HLA Class II-Restricted CD4\(^+\) T Cell Responses Directed Against Influenza Viral Antigens Postinfluenza Vaccination

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HLA Class II-Restricted CD4⁺ T Cell Responses Directed Against Influenza Viral Antigens Postinfluenza Vaccination

Nancy A. Danke and William W. Kwok

The memory T cell response is polyclonal, with the magnitude and specificity of the response controlled in part by the burst size of T cells expanded from effector/memory precursors. Sensitive assays using HLA class II multimers were used to detect low-frequency Ag-specific T cells directed against influenza viral Ags in subjects immunized with the influenza vaccine. Direct ex vivo tetramer staining of PBMC from five individuals identified frequencies of hemagglutinin (HA) 306-318 tetramer binding CD4⁺ T cells in the peripheral blood ranging from 1 in 600 to 1 in 30,000 CD4⁺ T cells. These frequencies were validated by counting CFSElow, tetramer-positive T cells after in vitro expansion. Low frequency of T cells directed to other influenza epitopes, including DRA1*0101/DRB1*0401-restricted matrix protein 60-73, DRA1*0101/DRB1*0101-restricted matrix protein 18-29 DRA1*0101/DRB1*0701-restricted HA 232-244 and DRA1*0101/DRB1*0101-restricted nucleoprotein 206-217 were also determined. T cells which occurred at a frequency as low as 1 in 350,000 could be ascertained by in vitro expansion of precursors. Peripheral HA306-318-responsive T cells expanded 2- to 5-fold following influenza vaccination. Examination of phenotypic markers of the CD4⁺ T cell response directed to influenza Ags before and after influenza vaccination. We also investigated the diversity and frequency of the CD4⁺ T cell response to influenza Ags before and after influenza vaccination. Frequencies of Ag-specific T cells, measured by direct staining with tetramers, were similar to those determined by measurement of tetramer-positive, CFSElow T cells after in vitro expansion. The CD4⁺ T cell responses against influenza Ag were diverse rather than focused on a single epitope, with surprisingly low expansion size of T cell populations directed to individual epitope specificities.

Materials and Methods

Donor samples and T cell clones

All donors were healthy subjects that had recently been immunized with the Trivalent Type A and B Influenza Virus FluZone Vaccine (Aventis, Bridgewater, NJ). PBMC were isolated from heparinized venous blood within a 60-day period after vaccination or as indicated. HLA class II typing of donors was performed by reverse dot blot hybridization (9). Isolation of DR0401/hemagglutinin (HA)³ 306-318 clone B16 was described earlier (10).

HLA class II tetramers

The generation of DRA1*0101/DRB1*0401 (DR0401)-soluble class II molecules has been described earlier (2). The peptide HA306-318 PRY VKQNTLKLAT was used to load the DR0401 molecule to generate the DR0401/HA306-318 tetramer. The procedure for peptide loading was identical to those described earlier (2), and a similar approach was used to load the matrix protein (MP) 60-73 peptide LGFVFLTVPSEGR to generate Borrelia Ags could be directly detected in the synovial fluid of a Lyme disease patient (7). In this particular case, 3% of CD4⁺ T cells in the synovial fluid were reactive with tetramers for the Borrelia protein OspA.

Limitations in the use of class II tetramers for direct ex vivo detection of CD4⁺ T cells in PBMC are mainly attributable to the low signal to background noise ratio in flow cytometric assays. In the current study, we demonstrated that T cells that occur at a frequency as low as 1 in 30,000 can be detected by direct staining. We also investigated the diversity and frequency of the CD4⁺ T cell response to influenza Ags before and after influenza vaccination. Frequencies of Ag-specific T cells, measured by direct staining with tetramers, were similar to those determined by measurement of tetramer-positive, CFSElow T cells after in vitro expansion. The CD4⁺ T cell responses against influenza Ag were diverse rather than focused on a single epitope, with surprisingly low expansion size of T cell populations directed to individual epitope specificities.

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2 Abbreviations used in this paper: HA, hemagglutinin; MP, matrix protein; NP, nucleoprotein; PSA, prostate-specific Ag; VP16, viral protein 16.
the DR0401/MP67-73 tetramer (11). Soluble DRAl*0101/DRB1*0101 (DR0101) and DRAl*0101/DRB1*0701 (DR0701) molecules were also produced from Schneider cells, and the HA306-318 peptide was used to generate DR0101/HA306-318 and DR0701/HA306-318 tetramers. The MP18-29 peptide GPLKAEIAQRL and nucleoprotein (NP) 206–217 peptide MPWRENKRKR were used to generate the DR0101/MP18-29 and DR0101/NP206-217 tetramers, respectively (12, 13). The HA232-244 peptide IYWTVKPGDILL was used to generate the DR0701/HA232-244 tetramer (14). The prostate-specific Ag (PSA) 64–78 peptide QFQVQSHSPHP-PLYD and the HSV-2 viral protein 16 (VP16) 209–228 peptide IADRYY- RETARLARVLPHL were used to generate DR0401/PSA64-78 and DR0101/VP16209-228 tetramers as control tetramers for staining. Either streptavidin-PE or streptavidin-allophycocyanin (BioSource International, Camarillo, CA) were used for cross-linking. All of the tetramers used for direct staining were filtered through a Sephadex G-50 size exclusion column before use.

Flow cytometry

For direct staining of PBMC with tetramers, both freshly isolated PBMC and cryopreserved PBMC were used. Cells were washed once in RPMI 1640 plus 10% serum. Two million cells were stained in a reaction volume of 150 μl with 1–3 μl/million tetramers at 37°C for 15 min. Abs were added and staining was conducted at 4°C for an additional 20 min. Cells were then washed once and analyzed on a FACSCalibur or a FACSVantage (BD Biosciences, San Jose, CA). At least 0.5 million cells were collected for analyses. The following Abs were used: CD3-FITC, Vβ-FITC, CD4- FITC, CD27-FITC, CD62L-FITC, CD14-PE, CD25-PE, CD27-PE, CD28- PE, CD45RA-PE, CCR7-PE, CD45RA-cychrome, CD45RO-cychrome, CD4-PerCP (BD Biosciences), and CD4-allophycocyanin-Cy7 (eBioscience, San Diego, CA).

Estimation of precursor frequency of Ag-specific T cells in PBMC through CFSE-stained cells and tetramer staining

PBMC were incubated in 24-well plates at a density of 5 × 10⁴/well for 1 h. Adherent cells were used as APC. Nonadherent cells were harvested and CD4⁺ T cells were purified by negative selection using the Miltenyi Biotec CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA). CD4⁺ T cells were labeled with 0.8 μM CFSE in PBS for 10 min at 37°C. Staining was stopped by adding 100% FBS and subsequent washing. Four × 10⁴ CFSE-labeled CD4⁺ T cells were added back to the adherent cells along with the Ag of interest at a concentration of 10 μg/ml. Staining and flow cytometry of T cells was conducted after 6 or 7 days. For estimation of precursor frequency, a portion of CFSE-labeled cells was stimulated with PHA, and the cells were analyzed by FACS on day 6 or 7. PHA stimulation results in cell division with distinct fluorescence peaks, allowing determination of the number of cell divisions (2).

ELISPOT

Freshly isolated PBMC were used. Five × 10⁴ PBMC were seeded per well onto polyvinylidenum difluoride membrane 96-well plates (Millipore, Bedford, MA) precoated with 4 μg/ml anti-IFN-γ mAb (Endogen, Cambridge, MA) in the presence or absence of 10 μg/ml HA306-318. After a 24-h incubation, cells were washed away with PBS plus 0.05% Tween 20. Secondary biotinylated anti-IFN-γ mAb (0.5 μg/ml) was added and incubation was conducted at 4°C overnight. Plates were washed again and cytokine was detected with avidin-peroxidase (Sigma-Aldrich, St. Louis, MO) and 3-aminobenzidine substrate solution (Pierce, Rockford, IL). The plate was counted using the Image J software obtained through the Research Service Branch of the National Institutes of Health (Bethesda, MD).

Results

Sensitivity of class II tetramers in detecting T cells

To evaluate the sensitivity of class II tetramers for Ag-specific T cell identification, DR0401-restricted influenza HA- specific T cells (clone B16) were added in decreasing amount to the PBMC of a non-DR0401 individual. The DR0401/HA306-318 tetramer was used to detect the presence of HA306-318-specific T cells (Fig. 1) in conjunction with anti-CD4, CD14, and CD3 Abs. As indicated in Fig. 1C, D, and D, DR0401/HA306-318 tetramer bound and successfully identified the Ag-specific T cells at a frequency of 5 in 100,000 PBMC (0.005%) and 3 in 100,000 PBMC (0.003%), respectively. For Fig. 1C, 22 tetramer-positive events were observed in 190,000 CD4⁺ T cells, which indicated a sensitivity of tetramer staining of 1 in 9,000 CD4⁺ T cells, and was in close agreement with the number of T cells being added into the PBMC of a non-DRB1*0401 subject in various percentages, as indicated in the top of each panel. Staining was conducted with the DR0401/HA306-318 tetramer for A–E and with DR0401/PSA64-78 for F as described in Materials and Methods. Live cells were gated by forward and side light scattering and additionally by CD3-positive and CD14-negative Ag determinants. Eight hundred thousand total events were collected and 300,000 gated events were analyzed as shown. The number of CD4⁺ T cells in the lower right quadrant of A–F were ~190,000. The number within each panel as indicated represents the number of tetramer-positive events.

Identification of HA306-318-specific T cells in PBMC through direct tetramer staining, CFSE/tetramer staining, and ELISPOT

For direct staining of CD4⁺ T cells ex vivo, PBMC were obtained from heparinized blood of healthy normal individuals within 60 days of immunization with the influenza virus vaccine. Individuals included in this study were either positive for HLA-DRB1*0401 (subjects 0313, 0405, and 0314) or DRB1*0101 (subject 2117). Two other vaccinated subjects lacking either the DRB1*0401 or DRB1*0101 allele were used as controls (subjects 0514 and 1819). PBMC were stained with either DR0401/HA306-318, DR0101/HA306-318, DR0401/PSA64-78, or DR0101/VP16209-228 tetramers. Results of staining from four different vaccinated subjects and two controls are shown in Fig. 2. Positive tetramer staining on the vaccinated subjects was specific, as staining with irrelevant tetramers (second and fourth rows, Fig. 2) and staining of control subjects 0514 and 1819 (non-DR0401, 0101) with either the DR0401/HA306-318 or the DR0101/HA306-318 tetramers, respectively, was negative. The frequency of HA306-318-specific T cells was determined by dividing the total gated CD4⁺ events by the
number of tetramer-positive events. The frequencies of DR0401/HA306-318 T cells for the DR0401 subjects ranged from 1 in 600 (subject 0314) to 1 in 11,000 (subject 0405) CD4+ T cells. The frequency of DR0101/HA306-318 T cells was determined to be 1 in 30,000 CD4+ T cells (subject 2117). These frequency estimates depend on the setting of the cytometry quadrants that distinguish the tetramer-positive cells and tetramer-negative cells established by comparing the staining pattern with irrelevant tetramers on the vaccinated subjects as well as the pattern of staining of control subjects with the relevant tetramers. The frequency as determined by direct staining falls within a 2-fold range of the frequency based on comparison with experiments using CFSE (see below). These results were reproducible, as repeated staining with the same samples provided frequency estimates within a 2-fold range (data not shown).

Most of the subjects studied had a very low frequency of HA306-318-specific T cells. To validate that the positive events being identified by direct staining are actual Ag-responsive T cells, samples from subject 0405 and 2117 were also subjected to CFSE and tetramer analysis after a 6-day in vitro stimulation with the HA306-318 peptide (Fig. 3). Another PHA-stimulated sample was used to calculate the number of cell divisions that the T cells had undergone in the 6-day period. For sample 2117, a total of 340 DR0101/HA306-318-positive events were observed of a total of 25,000 CD4+ cells, and the tetramer-positive T cells were estimated to have undergone eight cell divisions in that period. Using the formula \(2^n\), where \(n\) is the number of cell divisions, to calculate the frequency of HA precursors, these data indicate a frequency of 1 DR0101/HA306-318-specific T cell in 18,000 CD4+ cells, in comparison to the frequency of 1 cell in 30,000 cells estimated by direct staining.

For subject 0405, 466 tetramer-positive events were observed of 25,000 CD4+ T cells in the CFSE experiment, giving a precursor frequency of 1 in 14,000 CD4+ cells. Direct staining gave a frequency of 1 in 11,000 CD4+ cells. The data support the concept that both the direct staining approach and the CFSE approach can provide a fairly good estimation of the precursor frequency of Ag-specific T cells.

In another series of experiments, we compare the use of CFSE and ELISPOT in determining the frequency of HA306-317-specific T cells. The results are shown in Table I. The frequencies of HA306-318 as determined by either CFSE/tetramer staining were also very similar to the data provided by ELISPOT assays.

We concluded that all three assays, i.e., direct staining, CFSE/tetramer staining, and ELISPOT, could provide a good estimation of the CD4+ T cell frequencies in peripheral blood.

**Frequency of HA306-318 T cells before and after immunization**

The CFSE/tetramer staining approach was used to determine HA306-318 T cell frequency before and after influenza vaccination. PBMC were obtained the day before influenza vaccination and 10 days after the vaccination in five subjects and the frequencies of HA306-318 T cells were determined (Fig. 4). An increase of 2- to 5-fold was observed in three subjects. Changes were not observed in two subjects. The frequencies of HA306-318 T cells were also followed over a period of 200–300 days on two subjects. The results are shown in Fig. 5. For both subjects, after an initial increase in frequency after immunization, there was a relative sharp decline phase, followed by a steadier phase.

**Detection of other influenza-specific T cells with tetramers**

Subject 0313 carried both the HLA-DRB1*0401 and DRB1*0701 alleles. At least two different HA epitopes which are restricted by
the HLA-DR7 have been reported (14). CFSE labeling and specific tetramers DR0701/HA232–244, DR0701/HA306–318, DR0401/HA306–318, or DR0401/MP60–73 were used to estimate the viral-specific CD4+ T cell frequency in peripheral blood. The results are illustrated in Fig. 6A. Within the 6-day in vitro culture period, there were an average of seven division cycles as evaluated by CFSE dilution. The frequencies for DR0701/HA232–244, DR0701/HA306–318, DR0401/HA306–318, and DR0401/MP60–73 were calculated to be 1 in 6,000, 1 in 30,000, 1 in 8,000, and 1 in 12,000, respectively. Although the frequency of any single epitope tends to be very low, summation of all four epitopes led to a total of 1 in 11,000 in 4,000 T cells that were directed against the virus. Similar analyses were conducted in DR0401/DR0101 subject 1902. DR0101/HA306–318, DR0401/HA306–318, DR0101/MP18–29, and DR0101/NP206–217 tetramers were used to detect influenza-specific T cells. The results are shown in Fig. 6B. Within the 7-day period, there were an average of nine division cycles. The frequencies of DR0101/HA306–318, DR0401/HA306–318, DR0101/MP18–29, and DR0101/NP206–217 were determined to be 1 in 23,000, 1 in 26,000, 1 in 29,000, and 1 in 196,000, respectively. Summation of these T cell epitopes led to a total of 1 in 11,000 influenza-specific T cells.

**Phenotype of HA306–318 T cells in peripheral blood after vaccination**

The high frequency of DR0401-restricted HA306–318 in one of the subjects (subject 0314) provided an opportunity to examine the phenotypes of T cells in the peripheral blood. These data are shown in Figs. 7 and 8. Most of the HA306–318 T cells expressed Vβ3, showing a preferential usage of this particular Vβ chain in the recognition of the HA306–318 peptide. As expected, most of the HA306–318 T cells were CD45RA−, indicating that they are Ag-experienced T cells. A small population of CD45RA+ T cells was also present. The majority of these CD45RA+ T cells are CD62L− (data not shown), suggesting that they are also memory or effector T cells; most of these T cells were also CD27+ and CD28+.

**Comparison of ELISPOT assay and CFSE/tetramer assay in estimating HA306–318-specific T cell responses**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Average Spots/10⁶ PBMC without HA306–318 Peptide</th>
<th>Average Spots/10⁶ PBMC with HA306–318 Peptide</th>
<th>Frequency of CD4⁺ Cells as Estimated by ELISPOTa</th>
<th>Frequency of CD4⁺ Cells as Estimated by CFSE/Tetramerb</th>
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<tr>
<td>1</td>
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<td>4</td>
<td>1</td>
<td>53</td>
<td>1 in 9,000</td>
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ELISPOT assays were performed in quadruplicated wells with freshly isolated PBMC.

The frequency determination was based on the assumption that 50% of PBMC were CD4⁺ T cells.

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* The frequency determination was based on the assumption that 50% of PBMC were CD4⁺ T cells.

A few fold lower compared to the DR0401-restricted response and was not examined in these experiments.

**TABLE I. Comparison of ELISPOT assay and CFSE/tetramer assay in estimating HA306–318-specific T cell responses**

**FIGURE 5.** Frequency of HA306–318-specific CD4⁺ T cells after Flu-vaccine vaccination. Frequency of HA306–318 T cells in PBMC were examined the day before vaccination and at various time points after vaccination. A, Time points were taken at days −1, 20, 60, 180, and 300. Frequency of DR0101/HA306–318 T cells was estimated by the CFSE/tetramers approach. B, Time points were taken at days −1, 10, 20, 60, and 210, and the frequency of DR0401/HA306–318 T cells was estimated.

**FIGURE 4.** Frequency of HA306–318 CD4⁺ T cells 10 days after Flu-vaccine vaccination. Frequencies of HA306–318 T cells in PBMC were examined by the CFSE/tetramers staining approach the day before vaccination and 10 days after vaccination. Each graph represents results from a single individual. The tetramer used is indicated at the top of each panel.

**FIGURE 6.** T cell frequency in peripheral blood. The results are illustrated in Figs. 6A and 6B. Within the 6-day in vitro culture period, there were an average of seven division cycles as evaluated by CFSE dilution. The frequencies for DR0701/HA232–244, DR0701/HA306–318, DR0401/HA306–318, and DR0401/MP60–73 were calculated to be 1 in 6,000, 1 in 30,000, 1 in 8,000, and 1 in 12,000, respectively. Although the frequency of any single epitope tends to be very low, summation of all four epitopes led to a total of 1 in 11,000 in 4,000 T cells that were directed against the virus. Similar analyses were conducted in DR0401/DR0101 subject 1902. DR0101/HA306–318, DR0401/HA306–318, DR0101/MP18–29, and DR0101/NP206–217 tetramers were used to detect influenza-specific T cells. The results are shown in Fig. 6B. Within the 7-day period, there were an average of nine division cycles. The frequencies of DR0101/HA306–318, DR0401/HA306–318, DR0101/MP18–29, and DR0101/NP206–217 were determined to be 1 in 23,000, 1 in 26,000, 1 in 29,000, and 1 in 196,000, respectively. Summation of these T cell epitopes led to a total of 1 in 11,000 influenza-specific T cells.
and stimulated with the appropriate peptides at 10 μg/ml presented by peripheral blood adherent cells. T cells were analyzed 6 days after in vitro stimulation. Cell divisions within that period were estimated by CFSE dye dilution of a PHA-stimulated sample (data not shown), and it was estimated that cells in A underwent seven division cycles, while cells in B underwent nine division cycles. Cells were gated on forward and side light scattering, CD3+ and CD4+ events. 40,000 and 70,000 CD4+ and CD3+ T cells were analyzed in A and B, respectively. Tetramers used are indicated at the top of each panel. The number within the panel indicates the number of tetramer-positive events.

**Discussion**

We used sensitive class II tetramer staining to detect CD4+ T cells directed against influenza viral Ags. Ag-specific T cells were detected from PBMC without in vitro manipulation, demonstrating that T cells at a frequency of higher than 1 in 30,000 CD4+ T cells could be identified by class II tetramers through direct staining. Although the number of positive events by direct staining is low, we demonstrated that the events as estimated by direct positive staining were similar to those obtained by the combination of CFSE labeling and tetramer staining after in vitro expansion.

These experiments also validate the usefulness of the CFSE labeling approach for estimation of precursor T cell frequency providing that the staining was conducted within 6 or 7 days after the in vitro expansion. We found that the frequency of Ag-specific T cells as estimated by direct staining alone or CFSE in combination of tetramer staining correlated within a 2-fold range. In another series of experiments, comparable results in determining T cell frequency were obtained between the CFSE/tetramers approach and the ELISPOT approach. These experiments suggested that all three assays, i.e., direct staining, CFSE/tetramer staining, and ELISPOT, could provide an estimation of the frequency of Ag-specific CD4+ T cells.

We used the CFSE/tetramer approach to evaluate the responses of the HA\textsubscript{306–318} T cells after influenza vaccination in five individuals. Two- to 5-fold increases in frequency of HA\textsubscript{306–318} T cells were observed in three individuals 10 days after the vaccination. In a different set of experiments, we observed an increase in the frequency of T cells directed against the MP epitope in two of the three individuals examined (data not shown). The two individuals that showed increases in MP response also had increases in HA responses, while the individual that did not show an increase in MP responses also did not have a HA response. These experiments were in line with a previous report that increases in humoral responses after influenza vaccination are only observed in 70% of the general population (15). This modest increase in CD4+ T cell expansion is in marked contrast with the large oligoclonal expansions of CD8+ T cells documented in many systems. It is possible that the increases in frequency of HA\textsubscript{306–318} T cells reflected an expansion of pre-existing HA\textsubscript{306–318} memory T cells rather than the de novo expansion of naive HA\textsubscript{306–318} T cells. It may also be notable that the influenza vaccine contains only 15 μg of HA protein, a relatively small inoculum. The frequency of HA\textsubscript{306–318} T cells was estimated by ELISPOT, could provide an estimation of the frequency of Ag-specific CD4+ T cells.
cells increased after vaccination within the first 10–20 days, followed by a biphasic decline which can be characterized as an initial rapid decrease followed by a more gradual loss in cell numbers. The increase in frequency of HA306–318 T cells in one of the subjects at day 210 might represent an asymptomatic influenza infection (Fig. 5).

The use of tetramers in conjunction with CFSE also allows estimation of low frequency events. T cells that occur at a frequency of 1 in 350,000 could be detected after 7 days of in vitro culture. Thus, T cells that occur at a fairly low frequency, but are capable of dividing continuously for a few days by in vitro manipulation, could be detected by tetramers. Both nondividing Ag-specific T cells and apoptosis of Ag-specific T cells which occurs during the in vitro culture will lead to an underestimation of T cell precursor frequency. Thus, the observed frequency by the CFSE approach may only provide a rough estimation of the actual frequency. Other in vitro manipulation, which can promote Ag-specific expansion, should enhance the detection sensitivity of class II tetramers. In the CFSE analysis experiments, a population of CFSElow and tetramer-negative cells was observed. This particular population contains up to 75% of the CFSE-negative T cells. Previous experiments demonstrated that these T cells were Ag nonspecific (16). Further additional experiments are needed to confirm that these T cells are activated through bystander activation. The current studies also emphasize the fact that the frequency of individual epitope-specific CD4+ T cells is low. For individual 1902, the frequencies of DR0101/NP206–217 and DR0101/MP18–19 were determined to be 1 in 196,000 and 1 in 350,000, respectively. T cells that occur at that frequency are beyond the detection by direct tetramer staining of ex vivo PBMC.

Staining of CD8+ T cells with class I tetramers has demonstrated that Ag class I-restricted T cells directed against a particular antigenic epitope frequently occur at a very high frequency. In subjects with acute infectious mononucleosis, T cells specific for a particular EBV lytic protein epitope comprised 44% of the total CD8+ T cells (17). CD8+ T cells that are directed against other viral epitopes and tumor Ags have also been detected at a frequency of >1% of total CD8+ T cells (18–20). In contrast to results from CD8+ T cells, reports concerning the frequency of Ag-specific CD4+ T cells in humans are limited, and it has been speculated that the burst size of CD4+ T cells upon encountering Ags are much lower compared with CD8+ T cells (21). The mean frequency of CD4+ cells that are directed against varicella zoster virus and herpes simplex virus have been determined to be 0.1 and 0.2%, respectively, in healthy subjects by intracellular cytokine assays (22). In the same study, CMV infection elicited a strong CD4+ T cell response, and experiments showed that the majority of normal subjects have ~1% of CD4+ T cells in the periphery that can respond to the CMV viruses (22). Other studies show that the pp65 protein of CMV is a major antigenic protein in the majority of CMV-seropositive individuals, and the median frequency of pp65-reactive CD4+ T cells is 0.2% of the CD4+ T cell population in healthy subjects (23). The majority of the pp65-directed CD4+ T cell responses could also be attributed to single epitopes (24). However, the strong dominant CD4+ T cell responses against pp65 as observed may be unique to CMV infection and the frequency of the T cells that are directed against a specific epitope in other viral infections can still be relatively low.

We show that CD4+ T cells that are directed against a specific epitope of the influenza virus occur at a low frequency in PBMC. T cells that are directed against the HA proteins occurred at a frequency of between 1 in 600 and 1 in 30,000. Previous analyses of HA-specific T cells in two individuals also show frequencies in this range (2). In addition to HA and M1, other influenza viral proteins included M2, neuraminidase, nucleoprotein, three different transcriptases, and two nonstructural proteins. CD4+ T cells that are directed against some of these influenza Ags have also been reported (13, 25, 26). It is also likely that multiple epitopes exist for each viral protein and that these epitopes can be presented by multiple class II alleles. Thus, the frequency of CD4+ T cells in the PBMC that are directed against the virus is higher than the frequency of a single epitope. In the current study, examinations of two subjects for the DR0401/HA306–318 epitope alone showed a frequency of 0.004% and 0.01% viral-specific T cells; when four different viral epitopes were considered, the frequency of viral-specific T cells increased to 0.01% to 0.03%, respectively. These data imply that the frequency of CD4+ T cells directed against the whole influenza virus could be at least an order of magnitude higher than those determined with four epitopes. Although the CD4+ immune response against a particular epitope is low, the overall immune response against the virus as provided by the various restriction elements and multiple epitopes could be robust and appears to be sufficient for surveillance against the virus.

The frequencies as reported here are those being measured in peripheral blood after influenza vaccination, while the frequency of antiviral CD4+ T cells in the bronchoalveolar mucosal sites can be substantially higher in a natural influenza infection. Indeed, recent studies of antiviral responses during Sendai virus infection in a mouse model indicated that up to 13% CD4+ cells in the bronchoalveolar lavage were directed against a single viral epitope at the peak of infection (27).

Previous reports have indicated a diversity of Vβ usage among DR0101-restricted HA306–318 T cell clones or lines (28, 29). The current data provided a direct ex vivo analysis of the Vβ usage of DR0401 HA306–318 peripheral T cells. Although DR0101 HA306–318 T cells utilized a variety of Vβ, 75% (85/110) of the DR0401-restricted HA306–318 T cells in PBMC expressed Vβ3. The discrepancy of the current data with the previous published information can be attributed either to the differences in the restriction element or the different approaches in determining the Vβ usage.

Phenotyping of a typical HA306–318 T cell clone indicated that these T cells were CD45RA–, CD27+, CD25+, CCRI+, CD28+, and CD62L+, markers consistent with clones that have undergone repeated stimulation. In contrast, the phenotype of HA306–318 T cells in PBMC fit in with the expected phenotypes of early activated T cells. CD45RA–, CD27+, CD25+, CD28+, and CD62L+, with a heterogeneous expression of CCRI. CCRI expression allows the T cells to home to secondary lymphoid organs, including peripheral lymph nodes. Previous studies indicated that naive T cells and central memory T cells expressed CCRI, while terminal differentiated T cells lost CCRI expression (30, 31). The heterogeneous expression of CCRI as observed in the HA306–318 T cells in PBMC classifies these T cells as early differentiated T cells in accordance with the CD27+ phenotypes. Most of the HA306–318 T cells in peripheral blood were CD25+. We speculate that T cell expansion occurred in the lymph node, where the T cells were CD25+, and that by the time of re-entry into peripheral blood, down-regulation of CD25 had already occurred. The small population of CD45RA– and CD62L+ T cells may represent central memory HA306–318 CD4+ T cells.

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