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Dimerization of Soluble Disulfide Trap Single-Chain Major Histocompatibility Complex Class I Molecules Dependent on Peptide Binding Affinity

Eleni Kotsiou,[1,2] Joanna Brzostek,[1] Izabela Lenart,[3] Antony N. Antoniou,[3] Julian Dyson,[2,*] and Keith G. Gould[1,*]

Abstract

Stable presentation of peptide epitope by major histocompatibility complex (MHC) class I molecules is a prerequisite for the efficient expansion of $CD8^+$ T cells. The construction of single-chain MHC class I molecules in which the peptide, β_2-microglobulin, and MHC heavy chain are all joined together *via* flexible linkers increases peptide-MHC stability. We have expressed two T cell epitopes that may be useful in leukemia treatment as single-chain MHC class I molecules, aiming to develop a system for the expansion of antigen-specific $CD8^+$ T cells *in vitro*. Disulfide trap versions of these single-chain MHC molecules were also created to improve anchoring of the peptides in the MHC molecule. Unexpectedly, we observed that soluble disulfide trap single-chain molecules expressed in eukaryotic cells were prone to homodimerization, depending on the binding affinity of the peptide epitope. The dimers were remarkably stable and efficiently recognized by conformation-specific antibodies, suggesting that they consisted of largely correctly folded molecules. However, dimerization was not observed when the disulfide trap molecules were expressed as full-length, transmembrane-anchored molecules. Our results further emphasize the importance of peptide binding affinity for the efficient folding of MHC class I molecules. *Antioxid. Redox Signal*. 15, 635–644.

Introduction

Natural major histocompatibility complex (MHC) class I molecules surveyed by the immune system are heterodimeric glycoproteins consisting of an MHC encoded, polymorphic heavy chain comprised of three distinct domains (α1–α3) and the invariant, noncovalently associated β_2-microglobulin (β_2m) (20). The β-sheet platform formed by the α1 and α2 domains at the membrane distal part of the MHC class I molecule forms a cleft that accommodates endogenously generated peptides. The combined surface formed by the α1 and α2 helices of the class I molecule and the associated peptide interacts with the T cell receptor (34).

The assembly of trimeric MHC class I heavy chain/β_2m/peptide complexes takes place in the oxidizing environment of the endoplasmic reticulum (ER) by a complex and well-studied pathway (10, 14) (and see reviews in this issue). The fully assembled, mature MHC class I molecules then leave the ER and travel through the Golgi apparatus to the plasma membrane (32, 41). The overall efficiency of these processes is low, and it takes of the order of at least a thousand epitope-containing precursor polypeptides to obtain a single-peptide MHC at the cell surface (31). To improve the efficiency of antigen presentation, single-chain MHC molecules have been generated. The expression of an MHC class I trimeric complex as a single-chain molecule (known as a single-chain trimer [SCT]) (43) provides an attractive approach for the generation of highly stable reagents that can be used either for enumeration or for the expansion and isolation of antigen-specific $CD8^+$ T cell populations. Several groups have used mouse MHC class I (H-$2K^b$, H-$2D^b$) or human (human leukocyte antigen [HLA]-A2) SCTs in the form of DNA vaccines to induce efficient $CD8^+$ T cell responses *in vivo* (15–17, 19, 44), whereas others have used SCTs to create artificial antigen presenting cells for the expansion of CD8 T^+ cells *in vitro* (25).

We have expressed the HA-1 and HA-2 minor histocompatibility antigens (mHags) (11, 13) in the SCT format. These peptide epitopes are restricted by HLA-A2 and potentially offer two very useful targets for leukemia immunotherapy due to their pattern of expression, which is restricted to

[1]Department of Immunology, Wright-Fleming Institute, and [2]Section of Immunobiology, Hammersmith Hospital, Imperial College London, London, United Kingdom.
[3]Division of Infection and Immunity/Centre of Rheumatology, Department of Immunology and Molecular Pathology, University College London, London, United Kingdom.
*These two authors contributed equally to this work.

hematopoietic cells (40). Indeed HA-1 and HA-2-specific $CD8^+$ T cells were found to emerge after donor lymphocyte infusions in leukemic patients, correlating with disease remission (21). Therefore, *in vitro* or *in vivo* (after vaccination) generation of HA-1 and HA-2-specific $CD8^+$ T cells could be of great importance in the clinical setting. Unfortunately, HA-1 and HA-2 are only weakly immunogenic epitopes, and in addition to using the SCT format we attempted to increase their immunogenicity by incorporating a disulfide trap into the SCTs, as described recently by others (23, 38, 39). In these disulfide trap SCTs (dtSCTs), the peptide epitope is anchored into the peptide binding groove by an introduced disulfide bond, increasing the stability of the complex. Unexpectedly, we observed that while the full-length (including the transmembrane/cytoplasmic regions) SCTs and dtSCTs can assemble efficiently and reach the cell surface, the soluble versions (lacking transmembrane/cytoplasmic regions) of dtSCTs were prone to intermolecular disulfide bonding and the production of homodimers.

Materials and Methods

Cell culture, transfection, and retroviral transduction

Transporter associated with antigen processing (TAP) subunit 2-deficient Chinese hamster ovary (CHO) cells (36) were cultured in nutrient mixture F12 (Ham) supplemented with 5% fetal bovine serum (FBS). Human G10 T cells (18) (kindly supplied by Dr Tao Dong, University of Oxford) were cultured in RPMI 1640 medium supplemented with 10% human serum, and 100 U/ml recombinant human IL-2 (Roche Applied Sciences). Human T2 (HLA-A2 positive, TAP deficient) cells were cultured in RPMI 1640 medium supplemented with 10% FBS. The GP2 packaging cell line (Clontech) was cultured in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose) medium supplemented with 10% FBS, and human embryonic kidney (HEK) 293T cells in DMEM (2.5 g/l glucose) supplemented with 10% FBS. TAP2-deficient CHO cells and HEK293T cells were transfected with DNA using Superfect (Qiagen), according to the manufacturer's instructions. Transfectants of TAP2-deficient CHO cells were selected with and maintained in Geneticin (0.8 mg/ml; Invitrogen). The GP2 packaging cell line was transfected using Effectene (Qiagen) according to the manufacturer's instructions. After transfection, GP2 cells were incubated for 48 h, supernatant containing recombinant retrovirus was harvested, and 7 μg/ml polybrene (Sigma-Aldrich) was added. The retroviral supernatant was used to transduce HEK293T cells. All culture media contained 2 m*M* L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin, and were obtained from Invitrogen, unless stated otherwise.

Recombinant DNA constructs

The HA-1 and HA-2 HLA-A2 SCTs are analogous to an HLA-A2 SCT described previously (8), and present the epitopes VLHDDLLEA and YIGEVLVSV representing the HA-1 and HA-2 mHags, respectively (11, 29). They were produced by oligonucleotide-directed mutagenesis using a QuickChange® site-directed mutagenesis kit (Stratagene) with the previous HLA-A2 SCT as template, according to the manufacturer's instructions. Further changes to generate the dtSCTs, CD8 enhanced binding variants, and a high affinity HA-1 peptide variant were all made by oligonucleotide-directed mutagenesis in the same manner. All constructs were verified by full DNA sequencing. Oligonucleotides used for mutagenesis and DNA sequencing were purchased from Invitrogen. For cloning the SCT and dtSCT encoding sequences into the retroviral vector (pQCXIX; Clontech), the forward primer 5′-ATAAGAATGCGGCCGCCACCATGGCCCGTT CCGTGGCCTTAGCTGTG-3′ and reverse primer 5′-CAAG CCCCTCACCCTGAGATGGACCGGTGCAG-3′ were used in PCR to remove the cytoplasmic/transmembrane domain of HLA-A2 and introduce a *Not*I and *Age*I restriction site at the 5′ and 3′ ends of the amplified DNA sequence. The bicistronic retroviral expression vector pQCXIX, already encoding green fluorescent protein in one expression site and encoding a multiple epitope tag (MBH: c-Myc tag, biotin acceptor sequence, 6×his tag, amino acid sequence TGEQKLIS EEDLGLNDIFEAQKIEWHHHHHH) in the other expression site, was used for cloning the soluble forms of SCTs, generating fusions with a C-terminal MBH tag. These plasmids were used to generate protein-producing cell lines exactly as described previously (8).

Flow cytometry

Cells were incubated with the anti-HLA-A2 conformation-specific monoclonal antibody BB7.2 (BD Biosciences) or anti-HLA class I conformation-specific monoclonal antibody W6/32 (AbCam), both conjugated with fluorescein isothiocyanate, at a 1 in 25 dilution in phosphate-buffered saline (PBS) at 4°C for 30 min. After incubation with antibody, the cells were washed and analyzed on a FACS Calibur flow cytometer (Becton-Dickinson). Data were acquired using CellQuest software (Becton-Dickinson), and analyzed using FlowJo software (Tree Star).

Peptide competition assay

The peptide competition assay was performed as described previously (39). The HLA-A2-restricted gag p17 peptide epitope (SLYNTVATL) (18) (Proimmune) was used in concentrations ranging from 3×10^{-6} to 3×10^{-9} *M*. T cell cytotoxicity was measured using a standard 5 h ^{51}Cr release assay, as described previously (26).

Production of soluble SCT protein

Soluble SCT proteins were produced exactly as described previously (8). Briefly, the culture medium from transduced HEK293T cells was passed through a nickel-nitrilotriacetic acid agarose column. After extensive washing, his-tagged protein was eluted with an exponential gradient of imidazole (Sigma-Aldrich) ranging from 10 to 200 m*M*. After initial analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), fractions with the highest concentration of protein were pooled and concentrated. Fast protein liquid chromatography (FPLC) gel filtration was performed using an ÄKTA™explorer (GE Healthcare) and a Superdex 75 column (GE Healthcare) run in PBS.

Analysis of SCT dimerization in cells

About 1.5×10^6 cells per sample were treated with 20 m*M* N-ethylmaleimide (NEM; Sigma-Aldrich) for 10 min at 4°C, before being lysed in cell lysis buffer (1% NP-40, 150 m*M*

NaCl, 10 m*M* Tris-HCl [pH 7.5], 1×complete protease inhibitors [Roche Applied Sciences], and 1 m*M* phenylmethylsulfonyl fluoride [Sigma-Aldrich]), and 10 m*M* NEM for 30 min at 4°C. Lysates were electrophoresed on NuPAGE® 4%–12% Bis-Tris gels (Invitrogen), transferred onto Invitrolon™ polyvinylidine fluoride membranes (Invitrogen), blocked with 5% milk powder in PBS and 0.1% Tween-20 (Sigma-Aldrich), and probed with rabbit polyclonal anti-human β_2m (AbCam) followed by anti-rabbit HRP-conjugated antibody (AbCam), and observed using ECL (GE Healthcare).

Cell protein biotinylation assay

About 1.5×10^7 cells per sample were resuspended in PBS containing 10 m*M* EZ-Link NHS Biotin (cell permeable) or EZ-Link NHS–SS Biotin (cell impermeable), both from Thermo Scientific, and incubated at room temperature for 30 min. After washing with PBS, the cells were lysed in 1% NP-40 cell lysis buffer as described above and precleared with isotype control antibody (BD Biosciences), and folded HLA-A2 molecules were immunoprecipitated with BB7.2 antibody bound to protein A/G sepharose (Santa-Cruz Biotechnology). Immunoprecipitates were electrophoresed on NuPAGE® 4%–12% Bis-Tris gels, transferred onto Invitrolon™ polyvinylidine fluoride membranes, and blocked with 5% milk powder in PBS and 0.1% Tween-20, and biotinylated proteins were detected using streptavidin conjugated to an infrared dye (IRDye800CW, LI-COR).

Dithiothreitol/4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid assay

The dithiothreitol/4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid (DTT/AMS) assay was performed as described previously (4). Briefly, 5 μg FPLC-purified HA-1 dtSCT dimer was incubated with various concentrations of DTT (Sigma-Aldrich) for 10 min at 37°C. Samples were then incubated at room temperature for 20 min with nonreducing sample buffer in the presence or absence of 0.3 m*M* AMS (Invitrogen). Samples were analyzed by 8% SDS-PAGE and gels were stained for protein with SimplyBlue SafeStain (Invitrogen).

Pulse-chase experiments

About 10^7 cells per sample were incubated for 30 min at 37°C in methionine/cysteine-free RPMI 1640 medium (Sigma-Aldrich). Subsequently, 20 μl of 10 mCi/ml of [^{35}S]-translabel (MP Biomedicals) was added to each cell line, and incubation was continued for a further 5 min. Labeled cells were then incubated in complete DMEM for different periods of time, as indicated. At the end of each time period, cells were treated with 20 m*M* NEM for 10 min and then lysed in 1% NP-40 lysis buffer, as described above. Lysates were immunoprecipitated with mouse anti-c-Myc tag antibody (AbD Serotec) bound to protein A/G-Sepharose (Santa-Cruz Biotechnology). Immunoprecipitates were digested for 1 h at 37°C with 5 mU endoglycosidase H (Endo H; Roche Applied Sciences), reduced with 20 m*M* DTT, and analyzed by 8% SDS-PAGE.

BB7.2 ELISA

Different concentrations of proteins (ranging from 1 to 40 μM) were immobilized overnight on MaxiSorp 96-well-plates (Nunc), washed, and incubated overnight with 0.5 μg/ml BB7.2 antibody (BD Biosciences). Plates were incubated with biotinylated goat anti-mouse Ig (BD Biosciences), followed by incubation with streptavidin-conjugated alkaline phosphatase (BD Biosciences). The ELISA was developed with p-nitrophenyl phosphate substrate solution (Sigma-Aldrich).

Glycosidase treatment of HA-1 dtSCT dimer

Five micrograms of FPLC purified HA-1 dtSCT dimer was either left untreated or incubated with 5 mU of Endo H (Roche Applied Sciences) or 1 U peptide-N-glycosidase F (PNGase F, Roche Applied Sciences) for 1 h at 37°C. Samples were analyzed by 8% SDS-PAGE and gels were stained with SimplyBlue SafeStain (Invitrogen).

Results

HA-1 and HA-2 SCTs and dtSCTs assemble efficiently

We have expressed the HA-1 and HA-2 mHags in the format of SCTs, in which the peptide, β_2m, and HLA-A2 heavy chain are all covalently linked together in a single molecule with glycine/serine-rich linkers (Fig. 1A) (9, 43). Because peptide dissociation from the binding groove represents a significant obstacle for the generation of recombinant MHC molecules bearing suboptimally bound peptide such as the HA-1 and HA-2 epitopes, we also introduced mutations at position 2 of the first linker (L2C) and position 84 of the heavy chain (Y84C) to create disulfide bond trap SCT (dtSCT) versions (Fig. 1B) (39). Studies on a K^{b}OVA dtSCT showed that the introduction of these two cysteine residues leads to the formation of a new disulfide bond, anchoring the OVA peptide more efficiently into the binding groove of the MHC, and severely limiting the binding of exogenously added competitor peptides (38).

To test the ability of the HA-1 and HA-2 SCT and dtSCT molecules to fold correctly, they were stably expressed in TAP2-deficient CHO cells by transfection. In these cells there is no supply of endogenous peptides into the ER by TAP, so stable cell surface expression of folded molecules gives a good

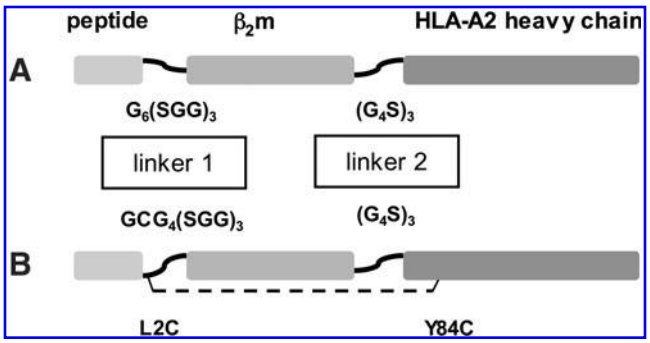

FIG. 1. Outline structure of single-chain HLA-A2 constructs. (A) Outline of a conventional major histocompatibility complex class I SCT. **(B)** Outline of a dtSCT, with two introduced cysteine residues. The dotted line represents the disulfide bond formed between the cysteine at position 2 of linker 1 (L2C), and the cysteine introduced instead of tyrosine at position 84 of the class I heavy chain (Y84C). dtSCT, disulfide trap single-chain trimer; HLA, human leukocyte antigen.

measure of how well the covalently linked peptide is able to bind. Cell surface expression was assessed by antibody staining with the conformation-specific anti-HLA antibodies BB7.2 (Fig. 2A) and W6/32 (data not shown). The expression levels of folded SCTs and dtSCTs showed that the single-chain HLA-A2 molecules reach the cell surface efficiently without the need for an endogenous peptide source, and therefore must be loaded with the covalently linked peptide (Fig. 2A). The HA-1 and HA-2 dtSCTs showed consistently higher levels of cell surface expression than the equivalent SCT.

The increased efficiency of the dtSCTs in peptide retention compared to SCTs was confirmed in a peptide competition assay, using an immunodominant, high affinity HLA-A2 binding peptide. Two transfected TAP2-deficient CHO cell clones with similar cell surface expression levels of HA-1 SCT or HA-1 dtSCT were incubated with different concentrations of exogenously added competitor peptide SLYNTVATL, derived from HIV-1 gag p17. These cells were then used as targets for a specific T cell clone in a cytotoxicity assay (Fig. 2B). At higher concentrations of gag peptide, the HA-1 dtSCT was clearly better at retaining the HA-1 peptide in the binding groove compared to the HA-1 SCT, leading to lower levels of lysis by the gag-specific T cells (Fig. 2B). These data provide further evidence that the dtSCT format enhances the stability of the bound peptide within the groove.

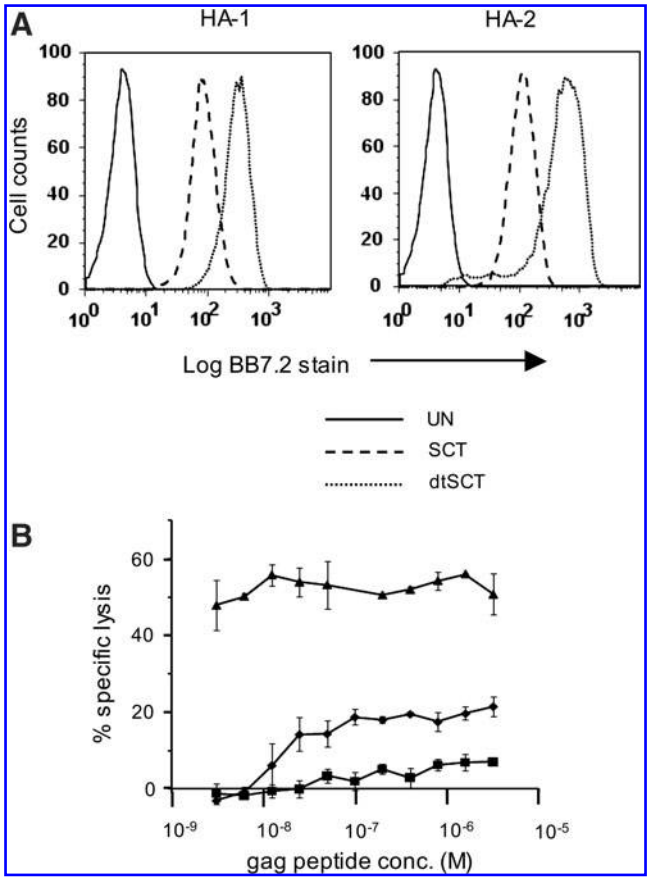

FIG. 2. Properties of single-chain HLA-A2 molecules presenting the HA-1 or HA-2 peptide. (A) Efficient cell surface expression of correctly folded single-chain HLA-A2 molecules in TAP2-deficient CHO cells. Staining profiles of representative, stable clones are shown. Untransfected cells (UN) were used as a negative control. **(B)** Clones of TAP2-deficient CHO cells expressing HA-1 SCT (◆) or HA-1 dtSCT (■) were incubated with the indicated concentrations of soluble gag peptide epitope, and used as targets in a cytotoxicity assay with a gag-specific T cell clone at an effector:target ratio of 5:1, as described in the Materials and Methods section. T2 cells (▲) were used as a positive control. Error bars show standard deviations of duplicates. CHO, Chinese hamster ovary; TAP, transporter associated with antigen processing.

Soluble HA-1 and HA-2 dtSCT protein expression by HEK293T cells

Having shown that the HA-1 and HA-2 SCTs assembled efficiently, we wished to express them as soluble proteins to develop an artificial culture system for the expansion of HA-1 and HA-2-specific T cells *in vitro*, which could be useful for adoptive transfer therapies (35). For protein production we adopted exactly the same methods that we had used previously for a different soluble HLA-A2 SCT (8). Because the dtSCT format gave the best and most stable expression in TAP2-deficient CHO cells, we focused on soluble versions of HA-1 dtSCT and HA-2 dtSCT. A further modification was also introduced into the HLA-A2 heavy chain in these constructs. The glutamine residue at position 115 was substituted with glutamic acid to create CD8 enhanced binding dtSCTs (Q115E, CD8 EB dtSCT). This mutation has been shown to increase the binding affinity of HLA-A2 for the CD8 T cell coreceptor (42). The HLA-A2 dtSCTs carrying the Q115E mutation would be expected to expand antigen-specific CD8 T cell populations *in vitro* more efficiently than their wild-type counterparts (19, 42).

The dtSCT proteins secreted by HEK293T cells were purified by nickel-nitrilotriacetic acid agarose chromatography, and analyzed initially by reducing SDS-PAGE. Yields of several mg per litre of culture supernatant were obtained, apparently of the predicted molecular weight and high purity (data not shown). For final purification, the proteins were subjected to gel filtration FPLC. Unexpectedly, the HA-1 dtSCT and HA-2 dtSCT proteins gave major peaks that eluted from the column at different volumes (Fig. 3A). The major peak of HA-1 dtSCT protein ran at a position corresponding to a molecular weight approximately twice that expected of SCT monomer. Nonreducing SDS-PAGE of material in the peaks confirmed that the HA-1 dtSCT was predominantly a dimer, with only a very small proportion secreted as monomer (Fig. 3A). By contrast, the HA-2 dtSCT was mainly secreted as correctly folded monomer, with only a small amount of dimer (Fig. 3A). The HA-1 dtSCT dimer resolved into monomer after reduction with DTT, showing that the high molecular weight form consists of disulfide-linked dimer. This dimer could not have been detected by our initial analysis using reducing SDS-PAGE. In conventional SCT format, HA-1 protein was found to be monomer (Fig. 3D), showing that the dimerization is mediated through the introduced cysteines of the disulfide bond trap.

The finding of distinct FPLC elution patterns for the HA-1 dtSCT and HA-2 dtSCT proteins was interesting because the only difference between them is the peptide moiety. Soluble HA-1 and HA-2 peptides have different binding affinities for HLA-A2 (IC_{50} 30 and 6.7 n*M*, respectively) (11, 13). Therefore,

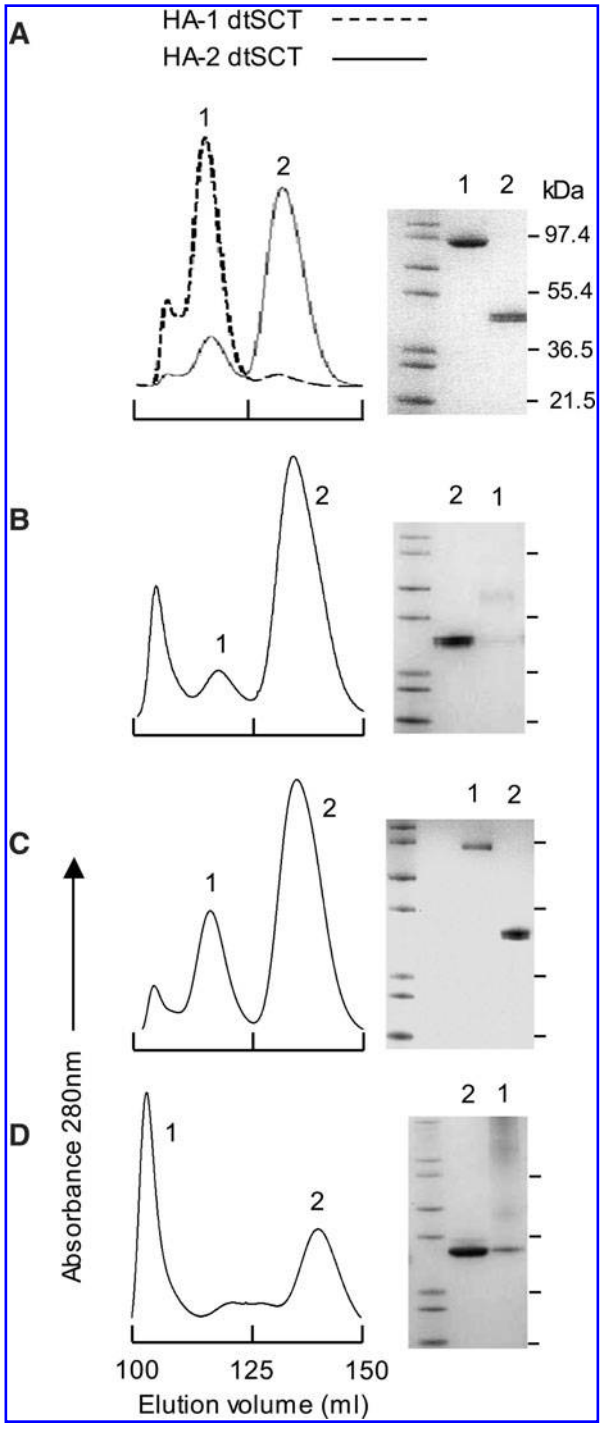

FIG. 3. Soluble forms of HA-1 and HA-2 dtSCTs can be secreted as dimers. Gel filtration FPLC chromatograms of nickel-nitrilotriacetic acid sepharose column purified soluble single-chain HLA-A2 molecules. *Left-hand panels* show FPLC chromatograms, and the corresponding *right-hand panels* show nonreducing SDS-PAGE gels of the peaks indicated, stained for protein. **(A)** HA-1 dtSCT (*dashed line*, peak 1) superimposed on HA-2 dtSCT (*solid line*, peak 2). **(B)** HA-1 dtSCT with HA-1 epitope amino acid residue 9 changed from alanine to valine (HA-1 AtoV dtSCT). **(C)** HA-1 dtSCT with enhanced CD8 binding (HA-1 CD8 EB dtSCT). **(D)** HA-1 SCT. Peak 1 was identified as nondisulfide bonded protein aggregates. FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

we tested whether peptide binding affinity for HLA-A2 influences folding and dimerization of the soluble forms of dtSCT. The affinity of the HA-1 peptide for HLA-A2 was increased by mutating the anchor residue at its C-terminus (position 9) from alanine to valine (12). This change at position 9 of the HA-1 peptide indeed greatly reduced the dimerization of soluble HA-1 dtSCT (Fig. 3B), suggesting that peptide binding affinity is a major factor in controlling dimer formation. Interestingly, the HA-1 CD8 enhanced binding dtSCT containing the wild-type HA-1 peptide sequence was also secreted mainly as a monomer (Fig. 3C), suggesting that the Q115E mutation may affect peptide epitope binding in addition to its effects on CD8 binding.

Properties of HA-1 dtSCT dimer

It was somewhat surprising that large amounts of HLA-A2 SCT disulfide bonded dimer were secreted from cells, suggesting that the dimer may consist of largely correctly folded molecules. Moreover, FPLC purified dimer was stored at 4°C for prolonged periods, and found to be highly stable. Therefore, experiments were carried out to establish the characteristics and structure of the HA-1 dtSCT dimer. The dimer was recognized by the HLA-A2-specific, conformation-sensitive antibody BB7.2 as efficiently as monomer (Fig. 4A), confirming that it consists of correctly folded molecules.

Previously, we applied rapid acidification/alkylation as a novel approach for analyzing the redox status and structure of MHC class I molecules (30). A similar approach using a DTT/AMS assay was used with purified HA-1 dtSCT dimer. Incubation with the alkylating reagent AMS reveals the presence of unpaired cysteine residues because after alkylation the polypeptide will increase in size by ~0.5 kDa per modified residue, and this can be observed by SDS-PAGE. Equal concentrations of dimer were left untreated or incubated with increasing concentrations of DTT ranging from 0.05 to 10 m*M*, and then incubated with or without AMS, as described in Materials and Methods. In the absence of DTT the dimers were not alkylated by AMS (Fig. 4B, lanes 1 and 2), showing that they do not have any accessible cysteine residues. Moreover, the dimers were compact and relatively resistant to reduction by DTT. This was apparent because incubation with a low concentration of DTT (0.05 m*M*) did not lead to the appearance of any monomer, and 0.5 or 1 m*M* DTT only gave partial reduction of dimer to monomer (Fig. 4B, lanes 7–12). Interestingly, as the concentration of DTT was increased the dimer showed an apparent increase in molecular weight (without AMS treatment), suggesting that the structure may have adopted a looser conformation, perhaps reflecting reduction of some disulfide bonds within the dimer not required to maintain the dimeric structure. The appearance of small amounts of monomer after incubation with intermediate DTT concentrations (Fig. 4B, lanes 7–10) suggested that dimers may consist of different populations in which either one or two disulfide bonds hold the two monomers together. Dimer was fully reduced to monomer by 10 m*M* DTT, but the protein only achieved full accessibility to AMS after additional heating at 95°C (Fig. 4B, lanes 3–6), again emphasizing that the dimers have a very compact structure.

MHC class I molecules are glycoproteins, and to determine whether the secreted HA-1 dtSCT dimer was in some way escaping ER quality control by the glycoprotein-dedicated

chaperone system due to incomplete glycosylation, purified dimer was incubated with Endo H or PNGase F, and analyzed with SDS-PAGE. Only PNGase F digestion caused a decrease in molecular size (Fig. 4C), confirming that the HA-1 dtSCT was N-glycosylated, but Endo H resistant, as expected for correctly folded molecules.

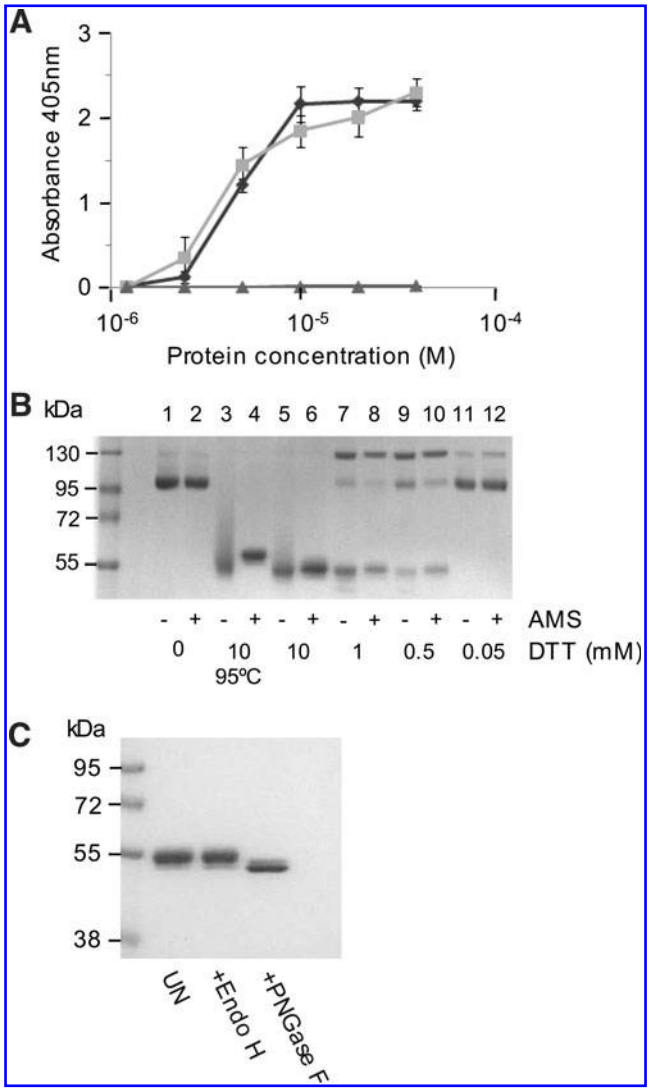

FIG. 4. Secreted HA-1 dtSCT dimer has a folded, compact structure, and is resistant to Endo H. (A) FPLC purified HA-1 dtSCT dimer (◆) or monomer (■) was used to coat wells of an ELISA plate, and tested for recognition by the BB7.2 monoclonal antibody in an ELISA. An irrelevant single-chain class I molecule (HFE) produced using the exactly the same methods was used as a negative control (▲). Error bars show standard deviations of triplicates. **(B)** FPLC purified HA-1 dtSCT dimer was treated with various concentrations of DTT, in the presence or absence of AMS, with or without heat denaturation at 95°C, and analyzed by nonreducing SDS-PAGE (*lanes 1–12*, different treatments as indicated). **(C)** FPLC purified HA-1 dtSCT dimer was incubated with Endo H or PNGase F or left untreated (UN), and analyzed by reducing SDS-PAGE, staining for protein. AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; DTT, dithiothreitol; Endo H, endoglycosidase H; PNGase F, peptide-N-glycosidase F.

Soluble HA-1 and HA-2 dtSCTs have different rates of maturation

To determine the extent of dimerization of soluble dtSCT molecules inside cells at the steady state, HEK293T cells producing proteins were lysed in the presence of NEM to prevent formation of disulfide bonds postlysis. Cell lysates were then separated on both reducing and nonreducing gels, and used for Western blotting, probing with anti-β_2m antibody (Fig. 5A). For cells expressing the dtSCT constructs there were significant amounts of both monomer and disulfide bonded dimer, demonstrating that dimerization takes place inside cells and not only after secretion. In agreement with the FPLC profiles of secreted proteins (Fig. 3), HA-1 dtSCT was predominantly dimer, and the two modifications that led to reduced dimerization, that is, enhancing peptide binding affinity (HA-1 AtoV dtSCT) or enhancing CD8 binding (HA-1 CD8 EB dtSCT), both led to significantly lower steady-state levels of intracellular dimer, similar to that found for HA-2 dtSCT (Fig. 5A). As expected, no dimer was detected for HA-1

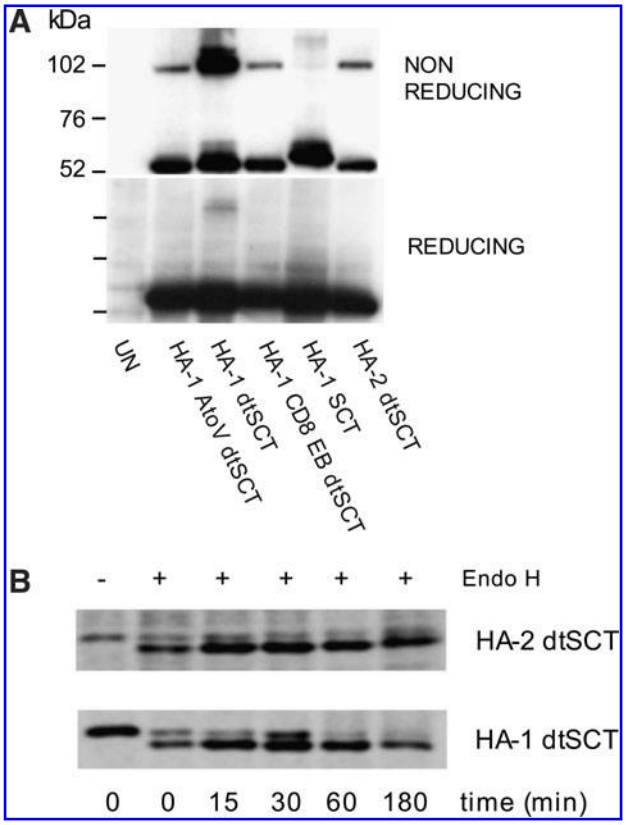

FIG. 5. Dimer formation and rate of secretion depend on SCT peptide-binding characteristics. (A) Lysates of HEK293T cells expressing the various different soluble SCT constructs or untransfected (UN) were treated with N-ethylmaleimide, separated by SDS-PAGE, reducing or nonreducing as indicated, and used for Western blotting with detection of β_2m. **(B)** Cells expressing the soluble dtSCT construct indicated were metabolically labeled with ^{35}S-methionine for 5 min and chased for up to 3 h. After cell lysis and immunoprecipitation with anti-myc tag antibody, precipitates were digested with Endo H (+) or left untreated (−), separated by reducing SDS-PAGE, and autoradiographed. β_2m, β_2-microglobulin; HEK, human embryonic kidney.

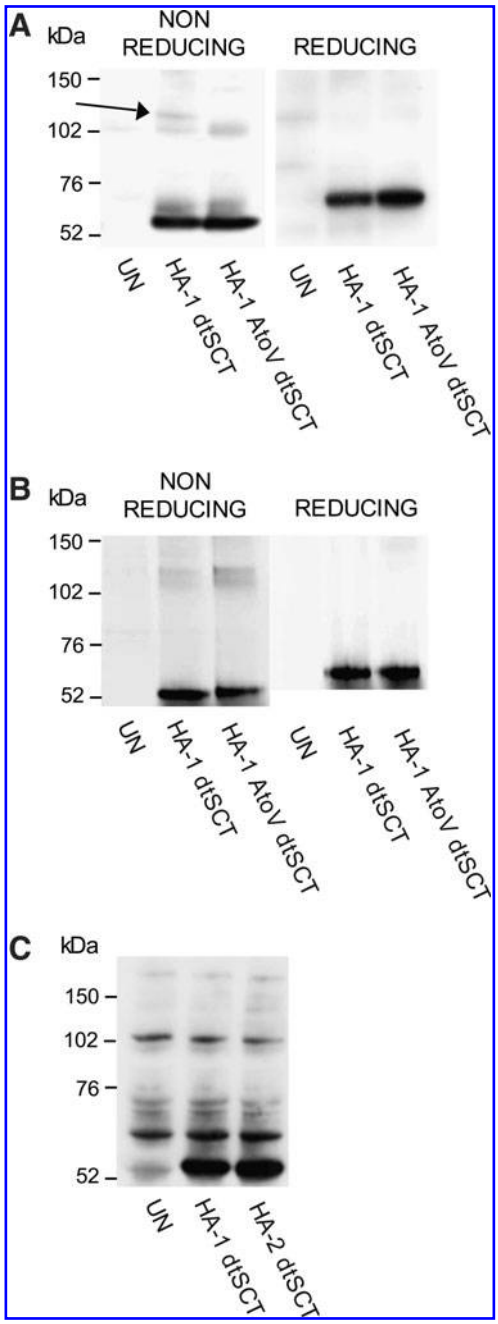

FIG. 6. Transmembrane-anchored dtSCTs do not form dimers. (A) Lysates of TAP2-deficient CHO cells expressing full-length, transmembrane-anchored HA-1 or HA-1 AtoV dtSCT or untransfected (UN) were treated with N-ethylmaleimide, separated by SDS-PAGE, reducing or nonreducing as indicated, and used for Western blotting with detection of β_2m. The *arrow* indicates possible dimer. Reduced dtSCT samples migrate more slowly in SDS-PAGE than nonreduced dtSCT samples. **(B)** Cells as in **(A)** were treated with NHS-biotin, lysed, and immunoprecipitated with BB7.2 anti-HLA-A2 antibody; precipitates were separated by SDS-PAGE, reducing or nonreducing as indicated, and used for Western blotting with detection of biotin. **(C)** Immunoblot analysis of cell lysates of HEK293T cells transiently transfected with full-length HA-1 dtSCT or HA-2 dtSCT, or untransfected (UN), with detection of β_2m (nonreducing SDS-PAGE).

SCT. The SCT monomers were found to migrate more slowly during nonreducing SDS-PAGE than the equivalent dtSCT monomers, suggesting that the additional introduced disulfide bond leads to a more compact structure, as reported previously (38).

Wild-type HLA-A2 has also been reported to dimerize when its rate of maturation from the ER was slowed down sufficiently by incubation of cells at 26°C, possibly due to prolonged exposure of accessible cysteines in the oxidizing environment of the ER (2). We therefore examined whether there are differences in the maturation rates of soluble HA-1 dtSCT and HA-2 dtSCT, correlating with their differences in tendency to dimerize. HEK293T cells expressing the soluble forms of HA-1 dtSCT or HA-2 dtSCT were metabolically labeled with a short pulse of radioactivity, chased for up to 3 h, lysed, and immunoprecipitated with anti-myc tag antibody (recognizes the C-terminal epitope tag on the soluble SCTs) to assess the complete pool of labeled single-chain molecules. To determine the rate of folding and maturation of the proteins, samples were treated with Endo H to assess exit from the ER and transit through the Golgi. Both HA-1 and HA-2 soluble dtSCTs trafficked very rapidly, as an Endo H-resistant fraction of protein was detected at the zero time point following just a 5-min pulse (Fig. 5B). Endo H-resistant soluble HA-2 dtSCT was present at time zero and decreased with time, suggesting that this fraction of protein had folded and been secreted from cells (Fig. 5B). Endo H-resistant soluble HA-1 dtSCT was also present at time zero, but was still prominent after 30 min (Fig. 5B), indicating that the HA-1 construct was less efficient at folding up than the HA-2 construct. For both constructs a pool of Endo H-sensitive material was still present after 3 h, suggesting that a significant proportion of the molecules was unable to fold up correctly for secretion.

Transmembrane-anchored HA-1dtSCT does not form dimers

TAP2-deficient CHO cells express membrane-anchored HA-1 dtSCT efficiently at the cell surface (Fig. 2A), and after finding that soluble HA-1 dtSCT is secreted predominantly as a dimer, we investigated possible dimer formation by membrane-anchored molecules. TAP2-deficient CHO cells expressing full-length HA-1 dtSCT or HA-1 AtoV dtSCT were treated with NEM before lysis, and used for Western blotting with anti-β_2m antibody. A very small amount of protein that may correspond to dimer was detected for HA-1 dtSCT, but not HA-1 AtoV dtSCT (Fig. 6A). This band was not present after running under reducing conditions. However, this material only represented a small fraction of the total material, and clearly there was a large difference in the ability of membrane-anchored and soluble versions of HA-1 dtSCT to dimerize. This result was confirmed by biotinylation of cells expressing the same full-length constructs, cell lysis, immunoprecipitation with BB7.2 antibody, Western blotting, and detection of biotin (Fig. 6B). Again, the vast majority of protein was detected as monomer.

The soluble dtSCT proteins found as dimers were produced in HEK293T cells, whereas the membrane-anchored dtSCT proteins were expressed in TAP2-deficient CHO cells. To rule out cell line-specific effects, full-length HA-1 dtSCT and HA-2 dtSCT constructs were transiently transfected into HEK293T cells. Dimers were not detected for either protein (Fig. 6C),

confirming that dimerization of dtSCTs is a feature only of the soluble versions.

Discussion

The data presented in this report describe a novel phenomenon of intracellular homodimerization of soluble MHC class I, dependent on the introduction of two cysteine residues in the sequence of single-chain HLA-A2 molecules. The introduction of these two cysteine residues, one located at position 2 of the linker between the peptide and β_2m and one in the F pocket of the HLA-A2 heavy chain, was designed to improve peptide binding *via* the formation of a new disulfide bond, and thereby increase immunogenicity for T cells. Interestingly, we observed that dimer formation was dependent on intrinsic peptide binding affinity, since dtSCT molecules containing lower affinity peptides formed a higher proportion of dimers.

MHC class I molecules bind antigenic peptides with lengths of 8–10 amino acid residues (37). For HLA-A2 molecules, the most important peptide anchor residues are leucine at position 2 (P2) and valine or leucine at position 9 (P9) (33). The HLA-A2-restricted HA-1 epitope, amino acid sequence VLHDDLLEA, has a relatively unfavorable residue at P9, which makes very few contacts with the HLA-A2 F pocket (24). Mutating P9 to a valine increases the binding affinity of the HA-1 peptide, and also led to a marked reduction in dimerization of soluble dtSCT protein, most probably because the mutation stabilized the peptide in a position to allow correct formation of the disulfide bond trap. Dimer formation clearly involved the introduced L2C and Y84C cysteine residues, because soluble HA-1 SCT molecules lacking these residues did not form dimers. Interestingly, the CD8 enhanced binding (Q115E) HA-1 dtSCT was secreted predominantly as a monomer. This mutation may limit dimer formation by accelerating folding of the SCT molecule, since a Q115A change in HLA-A2 has been shown to reduce TAP dependence and increase the rate of egress from the ER (3). Alternatively, the Q115E mutation may have had unanticipated effects on HA-1 peptide binding. In the HLA-B27 system, other residues neighboring position 115 were also associated with different degrees of homodimer formation (5).

When we assessed dimerization of membrane-anchored dtSCTs in transfected TAP2-deficient CHO cells, there was no intermolecular disulfide bonding between L2C and Y84C, suggesting that in these molecules the disulfide trap was properly formed and stable. This result was confirmed in HEK293T cells. Therefore, homodimerization must be limited by the physical constraints imposed when the molecules are correctly orientated on membranes. Such constraints may preclude close contact between the appropriate cysteine residues in neighboring molecules, so preventing formation of intermolecular disulfide bonds. It also suggests that dimers of soluble dtSCT molecules may consist of molecules in a head-to-head orientation, with the peptide epitope of one molecule sitting in the binding groove of the other molecule, and vice versa. This type of dimer could be stabilized by two disulfide bonds, and would appear to be correctly folded, at least as judged by BB7.2 antibody binding and by the fact that it is secreted efficiently, implying it has passed the ER quality control system. Membrane-anchored molecules would not be expected to be able to form this type of dimer.

The first report of MHC class I dimerization involved disulfide bonding through cytoplasmic tail cysteine residues conserved between human and mouse MHC class I proteins (7). Subsequently, the nonclassical MHC class I molecule HLA-G was found to dimerize on the cell surface through the unpaired cysteine residue 42 located in the α1 domain (6). Homodimerization in this case was dependent on the level of cell surface expression of HLA-G, but that did not appear to be the case in our system because the membrane-anchored dtSCT HLA-A2 proteins were expressed at high levels without forming dimers. Another extensively studied example of HLA multimer formation is the phenomenon of HLA-B27 heavy chain dimerization, not involving the β_2m light chain, which was initially thought to be mediated through the unpaired cysteine residue at position 67 located in the B pocket of the binding groove (1). Other studies have shown that the structural cysteine 164 located in the α2 domain may also participate in homodimer formation of HLA-B27 (2). The same study proposed that HLA-B27 dimerization was induced by the slow folding of the molecule, which led to an increased ER content of partially folded molecules. When the folding kinetics of soluble HA-1 and HA-2 dtSCTs were assessed, we observed that HA-2 dtSCT folds and is secreted more rapidly than HA-1 dtSCT, providing evidence for the role of peptide binding affinity in HLA SCT folding efficiency. Consistent with the HLA-B27 data, the slower folding of HA-1 dtSCT may result in more prolonged exposure of unpaired cysteine residues, leading to increased dimerization.

Homodimer formation of soluble dtSCts is most likely to be taking place in the ER due to the very high protein content and the oxidizing environment, which is favorable for disulfide bond formation in MHC class I molecules (28). Some dimer formation after secretion is theoretically possible, but seems unlikely as the secreted proteins were diluted in a large volume of culture medium. Our data provide further evidence for the crucial role of peptide binding affinity in the assembly of MHC class I molecules. Peptide binding affinity contributes to a great extent to the general stability of MHC class I molecules (27), and is an important factor in the design of tumor vaccines and in the development of efficient $CD8^+$ T cell expansion systems (22).

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Author Disclosure Statement

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Address correspondence to:
Dr. Keith G. Gould
Department of Immunology
Wright-Fleming Institute
Imperial College London
Norfolk Place
London W2 1PG
United Kingdom

E-mail: k.gould@imperial.ac.uk

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Abbreviations Used

AMS = 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid
β_2m = β_2-microglobulin
CHO = Chinese hamster ovary
DMEM = Dulbecco's modified Eagle's medium
dtSCT = disulfide trap single-chain trimer
DTT = dithiothreitol
Endo H = endoglycosidase H
ER = endoplasmic reticulum
FBS = fetal bovine serum
FPLC = fast protein liquid chromatography
HEK = human embryonic kidney
HLA = human leukocyte antigen
mHag = minor histocompatibility antigen
MHC = major histocompatibility complex
NEM = N-ethylmaleimide
PBS = phosphate-buffered saline
PNGase F = peptide-N-glycosidase F
SCT = single-chain trimer
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TAP = transporter associated with antigen processing