment persistently prevents anaphylaxis in response to peanut challenge

Challenge (time point)	Sham		FAHF-2		Naive	
	Individual score (N = 12-13)	Median score	Individual score (N = 8)	Median score	Individual score (N = 5-10)	Median score
1st (W14)	2,2,3,3 3,3,3,3 3,3,4,4,4	3	0,0,0,0,0 0,0,0	0*	0,0,0,0,0	0
2nd (W18)	3,3,3,3 3,3,4,4 4,4,4,4,4	4	0,0,0,0,0 0,0,0	0*	0,0,0,0,0	0
3rd (W22)	3,3,3,3,3 3,4,4 4,4,4,4,4	4	0,0,0,0,0 0,0,0	0*	NC	ND
4th (W28)	2,3,3,3,3 3,4,4,4 4,4,4,4	4	0,0,0,0,0 0,0,0	0*	NC	ND
5th (W34)	3,3,3,4,4 4,4,4,4 4,4,4,4	4	0,0,0,0,0 0,0,0	0*	NC	ND
6th (W40)	3,4,4,4,4 4,4,4 4,4,4,4,5	4	0,0,0,0,0 0,0,0	0*	NC	ND
7th (W50)	3,3,3,3,4 4,4,4,4 4,4,4	4	0,0,0,0,0 2,2,2	0*	0,0,0,0,0 0,0,0,0,0	0

NC, Nonchallenged; ND, no data of score.

Anaphylactic signs were assessed 25 to 30 minutes after each challenge by using the scoring system described in Methods. Anaphylactic scores in the sham and FAHF-2-treated groups are presented as individual scores and the median. The numbers of mice in each group are the same as indicated in legend for Fig 1.

* $P < .05$ vs sham.

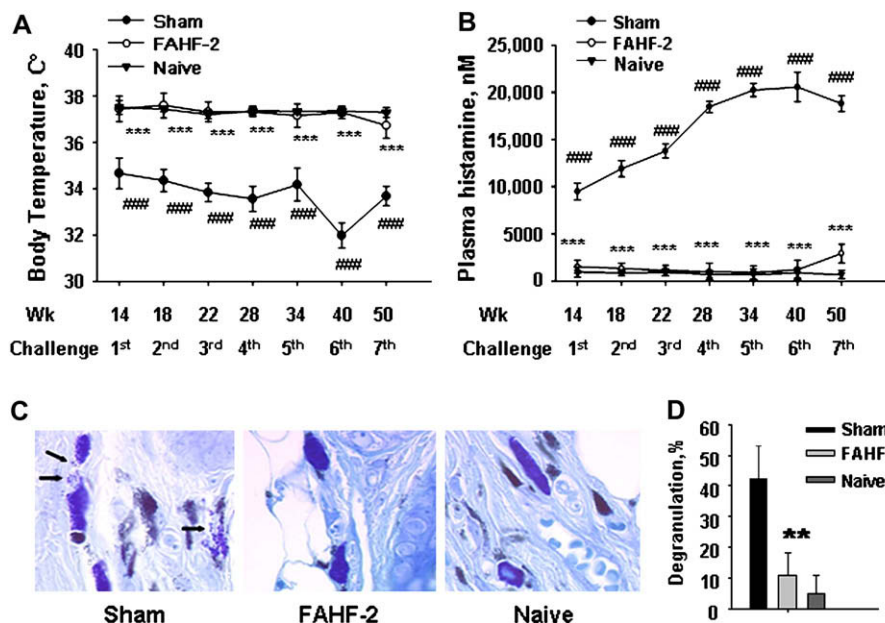


FIG 2. FAHF-2 treatment prevented a decrease in body temperature, histamine release, and mast cell degranulation. **A**, Postchallenge body temperatures. Body temperatures were measured immediately after evaluation of symptom scores. Data are means $\pm$ SEMs. The numbers of mice are as follows: sham (12-13), FAHF-2 (8), naive (5-10). *** $P < .001$ vs sham. **B**, Plasma histamine levels. Blood was collected immediately after scores and temperatures were recorded after each posttherapy challenge, and plasma was obtained. Histamine levels were measured by using an enzyme immunoassay. Data points indicate group means $\pm$ SEMs, $n =$ same as indicated in **A**. The scale of the x -axis is not linear. **C**, Illustration of mast cell degranulation. Ear tissue samples were taken 40 minutes after seventh challenge and stained with toluidine blue. Mast cells were considered degranulated if at least 5 granules appeared outside the cell body. Arrows indicate deregulated mast cells. **D**, Percent of degranulated mast cells. Mast cells were counted using light microscopy at $\times 100$. A total of 500 cells were counted from 3 sections of each ear sample for calculation of the percentage of degranulated cells. Data points indicate group means $\pm$ SEMs, $n = 4$ -5 mice/group from 1 set of experiments. ** $P < .01$; *** $P < .001$ vs sham; ### $P < .001$ vs naive.

hematoxylin. Degranulated mast cells were identified by the presence of at least 5 toluidine blue–positive granules outside the cell body. A total of 500 cells were counted from 3 sections of each ear sample for calculation of the percentage of degranulated cells as described previously.^{18,19}

Measurement of serum peanut specific IgE and IgG_{2a} levels

Blood was collected by retro-orbital bleeding using heparinized tubes (Fisher Scientific) 1 day before the week 3 sensitization and then periodically 1 day before boosting, treatment, or challenges. Harvested sera were stored at –80°C until used. Peanut-specific IgE and IgG_{2a} levels were determined by ELISA as previously described.¹⁵

Cell culture and cytokine measurements

Splenocytes and MLN cells from each group prepared as previously described¹⁵ were cultured in 24-well plates (4×10^6 /well/mL) in the presence or absence of crude peanut extract (CPE; 200 μ g/mL) or concanavalin A (2.5 μ g/mL). CD8⁺ or CD4⁺ T cells were isolated from spleens of mice from sham, FAHF-2, and naive groups by using magnetic bead separation (Stem Cell Technologies, Vancouver, British Columbia, Canada) by negative selection. Purity of isolations was routinely above 95%. Cells 5×10^5 were stimulated with 200 μ g/mL CPE in the presence of irradiated syngeneic splenocytes (2.5×10^6 cells) in 24-well plates. Supernatants were collected after 72 hours of culture. Cytokine levels were determined by ELISA in triplicate according to the manufacturer's instructions (IL-13, R&D Systems, Minneapolis, Minn; TGF- β , Promega, Madison, Wis; all others, BD Pharmingen, San Jose, Calif).

Neutralization of IFN- γ and depletion of CD4⁺ or CD8⁺ T cells

Mice received intraperitoneal injections of 200 μ g anti-IFN- γ (R&D Systems) or anti-CD4 (clone GK1.5) or anti-CD8 (clone H35-17.2; 100 μ g; Pharmingen) antibodies as described previously²⁰⁻²² once before initiating FAHF-2 treatment and then weekly throughout the duration of treatment. These antibodies resulted in virtually complete depletion of CD4⁺ and CD8⁺ T cells in the spleen (CD4⁺ T cells depleted by 99%; CD8⁺ T cells depleted by 95%) and MLN (CD4⁺ T cells and CD8⁺ T cells both depleted by more than 99%) 1 week after a single injection, as determined by flow cytometry. Appropriate isotype control antibodies (Rat IgG₂ for anti-CD8 and anti-CD4; hamster IgG for anti-IFN- γ) were also used.

Statistical analysis

Data were analyzed by using the SigmaStat statistical software package (SPSS Inc, Chicago, Ill). Data for histamine, temperature, immunoglobulins, and cytokines were evaluated for normality using the Kolmogorov-Smirnov test, and no need for log transformation was found because data were normally distributed. For these data, differences among groups were analyzed by 1-way ANOVA followed the Bonferroni multiple comparison procedure. For all time-course data, repeated-measures of ANOVA were applied. Because symptom score data were not normally distributed, differences between the groups were analyzed by Kruskal-Wallis 1-way ANOVA on ranks followed by the Dunn method for multiple comparisons to accommodate groups of unequal size. For repeat measurements of symptom scores, we used the Friedman repeated-measures ANOVA on ranks followed by the Dunn method for multiple comparisons. Tests were 2-tailed, and *P* values <.05 were considered significant.

RESULTS

FAHF-2 treatment provided prolonged protection against peanut-induced anaphylaxis

As shown in Table I, all sham-treated peanut-allergic mice (13 of 13) developed anaphylactic symptoms at first challenge

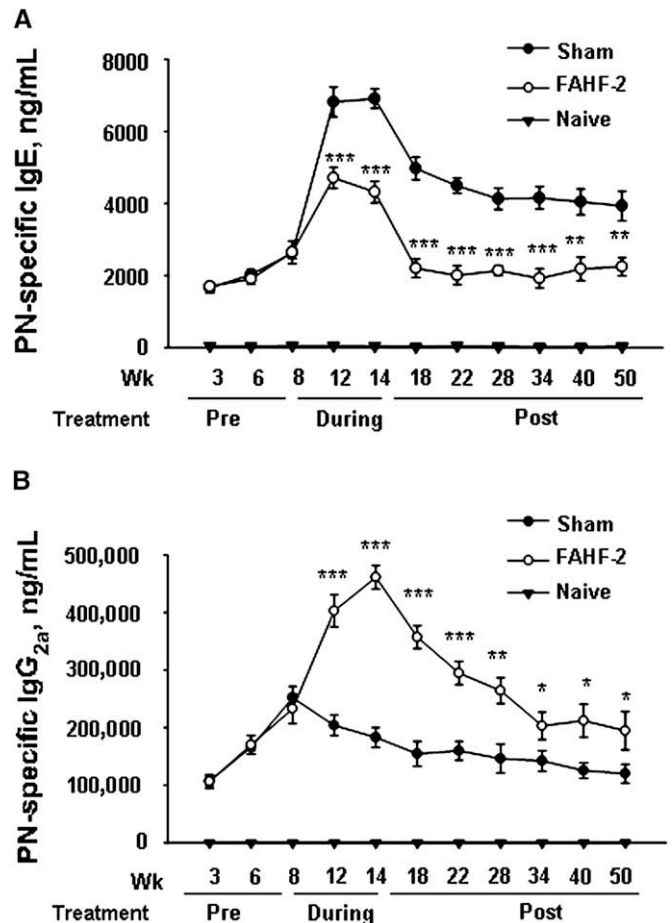


FIG 3. FAHF-2 treatment produced prolonged reduction in serum peanut (PN)-specific IgE and increased IgG_{2a} levels. Sera were harvested after blood collection by retro-orbital bleeding at indicated time points. Serum peanut-specific IgE levels (**A**) and specific IgG_{2a} levels (**B**) were determined by ELISA. Data points indicate group means $\pm$ SEMs, *n* as indicated in Fig 1. **P* < .05; ***P* < .01; ****P* < .001 vs sham. The scale of the x-axis is not linear.

(week 14) and each subsequent challenge until week 50 (seventh challenge). After the sixth challenge (week 40), 12 of 13 sham-treated mice developed near-fatal anaphylactic reactions (score 4), and 1 died. In contrast, none of the FAHF-2-treated mice (0 of 8) showed any anaphylactic signs at the sixth challenge (week 40). Even at the week 50 challenge, only 3 of 8 treated mice exhibited moderate reactions, whereas all sham-treated mice experienced severe anaphylaxis, 8 with near-fatal reactions. Naive mice showed no reactions to peanut challenge.

Decreased core body temperature correlates with the severity of systemic anaphylaxis. As shown in Fig 2, A, mean rectal temperatures of sham-treated mice decreased after each challenge. Consistent with the clinical observations, FAHF-2-treated mice maintained normal temperatures until the sixth challenge. Even after the seventh challenge, the mean body temperatures of FAHF-2-treated mice were not significantly different from naive mice (*P* < .001).

Plasma histamine levels of sham-treated mice were markedly increased 30 minutes after the first challenge compared with naive mice, and became substantially higher after each subsequent challenge. In contrast, FAHF-2-treated mice maintained histamine levels similar to naive mice through the sixth challenge

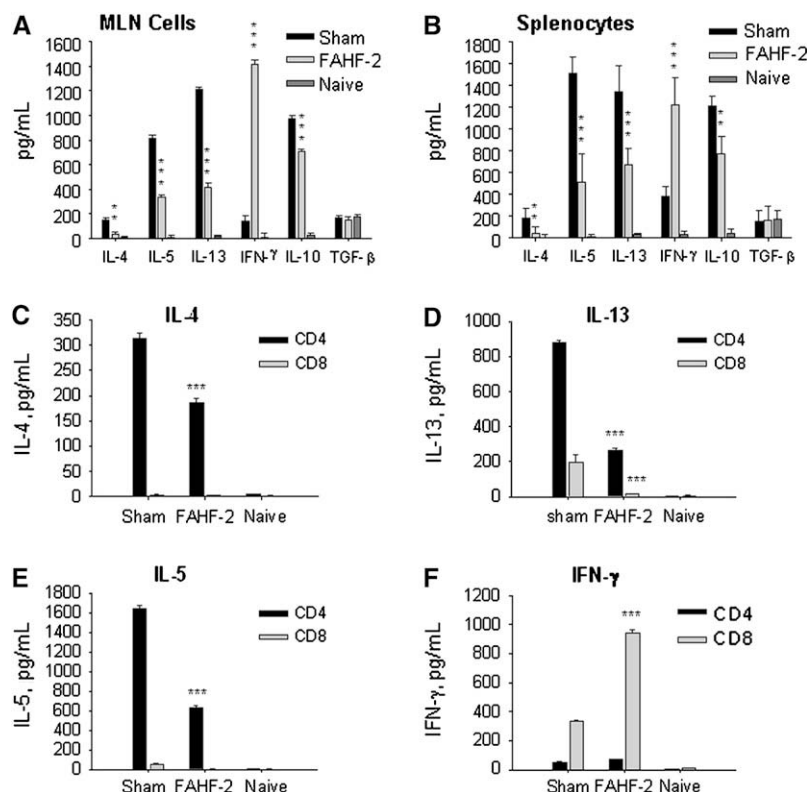


FIG 4. FAHF-2 treatment reduced T_H2 cytokine levels and increased IFN- γ secretion by specific modulation of $CD4^+$ and $CD8^+$ T cells. MLN cells and splenocytes were collected from each group of mice immediately after evaluation of clinical effect and blood drawing after the seventh challenge (week 50). MLN cells and splenocytes were prepared and stimulated with CPE for 72 hours. Cytokines in MLN culture supernatants (**A**) and splenocyte culture supernatants (**B**) were measured by ELISA. Data are shown as means $\pm$ SEMs of pooled cultures from a representative 1 of 2 experiments measured in triplicate ($n = 5$ mice per group). **C-F**, Cytokine production by isolated $CD4^+$ (solid bars) and $CD8^+$ T cells (open bars) after the seventh challenge. Splenic $CD8^+$ or $CD4^+$ T cells isolated from each group of mice after final challenge were restimulated with irradiated syngeneic splenocytes and CPE for 72 hours. Cytokines in culture supernatants were measured by ELISA. Data shown are means $\pm$ SEMs of pooled cultures measured in triplicate ($N = 5$ mice/group). ** $P < .01$; *** $P < .001$ vs sham.

(week 34; Fig 2, B). Even at the week 50 challenge, histamine levels were markedly lower than in sham-treated mice ($P < .001$). Consistently fewer degranulated mast cells were present in ear samples of FAHF-2-treated mice compared with those from sham-treated mice after the seventh challenge (Fig 2, C and D). Taken together, these results demonstrate that a single 7-week FAHF-2 treatment produced long-lasting posttherapy protection.

FAHF-2 treatment produced a persistent reduction of peanut-specific IgE levels and increased IgG_{2a} levels

To investigate persistent FAHF-2 effects on antibody production, serum peanut-specific IgE and IgG_{2a} levels were monitored over time. At week 8 (1 day before treatment), peanut-specific IgE levels were equally elevated in all peanut-allergic mice (Fig 3, A). These levels peaked at week 12 and remained elevated through week 50 in sham-treated mice. In contrast, peanut-specific IgE levels in FAHF-2-treated mice were significantly lower than in sham-treated mice by the fourth week of treatment (week 12, $P < .05$) and remained lower through week 50 ($P < .05$). Thus, there was no significant posttherapy rebound. Conversely, peanut-specific IgG_{2a} levels were significantly increased after 4 weeks of

FAHF-2 treatment and remained significantly elevated compared with sham-treated animals until week 50 (Fig 3, B).

FAHF-2 treatment produced long-term reduction of T_H2 cytokine secretion by $CD4^+$ T cells and increased IFN- γ secretion by $CD8^+$ T cells in response to recall antigen stimulation

To determine long-term recall cytokine responses, MLN cells and splenocytes were isolated from each group after the seventh posttherapy challenge at week 50. MLN cells and splenocytes from the sham-treated peanut-allergic mice showed marked increases in production of the T_H2 cytokines IL-4, IL-5, and IL-13 in response to CPE stimulation in culture. Cells from FAHF-2-treated mice produced significantly less IL-4, IL-5, and IL-13 (Fig 4, A and B; $P < .01$ -.001). In contrast, FAHF-2 treatment markedly elevated CPE-induced production of the T_H1 cytokine, IFN- γ , in both cultured MLN cells (Fig 4, A) and splenocytes (Fig 4, B) compared with that in sham-treated mice ($P < .001$). IL-10 production by MLN cells and splenocytes was slightly but significantly lower in cultures from FAHF-2-treated mice compared with sham-treated mice (Fig 4, A and B; $P < .01$ -.001). No effect of treatment on TGF- β levels was found. Cytokine profiles of the same cells cultured in the presence or absence of concanavalin A

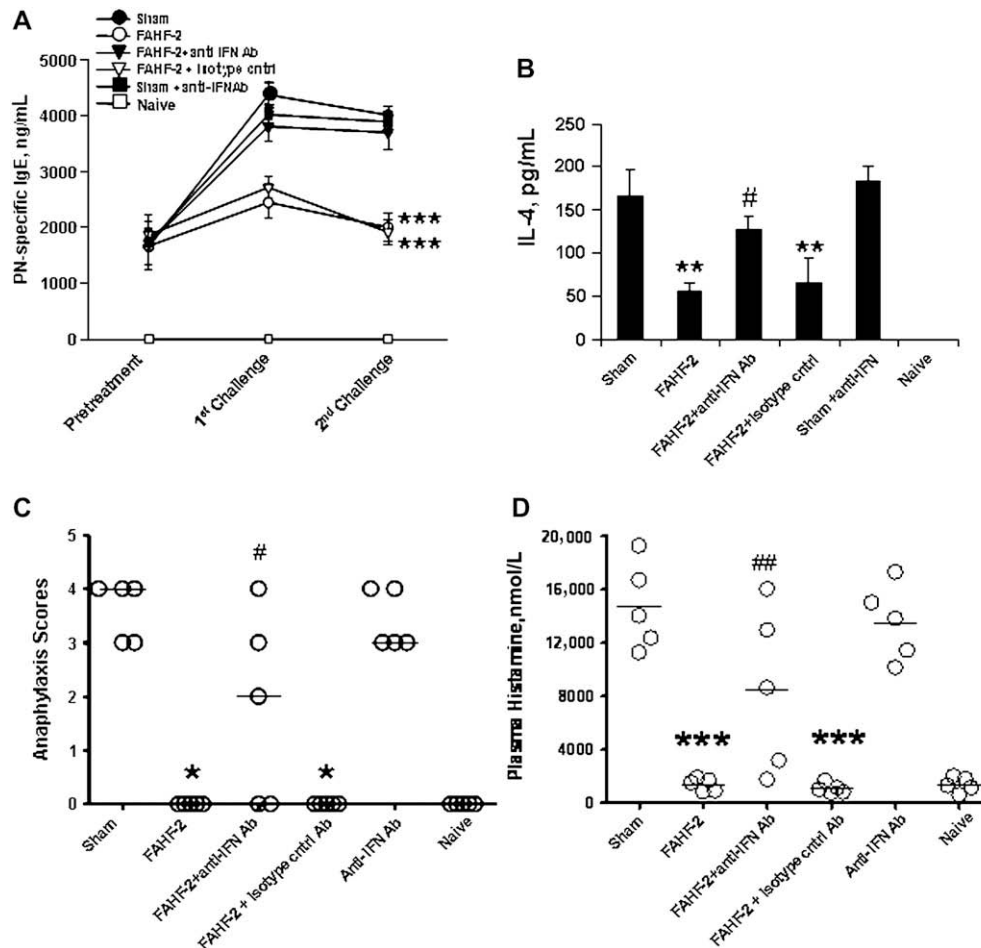


FIG 5. IFN- γ is necessary for FAHF-2 suppression of T_{H2} responses and anaphylaxis. **A**, Serum peanut (PN)-specific IgE levels. Blood was collected by retro-orbital bleed 1 day before treatment (week 8) and first and second posttherapy challenges (weeks 14 and 18). Serum peanut-specific IgE was measured by ELISA. Data include group means $\pm$ SEMs from 5 mice/group. **B**, IL-4 levels in splenocyte cultures. Splenocytes were prepared as in Fig 4 after the week 14 challenge. Cells were cultured in the presence or absence of CPE for 3 days. IL-4 levels were measured by ELISA. IL-4 is undetectable in unstimulated cell culture (data not shown). Data include group means $\pm$ SEMs, $n = 5$ mice/group. **C**, Anaphylaxis scores. Signs were visually assessed 25 to 30 minutes after the second challenge as described in the legend for Table I. Symbols indicate individual mice, and lines indicate group medians. **D**, Plasma histamine levels. Plasma was obtained after the second challenge immediately after anaphylactic score assessment. Histamine levels were measured by using an enzyme immunoassay. Symbols indicate individual mice. Lines indicate group means, $n = 5$ /group. * $P < .05$; ** $P < .01$; *** $P < .001$ vs sham; # $P < .05$ and ## $P < .01$ vs FAHF-2.

did not differ between groups (data not shown), suggesting that FAHF-2 has an antigen-specific immunomodulatory effect on T-cell responses.

Cytokine profiles produced by CD4⁺ versus CD8⁺ T-cell subtypes in response to FAHF-2 treatment were investigated. CD4⁺ T cells isolated from spleens of sham-treated mice after the seventh posttherapy challenge (week 50) were the predominant source of T_{H2} cytokines (IL-4, IL-5, and IL-13) after exposure to CPE *in vitro*, whereas CD8⁺ T cells were the predominant source of IFN- γ , although at low levels. CPE-stimulated CD4⁺ T cells from FAHF-2-treated mice exhibited substantially reduced production of IL-4, IL-5, and IL-13 compared with those of sham-treated mice, and no change in their minimal IFN- γ production. CD8⁺ T cells from FAHF-2-treated mice, in contrast, produced approximately 3 times more IFN- γ than cells from sham-treated mice (Fig 4, C-

F). Cells from naive mice did not respond to CPE. No cytokine responses were observed in cultures of irradiated APCs alone (data not shown).

IFN- γ mediates FAHF-2 suppression of peanut-induced T_{H2} responses and clinical protection

We next investigated the functional contribution of IFN- γ to the protective effects of FAHF-2 by administering IFN- γ neutralizing Ab. FAHF-2-treated mice given IFN- γ neutralizing Ab but not those given isotype control Ab during FAHF-2 treatment had levels of peanut-specific IgE similar to those in sham-treated mice, and significantly higher than in mice treated with FAHF-2 alone (Fig 5, A; $P < .01$). IL-4 (Fig 5, B), IL-5, and IL-13 levels (data not shown) in splenocyte cultures stimulated with CPE

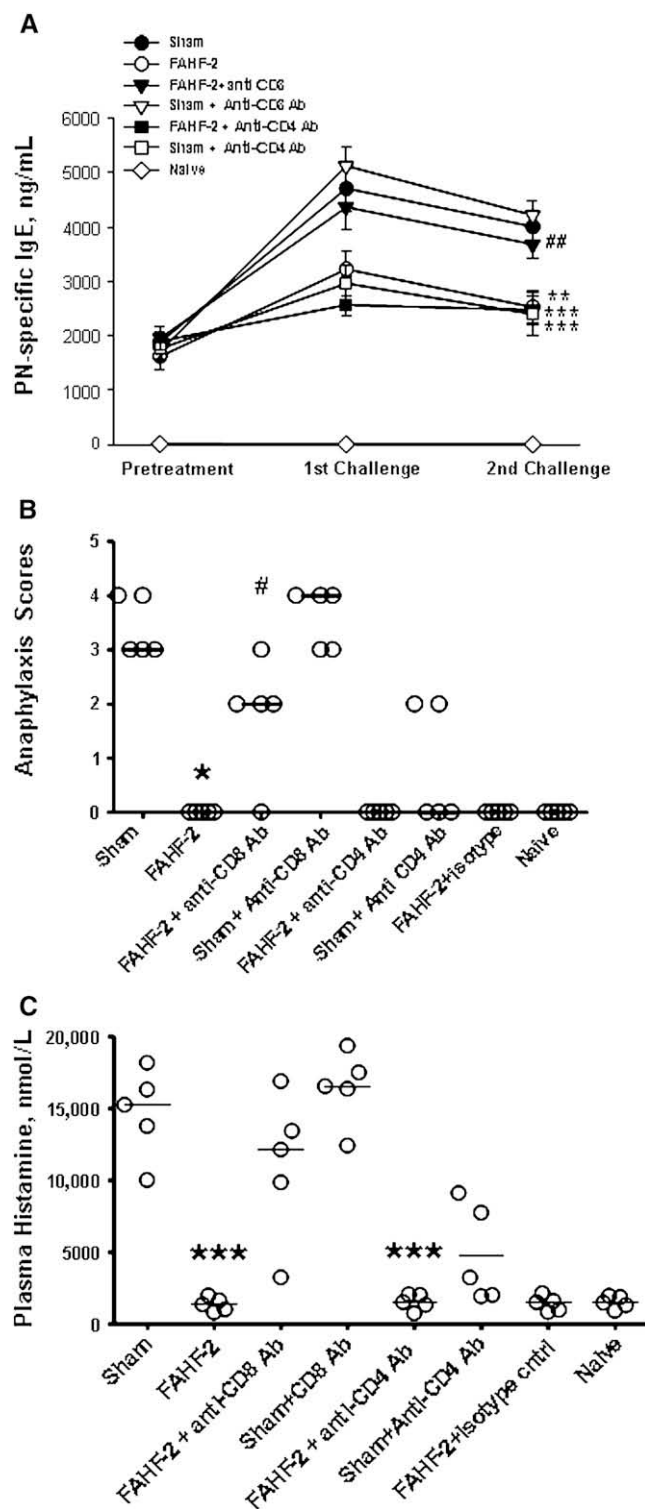


FIG 6. CD8⁺ T cells are important contributors to FAHF-2 inhibition of anaphylaxis and T_H2 responses. **A**, Peanut (PN)-specific IgE. Blood was collected by retro-orbital puncture 1 day before treatment (week 8) and first and second posttherapy challenges (weeks 14 and 18). Serum peanut-specific IgE was measured by ELISA. Data include group means $\pm$ SEMs, $n = 5$ mice/group. **B**, Anaphylaxis scores. Anaphylactic signs were visually assessed 25 to 30 minutes after the second challenge as in Table I. **C**, Plasma histamine levels. Histamine level measured by enzymatic assay in plasma obtained after the second challenge immediately after assessment of anaphylaxis. Symbols indicate individual mice. Lines indicate group medians

from FAHF-2-treated mice given IFN- γ neutralizing antibodies were also significantly higher than those of splenocytes from mice treated with FAHF-2 alone (Fig 5, B; $P < .05$). IFN- γ neutralizing Ab had no effect in sham-treated mice, which may be because of a negligible baseline of IFN- γ in sham-treated mice. The blocking effect of IFN- γ neutralizing antibodies on anaphylactic responses was not as evident after the first peanut challenge at week 14. Only 1 of 5 mice treated with FAHF-2 plus IFN- γ neutralizing antibodies showed anaphylactic signs, and plasma histamine levels were not significantly higher than in mice treated with FAHF-2 alone (data not shown). However, after peanut challenge 4 weeks later, mice in the FAHF-2 + anti-IFN- γ Ab group lost protection, with 3 mice showing anaphylactic signs, 2 of which were severe (scores 3 and 4), accompanied by significant elevation of plasma histamine (Fig 5, C and D). Because anti-IFN- γ Ab significantly attenuated FAHF-2-mediated protection and abolished FAHF-2 suppressive effects on IgE and IL-4 levels, observations were not continued beyond week 18. These observations suggest that increased IFN- γ is functionally important to FAHF-2 efficacy.

FAHF-2-induced IFN- γ -associated suppression of peanut hypersensitivity is CD8⁺ T-cell-dependent

On the basis of these results and our previous study,²⁸ we tested the role of CD8⁺ or CD4⁺ T cells in the response to FAHF-2 *in vivo* by treating mice with anti-CD4⁺ or anti-CD8⁺ T-cell Ab, or isotype control Abs. The effect of anti-CD8⁺ Ab was similar to that of anti-IFN- γ Ab in that it significantly attenuated FAHF-2-mediated suppression of peanut-specific IgE at both weeks 14 and 18 posttherapy (Fig 6, A; $P < .01$), but did not reverse FAHF-2 protection from anaphylaxis or reduction of histamine release at week 14 (data not shown). After the second challenge, however, 80% of mice in the FAHF-2 + anti-CD8⁺ Ab group developed anaphylactic signs and showed significantly higher plasma histamine levels than mice treated with FAHF-2 alone (Fig 6, B and C; $P < .01$ and $.001$, respectively). Administration of isotype control Ab during FAHF-2 treatment had no effect (Fig 6, B and C). Administration of anti-CD8⁺ Ab to sham-treated peanut-allergic mice appeared to increase peanut-specific IgE levels and anaphylactic reactions, but the effect did not reach statistical significance compared with sham-treated mice. As with the lack of effect of anti-IFN- γ Ab in sham-treated mice, this may reflect the low basal levels of IFN- γ in peanut-allergic mice (Fig 4). Table II shows cytokine profiles of the experimental groups after the week 18 peanut challenge. IFN- γ levels in cultured splenocytes from FAHF-2 + anti-CD8 Ab-treated mice were significantly lower, and IL-4, IL-5, and IL-13 production was significantly higher than that in splenocyte cultures from mice treated with FAHF-2 alone. In contrast, depletion of CD4⁺ T cells did not affect FAHF-2 efficacy and in fact inhibited allergic responses (Fig 6, A-C; $P < .05$ -.01), demonstrating a pathogenic role of CD4⁺ T cells in PNA. CD4⁺ cells are the predominant source of T_H2 cytokine production in our model (Fig 4), and as expected, treatment with anti-CD4 Ab reduced IL-4, IL-5, and IL-13 secretion without significantly altering IFN- γ (Table II). Isotype control Abs for anti-CD4 or CD8 Abs did not influence FAHF-2's effect.

for anaphylactic scores and group means for histamine, $n = 5$ /group. * $P < .05$; ** $P < .01$; *** $P < .001$ vs sham; # $P < .05$ and ## $P < .01$ vs FAHF-2.

TABLE II. Neutralization of INF- γ alters FAHF-2 effects on cytokine profiles

Treatment	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	INF- γ (pg/ml)
Sham	126.9 (27.9)	1513.5 (76.8)	1214.5 (132.3)	212.2 (67.8)
FAHF-2	69.8 (125.25)***	609.7 (112.1)***	476.3 (171)***	1096.5 (154.7)***
FAHF-2+anti-CD8 Ab	141.0 (19.5)#	1220.2 (121.3)###	1318.6 (107.1)###	626.5 (89.2)###
Sham+anti-CD8 Ab	156.0 (23.4)	1395.17 (87.3)	1426.7 (137.2)	141.3 (102.6)
FAHF-2+anti CD4 Ab	41.3 (16.2)***	337.6 (133.1)***	309.6 (121.4)***	1266.4 (163.1)***
Sham+anti-CD4 Ab	81.2 (15.9)*	937.1 (82.8)***	862.7 (142.7)**	276.2 (93.7)
FAHF-2+isotype control Ab	35.2 (26.4)***	703.1 (76.2)***	509.4 (118.2)***	1242.7 (109.8)***
Naive	3.2 (1.7)	11.5 (6.4)	11.1 (23.3)	26.9 (11.2)

Anti-IFN- γ Ab altered FAHF-2's effect on cytokine profiles. Splenocytes were prepared after week 18 challenge and cultured in the presence or absence of CPE. Supernatants were harvested after 72 hours in culture. Peanut-unstimulated cultures had no detectable or marginal levels of cytokines (data not shown). Isotype control antibody is rat IgG₂ for anti-CD8 and anti-CD4. Data are means $\pm$ SDs of peanut-stimulated cultures from spleens of 5 mice per group.

* $P < .05$ vs none; #vs FAHF-2.

** $P < .01$ vs none; vs FAHF-2.

*** $P < .001$ vs none; ###vs FAHF-2.

DISCUSSION

Peanut allergy is one of the most common food allergies provoking severe anaphylactic reactions, thereby imparting a significant psychological burden on individuals with allergy and their families.²³ An effective treatment would offer a life-altering option for those affected. The current study shows that a single 7-week course of FAHF-2 treatment prevented anaphylactic reactions for 6 months posttherapy (25% of the mouse life span), during which time 6 peanut challenges were administered. Even at approximately 9 months posttherapy, only 37% of mice showed moderate reactions, whereas all sham-treated mice showed severe anaphylactic reactions. These results were associated with persistent reduction of serum peanut-specific IgE levels throughout the 9-month period. FAHF-2 may prove to be more advantageous therapeutically than anti-IgE treatment, in which free-IgE increased 2 weeks after discontinuation of treatment with humanized monoclonal anti-IgE antibody.²⁴ We also found concomitant and persistent elevation in IgG_{2a} levels after FAHF-2 treatment. The value of IgG_{2a} as a blocking antibody, which interferes with mast cell degranulation via Fc ϵ RI ligation and antigen interception, has recently received attention.²⁵ Thus, in addition to sustained reduction of peanut specific IgE, increased peanut-specific IgG_{2a} may contribute to the long-term benefits of FAHF-2 treatment. These results also suggest that FAHF-2 does not induce overall immune suppression but a persistent immunomodulatory effect favorable to silencing peanut allergy. This is the first evidence that a herbal formula can produce long-term complete protection against peanut-induced anaphylaxis well beyond termination of therapy. The possibility that prolonged protection can be attained without continuous drug treatment represents a significant potential therapeutic advantage.

Mechanisms underlying the persistence of FAHF-2 benefits appear to include sustained suppression of IL-4, IL-13, and IL-5, all known to play a central role in the pathogenesis of allergic disorders.^{26,27} This was evident in the decreased T_H2 profile of CD4⁺ T cells in FAHF-2-treated mice observed 9 months after therapy had been stopped. CD4⁺ cells are the predominant producers of these T_H2 cytokines, as indicated by cytokine profiles of CD4⁺ T cells from sham-treated mice and the finding that depletion of these cells significantly attenuated peanut-specific IgE, T_H2 cytokines, and peanut hypersensitivity *in vivo*. In contrast, INF- γ levels were elevated in treated mice. INF- γ is a potent negative controller for T_H2 responses. It is known to reduce IgE production^{28,29} and T_H2 cytokine production.³⁰ Others have reported that the human

intestinal immune system preferentially responds with a dominant T_H1 profile against food antigens in the normal state.³¹ Studies with human Peyer patch T cells show that many cells spontaneously secrete cytokines, predominantly INF- γ , with negligible IL-4, IL-5, and IL-10³² or TGF- β .³³ Thus, we believe that FAHF-2 returns the immune responses to food toward a more normal state that is T_H1-biased. This possibility was further supported by the finding that *in vivo* neutralization of INF- γ during FAHF-2 treatment blocked FAHF-2 suppression of IgE, T_H2 cytokine production, and protection against peanut-induced anaphylaxis. TGF- β and IL-10, which suppress both T_H2 and T_H1 responses, have been suggested to be the mechanism underlying allergen immunotherapy.³⁴ However, we found no change in levels of TGF- β , and a reduction of IL-10 levels after FAHF-2 therapy, suggesting that the mechanisms of actions of FAHF-2 may differ from allergen immunotherapy.

We reported previously that FAHF-2 treatment increased the number of INF- γ -producing CD8⁺ T cells in mice.¹⁵ Our current results show that CD8⁺ T cells are the major source of INF- γ production induced by FAHF-2 treatment. CD8⁺ T cells have long been recognized as regulatory cells capable of inhibiting allergic responses by virtue of their production of INF- γ and IL-18.³⁵⁻³⁷ We show here that depletion of CD8⁺ T cells during FAHF-2 treatment attenuates FAHF-2-mediated INF- γ production, suppression of peanut-specific IgE, and clinical protection. Thus, enhancement of INF- γ production by CD8⁺ T cells may be a major mechanism underlying the long-term protection produced by FAHF-2. However, other mechanisms may coexist. As noted, INF- γ neutralization did not significantly affect the ability of FAHF-2 to protect mice from symptoms of anaphylaxis immediately after treatment (week 14) despite elevated IgE, although this protection was lost in a subsequent challenge 4 weeks later. This might reflect a direct effect of FAHF-2 on mast cell/basophil degranulation. Further studies are needed to understand FAHF-2's effect on mast cells through INF- γ -dependent³⁸ and independent mechanisms.

In conclusion, our studies with FAHF-2 have shown a beneficial effect that lasted long after the treatment regimen ended, a therapeutic effect not provided by other treatments. The long-term immunomodulatory effects of FAHF-2 on T_H1 and T_H2 responses, but not overall immune suppression, could provide the optimal immune milieu for establishing tolerance to peanut and other IgE-mediated food allergies. FAHF-2 is currently being

tested as a US Food and Drug Administration investigational new botanical drug in patients with food allergy including PNA. FAHF-2 might thus be the first available effective treatment for patients with PNA and other food allergies.

Key messages

- A single course of FAHF-2 treatment completely protected peanut-allergic mice from anaphylaxis for more than 6 months posttreatment, or approximately 25% of the mouse's life span.
- Persistent protection was associated with sustained reduction in peanut-specific IgE levels and T_H2 cytokines but elevated IgG_{2a} and IFN- γ production. FAHF-2 effects were reduced by IFN- γ neutralization and CD8⁺ T-cell depletion.
- FAHF-2, currently in clinical trials in the United States, is a promising candidate for development as a botanical drug for peanut and other IgE-mediated food allergies.

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