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Use of HLA class II tetramers in tracking antigen-specific T cells and mapping T-cell epitopes

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Abstract

The highly specific interaction between major histocompatibility complex (MHC)/peptide and its cognate T-cell receptor (TCR) directs to the specificity of the T-cell response. Although the interaction affinity of individual MHC/peptide and TCR molecules is low, multimerization of the MHC/peptide complexes increases the avidity of their interaction with TCRs, and enables the complexes to be used as staining reagents for antigen-specific T cells. Different approaches are used to generate class I and class II tetramer reagents, and these reagents have been demonstrated to be useful in identifying CD8+ and CD4+ T cells, respectively. This article focuses on the uses of human lymphocyte antigen (HLA) class II tetramers in tracking antigen-specific T cells. Class II tetramers can also be used to map antigenic epitopes of known antigens through a novel peptide screening procedure known as tetramer-guided epitope mapping (TGEM). In this article, we provide a description of the methodological approach of using HLA class II tetramers in tracking and isolating T cells, and the use of class II tetramers in mapping T-cell epitopes.

1. Introduction

Multimeric human lymphocyte antigen (HLA) class II major histocompatibility complex (MHC)/peptide complexes have been successfully used in enumeration of CD4+ T cells specific for viral antigens, bacterial antigens, and autoantigens [1–8]. One of the prerequisites for using class II tetramers in the detection of antigen-specific T cells is knowledge of the antigen and its immunodominant antigen-derived peptides. The specific peptide of interest can be covalently tethered to the MHC for the generation of class II/peptide complexes [4,5]. Peptide can also be loaded onto the empty soluble class II molecules exogenously [1,2]. The latter is the method of choice in our laboratory because of its versatility of providing a large number of different MHC/peptide complexes. The peptide-loaded class II molecules are then crosslinked by streptavidin to generate the tetramers. Tetramers generated by exogenous loading were found to be as effective in staining T-cell clones and hybridomas as tetramers with peptide covalently linked to the MHC (Kwok, unpublished). The detection of antigen-specific CD4+ T cells in peripheral blood has been challenging because of their low frequency in most situations. In our studies, in vitro stimulation of the peripheral blood mononuclear cells (PBMC) is used to expand the antigen-specific T cells prior to tetramer staining [1,2].

2. Production of soluble class II molecules

Soluble class II molecules with leucine zipper regions attached to the carboxyl terminals of the molecules are produced in the *Drosophila* system. A detailed description for the production of DR0401 molecules can be found elsewhere [1]. A general description is included in this article. For production of DR molecules, cDNAs encoding the extracellular domains of DRα and DRβ chains were attached to the acidic and basic portion of the leucine zipper cDNAs, respectively. A site-specific biotinylation sequence was also added to the 3′ end of the DRβ leucine zipper cassette, and the chimeric cDNAs were subcloned into a Cu-inducible *Drosophila* expression vector pRMHa3. DRα and DRβ expression

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vectors were cotransfected into Schneider S-2 cells. *Drosophila* cells were cloned by limiting dilution and screened for production of class II molecules. Clones that expressed a large amount of class II molecules were selected and further expanded for class II purification. Soluble DR molecules were purified by affinity chromatography with L243 antibody. A similar approach was also used successfully to produce soluble DQ molecules. Biotinylation of the class II molecule was carried out with the enzyme Bir A. Specific peptides of interest were loaded onto the empty class II molecules for 72 h in the presence of *n*-octyl-β-D-glucopyranoside, which facilitated the peptide loading process. A high molar ratio of peptide to MHC molecules was also used to improve the efficiency of loading the peptide onto the class II molecules. In the final step, tetramers were formed by incubating class II molecules with phycoerythrin (PE)-labeled streptavidin. Other fluorochrome labels can also be used on streptavidin. In contrast to class I MHC, the length of the peptide is not a critical factor for effective loading. Peptides ranging in length from 10 to 25 amino acids, have been used to generate functional tetramers for T-cell staining. Peptides longer than 25 amino acids have not been examined.

3. In vitro stimulation of PBMC and tetramer staining

CD4+ T cells that are directed against a specific antigen are present in small amounts in the peripheral blood. Specific expansion of these T cells by in vitro peptide stimulation facilitates their detection. PBMC from subjects of interest were separated from fresh heparinized blood (20–30 ml). The cells were resuspended in culture medium at the density of 2.5×10^6/ml, and cultured in the presence of the antigenic peptide at a concentration of 10 μg/ml in 24 wells. On Day 7, interleukin (IL)-2 was added to a final concentration of 10 units/ml. The medium was also replenished every 2 to 3 days. On Days 10 to 14, the stimulated cells were stained with 10 μg/ml of PE-labeled tetramer for 3 h at 37 °C, and subsequently with fluorochrome-labeled anti-CD4, and analyzed by flow cytometry. Fig. 1 shows an example of a herpes simplet virus type 2 (HSV-2)-infected subject who displayed a population of DQ0602-restricted T cells VP16 369–380 specific for the HSV epitope. This population was absent when the staining was carried out with a control tetramer.

Tetramer-positive T cells can be cloned through flow cytometry sorting as illustrated in Fig. 2; this is a rapid approach to the isolation of antigen-specific CD4 T-cell clones.

4. Assay protocols

4.1. Loading of peptides onto MHC molecules and crosslinking of MHC molecules with streptavidin

1. Adjust concentration of class II molecule to 0.5 mg/ml with 150 mM phosphate buffer (pH 6.) in 1.7 ml nonsticky surface microcentrifuge tube.

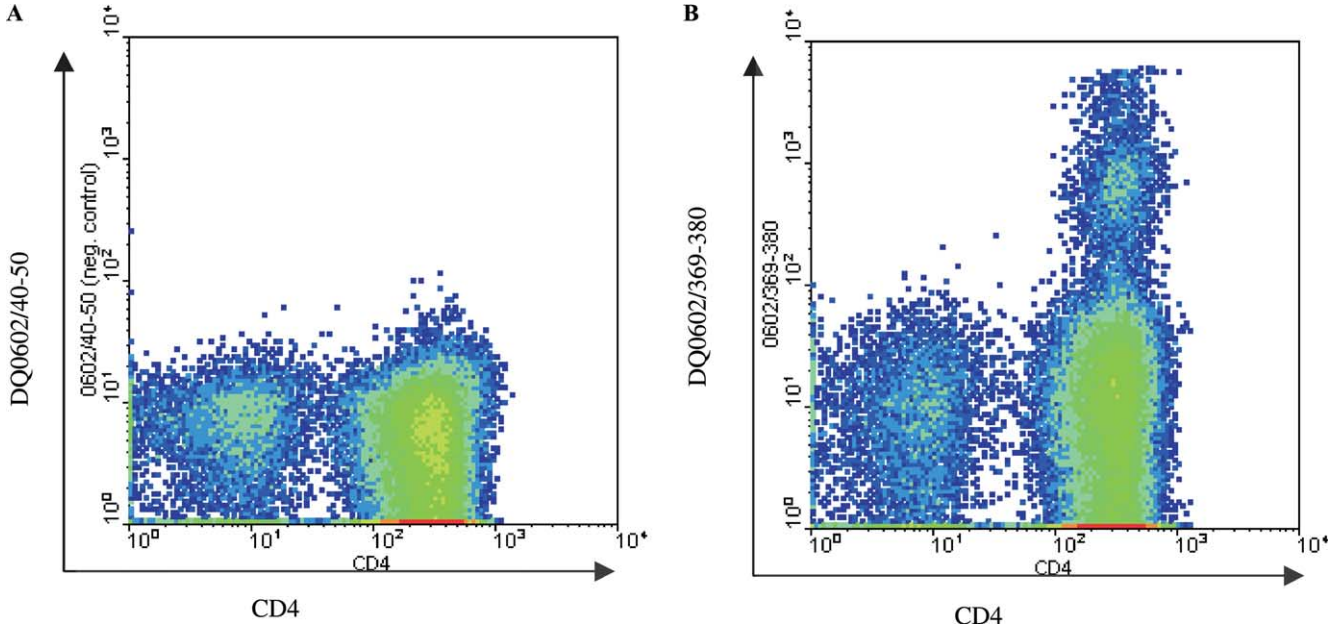

Fig. 1. Staining of DQ0602-restricted T cells from PBMC using specific tetramers. PBMC from a DQ0602 HSV-2 subject were stimulated with VP16 peptide 369–380 (10 μg/ml). Twelve days later, the cells were stained with either DQ0602/VP16 40–50 tetramers (A) or DQ0602/VP16 369–380 tetramers (B).

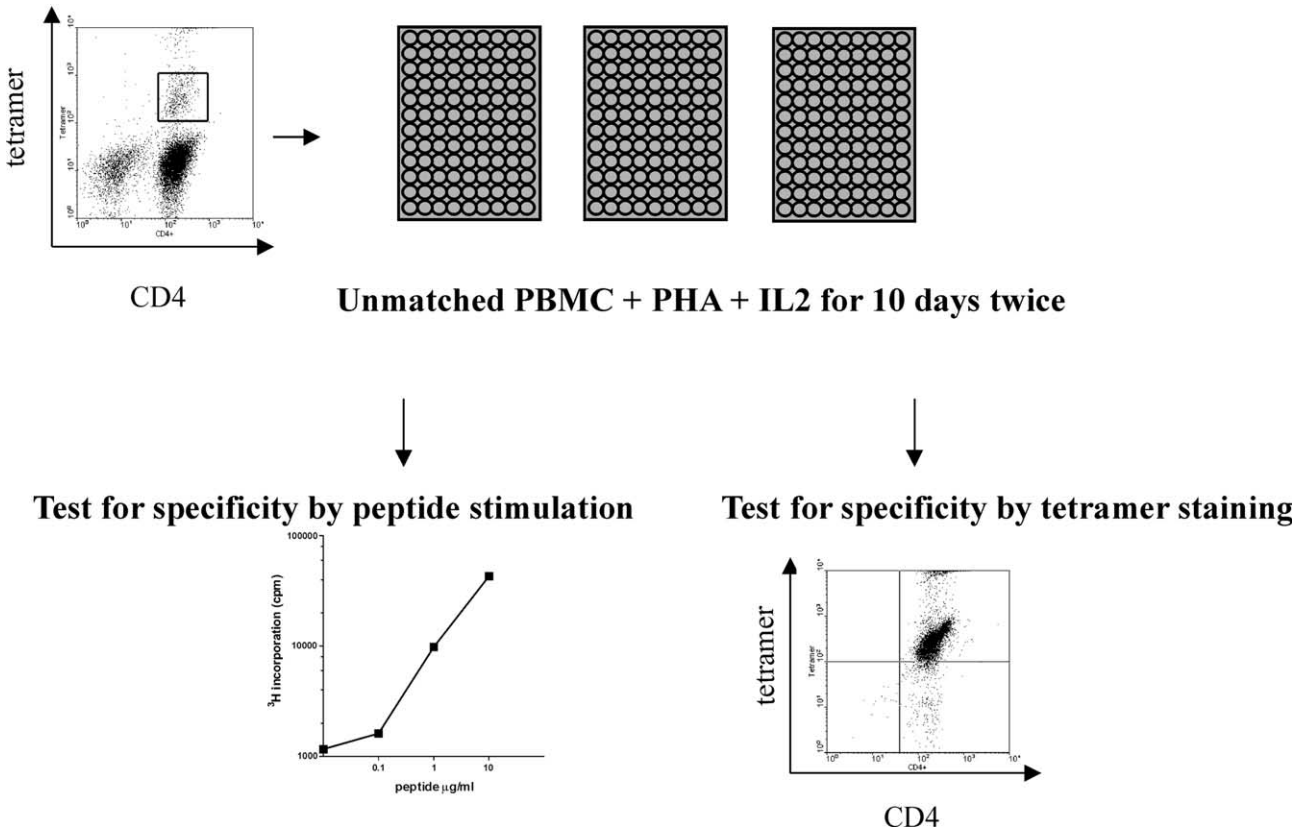

Fig. 2. Sorting of tetramer-positive T cells by flow cytometry. Tetramer-positive T cells can be single-cell sorted and expanded in the presence of PHA plus IL-2. Cells, which are cloned, can be reexamined for antigen specificity by proliferation assays and restaining with tetramers.

2. Add peptide to a final concentration of 0.5 mg/ml, *n*-octyl-β-D-glucopyranoside to a concentration of 0.25%, and Pefabloc SC to a concentration of 1 mg/ml.
3. Incubate at 37 °C for 72 h.
4. Add 20 μl of fluorochrome-conjugated streptavidin (0.25 mg/ml) per 100-μl volume of peptide-loaded MHC molecules and incubate at room temperature overnight.

4.2. Isolation of PBMC from peripheral blood

1. A volume of 20–30 ml freshly drawn heparinized blood is optimal for the assay.
2. Dilute 15 ml heparin blood 1:1 with 1× phosphate-buffered saline (PBS) in a 50-ml polypropylene conical tube.
3. Underlayer 10 ml Ficoll (Lymphoprep, Nycomed, Oslo, Norway) slowly with a 10-ml pipet.
4. Centrifuge for 20 min at 2000 rpm.
5. Collect the mononuclear cells at the interface.
6. Wash the cells three times with 1× PBS.
7. Resuspend the cells at a density of 2.5×10^6 cells/ml in culture medium. [RPMI-1640 (Gibco-BRL, Rockville, MD, USA) supplemented with 2 mM L-glutamine, 100 mg/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 10 to 15% pooled human serum (obtained from 20–25 healthy, nontransfused male donors)].

4.3. In vitro stimulation of PBMC with peptide

1. Divide the cells on 24-well plates, $\sim 5 \times 10^6$ cells in each well.
2. Add peptide at the concentration of 10 μg/ml and leave some wells without a peptide (control wells) and incubate the cells at 37 °C.
3. On Day 7, add in recombinant IL-2 such that the final concentration is 10 U/ml. Replenished cells with fresh medium plus IL-2 every 2 to 3 days and replate into additional wells when necessary.
4. Culture the cells for 14 days.

4.4. Tetramer staining

1. Between Days 10 and 14 of culture, collect at least one million cells and spin them down for 5 min at 1000 rpm.
2. Resuspend the cell pellet in 100 μl culture medium and divide the cell suspension into two 5-ml Falcon polystyrene tubes.
3. Add into one of the tubes 1 μl PE-labeled HLA-DR tetramer loaded with the same peptide as used in the specific stimulation (final concentration 10 μg/ml) and add into the second vial the same concentration of HLA-DR tetramer loaded with a control peptide.

4. Stain the cells in the dark with tetramer for 3 h at 37 °C.

5. Transfer the vials on ice and add 8 μl of antibodies specific for human CD4 labeled with fluorochromes other than PE (BD/Pharmingen, San Jose, CA, USA). Stain the cells for 15–30 min in the dark. Wash the cells with cold FACS buffer (1× PBS + 1% fetal bovine serum).

6. Add 200 μl FACS buffer, mix by tapping and analyze by flow cytometry.

4.5. Fluorescent single-cell sorting, T-cell cloning, and proliferation assay

1. Single-cell sort tetramer-positive T cells into round-bottom 96-well plates containing 100 μl culture medium/well using a FACS Vantage cell sorter (Becton-Dickinson).

2. Expand clones by stimulation with irradiated unmatched PBMC (1.5×10^5/well) and 5 μg/ml of phytohemagglutinin (PHA).

3. On the next day, add 10 U/ml IL-2.

4. Between Days 10 and 14, restimulate cells with 5 μg/ml PHA.

5. Transfer cells to 48- or 24-well plates as needed.

6. On day 20, screen cells for growth and test for antigen specificity. Set up the proliferation assay by stimulating 5×10^4 T cells with irradiated autologous PBMC (1×10^5/well) as antigen presenting cells (APC) with and without a specific peptide. Measure [^{3}H]thymidine incorporation at 72 h. Clones can also be screened by tetramer staining.

Note. For sorting, a short staining time and a minimum concentration of tetramers are preferred, as tetramers can induce T-cell apoptosis.

4.6. Tetramer-guided epitope mapping

Tetramers can also be used to identify novel T-cell epitopes. This approach is designated as tetramer-guided epitope mapping (TGEM) [8,9]. To apply TGEM, the class II HLA type of the subject has to be identified and a panel of overlapping peptides that encompass the antigenic protein of interest is synthesized. PBMC from the subject are stimulated with the peptide mixtures or the antigenic protein (if available). The panel of overlapping peptides are divided into different pools, with each pool composed of 5 to 10 different peptides. Rather than loading the soluble class II molecules of interest with individual peptides, tetramers are generated with the peptide mixtures in each pool. The protocol of loading is similar to that described earlier. The concentration of each individual peptide in the loading buffer is at least 0.05 mg/ml. Tetramers, which are generated with the peptide mixtures, are designated as pooled peptide tetramers. Ten to twelve days after the in vitro stimulation, the PBMC are stained with the pooled peptide tetramers. The particular pooled peptide tetramers that give positive staining are identified. Individual peptides from the pool are then used to generate single peptide tetramers, and the staining is repeated with the single peptide tetramers. Positive staining with a particular tetramer will identify the peptide containing the antigenic epitopes. A diagram that depicts this approach is shown in Fig. 3. The TGEM approach has been successfully used to identify T-cell epitopes of the HSV-2 VP16 protein for more than eight HLA class II alleles. We have successfully used TGEM with a peptide pool consisting of up to 10 peptides. It is quite likely that the TGEM approach can identify T-cell epitopes even when each pool consists of more than 10 peptides, depending on the relative competitive binding properties of peptides within the pool. The success of the TGEM approach can be accounted for by the following unique properties of class II/peptide interactions: (1) Class II molecules have an open-ended binding groove, allowing the MHC molecule to search for the correct binding register in a long peptide. This distinct binding property of class II molecules allows effective loading of peptides onto class II molecules even in the absence of the knowledge of the class II peptide binding motifs. (2) Binding of peptides to MHC molecules is highly selective. In a mixture of peptides that consist of a panel of overlapping peptides, the majority of the peptide cannot bind to the MHC, and only peptides with the correct binding motifs can bind to MHC. Thus effective tetramer formation can occur in the presence of peptide mixtures [8]. (3) Loading of peptide onto MHC molecules can be carried out in a very small reaction volume, allowing the possibility of generation of large numbers of pooled peptide tetramers and single peptide tetramers.

TGEM can also be used in combination with computer-assisted algorithms that predict class II-restricted T-cell epitopes [10]. For this particular approach, the putative CD4+ epitopes, as predicted from the computer-assisted algorithms, are first synthesized. PBMC are stimulated with the peptide mixtures, and tetramers loaded with the selected peptides are used to confirm the presence of the T cells specific for the antigenic epitopes. In this approach, only single peptide loaded tetramers are used. Tetramers generated from peptide mixtures, consisting of different high-affinity peptides as selected by the computer-assisted algorithms, are not as effective in T-cell staining. This combined TGEM and computer-assisted algorithm approach avoids the necessity of synthesizing the complete panel of peptides that encompass the antigen. In one set of experiments, the T-cell epitope program was used to select seven peptides for the putative DR0101-restricted T-cell epitopes of the pp65 protein of cytomegalovirus (CMV). The peptides were synthesized and the peptide mixtures were used to

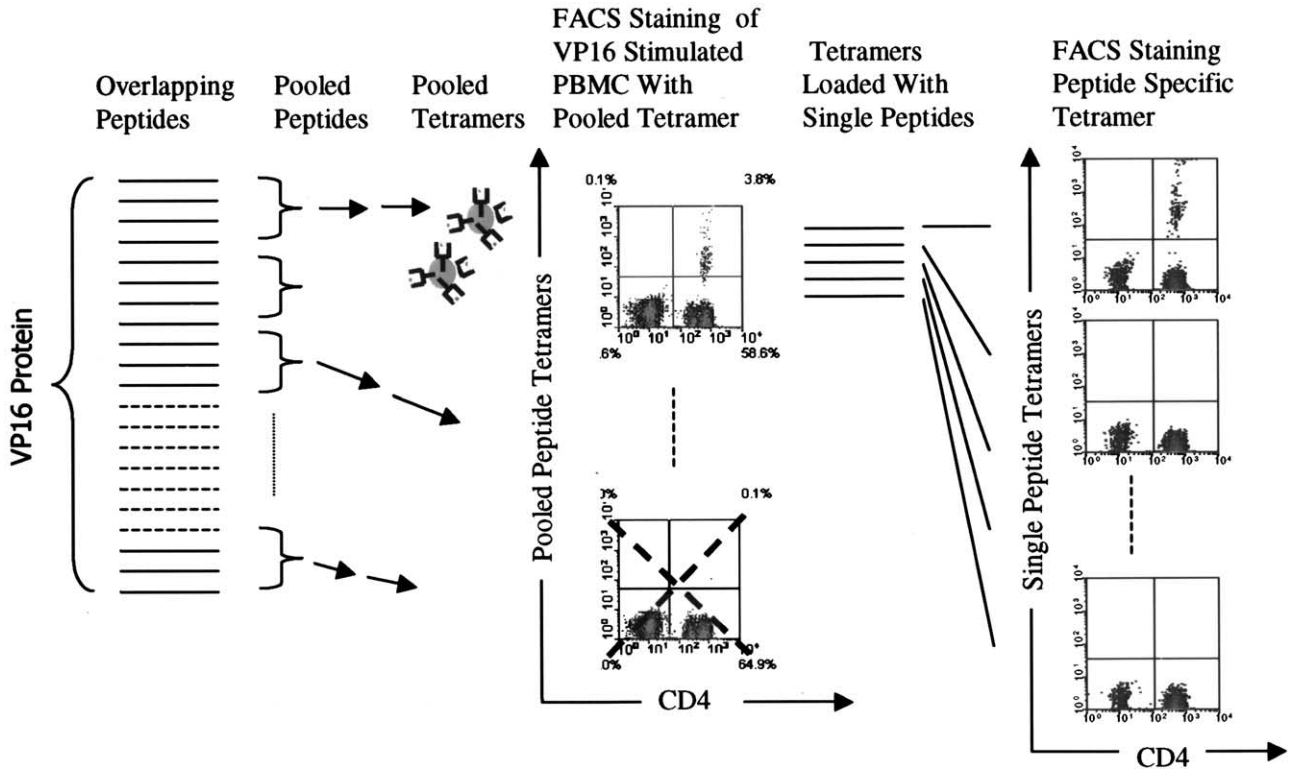

Fig. 3. Tetramer-guided epitope mapping (TGEM). A panel of overlapping peptides spanning the protein of interest are divided into pools, with each pool containing 5 to 10 peptides. Each peptide pool is loaded onto soluble MHC class II molecules to generate pooled peptide tetramers, and used to stain PBMC, which have been previously stimulated with the appropriate antigen. The pooled peptide tetramer that gives positive staining is identified, and peptides from that particular pool are used to generate tetramers loaded with individual peptides. Tetramers that positively stain in the second round of FACS analysis enable the identification of the MHC-restricted epitope.

stimulate PBMC of a DR0101 CMV serological positive individual. These peptides are listed in Table 1. Tetramers corresponding to all seven peptides were also generated and used to stain the PBMC 15 days after the in vitro stimulation. Positive staining was observed with the tetramers that were loaded with peptide CMV pp65 108–127 MSIYVYALPLKMLNIPSINVH (Fig. 4). Further truncation of the CMV pp65 108–127 peptide identified CMV pp65 115–127 LPLKMLNIPSINVH as the antigenic epitope. DR0101/pp65 115–127-specific. T cells were subsequently cloned by cell sorting, and the DR0101/pp65 115–127-specific T cells can be stained with the DR0101/pp65 115–127 tetramers (Fig. 4).

Table 1
CMV pp65 DR0101-binding peptides selected by TEPITOPE program at a threshold of 3[a]

Peptide	Sequence
CMV pp65 14–25	VLGPISGJVLKA
CMV pp65 47–57	HVRVSQPSLIL
CMV pp65 108–127	MSIYVYALPLKMLNIPSINVH
CMV pp65 337–349	VELRQYDPVAALF
CMV pp65 370–389	IVKPGDILVINSNGNLIA
CMV pp65 494–511	RNLVPMVATVQGQNLKYQ
CMV pp65 518–531	NDIYRIFAELEGVW

[a] Two other peptides, pp65 269–280 and 299–310, which were also selected by the program, were not being tested, as both of these peptides have a cysteine at position 5.

There are at least two distinct advantages to using TGEM compared with other epitope mapping approaches. Each individual expresses multiple class II alleles, and the investigator may only be interested in the T-cell epitope of the disease-associated HLA allele. The TGEM approaches focus on a particular HLA class II allele. TGEM also allows direct sorting of the CD4+ T cell of interest through flow cytometry. The T cells being sorted will be well defined for both antigen specificity and HLA restriction. In other techniques, T cells can be also sorted based on the cytokine secretion function, while TGEM allows the sorting of cells without this prior knowledge.

4.7. Troubleshooting for tetramer staining and TGEM

1. The culture conditions of the PBMC are critical for the success of detecting tetramer-positive T cells after in vitro stimulation and in the TGEM approach. In our laboratory, cells are usually plated at 4 to 5 million cells per well in a 24-well plate. Antigen-specific T cells that

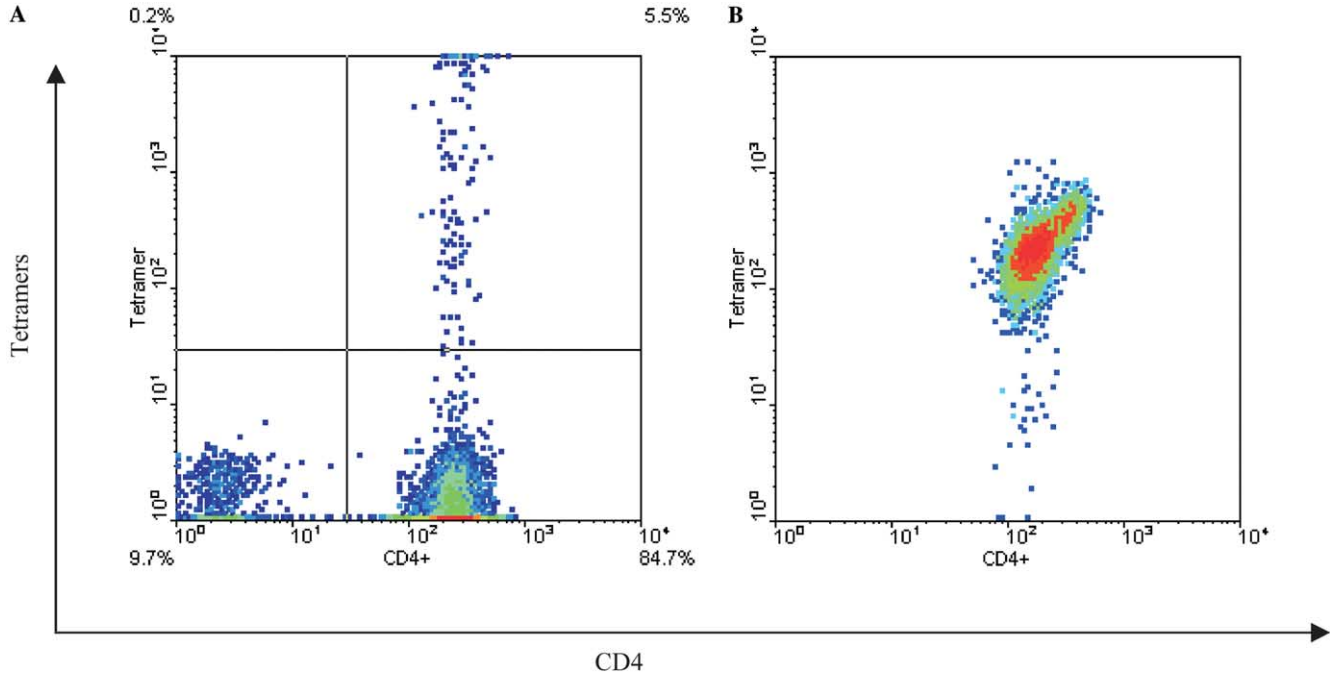

Fig. 4. Tetramer-guided epitope mapping of CMV pp65 epitope. An example of TGEM for the mapping of a DR0101-restricted pp65 epitope is shown. A panel of DR0101-binding peptides from the CMV pp65 protein as selected by the TEPITOPE program using a threshold of 3 as shown in Table 1 were used to stimulate the PBMC of a DR0101 CMV-seropositive individual. DR0101 tetramers corresponding to all seven peptides were also generated. Fifteen days later, PBMC were examined by staining with the panel of DR0101 tetramers. (A) Staining of cells with the DR0101/pp65 108–127 tetramers. (B) The tetramer-positive T cells were single-cell sorted by flow cytometry, expanded with PHA, and reexamined by tetramer staining.

are cultured at either too low or too high cell density will not expand.

2. All tetramer staining should be carried out within 10 to 16 day after in vitro stimulation. Antigen-specific T cells may undergo apoptosis after prolonged culture.

3. Aggregation of tetramers can cause high background staining. Purification of tetramers with size-exclusion chromatography will reduce the background.

4. Optimal staining time and optimal staining temperature for T cells of different specificity can vary.

5. Optimal pH for peptide loading depends on both the peptide and the MHC. For maximum loading of peptide into MHC, the investigator needs to run an experiment to examine each MHC and peptide pair.

4.8. Limitation of tetramers in staining T cells and epitope mapping

1. Peptides that have low affinity for MHC are not able to form tetramers; thus T cells that recognize the low-affinity peptide will not be detected. As a consequence, TGEM is also most effective in identifying antigenic epitopes that have high affinity for the MHC.

2. Formation of tetramers depends on the solubility of peptide antigens, and peptides that are insoluble in the loading buffer cannot form MHC/peptide complexes. T cells that recognize these complexes will not be detected by the TGEM approach.

3. PBMC in most cases will need to be stimulated with the peptide mixtures, and tetramer staining is carried out 10 to 14 days after the in vitro stimulation. T cells that are incapable of proliferating or that undergo apoptosis during that period will not be detected.

4. One major limitation of utilization of tetramers in staining and in TGEM is the availability of the soluble class II molecules that can be used for tetramer formation. Currently, only a limited number of soluble class II molecules are available, rendering it difficult to identify T-cell epitopes for uncommon class II alleles.

5. TGEM cannot be applied to mapping of class I-restricted epitopes.

5. Conclusions

The use of soluble class II MHC tetramers has enabled the identification of antigen-specific T cells in the peripheral blood. Our approach takes advantage of the efficient loading of the exogenous peptides onto empty soluble class II molecules. This method of choice allows the generation of large varieties of MHC/peptide complexes as staining reagents in a cost-effective manner.

Class II molecules can also be loaded with peptide mixtures rather than an individual peptide. The pooled tetramers can be used as reagents for screening CD4+ T-cell epitopes. The method can be used for rapid isolation of T cells that stain with the specific tetramer. The combination of computer-assisted algorithms for prediction of MHC binding and TGEM also provides an alternate choice for confirming the presence of the antigenic epitope as predicted by the algorithm. The utilization of tetramer techniques in the detection of T cells specific for foreign antigens and mapping epitopes is a powerful tool in the study of CD4+ T-cell responses against infectious viruses and bacteria. Success of tracking CD4+ T cells directed against autoantigens in insulin-dependent diabetes mellitus indicates that, in some cases, self antigens can also be detected with these tools. The use of tetramers in other autoimmune diseases and cancers and the applications of the TGEM approach in identifying T-cell epitopes directed against autoantigens remain largely unexplored, and will be the next challenge in applying the tetramer technology to examination of CD4+ T-cell responses.

References

[1] E.J. Novak, A.W. Liu, G.T. Nepom, W.W. Kwok, J. Clin. Invest 104 (1999) R63–R67.
[2] W.W. Kwok, A.W. Liu, E.J. Novak, J.A. Gebe, R.A. Ettinger, G.T. Nepom, S.N. Reymond, D.M. Koelle, J. Immunol. 164 (2000) 4244–4249.
[3] T.O. Cameron, J.R. Cochran, B. Yassine-Diab, R.P. Sekaly, L.J. Stern, J. Immunol. 166 (2001) 741–745.
[4] A.L. Meyer, C. Trollmo, F. Crawford, P. Marrack, A.C. Steere, B.T. Huber, J. Kappler, D.A. Hafler, Proc. Natl. Acad. Sci. USA 97 (2000) 11433–11438.
[5] B.L. Kotzin, M.T. Falta, F. Crawford, E.F. Rosloniec, J. Bill, P. Marrack, J. Kappler, Proc. Natl. Acad. Sci. USA 97 (2000) 291–296.
[6] H. Reijonen, E.J. Novak, S. Kochik, A. Heninger, A. Liu, W. Kwok, G. Nepom, Diabetes 51 (2002) 1375–1382.
[7] G.T. Nepom, J. Buckner, E.J. Novak, S. Reichstetter, H. Reijonen, J. Gebe, R. Wang, E. Swanson, W.W. Kwok, Arthritis Rheum. 46 (2002) 5–12.
[8] W. Kwok, N. Ptacek, A. Liu, J. Buckner, J. Immunol. Methods 268 (2002) 71.
[9] E.J. Novak, A.W. Liu, J.A. Gebe, B. Falk, G.T. Nepom, D.M. Koelle, W.W. Kwok, J. Immunol. 166 (2001) 6665–6670.
[10] W.W. Kwok, J. Gebe, A. Liu, S. Agar, N. Ptacek, J. Hammer, D. Koelle, G.T. Nepom, Trends Immunol. 22 (2001) 583–588.