

Article

Preparation and Characterization of HLA-A*0201 Tetramer Loaded with IE-1₃₁₆₋₃₂₄ Antigenic Peptide of Human Cytomegalovirus

Lihui Xu^{1,2}, Qingbing Zha^{1,3}, Hong Sun¹, Qiantao Jia¹, Fengyao Li¹ and Xianhui He^{1,4}

Major histocompatibility complex (MHC) class I tetramer technology has become the central technique for analyzing antigen-specific CD8⁺ T cell responses and it has been widely used to explore the differentiation and formation of memory CD8⁺ T cells. Previously, a simplified and efficient procedure for preparing high quality HLA-A*0201 tetramers has been established in our lab and the tetramers loaded with HCMV peptide pp65₄₉₅₋₅₀₃ has been successfully applied to investigate HCMV-specific CD8⁺ T cells in Chinese populations. Using similar procedure we reported here the construction of HLA-A*0201 tetramer loaded with another dominant epitope derived from immediate early (IE)-1₃₁₆₋₃₂₄ (VLEETSVML, VLE) of HCMV (A2-VLE) and characterization of this tetramer. After A2-VLE monomer was prepared and purified, its tetramer was then formed at a yield of 83%. The optimized amount of A2-VLE tetramer for staining 100 μ l whole blood was 0.5 μ g with incubation at 4°C for 1 h. Furthermore, the dissociation constant of the tetramer binding to the specific CD8⁺ T cells of one HLA-A2⁺ donor was estimated to be 32.7 nmol/L, which is markedly higher than that of MHC monomer. The construction of A2-VLE tetramer provides an alternative choice for investigating HCMV-specific CD8⁺ T cell responses and will deepen our understanding of the differentiation and formation of HCMV-specific memory CD8⁺ T cells. *Cellular & Molecular Immunology*. 2006;3(5):367-371.

Key Words: HLA, tetramer, cytomegalovirus, immediate early-1, dissociation constant

Introduction

Antigen-specific CD8⁺ T cells play an essential role in the control and elimination of virus infectious and tumor cells (1, 2) and thus direct characterization of these cells are critical for understanding the mechanism of cell-mediated immune responses (2). MHC class I tetramer technology has become the central technique for direct visualization of antigen-specific CD8⁺ T cells (3). Due to the application of tetramer technology in animal models and clinical studies, great progresses have recently been made in unveiling the

underlying mechanism of differentiation and formation of long-term memory CD8⁺ T cells (2, 4, 5). Such studies, undoubtedly, will provide new strategy for immunotherapy of tumors and shed new light on the design of new vaccines.

Although MHC I tetramer technology has been widely used to analyze CD8⁺ T cell phenotypes and functions (3), its application has been hampered due to either the complexity to construct a tetramer (6) or its expansiveness from commercial origin. Previously, we have established a simplified procedure for preparing HLA-A*0201 tetramers (7) and the tetramers loaded with HCMV peptide pp65₄₉₅₋₅₀₃ have been successfully applied to investigate HCMV-specific CD8⁺ T cells in Chinese populations (8). Using similar procedure we reported here the details of the construction of HLA-A*0201 tetramer loaded with another dominant epitope derived from IE-1₃₁₆₋₃₂₄ (VLEETSVML, VLE) of HCMV and characterization of this tetramer. These results provided an important basis for further investigation of HCMV-specific CD8⁺ T cells both in healthy and transplant patients.

Materials and Methods

Abbreviations: MHC, major histocompatibility complex; HLA, human leukocyte antigen; HCMV, human cytomegalovirus; IE-1, immediate early-1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

¹Institute of Tissue Transplantation and Immunology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China;

²Institute of Bioengineering, College of Life Science and Technology, Jinan University, Guangzhou 510632, China;

³Clinical Experimental Center of the First Affiliated Hospital, Jinan University, Guangzhou 510632, China;

⁴Corresponding to: Dr. Xianhui He, Institute of Tissue Transplantation and Immunology, College of Life Science and Technology, Jinan University, 601 Huangpu Dadao West, Guangzhou 510632, China. Tel: +86-20-8522-0679, Fax: +86-20-8522-0679, E-mail: thehx@jnu.edu.cn.

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Bacterial strains and reagents

Escherichia coli (*E. coli*) strain BL21 (DE3) was purchased from Novagen (Madison, WI, USA). MonoQ 5/50 GL column was obtained from Amersham (Uppsala, Sweden). Mouse anti-human monoclonal antibodies, CD3-FITC, CD8-APC, and HLA-A2-FITC were purchased from PharMingen (San Diego, CA, USA). R-phycoerythrin (PE) conjugate streptavidin (streptavidin-PE) was obtained from Molecular Probes (Eugene, OR, USA). Protein molecular weight (MW) markers were purchased from Sigma (St. Louis, MO, USA). The biotinylation enzyme, BirA, was purchased from Avidity (Denver, CO, USA). Isopropyl- β -D-thiogalactoside (IPTG) and all the other chemicals used were of analytical reagent grade. The recombinant plasmids harboring the genes for HLA-A*0201 heavy chain fused with BirA substrate peptide (BSP) or β_2 -microglobulin (β_2 m) were constructed as previously (7).

Peptide synthesis

The antigenic peptide VLE corresponding to residues 316-324 (VLEETSVML) of IE-1 protein of HCMV (9) was synthesized at Invitrogen Biotechnology Co. (Shanghai, China) and purified to purity of > 95%. The peptide was dissolved in dimethyl sulfoxide at a final concentration of 10 mg/ml and aliquots were stored at -70°C .

SDS-PAGE

Discontinuous SDS-PAGE was performed according to Laemmli (10), using a 15% polyacrylamide separating gel and a 5% stacking gel. In brief, samples were subjected to SDS-PAGE for 45 min at 200 V and then the gel was stained by Coomassie Brilliant Blue R250. Gel images were taken with FluorChem SP imaging system (Alpha Innotech, San Leandro, CA) and analyzed with AlphaEaseFC software (Alpha Innotech).

Inclusion bodies

Inclusion bodies of HLA-A*0201-BSP and β_2 m were purified as described previously (7) and the purified inclusion bodies were dissolved in 20 mmol/L 2-(*N*-morpholino) ethanesulfonic acid (pH6.0, containing 8 mol/L urea, 10 mmol/L EDTA and 0.1 mmol/L DTT), respectively. The protein concentration was determined by measuring absorbance at 280 nm and 260 nm, and calculated according to the empirical formula ($1.45 \times A_{280} - 0.74 \times A_{260}$ = protein concentration in mg/ml).

Reconstruction of HLA-A*0201-VLE (A2-VLE) monomers

A2-VLE monomeric complex was refolded as described previously (7). In brief, 2 mg VLE peptide was added into 200 ml of refolding buffer [0.1 mol/L Tris-HCl, pH 8.0, containing 0.4 mol/L L-arginine, 2 mmol/L EDTA, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.2 mmol/L phenylmethyl sulfonyl fluoride (PMSF)] prechilled to 10°C , and then 6 mg of HLA-A*0201-BSP and 5 mg of β_2 m were added to the stirring refolding reaction. The mixture was incubated at 10°C for 3 d

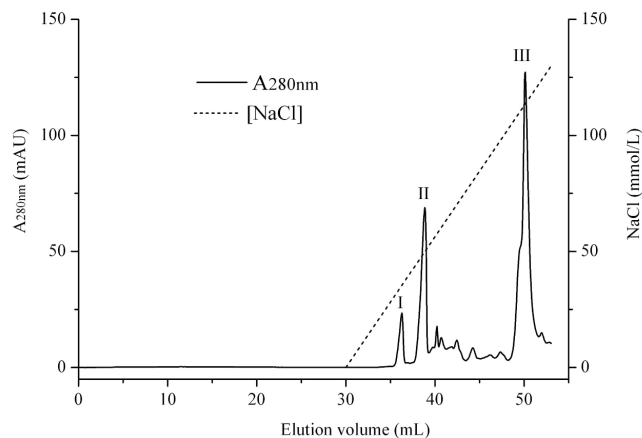


Figure 1. Purification of biotinylated A2-VLE monomer with MonoQ 5/50 GL column. The column was equilibrated with 10 mmol/L Tris-HCl (pH 8.0). The sample dialyzed against the same buffer was loaded onto the column and eluted with 0-150 mmol/L NaCl linear gradient at a flow rate of 1.0 ml/min. Fractions of 1 ml were collected. Peak III containing pure A2-VLE was pooled.

with continuous stirring and was then concentrated from 200 ml to about 5 ml using an ultrafiltration device (Amicon, Millipore) with 10 kD molecular mass cutoff membrane. The buffer was exchanged into 10 mmol/L Tris-HCl buffer (pH 8.0) by dialysis. After centrifugation at 13,000 rpm for 5 min to remove any pellets, the supernatant was collected for biotinylation.

Biotinylation of A2-VLE monomer

Refolded A2-VLE monomer was biotinylated at the specific site on BSP by BirA according to the recommended procedure. Biotinylated monomer was then dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.0) and loaded onto MonoQ 5/50 GL column preequilibrated with the same buffer. The column was eluted with a linear gradient of 0~150 mmol/L NaCl using Akta UPC9000 system (Amersham, Uppsala, Sweden). The fractions (about 1 ml) were collected and determined by SDS-PAGE. The peak containing both HLA-A*0201-BSP and β_2 m bands were collected and concentrated. The buffer was then changed to PBS (containing 0.2 mmol/L PMSF and 2 mmol/L EDTA) by

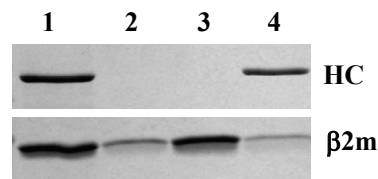


Figure 2. SDS-PAGE analysis of refolded A2-VLE monomers. Lane 1, A2-VLE monomer before purification; Lane 2, peak I of Figure 1; Lane 3, peak II of Figure 1; Lane 4, peak III of Figure 1. HC: heavy chain.

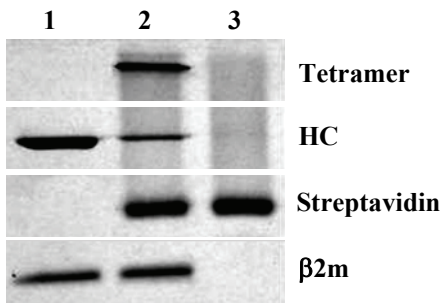


Figure 3. SDS-PAGE analysis of A2-VLE tetramer. Lane 1, A2-VLE monomer; Lane 2, A2-VLE monomer + streptavidin; Lane 3, streptavidin. HC: heavy chain.

ultrafiltration. The protein concentration was determined as described above and stored at 4°C.

A2-VLE tetramer

The tetramer was formed by mixing the biotinylated A2-VLE monomers with one-fourth of its molar amount of streptavidin-PE. SDS-PAGE analysis, under non-reducing conditions without boiling treatment, was used to estimate the multiplication extent. The final tetrameric complex was stored at 4°C.

Flow cytometry

Heparinized whole blood was collected from one healthy HLA-A2⁺ volunteer who was HCMV IgG positive (identified by IgG ELISA kit from Biocheck, USA). Whole blood (100 µl) was first incubated with A2-VLE tetramer/PE at 4°C for 1 h, followed by 20 min incubation with anti-CD3-FITC and anti-CD8-APC at 4°C in the dark. After lysing the red blood

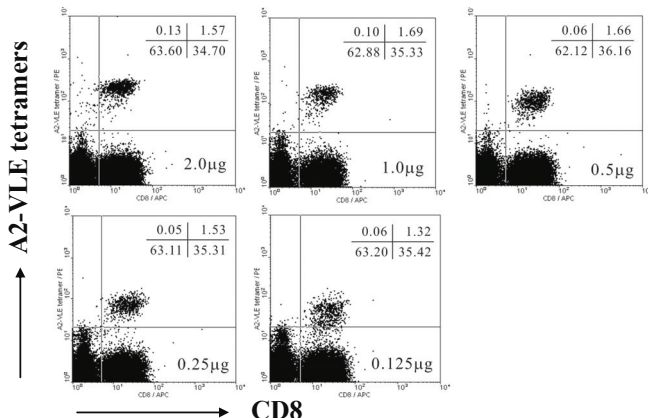


Figure 4. Flow cytometric analysis of HCMV-specific CD8⁺ T cells stained with A2-VLE tetramers. Different amounts of A2-VLE tetramers, as indicated in each panels, were used to stain 100 µl whole blood followed by anti-CD8-APC staining. The data represent the percentages of each quadrant. The cells were gated on lymphocytes.

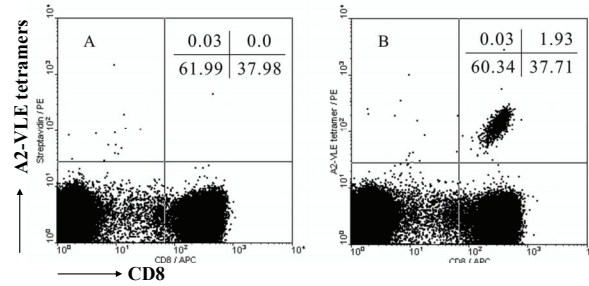


Figure 5. Flow cytometric analysis of HCMV-specific CD8⁺ T cells stained with optimized amount of A2-VLE tetramer. (A) Control; (B) A2-VLE tetramer (0.5 µg). About 100 µl whole blood was stained with tetramer followed by anti-CD3-FITC plus anti-CD8-APC staining. The cells were gated on CD3⁺ cells. The data represent the percentages of each quadrant gated on CD3⁺ T cells.

cells, nucleated cells were collected through centrifugation. The cell pellets were then washed twice with 2 ml PBS and fixed in 0.3 ml of 4% paraformaldehyde for 15 min. The samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and 200,000 events were collected for each sample and analyzed later with WinMDI version 2.8.

Results

Refolding, biotinylation and purification of A2-VLE monomeric complex

The A2-VLE monomers were constructed through *in vitro* refolding and subjected to anion-exchange chromatography. Figure 1 shows the elution profile of biotinylated A2-VLE monomers from MonoQ column. SDS-PAGE (Figure 2) showed that peaks I and II had β2m only while peak III contained both HLA-A*0201-BSP and β2m proteins, indicating that refolded A2-VLE monomers were eluted at peak III. This peak was collected and concentrated for preparing tetramers and the purity was estimated to be 97% by SDS-PAGE analysis.

A2-VLE tetramer formulation

The tetramer was subsequently prepared by mixing the biotinylated A2-VLE monomers with streptavidin-PE at a 4:1 molar ratio. SDS-PAGE analysis under non-reducing condition without boiling the sample revealed that more than 83% of monomeric complex formed tetramers, suggesting effective biotinylation and multiplication of the biotinylated A2-VLE monomers (Figure 3).

Optimization of A2-VLE tetramer staining

In order to obtain specific tetramer staining, it is required to determine the suitable amount of the tetramer. Different amount of tetramers was used to stain 100 µl whole blood and the samples were analyzed with flow cytometry under the same settings. As shown in Figure 4, the fluorescent

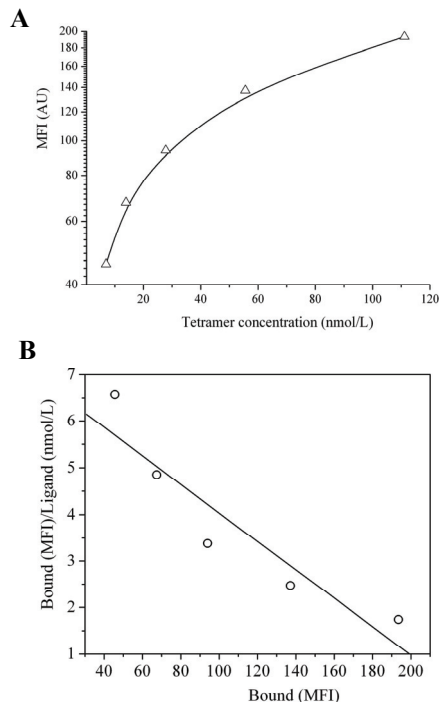


Figure 6. Saturation binding curve (A) and Scatchard plot (B) of A2-VLE tetramer with HCMV-specific CD8⁺ T cells from one HLA-A2⁺ donor.

intensity of tetramer-positive cells was reduced when the tetramer amount was decreased but the nonspecific staining of CD8-negative cells was decreased as well. When the tetramer amount was 0.5 μ g, the fluorescent intensity of tetramer-positive cells was still high enough to separate from the tetramer-negative cells while the nonspecific staining of CD8-negative cells was significantly lowered. Using this optimized tetramer amount, we showed that the frequency of VLE-specific CD8⁺ T cell in this donor was 1.93% within CD3⁺ T cell population (Figure 5).

Dissociation constant (Kd) of A2-VLE tetramer

The mean fluorescent intensity (MFI) of tetramer-positive cells was from the data shown in Figure 4 and the saturation binding curve was plotted by using the tetramer concentration versus the MFI of tetramer-positive cells (Figure 6A). The dissociation constant (Kd) was calculated to be 32.7 nmol/L by using Scatchard plot (Figure 6B).

Discussion

Primary HCMV infection is usually asymptomatic and leads to lifelong persistence in latency in immunocompetent hosts (11). However, in immunocompromised persons, including recipients of bone-marrow transplantation or organ allografts and patients with human immunodeficiency virus type I infections, HCMV infection or reactivation may cause severe diseases of a variety of organs, such as the lung, the retina,

and the liver (11). CD8⁺ T cells are believed to play a crucial role in both the elimination of active infection and maintenance of HCMV latency (11-13). High frequency of specific CD8⁺ T cells recognizing a single HCMV pp65₄₉₅₋₅₀₃ epitope has been observed in the peripheral blood of seropositive donors (8, 14, 15), suggesting that NLV is an immunodominant epitope. Apart from pp65, IE-1 is also a predominant antigen and one of the immunodominant epitope derived from IE-1, i.e., IE-1₃₁₆₋₃₂₄ (VLEETSVM, VLE), has been identified (9). We previously established a simplified procedure for preparing HLA-A*0201 tetramer incorporating pp65₄₉₅₋₅₀₃ peptide (7). By using similar procedure, in the present study we have successfully constructed another HLA-A*0201 tetramer loaded with VLE peptide, indicating this procedure being applicable for other HLA-A*0201 tetramers. In addition, our primary study showed that high frequency VLE-specific CD8⁺ T cells existed in one healthy individual although more subjects are needed to understand the specific CD8⁺ T cell responses in Chinese population.

Although high frequencies of pp65₄₉₅₋₅₀₃-specific CD8⁺ T cells are usually detected in the populations (8, 14, 15), a recent study suggested that high frequencies of IE-1 but not pp65-specific CD8⁺ T cells correlated with protection from HCMV disease in transplant recipients (16). This result indicates that IE-1 is also a dominant T cell target and detailed analysis of the phenotypes and functions of IE-1-specific CD8⁺ T cells would provide further information about the cellular response to HCMV. Unfortunately, no tetramer loaded with VLE peptide was included in that study (16). With the availability of A2-VLE tetramer, both the phenotypes and functions of the specific CD8⁺ T cells could be analyzed conveniently. Furthermore, comparison of VLE- and NLV-specific CD8⁺ T cells would provide us further information about their protection functions. These studies are presently underway in our lab.

Direct staining of CD8⁺ T cells with MHC class I tetramer depends on increased avidity of the tetramers due to multiplication. Here we applied flow cytometry to measure the avidity (Kd) of A2-VLE tetramer. The Kd of A2-VLE tetramers with the T cell receptors (TCRs) was nearly at nanomolar level, suggesting a significant increase in the avidity when compared with the affinity of monomer which is usually at micromolar level. As recently suggested, the avidity of TCRs recognizing an antigenic peptide presented on the MHC molecules is a reflection of their structural avidity (17). Thus flow cytometry measurement of Kd of tetramer binding to TCRs on CD8⁺ T cells would be a convenient way to estimate the functional avidity (the ability to secrete IFN- γ after stimulation with specific peptide) of antigen-specific cells. It is interesting when the structural avidity is compared with functional avidity of the specific T cells.

In summary, construction of A2-VLE tetramer provides an alternative choice for investigating HCMV-specific CD8⁺ T cell responses and will deepen our understanding of the differentiation and formation of HCMV-specific memory CD8⁺ T cells. In addition, the present approach for measuring

TCR avidity would be useful to investigate the relationship between structural and functional avidity.

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