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Direct ex vivo analysis of allergen-specific CD4⁺ T cells

William W. Kwok, PhD^{a,b}, Michelle Roti, BS^a, Jonathan H. DeLong, BS^a, Venus Tan, BS^a, Erik Wambre, PhD^a, Eddie A. James, PhD^a, and David Robinson, MD^c

^aBenaroya Research Institute at Virginia Mason

^bDepartment of Immunology, University of Washington

^cVirginia Mason Medical Center, Seattle. bkwok@benaroyaresearch.org.

To the Editor:

CD4⁺ T cells play essential roles in allergic sensitization and late-phase reactions. Previous studies identified epitopes within allergens such as the cat allergen Fel d 1.¹⁻⁵ However, in some cases, HLA restrictions were not clearly defined. Recent studies have applied tetramers to define HLA-restricted epitopes, studying subjects with cat and other allergies.⁴ In general, previous reports analyzed single, previously characterized epitopes and, with 1 exception,⁴ analyzed cells after *in vitro* expansion, which can alter cell phenotypes. Recent advances in MHC class II tetramer analysis enhance the efficiency of epitope identification and provide the sensitivity to examine phenotypes without *in vitro* expansion.^{6,7} We applied these methods to study allergen specific CD4⁺ T-cell responses in subjects with cat allergy.

To identify novel T-cell epitopes within Fel d 1, the Tetramer-Guided Epitope Mapping (TGEM) approach was applied to multiple subjects with allergy recruited with informed consent from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute. Overlapping peptides corresponding to both chains of Fel d 1 were pooled and used to stimulate T-cell cultures as described.⁶ Each peptide pool was loaded into purified class II molecules to generate tetramers.⁸ After 14 days, cultured cells were stained with corresponding pooled peptide loaded tetramers. Positive wells were stained again using tetramers loaded with single peptides (see this article's Fig E1 in the Online Repository at www.jacionline.org). Applying this approach, we identified novel Fel d 1 epitopes for 6 HLA types (Table I). Binding predictions⁹ and experiments using shorter peptides defined minimal epitopes. Responses to these Fel d 1 peptides were absent in subjects without allergy (Fig E1).

To determine frequencies for Fel d 1 specific T cells, we used a direct enrichment protocol, essentially as described by Day et al.⁷ For 9 subjects with allergy and 5 subjects without allergy, 20 to 30 million uncultured PBMCs were stained with phycoerythrin tetramers for 100 minutes. Cells were then stained with surface anti-bodies (anti-CD3 fluorescein isothiocyanate and anti-CD4 allophycocyanin from eBioscience, San Diego, Calif; anti-CD14 peridinin-chlorophyll-protein [PerCP] and anti-CD19 PerCP from BD Pharmingen, San Diego, Calif), incubated with anti-phycoerythrin magnetic beads (Miltenyi Biotec, Auburn, Calif), washed, and enriched by using a magnetic column (Miltenyi Biotec). Bound, phycoerythrin-labeled cells were stained with ViaProbe (BD Bioscience, San Jose, Calif), flushed, collected, and enumerated by flow cytometry (FACS Caliber cytometer, BD Bioscience; FlowJo software, Tree Star, Ashland, Ore). Frequencies were calculated using the following formula

$$\text{Frequency} = n/N$$

where n designates the number of tetramer positive cells in the bound fraction and N designates the total number of CD4^+ T cells in the sample. Fel d 1-specific CD4^+ T-cell frequencies in subjects with allergy ranged from 1 in 7,000 to 1 in 300,000 (Fig 1, A). Frequencies in subjects without allergy were barely detectable (Fig 1, B). Frequency measurements were reproducible in that frequencies for samples from the same subjects obtained at least 2 months apart were not significantly different ($P = .495$, paired t test with Welch correction).

To determine the phenotype of Fel d 1-specific T cells, parallel samples were analyzed by direct enrichment, using antibodies against surface markers of interest instead of anti-CD3. This allowed direct phenotyping with minimal manipulation. Phenotyping was carried out for 5 subjects with allergy (representative results shown in Fig 1, C). A comparison of the phenotype of Fel d 1-specific T cells and total CD4^+ T cells for all 5 subjects is summarized in Fig 1, D. As shown, more than 90% of Fel d 1-specific T cells were CD45RO , CD28 , CD62L , and CCR4 -positive. Most cells were CXCR3 and CCR6 -negative but showed heterogeneous expression of CRTH2 and CCR7 . In comparison with total CD4^+ cells, a higher percentage of Fel d 1-specific T cells expressed CCR4 and CRTH2 , and a lower percentage expressed CXCR3 . As an internal control, we examined the phenotype of influenza A-specific T cells (not shown). Influenza-specific T cells were CD45RO and CD28 -positive, but were CXCR3 -positive, CCR4 -negative, and CRTH2 -negative. Thus, allergen-specific T cells show a distinct phenotype compared with influenza-specific T cells and the total CD4^+ T-cell population.

In support of these phenotyping experiments, we conducted cytokine analysis of directly enriched Fel d 1-specific T cells using Miltenyi capture reagents after stimulation with tetramer, anti-CD28 (10 $\mu\text{g/mL}$), and anti-CD49d (2 $\mu\text{g/mL}$) for 4 hours at 37°C. Although 10% to 50% of the tetramer-positive cells secreted IL-5, no IFN- γ was detected (Fig 1, E). IL-5 secretion was limited to the tetramer positive population. To examine additional cytokines, cultured cells were reactivated by using plate-bound tetramer and soluble anti-CD28. Supernatants were harvested after 48 hours and assayed for cytokine content by using the Meso Scale Sector Imager, Gaithersburg, Md. In addition to IL-5, Fel d 1-specific T cells produced $\text{T}_\text{H}2$ cytokines such as IL-13 and IL-4 (not shown).

Previous reviews and studies have highlighted the attractive features of using MHC class II tetramers to simultaneously determine T-cell frequencies and phenotypes. Since the arrival of class II tetramers, technical advances have increased the utility of these reagents. First, the availability of additional alleles enables the study of larger segments of the population. Second, TGEM facilitates rapid identification of T-cell epitopes.⁶ Third, enrichment methods allow direct *ex vivo* detection of antigen-specific T cells.⁷ This study applied these methods to visualize and characterize Fel d 1-specific T cells in subjects with allergy. We identified epitopes restricted by multiple HLA-DR alleles. We observed frequencies ranging from 1 in 7,000 to 1 in 300,000 CD4^+ T cells in subjects with allergy. In subjects without allergy, allergen-specific T-cell frequencies were near background levels. However, tetramers may be limited in their ability to detect low-affinity cells. As such, tetramer measurements may underestimate T-cell frequencies in some cases.

In contrast with many published studies, we have directly examined the phenotype of allergen-specific T cells. Our observations indicated that nearly all Fel d 1-specific T cells (*ex vivo*) exhibit a memory phenotype. Fel d 1-specific T cells showed heterogeneous

expression of CCR7, a marker thought to be upregulated on central memory and downregulated on effector memory cells. A previous report observed enriched CCR4 expression by allergen-specific T cells.⁴ Our results were more dramatic in that almost all allergen-specific T cells were CCR4-positive (25%-30% of total CD4⁺ T cells were CCR4-positive). Expression of CCR4 is notable because CCR4 is a T_H2 marker that has been associated with trafficking to nonlymphoid sites, including the skin and airway mucosa. Thus, high levels of CCR4 expression may lead to rapid recruitment into relevant sites for allergic immune responses.

In contrast with CCR4 expression, the prostaglandin D2 receptor CRTH2 (another T_H2 marker)¹⁰ was expressed at variable frequencies (17%-88%) among subjects with cat allergy. Although variable, these frequencies were always higher than total CD4⁺ T cells. Regardless of CRTH2 expression level, tetramer-based cytokine assays indicated high levels of IL-5 and low levels of IFN- γ . These cytokine results reinforced the surface phenotype results. Cytokine levels were robust, which is typical of effector T cells. The absence of CXCR3 and CCR6 expression indicates that these peripheral cells do not belong to T_H1 or T_H17 lineages. However, these lineages could be present within specific tissues or during stages of allergy that were not reflected in our samples.

In conclusion, we used class II tetramers to rapidly define HLA-restricted epitopes within allergens and characterize allergen-specific T cells *ex vivo*. The application of tetramers to study allergen-specific CD4⁺ T cells may facilitate the development of peptide biologics for allergy treatment. Future studies using tetramers after immunotherapy should clarify the underlying mechanisms for allergen-specific immune tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG 1.

Ex vivo staining of Fel d 1 CD4⁺ T cells with tetramers. **A**, PBMCs from subjects with cat allergy were stained with phycoerythrin-labeled Fel d 1 tetramers (Tet) followed by the anti-phycoerythrin bead enrichment protocol. Each panel shows a representative staining result from a subject with cat allergy with the indicated HLA. The frequencies of Fel d 1 CD4⁺ T cells (per million) were as indicated. **B**, Staining of PBMCs from subjects without allergy with Fel d 1 tetramers using the magnetic bead enrichment protocol. Each panel shows a representative staining result from a subject without allergy with the indicated HLA. **C**, Phenotypes of Fel d 1-specific CD4⁺ T cells. PBMCs of a DR0401 subject with cat allergy were stained with DR0401/Fel d 1 tetramer *ex vivo* and a panel of antibodies. **D**, Comparison of phenotypes of Fel d 1 T cells (●) and total CD4⁺ T cells (Δ; total of 5 subjects). A Student *t* test was used in the statistical analysis. **P* < .01. **E**, Cytokine profiles of Fel d 1-reactive CD4⁺ T cells. PBMCs from subjects with cat allergy with the indicated HLA were stained with tetramers using the anti-phycoerythrin bead enrichment protocol and assayed for IL-5 and IFN- γ .

Table I

Fel d 1 T cell epitopes

HLA restriction	Epitope	AA sequence
DRB1*0101	Fel d 1 chain 1 ₂₅₋₄₄	VAQYKALPVVLENARILKNC
DRB1*0401	Fel d 1 chain 2 ₂₂₋₃₁	ELLLDLSLTK
DRB1*1101	Fel d 1 chain 1 ₅₈₋₆₇	LSLLDKIYTS
DRB1*1301	Fel d 1 chain 1 ₃₁₋₄₃	LPVVLENARILKN
DRB1*1401	Fel d 1 chain 1 ₅₅₋₆₇	ENALSLLDKIYTS
DRB5*0101	Fel d 1 chain 1 ₁₉₋₃₁	DEYVEQVAQYKAL