

# Allergen-Specific MHC Class II Tetramer<sup>+</sup> Cells Are Detectable in Allergic, but Not in Nonallergic, Individuals<sup>1</sup>

Claudia Macaubas,<sup>2\*</sup> Jan Wahlstrom,<sup>2\*</sup> Ana Paula Galvão da Silva,<sup>\*</sup> Thomas G. Forsthuber,<sup>†</sup> Grete Sønderstrup,<sup>‡</sup> William W. Kwok,<sup>§</sup> Rosemarie H. DeKruyff,<sup>¶</sup> and Dale T. Umetsu<sup>3¶</sup>

Allergen-specific cells are present in very low frequency in peripheral blood of humans, and differ in function in allergic and nonallergic individuals. We report in this study that soluble class II MHC tetramers can be used to directly identify and study such allergen epitope-specific CD4<sup>+</sup> T cells in humans. We identified the major antigenic epitope of rye grass allergen *Lol p 1* in HLA-DRB1\*0401 individuals using HLA-DR\*0401 transgenic mice and peripheral blood cells from HLA-DR\*0401 individuals. Using DRB1\*0401 tetramers loaded with this major epitope of *Lol p 1*, we detected allergen-specific CD4<sup>+</sup> T cells in the peripheral blood of DRB1\*0401 rye grass allergic individuals after ex vivo expansion with allergen. These tetramer-positive cells produced IL-4, but little IFN- $\gamma$ . In contrast, we were unable to detect rye grass tetramer-positive cells in cultures from HLA-DR\*0401 nonallergic individuals, even after expansion with IL-2. Thus, our results suggest that rye grass allergen-specific T cells in DR\*0401 nonallergic subjects are present at very low levels (e.g., because of deletion or suppression), differ in a fundamental way in their requirement for ex vivo expansion (e.g., they may be anergic), or use TCRs distinct from those of allergic individuals. Thus, analysis using DRB1\*0401 tetramers loaded with a major epitope of *Lol p 1* indicates that allergen-specific CD4<sup>+</sup> T cells in nonallergic individuals are distinct from those in allergic subjects. *The Journal of Immunology*, 2006, 176: 5069–5077.

Allergic diseases affect 25–40% of the general population in developed countries and have increased dramatically in prevalence over the past 2 decades (1). Allergic responses are characterized by the development of inappropriate immune responses to common environmental allergens, such as grass pollens, house dust mites, and animal dander, and are associated with allergen-specific Th2 CD4<sup>+</sup> Th cells producing IL-4, IL-5, and IL-13 (2). CD4<sup>+</sup> T cells are of fundamental importance in allergic diseases, regulating the production of IgE and the effector function of mast cells and eosinophils. Furthermore, depletion of CD4<sup>+</sup> T cells in animal models prevents the development of experimental asthma (3). In humans, the presence of CD4<sup>+</sup> Th2 cells correlates with disease severity in asthma (4, 5), suggesting that CD4<sup>+</sup> Th2 cells play a critical role in atopic diseases, including allergy and asthma.

Although allergic individuals develop Th2-dominant immunity, nonallergic individuals exposed to the same allergens develop immune responses that protect against the development of allergic symptoms. The specific nature of these protective responses is still largely unknown, and diverse mechanisms, such as Th1 cells (6) or IL-10-dependent regulatory T cell responses (7), have been pro-

posed to explain this protection. Studies of mouse models using Ag-specific TCR transgenic T cells suggest that allergic and nonallergic individuals might respond to inhaled allergens by recognizing identical antigenic epitopes of these allergens. These studies suggest that T cells of allergic and nonallergic individuals differ primarily in their cytokine profiles due to immune deviation, rather than at the level of the TCR of the responding T cells. Although the use of mouse TCR transgenic systems has greatly improved our understanding of immune deviation and Ag-specific protective immune responses (8), the precise mechanisms of protective immunity against allergy in humans remain unclear. The major reason for this uncertainty is that study of class II MHC-restricted, Ag-specific responses in humans is hampered by the inability to track allergen-specific cells due to their low frequency.

Presently, the examination of allergen-specific CD4<sup>+</sup> T cells requires ex vivo activation and expansion of Th2 cells present in the peripheral blood with various stimuli, including allergens as well as nonspecific stimuli, resulting in bystander activation of Ag-nonspecific cells. Despite the use of techniques designed to track allergen-responding cells using activation markers, cytokine production, and dyes such as CFSE, these methods do not clearly discriminate between Ag-specific cells and activated Ag-nonspecific cells. Thus, the difficulty in studying and tracking human Ag-specific CD4<sup>+</sup> T cells present in peripheral blood at very low frequency has hampered our understanding of the critical role of allergen-specific CD4<sup>+</sup> T cells in allergy and protective immunity.

The development of class II MHC tetramers provides a significant opportunity to directly identify and characterize allergen-specific CD4<sup>+</sup> T cells (9, 10). Tetramers are multimeric forms of soluble recombinant MHC molecules associated with specific bound Ag peptides (11) and have been used to track epitope-specific T cells in complex systems (12, 13). The use of tetramers also involves identification of the determinants that bind to the TCR in an HLA-specific context. Class I MHC tetramers have been used to identify circulating specific CD8<sup>+</sup> T cells to the major allergen from the house dust mite, *Der p 1*, in allergic individuals with

\*Department of Pediatrics, and ‡Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305; †Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106; §Benaroya Research Institute, Virginia Mason, Seattle, WA 98101; and ¶Children's Hospital, Harvard Medical School, Karp Laboratories, Boston, MA 02115

Received for publication September 14, 2005. Accepted for publication January 25, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by U.S. Public Health Service Grants R01AI52256 and R01AI26322 and M01-RR00070.

<sup>2</sup> C.M. and J.W. contributed equally to these studies.

<sup>3</sup> Address correspondence and reprint requests to Dr. Dale T. Umetsu, Children's Hospital, Harvard Medical School, Karp Laboratories, Room 10127, One Blackfan Circle, Boston, MA 02115. E-mail address: dale.umetsu@childrens.harvard.edu

atopic dermatitis and in asymptomatic allergic individuals (14). An association between the level of disease and the frequency of CD8<sup>+</sup> tetramer-positive cells was observed, showing the potential of using tetramers to identify and characterize allergen-specific T cells. However, class II-restricted CD4<sup>+</sup> cells, rather than class I CD8<sup>+</sup> cells, are thought to play the primary role in regulating immune responses to allergens (15). Therefore, examination of MHC class II Ag-specific T cells could provide important information regarding immune regulation in allergy and the nature of protective immunity and tolerance in allergy and could lead to improved new therapies for allergy and asthma.

In this study we characterized rye grass allergen-specific, MHC class II-restricted CD4<sup>+</sup> T cells in allergic and nonallergic individuals, using MHC class II tetramers. We identified the dominant HLA-DR epitope of the major rye grass allergen *Lol p 1* in HLA-DRB1\*0401 individuals using HLA-DR\*0401 transgenic mice and peripheral blood T cells from HLA-DR\*0401 individuals. Using soluble DRB1\*0401 tetramers loaded with this epitope, we studied whether allergic and nonallergic individuals differed in their frequency of CD4<sup>+</sup> rye grass tetramer cells and in the cytokines produced by these rye grass tetramer<sup>+</sup> cells.

## Materials and Methods

### Subjects

Two hundred sixty-two individuals were recruited for this study. Serum was assayed by the clinical laboratories of Stanford Hospital for total IgE and specific IgE (by IgE radioallergosorbent test (CapRAST; Pharmacia Diagnostics); assay range, 0–6) against 16 common, local aeroallergens: alder tree, oak tree, olive tree, Bermuda grass, rye grass, timothy grass, dock weed, sage weed, western ragweed, alternaria mold, *Aspergillus* mold, *Cladosporium* mold, cockroach, cat dander, dog dander, and dust mite. Genomic DNA from all individuals was typed for HLA-DRB1\*0401 by sequence specific oligonucleotide probes by Stanford Medical School Blood Center. From these 262 individuals, 28 were found to be positive for HLA-DRB1\*0401, representing 11% of the total donor sample. From these, 12 individuals (5% of the original sample) were rye grass positive by CapRAST (minimum test score, 2), and 11 were negative for all 16 allergens tested (4% of the total). Five individuals (2% of the total) were found to be negative for rye grass, but positive for other allergens (designated intermediate allergic). From the eligible donors, six rye grass allergic, six nonallergic, and two intermediate individuals donated additional blood for analysis. All allergic subjects analyzed had IgE to one or more perennial allergen (house dust mite, cat, dog, and cockroaches), and with the exception of one intermediate subject (I-1), all allergic subjects were polysensitized. The average total IgE was 111 kU/l (range, 58–170) for allergic subjects, 14 kU/l (range, 4–30) for nonallergic subjects, and 36 kU/l for intermediate allergic subjects. The allergic group comprised five men and one woman, the nonallergic group comprised two males and four females, and the intermediate allergic subjects were both women. The gender difference was not statistically significant between the allergic and nonallergic groups, and the majority of DRB1\*0401 men were allergic, with only two nonallergic men. Two allergic individuals were of Asian ethnicity; all others were Caucasian. The average age was 33 years (range, 25–49) for the allergic group and 39 years (range, 28–63) for the nonallergic group; this difference was not statistically significant. Intermediate allergic individuals were 34 and 44 years of age. The Stanford University human subjects committee approved the study, and all subjects gave written informed consent before enrolling in the study.

### Antigens

Purified rye grass Ag group I from *Lolium perenne* pollen (purified *Lol p 1*) was obtained from the National Institute of Allergy and Infectious Diseases Reference Reagent Repository. Peptides were synthesized at Stanford University Protein and Nucleic Acid Facility by F-moc chemistry, as free carboxyl and nonacetylated, and were analyzed by reverse phase HPLC and mass spectrometry. For the tetramer synthesis, the peptides were purified, and only preparations with >80% purity were used.

### Tetramer synthesis

Biotinylated DRB1\*0401 molecules were produced as described previously (9). Rye grass peptides were loaded onto soluble DRB1\*0401 mol-

ecules. Loading of peptide into the tetramers was conducted with 1 mg/ml class II MHC molecules, 25 mg/ml peptides in 100 mM sodium phosphate (pH 6.0), and 0.2% *h*-octyl-*D*-glucopyranoside for 48 h at 37°C. Tetramers were formed by incubation of class II MHC molecules with PE-labeled streptavidin (BioSource International) at a molar ratio of 8:1 for 6 h at room temperature. Empty tetramer or tetramer loaded with myelin basic protein served as negative controls.

### Immunization of mice

BALB/c mice were obtained from The Jackson Laboratory, and HLA-DR4 (DRA1\*0101, DRB1\*0401) transgenic mice were generated at Stanford University (16) or were provided by the animal facility at Case Western Reserve and have previously been used for extensive characterization of T cell epitopes in autoimmune diseases. Mice were immunized s.c. in the footpad with 100 µg of purified *Lol p 1*/mouse in IFA. On day 7, mice were killed, and cells from draining lymph nodes were used in proliferation assays with *Lol p 1*-derived peptides. The Stanford administrative panels on laboratory animal care approved all mouse protocols.

### Cytokine ELISPOT assay

PBMC from DRB1\*0401 rye grass-allergic subjects were isolated by Ficoll-Hypaque gradient centrifugation from freshly drawn blood; resuspended at  $2 \times 10^6$  cells/ml in DMEM supplemented with 10% human AB<sup>+</sup> sera, 2 mM glutamine, and 10 µg/ml gentamicin (complete DMEM); and incubated with 100 µg/ml purified *Lol p 1* for 6 days. Nonadherent cells were harvested on day 6 and incubated with freshly obtained, adherent, irradiated, autologous APCs and Ag at the same concentration on ELISPOT plates (ImmunoSpot; Cellular Technology) previously coated with IFN-γ- or IL-5-specific capture Ab and blocked with 1% BSA in PBS. The cells were cultured for 24 h for IFN-γ and 48 h for IL-5. Subsequently, cells were removed by washing, and biotinylated detection Abs were added and incubated overnight. Spots were visualized by adding streptavidin-alkaline phosphatase and NBT/5-bromo-4-chloro-3-indolyl-phosphate substrate. Image analysis of ELISPOT assays was performed on a series 1 ImmunoSpot Image Analyzer (Cellular Technology) customized for analyzing ELISA spots to meet objective criteria for size, chromatic density, shape, and color and suited to measure the cytokine production of low frequency, Ag-specific, memory T cells.

### Cell culture for tetramer detection and intracellular cytokine detection

Freshly isolated PBMCs ( $2 \times 10^6$  cells/ml) in complete DMEM were stimulated with peptide (final concentration, 10 µg/ml) or purified *Lol p 1* (final concentration, 100 µg/ml) in 24-well plates (1 ml/well). Optimal concentrations of peptide and *Lol p 1* were previously determined by [<sup>3</sup>H]thymidine cell proliferation assays. The cells were cultured for 6 days in 10% CO<sub>2</sub> at 37°C. On day 6, the nonadherent cells were harvested and incubated with Ag plus freshly obtained irradiated autologous PBMCs. Except as noted, the cells were restimulated for 3 days. The nonadherent cells were then harvested and resuspended at  $5 \times 10^6$  cells/ml in complete DMEM for tetramer staining and intracellular cytokine assays.

### Tetramer staining

Cells ( $5 \times 10^5$ ) in 100 µl complete DMEM were distributed in 96-well, round-bottom plates. One microliter of rye grass or control tetramer (0.5 mg/ml) was added, and the plate was incubated in 10% CO<sub>2</sub> at 37°C for 2 h. After the incubation, surface staining was performed for 15 min at room temperature. The cells were then washed in complete DMEM, resuspended in PBS plus 1% heat-inactivated FCS, and analyzed by a FAC-Scan (BD Biosciences) on the same day or, alternatively, fixed by addition of paraformaldehyde (1% final concentration) and analyzed 24 h later. Preliminary analysis showed that fixation did not interfere with the detection of tetramer staining. Approximately  $1 \times 10^5$  lymphocytes were collected for each sample.

### Tetramer staining and intracellular cytokine detection

Cells ( $1 \times 10^6$ /ml) in 200 µl of complete DMEM were distributed in 5-ml polypropylene tubes. Five microliters of rye grass or negative tetramer was added, and cells were incubated in 10% CO<sub>2</sub> at 37°C for 2 h. After this incubation, 800 µl of complete medium with brefeldin A (BD Biosciences; final concentration, 1/1000), PMA (final concentration, 40 ng/ml; Sigma-Aldrich), and ionomycin (final concentration, 1 µg/ml; Sigma-Aldrich) was added to the tubes, and the cells incubated for an additional 4 h at 37°C. The cells were then stained for surface markers, fixed with 1% paraformaldehyde in PBS, and permeabilized with PermWash (BD Biosciences). Abs for intracellular cytokine staining were added for 30 min,

followed by washes and resuspension in PBS with 1% FCS and 1% paraformaldehyde, and were analyzed by flow cytometry within 24 h. Approximately  $5 \times 10^5$  lymphocytes were collected from each sample.

#### Abs and flow cytometry

Cells were stained with PE-Cy5-labeled anti-CD4, and B cells were excluded using FITC-labeled anti-CD19 or PerCP-labeled anti-CD19. For detection of intracellular cytokines, anti-IL-4, IL-10, and IFN- $\gamma$  (all FITC labeled) and their respective isotype controls were used. All Abs were obtained from BD Biosciences. A FACScan (BD Biosciences) was used for analytical flow cytometry, and data were processed with CellQuest Pro (BD Biosciences) and FlowJo (Tree Star) software.

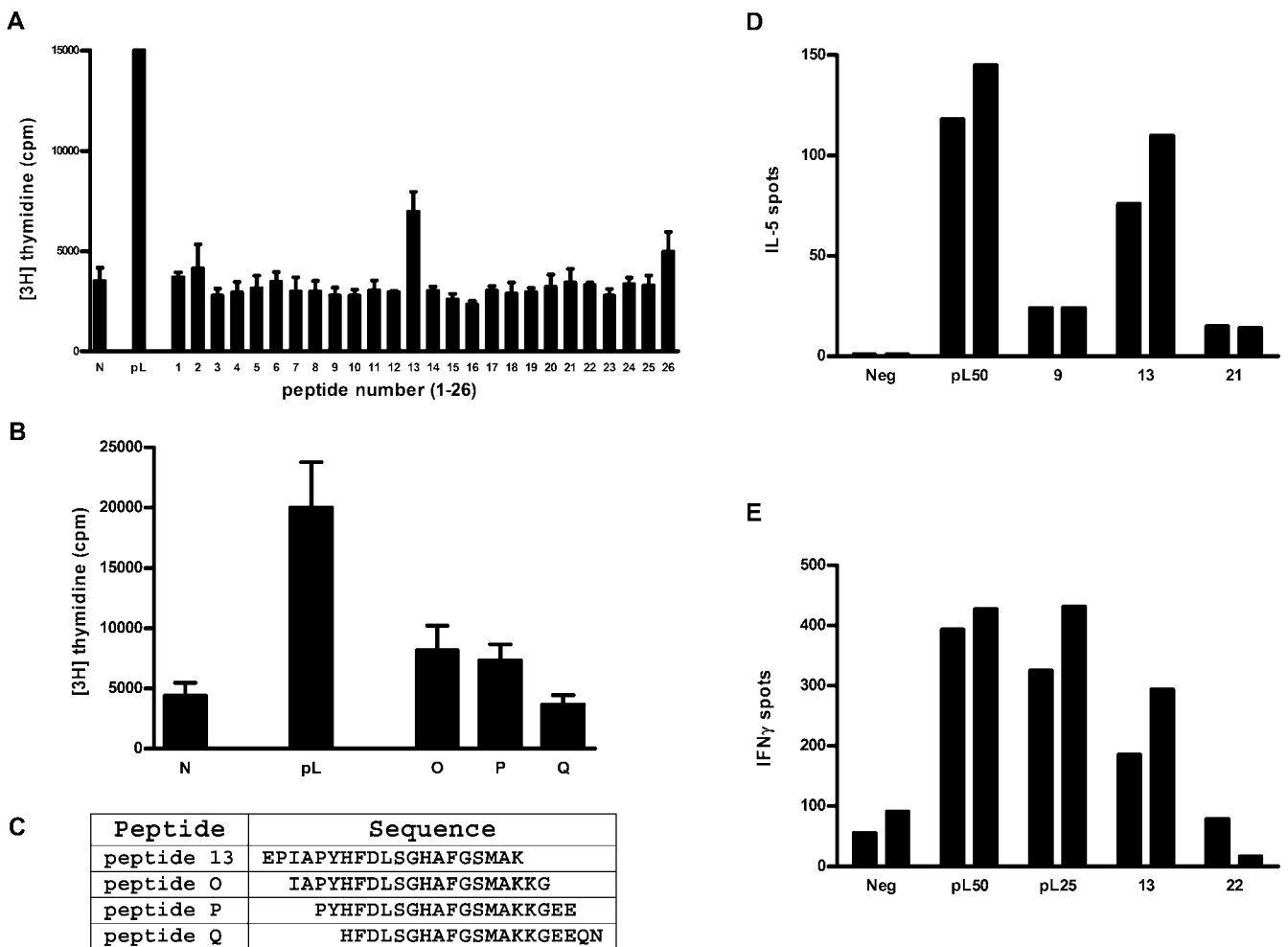
#### Statistical analyses

For statistical analysis, rye grass-allergic and intermediate allergic subjects were considered as one group. Paired data were analyzed by Wilcoxon test, and nonpaired data were analyzed by Mann-Whitney *U* test, both two-tailed. PRISM software (version 4; GraphPad) was used for the analysis.

## Results

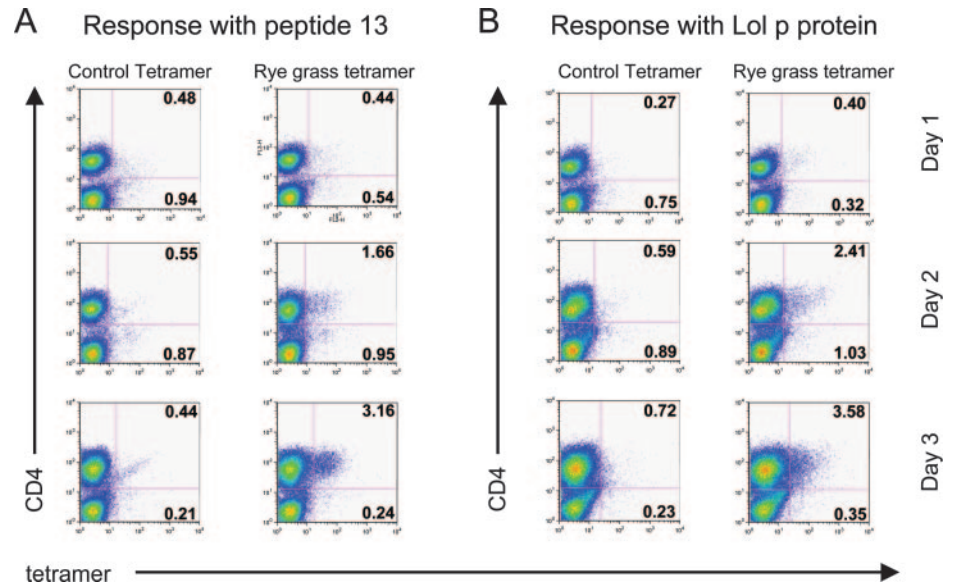
### Identification of the dominant T cell epitope of rye grass allergen protein *Lol p 1* in the context of HLA-DRB1\*0401

We initially determined the dominant T cell epitope of *Lol p 1* in the context of HLA-DRB1\*0401 using HLA-DR4 (DRA1\*0101 and DRB1\*0401) transgenic mice. Mice were immunized s.c. with purified *Lol p 1*, and the response to a panel of overlapping *Lol p 1* peptides (17) was determined by [ $^3$ H]thymidine incorporation. The panel consisted of 26 peptides spanning the entire length of the *Lol p 1* protein (20-mer overlapping by 10 aa). Immunization conditions for these experiments, including allergens and allergen dose, adjuvant, route of administration, lymphoid organ used as source of T cells, and conditions of the proliferation assay, were optimized using control BALB/c mice (data not shown). Fig. 1A shows



**FIGURE 1.** Identification of T cell epitopes in rye grass allergen protein *Lol p 1*. **A**, HLA-DR4 transgenic mice were immunized s.c. with purified *Lol p 1*, and the proliferation of lymph node cells in response to purified *Lol p 1* and a panel of 26 overlapping *Lol p 1* peptides was determined by [ $^3$ H]thymidine incorporation. N, negative control (medium alone); pL, positive control (purified *Lol p 1*). **B**, Determination of the HLA-DR4 binding motif in peptide 13. Proliferation in response to p13-derived peptides O and P, but not Q, demonstrates that the DR4 binding motif is YHFDLSGHA (see text). **C**, Sequence of immunodominant peptide 13 and peptides O, P, and Q. Peptides O, P, and Q were shifted by two amino acids toward the C terminus and were used to determine the critical DR4 binding motif in peptide 13. **D**, Determination of the HLA-DR4 binding motif in peptide 13. Proliferation in response to p13-derived peptides O and P, but not Q, demonstrates that the DR4 binding motif resides is YHFDLSGHA (see text). **E**, ELISPOT assay of IL-5 production. PBMCs from rye grass-allergic DRB1\*0401 subjects were stimulated for 6 days with medium alone (Neg), *Lol p 1* protein (pL), or peptides, followed by 48-h restimulation with irradiated APCs under the same respective conditions as in the primary stimulation. The graph depicts the number of IL-5-producing cells after 48-h restimulation with medium alone (Neg), *Lol p 1* protein at 50  $\mu$ g/ml (pL 50), and peptides 13, 9, and 21. **F**, ELISPOT assay of IFN- $\gamma$  production. PBMCs from rye grass-allergic DRB1\*0401 subjects were stimulated for 6 days with *Lol p 1* protein, followed by 24-h restimulation with irradiated APCs and medium alone (Neg), *Lol p 1* protein (pL), or peptides. The graph depicts the number of IFN- $\gamma$ -producing cells after 24-h restimulation with medium alone (Neg), *Lol p 1* protein at 50  $\mu$ g/ml (pL 50) and 25  $\mu$ g/ml (pL 25), and peptides 13 and 22.

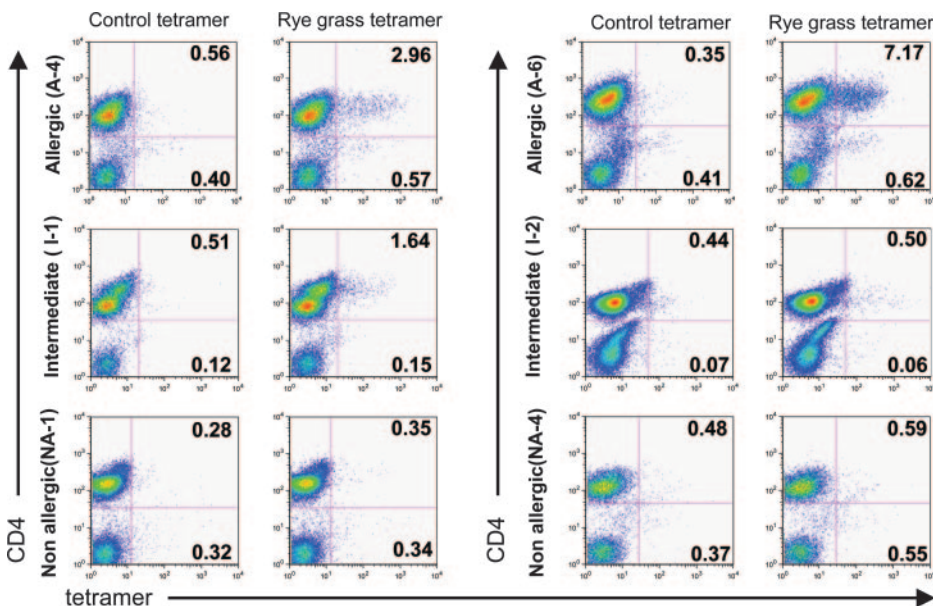
**FIGURE 2.** Establishing the conditions for detection of CD4<sup>+</sup> rye grass tetramer-positive T cells. Freshly isolated PBMCs from a DRB1\*0401 rye grass-allergic individual (A-1) were stimulated with p13 peptide (10  $\mu$ g/ml; A) or purified *Lol p 1* protein (100  $\mu$ g/ml; B) for 6 days. On day 6, nonadherent cells were collected and restimulated for 3 days in the presence of an equal amount of fresh autologous irradiated PBMCs as APCs and the same Ag as before. Cells were assayed by flow cytometry, with rye grass tetramer or empty tetramer as the control tetramer, every 24 h during restimulation. For flow analysis, live total lymphocytes were gated by forward and side light scattering and additionally by CD19<sup>-</sup> cells to exclude B cells.



that of the 26 peptides, peptide 13 contains the dominant *Lol p 1* epitope in the context of HLA-DR4. Three putative DR4 binding motifs (IAPYHFDLS, YHFDLSGHA, and FDLSGHAFG) were present within the 20-mer peptide 13. To identify the DR4 binding motif in peptide 13, additional 20-mer peptides (peptides O, P, and Q; Fig. 1C) sequentially shifted in the C-terminal direction were synthesized. T cell proliferation was induced with peptides O and P, but not Q (Fig. 1B), indicating that in DR4 transgenic mice, the motif YHFDLSGHA is the critical one.

We confirmed that the peptide 13 is also the dominant peptide in DR4 rye grass-allergic subjects, who were identified by total IgE, radioallergosorbent tests, and HLA-DR typing from an initial pool of 262 volunteers. PBMCs from DRB1\*0401 individuals allergic to rye grass were stimulated with purified *Lol p 1*, peptide 13, and several rye grass peptides as negative controls, and cytokine production was measured by ELISPOT assay. Fig. 1 also shows that peptide 13, but not peptide 9 or 21, induced

T cells from DRB1\*0401 individuals to produce IL-5 (Fig. 1D), and that peptide 13, but not peptide 22, induced production of IFN- $\gamma$  (Fig. 1E), confirming that peptide 13 is immunogenic in humans. Together with the results from the HLA-DR\*0401 transgenic mice, these results indicate that peptide 13 contains the immunodominant *Lol p 1* epitope in the context of HLA-DRB1\*0401. Peptide 13 was therefore chosen for the construction of a DR4 rye grass tetramer. We compared the efficacy of DRB1\*0401 tetramers loaded with the original peptide 13 20-mer sequence and tetramers loaded with a shorter (13-mer) derived peptide (sequence: APYHFDLSGHAFG). The core nine amino acids of peptide 13 (underlined) consist of the DR4 binding motif previously identified using the DR4 transgenic mice. We found that staining of CD4<sup>+</sup> cells with the tetramer loaded with the 13-mer peptide was 15–20% higher than that with the tetramer loaded with the 20-mer peptide. All subsequent experiments were conducted using 13-mer loaded tetramer.

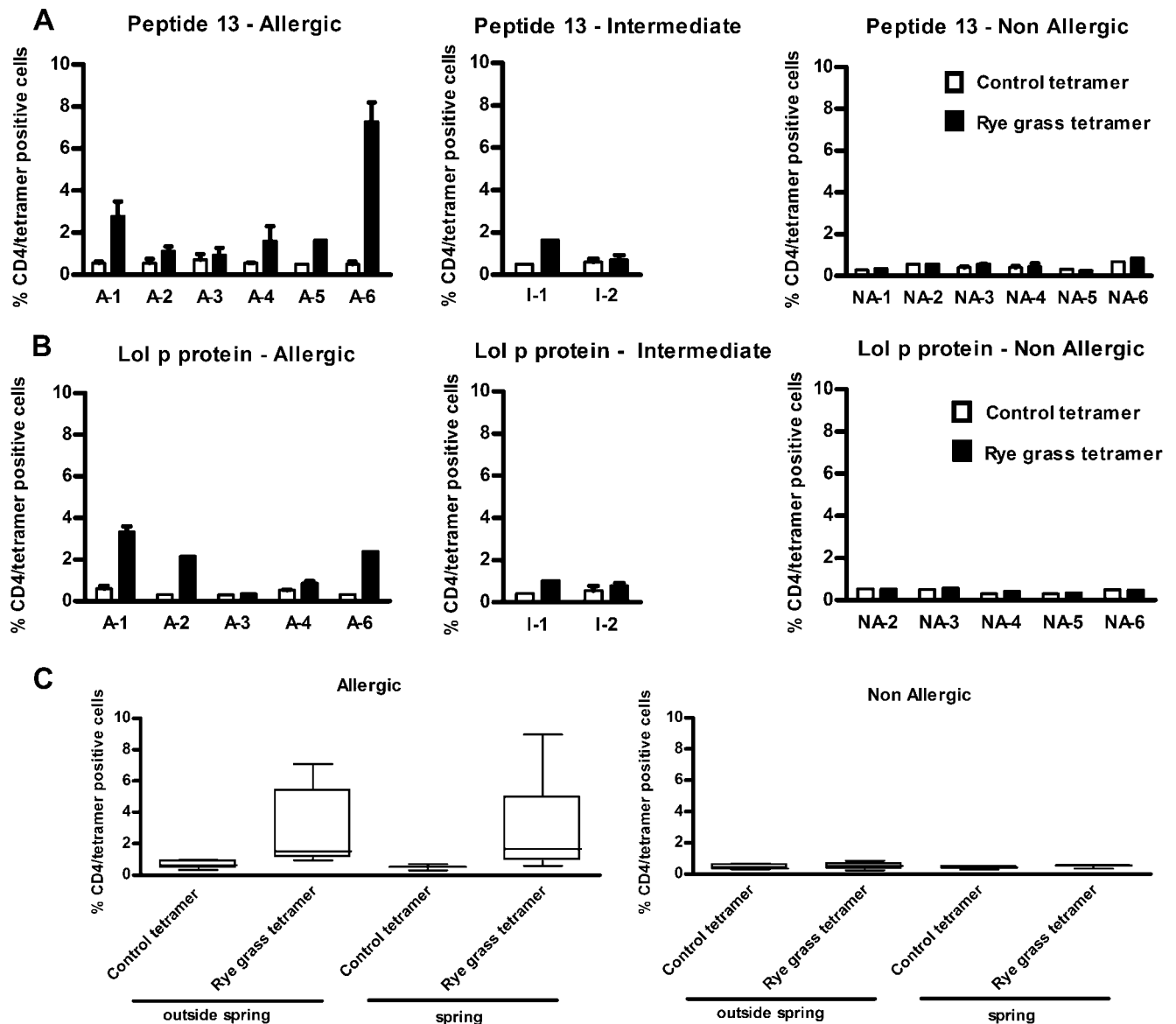


**FIGURE 3.** Representative examples of tetramer staining in single individuals. Freshly isolated PBMCs from DRB1\*0401 rye grass-allergic, intermediate allergic, and nonallergic individuals were stimulated with p13 peptide (10  $\mu$ g/ml) for 6 days and restimulated for 3 days. Cells were then assayed by flow cytometry with rye grass tetramer or empty tetramer as the control.

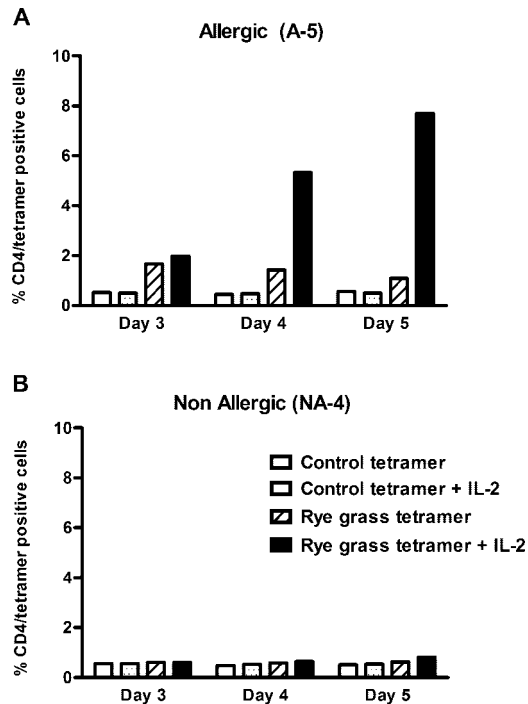
*CD4<sup>+</sup> rye grass tetramer-positive cells are detected after ex vivo expansion*

In initial experiments we were unable to detect rye grass tetramer-positive cells in freshly isolated PBMCs from DRB1\*0401 rye grass-allergic individuals (data not shown), presumably because rye grass-positive cells are present in the peripheral blood in too low a frequency. Therefore, we expanded rye grass Ag-specific cells by ex vivo stimulation with Ag. Different expansion schemes were tested using peptide 13 or purified *Lol p 1*, and the best results were obtained by culturing the cells for 6 days, followed by 3-day restimulation with fresh irradiated APCs from autologous PBMC and the same Ag. Fig. 2 shows the results for tetramer detection by flow cytom-

etry of PBMCs from an allergic individual (A1) cultured with either peptide 13 (Fig. 2A) or *Lol p 1* protein (Fig. 2B). B cells were excluded from all analysis by gating out CD19<sup>+</sup> cells. No difference from the control tetramer staining was observed for cells stimulated with peptide 13 or *Lol p 1* on day 1. On day 2 of restimulation, CD4<sup>+</sup> rye grass tetramer-positive cells were detectable in both cultures. The percentage of CD4<sup>+</sup> rye grass tetramer-positive cells increased on day 3, whereas staining with the tetramer control remained stable, such that by day 3, rye grass tetramer-positive cells for both peptide and *Lol p 1* protein cultures were mainly CD4<sup>+</sup> and were confined to cells in the large lymphocyte gate (defined on flow cytometry by forward and side scatters; data not shown), presumably blasts.



**FIGURE 4.** Percentage of CD4<sup>+</sup> tetramer-positive cells in total lymphocytes from single individuals. Freshly isolated PBMCs from DRB1\*0401 rye grass-allergic (A-1 to A-6) and intermediate allergic (I-1 and I-2) individuals or nonallergic (NA-1 to NA-6) individuals were stimulated with p13 peptide (10 μg/ml; A) or purified *Lol p 1* protein (100 μg/ml; B) for 6 days and restimulated for 3 days. Cells were assayed by flow cytometry with rye grass tetramer or empty tetramer as the control. Bars with error bars represent an average of two to four experiments. Bars without error bars represent single experiments. C, Percentage of CD4<sup>+</sup> tetramer-positive cells during spring and not during spring. Freshly isolated PBMCs from DRB1\*0401 rye grass-allergic (left panel; n = 5) and nonallergic (right panel; n = 5) individuals were stimulated with p13 peptide (10 μg/ml) for 6 days and restimulated for 3 days. Cells were assayed by flow cytometry with rye grass tetramer or empty tetramer as the control. Graph boxes show the median and the 25th and 75th percentiles. The whiskers of the graph show the largest and smallest values. Data from intermediate allergic subjects were not included, because only one subject was tested during and not during spring, with equivalent results.

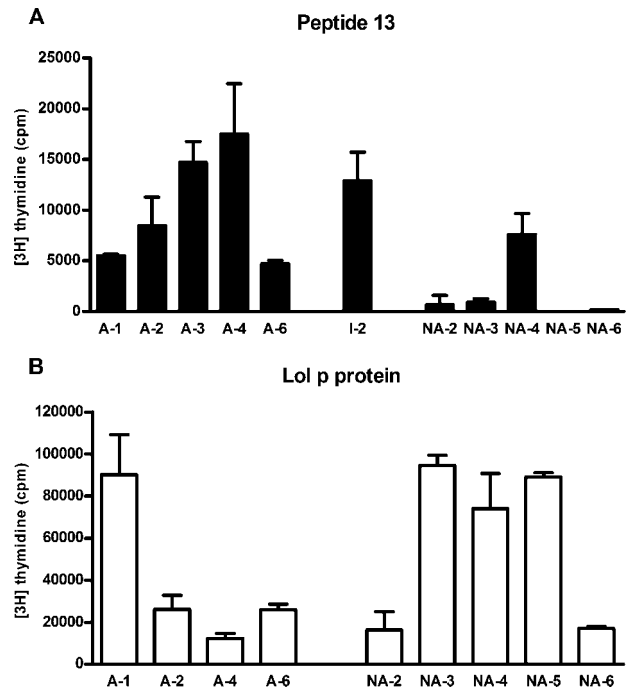


**FIGURE 5.** Effect of IL-2 on the percentage of CD4<sup>+</sup> tetramer-positive cells in the total lymphocyte population. Freshly isolated PBMCs from a DRB1\*0401 rye grass-allergic (A-5; A) and a nonallergic individual (NA-4; B) were stimulated with p13 peptide (10  $\mu$ g/ml) for 6 days and restimulated for 3 days with or without 5 U/ml IL-2. Cells were assayed by flow cytometry with rye grass tetramer or DRB1\*0401/myelin basic protein as the control tetramer every 24 h during restimulation.

#### CD4<sup>+</sup> rye grass tetramer-positive cells are present in allergic, but not nonallergic, individuals

We next compared the percentage of CD4<sup>+</sup> tetramer-positive cells in PBMCs from DRB1\*0401 rye grass-allergic, nonallergic and intermediate allergic individuals, identified as described in *Materials and Methods*. Fig. 3 shows a representative example of tetramer staining for both rye grass and control tetramers after 6-day culture and 3-day restimulation with peptide 13 in cells from allergic, intermediate, and nonallergic individuals. CD4<sup>+</sup> rye grass tetramer-positive cells were clearly identified in cells from allergic individuals and those from one intermediate individual, but not in those from nonallergic individuals.

Fig. 4 shows the percentage of CD4<sup>+</sup> rye grass tetramer-positive cells in the total lymphocyte population of single individuals in cultures stimulated with peptide 13 (Fig. 4A) or with purified *Lol p 1* protein (Fig. 4B). After culture with the peptide, cells from rye grass-allergic individuals showed an increased frequency of CD4<sup>+</sup> rye grass tetramer-positive cells compared with control tetramer. The increased frequency in cells from allergic individuals was, on the average, 5-fold, from a minimum of 2- to 14-fold above control tetramer staining. This variation was subject specific, but reproducible, because repeated cultures of cells from the same individual on different occasions gave similar results. Another source of variation could be the relationship between the timing of blood drawing and the rye grass pollen season. However, when examined both during and not during spring, the percentages of CD4<sup>+</sup> tetramer-positive cells in high and lower responders were equivalent regardless of the season (Fig. 4C). Interestingly, the poorest responder allergic individual (A-3) had the highest percentage of CD4<sup>+</sup>/CD19<sup>-</sup> among all subjects tested, suggesting a preferential expansion of CD8<sup>+</sup> T cells (data not shown). The increase in the

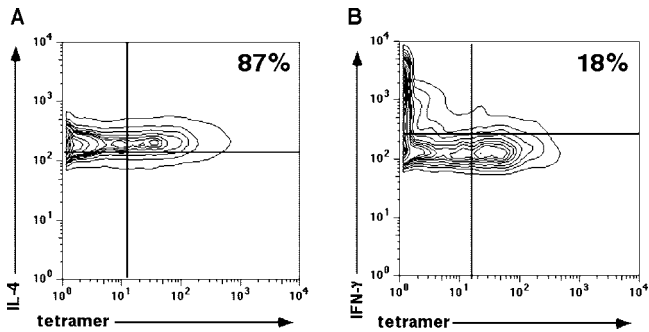


**FIGURE 6.** PBMC proliferation in response to rye grass peptide 13 and purified *Lol p 1* protein. Freshly isolated PBMCs from DRB1\*0401 rye grass-allergic (A) and intermediate allergic (I) individuals or nonallergic individuals (NA) were stimulated with p13 peptide (10  $\mu$ g/ml; A) or purified *Lol p 1* protein (100  $\mu$ g/ml; B) for 6 days and restimulated for 3 days. Radioactive [<sup>3</sup>H]thymidine was added 16 h before completion of the third day of restimulation. Results are expressed as the mean  $\pm$  SD of triplicate determinations.

percentage of CD4<sup>+</sup> rye grass tetramer-positive cells above that in the control tetramer was statistically significant ( $p < 0.01$ ) in cultures from allergic subjects. One intermediate allergic individual (negative for rye grass-specific IgE) also showed an increased percentage of CD4<sup>+</sup> rye grass tetramer-positive cells above that of the control tetramer. In contrast, nonallergic individuals showed no increase in CD4<sup>+</sup> rye grass tetramer-positive cells compared with the control tetramer ( $p > 0.05$ ). As in allergic subjects, nonallergic subjects tested during and not during the pollen season gave equivalent results (Fig. 4C). Importantly, the percentage of CD4<sup>+</sup> rye grass tetramer-positive cells in cultures from allergic subjects was higher than that in cell cultures from nonallergic subjects ( $p < 0.01$ ).

Similar results were observed with cultures stimulated with purified *Lol p 1* protein (Fig. 4B). Allergic subjects showed a significant increase in the percentage of CD4<sup>+</sup> rye grass tetramer-positive cells above that of control tetramer ( $p < 0.02$ ), whereas nonallergic subjects did not show such an increase ( $p > 0.05$ ). The increase in the percentage of CD4<sup>+</sup> rye grass tetramer-positive cells in allergic individuals was much greater than that of cells from nonallergic subjects ( $p < 0.05$ ). The percentage of control tetramer-positive cells was similar in all groups ( $p > 0.05$ ).

Given the absence of rye grass-positive cells in nonallergic individuals, we investigated whether addition of IL-2 could promote the expansion of rye grass tetramer-positive cells from nonallergic individuals. Fig. 5 shows that addition of IL-2 during the 3-day restimulation induced expansion of the CD4<sup>+</sup> rye grass tetramer-positive population in cells from the allergic individual, but had no effect on cells from the nonallergic individual.



**FIGURE 7.** Cytokine production in CD4<sup>+</sup> rye grass p13 tetramer-positive T cells. Freshly isolated PBMCs from a DRB1\*0401 rye grass-allergic (A-1) were stimulated with p13 peptide (10  $\mu$ g/ml) for 6 days. On day 6, nonadherent cells were collected and restimulated for 3 days. Cells were assayed with rye grass tetramer and for intracellular synthesis of IL-4 (A) and IFN- $\gamma$  (B) after stimulation with PMA and ionomycin. For flow analysis, tetramer-positive cells were confined in a large lymphocyte population (defined by forward and side light scattering). Negative quadrants were defined using the respective isotype controls for each cytokine.

Addition of IL-2 had no effect on the percentage of cells positive for control tetramer in both allergic and nonallergic individuals.

#### Proliferative responses of PBMC to peptide and purified *Lol p 1*

To investigate the proliferative responses of PBMC to peptide 13 and purified *Lol p 1*, we performed [<sup>3</sup>H]thymidine incorporation assays after the 3-day restimulation (Fig. 6). Proliferative responses to both peptide 13 and *Lol p 1* were observed in cell cultures from all allergic individuals tested. Cells from only one intermediate individual were tested with peptide 13, and proliferation was also observed. In contrast, only one of five cell cultures from nonallergic individuals proliferated in response to peptide 13 (Fig. 6A). Interestingly, cell cultures from all nonallergic individuals tested showed strong proliferative responses to *Lol p 1* at levels similar to those in the allergic individuals, indicating that the rye grass nonallergic individuals were sufficiently exposed to rye grass pollen and had rye grass-specific memory T cells (Fig. 6B).

#### Cytokine profile of CD4<sup>+</sup> rye grass tetramer-positive cells

To examine the cytokine profile of CD4<sup>+</sup> tetramer-positive cells, we combined tetramer staining with intracellular cytokine staining. After the 3-day restimulation, cells were initially labeled with the tetramer, then stimulated with PMA/ionomycin. A difficulty in this type of experiment is to balance the decrease in tetramer staining that occurs after stimulation with PMA/ionomycin to induce cytokine production (due to TCR down-modulation) and the low

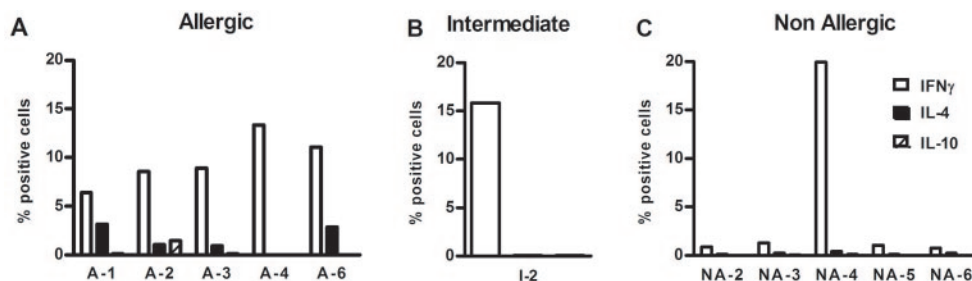
level of cytokine production if PMA/ionomycin is not used. Cytokine production was not detectable in the absence of PMA/ionomycin stimulation. Two individuals had a detectable number of rye grass tetramer-positive cells after PMA/ionomycin treatment, so we could analyze cytokine responses. These individuals had previously showed some of the highest tetramer-positive responses (A1 and A6; Fig. 4). Interestingly, we found that 87% of rye grass tetramer-positive cells produced IL-4, whereas only 18% of tetramer-positive cells produced IFN- $\gamma$  for A-1 (Fig. 7). Similarly, for A6, 81% of tetramer-positive cells produced IL-4, and 37% produced IFN- $\gamma$  (data not shown). We did not detect IL-10-producing, rye grass tetramer-positive cells in any of the subjects.

We also analyzed cytokine production of IL-4, IFN- $\gamma$ , and IL-10 from total lymphocytes to peptide 13 without a tetramer gate (Fig. 8). As expected, IL-4-producing cells were observed only in cell cultures from allergic individuals, together with IFN- $\gamma$ -producing cells, but IL-10-producing cells were observed in only one allergic individual (A-2). Cells from one intermediate subject produced only IFN- $\gamma$  and no detectable IL-4 or IL-10. In cultures from nonallergic individuals, production of IFN- $\gamma$  was observed in cells from only one subject (NA-4); cells from this individual also showed a proliferative response to the peptide. No IL-4- or IL-10-producing cells were found in culture of cells from nonallergic individuals.

## Discussion

In this article, we demonstrated for the first time that allergen-specific CD4<sup>+</sup> T cells can be identified using class II MHC tetramers in rye grass-allergic individuals. First, we identified the major antigenic epitope of rye grass allergen *Lol p 1* in HLA-DRB1\*0401 individuals using HLA-DR\*0401 transgenic mice and peripheral blood T cells from HLA-DR\*0401 individuals. Using DR\*0401 tetramers loaded with this peptide, we found that rye grass-allergic individuals consistently exhibited detectable numbers of CD4<sup>+</sup> rye grass tetramer-positive cells in peripheral blood after ex vivo expansion with allergen. In contrast, cultures of nonallergic individuals stimulated with rye grass peptide or with the purified *Lol p 1* protein contained few or no CD4<sup>+</sup> rye grass tetramer-positive cells. Interestingly, individuals who were not allergic to rye grass, but had measurable IgE responses to other allergens, exhibited CD4<sup>+</sup> rye grass tetramer-positive cells after expansion, although at levels lower than in rye grass-allergic individuals, demonstrating the presence of allergen-specific cells in these individuals despite undetectable levels of serum rye grass-specific IgE.

The rye grass tetramer-positive cells that we identified in allergic individuals produced IL-4, but little IFN- $\gamma$ . This definitively



**FIGURE 8.** Percentage of cytokine producing cells without a tetramer gate in PBMC from single individuals after cell culture with peptide 13. Freshly isolated PBMCs from DRB1\*0401 rye grass-allergic (A; A), intermediate allergic (I; B), and nonallergic (NA; C) individuals were stimulated with p13 peptide (10  $\mu$ g/ml) for 6 days and restimulated for 3 days. Cells were assayed by flow cytometry for intracellular synthesis of IL-4, IFN- $\gamma$ , and IL-10 after stimulation with PMA plus ionomycin. Results are expressed as the percentage of cytokine production from both CD4<sup>+</sup> and CD4<sup>-</sup> cells due to CD4 down-regulation after PMA/ionomycin stimulation.

demonstrated that CD4<sup>+</sup> T cells specific for the immunodominant peptide of rye grass allergen expressed a Th2 cytokine profile, suggesting a role for such cells in the induction and amplification of allergic inflammatory responses. Unfortunately, we did not isolate sufficient numbers of cells from the intermediate allergic individuals to examine the cytokine profile of these rye grass-specific T cells. Such rye grass-specific cells from the intermediate allergic individuals might produce suppressive cytokines, such as IL-10, but not IL-4. These rye grass-specific, non-Th2 cells might suppress the production of rye grass-specific IgE in these individuals and perhaps protect against the development of symptoms in response to rye grass allergen challenge.

Our data demonstrating that nonallergic individuals have undetectable numbers of T cells that recognize the immunodominant rye grass peptide are important because they provides clues regarding the mechanisms by which nonallergic individuals remain tolerant to allergens. Our results are similar to those reported by Seneviratne et al. (14), who showed, using a class I MHC tetramer, that a high frequency of *Der p 1*-specific CD8<sup>+</sup> T cells was present in allergic individuals, but not in nonallergic individuals. The low frequency of rye grass tetramer-positive cells in nonallergic individuals in our study suggests several possible mechanisms. First, Ag-specific CD8<sup>+</sup>, rather than CD4<sup>+</sup>, cells might predominate and proliferate in nonallergic individuals. This possibility is unlikely, because we did not notice an expansion of CD3<sup>+</sup>CD4<sup>-</sup> cells in cultures from nonallergic subjects, although CD19<sup>+</sup> cells appeared to increase in number in these cultures. Indeed, we observed a positive correlation between the percentage of CD19<sup>+</sup> cells and the proliferative response in nonallergic ( $r = 0.8$ ), but not allergic, individuals, suggesting that B cells might be responsible in part for the vigorous proliferative response to *Lol p 1* protein in nonallergic individuals (Fig. 6B). A second possibility is that the rye grass tetramer-positive cells in rye grass nonallergic individuals might be anergic and/or deleted and cannot expand *ex vivo* to a frequency that is detectable using the available tetramer technology. The addition of IL-2 to the rye grass peptide cultures did not reveal rye grass tetramer-positive cells in the peripheral blood of nonallergic individuals, suggesting that anergic T cells are not present in nonallergic individuals. Nevertheless, we cannot fully eliminate the possibility that rye grass tetramer-positive cells proliferate and expand poorly *ex vivo* and/or that they are deleted from and therefore are undetectable in nonallergic individuals.

A third possible reason for the low frequency of rye grass tetramer-positive cells in nonallergic individuals is that HLA-DR4 rye grass nonallergic individuals may use a DR4 immunodominant peptide of rye grass Ag distinct from that used by allergic individuals. However, because the selection of immunodominant *Lol p 1* peptides for HLA-DR4 is primarily determined by the binding affinity of the peptide for HLA-DR4, and because we identified the immunodominant *Lol p 1* peptide using HLA-DR4 transgenic mice, it is likely that the *Lol p 1* DR4 peptide we identified is indeed immunodominant in humans and important in both allergic and nonallergic individuals. This would be consistent with several studies that analyzed the specificities of allergen-specific T cell clones and showed that allergic and nonallergic individuals recognize the same allergenic epitopes, for example, of *Bet v 1* (18) and *Fel d 1* (19). Nevertheless, because the IgE Ab response to *Lol p 1* has been shown to be associated with HLA-DR3 (20), it is possible that allergic and nonallergic individuals use distinct HLA-DR molecules as well as distinct Ag peptides in responding to *Lol p 1*. Additional studies with other HLA-DR tetramers, therefore, are necessary to resolve this issue.

A study by Akdis et al. (7), using a very different approach, showed that allergen-specific/activated cells producing IL-10

could be identified in the blood of both allergic and nonallergic individuals. In their study, cells isolated and expanded from peripheral blood from allergic and nonallergic individuals produced IL-4, IFN- $\gamma$ , or IL-10, as analyzed with a cytokine secretion assay (Miltenyi Biotec). IL-4 production distinguished the T cells of allergic individuals from those of nonallergic individuals, whereas IL-10 production with inhibitory function was observed in T cells from nonallergic individuals. However, the IL-10-producing cells had many features of CD25<sup>+</sup> natural regulatory T cells (expression of CTLA-4, TGF- $\beta$ RI and -II, and activity dependent on either IL-10 or TGF- $\beta$ ), rather than adaptive Ag-specific regulatory T cells, although the expression of Forkhead/winged helix transcription factor was not assessed. In contrast, in another study, examination of cells from a large population of allergic and nonallergic individuals indicated that allergen-responsive cells have a very wide spectrum of cytokine profiles, and that the human population is extremely heterogeneous (21). Because whole protein Ags were used in both of these studies, there was no assessment of immunodominant peptide use. Furthermore, allergen specificity of the analyzed cells was assumed, because the cells developed in cultures responding to allergen stimulation, but it is possible that a number of responding cells, including the IL-10-producing regulatory T cells, were Ag nonspecific. This possibility is consistent with our finding (Fig. 8) that IFN- $\gamma$  production is very prominent in supernatants from cultures of total cells, but is modest as assessed by intracellular cytokine staining of rye grass tetramer-positive cells.

In summary, we demonstrated that in allergic individuals, class II MHC tetramer technology provides a method to identify allergen-specific cells in conjunction with assessment of their cytokine profile. HLA-DRB1\*0401 rye grass-allergic individuals exhibited CD4<sup>+</sup> cells that bound HLA-DR4 tetramers loaded with the immunodominant rye grass peptide, and these T cells produced predominantly IL-4. In contrast, HLA-DR4 nonallergic individuals exhibited a very low frequency of such tetramer-specific cells even after *ex vivo* allergen stimulation and cell expansion. This result suggests that the response in nonallergic individuals is fundamentally different from that in allergic individuals, and that allergen-specific cells are present at very low frequency or are deleted or anergic in nonallergic individuals. Alternatively, allergen-specific cells in nonallergic individuals may respond to allergen epitopes distinct from those recognized by T cells in allergic individuals. Additional study of allergen epitopes in the context of defined class II specificities and the use of class II tetramers is required to fully understand the nature of the nonallergic phenotype. Nevertheless, the use of class II tetramers to identify allergen-specific cells opens a new avenue in allergy research and should contribute to better definition of the cells involved in the allergic inflammatory response.

## Acknowledgments

We thank Dr. Gerald Nepom for very helpful advice. In addition, we thank all our volunteer blood donors for their cooperation and understanding.

## Disclosures

The authors have no financial conflict of interest.

## References

- Umetsu, D. T., J. J. McIntire, O. Akbari, C. Macaubas, and R. H. DeKruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3: 715–720.
- Herrick, C. A., and K. Bottomly. 2003. To respond or not to respond: T cells in allergic asthma. *Nat. Rev. Immunol.* 3: 405–412.
- Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4<sup>+</sup> T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10: 587–593.

4. Robinson, D. S., Q. Hamid, S. Ying, A. Tscopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326: 298–304.
5. Robinson, D. S., S. Ying, A. M. Bentley, Q. Meng, J. North, S. R. Durham, A. B. Kay, and Q. Hamid. 1993. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J. Allergy Clin. Immunol.* 92: 397–403.
6. Romagnani, S. 1994. Lymphokine production by human T cells in disease states. *Annu. Rev. Immunol.* 12: 227–257.
7. Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Cramer, S. Thunberg, G. Deniz, 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.
8. Akbari, O., P. Stock, R. H. DeKruyff, and D. T. Umetsu. 2003. Role of regulatory T cells in allergy and asthma. *Curr. Opin. Immunol.* 15: 627–633.
9. Novak, E. J., A. W. Liu, G. T. Nepom, and W. W. Kwok. 1999. MHC class II tetramers identify peptide-specific human CD4<sup>+</sup> T cells proliferating in response to influenza A antigen. *J. Clin. Invest.* 104: R63–67.
10. Crawford, F., H. Kozono, J. White, P. Marrack, and J. Kappler. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity* 8: 675–682.
11. Nepom, G. T., J. H. Buckner, E. J. Novak, S. Reichstetter, H. Reijonen, J. Gebe, R. Wang, E. Swanson, and W. W. Kwok. 2002. HLA class II tetramers: tools for direct analysis of antigen-specific CD4<sup>+</sup> T cells. *Arthritis Rheum.* 46: 5–12.
12. Tatsumi, T., L. S. Kierstead, E. Ranieri, L. Gesualdo, F. P. Schena, J. H. Finke, R. M. Bukowski, J. Mueller-Berghaus, J. M. Kirkwood, W. W. Kwok, et al. 2002. Disease-associated bias in T helper type 1 (Th1)/Th2 CD4<sup>+</sup> T cell responses against MAGE-6 in HLA-DRB10401<sup>+</sup> patients with renal cell carcinoma or melanoma. *J. Exp. Med.* 196: 619–628.
13. Wong, R., R. Lau, J. Chang, T. Kuus-Reichel, V. Brichard, C. Bruck, and J. Weber. 2004. Immune responses to a class II helper peptide epitope in patients with stage III/IV resected melanoma. *Clin. Cancer Res.* 10: 5004–5013.
14. Seneviratne, S. L., L. Jones, A. S. King, A. Black, S. Powell, A. J. McMichael, and G. S. Ogg. 2002. Allergen-specific CD8<sup>+</sup> T cells and atopic disease. *J. Clin. Invest.* 110: 1283–1291.
15. Corry, D. B., and F. Kheradmand. 1999. Induction and regulation of the IgE response. *Nature* 402: B18–23.
16. Sonderstrup, G., A. P. Cope, S. Patel, M. Congia, N. Hain, F. C. Hall, S. L. Parry, L. H. Fugger, S. Michie, and H. O. McDevitt. 1999. HLA class II transgenic mice: models of the human CD4<sup>+</sup> T-cell immune response. *Immunol. Rev.* 172: 335–343.
17. Rosloniec, E. F., D. D. Brand, L. K. Myers, K. B. Whittington, M. Gumanovskaya, D. M. Zaller, A. Woods, D. M. Altmann, J. M. Stuart, and A. H. Kang. 1997. An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. *J. Exp. Med.* 182: 1113–1122.
18. Ebner, C., S. Schenk, N. Najafian, U. Siemann, R. Steiner, G. W. Fischer, K. Hoffmann, S. Szepfalusi, 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.* 154: 1932–1940.
19. Reefer, A. J., R. M. Carneiro, N. J. Custis, T. A. Platts-Mills, S. S. Sung, J. Hammer, and J. A. Woodfolk. 2004. A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *J. Immunol.* 172: 2763–2772.
20. Freidhoff, L. R., E. Ehrlich-Kautzky, D. A. Meyers, A. A. Ansari, W. B. Bias, and D. G. Marsh. 1988. Association of HLA-DR3 with human immune response to Lol p I and Lol p II allergens in allergic subjects. *Tissue Antigens* 31: 211–219.
21. Heaton, T., J. Rowe, S. Turner, R. C. Aalberse, N. de Klerk, D. Suriyaarachchi, M. Serralha, B. J. Holt, E. Hollams, S. Yerkovich, et al. 2005. An immunoepidemiological approach to asthma: identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children. *Lancet* 365: 142–149.