

Targeting Fel d 1 to Fc γ RI induces a novel variation of the T_H2 response in subjects with cat allergy

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Background: Induction of CD4⁺ T cells that produce IL-10 or IFN- γ is central to the protective effects of conventional allergen immunotherapy.

Objective: We examined the T-cell modulatory capacity of a fusion protein (H22–Fel d 1) that targets Fel d 1 to the high-affinity IgG receptor (Fc γ RI) on antigen-presenting cells.

Methods: Monocyte-derived dendritic cells pulsed with H22–Fel d 1 were analyzed for surface phenotype and cytokine secretion by flow cytometry and cytometric bead assay, respectively.

CD4⁺ T cells generated after coculture with H22–Fel d 1—pulsed dendritic cells were analyzed at the single-cell level by flow cytometry after intracellular cytokine staining. The T-cell repertoire was compared for subjects with (IgE⁺) and without cat allergy (IgE^{neg}IgG^{neg}), including subjects with a modified T_H2 response (IgE^{neg}IgG⁺).

Results: H22–Fel d 1 induced a semimature phenotype in dendritic cells in conjunction with a selective increase in IL-5⁺ and IL-10⁺ CD4⁺ T cells compared with nonreceptor-targeted Fel d 1. Amplified T cells included diverse subtypes characteristic of T_H0 (IL-5⁺IFN- γ ⁺), regulatory T_H1 (IL-10⁺IFN- γ ⁺) and regulatory T_H2 (IL-10⁺IL-5⁺) cells. T-cell qualitative changes were restricted to subjects with allergy and were distinct from a modified T_H2 response. Blocking IL-10 induced by H22–Fel d 1 selectively increased IL-5⁺ CD4⁺ T cells, suggesting that T_H2 responses were controlled.

Conclusion: Targeting Fel d 1 to Fc γ RI induces a novel variation of the T_H2 response that incorporates major elements of a protective T-cell response.

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Key words: CD64, IL-10, IL-5, IFN- γ , H22–Fel d 1, dendritic cells, CD4⁺ T cells, regulatory T cells, immunotherapy

Abbreviations used

APC: Antigen-presenting cell
MCP: Monocyte chemoattractant protein
MDDC: Monocyte-derived dendritic cell
MIP: Macrophage inflammatory protein
TARC: Thymus and activation-regulated chemokine

Conventional immunotherapy, which involves repeated injection of allergen extracts, remains the mainstay of treatment for allergy to a variety of inhalant allergens. Although there is considerable evidence for its efficacy, this approach has several drawbacks, including a risk for anaphylaxis and prolonged treatment regimens. Although the immune mechanisms which underlie the protective effects of immunotherapy remain ill-defined, there is evidence that induction of CD4⁺ T cells, which secrete IL-10 or IFN- γ , is a prerequisite. This suggests the involvement of T cells that are either regulatory or T_H1-like.^{1–4} Consequently, recombinant allergens or allergen variants, which may provide a more tailored approach to immunotherapy, have been engineered with a view to activating specific T-cell subsets preferentially.^{5–7} Unfortunately, many allergens induce weak T-cell responses *in vitro*, which may reflect the low precursor frequency of allergen-specific T cells in the T-cell repertoire. As a result, defining T-cell mechanisms that underlie the protective effects of immunotherapy has proved to be a challenge. Moreover, *in vivo* presentation of allergen to T cells during conventional immunotherapy is likely to be inefficient.

The approach described here uses a fusion protein designed to target allergen to the surface of antigen-presenting cells (APCs) as a method of improving allergen presentation to T cells. This protein (designated H22–Fel d 1) is composed of the major cat allergen, Fel d 1, linked to a single chain fragment of the variable region (sFv) of the humanized anti-CD64 mAb, H22. This molecule has been shown to bind to the high-affinity IgG receptor, Fc γ RI (CD64), on APCs.⁸ Targeting to CD64 has been shown to increase uptake of diverse antigens by APCs and to increase T-cell proliferative responses.^{8–11}

A unique feature of the major cat allergen, Fel d 1, is its ability to induce a modified T_H2 response (IgG⁺IgE^{neg}) in a subset of individuals with high-level environmental exposure.¹² This protective response is associated with increased IL-10 production in PBMC cultures stimulated with major T-cell epitopes of Fel d 1.⁴ Because targeting Fel d 1 to CD64 on APCs may enhance presentation of major T-cell epitopes, we hypothesized that H22–Fel d 1 would promote induction of IL-10-producing CD4⁺ T cells.

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TABLE I. Serum antibody profiles in subjects with distinct immune responses to cat allergen*

Group	Total IgE† (IU/mL)	IgE to cat† (IU/mL)	Fel d 1	
			IgE‡ (IU/mL)	IgG§ (U/mL)
Allergic (n = 11)	119 (60-235)	13 (7-25)	7 (0.5-99)	3168 (1392-7213)
Modified TH ₂ (n = 9)	22 (10-46)	<0.35	<0.35	4917 (1923-12573)
Control (n = 5)	14 (4-45)	<0.35	<0.35	<125

*Values represent geometric means (95% CIs).

†Measured by CAP assay.

‡Measured by streptavidin CAP assay.

§Measured by radioimmunoprecipitation assay.

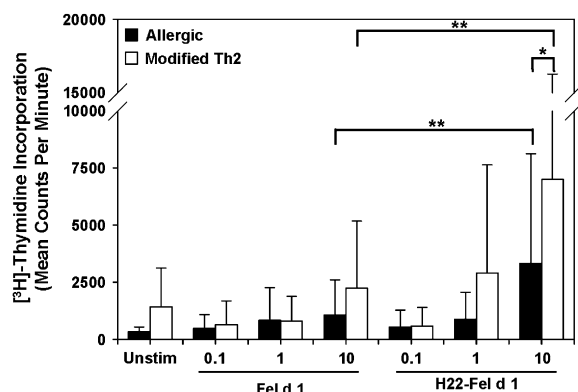


FIG 1. PBMC proliferation to H22–Fel d 1. PBMCs were stimulated with allergen and proliferation measured on day 7 (n = 7 per group). Values correspond to mean counts per minute + SDs. **P* < .01; ***P* < .001. Subjects with cat allergy: *P* < .04 for unstim versus all other conditions. Modified TH₂ responders: *P* < .02 for unstim versus Fel d 1 (10 μg/mL) and H22–Fel d 1 (1 and 10 μg/mL). Unstim, Unstimulated.

The current study was designed to examine the immunomodulatory effects of H22–Fel d 1 at both the APC and the T-cell level. To this end, we analyzed patterns of cytokine secretion by dendritic cells and performed rigorous single-cell analyses of cytokine production by CD4⁺ T cells. Our findings provide evidence of a new approach to immunotherapy and present a new paradigm for variations of a TH₂ response.

METHODS

Classification of subjects

Patients were recruited from the University of Virginia Allergic Diseases Clinic or by advertisement. Subjects with cat allergy were classified on the basis of high-titer serum IgE antibodies to cat extract (CAP > 0.7 IU/mL) and anti–Fel d 1 IgE antibodies measured by streptavidin CAP assay.¹³ Subjects with a modified TH₂ response to cat allergen were identified on the basis of high-titer anti–Fel d 1 IgG antibodies measured by antigen binding radioimmunoassay (>500 U/mL) without anti–Fel d 1 IgE antibodies.^{12,14} Control subjects had no measurable serum IgG or IgE antibodies to Fel d 1. Serum antibody profiles are shown in Table I. All studies were approved by the University of Virginia Human Investigations Committee.

Recombinant allergens and LPS

Recombinant Fel d 1 and Fel d 1 targeted to the high-affinity IgG receptor, FcγRI (H22–Fel d 1),⁸ were expressed in *Pichia pastoris* and purified by multistep chromatography. The purity was >90% as determined by SDS-PAGE. The endotoxin content of Fel d 1 and H22–Fel d 1 was comparable (145 EU/mL and 153 EU/mL, respectively). LoTox Der p 1 (Indoor

Biotechnologies Inc, Charlottesville, Va) (endotoxin content < 0.05 EU/μg) was purified from mite culture by affinity chromatography (>90% purity by SDS-PAGE) and endotoxin removed by proprietary techniques. LPS was obtained from Sigma-Aldrich (St Louis, Mo).

Proliferation assays

For a description of proliferation assays, see this article's Methods section in the Online Repository at www.jacionline.org.

Induction of cytokines in monocyte-derived dendritic cells

Dendritic cells were generated from CD14⁺ monocytes (see this article's Methods section in the Online Repository at www.jacionline.org). Monocyte-derived dendritic cells (MDDCs)¹⁵ were stimulated for 24 to 48 hours in medium containing 10% autologous human serum with allergen (10 μg/mL) or LPS (1 μg/mL). Cytokines were measured by cytometric bead assay (human 27-plex kit; BioRad, Hercules, Calif).

CD4⁺ T-cell/MDDC cocultures

CD4⁺ T cells were purified by negative selection using magnetic-activated cell sorting (Miltenyi Biotec, Auburn, Calif), and purity was assessed by flow cytometry (>95%). MDDCs were pulsed with allergen (10 μg/mL) for 48 hours before coculture (2 × 10⁵) with purified CD4⁺ T cells (8 × 10⁵) for 10 days in 24-well plates. Cultures were supplemented with recombinant human IL-2 (12 U/mL; BioSource International, Carlsbad, Calif) on day 5, and intracellular cytokines were measured on day 10. In some experiments, a blocking anti–IL-10 receptor mAb (clone 3F9; BioLegend, San Diego, Calif) or isotype control (clone RTK2758; BioLegend) was added (20 μg/mL) on day 0 of coculture.

Flow-cytometry analysis

For a complete list of fluorescently conjugated mAbs and instrumentation, see this article's Methods section in the Online Repository at www.jacionline.org.

Dendritic cell phenotyping

Monocyte-derived dendritic cells were collected immediately after generation or allergen pulsing (24–48 hours) and washed in cold FACS buffer (0.5% BSA, 2 mmol/L EDTA in PBS). Cells were stained for surface markers, washed, and analyzed by flow cytometry.

Intracellular cytokine staining

CD4⁺ T-cell/MDDC cocultures were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (2 μg/mL; Sigma-Aldrich) for 5 hours, and Brefeldin A (BD Biosciences, San Jose, Calif) was added during the final 4 hours. Cells were stained for surface and intracellular cytokines according to the manufacturer's instructions (Caltag International Inc, Carlsbad, Calif) and analyzed by flow cytometry.

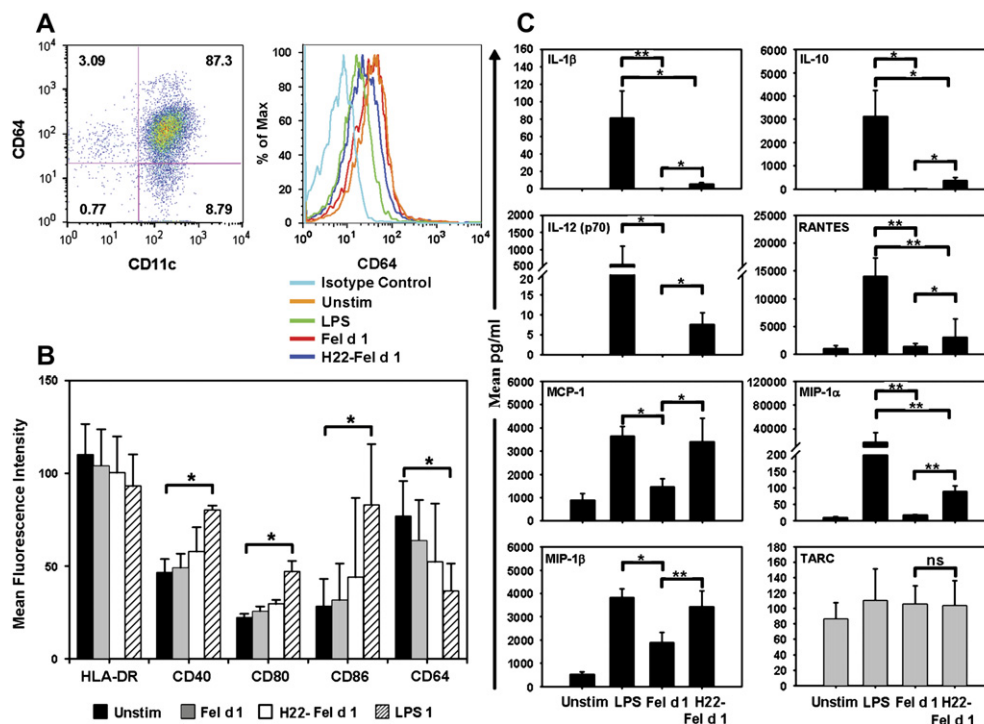


FIG 2. Effects of H22-Fel d 1 on MDDCs. MDDCs from subjects with cat allergy ($n = 6$) were pulsed with antigen (24 hours) and analyzed for expression of surface antigens and cytokine secretion. **A**, Representative data showing expression of CD64. **B**, Effects of different stimuli on maturation markers and CD64. * $P < .01$. **C**, Effects of different stimuli on cytokine secretion. Values represent the means $\pm$ SDs. * $P < .05$; ** $P < .01$.

Statistical analysis

The Student t test (SPSS 14.0, SPSS Inc, Chicago, Ill) was used to compare proliferation, cytokines, and mean fluorescence intensity levels. The percentages of cytokine-positive CD4⁺ T cells induced by allergen were analyzed by general linear mixed-effects ANOVA models after transforming data to the natural logarithmic scale (for details, see this article's [Methods](#) section in the Online Repository at www.jacionline.org).

RESULTS

H22-Fel d 1 induces increased proliferation in PBMC cultures

Targeting antigens to the high-affinity IgG receptor, Fc γ RI (CD64), using the mAb H22 has been shown to enhance T-cell proliferative responses *in vitro*.¹¹ Initially we compared proliferation to receptor-targeted Fel d 1 (H22-Fel d 1) with Fel d 1 in PBMC cultures established from cat-allergic subjects and nonallergic subjects with a modified T_H2 response to cat ($n = 7$ per group). Stimulation with H22-Fel d 1 resulted in increased proliferation compared with Fel d 1 at a dose of 10 μ g/mL in both subject groups ($P < .001$), and this effect was strongest for cultures from subjects with a modified T_H2 response ($P < .01$; Fig 1).

H22-Fel d 1 induces production of inflammatory cytokines by dendritic cells without increasing surface markers of maturation

Dendritic cells are essential for generating T-cell responses to allergens. These responses may be influenced by the degree of maturation of the dendritic cell as well as by the cytokines these cells secrete.^{5,16,17} We investigated whether binding of H22-Fel d 1 to Fc γ RI on dendritic cells alters the properties of these cells.

Flow-cytometry analysis showed that MDDCs obtained from subjects with cat allergy expressed a phenotype characteristic of myeloid dendritic cells including high-level expression of CD11c, CD1a, and HLA-DR and lack of expression of CD14 (data not shown). Importantly, these cells also expressed CD64 (Fig 2, A). As expected, stimulation with LPS led to increased expression of the costimulatory molecules CD40, CD80, and CD86 on MDDCs, consistent with maturation, whereas expression of CD64 was decreased ($P \leq .01$). However, stimulation with H22-Fel d 1 or with Fel d 1 had no significant effect on these markers (Fig 2, B). By contrast, H22-Fel d 1 induced increased secretion of T_H1-promoting and inflammatory cytokines (IL-12, RANTES, IL-1 β , monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1 β , MIP-1 α) by MDDCs compared with Fel d 1 ($P < .05$). Moreover, levels for selected inflammatory cytokines (MCP-1, MIP-1 β) were comparable to those induced by LPS (Fig 2, C). H22-Fel d 1 also induced increased IL-10 compared with Fel d 1 ($P = .04$), whereas no change in the levels of T_H2-attracting chemokines (thymus and activation-regulated chemokine [TARC] and eotaxin) was observed for any stimuli (Fig 2, C, and data not shown). Induction of inflammatory cytokines in dendritic cells by H22-Fel d 1, without increased expression of maturation markers, indicates a semi-mature phenotype.¹⁸

H22-Fel d 1 induces increased IL-5⁺ and IL-10⁺ CD4⁺ T cells, and this effect is specific to subjects with allergy

Next we examined whether changes induced by H22-Fel d 1 at the APC level were reflected at the T-cell level. Single-cell

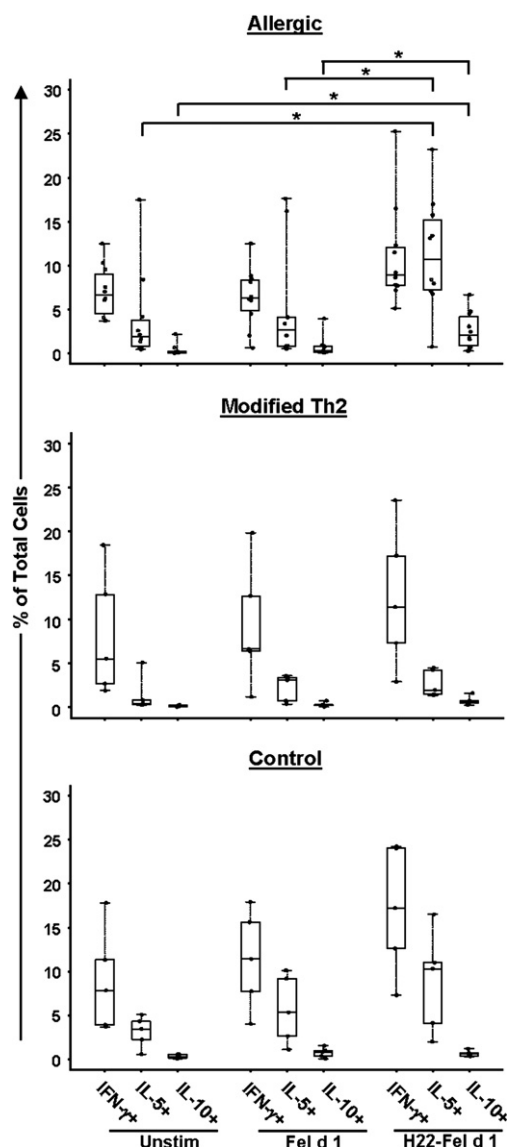


FIG 3. Changes in cytokine-positive CD4 $^+$ T cells induced by H22–Fel d 1 compared with Fel d 1. CD4 $^+$ T cells were cocultured with antigen-pulsed MDDCs for 10 days, and the percentage of total CD4 $^+$ T cells that were IFN- γ^+ , IL-5 $^+$, and IL-10 $^+$ was analyzed by flow cytometry. Experiments were performed in subjects with cat allergy ($n = 10$), modified T $_H$ 2 ($n = 5$), and control ($n = 5$) subjects. Boxes represent medians and interquartile ranges. *Significant after adjusting for multiple comparisons.

analysis of cytokine-producing CD4 $^+$ T cells was performed by using flow cytometry after culture of purified CD4 $^+$ T cells with allergen-pulsed dendritic cells for 10 days. Experiments were performed in 3 groups of subjects with distinct immune responses to cat allergen: (1) cat allergy, (2) modified T $_H$ 2, and (3) serum antibody-negative controls (Table I). Our objectives were to evaluate changes in the frequency of cytokine-positive CD4 $^+$ T cells and to assess the relevance of these changes to allergic status. We performed a rigorous multiple comparison statistical test to evaluate these changes to provide strong statistical power. H22–Fel d 1 induced increased IL-10 $^+$ and IL-5 $^+$ CD4 $^+$ T cells compared with Fel d 1, and this effect was restricted to subjects with allergy ($P \leq .05$; Fig 3). Interestingly, although H22–Fel d 1 induced an increased frequency of IFN- γ^+ cells compared

with Fel d 1, this effect was not statistically significant for any group (Fig 3).

H22–Fel d 1 induces cytokine-secreting subtypes consistent with T $_H$ 0 and regulatory T $_H$ 1 cells

Single-cell analysis of CD4 $^+$ T cells from subjects with allergy revealed that a significant proportion of cytokine-positive cells induced by H22–Fel d 1 expressed more than 1 cytokine (Fig 4). To assess further the nature of these cells, we analyzed the following 6 subtypes: (1) IFN- γ^+ only, (2) IL-5 $^+$ only, (3) IFN- γ^+ IL-5 $^+$, (4) IL-10 $^+$ only, (5) IL-10 $^+$ IFN- γ^+ , and (6) IL-10 $^+$ IL-5 $^+$. Before adjusting for multiple comparisons, H22–Fel d 1 induced significant increases in all subtypes compared with Fel d 1 ($P \leq .02$) with the exception of CD4 $^+$ T cells expressing IFN- γ only ($P = .25$). After adjusting for multiple comparisons, changes in 3 subtypes remained significant (IFN- γ^+ IL-5 $^+$, IL-10 $^+$ only, and IL-10 $^+$ IFN- γ^+ ; see this article's Fig E1 in the Online Repository at www.jacionline.org). Thus, although CD4 $^+$ T cells expressing IFN- γ alone were not significantly increased, cells expressing IFN- γ together with IL-5 or IL-10 were increased, pointing to the production of IFN- γ by T $_H$ 0 (IL-5 $^+$ IFN- γ^+) and regulatory T $_H$ 1 (IL-10 $^+$ IFN- γ^+) cells. Fig 5, A, is a color graphic summarizing geometric mean data for all cytokine-positive CD4 $^+$ T-cell subtypes in each of the 3 groups of subjects. This figure shows that the T-cell repertoire induced by H22–Fel d 1 was distinct from that induced by Fel d 1 in subjects with a modified T $_H$ 2 response (compare *third bar, upper panel*, with *second bar, middle panel*, Fig 5, A). In summary, H22–Fel d 1 induced a selective increase in T $_H$ 0 cells and IL-10-expressing T-cell subtypes in CD4 $^+$ T cells from subjects with allergy.

H22–Fel d 1 does not induce a proallergic CD4 $^+$ T-cell repertoire

Given that H22–Fel d 1 stimulated strong T-cell proliferation and induced an increased frequency of IL-5 $^+$ CD4 $^+$ T cells (Figs 1 and 3), we investigated whether the repertoire of cytokine-secreting T cells induced by this molecule was similar to that induced by the prototypic allergen, Der p 1. Five patients were selected for study who were cosensitized to cat and dust mite allergen, and cytokine expression was analyzed in CD4 $^+$ T cells after stimulation with H22–Fel d 1 or Der p 1. After adjusting for multiple comparisons, H22–Fel d 1 induced significant increases in T $_H$ 0 cells (IFN- γ^+ IL-5 $^+$) and all 3 IL-10-expressing subtypes (IL-10 $^+$ only, IL-10 $^+$ IFN- γ^+ , and IL-10 $^+$ IL-5 $^+$) compared with Der p 1 (Fig 5, B; see this article's Fig E2 in the Online Repository at www.jacionline.org). Thus, the T-cell repertoire induced by H22–Fel d 1 is distinct from a proallergic repertoire, comprising a higher proportion of T $_H$ 0 cells and diverse IL-10-expressing subtypes.

IL-10 induced by H22–Fel d 1 controls IL-5 $^+$ CD4 $^+$ T cells

Our previous work suggested a role for IL-10 in the development of nonallergic protective responses (modified T $_H$ 2) to cat allergen.⁴ Although IL-10 $^+$ CD4 $^+$ T cells were increased after stimulation with H22–Fel d 1 in cultures from subjects with allergy, IL-10-expressing T cells represented only a small fraction of total cytokine-positive T cells. This prompted us to question

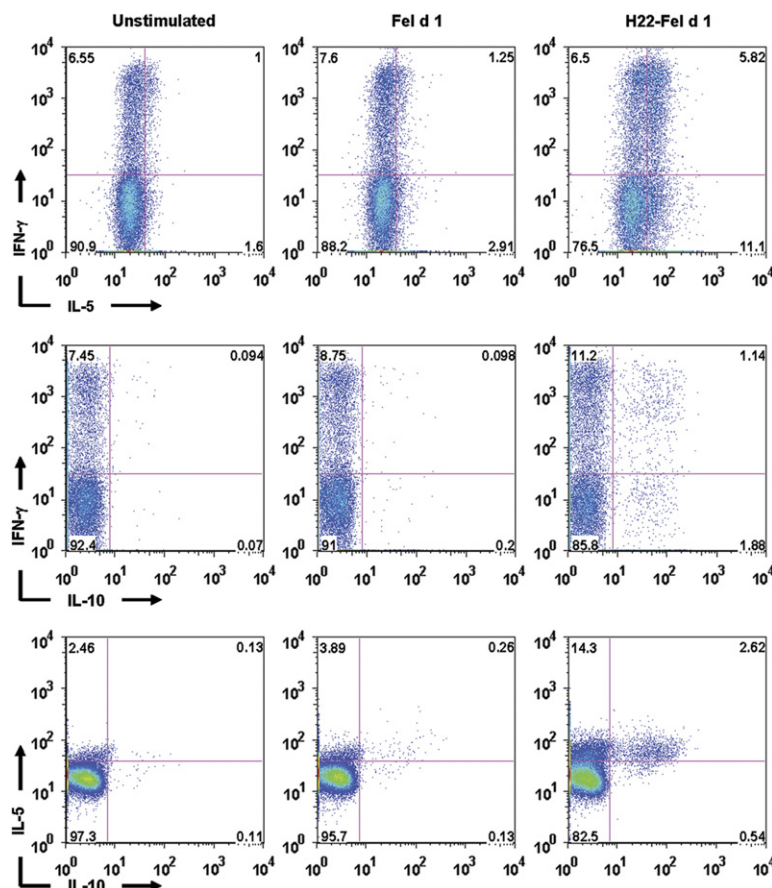


FIG 4. Representative data showing changes in cytokine-positive CD4⁺ T cells induced by H22–Fel d 1. Flow cytometry dot plots are shown for 1 of 10 patients with allergy.

whether T-cell responses induced by H22–Fel d 1 were controlled. Thus, we examined the effects of IL-10 blockade on the frequency of IL-5⁺ and IFN-γ⁺ CD4⁺ T cells. In the presence of antibody to the IL-10 receptor, stimulation with H22–Fel d 1 resulted in an increased frequency of IL-5⁺ CD4⁺ T cells in cultures from 4 of 5 subjects with allergy. By contrast, this antibody had little or no effect on IFN-γ⁺ or IL-10⁺ CD4⁺ T cells (Fig 6; see this article's Fig E3 in the Online Repository at www.jacionline.org). The increase in IL-5⁺ CD4⁺ T cells observed in the presence of anti-IL-10 receptor antibody suggests that the response to H22–Fel d 1 reflects a controlled T_H2 response.

DISCUSSION

Defining the effects of H22–Fel d 1 on the allergic T-cell response may provide a new approach for treatment of patients who have allergy to cats. We have shown that targeting Fel d 1 to FcγRI alters the quality of the CD4⁺ T-cell repertoire induced by Fel d 1 in subjects with cat allergy. Our findings showed a preferential increase in CD4⁺ T cells expressing the T_H2 cytokine, IL-5, which included T_H0 (IL-5⁺IFN-γ⁺) cells. These changes occurred in concert with increased IL-10-expressing T-cell subtypes. Moreover, we found evidence that IL-5⁺ CD4⁺ T cells induced by H22–Fel d 1 were controlled by IL-10. These observations point to complex changes in the T-cell repertoire at

the single-cell level and provide a new variation on existing paradigms related to T-cell modulation.

Other studies have described a fusion protein of Fel d 1 that targets allergen to the inhibitory receptor, FcγRIIb, on mast cells and basophils¹⁹; however, the effects of that molecule on T cells were not investigated. In the current study, H22–Fel d 1 selectively enhanced total IL-5⁺ and IL-10⁺ CD4⁺ T cells, as well as the proportion of these cells coexpressing IFN-γ. However, no change in CD4⁺ T cells which secreted IFN-γ alone was observed. Thus, H22–Fel d 1 induced an altered T_H2 response that did not fit with typical T_H1 skewing. Furthermore, although IL-5⁺ CD4⁺ T cells were a prominent feature of T-cell responses to H22–Fel d 1 in subjects with allergy, these cells were suppressed by IL-10 on the basis of IL-10 blocking experiments. The capacity for H22–Fel d 1 preferentially to increase IL-5⁺ T cells (and different T-cell subtypes) in cultures from subjects with cat allergy may reflect more efficient antigen uptake and presentation to T cells coupled with a higher precursor frequency of Fel d 1–specific T cells in subjects with compared with subjects without allergy.²⁰

If the T-cell response to H22–Fel d 1 does not signify T_H1 skewing per se, then what does it represent? Despite the presence of IL-5⁺ T cells, the T-cell repertoire induced by this molecule differed from that induced by the prototypic allergen, Der p 1. Moreover, the repertoire did not resemble the Fel d 1–specific repertoire observed in subjects with a modified T_H2 response or subjects without allergy (serum antibody-negative; Fig 5; see

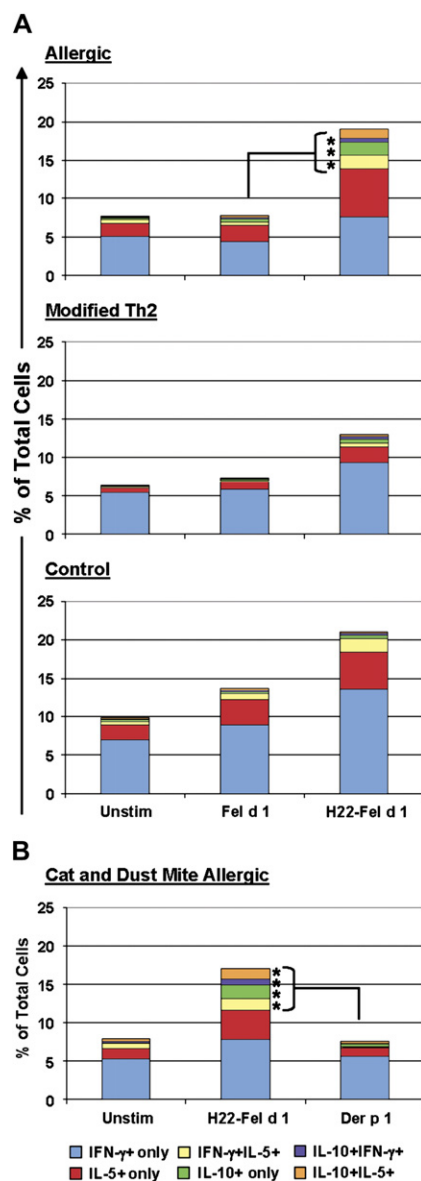


FIG 5. Summary of cytokine-positive CD4⁺ T-cell subtypes induced by H22-Fel d 1. **A**, Comparison of 6 CD4⁺ T-cell subtypes induced by Fel d 1 and H22-Fel d 1 in subjects with cat allergy (n = 10), modified TH2 (n = 5), and control (n = 5) subjects. **B**, Comparison of CD4⁺ T-cell subtypes induced by H22-Fel d 1 with Der p 1 in subjects cosensitized to cat and dust mite allergens (n = 5). Bars represent the geometric mean of the percentage of total CD4⁺ T cells for each subtype. *Significant after adjusting for multiple comparisons.

this article's Figs E1 and E2 in the Online Repository at www.jacionline.org. For all comparisons, the prevailing difference was induction of IL-10⁺ CD4⁺ T cells. The population of IL-10-expressing cells induced by H22-Fel d 1 included cells that expressed IL-10 alone, or with IFN- γ or IL-5. Such coexpression of IFN- γ or IL-5 is consistent with a regulatory TH1 or regulatory TH2 subtype, respectively. Similar types of IL-10-secreting cells have been previously described by Umetsu's group.^{21,22} In those studies, induction of regulatory TH1 and TH2 cells was mediated by distinct types of dendritic cells, and both types of regulatory T cells were shown to inhibit the development of TH2-driven airway

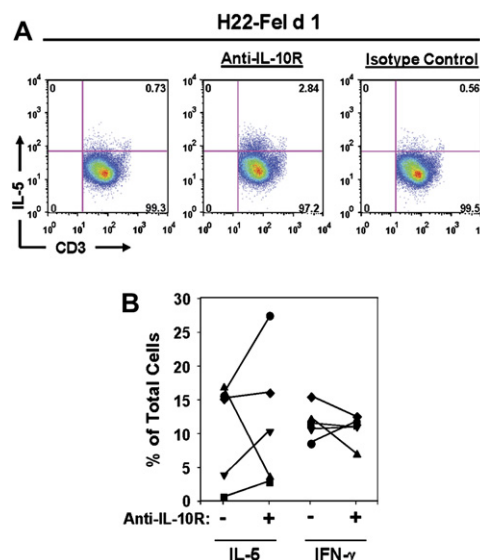


FIG 6. Effect of blocking IL-10 on cytokine-positive CD4⁺ T cells induced by H22-Fel d 1. CD4⁺ T cells were cocultured with MDDCs pulsed with H22-Fel d 1 in the presence of a blocking anti-IL-10 receptor mAb or isotype control (n = 5 subjects with cat allergy). **A**, Representative flow cytometry dot plots. **B**, Frequency of IL-5⁺ and IFN- γ ⁺ CD4⁺ T cells in the presence or absence of anti-IL-10 receptor (IL-10R) mAb for each subject.

hyperreactivity and inflammation in mice. Specifically, regulatory TH1 cells were induced by dendritic cells producing both IL-10 and IL-12.²² In the current study, H22-Fel d 1 induced increased production of IL-10 and IL-12 by dendritic cells from subjects with allergy in conjunction with increased IFN- γ ⁺IL-10⁺ CD4⁺ T cells. Thus, H22-Fel d 1 may condition dendritic cells to prime regulatory TH1 cells in individuals with allergy.

Mature dendritic cells are very efficient at T-cell priming. Such dendritic cells are characterized by high expression of costimulatory molecules (CD40, CD80, and CD86) and production of inflammatory cytokines. Dendritic cells with an immature or semimature phenotype are characterized by lower degrees of expression of costimulatory molecules and/or cytokine production.¹⁸ H22-Fel d 1 induced production of inflammatory cytokines by dendritic cells without increased expression of costimulatory molecules, consistent with a semimature phenotype. Such cells have been shown to exhibit a potent ability to induce regulatory T cells.¹⁸ Interestingly, H22-Fel d 1 did not induce increased secretion of TH2-promoting cytokines (TARC and eotaxin) by dendritic cells. Thus, these factors are unlikely to promote the generation of IL-5⁺ CD4⁺ T cells that we observed. Involvement of receptors expressed on dendritic cells (eg, OX40 ligand) provides an alternate mechanism and warrants further investigation.²³ Taken together, our findings suggest that H22-Fel d 1 induces a dendritic cell phenotype that is intermediate to that induced by Fel d 1 and LPS. This semimature phenotype may explain the selective enhancement of IL-5⁺ CD4⁺ T cells, including TH0 cells, in parallel with IL-10-expressing subtypes.

Although H22-Fel d 1 induced enhanced proliferative responses in PBMC cultures from subjects with allergy compared with Fel d 1, proliferation was even higher for cultures from subjects with a modified TH2 response. Despite this, the lowest increase in cytokine-positive T cells was observed for the modified TH2 group. Thus, cytokine production appeared to be dissociated from proliferation. This could arise from altered signaling to

T cells mediated at the APC/T-cell interface, which results in uncoupling of distinct signaling pathways within the T cell that are required for cell division and cytokine production.²⁴ Because the modified Th2 response to Fel d 1 may be controlled at the T-cell level,⁴ it is possible that targeting H22–Fel d 1 to FcγRI is not sufficient to overcome those regulatory mechanisms. Interestingly, <3% of total cytokine-secreting CD4⁺ T cells were IL-10⁺ in cultures from subjects with a modified Th2 response. Thus, low frequencies of IL-10⁺ T cells may be effective in controlling T-cell responses to allergen.

In summary, we describe a novel variation of a Th2 response to Fel d 1 in subjects with cat allergy. This response, which is induced by targeting Fel d 1 to FcγRI on dendritic cells, incorporates major elements of a protective T-cell response. We propose that targeting allergen to the high-affinity IgG receptor on APCs provides a new approach to T-cell-based vaccines for allergic disease.

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Clinical implications: Targeting allergen to FcγRI provides a new approach to T cell-based vaccines for allergic disease.

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METHODS

Proliferation assay

PBMCs were cultured for 7 days (2×10^5 cells/well in 96-well plates) in the presence of Fel d 1 or H22–Fel d 1 (0.1, 1, or 10 $\mu\text{g/mL}$; ≥ 6 replicate wells per condition).^{E1} Proliferation was measured by [^3H]-thymidine incorporation.

Generation of MDDCs

CD14⁺ monocytes were purified by magnetic-activated cell sorting (Miltenyi Biotec, Auburn, Calif) and cultured for 6 days in RPMI-1640 with 10% heat-inactivated FBS, L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% sodium pyruvate, 100 ng/mL recombinant human GM-CSF, and 100 ng/mL recombinant human IL-4 (BioSource International, Inc, Carlsbad, Calif) to generate MDDCs. Half of the media in each well was changed every 2 to 3 days.

Flow cytometry

All flow cytometry was performed on a Becton Dickinson FACSCalibur machine equipped with CellQuest software version 5.2 (BD Biosciences, Mountain View, Calif) and upgraded with a 635-nm red diode laser run by Rainbow software version 1.2b3 (Cytek Development, Gordonville, Va). All experiments were analyzed by using FlowJo version 6.4.1 (Tree Star Inc, Ashland, Ore).

Unless otherwise stated, all fluorescently conjugated mAbs were purchased from BD Biosciences. MDDC surface mAbs included phycoerythrin–anti-CD1a (HI149), peridinin-chlorophyll-protein–anti-CD14 (M ϕ P9), APC–anti-CD11c (B-ly6), biotin conjugated anti-CD64 (10.1) then avidin-APC-Cy7, and fluorescein isothiocyanate–anti-HLA-DR (L2234), anti-CD40 (5C3), anti-CD80 (L307.4), or anti-CD86 (2331). T-cell surface mAbs included

peridinin-chlorophyll-protein–anti-CD8 (SK1) and APC-Cy7–anti-CD3 (SK7), and intracellular mAbs included fluorescein isothiocyanate–anti-IL-5 (R&D Systems Inc, 22026, Minneapolis, Minn), phycoerythrin–anti-IL-10 (JES3-9D7), and APC–anti-IFN- γ (25723.11). For all multicolor analyses, compensation controls (single stains, 1 for each fluorochrome) and gating controls (cells stained with all reagents minus 1) were included.^{E2}

Statistical analysis

General linear mixed effects ANOVA models. Each ANOVA model was specified as a hierarchical model with respect to the model factors. For each ANOVA, the stimulus and cytokine type represented the highest and lowest level factor of the 2-level hierarchical model, respectively. Data from each subject represented an independent random block of correlated information within the model. ANOVA model parameters were estimated via restricted maximum likelihood, and the variance components were modeled in the compound symmetry variance-covariance matrix form. Linear contrasts of the ANOVA least-squares means were used to analyze the log-transformed data. All hypothesis tests were 2-sided, and the Tukey multiple comparison type I error rate adjustment was used so that the cumulative type I error rate (α) was ≤ 0.1 for the entire set of hypothesis tests. This value was selected on the basis of the conservative nature of the Tukey adjustment. The models were fit using PROC MIXED in SAS, version 9.1 (SAS Institute Inc, Cary, NC).

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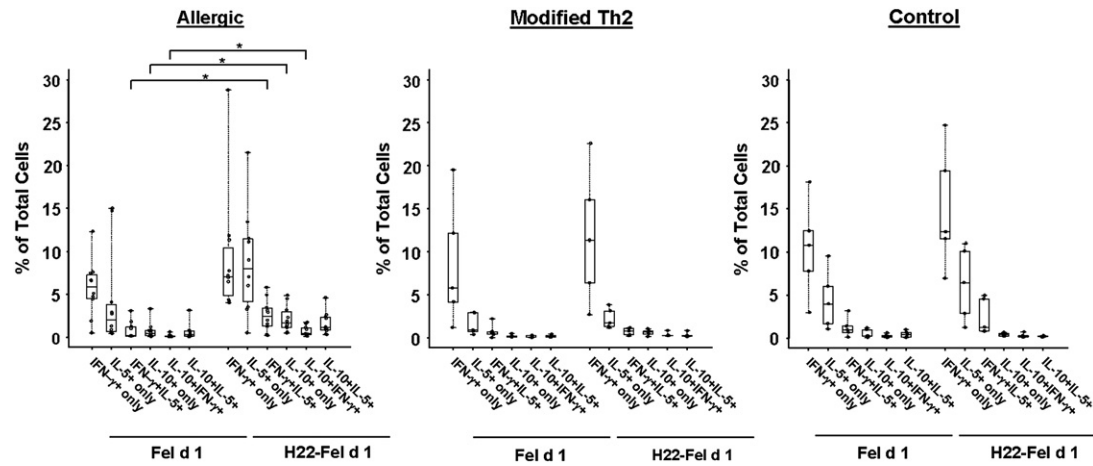


FIG E1. Changes in cytokine-positive CD4⁺ T-cell subtypes induced by H22-Fel d 1 compared with Fel d 1. CD4⁺ T cells were cocultured with MDDCs pulsed with Fel d 1 or H22-Fel d 1 for 10 days, and the percentage of the following 6 CD4⁺ T-cell subtypes was analyzed by flow cytometry: (1) IFN- γ ⁺ only; (2) IL-5⁺ only; (3) IFN- γ ⁺IL-5⁺; (4) IL-10⁺ only; (5) IL-10⁺IFN- γ ; and (6) IL-10⁺IL-5⁺. Experiments were performed in subjects with cat allergy (n = 10), modified T_H2 (n = 5), and control (n = 5) subjects. Boxes represent medians and interquartile ranges. *Significant after adjusting for multiple comparisons.

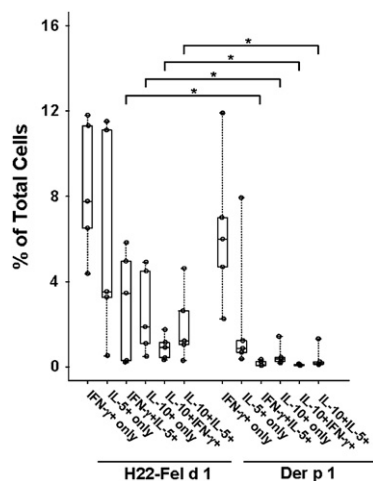


FIG E2. Comparison of T-cell repertoires induced by H22-Fel d 1 and Der p 1. CD4⁺ T cells were cocultured with MDDCs pulsed with H22-Fel d 1 or Der p 1 for 10 days, and the percentage of the following 6 CD4⁺ T-cell subtypes was analyzed by flow cytometry: (1) IFN- γ ⁺ only; (2) IL-5⁺ only; (3) IFN- γ ⁺IL-5⁺; (4) IL-10⁺ only; (5) IL-10⁺IFN- γ ; and (6) IL-10⁺IL-5⁺. Experiments were performed in 5 subjects who were cosensitized to cat and dust mite allergens. Boxes represent medians and interquartile ranges. *Significant after adjusting for multiple comparisons.

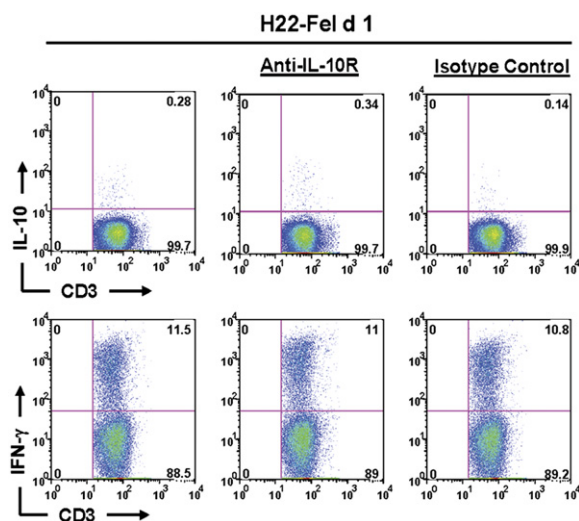


FIG E3. Effect of blocking IL-10 on cytokine-positive CD4⁺ T cells induced by H22-Fel d 1. CD4⁺ T cells were cocultured with MDDCs pulsed with H22-Fel d 1 in the presence of a blocking anti-IL-10 receptor mAb or isotype control. Flow cytometry *dot plots* showing effects of IL-10 blockade on IL-10⁺ and IFN-γ⁺ cells (representative of 5 subjects with cat allergy).