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A chimeric human-cat fusion protein blocks cat-induced allergy

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Animal allergens are an important cause of asthma and allergic rhinitis. We designed and tested a chimeric human-cat fusion protein composed of a truncated human IgG Fcγ1 and the major cat allergen Fel d1, as a proof of concept for a new approach to allergy immunotherapy. This Fcγ-Fel d1 protein induced dose-dependent inhibition of Fel d1-driven IgE-mediated histamine release from cat-allergic donors' basophils and sensitized human cord blood-derived mast cells. Such inhibition was associated with altered Syk and ERK signaling. The Fcγ-Fel d1 protein also blocked *in vivo* reactivity in FcεRIα transgenic mice passively sensitized with human IgE antibody to cat and in Balb/c mice actively sensitized against Fel d1. The Fcγ-Fel d1 protein alone did not induce mediator release. Chimeric human Fcγ-allergen fusion proteins may provide a new therapeutic platform for the immune-based therapy of allergic disease.

Traditionally, immune-based therapy for inhalant allergens relies upon frequent injection of gradually increasing amounts of allergens. This approach is time-consuming, protracted and marred by serious treatment reactions. Immunotherapy for life-threatening food allergy (*e.g.*, peanut allergy) has proven unsuccessful[1]. Allergen-induced IgE-driven mediator release from mast cells and basophils is a key contributor in asthma, allergic rhinitis and severe food reactions[2]. Cross-linking mast cell and basophil FcεRI by multivalent antigen activates tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the β- and γ-FcεRI subunit cytoplasmic tails, thereby initiating downstream signaling through Syk[3]. Mast cells and basophils also express FcγRIIb, which contains a single conserved immunoreceptor tyrosine-based inhibition motif (ITIM) within its cytoplasmic tail[4,5]. Studies indicate that aggregating FcγRIIb to FcεRI leads to rapid tyrosine phosphorylation of the FcγRIIb ITIM tyrosine by FcεRI-associated Lyn and inhibition of FcεRI signaling[6–9]. Experiments using a human Ig Fcγ-Fcε fusion protein that directly cross-links FcεRI and FcγRIIb on human basophils support this hypothesis[10–13]. We have developed and tested a new form of immune therapy based on a chimeric fusion protein (GFD) comprised of the human Fcγ plus the cat (*Felis domesticus*) allergen[14] Fel d1. This molecule is specifically designed to coaggregate FcγRIIb with FcεRI-bound IgE, thereby inhibiting mediator release while serving as allergen immunotherapy that could be given as a safe series of high-dose injections.

We cloned human IgGγ1 constant region genomic DNA from the hinge through the CH3 domain into a mammalian expression vector with a cytomegalovirus promoter and a mouse immunoglobulin κ-chain leader sequence. We placed the cDNA encoding a Fel d1 construct[15] containing both chain 1 and chain 2 after the CHγ3 domain with a 15–amino acid $(Gly_4Ser)_3$ linker[16] between Fcγ1 and Fel d1. The fusion protein contained the binding site for FcγRII[17]. The Fcγ1-Fel d1 fusion protein was expressed as the predicted dimer of 140 kDa. Western blot and ELISA testing showed that antibodies to human gamma chain and Fel d1 recognize GFD. GFD binding to the human FcγRII was shown using HMC-1, a human mast cell–like line that expresses FcγRII but not FcεRI[18]. GFD bound to FcγRII in a fashion equivalent to human IgG, as assessed by flow cytometry. ELISA results indicate that specific IgE from cat-allergic patients' sera recognized GFD (data not shown). These results show that GFD is properly folded, has preserved FcR binding and is recognized by human IgE antibody to cat.

We purified basophils from cat-allergic subjects[19] and cultured them with 1 ng/ml to 1 μg/ml of GFD. Purified human IgG served as a control. Two hours later, we centrifuged the cells and assayed the histamine in supernatants as 'prerelease.' We then washed and challenged the cells with an optimal dose of Fel d1 (1.0 μg/ml) for 30 min and measured the resulting histamine release. GFD inhibited release by more than 75% ($P < 0.002$) at 10 ng/ml, whereas at 100 ng/ml inhibition was >90% ($P < 0.001$) (**Fig. 1a**). Results without autologous serum during the first incubation were similar, except overall histamine release was about 15% less. We observed similar inhibition in cat allergen–sensitized cord blood–derived mast cells wherein GFD (10 μg/ml) reduced degranulation by an average of 77% ($P < 0.05$) (**Fig. 1b**). Thus, GFD inhibited allergen-driven histamine release in a dose-dependent fashion. Notably, these results also show that GFD does not function as an allergen because mediator prerelease was not observed with GFD-incubated cat allergen–sensitized basophils.

Tyrosine phosphorylation is a key event connecting FcεRI cross-linking to downstream signaling in human mast cells and basophils. IgE stimulation in human FcεRI-positive cells quickly leads to phosphorylation of ERK1/2 and Syk[12,20]. Cross-linking FcεRI on cord

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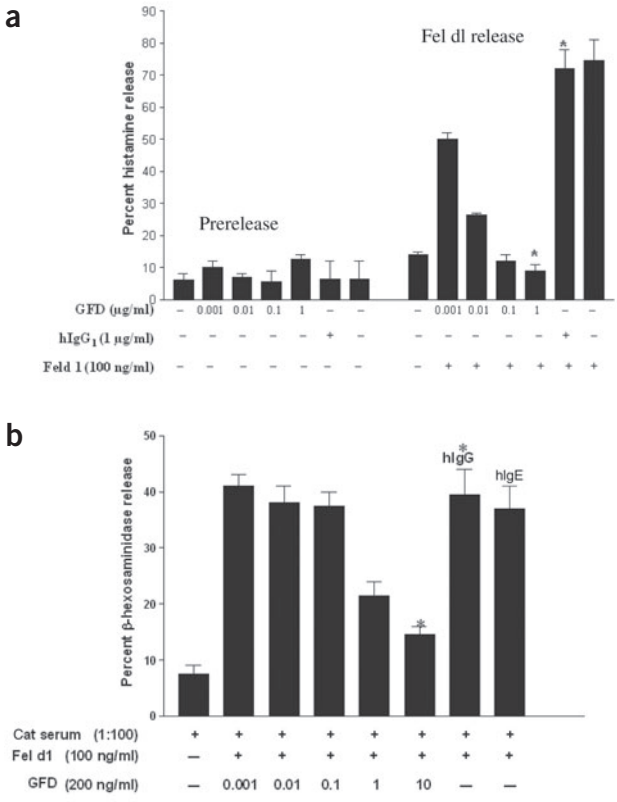


Figure 1 GFD inhibits human basophil and mast cell degranulation. Basophils from an atopic donor (**a**) or cat serum–sensitized, cord blood–derived mast cells (**b**) were incubated for 2 h with GFD and the supernatant assayed for prerelease of histamine (**a**) or β-hexosaminidase (**b**). Washed cells were challenged with Fel d1 and histamine or β-hexosaminidase measured in the supernatant (Fel d1 release). Nonspecific human IgG (hIgG) and IgE (hIgE) were used as controls. The results from one experiment are representative of three separate experiments. The asterisk indicates a statistically significant difference when comparing the two conditions. $^{*}P < 0.05$.

blood mast cells with IgE directed to Fel d1 induces substantial tyrosine phosphorylation of Syk and ERK, which was markedly reduced in cells preincubated with GFD (**Fig. 2**). We observed inhibition 2 min after antigen stimulation; inhibition persisted for 15 min. Thus, GFD coaggregation of FcεRI and FcγRII through an Fcγ-Fel d1-IgE linkage inhibits IgE-mediated Syk and ERK phosphorylation, which probably contributes to inhibition of basophil and mast cell function.

Transgenic mice expressing the human FcεRIα chain (hFcεRIα^{+} mice) show allergic reactivity after administration of human IgE antibody and challenge with the appropriate antigen[21,22]. Mast cells in these transgenic mice also express the mouse FcγRIIb that can bind human IgG. Using passive cutaneous anaphylaxis (PCA) in the hFcεRIα^{+} mice, we tested the predicted inhibitory effects of GFD by co-cross-linking the humanized FcεRI and the mouse FcγRIIb.

We primed transgenic mice (n = 12) intradermally with human serum containing high-titer IgE antibody to Fel d1 (118 kU/L) and measured PCA reactivity at sites injected with varying doses of GFD, after intravenous challenge with purified Fel. We measured each PCA reaction as the size of blue staining reaction of the skin at 30 min. GFD at 100 ng/spot consistently and completely blocked PCA reactivity (**Fig. 3a**). The GE2 fusion protein, which directly crosslinks FcεRI-FcγRII[23] gave analogous inhibition (**Fig. 3b**). GFD blocked PCA reactivity with 10-fold greater efficiency compared to GE2 (**Fig. 3b**). GFD blocked PCA reactivity equally well when injected 4 h after or simultaneously with cat-allergic donors' serum (**Fig. 3b**). PCA reactivity of purified cat-allergic donors' serum was destroyed by heat inactivation at 56 °C for 30 min[23] (data not shown). To test the specificity of GFD, we sensitized hFcεRIα^{+} mice with chimeric human IgE antibody to NP (4-hydroxy-3-nitrophenylacetyl) and induced PCA reactivity by intravenous challenge with NP–bovine serum albumin. GFD did not block IgE-induced reactivity to NP (data not shown). These data show that the GFD specifically inhibits cat allergen–induced IgE-mediated mediator release *in vivo*. To prove that GFD was not functioning as an allergen, we gave mice Evans blue dye 15 min after local administration of GFD at sites initially injected with cat-allergic donors' serum or purified IgE from cat-allergic subjects' serum (data not shown). We observed no reaction as evidenced by lack of dye extravasation, which shows that GFD itself did not induce mast cell release and such an effect does not account for the failure of GFD-treated sites to react upon later systemic allergen challenge.

We developed a model of systemic reactivity to Fel d1 in actively sensitized Balb/c mice to test the immunotherapeutic potential of GFD. The value of this model is based on the binding of human Fcγ by the mouse FcγRs[11] (data not shown). Thus, the Fcγ portion of GFD binds to mouse FcγRs, including FcγRIIb, and drives inhibitory signaling through its ITIM motif. Simultaneously, the Fel d1 portion of GFD will bind to mouse Fel d1-specific IgE and/or IgG1 on the surface of sensitized mast cells or basophils with the expectation that GFD-mediated cross-linking of these FcεRIs and FcγRs will generate a negative signal for mast cells or basophils.

We sensitized BALB/c mice with Fel d1 and treated them up to day 21 (**Fig. 4a**). GFD treatment completely blocked Fel d1-induced airway hyper-responsiveness at days 30 (**Fig. 4b**) and 44 (data not shown), assessed by increased pulmonary resistance after methacholine challenge. Similarly, we observed blunted eosinophilic airway inflammation in Fel d1-sensitized and intratracheally challenged mice, evident as decreased eosinophils in bronchoalveolar lavage fluid after GFD treatment (**Fig. 4c**). Using a sensitization and GFD immunotherapy model that used intense

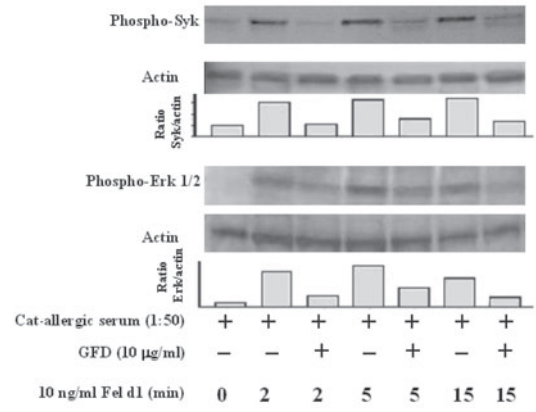


Figure 2 GFD inhibits FcεRI-mediated Syk and ERK phosphorylation. Cord blood–derived mast cells were sensitized with cat-allergic donors' serum, washed, activated as described, and western blotted with the indicated antibodies. The top panel (Syk) represents an independent experiment from the bottom panel (Erk). Results are representative of at least three separate experiments.

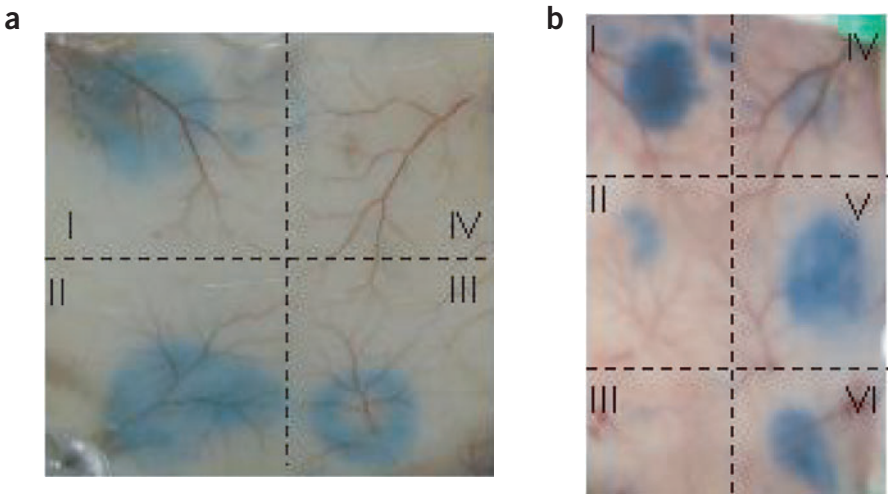
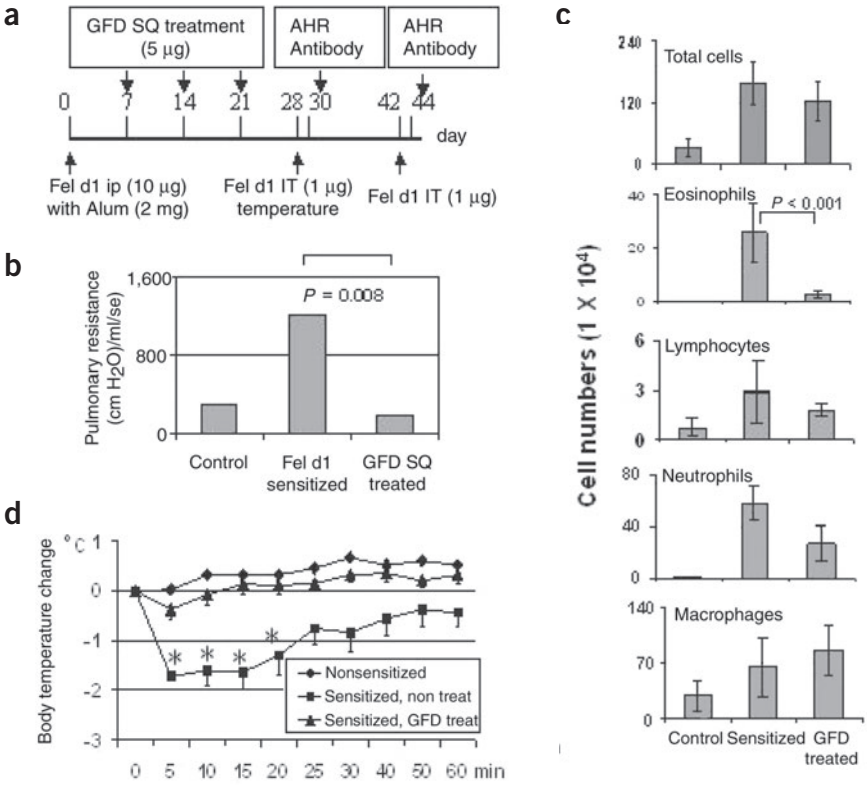


Figure 3 GFD inhibits IgE-mediated degranulation in FcεRIα transgenic mice. (**a**) Dose-dependent inhibition of PCA by GFD. The skin sites were sensitized with cat-allergic donors' serum, followed by the administration of: (I) saline; (II–IV), GFD 1, 10 and 100 ng, respectively. (**b**) Comparison of GFD and GE2 for inhibiting PCA. The skin sites were sensitized and treated as follows: (I) saline 4 h later; (II) 100 ng GFD 4 h later; (III) 100 ng GFD simultaneously with serum; (IV) 1 μg GE2 simultaneously with serum; (V) 100 ng GE2 4 h later; (VI) 100 ng GE2 simultaneously with serum.

Fel d1 sensitization, we tested GFD for its ability to block systemic allergic reactivity as indicated by a decrease in challenged animals' core body temperature[24]. Core temperatures dropped an average of 1.7±0.2 °C over the hour after intratracheal Fel d1 challenge. This was completely blocked following GFD treatment ($P < 0.001$) (**Fig. 4d**). These results indicate that GFD administrated in a regime similar to allergen immunotherapy ameliorates allergic responses to Fel d1 in previously sensitized animals.

Recognition of the Fel d1 portion of GFD by cat allergen-specific IgE is predicted to lead to the formation of FcγR-(GFD)-IgE-FcεRI complexes on basophils and mast cells. This probably accounts for the ability of GFD to inhibit mediator release from basophils of cat allergen–sensitive humans and to inhibit PCA reactivity in transgenic mice, and is analogous to what we found with a human bifunctional Fcγ-Fcε protein (GE2), which directly cross-links FcγRIIb and FcεRI to induce antigen nonspecific inhibitory signaling[10,23]. In contrast to GE2, GFD indirectly cross-links FcγRIIb and FcεRI through antigen-specific IgE and inhibits basophil and mast cell reactivity in an allergen-specific fashion. GFD also contains the allergen (Fel d1) that should induce a protective immune response, as seen with standard immunotherapy[25,26] and as observed in mice with Fel d1-induced systemic and airway reactivity (**Fig. 4**). The advantage of an Fcγ-allergen construct (*e.g.*, GFD) versus allergen is that the chimeric protein does not drive mediator release.

Overall, GFD inhibited allergen-driven IgE-mediated mediator release *in vitro* from human basophils and cord blood–derived mast cells, and *in vivo* from both passively cat allergen–sensitized FcεRIα transgenic mice and in actively cat allergen–sensitized mice. Chimeric human Fcγ-allergen proteins such as GFD provide a new platform for antigen-specific immunotherapy in a host of human allergic diseases and may be a particularly powerful approach for treatment of severe food-induced allergy.



METHODS

GFD construction and expression. To construct the human Fcγ-Fel d1 chimeric gene, we amplified cDNA encoding Fel d1 from a recombinant Fel d1 cDNA clone (chain 1 + chain 2)[15]. The 5′-end primer contained a flexible linker sequence. We cloned amplified products into pCR2.1 vector (Invitrogen), sequenced and inserted the Sal I-Not I fragment into pAN expression vector (Invitrogen pDisplay). We transfected the expression vector containing the new Ig Fcγ-Fel d1 chimeric gene into SP2/0 cells and purified the Fcγ-Fel d1 fusion protein in the culture supernatants by protein A affinity chromatography.

Figure 4 GFD blocks Fel d1-induced allergic response in mice. (**a**) Schematic diagram of the experimental protocol. (**b**) Inhibitory effect of GFD on Fel d1-induced AHR. The numbers represent the average values from three measurements of airway resistance. (**c**) Inhibitory effect of GFD Fel d1-induced pulmonary eosinophilic inflammation. Total and differential numbers of bronchoalveolar lavage fluid cells were counted. (**d**) Inhibitory effect of GFD on Fel d1-induced systemic allergic reactivity evidenced as core body temperature. Core body temperature change was measured at 5-min intervals immediately after Fel d1 challenge. The asterisk indicates a statistically significant difference between the two conditions. *$P < 0.05$.

Western blots. We ran purified GFD on SDS PAGE and transferred it to membranes where we probed it with either mouse antibody to human IgG (γ chain–specific) or antibody to Fel d1 and goat antibody to mouse IgG conjugated to horseradish peroxidase. For Syk and Erk measurements, we treated IgE-sensitized cord-blood mast cells with GFD or control materials, and followed with activation, lysis and blotting as described[5]. We probed blots with rabbit antibodies that recognize phosphorylated Syk (Cell Signaling) or mouse antibodies to phosphorylated ERK 1/2 (Upstate). We developed blots using enhanced chemiluminescence reagents (ECL, Amersham Biosciences) and exposed them to BioMax film (Eastman Kodak). We quantified band intensities using a molecular imaging package (Bio-Rad, Discovery Series; Quantity One, Quantitative Software) and presented them as a ratio of Syk or Erk to actin to compensate for gel loading and blot stripping-reprobing variations.

Basophil and cord blood–derived mast cell purification. We collected blood from donors who were skin test–positive to Fel d1 and had a self-reported history of cat allergy. The basophils were purified by Percoll gradient centrifugation, followed by negative selection using magnetic beads. Basophil purities were ≥95% as determined with Wrights-Giemsa stain. Cord blood–derived mast cells were derived as described[12]. We obtained informed consent for all human subjects as approved by the Institutional Review Board at Virginia Commonwealth University.

Measurement of degranulation. We sensitized cord blood–derived mast cells with human IgE antibody to Fel d1 in serum from cat-allergic donors (Plasma Labs) for 24 h. After washing the mast cells or basophils, we added GFD (0–10 μg/ml) for 2 h at 37 °C and centrifuged the cells and used the supernatants to determine any mediator prerelease. We washed and activated cells in Tyrodes (mast cells) or DMEM (basophils), with or without optimal concentrations of Fel d1 (Indoor Biotechnologies) (10–200 ng/ml). As a control, we substituted nonspecific human IgG for GFD. After 30 min, we centrifuged cells and removed the supernatant for β-hexosaminidase or histamine analysis, as described[27].

Passive cutaneous anaphylaxis. We injected transgenic mice intradermally with 50 μl of 1:5 diluted cat allergic serum or purified IgE from that serum. We injected different doses of purified GFD at the same sites 4 or 24 h later. We injected GFD simultaneously with the allergic serum (or purified IgE) in selected experiments. We injected mice intravenously with 10 μg of purified Fel d1, plus Evans blue dye, 4 or 24 h later. The mice were generally killed 30 min after the intravenous challenge although in some experiments, this was done at 60 and 120 min to assess and confirm the blocking activity of GFD.

Mouse models. To measure airway changes shown in **Fig. 4b** and **4c**, we sensitized and treated 6–8-week-old BALB/c mice using the protocol diagrammed in **Fig. 4a**. To assess systemic allergic reactivity, we sensitized mice by intraperitoneal injection with 5 μg of Fel d1 on d 1 and 14, then boosted them intratracheally with 1 μg of Fel d1 on days 28, 29, 30 and 33. We treated mice subcutaneously with 5 μg of GFD or saline on days 37, 38 and 39. We subjected the mice then to intratracheal challenge with 1 μg of Fel d1 on day 40 (**Fig. 4d**) and monitored the animals' core temperature rectally using a rectal probe digital thermometer (YSI Inc.). We obtained approval from the Animal Research Committee at UCLA for all the animal experiments performed.

Airway response to methacholine. We measured airway responsiveness 48 h after intratracheal challenge using a modified forced oscillation method[28]. We connected anesthetized mice to a computer-controlled small-animal ventilator (FlexiVent, SCIREQ) and calibrated it to remove resistance of the tracheal, cannula and tubing. We obtained measurements of pulmonary resistance at 10-s intervals, both before and after intravenous administration of acetyl-β-methylcholine-chloride (1.67 μg/g body weight) by using the forced oscillatory technique with the conventional primewave 8 with a peak to peak amplitude of 0.17442 ml.

Bronchoalveolar lavage. Two days after intratracheal challenge, the mice were killed, their lungs lavaged and total numbers of bronchoalveolar lavage cells counted after cells were stained with trypan blue. Differential cell counts measured on at least 300 cells stained with Wright-Giemsa.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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