Cutting Edge: In Situ Tetramer Staining of Antigen-Specific T Cells in Tissues

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The introduction by Altman et al. of a method to identify and phenotypically characterize Ag-specific T lymphocytes has quickly advanced understanding of the immune response in general and particularly the virus-specific CTL response to a number of acute and chronic infections that include influenza virus, lymphocytic choriomeningitis virus, EBV, human T cell leukemia virus-1, hepatitis C, Listeria monocytogenes, and HIV-1 and SIV infections (1–11). Enumerating virus-specific CD8+ T lymphocytes in cross-sectional or longitudinal studies has made it possible to track expansion and contraction of the CTL response in these infections and, in HIV-1 infection, to document the inverse correlation between the CTL response and viral load and progression to disease (4). Thus far, however, these studies have been directed to cells isolated from blood, semen, or tissues that were stained with tetramer-peptide complexes and then analyzed by flow cytometry. With the eventual goal of investigating the spatial and temporal relationships between viral replication and the specific CTL response in tissue, we sought to adapt the tetramer staining technology to tissue analysis. To that end, we set out to find conditions in an optimal well-characterized transgenic model for directly staining Ag-specific T cells in tissues. In this report, we describe such a technique that should be generally applicable to visualizing Ag-specific T cells in tissues.

**Materials and Methods**

**Generation of MHC tetramers/multimers**

Biotinylated K\(^{b}\)/\(\beta_{2}\)-microglobulin/peptide molecules were generated with either OVA (SIINFEKL) or SIY (SIYRYYL) peptides as previously described (1, 12). Tetramers/multimers were generated by adding six aliquots of FITC-labeled ExtraAvidin (Sigma, St. Louis, MO) over the course of 8 h to either K\(^{b}\)/\(\beta_{2}\)-microglobulin/SIY or K\(^{b}\)/\(\beta_{2}\)-microglobulin/OVA to a final molar ratio of 4.5:1.

**Generation of spleen sections**

2C (13) or OT-I (14) TCR transgenic mice on a C57BL/6 background or wild-type C57BL/6 mice were used in these experiments. The adoptive transfer of 2C transgenic cells into a C57BL/6 recipient was performed as described (15). Fresh spleens or spleens stored overnight in PBS at 4°C were cut into three pieces and embedded in 4% low-melt agarose, patted dry, and secured to vibratome tissue adhesive (Ted Pella, Redding, CA). After letting the glue set for at least 3 min, the blocks were placed in a vibratome bath containing 0°C PBS. A Vibratome 3000 (Technical Products International, St. Louis, MO) was used to cut the tissue (16, 17). Vibratome sections, 200 \(\mu\)m thick, were generated with a dead slow speed and maximum amplification using a standard double-edged razor blade set at an angle of 27°.

**Detection of Ag-specific T cells in situ**

Fresh sections were stained free floating in 1 ml solution with four parts per well in 24-well tissue culture plates, and incubations were conducted at 4°C on a rocking platform. Tetramers were added at a concentration of 0.5 \(\mu\)g/ml with 2% normal goat serum (NGS)\(^1\), and 0.5 \(\mu\)g/ml rat anti-CD8a Abs clone 53-6.7 (PharMingen, San Diego, CA) or CTCD8a (Caltag, South San Francisco, CA) and incubated overnight. Sections were washed with PBS and then fixed with PBS-buffered 2% formaldehyde for 30 min at room temperature. Sections were then incubated with rabbit anti-FITC Abs (BioDesign, New York, NY or Zymed, San Francisco, CA) diluted 1:10,000 in PBS with 2% NGS and incubated overnight. Sections were washed three times with PBS for at least 20 min and then incubated with Cy3-conjugated goat anti-rabbit Abs and Cy5-conjugated goat anti-rat Abs (Jackson ImmunoResearch, West Grove, PA) both

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\(^{4}\)Abbreviations used in this paper: NGS, normal goat serum; IST, in situ tetramer.
diluted 1:1000 in PBS with 2% NGS overnight. Finally, sections were washed three times for at least 20 min and then mounted to slides with warmed glycerol gelatin (Sigma) containing 4 mg/ml n-propyl galate. Stained sections were analyzed using a Bio-Rad 1000 confocal microscope (Richmond, CA).

In some experiments, spleens were fixed in 2% formaldehyde buffered with PBS for 30 min at room temperature before sectioning and staining. The prefixed sections were stained as described for fresh sections with the exception that they were not fixed after the tetramer incubation. Frozen tissue sections, 10 μm thick, were also generated from spleen tissue that had been frozen in OCT freezing medium (Tissue-Tek, OT Embedding Medium, Sakura Finetek, Torrance, CA). These sections were stained as described for fresh sections with the exception that sections were mounted onto silane-coated slides before staining, incubations were performed with 200 μl of solution, PBS wash times were reduced to 5 min, and secondary incubations were reduced to 3 h.

Results and Discussion

We chose 2C TCR transgenic mice (13) to initiate our attempts to develop a method using MHC class I tetramers to identify Ag-specific T cells in situ because 2C receptors bind several ligands including the K\(^b\)/SIY molecule with relatively high affinity and K\(^b\)/SIY tetramers have been used to identify 2C T cells ex vivo (12, 18, 19). Most T cells in 2C transgenic mice are CD8\(^+\) but there are some CD8\(^-\) 2C\(^+\) T cells (13, 20) (data not shown). We conjugated biotinylated K\(^b\)/SIY monomers with FITC-labeled ExtrAvidin to make K\(^b\)/SIY tetramers. Because tetramer staining has thus far only been successfully applied to viable cells, we initiated our studies in tissues sectioned with a vibratome (16, 17). Using the K\(^b\)/SIY tetramers, we stained 200-μm fresh spleen sections from 2C transgenic mice. To detect bound tetramers, we amplified the signal using purified rabbit Abs directed against FITC followed by anti-rabbit Abs conjugated to Cy3 (Fig. 1, A and C). Sections were counterstained with anti-CD8 Abs as a positive control (Fig. 1, B and C). The merged image of Kb/SIY staining and anti-CD8 staining (Fig. 1C) shows that the tetramer-positive cells counterstained with anti-CD8 Abs. Without amplification, no tetramer signal was detected (not shown). As a negative control, spleen sections from 2C transgenic mice were stained with tetramers of K\(^b\) molecules loaded with an irrelevant peptide SIINFEKL from OVA (Fig. 1, D and F) and counterstained with anti-CD8 Abs (Fig. 1, E and F). In contrast to the Kb/SIY tetramers, the Kb/OVA tetramers did not stain the CD8\(^+\) 2C transgenic T cells (Fig. 1, D–F). As an additional negative control, spleen sections from wild-type C57BL/6 were stained with Kb/SIY tetramers, and these did not show tetramer-specific staining (not shown). However, the anti-FITC/anti-rabbit-Cy3 Abs, used to amplify the tetramer signal, did label a small subset of cells that were not further evaluated because they typically were outside of the T cell-rich white pulp of the spleen, where they would not be confused with tetramer-positive T cells. Because this subset was also CD8\(^+\), in all experiments sections were counterstained with anti-CD8 Abs so that doubly stained cells that bound the labeled peptide/MHC complex could be unequivocally distinguished from background staining. Using a confocal microscope, tetramer-stained cells could be detected as far as 120 μm into the tissue. This technique has considerable analytical and sampling power because MHC class I tetramers can stain Ag-specific T cells in situ through this depth of tissue.

We subsequently investigated whether in situ tetramer (IST) staining would work using a different system. We used K\(^b\)/OVA tetramers to stain OVA-specific T cells from OT-I transgenic mice (14) (Fig. 2A). As a negative control, parallel sections were stained with Kb/SIY tetramers and did not show tetramer staining (Fig. 2B). Thus, the IST staining technique was used successfully to stain Ag-specific T cells in tissues from 2C as well as OT-I transgenic mice.

To see if we could use MHC tetramers to detect Ag-specific T cells in a system in which only a fraction of a percentage of the T cells are transgenic, we adoptively transferred lymphocytes from 2C transgenic mice into a wild-type C57BL/6 mouse (21, 22). Five days after the adoptive transfer, spleen and lymph tissue was collected. Flow

![FIGURE 1. MHC tetramers used to stain fresh spleen sections from 2C transgenic mice. A–C show a section that was stained with Kb/SIY tetramers (A, red) and anti-CD8 clone 53-6.7 Abs (B, green); C shows the images from A and B merged. D–F show a section that was stained with Kb/OVA multimers (D, red) and anti-CD8 clone 53-6.7 (E, green); F shows the images from D and E merged. The images shown in A and D were collected using the same confocal parameters. The spleen used in this experiment was stored in PBS at 4°C for 24 h before sectioning and staining.](http://www.jimmunol.org/content/vol72/issue6/614/fig1)
cytometric analysis revealed that the transferred transgenic T cells comprised 0.2–0.4% of the total lymphocytes (not shown). Spleen sections stained with Kb/Siy tetramers (Fig. 3, A and C) and anti-CD8 Abs (Fig. 3, B and C) showed staining of the 2C transgenic T cells. The merged image of Fig. 3, A and B shows that the Kb/Siy tetramer-stained cells were CD8$^+$ (Fig. 3C). In contrast, control sections from a mouse in which no 2C cells were transferred were stained with Kb/Siy and did not show tetramer staining (not shown). Quantitation revealed that 23 of 2306 or 1.0% of the CD8$^+$ T cells were stained with Kb/Siy tetramers in the adoptively transferred spleen. In contrast, 1 of 2742 or 0.04% of the CD8$^+$ T cells were stained with Kb/Siy in the control sections. To corroborate these findings, flow cytometry was used to calculate the percentage of transgenic T cells from a portion of the same spleen presented in Fig. 3. Flow cytometry identified a comparable stained population of 1.3% of CD8$^+$ T cells (not shown). Thus, these data demonstrate that IST staining can be used to stain small populations of Ag-specific T cells at a sensitivity that is comparable to flow cytometry.

Recently, Daniels et al. reported that anti-CD8 Abs, depending on the clone, either interfere with or enhance tetramer binding its TCR ligand (12). Clone CTCD8a was shown to inhibit Kb/Siy tetramer binding. In contrast, clone 53-6.7 was shown to enhance tetramer binding. To assess this effect in the IST staining technique, we stained spleen sections from 2C transgenic mice with Kb/Siy tetramers and the anti-CD8 clone CTCD8a at a concentration of 0.5 μg/ml. We found that anti-CD8 clone CTCD8a had a negative effect on tetramer staining (not shown). Given that some anti-CD8 Abs can interfere with tetramer binding, care should be taken when selecting a CD8 Ab to counterstain cells, and minimal amounts of anti-CD8 should be used.

Frequently, investigations involve collaborations in which tissue is collected at one institution and analyzed at another. Also, investigations sometimes involve limited amounts of tissue and/or tissue that has been frozen and archived. To facilitate the investigation of Ag-specific T cells in these circumstances, we tested whether IST staining could be performed on tissue that was either

FIGURE 2. MHC tetramers used to stain fresh spleen sections from OT-1 transgenic mice. A–C shows a section that was stained with Kb/OVA tetramers (A, red) and anti-CD8 (B, green); C shows the images from A and B merged. D–F shows a parallel section that was stained with Kb/Siy tetramers (D, red) and anti-CD8 (E, green); F shows the images from D and E merged. The same confocal parameters were used to capture both images.

FIGURE 3. Adoptively transferred T cells detected in situ using MHC tetramers. T cells from a 2C transgenic mouse were adoptively transferred into a wild-type mouse. After euthanizing this mouse, fresh spleen sections were generated and stained with Kb/Siy tetramers (A, red) and anti-CD8 Abs (B, green). C shows the images from A and B merged.
stored overnight in PBS at 4°C, lightly fixed with 2% formaldehyde, lightly fixed with 50% acetone and 50% methanol, or frozen. Spleens from 2C transgenic mice were either stored at 4°C in PBS overnight before sectioning and staining or sectioned and stained shortly after dissection. The sections from spleens that were stored overnight showed identical staining as the spleens that were sectioned and stained promptly after dissection. An example of tissue that was stored overnight before sectioning and staining is shown in Fig. 1. We were also successful in staining sections from spleens prefixed for 30 min in 2% formaldehyde (Fig. 4) or prefixed in 50% acetone and 50% methanol at −20°C for 5 min (not shown) that were stored in PBS overnight at 4°C prior to sectioning and staining. Finally, we were also successful in staining 2C transgenic T cells in 10-μm thick frozen sections from spleens from 2C transgenic mice with K$^b$/SIY tetramers (Fig. 5). The best quality staining with regard to cellular morphology was achieved with tissue that was not fixed or frozen before staining. In addition, the 200-μm thick sections offered more information than the 10-μm frozen sections as about 10 times more cells could be analyzed per section. Accordingly, when examining frozen tissue from a system with relatively low levels of Ag-specific T cells in situ, thick sections should be generated to increase the number of positive cells per section.

In summary, we have developed a technique to stain Ag-specific T cells in situ. We have successfully used MHC class I tetramers to stain Ag-specific T cells from 2C and OT-1 transgenic mice, as well as 2C transgenic T cells that were adoptively transferred into a wild-type mouse. We point out that careful selection of anti-CD8 Abs is important when colabeling with tetramers, as anti-CD8 Abs can alter tetramer binding in situ. Finally, we demonstrated that our technique can be used to stain Ag-specific T cells in tissue that has been stored overnight, lightly fixed, or frozen. We think this technique, the first to be described that allows the detection of Ag-specific T cells in tissues, promises to be a valuable tool for studies of Ag-specific T cells in vivo.

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References


