Article

Preparation and Identification of HLA-A*1101 Tetramer Loading with Human Cytomegalovirus pp65 Antigen Peptide

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MHC/peptide tetramer technology has been widely used to study antigen-specific T cells, especially for identifying virus-specific $CD8^+$ T cells in humans. The tetramer molecule is composed of HLA heavy chain, β_2-microglobulin (β_2m), an antigenic peptide, and fluorescent-labeled streptavidin. To further investigate the HLA-A*1101-restricted $CD8^+$ T cell responses against human cytomegalovirus (HCMV), we established an approach to prepare HLA-A*1101 tetramer complexed with a peptide from HCMV. The cDNA encoding HLA-A*1101 heavy chain was cloned and the prokaryotic expression vector for the ectodomain of HLA-A*1101 fused with a BirA substrate peptide (HLA-A*1101-BSP) at its carboxyl terminus was constructed. The fusion protein was highly expressed as inclusion bodies under optimized conditions in *Escherichia coli*. Moreover, HLA-A*1101-BSP protein was refolded in the presence of β_2m and an HCMV peptide $pp65_{16-24}$ (GPISGHVLK, GPI). Soluble HLA-A*1101-GPI monomer was biotinylated and purified to a purity of 95%, which was subsequently combined with streptavidin to form tetramers at a yield of > 80%. The HLA-A*1101-GPI tetramers could bind to virus-specific $CD8^+$ T cells, suggesting soluble HLA-A*1101-GPI tetramers were biologically functional. This study provides the basis for further evaluation of HLA-A*1101-restricted $CD8^+$ T cell responses against HCMV infection. *Cellular & Molecular Immunology*. 2007;4(2):141-146.

Key Words: HLA-A*1101, tetramer, cytomegalovirus, $CD8^+$ T cell, epitope

Introduction

The $CD8^+$ T cells are an important T cell subset that contributes to the elimination of virus infection and tumor cells (1). Enumeration and characterization of antigen-specific T cells provide critical information for understanding the differentiation and formation of effector and memory $CD8^+$ T cells (2, 3). Soluble tetrameric MHC class I complexes are able to stain T cell receptors (TCR) on antigen-specific T cells (4). In combination with flow cytometry, tetramer technology has recently been widely used to identify and quantify the activation and differentiation status of antigen-specific $CD8^+$ T cells. Directly *ex vivo* detection and quantification of antigen-specific $CD8^+$ T cells present in peripheral blood samples should greatly facilitate the development of individualization strategy for immunotherapy of chronic virus infections and tumors (5).

To further investigate the HLA-A*1101-restricted $CD8^+$ T cell responses against human cytomegalovirus (HCMV), we here reported an approach to prepare HLA-A*1101 tetramers complexed with a peptide from HCMV. MHC class I molecules are composed of noncovalently associated polypeptide chains, HLA heavy chain (45 kDa) and human β_2-microglobulin (β_2m, 12 kDa) (4, 6, 7). In order to obtain soluble MHC I molecules, the prokaryotic expression vector harboring the cDNA encoding the ectodomain of HLA-A*1101 heavy chain fused with a BSP sequence (HLA-A*1101-BSP) was constructed. HLA-A*1101-BSP fusion protein was overexpressed as inclusion bodies in *E. coli*. Highly purified soluble HLA-A*1101 monomers and tetramers loaded with HCMV $pp65_{16-24}$ peptide (GPISGHVLK, GPI) were prepared according to a simplified

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

approach described previously (7). The functional HLA-A*1101 tetramers provide a basis for further investigation of HLA-A*1101-restricted $CD8^+$ T cell responses against HCMV infection.

Materials and Methods

Bacterial strains, enzymes and chemical reagents

E. coli strain BL21 (DE3) and plasmid pET-3d were purchased from Novagen (Madison, WI, USA). Restrictive endonucleases *EcoR* I, *Nco* I, *BamH* I, T4 DNA ligase and PrimeSTAR™ HS polymerase were purchased from Takara (Dalian, China). The TRIzol reagent and ThermoScript reverse transcription polymerase chain reaction (RT-PCR) system were obtained from Invitrogen (Carlsbad, USA). MonoQ 5/50 GL column was obtained from Amersham (Uppsala, Sweden). Mouse anti-human monoclonal antibodies, CD3-FITC, and CD8-APC were purchased from PharMingen (San Diego, USA). R-phycoerythin (PE)-conjugated streptavidin (streptavidin-PE) was obtained from Molecular Probes (Eugene, USA). Protein molecular weight (MW) markers were purchased from Sigma (St. Louis, USA). The biotinylation enzyme, BirA, was purchased from Avidity (Denvor, USA). Isopropyl-β-D-thiogalactoside (IPTG) and all the other chemicals used were analytically pure. β_2-microglobulin (β_2m) was constructed as previously described (8). The antigenic peptide GPI_{16-24} (GPISGHVLK) derived from pp65 protein of HCMV was synthesized at Invitrogen Biotechnology Co. (Shanghai, China) and purified to purity of > 95%. GPI peptide was dissolved in dimethyl sulfoxide at a final concentration of 10 mg/ml and stored at -70°C.

*Cloning of HLA-A*1101 heavy chain*

Heparinized human peripheral blood was collected from three HLA-A11 positive (identified by sequence-specific primers amplification refractory mutation system-PCR, ARMS-PCR) donors by venipuncture. Total RNA was extracted from freshly isolated peripheral blood mononuclear cells (PBMCs) using the TRIzol reagent. cDNAs were synthesized from the isolated RNA using the ThermoScript RT-PCR system according to the recommended procedure. PCR amplification of the resultant cDNA was performed in a total volume of 50 μl containing high fidelity PrimeSTAR™ HS polymerase. The PCR protocol was performed with an initial denaturation for 2 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, and a final extension for 10 min at 72°C and the primers (the forward primer 5'-ACT ATT CGC CAC CAT GGC CGT CAT GGC GCC-3' and the reverse primer 5'-CGC GGG ATC CCG CAC TTT ACA AGC TGT GAG AGA CAC-3') were designed to amplify the entire mature A11 coding sequence. The amplified PCR products were inserted into the *EcoR* I and *BamH* I sites of the pEGFP-N1 vector. Randomly selected clones were *EcoR* I and *BamH* I digested and screened for the presence of a correctly sized insert. Several independent clones were submitted for DNA sequencing of the HLA-A*1101 heavy chain through the dye-labeled deoxy-terminator protocol on a 377 automated DNA sequencer (Applied Biosystems).

Construction of expression vector for extracellular domain of HLA-A11 heavy chain fused with BirA substrate peptide (BSP)

To construct an expression vector in which the HLA-A11 heavy chain was fused with BSP, the DNA fragment encoding a Gly-Ser linker and a BSP (LHHILDAQKMV WNHR) was fused to the 3' end of the cDNA encoding the ectodomain of the HLA-A11 heavy chain (1-275) by PCR amplification from cloned HLA-A11 heavy chain cDNA with specific primers (the forward primer 5'-ATA TCC ATG GGT TCT CAC TCC ATG AGG TAT TTC-3' and the reverse primer 5'-GCG CGG ATC CTT AAC GAT GAT TCC ACA CCA TTT TCT GTG CAT CCA GAA TAT GAT GCA GAG AGC CCG GCT CCC ATC TCA GGG T-3'). The amplified PCR product was *Nco* I/*BamH* I digested and inserted into plasmid pET-3d. Clones with correct-size inserts were verified by direct sequencing, and the recombinant plasmid was named as pET-A11-BSP.

SDS-PAGE and Western blotting

Discontinuous SDS-PAGE was performed according to Laemmli (9), using a 15% polyacryamide 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) and analyzed with AlphaEaseFC sofeware (Alpha Innotech). Western blotting was performed as described previously (10). Anti-HLA-A*0201 antiserum raised in mice was used as primary antibody.

*Expression and purification of recombinant HLA-A*1101-BSP*

The expression vector pET-A11-BSP was transformed into *E. coli* BL21 (DE3). Single transformed colony was inoculated into 5 ml of LB medium and rocked at 37°C overnight. Each culture was diluted 10-fold in fresh LB and incubated at 37°C for about 2-3 h. After 0.4 mmol/L IPTG was added, the culture was rocked for another 4 h to induce the expression of target protein, and the cells were collected by centrifugation. Inclusion bodies of HLA-A*1101-BSP were purified as described previously (7) and the purified inclusion bodies were dissolved in 20 mmol/L 2-(N-morpholino) ethanesulfonic acid (pH 6.0, containing 8 mol/L urea, 10 mmol/L EDTA and 0.1 mmol/L DTT). 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).

*Refolding of HLA-A*1101-GPI monomers*

HLA-A*1101-GPI monomeric complex was refolded as described previously (7). First, 2 mg GPI peptide was added into 200 ml prechilled refolding buffer (100 mmol/L Tris-HCl, pH 8.0, containing 400 mmol/L L-arginine, 2

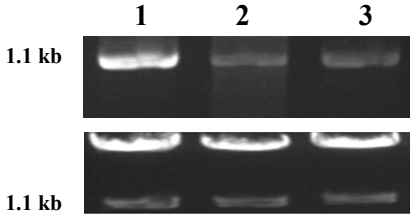

Figure 1. Electrophoresis of cDNA of HLA-A*1101 heavy chain and pEGFP-A11 digested by *EcoR* I and *BamH* I. The cDNA of HLA-A*1101 was amplified from total RNA of human white blood cells, the recombinant plasmid pEGFP-A11 was identified by restrictive enzyme digestion. Upper, HLA-A*1101 cDNA products; lower, the recombinant plasmid pEGFP-A11 digested by *EcoR* I and *BamH* I. Lanes 1-3, samples from three independent donor.

mmol/L EDTA, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione and 0.2 mmol/L PMSF). Then 6 mg of HLA-A*1101-BSP and 5 mg of β_2m were added to the stirring refolding reaction. The mixture was incubated at 10°C for 3 days, and finally, 200 ml of the refolding mixture was concentrated to about 5 ml through an ultrafiltration apparatus with 10 kDa molecular mass cut-off membrane, and dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.0). By centrifugation precipitates was eliminated, and then refolded HLA-A*1101 monomer was ready for biotinylation.

*Biotinylation of HLA-A*11011-GPI monomer*

Refolded HLA-A*11011-GPI monomer was biotinylated on BSP by BirA according to the recommended procedure and then dialyzed against 10 mmol/L Tris-HCl buffer (pH 8.0, with 0.2 mmol/L PMSF) and subjected onto MonoQ 5/50 GL column pre-equilibrated with the same buffer. The column was eluted with a 0~150 mmol/L NaCl linear gradient using AKTA UPC9000 system (Amersham, Uppsala, Sweden). The fractions containing both HLA-A*1101-BSP and β_2m bands were harvested and analyzed by SDS-PAGE. After concentration, the buffer was changed to PBS (containing 0.2 mmol/L PMSF and 2 mmol/L EDTA) by ultrafiltration and stored at 4°C.

Flow cytometry

Heparinized peripheral whole blood was collected from three healthy HLA-A*1101 volunteers with HCMV IgG seropositive (identified by IgG ELISA kit from Biocheck, USA). Whole blood (100 μl) was first incubated with HLA-A*1101-GPI tetramer/PE at 37°C for 20 min in the dark, then incubated with anti-CD3-FITC and anti-CD8-APC at 4°C for 20 min in the dark. The red blood cells were eliminated with lysing buffer and the nucleated cells were collected through centrifugation. The cells were then washed twice with 2 ml PBS and fixed in 200 μl PBS containing 1% paraformaldehyde for 15 min. For each sample, 200,000 events were acquired with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA) and the data were analyzed with WinMDI version 2.8 (http://facs.scripps.edu/software.html).

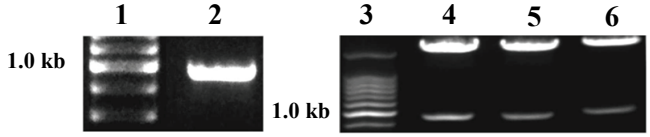

Figure 2. Electrophoresis of HLA-A*1101 sub-clone PCR products and pET-A11-BSP digested by *Nco* I and *BamH* I. Lanes 1 and 3, 200 bp DNA ladder; Lane 2, PCR product; Lanes 4-6, pET-A11-BSP recombinant plasmids digested by *Nco* I and *BamH* I.

Results

*Cloning of HLA-A*1101 heavy chain cDNA*

The cDNA of HLA-A*1101 gene was amplified using the total RNA as a template and the DNA fragments were inserted into pEGFP-N1 vector. Then the ligation mixture was transformed into DH5α cells and the clones with expected DNA insert (1100 bp) were identified by agarose gel electrophoresis analysis (Figure 1). DNA sequence analysis of these clones confirmed that 3 clones from donor 2 were consistent with HLA-A*1101 heavy chain gene, and the cDNA sequence for HLA-A*1101 was submitted to GenBank (accession number DQ327721). This recombinant plasmid was named as pEGFP-A11.

*Construction of expression vector for ectodomain of HLA-A*1101 heavy chain fused with BSP*

The DNA fragment encoding the ectodomain of HLA-A*1101 fused with BSP was PCR amplified and the PCR product was inserted into pET-3d vector. The recombinant plasmids with a correct insert were screened out and verified by DNA sequencing. This expression vector was called pET-A11-BSP (Figure 2).

*Refolding, biotinylation and purification of HLA-A*1101-GPI monomer*

The expression vector pET-A11-BSP was transformed into *E. coli* BL21 (DE3). Expression of the recombinant protein was analyzed by SDS-PAGE. The results showed that the transformed cells produced the expected recombinant proteins (35 kDa) which account for about 20% of the total

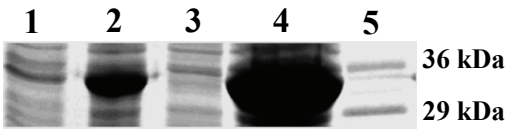

Figure 3. SDS-PAGE analysis of recombinant HLA-A*1101-BSP proteins expressed in *E. coli* BL21 (DE3). Lane 1, BL21 (pET-A11-BSP) without IPTG induction; Lane 2, BL21 (pET-A11-BSP) with IPTG induction; Lane 3, supernatant of BL21 (pET-A11-BSP) lysate; Lane 4, washed insoluble inclusion body of HLA-A*1101-BSP; Lane 5, MW marker.

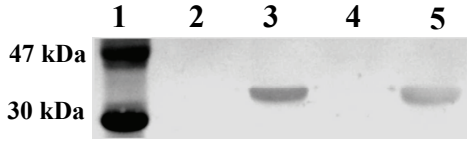

Figure 4. Western blotting analysis of IPTG-induced expression of HLA-A*1101-BSP in BL21 (DE3). After 15% SDS-PAGE, the proteins were transferred to PVDF membrane, and the membrane was incubated with anti-HLA-A*0201 antiserum and followed by the HRP-labeled secondary antibody. The specific bands were visualized using the DAB as a substrate. Lane 1, MW marker; Lane 2, BL21 (pET-A11-BSP) without IPTG induction; Lane 3, BL21 (pET-A11-BSP) with IPTG induction; Lane 4, supernatant of BL21 (pET-A11-BSP) lysate; Lane 5, washed insoluble inclusion body of HLA-A*1101-BSP.

bacterial proteins (Figure 3, Lane 2). The HLA-A*1101-BSP protein was mainly expressed in the insoluble fraction (inclusion bodies). Western blotting analysis showed that there was a strong band corresponding to HLA-A*1101-BSP (Figure 4), while no such band appeared in the cells before induction.

Next, the HLA-A*1101-GPI monomers were constructed through *in vitro* refolding and purified by anion-exchange chromatography on a MonoQ column. SDS-PAGE analysis showed that the refolded soluble monomers contained both heavy chain and β_2m (Figure 5, Lane 2). After biotinylation and column purification, the purity of HLA-A*1101-GPI monomers was estimated to be 95% (Figure 5, Lane 3).

*HLA-A*1101-GPI tetramer formulation*

Subsequently, HLA-A*1101-GPI tetramers were prepared by mixing the biotinylated HLA-A*1101-GPI monomers with streptavidin-PE at a 4:1 molar ratio. The tetramers were subjected to SDS-PAGE analysis without boiling treatment and reducing reagent. As shown in Figure 6, HLA-A*1101-GPI tetramers appeared as a larger complexes and more than 80% of monomers formed tetramer complexes.

*HLA-A*1101-GPI tetramer staining*

The biological function was determined by flow cytometric analysis. HLA-A*1101-GPI tetramers were used to stain

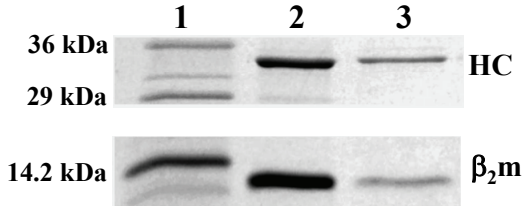

Figure 5. SDS-PAGE analysis of refolded HLA-A*1101-GPI monomers. Lane 1, MW marker; Lane 2, HLA-A*1101-GPI refolding mixtures before purification; Lane 3, MonoQ column purified HLA-A*1101-GPI monomers. HC, heavy chain.

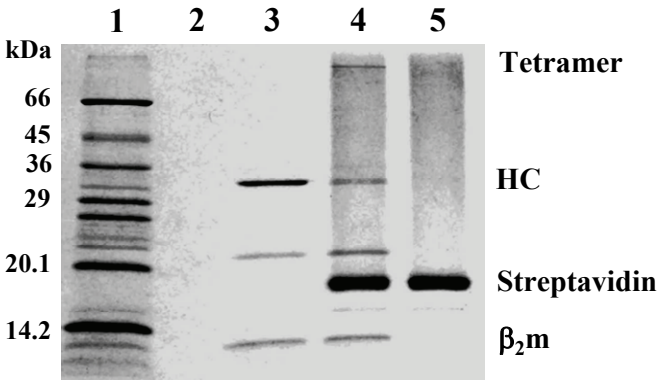

Figure 6. SDS-PAGE analysis of HLA-A*1101-GPI tetramer. Lane 1, MW marker; Lane 2, empty; Lane 3, HLA-A*1101-GPI monomer; Lane 4, HLA-A*1101-GPI monomer + streptavidin; Lane 5, streptavidin. HC, heavy chain.

whole blood from 3 HLA-A*1101 positive donors with HCMV latent infection. The samples were analyzed with multicolor-flow cytometry. Firstly $CD3^+$ lymphocytes were gated and then $CD8^+$ T cells further gated out for assessing tetramer-positive T cells. As shown in Figure 7, HLA-A*1101-GPI tetramer-specific $CD8^+$ T cells were detectable in all donors and the frequency was from 0.02% to 0.12% within $CD8^+$ T cell population. This result showed that HLA-A*1101-GPI tetramer was biologically functional.

Discussion

HCMV is the common cause of deafness and mental retardation in newborn infants, the infectious mononucleosis

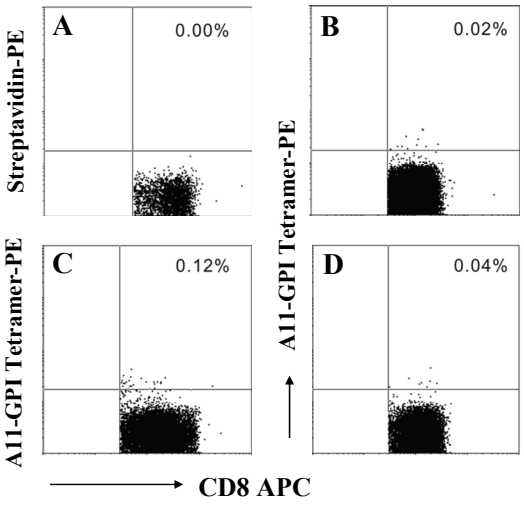

Figure 7. Flow cytometric analysis of HCMV-specific $CD8^+$ T cells stained with HLA-A*1101-GPI tetramers. (A) Control; (B-D) 100 µl whole blood from three HLA-A*1101 donors was stained with HLA-A*1101-GPI tetramers.

syndrome in young adults and devastating disseminated infection and enhances graft rejection in transplant patients (11). The $CD8^+$ T cells specific for HCMV pp65 provide protective immunity against the development of HCMV disease after transplantation (12). Moreover, recent studies indicate that HCMV infection is strongly associated with immunosenescence (13). Thus, quantitative analyses of antigen-specific $CD8^+$ T cell populations have provided critical information on cellular immune responses against HCMV infections (2). MHC class I tetramer technology has become the essential method for assessing virus-specific $CD8^+$ T cells. Its applications in the assessment of HCMV-specific cellular immune responses have also been widely adopted together with multiparametric flow cytometry (5, 14). The benefit of tetramer technology is much more than enumeration and sorting of antigen-specific T cells (15), it affords additional information about their phenotypes and functions by co-staining for cell surface markers and intracellular molecules (16). On the other hand, tetramers of different HLA alleles should be prepared in order to study different populations because HLA is a highly polymorphic gene in humans and the distribution frequency of HLA alleles is variable in different population. As HLA-A*1101 is one of the high frequency alleles in Chinese population, its tetramers are preferable to be used in any clinical studies. In the present study, we had prepared HLA-A*1101 tetramers loaded with a peptide $pp65_{16\text{-}24}$ (GPISGHVLK, GPI) derived from HCMV. The functional HLA-A*1101 tetramers thus will provide a critical reagent for further investigation of HLA-A*1101-restricted $CD8^+$ T cell responses against HCMV infection in Chinese population.

To study HCMV-specific $CD8^+$ T cells, antigenic peptide of $pp65_{495\text{-}503}$ is usually used to prepare HLA-A* 0201 tetramer, because this peptide is the strongest immunodominant epitope in HCMV (17, 18). However, this tetramer can only be used to study HLA-A*0201-restricted $CD8^+$ T cell responses. In order to analyze the HCMV-specific immune response in HLA-A*1101 individuals, HLA-A*1101 tetramers with immunodominant peptide epitope of HCMV are urgently needed because HLA-A*1101 is a very high frequency alleles in Chinese population especially in southern Chinese population. In our study, antigenic peptide $pp65_{16\text{-}24}$ of HCMV was loaded into the cleft of recombinant HLA-A*1101 molecule. Comparing to HLA-A*0201 restricted peptide antigen $pp65_{495\text{-}503}$ (17), the frequency of HLA-A* 1101-GPI tetramer-positive $CD8^+$ T cells in HCMV seropositive individuals was very low, indicating $pp65_{16\text{-}24}$ is not an immunodominant epitope to T cells. This result is consistent with previous study which also revealed by ELISPOT very low frequency of GPI-specific $CD8^+$ T cells in healthy individuals (19). Thus, for analyzing HCMV-specific immune responses in HLA-A*1101 donors, other HLA-A*1101-restricted immunodominant epitopes derived from HCMV remain to be identified. On the other hand, GPI-specific $CD8^+$ T cells can be firstly stimulated with this peptide *in vitro* to stimulate the proliferation of specific cells into a level which could be easily analyzed using HLA-A*1101-GPI tetramers.

In conclusion, we have expressed the HLA-A*1101 heavy chain fused with BSP at high levels in *E. coli.* Soluble HLA-A*1101-GPI monomers and tetramers were successfully refolded and purified. The preparation of HLA-A* 1101-GPI tetramer has offered the possibility to enumerate and identify the HCMV $pp65_{16\text{-}24}$-specific $CD8^+$ T cells in HLA-A*1101 individuals.

Acknowledgements

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