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Disulfide Bond Engineering to Trap Peptides in the MHC Class I Binding Groove

Steven M. Truscott,* Lonnie Lybarger,† John M. Martinko,‡ Vesselin E. Mitaksov,* David M. Kranz,§ Janet M. Connolly,* Dave H. Fremont,* and Ted H. Hansen²*

Immunodominant peptides in CD8 T cell responses to pathogens and tumors are not always tight binders to MHC class I molecules. Furthermore, antigenic peptides that bind weakly to the MHC can be problematic when designing vaccines to elicit CD8 T cells in vivo or for the production of MHC multimers for enumerating pathogen-specific T cells in vitro. Thus, to enhance peptide binding to MHC class I, we have engineered a disulfide bond to trap antigenic peptides into the binding groove of murine MHC class I molecules expressed as single-chain trimers or SCTs. These SCTs with disulfide traps, termed dtSCTs, oxidized properly in the endoplasmic reticulum, transited to the cell surface, and were recognized by T cells. Introducing a disulfide trap created remarkably tenacious MHC/peptide complexes because the peptide moiety of the dtSCT was not displaced by high-affinity competitor peptides, even when relatively weak binding peptides were incorporated into the dtSCT. This technology promises to be useful for DNA vaccination to elicit CD8 T cells, in vivo study of CD8 T cell development, and construction of multivalent MHC/peptide reagents for the enumeration and tracking of T cells—particularly when the antigenic peptide has relatively weak affinity for the MHC. The Journal of Immunology, 2007, 178: 6280 – 6289.

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3 Abbreviations used in this paper: pMHC, peptide/MHC; 3KO, Kb−/−/Db−/−; D9/−/−; β2m−/−; fibroblast; β2m, β2-microglobulin; dtSCT, disulfide-trap single-chain trimer; EndoH, endoglycosidase H; ER, endoplasmic reticulum; [G4S]n, n multiples of Gly-Gly-Gly-Ser; HPV, human papillomavirus; MCMV, murine cytomegalovirus; MFI, mean fluorescence intensity; SCT, single-chain trimer.

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to compensate for disruption of F pocket anchoring. This explains the improved cell surface stability of the OVA,β2, m,Ko SCT vs native Ko/OVA: the SCT is able to quickly rebind the covalently attached high-affinity OVA peptide. Consistent with this finding, we also determined that SCTs constructed with tight binding peptides were able to attain higher levels of steady-state folding and surface expression compared with SCTs constructed with weak binding peptides (13). Therefore, to re-engineer the SCT so that the peptide remained bound and to enhance the binding of weaker peptides, we investigated the possibility of strategically positioning a disulfide bond in the SCT.

In this study, we report the successful introduction of a disulfide trap into a MHC class I SCT. Using structure-based comparisons, we predicted that a disulfide bond would effectively trap peptide in the class I groove if one cysteine were placed in the Gly-Ser linker extending from the C terminus of the peptide and the other in a proximal H chain position. The engineered SCT molecules oxidized properly in the endoplasmic reticulum (ER) and were recognized at the cell surface by both Abs and T cells specific for the Ko/OVA complex. The disulfide-trap SCTs, or dtSCTs, did not succumb to high concentrations of competitor peptide, even when the dtSCT construct was based on a low-affinity complex. Similar results were obtained with a second MHC allele, H-2Ld, known for its relatively poor peptide-binding capacity (14, 15). SCTs have recently been found to have unique applications as DNA vaccines to tumors, tetramers for enumerating T cells, and as probes of lymphocyte development (4, 10, 11, 13, 16–19). We discuss how the incorporation of the disulfide trap should have a significant impact on SCT applications.

### Materials and Methods

#### Cell lines and Abs

Triple-knockout fibroblasts (Koβ-/- Dko β-/- β2, m-/-; 3KO) are a transformed murine embryo fibroblast line derived from 3KO mice (20). B6/WT3 (21) is a C57BL/6 (H-2b) murine embryo fibroblast line and was a gift from S. Jennings (Louisiana State University Health Sciences Center, Shreveport, LA). LM1.8 cells are L cells (H-2K) transfected with ICAM (22) and were a gift from Dr. P. Kourilsky (Institut National de la Santé et de la Recherche Médicale, Institut Pasteur. Paris, France). mAbs used in this study include the following: B8-24-3, which recognizes folded Ko (American Type Culture Collection); Y3, which recognizes folded H-2K molecules (23); 25-D1.16 (a gift from Dr. J. Yewdell, National Institutes of Health, Bethesda, MD), which recognizes Ko plus SIINFEKEL peptide (24); and 64-3-7, which recognizes open (peptide-free) forms of Ld and other class I molecules tagged with the 64-3-7 epitope (25).

#### Peptides

The OVA,β2, m,Ko (SIINFEKL) (26), SIYR peptide (SIYRYYGL) (27), VSV peptide (RGYYYVGL) (28), the QL9 peptide (QLSPPFDL) (29–31), and the murine CMV (MCMV) pp69 peptide (YPYDPPTNL) (32) were synthesized on an Applied Biosystems Model 432A peptide synthesizer. Peptides were dissolved directly in culture medium and incubated with cells for 5 h or overnight before cytotoxicometry.

#### DNA constructs and retroviral transduction

Constructs were generated using standard techniques and confirmed by DNA sequence analysis. The OVA,β2, m,Ko SCT sequence was previously described (10), and the QL9,β2, m,Ld SCT was constructed similarly as follows. The secretion signal sequence of β2, m is followed immediately by the peptide sequence, then a linker of 15 residues, [G4S]15. An alanine residue necessarily occupies the fourth position of the first linker (Table I) as a result of the placement of a convenient restriction site in the SCT construct. This first linker is followed by the mature β2, m sequence, the second linker of 20 residues, [G4S]20, then the mature class I Sequence. The Y84A mutation, first described in the OVA,β2, m,Ko SCT (11), was also introduced into the QL9,β2, m,Ld SCT. This mutation and the cysteine mutations shown in Table I were introduced by site-directed mutagenesis (QuickChange II XL; Stratagene). Retroviral expression vectors pMSCV-IRESHyogromycin and pMSCV-IRESYmogromycin were constructed in our lab (33) and used for concomitant expression of class I SCTs and drug resistance genes. Retrovirus-containing supernatants were generated using the Vpack vector system (Stratagene) for transient transfection of 293T cells to generate ecotropic virus for infection of 3KO and B6/WT3 cells or amphotropic virus for infection of LM1.8 cells.

#### Flow cytometry

Viable cells, gated by forward and side scatter, were analyzed on a FACSCalibur (BD Biosciences), and data (10,000 events/sample) were analyzed using CellQuest software (BD Biosciences). Staining with anti-class I mAbs was visualized using PE-conjugated goat anti-mouse IgG (BD Biosciences). Soluble, recombinant m6 TCR was used for flow cytometry directly from the supernatant of insect cells (34–36). Secondary reagents used to detect binding of the m6 TCR included biotinylated anti-mouse TCR β-chain mAb H57 (BD Biosciences) and PE-conjugated streptavidin (BD Biosciences).

#### Immunoprecipitation and Western blotting

Immunoprecipitations were performed essentially as described previously (20). Briefly, cells were treated with 1% Nonidet P-40 (IGEPAL CA-630; Sigma-Aldrich) lysis buffer, including 20 mM iodoacetamide, protease inhibitors, and a saturating concentration of precipitating mAb. After 30 min on ice, postnuclear lysates were incubated with protein A-Sepharose beads, and 2-ME (Sigma-Aldrich) was either added to a final concentration of 20 mM. Lysates were resolved on SDS-PAGE and transferred to nitrocellulose. Detection of β2, m was performed using polyclonal anti-human MHC class I mAb H57 (BD Biosciences) and PE-conjugated streptavidin (BD Biosciences). Lysates were visualized using CellQuest software (BD Biosciences). Staining with anti-class I mAbs was visualized using PE-conjugated goat anti-mouse IgG (BD Biosciences). Biotin-conjugated secondary reagents (Invitrogen Life Technologies) were added to the beads, and 2-3% (Sigma-Aldrich) was either added to a final concentration of 1% or excluded for nonreducing gels. Western blotting was performed after SDS-PAGE separation of precipitated proteins as described previously (37). Rabbit serum for blotting Ko was generated in our lab by peptide immunization using an amino acid sequence from the cytoplasmic tail of Ko. Rabbit serum for detecting β2, m was also generated in our lab by immunizing with denatured, recombinant mouse β2, m. Biotin-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as a secondary staining reagent, followed by streptavidin-HRP (Zymed Laboratories). Biotinylated mAb 64-3-7 was used for blotting Ld. Specific proteins were visualized by chemiluminescence with the ECL System (Amersham Biosciences).

#### CTLs

LM1.8 target cells transduced with the indicated class I constructs were labeled with Na2H1CrO4 for 1 h. OT-1 or 2C T cells were plated at various concentrations onto 96-well microtiter plates and incubated with target cells for 4 h at 37°C in 5% CO2 with or without 10 μM exogenous peptide. Activity of supraclonal Abs was measured in an Isospec gamma counter (JCN Biomedicals). The mean of triplicate samples was calculated, and percent specific 31Cr release was determined as follows: percentage of 31Cr release = 100 × (experimental 31Cr release – control 31Cr release)/(max-imum 31Cr release – control 31Cr release), where experimental 31Cr release represents counts from target cells mixed with effector cells, control

<p>| Table I. SCT constructs with zero, one, or two engineered cysteines |
|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>OVA,β2, m,Ko Construct</th>
<th>Sequence at N Terminus</th>
<th>Heavy Chain Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>SIINFEKLggaasggggs</td>
<td>None</td>
</tr>
<tr>
<td>Y84A</td>
<td>SIINFEKLggaasggggs</td>
<td>Y84A</td>
</tr>
<tr>
<td>T80C only</td>
<td>SIINFEKLggaasggggs</td>
<td>T80C, Y84A</td>
</tr>
<tr>
<td>T80C, L1C</td>
<td>SIINFEKLggaasggggs</td>
<td>T80C, Y84A</td>
</tr>
<tr>
<td>T80C, L2C</td>
<td>SIINFEKLggaasggggs</td>
<td>T80C, Y84A</td>
</tr>
<tr>
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<td>Y84C</td>
</tr>
<tr>
<td>Y84C, L2C (dtSCT)</td>
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<td>Y84C</td>
</tr>
<tr>
<td>Y84C, L3C</td>
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<td>Y84C</td>
</tr>
<tr>
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<td>Y84C</td>
</tr>
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<td>Y84A, N86C</td>
</tr>
<tr>
<td>OVA, Y84A</td>
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<td>Y84A</td>
</tr>
<tr>
<td>OVA, Y4A, Y84C, L2C (dtSCT)</td>
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<td>Y84C</td>
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</table>

<table>
<thead>
<tr>
<th>QL9, β2, m,Ld Construct</th>
<th>Sequence at N Terminus</th>
<th>Heavy Chain Mutation(s)</th>
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<td>SCT</td>
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<td>None</td>
</tr>
<tr>
<td>Y84A</td>
<td>QLSPPFDLggaasggggs</td>
<td>Y84A</td>
</tr>
<tr>
<td>Y84C, L2C (dtSCT)</td>
<td>QLSPPFDLggaasggggs</td>
<td>Y84C</td>
</tr>
</tbody>
</table>

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31Cr release represents counts from target cells in medium alone, and maximum 51Cr release represents counts from target cells lysed with 5% (v/v) Triton X-100 (Sigma-Aldrich).

**N15 hybridoma assays**

The TCR- mouse T cell hybridoma 58α-β (38) transfected with the α- and β-chains for the N15 TCR was a gift from Dr. H.-C. Chang (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). This cell line is also transfected with CD3ζ and CD8αβ CDNAs to optimize TCR expression and recognition of class I (39). Biological activity of IL-2 secreted by the N15 hybridoma was used to detect Kβ/VS/V8 complexes. B6/Wt3 cells or 3KO cells (5 x 10^7/200 μl/well) expressing endogenous Kβ or Kα SCT constructs were incubated for 1 h at 37°C with the indicated concentrations of VS/V8 peptide in a flat-bottom 96-well plate. The cells were washed, fixed in 1% paraformaldehyde for 15 min at room temperature, then washed again. The N15 hybridoma cells (10^5/200 μl/well) in fresh medium were then added to the plate and cultured for 24 h. Supernatants were harvested and frozen at -80°C for at least 1 h to lyse trace cells that may have carried over. CTLL-2 cells were washed and added to supernatants at a final cell density of 10^4/200 μl/well in a 96-well plate. After 18–24 h of incubation, Alamar blue (BioSource International) was added at 20 μl/well, and relative amounts of IL-2 in each supernatant were determined by fluorescence on a multidetection microplate reader (Bio-Tek Instruments).

**Results**

**Disulfide bond engineering strategy**

To secure the SCT peptide firmly in the class I peptide-binding groove, we set out to covalently link the peptide directly to the H chain α1 helix using a disulfide bond. As in our previous characterization of SCT molecules (10, 11), we again chose the Kβ/ OVA system, for which there are excellent reagents to monitor both MHC conformation and peptide occupancy (24, 40). Our approach was to introduce cysteine residues by site-directed mutagenesis, one in the H chain of Kβ and one in the first linker (Fig. 1A), which extends from the C terminus of the SIINFEKL peptide to the N terminus of mature β2m. Placing the second cysteine in the linker, rather than within the peptide itself, we hypothesized would minimize perturbations to the pMHC conformation and also allow us to more readily translate this disulfide-binding approach to SCT constructs based on other pMHC complexes.

To select the exact positions for cysteine mutations, we superimposed the crystal structures of Kβ/ OVA (41) and the MHC class II molecule I-Ek bound to a hemoglobin peptide (Hb64–76) (42). This latter structure features the Hb64–76 Peptide covalently attached with a Gly-Ser linker to the β-chain of class II (43, 44). The linker extends from the open class II groove from the C-terminal residue of the Hb64–76 peptide. Thus, overlaying these structures provided a framework for determining distances between Kβ H chain residues and specific positions along the first SCT linker (Fig. 1B). Three residues on the Kβ H chain were selected for substitution of a cysteine residue: Thr80, Tyr84, and Asn86. Importantly, the Y84A H chain mutation was also incorporated into the SCTs with the T80C and N86C mutations since we previously found that the Y84A mutation allowed for better linker accommodation (11). Based on the superimposition shown in Fig. 1B, the locations for various linker cysteine residues were selected. Thus, several constructs were made and tested for potential disulfide bond formation to trap the peptide into the class I-binding groove (Table I). SCT mutants with single cysteine mutations at the three abovementioned H chain positions were also constructed to control for the consequences of having an unpaired cysteine.

**Characterization of SCT cysteine variants**

Retroviral transduction was used to introduce each of the SCT variants into 3KO (20). This permitted the unambiguous analysis of the transduced class I constructs. It should be noted that cell surface expression of SCTs with disulfide traps was also confirmed in wild-type cells (see Fig. 5). Each of the SCT cysteine variants was detected at the cell surface by flow cytometry (Fig. 2A), both with mAb B8-24-3, which detects folded, peptide-occupied Kβ, and with mAb 25-D1.16, which binds specifically to the Kβ/ OVA complex (24). Kβ-reactive mAb Y3 also recognized the SCT variants (data not shown). The engineered molecules were expressed at high levels similar to the control SCT Y84A, suggesting that they had folded properly in the ER, passed ER quality control mechanisms, and efficiently transited to the cell surface. Importantly, the level of staining with the Kβ/ OVA-specific Ab was constant relative to the amount of folded Kβ with the exception of the control SCT variant T80C. The mAb 25-D1.16 is known to bind near the C terminus of the peptide (45), and thus, the unpaired cysteine or the loss of threonine at position 80 could impair mAb 25-D1.16 detection of this variant SCT. However, when the T80C H chain mutation

![FIGURE 1. A structure-based approach to engineering a disulfide bond in single-chain MHC class I. A, Schematic representation of the SCT based on the published structure for Kβ/ OVA (PDB code: 1VAC), including Gly-Ser linkers (red) and hypothetical location of the engineered disulfide bond (green). B, Analysis of superimposed class I and class II structures to select residues for cysteine mutagenesis. Kβ H chain (blue) aligned with the I-Ek-bound hemoglobin peptide (yellow; PDB code: 1FNE), which features covalent attachment with a Gly-Ser linker to the β-chain of class II. This structure-based visual provided a framework for examining distances between residues on the class I H chain and along the first SCT linker. Three residues on the Kβ H chain were selected for mutagenesis (purple): T80, Y84, and N86. Appropriately spaced residues in the linker were matched with each of these three H chain positions (see Table I and Fig. 2). The second linker position was chosen for further characterization of dtSCT constructs (Y84C, L2C).](http://www.jimmunol.org/content/199/3/6282.full.html)
was combined with a cysteine mutation in the linker, efficient recognition by mAb 25-D1.16 was restored. The comparable detection of the other variant SCTs with mAbs 25-D1.16 and B8-24-3 suggests that the introduction of cysteines at these various locations in the SCT spacer and/or H chain had no significant impact on the pMHC conformation detected by mAb 25-D1.16.

Our next objective was to determine biochemically which constructs actually had the additional disulfide bond, for it was unknown a priori how engineered cysteine residues would interact with ER thioreductases (46–48). In nonreducing SDS-PAGE, the presence of disulfide bonds can reduce the radius of gyration of denatured proteins, causing them to migrate faster through the gel matrix. Thus, the variant OVA\(^{\beta, m, K^\alpha}\) SCT constructs were compared in reduced vs nonreduced SDS-PAGE gels (Fig. 2B). As expected, all of the constructs migrated the same distance when the samples were reduced with 2-ME. (One exception was the N86C, L5C mutant in lane 10, which migrated faster than the other mutants because it no longer bears its N-linked glycan.) However, in the nonreducing gel, each of the constructs with engineered paired cysteines (lanes 3–5, 7, 8, and 10) migrated faster than their counterparts with either zero or one additional cysteine. This provided clear biochemical evidence that the cells had catalyzed the engineered disulfide bond in each of these six constructs. The oxidation appeared to be complete because reduced forms were not observed. Postlysis formation of disulfide bonds is not likely to account for these results since the sulfhydryl-reactive compound iodoacetamide was included in the sample preparation. The observation that cysteines at different linker locations formed disulfide bonds with the same H chain residues (Y84C and T80C) was probably a reflection of the flexibility of the Gly-Ser linker.

For a number of reasons, we focused our subsequent analyses on the Y84C, L2C disulfide bond position. First, we had previously engineered position 84 in the SCT H chain, and T cells recognized the Y84A mutant of the SCT, perhaps even more efficiently (11). Second, position 84 is highly conserved among class I alleles, increasing the likelihood that engineering at that position would easily transfer to other class I molecules (see Figs. 5 and 6). Furthermore, it was anticipated that placement of a disulfide bond at position 84 would substitute for hydrogen bonding between the C terminus of the peptide and Y84 in the F pocket of native class I molecules. We chose the second linker position (L2C) downstream of the SCT peptide to match with Y84C, reasoning that a disulfide bond closer to the SCT peptide would provide the best anchor. Thus, the Y84C, L2C construct was chosen for further study and designated dtSCT.

T cell recognition of the dtSCT

Given that T cells are highly sensitive to perturbations in pMHC structure and/or conformation, it was imperative to determine whether the mutations in the dtSCT constructs affected T cell recognition. The dtSCT and each of the previous versions of the OVA\(^{\beta, m, K^\alpha}\) SCT were transduced into LM1.8 cells (H-2\(^k\) fibroblast expressing ICAM) and tested in chromium release assays as targets for K\(^\alpha\)/OVA-reactive OT-1 T cells (Fig. 3A). Cells expressing dtSCT and SCT constructs were strongly recognized by OT-1 in cytolytic assays while LM1.8-K\(^k\) cells were not. Additionally, the B3Z T cell hybridoma (49), which is also specific for the K\(^\alpha\)
OVA complex, responded robustly to 3KO fibroblasts expressing the dtSCT construct (Y84C, L2C) or any of the other cysteine variants (data not shown). Thus, disulfide bond engineering of the single-chain class I molecule preserved the ability to present the OVA epitope to two of two distinct T cell clones. As further evidence of the integrity of the interaction between TCR and SCT molecules, we have found that SCT tetramers and conventional tetramers detect the same polyclonal Ag-specific T cells elicited by pathogen infection; moreover, the same holds true for tetramers built with the engineered disulfide trap—they retain the ability to bind the same T cells as conventional tetramers (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication) (4).

dtSCT molecules exclude high-affinity competitor peptides

A crucial test of the use of the dtSCT was to measure its capacity to exclude competing peptides. We had previously found that the SCT format without the disulfide trap excluded high-affinity competitor peptides to a great extent (10), ~1000 times more effectively than native Kb loaded with endogenous peptides. This exclusion of competitor peptides was enhanced by the Y84A mutation in the SCT that opens the groove and allows for better linker accommodation (11) (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication) (4).

FIGURE 4. The disulfide trap effectively prevents binding of exogenous competitor peptides. A. Loss of mAb 25-D1.16 reactivity to monitor displacement of OVA or OVAp5Y from K\(^\beta\) SCT constructs. 3KO cells expressing the indicated constructs were incubated with increasing concentrations of exogenous SIYR peptide and analyzed by flow cytometry with Abs specific for K\(^\beta\) (mAb B8-24-3, gray) or K\(^\beta\)/OVA complex (mAb 25-D1.16, black). Mean fluorescence intensity (MFI) at each dose of competitor is plotted as a percentage of the starting signal (no competitor). B, N15 hybridoma activation to detect VSV8 competitor peptide binding to SCT constructs. 3KO cells expressing the indicated constructs, including OVA\(\beta_2m\)K\(^\beta\) SCTs or OVAp5Y\(\beta_2m\)SCTs, were incubated with increasing concentrations of exogenous VSV8 peptide, washed, and cultured with the N15 hybridoma. IL-2 produced upon hybridoma activation was detected measuring CTLL-2 proliferation. N15 activation as a result of VSV8 binding to native K\(^\beta\) (loaded with endogenous peptides in B6/WT3 fibroblasts) is shown for comparison.

However, this assay will not detect very low levels of competitor binding, which could be sufficient for T cell recognition. Thus, as a complementary approach, we also monitored exogenous peptide binding using a gain of recognition T cell assay. For this assay, we used the highly sensitive T cell hybridoma N15 (39). The N15 hybridoma, derived from the TCR\(^-\) murine hybridoma 58\(\alpha^-\)B\(-\) (38), expresses CD8 \(\alpha\)- and \(\beta\)-chains, as well as the N15 TCR, which is specific for the K\(^\beta\)/VSV8 complex. As shown in Fig. 4B, the N15 hybridoma was activated of K\(^\beta\)/OVA complexes. In control cells expressing the Y84A SCT, half of the K\(^\beta\)/OVA complexes were displaced by the addition of 125 \(\mu\)M competitor (Fig. 4A, upper left). The cells expressing the OVA\(\beta_2m\)K\(^\beta\) dtSCT displayed no diminution of reactivity with the 25-D1.16 Ab at the highest concentration tested (500 \(\mu\)M) (Fig. 4A, upper right). Thus, the preponderance of dtSCT molecules retained the OVA peptide moiety in the face of high concentrations of competitor, which was clear evidence that the engineered disulfide bond was present and functioning as expected to lock in the SCT peptide.
readily when cells expressing K\(^b\) were fed 10\(^{-9}\) M exogenous VSV8 peptide. On the order of 10\(^{-6}\) M, VSV8 peptide was required to attain the same level of N15 activation in the context of the OVA.\(\beta\_m\).K\(^b\) SCT Y84A construct. Yet, most striking was the disulfide trap, which enabled the OVA.\(\beta\_m\).K\(^b\) dtSCT to effectively exclude even 10\(^{-4}\) M VSV8 competitor peptide. Importantly, the peptide competition experiments based on N15 recognition in this study used fixed cells. Thus, peptide competition presumably occurred only at the cell surface. However, we have also performed similar competition studies using non-fixed cells and attained very similar results, suggesting that dtSCT are refractory to exogenous peptide binding both at the cell surface and intracellularly (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication). These combined biochemical (Fig. 2B), serological (Fig. 4A), and T cell (Fig. 4B) assays provide compelling evidence that the engineered disulfide bond is in place and functions to exclude the binding of exogenous peptides. The disulfide trap extends the remarkable ability of SCT to prevent the binding of other peptides in comparison with K\(^b\) loaded with endogenous peptides. However, it should be noted that, while the original SCT design appears to prevent exogenous peptide binding by rapidly rebinding the SCT peptide (11), the dtSCT format clearly secures the SCT peptide permanently in the groove, such that no other peptide can gain access.

A poor binding OVA analog is tethered to the MHC groove using the dtSCT approach

The above results demonstrated that the dtSCT approach can result in stronger binding of the covalently attached peptide. Based on this finding, we hypothesized that a disulfide trap could potentially be exploited to enhance presentation of relatively poor binding peptides. As a first test of this hypothesis we investigated the previously described OVA analog SIINFEKLY, or OVAp5Y, which binds poorly to K\(^b\) (50–52) (boldface was used to emphasize that these experiments were conducted with the SIINFEKL and not the SIINFEKLY peptide). Howarth et al. (50) showed that the OVAp5Y analog was still recognized in the context of K\(^b\) by the 25-D1.16 mAb. Furthermore, they demonstrated that the surface half-life of K\(^b\)/OVAp5Y complexes was reduced 3-fold and efficiency of ex-pression in B6-derived fibroblasts. As a control, a QL9.\(\beta\_m\).L\(^d\) SCT with and without the disulfide trap were subcloned individually into a retroviral vector for expression in B6-derived fibroblasts. As a control, a QL9.\(\beta\_m\).L\(^d\) SCT with the Y84A mutation was also included. This mutation opens the groove and reduces binding of exogenous peptides (11). As shown in Fig. 5, L\(^d\) and the three different L\(^d\)/QL9 SCTs were detected at high levels on the cell surface by the mAb 30-5-7, which is specific for folded (peptide-associated) L\(^d\) molecules (55). This, combined with the staining pattern of mAb 64-3-7 on each of these transduced cell lines, was especially informative because it provided a relative measure of peptide-induced folding. Native L\(^d\) (Fig. 5B) had the highest ratio of open conformers to folded conformers detected by mAb 30-5-7 (open:folded = 1.01), which was an indication of its characteristically weak association with endogenous peptides. Expressing L\(^d\) in single-chain format with the QL9 peptide (Fig. 5C) resulted in a lower level of staining with mAb 64-3-7 (open:folded = 0.504) and introducing the Y84A mutation (Fig. 5D) lowered the ratio even further (open:folded = 0.250). By comparison, when the disulfide trap was present in the QL9.\(\beta\_m\).L\(^d\) SCT (Fig. 5E), peptide-empty (mAb 64-3-7) conformers were barely detectable (open:folded = 0.012). We concluded from this experiment that the disulfide trap was clearly functional and significantly increased peptide occupancy of this relatively unstable class I/peptide complex.

Introducing two additional cysteine residues, as we had done by engineering the disulfide trap, could theoretically decrease the efficiency of intracellular folding/assembly of these MHC molecules. To test this possibility, both open and folded L\(^d\) conformers were precipitated from cell lines expressing native L\(^d\) or L\(^d\) SCTs

A second, unstable H-2 complex is secured when expressed as a dtSCT

To test the applicability of the dtSCT technology on an additional class I/peptide complex and to further test its ability to enhance presentation of a complex that turns over rapidly at the cell surface, we used the L\(^d\)/QL9 complex. The QL9 peptide (QLSPFPFDL) is derived from an endogenous dehydrogenase and binds to H-2L\(^d\) (29–31), a class I allele that is unique due to its relatively weak association with peptide and \(\beta\_m\) (15, 53). Importantly for this study, the L\(^d\)/QL9 complex is relatively unstable based on its limited cell surface half-life (~0.5 h) and the difficulty of constructing L\(^d\)/QL9 tetramers (T. H. Hansen, unpublished observations). Thus, the L\(^d\)/QL9 complex was ideal for testing the stabilizing effects on class I molecules conferred by the single-chain format itself and by the introduction of a disulfide bond. A second advantage of studying the L\(^d\) molecule is the ability to directly measure relative amounts of peptide-occupied vs peptide-empty class I conformers at the cell surface with mAb 64-3-7, which specifically detects peptide-empty class I molecules (25, 54–56). A third reason to construct SCTs of the L\(^d\)/QL9 complex was its well-defined reactivity with the 2C TCR (31, 57, 58) and the availability of a soluble recombinant high-affinity 2C-derived mutant TCR designated m6\(\alpha\) (34–36), which is an excellent peptide-specific L\(^d\)/QL9 staining reagent for flow cytometry.

Native L\(^d\) and QL9.\(\beta\_m\).L\(^d\) SCTs with and without the disulfide trap were subcloned individually into a retroviral vector for expression in B6-derived fibroblasts. As a control, a QL9.\(\beta\_m\).L\(^d\) SCT with the Y84A mutation was also included. This mutation opens the groove and reduces binding of exogenous peptides (11). As shown in Fig. 5, L\(^d\) and the three different L\(^d\)/QL9 SCTs were detected at high levels on the cell surface by the mAb 30-5-7, which is specific for folded (peptide-associated) L\(^d\) molecules (55). This, combined with the staining pattern of mAb 64-3-7 on each of these transduced cell lines, was especially informative because it provided a relative measure of peptide-induced folding. Native L\(^d\) (Fig. 5B) had the highest ratio of open conformers to folded conformers detected by mAb 30-5-7 (open:folded = 1.01), which was an indication of its characteristically weak association with endogenous peptides. Expressing L\(^d\) in single-chain format with the QL9 peptide (Fig. 5C) resulted in a lower level of staining with mAb 64-3-7 (open:folded = 0.504) and introducing the Y84A mutation (Fig. 5D) lowered the ratio even further (open:folded = 0.250). By comparison, when the disulfide trap was present in the QL9.\(\beta\_m\).L\(^d\) SCT (Fig. 5E), peptide-empty (mAb 64-3-7) conformers were barely detectable (open:folded = 0.012). We concluded from this experiment that the disulfide trap was clearly functional and significantly increased peptide occupancy of this relatively unstable class I/peptide complex.
with or without the disulfide trap. To compare the relative efficiencies of folding/assembly, immunoprecipitates were treated with endoglycosidase H (EndoH) as shown in Fig. 5. Clearly, each stage of SCT engineering resulted in progressively fewer EndoH-sensitive (ER-resident) and more EndoH-resistant (post-ER) molecules, demonstrating more rapid assembly and egress from the ER. Furthermore, we quantified the /H9252 2m that coimmunoprecipitated with Ld or Ld SCTs and found that /H9252 2m exchange was eliminated effectively in the dtSCT (Fig. 5G). Thus, introducing the disulfide trap resulted in a dramatic improvement in the efficiency and quality of folding and assembly of class I.

Peptide occupancy determines the half-life of class I molecules at the cell surface (59). Ld, being a relatively poor peptide binder, is thus highly inducible at the cell surface by incubation with exogenous ligands (15). Given this, we reasoned that susceptibility to peptide induction would be informative for comparing the relative peptide occupancy of the Ld/QL9 SCT complexes. As shown in Fig. 6A, culture of cells with exogenous Ld-binding peptide from MCMV (32) increased surface expression of native Ld//H11022 3-fold. Furthermore, exogenous MCMV peptide increased the expression of the QL9.//H9252 2m.Ld SCT (/H11011 3-fold) and, to a lesser extent, the QL9.//H9252 2m.Ld Y84A SCT (/H11011 1.5-fold). The improved refractoriness to exogenous peptide of the SCT with the Y84A mutation was expected based on our previous findings (11). However, the most striking result of this experiment was the constant expression level of the dtSCT at all exogenous peptide concentrations tested. Thus, for relatively unstable pMHC complexes such as Ld/QL9, the disulfide trap clearly renders the SCT more refractory to exogenous peptide binding as monitored by surface stabilization.

We next wanted to extend these findings with Ld/QL9 SCTs with more physiologically relevant TCR-based assays. First, we found that 2C T cells detected target cells expressing QL9.//H9252 2m.Ld dtSCT or QL9 peptide-fed targets comparably (Fig. 3B). Then, to detect Ld/QL9 complexes on the cell surface by flow cytometry,
we stained these cells with the rTCR m6α (Fig. 5). This high-affinity TCR variant was selected in vitro by yeast display of a library of 2C TCR mutants; its affinity for L2/QL9 (Kd ~ 9 nM) (35) is >100-fold greater than that of wild-type 2C (Kd ~ 1.5 μM) and thus approaches the affinity of Ab/Ag complexes. Interestingly, m6α bound relatively poorly to the QL9.β2m.L4 SCT (Fig. 5), likely reflecting the fact that residue Y84 in this construct forces the linker extending from the C terminus of the peptide to bulge, hindering m6α binding. Consistent with this conclusion, the SCTs with the Y84A mutation or the disulfide trap have a more relaxed linker (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication) and better detection by rTCR m6α (Fig. 5). In any case, the disulfide-trap form of the QL9.β2m.L4 SCT displayed strong engagement with both the 2C TCR (Fig. 3B) and its derivative, high-affinity rTCR m6α (Fig. 5).

The strong reactivity of m6α TCR for the dtSCT allowed us to determine the extent to which disulfide trapping of the QL9 peptide to L4 excluded competitor peptides relative to native L4/QL9 complexes. Cells expressing native L4 were fed exogenous QL9 peptide overnight. The next morning, these cells, along with cells expressing each generation of QL9.β2m.L4 SCT, were incubated with increasing concentrations of competitor peptide YPHF. MPTNL derived from MCMV pp89 (32), which binds to Ld with increased affinity rTCR m6α (Fig. 5).

In this study, we demonstrate the engineering of a disulfide bond to lock peptide into the MHC class I binding groove. This disulfide trap was introduced into class I molecules expressed as single-chain trimers or SCTs, which have now undergone three distinct generations of molecular engineering. Each of these generations of SCT was recently crystallized and high resolution structures analyzed in collaboration with Dr. Daved Fremont’s lab (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication). These structures verify the native class I conformation of the SCTs, as well as provide an anatomical basis for our biochemical and functional characterizations of each of the three generations of SCTs.

First-generation SCTs are class I MHC molecules expressed in the format: peptide–[G4S]3–mature β2m–[G4S]3–mature class I H chain (Fig. 1A). These SCTs exhibited extended cell surface half-life and resistance to exogenous peptide binding, compared with Kb loaded with endogenous peptides (10). However, they were found to be more susceptible to peptide displacement when compared with K4 loaded with OVA peptide (11). These combined observations strongly support the model that the cell surface stability of the SCT and its refractoriness to exogenous peptide binding both reflect the ability of the SCT to quickly rebind the covalently attached peptide. To improve spacer accommodation, the second-generation SCTs included the H chain substitution Y84A, which opened the peptide binding groove where the linker exits (11). As evidence for better linker accommodation, the SCT Y84A displayed improved detection by mAb 25-D1.16 (11) and by the TCR-staining reagent m6α (Fig. 5) compared with SCTs lacking the Y84A mutation.

Although functional SCTs have been constructed from a number of different mouse and human class I pMHC complexes, SCTs constructed with high-affinity peptides are clearly superior. The reason for this is now clear. As noted above, SCTs are dependent upon rebinding of the linker-attached peptide. Consequently, SCTs made with lower affinity peptides have higher steady-state levels of peptide-empty conformers at the cell surface (Fig. 5 and unpublished observations). Thus, further engineering of the SCT was required so that the SCT format would be more applicable to pMHC complexes with lower affinity peptides. Therefore, we began a third round of engineering in the SCT to strategically position a disulfide bond.

The data presented in this article, along with the crystal structure of the dtSCT (V. E. Mitaksov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication), confirm that the engineering of the disulfide bond was achieved at H chain position 84 and the second position on the linker extending from the C terminus of the peptide. Migration of the dtSCT in nonreducing SDS-PAGE, as well as the ability of dtSCTs to exclude formidable concentrations of high-affinity competitor peptide, clearly argues in favor of formation of the disulfide bond in cells. Furthermore, the presence of the disulfide bond in the electron density data from dtSCT crystals was unquestionable. Corroborating the native structure revealed in the dtSCT crystal analyses, we show here that dtSCTs of two distinct pMHC complexes are recognized by Abs, TCR reagents, and T cells specific for native pMHC complexes. Due to linker flexibility and use of a conserved position for the H chain cytostine, the disulfide-trap approach presented here will likely beft other H-2 and HLA complexes. Moreover, we have shown that dtSCT construction offers great usage for studying lower affinity pMHC complexes, as weak K4/peptide and L4/peptide complexes could both be expressed with a disulfide trap and, more importantly, excluded competitor peptides.

As revealed by its crystal structure, our engineering of cytostine residues in the dtSCT provided not only a disulfide bond but also better linker accommodation and fortuitous acquisition of hydrogen bonds for improved peptide anchoring in the F pocket of the dtSCT groove.

Recent applications of SCTs have begun to provide unique insights into our understanding of the complex role of MHC class I molecules in lymphocyte development and function (17–19). In a seminal study of the role of class I in NK cell development, Kim et al. (17) examined NK cells from mice that expressed the OVA.β2m.K4 SCT as a transgene, but not β2m or other class I genes. The experiments clearly established that expression of a single MHC class I molecule selectively licenses NK functional activity only in NK precursors with an inhibitory receptor specific for this MHC class I molecule. The SCT also promises to play a pivotal role in studies of CD8 T cell development. To study the development of CD8 T cell in the context of a single MHC class I/peptide complex, we have expressed the OVA.β2m.K4 SCT as a transgene and bred to a K4-, D4-, β2m-deficient background. Importantly, the covalently attached OVA excludes the binding of endogenous peptides, as demonstrated by the strong primary CTL.
response of T cells from these mice when cultured with cells expressing K\(^+\) loaded with endogenous peptides (J. M. Connolly, unpublished observation). However, previous studies of CD4 \(^+\) T cell development made it clear that it is imperative to absolutely ablate endogenous peptide binding to make definitive conclusions about the role of peptide in thymic selection (61–63). Thus, transgenic mice that express a disulfide-trap OVA,\(\beta\)-m.K\(^+\) SCT could be important tools for approaching a key outstanding question in \(\beta\) T cell development, i.e., the relationship between the MHC-bound selecting peptide in the thymus vs the activating peptide in periphery.

Beyond basic science, the engineering of the disulfide trap holds great promise for SCT clinical applications. There are already four reports demonstrating that vaccination with plasmid DNA encoding an SCT leads to generation of specific Abs and/or CTL. In the report by Yu et al. (10), DNA encoding the OVA,\(\beta\)-m.K\(^+\) SCT was used to vaccine BALB/c mice. BALB/c mice vaccinated with plasmid encoding SCT were found to generate anti-K\(^+\)/OVA Ab, clearly demonstrating that the SCTs are expressed as intact structures by DNA vaccination. Furthermore, in a recent report by Primeau et al. (13), syngeneic immunization of plasmid DNA encoding SCT was found to elicit Ag-specific CTLs. Extending these findings are two exciting recent studies showing that SCT DNA vaccination is highly effective for priming T cells in clinically relevant model systems. In one of these studies, mice doubly transgenic for HLA-A2 and human CD8 were vaccinated with DNA encoding an SCT that included HLA-A2 and a breast cancer-associated peptide derived from mammaglobin (16). This vaccination with SCT DNA was reported by Jaramillo et al. (16) to induce a significant expansion of CTLs capable of specific detection of A2-positive human breast cancer cells. Another recent study by Huang et al. (4) tested SCT DNA vaccination in a mouse model of human papillomavirus (HPV)-induced tumors such as cervical cancer. The HPV oncoprotein E6 is responsible for malignant transformation and is consistently expressed in HPV-associated tumors. Peng et al. (64) identified an immunodominant CTL epitope of the E6 protein that binds to the mouse class I molecule K\(^+\), and this E6 epitope was then incorporated into an SCT (E6p,\(\beta\)-m.K\(^+\)). B6 mice were vaccinated with plasmid DNA encoding either the SCT or the intact E6 protein alone. Importantly, DNA vaccination of B6 mice with the SCT elicited high levels of CTL, whereas DNA vaccination with only full-length E6 displayed low response over background. Furthermore, DNA vaccination with SCT protected mice against a lethal tumor challenge whereas vaccination with DNA-encoding E6 or OVA,\(\beta\)-m.K\(^+\) SCT did not. These experimental findings represent the first evidence that SCTs can have a significant advantage over protein-based vaccine approaches in a clinically relevant model system. The success of SCTs was credited to the fact they are Ag processing independent, do not have to compete with endogenous peptide, and are more stable at the cell surface due to their covalent nature (4). Most recently, it was shown that SCT-based DNA vaccination generated protection in a model of human ovarian cancer in HLA-A2 transgenic mice (65). Each of these DNA vaccination approaches used first generation SCTs. Although SCT-based DNA vaccines show great promise, they may be improved even further by incorporation of the disulfide trap. Certain vaccines may elicit T cell responses to peptide determinants that bind relatively poorly to the MHC, in which case the use of disSCTs may have a significant impact on the stability of antigenic determinants, and thus the level of vaccine protection.

We are exploring yet another potential application of our disulfide trap technology in the context of rMHC class I molecules. Rapid dissociation of a peptide from the MHC is a critical limitation in the construction of MHC multimers as FACS staining reagents. Thus, tetramers for several defined CD8 \(^+\) T cell epitopes have been difficult to construct or are very unstable once constructed (66). Furthermore, in vivo applications of rMHC molecules to modulate CD8 responses may require reagents with longer in vivo half-lives and greater thermal stability (67–69). It was clear from the crystal structure of the diSCT that an analogous disulfide trap might also be incorporated into soluble, recombinant class I molecules without the SCT linkers. This was done by refolding the class I H chain harboring a Y84C mutation with \(\beta\)-m and a synthetic peptide having a GC extension. Full characterization of these disulfide-trap pMHC molecules is in progress; however, the approach clearly works for K\(^+\)/OVA complexes. The disulfide trap forms properly upon in vitro refolding, and disulfide-trap pMHC tetramers bind T cells specific for the native complex (V. E. Mitakov, S. M. Truscott, L. Lybarger, J. M. Connolly, T. H. Hansen, and D. H. Fremont, submitted for publication). Using disulfide-trap approaches should enable the construction of stable multimers of lower affinity pMHC complexes and thus make possible more comprehensive analyses of T cell responses to pathogens and tumors.

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Disclosures


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