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Ultrasensitive Detection and Phenotyping of CD4+ T Cells with Optimized HLA Class II Tetramer Staining

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HLA class I tetramers have revolutionized the study of Ag-specific CD8+ T cell responses. Technical problems and the rarity of Ag-specific CD4+ T cells have not allowed the potential of HLA class II tetramers to be fully realized. Here, we optimize HLA class II tetramer staining methods through the use of a comprehensive panel of HIV-, influenza-, CMV-, and tetanus toxoid-specific tetramers. We find rapid and efficient staining of DR1- and DR4-restricted CD4+ cells and clones and show that TCR internalization is not a requirement for immunological staining. We combine tetramer staining with magnetic bead enrichment to detect rare Ag-specific CD4+ T cells with frequencies as low as 1 in 250,000 (0.0004% of CD4+ cells) in human PBMCs analyzed directly ex vivo. This ultrasensitive detection allowed phenotypic analysis of rare CD4+ lymphocytes that had experienced diverse exposure to Ag during the course of viral infections. These cells would not be detectable with normal flow-cytometric techniques. The Journal of Immunology, 2005, 175: 6334–6343.

Lymphocytes recognize antigenic peptides presented by MHC molecules on the surface of APCs with their unique TCR. The interaction between the TCR complex and peptide-MHC (pMHC) is weak and has a fast dissociation rate (1–3). As a result, soluble recombinant monomeric pMHC molecules do not stably adhere to the surface of cognate T cells. This limitation can be overcome by increasing the valency of interactions by multimerization of biotinylated pMHC molecules. Multimerized pMHC can be conjugated to fluorochromes to allow detection by FACS (4). Such multimeric pMHC complexes were able to bind multiple TCRs on Ag-specific cells and thus lowered the dissociation rate sufficiently to be used as an immunological stain. The development of these peptide-HLA tetramer complexes marked the beginning of a new era in the study of Ag-specific T cells, and the capacity to physically detect Ag-specific T cells in diverse states of activation or function makes the use of HLA class I, and potentially class II tetramers, more powerful than techniques that rely on the ability of cells to respond to synthetic Ags; a quality that may define only a subset of the relevant cell population (5, 6).

The application of this powerful technology to the study of Ag-specific CD4+ T cells has not been fully realized due to numerous technical issues. Production of MHC class II (MHCI) complexes has been more difficult than MHCI. The MHCI H chain can be expressed in Escherichia coli as a soluble product by truncation of the membrane-spanning domain and folded readily around the antigenic peptide in the presence of β2-microglobulin (4). pMHCI molecules consist of two polymorphic chains (α and β), which have been more difficult to refold around the antigenic peptide. E. coli expression of the of MHCI alleles has been inefficient (7). However, correctly folded molecules can be manufactured in insect and mammalian cells (8, 9). These efforts allowed successful staining of in vitro-stimulated CD4+ T cell lines and clones (9–16). Despite these advances, the direct detection of Ag-specific CD4+ T cells in PBMC remained difficult, mainly because the cells are rare in peripheral blood (17–20).

Virtually all studies reporting MHCI tetramer staining used high concentrations of tetramer (20 μg/ml or more), long incubation times (1–5 h) and temperatures of 23 or 37°C (8–10, 13–17, 21–27). In these studies, HLA class II tetramers stained cognate T cell clones poorly or not at all at 4°C (12, 21, 25). Cameron et al. (25) further suggested that HLA class II tetramer staining was dependent on active cellular processes by demonstrating blockade of tetramer staining when endocytosis and cytoskeletal rearrangements were disrupted. Because the association rates of tetrameric TCR/pMHCI interactions fall within a similar range to those seen for TCR/pMHCI (28), the kinetics of HLA class II tetramer staining should resemble those of HLA class I tetramers. Efficient staining of CD8+ T cells was reported at 4°C, and incubation of cells with tetramer for 10 min is sufficient for complete staining (29, 30).

In this study, we determine the conditions required for optimal HLA class II tetramer staining of DR1- and DR4-restricted CD4+ T cells specific for HIV, CMV, tetanus toxoid (TT), and influenza virus. We find rapid and efficient staining of CD4+ T cells in conditions similar to those reported for HLA class I staining of CD8+ T cells. Active cellular processes such as internalization are...
not a requirement for staining. We test and apply an optimized method in combination with a magnetic bead enrichment technique to detect HIV- and CMV-specific CD4+ T cells at frequencies below 1 in 100,000 in direct ex vivo human PBMC samples. This technology allowed us to compare the phenotypic profile of rare virus-specific CD4+ T cells from different infections.

Materials and Methods

Patients and HLA typing

Heparinized blood was obtained from HIV-infected patients who were recruited at St Mary’s Hospital, London, as described elsewhere (20), the SPARTAC Trial or from healthy, HIV-negative donors. Informed consent was obtained from all participants, and the study had full ethical approval. PBMC were isolated by density gradient centrifugation of blood layered over Lymphoprep (Axis Shield). High-resolution HLA class II genotypes were determined for each study participant by PCR using sequence-specific primers (31).

Cell lines and clones

Fresh PBMC were depleted of CD8+ cells using anti-CD8-conjugated magnetic beads (Dynal) and stimulated with 1–2 μM peptide in RPMI 1640 medium containing 10% heat-inactivated human AB+ serum. When lines were cultured from HIV-infected patients, 0.4 μM indinavir (Merck) and 0.5 μM zidovudine (GlaxoSmithKline) were added to the medium. The plates were incubated at 37°C in 5% CO2, and after 3 days, the medium was supplemented with 100 U/ml IL-2 (Proleukin, Chiron) and 5% human T cell culture supplement (T-STIM) (BD Biosciences). Medium was exchanged as necessary. Cell lines were resuspended with peptide, IL-2, human T cell culture supplement (T-STIM), and irradiated autologous PBMC every 12–14 days. For generation of CD4+ T cell clones, cell lines were stimulated with 4 μM peptide for 4 h, and IFN-γ and IL-2-producing cells were selected using a cytokine detection and enrichment method (Millenyi Biotech). Ag-specific (IFN-γ and IL-2)-producing CD4+ cells were plated at limiting dilution in medium containing 10% human AB+ serum, 2 × 105 irradiated mixed lymphocytes/ml, 100 U/ml IL-2, 2% T-STIM, and 1 μg/ml PHA (Sigma-Aldrich). Cell lines and clones were tested for Ag specificity by IFN-γ ELISPOT, intracellular cytokine staining (ICS), or HLA class II tetramer staining. ELISPOT and ICS assays were performed with either peptides or recombinant proteins as Ag as described (20). The cell lines and clones used in this study are listed in Table I. All cell lines were used for experiments after two or three rounds of antigenic stimulation, and all experiments were done at least 7 days after restimulation of cell lines and clones. The influenza H1N1-319-specific CD4+ T cell clone HA1.17 (32) was kindly donated by J. Lamb (Medical Research Council for Inflammation Research, University of Edinburgh, Edinburg, U.K.).

MHCI tetramers

DRB1*0101 and DRB1*0401 tetramer MHCII tetramers were purchased from Beckman Coulter. DRB1*0101 tetramers were complexed to the HIV p24 Gag peptide p24.17 (aa 294–313; FRDYVRDVFYKTLRAEQASQD), the p24 Gag peptide p24.14 (aa 264–283; KRWILGLNKIVRMYSPTSI), the TT830–843 peptide (QYIKANSKFIGITE), the influenza hemagglutinin (HA)307–319 peptide (PKYVKQNTLKLAT) and the CMV pp65 108–127 peptide (PKYVKQNTLKLAT) and the CMV pp65 108–127 (aa 1248–1261; GYKVLNLPSVATL) was used as control. These tetramers are referred to as p24.4-DR4 and HCV1-DR4, respectively. All tetramers were PE-conjugated and supplied at a concentration of 100 μg/ml.

Tetramer staining and flow cytometry

Unless stated otherwise, cell lines and clones were incubated with 2 μg/ml HLA class II tetramer for 30 min at 37°C in PBS/1% FCS or PBS/0.5% BSA buffer. The cell surface marker Abs CD4-allophycocyanin or CD4- FITC and 7-aminoactinomycin D (7-AAD) (all BD Pharmingen) were added for the last 20 min or for 20 min after the cells were washed with cold PBS/1% FCS. Stained cells were washed with cold PBS/1% FCS and fixed in 1% PBS/formaldehyde. At least 30,000 cells were acquired on a FACS Calibur flow cytometer (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences).

Cell surface tetramer decay assay

CD4+ T cells were precultivated on ice for 5 min with chilled azide buffer (0.1% NaN3, 0.5% BSA in PBS) and incubated with 1 μg/ml tetramer in azide buffer for 2 h on ice. Stained cells were washed twice in chilled azide buffer, split into two aliquots, and placed at room temperature. To one aliquot, 100 μg/ml anti-HLA-DR Ab (clone L243; BD Biosciences) was added, and cells were taken at time points 0, 1, 2, 5, 10, and 30 min, resuspended in PBS, and acquired on a flow cytometer.

Microscopy

For microscopy, CD4+ T cells were stained at 37°C or on ice in azide buffer as described for each experiment and washed with PBS/0.5% BSA. For imaging, stained cells were suspended in PBS/0.5% BSA in eight-well chambers (Labtek; Nunc). A Zeiss 200M/Universal Imaging system with a x63 objective was used for wide-field fluorescence microscopy. PE fluorescence was detected using a 535/50 excitation, 610/75 emission, and 565LP dichroic filter set (Chroma). To cover the entire three-dimensional surface of the cell, z-stack fluorescent images were taken (21 individual planes; 1 mm apart). Data were analyzed using Metamorph software. Data were evaluated for at least 20 cells in each experimental condition.

Detection of rare cells by magnetic bead enrichment and phenotypic staining

Magnetic bead enrichment of tetramer-positive CD4+ T cells was done as described previously (20). Briefly, the cells were incubated with 5 μg/ml HLA class II tetramer (to compensate for large cell numbers). Anti-CD4-allophycocyanin, anti-CD19-PerCP, and anti-CD14-PerCP Abs and 7-AAD (all from BD Biosciences) were added during the last 20 min for the 120-min staining at 23°C and for 20 min after a wash for the 20-min staining at 37°C. For phenotypic staining of tetramer-positive CD4+ cells, anti-CCR7-FITC (R&D Systems) and anti-CD27-FITC and anti-CD28-FITC (BD Pharmingen) were added, and cells were taken at time points 0, 1, 2, 5, 10, and 30 min, resuspended in PBS, and acquired on a flow cytometer.

Table I. Ag-specific CD4+ T cell lines and clones

<table>
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<th>Cell Line/Clone</th>
<th>Line/Clone</th>
<th>HLA-DRB1 Typea</th>
<th>Epitope Specificty</th>
<th>Specific Tetramer</th>
<th>Epitope Sequence</th>
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<td>HIV Gag p2429a–313</td>
<td>p24.17-DR1</td>
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<td>p24.17-DR1</td>
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<tr>
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<td>Line</td>
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<td>T230-DR1</td>
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<td>Clone</td>
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a HLA restriction of epitope specificity is in boldface type.
Results

Staining of Ag-specific CD4+ T cells with HLA class II tetramers is highly specific

We studied the labeling of Ag-specific CD4+ T cells with HLA class II tetramers at different experimental conditions. Cell lines and clones specific for antigenic peptides encoded by HIV type 1 (HIV-1) p24 Gag, influenza HA, TT, or human CMV pp65 were generated and tested for specificity by IFN-γ ELISPOT assay or ICS (Fig. 1A). Clones and CD4+ T cell lines were stained with their corresponding peptide-HLA class II tetramer (Fig. 1B). Although not identical, the frequencies of HLA class II tetramer-positive CD4+ cells were comparable with the frequencies of IFN-γ-producing cells as measured by ICS (Fig. 1 and data not shown). Staining of all cell lines and clones with peptide-mismatched, DRB1-allele-matched tetramers yielded CD4+ tetramer+ populations that never exceeded 0.2% (Fig. 1B). The cell lines and clones and peptide-matched HLA class II tetramers are shown in Table I.

Optimizing the use of HLA class II tetramers for staining T cells

A wide range of HLA class II tetramer concentrations are reported to be required for adequate staining of specific CD4+ T cells. Some investigators have used tetramer concentrations of 20 μg/ml or more (8–10, 12, 13, 15–18, 21, 23–26). Others have used lower concentrations (11, 14, 20, 22, 33, 34). Meyer et al. (17) found that certain Borrelia-specific CD4+ T cell clones could be stained with as little as 0.2 μg/ml. To assess the optimal HLA class II tetramer staining concentrations for a range of cell lines and clones, we incubated CD4+ T cells with tetramer ranging from 0.01 to 10 μg/ml in a total volume of 100 μl (Fig. 2) for 30 min at 37°C. Although some variation was seen, 2 μg/ml tetramer was sufficient to label Ag-specific cells within their respective cell lines and to label true clones as well. At 5–10 μg/ml and higher concentrations, background staining increased for all tetramers as nonspecific cells were also stained (Fig. 2A and data not shown).

HLA class II tetramer staining is rapid

CD8+ T cells can readily be labeled with HLA class I tetramers by incubation at 37°C for 10 min (29, 35) and staining can be detected as early as after 30 s (29). To test the rate at which HLA class II tetramers stained CD4+ T cells, we incubated all cell lines and clones with 2 μg/ml HLA class II tetramer at 37 and 23°C for 1–120 min. We found efficient staining of CD4+ T cells of all specificities within 10 min at 37°C. This was observed with the TT, the influenza HA and CMV pp65 DR1 tetramers as well as the HIV Gag p24 DR1 and DR4 tetramers when used to stain all CD4+ cell lines and clones (Fig. 3, A and B). Although all cells were stained after 10 min, the mean fluorescence intensity (MFI) continued to increase up to 120 min after starting incubation with the tetramer (data not shown). Notably, the proportion of tetramer+ CD4+ cells also increased with time, suggesting that internalization of pMHCII-TCR complexes was taking place (data not shown). At 23°C, tetramer staining was slower for most specificities, although the HIV-specific Ox24-p24.17 and K37-p24.4 cell lines and the influenza-specific HA1.7 clone were fully stained after 10 min. At 23°C, complete staining of all CD4+ T cells was seen only after 30–60 min for the other cell lines and clones (Fig. 3C), and the MFI was generally lower than when cells were stained at 37°C (data not shown).

HLA class II tetramer staining does not require internalization

Labeling of Ag-specific CD8+ T cells with HLA class I tetramers can be efficient at 4°C (30, 35). However, some earlier investigators failed to label Ag-specific CD4+ T cells with HLA class II tetramers.
FIGURE 2. Concentration titration of HLA class II tetramers. A, FACS dot plots of the CD4⁺ T cell line Ox24-p24.17 stained with the indicated concentrations of p24.17-DR1 tetramer at 37°C for 30 min. The percentage of cells falling into the upper right quadrant is indicated in each plot. B, Graphical representation of the MFI obtained from concentration titrations of five HLA class II tetramers. C, Graphical representation of the percentage CD4⁺ tetramer⁺ cells recorded for each tetramer.

FIGURE 3. HLA class II tetramer staining is rapid. FACS density plots (A) and histograms (B) showing efficient HLA class II tetramer staining of Ag-specific CD4⁺ cell lines and clones after a 10-min incubation at 37°C. C, Graphical representation of the percent maximum CD4⁺ tetramer⁺ cells measured when cells were stained at 37°C (upper chart) and 23°C (lower chart) for the indicated time periods.
tetramers at 4°C (12, 21, 25). By contrast, we stained CD4⁺ T cell lines and clones with tetramer at 4°C (Fig. 4). The HIV p24-specific cell lines Ox24-p24.17 and K37-p24.4 and Ox97-clone10 could be stained at 4 and at 0°C. Successful HLA class II tetramer staining was also seen when the TT-specific cell line JF-TT830 and the influenza-specific cell line JF-HA were incubated with their cognate tetramers at 4°C. Staining at 4°C was markedly slower than at 23 or 37°C and resulted in considerably lower fluorescence intensities (Fig. 4C).

Successful staining at 4°C suggests that CD4⁺ T cells can be labeled with surface-bound tetramer and that this process does not require receptor internalization. To assess this further, we stained prechilled, azide-treated cells with HLA class II tetramer in azide buffer on ice. Following removal of unbound tetramer and blocking of tetramer rebinding, the decay of cell surface-bound tetramer was followed by monitoring the decrease of the PE-fluorescence. Azide-treated CD4⁺ T cells from the HIV-Gag p24-specific Ox97-clone10 and JF-TT830 were labeled with HLA class II tetramer on ice (Fig. 5 and not shown). When rebinding of unbound tetramer was blocked with anti-HLA-DR Ab, the fluorescence of tetramer-labeled cells fell, suggesting tetramer dissociation from the cell surface. This effect was seen to a lesser degree when no competitor Ab was added (Fig. 5).

To confirm that HLA class II tetramer can bind CD4⁺ T cells without being internalized, we performed fluorescence microscopy of HLA-DR1-restricted, HIV p24-specific Ox97-clone10 stained with tetramer at 37°C or in azide buffer on ice. When cells were labeled at 37°C for 60 min, clear PE-clusters were visible that were not associated with the cell membrane when the mid-cell z-section of PE-fluorescence and differential interference contrast (DIC) images were overlaid (Fig. 6). These clusters typically localized to compartments within the cytoplasm, suggesting internalized TCR/tetramers. In contrast, when azide-treated cells were stained on ice, PE-clusters of lower fluorescence intensity than those seen at 37°C were only visible along the outer perimeter of the cells. These clusters aligned with the plasma membrane when the DIC and mid-cell z-section of PE-fluorescence images were overlaid, suggesting that visible tetramers were membrane-bound and not internalized.

Sensitive detection of rare CD4⁺ T cells

The frequencies of most CD4⁺ Th cell populations are markedly lower than those seen for CD8⁺ T cells (36). Because the lower detection limit of flow cytometric techniques is ~0.02% (17, 37, 38), confident detection of many Ag-specific CD4⁺ T cell responses with conventional HLA class II tetramer staining and flow cytometry is impossible. Recently, the use of HLA class I and II tetramers and a magnetic bead enrichment technique has improved

**FIGURE 4.** Staining of cell lines and clones with HLA class II tetramer at 4°C. A, FACS density plots showing staining of cell lines for 120 min at 4°C. B, Histograms of the DR1-restricted CD4⁺ clones Ox97-clone10 and HA1.7 showing staining for 120 min at 4°C. C, Graphical representation of the percent CD4⁺ tetramer⁺ cells (upper chart) and the MFI (lower chart) when cells were stained at 4°C for the indicated time periods expressed as a percentage of the maximum recorded at 37°C.
the sensitivity of detection considerably (18–20, 37). This technique enriches the cells of interest and reduces background staining by ensuring that only cell surface-stained cells are enriched (thereby eliminating nonspecific tetramer internalization). We previously demonstrated that combination of these techniques yielded a linear enrichment recovery from human PBMC (20). The application of the optimized tetramer labeling techniques developed in this report to the study of CD4⁺ T cell populations directly ex vivo may allow HLA class II tetramers to become more useful. To optimize the combination of HLA class II tetramer staining and magnetic bead enrichment, a balance must be met between obtaining tetramer staining intense enough to allow discrimination between tetramer-positive and -negative populations. Internalization of tetramers must also be prevented so that magnetic beads can bind to cell surface-associated tetramers. To address this, we compared the detection of Ag-specific CD4⁺ T cells by HLA class II tetramer staining and magnetic bead enrichment with two methods: staining at 23°C for 120 min and 37°C for 20 min. The mean and SEs obtained for triplicate stainings at two defined spike frequencies (0.8 and 1.6%) before and after magnetic bead enrichment are shown in Fig. 7. The mean pre-(Fig. 7A) and postenrichment (B) CD4⁺ tetramer² frequencies obtained with the different staining protocols were virtually identical. When the enrichment recovery was determined (expected postenrichment frequency of CD4⁺ tetramer² cells as a percentage of observed postenrichment frequency), the two methods were also indistinguishable (Fig. 7C). Notably, the recovery of tetramer⁻CD4⁺ cells was <50%, indicating that a considerable number of tetramer-stained cells are lost during the enrichment process.

Direct ex vivo HLA class II tetramer staining of Ag-specific CD4⁺ cells from PBMC at frequencies that fall below the typical threshold of detection (0.02%) are shown in Fig. 8. Although a few CD4⁺ tetramer² cells can be identified by normal flow cytometric detection, the very low frequency does not allow confident quantification of such responses. However, after magnetic bead enrichment, the number and proportion of CD4⁺ tetramer² cells is increased, greatly enhancing the detection sensitivity and reducing background staining (Fig. 8A). We performed cross-sectional staining of HIV-infected individuals with the HIV-specific tetramers p24.4-DR4, p24.14-DR1, and p24.17-DR1, as well as the CMV-specific pp65-DR1 tetramer. Patients with CD4⁺ tetramer⁺ T cell responses <0.02% were studied (Fig. 8). Tetramer staining combined with magnetic bead enrichment detected Ag-specific CD4⁺ responses reliably at frequencies as low as 0.0004% (1 in 250,000 CD4⁺ cells; Fig. 8B). No HLA class II tetramer staining of HLA-mismatched or HLA-matched, HIV-negative and CMV-negative control PBMCs was seen with the p24.17-DR1 and p24.4-DR4 or pp65-DR1 tetramers, respectively (data not shown and Ref. 20).

**Phenotypic analysis of virus-specific CD4⁺ T cells**

Memory virus-specific CD4⁺ T cells have different functions and different phenotypes that are thought to be associated with virus persistence and re-exposure to Ag. In infections such as influenza where virus is cleared from the host, virus-specific CD4⁺ T cells have been shown to predominantly produce IL-2 and display a CCR7⁺ phenotype. In contrast, the persistent Ag exposure seen in

**FIGURE 5.** HLA class II tetramer can dissociate from the cell surface. Azide-treated JF-TTox cells were stained with the TT830-DR1 tetramer in ice. The fluorescence of surface-bound tetramer was then measured 1, 2, 5, 10, 20, and 30 min later in the presence or absence of a competitor Ab (anti-HLA-DR). A, FACS density plots showing TT830-DR1 staining of the JF-TTox cell line 30 min after the competitor was added. The percentage of CD4⁺ tetramer⁺ cells is indicated in the top right quadrant of each plot. B, Graphical representation of the percent CD4⁺ tetramer⁺ cells detected at each time point.

**FIGURE 6.** HLA class II tetramers are internalized at 37°C but remain surface bound at 0°C. A, FACS histogram representing the fluorescence intensity seen when Ox97-clone10 cells were stained with p24.17-DR1 tetramer for 60 min at 37°C or on ice in the presence of azide. B, Fluorescence microscopy of Ox97-clone10 cells stained with the PE-labeled p24.17-DR1 tetramer at 37°C or on ice in the presence of azide. The DIC and mid-cell z-section of PE-fluorescence recordings as well as an overlay of the two are shown. Images were recorded at a ×63 magnification.
chronic, viremic HIV infection is most often associated with CCR7\textsuperscript{+} HIV-specific CD4\textsuperscript{+} T cells that produce IFN-\(\gamma\) (39, 40). Most studies that have compared phenotypes of virus-specific CD4\textsuperscript{+} T cells have relied on techniques that identify Ag-specific cells by the detection of cytokines in response to Ag. These methods may fail to detect all of the cells with the defined specificity (5, 6). These methods may also alter the phenotype of the specific cells during Ag-induced activation (41, 42). By using the sensitive combination of HLA class II tetramer staining and magnetic bead enrichment, we compared the phenotype of rare CD4\textsuperscript{+} T cells specific for HIV p24 Gag, CMV pp65, influenza HA, and HCV by costaining with HLA class II tetramers and surface-marker Abs. The phenotypes of CD4\textsuperscript{+} T cells were previously analyzed with the DR1-restricted, HIV p24 Gag-specific p24.17-DR1 tetramer in viremic HIV infection (20); the DR1-restricted, influenza HA-specific HA307-DR1 tetramer in resolved influenza infection (19); and the DR4-restricted, HCV nonstructural protein 3- and 4-specific DR4-HCV 1248, 1579, 1770 tetramers in resolved HCV infection (18). In this study, we measured the expression of CCR7, CD27, and CD28 on CD4\textsuperscript{+} T cells with the DR4-restricted, HIV p24 Gag-specific tetramer p24.4-DR4 in viremic HIV infection, and the DR1-restricted, CMV pp65-specific pp65-DR1 tetramer in HIV-negative individuals (Fig. 9). Memory CD4\textsuperscript{+} T cells of all specificities expressed CD28 (HCV NS3- and NS4-specific tetramer data were not available). CD4\textsuperscript{+} Th cells specific for both HIV p24 epitopes (p24.17-DR1 and p24.4-DR4), the influenza HA epitope 307–319, and the HCV NS3 and NS4 epitopes were predominantly CD27-positive. However, a lower proportion of CMV pp65-specific CD4\textsuperscript{+} Th cells expressed CD27. Interpatient variation of CD27 expression on CMV-specific CD4\textsuperscript{+} T cells (as indicated by error bars representing SEM) was also markedly higher.
class II tetramer staining of Ag-specific CD4\(^+\) T cells should be rapid. Yet, most studies which report HLA class II tetramer staining used long incubation times (1–5 h) (8–10, 13–17, 21–27, 33). We find efficient and rapid staining of CD4\(^+\) T cells with HLA class II tetramers. Specifically, incubation at 37°C for 10 min was sufficient to allow complete labeling of all Ag-specific cells. At 23°C, staining was slower, and although some lines and clones were stained after 10 min, complete staining for all cell specificities required 30–60 min.

Ag-specific CD8\(^+\) T cells can also be labeled with HLA class I tetramers at 4°C (30, 35). Moreover, MHCI tetramers bind Ag-specific T cells independent of a cellular response and do not require an active cellular process (4, 43, 44). However, little or no HLA class II tetramer staining of CD4\(^+\) T cell clones was observed at 4°C in previous studies (12, 21, 25). These findings led to the hypothesis that HLA class II tetramer staining is dependent on active cellular processes. This was supported by experiments that showed blockade of tetramer staining of the HA1.7 clone when endocytosis and cytoskeletal rearrangements were disrupted (25). In contrast to these studies, we demonstrate successful staining of the TT-, influenza HA-, and HIV Gag-specific cell lines and the HIV Gag-specific Ox97-clone10 at 4°C. The JF-TTox cell line and Ox97-clone10 were also labeled with tetramer in the presence of azide on ice as detected by flow cytometry and wide-field microscopy. Furthermore, when the JF-TTox cell line and Ox97-clone10 were prestained with HLA class II tetramer, PE-fluorescence decreased upon addition of the competitor Ab anti-HLA-DR, suggesting dissociation of cell surface-bound tetramer. These results show that HLA class II tetramer can be surface-bound and that active cellular processes such as TCR/pMHCII internalization are not an absolute requirement for HLA class II tetramer staining of Ag-specific CD4\(^+\) T cells.

Fluorescence microscopy showed a clear accumulation of PE-conjugated HLA class II tetramer clusters that are internalized at 37°C. This finding is in agreement with Cameron et al. (25), who reported tetramer clusters that colocalized with endocytic compartments. At 4°C or on ice, bound tetramer remained on the cell surface. These surface-bound tetramers clustered. This suggested that the TCRs were in close apposition before the tetramers were added. This is in agreement with previous work, demonstrating that TCRs are asymmetrically localized to distinct regions of the plasma membrane that contain the highest concentration of lipid rafts (45).

We were unable to completely stain some CD4\(^+\) T cells at 4°C. Specifically, the HA-specific clone HA1.7 could only be partially stained and the CMV-specific line LA-pp65 could not be stained. These findings are in accordance with previous studies (15, 25, 27). The differential staining at low temperatures may be due to differences in the binding affinities of the TCR/pMHC complexes as suggested by Reichstetter et al. (27). Data supporting the hypothesis that only "high"-affinity TCRs are bound by tetramers was published by Fatta et al. (46). They reported a strong correlation between functional avidity and intensity of HLA class II tetramer staining in human cartilage-specific T cell hybridomas.

Discussion

In this study, we optimized the conditions required to label Ag-specific CD4\(^+\) T cells with HLA class II tetramers and developed techniques for detection of HLA class II staining in direct ex vivo samples. Many investigators have reported HLA class II tetramer-staining methods that differ substantially from those used to stain HLA class I-restricted CD8\(^+\) T cells. CD8\(^+\) T cells can readily be labeled with HLA class I tetramers by incubation with them at 37°C for 5–10 min as shown by FACS analysis and confocal microscopy (29, 30, 35). Because the association rates of tetrameric TCR/pMHCI and TCR/pMHCI\(\alpha\) interactions are similar (28), HLA HLA class I-restricted CD8\(^+\) samples. Many investigators have reported HLA class II tetramer-staining techniques for detection of HLA class II staining in direct ex vivo samples.
cells. We visualized HIV-specific and CMV-specific CD4+ T cells that were undetectable with direct tetramer staining when we combined tetramers with magnetic bead enrichment. This technique was sufficiently sensitive to detect reliably 1 in 250,000 CD4+ cells, corresponding to a frequency in PBMC populations of almost 1 in a million. Previous experiments also demonstrated that this technique offered a quantitative readout when a range of low cell frequencies were stained (20). The enrichment technique also reduces background staining. However, this may reduce detection of low-avidity cells, because the HLA class II tetramer may not adhere strongly enough to remain bound after magnetic bead enrichment. Evidence for such an effect is suggested by the postenrichment preference for cells with high fluorescence intensity, which probably represent high-avidity T cells.

We then used this sensitive technique to compare the phenotypes of Ag-specific Th cells with different Ag exposure in viral infections of varying persistence. These analyses might, in principle, allow further insights into the function, survival, and in vivo recirculation patterns of different antiviral T cell populations. The proportion of HLA class II tetramer-positive cells that expressed the surface markers CCR7, CD27, and CD28 was measured on HIV-specific CD4+ cells in viremic HIV infection and CMV-specific CD4+ cells in HIV-negative individuals. These characteristics were compared with influenza HA-specific CD4+ cells in cleared influenza infection and HCV-specific CD4+ cells in resolved HCV infection (18–20). We show that CMV-specific cells are less likely to express CD27 than HIV-, influenza- or HCV-specific CD4+ Th cells. The proportion of HIV-specific and CMV-specific CD4+ cells that expressed CCR7 was also lower than the proportion of influenza virus-specific or HCV-specific CD4+ T cells. This difference between the HIV-specific and influenza- or HCV-specific CD4+ T cells was statistically significant. Notably, the CD27−CD28+ phenotype of HIV-specific CD4+ T cells contrasts with the CD27−CD28− phenotype reported for HIV-specific CD8+ T cells (47). Our data are in accord with a report that, on CD4+ T cells, CD27 appears to be down-regulated before CD28 (48); the reverse applies to CD8+ T cells (47). The CD27−/CD28− phenotype detected on CMV-specific CD4+ cells in our analysis also contrasts with the large proportion of CD27−CD28− CMV-specific CD4+ T cells identified by intracellular IFN-γ staining in healthy individuals (49). The 18-h incubation with CMV Ag used by Amyes et al. (49) to identify IFN-γ-producing, Ag-specific cells may artifactualy down-regulate the costimulatory molecule CD28 on these cells (50, 51).

Our data from these scenarios of varying Ag exposure and viral persistence do support the central/effector T cell model described by Sallusto et al. (52). This model suggests that, upon Ag exposure, central memory CD4+ T cells (represented, for example, by the HA- and HCV-specific cells in resolved infection) lose CCR7 expression and assume an effector phenotype (reminiscent of the HIV- and CMV-specific cells in persistent infection). Effector CD4+ T cells can be subdivided into different states of maturation (49). The sequential loss of CD27 and CD28 expression demarcate increasingly "mature" phenotypes. Our results underscore the idea that CMV-specific CD4+ T cells (CD27+/CD28−) display a more "mature" phenotype than HIV-specific CD4+ cells (CD27−CD28−). In agreement with Amyes et al. (49), these data do not support the idea that CD27 and CD28 expression can be used as a means of distinguishing between central memory and effector T cells.

Until recently, the study of Ag-specific CD4+ T cells with HLA class II tetramers was limited by the insensitivity of detection in ex vivo samples and paucity of defined CD4+ Th cell epitopes with known HLA restrictions. However, more epitopes are being defined, so the utility of HLA class II tetramers is expanding. This technique will advance our knowledge of the Th immune response. This form of immunity can be critical as a means of curbing infections but remains poorly defined. It is certainly part of antiviral immune control and should be evaluated when protective immunity is the aim of vaccination.

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Disclosures

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References


