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A chimeric human-cat Fcγ-Fel d1 fusion protein inhibits systemic, pulmonary, and cutaneous allergic reactivity to intratracheal challenge in mice sensitized to Fel d1, the major cat allergen

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Abstract Co-aggregation of FcRI with FcγRIIb can block FcRI-mediated reactivity and Fc gamma:allergen chimeric proteins, by co-crosslinking FcγRIIb to allergen-specific IgE bound to the FcRI can block allergen-specific reactivity. We evaluated whether a human cat chimeric fusion protein (GFD) composed of part of the human Ig G1 Fc fused to the major cat allergen (Fel d1) would function as allergen immunotherapy while not inducing acute allergic reactivity in mice sensitized to Fel d1. Injection of GFD 6 h prior to Fel d1 challenge acutely blocked systemic and skin reactivity to Fel d1 challenge while mice given subcutaneous immunotherapy with GFD at days 37, 38, and 39 showed inhibition of systemic, lung, and cutaneous reactivity to Fel d1 2 weeks later. GFD immunotherapy did not induce systemic reactivity. Overall, the Fcγ-Fel d1 chimeric fusion protein blocked Fel d1-induced IgE-mediated reactivity but did not induce in vivo mediator release on its own; suggesting that this approach using allergen combined with Fc gamma1 so as to achieve inhibitory signaling may provide an enhanced form of allergen immunotherapy.

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Introduction

For nearly 100 years, parenteral administration of allergens as immunotherapy has been used as a disease modifying therapy in allergic respiratory diseases [1,2]. However, even when given in a cautious and protracted schedule, standard allergen immunotherapy gives rise to local and systemic allergic reactions and carries the risk of eliciting rare but life-threatening reactions [3,4]. Furthermore, immunotherapy with food allergens has proven to be both dangerous and of limited efficacy [5,6]. Thus, there is great interest in the development of novel forms of allergen immunotherapy.

Allergen exposure, be it from natural sources or as a result of immunotherapy, results in cross-linking of FcRI, which activates tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the β- and γ-FcRI subunit cytoplasmic tails and leads to cell activation and degranulation in basophils and mast cells [7]. This, in turn, leads to the classic immediate hypersensitivity reaction. Human mast cells and basophils also express FcγRIIb, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIMs) within its cytoplasmic tail [8,9]. Co-aggregation of FcRI to FcγRIIb has been shown to block in vitro and in vivo human basophil and mast cell function [10—13]. Our previously reported studies experiments have shown that this inhibition is mediated via the reduction in the tyrosine phosphorylation of Syk, ERK, and several other cellular substrates and increased tyrosine phosphorylation of the adapter protein downstream of kinase (Dok) growth factor receptor-bound protein 2 (Grb2) and SH2 domain containing inositol 5-phosphatase (SHIP) [8,14].

We set out to test in vivo whether, by driving co-aggregation of FcγRIIb and FcRI, an allergen molecule combined with a human Fcγ region would: (1) block allergen driven reactivity, (2) fail to function as an allergen, and (3) serve as an immunogen for allergy immunotherapy. We employed a novel chimeric human: cat protein composed of part of the human IgGγ1 Fc region fused to Fel d1, the major allergen produced by domestic cats. Previous experiments had shown that Fcγ-Fel d1 chimeric fusion protein (GFD) inhibited allergen-driven IgE-mediated mediator release in vitro from human basophils and cord blood-derived mast cells, and in vivo in passive cutaneous anaphylaxis in FcRIα transgenic mice sensitized with human IgE to Fel d1 [14].

In the current study, we tested whether mice actively sensitized to Fel d1, when treated with the gamma-Fel d1 fusion protein, were protected from local, airway, and systemic reactivity. A single systemic administration of GFD could block type I allergic reactivity acutely, while subcutaneous immunotherapy with GFD at days 37, 38, and 39 showed inhibition of systemic, lung, and skin test reactivity, at the same time. GFD itself did not trigger allergic reactivity in Fel d1-sensitized mice even when given in up to 5-fold higher levels than Fel d1.

Materials and methods

Animals

Male Balb/c mice were purchased from Harlan Sprague—Dawley Inc. (Indianapolis IN) and housed in the UCLA Vivarium under specific pathogen-free conditions. The Chancellor's Animal Research Committee approved all of the animal studies as adhering to the guidelines set forth by the National Institutes of Health. Mice were 6—8 weeks of age at the initiation of each experiment.

Gamma Fel d1 (GFD) fusion protein

The Fcγ-Fel d1 chimeric fusion protein was produced by expression of a chimeric gene consisting of the genomic human IgGγ1 constant region from the hinge through CH3, a 15 amino acid linker, and a combined Fel d1 chain 1 and 2 construct kindly provided by Drs. Amanda Sun and Paul Guyre at Dartmouth Medical School as described [14]. SDS-PAGE demonstrated that the Fcγ-Fel d1 fusion protein was expressed as the predicted ~140 dimer. Western-blot analysis and ELISA demonstrated that both antibodies specific for the human γ chain and Fel d1 antigen recognized the GFD protein.

Fel d1 sensitization and GFD treatment protocols

Protocol 1

On days 0 and 14, mice were sensitized by means of an IP injection of 5 μg of purified natural Fel d1 (Indoor Biotechnology Inc., Charlottesville, VA) emulsified in 2 mg of alum hydroxide (Alhydrogel; Brenntag Biosector, Denmark) in a total volume of 160 μl. On days 28, 29, 30, and 33, the sensitized mice were boosted with an intratracheal (IT) administration of 1 μg of native Fel d1. As immunotherapy in this protocol, native Fel d1 (5, 10, and 20 μg/100 μl) or GFD (10, 20, 40 μg/100 μl) in normal saline was given SQ on days 37, 38, and 39, while control treatment consisted of the same volume of saline alone (Fig. 1A). Twice the dose of GFD was used for Fel d1, as the molecular weight of GFD is approximately twice that of Fel d1. Animals were given an IT challenge with 1 μg of native Fel d1 on days 40, 47, and 54. Systemic reactivity (temperature) was examined at day 54, airway hyper-responsiveness (AHR), airway, and lung allergic inflammation (e.g., eosinophilia) were tested on day 56, and skin sensitization was examined on day 60.

Protocol 2

Mice were sensitized by IP injection of either 1 or 10 μg of purified natural Fel d1 emulsified in 2 mg of alum hydroxide in a total volume of 160 μl at day 0 (Fig. 1B), boosted IT with 1 μg of Fel d1 on day 28, and challenged IT with 1 μg of Fel d1 on days 35 and 49. As an acute treatment regimen, 5 μg of GFD in 50 μl of 0.9% NaCl was given by IP injection 6 h before the IT challenge with Fel d1 on day 35. For sham treatment, the same volume of saline was given IP.

Measurement of airway response to methacholine

Airway responsiveness was measured 48 h after IT challenge using a modified forced oscillation method as previously described [15—19]. Briefly, mice were anesthetized with 90 mg/kg of pentobarbital sodium injected IP and anesthesia was confirmed by the absence of response to paw pinch. The mice were then tracheostomized with a 22-gauge IV catheter (Terumomedical Corp., Elkton, MD) that was firmly tied in place. Mice were then connected to a computer-

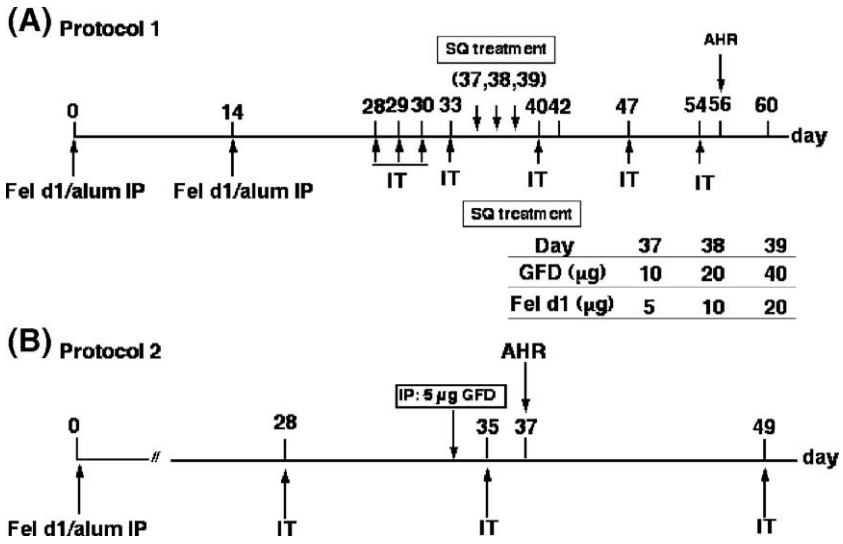

Figure 1 Sensitization protocols for Fel d1. (A) Protocol 1. On day 0 and 14, mice were sensitized by means of an IP injection of 5 μg of Fel d1 in alum. On days 28, 29, 30, 33, 40, and 47, the mice were boosted with intratracheal (IT) administration with 1 μg of Fel d1. Treatment with increasing amounts of native Fel d1 (5, 10, and 20 μg/100 μl) or GFD (10, 20, 40 μg/100 μl) in normal saline was given SQ on days 37, 38, 39, respectively. Control treatment consisted of the same volume of saline alone. Animals were challenged IT with 1 μg of Fel d1 on day 54. (B) Protocol 2. On day 0, mice were sensitized by means of an IP injection either of 1 or 10 μg of Fel d1 in alum. On days 28, 35, and 49, the sensitized mice were challenged by intratracheal (IT) administration of 1 μg of Fel d1. GFD or the vehicle (saline) was administered by IP injection 6h before the 2nd IT challenge.

controlled small animal ventilator (flexiVent®, SCIREQ, Montreal, PQ, Canada). Animals were then paralyzed with an IP injection of pancuronium bromide (0.1 μg/kg) and mechanically ventilated (100 breaths/min, tidal volume 0.4 ml against a PEEP of 1 cm H_2O, inspiratory time of 0.12 s, and expiratory time of 0.48 s). The flexiVent® ventilator was used for both regular ventilation and delivery of an oscillatory signal without the need to disturb the mice. A calibration procedure was used to remove resistance of the tracheal cannula and other tubing. Measurements of pulmonary resistance (RL) were collected by using the forced oscillatory technique (0.25—19.63 Hz). RL was obtained using the conventional primewave 8 with a peak to peak amplitude of 0.17442 ml (flexiVent®, SCIREQ, Montreal, PQ, Canada). Three measurements of RL were obtained at 10 s intervals both pre- and post-IV administration of acetyl-β-methylcholine-chloride (Methacholine, 1666 μg/kg).

Monitoring of body temperature

Changes in core body temperature associated with systemic anaphylaxis were determined by rectal temperature using a rectal probe coupled to a digital thermometer (YSI Inc., Beavercreek, OH).

Bronchoalveolar lavage (BAL)

Two days after IT challenge, mice were sacrificed and the lungs lavaged twice with 0.8 ml of PBS plus 2% fetal calf serum (FCS). The BAL fluid cells were washed with PBS (400 × *g*, 4°C, 5 min) and the pellet was resuspended in 500 μl PBS. Total numbers of BAL cells were counted in a hemacytometer after staining cells with trypan blue. We performed differential cell counts by enumerating at least 300 cells on cytocentrifuge preparations (Cytospin 3; Cytospin, Shandon Ltd., Runcorn, Cheshire, United Kingdom) stained with Wright-Giemsa staining (HEMA 3 STAIN SET, Fisher Scientific Company, Middletown, VA).

Measurement of murine anti-Fel d1 antibodies

The levels of Fel d1-specific IgE, IgG1, and IgG2a present in blood samples collected 2 days after the final Fel d1 challenge in Protocol 1 were determined by ELISA. To determine the serum levels of Fel d1-specific IgE, 96-well microtiter plates (Corning Glass, Corning, NY) were coated overnight at 4°C with 1 μg/ml Fel d1. After washing and blocking with 1% BSA-PBS, serum samples (10—40 × dilutions) and twofold dilutions of a Fel d1-specific reference standard in duplicate were placed in wells overnight. As a standard, pooled serum obtained from sensitized mice was used. Plates were then incubated with 2 μg/ml biotinylated rat anti-mouse IgE monoclonal antibody (clone R35-118, BD Pharmingen, San Diego, CA), diluted in 1% BSA-PBS for 1 h at room temperature followed by addition of avidin—horseradish peroxidase conjugate. After washing, 3,3′ ,5,5′ -tetramethylbenzidine substrate with hydrogen peroxide was added, and absorbance at 450 nm was measured by a microplate reader (Molecular Devices, Sunnyvale, CA).

To determine Fel d1-specific IgG levels, plates were coated with 1 μg/ml Fel d1. For detection, 2 μg/ml of biotinylated rat anti-mouse IgG1 monoclonal antibody (clone A85-1, BD Pharmingen, San Diego, CA) or 2 μg/ml biotinylated rat anti-mouse IgG2a monoclonal antibody (clone R19-15, BD Pharmingen, San Diego, CA) was used. All samples were compared with high titer anti-Fel d1 IgE, IgG1, and IgG2a standards consisting of a serum pool from mice highly immunized to Fel d1. Absorbance values were converted into units(U)/ml by assigning an arbitrary unit

value to this reference sera pool that contained allergen-specific IgE, IgG1, and IgG2a.

Measurement of mouse antibody to GFD

A standard solid phase immunoassay was employed to measure the level of mouse antibody to the IgG Fc portion of GFD. Human IgG1 (2 μg/ml) was used for coating plates, and mouse anti-human IgG1 (Sigma) was served as reference. Sera from untreated, Fed d1, and GFD-treated mice at various dilutions as well as the reference serum were added to wells on the coated plate and incubated for 2 h at room temperature. After washing, goat anti-mouse IgG-AP, which was used as the secondary detecting antibody, was incubated for 2 h, followed by the addition of the substrates for color development.

Assessment of active cutaneous type I hypersensitivity

In Protocol 1 (Fig. 1A), four days after the seventh IT challenge (day 60), intradermal skin testing was performed. Fel d1 (0.1 μg) or GFD (0.2 μg) was injected intradermally into the shaved skin on the back followed by IV injection of 150 μl of 0.5% Evans blue (ICN Pharmaceuticals, Inc., Costa Mesa, CA). The mast cell degranulation compound 48/80 (0.6 μg of a 1 mg/ml solution, Sigma) was used as a positive control, and saline was injected as a negative control. In Protocol 2, two days after day 37, IT challenge intradermal skin testing was performed.

Histological studies

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) or periodic acid schiff (PAS) for light microscopic examination. Pulmonary inflammation was scored by an observer who was blinded as to the nature of the samples (Grade 0; not present: Grade 1; very slight: Grade 2; moderate: Grade 3; severe).

Statistical analysis

All results except serologic responses to Fel d1 are expressed as mean ± SD. ANOVA was used to determine the levels of difference among groups. Pairs of groups were compared with the unpaired 2-tailed Student's *t* test. $P < 0.05$ was considered significant. Anti-Fel d1 antibody responses, being non-parametric, were expressed as geometric means plus the range and were analyzed by the Mann—Whitney *U* test.

Results

Mouse models of native allergen Fel d1-induced allergic airway hypersensitivity

We have previously shown with an experimental regimen that induced moderate allergic responses that GFD is capable of blocking Fel d1-mediated allergic responses in a Balb/c mice model [14]. In the current study, we set out to test: (1) whether GFD, when administered in a protocol to mimic rush immunotherapy (e.g., high dose GFD administrated in a short period), was able to inhibit Fel d1-dependent allergic responses in already highly sensitized animals, and (2) whether a single administration of GFD is sufficient to acutely block reactivity in animals with established Fel d1-induced allergic responses. For these purposes, we developed two experimental protocols. Sensitization of animals via Protocol 1 and Protocol 2 induced cutaneous reactivity, systemic allergic reactivity as measured by body temperature decrease, Fel d1-dependent AHR, and allergic airway inflammation as determined by the lung histological/BAL changes (Protocol 1 only) as shown in sensitized, untreated, and challenged mice in the experiments that follow. Protocol #1 employed multiple boosters to induce potent allergic reactivity that could primarily be employed to see if repeated GFD administration would have immunomodulatory effects. Protocol #2 induced weaker sensitization and was used primarily to examine the acute effects of GFD administration.

GFD does not induce allergic reactivity in sensitized animals

Since GFD is a fusion protein containing Fel d1, it was important to determine whether GFD itself would function as Fel d1 to induce allergic reactivity. We undertook several approaches to examine this issue in mice actively sensitized to Fel d1. As shown in Fig. 2A, IT administration of Fel d1 (1 μg) in Protocol 2-sensitized mice induced a systemic allergic reactivity as evidenced by significant body temperature drop (2.58 ± 0.4°C). In contrast, an equimolar amount of GFD (2 μg) and a fivefold higher GFD dose (10 μg) also induced a significant temperature drop in sensitized animals. In addition, IT administration of GFD did not induce AHR as was seen with Fel d1 (data not shown). Intradermal injection of Fel d1 (0.1 μg) induced mast cell degranulation in the skin of Fel d1-sensitized Balb/c mice (Fig. 2B, panel D). GFD (0.2 μg) did not induce the skin reactivity in sensitized animals (Fig. 2B, panel C). Thus, GFD failed to elicit allergic reactivity systemically in the skin or the airways of Fel d1-sensitized animals.

In Protocol 1, the animals were sensitized to Fel d1, boosted, and then treated at days 37—39 with subcutaneous therapy using Fel d1 or GFD. Change in core body temperature following treatment was assessed at day 39 to determine if the animal was adversely reacting to either treatment. Sensitized animals given Fel d1 treatment demonstrated a significant fall in body temperature (sensitized Fel d1-treated mice, Fig. 2C, $^{**}P < 0.01$, $^{***}P < 0.001$) as compared to non-treated mice. The fall in temperature was less, but far more prolonged, than that which occurred in Protocol 2. In contrast, GFD SQ treatment did not induce a fall in body temperature.

GFD blocks Fel d1-induced systemic reactivity

When Protocol 1-sensitized animals were treated with saline at days 37—39 and challenged at day 40 with IT Fel d1, they demonstrated a significant fall in body temperature (sensitized challenged mice) as compared to challenged non-

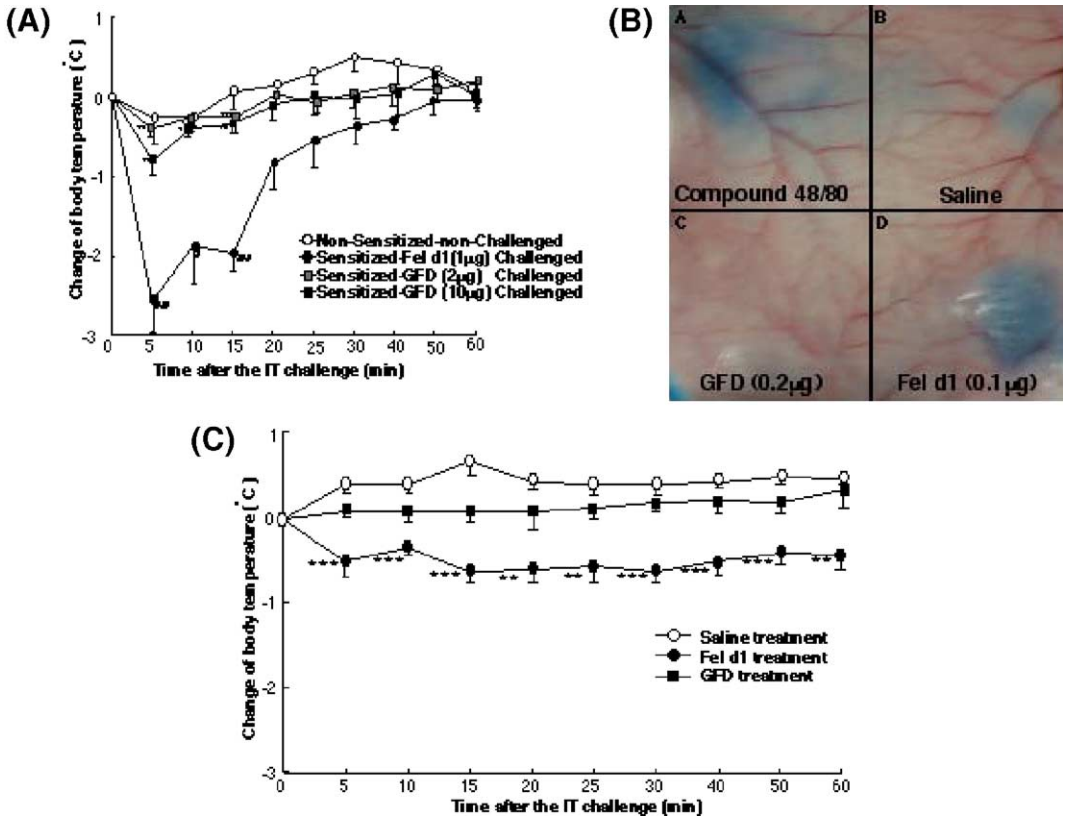

Figure 2 GFD does not induce systemic reactivity in sensitized animals. Changes in rectal temperature in non-sensitized non-challenged mice, Fel d1-sensitized and 1 μg Fel d1-challenged mice, Fel d1-sensitized and 2 μg or 10 μg GFD-challenged mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in comparison with sensitized Fel d1-challenged mice. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ in comparison with non-sensitized non-challenged mice. $n = 4$ for each group except non-sensitized non-challenged group where $n = 3$. Mice were sensitized in Protocol 2 with 10 μg of Fel and challenged IT at day 28. GFD does not induce skin reactivity in Fel d1-sensitized mice. Skin reactivity to Fel d1 (0.1 μg, panel D), GFD (0.2 μg, panel C), histamine releasing compound 48/80 as a positive control (0.6 μg, panel A), and saline as a negative control (panel B) was measured 20 min after intradermal injection. Mice were sensitized in Protocol 2 with10 μg of Fel and the testing was done at day 37. GFD SQ treatment at day 39 did not induce systemic reactivity as assessed by core body temperature. Changes in rectal temperature (mean ± SD) in saline-treated, Fel d1 SQ-treated, or GFD SQ-treated-sensitized mice are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, in comparison saline-treated mice.

sensitized mice (sensitized challenged mice, Fig. 3A). The temperature fall was maximal at 5 min (1.42 ± 0.30°C, $P < 0.01$); body temperature was gradually recovered over the ensuing 60 min. Associated with the fall in body temperature, the mice remained motionless in comparison to challenged non-sensitized control mice while piloerection of hunching over was not observed. Treatment with SQ GFD but not Fel d1 on days 37—39 completely blocked this decrease in body temperature as measured at day 40 (Fig. 3A) when compared to the sensitized challenged non-treated mice.

A similar pattern of temperature decrease was observed at day 54 in saline-treated animals following IT Fel d1 challenge (Fig. 3B). However, at that time, the animals that had been treated with either GFD or Fel d1 on days 37—39 were protected. Thus, GFD demonstrated an acute effect in its ability to block the temperature drop (day 40), while both GFD and Fel d1 treatment had a delayed effect at day 54. Given this distinction, the acute and delayed effects are likely mediated by different mechanisms.

When animals were sensitized by Protocol 2 and challenged at day 35, they also demonstrated a significant fall in body temperature immediately following the IT Fel d1 challenge, as compared to the control non-sensitized challenged animals. The maximal temperature fall occurred at 5 min (0.93 ± 0.23°C, $P < 0.05$), after which the body temperature gradually recovered over the ensuing 60 min. A similar pattern of temperature fall was observed following Fel d1 challenge in sensitized animals at day 49 as well (data not shown). Treatment with a single IP dose of 5 μg of GFD 6 h prior to the day 35 Fel d1 challenge completely blocked the decrease in body temperature ($P < 0.05$). However, administration of GFD at day 35 had no inhibitory effect on the temperature drop measured at day 49 when the mice were IT rechallenged with Fel d1; this indicates that the inhibitory effect of GFD on temperature drop did not last over 14 days. Thus, in contrast to the three subcutaneous doses of GFD in Protocol 1, the single dose of GFP was able to induce an acute effect but failed to have a delayed effect on systemic reactivity to IT Fel d1 challenge.

GFD inhibits Fel d1 driven skin test reactivity

Mice sensitized using Protocol 2 showed immediate skin reactions when injected subcutaneously with 0.1 μg of Fel

d1. When mice were given an IP injection of 5 μg GFD 6 h prior to skin testing, there was marked inhibition of this Fel d1-induced skin test reactivity, as shown by loss of bluing following Fel d1 injection (Fig. 4D, compared to Fig. 2B, panel D).

Mice sensitized using Protocol 1 also showed immediate skin reactions when injected subcutaneously with 0.1 μg of Fel d1 (Fig. 5A, panel A, sensitized challenged), while non-sensitized control mice did not. Mice treated with GFD SQ at days 37—39 failed to show Fel d1-induced skin test reactivity (Fig. 5B, panel A, GFD SQ-treated). Fel d1 treatment failed to statistically decrease skin test reactivity. The decrease in the area of bluing following Fel d1 injection in the various treated animals is shown in Fig. 5C, in which only GFD treatment has an inhibitory effect ($P < 0.05$). Notably, intradermal injection of GFD (0.2 μg) in sensitized non-treated and sensitized Fel d1-treated animals failed to elicit local allergic reactions above saline controls (Fig. 5A, panel C and data not shown).

GFD blocks Fel-d1-induced airway responsiveness

In Protocol 1, following an IT Fel d1 challenge at day 54, airway responsiveness to intravenously administered methacholine (1666 μg/kg) was significantly increased at day 56 when compared to that of non-sensitized challenged animals (Fig. 6). Treatment with GFD, as opposed to Fel d1, at days 37—39 completely reversed this Fel d1-induced increased airway hyper-responsiveness to methacholine (Fig. 6, $P < 0.001$). Neither non-sensitized mice nor sensitized non-challenged animals show airway hyper-responsiveness following IT Fel d1 challenge.

Airway responsiveness was also assessed in Protocol 2 following an IT Fel d1 challenge at day 35. Airway responsiveness was significantly increased in sensitized challenged animals, with airway resistance increasing from a mean of 198 (±15) $cmH_2O/l/s$ to 550 (±194) ($P = 0.034$) as compared to non-sensitized challenged animals. Administration of 5 μg of GFD IP 6 h prior to IT Fel d1 challenge completely ablated any increase in airway responsiveness to methacholine (resistance = 124(±34) $cmH_2O/l/s$, $P > 0.05$ compared to non-sensitized challenged animals and $P < 0.01$ compared to sensitized challenged animals.

Effects of GFD as immunotherapy on Fel d1-induced airway inflammation

Sensitization and challenge with Fel d1 elicited airway inflammation, as shown by an increase in the percent of eosinophils ($P < 0.001$) and lymphocytes ($P < 0.01$) in the BAL of the sensitized and challenged animals at day 56 (Protocol 1) compared to BAL in non-sensitized IT challenge

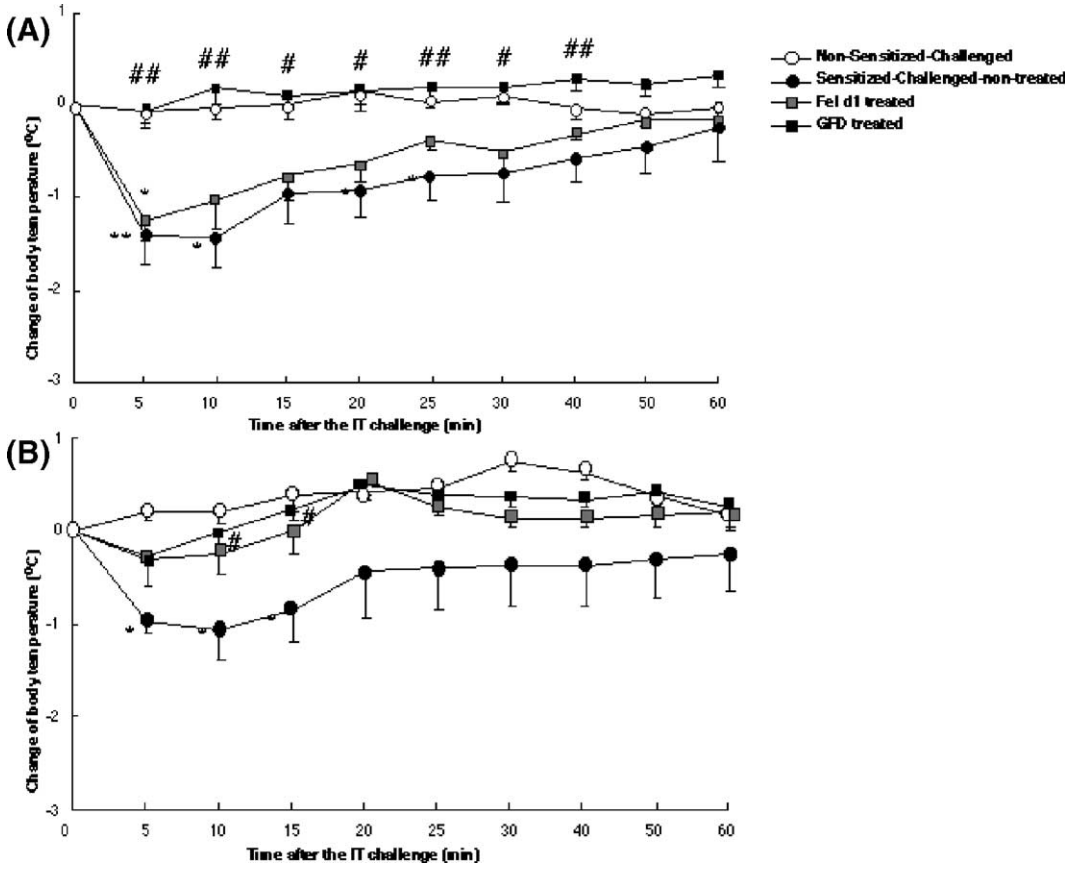

Figure 3 Effect of treatment on systemic reactivity. Effect of GFD or Fel d1 SQ immunotherapy on body temperature at days 40 (A) and 54 (B). Each value indicates mean ± SD of 4—6 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with non-sensitized challenged animals. GFD treatment inhibited the fall in body temperature at day 40 in comparison with sensitized challenged non-treated animals $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. At day 54, both GFD and Fel d l treatments lead to blocking of the fall in temperature following IT Fel d1 challenge.

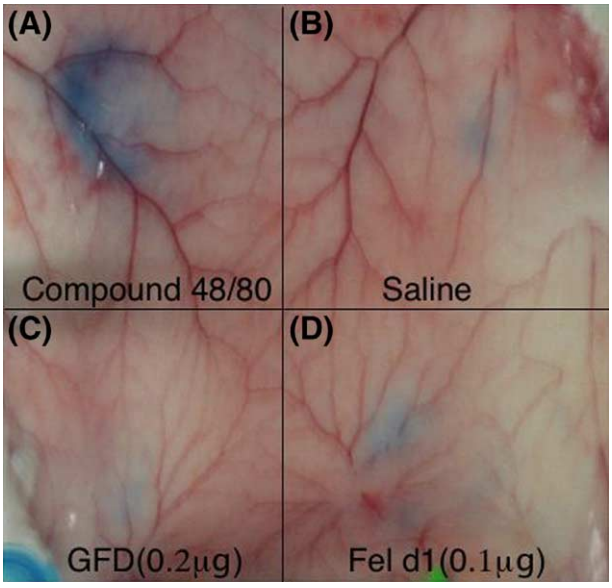

Figure 4 Effect of day GFD treatment on skin reactivity in Fel d1-sensitized mice. An animal sensitized with Fel d1 was injected i.p. with 5 μg of GFD and 6 h later, skin reactivity was tested to (A) the histamine-releasing compound 48/80 (0.6 μg), (B) saline, (C) GFD (0.2 μg), and (D) Fel d1 (0.1 μg). The skin on the inside of the back was examined 20 min after intradermal injection and an i.v. injection of Evans blue dye.

animals (Figs. 7A and B) as well as the absolute numbers of all cell types (data not shown). Animals that received SQ treatment with GFD or Fel d1 immunotherapy at days 37, 38, and 39 showed a far smaller percentage of eosinophils in the BAL fluid ($P < 0.001$) at day 56 compared to the saline-treated animals. GFD treatment also led to a decrease in lymphocytes when compared to Fel d1 treatment. Compared to the challenged sensitized animals, absolute number of eosinophils was significantly decreased by GFD treatment but not by Fel d1 treatment (mean $\pm$ SD = 13.5 $\pm$ 3.4 vs. 3.0 $\pm$ 1.2 vs. 8.6 $\pm$ 4.5 $\times$ 10^4 cells) while the absolute number of lymphocytes was not decreased statistically by either treatment (data not shown).

Histological examination at day 56 revealed that Fel d1 IT in sensitized saline-treated mice caused moderate pulmonary inflammation, which mainly consisted of eosinophils, a result that was not seen in non-sensitized challenged mice (Fig. 8A). Both Fel d1 and GFD treatment significantly inhibited the Fel d1-induced eosinophil accumulation in the lung (Fig. 8B, $P < 0.001$), which is consistent with the observations in the BAL fluid. Marked goblet cell metaplasia was observed in the large airways of sensitized mice and Fel d1-treated mice, while GFD treatment inhibited this goblet cell metaplasia (Fig. 8C).

Effects of immunotherapy on the serologic response to Fel d1

Blood samples were obtained 2 days after the final IT challenge (day 56) in Protocol 1 for analysis of Fel d1-specific IgG1, IgG2a, and IgE. As expected, sensitization and challenge induced significant increases in serum Fel d1 antibodies in the positive control animals with levels going from undetectable (<1.0 U/ml) for non-sensitized animals to geometric means of 57,859, 2, and 113 U/ml for IgG1, IgE, and IgG2, respectively ($P < 0.01$ for all). GFD treatment led to an increase in IgG1 antibodies to Fel d1 as compared to untreated ($P < 0.05$, 186,167 vs. 57,859 U/ml) and Fel d1-treated animals ($P < 0.02$ 186,167 vs. 33,157 U/ml). GFD did not alter IgE or IgG2 antibodies (geometric means of 34.4 and 166 U/ml, respectively) and Fel d1 treatment did not significantly alter any antibodies levels (geometric means of 33,157, 14.2, and 140 U/ml for IgG1, IgE, or IgG2a) compared to untreated animals. While there was a relative increase in IgE with GFD and a decrease with Fel d1 treatments, due to the large variation in responses, these did not reach statistical significance.

GFD-treated mice develop an anti-human Fc gamma response

Mice were examined for the development of an anti-human gamma 1 antibody response at day 56. We predicted that GFD-treated mice would make such a response in addition to that to the Fel d1 component of GFD. Indeed, at day 56, there was a robust anti-human IgG1 response in GFD-treated mice with a mean level of level 690 ng/ml while the levels in the untreated controls and Fel d1-treated groups were both background at less than 5 ng/ml.

Discussion

A number of approaches to allergen immunotherapy, including the use of peptides [20] and allergens coupled to immune stimulatory sequences [21] or other carriers that may lead to a modified immune response, are undergoing development. We designed GFD as a chimeric Fcγ-Fel d1 fusion protein so that it potentially could be given with greater rapidity and safety than standard allergen immunotherapy and thereby allow for treatment with high levels of allergen in a short time frame. Allergen immunotherapy has been demonstrated to require specific allergen dose thresholds that must be reached in order to achieve therapeutic benefit. Secrist et al. [22] reported that CD4+ T cells from allergic donors produced little interleukin 4 (IL-4) when stimulated with high concentrations of allergen, although they produced high levels of IL-4 when stimulated with low concentrations of allergen, which suggests that the delivery of high doses of antigen has maximum therapeutic effects. One of the main drawbacks of standard allergen immunotherapy is the long time that it takes to achieve it due to safety concerns. Rapid administration of standard allergen(s), so-called "rush immunotherapy," has been shown to be effective but has not gained wide acceptance due to the relatively high frequency of adverse events [23,24]. Herein, we demonstrate that GFD, when given acutely or as subcutaneous "rush" immunotherapy, can inhibit Fel d1-induced immediate hypersensitivity reactions in the skin, lungs, and systemically in Fel d1-sensitized mice. At the same time, we show that GFD, as opposed to Fel d1, when given as immunotherapy or as a deliberate challenge, did not induce an effector allergic response, presumably because of its ability to co-aggregate FcγRII with FcRI on basophils and mast cells.

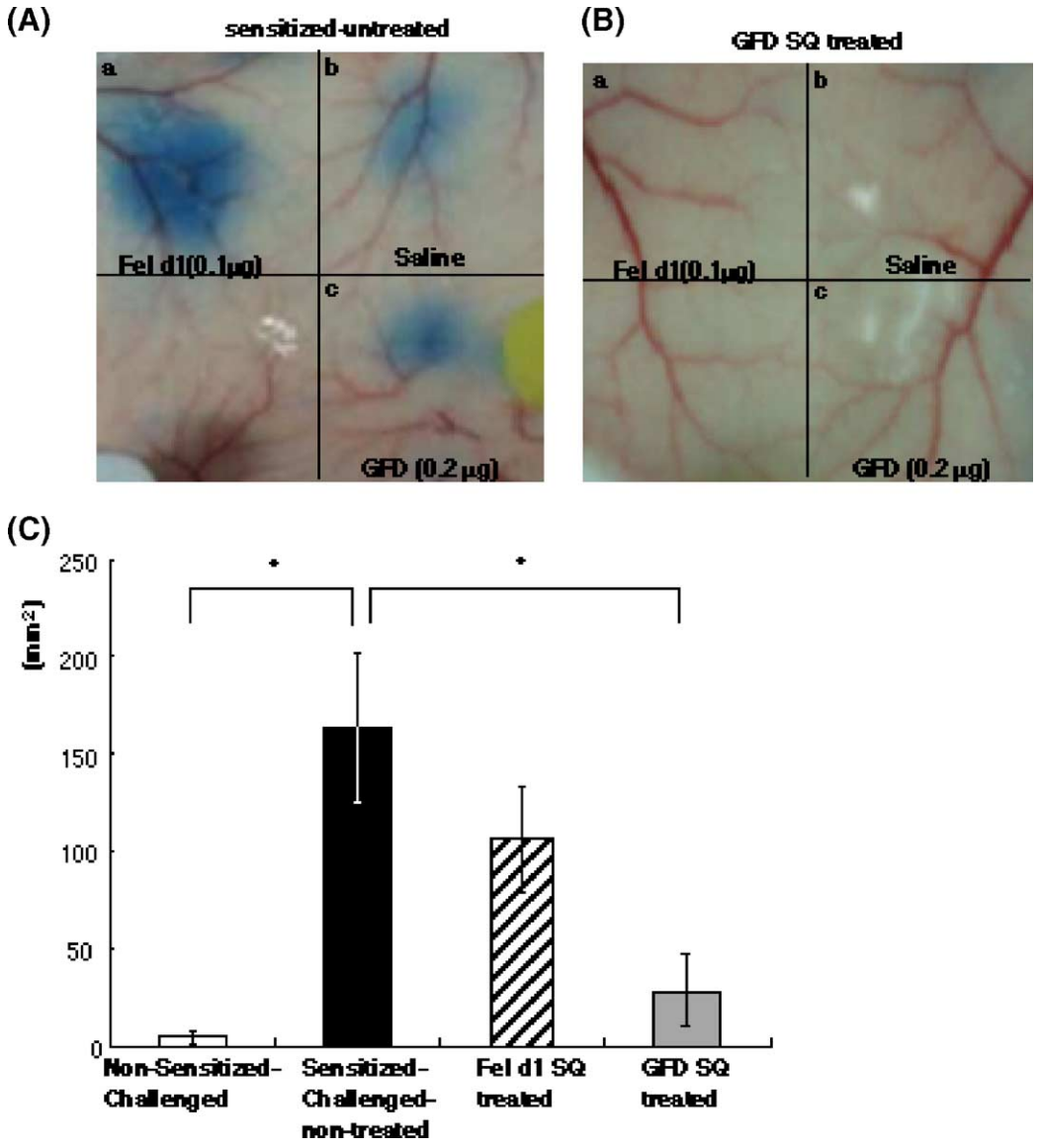

Figure 5 Effect of GFD treatment on skin reactivity in Protocol 1 Fel d1-sensitized mice. At day 60, 4 days after the final IT challenge, skin test reactivity was tested to (a) Fel d1 (0.1 μg), (b) saline, (c) GFD (0.2 μg). Panel A is a sensitized untreated animal while panel B shows a GFD-treated animal. The skin on the back was examined 20 min after intradermal injection along with an i.v. injection of Evans blue dye. Panel C shows the mean of the area ± SD of bluing at sites injected with 0.1 μg of Fel d1in the different type of mice noted. GFD-treated mice showed a decrease in the size of reactivity to Fel d1 injection (*$P < 0.05$ between the groups).

The mouse models of native Fel d1-induced allergic reactivity that we have developed combine Fel d1 IP immunization followed by IT boost and challenge, which allowed us to test the ability of GFD to modify cat allergen-induced allergic reactivity acutely and over the short term with one IP or three subcutaneous GFD treatments. This is based on evidence that the murine FcγRs will bind human IgG Fc [25]. Thus, the human Fc portion of GFD is expected to bind murine FcγRs, including FcγRIIb that contains an ITIM inhibitory signaling motif, while the Fel d1 portion of GFD will bind murine Fel d1-specific IgE and/or IgG on the surface of basophils/mast cells in the sensitized mice. The prediction was that GFD focused onto the basophil and mast cell surface would cross-link FcRs and FcγRs and, thereby, inhibit allergic reactivity. End points of allergic response in the sensitized and challenged animals included systemic reactivity manifested by core temperature drop, AHR, airway and lung inflammation, and Fel d1-induced mast cell degranulation in the skin. Long-term immunotherapy with our current human Fcγ-Fel d1 GFD molecule in these mouse models was not undertaken because we showed that the GFD induced the development of a strong anti-human Fcγ response that complicates the results and their interpretation. While use of a murine Fcγ in GFD would overcome this issue for mice, another caveat is that, in mice, IgG1 and IgE antibodies mediate immediate hypersensitivity while in humans only IgE does and, therefore, one cannot directly extrapolate any results to humans. Development of antibodies against the human Fcγ is not predicted to be an issue in humans, in whom GFD is expected to have a longer half-life compared to in mice, based on the known differences in IgG turnover between mice and humans.

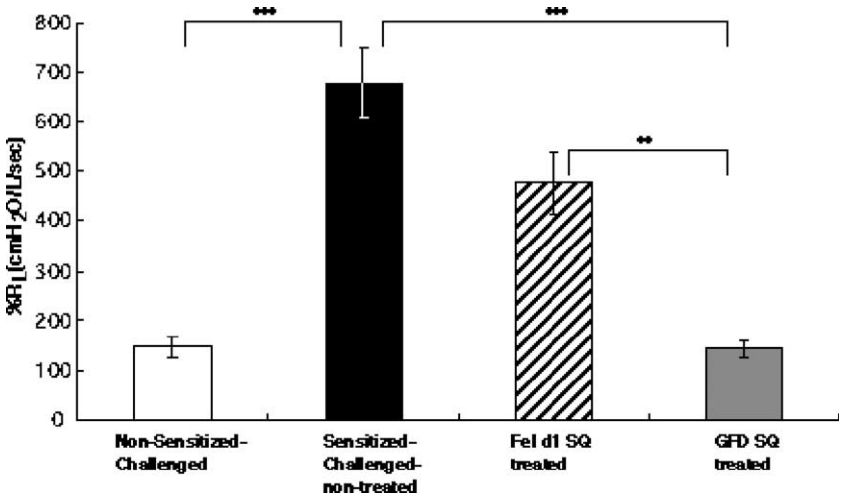

Figure 6 Effect of GFD or Fel d1 SQ immunotherapy on airway responsiveness at day 56. Changes in airway resistance to methacholine challenge 2 days after IT Fel d1 challenge are shown in control non-sensitized challenged animals and Fel d1-sensitized animals treated with saline, GFD, or Fel d1. Each value indicates mean ± SD of 4 to 5 animals. $^{**}P < 0.01$, $^{***}P < 0.001$ between the groups.

In this report, we demonstrate that a single IP injection of GFD acutely inhibited skin (Fig. 4), lung, and systemic reactivity to an IT Fel d1 challenge in mice sensitized to and boosted with Fel d1. This extends our findings that GFD can inhibit Fel d1-mediated allergic responses in actively sensitized Balb/c mice and also passive cutaneous anaphylaxis mediated by Fel d1 specific human IgE in human FcRIα transgenic mice [14]. Treatment with three subcutaneous doses of GFD on days 37—39 also blocked systemic reactivity to Fel d1 challenge on day 40 (Fig. 3A), an effect that is also likely due to the presence of GFD itself actively and directly blocking Fel d1 reactivity. The mechanism underlying GFD's acute therapeutic effect on allergic responses is expected to be mediated primarily through a mechanism similar to that seen with our previously tested GE2 fusion protein, e.g., cross-linking of FcγRIIb with FcRI to negatively signal the FcRI-bearing basophils/mast cells [8,26]. Indeed, we have shown that GFD treatment alters Syk and Erk signaling, although whether the ultimate inhibition of reactivity depends on ongoing negative signaling or the depletion of critical signaling components, e.g., Syk, remains to be determined [14]. These acute effects of GFD can be thought of as immunopharmacological outcomes to the direct presence and action of GFD as opposed to immunomodulatory outcomes that represent longer term indirect changes related to the immune response directed against Fel d1.

We also observed non-acute effects of GFD (and Fel d1) on allergic responses that appear to be mediated through immunomodulatory effects of GFD that are distinct from its direct negative regulation of FcRI activation. Treatment with GFD as 3 days of subcutaneous "rush" immunotherapy in Protocol 1 led to inhibition of skin, lung, and systemic reactivity 2 weeks after treatment was stopped (Figs. 5—8). Treatment with equimolar amounts of Fel d1 at days 37—39 also was able to block the late systemic reactivity and lung eosinophilia but not AHR, goblet cell metaplasia, or cutaneous reactivity. That the effect on eosinophils and systemic reactivity was seen with Fel d1 treatment, as well

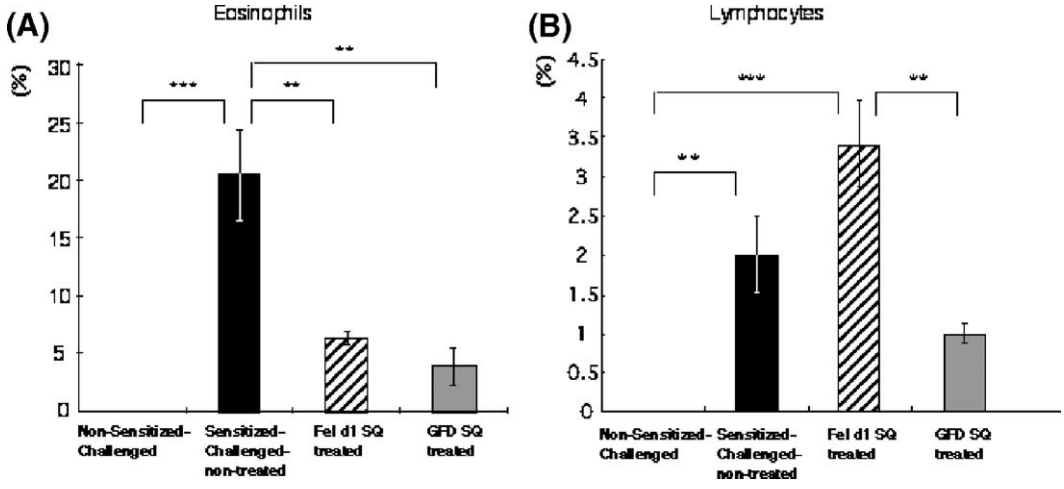

Figure 7 Effects of sensitization and treatment on BAL composition after challenge. Percentage of eosinophils (A) and lymphocytes (B) in BAL fluids from non-sensitized animals and those receiving different treatment as indicated. Results of each group are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between the groups.

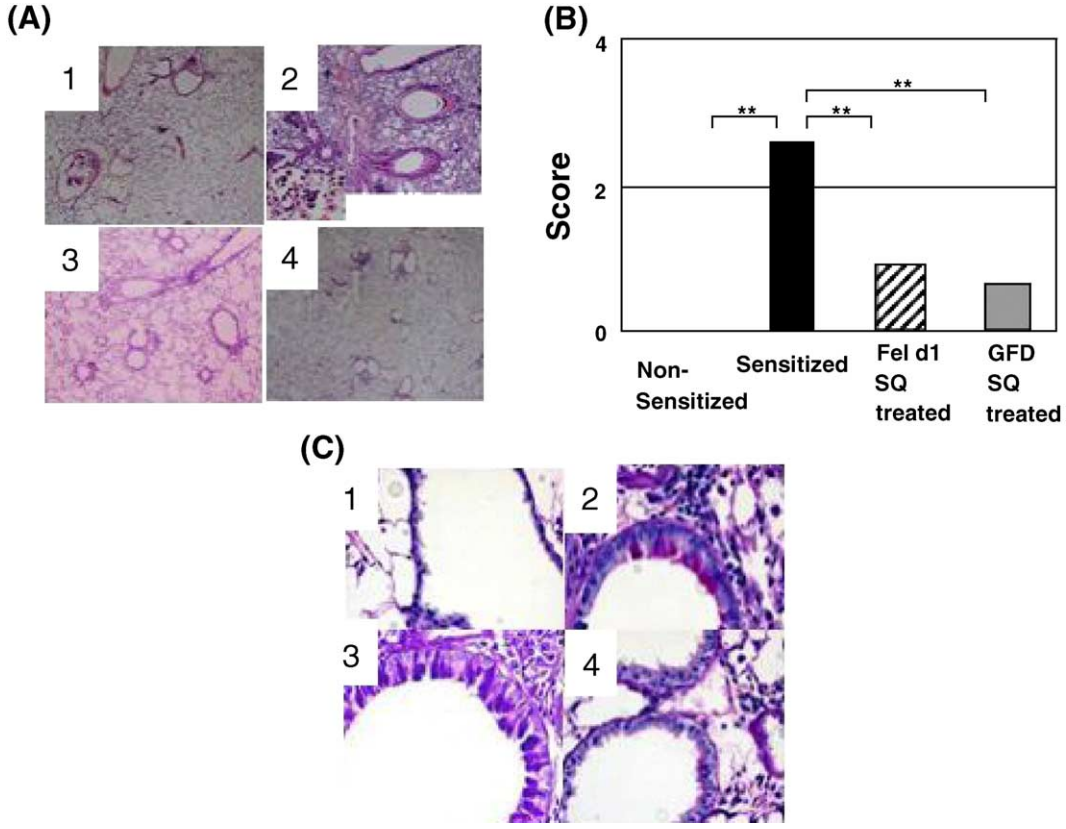

Figure 8 Histological changes the lung in sensitized challenged mice receiving various treatments and in control non-sensitized mice in Protocol 1. (A) Light photomicrographs of H&E-stained sections of lung tissue obtained at day 56 from non-sensitized mice and mice receiving the different treatments. Few leukocytes were observed in the lung of non-sensitized mice (1). In contrast, numerous eosinophils were present 2 days after the day 54 IT Fel d1 challenge in the lung of saline-treated sensitized mice (2). Both Fel d1 and GFD treatments (3 and 4) lead to markedly decreased eosinophil accumulation in the lung. (B) Semi-quantitative analysis of the eosinophil accumulation in the lung of animals challenged IT with Fel d1 at day 54. Results of each group are expressed as mean $\pm$ SD. ***$P < 0.001$ between the groups. (C) Light photomicrographs of PAS-stained sections of lung tissue obtained at day 56 from non-sensitized mice (panel 1) and mice receiving the different treatments. Marked goblet cell metaplasia was observed in large airways in the sensitized challenged animals (2). GFD treatment (4) but not Fel d1 (3) clearly inhibited this goblet cell metaplasia.

as GFD, indicates that these late beneficial outcomes were not just due to the treatment itself directly blocking reactivity because Fel d1 does not possess this ability. Furthermore, given the half-life of IgG in the mouse, much less that of GFD in which the Fel d1 portion has become recognized as an antigen, it is unlikely that GFD would be systemically present at the time of the late challenges. Increases in the level of Fel d1-specific IgG1 and possibly IgE in the GFD-treated animals are further evidence of an immune modulatory effect of a 3-day subcutaneous GFD treatment regimen. It was expected that GFD would serve as an immunogen for Fel d1 and thus the GFD-driven increase in allergic antibody observed is typically seen early on with allergen immunotherapy [27]. In spite of this, the GFD-treated animals had decreased in vivo AHR and inflammation showing that a loss of allergic antibody is certainly not the mechanism that accounts for GFD's beneficial in vivo effects. Feld1 treatment on day 37—39 did not alter the IgG1 or IgE antibody to Fel d1 as compared to untreated animals at day 56, possibly as these animals had received doses of Fel d1 alone on multiple occasions earlier. However, the subcutaneous Fel 1 treatment did have salutary although less profound effects than GFD on AHR and lung inflammation at day 56. Thus, it appears that GFD was a better immunogen than Fel d1. This outcome is likely related to an effect of GFD via the FcG focusing to antigen presenting cells. Overall, the effects of GFD and Fel d1 treatment on the 2-week outcomes appear to represent an immunomodulatory as opposed to immunopharmacological effect. The exact nature of any immunomodulatory effect will require further investigation as to timing, nature of the antibody response, and effects on various T cells populations. It is not surprising that a single IP injection of GFD in Protocol 2 did not block responsiveness to IT challenge two weeks later because the GFD would be gone and a single systemic administration is not expected to serve as an adequate immunotherapy in already sensitized animals.

Due to its ability to simultaneously bind FcγRII and anti-Fel d1 IgE bound to FcRI, we predicted that GFD would no longer function as an allergen in vivo or in vitro while still maintaining its immunogenic capacity. We have previously shown that GFD, as opposed to Fel d1, failed to induce histamine release from human mast cells and basophils in vitro and in PCA testing of human IgE to Fel d1-sensitized

FcRIα transgenic mice [14]. Evidence for this lack of induction of allergenic reactivity by GFD was extended in the present paper using mice actively sensitized to Fel d1, in which we found that GFD failed to induce skin test or systemic reactivity even when given in fivefold greater amount as compared to Fel d1. Thus, GFD has now shown a lack of elicitation of allergic reactivity in various in vitro and in vivo experimental models, which gives strong supporting evidence for the proposal that GFD does not function as an allergen to trigger either human IgE or murine IgG1/IgE-dependent allergic effector responses. This provides a potentially attractive feature for GFD as a modified Fel d1 "antigen" for immunotherapy. This would be similar in effect but different in mechanism to approaches that remove B cell epitopes or couple the allergen to immune stimulatory sequences, e.g., Ragweed coupled CpG, approaches that also have been shown to be diminished the allergenicity of the immunogen [28].

As noted above, the bifunctional nature of GFD which can simultaneously bind mast cell-bound Fel d1-specific IgE (or other isotypes such as IgG1 in mice) and FcγRII is predicted to be the underlying mechanism for the observation that GFD does not function as an allergen. While conformational changes due to the fusion of Fel d1 to the human IgG Fc portion in GFD could theoretically be responsible for GFD's lack of allergenicity while remaining active as an immunogen, this is unlikely as in our initial characterization of GFD we could show that the GFD binds to human anti-Fel d1 IgE (data not shown). Similarly, it is highly unlikely that biochemical alterations, e.g., glycosylation differences between the natural Fel d1 and the Fel d1 portion in GFD, can account for the differences of the biological effects observed between natural Fel d1 and GFD since they are both made in mammalian systems and Fel d1 reactivity is not felt to be carbohydrate-dependent. Whatever the mechanism underlying the failure of GFD to act as an allergen, our results suggest that this platform of IgG Fc-allergen fusion proteins will provide a markedly improved safety profile for allergy immunotherapy. This approach is predicted to be particularly valuable for immunotherapy with food allergens, which is at high risk for triggering fatal allergic responses during standard immunotherapy protocols, or for use with rapid or "rush" forms of allergy immunotherapy.

In summary, we demonstrated that GFD, when given as a 3-day rush immunotherapy, was able to block Fel d1-induced acute systemic allergic reactivity, degranulation of skin mast cells, AHR, and lung inflammation in mice already actively sensitized to Fel d1. At the same time, GFD itself, even in a significantly higher dose than Fel d1, did not function as an allergen to induce allergic reactivity by Fel d1-sensitized mast cells/basophils in vivo or in vitro. These salutary in vivo effects were accompanied by a decrease in Fel d1-specific IgG1, an indicator of a decreased Th2-like response as it is IgG1 that is the major anaphylactic antibody in mice. Thus, GFD functioned as an immunotherapeutic, not only to acutely block Fel d1-induced allergy but also as a novel form of allergen rush immunotherapy. While standard immunotherapy with unmodified antigens has been shown to be effective in human airway allergic disease [29—33], treatment is quite prolonged and has a relatively narrow therapeutic index. In contrast, treatment with gamma-allergen proteins such as GFD is predicted to have enhanced safety due to an inability to trigger mast cells and basophils. If this turns out to be the case, it will be possible to use gamma-allergens to achieve rapid high dose immunotherapy. An obvious target for this approach is severe food allergy, in which case there is no acceptable immunotherapy and the safety concerns are paramount.

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