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

Carla Oseroff,* John Sidney,* Maya F. Kotturi,* Ravi Kolla,† Rafeul Alam,* David H. Broide,‡ Stephen I. Wasserman,§ Daniela Weiskopf,* Denise M. McKinney,* Jo L. Chung,* Arnd Petersen,§ Howard Grey,* Bjoern Peters,* and Alessandro Sette*

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: 943–955.

It is generally recognized that T cells play a central role in the pathogenesis of allergic diseases. One of the initial events in the development of allergic disease is the generation of CD4+ Th cells. Under the influence of IL-4, naive T cells differentiate into Th2 cells (1, 2), which produce cytokines essential in the pathogenesis of allergy. The importance of Th2 cells is underlined by studies that compared the Ag-specific T cell phenotypes from allergic and nonallergic individuals. Although allergen-specific T cell clones from nonatopic individuals were mostly associated with a Th1/Th0 phenotype, high proportions of Th2 clones were obtained from allergic individuals (3–5). Furthermore, some previous reports have shown that specific immunotherapy treatment (SIT) shifts the allergic Th2 response toward a nonallergic Th1 response (6, 7), although other reports have not supported this conclusion (8–10).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: Phl p, *Phleum pratense* allergen; RAST, radioallergosorbent test; SFC, spot-forming cell; SI, stimulation index; SIT, specific immunotherapy treatment; Treg, regulatory T cell.
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.

**Table 1. Study donor cohort**

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*Wheat response to Timothy grass extract in millimeters.  RAST response to Timothy grass extract in kilounits per liter. A dash indicates <0.1 kilounits per liter. –, Wheat <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 y 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.go.jp/ADFS), Food Allergy Research and Resource Program (www.farrp.org), Allergen Nomenclature (www.allergen.org/Allergen.aspx), Allergome (www.allergome.org), Swissprot (www.expasy.ch/sprot), Structural Database of Allergenic Proteins (http://ferma.utmhb.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 allergic 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 disproportionate 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 labels 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 of protease inhibitors. Following a 2–4-d incubation, the percent of MHC-pulsed EBV cell lines and fibroblasts used.

**PBMC isolation and HLA typing**

PBMCs were obtained by density gradient centrifugation (Ficoll-Hypaque, Amerham 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 radioallergosorbent test (RAST) IgE and IgG analyses (Phadia, Uppsala, Sweden; performed at National Jewish Medical Research Center, Denver, CO).

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

Serum was collected from each donor as described above. IgE titers specific to Timothy grass extract, rPhl p 1, 2, 3, 4, 5, 6, 7, 11, and 12 were performed by the National Jewish Medical Research Center using ImmunoCAPs (Phadia). rPhl p 3 was provided by Dr. Arnd Petersen (Borstel, Germany), and custom ImmunoCAPs were prepared by Phadia. rPhl p 3-specific IgE and all Ag-specific total IgG and IgG4 assays were performed by Phadia.

**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 (Celigro, Herndon, VA) at a density of 2 x 10^6 cells/ml in 24-well plates (BD Biosciences, San Jose, CA) and stimulated with Timothy grass pollen extract (50 µg/ml), rPhl p proteins (10 µg/ml) (Greer, Lenoir, NC), or individual peptides. Cells were kept at 37°C, 5% CO2, and additional IL-2 (10 U/ml), IL-10, 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

**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

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; Matibech, Cincinnati, OH) and anti-human IFN-γ (clone 1-D1K; Matibech) or 10 µg/ml anti-human IL-10 (clone 9D7; Matibech) and anti-human IL-17 (clone 14G7187; Bioscience). Cells were then incubated at a density of 1 x 10^5/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-age of DMSO in the pools/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 (Matibech) and FITC-conjugated anti-human IFN-γ Ab (Matibech) or 2 µg/ml biotinylated anti-human IL-10 Ab (Matibech) and FITC-conjugated anti-human IL-17 Ab (Bioscience) 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-amin-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-ELISPOT 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)/10^5 PBMC, p < 0.05, and a stimulation index (SI) ≥ 2, whereas criteria for individual peptide positivity were ≤ 20 SFC/10^5 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 (SVFL3) 30 min prior to peptide stimulation. Cytokine production in response to positive peptides was then measured in ELISPOT assays as described above. The pan HMC 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 epitope peptide and were then subsequentially 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.
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 100 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 epitopic 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 donors.
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 IL-5 responses, virtually absent IFN-γ responses, and no IL-17 or TH1- to TH1-type response. Rather, these data suggest that SIT is associated with an overall downregulation of the response against a Th2- to Th1-type response. Furthermore, our data are not consistent with SIT inducing IL-10 production against the main allergens investigated. There-fore, nonallergic individuals had minimal T cell responses recorded.

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

<table>
<thead>
<tr>
<th>Region Number</th>
<th>Phl p</th>
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<th>Length</th>
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<td>260</td>
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</table>

$^a$The 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 region is identified as Phl p 5a/b.

$^b$Indicates the number of donors responding to the indicated Phl p region. The total reflects the entirety of donor/region responses recorded.

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 significant IgE and IgG titers. In contrast, nonallergic donors demonstrated higher IgG levels, but no detectable allergen specific IgE, as might be expected. SIT donors had lower allergen specific IgE than the allergic cohort and dramatically higher allergen specific IgG levels.

**HLA restriction of identified epitopes**

To more fully characterize the responses associated with nonallergic and SIT donors, we also measured the RAST levels for both IgE and IgG and compared them to the measurements in allergic donors (Fig. 2C). Allergic individuals were associated with significant IgE and IgG titers. In contrast, nonallergic donors demonstrated higher IgG levels, but no detectable allergen specific IgE, as might be expected. SIT donors had lower allergen specific IgE than the allergic cohort and dramatically higher allergen specific IgG levels.

Taken together, nonallergic individuals had minimal T cell responses. SIT donors were associated with lower and less frequent IL-5 responses, virtually absent IFN-γ responses, and no IL-17 or IL-10 production against the main allergens investigated. Therefore, our data are not consistent with SIT inducing IL-10 producing Tregs as an immunomodulatory mechanism nor a switch from a Th2- to Th1-type response. Rather, these data suggest that SIT is associated with an overall downregulation of the response against these allergens by an as yet unknown mechanism.

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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 recognition 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 cell responses 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 ∼ 250 nM was associated with ∼ 70% of the determined restrictions of microbial epitopes, and 1000 nM accounts for ∼ 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 ∼ 4-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 ∼ 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 an affinity of ≥ 1000 nM.

It follows from the data above that ∼ 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 representing those predicted to bind HLA class II restriction was known, as shown in Fig. 3, an affinity threshold of ∼ 250 nM was associated with ∼ 70% of the determined restrictions of microbial epitopes, and 1000 nM accounts for ∼ 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 ∼ 4-fold lower affinity.

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It follows from the data above that ∼ 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 ∼ 1 in 50 peptides with an affinity ≥ 1000 nM would be recognized by T cells.

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 ≥ 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,
Nine antigenic regions account for about half of the Phl p response.

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

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<td>45,863</td>
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</tr>
</tbody>
</table>

Bold font highlights the totals for the nine most frequently recognized regions.

used to stimulate PBMCs from allergic donors. The results confirmed that Phl p 1 and 5 were highly immunogenic for allergic donors, 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 to

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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 in terms of their Ag of provenance.

Low 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. 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 to stimulate PBMCs from allergic donors. The results confirmed that Phl p 1 and 5 were highly immunogenic for allergic donors, 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.
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.
production of both IL-5 and IFN-γ, and 13 Ags were associated with all four cytokines tested. The Phl p 2, 5 were associated with production of IL-5 production, whereas Phl p 4 and Ags were associated with exclusive Ag was tabulated. The Phl p 1 and 11 specific for each cytokine and Phl p 11 allergens. The total response (SFC) patterns vary against different Phl p allergens 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 during SIT. Thus, the 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).
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 schemes 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 reactivity. However, although binding to HLA is an important factor, it is not by itself sufficient for recognition by allergen-specific T cells.
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 the production of tetrameric reagents and conduct more in-depth 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 the production of tetrameric reagents and conduct more in-depth 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 the production of tetrameric reagents and conduct more in-depth restriction elements. 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It will now be possible to use these epitopes for the production of tetrameric reagents and conduct more in-depth studies. For example, tetramer...


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Supplemental Table I. Phl p peptide responses identified in the timothy grass allergic donor cohort.

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**Supplemental Table II. Phl p peptide responses identified in the non-allergic donor cohort.**

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**Supplemental Table III. Phl p peptide responses identified in the SIT donor cohort.**

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Supplemental Table V. Representative HLA molecules used for binding assays.

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* Phenotypic frequencies are based on data available at dbMHC for 11 major population groups, to include Australia, Europe, North Africa, North America, North-East Asia, Oceania, Other, South America, South-East Asia, South-West Asia, and Sub-Saharan Africa. Frequency data are not available for DRB3/4/5 alleles and antigens. However, because of linkage with DRB1 alleles, coverage for these specificities at the antigen level may be assumed as follows: DRB3 with DR3, DR11, DR12, DR13 and DR14; DRB4 with DR4, DR7 and DR9; DRB5 with DR15 and DR16. For alleles sharing the same serological antigen (DR4: DRB1*0401 and DRB1*0405; DQ3: DQB1*0301 and DQB1*0302; DPB1*0401 and DPB1*0402), the antigen frequency has been noted only under the first allele.