

Specific immunotherapy modifies allergen-specific CD4⁺ T-cell responses in an epitope-dependent manner

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Background: Understanding the mechanisms by which the immune system induces and controls allergic inflammation at the T-cell epitope level is critical for the design of new allergy vaccine strategies.

Objective: We sought to characterize allergen-specific T-cell responses linked with allergy or peripheral tolerance and to determine how CD4⁺ T-cell responses to individual allergen-derived epitopes change over allergen-specific immunotherapy. **Methods:** Timothy grass pollen (TGP) allergy was used as a model for studying grass pollen allergies. The breadth, magnitude, epitope hierarchy, and phenotype of the DR04:01-restricted TGP-specific T-cell responses in 10 subjects with grass pollen allergy, 5 nonatopic subjects, and 6 allergy vaccine-treated subjects was determined by using an *ex vivo* peptide–MHC class II tetramer approach.

Results: CD4⁺ T cells in allergic subjects are directed to a broad range of TGP epitopes characterized by defined immunodominance hierarchy patterns and with distinct functional profiles that depend on the epitope recognized. Epitopes that are restricted specifically to either T_H2 or T_H1/T_R1 responses were identified. Allergen-specific immunotherapy was associated with preferential deletion of allergen-specific

T_H2 cells and without a significant change in the frequency of T_H1/T_R1 cells.

Conclusions: Preferential allergen-specific T_H2 cell deletion after repeated high-dose antigen stimulation can be another independent mechanism to restore tolerance to allergen during immunotherapy. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Immunotherapy, allergy, epitope, pollen, T cells, CD4, peptide–MHC class II tetramer, peripheral tolerance, *ex vivo*

Allergen-specific immunotherapy (ASIT) is the only disease-modifying treatment for allergy.^{1,2} However, effective limited understanding of the immunologic mechanisms underlying ASIT has hampered its broad applicability and the development of novel targeted vaccines with improved efficacy and safety. Many reports have described the pivotal role of T_H cells in both the induction and regulation of allergic immune responses.^{3–6} However, the precise epitope identification and functional characterization of allergen-specific CD4⁺ T cells at the single-epitope level are still lacking. Advancement in this area will facilitate the design of new allergy vaccine strategies and help us understand how epitope recognition by T cells influences the magnitude, fate, and quality of immune responses.

Timothy grass is one of the most prevalent types of grasses in the world and contains several allergens, of which Phl p 1, Phl p 5a, and Phl p 5b are major allergens for grass pollen-sensitive patients,⁷ accounting for most of the IgE-binding capacity of crude pollen extracts. Immunotherapy with Timothy grass pollen (TGP) alone is effective for treating allergies caused by all grass species.^{8,9} Therefore in this study we used Timothy grass (*Phleum pratense*) pollen allergy as a model for studying grass pollen allergies. We used an *ex vivo* peptide–MHC class II (pMHCII) tetramer approach to provide a complete description of the DR04:01-restricted TGP-specific CD4⁺ T-cell responses in both allergic and nonatopic subjects, including the determination of the breadth, magnitude, epitope hierarchy, and phenotype of response. We also assessed responses in ASIT-treated patients to correlate the induced T-cell response with clinical benefit, providing detailed information about the pathogenic and nonpathogenic responses in allergic and nonallergic subjects and the effects of conventional extract-based allergy vaccine on allergen-specific T-cell responses. Results show that CD4⁺ T cells in allergic subjects are directed to a broad range of TGP epitopes characterized by defined immunodominance hierarchy patterns and with distinct functional profiles that depend on the epitope recognized. ASIT does not specifically increase allergen-specific T_H1/T_R1 cell responses. Instead, we identified the preferential allergen-specific T_H2 cell deletion as the main mechanism that drives the change in T_H1/T_H2 allergen-specific T-cell ratios and governs the restoration of tolerance to allergen

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Abbreviations used

ASIT: Allergen-specific immunotherapy
 PE: Phycoerythrin
 pMHCII: Peptide–MHC class II
 TGP: Timothy grass pollen

during immunotherapy. Overall, these results elucidate what we believe to be a primary mechanism for ASIT that suggests new approaches for designing improved allergy vaccines.

METHODS**Subjects**

Subjects with DR04:01 or DR07:01 haplotypes were recruited at the allergy clinic at Virginia Mason Medical Center (Seattle, Wash) after providing written consent as part of an institutional review board–approved study. Subjects with TGP allergy ($n = 12$) were selected based on their clinical symptoms, a positive skin prick test response, and positive IgE reactivity, as determined by using the ImmunoCAP test (Phadia AB, Uppsala, Sweden), with TGP extracts (test score, ≥ 3). For subjects with no history of allergy ($n = 5$), nonatopic status was confirmed by a lack of IgE reactivity to grass pollen extracts (see Table E1 in this article's Online Repository at www.jacionline.org). Patients who responded successfully to subcutaneous ASIT ($n = 6$) were also recruited. These subjects had a clinical history, positive skin prick test responses, and IgE scores to TGP before ASIT and then underwent ASIT for a minimum of 3 years. Treatment was considered

efficacious when patients had a significant reduction in clinical symptoms and when their drug use needs during the pollen season decreased significantly.

Peptides and pMHCII tetramer reagents

A peptide library was generated based on the Phl p 1, Phl p 5a, and Phl p 5b sequences. The library consisted of overlapping peptides spanning the entire allergen, each 20 amino acids long with a 12-amino-acid overlap synthesized by Mimotopes (Clayton, Australia). Peptide-loaded DR04:01 and DR07:01 proteins were generated, as previously described,¹⁰ and subsequently conjugated as tetramers by using R-PE streptavidin (Biosource International, Camarillo, Calif). The tetramer-guided epitope mapping used to determine CD4⁺ T-cell epitopes within TGP major allergens is described in the [Methods](http://www.jacionline.org) section in this article's Online repository at www.jacionline.org.

Ex vivo epitope-specific CD4⁺ T-cell analysis

Forty million PBMCs in culture medium at a concentration of 150 million/mL were treated with dasatinib¹¹ for 10 minutes at 37°C, followed by staining with 20 μ g/mL phycoerythrin (PE)–labeled tetramers at room temperature for 100 minutes. After tetramer staining, cells were labeled with anti-PE magnetic beads and enriched by using a magnetic column, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, Calif). Frequency was calculated as previously described.¹² Magnetically enriched cells were next stained with antibodies against markers of interest or corresponding isotype-matched mAbs. Data acquisition was performed on a BD LSR II instrument and analyzed with FlowJo software (TreeStar, Ashland, Ore).

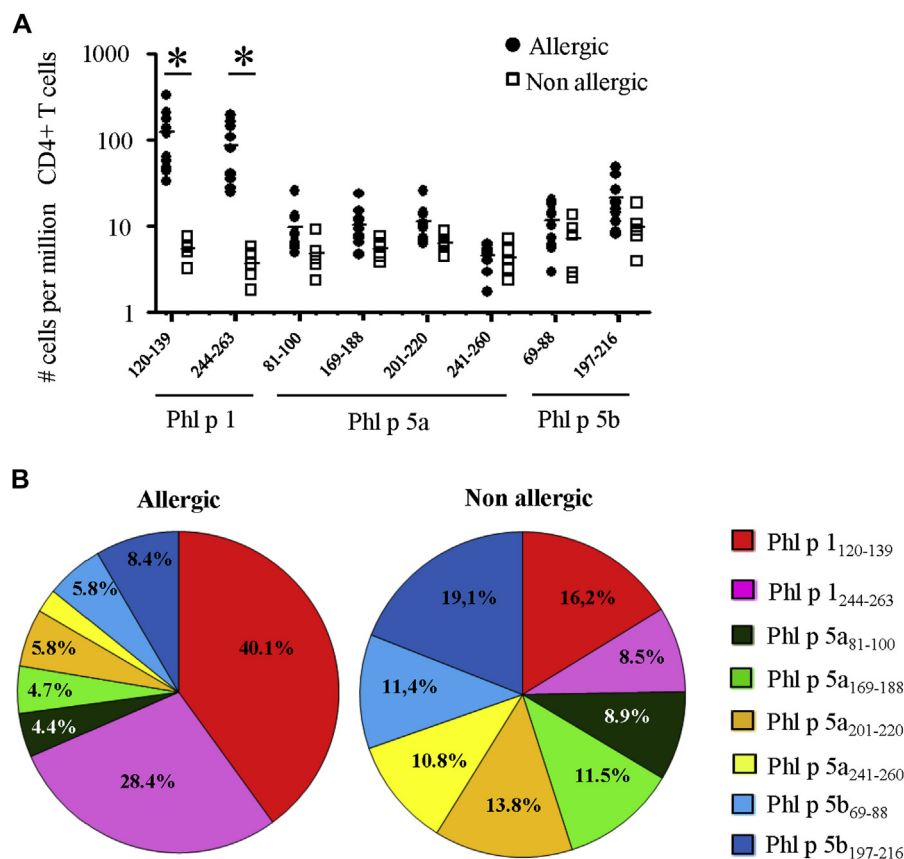


FIG 1. A, *Ex vivo* frequencies of group 1 and group 5 TGP-specific T cells in allergic subjects (solid circles) and nonatopic subjects (open squares). * $P < .001$. B, Contribution of each epitope to the global DR04:01-TGP allergen-specific T-cell response in allergic ($n = 10$) and nonatopic ($n = 5$) subjects. Data are presented as mean values from each group in pie charts.

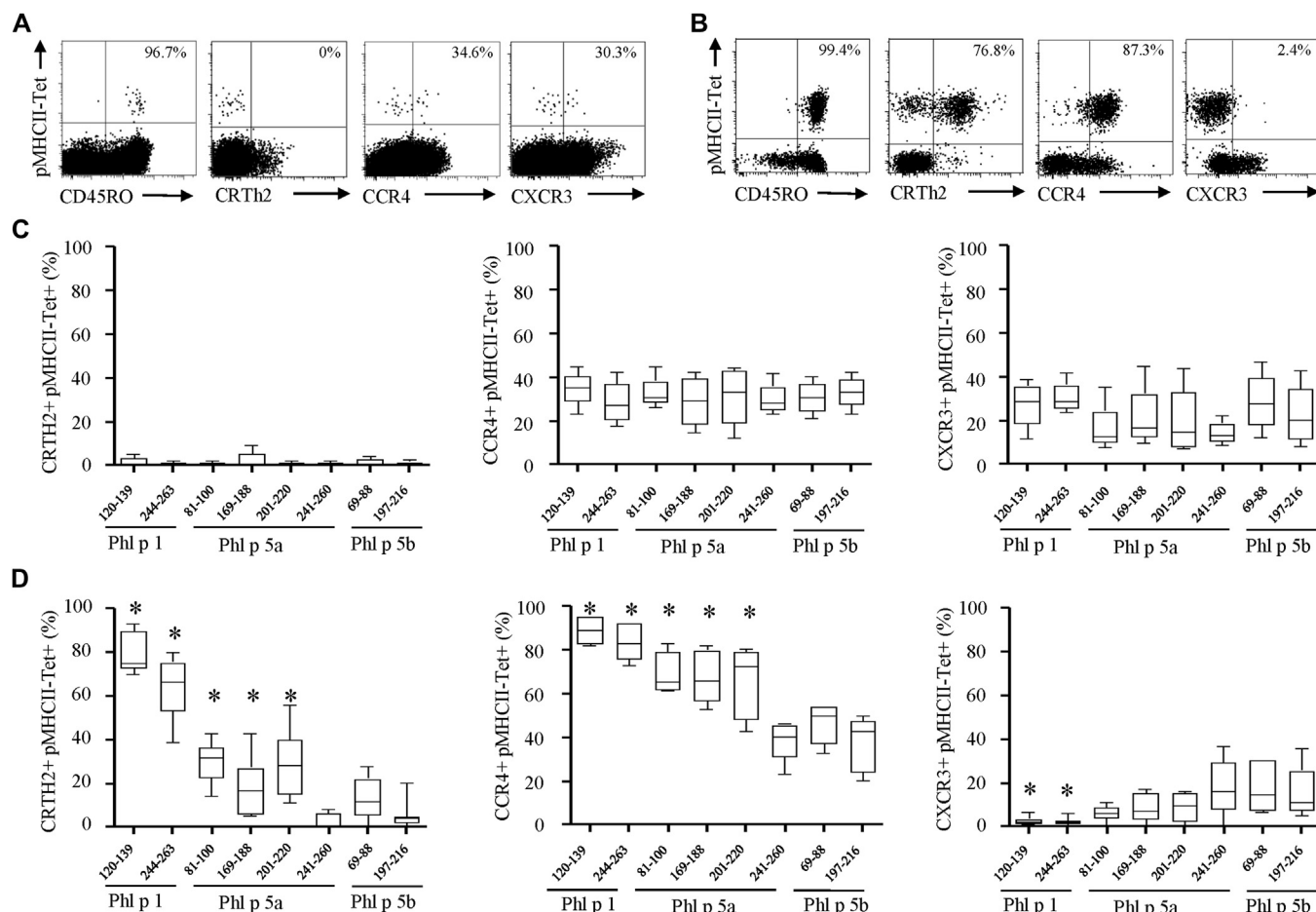


FIG 2. **A** and **B**, Representative *dot* plots showing *ex vivo* multicolor phenotyping of Phl p 1₁₂₀₋₁₃₉-specific CD4⁺ T cells in DR04:01-restricted nonatopic (**A**) and allergic (**B**) subjects. **C** and **D**, Side-by-side comparison of the *ex vivo* phenotype of group 1 and group 5 TGP-specific CD4⁺ T cells in 5 nonatopic subjects (**C**) and 10 subjects with TGP allergy (**D**). **P* < .01.

Intracellular cytokine staining

Intracellular cytokine staining is described in the [Methods](#) section in this article's Online Repository.

Statistical analysis

The nonparametric Mann-Whitney *U* test was used for unpaired comparisons between groups, whereas the nonparametric Wilcoxon matched-pairs test was used for paired comparison. All statistical analysis was performed with GraphPad Prism software 150, version 5.0a (GraphPad Software, La Jolla, Calif).

RESULTS

Differences in the magnitude of the T-cell responses to allergen are correlated with the allergic immune response

We used the tetramer-guided epitope mapping approach to determine CD4⁺ T-cell epitopes within TGP major allergens (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). These experiments focus on HLA-DRB1*04:01 because this allele was prevalent in our cohort of subjects with TGP allergy. We identified a total of 8 immunogenic DR04:01-restricted CD4⁺ T-cell epitopes from group 1 (Phl p 1) and group 5

(Phl p 5a and Phl p 5b) TGP major allergens. These epitopes are listed in [Table E2](#) in this article's Online Repository at www.jacionline.org and were loaded on pMHCII tetramers to track TGP allergen-specific CD4⁺ T cells *ex vivo*. We consistently detected DR04:01-restricted CD4⁺ T cells specific for all group 1 and group 5 TGP epitopes *ex vivo* using specific pMHCII tetramers (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). In nonatopic subjects the magnitude of each TGP epitope-specific T-cell response was very low, and no distinct immunodominance hierarchy of TGP-derived epitope patterns was observed ([Fig 1](#)). In the allergic group Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃ elicited a 20- to 30-fold greater T-cell immune response (average, 122.5 ± 95.9 and 86.3 ± 63.1 vs 5.4 ± 1.6 and 3.7 ± 1.6 specific T cells per 10^6 CD4⁺ T cells, respectively; *P* < .005) than in the healthy group ([Fig 1, A](#)). Conversely, all group 5 TGP-derived epitopes remain poorly immunogenic *ex vivo* (average, 11.5 ± 8.7 specific T cells per 10^6 CD4⁺ T cells). Consequently, Phl p 1₁₂₀₋₁₃₉- and Phl p 1₂₄₄₋₂₆₃-specific CD4⁺ T cells dominate the DR04:01-restricted T-cell repertoire in subjects with TGP allergy, constituting approximately 70% of the global DR04:01 TGP-specific CD4⁺ T-cell response ([Fig 1, B](#)). Importantly, this epitope hierarchy was consistent among DR04:01-restricted allergic subjects, and no difference

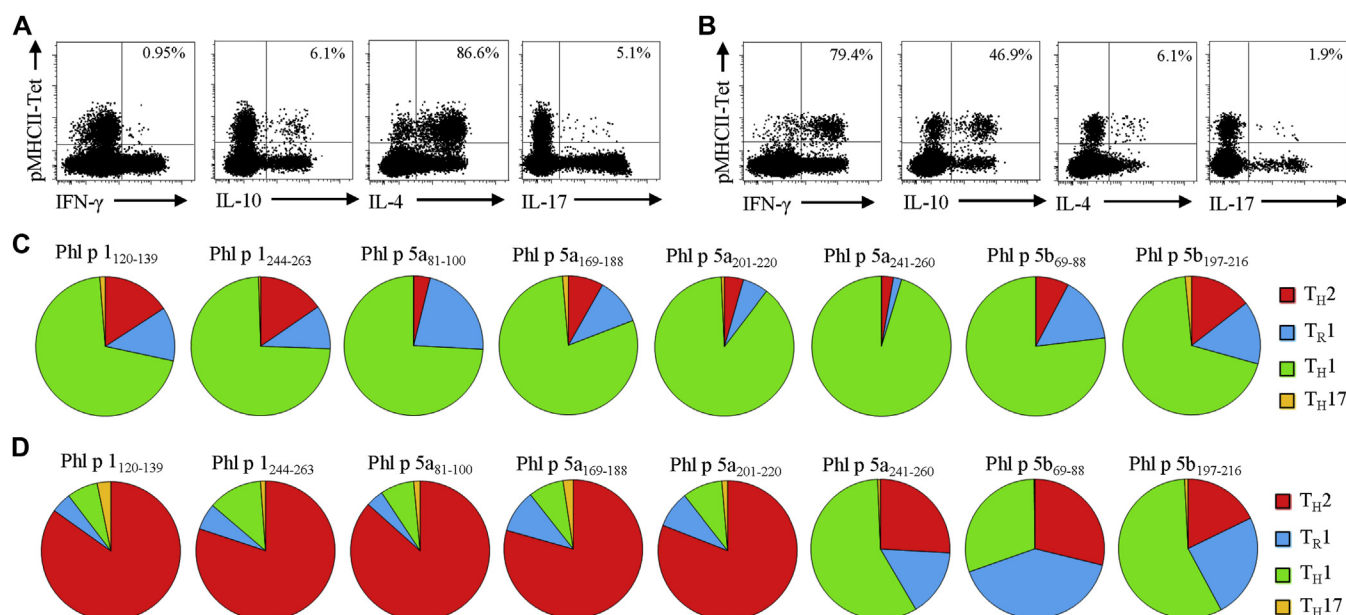


FIG 3. **A** and **B**, Cytokine expression of Phl p 1₁₂₀₋₁₃₉-specific (Fig 3, **A**) and Phl p 5b₁₉₇₋₂₁₆-specific (Fig 3, **B**) T cells in DR04:01-restricted allergic subjects. **C** and **D**, Cytokine profiles of DR04:01-restricted TGP epitope-specific T cells in nonatopic (Fig 3, **C**) and allergic (Fig 3, **D**) subjects. Data are representative of at least 5 subjects per group and are presented as mean values from each group in pie charts.

was observed between the binding affinities of the dominant epitopes Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃ (75.9 and 232.3 nmol/L, respectively) and the nondominant epitopes (range, 104-595 nmol/L) to DR04:01 protein (see Table E2). This suggests that the efficiency of the pMHCII tetramer staining was equivalent for the different TGP epitopes tested and that this epitope hierarchy was not due to differences in binding affinities.

To next explore whether Phl p 1 was intrinsically more immunogenic than Phl p 5a and Phl p 5b, we assessed the TGP-specific CD4⁺ T-cell responses restricted by another HLA, HLA-DRB1*07:01, which was the second most prevalent allele in our cohort of allergic subjects. We observed that the DR07:01-restricted immunodominant T-cell epitopes in allergic subjects are only derived from group 5 TGP major allergens (Phl p 5a₁₂₁₋₁₄₁ and Phl p 5b₈₉₋₁₀₈ epitopes), whereas no immunogenic CD4⁺ T-cell epitope was found in Phl p 1 for this allele (see Fig E1). Thus different grass pollen allergens contain multiple T-cell epitopes with different immunogenicity depending on HLA restriction, meaning that there will be differences in the allergen-derived peptides that are recognized by different patients' T cells.

Phenotypes of allergen-reactive T cells depend on the epitope they recognize

To investigate whether differences in epitope-specific T-cell frequency correlate with differences in phenotype, we made a side-by-side comparison of the cytokine profile and the *ex vivo* surface marker expression of DR04:01-restricted TGP epitope-specific T cells. In all allergic subjects and nonatopic subjects tested, TGP allergen-specific CD4⁺ T cells displayed a predominantly CD45RO⁺ memory phenotype, implying that they are antigen-experienced T cells (Fig 2, **A** and **B**). In nonatopic subjects peripheral tolerance to all TGP-derived epitopes was

associated with IFN-γ-producing CD4⁺ T cells (T_H1 profile) and IL-10-producing CD4⁺ T cells (T_R1 profile) that expressed the T_H1-associated marker CXCR3 (average, 22.8% ± 12.7%; Figs 2, **C**, and 3, **C**).^{13,14} Conversely, in DR04:01-restricted allergic subjects certain epitopes elicited strong IL-4 responses, whereas other TGP-derived epitopes elicited IFN-γ and IL-10 responses in the same subject. Costaining for cytokines in tetramer-positive cells showed that IL-4-producing cells did not coproduce IFN-γ, IL-10, or IL-17, implicating that these IL-4-producing cells were *bona fide* T_H2 cells. Specifically, 3 basic patterns of surface marker expression can be discerned (Figs 2 and 3, **D**). The first pattern is exemplified by the immunodominant epitopes Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃. CD4⁺ T cells specific for these 2 epitopes highly expressed CRTH2 and CCR4, markers usually associated with T_H2 cells (average, 71.5% ± 13.7% and 85.9% ± 7.2%, respectively),^{15,16} which is in marked contrast to nonatopic subjects (average, 0.8% ± 1.2% and 31.6% ± 9.7%, respectively; *P* < .0001; Fig 2). Interestingly, these 2 epitopes also elicited a distinct protective T_H1/T_R1 response in allergic subjects but at a dramatically lower proportion than the pathogenic T_H2 response (Fig 3). The second pattern is exemplified by the nondominant epitopes Phl p 5a₂₄₁₋₂₆₀, Phl p 5b₆₉₋₈₈, and Phl p 5b₁₉₇₋₂₁₆ and is characterized by a higher proportion of CXCR3 than cells of the first pattern (average, 16.9% vs 2.4%, respectively; *P* < .0001), low levels of CRTH2 and CCR4 (average, 6.3% and 40.8%, respectively), and a dominant IFN-γ/IL-10-mediated immune response. Finally, a third pattern is exemplified by the nondominant epitopes Phl p 5a₈₁₋₁₀₀, Phl p 5a₁₆₉₋₁₈₈, and Phl p 5a₂₀₁₋₂₂₀, which preferentially elicit a T_H2 response in allergic subjects but are characterized by intermediate expression of CRTH2 and CCR4 compared with levels observed for the first and second patterns. In Table E2 we have categorized each of the TGP epitopes evaluated in this study as T_H2 (allergy-associated) or T_H1/T_R1 epitopes.

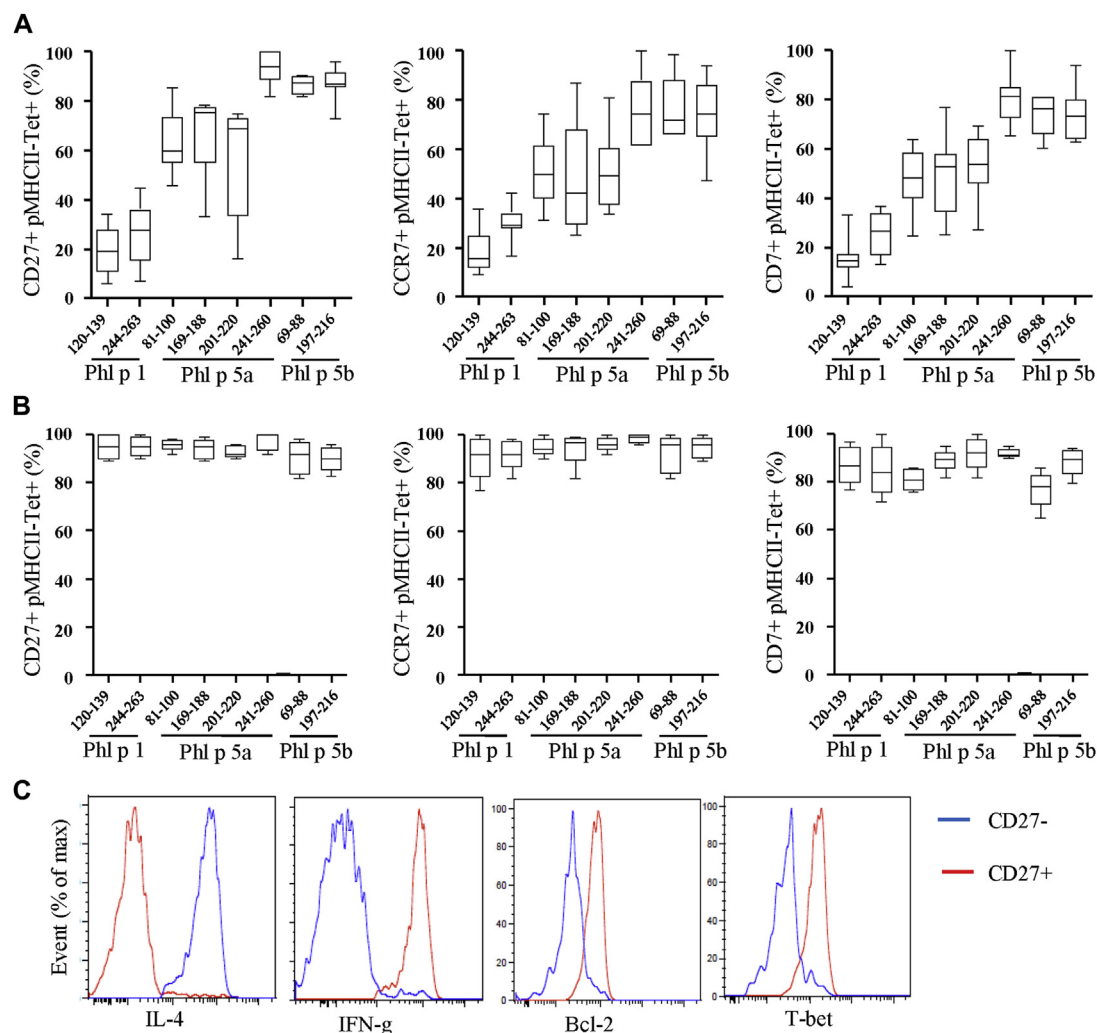


FIG 4. **A** and **B**, *Ex vivo* expression of CD27, CCR7, and CD7 of DR04:01-restricted TGP epitope-specific T cells in 10 allergic subjects (Fig 4, **A**) and 5 nonatopic subjects (Fig 4, **B**). * $P < .01$. **C**, IL-4, IFN- γ , Bcl-2, and T-bet expression by CD27⁺ (red histogram) and CD27⁻ (blue histogram) Phl p 1₁₂₀₋₁₃₉-specific T cells in allergic subjects. Data are representative of at least 4 DR04:01-restricted allergic subjects.

Dominant allergen-derived epitope-specific CD4⁺ T cells are more sensitive to T-cell deletion

Consistent with previous results in subjects with alder allergy,¹⁷ we also observed that CRTH2 expression within allergen-specific CD4⁺ T cells coincided with the lack of CD27 expression in subjects with TGP allergy (see Fig E3, **A**, in this article's Online Repository at www.jacionline.org). Interestingly, this is concomitant with a positive relationship between the frequency of allergen-specific CD4⁺ T cells elicited by an epitope and the level of CRTH2 expression (see Fig E3, **B**). Hence the 2 immunodominant T_H2 epitopes Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃ elicited significantly greater proportions of terminally differentiated cells (ie, CD7⁻, CCR7⁻, and CD27⁻) compared with the T_H1/T_R1 epitopes Phl p 5a₂₄₁₋₂₆₀, Phl p 5b₆₉₋₈₈, and Phl p 5b₁₉₇₋₂₁₆ (Fig 4, **A**). Conversely, allergen-specific CD4⁺ T cells in healthy subjects were exclusively CD7⁺, CCR7⁺, and CD27⁺ (Fig 4, **B**). Loss of CD27 expression has been associated with cells with a shorter lifespan.¹⁸⁻²⁰ To confirm the increased susceptibility to apoptosis of allergen-specific T_H2 cells, we next investigated the expression of Bcl-2, a key inhibitor of

apoptotic cell death, between CD27⁺ and CD27⁻ allergen-specific CD4⁺ T cells. Along with the fact that IL-4 production was largely restricted to the allergen-specific CD4⁺ T cells lacking CD27 expression, those cells express less Bcl-2 than did the CD27⁺ subset of the same specificity (Fig 4, **C**). Together, these results indicate that CD27⁻ allergen-specific T cells are functionally connected with allergic disease and display classical features of short-lived cells.

Allergen-specific T_H2 cells represent the main subset affected by ASIT

We next sought to determine the immunologic changes induced by grass pollen mixed extract ASIT by comparing the DR04:01-restricted TGP epitope-specific CD4⁺ T-cell responses between allergic subjects and patients after successful ASIT. In post-ASIT patients the mean numbers of total group 1 and group 5 TGP-reactive CD4⁺ T cells were significantly lower (32.4 ± 7.9) than those found in allergic subjects (280 ± 181.6 cells per 10^6 CD4⁺ T cells, $P < .001$). Specifically, a 20- to

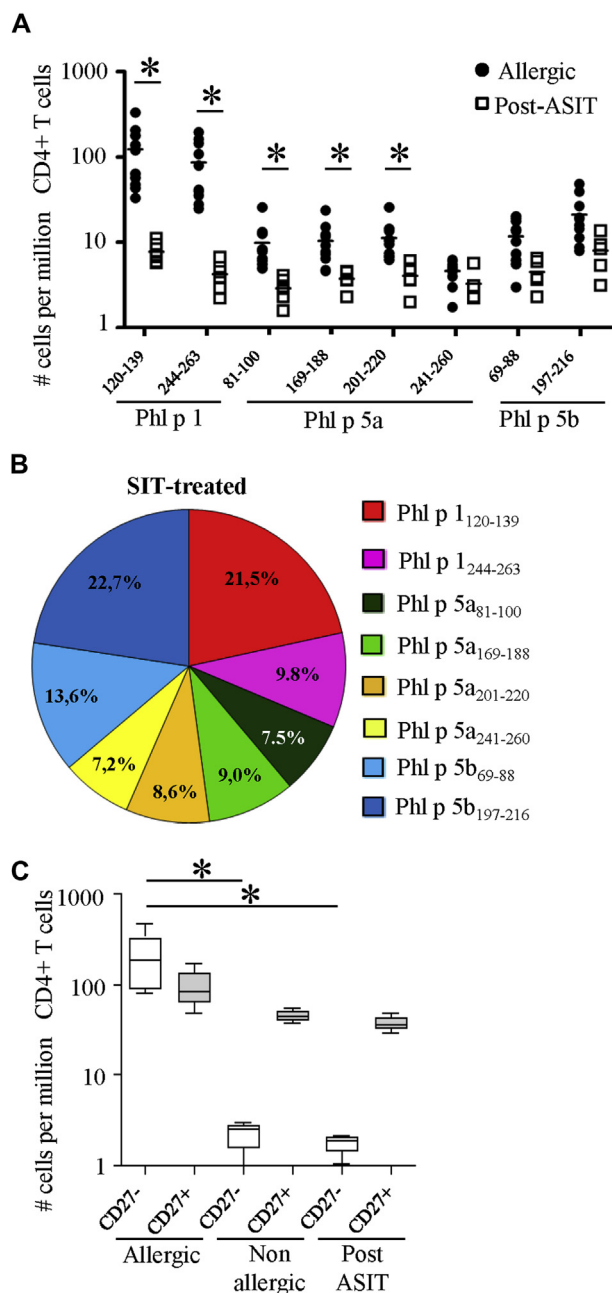


FIG 5. A, *Ex vivo* frequencies of DR04:01-restricted TGP-specific CD4⁺ T cells in allergic subjects and ASIT-treated subjects. **B,** Contribution of each DR04:01-restricted TGP epitope to the global allergen-specific CD4⁺ T-cell response in ASIT-treated subjects. **C,** Overall frequencies of CD27⁻ and CD27⁺ DR04:01-restricted TGP allergen-specific T cells in allergic subjects (n = 10), nonatopic subjects (n = 5), and subjects after ASIT (n = 6).

30-fold lower *ex vivo* frequency of Phl p 1₁₂₀₋₁₃₉- and Phl p 1₂₄₄₋₂₆₃-specific T cells was observed in all ASIT-treated patients (average, 7.7 ± 2.2 and 4.2 ± 1.6 vs 122.5 ± 95.9 and 86.3 ± 63.1 cells per 10^6 CD4⁺ T cells, respectively; $P < .001$) when compared with the allergic group (Fig 5, A). In contrast, the frequencies of allergen-specific CD4⁺ T cells specific for nondominant T_H2 epitopes only decreased by 2-fold after ASIT, and those elicited by T_H1/T_R1 epitopes were unaltered (average, 12.1 ± 9.2 vs 8.9 ± 5.7 cells per 10^6 CD4⁺ T cells; $P > .05$)

compared with those seen in allergic subjects. Thus tolerance induction during ASIT was accompanied by a dramatic change in the TGP allergen-specific CD4⁺ T-cell immunodominance hierarchy (Fig 5, B). Notably, CD27⁻ allergen-specific CD4⁺ T cells represent the main subset affected by ASIT, with no significant change in their CD27⁺ counterparts when compared with allergic subjects (Fig 5, C). Consequently, the previously subdominant CD27-expressing allergen-specific CD4⁺ T-cell subset becomes dominant because the allergen-specific CD4⁺ T-cell subset lacking CD27 expression is deleted, thereby changing the ratio of allergen-specific T_H2/T_H1 and T_H2/T_R1 cells. Indeed, in contrast to the functional heterogeneity observed in allergic subjects, all DR04:01-restricted TGP epitope-specific CD4⁺ T-cell responses observed in post-ASIT patients were dominated by T_H1 and T_R1 cells (Fig 6, A, and see Fig E4 in this article's Online Repository at www.jacionline.org). These results are also consistent with our *ex vivo* phenotyping showing minimal expression of CCR2 and CCR4 along with proportionately higher expression of CXCR3 within DR04:01-restricted TGP-specific T cells after ASIT (Fig 6, B). However, we did not observe any significant differences over the course of ASIT in the percentage of forkhead box protein 3-positive cells within allergen-specific CD4⁺ T cells (data not shown). Together, our data indicate that allergen-specific T_H2 cell deletion might be a crucial factor involved in the restoration of peripheral tolerance to allergen during ASIT.

DISCUSSION

The design of optimal peptide-based allergy vaccines is dependent on an accurate assessment of their ability to restore protective immune responses. In the present study we used an *ex vivo* pMHCII tetramer approach to characterize the TGP-specific CD4⁺ T-cell response at the epitope-specific level in DR04:01-restricted allergic subjects, ASIT-treated patients, and nonallergic donors. Consistent with previous studies,^{21,22} we found a broad range of TGP allergen-derived epitopes able to elicit DR04:01-restricted CD4⁺ T-cell responses. Critical for clinically relevant peptides, we also found that DR04:01-restricted allergic subjects recognize exactly the same allergen-derived epitopes as allergen-tolerant subjects. However, although all those epitopes strongly bind the DR04:01 molecules, our *ex vivo* approach reveals that the allergen-specific CD4⁺ T-cell repertoire in allergic subjects falls into a highly reproducible immunodominance hierarchy that is absent in healthy control subjects. Notably, of the 8 DR04:01 immunoprevalent epitopes that drive the CD4⁺ T-cell responses to group 1 and group 5 TGP allergens, only Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃ elicit strong responses in allergic subjects. These responses are so robust that they dominate the T-cell repertoire and suggest that the induction and regulation of allergic immune responses depend on a change in the magnitude but not the breadth of the T-cell response to allergen. Another finding of this study is that allergen-specific CD4⁺ T cells in allergic subjects have a range of phenotypes and frequencies that depend on the epitope they recognize. Conversely, all the allergen-specific T cells in nonatopic subjects are present at low frequencies, and CD27-expressing T_H1/T_R1 cells are the dominant phenotype, regardless of the epitopes they recognize.

Prior studies show that the cat allergen Fel d 1 has epitopes that preferentially elicit a T_H1 cytokine pattern.²³ More recently,

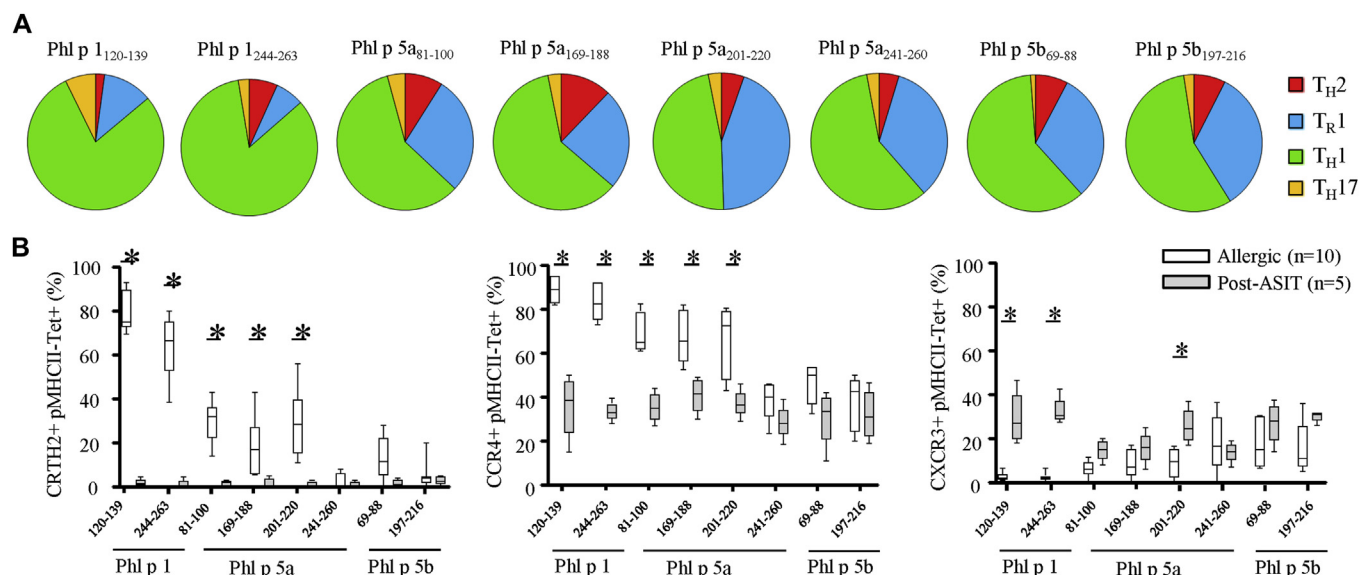


FIG 6. A, Cytokine profiles of DR04:01-restricted TGP epitope-specific T cells in ASIT-treated subjects. Data are representative of at least 4 subjects per group. **B**, *Ex vivo* phenotype of DR04:01-restricted TGP epitope-specific CD4⁺ T cells in allergic subjects and ASIT-treated subjects. Differences between groups were analyzed by using the Mann-Whitney *U* test. **P* < .01.

pathogen-specific epitopes targeted by regulatory T cells have been described.^{24,25} In agreement with this, we also found TGP epitopes (ie, Phl p 5a₂₄₁₋₂₆₀, Phl p 5b₆₉₋₈₈, and Phl p 5b₁₉₇₋₂₁₆) that preferentially elicit a T_H1/T_R1 cytokine pattern regardless of the allergic status of the donor. Alternately, we also found allergy-associated TGP epitopes (ie, Phl p 1₁₂₀₋₁₃₉ and Phl p 1₂₄₄₋₂₆₃) that preferentially elicit a T_H2 cytokine pattern in allergic subjects but not in nonatopic subjects. Of note, these epitopes remained able to elicit IFN- γ - and IL-10-expressing CD4⁺ T cells in allergic subjects, although at dramatically lower numbers than T_H2 cells of the same specificity. Therefore the effects of these concurrent responses in exacerbating or downregulating the immune response to an allergen seem to depend on their frequencies. These findings confirm the idea that the balance between pathogenic and protective T cells is decisive in the development of allergic disease or a healthy immune response.²⁶

The above observations raise questions about the mechanisms that cause epitopes that normally elicit low-frequency allergen-specific CD4⁺ T-cell responses to become immunodominant and pathogenic in atopic subjects. It also remains to be determined why the phenotype displayed by allergen-specific CD4⁺ T cells can depend on both the atopic status and the allergen epitope. A combination of factors can shape immunodominance hierarchies, including some related to antigen processing and others related to CD4⁺ T-cell dynamics.^{27,28} Differences in the magnitude of *ex vivo* allergen-derived epitope-specific T-cell responses between allergic and nonallergic subjects and within allergic subjects might arise from differences in antigen-processing machinery. As a consequence, some epitopes might be more effectively processed and presented on the surface of the antigen-presenting cell, leading to better recruitment and memory expansion of the reactive CD4⁺ T cells that are present in the normal repertoire. Extrinsic factors, such as danger signals and tissue-specific inflammation, might also be important.²⁹ The quantity and strength of signal received on

cognate recognition of the T-cell receptor also might dictate in part the fate and function of CD4⁺ T cells,^{30,31} and there is mounting evidence that CD27 is irreversibly lost from T cells only after repetitive antigenic stimulation.^{32,33} Taken together with our observation that only terminally differentiated (CD27⁻) allergen-specific CD4⁺ T cells display a T_H2 phenotype, this raises the possibility that repetitive T-cell receptor triggering biases the immune response in atopic subjects. Differences in the capacity to process and present peptide would be consistent with these observations and underlie the complexity of the cellular mechanisms that govern the allergic response.

It has been suggested that the primary mechanism of successful immunotherapy in atopic patients might be an increase in the number of CD4⁺CD25⁺ forkhead box protein 3-positive Treg cells and specific T_R1 cells to suppress allergic responses. Therefore for clinical applications, it has been speculated that vaccination strategies targeting T_H1/T_R1 cell target epitopes might most efficiently induce allergen tolerance. However, the absence of an increased number of CD4⁺ T cells directed to such nondominant epitopes in the post-ASIT group argues against this. In addition, although the use of single peptides might bypass allergen processing, epitopes that are not efficiently processed, such as T_H1/T_R1 epitopes, can target T cells with a limited role in future adaptive immune responses because they might not be efficiently recruited and activated during natural allergen exposure. With the assumption that the mechanisms underlying the efficacy of peptide-based allergy vaccine are similar to those for current extract-based ASIT, our results suggest that a combination of immunodominant allergy-associated (T_H2) epitopes would result in better clinical efficacy during peptide-based vaccine than nondominant allergen-derived epitopes that selectively induce T_H1 and T_R1 cells. First, we found that successful ASIT was associated with a markedly lower number of memory T cells reacting to previously immunodominant epitopes. Second, although we observed an increase in allergen-specific T_H1/T_H2 and T_R1/T_H2 cell ratios, the overall frequencies

of allergen-specific T_H1/T_R1 cells do not significantly increase when compared with those seen in allergic subjects. Third, we observed that allergen-specific T_H2 cells express low levels of the survival protein Bcl-2 compared with allergen-specific T_H1/T_R1 cells of the same specificity. Hence deletion of the previously dominant pathogenic T_H2 response during ASIT is a crucial factor in the restoration of peripheral T-cell tolerance to allergen, allowing other responses to emerge. This is consistent with a recent study by Mobs et al,³⁴ suggesting that allergen tolerance induced by ASIT is accompanied by long-term loss of allergen-specific T_H2 cells.

Collectively, our study supports the notion that ASIT might not exclusively function by inducing allergen-specific regulatory T cells and suggests that preferential allergen-specific T_H2 cell deletion after repeated high-dose antigen stimulation can be another independent mechanism to restore tolerance to allergen during immunotherapy.

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Key messages

- Functional patterns of allergen-reactive T cells depend on the epitope they recognize.
- Differences in the magnitude of the T-cell responses to allergen-derived epitopes are correlated with the allergic immune response.
- Allergen-specific T_H2 cell deletion by prolonged high-dose stimulation is a crucial factor in the restoration of peripheral T-cell tolerance to allergen.

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METHODS

Tetramer reagents and tetramer-guided epitope mapping

For epitope mapping, Phl p 1–, Phl p 5a–, and Phl p 5b–derived peptides were divided into pools of 5 peptides each, as previously described.^{E1} Cells were cultured with peptide pools for 14 days and then stained with pooled peptide tetramers. Cells from wells that resulted in positive staining were stained again with individual pMHCII tetramers from the positive pool. pMHCII tetramers loaded with irrelevant peptides were used as negative controls.

Intracellular cytokine staining

CD4⁺ T cells cultured for 14 days with specific immunodominant peptide were stained with corresponding PE-conjugated pMHCII tetramers for 60 minutes at 37°C. Cells were then restimulated with 50 ng/mL phorbol

12-myristate 13-acetate and 1 mg/mL ionomycin in the presence of 10 mg/mL monensin in 1 mL of complete medium for 6 hours at 37°C in a 5% CO₂ atmosphere. After restimulation, surface staining was performed first, followed by fixation/permeabilization, as per the manufacturer's protocol (eBioscience, San Diego, Calif). Cells were then stained with various combinations of antibodies for IFN- γ , IL-17, IL-10 (all from BioLegend, San Diego, Calif), and IL-4 (eBioscience) or corresponding isotype-matched mAbs. After 30 minutes at 4°C, cells were washed and immediately analyzed by using flow cytometry.

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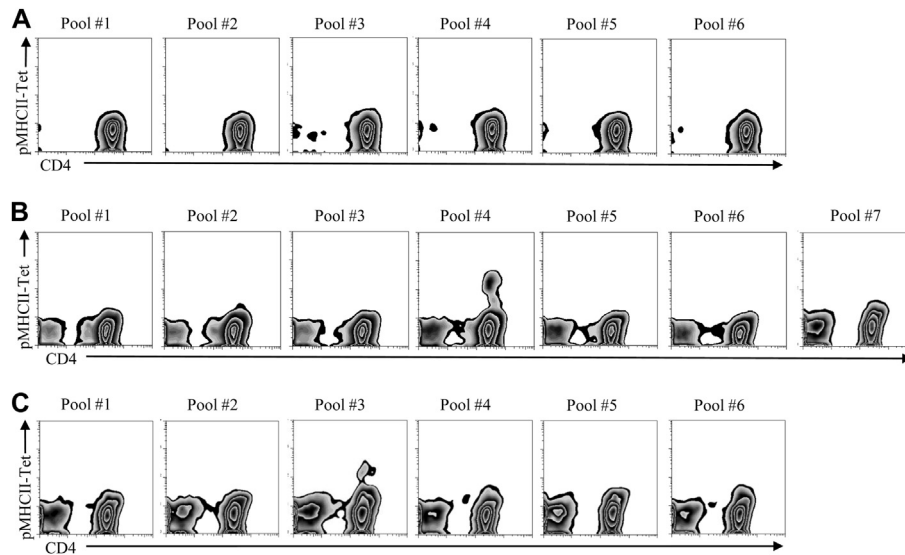


FIG E1. Tetramer-guided epitope mapping of the TGP major allergen. T cells from DRB1*07:01-restricted subjects with TGP allergy were stimulated with overlapping peptide mixtures spanning the entire Phl p 1 (A), Phl p 5a (B), or Phl p 5b (C) protein and stained with pooled peptide-loaded tetramers after 2 weeks. Data are representative of 3 DRB1*07:01-restricted subjects with TGP allergy.

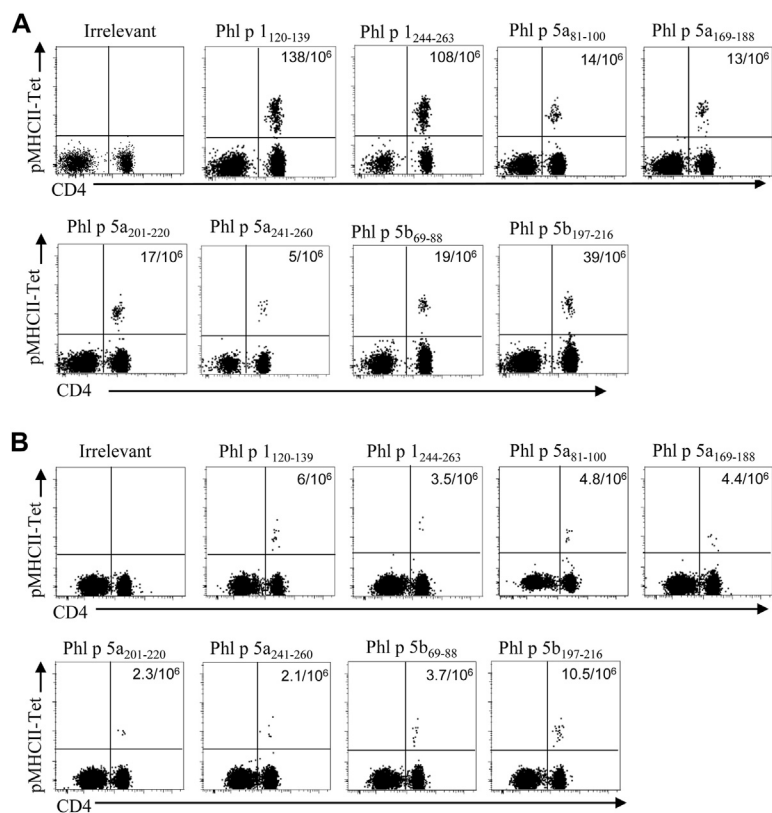


FIG E2. Representative flow cytometric analysis of *ex vivo* PE-conjugated DRB1*04:01/Phl p 1, DRB1*04:01/Phl p 5a, and DRB1*04:01/Phl p 5b pMHCII tetramer staining in allergic (**A**) and nonallergic (**B**) subjects after magnetic bead enrichment for PE-positive cells. Plots are gated on live CD3⁺CD14⁻CD19⁻ T lymphocytes. Frequencies of specific T cells per million CD4⁺ T cells are as indicated.

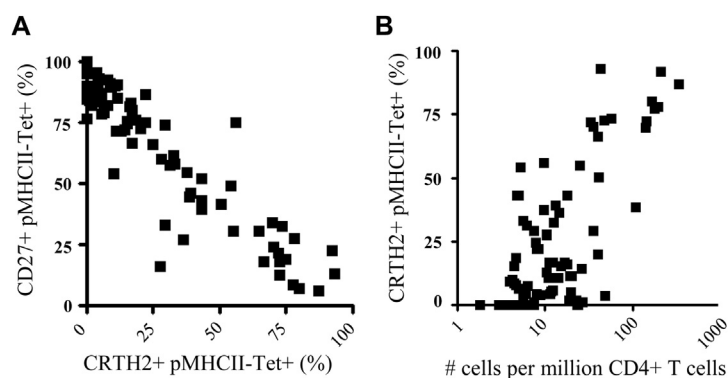


FIG E3. **A**, Correlation between the proportion of CRTH2⁺ cells and the proportion of CD27⁺ cells in TGP allergen-specific T cells. **B**, Scatter plot showing the correlation between the *ex vivo* frequency and the proportion of CRTH2⁺ cells in TGP allergen-specific T cells. Each data point represents the value for each TGP epitope from 10 DR04:01-restricted donors with TGP allergy.

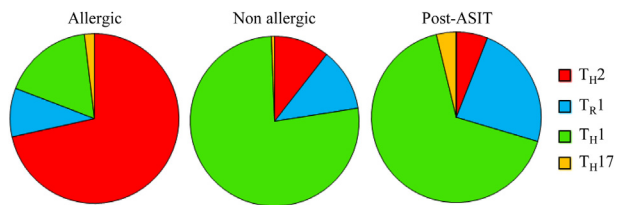


FIG E4. Global DR04:01-restricted TGP-specific CD4⁺ T-cell response in allergic, nonallergic, and post-ASIT subjects. Data are representative of at least 5 subjects and are presented as the mean values from each group in pie charts.

TABLE E1. Clinical characteristics of the DRB1*04:01-restricted subjects enrolled in the study

Group	ID	HLA-DR typing	Age (y)	Sex	Phl p 1 (IgE, kU _A /L)	Phl p 5 (IgE, kU _A /L)	Skin prick test	Asthma
Grass pollen allergy	GP-1	DR4/DR15	33	F	16.9	26.2	4++	Yes
	GP-2	DR4/DR13	58	M	1.97	2.06	4+	No
	GP-3	DR4/DR3	36	F	>100	41.3	4+	No
	GP-4	DR4/DR3	60	F	5.72	6.06	4+	No
	GP-5	DR4/DR13	26	M	45.4	31.3	4+	No
	GP-6	DR4/DR7	36	M	18.8	21.5	4+	Yes
	GP-7	DR4/DR1	38	F	15.7	8.14	4+	No
	GP-8	DR4/DR3	38	M	—	—	4+	No
	GP-9	DR4/DR1	33	M	19.3	21.9	4+	No
	GP-10	DR4/DR4	34	F	6.57	8.38	3+	Yes
	GP-11	DR7/DR15	27	M	—	—	4+	Yes
	GP-12	DR7/DR1	29	M	17.2	26.7	4++	Yes
Nonallergic	H-1	DR4/DR15	28	M	—	—	0	No
	H-2	DR4/DR11	36	M	—	—	—	No
	H-3	DR4/DR3	29	F	—	—	—	No
	H-4	DR4/DR14	33	F	—	—	0	No
	H-5	DR4/DR13	36	M	—	—	—	No
Post-ASIT	ASIT-1	DR4/DR15	56	M	—	—	—	No
	ASIT-2	DR4/DR7	61	M	—	—	1+	Yes
	ASIT-3	DR4/DR11	30	M	—	—	2+	Yes
	ASIT-4	DR4/DR15		F	—	—	—	No
	ASIT-5	DR4/DR11		M	—	—	2+	Yes
	ASIT-6	DR4/DR7		F	—	—	2+	No

TABLE E2. Binding capacities of Phl p 1, Phl p5a, and Phl p 5b peptides to immunopurified HLA-DR04:01 and HLA-DR07:01 molecules

HLA-DRB1	Allergen epitope	Sequence	IC ₅₀ (nmol/L)	Immunogenicity	Induced response
*04:01	Phl p 1 ₁₂₀₋₁₃₉	EEPIAPYHFDLSGHAFGAMA	76	Dominant	T _H 2 epitope
	Phl p 1 ₂₄₄₋₂₆₃	TEAEDVIPEGWKADTSYESK	232	Dominant	T _H 2 epitope
	Phl p 5a ₈₁₋₁₀₀	FAEGLSGEPKGAAESSSKAA	297	Nondominant	T _H 2 epitope
	Phl p 5a ₁₆₉₋₁₈₈	DAAFKVAATAANAAPANDK	177	Nondominant	T _H 2 epitope
	Phl p 5a ₂₀₁₋₂₂₀	KASTGGAYESYKFIPALEAA	565	Nondominant	T _H 2 epitope
	Phl p 5a ₂₄₁₋₂₆₀	ETALKKAITAMSEAQKAAKP	595	Nondominant	T _H 1/T _R 1 epitope
	Phl p 5b ₆₉₋₈₈	KFKTFEAAFTSSSKAAAAKA	104	Nondominant	T _H 1/T _R 1 epitope
	Phl p 5b ₁₉₇₋₂₁₆	GGAYDTYKCIPSLEAAVKQA	123	Nondominant	T _H 1/T _R 1 epitope
*07:01	Phl p 5a ₁₂₁₋₁₄₀	PEAKYDAYVATLSEALRIIA	81	Dominant	T _H 2 epitope
	Phl p 5b ₈₉₋₁₀₈	ATPEAKFDSFVASLTEALRV	194	Dominant	T _H 2 epitope

IC₅₀, Inhibitory concentration of 50%.