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*J Immunol* published online 16 June 2010
http://www.jimmunol.org/content/early/2010/06/16/jimmunol.1000405

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/16/jimmunol.1000405.DC1

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Molecular Determinants of T Cell Epitope Recognition to the Common Timothy Grass Allergen

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We investigated the molecular determinants of allergen-derived T cell epitopes in humans utilizing the Phleum pratense (Timothy grass) allergens (Phl p). PBMCs from allergic individuals were tested in ELISPOT assays with overlapping peptides spanning known Phl p allergens. A total of 43 distinct antigenic regions were recognized, illustrating the large breadth of grass-specific T cell epitopes. Th2 cytokines (as represented by IL-5) were predominant, whereas IFN-γ, IL-10, and IL-17 were detected less frequently. Responses from specific immunotherapy treatment individuals were weaker and less consistent, yet similar in epitope specificity and cytokine pattern to allergic donors, whereas nonallergic individuals were essentially nonreactive. Despite the large breadth of recognition, nine dominant antigenic regions were defined, each recognized by multiple donors, accounting for 51% of the total response. Multiple HLA molecules and loci restricted the dominant regions, and the immunodominant epitopes could be predicted using bioinformatic algorithms specific for 23 common HLA-DR, DP, and DQ molecules. Immunodominance was also apparent at the Phl p Ag level. It was found that 52, 19, and 14% of the total response was directed to Phl p 5, 1, and 3, respectively. Interestingly, little or no correlation between Phl p-specific IgE levels and T cell responses was found. Thus, certain intrinsic features of the allergen protein might influence immunogenicity at the level of T cell reactivity. Consistent with this notion, different Phl p Ags were associated with distinct patterns of IL-5, IFN-γ, IL-10, and IL-17 production. The Journal of Immunology, 2010, 185: 000–000.

Over the past several years, the concept of T cell subsets has been modified and expanded. It has been proposed that naturally occurring regulatory T cells (Tregs) (11–13) may regulate allergic diseases (14, 15). Furthermore, inducible Tregs, designated Tr1 cells, which function predominantly through the secretion of the regulatory cytokines IL-10 and/or TGF-β (16–21), have also been invoked as regulators of allergic reactions. The emerging recognition of the importance of Tregs led to the hypothesis that the pathogenesis of allergic disease may also involve an imbalance between Th2 cells and Tregs (22, 23). Furthermore, successful SIT has been shown to be associated with an increased production of IL-10 and IL-10–producing T cells (8, 24).

Recently, Th cells that produce IL-17 (Th17) have been described in both mice (25, 26) and humans (27, 28) as a distinct Th subset. Th17 cells require IL-6 and TGF-β to differentiate from naïve T cells and express the retinoic acid receptor-related orphan receptor-γ transcription factor. Accumulating data suggest that Th17 cells are highly proinflammatory and might play a role in allergic asthmatic disease (29–31).

In contrast to this wealth of information regarding Th cell phenotypes in allergic disease, a comprehensive characterization of the epitopes recognized by human T cells in most clinically relevant allergens is lacking. Thus, the exact mapping of the epitopes involved, their restriction and binding affinity, Ag of origin, and patterns of associated Th cell responses are yet to be fully elucidated. First, it is unclear to what extent the mechanisms involving immunodominance and immunoprevalence of T cell responses in microbial diseases are also active in allergy. In microbial diseases, it is well established that responses to complex Ags are broad and involve a large number of epitopes (32). It is unclear whether the same situation applies to allergic diseases. Additionally, in allergic disease, the molecular mechanisms involved in establishing Ag/epitope prominence are unknown. In microbial
systems, it is known that HLA binding affinity plays an important role in determining immunodominance, but it has been hypothesized that allergic epitopes might be less dependent on high HLA affinity, because of differences in amount, frequency, and modality of Ag encounter (33, 34). To date, a molecular evaluation of HLA binding capacity of HLA-restricted allergen epitopes is lacking.

It has been described that, in many instances, HLA-restricted epitopes are associated with promiscuous HLA binding capacity or that certain protein regions are hot spots for T cell recognition, with multiple HLA types recognizing largely overlapping epitopes. These two mechanisms provide alternative molecular explanations for the potential dominance of discrete protein sequences in T cell responses, but lack of HLA restriction data on most allergen epitopes has not allowed this issue to be sufficiently addressed. If few discrete epitopes/regions could be defined that account for a majority of responses, these could be of significant practical interest for diagnostic and immunotherapeutic applications.

An additional key issue that remains to be addressed in the study of immunodominance in allergic responses is what determines which allergen proteins are going to be recognized by T cell responses. In other systems, it has been shown that the proteins most frequently recognized by T cell responses are those that are most abundant and/or most frequently recognized by Ab responses (32). Whether this applies to allergens as well is currently unknown. This issue is of particular relevance, as proteins from allergens for which sequences are available and have been therefore characterized at the level of T cell responses have been in most cases selected on the basis of IgE reactivity. It is therefore important to test the assumption that the Ags recognized by dominant IgE responses are also the major targets of T cell reactivity.

An additional issue that requires clarification is the relation between the epitopes recognized by effector T cells (Th1, Th2, or Th17 cells) and the epitopes that are recognized by Tregs. Finally, it is, in general, unclear how T cell reactivity in exposed nonallergic individuals relates to reactivity identified in allergic individuals and in those allergic individuals who have undergone successful immunotherapy.

In this study, we selected the *Phleum pratense* (Timothy grass) allergen (Phl p) system to rigorously address these issues and probe in detail the breadth of the repertoire of T cell specificities recognizing different Phl p allergens. This system also permitted investigation of the mechanisms involved in immunodominance, with a particular emphasis on promiscuous epitopes and on the analysis of epitopes recognized by both effector Th cells and Tregs. This system was chosen because Timothy is a clinically important allergen, and it has many well-characterized protein allergen targets of IgE responses. In this study, we also set out to address whether T cell reactivity can be detected in nonallergic donors and individuals undergoing SIT and how any such reactivity compares to that observed in allergic individuals in terms of magnitude, the phenotype(s) of responding cells, the specific epitopes recognized, and whether T cells with a Th1, Th2, Th17, or Treg phenotype recognize the same or different epitopes.

**Materials and Methods**

**Characteristics of the patient population**

T cell reactivity against Phl p was analyzed utilizing a cohort of 43 donors (Table I). Each donor was recruited following Institutional Review Board approval (Federal Wide Assurance number 0000032) and informed consent and assigned a study identification number. Clinical case histories and other information were collected and recorded by clinical investigators. Skin test reactivity to a panel of extracts from 32 common allergens, including Timothy grass, was determined by standard methods. Both wheal (millimeter) and flare (millimeter) were measured. All volunteers were asked to provide a 5-ml serum sample and a unit of peripheral blood.

**Selection of 15-mer peptides from Timothy grass sequences**

Phleum pratense, or different epitopes. Of responding cells, the specific epitopes recognized, and whether served in allergic individuals in terms of magnitude, the phenotype(s) undergoing SIT and how any such reactivity compares to that ob-

| Donor | Allergic Status | Wheal | RAST |
|-------|----------------|------|------|
| D00004 | Allergic | 23 | 42 |
| D00012 | Allergic | 13 | 2.42 |
| D00020 | Allergic | 15 | – |
| D00058 | Allergic | 15 | 20.6 |
| D00042 | Allergic | 20 | 8.62 |
| U00032 | Allergic | 30 | 10.9 |
| D00036 | Nonallergic | – | – |
| D00017 | Allergic | 14 | 1.68 |
| D00063 | Allergic | 15 | 37.99 |

aWheal response to Timothy grass extract in millimeters.  
RAST response to Timothy grass extract in kiloliters per liter. A dash indicates <0.1 kiloliters per liter.  
–, Wheal <3 mm.

An allergic donor was defined as a donor yielding a reaction to the Timothy grass extract with a wheal at least 3 mm in diameter and a clinical history consistent with allergic symptoms during the grass pollen season. A total of 33 allergic donors was investigated, including a cohort of eight individuals undergoing maintenance SIT. A control group of 10 nonallergic donors, identified by a negative skin test to the entire panel of 32 allergens tested, lack of any clinical symptoms consistent with allergic asthma and/or rhinitis, and no history of SIT, was also included in the study. The donor cohort included 32 females and 11 males and ranged between 20 and 63 yr of age. Of the 43 donors, a total of 21 allergic donors had rhinitis, and 7 individuals were categorized as having rhinitis and asthma.

**Selection of 15-mer peptides from Timothy grass sequences**

The Timothy grass model in the current study contains 10 allergens, denominated Phl p 1, 2, 3, 4, 5, 6, 7, 11, 12, and 13. At the initiation of the study, these represented all Phl p allergens with reported amino acid sequences. Peptides were selected from collected sequences of Phl p allergens from seven databases: Allergen Database for Food Safety (http://allergen.nihs.jp/ADFS), Food Allergy Research and Resource Program (www.farrp.org), Allergen Nomenclature (www.allergen.org/Allergen.aspx), Allergen (www.allergome.org), Swissprot (www.expasy.ch/sprot), Structural Database of Allergenic Proteins (http://fermi.umn.edu/SDAP), and the Biotechnology Information for Food Safety Database (35).

Sets of peptides of 15 aa in length, overlapping by 10 residues, were generated to cover the allergen protein sequences. Where variability
between sequences for the same allergen was encountered, all peptide variants were included. In the case of Phl p 5, a relatively large number of unique sequences were retrieved, and, as a result, a disproportionately number of peptides would be required to cover all sequence variants of the Ag. To reduce the number of peptides to a level more in line with the relative size of the protein sequence compared with other Phl p Ags, an analysis was performed using CD-HIT (36). From this analysis, four Phl p 5 sequences were selected as being representative. Using these representative sequences, the number of resulting 15-mer peptides necessary to span the Ag was reduced from 366 to 145.

Peptide synthesis

Peptides for screening studies were purchased from Mimotopes (Clayton, Victoria, Australia) and/or A and A (San Diego, CA) as crude material on a small (1-mg) scale. Peptides used as radiolabeled ligands were synthesized on larger scale and purified (>95%) by reversed phase HPLC. The Timothy grass-specific CD4+ T cell epitope data and peptide sequence information has been submitted to the Immune Epitope Database (www.iedb.org), and has been assigned the submission identification number 1000440.

MHC purification

MHC molecules were purified from EBV-transformed homozygous cell lines by mAb-based affinity chromatography, essentially as described in detail elsewhere (37). HLA-DR, DQ, and DP molecules were captured by repeated passage of lysates over LB3.1 (anti–HLA-DR), SPV-L3 (anti–HLA-DQ), and B7/21 (anti–HLA-DP) columns.

MHC-peptide binding assays

Assays to quantitatively measure peptide binding to MHC class II molecules are based on the inhibition of binding of a high-affinity radiolabeled peptide to purified MHC molecules and have been described in detail elsewhere (37). Briefly, 0.1–1 nM radiolabeled peptide was coincubated at room temperature or 37°C with 1 µM to 1 nM of purified MHC in the presence of a mixture of protease inhibitors. Following a 2–4-d incubation, the percent of MHC-bound radioactivity was determined by capturing MHC/peptide complexes on LB3.1 (DR), L243 (DR), HB180 (DR/DQ/DP), SPV-L3 (DQ), or B7/21 (DQ) Ab-coated Optiplates (Packard Instrument, Meriden, CT) and bound cpm measured using the TopCount (Packard Instrument) microscintillation counter. Under the conditions used, where [label] ≤ [MHC] and IC50 ≥ [MHC], the measured IC50 values are reasonable approximations of the true KD values (38, 39).

PBMC isolation and HLA typing

PBMCs were obtained by density gradient centrifugation (Ficoll-Hypaque, AmerHash Biosciences, Uppsala, Sweden) from one unit of blood (450 ml) according to the manufacturer’s instructions and cryopreserved for further analysis. HLA typing was performed according to standard methods (Blood System Laboratories, Tempe, AZ), and an aliquot of serum was obtained for analysis. HLA typing was performed according to standard methods (Blood System Laboratories, Tempe, AZ), and an aliquot of serum was obtained for analysis. HLA typing was performed according to standard methods (Blood System Laboratories, Tempe, AZ), and an aliquot of serum was obtained for analysis. HLA typing was performed according to standard methods (Blood System Laboratories, Tempe, AZ), and an aliquot of serum was obtained for analysis.

Ag-specific determination of Timothy grass-specific IgE, IgG, and IgG4 titers

Sequencing was collected from each donor as described above. IgE titers specific to Timothy grass extract, rPhl p 1, 2, 4, 5, 6, 7, 11, and 12 were performed by the National Jewish Medical Research Center, Denver, CO.

In vitro expansion of Timothy grass pollen extract-specific T cells

PBMCs were cultured in RPMI 1640 (Q Scientific, Tarzana, CA) supplemented with 5% human serum (Cellogiro, Herrndon, VA) at a density of 2 × 10⁶ cells/ml in 24-well plates (BD Biosciences, San Jose, CA) and stimulated with Timothy grass pollen extract (50 µg/ml), rPhl p 10 proteins (10 µg/ml) (Greer, Lenoir, NC), or individual peptides. Cells were kept at 37°C, 5% CO₂, and an additional IL-2 (10 U/ml; ebioscience, San Diego, CA) was added every 3 d after initial antigenic stimulation. On day 17, cells were harvested and screened for reactivity against Timothy grass-specific peptide pools or individual peptides.

Dual ELISPOT assays

The production of IL-5, IFN-γ, IL-10, and IL-17 poststimulation with Timothy grass pollen extract was analyzed in dual ELISPOT assays. Flat-bottom 96-well nitrocellulose plates (Millipore, Bedford, MA) were prepared according to the manufacturer’s instructions and coated with either 10 µg/ml anti-human IL-5 (clone TRFK5; Mabtech, Cincinnati, OH), or anti-human IFN-γ (clone 1-D1K; Mabtech) or 10 µg/ml anti-human IL-10 (clone 9D7; Mabtech) and anti-human IL-17 (clone 147178; ebioscience). Cells were then incubated at a density of 1 × 10⁶/well either with peptide pools or individual peptides (10 µg/ml), Timothy grass extract (50 µg/ml), PHA (10 µg/ml), or medium containing 1% DMSO (corresponding to the percent of DMSO in the pool/peptides) as a control. After 24 h, cells were removed, and plates were incubated with either 2 µg/ml biotinylated anti-human IL-5 Ab (Mabtech) and FITC-conjugated anti-human IFN-γ Ab (Mabtech) or 2 µg/ml biotinylated anti-human IL-10 Ab (Mabtech) and FITC-conjugated anti-human IL-17 Ab (ebioscience) at 37°C. After 2 h, spots corresponding to the biotinylated Abs (IL-5, IL-10) were developed by incubation with avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) followed by incubation with 3-aminio-9-ethylcarbazole solution (Sigma-Aldrich, St. Louis, MO). Spots corresponding to the FITC-conjugated Abs (IFN-γ, IL-17) were incubated for 1 h with HRP-conjugated anti-FITC in Avidin-Peroxidase-Complex and then visualized by applying the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories) according to the manufacturer’s instructions. Spots were counted by computer-assisted image analysis (KS-ELISPTOP Reader, Zeiss, Munich, Germany).

Each assay was performed in triplicate. The level of statistical significance was determined with a Student t test using the mean of triplicate values of the response against relevant pools or individual peptides versus the response against the DMSO control. Criteria for peptide pool positivity were 100 spot-forming cells (SFCs)/10⁶ PBMC, p ≤ 0.05, and a stimulation index (SI) ≥ 2, whereas criteria for individual peptide positivity were ≥20 SFC/10⁶ PBMC, p ≤ 0.05, and an SI ≥ 2.

HLA restriction

To determine the HLA locus restriction of identified epitopes, Ab inhibition assays were performed. After 14 d of stimulation with Timothy grass pollen extract (50 µg/ml) or specific peptide (10 µg/ml), PBMCs were incubated with 10 µg/ml of Abs (Strategic Biosolutions, Windham, ME) against HLA-DR (LB3.1, DP (B7/21), or DQ (SVPL3) 30 min prior to peptide contact. Cytokine production in response to positive peptides was then measured in ELISPOT assays as described above. The pan MHC class I Ab (W6/32) was used as a control.

To determine the specific HLA allele restriction, donor-derived T cells were expanded for 10 d using a single peptide epitope and were then subsequently incubated with peptide-pulsed EBV cell lines and/or fibroblasts expressing known HLA molecules also expressed in the donor from whom T cells were derived. Cytokine-specific ELISPOT assays were performed as described above to determine cytokine production and allele restriction determined by analyzing a matrix of negative and positive cytokine responses with the HLA-expressing EBV lines and fibroblasts used.

Results

Identification of T cell epitopes

To map the epitopes recognized by T cell responses specific for Timothy grass, we synthesized a panel of 687 overlapping 15-mer peptides spanning the Phl p 1, 2, 3, 4, 5, 6, 7, 11, 12, and 13 allergens. These allergens were selected on the basis of the availability of protein sequence information and previous literature reports that identified these proteins as targets for IgE responses (40–42).

Next, utilizing a cohort of 43 donors (Table I), we analyzed T cell reactivity against Timothy grass pollen allergens by measuring in vitro production of IL-5, IFN-γ, IL-17, and IL-10. These cytokines were chosen as representative cytokines produced by Th2, Th1, Th17, and Treg subsets of CD4+ T cells, respectively. Responses against the entire peptide panel were determined utilizing dual ELISPOT assays. The ELISPOT assay was used based on previous experience in other systems (43–45) that demonstrated that this assay is sensitive and also most amenable to screens involving relatively large numbers of candidate epitopes (46). The overlapping peptides were arranged in 35 pools of ~20 peptides each and then the entire set screened for recognition by PBMCs from the various donors.

Preliminary experiments evaluated different screening strategies. Direct screening of the peptide pools followed by deconvolution yielded erratic and weak responses (data not shown). As a result, we...
adopted a strategy in which PBMCs from each donor were first restimulated in vitro with Timothy grass pollen extract for 14–17 d to expand Timothy grass-specific T cells, and then the panel of peptide pools was screened. Subsequently, each positive pool was deconvoluted to identify individual reactive peptides. This strategy was found to give consistent results. Based on an interim analysis performed after screening five donors, 100 SFCs/10⁶ PBMCs was determined as the minimum response value to attempt pool deconvolution, as experiments with pools associated with responses >100 SFCs failed to clearly define any epitopes. A representative example of pool screening is shown in Fig. 1. In all, PBMCs from 25 allergic donors were screened for reactivity to the pools, and 19 donors generated positive peptide responses. Also, 3 out of 10 nonallergic donors and 3 out of 8 individuals undergoing SIT had epitope-specific T cell responses.

Responses to individual peptides and antigenic regions in allergic donors

Overall, our screening of allergic individuals identified a total of 183 distinct positive recognition events, defined as a unique peptide/donor/cytokine response. These responses mapped to 108 different peptides. Because the peptides screened were overlapping and included peptide variants from different isoforms of the same P. pratense proteins, it was frequently found that donors recognized multiple peptides with high sequence homology and/or sequence overlap. These sets of homologous and overlapping peptides are herein referred to as antigenic regions. For each donor, the most highly positive peptide in an antigenic region was considered the epitope recognized. The totality of the responses to the 108 peptides could be encompassed by 43 distinct antigenic regions (Table II), ranging in size from 15–30 aa residues.

The number of donors recognizing each region and the magnitude of responses measured in the ELISPOT assay (calculated as the SFC sum for any detected cytokine for all donors to their respective epitopes in any given region) is shown in Table II. As discussed in more detail in the following sections, each region was recognized by a variable number of donors, ranging from one to seven, with an average response per donor of 533 SFCs, and was associated with an average total SFC response of ~1000 SFCs/region (range of 37–10,631). A complete account of all responses and the corresponding cytokine associated with each donor/peptide combination is provided in Supplemental Table I.

The most commonly detected cytokine was IL-5, which accounted for 69.9% of the events and 83.1% of the SFC responses from the allergic donors. Much less frequent responses were detected for IFN-γ, IL-10, and IL-17, which accounted for 23.7, 3.2, and 3.2% of the events and 12.3, 0.7, and 3.8% of the responses, respectively (Fig. 2A, 2B). We did not detect any significant numbers of Timothy grass-specific T cells producing more than one cytokine, thus the reported SFCs represent polarized T cells of the various Th cell subsets. Similarly, intracellular cytokine staining for IFN-γ, IL-5, IL-10, and IL-17 further supported this finding and demonstrated that cytokine production stimulated by the Timothy grass extract was indeed CD4⁺ T cell specific (data not shown). In conclusion, these results illustrate the diversity of the repertoire of epitopes recognized by Timothy grass-specific T cells. The data presented in this study also demonstrate that as expected, a Th2 cytokine (i.e., IL-5) represents the predominant cytokine associated with Timothy grass-specific T cell responses from allergic individuals.

**Epitope and Ag reactivity in nonallergic and SIT donors**

In contrast to the results with allergic donors, when the same complete peptide panel and screening strategy was applied to PBMCs from a cohort of nonallergic donors, positive epitopes were only rarely identified. Of the 10 nonallergic donors screened, peptide responses were observed in only 3. From these three individuals, only four Phl p antigenic regions were identified. Two of these regions were also identified in allergic donors, and two were uniquely identified in nonallergic donors. All of the responses in the nonallergic donors were low, with an average of 110 SFCs, and none were ≥207 SFCs (Supplemental Table II, Table III). Of the cytokines associated with the responses, IL-5 represented 47% of the total response in terms of SFCs (Fig. 2A, 2B), IFN-γ responses accounted for another 35% and IL-10 for ~18%. No IL-17 responses were detected in the nonallergic donors. The data obtained suggest that nonallergic individuals have weak and inconsistent epitope-specific responses, at least with the assay conditions used. This weak response was skewed toward IFN-γ and IL-10, when compared with the allergies, and two of the four peptides identified were not previously identified in allergic donors.

In individuals undergoing grass SIT, epitope-specific responses were detected in three of the eight donors (38%) examined (Table IV, Supplemental Table III). These responses mapped to nine epitope regions, seven of which were also identified in the allergic cohort not undergoing SIT. In terms of magnitude, responses were intermediate between those in allergic and nonallergic donors, with an average of 390 SFCs. IL-5 was again the most prevalent and dominant cytokine, accounting for almost the entirety (95%) of the total response. IFN-γ accounted for the small residual fraction (5%) of the response; IL-17 and IL-10 were not detected in any epitope-specific responses (Fig. 2A, 2B). In summary, considering the main Phl p allergens analyzed and the assay conditions used in this study, SIT donors manifested weaker and less consistent epitope-specific T cell responses when compared with non-SIT...
allergic individuals. However, these responses appear similar in terms of epitope specificity and the pattern of cytokines produced to those observed in allergic donors.

Taken together, nonallergic individuals had minimal T cell responses. SIT donors were associated with lower and less frequent allergen specific IgE and IgG responses, and no IL-17 or IFN-γ responses, than the allergic cohort and dramatically higher allergen specific IgE levels, as might be expected. SIT donors had lower allergen specific IgE than the allergic cohort and dramatically higher allergen specific IgE levels.

**HLA restriction of identified epitopes**

We next examined the nature of Timothy grass epitope-specific responses in terms of the specific epitopes recognized in allergic donors, in more detail, to determine whether there was any association between particular HLA class II loci or alleles and the T cell responses of allergic donors and thereby to gain insight into the mechanisms underlying the frequent recognition of certain peptide regions. To define the HLA restriction of the identified epitopes, for each antigenic region/donor combination, we derived short-term T cell lines by either extract or peptide stimulation and determined the HLA restriction of identified epitopes.

| Region | Position | Sequence | Length | Donors | SFC |
|--------|----------|----------|--------|--------|-----|
| Phl p 1 | 46       | STWYGKPTAGPKDN | 15     | 2     | 860 |
| Phl p 1 | 71       | KPPFSGNTCGNTP1 | 15     | 2     | 540 |
| Phl p 1 | 96       | FRIKCTKPERACGEGPVVPHI | 20  | 2     | 124 |
| Phl p 1 | 111      | VVHHITTDDNEEPIAP | 15     | 1     | 153 |
| Phl p 1 | 121      | EPIAPYHFDLIGHAF | 15     | 1     | 647 |
| Phl p 1 | 131      | SGIAFGSMARKQDQ | 15     | 2     | 1,823 |
| Phl p 1 | 151      | GEELQFRVXKXEY | 15     | 2     | 1,410 |
| Phl p 1 | 161      | KCKYPEGKTVFHV | 15     | 1     | 37  |
| Phl p 1 | 171      | TPHVEKGSNPNYLALLLVKYVNGDGD | 25  | 2     | 803 |
| Phl p 1 | 196      | VVAVDIKEKKGKWI | 15     | 1     | 277 |
| Phl p 1 | 211      | ALKESWGA1WRIDTP | 15     | 1     | 997 |
| Phl p 1 | 241      | GTKEEAEDVIPEGMNX | 15  | 1     | 690 |
| Phl p 1 | 249      | VIPFWKOADTAYESEK | 15  | 1     | 447 |
| Phl p 2 | 61       | EHKSDDEVAMVTKEGEGVMTF | 20  | 2     | 1,874 |
| Phl p 2 | 76       | GVMTFDSEELPLQGPF | 15     | 1     | 457 |
| Phl p 2 | 86       | LQGPNNFRLTEKMNKFFFVPEKYYTG | 30  | 4     | 280 |
| Phl p 3 | 1        | AVQVTFTVQKGDPKVLVIKTYRGPDSL | 30  | 5     | 3,037 |
| Phl p 3 | 41       | EEWIPEITKGMNVEW | 15     | 3     | 706 |
| Phl p 3 | 51       | NWFERNRSKPLGQPF | 15     | 5     | 1,950 |
| Phl p 3 | 61       | LVGPFPNRFMSKGMNNVDFEVPIPT | 25  | 4     | 913 |
| Phl p 4 | 191      | MLLKRYGIAAENVID | 15     | 1     | 140 |
| Phl p 4 | 221      | GIVVAMKVLPLVP | 15     | 1     | 53  |
| Phl p 4 | 321      | FVHLGHDNNIEDDON | 15     | 1     | 73  |
| Phl p 4 | 336      | NRRNTFKPFAEYKSDYWQYPFPFK | 23  | 2     | 2,610 |
| Phl p 4 | 356      | FPKEVNVQIIFSTWLL | 15     | 1     | 97  |
| Phl p 5a/b | 70 | INADFKAALAAAGAVVPPAFDY | 20  | 4     | 1,200 |
| Phl p 5b | 76       | PAAAPKFTPEAATPS | 15     | 1     | 160 |
| Phl p 5a | 76       | PKGGAESSKSAALT | 15     | 1     | 97  |
| Phl p 5b | 76       | TPEAKDFSFAQSLTE | 15     | 1     | 220 |
| Phl p 5a | 111      | KYDAVATLSEALRI | 15     | 1     | 2,047 |
| Phl p 5a | 126      | TSKLDAAAYKLAYTAA | 15     | 1     | 1,103 |
| Phl p 5b | 136      | ALRVIGALEVHAVK | 15     | 1     | 543 |
| Phl p 5a | 181      | VIPAGELOQVIEKDAFKNVA | 20  | 2     | 2,047 |
| Phl p 5a | 196      | AFKVAATAAANAPAN | 15     | 7     | 10,631 |
| Phl p 5a/b | 208 | PANDKPTVEFAQNDAIKE | 19  | 5     | 1,170 |
| Phl p 5a/b | 231 | AYESYKFIAPELAAVAKQYAATVA | 26  | 2     | 2,246 |
| Phl p 5a/b | 251 | ATVATAAPKVTVEFATKAIATAM | 26  | 4     | 3,011 |
| Phl p 5b | 251      | ITANSBQVKSQPAAT | 15     | 1     | 33  |
| Phl p 5b | 111      | RYANFP1AFRKEPLK | 15     | 1     | 927 |
| Phl p 12 | 26       | LGHDZTNPQADGDFP | 15     | 1     | 30  |
| Phl p 13 | 36       | KTDCCTKVEEAWASA | 15     | 1     | 110 |
| Phl p 13 | 96       | LAKYKANWIEIRIK | 15     | 2     | 260 |
| Phl p 13 | 126      | AVWIKGNCSKACNICK | 15     | 1     | 330 |

| Total | 86 | 45,863 |

Table II. Phl p antigenic regions identified in the Timothy grass-allergic donor cohort

aThe Phl p 5 allergen consists of two isoforms, Phl p 5a and 5b. Although the isoforms contain highly homologous regions, each is also unique enough over large stretches to be considered individually. Thus, representative sequences of both isoforms were used in the current study. In cases in which homologous epitopes were identified from both isoforms, the corresponding number of donors responding to the indicated Phl p region. The total reflects the entirety of donor/region responses recorded.

bIndicates the number of donors responding to the indicated Phl p region. The total reflects the entirety of donor/region responses recorded.
the HLA locus that restricted the response by the capacity of Abs specific for HLA-DR, DP, or DQ to inhibit the response. Locus restriction could be determined for 122 out of 138 (88%) of the recognized events analyzed. These accounted for ~95% of all SFC responses. For the remainder, either scarcity of cells, lack of a sufficiently strong response, or an inconclusive inhibition pattern precluded a locus definitive assignment. In terms of the locus distribution of those events, no event was found to be restricted by HLA class I molecules. Where the class II restricting locus could be determined, DR restricted 61% of the responses (49% of the total SFC response), DP 21% (35% of the total SFC response) and DQ 18% (16% of the total SFC response). As also discussed in more detail below, several antigenic regions were restricted by multiple loci.

Next, for each of the cases in which the restricting HLA locus could be assigned, we attempted to define the allelic molecule restricting the response. For this purpose, we used partially matched EBV lines or, as available, single HLA molecule-transfected fibroblasts. The results are detailed in Supplemental Table IV and summarized in Table V. These data represent, to the best of our knowledge, the first systematic characterization of the locus and allelic molecule restricting human T cells directed against a clinically relevant allergen. The implications of these results are discussed in more detail in the following sections.

**A few promiscuous antigenic regions account for the majority of the responses**

As described above, we identified a total of 108 different Phl p peptides that could induce significant cytokine responses in allergic donors. These 108 epitopes could be mapped to 43 different epitope/antigenic regions. This result underlines the significant heterogeneity of responses that exist in allergic individuals reactive to the Timothy grass extract. However, upon closer inspection of the data, several important observations can be made. First, within a given donor, few epitopes account for the majority of the response (immunodominance). Second, certain epitopes tend to be recognized in multiple individuals (immunoprevalence).

Indeed, when the fraction of the total response was calculated, by summing all SFCs directed against each antigenic region in each allergic donor, it was found that nine antigenic regions, each recognized in three or more donors, accounted for 51% of the total response (Table VI). Interestingly, these same nine regions accounted for 66% of the responses seen in nonallergic and SIT donors, further highlighting their dominance in Timothy grass-specific T cell responses. The 20 regions recognized in two or more donors accounted for 79% of the response. This immunodominance could be due to several different, non-mutually exclusive mechanisms. Specifically, multiple HLA types could restrict the same region, the restricting HLA type could be a frequent one, and/or the epitope could be immunodominant in terms of the magnitude of responses.

To address these issues, the patterns of restrictions associated with each antigenic region were investigated in more detail. Overall, as shown above, nine regions restricted by three or more different allergic donors accounted for about half of the responses in terms of magnitude. Interestingly, we found (Table V) that multiple HLA molecules and loci restricted all of these most dominant regions. On average, three different HLA molecules restricted each region. For six of the nine regions, multiple loci (DR/DP/DQ) were involved in their recognition. These data establish that a few antigenic regions account for the majority of the response. Even within a given locus, multiple HLA allelic variants restrict the most immunodominant epitopes. These data provide an insight into the molecular mechanisms involved in the dominance of these particular epitopes.

**HLA binding affinity of identified epitopes**

The data presented above define the HLA molecules restricting T cell responses directed against Timothy grass-specific epitopes. This allowed us to probe the relationship between binding affinity and recognition by Timothy grass-specific T cells and ask whether this relationship would be similar or different from that previously reported for epitopes of microbial origin. Accordingly, the HLA binding capacity of epitopes of known restriction, and for which a molecular assay was available, was determined next (Supplemental Table IV). Overall, an affinity threshold of 1000 nM accounts for ~70% of the determined restrictions (Fig. 3) and ~80% of the total SFC responses for which restriction was determined (data not shown). No significant difference was apparent between the HLA binding affinity thresholds for epitopes restricted by different loci or in terms of the cytokine associated with recognition (data not shown).
When these results were compared with the affinity values observed for a panel of 46 epitopes of microbial origin for which HLA class II restriction was known, as shown in Fig. 3, an affinity threshold of $\sim 250 \text{nM}$ was associated with $\sim 70\%$ of the determined restrictions of microbial epitopes, and 1000 nM accounts for $\sim 95\%$ of known HLA class II restrictions, as previously noted (47). Thus, the distribution of affinities of microbial epitopes is significantly different from that of the allergen-derived epitopes ($p = 0.03$ according to the Kolmogorov-Smirnov test), which are associated with a $\sim 4\times$ fold lower affinity.

To further investigate the relationship between binding capacity and recognition by allergen-specific T cells, we assembled a peptide-binding assay panel of 23 common HLA molecules, selected as representative of the main DR, DP, and DQ molecules expressed in the general population (Supplemental Table V). These molecules allow coverage of 75–98% of the general population at each locus. Each of the overlapping peptides tested for immunogenicity was tested for HLA binding capacity in molecular assays utilizing purified HLA molecules (the full data set is available at www.iedb.org). We found that $\sim 30.3\%$ ($n = 4782$) of the 15,801 possible Phl p peptide/HLA class II molecule binding events (23 different class II specificities times 687 Phl p peptides) were associated with a $\sim 1000 \text{nM}$ affinity.

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It follows from the data above that $\sim 70\%$ of the Phl p epitopes would be in the top 30th percentile of the binding peptides, which indicates, as expected, that epitopes have higher binding affinities than average peptides. In the comprehensive immunogenicity screen of the panel of 687 Phl p peptides, 108 (15.7%) peptides were identified as epitopes, yielding 183 unique donor/cytokine responses restricted by 31 different alleles. Given these rates, it is estimated that $\sim 1$ in 50 peptides with an affinity $\leq 1000 \text{nM}$ would be in the top 30th percentile of the binding peptides, which accounts for half of the total T cell response (SFCs).

Taken together, these data further support the restriction data presented above and also provide a biologically relevant affinity threshold to be used in subsequent analyses. Furthermore, these results also reveal a significant difference in HLA binding affinity of epitopes recognized in the context of infectious diseases and allergy. Finally, these data emphasize how HLA binding is by no means sufficient to ensure antigenicity for allergen-specific T cells.

### Prediction of promiscuous binding regions

Based on the data presented above, we hypothesized that promiscuous immunodominant epitopes might be predictable using bioinformatic methods based on their HLA binding characteristics. The advantage of this approach is that it would identify the optimal set of peptide candidates for immunogenicity testing, eliminating the necessity of synthesis of a large number of overlapping peptides and, more importantly, circumvent the need to test each one of them for binding to numerous HLA class II molecules in vitro. For this purpose, we generated updated versions of the prediction algorithms in Ref. 48 for our panel of different HLA molecules representative of the main DR, DP, and DQ types expressed in the general human population. To accurately access prediction performance in a blinded fashion, predictions were made in a 5-fold cross-validated format.

Using these predicted affinities, for each peptide, we recorded the number of HLA molecules for which the predicted binding affinity was in the top 20th percentile. It was found that two peptides were predicted to bind 19 of 23 alleles, whereas at the other extreme, 48 peptides were predicted to bind no allele. Using the number of bound alleles as the selection criteria, we recorded how many of the total SFCs would have been accounted for by selecting a given number of peptides (Fig. 4). Selecting the top 20th percentile of peptides, representing those predicted to bind $\geq 10$ alleles, would have accounted for half of the total T cell response (SFCs).

Thus, predictions of promiscuous binders identify about half of the T cell responses in individual donors. In light of the fact that our epitope set encompassed a peptide every five residues of sequence, threshold to be used in subsequent analyses. Furthermore, these results also reveal a significant difference in HLA binding affinity of epitopes recognized in the context of infectious diseases and allergy. Finally, these data emphasize how HLA binding is by no means sufficient to ensure antigenicity for allergen-specific T cells.

### Table III. Phl p antigenic regions identified in the non-allergic donor cohort

| Region Number | Region | Ag Position | Sequence | Length | Donors | SFC |
|---------------|--------|-------------|----------|--------|--------|-----|
| 34            | Phl p 5a | 196         | AFKVAATAAAAPAN | 15     | 1      | 207 |
| 35            | Phl p 5a | 206         | AAPANDKPTVFEAAF | 15     | 1      | 37  |
| 44            | Phl p 4  | 181         | GGNFAGGGFGMLRKR | 15     | 1      | 157 |
| 45            | Phl p 5a | 201         | KAICESTGGAYYTK | 15     | 1      | 40  |
| Total         |         |             |           |        | 4      | 441 |

See footnotes to Table II.

### Table IV. Phl p antigenic regions identified in the SIT donor cohort

| Region Number | Region | Ag Position | Sequence | Length | Donors | SFC |
|---------------|--------|-------------|----------|--------|--------|-----|
| 26            | Phl p 5a/5b | 70          | INAGFKAAVAAAAASVPAADK | 20     | 1      | 1904 |
| 29            | Phl p 5b   | 126         | FDSFVASLTEALRVI | 15     | 1      | 117  |
| 32            | Phl p 5a/5b | 131         | ASLITEALRVIAGALEVH | 17     | 1      | 163  |
| 34            | Phl p 5a/5b | 196         | AFKVAATAAAAPAN | 15     | 1      | 90   |
| 35            | Phl p 5a/5b | 176         | PTVFEAAFPNKIAEKG | 15     | 1      | 110  |
| 36            | Phl p 5a/5b | 231         | AYESYKFIPALEAAV | 15     | 2      | 583  |
| 37            | Phl p 5b   | 241         | AVFREA/LTKATAMS | 15     | 1      | 870  |
| 46            | Phl p 5a   | 298         | AATGAAATAAGGKYK | 15     | 1      | 30   |
| 47            | Phl p 6    | 6           | FMVAMFLAVAVVGL | 15     | 1      | 33   |
| Total         |         |             |           |        | 10     | 3900 |

See footnotes to Table II.
selecting the top 20% promiscuous peptides would require one peptide every 25 residues of sequence, thus allowing coverage of a 200-residue protein with eight synthetic 15-mer peptides.

Allergen immunodominance at the level of T cell responses

The peptide-based screens of Timothy grass-specific responses allow mapping, in a relatively unbiased fashion, of global patterns of reactivity and thus can be used to probe the mechanisms of immunodominance in T cell responses. Accordingly, we examined the responses in the allergic donors in terms of their Ag of provenance. We found a heavily skewed distribution with 51.5, 19, and 14% of responses in the allergic donors. Furthermore, using rPhl p 1, 2, 4, and 5 to stimulate PBMCs from allergic donors, the results confirmed that Phl p 1 and 5 were highly immunogenic for T cells derived from allergic patients, whereas Phl p 6 was only marginally immunogenic (data not shown). These results demonstrate that allergen abundance in the Timothy grass extract/pollen does not accurately predict relative reactivity for T cell responses.

Relationship between allergen specific IgE and IgG abundance and antigenicity for T cell responses

As an alternative explanation for why certain allergens were more important T cell Ags, we next considered whether allergen specific IgE levels were potential predictors of T cell reactivity. It has been frequently assumed that antigenicity for Ab responses, and in particular for IgE responses, might also be predictive of antigenicity for T cell responses. To test this assumption, for each donor, the RAST IgE levels directed against the available rPhl p Ags for this assay were measured (Phl p 1, 2, 4, 5, 6, 7, 11, and 12).

As shown in Fig. 5, no T cell response was measured in the case of Phl p 6 and 7, and only minor positive responses were detected to Phl p 11, 12, and 13. In contrast, highly positive IgE responses were detected against these same allergens in a large fraction of the allergic donors. Furthermore, using rPhl p 1, 2, 4, and 5 used to stimulate PBMCs from allergic donors. The results confirmed that Phl p 1 and 5 were highly immunogenic for T cells derived from allergic patients, whereas Phl p 6 was only marginally immunogenic (data not shown). These results demonstrate that allergen abundance in the Timothy grass extract/pollen does not accurately predict relative reactivity for T cell responses.

Table V. The dominant Phl p epitope regions are promiscuous

| Region Number | Phl p Region | Ag Position | Donors | Locus | Restricting Alleles |
|---------------|--------------|-------------|--------|-------|---------------------|
| 34            | Phl p 5a     | 196         | 7      | DP (6), DQ (2) | DPB1*0501, DPB1*1401 (3), DQB1*0301 (2) |
| 17            | Phl p 3      | 1           | 5      | DP (2), DQ, DR (3) | DPB1*0501, DPB1*1401, DQB1*0301, DRB1*0301 (2), DRB1*1101 (2), DRB1*1301, DRB3*0101 |
| 19            | Phl p 3      | 51          | 5      | DQ, DR (4) | DRB1*0801, DRB1*1101, DRB3*0202, DRB4*0103 |
| 35            | Phl p 5a/5b  | 208         | 5      | DP, DR (2) | DPB1*0501, DPB1*1401, DRB1*0101 |
| 16            | Phl p 2      | 86          | 4      | DQ, DR | DRB1*0201, DRB5*0101 |
| 20            | Phl p 3      | 61          | 4      | DQ, DR (2) | DRB1*1101, DRB1*1501 |
| 26            | Phl p 5a/5b  | 70          | 4      | DQ (2), DR (3) | DQB1*0501 (2), DRB1*1501, DRB3*0101, DRB5*0101 |
| 37            | Phl p 5a/5b  | 251         | 4      | DP (2), DR (2) | DPB1*0402, DRB1*0701 |
| 18            | Phl p 3      | 41          | 3      | DR (3) | DRB1*0801, DRB1*1101 |

*If more than one donor used the indicated locus or allele, the number of donors is shown in parentheses.

Table VI. Nine antigenic regions account for about half of the Phl p response

| Number of Positive Donors | Number of Regions | Total Spots (Region) | Fraction of Total Spots | Cumulative Fraction of Spots |
|---------------------------|-------------------|----------------------|-------------------------|-----------------------------|
| 7                         | 1                 | 10,631               | 0.232                   | 0.232                       |
| 5                         | 3                 | 6,157                | 0.134                   | 0.366                       |
| 4                         | 4                 | 5,951                | 0.130                   | 0.496                       |
| 3                         | 1                 | 706                  | 0.015                   | 0.511                       |
| 2                         | 11                | 12,750               | 0.278                   | 0.789                       |
| 1                         | 23                | 9,667                | 0.211                   | 1.000                       |
| Total                     | 43                | 45,863               |                         |                             |

Bold font highlights the totals for the nine most frequently recognized regions.
allergens, we also examined at the level of individual donors whether a correlation existed between the IgE amount and T cell antigenicity as detected by the epitope screen (Fig. 6). It is apparent that very little correlation exists, and many donors for whom no T cell reactivity against a given allergen was detected had highly positive IgE responses. Conversely, several instances of vigorous T cell reactivity in the absence of detectable allergen specific IgE were also observed.

Considering the reactivity of each allergic donor against a given allergen as an individual data point, out of 104 donor/allergen combinations in which significant IgE was detected, 66 were associated with no significant T cell responses directed against the same allergen. Conversely, of 136 donor/allergen combinations in which no significant IgE was detected, 14 were associated with significant T cell responses directed against the same allergen. This association, albeit significant ($p < 0.01$ by Fisher test), is far from being deterministic. When allergen specific IgG levels were analyzed employing a similar approach, no significant correlation was detectable. In conclusion, these data illustrate that there is little or no correlation between IgE levels against the Phl p allergens and the antigenicity of these same allergens for T cells as measured by SFC reactivity.

Differential patterns of cytokine production are associated with Phl p allergens

The data presented above demonstrate that certain Ags are immunodominant for T cell responses. Although Ag abundance and IgE levels might factor in the prediction of immunodominance, neither of them appears to satisfactorily predict T cell immunodominance at the individual allergen level. This suggests that certain intrinsic features of the allergen protein might influence its immunogenicity and prominence at the level of T cell reactivity.

Consistent with this notion, when the pattern of cytokine production in allergic donors across the various Ags was scrutinized (Fig. 7), it was found that certain Ags were associated exclusively with IL-5 production (Phl p 1 and 11), whereas others (Phl p 4 and 5) were associated with production of all four cytokines tested. In contrast, production of IL-10 and IL-17 was limited to epitopes derived from these two allergens. The Phl p 2, 3, and 13 Ags were associated with production of both IL-5 and IFN-γ, but not IL-10 or IL-17.
Discussion

In this study, we report an in-depth analysis of the molecular determinants recognized by T cell responses specific for common Timothy grass Phl p allergens. Our comprehensive approach used overlapping peptides spanning 10 different IgE-reactive Phl p allergens to examine production of cytokines representative of Th1, Th2, Th17, and Treg cell subsets in allergic individuals, patients undergoing SIT, and nonallergic controls. These studies identified a total of 43 different epitopic regions recognized in allergic individuals. Thus, these results underline the great breadth and complexity of Timothy grass allergen responses in humans and are consistent with and significantly extend previous analyses (54).

Some of the antigenic regions identified in this study overlap with regions previously described in the literature. In particular, Muller et al. (54) described three main Phl p 5 regions, 16–48, 133–150, and 181–207, according to their numbering system. These regions roughly correspond to the regions 33/34, 36/37, and 26/27/28 in our study, respectively.

In allergic donors, it was found that, as expected, Th2 cytokines, such as IL-5, were most prevalent responses. This cytokine was measured as representative of Th2 responses, rather than IL-4 or IL-13, because in our experience IL-5 is the Th2 cytokine detected with the most sensitivity and reliability by ELISPOT assays. Through intracellular cytokine staining, we confirmed that Timothy grass-specific production of IL-5 correlated well with concurrent IL-13 production in allergic donors (data not shown).

In our hands, primary ex vivo detection of lymphokine production yielded weak signals, and an in vitro restimulation step was necessary to obtain a signal sufficiently robust to characterize responses in detail. Other studies have reported success using overlapping peptides to directly define cytokine production (55–57). The reason for this apparent discrepancy is not clear but might perhaps reflect differences in the specific allergens investigated.

Clearly, there is potential that some specificities respond differently to the 17-d expansion protocol used. However, the signal observed directly ex vivo was too low to allow reproducible deconvolutions. Furthermore, the epitopes identified after the 17-d extract expansion were also recognized following the shorter (1 wk) peptide stimulations used for the restriction experiments. Nevertheless, our data should be interpreted with this caveat in mind. Similarly, all of our data were generated utilizing commercially available Timothy grass extracts. It is possible that there are differences in epitope-specific expansion between stimulation with extract as compared to, for example, recombinant protein.

In our study, we have not measured the percent of Tregs present, either at the onset or at the end of the restimulation step. Because we merely measured the production of IL-10, one lymphokine associated with one type of Tregs, no conclusion can be drawn regarding a lack of Tregs in general or their absence under the culture conditions used.

A large number of grass species exist that cross-react extensively. It is possible that at least some of the subjects studied were primarily sensitized by exposure to other grasses. To address this issue, we have examined the skin test results of our donor cohort. All of our donors were tested for reactivity against a panel of 32 common allergens including 8 grasses: Bermuda, Canary, Kentucky, Orchard, Rye, Sweet Vernal, Timothy, and Wheat grass. In all cases, each Timothy grass-allergic individual also had >3 mm wheal response to at least two other grasses, and 68% of these donors were allergic to all eight grasses. Wheal responses to other grasses were similar in size to those measured for Timothy grass. Thus, the origin of the grass allergens that sensitized our allergic donor population cannot be definitively established.

We found that SIT donors were associated with lower and less frequent IL-5 responses and virtually absent IL-17, IFN-γ, or IL-10 production. These data are not consistent with SIT inducing IL-10–producing Tregs as an immunomodulatory mechanism, but rather suggest that SIT is associated with a generalized downregulation of Timothy grass reactivity, possibly as a result of induction of T cell anergy/deletion of allergen–specific T cells or induction of Tregs not associated with IL-10 production. However, these conclusions must be considered preliminary, as Tregs may not have expanded in vitro following the conditions we employed for Timothy grass restimulation, although direct ex vivo experiments also failed to detect production of IL-10, or any other of the cytokines measured, from either allergic or SIT donors. Our results and conclusions are at variance with a report by Akdis et al. (24), in which bee venom SIT patients were associated with IL-10 production. It is possible that this apparent contradiction might be explained by the different allergen studied and/or by the different route and doses of allergen exposure. By contrast, our results support the notion that SIT patients are associated with significant increases in levels of allergen specific IgG, thus supporting the hypothesis that SIT therapy might be associated with competitive inhibition of allergen binding to IgE (58).

FIGURE 7. Cytokine response patterns vary against different Phl p allergens. The total response (SFC) specific for each cytokine and Phl p Ag was tabulated. The Phl p 1 and 11 Ags were associated with exclusive IL-5 production, whereas Phl p 4 and 5 were associated with production of all four cytokines tested. The Phl p 2, 3, and 13 Ags were associated with production of both IL-5 and IFN-γ.
Likewise, nonallergic individuals were essentially nonreactive. These results are at variance with the results of earlier data utilizing proliferation assays (59, 60). It is likely that ELISPOT assays might be a more reliable quantitative cellular assay for detecting specific responses associated with allergic responses than proliferation assays. It is also possible that a significant Th0 response by nonallergic individuals would go undetected by the ELISPOT analyses we performed but could be detected in proliferation assays. Finally, when responses from nonallergic and SIT donors were compared with the responses observed in allergic donors, it appears that similar epitopic regions are recognized. These results are consistent with previous reports in other allergens, such as those from Ebner et al. (7, 61) in the birch pollen system. This observation suggests that it is unlikely that differential recognition of epitopes derived from the main known Timothy grass allergens could be used as the basis for diagnostic or immunotherapy purposes in the case of Timothy grass.

Our experimental approach precisely mapped the MHC class, locus, and allele restricting the T cell responses in the majority of cases. Not surprisingly, all responses were class II restricted. In terms of the specific loci, DR was the most prevalent restriction element, followed by DP, with DQ a more distant third. This represents, to the best of our knowledge, the first in-depth analysis of the relative role of the three different loci in restricting allergen responses. In practical terms, these data suggest that, although as expected, DR is predominant, DP also restricts a significant fraction of the Timothy grass responses, and its contribution should not be overlooked in future studies.

It is possible that the in vitro culture conditions we used might be responsible for the skewed restriction pattern toward DR molecules that are expressed more abundantly than DP or DQ molecules. Even so, our study highlights the important contribution that DQ and DP molecules make as restriction elements for Timothy grass responses.

Because our comprehensive approach mapped responses independent of bioinformatic predictions, restriction locus, or phenotype of responding T cells, it allowed us to establish that the majority of responses (>50%) observed in allergic individuals can be mapped to nine different epitopic regions. This observation has relevance for potential peptide-based immunotherapies, as it suggests that meaningful interventions could be designed based on a limited number of synthetic peptides.

What is the mechanism that determines and selects for the antigenic regions that dominate the response? To gain insights into this question, we considered two alternative hypotheses. First, it is possible that each of the regions is restricted by a specific HLA molecule, perhaps on the basis of binding affinity. Alternatively, these prevalently recognized regions could correspond to hot spots presented by multiple HLA molecules. The binding and restriction data clearly demonstrated that the second hypothesis is likely to be correct. This is further underlined by the fact that three of the nine regions are actually contiguous or overlapping with each other or with other less frequently recognized regions. The exact molecular mechanisms selecting for these hot spots is currently unclear, but it has been hypothesized that they might reflect processing propensity preferentially generating certain protein fragments.

The determination of the specific allelic variants restricting the responses allowed us to also measure the levels of HLA binding associated with allergic epitopes. We demonstrate a clear difference when the HLA binding affinity of allergic epitopes was compared with the binding of class II-restricted epitopes of microbial origin. The weaker binding of allergen-derived epitopes had been hypothesized, but until now had never been conclusively demonstrated. Furthermore, the data show that only 1 out of 50 peptides binding to a given HLA molecule with an affinity of 1000 nM is recognized as an epitope. These results emphasize that although HLA binding is an important factor, it is not by itself sufficient for recognition by allergen-specific T cells.

Based on the observation that promiscuous recognition is associated with the more prevalent epitopes, we further showed that promiscuous HLA-binding capacity could be used to identify and predict a subset of epitopes associated with a large fraction of responses. We anticipate that the results of this study will greatly enhance our molecular knowledge of the targets of allergic T cell responses.

Predicted promiscuous binding can help in predicting allergen reactivity. However, although binding to HLA is an important factor, on the basis of the present data, it is also clearly not the only factor determining responses. Thus, in this context, a bioinformatics approach should be limited to selection of likely candidates, and experimental testing will still be necessary.

As neither epitope binding, allergen abundance, nor IgE reactivity by themselves alone appear to predict T cell dominance, additional factors need to be considered. For example, dominance may be a function of the nature and composition of the T cell repertoire recognizing the various epitopes, as well as the particular history of exposure to the allergen and related species. It is possible that predictive schema simultaneously incorporating all these factors will be successful in predicting allergen immunodominance.

In terms of the specific Ags targeted by Timothy grass-specific T cell responses, not all Phl p allergens studied were equally recognized. It is known that, albeit variable, the most abundant proteins in the pollen extract are the Phl p 1, 2, 3, 5, and 6 (49, 50, 52). Most of these proteins (but not Phl p 2 and 6) were also among the most abundantly recognized by T cells in our study. The Phl p 6 allergen is abundant in Timothy grass extracts in general (53) and in the specific Timothy grass extract used in our experiments (A. Petersen, unpublished observation). Phl p 4, 7, and 13 Ags are known to be less abundant in allergen extracts (62). Although Phl p 7 and 13 were not recognized in our assays, Phl p 4 was recognized at levels similar to those noted for the much more abundant Phl p 2 allergen. Thus, although the hierarchy of recognition of the various allergens seems to correlate with their abundance, this correlation is far from being absolute or predictive of T cell reactivity.

Interestingly, the areas of Phl 5 that appear to be most dominant are also the areas that are least variable among the various isolates, as well as between the a and b variants. Future studies will address this point in more depth, but on the basis of these observations, it is possible that the T cell reactivity against Phl 5 might be even greater than detected by the current study.

In our studies, we measured IgE concentrations against most of the allergens also investigated at the level of T cell responses. This allowed us to critically test the assumption that the allergens associated with the highest IgE titers are also the ones most antigenic for Ag-specific T cells. This assumption proved to be incorrect. Most notably, in many cases, we were able to detect strong T cell responses in the absence of detectable IgE. The poor correlation between IgE Abs to specific Timothy grass allergens and T cell responses is surprising, because clearly, IgE Ab responses are fully T cell dependent. This finding may reflect methodological issues. Alternatively, it is possible that T cell specificities directed toward other pollen-derived proteins could provide help for the IgE responses against some Timothy grass allergens.

Another important difference between IgE reactivity and the reactivity at the T cell level is that, in contrast with the well-known cross-reactivity between certain allergens at the Ab level, little or no cross-reactivity could be observed at the level of T cell epitopes, with the exception of the Phl p 5a and 5b allergens, which cross-reacted as expected on the basis of the extensive sequence identity. These findings have potentially profound implications in terms of
identification of T cell Ags. This is because the proteins available for the T cell epitope mapping were originally identified and characterized on the basis of IgE reactivity. Thus, our results suggest important targets of T cell reactivity in Timothy grass might remain to be discovered. This notion is also consistent with the fact that although SIT treatment was associated with impressive increases in allergen specific IgG, the T cell responses against the main allergens were actually decreased in SIT donors. In conclusion, our data are consistent with the possibility that SIT therapy might be associated with a switch or modulation in the type of Timothy grass proteins recognized.

We observed that different allergens are apparently associated with distinct patterns of cytokine production. This finding might be relevant for our understanding of the mechanisms of allergen responses and might have further influence on the allergen spectrum recognized by patients. It has been recently suggested that allergens might have TLR signaling capacities (63, 64) and that different allergens might provide differential signals leading to the generation of different patterns of cytokine production. In fact, it has been recently shown that different fungal components of the common mold Aspergillus fumigatus differentially activate distinct Th cell subsets (65). Fungal membrane proteins activate Th1 cells and Tregs, whereas secreted proteins induce Th2 cell activation and glycolipid Th17 (65). In any case, our data suggest that SIT treatments specifically targeting allergen proteins lacking, or less prone to induce Th2 responses, might be a feasible strategy to improve SIT efficacy.

The present study identifies several epitopes and associated restriction elements. It will now be possible to use these epitopes for the production of tetrameric reagents and conduct more in-depth studies. For example, tetramers could be used prior to and following invitrocultureandexpansiontodeterminewhethersomelevelofbias is introduced.

In conclusion, our studies shed light on the mechanisms involved in establishing which particular peptide regions within a given allergen are most dominantly recognized by T cell responses and also establish bioinformatic methods to aid in the prediction of such sequences. Furthermore, our study probes the mechanism involved in determining which particular allergen proteins are dominantly recognized. Surprisingly, neither allergen abundance nor IgE reactivity satisfactorily predict dominance in T cell recognition, suggesting that further studies might be needed to investigate in detail which factor(s) determine dominant recognition at the T cell level for a given allergen protein.

Acknowledgments
We thank Louis Huynh, Carrie Moore, Sandy Ngo, and Amiyah Steen for performing the MHC binding assays and Jean Glenn for assistance with the cellular assays. We also thank Hilda Grey, Deborah Broide, and Linda Bannister for clinical coordination and processing of donor samples.

Disclosures
A preliminary patent has been filed on the identification of the allergen-specific T cell epitopes.

References
1. Romagnani, S. 1994. Regulation of the development of type 2 helper cells in allergy. Curr. Opin. Immunol. 6: 838–846. 5. Del Prete, G. F., M. De Carli, M. M. D’Elia, P. Maestrelli, M. Ricci, L. Fabbrini, and S. Romagnani. 1993. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. Eur. J. Immunol. 23: 1445–1449.
6. Jutel, M., W. J. Pichler, D. Skrbic, A. Uwerylz, C. Dahindin, and U. R. Muller. 1995. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J. Immunol. 154: 4187–4194.
7. Ebner, C., U. Siemann, B. Bohle, M. Willheim, U. Wierdemann, S. Schenk, F. Klotz, H. Ebner, D. Kraft, and O. Scheiner. 1997. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from Th2 to Th1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin. Exp. Allergy 27: 1007–1015.
8. Francis, J. N., S. J. Till, and S. R. Durham. 2003. Induction of IL-10+CD4+ CD25+ T cells by grass pollen immunotherapy. J. Allergy Clin. Immunol. 111: 1255–1261.
9. Till, A. S., Walker, R. Dickason, D. Huston, F. O’Brien, J. Lamb, A. B. Kay, C. Corriigan, and S. Durham. 1997. IL-5 production by allergen-stimulated T cells following grass pollen immunotherapy for seasonal allergic rhinitis. Clin. Exp. Immunol. 110: 114–121.
10. Wachholz, P. A., K. T. Nouri-Aria, A. R. Wilson, S. M. Walker, M. Verhoef, S. J. Till, and S. R. Durham. 2002. Grass pollen immunotherapy for hayfever is associated with increases in local nasal but not peripheral Th1:Th2 cytokine ratios. Immunology 105: 56–62.
11. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. 4: 330–336.
12. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299: 1057–1061.
13. Khattri, R., T. I., S. A. Yosayko, and D. Macchia. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat. Immunol. 4: 337–342.
14. Ling, E. M., T. Smith, X. D. Nguyen, C. Pridgeon, M. Hallman, J. Arbery, V. A. Carr, and D. S. Robinson. 2004. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 363: 608–615.
15. Shi, H. Z., and X. J. Qin. 2005. CD4CD25 regulatory T lymphocytes in allergy and asthma. Allergy 60: 986–995.
16. Chen, T. V., K. Kochroo, J. Irobe, D. A. Hafer, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265: 1237–1240.
17. Bacchetta, R., M. Bigler, J. L. Touraine, R. Parkman, P. A. Tovo, J. Abrams, R. de Waal Malefyt, J. E. de Vries, and M. G. Roncarolo. 1994. High levels of interleukin-10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. J. Exp. Med. 179: 493–502.
18. Groux, H., A. O’Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T responses and prevents colitis. Nature 389: 737–742.
19. Hawrylowicz, C. M., and A. O’Garra. 2005. Potential role of interleukin-10- secreting regulatory T cells in allergy and asthma. Nat. Rev. Immunol. 5: 271–283.
20. Levingas, M. K., R. Sangregorio, F. Galbiati, S. Squadrono, R. de Waal Malefyt, and M. G. Roncarolo. 2001. IFN-alpha and IL-10 induce the differentiation of human type 1 regulatory T cells. J. Immunol. 166: 5530–5539.
21. Sundstedt, A., E. J. O’Neill, S. K. Nicolson, and D. C. Wrath. 2003. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. J. Immunol. 170: 1240–1248.
22. Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Caragianiadis, R.rameri, S. Thumberg, G. Denuz, R. Valenta, H. Fiebig, et al. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J. Exp. Med. 199: 1567–1575.
23. Heaton, T., J. Rowe, S. Turner, R. C. Aalberse, N. de Klerk, D. Suryaarachchi, M. Serralha, B. J. Holt, E. Hollams, S. Verkovich, et al. 2005. An immunoprotective immunological approach to asthma: identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children. Lancet 365: 142–149.
24. Akdis, C. A., T. Blesken, M. Akdis, B. Wuthrich, and K. Blaser. 1998. Role of interleukin 10 in specific immunotherapy. J. Clin. Invest. 102: 98–106.
25. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, H. Y. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol. 6: 1133–1141.
26. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. 6: 1123–1132.
27. Ammassariato, P. L., C. Comsi, V. Santarasci, L. Maggi, F. Liotta, B. Mazzinghi, A. Parente, L. Filı`, S. Ferri, F. Frosali, et al. 2007. Phenotypic and functional features of human Th17 cells. J. Exp. Med. 204: 1849–1861.
28. Brucklacher-Waldert, V., K. Steinbach, M. Lizoznov, M. Kolster, C. Hölscher, and E. Tofol. 2009. Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. J. Immunol. 183: 5494–5501.
29. Molet, S., Q. Hamid, F. Davoine, E. Nukui, R. Taha, N. Pagé, R. Olivestein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J. Allergy Clin. Immunol. 108: 430–438.

30. Vrtala, S., W. R. Sperr, I. Reimitzer, R. van Ree, S. Laffer, W. D. Müller, R. Ronquillo, A. Varma, S. G. Deeks, J. M. McCune, D. F. Nixon, and W. M. Becker. 2006. Phl p 3: structural and functional properties. J. Allergy Clin. Immunol. 118: 307–314.

31. Karlsson, A. C., J. N. Martin, S. R. Younger, B. M. Bredt, L. Epling, Z. Szépfalusi, O. Scheiner, and D. Kraft. 1995. Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. J. Immunol. Methods 187: 87–96.

32. Tassignon, J., W. Burny, S. Dahmani, L. Zhou, P. Stordeur, B. Byl, and D. De Groote. 2005. Monitoring of cellular responses after vaccination against tetanus toxoid: comparison of the measurement of IFN-gamma production by ELISA, ELISPOT, flow cytometry and real-time PCR. J. Immunol. Methods 305: 188–198.

33. Zába, D., P. Vágó, Z. Szelepessy, B. Trócsányi, and I. Miklós. 2009. Immune response to the major allergens of timothy grass (Phleum pratense) pollen. J. Immunol. Methods 346: 90–97.

34. Lam, D., N. Ng, S. Lee, G. Batzer, and A. A. Horner. 2008. Airway house dust extract exposures modify allergen-induced airway hyperresponsiveness by TLR4-dependent and independent pathways. J. Immunol. 181: 2925–2932.

35. Bozza, S., C. Clavaud, G. Giovannini, T. Fontaine, A. Beaussais, J. Sarfati, C. D’Angelo, K. Perruccio, P. Bonifazi, S. Zagarella, et al. 2009. Immune sensing of Aspergillus fumigatus proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. J. Immunol. 183: 2407–2414.