

A Role for IL-10-Mediated HLA-DR7-Restricted T Cell-Dependent Events in Development of the Modified Th2 Response to Cat Allergen¹

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Although high dose exposure to inhaled cat allergen (Fel d 1) can cause a form of tolerance (modified Th2 response), the T cell mechanism for this phenomenon has not been studied. T cell responses to Fel d 1 were characterized in both allergic (IgE^{pos}) and modified Th2 (IgE^{neg}IgG^{pos}) responders as well as serum Ab-negative controls (IgE^{neg}IgG^{neg}). Fel d 1 stimulated high levels of IL-10 in PBMC cultures from all individuals, with evidence of Th2 and Th1 cytokine skewing in allergic and control subjects, respectively. Using overlapping peptides, epitopes at the N terminus of Fel d 1 chain 2 were shown to stimulate strong T cell proliferation and to preferentially induce IL-10 (peptide 2:1 (P2:1)) or IFN- γ (P2:2) regardless of the allergic status of the donor. Injection of cat extract during conventional immunotherapy stimulated expansion of IL-10- and IFN- γ -producing chain 2 epitope-specific T cells along with increased Fel d 1-specific serum IgG and IgG4 Ab. Six of 12 modified responders expressed the major HLA-DRB1 allele, *0701, and both P2:1 and P2:2 were predicted ligands for this allele. Cultures from DR7-positive modified responders produced the highest levels of IL-10 to P2:1 in addition to other major and minor epitopes within chains 1 and 2. In the presence of anti-IL-10 mAb, both T cell proliferation and IFN- γ production were enhanced in a Fel d 1- and epitope-specific manner. We conclude that IL-10-producing T cells specific for chain 2 epitopes are relevant to tolerance induction, and that DR7-restricted recognition of these epitopes favors a modified Th2 response. *The Journal of Immunology*, 2004, 172: 2763–2772.

There is now extensive evidence that children raised in a house with animals do not have an increased risk of sensitization to allergens (1–4). Furthermore, living with a cat and exposure to high levels of the major cat allergen, Fel d 1, can induce a form of tolerance described as a modified Th2 response (1). This immune response is characterized by high titer Fel d 1-specific serum IgG and the IL-4-regulated Ab isotype IgG4 without IgE; this response is not associated with an increased risk of allergic symptoms or asthma (1, 2). In humans, s.c. administration of allergen results in improvement of allergic symptoms and down-regulation of allergen-specific T cell responses (5–9). This response has been attributed to increased production of IL-10 or induction of regulatory T cells measured both systemically (i.e., in circulating T cells) and locally at sites of allergen challenge (i.e., skin and nasal mucosa) (6, 10–13). To date, no studies have examined the relevance of IL-10 to high dose tolerance induced by natural exposure to an inhalant allergen. In murine models of asthma, respiratory tolerance to high dose OVA has been attributed to the induction of IL-10-producing dendritic cells, which drive differentiation of T regulatory cells within the lung (14). In keeping

with this, adoptive transfer of CD4⁺ Th cells engineered to express IL-10 abolished airway hyper-reactivity and eosinophilia (15). Nevertheless, there is evidence against a protective role for IL-10 in development of asthma. In some animal models, IL-10 appears to be necessary for development of airway hyper-reactivity (16, 17). Furthermore, elevated levels of IL-10 production or IL-10 mRNA expression have been reported in PBMC and lung-derived lymphocytes or monocytes from asthmatic patients (18–22).

In an attempt to reconcile these observations, we analyzed the relevance of IL-10 to allergic (IgG^{pos}, IgG4^{pos}, IgE^{pos}), nonallergic (IgG^{neg}, IgG4^{neg}, IgE^{neg}), and tolerant (IgG^{pos}, IgG4^{pos}, IgE^{neg}) responses to cat allergen. Our findings show that although IL-10 is a feature of both allergic and nonallergic responses to cat allergen, major epitopes of Fel d 1 that selectively induce IL-10 or IFN- γ fulfill a tolerogenic role in both allergic patients receiving conventional immunotherapy and in modified Th2 responders. Furthermore, although recognition of these epitopes is not unique to one group of patients, our findings point toward a central role for DR7-restricted recognition of these epitopes and IL-10 production in mediating tolerance to cat allergen.

Materials and Methods

Classification of allergic, modified Th2, and nonallergic subjects

Study participants were recruited from patients attending the University of Virginia Allergic Diseases Clinic or by advertisement. Seventy-four patients were skin tested with commercial cat extract (Greer, Lenoir, NC) and with affinity-purified natural Fel d 1 obtained from cat hair (23, 24). Natural Fel d 1 was filtered through a 0.2- μ m pore size, sterile, nitrocellulose disposable filter (Micron Separations, Westborough, MA) and diluted for prick testing to 20 μ g/ml in 0.05% human serum albumin in phenol-saline solution. Skin test sites were examined 10 min after injection of Ag and compared with a diluent control. Criteria for classifying patients in the allergic experimental group ($n = 14$) were a positive immediate skin prick test to cat extract and to Fel d 1 and the presence of serum IgE Ab to cat (>0.7 IU/ml). Subjects with a modified Th2 response ($n = 12$) had a

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negative skin test to cat and Fel d 1, no measurable IgE Ab to cat, and high titer anti-Fel d 1 IgG Ab (>500 U/ml). Nonallergic subjects (controls, $n = 11$) were identified on the basis of negative skin test reactivity and no measurable serum IgG (<125 U/ml) or IgE (<0.35 IU/ml) Ab to cat. These studies were approved by the University of Virginia human investigation committee.

Immunoassays for IgG, IgG4, and IgE Abs to cat

Serum IgG and IgG4 Ab to Fel d 1 were measured using an Ag binding radioimmunoprecipitation assay by methods described previously (1, 25). Quantitation of anti-Fel d 1 IgG and IgG4 Ab was conducted using separate control curves established with serum obtained from cat-allergic patients assigned to contain 2000 U of IgG or 1500 U of IgG4 Ab/ml. IgE Ab to cat was measured by CAP assay (Pharmacia Biotech, Uppsala, Sweden).

Measurement of Fel d 1 levels in floor dust samples

Floor dust samples were collected from the living rooms of the homes of study patients using standard techniques, and samples were assayed for Fel d 1 content (micrograms per gram of dust) by ELISA (23).

Preparation of Ag and peptides for T cell studies

A set of 20 peptides was synthesized by standard F-moc chemistry (University of Virginia Biomolecular Research Facility) using a Symphony automated peptide synthesizer (50 μ m scale; Rainin, Woburn, MA), and peptides were purified to $>90\%$ by reverse phase HPLC. Peptides spanning both polypeptide chains of the heterodimeric Fel d 1 molecule were designed with a 10-aa overlap (26). Peptides spanning chain 1 were designated P1:1 through P1:9, and those spanning chain 2 were designated P2:1 through P2:11. Peptides contained 17 aa, with the exception of P1:9 (14-mer) and P2:11 (19-mer), which spanned the C termini of chains 1 and 2. Stock solutions of peptides (1 mM) were solubilized in sterile water or 50% DMSO. All peptides were determined to be free of endotoxin by quantitative chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD). Fel d 1, tetanus toxoid (TT),³ and PHA were sterile-filtered before use.

PBMC cultures

PBMC were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech) from 90 cc of blood and cultured in complete medium (C-RPMI) containing RPMI 1640 (Life Technologies, Gaithersburg, MD) with L-glutamine, 10% autologous human serum (heat-inactivated), 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Proliferation assays. PBMC were cultured in the presence of 10 μ g/ml Fel d 1 (2×10^5 cells in quadruplicate wells) or 7.5 μ M Fel d 1-derived peptides (3×10^5 cells in 12 replicate wells) for 6 days at 37°C in 96-well plates. TT (10 μ g/ml) and PHA (20 μ g/ml) in quadruplicate wells were used as positive controls, and 60 unstimulated wells (12 wells on each assay plate) were negative controls. Cells were pulsed with 1 μ Ci of [³H]thymidine/well during the final 8 h of culture before harvesting and counting (Topcount NXT; Packard Instrument, Meriden, CT). Results were expressed as stimulation indexes for Fel d 1 and TT. Data for peptides were log-transformed to derive a standardized index value as described previously (27).

Cytokine assays. Cells were cultured in 24-well plates (4×10^6 cells/well in 1-ml volume) in the presence of Fel d 1 (10 μ g/ml) or peptide (7.5 μ M). Positive control wells included PHA and TT, whereas unstimulated and medium-only wells served as negative controls. Culture supernatants were harvested on day 6 and assayed by ELISA for IL-4, IL-5, IL-10, IL-13, and IFN- γ content using matched mAb pairs (Pierce-Endogen, Woburn, MA). Values were derived from standard curves to a sensitivity of 2 pg/ml for IL-4, IL-5, IFN- γ , and IL-13, and 4 pg/ml for IL-10.

HLA class II typing

Genomic DNA was isolated from 2 ml of whole blood, and HLA typing was conducted in the Molecular Pathology Laboratory at University of Virginia as described previously (27). Sequences were evaluated using HLA SequiTyper software (Pharmacia Biotech) and compared with the European Bioinformatics Institute HLA database (European Bioinformatics Institute, Cambridge, U.K.) for allele assignments.

Computer prediction of CD4⁺ T cell epitopes using TEPITOPE

The primary amino acid sequence of Fel d 1 chains 1 and 2 was loaded into the HLA-DR ligand prediction software, TEPITOPE (Vaccinome, Kearny, NJ), to predict promiscuous HLA-DR ligands. HLA-DR alleles most frequent in the Caucasian population (*0101, *0301, *0401, *0701, *0801, *1101, *1501) were selected, and the TEPITOPE prediction threshold was set at 5% (28, 29). Peptides binding to three or more different allotypes were defined as promiscuous.

Studies using anti-IL-10 mAb

Freshly isolated PBMC were stimulated with Fel d 1 (10 μ g/ml), pooled chain 2 peptides (P2:1, P2:2, P2:3, and P2:4; 7.5 μ M each), or TT (1 μ g/ml) for 7 days, with or without anti-IL-10 mAb (10 μ g/ml; rat IgG2a κ ; Pierce-Endogen). Unstimulated cultures and cultures containing an isotype control (BioSource International, Camarillo, CA) were included as negative controls. For proliferation assays, cultures incorporated four replicate wells (Fel d 1 and TT) or 12 replicate wells (for each peptide), with 12 unstimulated wells on each 96-well plate. For cytokine measurements, assays were conducted in 24-well plates, and culture supernatants were assayed for IL-5, IL-10, IL-13, and IFN- γ by ELISA.

Flow cytometric analysis

IL-10-producing cells were isolated by cytokine secretion assay (Miltenyi Biotec, Auburn, CA). Briefly, freshly isolated PBMC (7×10^7) were cultured in C-RPMI for 16 h in six-well plates in the absence or the presence of pooled Fel d 1 peptides (P2:1, P2:2, P2:3, and P2:4; 15 μ M each). Cells were washed, resuspended ($\sim 10^8$ cells/ml), and labeled with IL-10 Catch Reagent (5 min on ice) before incubating for 45 min in humidified 5% CO₂ at 37°C in warm C-RPMI (1×10^6 cells/ml). After washing and labeling with PE-conjugated IL-10 detection Ab (20 μ l label/ 10^7 cells), cells were enriched using anti-PE microbeads (20 μ l/ 10^7 cells), passing them through a 30- μ m pore size mesh nylon filter and applying them to an LS column (VarioMACS; Miltenyi Biotec). Isolated cells were counterstained using FITC-labeled anti-CD4 mAb (Miltenyi Biotec) before analyzing by flow cytometry (FACScan equipped with CellQuest software; BD Biosciences, Mountain View, CA).

Studies of patients receiving conventional immunotherapy

Serum Abs and T cell responses to cat were monitored for 4 mo in five cat-allergic patients who started conventional immunotherapy (IT) as part of their routine care in the Allergic Diseases Clinic. All patients demonstrated positive skin prick tests and serum IgE Ab to cat (≥ 0.35 IU/ml). Blood (90 cc) was collected before starting IT (baseline) and at monthly intervals thereafter.

Cat IT. Commercial cat extract (10,000 bioequivalent allergy units; Greer) containing 0.154 mg of Fel d 1/ml was administered s.c. in the upper arm. The first injection contained 0.0077 μ g of Fel d 1, and injections were received twice weekly with stepwise increments to an ideal maximum dose containing 77 μ g of Fel d 1. The total cumulative dose of Fel d 1 received after 4 mo ranged from 4.8 μ g (patient 62) to 451 μ g (patient 53).

Statistical analysis

Proliferation to whole allergen and to each of the 20 peptides was compared between groups using mean stimulation indexes and mean standardized indexes, respectively. Cross-validated linear discriminant analysis was conducted to identify peptide markers associated with distinct immune responses based on proliferation data (27, 30, 31). Between-group cytokine responses to whole allergen and to each peptide were analyzed by Mann-Whitney *U* test and independent two-sample Student's *t* test, respectively. One-way ANOVA was used to compare the mean of the sum of all 20 peptides in DR7-positive and DR7-negative modified Th2 subjects as well as allergic and control groups. The pattern of the distribution of mean levels of cytokine responses to all 20 peptides was compared by Wilcoxon matched pairs, signed ranks test. Linear regression analysis was used to determine relationships between serum Ab titers and Fel d 1 levels in floor dust. Between-group frequencies of HLA-DR7 expression and the prevalence of measurable Th2 cytokines to Fel d 1 were compared using the Exact binomial test and Pearson χ^2 test, respectively. Statistical tests were

³ Abbreviations used in this paper: TT, tetanus toxoid; IT, immunotherapy.

conducted using S-Plus version 2000 (Insightful, Seattle, WA) and SPSS 10.1 (SPSS, Chicago, IL) software packages.

Results

Tolerance to cats is associated with high dose exposure to cat allergen

The relationship among exposure to Fel d 1, distinct allergic phenotypes, and Ab responses to cat allergen was examined (Table I). The significantly higher level of Fel d 1 measured in the homes of

modified Th2 compared with allergic responders (mean, 711 vs 17 $\mu\text{g/g}$ floor dust; $p = 0.024$) was consistent with the high prevalence of cat ownership in the modified group (>90%) compared with other experimental groups (<40%). In individuals who had an allergen-specific IgG Ab response, concentrations of Fel d 1 in floor dust were strongly correlated with Ab titers ($r = 0.77$; $p < 0.0001$); however, the absence of a humoral response was not restricted to individuals with low exposure to Fel d 1 (Table I).

Table I. Characteristics of patients with distinct immune responses to cat

Group	Age (year), Sex, Race ^a	Fel d 1 ($\mu\text{g/g}$) and (No. of Cats)	Anti-Fel d 1 Ab ^b (U/ml)		IgE to Cat ^c (IU/ml)	DRB1
			IgG	IgG4		
Allergic						
6 ^d	22 FW	4.3 (0)	153	<140	5.12	0901 1501
7 ^e	26 FW	9.5 (0)	4,878	208	17	0301 0701
8 ^d	29 FW	0.3 (0)	421	<140	2.59	0301 0404
9 ^d	28 FA	0.4 (0)	233	<140	1.35	0301 0405
11 ^e	28 MW	112 (0)	1,410	<140	14.7	0301 1501
12 ^d	23 FB	0.7 (0)	338	<140	0.94	0404 1301
13 ^e	24 MW	1,272 (1)	9,159	5,733	17.1	0301 0407
14 ^{d,f}	31 FW	0.4 (0)	384	<140	0.77	1104 1302
15 ^d	24 MA	44 (0)	849	<140	3.86	0801 1308
45 ^e	32 FW	1,752 (4)	43,700	34,145	1.99	0103 1104
46 ^{d,e}	53 FB	1,907 (12)	11,443	5,230	31.1	0301 1501
47 ^d	26 FW	401 (3)	1,987	1,242	6.46	0301 0401
49 ^{d,e,f}	36 FW	3.4 (0)	1,609	<140	4.15	0401 1201
53 ^{e,g}	50 FW	3.5 (0)	2,432	1,406	2.89	0404 1101
Mean	31	17 ^h	1,536 ^h	496 ^h	4.40 ^h	
Mod. Th2						
1	48 FW	145 (2)	8,962	<140	<0.35	0301 1501
2	42 FW	4,700 (2)	2,374	1,064	<0.35	0404 0701
3	22 MW	166 (0)	2,916	1,273	<0.35	0701 1001
4 ^d	59 FW	71 (1)	786	<140	<0.35	0103 0405
5 ^d	34 FW	271 (1)	5,603	1,998	<0.35	0701 1302
30	24 MW	2,380 (3)	5,418	2,060	<0.35	0701 1501
31 ^d	28 FW	3,004 (3)	5,883	2,349	<0.35	0701 1501
32	26 MB	68 (2)	2,025	<140	<0.35	0401 0901
33 ^e	26 MW	563 (3)	1,669	964	<0.35	0101 0401
43	50 FW	3,700 (7)	10,239	10,876	<0.35	0102 1401
44 ^d	59 FW	2,605 (14)	50,500	43,472	<0.35	0401 0701
48	37 FW	2,908 (3)	9,935	3,569	<0.35	0301 1101
Mean	38	711 ^h	4,804 ^h	1,404 ^h	<0.35	
Control						
21	24 FW	1,272 (1)	<60	<140	<0.35	0301 0701
22	21 FW	2.3 (0)	<60	<140	<0.35	0101 1301
23	26 FW	4,656 (3)	<60	<140	<0.35	0101 1501
24	32 MW	5.6 (0)	<60	<140	<0.35	1302 1501
26	24 MW	24.5 (0)	<60	<140	<0.35	0701 1101
27	22 FW	1.1 (0)	<60	<140	<0.35	0301 1501
35 ^d	28 FB	2.4 (0)	<60	<140	<0.35	0301 0901
36	25 MW	1,197 (1)	<60	<140	<0.35	0301
37	19 MA	132 (1)	<60	<140	<0.35	0102 0403
42 ^d	25 MW	5.3 (0)	<60	<140	<0.35	0701 1301
50	34 FW	738 (4)	<60	<140	<0.35	0101 0701
Mean	26	48 ^h	<60	<140	<0.35	
Immunotherapy						
52 ^{d,e}	31 MW	ND (0)	3,395	211	0.43	0102 0401
60 ^e	69 MW	ND (0)	1,229	<140	0.35	1101 1501
62 ^d	37 FW	ND (1)	14,893	6,083	1.01	0301 0404
63 ^d	25 MW	ND (0)	2,706	<140	0.35	0408 0701

^a F, female; M, male; W, white; B, black; A, Asian.

^b Measured by Ag binding radioimmunoprecipitation assay.

^c Measured by Pharmacia CAP assay.

^d Patients with rhinitis.

^e Patients with asthma.

^f Patients with hives.

^g Patient received immunotherapy.

^h Geometric mean values.

Fel d 1 induces high levels of IL-10 in allergic and nonallergic donors

Fel d 1-specific T cell responses were compared between allergic and nonallergic responders. T cell proliferation in the modified group was comparable to that in the allergic group, with lowest levels in the control group (Fig. 1A). Surprisingly, Fel d 1 induced high levels of IL-10 in PBMC cultures established from all patient groups, with slightly lower levels in modified responders ($p < 0.023$; Fig. 1, B and C). The level of IFN- γ was significantly increased in controls compared with allergic subjects ($p = 0.025$). In addition, there was a trend toward decreased IL-5 production in modified (mean, 4.6 ± 2.2 pg/ml) and control (mean, 2.2 ± 0.2 pg/ml) subjects compared with allergics (mean, 16 ± 7 pg/ml), with a significant difference between allergics and controls ($p = 0.025$; Fig. 1, B and C). No IL-4 was measurable in any of these primary cultures stimulated with Fel d 1. The cytokine profiles observed in this study provide evidence of Th1 and Th2 skewing in nonallergic and allergic subjects, respectively, but do not fit classical Th1/Th2 patterns. Furthermore, cytokine profiles in Fel d 1-stimulated cultures were in marked contrast to the IFN- γ -dominated profile observed in cultures stimulated with TT (Fig. 1B).

Novel chain 2 major epitopes of Fel d 1 selectively induce IL-10 and IFN- γ

To resolve the significance of cytokine profiles induced in cultures stimulated with cat allergen, peptides spanning chains 1 and 2 of the Fel d 1 molecule were used to stimulate T cell responses in vitro. Major epitopes of Fel d 1 (defined as those with a mean standardized index ≥ 1.5) localized to chain 1 (P1:2 and P1:6) as described previously (32, 33); however, a new immunodominant region was also identified, which mapped to the N terminus of chain 2 (P2:1, P2:2, P2:3, P2:4; Fig. 2A). By cross-validated linear discriminant analysis, P1:2 was the best single peptide marker for distinguishing allergic and control groups (68% of patients classified correctly) (Student's t test, $p = 0.038$) compared with P1:1 for allergic and modified groups (69% of patients classified correctly) (Student's t test, $p = 0.007$). Induction of IL-5 also localized to P1:1 and P1:2 (Fig. 2B), with a trend toward significantly increased levels in allergics compared with controls ($p = 0.098$ and $p = 0.072$ for P1:1 and P1:2, respectively). Furthermore, mean production of IL-5 to all 20 peptides was significantly increased in the allergic group compared with modified and control groups ($p < 0.04$), but not in the modified group compared with controls

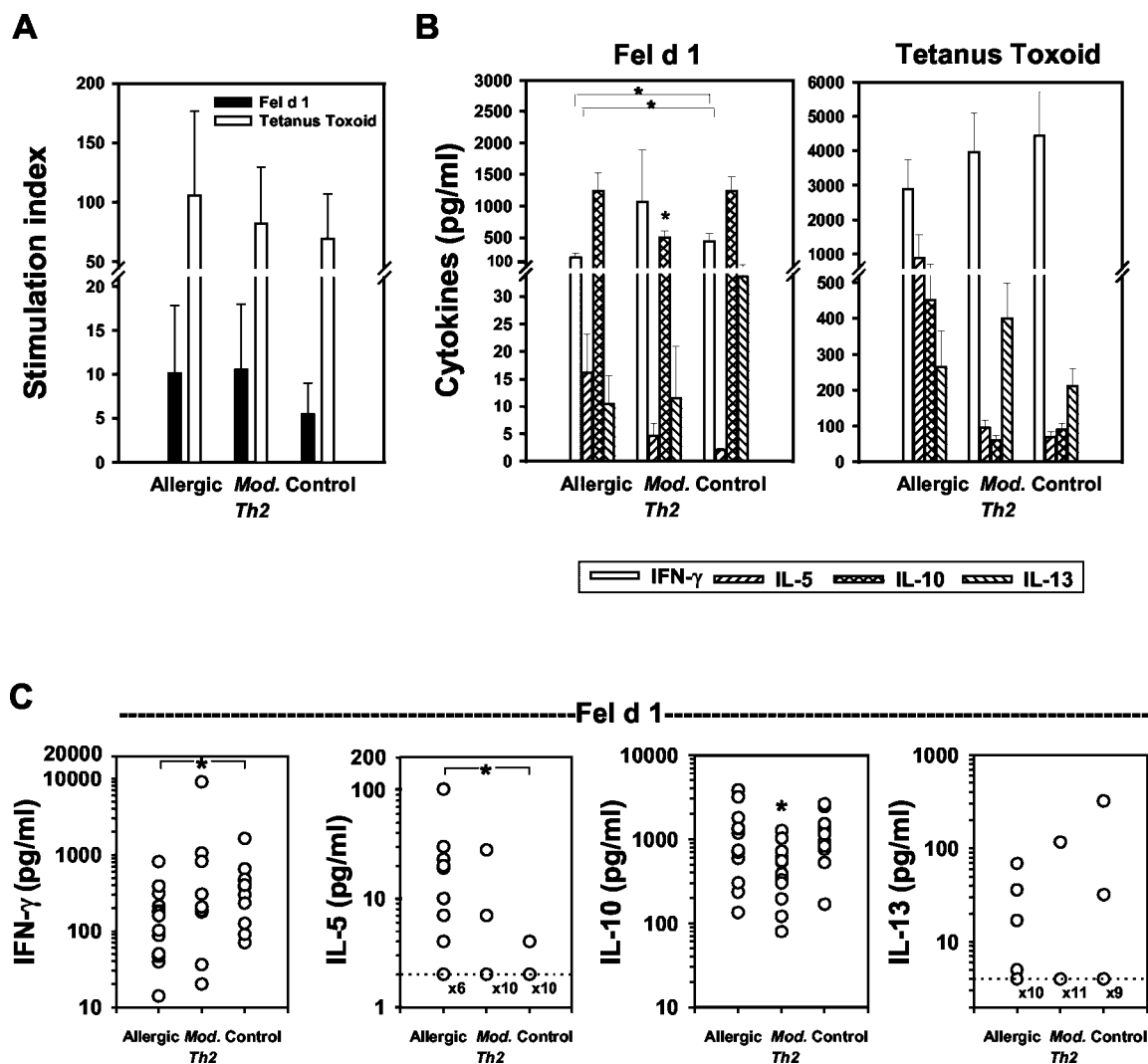


FIGURE 1. Fel d 1 induces IL-10-dominated responses in allergic and modified responders. Freshly isolated PBMC were stimulated for 6 days with 10 μ g/ml Fel d 1 or TT. A, Proliferation was measured by [^{3} H]thymidine incorporation (cpm $\times$ 1000) and expressed as the mean stimulation index $\pm$ SEM. B, Culture supernatants were assayed for cytokines, and values expressed as the mean $\pm$ SEM. C, Comparison of the distribution of Fel d 1-induced cytokines between patient groups. Dotted lines represent the sensitivity of the ELISA. Allergics, $n = 14$; modified Th2, $n = 12$; controls, $n = 11$. *, $p \leq 0.025$.

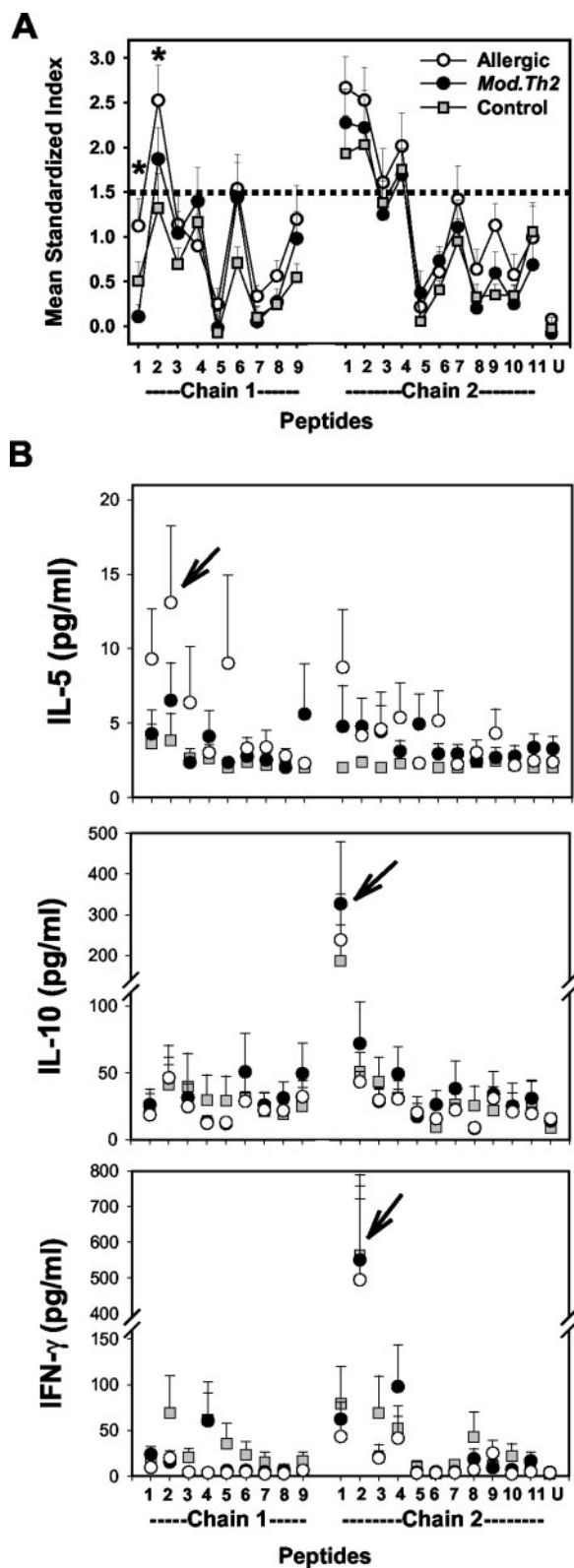


FIGURE 2. Chain 2 epitopes of Fel d 1 selectively induce IL-10 and IFN- γ . PBMC were stimulated for 6 days in the presence of Fel d 1 peptides spanning chains 1 and 2. **A**, Proliferation assays incorporated 12 replicate wells for each peptide ($n = 20$), and the mean standardized index $\pm$ SEM was used for between-group comparisons. The dotted line represents an arbitrary reference line corresponding to a mean standardized index of 1.5. *, $p = 0.007$ for P1:1 (allergics vs modified responders) and $p = 0.038$ for P1:2 (allergics vs controls). **B**, Cytokine values represent the mean $\pm$ SEM. The arrows in three panels denote peptides that induce IL-5 (P1:2), IL-10 (P2:1), or IFN- γ (P2:2), respectively. U, unstimulated wells.

($p = 0.054$). In contrast, the novel chain 2 major epitopes, P2:1 through P2:4, stimulated strong T cell reactivity in all patient groups, with overlapping peptides P2:1 and P2:2 inducing the highest levels of IL-10 and IFN- γ , respectively (Fig. 2B). Mean levels of IL-10 in P2:1-stimulated cultures were highest in the modified group (326 ± 152 pg/ml vs 238 ± 113 pg/ml for allergics and 187 ± 88 pg/ml for controls), whereas P2:2 induced comparable levels of IFN- γ in all patient groups (allergic, 494 ± 226 pg/ml; modified, 562 ± 215 pg/ml; controls, 563 ± 227 pg/ml; Fig. 2B). Flow cytometric analysis of lymphocytes from subjects with distinct immune responses identified IL-10-producing CD4 $^{+}$ T lymphocytes in cultures stimulated with pooled chain 2 peptides (P2:1 through P2:4; Fig. 3).

Subcutaneous injection of whole allergen is associated with activation of T cells that target chain 2 epitopes

Injection of proteins from diverse sources, including cat, during conventional allergen immunotherapy has been reported to induce T cell hyporesponsiveness mediated by IL-10 and/or immune deviation from Th2 to Th1 responses (5–7, 10, 34, 35). In addition, elevated allergen-specific IgG4 Ab and decreased IgE Ab frequently accompany successful IT (8, 36–38). We theorized that, by virtue of their ability to induce IL-10 and IFN- γ , chain 2 epitopes of Fel d 1 would fulfill an important role in tolerance induction during IT. T cell cultures were studied at monthly intervals for 4 mo in five cat-allergic patients (no. 52, 53, 60, 62, and 63) starting IT. Immunotherapy was associated with increases in production of IgG and IgG4 Ab specific for Fel d 1 in all patients

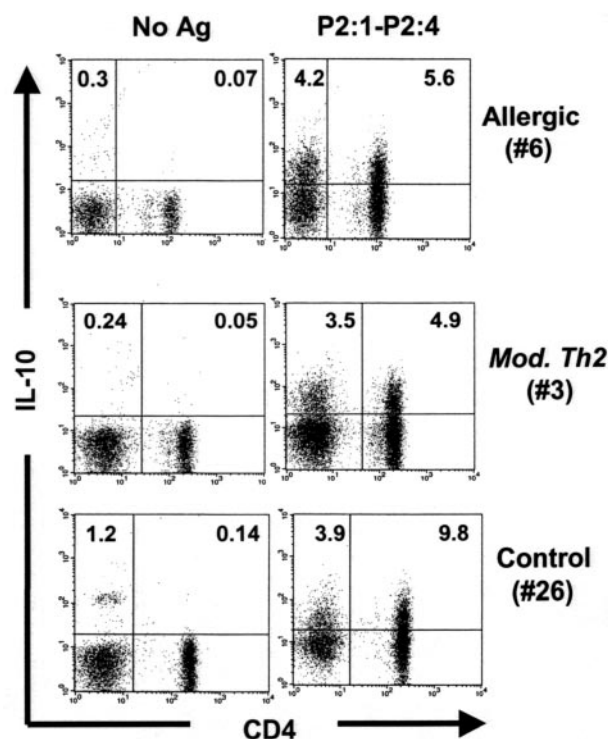


FIGURE 3. IL-10 $^{+}$ CD4 $^{+}$ T lymphocytes that recognize chain 2 major epitopes are a component of the Fel d 1-specific T cell repertoire in patients with diverse serological responses. Detection of live IL-10-secreting T cells was conducted after in vitro culture of PBMC with pooled chain 2 peptides (16 h). MACS was used to separate cells labeled with PE-conjugated anti-IL-10 mAb using anti-PE microbeads, and cells were counterstained with FITC-CD4 mAb before analysis. Live lymphocytes were gated according to light scatter properties and propidium iodide exclusion. Representative data from experiments on six patients is shown.

(Fig. 4A). Maximal increases in production of peptide-induced IL-10 and IFN- γ localized to the N-terminal region of Fel d 1 spanned by peptides 2:1, 2:2, 2:3, and 2:4 (Fig. 4B). Strong T cell reactivity to chain 2 epitopes persisted, whereas levels of IL-5 and IL-13 generally remained unchanged throughout the course of therapy (data not shown). The results show that enhancement of in vitro T cell responses to these chain 2 epitopes is a consistent feature of the response to cat allergen injected in vivo.

IL-10 production in modified Th2 responders is associated with expression of DR7

The ability for the IL-10- and IFN- γ -inducing chain 2 epitopes, P2:1 and P2:2, to induce strong T cell responses in individuals with diverse HLA-DR types (Table I) was consistent with the presence of multiple DR ligands within these peptides (Fig. 5A). HLA analysis revealed an increased frequency of HLA-DR7 in the modified group (6 of 12 patients) compared with allergics (one of 14; $p = 0.002$; Table I). Of the four DR7 peptide binding motifs identified within Fel d 1, two localized to the major epitopes P2:1 and P2:2 (Fig. 5A), whereas the remaining two mapped to weakly stimulatory peptides (P1:5 and P2:9), suggesting that these epitopes were subdominant or not relevant in vivo. IL-10 production to P2:1 was markedly increased in DR7-positive modified responders compared with their DR7-negative counterparts (mean, 560 ± 278 vs 93 ± 49 pg/ml) and compared with allergic (238 ± 113 pg/ml) and control (187 ± 88 pg/ml) groups (Fig. 5B). Furthermore, P2:1 induced increased IL-10 production in DR7-positive subjects with an allergic or modified response (711 ± 280 pg/ml; $n = 7$) compared with DR7-negative allergic or modified responders (119 ± 32 pg/ml; $n = 19$; $p < 0.01$). Mean levels of IL-10 production in

response to all 20 peptides were enhanced in DR7-positive vs DR7-negative modified subjects ($p = 0.029$). Differences in the pattern of peptide-induced IL-10 induction were also observed for DR7⁺ modified subjects compared with each of the other patient groups ($p < 0.001$; Fig. 5B).

IL-10 down-regulates Fel d 1- and epitope-specific Th responses independent of allergic phenotype

The association between IL-10 production and the modified Th2 response coupled with activation of chain 2 epitope-specific T cells during IT support a role for IL-10 in tolerance to cat allergen. The effects of anti-IL-10 mAb on Fel d 1-specific T cell responses were examined in cultures from two allergic, two modified, and two nonallergic high IL-10 producers (>200 pg/ml IL-10 in Fel d 1-stimulated cultures). Anti-IL-10 mAb caused a significant increase in T cell proliferation in response to Fel d 1 and pooled chain 2 epitopes in all cultures (Fig. 6A). Marked increases in IFN- γ production were also observed for all cultures in the presence of anti-IL-10 mAb, whereas increases in IL-5 and IL-13 were restricted to allergic and modified responders (Fig. 6B). There was generally no effect of anti-IL-10 mAb on cultures stimulated with TT (Fig. 6A and data not shown). Findings support the view that IL-10 down-regulates Fel d 1-specific T cell responses associated with distinct allergic phenotypes.

Discussion

We have identified major T cell epitopes localizing to chain 2 of the cat allergen, Fel d 1, which selectively induce IL-10 and IFN- γ .

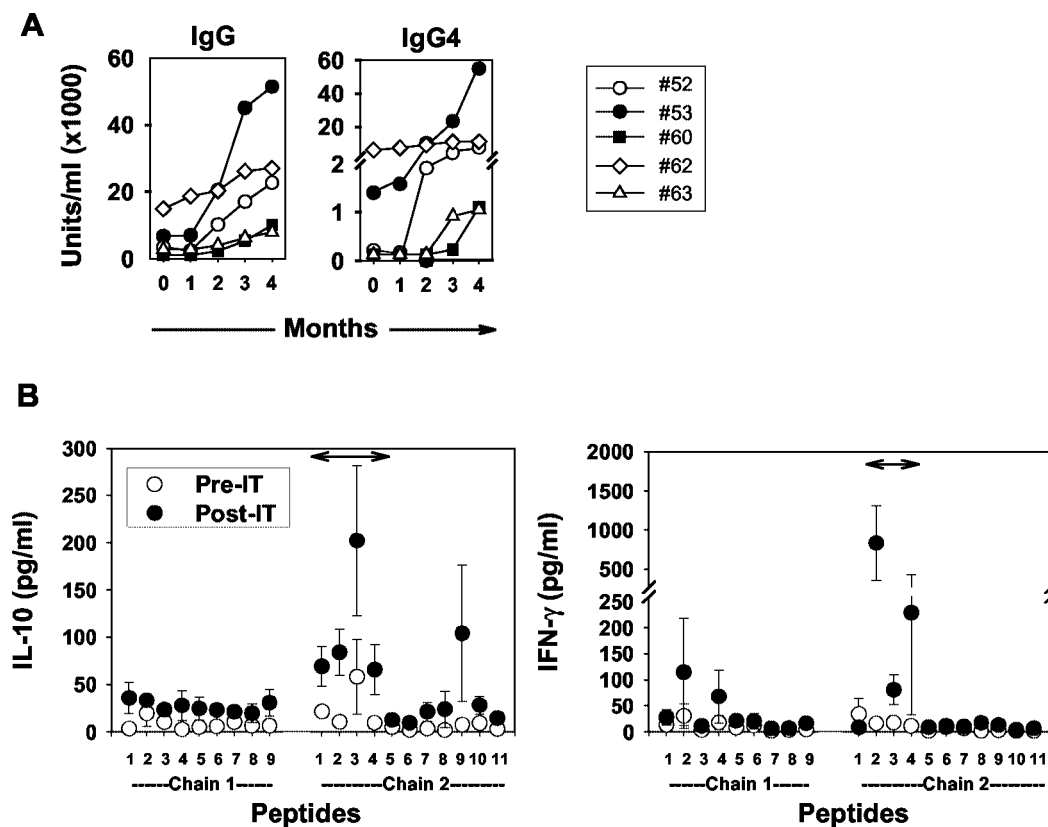


FIGURE 4. Increased production of chain 2 epitope-specific IL-10 and IFN- γ is associated with injection of whole allergen. **A**, Fel d 1-specific serum IgG and IgG4 Ab were monitored at monthly intervals for 4 mo in five cat-allergic patients receiving IT. **B**, Mean maximal change in peptide-induced IL-10 and IFN- γ in PBMC cultures measured within the first 4 mo of therapy. Values represent the mean $\pm$ SEM for each peptide after correction for background levels. Pre-IT, baseline levels; Post-IT, maximal levels measured within the first 4 mo of immunotherapy.

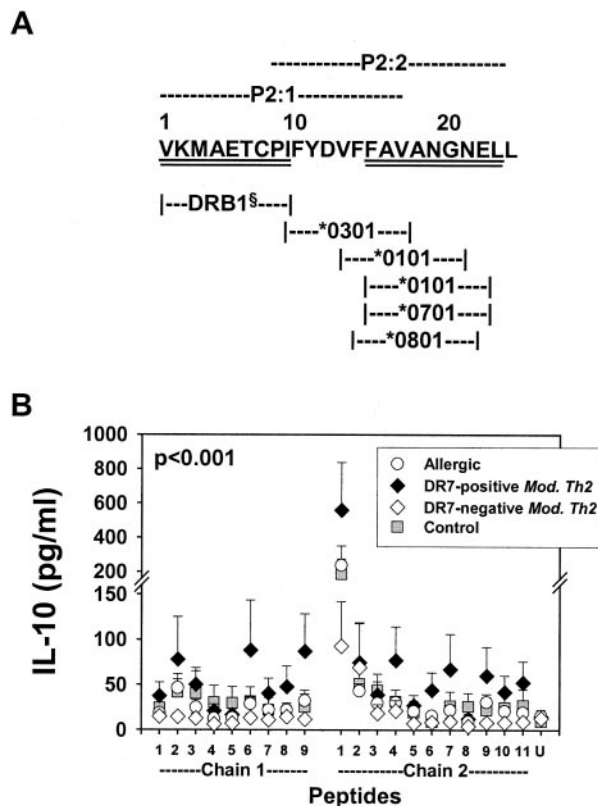


FIGURE 5. Relationship between expression of HLA-DR7 and peptide-induced IL-10 in modified responders. **A**, HLA-DR peptide binding motifs mapping to chain 2 epitopes. §, The motif, VKMAETCP, within P2:1 was predicted to bind to DRB1 alleles *0101, *0301, *0401, *0701, *0801, *1101, and *1501. The double underline denotes DR7 binding motifs. **B**, Analysis of IL-10 production among DR7-positive modified responders ($n = 6$), DR7-negative modified responders ($n = 6$), allergics ($n = 14$), and controls ($n = 11$). Values represent the mean $\pm$ SEM. U, unstimulated wells.

Lymphocytes that target this site were activated after systemic administration of whole allergen as part of a conventional IT regimen, suggesting that chain 2 epitopes fulfill a tolerogenic role. These results provide strong support for the argument that the response to allergens is influenced by both the dose received and intrinsic properties of the allergen molecule. The ability of an immunodominant region of an Ag to induce both IL-10 and IFN- γ has been described previously for the hepatitis C virus core protein (39). In that study the authors speculated that suppression of IFN- γ production by IL-10 could contribute to the persistence of infection, because blocking IL-10 had been shown to restore IFN- γ production to hepatitis C virus Ags (40). Similarly, we have shown that blocking IL-10 enhances IFN- γ production in response to Fel d 1 and chain 2 epitopes. The potent capacity for chain 2 peptides to induce IFN- γ in the absence of IL-10 (Fig. 6) suggests that responses to the N-terminal region of chain 2 are regulated by IL-10. To our knowledge, the current study is the first report of an immunoregulatory region within an allergen molecule that could play a central role in determining allergic phenotype.

The similar patterns of T cell epitope recognition observed for all patient groups implicates involvement of similar Ag processing and presentation pathways in distinct immune responses to Fel d 1. Given that the primary mode of exposure to cat allergen in patients studied is via the respiratory route, we would anticipate that similar T cell and non-T cell populations participate in the generation and recognition of Fel d 1-derived antigenic determinants. In contrast,

we recently showed that T cell responses to chain 2 epitopes are altered in patients with atopic dermatitis (41). In that study we speculated that delivery of cat allergen through the skin and involvement of distinct APC types (e.g., Langerhans cells or inflammatory dendritic epidermal cells) from those in the respiratory tract could contribute to these differences. Lung dendritic cells have a propensity to secrete IL-10, which may favor the induction of IL-10-producing T regulatory cells (14). Indeed, we have confirmed that chain 2 epitope-specific, IL-10-producing CD4⁺ T cells are a component of the T cell repertoire associated with both allergic and nonallergic responses. The ability for IL-10 to exert a regulatory effect in allergic subjects may seem paradoxical, especially in light of our study of highly allergic atopic dermatitis patients (mean IgE Ab, 20.1 IU/ml), in whom blocking IL-10 had little or no effect (41). However, given that allergic subjects in the present study had relatively low titer IgE Ab to cat (mean, 4.4 IU/ml), IL-10 may fulfill a central role in controlling the magnitude or quality of the allergic response.

Definition of an Ab response that is not associated with IgE Ab or allergic symptoms has provided a new avenue for investigating immune responses associated with high dose allergen exposure. Based on the presence of the Th2-dependent Ab isotype IgG4, it seems likely that the modified response reflects a variation of the allergic response. Thus, in those subjects with an allergic predisposition and the appropriate genetic type (i.e., DR7-positive), high dose exposure to cat allergen could favor production of IL-10 by chain 2 epitope-specific T cells within the lung beyond a critical threshold, resulting in tolerance. In keeping with this allergic-modified Th2 balance model, enhanced IL-10 secretion to chain 2 epitopes along with increased IgG4 Ab in patients receiving IT demonstrates plasticity of the allergic response consistent with a shift toward a modified phenotype. Furthermore, when analyzing IL-10 production to P2:1 among allergic and modified responders collectively, significant differences were observed for DR7-positive and DR7-negative subjects. These data support the view that DR7 expression favors IL-10 production to specific epitopes of Fel d 1 in both allergic and modified responders. Our findings do not support a role for IL-10-mediated T cell hyporesponsiveness in tolerance to cat allergen induced either by inhalation or systemically. To the contrary, strong persistent CD4⁺ T cell responses that target chain 2 epitopes appear to be important.

Expression of HLA-DR7 in modified Th2 responders was associated with increased IL-10 production, not only to P2:1, but also to Fel d 1 peptides in general. This observation is consistent with our view that DR7-restricted recognition of chain 2 epitopes in individuals exposed to high dose cat allergen favors IL-10 induction to the whole molecule by intramolecular epitope spreading (42). This phenomenon has been described in relation to cancer treatment and, more recently, induction of tolerance to cat allergen, where vaccination with specific peptides resulted in an immune response to peptides or Ags not included in the vaccine (13, 43, 44). For such an event to occur during natural exposure, a high dosage of allergen is likely to be critical. Exactly why DR7 expression would favor such a response is more difficult to answer. Although two DR7 peptide ligands reside within the regulatory region, major epitopes of chain 2 also stimulate strong T cell responses in allergic subjects, presumably by virtue of their ability to bind to multiple HLA-DR molecules. However, increased affinity and/or stability of chain 2 peptide binding to DR7 coupled with increased density of DR7-peptide complexes at the APC surface could alter T cell signaling events to favor production of regulatory T cells.

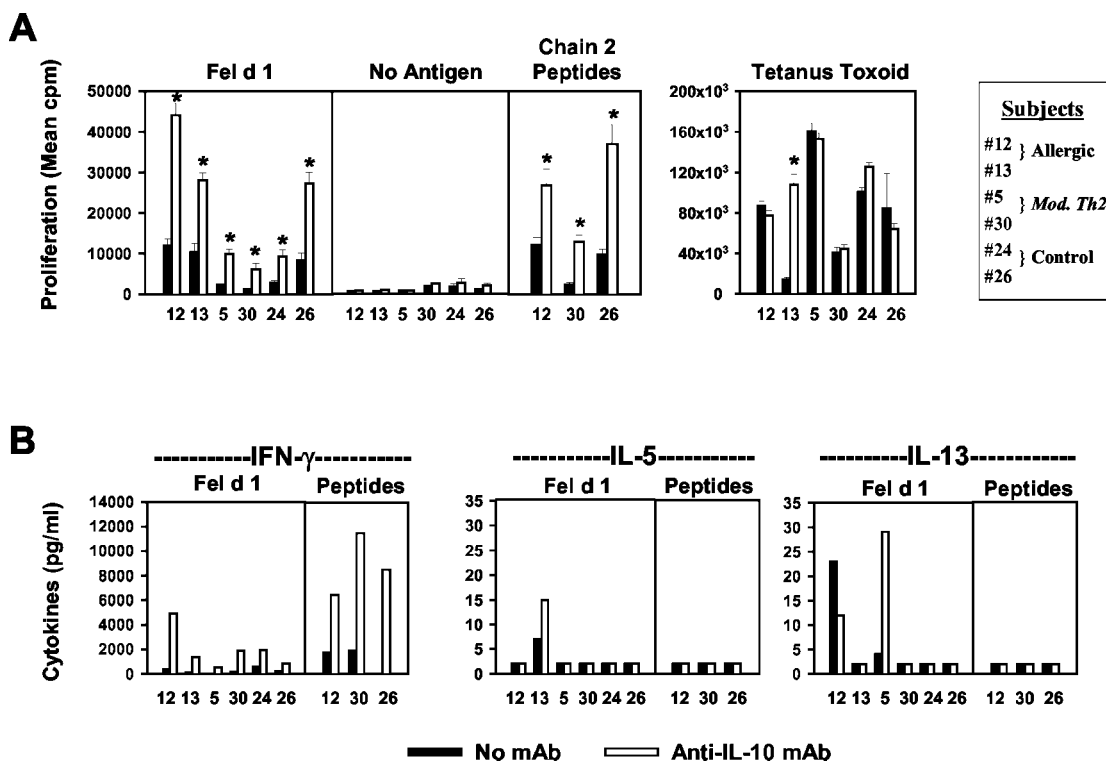


FIGURE 6. Blocking IL-10 enhances Fel d 1- and chain 2 epitope-specific T cell proliferation and cytokine responses regardless of allergic phenotype. PBMC from six donors with distinct immune responses were stimulated with Fel d 1 or pooled chain 2 peptides (P2:1, P2:2, P2:3, and P2:4) with or without 10 μ g/ml anti-IL-10 mAb. Proliferation (A) and cytokine (B) responses were measured on day 7. Proliferation values represent the mean cpm for 12 replicate wells $\pm$ SEM. *, $p < 0.05$. Data are representative of duplicate experiments.

The increased expression of HLA-DR7 observed in the modified group compared with allergic subjects was striking. Although decreased expression of HLA-DR7 has been reported among atopic patients compared with nonallergic healthy controls (45), other studies report an association between DR7 and sensitization to certain allergens (46–50). Our interpretation of these findings is that development of the modified Th2 response to cats is an allergen-specific phenomenon that does not preclude sensitization to other allergens (51). The ability for IL-10 to selectively enhance IgG4 Ab while suppressing IgE Ab (6, 52) formed the basis for our initial hypothesis that the modified response to cats was mediated by IL-10. Indeed, we have shown that the production of Fel d 1-specific IgG and IgG4 Ab in patients receiving IT is accompanied by enhancement of epitope-specific IL-10. Nevertheless, it is clear that some individuals living with cats develop an IgG Ab response independent of DR7 expression. The lack of IgG4 Ab in some of these DR7-negative modified responders coupled with low levels of IL-10 suggest an alternate mechanism governing the immune response in these individuals.

A notable finding in our study was the discrepancy in IL-10 induced by whole allergen and peptides in the modified group compared with other groups. These data highlight the importance of analyzing allergen- and epitope-specific responses in parallel in individuals with defined HLA haplotypes (27, 53). Although Fel d 1 stimulated reduced IL-10 in PBMC from modified responders (Fig. 1, B and C), levels of IL-10 were nevertheless very high. Recent studies in our laboratory that have compared endotoxin-high and endotoxin-low preparations of Fel d 1 showed that endotoxin associated with Fel d 1 can contribute, at least in part, to IL-10 production by PBMC (our unpublished observations); however, induction of IL-10 appears to be an intrinsic property of the Fel d 1 molecule itself. This latter statement is supported by the

observation that in cultures stimulated with endotoxin-free Fel d 1 peptides, IL-10 production localizes to a defined region of the molecule, and peptides spanning this region induce IL-10 secretion by CD4⁺ T cells. It remains to be determined which other allergens exhibit similar features.

Characterization of tolerogenic epitopes of Fel d 1 chain 2 has important implications for therapy. It is of note that previous epitope-mapping studies identified immunodominant epitopes of Fel d 1 in chain 1, but not chain 2, of the molecule (32, 33). It was largely as a result of those studies that a subsequent vaccine incorporated only chain 1 peptides (54). More recent studies examined the effects of injecting short peptides (~16 mer) spanning chain 1 or both chains of the Fel d 1 molecule (9, 13, 55). In those studies a subset of cat-allergic patients receiving peptides experienced late asthmatic reactions that were attributed to MHC-restricted T cell-dependent events; however, diminished late asthmatic reactions and peptide-specific T cell hypo-responsiveness resulted from a second injection of peptides. Notably, peptides spanning the N terminus of Fel d 1 chain 2 were not administered as part of the peptide preparations, although T cell hypo-responsiveness to all four noninjected peptides was observed (13). The strong reactivity to IL-5-inducing chain 1 epitopes in allergic responders with asthma suggests that chain 1 peptides may be relevant to peptide vaccine-related adverse events. Furthermore, the lack of evidence for T cell hypo-responsiveness related to either IT or the modified Th2 response in our study argues against diminished T cell proliferation as the primary goal of therapy. Instead, identification of regulatory epitopes may provide an opportunity for developing a more tailored approach to peptide-based treatments for allergy by preferentially inducing T cell responses that target defined regions of the allergen molecule.

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