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Structural and functional characterization of peptide–β_2m fused HLA-A2/MART1$_{27–35}$ complexes ☆

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Abstract

The uses of soluble HLA class I/peptide complexes to monitor antigen reactive T cells are often hampered by their low-yield and high-cost production. As an alternative strategy, the peptide–β_2m fused, 2-component (2C) HLA class I/peptide complex has been developed, but its application is limited due to the lack of the comparison of its structural and functional characteristics with those of its conventional 3-component (3C) counterpart. In this study, we have demonstrated that the 2C and 3C HLA-A2/MART1$_{27–35}$ complexes have a similar chromatographical profile and comparable stability, but the former has 2.5 times higher yield and significantly higher binding ability with HLA-A2/MART1$_{27–35}$ complex-specific receptors than the latter. Furthermore, the 2C complex has a comparable ability to stimulate specific CTL proliferation, but appears to be more effective in eliciting the cytotoxicity of antigen-specific CTL, as compared to its 3C counterpart.

Keywords: HLA/peptide complex; HLA/peptide tetramer; Antigen-specific CTL

Human leukocyte antigen (HLA) class I molecules are a 3-component complex composed of a 45 kDa heavy chain, a 12 kDa light chain (β_2 microglobulin, β_2m), and a 8–9 residue antigenic peptide. These three components are non-covalently associated. The heavy chain α1 and α2 domains form a groove structure to anchor the antigenic peptide, while the heavy chain α3 domain, together with the underside of the α1 and α2 domains, heterodimerizes with β_2m. The function of HLA class I molecules is mainly interacting with T cell receptor (TCR) on CD8$^+$ T lymphocytes, thereby triggering T cell activation and antigen-specific T cell responses. The specificity of a given HLA class I-TCR interaction is largely dependent on a unique epitope expressed on the antigenic peptide in the context of a HLA class I allospecificity. This phenomenon prompted the idea of using soluble HLA class I molecules as a probe to identify antigen-specific T cell subpopulations [1].

Ever since the first application of soluble HLA class I tetramers for the determination of the frequencies of antigen-specific CD8$^+$ T cells in a mixed T cell population [1–4], soluble HLA class I molecules have also been used in other areas of research, such as eliciting antigen-specific CD8$^+$ cytotoxic T cell responses in vitro and in vivo [5–7], and measuring the relative affinity of a given TCR for a HLA class I/peptide complex [8–10]. For all these applications, recombinant HLA class I heavy chains and β_2m need to be expressed and purified to high homogeneity from bacterial hosts before being refolded in a cell-free system in the presence of synthetic peptides, in order to generate

☆ *Abbreviations:* β_2m, β_2 microglobulin; BSP, biotin protein ligase BirA substrate peptide; 2C, 2-component; DDIA, double determinant immunosorbent assay; ELISA, enzyme-linked immunosorbent assay; FPLC, fast performance liquid chromatography; PE, *R*-phycoerythrin; scFv, single chain antibody variable region fragments.

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properly conformed HLA class I molecules [1]. Although as a standard procedure, this approach has suffered from the low yield and high cost associated with the experimental steps involved and thus has limited the use of soluble HLA class I/peptide complexes in immunological research.

To facilitate the production of HLA class I/peptide complexes, the peptide–β_2m fusion strategy has been developed [11–13]. In this strategy, the antigenic peptide and β_2m are linked with a Gly-Gly-Ser motif-containing linker. This linker, usually 8–15 residues long, provides the flexibility of the linker structure without affecting the association of the peptide and β_2m with the MHC class I heavy chain. The resulting HLA class I/peptide complexes are 2-component (2C), as opposed to the 3-component (3C) composition of the conventional HLA class I molecules.

Tafuro et al. [13] were the first to demonstrate that 2C HLA class I/peptide complexes could be properly refolded utilizing the HLA-A2-restricted influenza A matrix protein-derived peptide GILGFVFTL ($MA_{58–66}$), and tetramers of the 2C HLA class I molecules could be generated to replace the conventional 3C tetramers for antigen-specific CTL staining in flow cytometry. However, the information about the comparative structural and functional characteristics of the two types of HLA class I/peptide complexes has been scanty thus far. The lack of this information may account, at least in part, for the limited use of the 2C HLA class I/peptide complex in spite of its economical advantage. In the present study, using the HLA-A2-restricted melanoma antigen MART1-derived peptide AAGIGILTV as a model antigen, we reported the first comprehensive analysis of the structural and functional characteristics of the 2C and 3C HLA-A2/$MART1_{27–35}$ complexes.

Materials and methods

Monoclonal and polyclonal antibodies and scFv fragment. The monoclonal antibody (mAb) W6/32 [14] which recognizes a framework determinant expressed on β_2m-associated HLA-A, -B, -C, -E, and -G heavy chain; mAb B1.23.1 [15] which recognizes β_2m-free and β_2m-associated HLA-B and -C heavy chains; HLA-A2, -A24, and -A28-specific mAb CR11-351 [16]; mAb HCA-2 [17,18] which recognizes β_2m-free HLA-A (excluding -A24), -B7301, and -G heavy chain; β_2m-specific mAb NAMB-1 and L368 [19,20]; and c-myc-specific mAb 9E10 [21] were developed as described and kindly provided by Dr. Ferrone (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA). HLA-A2/$MART1_{27–35}$-specific scFv8.3 was isolated from the human synthetic VH + VL scFv library (Griffin.1 library) by panning with HLA-A2/$MART1_{27–35}$ peptide complexes. The detailed characterization of scFv8.3 will be described elsewhere. Fluorescein-5-isothiocyanate (FITC)-labeled mouse anti-human CD8 mAb RPA-T8 and mouse anti-His_6 mAb were purchased from BD PharMingen (San Diego, CA, USA) and from Amersham Biosciences (Piscataway, NJ, USA), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc antibodies were purchased from Sigma (St. Louis, MO, USA).

Synthetic oligonucleotide primers and peptides. The human β_2m-specific primers BATE1 (5′-GGCCCATGGATATCCAGCGTACTCCAAAG-3′) containing a *Xho*I site and BATE2 (5′-CAACTCGAGCATGTCTCGATCCCACTTAAC-3′) containing a *Nco*I site; the 5′ primers for construction of $MART1_{27–35}$–β_2m fusion plasmid BATE3 (5′-GGAGGTGGTGGTGGCGGATCAGGAGGCTCAGGAGGTTCAGGAGGCATCCAGCGTACTCCAAAGATT-3′) containing the sequence for the 15-aa-long Gly/Ser linker (underlined) and BATE4 (5′-GGCCCATGGCAGCAGGAATTGGTATCTTAACGGTAGGAGGTGGTGGTGGCGGATCA-3′) containing the $MART1_{27–35}$ epitope and an *Xho*I overhang site; and the 3′ primer for construction of $MART1_{27–35}$–β_2m fusion plasmid BATE5 (5′-CAACTCGAGCATGTCTCGATCCCACTTAAC-3′) containing a *Nco*I site were purchased from Integrated DNA Technologies (Coralville, IA, USA). The synthetic peptides corresponding to residues 27–35 (N′-AAGIGILTV-C′) of MART1 and residues 369–377 (N′-KIFGSLAFL-C′) of HER2/neu were purchased from the Advanced Protein Technology Centre at The Hospital for Sick Children (Toronto, Ontario, Canada).

Cells. HLA-A2-restricted tumor infiltrating lymphocyte (TIL)1 population, isolated from a melanoma tissue that is HLA-A2 positive and MART1 antigen positive, was maintained in RPMI 1640 medium supplemented with 10% human AB serum (SeraCare Life Sciences, Oceanside, CA, USA) and 5000 IU/ml IL-2 (BD Pharmingen, San Diego, CA, USA) at 37 °C in a 5% CO_2 atmosphere. TIL2 population is derived from TIL1 following incubation with $MART1_{27–35}$ peptide for 3 weeks. HLA-$A2^+$, MART1, and HER2/neu antigen-irrelative peripheral blood mononuclear cells (PBMCs) were obtained from a healthy donor. Antigen processing deficient T2 cells (HLA-$A2^{+/+}$ $TAP^{-/-}$), K562 cells, a melanoma cell line 501 (HLA-$A2^+$, $MART1^+$, and $HER2/neu^-$), and breast cancer cell line MDA-MB231 (HLA-$A2^+$, $MART1^-$, and $HER2/neu^+$) were obtained from American Type Culture Collection (Manassas, VA, USA).

Construction of recombinant β_2m-His_6, $MART1_{27–35}$-linker-β_2m-His_6, and HLA-A2(α1, α2, α3)-BSP-His_6 proteins. The human β_2m cDNA without the signal sequence was cloned by RT-PCR using the primer combination BATE1/BATE2 from mRNA isolated from PBMC. The PCR product was ligated to the pET28a plasmid (Novagen, Madison, WI, USA) and the resulting plasmid, pET28a-β_2m, was used as a template to construct the $MART1_{27–35}$–β_2m fusion plasmid with two sequential PCR using the primer combinations BATE3/BATE4 first and BATE5/BATE4 later. The resulting PCR product ($MART1_{27–35}$-linker-β_2m) was cloned into the *Xho*I and *Nco*I sites in pET28a. The plasmid pET28a encoding the HLA-A*0201 α1, α2, α3 domain with a C terminal biotin protein ligase BirA substrate peptide (BSP) was constructed as described [1]. Expression of the recombinant HLA-A2-BSP-His_6, β_2m-His_6, and $MART1_{27–35}$-linker-β_2m-His_6 proteins was induced in BL21 (DE3) bacteria (Novagen) at OD = 0.4 with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM) after a 3-h incubation at 37 °C. The extracted inclusion bodies were dissolved in 6 M urea buffer and purified by passing through a Ni^+-chelating affinity column Ni^+–NTA-Sepharose 6BL (Novagen). The resulting purified protein was analyzed by 12% SDS–PAGE and Western blot with mAb HCA-2, mAb L368, and anti-His_6 mAb.

Generation of 2C, 3C HLA-A2/peptide monomer, and PE-labeled tetramer. The 2C and 3C HLA-A2/$MART1_{27–35}$ monomers and the 3C HLA-A2/HER2/$neu_{369–377}$ monomer were refolded and purified as described [1] except for the injection of the purified HLA-A2-BSP-His_6 and $MART1_{27–35}$-linker-β_2m-His_6 fusion proteins into the refolding buffer for generating 2C HLA-A2/$MART1_{27–35}$ monomer. After biotinylation in the presence of BirA and d-biotin (Avidity, LLC, Denver, CO, USA), the 2C and 3C monomers were purified by FPLC (Pharmacia Fine Chemicals, Uppsala, Sweden) with the Hiload16/60 Superdex 75-gel filtration column (Amersham Biosciences). Samples were run at a speed of 1 ml/min and fractions of 1 ml each were collected. Proteins were detected with an UV monitor at 280 nm. Each fraction was analyzed by 12% SDS–PAGE and Western blotting with mAb HCA-2 and mAb L368.

The tetrameric form of 2C and 3C HLA-A2/$MART1_{27–35}$ monomers and of 3C HLA-A2/HER2/$neu_{369–377}$ monomer was generated by linking each monomer with PE-conjugated streptavidin (premium grade) (Molecular Probes, Eugene, OR, USA) as described [1].

Enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Costar, Cambridge, MA, USA) were coated overnight at 4 °C with 2C or 3C HLA-A2/$MART1_{27–35}$ monomer, 3C HLA-A2/HER2/$neu_{369–377}$ monomer, recombinant HLA-A2 heavy chain or recombinant β_2m prepared in FPLC wash buffer (20 mM Tris–HCl, 200 mM NaCl, pH 8.0) at various concentrations. After blocking the plate with 2% BSA in 1× PBS for 4 h at 4 °C, serial 2-fold dilutions of mAb W6/32 or CR11-351

preparation was added. Following a 2-h incubation at 4 °C, HRP-conjugated goat anti-mouse IgG Fc fragment antibodies were added and incubation was continued for one additional hour at 4 °C. The color was developed using TMB peroxidase as substrate (KPL, Gaithersburg, MD, USA). Absorbance was measured at 450 nm after the reaction was stopped by the addition of 0.18 M H_2SO_4. Results are expressed as optical density (OD) values.

Double determinant immunosorbent assay (DDIA). Ninety-six-well micro-titer plates (Costar) were coated overnight at 4 °C with streptavidin (100 μg/ml in 0.1 M $NaHCO_3$ buffer, pH 9.6) and then blocked with 2% BSA in 1× PBS for 2 h at room temperature. Biotinylated 2C, 3C HLA-A2/MART1$_{27–35}$ monomers or 3C HLA-A2/HER2/neu$_{369–377}$ monomers at various concentrations in FPLC wash buffer were then added to the wells and incubation was continued for an additional 4 h at 4 °C. Then HLA-A2/MART1$_{27–35}$-specific scFv8.3 (40, 20 or 10 μg/ml), which include c-myc sequence, and c-myc-specific mAb 9E10 (10 μg/ml) were added and incubation was continued for an additional 4 h at 4 °C. Finally HRP-conjugated goat anti-mouse IgG Fc fragment antibodies were added and incubation was continued for one additional hour at 4 °C. Absorbance was measured at 450 nm after the reaction was stopped by the addition of 0.18 M H_2SO_4. Results are expressed as optical density (OD) values.

Tetramer staining of TILs and PBMC. The growing TIL1, TIL2 or MART1 and HER2/neu antigen-irrelative PBMC were harvested, re-suspended in 2% BSA–PBS (2×10^5 cells/20 μl/well), and incubated with PE-labeled 2C HLA-A2/MART1$_{27–35}$ tetramers, 3C HLA-A2/MART1$_{27–35}$ tetramers or 3C HLA-A2/HER2/neu$_{369–377}$ tetramers for 30 min at 25 °C. FITC-labeled anti-CD8 mAb (10 μl/well) was then added to the wells and incubation was continued for one additional hour at 4 °C. Isotype control of FITC-labeled mouse IgG and PE-labeled streptavidin were stained with TILs and PBMC in parallel. After washing three times with 1% BSA–PBS, cells were re-suspended in 0.5% paraformaldehyde-PBS and analyzed on a FAScan flow cytometer (BD Biosciences). Cells (5×10^4/well) were collected and analyzed with the CellQuest and WinMDI 2.8 software.

Proliferation of antigen-specific CTL stimulated by HLA-A2/MART1$_{27–35}$ monomers. The growing lymphocytes TIL1 re-suspended in RPMI 1640 medium supplemented with 10% human AB serum (SeraCare Life Sciences) and IL-2 (100 IU/ml) (BD Pharmingen) were incubated in 24-well plate at the concentration of 1×10^6 cells/ml/well at 37 °C in a 5% CO_2 atmosphere with the 2C or 3C HLA-A2/MART1$_{27–35}$ monomer (0.0245 or 0.245 nM). The culture medium was replaced with fresh medium every 4 days and supplemented with 2C or 3C HLA-A2/MART1$_{27–35}$ monomer. At the end of a 3-week stimulation, the TIL1 cells were stained with PE-labeled 2C HLA-A2/MART1$_{27–35}$ tetramers or 3C HLA-A2/HER2/neu$_{369–377}$ tetramers and FITC-labeled anti-CD8 mAb and analyzed by flow cytometry as described.

Cytotoxicity assay. The standard 4-h ^{51}Cr release assay was performed except that the target cells, antigen processing deficient T2 cells (HLA-A2$^{+/+}$ TAP$^{-/-}$), were pulsed with the MART1$_{27–35}$ (0.1 mg/ml) for 2 h at room temperature before being labeled with $Na^{51}Cr$ (100 μCi/10^7 cells) (Amersham Biosciences). Cold K562 cells (5×10^4 cells/well) were added to prevent lysis of target cells by the NK cells present in the TIL population. Then TIL1 cells stimulated with 2C or 3C HLA-A2/ MART1$_{27–35}$ monomer for 3 weeks were added at the concentration of 4×10^5 cells/well.

Statistical analysis. Student's *t* test was used to determine the significance between 2C HLA-A2/MART1$_{27–35}$ monomer group and 3C HLA-A2/MART1$_{27–35}$ monomer group. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

Construction and biochemical characterization of 2C and 3C HLA-A2/MART1$_{27–35}$ complexes

Recombinant HLA-A2 heavy chain, β_2m and MART1$_{27–35}$-linker-β_2m proteins were purified from BL21 bacteria inclusion bodies using Ni^+-chelating affinity chromatography as recommended by the manufacturer (Novagen). The size and purity of each recombinant protein was confirmed by Western blotting with anti-HLA class I heavy chain (HCA-2), anti-β_2m (L368), and anti-His$_6$-specific mAbs (data not shown). 2C and 3C HLA-A2/MART1$_{27–35}$ complexes were then refolded, biotinylated, and purified by FPLC as described [1]. Three major peaks were seen on the FPLC histogram (Fig. 1). Western blot analysis with mAb HCA-2 and L368 indicated that the first peak, which appeared after 40 min, represented correctly refolded HLA-A2/peptide complexes (data not shown). The OD_{280} of this first peak containing the 2C complexes was greater than the OD_{280} of the first peak containing the 3C complexes (1.307 vs. 0.728), indicating the yield of 2C HLA-A2/MART1$_{27–35}$ complexes was much higher than its 3C counterpart (10.5 mg vs.

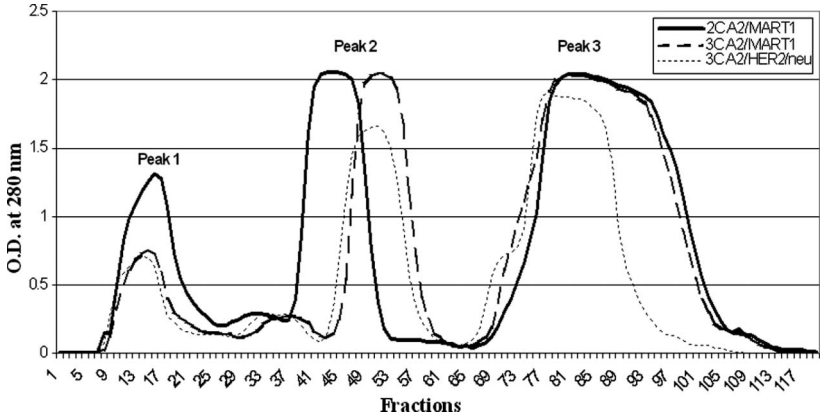

Fig. 1. FPLC profile of 2C and 3C HLA-A2/peptide monomers. (—) 2C A2/MART1, 2C HLA-A2/MART1$_{27–35}$ monomer; (-·) 3C A2/MART1, 3C HLA-A2/MART1$_{27–35}$ monomer; (---) 3C A2/HER2/neu, 3C HLA-A2/HER2/neu$_{369–377}$ monomer. For the generation of 2C monomer, 87.6 mg HLA-A2-BSP-His$_6$ and 70.2 mg MART1$_{27–35}$-linker-β_2m-His$_6$ were refolded in 800 ml refolding buffer. For the generation of 3C monomer, 87.6 mg HLA-A2-BSP-His$_6$, 54 mg β_2m-His$_6$, and 48 mg synthetic MART1$_{27–35}$ peptide or HER2/neu$_{369–377}$ peptide, were refolded in 800 ml refolding buffer.

4.0 mg). The second peak contained free MART1$_{27–35}$-linker-β_2m-His$_6$ fusion protein or β_2m-His$_6$ protein that failed to associate with HLA-A2-BSP-His$_6$. The second peak in the 2C HLA-A2/MART1$_{27–35}$ FPLC profile appeared earlier than the corresponding second peak in the 3C HLA-A2/MART1$_{27–35}$ or 3C HLA-A2/HER2/neu$_{369–377}$ FPLC profile, which was consistent with the difference of molecular weight between free MART1$_{27–35}$-linker-β_2m-His$_6$ (14.6 kDa) and β_2m-His$_6$ (12 kDa) proteins. The third peak most likely contained unbound d-biotin, ATP, peptide, and other unidentified moieties. Therefore, based on FPLC and Western blot analysis, the 2C form of the HLA-A2/MART1 complex had comparable molecular size as the 3C forms of the HLA-A2/MART1$_{27–35}$ and HLA-A2/HER2/neu$_{369–377}$ complexes.

The approximately 2.5 times higher yield of 2C monomer versus its 3C counterpart indicates that the fusion of the peptide with β_2m can largely facilitate the production of HLA class I/peptide complexes. This finding may reflect two reasons. First, the solubility of the peptide, fused to

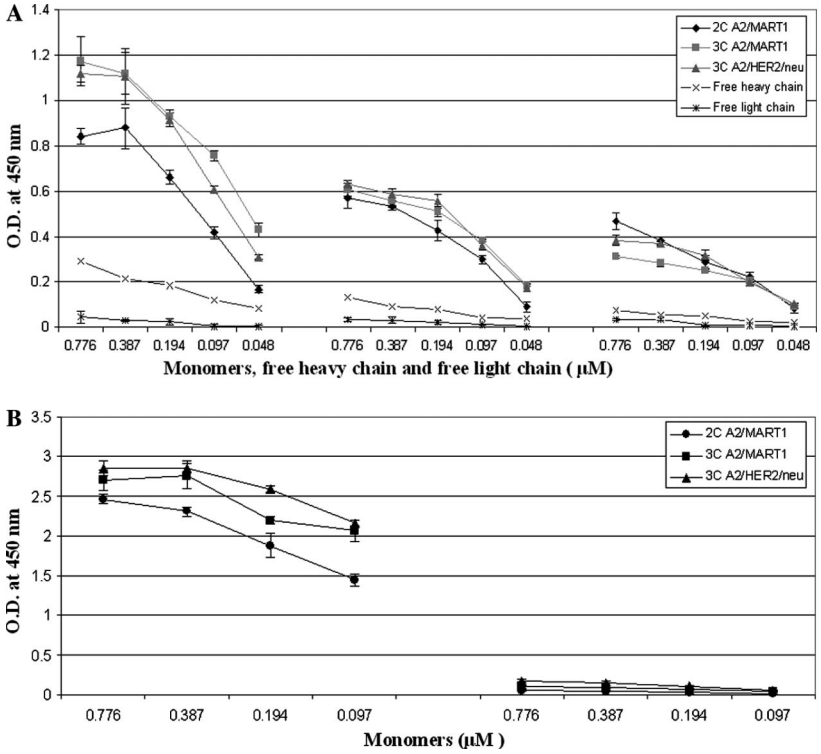

Fig. 2. Reactivity of 2C and 3C HLA-A2/peptide monomers with mAb W6/32 and CR11-351 in ELISA. –♦– or –●– 2CA2/MART1, 2C HLA-A2/MART1$_{27–35}$ monomers; —■— or –■– 3C A2/MART1, 3C HLA-A2/MART1$_{27–35}$ monomer; —▲— or –▲– 3C A2/HER2/neu, 3C HLA-A2/HER2/neu$_{369–377}$ monomer; —╳— free heavy chain: β_2m-free HLA-A2 heavy chain-BSP; —☼— free light chain, HLA-A2 heavy chain-free MART1$_{27–35}$-linker-β_2m. (A) Reactivity of the 2C and 3C HLA-A2/MART1$_{27–35}$ monomer with mAb W6/32 in ELISA. Primary mAb W6/32 was added to wells at concentrations of 10 μg/ml (left panel), 5 μg/ml (middle panel), and 2.5 μg/ml (right panel). (B) Reactivity of the 2C and 3C HLA-A2/MART1$_{27–35}$ monomer with mAb CR11-351 in ELISA. Primary mAb CR11-351 (left panel) and B1.23.1 (right panel) were added to wells at concentrations of 10 μg/ml.

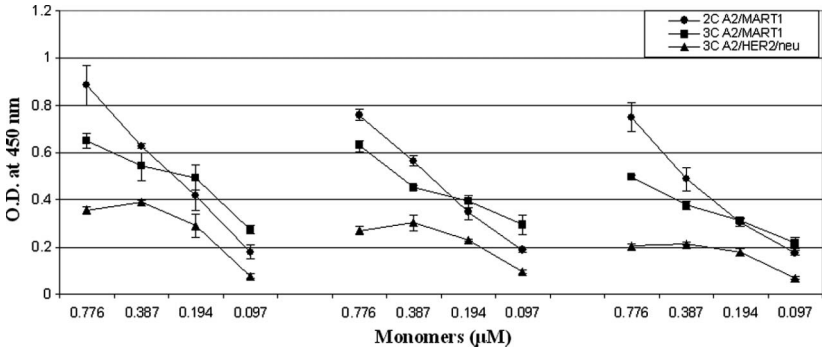

Fig. 3. Reactivity of 2C and 3C HLA-A2/MART1$_{27–35}$ monomer with scFv8.3 in DDIA. –●– 2C A2/MART1, 2C HLA-A2/MART1$_{27–35}$ monomers; –■– 3C A2/MART1, 3C HLA-A2/MART1$_{27–35}$ monomer; –▲– 3C A2/HER2/neu, 3C HLA-A2/HER2/neu$_{369–377}$ monomer. scFv8.3 was added to wells at concentrations of 40 μg/ml (left panel), 20 μg/ml (middle panel), and 10 μg/ml (right panel). mAb 9E10 was added at a concentration of 10 μg/ml.

β_2m, appears to increase the refolding efficiency of the 2C complex compared with the HLA class I-binding synthetic peptides that, in many cases, are poorly soluble. Second, the β_2m-fused peptide may have more opportunities to contact the antigen-binding groove and cannot be easily freed from the complex.

Size similarity is not adequate to verify that the 2C and 3C forms of the HLA-A2/MART1$_{27–35}$ complex are comparable for immunological studies. Therefore, we wished to further characterize the 2C form of the HLA-A2/MART1$_{27–35}$ complex to determine if it is equivalent to the 3C form. As shown in Fig. 2A, the conformation sensitive mAb W6/32 strongly binds to 2C and 3C HLA-A2/MART1$_{27–35}$ complexes as well as to 3C HLA-A2/HER2/neu$_{369–377}$ complexes in a dose-dependent manner, whereas it reacts neither with the HLA-A2 heavy chains nor with β_2m. In addition, the HLA-A2, -A24, and -A28-specific mAb CR11-351 also strongly reacts with the 2C and 3C HLA-A2/peptide complexes in a dose-dependent manner. In contrast, no binding of mAb B1.23.1, which recognizes a determinant restricted to HLA-B and -C antigens, was detected (Fig. 2B). It should be noted that the reactivity of 2C HLA-A2/MART1$_{27–35}$ complexes with mAb W6/32 and mAb CR11-351 is significantly lower than that of its 3C counterpart ($p < 0.05$ and $p < 0.01$, respectively), indicating that the 2C form of the HLA-A2/

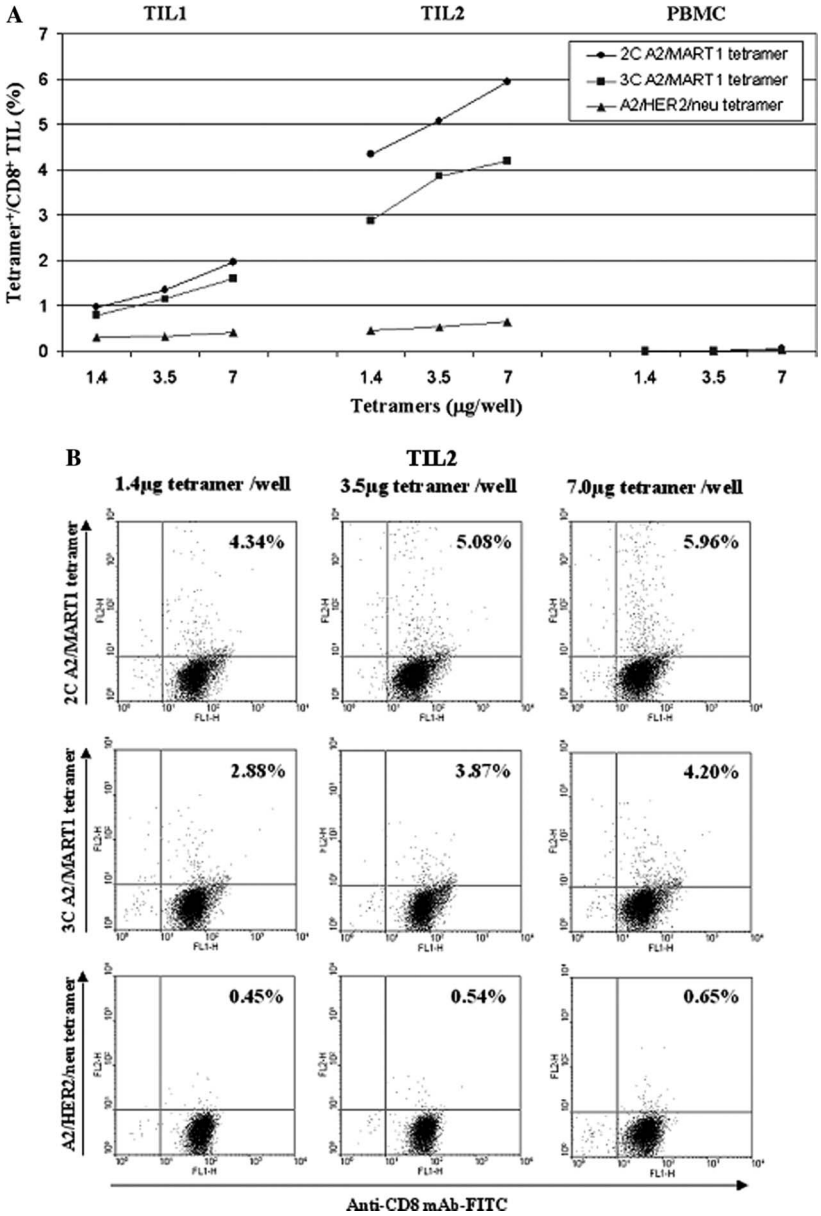

Fig. 4. Tetramer staining of TIL1, TIL2, and PBMC. 2C A2/MART1 tetramer: the PE-labeled tetramer of 2C HLA-A2/MART1$_{27–35}$ monomers; 3C A2/MART1 tetramer: the PE-labeled tetramer of 3C HLA-A2/MART1$_{27–35}$ monomers; A2/HER2/neu tetramer: the PE-labeled tetramer of 3C HLA-A2/HER2/neu$_{369–377}$ monomers. TIL1, TIL2 as well as MART1 antigen- and HER2/neu antigen-irrelative PBMC population were stained, respectively, with 2C, 3C HLA-A2/MART1$_{27–35}$ tetramer or 3C HLA-A2/HER2/neu$_{369–377}$ tetramer along with FITC-conjugated anti-human CD8 mAb RPA-T8 (10 μl/well).

MART1 complex resembles, but is not identical to its 3C counterpart in its antigenic profile or conformational epitopes expressed by HLA class I heavy chain/β_2m complex. The presence of 15-aa-long linker may cause, at least in part, interference with the binding of the mAb W6/32- and mAb CR11-351-recognized epitopes with the respective mAb. This possibility is supported by the location of mAb W6/32- and mAb CR11-351-recognized epitope (α3 domain and α2 domain, respectively).

Binding of 2C and 3C HLA-A2/MART1$_{27–35}$ complexes to HLA-A2/MART1$_{27–35}$ complex-specific receptors

The most critical feature of an MHC/peptide complex is its ability to bind to T cell receptors. Therefore, we measured the ability of our 2C HLA-A2/MART1$_{27–35}$ complexes to bind an HLA-A2/MART1$_{27–35}$ complex-specific single chain receptor (scFv8.3). As shown in Fig. 3, both the 2C and 3C forms of the HLA-A2/MART1$_{27–35}$ complex react well with scFv8.3 in DDIA in a dose-dependent manner. These bindings are epitope specific since scFv8.3 does not bind or only weakly bind to HLA-A2/HER2/neu$_{369–377}$ complexes. The optimal concentration of monomer and scFv8.3 for this DDIA is 0.776 μM and 20 μg/ml, respectively. Interestingly, the reactivity of the 2C HLA-A2/MART1$_{27–35}$ complexes with scFv8.3 is significantly higher than that of its 3C counterpart ($p < 0.05$ at the 0.776, 0.387, and 0.194 μM monomer data points) with the exception of the 0.097 μM monomer data point, suggesting the 2C HLA-A2/MART1$_{27–35}$ complex has higher binding ability to its soluble receptor as compared to its 3C counterpart though both the 2C and 3C complexes express the epitope corresponding to the single chain antibody.

Furthermore, we assessed the binding capacity of our 2C HLA-A2/MART1$_{27–35}$ complexes to nature T cell receptors on cell surface. PE-labeled tetramers of 2C and 3C HLA-A2/MART1$_{27–35}$ complexes and 3C HLA-A2/HER2/neu$_{369–377}$ complexes were prepared and used to stain MART1-relative TIL1 and TIL2 population as well as healthy donor's PBMC. As shown in Figs. 4A and B, the frequency of tetramer$^+$/CD8$^+$ cells as detected by the 2C HLA-A2/MART1$_{27–35}$ tetramer in TIL1 or TIL2 population is always little higher than that detected by the 3C HLA-A2/MART1$_{27–35}$ tetramer at various concentrations of tetramer, particularly in the TIL2 population. These results are consistent with the reactivity of 2C, 3C HLA-A2/MART1$_{27–35}$ monomers with soluble single chain receptor (scFv8.3) in DDIA. Considering the percentage of MART1$_{27–35}$-specific CTL in TIL1 or TIL2 population is as low as 1–6%, the slightly higher frequency detected by 2C tetramer might support the idea that 2C tetramer may be better than conventional 3C tetramer for enumerating the antigen-specific CTL.

As a negative control, MART1 and HER2/neu-irrelative PBMC population from an HLA-A2$^+$ healthy donor was stained with tetramers in parallel. As shown in Fig. 4A, both HLA-A2/MART1$_{27–35}$ tetramers and HLA-A2/HER2/neu$_{369–377}$ tetramer only can detect tetramer$^+$/CD8$^+$ cells with a very low frequency (0.00% up to 0.05%) at increasing concentrations of tetramer (1.4–7.0 μg/well). Of note is that the HLA-A2/HER2/neu$_{369–377}$ tetramer also can detect tetramer$^+$/CD8$^+$ cells in TIL1 and TIL2 population with a much lower frequency

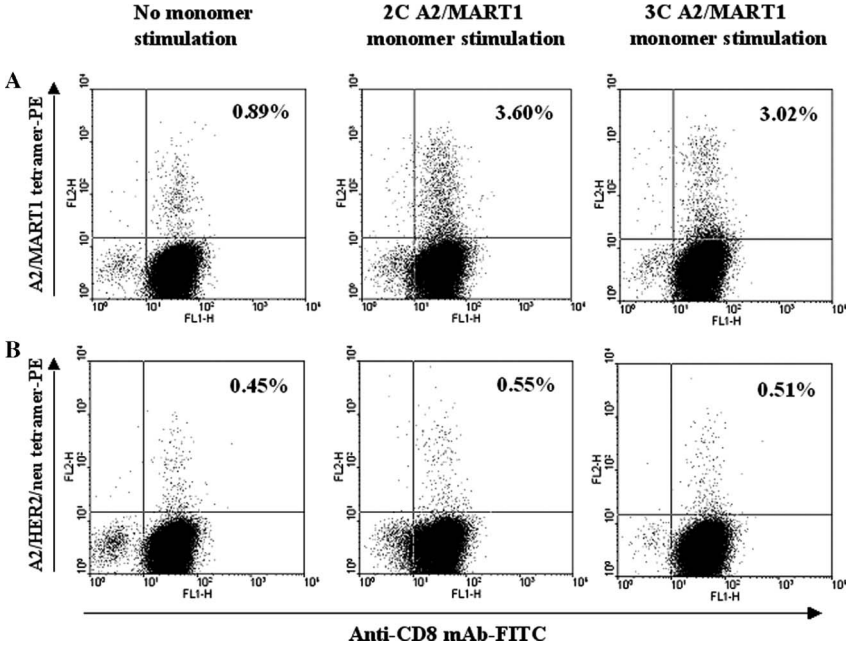

Fig. 5. Activity of 2C and 3C HLA-A2/MART1$_{27–35}$ monomer to elicit antigen-specific CTL proliferation. TIL1 population was incubated with or without 2C or 3C HLA-A2/MART1$_{27–35}$ monomer (0.245 nM) for 3 weeks followed by staining with PE-labeled 2C HLA-A2/MART1$_{27–35}$ tetramer (1.4 μg/well) (A) or PE-labeled 3C HLA-A2/HER2/neu$_{369–377}$ tetramer (1.4 μg/well) (B) and FITC-conjugated anti-human CD8 mAb RPA-T8 (10 μl/well).

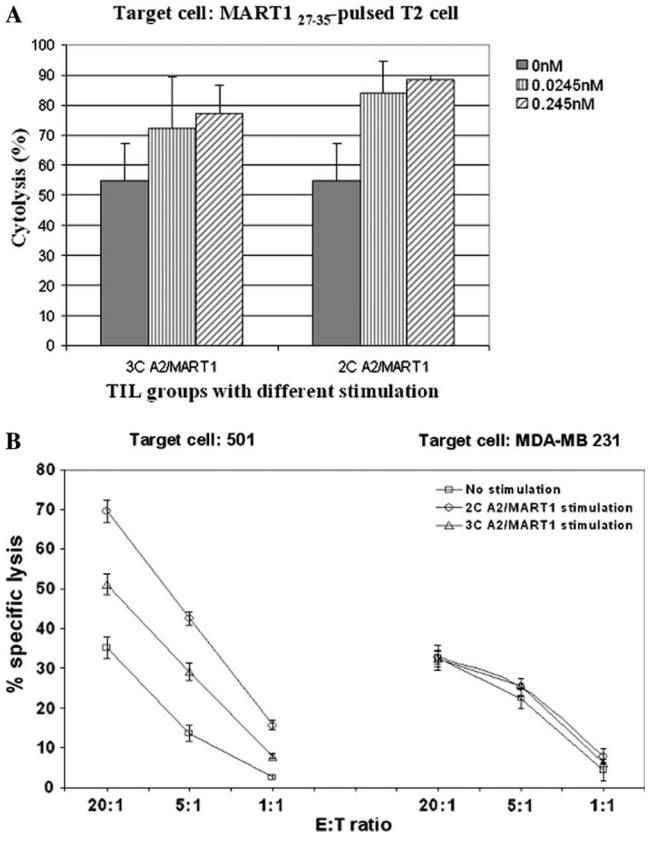

Fig. 6. Cytolysis of 501 cells, MDA-MB231 cells, and MART1$_{27–35}$ peptide-pulsed T2 cells by TIL1 cells stimulated by 2C or 3C HLA-A2/MART1$_{27–35}$ monomers in a ^{51}Cr release assay. (A) TIL1 cells, incubated with 2C or 3C HLA-A2/MART1$_{27–35}$ monomers (0, 0.0245 or 0.245 nM) for 3 weeks, were used to lyse MART1$_{27–35}$ peptide-pulsed T2 cells at the 40:1 ratio (E:T). 3C A2/MART1, TIL1 groups stimulated by 3C HLA-A2/MART1$_{27–35}$ monomers. 2C A2/MART1, TIL1 groups stimulated by 2C HLA-A2/MART1$_{27–35}$ monomers. (B) TIL1 cells, incubated with 2C or 3C HLA-A2/MART1$_{27–35}$ monomers (0.245 nM) for 3 weeks, were used to lyse 501 cells and MDA-MB231 cells at various E:T ratios.

than HLA-A2/MART1$_{27–35}$ tetramers. This may be attributable to two reasons. One is the background staining at least in part. Another reason is the possible low expression of HER2/neu antigen in the melanoma tissue from which the TIL1 and TIL2 were isolated and generated. Although the latter possibility cannot be directly tested due to the unavailability of the original melanoma tissue, it may be supported in part by the finding that the TIL1 population can lyse up to 32% of the breast carcinoma cell line MDA-MB231 (HLA-A2$^+$/HER2/neu$^+$/MART1$^-$) (Fig. 6B). The cytolysis observed was not caused by NK cells, since the activity of NK cells had been neutralized by the addition of unlabeled K562 cells (5×10^4 cells/well) as a cold target in the assay. Our previous data also reflect HER2/neu antigen, a member of human EGF-receptor family of receptor tyrosine kinases, as a widely expressed tumor antigen in many tumor types. Over-expression of HER2/neu antigen has been found in more than 5% melanoma specimens [22].

Activity of 2C and 3C HLA-A2/MART1$_{27–35}$ complexes to elicit specific CTL proliferation and cytotoxicity

In light of the higher reactivity of 2C HLA-A2/MART1$_{27–35}$ complexes to specific receptors in DDIA and flow cytometry than its 3C counterpart, it is reasonable to believe that the 2C complex may be more functional in stimulating antigen-specific CTL proliferation or in eliciting their cytotoxicity since HLA/peptide complexes exert their stimulatory effect on T lymphocytes by binding to specific TCR. We therefore cultured TIL1 in the presence of either type of the 2C or 3C HLA-A2/MART1$_{27–35}$ monomers followed by analyzing the frequency of MART1$_{27–35}$-specific CD8$^+$ cells. The frequency of tetramer$^+$/CD8$^+$ cells in the 0.245 nM 2C monomer- and 3C monomer-stimulated TIL1 population increased from 0.89% (no stimulation group) to 3.60% and to 3.02%, respectively (Fig. 5A, upper row). The proliferation of CTL is antigen-specific, since the frequency of the HER2/neu$_{369–377}$-specific CD8$^+$ cells remained unchanged following stimulation of the 2C or 3C HLA-A2/MART1$_{27–35}$ monomers (Fig. 5B, down row). These data imply that both the 2C monomer and 3C monomer can exert their proliferative effect on antigen-specific CTL in vitro, but whether the 2C complex is more functional

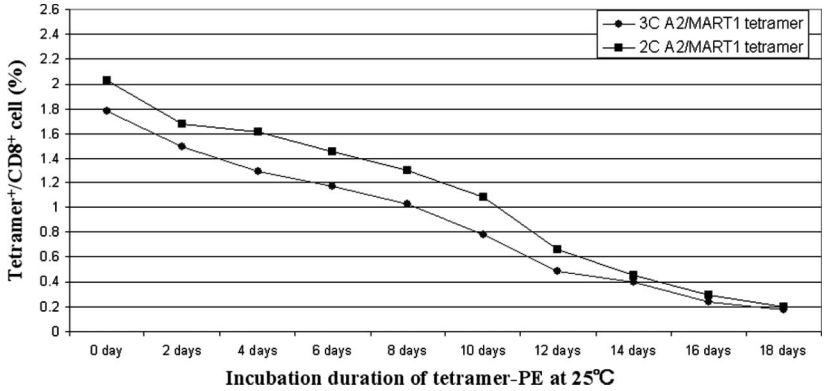

Fig. 7. Stability of 2C and 3C HLA-A2/MART1$_{27–35}$ tetramer. TIL1 cells were stained with PE-labeled 2C or 3C HLA-A2/MART1$_{27–35}$ tetramer (7.0 μg/well), which was incubated at 25 °C up to 18 days, along with FITC-conjugated anti-human CD8 mAb RPA-T8 (10 μl/well). –●– 3C A2/MART1 tetramer and –■– 2C A2/MART1 tetramer, the PE-labeled tetramers of 3C HLA-A2/MART1$_{27–35}$ monomers and 2C HLA-A2/MART1$_{27–35}$ monomers, respectively.

than its 3C counterpart remains to be further proven since the frequency of $MART1_{27–35}$-specific $CD8^+$ cells in the 2C monomer-stimulated TIL1 population is not too much higher than that in the 3C monomer-stimulated TIL1 population (3.60% vs. 3.02%). Of note is that we did not optimize the stimulation conditions such as cell density, supplement of anti-CD28 mAb, and immobilization of the soluble 2C or 3C monomers by coating plates or beads. It was reported that the conventional 3C MHC class I/peptide complexes which were co-coated on the micro-magnetic beads with anti-CD28 mAb can strongly elicit antigen-specific CTL response ex vivo or in vivo [5–7]. Therefore, the optimized stimulation conditions should prove to be more sensitive in determining the higher ability to excite specific CTL proliferation of the 2C HLA/peptide complexes over its 3C counterpart.

A standard ^{51}Cr release assay was performed to further analyze the function of the 2C and 3C complexes in eliciting cytotoxicity of specific CTL. Fig. 6A showed that the 2C complex-stimulated TIL1 had a significant higher cytolysis against the $MART1_{27–35}$-pulsed T2 target cells than the 3C complex-stimulated TIL1 ($p < 0.05$), while the cytolysis of both TIL1 populations stimulated by 2C, 3C complexes was apparently stronger than that of no-stimulated TIL1 ($p < 0.001$), even at the 0.0245 nM monomer data groups. In Fig. 6B, a significant better ability of the 2C complex-stimulated TIL1 to lyse melanoma cell line 501 also was detected at various E/T ratios as compared to the 3C complex-stimulated TIL1 ($p < 0.01$). The cytolysis is antigen specific since the TIL1 with or without stimulation showed similar level of cell lysis against breast cell line MDA-MB231 that is MART1 negative, HLA-A2 and HER2/neu positive. If these data imply stronger antigenicity or stimulatory function of 2C complex versus the 3C complex, the peptide–β_2m fused HLA class I/peptide complex may represent a more functional tool to prime antigen-specific CTL response in vivo or ex vivo than the conventional 3C HLA class I/peptide complex.

Stability of the 2C and 3C HLA-A2/$MART1_{27–35}$ complexes

The tetrameric forms of 2C and 3C HLA-A2/$MART1_{27–35}$ monomers were kept at 25 °C for up to 18 days. The tetramers were used to stain TIL1 population every 2 days for comparing the stability of 2C and 3C monomers. As shown in Fig. 7, both percentages of 2C tetramer$^+$/$CD8^+$ cell and 3C tetramer$^+$/$CD8^+$ cell decreased in a similar manner, suggesting their stability decreased gradually and was comparable, at least under such experimental conditions. It is noteworthy that both tetramers of 2C and 3C monomer remain stable during the incubation up to 14 h at 25 °C, since the frequency of tetramer$^+$/$CD8^+$ cells did not decrease (data not shown).

After comparing their yield, running behavior in gel filtration, stability, expression of polymorphic and monomorphic antigen determinants, binding ability to specific receptors, and function to stimulate CTL proliferation and cytotoxicity, the peptide–β_2m fused HLA class I/peptide complex is supposed to be a reliable, economical, and more functional substitute for the conventional 3C HLA class I/peptide complex. This finding will accelerate the application of peptide–β_2m fused HLA class I/peptide complexes in immunological studies.

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