

Food allergy herbal formula 2 protection against peanut anaphylactic reaction is via inhibition of mast cells and basophils

Ying Song, MD,^{a*} Chunfeng Qu, PhD,^{a*} Kamal Srivastava, MPhil,^a Nan Yang, PhD,^a Paula Busse, MD,^a Wei Zhao, MD, PhD,^b and Xiu-Min Li, MD^a *New York, NY, and Richmond, Va*

Background: Mast cells and basophils are key effector cells of IgE-mediated anaphylactic reactions. The Chinese herbal formula, food allergy herbal formula 2 (FAHF-2), protects against peanut anaphylaxis in mice. However, the mechanisms underlying this effect are not fully elucidated.

Objective: To investigate whether FAHF-2 inhibits mast cell/basophil numbers and IgE-mediated activation.

Methods: Mice with peanut allergy (PNA mice) were treated with FAHF-2 intragastrically for 7 weeks and challenged intragastrically with peanut 1 day and 4 weeks posttreatment. Peripheral blood basophil numbers and peritoneal mast cell numbers and FcεRI expression were determined. Direct effects of FAHF-2 on the murine mast cell line MC/9, and effects of 4 fractions and 3 compounds isolated from FAHF-2 on rat basophilic leukemia cells (RBL-2H3) and human skin mast cells degranulation and on the IgE-mediated spleen tyrosine kinase signaling pathway, were determined.

Results: Although all sham-treated PNA mice developed anaphylaxis, FAHF-2-treated PNA mice were protected against anaphylaxis after peanut challenge at 1 day and 4 weeks posttherapy. Reduction of peripheral blood basophils began after 1 week of treatment and continued for at least 4 weeks posttherapy. The number and FcεRI expression of peritoneal mast cells were also significantly decreased 4 weeks posttherapy. FAHF-2-treated MC/9 cells showed significantly reduced IgE-induced FcεRI expression, FcεRI γ mRNA subunit expression, proliferation, and histamine release on challenge. Fraction 2 from FAHF-2 inhibited RBL-2H3 cell and human mast cell degranulation. Three compounds from fraction 2—berberine,

palmitine, and jatrorrhizine—inhibited RBL-2H3 cell degranulation via suppressing spleen tyrosine kinase phosphorylation.

Conclusion: Food allergy herbal formula 2 reduction of basophils and mast cell numbers as well as suppression of IgE-mediated mast cell activation may contribute to FAHF-2's persistent protection against peanut anaphylaxis. (*J Allergy Clin Immunol* 2010;126:1208-17.)

Key words: Peanut anaphylaxis, Chinese herbal medicine, mast cells/basophils, FcεRI

Anaphylaxis is a potentially life-threatening allergic reaction of rapid onset. Food allergy reactions account for one third to one half of anaphylaxis cases in emergency departments.¹

Food-induced anaphylaxis is an IgE-mediated type I hypersensitivity reaction in which mast cells and basophils are key effector cells. Mast cells, released from the bone marrow as undifferentiated precursor cells mature in tissues, where they reside. In contrast, basophils complete their differentiation in the bone marrow and subsequently circulate in the bloodstream.² Both cells express high-affinity IgE receptors (FcεRI), which bind to specific IgE molecules. On exposure, specific allergen cross-links the surface IgE molecules, which stimulate mast cells and basophils to release potent preformed mediators such as histamine. Reduction of FcεRI expression is an active research area with the goal of preventing or treating allergic disorders.³ Animal models are important tools for testing potential therapeutic modalities for food allergy and exploring novel pharmacologic agents. By using a well established murine model of peanut anaphylaxis, we found that the food allergy herbal formula 2 (FAHF-2) abrogated anaphylactic reactions in mice, and this protection persisted for at least 36 weeks after therapy was discontinued.⁴⁻⁶ The increased IFN-γ production by CD8⁺ T cells, as shown previously, may be an important mechanism underlying FAHF-2–modulated potent and long-term protection.^{4,6} However, neutralization of IFN-γ or depletion of CD8⁺ T cells blocked the suppression of IgE and T_H2 cytokine production induced by FAHF-2, whereas protection from anaphylaxis still existed up to 4 weeks posttherapy.⁶ We therefore hypothesized that FAHF-2 may also directly inhibit mast cells/basophils.

In this article, we investigated the effect of FAHF-2 on the number of peripheral blood basophils and peritoneal mast cells, as well as mast cell FcεRI expression in mice with peanut allergy. We further investigated FAHF-2 *in vitro* effects on murine mast cell (MC/9 cells) proliferation, histamine release, and FcεRI subunits expression in response to IgE. Using preparative HPLC (prep-HPLC), we identified and tested the effect of 4 fractions and 3 major alkaloid compounds on rat mast cells (rat basophilic leukemia cell 2H3 [RBL-2H3]) and

From ^athe Division of Allergy and Immunology, Department of Pediatrics, Mount Sinai School of Medicine, New York; and ^bthe Division of Allergy and Immunology, Department of Pediatrics, Virginia Commonwealth University.

*These authors contributed equally to this work.

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A US provisional patent application regarding FAHF-2 (reference no. 60554775) has been filed by X.-M. Li and Herbal Springs LLC.

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Reprint requests: Xiu-Min Li, MD, Pediatric Allergy and Immunology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574.

E-mail: xiu-min.li@mssm.edu.

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Abbreviations used

CT:	Cholera toxin
DMEM:	Dulbecco modified Eagle medium
FAHF-2:	Food allergy herbal formula 2
FITC:	Fluorescein isothiocyanate
MFI:	Mean fluorescent intensity
PBLC:	Peripheral blood leukocyte
PE:	Phycoerythrin
prep-HPLC:	Preparative HPLC
qPCR:	Quantitative PCR
RBL-2H3:	Rat basophilic leukemia cell 2H3
Syk:	Spleen tyrosine kinase

human skin mast cells. In addition, we determined the mechanism by which the isolated compounds inhibit mast cell degranulation.

METHODS

Mice and reagents

C3H/HeJ mice (female, 5 weeks old) purchased from 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. Freshly ground, whole roasted peanuts were prepared as previously described.^{7,8} Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, Calif). MC/9 cells (mouse mast cell line) and RBL-2H3 cells (rat basophilic leukemia cell line) were obtained from American Type Culture Collection (Manassas, Va). FAHF-2 is dried powder of aqueous extract of 9 herbs. The formula and process of preparation of the final product are the same as described in previous publications^{6,9} and included in the section “FAHF-2 formula and product” in this article’s [Methods](#) in the Online Repository at www.jacionline.org. To identify active compounds, FAHF-2 was first extracted with butanol, then dried, and then further divided into 4 fractions on the basis of polarity by prep-HPLC. Compounds were identified by liquid chromatography mass spectrometry with standard compounds as described in detail in the section “Extraction and isolation of fractions and compounds from FAHF-2” in this article’s [Methods](#) in the Online Repository.

Antibodies

Phycoerythrin (PE)-conjugated mAbs specific for mouse FcεRI mAb (MAR-1), allophycocyanin (APC)-conjugated anti-mouse Gr-1 (RB6-8C5), anti-c-kit (2B8), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE (23G3) and FITC-conjugated control hamster IgG were obtained from eBioscience (San Diego, Calif). FITC-conjugated anti-mouse CCR3 mAb (83101) was purchased from R&D Systems (Minneapolis, Minn). PE-conjugated anti-mouse CD3ε(145-2C11), PE-conjugated anti-mouse B220(RA3-6B2), APC-conjugated anti-mouse CD49b(DX5), FITC-conjugated anti-mouse I-A^k (AbK, 10-3.6) and purified anti-mouse CD16/32 mAb (2.4G2) were purchased from BD Biosciences (San Diego, Calif). Mouse anti-dinitrophenol (DNP) IgE was purchased from Sigma-Aldrich (St Louis, Mo).

Protocol for peanut sensitization/challenge, FAHF-2, treatment and assessment of anaphylaxis

Mice were sensitized intragastrically with ground peanut (10 mg/mouse) weekly for 5 weeks and boosted at weeks 6 and 8 (50 mg/mouse; [Fig 1, A](#)) as previously described.⁴ FAHF-2 was administered 24 hours after the week 8 boost, at which time peanut hypersensitivity was established.⁴ FAHF-2 was administered i.g. at 32 mg in 0.5 mL water, twice daily for 7 weeks. The daily dose of FAHF-2 used in this study was calculated by normalizing the daily human dose to mouse body weight as previously described.^{4,5} An additional group of mice with peanut allergy received equal volume of water (sham). Both groups exhibited equal levels of peanut-specific IgE before treatment. Naive mice served as normal controls. All mice were peanut-challenged i.g. (200 mg/mouse) 1 day (week 14) and 4 weeks posttreatment (week 18). CT

as mucosal adjuvant at 20 μg/mouse was coadministered with peanut at all times except at final challenge. Anaphylactic symptoms were evaluated approximately 30 minutes after the challenge dose, using a previously described scoring system^{4,5} (see the section “Assessment of systemic anaphylactic symptoms” in this article’s [Methods](#) in the Online Repository) in a blind manner. Rectal temperatures were measured approximately 30 minutes after challenge by using a thermal probe (Harvard Apparatus, Newark, NJ). Plasma histamine levels were determined 30 minutes after the second challenge and analyzed by using an enzyme immunoassay kit (ImmunoTECH Inc, Marseille, France) as described by the manufacturer.

Flow cytometry measurement of basophil number, peritoneal mast cell number, and FcεRI expression

Peripheral blood leukocytes (PBLs) were obtained before FAHF-2 treatment, then weekly during treatment and 4 weeks posttherapy from all mice, as shown in [Fig 1, A](#). Approximately 150 μL EDTA anticoagulated blood samples per mouse were pooled and subjected to red cell lysis by using Pharmlyse (BD Biosciences). PBLs (1×10^6) were incubated in 100 μL staining buffer (HBSS containing 0.02% NaN₃ and 2 M EDTA, 0.1% BSA, 2% FBS, 1% normal mouse serum, 1% normal rat serum) and 20 μg/mL purified anti-mouse CD16/32 mAb (2.4G2) as Fcγ receptor–blocking mAb for 30 minutes at 4°C. PE-conjugated anti-mouse FcεRI, FITC-conjugated anti-mouse I-A^k, and APC-conjugated Gr-1 were then added to the cell suspension in the presence of Fcγ receptor–blocking mAb on ice for 30 minutes. After washing, cells were acquired on an LSR-II flow cytometer (BD Biosciences), and data were analyzed by using Flowjo software (Tree Star, Inc, Ashland, Ore). FcεRI⁺CCR3⁺Gr-1⁺ is defined as mouse blood basophils.^{10–12}

Two days after the final challenge at week 18, mice were killed, and peritoneal cavity cells were lavaged by using 10 mL cold HBSS. Peritoneal cavity cells from every 3 to 4 mice in each group were combined in a total of 3 subsets. A total of 5×10^5 cells was incubated in 100 μL HBSS staining buffer containing 20 μg/mL purified anti-CD16/32 mAb (2.4G2) as Fcγ receptor–blocking mAb for 30 minutes at 4°C. The cells were then incubated with the following antibodies for 30 minutes: PE-conjugated anti-mouse FcεRI, FITC-conjugated anti-I-A^k, and APC-conjugated anti-mouse c-kit. After washing, cell acquisition and data analysis were performed as described.

Flow-cytometric determination of FAHF-2 effects on murine MC/9 mast cell FcεRI expression *in vitro*

MC/9 cells cultured at 5×10^5 /mL in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS were incubated with 2 μg/mL mouse anti-DNP IgE (Sigma-Aldrich) in the presence or absence of different dose of FAHF-2 (at 10 or 20 μg/mL) for 48 hours. After washing, cells were then incubated in 100 μL HBSS buffer containing 20 μg/mL purified anti-CD16/32 mAb (2.4G2) as Fcγ receptor–blocking mAb for 30 minutes at 4°C as previously described.¹³ Purified mouse anti-DNP IgE (2 μg/mL) was then added to the cell suspension in the presence of Fcγ receptor–blocking antibody and incubated on ice for another 30 minutes to saturate any FcεRI expressed by these cells as previously described.¹³ After washing twice, 1 μg FITC-conjugated rat anti-mouse IgE was added to 100 μL cell suspension in HBSS containing 0.1% BSA and incubated for 30 minutes at 4°C. FITC-conjugated rat IgG₁ was used as isotype control. After washing twice, cell acquisition and data analysis were performed as described.

Determination of the effect of FAHF-2 on mast cell proliferation and degranulation *in vitro*

To examine the effect of FAHF-2 on mast cell growth, 1×10^5 MC/9 cells were cultured in 200 μL DMEM containing 10% FBS in the presence or absence of 2 μg/mL mouse anti-DNP IgE with or without 20 μg/mL FAHF-2. Cell viability was assessed 24 hours, 48 hours, and 72 hours after treatment by trypan blue exclusion.¹⁴ The fold of cell growth was calculated (number of viable cells at 24 hours, 48 hours, and 72 hours divided by the number of cells on day 0). The percent of trypan blue–positive cells was used to assess toxicity.

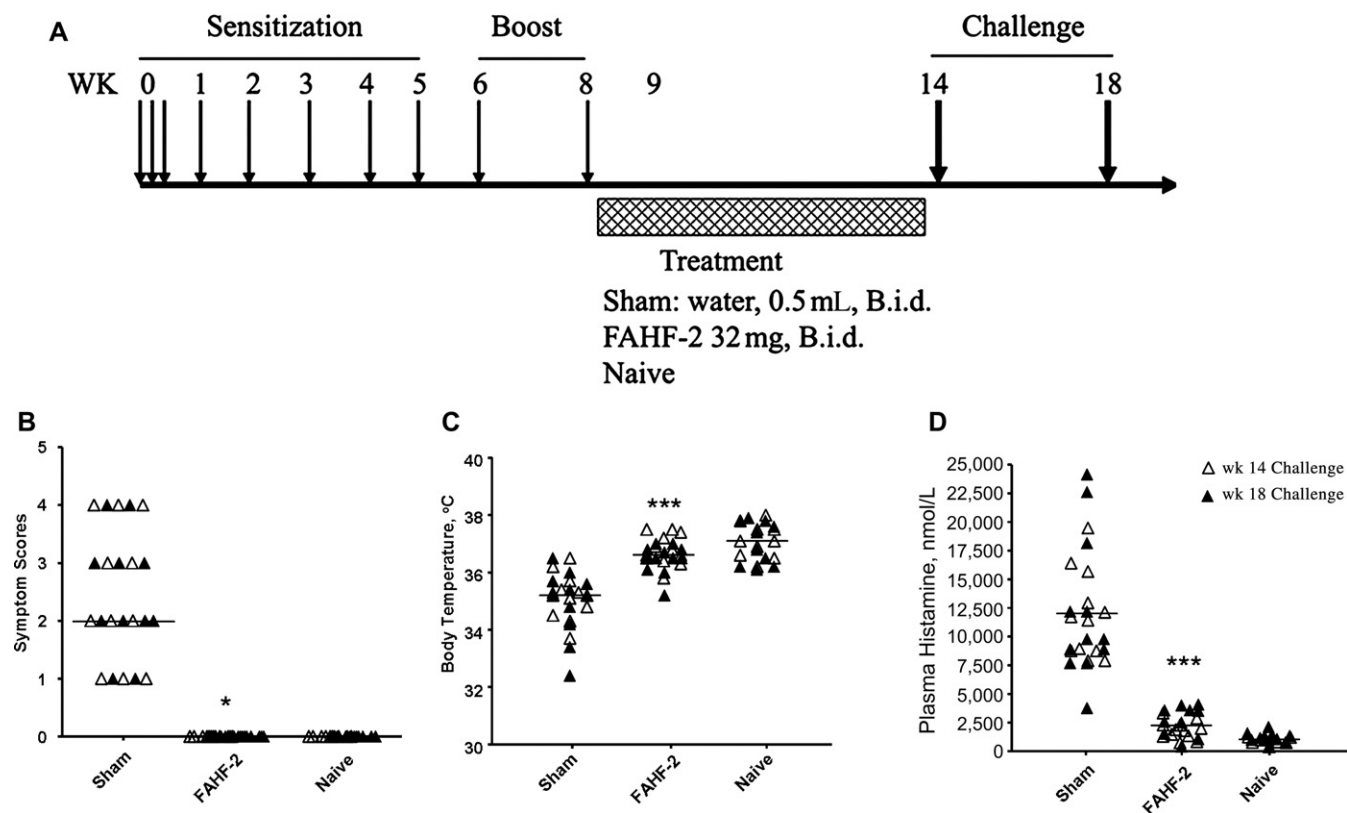


FIG 1. A, Experimental protocol. Female C3H/HeJ mice were sensitized intragastrically with freshly ground roasted peanut together with cholera toxin as indicated and treated with FAHF-2 ($n = 10$) or water (sham, $n = 10$). All mice were orally challenged with peanut at weeks 14 and 18. Naive mice served as controls ($n = 10$). B, Anaphylactic symptom scores. C, Postchallenge body temperatures. D, Postchallenge plasma histamine levels. Bars in B indicates median of scores of mice from each group with combined data at week 14 (open triangles) and week 18 challenges (solid triangles). (Sham, $n = 20$; FAHF-2, $n = 20$; and naive, $n = 20$). Bars in C and D are means of each group of mice from combined data at week 14 (open circles) and week 18 (open triangles) challenges. * $P < .05$; *** $P < .001$ versus sham. B.i.d., Twice daily.

Mast cell degranulation was determined by measuring histamine release as previously described.^{15,16} Briefly, 1×10^5 MC/9 cells were cultured in 200 μ L DMEM containing 10% FBS in the presence of 2 μ g/mL mouse anti-DNP IgE. Cells were cocultured with or without FAHF-2 at 20 μ g/mL for 48 hours. This concentration was used on the basis of the *in vitro* study showing FAHF-2 markedly reduced Fc ϵ R1 expression by MC/9 mast cells at 20 μ g/mL. The cells were then collected, washed with DMEM, and resuspended in 100 μ L Tyrode solution containing 1 mg/mL BSA and 1 M CaCl_2 . Fc ϵ R1 cross-linking was performed by stimulation with specific antigen, 100 ng/mL DNP conjugated with human serum albumin (Sigma-Aldrich) for 45 minutes at 37°C, as previously described.^{15,16} After centrifugation at 300g for 5 minutes, supernatant was collected, and cell pellets were lysed with 100 μ L distilled water, followed by freeze-thaw cycle twice. Histamine levels in the supernatants and cell lysates were measured by using ELISA as described. Additional control cultures included MC/9 cells cultured in the media alone, with anti-DNP IgE but without DNP stimulation, or with irrelevant antigen, conalbumin (100 ng/mL), stimulation.

Quantifying Fc ϵ R1 subunit mRNA levels by real-time PCR (quantitative PCR)

Real-time reverse transcription quantitative PCR (qPCR) was performed by using a SYBR green protocol and an ABI7900 HT machine (Applied Biosystems, Foster City, Calif) as previously described¹⁷ (see the section "Quantifying Fc ϵ R1 subunit mRNA levels by real-time PCR (qPCR)" in this article's Methods in the Online Repository).

Testing isolated fractions and identified compounds from FAHF-2 on RBL-2H3 and human skin mast cells

RBL-2H3 cells were plated at 2.5×10^5 cells/well in 24-well plates for experiments described. For β -hexosaminidase assays, RBL-2H3 cells were pretreated with FAHF-2 fraction 1 or 4 (18, 36, 72 μ g/mL); fraction 2 or 3 (9, 18, 36 μ g/mL); and 3 compounds from fraction 2, berberine (1.25, 2.5, 5, 10 μ g/mL), palmatine, or jatrorrhizine (1.25, 2.5, 5, 10, 20, 40 μ g/mL) for 24 hours. The doses used were based on our preliminary studies. Cells were then sensitized with anti-DNP IgE (72 ng/mL) and challenged with DNP-BSA (150 μ g/mL). β -Hexosaminidase was assayed according to a previously published method.¹⁸ Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assay following the methods of previous publications.¹⁹

Human skin-derived mast cells were prepared as previously described.²⁰ Mast cells (1×10^5) were treated with FAHF-2 fraction 2 at 200 μ g/mL for 24 hours. The cells were then washed and stimulated with Fc ϵ R1 α -specific mAb 22E7 (1 μ g/mL) or complement pathway activator C5a (100 ng/mL). Degranulation was assessed by measuring β -hexosaminidase in culture supernatants and cell lysates as previously described.²⁰

Western blot

IgE-sensitized RBL-2H3 cells (7×10^6) were cultured with or without berberine, palmatine, and jatrorrhizine for 24 hours and stimulated with DNP (150 μ g/mL) for 5 minutes. The cells were then lysed, and Western blot analysis for determination of early Fc ϵ R1-mediated signal events was performed by using anti-spleen tyrosine kinase (Syk) or antiphosphorylated Syk

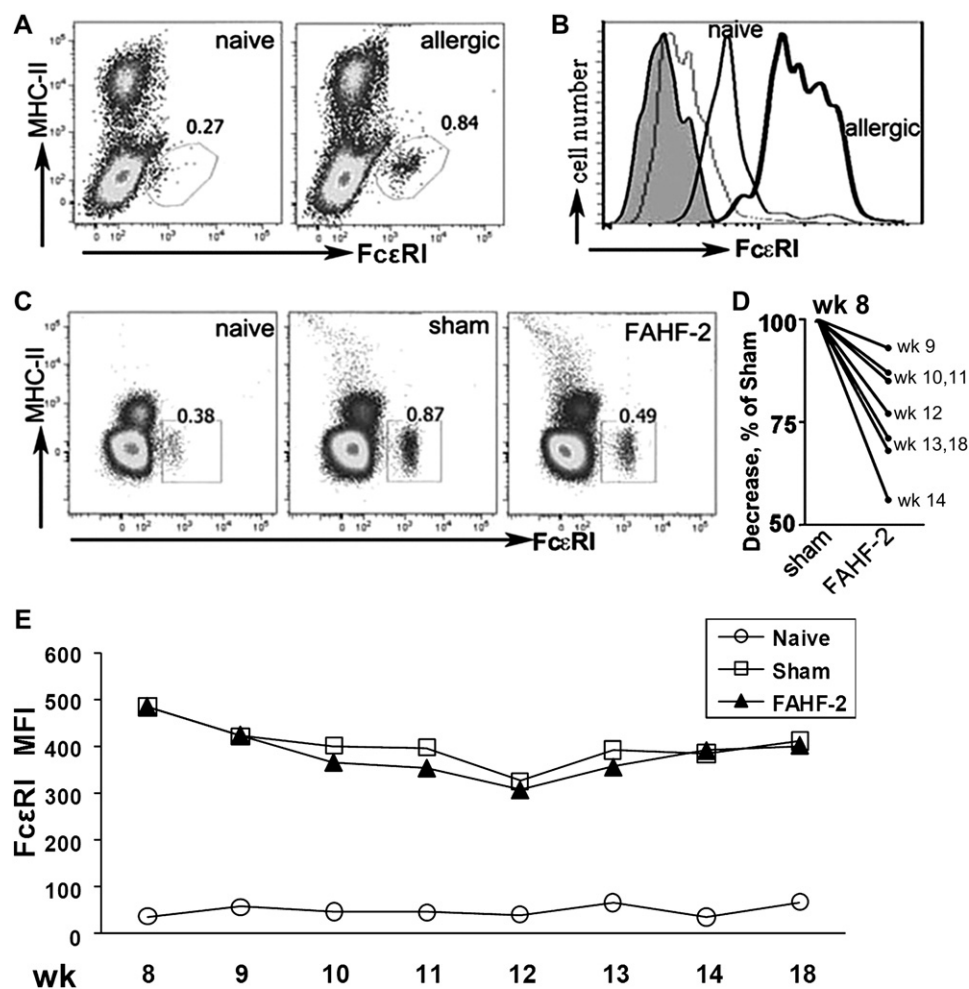


FIG 2. FAHF-2 reduced the number of peripheral blood basophils. **A**, Dot plots show an increased percentage of basophils in mice with peanut allergy at week 8 after the last boost compared with naive mice. **B**, Histogram shows labeled FcεRI expression on cells from mice with allergy (bold line) and naive mice (thin line). Shaded, Unstained cells; gray, isotype controls. **C**, Percentage of basophils in peripheral blood from each group at week 14 immediately after treatment. **D**, Reduction of basophil numbers as determined weekly at different time points as indicated. Numbers were normalized to and expressed as percentages of sham. **E**, FcεRI MFI of blood basophils from each group over time as in **D**.

antibodies (Cell Signaling, Danvers, Mass) as described previously²¹ (see the section “Western blot” in this article’s **Methods** in the Online Repository).

Statistical analysis

Data were analyzed by using the SigmaStat statistical software package (Systat, Chicago, Ill). For normally distributed data, differences between multiple groups were analyzed by 1-way ANOVA followed by the Bonferroni *t* test for all pairwise comparisons. For any data that failed the normality test, 1-way ANOVA on ranks followed by all pairwise comparisons was used. The differences between 2 groups were analyzed by *t* test and Mann-Whitney rank-sum test for normally distributed data and nonnormally distributed data, respectively. *P* values <.05 were considered significant.

RESULTS

FAHF-2 prevented peanut-induced anaphylactic reactions in the chronic peanut-allergic murine model

To investigate the mechanisms by which FAHF-2 abrogates peanut-induced anaphylaxis, we used the previously described model

(Fig 1, A).⁴ As expected, all sham-treated mice developed anaphylactic reactions after the first oral peanut challenge at week 14 (median symptom score, 3; Fig 1, B). Concurrently, body temperatures of sham-treated mice decreased significantly compared with those of naive mice, a sign of systemic anaphylaxis (*P* < .001; Fig 1, C). Plasma histamine was also markedly elevated in all sham-treated mice (*P* < .001; Fig 1, D). In addition, all sham-treated mice responded to peanut rechallenge 4 weeks later at week 18 (Fig 1, C and D; *P* < .001). In contrast, FAHF-2-treated mice showed no anaphylactic symptoms immediately after treatment (at week 14) or 4 weeks posttherapy (at week 18), maintained normal body temperatures, and showed suppressed histamine levels (Fig 1, B-D).

FAHF-2 reduced the number of peripheral blood basophils

With the model described, we used flow cytometry to determine the number of peripheral blood basophils before and after FAHF-2 treatment. As shown in Fig 2, A, the FcεRI-positive cells were MHC-II-negative, thereby excluding B cells, monocytes, and

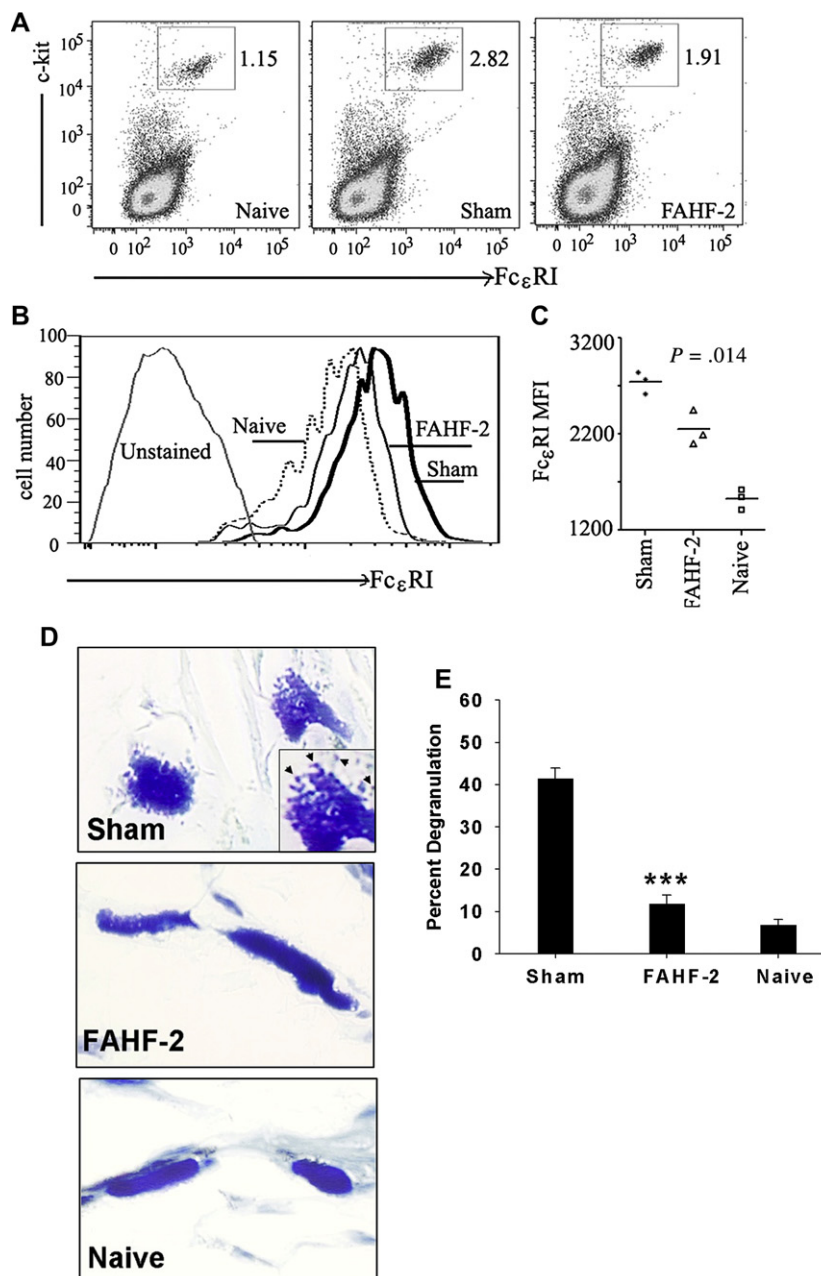


FIG 3. FAHF-2 reduced the number of and FcεRI expression of peritoneal cavity mast cells. **A**, Data show 1 representative result with mast cells within the gated area. Numbers indicate percentages. **B**, Mast cells (c-kit and FcεRI double-positive) were gated to show FcεRI levels in each group as indicated. **C**, FcεRI MFI. Each dot indicates 1 set of pooled cells from each group. Bars indicate the mean values of each group. **D**, Representative images of cutaneous mast cells in skin tissue of each group of mice. **E**, Percentage of degranulated cutaneous mast cells in skin samples postchallenge. Data are shown as means $\pm$ SEMs ($n = 5$). *** $P < .001$ vs naive.

dendritic cells; and CCR3-negative, excluding eosinophils. This phenotype is characteristic of blood basophils.¹⁰⁻¹² Blood from mice with peanut allergy contained significantly more FcεRI-positive peripheral blood cells (0.84%) than naive mice (0.27%) when treatment was initiated at week 8 (Fig 2, A). Mean fluorescent intensity (MFI) was also increased in mice with peanut allergy (Fig 2, B), reflecting increased FcεRI expression. However, basophil numbers in FAHF-2-treated mice were significantly reduced compared with sham-treated mice immediately after completing treatment (week 14, 0.49% and 0.87%, respectively; Fig 2, C). In fact,

they were essentially the same as naive mice (0.38%). Fig 2, D, shows the reduction of basophils between weeks 9 to 14 and after FAHF-2 therapy (week 18). This reduction persisted until 4 weeks posttherapy. However, FAHF-2 treatment did not affect the density of FcεRI expression (Fig 2, E). The CD49b+FcεRI+ basophil criteria were also employed to detect the difference between FAHF-2 treated PNA mice and PNA sham mice and the basophil results were similar to what was observed using FcεR+CCR3-Gr- as markers (see the Results section and Fig E1 in this article's Online Repository at www.jacionline.org).

FAHF-2 reduced the number and FcεRI expression of peritoneal mast cells

To determine the effect of FAHF-2 on mast cell numbers and FcεRI expression, mice were killed after the final challenge (week 18), and peritoneal cavity cells were lavaged from naive, sham-treated, and FAHF-2-treated mice. The number of mast cells (c-kit⁺ cells) and their FcεRI expression were determined. As shown in Fig 3, A, the percentage of mast cells in peritoneal cavities of sham-treated mice was increased compared with naive mice ($2.68\% \pm 0.27\%$ in sham-treated vs $0.98\% \pm 0.14\%$ in naive mice). Peritoneal mast cells in FAHF-2-treated mice were $1.9\% \pm 0.16\%$, significantly lower than in sham-treated mice ($P = .014$; Fig 3, A). FcεRI expression was also reduced (Fig 3, B and C).

In addition, we determined mast cell numbers and extent of degranulation in skin and intestinal tissues histologically (see the section "Histologic assessment of mucosal and cutaneous mast cells degranulation" in this article's Methods in the Online Repository). Unlike peritoneal mast cells, there were no differences in numbers of intestinal or cutaneous mast cells between FAHF-2-treated and sham-treated groups (data not shown). Consistent with our previous findings,^{8,22} the percentage of degranulated mast cells in tissues from FAHF-2-treated mice was significantly lower than in sham-treated mice ($11.7\% \pm 2.1\%$ vs $41.3\% \pm 2.5\%$; $P < .001$; Fig 3, D and E). We also found a trend of reduction of percentage of degranulation of intestinal mast cells (FAHF-2 vs sham, $P = .053$; data not shown).

FAHF-2 inhibited IgE-mediated MC/9 mast cell proliferation and degranulation

Binding of monomeric IgE to its high-affinity receptor (FcεRI) promotes mouse mast cell survival and growth.^{13,14,23} We hypothesized that FAHF-2 may inhibit mast cell proliferation. As found in our preliminary study, FAHF-2 at 20 μg/mL was not cytotoxic to MC/9 cells. Therefore, this concentration was used in the following experiments. MC/9 cells were incubated with FAHF-2 for 24, 48, and 72 hours, and viable cells were counted at each time point. As shown in Fig 4, A, the number of mast cells increased at each time point, demonstrating proliferation. Addition of FAHF-2 caused no statistically significant decrease of proliferation. Addition of anti-DNP IgE significantly increased mast cell proliferation at both 48 hours and 72 hours compared with medium alone ($P < .01$; Fig 4, A). Coculture with FAHF-2 significantly reduced anti-DNP IgE-induced proliferation at 72 hours ($P < .01$; Fig 4, A). These results indicate that FAHF-2 specifically inhibited IgE-stimulated mast cell proliferation. No cytotoxicity was detected by using trypan blue exclusion assay (data not shown).

We next determined the effect of FAHF-2 on IgE receptor cross-linking-induced mast cell degranulation. MC/9 cells were sensitized by incubation with FAHF-2 in the presence of mouse anti-DNP IgE. Cells were then activated by addition of 100 ng/mL anti-DNP IgE. Histamine levels were determined by ELISA. As shown in Fig 4, B, anti-DNP IgE (first bar) after DNP-challenge induced significant release of histamine. FAHF-2 (second bar) significantly decreased specific antigen-mediated histamine release ($14.5\% \pm 1.2\%$ vs $35.8\% \pm 2.9\%$; $P < .001$). Spontaneous release after DNP only (third bar), nonspecific antigen conalbumin challenge (fourth bar), unspecific antigen conalbumin plus FAHF-2 (fifth bar), and conalbumin only (sixth bar) were all less than 5%.

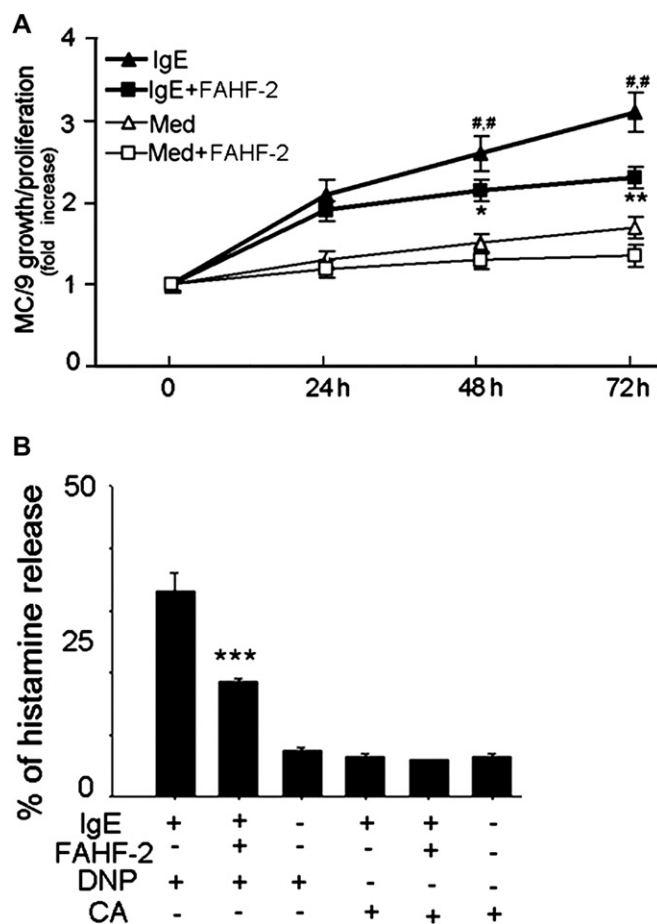


FIG 4. FAHF-2 inhibited mast cell proliferation and degranulation. **A**, Mast cell proliferation. MC/9 cells were cultured, and viable cells were counted at indicated time points. Cell numbers are expressed as fold increase versus day 0. Data are shown as means $\pm$ SEMs of 3 individual experiments. * $P < .05$, ** $P < .01$ versus IgE alone. ## $P < .01$ versus medium (Med) alone. **B**, Mast cell degranulation. MC/9 cells were cultured and then challenged at different conditions as indicated. Supernatant and total cell histamine levels were determined. Data expressed as percentage of histamine release (means $\pm$ SEMs of 3 individual experiments). *** $P < .001$ versus IgE + DNP without FAHF-2 (first bar). CA, Conalbumin.

FAHF-2 blocked IgE-induced upregulation of FcεRI expression by MC/9 cells

IgE upregulates mast cell FcεRI expression *in vitro* and *in vivo*.^{13,24} We hypothesized that FAHF-2 might directly suppress IgE-mediated upregulation of FcεRI expression. To address this possibility, we used the well characterized murine mast cell line MC/9 treated with DNP-specific IgE. As expected, IgE augmented FcεRI expression by MC/9 cells (Fig 5, A). Addition of FAHF-2 to the cultures attenuated this augmentation. FcεRI MFI was reduced 37% by 10 μg/mL and 53% by 20 μg/mL FAHF-2 compared with controls (Fig 5, A).

We next determined FcεRI subunit mRNA expression by real-time qPCR. Receptor surface expression could not be measured because of the lack of a commercially available antibody against murine FcεRI subunits. As shown in Fig 5, B, mRNA levels of the FcεRI γ subunit were significantly enhanced by anti-DNP IgE compared with the medium-alone group ($P < .001$). IgE-mediated upregulation of the γ subunit was significantly attenuated by FAHF-2 ($P < .05$). FcεRI α and β subunit mRNA

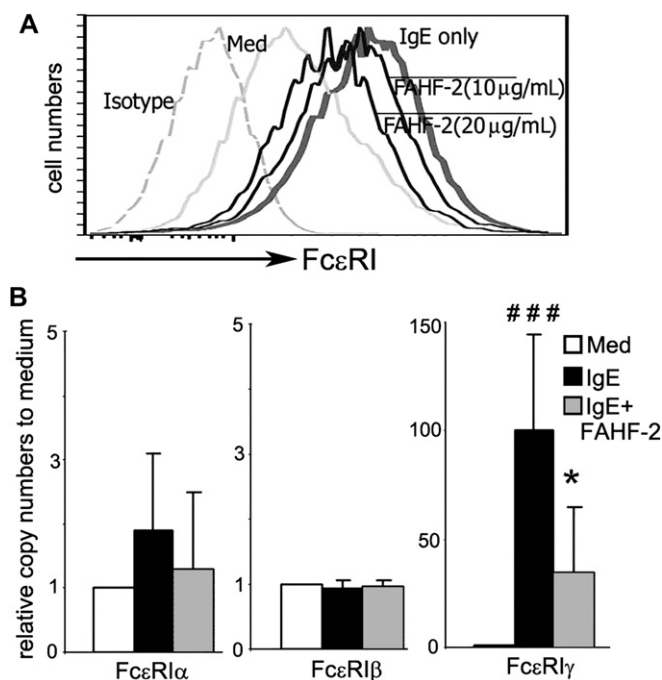


FIG 5. FAHF-2 treatment *in vitro* reduced the expression of surface FcεRI and mRNA levels of FcεRI γ subunit by the mast cell line MC/9. **A**, Flow-cytometry detection of mast cell surface FcεRI expression. Dashed line, Isotype control; gray line, medium (Med) alone; bold line, 2 μg/mL IgE (IgE only); thin lines, IgE plus 10 μg/mL and 20 μg/mL FAHF-2. **B**, Messenger levels of FcεRI subunits of MC/9 cells in the presence or absence of anti-DNP IgE with or without FAHF-2. Data are expressed as means ± SDs of 3 individual experiments (* $P < .05$ vs IgE alone, ### vs Med alone).

expression was not affected by the presence of IgE with or without FAHF-2 treatment.

FAHF-2 fraction 2 inhibited β-hexosaminidase release by RBL-2H3 and human skin mast cells

To identify active compounds, FAHF-2 was first extracted with butanol, then further divided into 4 fractions on the basis of polarity by prep-HPLC. Fraction 1 contains water soluble compounds, fraction 2 mainly alkaloids compounds, and fractions 3 and 4 mainly flavonoids and terpenoids (see the section “Extraction and isolation of fractions and compounds from FAHF-2” in this article’s [Methods](#) in the Online Repository). The RBL-2H3 cell line has been widely used as a mast cell model for IgE-mediated degranulation studies but may be more similar to basophils than other histamine-releasing cell types.¹⁸ The β-hexosaminidase assay has been widely used to monitor RBL-2H3 mast cell degranulation. We therefore used the RBL-2H3 cell line as an *in vitro* model and the β-hexosaminidase assay for testing effects of isolated fractions and identified compounds from FAHF-2 on degranulation. RBL-2H3 cells were treated with varying doses of FAHF-2 fractions (1, 2, 3, and 4) for 24 hours before sensitization by anti-DNP IgE. **Fig 6, A**, shows that fraction 2 at 18 μg/mL and 36 μg/mL (third and fourth bars) significantly reduced β-hexosaminidase release compared with untreated control (first bar; $P < .01$ -.001; **Fig 6, A**) in a nontoxic manner (data not shown). Fractions 1, 3, and 4 did not result in significant inhibition (**Fig 6, A**). In view of the inhibitory effects of fraction 2 on RBL-2H3 cells, we examined its effects on human skin mast cells. Fraction 2 also

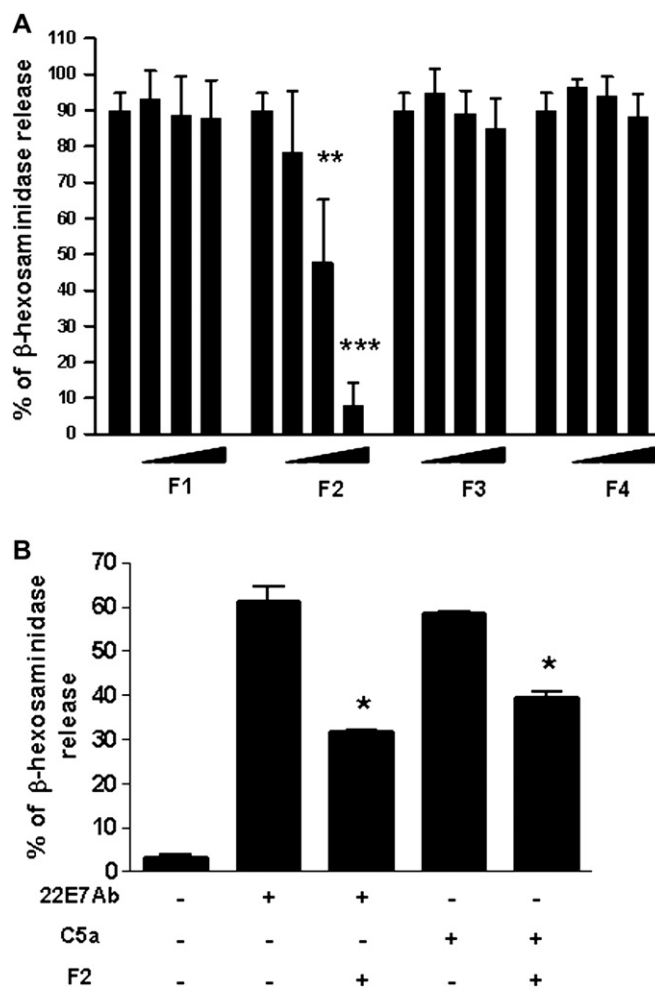


FIG 6. Effect of active fractions of FAHF-2 on RBL-2H3 cells and human skin mast cells. **A**, Effect of fractions at different doses fraction 1 (F1; 0, 18, 36, 72 μg/mL), fractions 2 and 3 (F2, F3; 0, 9, 18, 36 μg/mL), and fraction 4 (F4; 0, 18, 36, 72 μg/mL) of FAHF-2 on β-hexosaminidase released by RBL-2H3. **B**, Effect of F2 on human skin mast cells. β-Hexosaminidase release was triggered by mAb 22E7 or C5a. Data are means ± SDs of 3 experiments. * $P < .05$, ** $P < .01$, *** $P < .001$ versus untreated control.

inhibited human skin mast cell degranulation triggered by IgE cross-linking and C5a ($P < .05$ for both; **Fig 6, B**).

Berberine, palmatine, and jatrorrhizine effects on β-hexosaminidase release, Syk signaling pathway, and IgE binding in RBL-2H3 cells

By using liquid chromatography mass spectrometry, we identified 3 major alkaloid compound peaks (11, 13, and 15) that corresponded to jatrorrhizine, palmatine, and berberine (**Fig 7, A**) in fraction 2. We then tested effects of these compounds on RBL-2H3 cells. **Fig 7, B**, shows that β-hexosaminidase release in the presence of berberine at 5 μg/mL and 10 μg/mL (fourth and fifth bars), palmatine at 40 μg/mL (seventh bar), and jatrorrhizine at 20 μg/mL and 40 μg/mL (sixth and seventh bars) were significantly inhibited compared with untreated cells (first bars; $P < .05$ -.001), in a nontoxic dose-dependent manner (**Fig 7, C**). Furthermore, we determined effects of the combination of berberine, palmatine, and jatrorrhizine on Syk, an IgE-mediated FcεRI early signaling event, focusing on Syk, which is required for mast cell

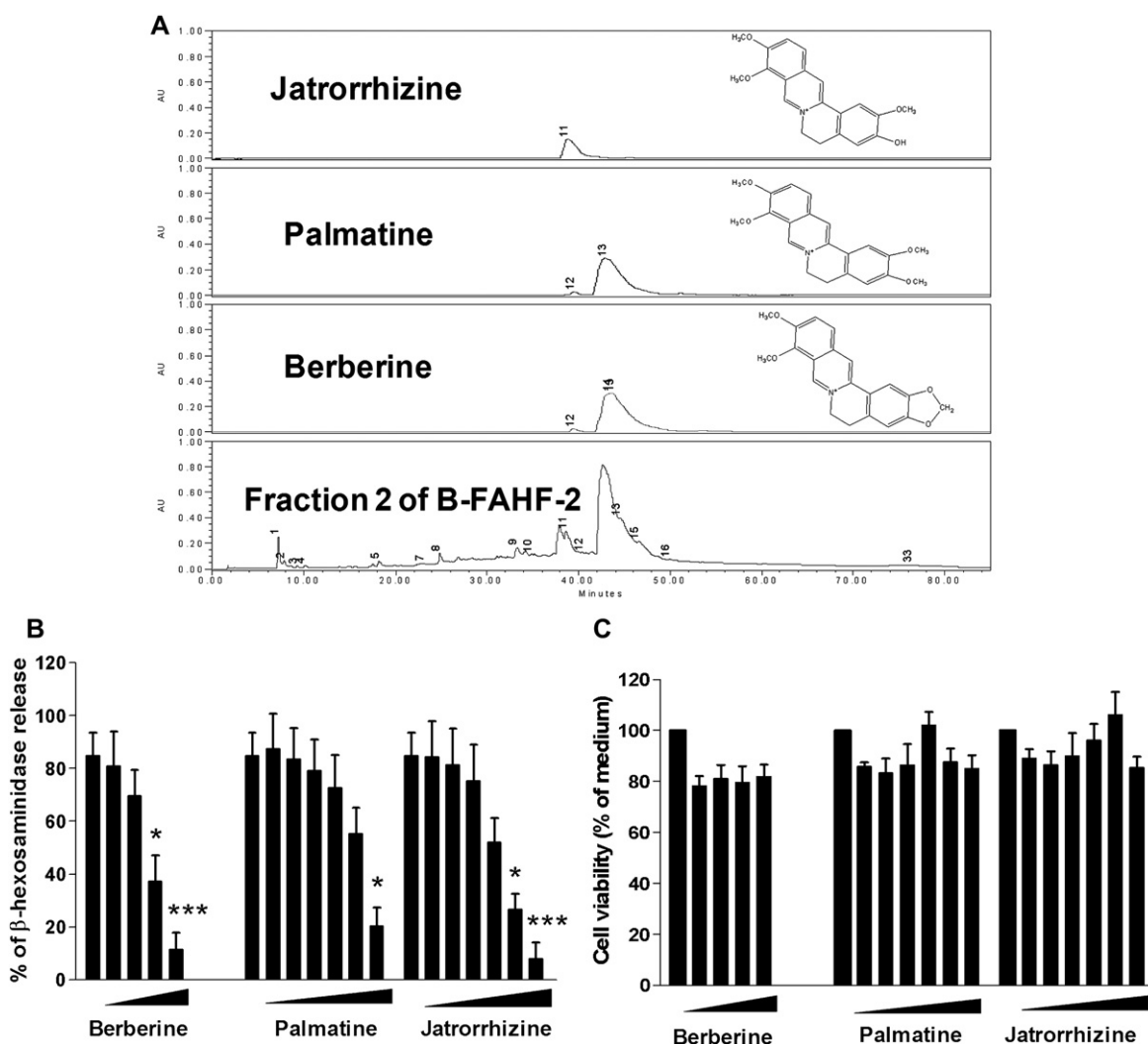


FIG 7. Major alkaloid compounds in fraction 2-inhibited mast cell degranulation in RBL-2H3 cells. **A**, Identification and chemical structures of berberine, palmatine, and jatrorrhizine in fraction 2 of butanol FAHF-2 (B-FAHF-2). **B**, Dose-dependent responses to berberine, palmatine, and jatrorrhizine by RBL-2H3 cells. **C**, Cell viability. Berberine, palmatine, and jatrorrhizine toxic effects on RBL-2H3 cells using MTT assay. * $P < .05$, *** $P < .001$ versus untreated control.

degranulation. Phosphorylation of Syk in antigen-stimulated RBL-2H3 cells was significantly inhibited by the combination of these compounds (Fig 8, A and B). To clarify further the mechanisms of inhibition of RBL-2H3 cell degranulation by berberine, palmatine, and jatrorrhizine, we examined the effect of these compounds on IgE binding to RBL-2H3 cells by using the method as previously established²⁵ and described in the section “IgE binding test by flow cytometry” in this article’s [Methods](#) in the Online Repository. Coculture of cells with mouse IgE in the presence of berberine, palmatine, and jatrorrhizine (Fig 8, C, purple, brown, and blue lines) showed no difference in IgE binding to mast cells from cells cultured with mouse IgE alone (Fig 8, C, green line), demonstrating that berberine, palmatine, and jatrorrhizine did not inhibit IgE binding to the mast cells.

DISCUSSION

Peanut allergy is a type I hypersensitivity. During the sensitization phase, peanut allergen presented by antigen-presenting cells to

T cells stimulates B cells to produce specific IgE that binds to effector cells such as basophils and mast cells. During the activation phase, after antigen challenge, effector cells degranulate and release mediators such as histamine that lead to clinical symptoms. In this study, we demonstrated that FAHF-2 protected mice with peanut allergy against oral peanut challenge-induced anaphylaxis, and that this effect is persistent, as previously reported.⁴ FAHF-2-treated animals exhibited normal body temperature, no allergy symptoms, and no increased histamine release. In this study, FAHF-2 was administered at week 8, at which time peanut hypersensitivity was established.⁴ This simulates a potential human treatment regimen to prevent food-induced anaphylaxis. We further demonstrated that FAHF-2 treatment reduced the number of peripheral blood basophils and peritoneal mast cells and cutaneous mast cell degranulation. Basophil numbers began to decrease after 1 week of treatment, reached their nadir at the end of treatment (week 14), and remained lower for at least 4 weeks posttreatment (week 18). At that time, the number of peritoneal mast cells was also significantly reduced, as was their expression of FcεRI.

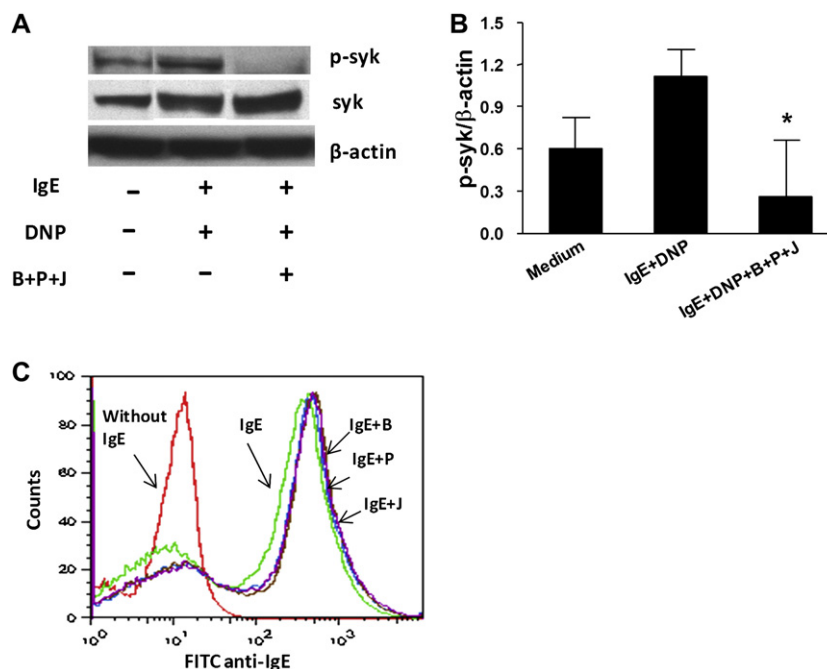


FIG 8. Berberine (B), palmatine (P), and jatrorrhizine (J) effects on Syk signaling pathway and IgE binding in RBL-2H3 cells. **A**, Downregulation of Syk-phosphorylation in IgE-triggered RBL-2H3 cells by berberine, palmatine, and jatrorrhizine (B+P+J). Bands are representative of 3 individual Western blot experiments. **B**, Quantitative assay by densitometry of syk-phosphorylation (p-syk)/β-actin ratio. All data are means ± SDs of 3 individual experiments. **P* < .05, DNP + IgE + B+P+J versus DNP + IgE. **C**, Effects of B, P, or J on IgE binding to RBL-2H3 cells. Red, Cells only without IgE; green, cells with IgE; purple, brown, blue, B, P, J + IgE.

In our previous publications, and in this study (data not shown), we found a significant reduction in peanut-specific IgE levels after 4 weeks of treatment.⁴⁻⁶ Previous publications reported that binding of monomeric IgE to FcεRI promotes mouse mast cell/basophil survival and function.^{3,14,23} Therefore, decreased peanut-specific IgE levels could play a role in decreased mast cell and basophil levels after 7 weeks of treatment with FAHF-2 in this model. However, the finding that peripheral blood basophil numbers reduced as early as 1 week of treatment, before IgE reduction, suggests that additional mechanisms may coexist. To test the possibility that FAHF-2 may also directly suppress basophil and mast cell activities, we used the MC/9 mast cell line as an *in vitro* model and showed that MC/9 cells treated with FAHF-2 showed significantly suppressed anti-DNP IgE-induced cell proliferation and histamine release on DNP challenge in a noncytotoxic manner. FAHF-2 also significantly suppressed anti-DNP IgE-induced FcεRI expression. These results suggested that FAHF-2 suppression of mast cell and basophil numbers and activation contributes to FAHF-2's persistent protection against peanut anaphylaxis.

Mast cell activation is triggered by binding of IgE to the high-affinity receptor, FcεRI. Rodent FcεRI is a tetrameric receptor that consists of 1 α chain, unique to this receptor, 1 β chain, and 2 disulfide-linked γ chains. Our *in vitro* study showed that FAHF-2 treatment suppressed γ subunit FcεRI expression by MC/9 cells. These data together with the *in vivo* findings that peritoneal mast cells exhibited significantly reduced FcεRI expression suggest that FAHF-2 suppression of mast cell growth and activation may be through suppression of mast cell high-affinity receptors and specific suppression of the signaling γ chain. In this study,

we found FAHF-2 reduced mast cell FcεRI expression, but peripheral blood basophil FcεRI expression was unaffected, although basophil numbers were significantly reduced. The mechanisms of basophil reduction were not determined. One possibility is FAHF-2 suppression of IL-3 and/or IL-33 receptors, because activation of these receptors are important for basophil maturation, survival, and migration.²⁶ Further investigation is required.

FAHF-2 is a water extract of 9 herbs. Previously, we began to determine how FAHF-2 acts on the allergic immune response in the murine model of peanut allergy at a single-component herb level.⁹ While FAHF-2 blocked histamine release, significantly reduced peanut-specific serum IgE and IL-4, IL-5, and increased IgG_{2a} and IFN-γ levels, no individual herb affected all these aspects. These results suggested that component herbs of FAHF-2 may work synergistically to produce the therapeutic effects produced by the whole formula. In this study, we focused on identification of active compounds in FAHF-2 that affect mast cells by using rat RBL-2H3 cells, widely used in previous studies.^{18,19} We found that the alkaloid-rich fraction 2 inhibited RBL-2H3 cell degranulation and human skin mast cell degranulation activated by FcεRIα-specific mAb 22E7 as well as the complement pathway activator C5a. We demonstrated for the first time that 3 major alkaloid compounds (berberine, palmatine, and jatrorrhizine) in FAHF-2 showed dose-dependent responses, with berberine the most effective. Interestingly, the mixture of 3 compounds showed a synergistic action (data not shown), which was associated with suppression of the phosphorylation of Syk in antigen-stimulated RBL-2H3 cells. A previous study showed honeybee-collected pollen inhibition of mast cell degranulation associated with

inhibition of IgE binding to mast cells, but no effect on FcεRI expression.²⁵ However, our study showed that FAHF-2 compounds did not affect IgE binding to mast cells. This finding suggests that FAHF-2 inhibition of IgE-mediated mast cell degranulation is less likely via “a steric hindrance” or “competes off the IgE” phenomena.^{27,28}

In summary, we demonstrated for the first time that FAHF-2 reduces basophil and mast cell numbers in a murine model of peanut allergy. FAHF-2 also suppressed FcεRI expression *in vivo* and directly suppressed IgE-induced mast cell growth, and activation *in vitro*, which may be a result of suppression of FcεRI γ subunit expression. The 3 major alkaloid compounds may contribute to FAHF-2 inhibition of mast cells/basophils activation.

We acknowledge Dr H. A. Sampson for his support for this study.

Clinical implications: FAHF-2 blocked peanut anaphylaxis. This effect was associated with reduction of mast cells/basophils activation and proliferation. FAHF-2 may be a novel antipeanut anaphylaxis therapy.

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METHODS

FAHF-2 formula and product

Dried aqueous extract of FAHF-2, produced in a good processing practice-certified facility (Xiyan Chinese Medicine Research Pharmaceutical Manufacturer, Beijing, China), was obtained from Beijing Shen Hua Shi Di Medical Technology, Beijing, China, and stored at room temperature. The quality of the raw herbs was established and described in detail in previous publications.^{E1,E2} In brief, on the basis of organoleptic and microscopic examination, the raw herbal materials used in FAHF-2 were identified as the fruits of *Prunus mume*, the skin of the fruits of *Zanthoxylum schinifolium*, the roots of *Angelica sinensis*, the rhizome of *Zingiber officinalis*, the twigs of *Cinnamomum cassia*, the bark of *Phellodendron chinense*, the rhizome of *Coptis chinensis*, the roots of *Panax ginseng*, and the fruiting body of *Ganoderma lucidum*. Product quality was monitored by HPLC fingerprinting according to the US Food and Drug Administration's Guidance for Industry Botanical Drug Products^{E3} and as described in a previous publication.^{E2} The results of safety testing, including heavy metals, pesticide residue, and microbes all met the standards for botanical products.^{E4-E6} FAHF-2 and peanut preparations were tested for endotoxin by using the Pyrogen Plus assay kit (Lonza, Mass). They were both undetectable with a low limit of detection of <0.03 endotoxin unit/mL.^{E2}

Extraction and isolation of fractions and compounds from FAHF-2

Powered FAHF-2 extract (5 g) was dissolved into distilled water (125 mL). The solution was sonicated for 10 minutes followed by vortexing. This procedure was repeated 3 times. The solution was transferred to a separatory funnel and extracted with an equal volume of butanol (125 mL; Fisher Scientific, Pittsburgh, Pa). After the separation, the organic layer was removed and saved. The aqueous layer was re-extracted with butanol 3 more times. All separated butanol extracts were combined and washed with distilled water at a ratio of 3:1. The butanol extracts were then evaporated by using the Rotorvapor R-210 (Buchi Corp, New Castle, Del) under reduced pressure. The dried extract butanol FAHF-2 (B-FAHF-2) was collected and stored at 4°C.

Subfractions of B-FAHF-2 were collected by using the prep-HPLC system coupled with a UV detector (Waters, Milford, Mass). Briefly, methanol-dissolved B-FAHF-2 was loaded onto prep-HPLC with an Xbridge C18 reverse-phase column 150 mm × 19 mm × 5 μm (particle size) from Waters. The mobile phases used were 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The separation gradient started at 97% of A to 75% of A for 10 minutes and to 55% of A in another 20 minutes at a flow rate of 20 mL/min. Four subfractions were collected on the basis of their polarities.

The liquid chromatography mass spectrometry system and commercially available standards were used to analyze the constituents in subfraction 2 of B-FAHF-2. Berberine and palmatine were purchased from Sigma-Aldrich (St Louis, Mo). Jatrorrhizine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). The exact mass of each constituent was measured by a high-resolution micromass LCT premier time-of-flight mass spectrometer (Waters) coupled to a Waters Alliance 2695 HPLC system. The mass spectra range was set to be mass-to-charge ratio 50 to 1000. The mass spectrometer was set in the positive mode with source conditions as follows: capillary voltage, 3200 V; cone voltage, 25 V; desolvation gas flow, 500 L/h; cone gas flow, 40 L/h; desolvation temperature, 350°C; and source temperature, 110°C. All spectra were obtained with an internal calibrant (lock mass with leucine enkephalin as reference).

Assessment of systemic anaphylactic symptoms

Anaphylactic symptoms were evaluated approximately 30 minutes after the challenge dose by using a previously described scoring system^{E7}: 0, no signs; 1, scratching and rubbing around the snout and head; 2, puffiness around the eyes and snout, redness around snout, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsions; and 5, death. Rectal temperatures were measured approximately 30 minutes after challenge by using a thermal probe (Harvard Apparatus, Newark, NJ).

Histologic assessment of mucosal and cutaneous mast cells degranulation

For assessment of mucosal mast cell degranulation, sham-treated and FAHF-2-treated mice were killed 40 hours after the final challenge at week 18. The proximal jejunum from each mouse was snap-frozen in optimal cutting temperature medium, and 8-μm transverse sections each were fixed in Mota fixative containing lead acetate and stained with acidic toluidine blue. Mast cells were counted at ×400 magnification. Cutaneous mast cell degranulation during systemic anaphylaxis was assessed by examination of skin samples collected after challenge as described previously.^{E7}

Flow-cytometry measurement of peripheral blood basophil numbers by using FcεRI +CD49b⁺ as a marker to identify mouse basophils

In addition to the previous method using the FcεRI +CCR3⁺Gr⁻,^{E8-E10} other methods such as FcεRI +CD49b⁺ have been used recently as markers to identify mouse basophils.^{E11-E13} To validate further the study on peripheral blood basophils, and to determine prolonged inhibitory effect of FAHF-2 on basophils, we conducted additional sets of experiments in which mice were sensitized with peanut and CT as previously described.^{E2,E14} FAHF-2 treatment began at week 16 (32 mg in 0.5 mL water, twice a day i.g) for 10 weeks. Blood samples of approximately 100 μL were collected from each mouse 23 weeks after FAHF-2 therapy. PBLs were processed, and Fcγ receptors were blocked with anti-CD16/32 mAb (2.4G2). PE-conjugated anti-CD3, PE-conjugated anti-B220, FITC-conjugated FcεRI, and APC-conjugated CD49b were added to the cell suspension. CD49b⁺ FcεRI + CD3⁻B220⁻ cells were defined as mouse blood basophils.^{E13} Data were analyzed by using Flowjo software (Tree Star, Inc, Ashland, Ore).

Quantifying FcεRI subunit mRNA levels by real-time PCR (qPCR)

Real-time reverse transcription qPCR was performed by using a SYBR green protocol and an ABI7900 HT machine (Applied Biosystems, Foster City, Calif) as previously described.^{E15} Briefly, 1 × 10⁶ MC/9 cells were cultured in the presence or absence of FAHF-2 (20 μg/mL) for 24 hours. Cells were then exposed to 2 μg/mL mouse anti-DNP IgE for 5 hours. Cells were harvested, and RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. The RNA was then deoxyribonuclease-treated (Invitrogen). cDNA was prepared from these samples by using oligo-dT primers (Invitrogen) and Omniscript reverse transcript (QIAGEN, Valencia, Calif). The cDNA was used to quantify the mRNA levels of FcεRI α, β, and γ subunits by using real-time PCR. The primers used were as follows: FcεRIα (sense primer, 5'-TGGGAACAATCACCTTCAAA-3, and antisense, 5'-CAGCCAATCTT GCGTTACAT-3'), FcεRIβ (sense, 5'-CATTAAAGGTC-CAGACACTCCA-3', and antisense, 5'-CGTCCCATATCAATTACCCA-3'), and FcεRIγ (sense, 5'-CGCAGCTCTGTATATCTTG-3', and antisense, 5'-ACAGCATCTGCTTTCTCACG-3'). The primers were designed on the basis of National Center for Biotechnology Information accession numbers NM01084 (α subunit), AB033617 (β subunit), and NM0185 (γ subunit).

Western blot

Determination of early FcεRI-mediated signal events was performed by Western blot analysis using anti-Syk and antiphospholated Syk antibodies (Cell Signaling, Danvers, Mass) as described previously.^{E16} Briefly, IgE-sensitized RBL-2H3 cells (7 × 10⁶) were cultured with or without berberine, palmatine, and jatrorrhizine for 24 hours and stimulated with DNP (150 μg/mL) for 5 minutes. The cells were then lysed following the manufacturer's instructions for the Nuclear/Cytoplasmic Extract Kit (Active Motif, Carlsbad, Calif), and 50 μg whole-cell proteins was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. After overnight blocking with 5% fat-free milk in phosphate buffered saline Tween-20, the membranes were incubated with anti-Syk/phospho antibody for 2 hours. After washing with PBST, the membranes were incubated with peroxidase-conjugated antirabbit IgG. Immunoreactive bands were visualized by using the ECL Western detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

IgE binding test by flow cytometry

An IgE binding test was performed as described previously.^{E17} RBL-2H3 Fc γ receptors were first blocked with anti-Fc γ R2/3(2.4G2). A 20 μ g/mL volume of berberine, palmatine, or jatrorrhizine and then 1 μ g/mL mouse IgE antibody were incubated with RBL-2H3 (1×10^6 /mL) at 4°C for 1 hour. Cells were washed twice with PBS. FITC-antimouse IgE was added to detect binding of IgE on RBL-2H3 cells (4°C, 30 minutes) and then analyzed. Cells were incubated without IgE in the presence or absence of berberine, palmatine, or jatrorrhizine, and then stained with FITC-antimouse IgE for use as negative controls.

RESULTS

Reduction of basophil number and anaphylactic reactions

In additional set of experiments, using the CD49b⁺Fc ϵ RI⁺ basophil criteria, numbers of basophil in FAHF-2-treated mice remained significantly lower than sham-treated mice 23 weeks after treatment (Fig E1, A and B; $P < .01$). Consistent with these findings, FAHF-2-treated mice were protected after peanut challenge at this time point (Fig E1, C-E; $P < .05$ for all).

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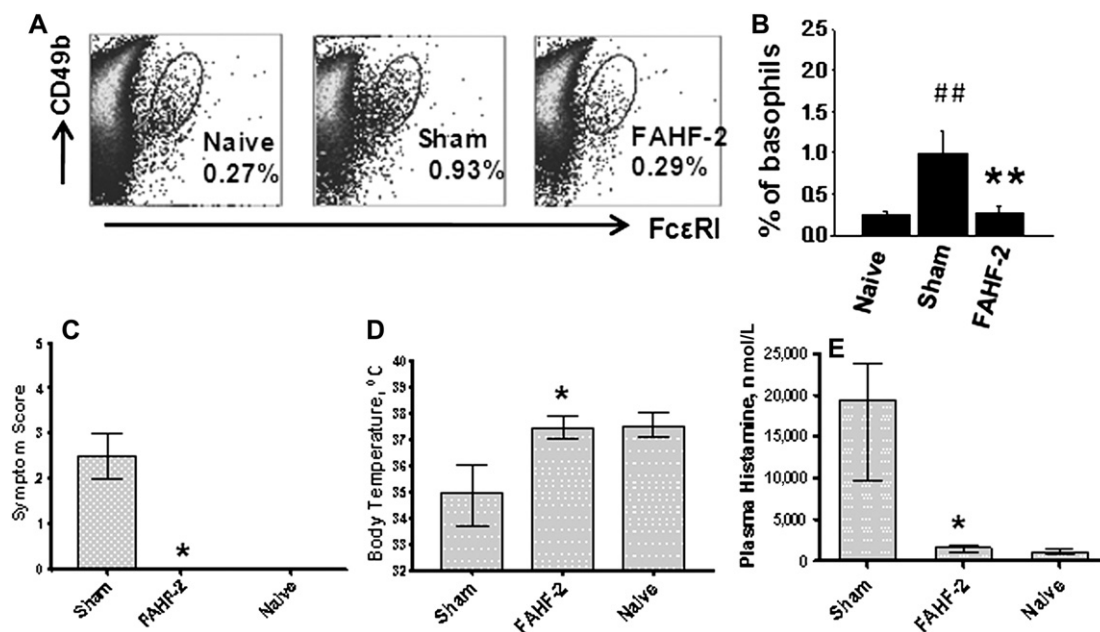


FIG E1. FAHF-2 induced prolonged inhibition of peripheral basophils and protection against peanut anaphylaxis. **A**, Dot plots are representative of individual mouse blood basophils from each group 23 weeks posttherapy. **B**, Bar graph shows means $\pm$ SEMs of 3 individual mice from each group. Data were analyzed by 1-way ANOVA followed by the Bonferroni test. $**P < .01$, sham versus FAHF-2; $##P < .01$, sham versus naive. **C-E**, Clinical reactions 30 minutes after oral peanut challenge. **C**, Anaphylactic symptom score. **D**, Rectal temperature. **E**, Plasma histamine levels. Data were analyzed by Kruskal-Wallis 1-way ANOVA on ranks followed by the Student-Newman-Keuls method. Bars show median; error bars show range. $n = 4$. $*P < .05$, sham versus FAHF-2.