

## Article

# High Level Expression of HLA-A\*0203-BSP Fusion Protein in *Escherichia coli* and Construction of Soluble HLA-A\*0203 Monomer and Tetramer Loaded with Epstein-Barr Virus Peptide

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Major histocompatibility complex (MHC) tetramer technology is critical for characterization of antigen-specific T cells. In the present study we reported the successful generation of HLA-A\*0203 tetramer loaded with Epstein-Barr virus EBNA3<sub>596-604</sub> peptide (SVRDRLARL, SVR). Prokaryotic expression vector for the ectodomain of the heavy chain of HLA-A\*0203 fused with a BirA substrate peptide (HLA-A\*0203-BSP) was constructed and the expression conditions of the fusion protein in *Escherichia coli* (*E. coli*) were optimized. The fusion protein was highly expressed in inclusion bodies within *E. coli*. It was then refolded in the presence of  $\beta_2$ -microglobulin and SVR peptide to form a soluble HLA-A\*0203-SVR monomer. After biotinylation with BirA, the monomer was purified by anion-exchange chromatography and its purity was up to 95%. The tetramer was then formulated by mixing the biotinylated monomer with streptavidin-PE at a ratio of 4:1. Flow cytometry showed that this tetramer could specifically react with antigen-specific CD8<sup>+</sup> T cells, indicating that it was biologically functional. These results provide a foundation for further characterization of antigen-specific CD8<sup>+</sup> T cells from HLA-A\*0203 subjects. *Cellular & Molecular Immunology*. 2007;4(4):301-308.

**Key Words:** HLA-A\*0203, tetramer, Epstein-Barr virus, prokaryotic expression, biotinylation

## Introduction

The CD8<sup>+</sup> T cells play an essential role in the control of cancer and infectious diseases (1, 2). These cells recognize antigenic peptides of 8-11 amino acid residues in the context of major histocompatibility complex (MHC) class I molecules on the surface of target cells *via* T cell receptors (TCR) (2, 3) and subsequently lyse the target cells (4). Direct *ex vivo* visualization and quantification of antigen-specific CD8<sup>+</sup> T cells thus critical for the characterization of cellular immune responses (5, 6). However, previously employed assays including limiting dilution assays and ELISPOT are indirect, laborious and time-consuming (6, 7). Previous attempts to stain CD8<sup>+</sup> T cells using soluble monomeric

forms of peptide-MHC class I complexes have been proved unsuccessful due to the low affinity and fast off-rate of TCR peptide-MHC interaction (8, 9) which is necessary to enable serial contact of each TCR molecule with multiple MHC-peptide ligands on target cells (10). To overcome this problem, researchers focused on increasing the overall avidity by increasing the number of MHC class I molecules available to bind TCR. Consequently, in 1996, after more than 20 years of abortive efforts, peptide-MHC class I tetramer technology was introduced for the identification and enumeration of antigenic-specific CD8<sup>+</sup> T cells, thus initiating a profound revolution in the field of cellular immunology (11). These tetrameric molecules are thought to compensate for the low affinity and relative fast dissociation rate of the TCR/MHC-peptide interaction by increasing the avidity of this interaction, thus allowing the stable binding of MHC-peptide tetramers to TCR expressing cells (12, 13).

In recent years, tetramer technology has been widely used to examine T cell phenotype and function and has become the central technology for studying cell-mediated immune responses (6, 11, 14). Its applications, however, are limited to high frequency alleles owing to the availability of HLA matched donors. Due to the distinct distribution of HLA class I alleles in different populations, high frequency alleles are various in different ethnic populations (15). HLA-A\*0203 is one of the high frequency alleles in southern

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China (16) and its tetramer has not been prepared up to now. In the present study, we reported the successful generation of HLA-A\*0203 tetramer loaded with Epstein-Barr virus (EBV) EBNA3<sub>596-604</sub> peptide (SVRDRLARL, SVR) (17). Initially, the prokaryotic expression vector for the ecto-domain of heavy chain of HLA-A\*0203 fused with a BirA substrate peptide (BSP) was constructed and transformed into *Escherichia coli* (*E. coli*). Subsequently the temperature, IPTG concentration, and induction duration for the expression of the fusion protein in *E. coli* transformant were optimized. The fusion protein was efficiently expressed in insoluble inclusion bodies under the optimized expression conditions. The monomer of soluble MHC-peptide was generated by *in vitro* refolding of HLA-A\*0203-BSP in the presence of  $\beta_2$ -microglobulin ( $\beta_2m$ ) (18) and SVR peptide (17). Finally, the refolded HLA-A\*0203-SVR monomer was biotinylated with BirA and the tetramer was produced by mixing the biotinylated monomer with PE-conjugated streptavidin. The tetramer was biologically functional and could be used to characterize EBV-specific CD8<sup>+</sup> T cells from HLA-A\*0203 subjects.

## Materials and Methods

### Reagents and bacterial strains

Plasmid pET-3d and *E. coli* strain DH5 $\alpha$  and BL21(DE3) were purchased from Novagen (Madison, WI, USA). *EcoR* I, *Nco* I, *BamH* I, T<sub>4</sub> DNA ligase and high fidelity PyroBest DNA Taq polymerase were purchased from TaKaRa (Dalian, China). The TRIzol reagent and ThermoScript reverse-transcription polymerase chain reaction (RT-PCR) kit were obtained from Invitrogen (Carlsbad, CA, USA). QIAquick Gel Extraction kit was purchased from QIAGEN (Germany). Lymphocyte separation medium was purchased from NYCOMED (Norway). 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) conjugated streptavidin (streptavidin-PE) and protein molecular weight (MW) markers (DALTON MARK VII-L) were purchased from Sigma (St. Louis, MO, USA). Pre-stained protein MW standard was purchased from TIANGEN (China). The biotinylation enzyme, BirA, was purchased from Avidity (Denver, CO, USA). Isopropyl- $\beta$ -D-thiogalactoside (IPTG), diaminobenzidine (DAB) and all the other chemicals used were of analytical reagent grade. Anti-human HLA-A\*0201 serum was raised in mouse with soluble HLA-A\*0201 antigen prepared previously (19).

### Peptide

The synthetic peptide corresponding to residue 596-604 (SVRDRLARL, SVR) (17) derived from EBNA3 of EBV was synthesized at Invitrogen Biotechnology Co. (Shanghai, China) and the purity > 95%. The peptide was dissolved in dimethyl sulfoxide with a final concentration of 10 mg/ml and aliquots were stored at -70°C.

### Cloning of HLA-A\*0203 heavy chain cDNA from PBMC

Firstly, total RNA was extracted using TRIzol reagent from the peripheral blood mononuclear cells (PBMCs) isolated from 3 ml human heparinized venous blood of three HLA-A2<sup>+</sup> donors (identified by anti-human HLA-A2-FITC staining and flow cytometric analysis). Single-chain cDNAs were then synthesized from the total RNA using the Thermo Script RT-PCR system according to the recommended procedures. The cDNA of HLA-A\*0203 heavy chain was amplified by PCR (94°C for 2 min, 35 cycles [94°C for 30 s, 55°C for 30 s and 72°C for 90 s] and a final extension at 72°C for 10 min) using the resultant cDNA as a template with the forward primer 5'-ACT AGA ATT CGC CAC CAT GGC CGT CAT GGC GCC-3', containing *EcoR* I restriction enzyme-cleavage site and the reverse primer 5'-CGC GGG ATC CCG CAC TTT ACA AGC TGT GAG AGA CAC-3', containing *BamH* I restriction enzyme-cleavage site. The PCR product digested with *EcoR* I/*BamH* I was inserted into pEGFP-N1 vector and transformed the competent DH5 $\alpha$  cells. The clones with a correct insert were selected with *EcoR* I/*BamH* I digestion and those containing the cDNA of HLA-A\*0203 heavy chain were further identified by DNA sequencing (Invitrogen Shanghai).

### Construction of the expression vector

The fragment encoding the extracellular domain of HLA-A\*0203 heavy chain fused with a BirA substrate peptide (HLA\*0203-BSP) was amplified using cloned HLA-A\*0203 cDNA as a template with the forward primer 5'-ATA TCC ATG GGT TCT CAC TCC ATG AGG TAT TTC-3', containing *Nco* I restriction enzyme-cleavage site and the initiation codon, and the reverse primer 5'-AAT AGG ATC CTT AAC GAT GAT TCC ACA CCA TTT TCT GTG CAT CCA GAA TAT GAT GCA GAG AGC CGG GCT GGG AAG ACG GCT CCC ATC TCA GGG TGA-3', containing the stop codon, Gly-Ser linker, a BSP (LHHILDAQK MVWNHR) recognized by BirA, and *BamH* I restriction enzyme-cleavage site. PCR was performed with an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 10 min. The resultant PCR product was *Nco* I/*BamH* I digested and subcloned into plasmid pET-3d. Clones with a correct sized insert were verified by a DNA sequencing (Invitrogen Shanghai).

### Optimization for high expression of HLA-A\*0203-BSP

The plasmids containing HLA-A\*0203-BSP fusion protein were introduced into *E. coli* strain BL21(DE3). The temperature, IPTG concentration, and induction duration for the expression of the fusion protein in *E. coli* BL21(DE3) transformant were optimized. When the optical density ( $A_{600\text{nm}}$ ) of the liquid culture reached 0.8 after vigorous shaking at 37°C, the culture was divided into different tubes and IPTG was added to each subculture with different final concentrations (0.05, 0.1, 0.2, 0.4, and 0.8 mM). All the subcultures were continued to incubate for an additional 4 h at 37°C for the optimization of IPTG concentration. For optimization of expression temperature, the divided tubes

were continued to incubate for an additional 4 h at 28°C, 32°C, and 37°C, respectively, with a final 0.4 mM IPTG concentration. For optimization of induction duration, the cultures were incubated for an additional 1, 2, 4, 8 h and overnight respectively, with 0.4 mM IPTG. Cells were harvested by centrifugation (3 min, 13,000 rpm) and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### *SDS-PAGE*

SDS-PAGE was performed as described by Laemmli (20), using a 15% polyacrylamide separating gel and a 5% stacking gel. In brief, samples from each preparation were subjected to SDS-PAGE for 45 min at 200 V and then the gel was stained by Coomassie Brilliant Blue R250. Protein molecular weight markers (DALTON MARK VII-L) were included on each gel as a reference and images were captured with FluorChem SP imaging system (Alpha Innotech, San Leandro, CA) and analyzed with AlphaEaseFC software (Alpha Innotech).

#### *Western blot analysis*

The separated protein by SDS-PAGE were electrotransferred to a nitrocellulose membrane for 16 h at 15 V and the membrane was blocked for 30 min at 37°C with TBST (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20) containing 3% calf serum. The membrane was then incubated with mouse anti-human HLA-A\*0201 serum (diluted at a ratio of 1:3,000 in TBST with 3% calf serum) for 2 h at 37°C. After washed with TBST at room temperature for 3 times, the membrane was incubated with the HRP-conjugated goat anti-mouse IgG antibodies (diluted at a ratio of 1:2,000 in TBST with 3% calf serum). After washed with TBST for 3 times, DAB solution was added as the substrate to develop the band and the images were captured by FluorChem SP imaging system (Alpha Innotech).

#### *Purification of HLA-A\*0203-BSP*

Insoluble protein aggregates (inclusion bodies) were purified as previously described (19). The washed inclusion bodies were dissolved in 20 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.0, containing 8 M urea, 10 mM EDTA and 0.1 mM DTT). The protein concentration was determined by measuring absorbance at 280 nm and 260 nm, and calculated following the empirical formula ( $1.45 \times A_{280} - 0.74 \times A_{260}$  = protein concentration in mg/ml). The protein was then divided into small aliquots for storage at -70°C.

#### *Refolding of HLA-A\*0203-SVR monomers*

The construction of monomeric complex was carried out essentially according to dilution refolding method as described previously (19). In brief, 2 mg SVR peptide was added to 200 ml of refolding buffer (0.1 M Tris-HCl pH 8.0, containing 0.4 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.2 mM phenylmethyl sulfonyl fluoride [PMSF]) prechilled to 10°C, and then 6 mg of HLA-A\*0203-BSP (dissolved in 0.5 ml

injection buffer [3 M guanidine HCl pH 4.2, containing 10 mM sodium acetate, 10 mM EDTA]) was injected quickly to the stirring refolding reaction. After 5 mg of  $\beta_2m$  was added, the refolding mixture was incubated at 10°C for 3 d with stirring (18). Finally the refolding mixture was concentrated from 200 to 5 ml using an ultrafiltration device (Amicon, Millipore) with 10 kDa molecular mass cutoff membrane. The buffer was exchanged into 10mM Tris-HCl buffer (pH 8.0, containing 0.2 mM PMSF) by dialysis. The resulting monomeric complex was centrifuged (Eppendorf, Germany) at 13,000 rpm for 10 min and the supernatant was collect for biotinylation.

#### *Biotinylation and purification of the monomer*

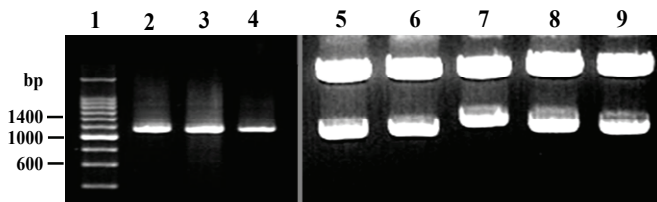
The refolded monomer was enzymatically biotinylated by incubation with BirA according to the manufacturer's recommendations. Then the biotinylated monomer was dialyzed against 10 mM Tris-HCl buffer (pH 8.0, containing 0.2 mM PMSF) and loaded onto MonoQ 5/50 GL column preequilibrated with the same buffer. The column was eluted with a linear gradient of 0~300 mM NaCl using Akta UPC9000 system (Amersham, Uppsala, Sweden). Fractions of 1 ml were collected and determined by SDS-PAGE. The fractions containing both HLA-A\*0203-BSP and  $\beta_2m$  bands by SDS-PAGE were pooled and concentrated to about 300  $\mu$ l using an Amicon Ultra-4 (MWCO10000) ultrafiltration. The buffer was then changed to PBS (containing 0.2 mM PMSF and 2 mM EDTA) by ultrafiltration. The purified protein concentration was determined according to the process as described above and stored at 4°C.

#### *HLA-A\*0203-SVR tetramer formulation*

The tetramer was formulated by mixing the purified biotinylated HLA-A\*0203-BSP monomers with streptavidin-PE at a 4:1 molar ratio. SDS-PAGE analysis, under non-reducing conditions without boiling the sample, was employed to identify the multiplication. The final tetrameric complex was stored at 4°C.

#### *Flow cytometric analysis*

Heparinized whole blood (100  $\mu$ l) from healthy HLA-A2<sup>+</sup> donors which were EBNA1 IgG positive (identified by EBNA1 IgG ELISA kit from Virion\Serion, Germany) was first stained with 0.5  $\mu$ g HLA-A\*0203-SVR tetramer/PE or equal amount of streptavidin-PE was used to as negative control and incubated at 4°C in the dark for 1 h, followed by a 20 min incubation with anti-CD3-FITC and anti-CD8-APC at 4°C in the dark. To lyse red blood cells, 3 ml lysing solution (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) was added to the blood, followed by 6-8 min at room temperature in the dark. The nucleated cells were collected through centrifugation at 1,500 rpm for 5 min. The cell pellets were then washed twice with 2 ml PBS-1%BSA and fixed in 0.3 ml of 4% paraformaldehyde for 15 min. The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and 300,000 events were collected for each sample and analyzed with CELLQuest



**Figure 1. Agarose gel electrophoresis of RT-PCR products of HLA-A\*0203 cDNA and restrictive enzyme digestion of the recombinant vectors containing HLA-A\*0203 cDNA.** Lane 1, 200 bp DNA ladder; Lanes 2-4, RT-PCR products from 3 donors; Lanes 5-9, five independent recombinant vectors digested with *EcoR* I and *BamH* I.

software.

## Results

### Cloning of cDNA of HLA-A\*0203 heavy chain

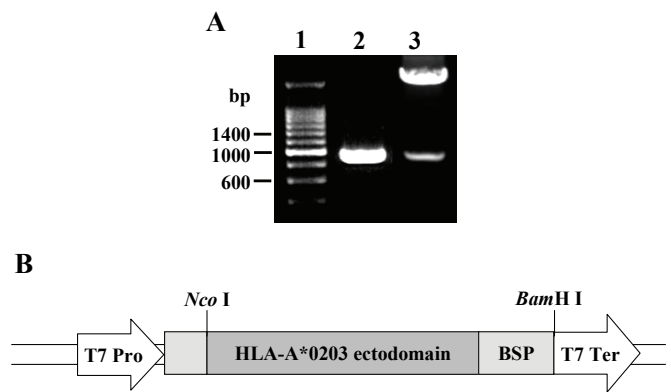
The cDNA encoding HLA-A\*0203 heavy chain was RT-PCR amplified from PBMC of three HLA-A2 donors. The PCR products with the expected length (1,100 bp) (Figure 1, Lanes 2, 3 and 4) were inserted into pEGFP-N1 vector. The recombinant plasmid was then verified with restrictive enzyme digestion and five independent clones were identified to have the correct insert as determined by agarose gel electrophoresis (Figure 1, Lanes 5-9). DNA sequencing (date not shown) confirmed all clones from donors 1 and 3 were consistent with the cDNA for HLA-A\*0201 heavy chain in GenBank as analyzed by BLAST program at NCBI website while those from donor 2 contained the cDNA for HLA-A\*0203 heavy chain. The cDNA sequence for HLA-A\*0203 was submitted to GenBank (accession number DQ336693).

### Construction and identification of HLA-A\*0203-BSP

DNA fragment encoding a Gly-Ser linker and a BSP (LHH ILDAQKMVWNHR) was fused to the 3' end of the ectodomain of HLA-A\*0203 heavy chain (residues from 1 to 280) by PCR with the sequenced recombinant pEGFP-N1/HLA-A\*0203 as a template. The amplified DNA fragment with expected length (900 bp) (Figure 2A, Lane 2) was digested with *Nco* I plus *BamH* I and inserted into pET-3d. The clone was identified by double enzyme cleavage to have the correct insert (Figure 2A, Lane 3) and confirmed by DNA sequencing, indicating that the recombinant plasmid (designated as pET/HLA-A\*0203-BSP which was illustrated in Figure 2B) was constructed correctly.

### Optimization of HLA-A\*0203-BSP expression

The plasmid pET/HLA-A\*0203-BSP was introduced into *E. coli* strain BL21(DE3) and the expression of the fusion protein was induced by IPTG. The expression levels of HLA-A\*0203-BSP under different IPTG concentrations (Figure 3A), induction durations (Figure 3B), and temperature conditions (Figure 3C) were examined by SDS-PAGE and

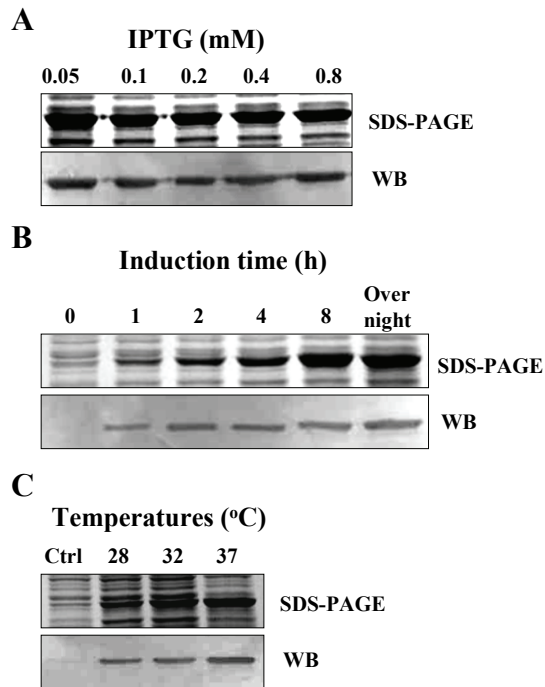


**Figure 2. Restriction endonuclease analysis of the recombinant plasmid and schematic diagram of HLA-A\*0203-BSP gene in pET-3d expression vector.** (A) Agarose gel electrophoresis of PCR product for HLA-A\*0203-BSP and restrictive enzyme digestion of the recombinant vector. Lane 1, 200 bp DNA ladder; Lane 2, PCR product for HLA-A\*0203-BSP; Lane 3, recombinant vector digested with *Nco* I and *BamH* I. (B) A schematic diagram of pET/HLA-A\*0203-BSP. T7 Pro, T7 promoter; T7 Ter, T7 terminator; BSP, BirA substrate peptide.

Western blot analyses. The results indicated that the expression of the fusion protein was not affected by IPTG concentration, whereas it was significantly influenced by temperature and inducing duration. The optimal growth temperature was determined to be 37°C and the optimal inducing duration was 8 h. Prolonged induction time (from 8 h to overnight) did not result in a significant increase in the yield of the recombinant protein (Figure 3B). The expressed protein accumulated up to about 30% of total bacterial proteins under the optimized expression conditions. Furthermore, Western blot showed that the recombinant protein mainly existed in the inclusion bodies (Figure 4). The fusion protein had an apparent molecular mass of 34 kDa as determined by SDS-PAGE, which is in accordance with the theoretical molecular mass. These results demonstrated that the fusion protein was efficiently expressed in *E. coli* strain BL21(DE3) as inclusion bodies under the optimized expression conditions.

### Refolding and biotinylation of monomeric complex

The fusion protein in the inclusion bodies was purified through simple washing and centrifugation as described (19). The monomer of soluble MHC-peptide was generated by *in vitro* refolding of HLA-A\*0203-BSP and  $\beta_2m$  (18) in the presence of HLA-A\*0203 restricted EBNA3<sub>596-604</sub> peptide (SVRDLARL, SVR) (17). SDS-PAGE analysis showed that the refolded HLA-A\*0203-SVR was composed of heavy chain (HLA-A\*0203-BSP) and light chain ( $\beta_2m$ ) (Figure 5, Lane 2). The yield of refolded monomeric complexes was estimated to be ~50%. The refolded HLA-A\*0203-SVR monomer was then biotinylated with BirA and purified by anion-exchange chromatography. The fractions containing HLA-A\*0203-SVR monomer were pooled and concentrated

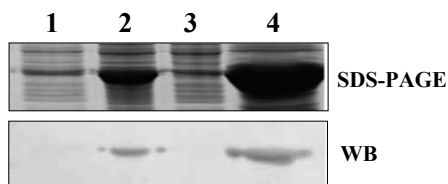


**Figure 3. Optimization of HLA-A\*0203-BSP expression in BL21(DE3).** SDS-PAGE and Western blot analyses of the expression of HLA-A\*0203-BSP in BL21(DE3) induced with different IPTG concentrations (A), times (B) and temperatures (C). For optimization of IPTG concentrations, BL21(DE3) were incubated with different IPTG concentrations (0.05, 0.1, 0.2, 0.4, and 0.8 mM) for an 4 h at 37°C. For optimization of expression temperature, BL21(DE3) were incubated for 4 h at 28°C, 32°C or 37°C, respectively, with a final 0.4 mM IPTG concentration. For optimization of induction duration, BL21(DE3) were incubated for 1, 2, 4, 8 h or overnight respectively, with 0.4 mM IPTG.

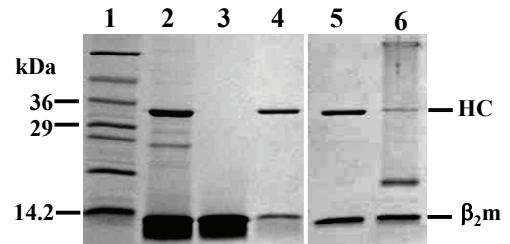
(Figure 5, Lane 4). Purified HLA-A\*0203-BSP was concentrated to 1 mg/ml by ultrafiltration.

#### Preparation of HLA-A\*0203-SVR tetramer

The tetramer was formulated by mixing the biotinylated HLA-A\*0203-SVR monomer with streptavidin-PE at a 4:1 molar ratio. Comparison of monomer and tetramer by



**Figure 4. SDS-PAGE and Western blot analyses of HLA-A\*0203-BSP expressed in BL21(DE3) under optimized conditions.** Lane 1, BL21(DE3) before IPTG induction; Lane 2, BL21(DE3) induced with IPTG for 8 h; Lane 3, supernatant of IPTG-induced BL21(DE3) bacterial lysates; Lane 4, washed inclusion body of IPTG-induced BL21(DE3).



**Figure 5. SDS-PAGE analysis of HLA-A\*0203-SVR monomer and tetramer.** Lane 1, protein MW standard; Lane 2, refolded HLA-A\*0203-SVR monomer; Lane 3,  $\beta_2m$ ; Lane 4, purified biotinylated HLA-A\*0203-SVR monomer; Lane 5, HLA-A\*0203-SVR monomer under non-reducing condition; Lane 6, HLA-A\*0203-SVR tetramer under non-reducing condition. HC: heavy chain.

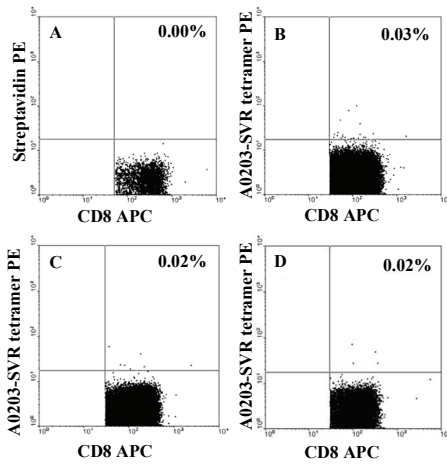
SDS-PAGE analysis under non-reducing condition without boiling the sample demonstrated that more than 85% of monomeric complex formed tetramers, indicating the biotinylated HLA-A\*0203-SVR monomer was effectively biotinylated and multiplied (Figure 5, Lane 6).

#### Staining of antigen-specific CD8<sup>+</sup> T cells with HLA-A\*0203-SVR tetramer

Flow cytometric analysis was performed to determine binding activity of the tetramer with antigen-specific CD8<sup>+</sup> T cells. Heparinized whole blood from healthy EBNA1 IgG-positive HLA-A2<sup>+</sup> donors was stained with HLA-A\*0203-SVR tetramer plus anti-CD3-FITC and anti-CD8-APC. As a negative control, streptavidin-PE was added instead of the tetramer. Lymphocytes were identified and gated on a dot plot where forward scatter (FSC) versus side scatter (SSC) was displayed; total T cells were further gated on the region of CD3 and CD8 double positive events. The gated cells were analyzed for the percentage of tetramer positive CD8<sup>+</sup> cells. HLA-A\*0203-SVR tetramer-reactive CD8<sup>+</sup> T cells could be detected in three donors with a low percentage (0.02~0.03% within CD8<sup>+</sup> T cells), but no positive event was detected in the negative control sample (Figure 6). This result indicated that HLA-A\*0203-SVR tetramer was biologically functional and could be used for analysis of EBV specific CD8<sup>+</sup> T cells from HLA-A\*0203 donors.

## Discussion

As recognition of peptide-MHC class I molecules by CD8<sup>+</sup> T cells is in a HLA-restricted manner (3), corresponding tetramers are needed for the study of antigen-specific CD8<sup>+</sup> T cells restricted by the same HLA allele. The heterogeneous frequency distributions of HLA class I alleles in different populations (15) together with the HLA-restricted CD8<sup>+</sup> T cell response (3) hampers the universal application of tetramer technology. To address this issue, tetramers of different HLA alleles should be constructed for the heterogeneous populations. Although HLA-A\*0201 is the



**Figure 6.** Flow cytometric analysis of EBV-specific CD8<sup>+</sup> T cells stained with HLA-A\*0203-SVR tetramer. (A) Control; (B, C, D) HLA-A\*0203-SVR tetramer staining of whole blood from three HLA-A2<sup>+</sup> donors, respectively. The data represent the percentages of HLA-A\*0203-SVR tetramer-positive CD8<sup>+</sup> T cells.

most frequent HLA class I allele in all populations worldwide, the other high frequency alleles of the HLA-A2 supertype (including HLA-A\*0203, HLA-A\*0205 and HLA-A\*0207, etc.) have significant difference in the frequencies among different populations (15). The allele frequency distributions for HLA-A2 between northern and southern Chinese are heterogeneous: A\*0201 is the most frequently observed allele in all Chinese populations, followed by A\*0207 allele (16). However, the gene frequency of A\*0203 allele is 9.8% in the population of southern China, whereas its frequency in the population of northern China is only 0.8% (16). Due to its high frequency, HLA-A\*0201 tetramers loaded with a variety of antigenic peptides have been widely used to investigate the specific CD8<sup>+</sup> T cell responses (7, 21, 22). Recently, HLA-A\*0205 tetramers are also available in the Tetramer Facility sponsored by Emory University (23). However, to our knowledge, HLA-A\*0203 tetramer has not been reported up to now. In order to study the CD8<sup>+</sup> T cell responses against EBV in HLA-A\*0203 individuals in southern China, the HLA-A\*0203 tetramers loaded with an SVR peptide (17) derived from EBNA3 were prepared and verified by flow cytometry. This tetramer is a powerful tool for direct visualization and quantification of antigen-specific CD8<sup>+</sup> T cells from HLA-A\*0203 donors.

Since high-level expression of HLA heavy chain is critical for the tetramer preparation (19), it is necessary to optimize the culture conditions of the *E. coli* cells harboring the expression vector. The expression conditions of HLA-A\*0203-BSP fusion protein were optimized under different IPTG concentrations, induction durations and temperature conditions. The optimized expression condition was incubating the culture for an additional 8 h at 37°C after IPTG was added. Under these conditions, the fusion protein accumulated up to about 30% of total protein of bacterial

cells. As frequently observed in other high level expression cases, the highly expressed protein was found in insoluble form within inclusion bodies (19, 24). However, the inclusion bodies can easily be purified to > 80% of purity by simple washing steps, greatly facilitated the following steps involved for tetramer preparation (19, 24). The monomer of soluble HLA-A\*0203-SVR was then generated and the tetramer was formed by incubation biotinylated monomer with streptavidin-PE at a ratio of 4:1. SDS-PAGE analysis showed that > 85% of the monomer was bound to streptavidin, suggesting biotinylation of BSP sequence fused at the carboxyl terminus of the heavy chain was highly efficient. Taken together, in agreement with previous study, high yield expression of HLA-A\*0203-BSP heavy chain greatly simplified the procedure for tetramer preparation and thus would facilitate application of tetramer technology (19, 24).

EBV, a  $\gamma$  herpes virus detected in over 90% of the world population, is widely studied due to its clinical and oncogenic importance (25-28). Although EBV usually behaves as a harmless passenger and in rare cases, the virus causes infectious mononucleosis in normal adolescents and lymphoproliferative disease in immunocompromised individuals; it also associates with several human malignancies, particularly with Burkitt's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disease (25-27, 29). Its strong association with nasopharyngeal carcinoma in southern China population (30-32) arouses general interests for this virus. As CD8<sup>+</sup> T cell responses are believed to play a critical role in the control and elimination of EBV infection (27, 33, 34), tetramer technology has been extensively used to study the EBV-specific CD8<sup>+</sup> T cell responses both in acute and in latent infected subjects (28, 35). HLA-A\*0201 (22, 28, 36) and A\*1101 (34) loaded with immunodominant peptides of EBV are usually adopted in these studies due to the high frequencies of these two alleles in the population (15). In the present study, successful preparation of HLA-A\*0203-SVR tetramer provides the critical reagent for evaluate CD8<sup>+</sup> T cell responses against EBV in HLA-A\*0203-restricted manner. The reason for selection of SVR peptide for the tetramer preparation is that this peptide is the only epitope which is known to be HLA-A\*0203 restrictive (17). The efficient refolding of HLA-A\*0203 in the presence of SVR peptide in this study demonstrated that the peptide can fit to the groove of A\*0203, providing further evidence that SVR is indeed HLA-A\*0203 restrictive.

With HLA-A\*0203-SVR tetramer, antigen-specific CD8<sup>+</sup> T cells were analyzed by flow cytometry. The results showed low frequency (0.02~0.03%) of HLA-A\*0203-SVR tetramer-positive CD8<sup>+</sup> T cells detected in three HLA-A2<sup>+</sup> donors. The result is consistent with previous findings by Hollsberg et al. (33), which also showed low frequency of SVR-specific T cells (about several spots per 10<sup>6</sup> PBMCs) by ELISPOT. Together, these results suggest that SVR peptide may not be an immunodominant epitope of EBV in HLA-A\*0203 individuals. Thus, *in vitro* amplification of SVR-specific CD8<sup>+</sup> T cells by SVR peptide is necessary for analyzing the phenotypes and functions of these cells. On the other hand, more HLA-A\*0203-restricted epitopes remain to be

identified for investigating CD8<sup>+</sup> T cell responses against EBV in HLA-A\*0203 individuals.

In conclusion, the present study showed that high level expression of HLA-A\*0203-BSP fusion protein was achieved under optimized conditions and soluble HLA-A\*0203-SVR monomer was produced in the presence of  $\beta_2m$  plus SVR peptide. Furthermore, HLA-A\*0203-SVR tetramer was formulated from the biotinylated monomers with high efficiency and it was functional. This tetramer provides a foundation for further characterization of antigen-specific CD8<sup>+</sup> T cells from HLA-A\*0203 subjects.

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