

MHC class II tetramers identify peptide-specific human CD4⁺ T cells proliferating in response to influenza A antigen

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Antigen-specific T helper cells present in peripheral blood at very low frequencies are capable of rapid clonal expansion during antigenic challenge. The exquisite specificity of this response provides for activation and expansion of a very select cohort of T cells, a feature we have used to directly identify and quantify human epitope-specific T helper cells from peripheral blood. Soluble tetramerized class II MHC molecules, loaded with an immunodominant peptide from hemagglutinin (HA) and labeled with fluorescent dyes, were constructed and used to directly identify antigen-specific T cells from influenza-immune individuals. After 7 days of proliferation in response to stimulation by HA peptide or whole influenza vaccine, cells staining positive with the HA tetramer had undergone between 6 and 9 divisions and were CD3⁺, CD4⁺, CD25⁺, and CD8⁻, characteristic of activated T helper cells responding to antigen. The HA epitope-specific component of the complex response to whole influenza vaccine represented a major subset of proliferating T helper cells. Soluble class II tetramers allow a direct approach for the analysis of immunodominant antigenic specificities. The identification of antigen-specific T helper cells in the peripheral blood provides a means for tracking the immune response against infectious agents and in autoimmune disease.

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Introduction

Selective expansion and activation of a very small number of antigen-specific precursor cells is a remarkable and essential property of the adaptive immune response. Techniques for assessing human antigen-specific T-cell responses are hampered by the requirement for specificity and sensitivity needed to detect such small cohorts of reactive cells. In model systems mice transgenic for single T-cell receptor (TCR) molecules have been used successfully to follow the evolution of the antigen-specific response and have provided much insight into mechanisms of antigen-specific expansion (1–5). Nonetheless, these approaches are limited to the study of a fixed TCR and do not solve the problem of following TCR repertoire evolution or identifying antigen-specific T cells in complex systems. Novel approaches are especially impor-

tant for studying TCR repertoire evolution in humans where the pattern of epitope-specific TCR development can determine the response to disease and risk for autoimmune disease (6, 7).

Recently, a key approach has been developed using MHC class I ligands for detecting T cells specific for soluble multimeric peptide-MHC complexes (8). A number of studies have employed soluble MHC class I molecules in identification, enumeration, and phenotyping of antigen-specific CD8⁺ T cells from peripheral blood (9–11). Comparable studies of class II-dependent CD4⁺ T-cell responses, however, have been lacking because of difficulties in the preparation of soluble class II-peptide complexes, low frequencies of antigen-specific CD4⁺ T cells, and low intermolecular affinities for MHC-peptide-TCR binding.

Previously, Crawford et al. described

an approach in designing class II molecules where the peptide of interest is covalently linked to the β -chain of the MHC molecule to ensure its placement in the peptide-binding groove during the synthesis process (12). Peptide-MHC multimers produced in this manner have been used to identify T cells from mice transgenic for an α/β TCR specific for moth cytochrome *c*. Because of the introduction of the TCR transgene, the majority of T cells are bound by the class II tetramer in this system. In contrast, frequencies of epitope-specific T cells are significantly lower in humans, necessitating a much more sensitive system to successfully follow CD4⁺ T-cell responses. CD4⁺ T cells play a critical role in initiating and guiding antigen recognition in most adaptive immune responses, as well as in response to vaccine and autoimmune stimuli. We therefore developed a general method for detection of such cells using soluble human class II tetramers and describe the properties of an epitope-specific component of the complex immune response to influenza A virus.

Methods

Construction of DR0401-leucine zipper-biotinylation site-expression vectors. Chimeric cassettes containing the coding regions for DR/leucine zipper (LZ) were made using the PCR-mediated splicing overlap technique (13, 14). To generate the soluble DRA1 chain, cDNA of DRA1*0101 was amplified in the first round using the primer pair (+) 5'-AGAATTCATGGCCATAAGTGGAGTCC-3' and (-) 5'-CCAGGTCTGCTGAC-GACTCTGTAGTCTCTGGG-3' (sharing homology with the 5' end of the basic LZ), at a concentration of 4 nM for each

Figure 1

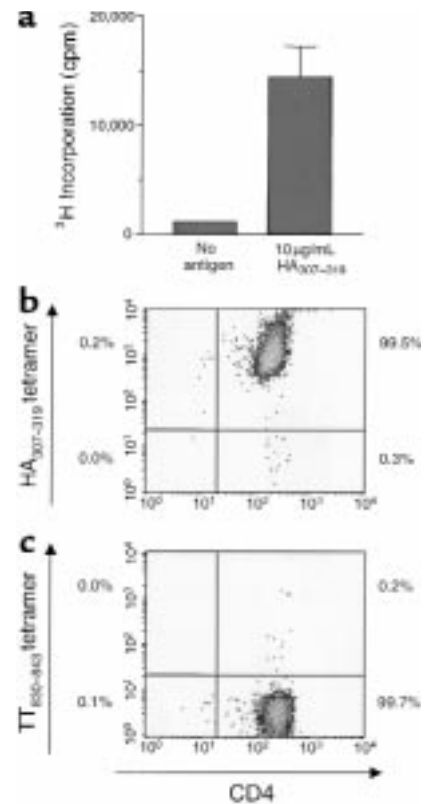
Specificity and HLA restriction of HA₃₀₇₋₃₁₉ tetramer. (a) Comparison with thymidine incorporation at 72 hours between the T-cell clone cultured with the DRA1*0101/DRB1*0401 transfected BLS-1 pulsed with no antigen (left bar) and 10 μg/mL of HA₃₀₇₋₃₁₉ peptide (right bar). Error bars indicate SEM of triplicates. In b and c, clonal cells were stained with PE-labeled tetramer for 3 hours at 37°C, washed, stained with anti-CD4, washed again, and analyzed by flow cytometry. Staining with tetramer loaded with HA₃₀₇₋₃₁₉ is shown in b, and tetramer loaded with TT₈₃₀₋₈₄₃ peptide is shown in c. In both, cells are gated on forward and side scatter, the vertical axis shows tetramer fluorescence, and the horizontal axis shows CD4 fluorescence. Percentages shown in the margins of each panel represent the percent of total cells present in each quadrant.

primer. For the second round of amplification, the first-round product was used as the initial (+) primer on the pN15LZα template, which contains the basic LZ cDNA motif, at a concentration of 10 pM, to form the DRA1/LZ chimera (pN15LZα and pN15LZβ were gifts from D. Ostrov and S. Nathenson, Albert Einstein College of Medicine, Bronx, New York, USA). The primer pair (+) 5'-AGAATTCATGGCCATAAGTGAGTCCC-3' and (-) 5'-CTGGTACCATCTACTGGGCGAGTT-3' (sharing homology with the 3' end of basic LZ), was then used to amplify the chimera. The fragment was TA cloned into pCR2.1-TOPO (Invitrogen Corp., San Diego, California, USA), sequenced, and then subcloned into the Cu-inducible *Drosophila* expression vector pRmHa-3 (gift from L.S.B. Goldstein, Howard Hughes Medical Institute, LaJolla, California, USA) using *EcoRI* and *KpnI* sites engineered into the second-round primer (underlined). To generate the soluble DRB1 chain, cDNA of DRB1*0401 was amplified in the first round using the primer pair (+) 5'-ACTCGAGCCATGGTGTGTCTGAAGTCCC-3' and (-) 5'-CCAGGTCTGCTGACGACTGCTCTGT-3' (sharing homology with the 5' end of acidic LZ), at a concentration of 4 nM for each primer. For second-round amplification, the first-round product was used as the initial (+) primer on the pN15LZβ template containing the acidic LZ cDNA motif at a concentration of 10 pM to form the DRB1/LZ chimera. The primer pair (+) 5'-ACTCGAGCCATGGTGTGTCTGAAGTCCC-3' and (-) 5'-ACAAGCTTGCCCTGAGCCAGTTCCTTTCC-3' was then used to amplify the chimera. The DRB1/LZ cassette was cloned in-frame 5' of the biotinylation sequence present in the vector pAC1 (Avidity, Denver, Colorado, USA) using *XhoI* and *HindIII* sites (underlined

in second-round primer pair). The complete DRB1/LZ/biotinylation site cassette was then amplified out using the primer pair (+) 5'-AGAATTCATGGTGTGTCTGAAGTCCC-3' and (-) 5'-CTGGTACCATCTACTGGGCGAGTT-3'. The fragment was TA cloned into pCR2.1-TOPO, sequenced, and then subcloned into the *Drosophila* expression vector pRmHa-3 using *EcoRI* and *KpnI* sites (underlined).

Generation of DRA1*0101/DRB1*0401 tetramers. The chimeric cDNAs in the Schneider expression vectors pRmHa-3, together with the plasmid pUCHsneo (gift from M. McKeown, Salk Institute, San Diego, California, USA), which carries the neomycin resistance marker, were cotransfected into Schneider cells S-2 (gift from D. Zaller, Merck Research Laboratories, Rahway, New Jersey, USA) by standard calcium phosphate transfection techniques. Cells were selected with G418 at 2 mg/mL. Cells were expanded and grown to a density of 10⁷ cells/mL. CuSO₄ was added at a concentration of 1 mM to induce the production of soluble class II molecules. The DR0401 molecules were purified by affinity chromatography using L243 as described previously (15).

The class II molecules were concentrated to 2 mg/mL and then dialyzed against 10 mM Tris, pH 8.0, 10 mM NaCl. The protein was then biotinylated using the Bir A enzyme according to the manufacturer's conditions (Avidity, Denver, Colorado, USA) (16). The excess biotin was removed by dialysis. The biotinylated DR0401 molecules were then loaded with peptide by incubation for 72 hours at 37°C with 10-fold molar excess of either hemagglutinin peptide residues 307–319 (HA₃₀₇₋₃₁₉) or tetanus toxoid peptide residues 830–843 (TT₈₃₀₋₈₄₃) in 100 mM NaPO₄, pH 5.5, and 0.2% *n*-octyl-



–D-glucopyranoside. Class II molecules were then incubated overnight at room temperature with phycoerythrin (PE)-streptavidin (BioSource International, Camarillo, California, USA) at an 8:1 molar ratio to allow the formation of tetrameric class II peptide complexes.

Characterization and staining of HA₃₀₇₋₃₁₉-specific T-cell clone. HA₃₀₇₋₃₁₉-specific T cells were cloned from a DRB1*0401, DRB1*0101 individual who had been immunized against influenza virus 8 months earlier by limiting dilution against autologous irradiated antigen-presenting cells (APCs) as described previously (17). To confirm the specificity of the clone, clonal cells were stimulated with 10 μg/mL HA₃₀₇₋₃₁₉ using a transfected bare lymphocyte syndrome cell line (BLS-1), which expresses the sole class II molecule DRA1*0101/DRB1*0401 (18). ³H-thymidine incorporation was measured at 72 hours. Clonal cells were stained with 1 μg PE-labeled tetramer for 3 hours at 37°C in 50 μL of culture media. Cells were then washed in PBS containing 1% FBS and 0.1% NaN₃, and stained with fluorochrome-labeled anti-CD4 (PharMingen, San Jose, California, USA). After a 30-

minute incubation, cells were washed again and analyzed using a Becton Dickinson FACSCalibur flow cytometer (San Jose, California, USA).

Isolation, stimulation, and staining of PBMC. PBMC from the same DRB1*0401, DRB1*0101 individual from which the clone was derived and a second individual (DRB1*0401, *0401) were separated from heparinized venous blood by gradient centrifugation (Lymphoprep; Nycomed Pharma AS Diagnostics, Oslo, Norway). Cells were cultured in RPMI-1640 (GIBCO BRL, Rockville, Maryland, USA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/mL penicillin/streptomycin and 15% vol/vol pooled human serum. Adherent cells were prepared by plating out PBMC at 5×10^6 cells per well in 24-well plates for 1 hour. Nonadherent cells were removed using a transfer pipette. Adherent cells were incubated for 3 hours with either

10 µg/mL HA₃₀₇₋₃₁₉ peptide, whole influenza vaccine containing 11 µg/mL HA (Connaught Laboratories Inc., Swiftwater, Pennsylvania, USA), or a maximally stimulating dose of whole tetanus toxoid. The nonadherent fraction was passed through a nylon wool column, washed twice with serum-free PBS, and stained with 0.8 µM 5- (and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon, USA) for 10 minutes at 37°C. Staining was stopped by adding 100% FBS and subsequently washing the cells twice in RPMI-1640 culture media. CFSE-stained nylon wool-purified T cells were then added back to the adherent cells at a density of 2.5×10^6 cells per well. Following 7 days of culture, cells were stained with PE-labeled tetramer and combinations of fluorochrome-labeled anti-CD3, -CD4, -CD8, and -CD25 (PharMingen; Becton Dickinson Immunocytometry

Systems, San Jose, California, USA) as described above and analyzed by flow cytometry.

Calculation of cell divisions and precursor frequency. A portion of CFSE-stained cells was stimulated with 2.5 µg/mL phytohemagglutinin (PHA) and 10 U IL-2 and examined by FACS on day 7. Polyclonal stimulation of T cells with PHA and IL-2 results in cell division with distinct CFSE fluorescence peaks, allowing determination of the mean CFSE fluorescence for each generation. These values were used to calculate the average number of cell divisions in cells stimulated with antigen. Precursor frequency was estimated by dividing the number of tetramer-positive cells by 2^x , where x is the average number of cell divisions, to determine the absolute number of precursors for the tetramer-positive cells, and then dividing this value by the total number of cells analyzed.

Figure 2

HA₃₀₇₋₃₁₉ tetramer identification of antigen-specific cells in relation to CFSE fluorescence. Nylon wool-purified T cells, labeled with CFSE before culture with autologous adherent cells and antigen, were stained on day 7 with PE-labeled HA₃₀₇₋₃₁₉ tetramer and analyzed subsequently by flow cytometry. Each row shows cells from a different stimulating antigen for 2 different individuals: (a) 10 µg/mL HA₃₀₇₋₃₁₉ peptide, (b) whole influenza vaccine containing 11 µg/mL HA, (c) whole TT at a maximally stimulating dose. In all panels cells are gated on forward and side scatter, the vertical axis shows PE fluorescence of the HA₃₀₇₋₃₁₉ tetramer, and the horizontal axis shows CFSE fluorescence over a 4-decade logarithmic scale. In addition, the horizontal axis shows the corresponding number of cell divisions, with “P” depicting the undivided parent population. This scale was calculated from the distinct CFSE fluorescence peaks produced by polyclonal stimulation with PHA and IL-2 as described in Methods. Percentages shown in the margins of each panel represent the percent of total cells present in each quadrant. The panels depict results from representative individual experiments.

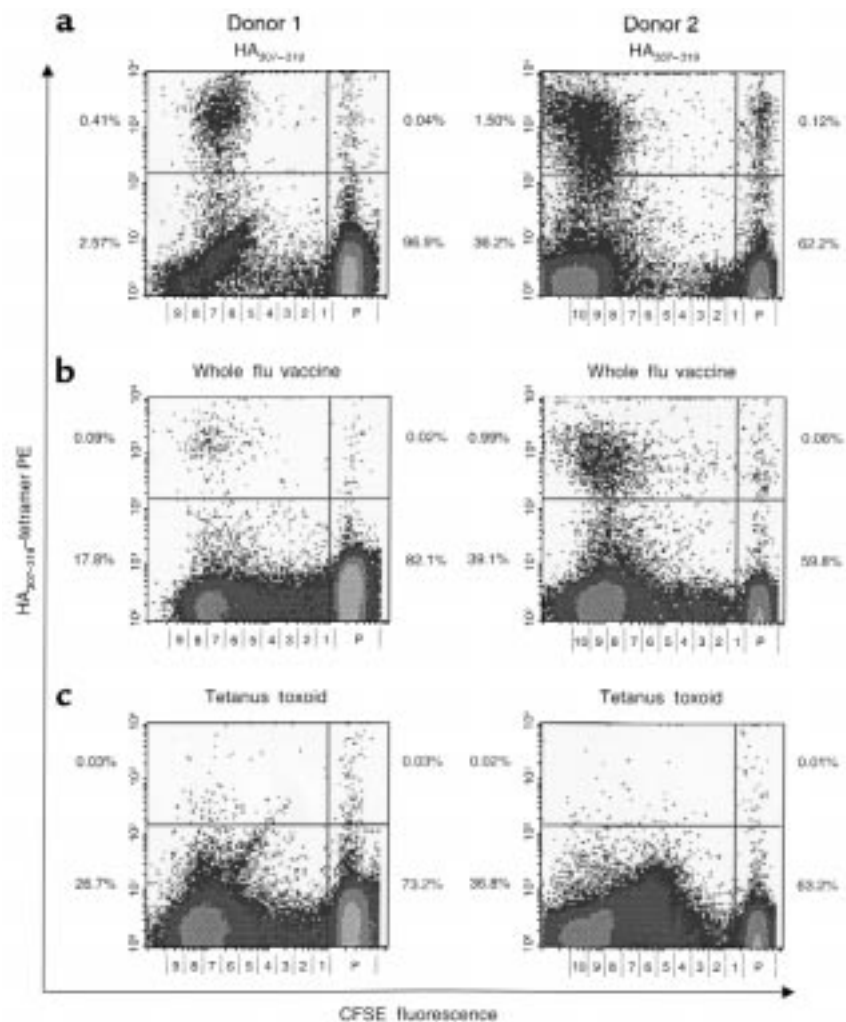
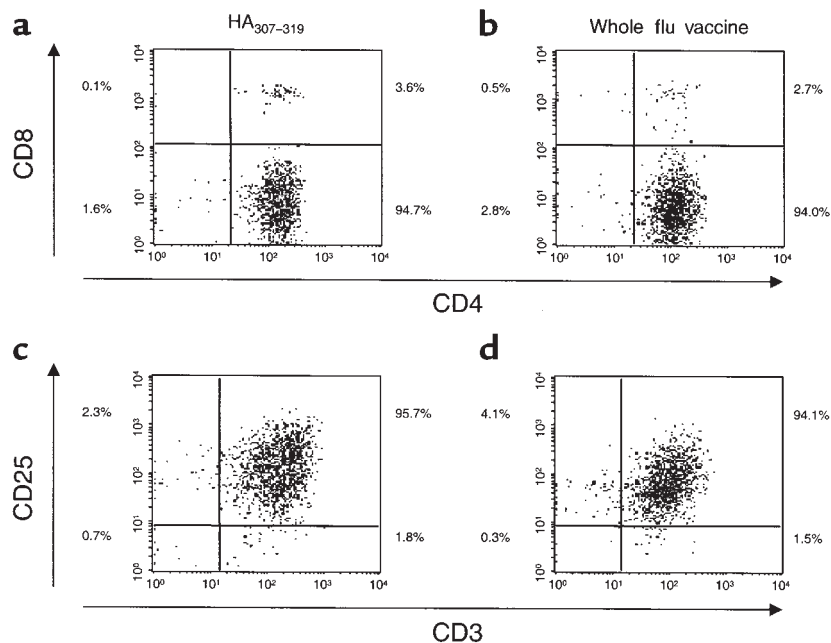


Figure 3

Phenotypic characterization of HA₃₀₇₋₃₁₉ tetramer-specific cells. Nylon wool-purified T cells were labeled with CFSE before culture with autologous adherent cells and antigen. On day 7, cells were stained with PE-labeled tetramer and then stained with combinations of fluorochrome-labeled anti-CD3, -CD4, -CD8, and -CD25 antibodies before flow-cytometry analysis. All panels are gated on tetramer-positive cells. Upper panels show CD8 versus CD4 staining in cells stimulated with (a) HA₃₀₇₋₃₁₉ peptide, and (b) whole influenza vaccine, whereas lower panels show CD25 versus CD3 staining in cells stimulated with (c) HA₃₀₇₋₃₁₉ peptide, and (d) whole influenza vaccine. Boundaries for positive and negative populations were determined by controls. Percentages shown in the margins of each panel represent the percent of total cells present in each quadrant. The panels depict results from a representative individual experiment.



Results and Discussion

Earlier studies have shown that peripheral blood lymphocytes from individuals with previous exposure to influenza A virus generate a class II DR-restricted T-cell proliferative response to the HA₃₀₇₋₃₁₉ epitope (19). This conserved peptide can induce a proliferative response in a number of different DR haplotypes, including DR1, DR4, DR5, and DR7 (20).

To detect T cells specific for the HA₃₀₇₋₃₁₉ peptide presented in the context of DR4, we synthesized class II DRA1*0101/DRB1*0401 tetramers loaded with HA₃₀₇₋₃₁₉ peptide. We tested the specificity of the HA₃₀₇₋₃₁₉ tetramer using a DRB1*0401-restricted human T-cell clone specific for HA₃₀₇₋₃₁₉. As shown in Figure 1a, the clone demonstrated antigen-specific proliferation against HA₃₀₇₋₃₁₉ in the context of DRB1*0401 expressed in a BLS-1 cell line (18). We stained the HA₃₀₇₋₃₁₉-specific clone using DRB1*0401 tetramers loaded with the HA₃₀₇₋₃₁₉ peptide. As shown in Figure 1b, virtually all the cells stained positive for the HA₃₀₇₋₃₁₉ tetramer and CD4, consistent with the phenotype of the clone. As a control we also constructed a DRB1*0401 tetramer loaded with TT₈₃₀₋₈₄₃ peptide (21). As illustrated in Figure 1c, none of the HA₃₀₇₋₃₁₉ clonal cells stained positive for the TT₈₃₀₋₈₄₃ tetramer.

We then tested the ability of the HA₃₀₇₋₃₁₉ tetramer to detect antigen-specific T cells collected from the peripheral blood of 2 DRB1*0401 donors, including the same donor from which the HA-specific clone was derived. Nylon wool-purified T cells from peripheral blood were stained with CFSE, a fluorescent dye that stably binds cytoskeletal actin (22). CFSE-stained cells were cultured with autologous adherent cells pulsed with HA₃₀₇₋₃₁₉ peptide, whole influenza vaccine, or TT. After 7 days of culture, HA₃₀₇₋₃₁₉ tetramers were used to stain the cells before analysis with flow cytometry. Each time a cell divides, CFSE is apportioned equally among daughter cells, resulting in a halving of CFSE fluorescence. Therefore, the number of cell divisions can be determined by comparing the resultant CFSE fluorescence to the original fluorescence of the parent population. As shown in Figure 2, all 3 antigens induced cell proliferation as indicated by populations of cells with decreased CFSE fluorescence, shown on the horizontal axis. The vertical axis shows the fluorescence from the PE-labeled HA₃₀₇₋₃₁₉ tetramers, and the 2 columns represent equivalent experiments done on cells from the 2 donors. As shown in the upper-left quadrants of Figure 2, a and b, the labeled tetramer clearly identified a significant number of HA₃₀₇₋₃₁₉-

specific cells in both the HA₃₀₇₋₃₁₉ and whole influenza vaccine samples in both donors. To our knowledge, this is the first time epitope-specific T helper cells have been directly seen in a stimulation of human lymphocytes taken from the peripheral blood.

Examination of the cells stimulated with HA₃₀₇₋₃₁₉ peptide showed that in both individuals around 90% of the tetramer-binding cells were in the divided population, reflecting the specific expansion of this cohort of T cells. The observed number of cell divisions for a given CFSE fluorescence is shown along the horizontal axis for all figure parts. In donor 1, the tetramer-binding population divided an average of 6.5 times during the 7-day culture as calculated by CFSE fluorescence, whereas in donor 2 the tetramer-binding cells divided an average of 9 times. This difference in the numbers of divisions accounts for the greater number of divided cells seen in donor 2. The calculated precursor frequency of DRB1*0401 tetramer-specific cells is similar for the 2 individuals, ranging between 3 and 5 per 100,000 cells, depending on the individual experiment.

The tetramer-binding population of dividing cells comprised a distinct portion of the total dividing cells in the HA₃₀₇₋₃₁₉ stimulated sample. In donor 1, some of the divided cells that are tetramer negative likely represent T

cells specific for HA₃₀₇₋₃₁₉ in the context of DRB1*0101 because the donor haplotype is DRB1*0401, DRB1*0101, and DR1 has been described as capable of presenting the peptide (23). In addition, background levels of proliferation of between 1% and 7% of the total cells were seen even in control samples where antigen was not added, depending on the individual (data not shown). IL-2 and other cytokines liberated by the antigen-specific HA₃₀₇₋₃₁₉ cells would likely increase this background through increased bystander activation and proliferation.

In cells stimulated with whole influenza vaccine (Figure 2b), there was likewise a definite population of tetramer-positive cells for both individuals, shown in the upper-left quadrant. Therefore, even in the context of a vigorous and complex proliferative T-cell response to viral antigens, the specific T cells corresponding to an immunodominant epitope are readily identified. There was no tetramer labeling of cells stimulated with TT despite vigorous proliferation (Figure 2c). These results indicate that the tetramer detection method is both specific and sensitive for the population of T cells reactive toward HA₃₀₇₋₃₁₉.

The use of tetramer staining, together with flow cytometry, to identify antigen-specific cells permits simultaneous analysis of cells using additional fluorochromes. This additional phenotypic analysis can provide important information about an antigen-specific response such as the type of T cell involved, presence of activation or other markers, and cytokine production through intracellular staining. In this study we examined surface expression levels of CD3, CD4, CD8, and CD25 to further characterize the cells identified by the HA₃₀₇₋₃₁₉ tetramer. The data in Figure 3 is gated to show only cells identified as HA₃₀₇₋₃₁₉-tetramer positive. Figure 3, a and b, shows that almost all the tetramer-positive cells in both the HA₃₀₇₋₃₁₉ peptide-stimulated and whole influenza vaccine-stimulated samples are CD4⁺ CD8⁻. Similarly, Figure 3, c and d, shows that the majority of tetramer-positive cells are CD3⁺ CD25⁺ for both samples. We conclude that the DRB1*0401-HA₃₀₇₋₃₁₉ tetramer is

bound almost entirely to class II-restricted, activated, and proliferating T cells, as expected, lending further support to the specificity of the tetramer detection method.

The concomitant use of peptide-MHC class II tetramers and CFSE staining provides a powerful tool to assess and dissect antigen-specific T-cell responses. Direct identification of peptide-specific proliferation in primary cultures avoids the need for limiting dilution analysis to calculate precursor frequencies and permits simultaneous phenotypic analysis of the antigen-specific cells using flow cytometry. The present study demonstrates the immunodominance of the HA₃₀₇₋₃₁₉ epitope in the context of the complex response against whole influenza vaccine and illustrates how tetramers can be used to directly identify immunodominant antigenic specificities. The potential for loading different peptides into the tetramers suggests multiple applications, although the stability of specific peptide-class II tetramers is likely to vary among peptides. The use of class II tetramers provides a means for understanding on a much more detailed level the immune response against infectious agents and in autoimmune disease.

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