T-cell epitope conservation across allergen species is a major determinant of immunogenicity

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Background: Patients with pollen allergies are frequently polysensitized. Pollens contain epitopes that are conserved across multiple species.

Objective: We sought to demonstrate that cross-reactive T cells that recognize conserved epitopes show higher levels of expansion than T cells recognizing monospecific epitopes because of more frequent stimulation.

Method: RNA was sequenced from 9 pollens, and the reads were assembled *de novo* into more than 50,000 transcripts. T-cell epitopes from timothy grass *(Phleum pratense)* were examined for conservation in these transcripts, and this was correlated to their ability to induce T-cell responses. T cells were expanded *in vitro* with *P pratense*–derived peptides and tested for cross-reactivity to pollen extracts in ELISpot assays.

Results: We found that antigenic proteins are more conserved than nonimmunogenic proteins in *P pratense* pollen. Additionally, *P pratense* epitopes that were highly conserved across pollens elicited more T-cell responses in donors with grass allergy than less conserved epitopes. Moreover, conservation of a *P pratense* peptide at the transcriptomic level correlated with the ability of that peptide to trigger T cells that were cross-reactive with other non–*P pratense* pollen extracts.

Conclusion: We found a correlation between conservation of peptides in plant pollens and their T-cell immunogenicity within *P pratense*, as well as their ability to induce cross-reactive T-cell responses. T cells recognizing conserved epitopes might be more prominent because they can be stimulated by a broader range of pollens and thereby drive polysensitization in allergic donors. We propose that conserved peptides could potentially be used in diagnostic or immunomodulatory approaches that address the issue of polysensitization and target multiple pollen allergies. (J Allergy Clin Immunol 2016;138:571-8.)

Key words: *T cell, epitope, timothy grass allergy, pollen allergy, cross-reactivity, RNA sequencing, sequence conservation, transcriptome*

Patients with pollen allergies are often polysensitized, as evidenced by positive RAST and/or skin prick test results to multiple pollen allergens. The relatively low frequency of monosensitizations can be explained by the presence of cross-reactive IgE epitopes conserved across multiple pollens, which result in immune reactivity to homologous regions in allergens to which the patient was not originally sensitized.[1] In the context of immunotherapy, the high degree of polysensitization in subjects suggests that a single allergen administered in therapeutic mode could be sufficient to induce tolerance. In fact, several investigators have suggested that immunotherapy with a single grass species, such as *Phleum pratense*, is sufficient to also treat allergies to other grass pollens caused by observed cross-reactivity at the IgE level.[2,3] On the other hand, it is firmly established that allergen-specific T cells play an important role in allergic inflammation[4] and that induction of antigen-specific regulatory T cells[5] or elimination of allergen-specific T_H2 cells might be a prerequisite for the induction of specific tolerance.[6] Yet evaluation of cross-reactivity at the T-cell level has been less documented.

A recent study using tetramer costaining of 6 different MHC-epitope complexes found limited cross-reactivity of these epitopes with homologs in other Pooidea grasses and concluded that multiple grass pollen species immunotherapy is likely to be more beneficial than single-species immunotherapy.[7] Although that study was limited to epitopes from 2 major allergens (Phl p 1 and 5), we have recently shown that a large fraction of *P pratense*–specific T cells target epitopes contained in timothy grass T-cell antigens (TGTAs) unrelated to the known *P pratense* allergens that are also major targets of T-cell responses but were initially identified based on their high IgE reactivity.[8] On the basis of these data, we reasoned that a broader evaluation of T-cell cross-reactivity including more epitopes and also those from TGTAs would be of interest, particularly because clinical studies have shown a good degree of success for single-species immunotherapy[9,10] contrary to what might be expected based on the data presented in the tetramer costaining study for a selected set of epitopes. To gain a comprehensive picture of conservation between different grass, weed, and tree pollens, we sequenced the

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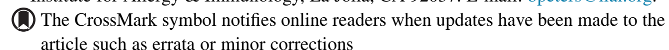

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Abbreviations used
IUIS: International Union of Immunological Societies
SIT: Specific immunotherapy
TGTA: Timothy grass T-cell antigen

transcriptome of 9 allergenic pollen species and determined how the conservation between *P pratense* T-cell epitopes and other pollen transcriptomes related to their immunogenicity and their ability to elicit cross-reactive T-cell immune responses.

METHODS

Patient population

Study participants were recruited, as previously described.[11] We drew from a cohort of 55 patients between the ages of 19 and 62 years, and 28 of our patients were female. We only included participants with timothy grass allergy who had skin reactions with wheals of 5 mm or greater in diameter after skin prick testing or *P pratense*–specific IgE levels of 0.35 kU/L or greater, as determined by RAST, and had a history of seasonal grass pollen allergy symptoms. We did not control for other allergies, and many patients were polysensitized. We included patient samples collected both in and out of the season. Further details about the age, sex, and allergy status of the patients used in our studies can be found in Table E1 in this article's Online Repository at www.jacionline.org. Patients' PBMCs used in T cell cross-reactivity studies were further prescreened based on their reactivity to *P pratense* peptides after timothy grass culture.

RNA sequencing and *de novo* transcriptome assembly

Total RNA of the pollen extract was isolated, as previously described.[12] RNA was sequenced on a HiSeq 2500 Sequencer (Illumina, Sn Diego, Calif). Replicate samples were run across all lanes of the sequencer to generate paired reads of 100 bp in each direction. Before assembly, several preprocessing steps were performed: (1) reads not passing Illumina filters were discarded, (2) portions of reads matching adapter/primer sequences were trimmed, (3) 3′ regions of reads following a low-quality score ($Q < 20$) were discarded, and (4) remaining reads of less than 30 bp in length were discarded. These preprocessing steps were performed with a combination of FASTX-toolkit (0.0.13.2)[13] and cutadapt (1.3).[14] High-quality reads were assembled into transcripts by using Trinity (r2012-10-05),[15] specifying "–min_kmer_cov 2" to ensure each sequence was observed at least twice.

In vitro expansion of allergen-specific T cells from PBMCs

PBMCs were isolated from whole blood by means of density gradient centrifugation and cryopreserved, as previously described.[8] For *in vitro* expansion, cells were thawed and cultured with RPMI 1640 (Omega Scientific, Tarzana, Calif) supplemented with 5% human AB serum (Cellgro, Manassas, Va) at a density of 2×10^6 cells/mL in 24-well plates (BD Biosciences, San Jose, Calif) and stimulated with peptide (0.5 μg/mL). Cells were kept at 37°C in a 5% CO_2 atmosphere, and additional IL-2 (10 U/mL; eBioscience, San Diego, Calif) was added every 3 days after initial antigenic stimulation. On day 14, cells were harvested and screened for cytokine production by means of ELISpot after restimulation with peptides or pollen extracts.

ELISpot assays

Production of IL-5 from *in vitro*–expanded PBMCs in response to peptide pool or extract stimulation was measured with an ELISpot, as previously described.[16] Briefly, 1×10^5 cells per well were incubated with a peptide pool (5 μg/mL) or extract (50 μg/mL, except for oak, which was tested at 25 μg/mL). After 22 hours, cells were removed, and plates were washed and incubated with 2 μg/mL biotinylated anti-human IL-5 antibody (Mabtech, Cincinnati, Ohio) at 37°C. After 2 hours, plates were washed, and avidin-peroxidase complex was added (Vector Laboratories, Burlingame, Calif) for 1 hour at room temperature. Peroxidase-conjugated spots were developed with 3-amino-9-ethylcarvazole solution (Sigma-Aldrich, St Louis, Mo).

T-cell clones

PBMCs were labeled with carboxyfluorescein succinimidyl ester and cultured in complete RPMI 1640 supplemented with 2 mmol/L glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate, penicillin (50 U/mL), streptomycin (50 μg/mL), and 5% human serum (Swiss Red Cross, Bern, Switzerland) at a density of 2×10^6 cells/mL in 24-well plates. Cells were stimulated with pools of peptides (0.5 μg/mL per peptide), and rIL-2 (10 U/mL) was added on day 3 after initial antigenic stimulation. At day 11, cells were stained with Pacific blue–labeled anti–inducible costimulator mAb (C398.4A; BioLegend, San Diego, Calif) and BV785-labeled anti-CD25 mAb (BC96; BioLegend). carboxyfluorescein succinimidyl ester–low, inducible costimulator–positive, $CD25^+$ cells were sorted and cloned by means of limiting dilution, as previously described.[17] T-cell clones were screened at day 20 after initial stimulation by culturing 3×10^4 T cells/well with autologous irradiated (95 Gy) EBV-transformed B cells (2×10^4) in the absence or presence of allergen extracts (50 μg/mL) or peptides (0.5 μg/mL). *Mycobacterium tubercolosis* lysate (5 μg/mL) was used as a negative control. Proliferation was measured on day 3 after a 16-hour pulse with tritiated thymidine (GE Healthcare, Fairfield, Conn).

RESULTS

Determining sequence conservation among a diverse selection of pollen species

To address the potential effect of sequence conservation and T-cell cross-reactivity on allergic responses, we selected 9 pollen species that represent the 3 major groups of pollen allergens (grasses, weeds, and trees). Specifically, we included 4 grasses (sweet vernal *[Anthoxanthum odoratum]*, rye *[Lolium perenne]*, Kentucky blue *[Poa pratensis]*, and Bermuda *[Cynodon dactylon]*), 3 trees (ash *[Fraxinus excelsior]*, olive *[Olea europaea]*, and oak *[Quercus alba]*), and 2 weeds (western ragweed *[Ambrosia psilostachya]* and English plantain *[Plantago lanceolata]*). Because no pollen transcriptomic data were available for any of these, we isolated and sequenced pollen RNA and assembled the reads *de novo* into transcripts, resulting in more than 50,000 transcripts for each pollen. Table E2 in this article's Online Repository at www.jacionline.org provides an overview of the read counts and assembly statistics.

As a quality control, for each pollen transcriptome, we examined whether the known IgE-reactive allergens listed by the International Union of Immunological Societies (IUIS) were reidentified in our analysis. A total of 26 allergens are listed by IUIS (minimum length, 50 residues), covering all pollen species we sequenced (except for sweet vernal grass). For 23 of these allergens, we identified transcripts that had more than 90% sequence identity for more than 50% of the length of previously described allergens (Table I). For 2 of the 3 remaining allergens (Poa p 5 and Que a 1), isoforms of the IUIS allergens that met these criteria were listed in Allergome.[18] Thus our transcriptomic analysis reidentified isoforms of all but 1 (Amb p 5) of the known allergens from the pollen species we sequenced.

Next, we wanted to determine the degree of conservation of the 10 IgE-reactive *P pratense* allergens listed by the IUIS. We

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