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Engineering superior DNA vaccines: MHC class I single chain trimers bypass antigen processing and enhance the immune response to low affinity antigens

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Abstract

It is commonly believed that delivery of antigen into the class I antigen presentation pathway is a limiting factor in the clinical translation of DNA vaccines. This is of particular concern in the context of cancer vaccine development as many immunodominant peptides derived from self tumor antigens are not processed and presented efficiently. To address this limitation, we have engineered completely assembled peptide/MHC class I complexes whereby all three components (class I heavy chain, β_2m , and peptide) are attached by flexible linkers and expressed as a single polypeptide (single chain trimers or SCT). In this study, we tested the efficacy of progressive generations of SCT DNA vaccines engineered to (1) enhance peptide binding, (2) enhance interaction with the CD8 coreceptor, and/or (3) activate CD4⁺ helper T cells. Disulfide trap SCT (dtSCT) have been engineered to improve peptide binding, with mutations designed to create a disulfide bond between the class I heavy chain and the peptide linker. dtSCT DNA vaccines dramatically enhance the immune response to model low affinity antigens as measured by ELISPOT analysis and tumor challenge. SCT engineered to enhance interaction with the CD8 coreceptor have a higher affinity for the TCR/CD8 complex, and are associated with more robust CD8⁺ T cell responses following vaccination. Finally, SCT constructs that coexpress a universal helper epitope PADRE, dramatically enhance CD8⁺ T cell responses. Taken together, our data demonstrate that dtSCT DNA vaccines coexpressing a universal CD4 epitope are highly effective in generating immune responses to poorly processed and presented cancer antigens.

Introduction

The observation that direct administration of recombinant DNA can generate potent immune responses in rodents established the field of DNA vaccines in the early 1990s (Tang *et al.*, 1992; Cox *et al.*, 1993; Davis *et al.*, 1993; Fynan *et al.*, 1993; Ulmer *et al.*, 1993; Wang *et*

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Author Disclosure Statement

S.M. Truscott and T.H. Hansen are listed on a pending patent based on single chain format and disulfide traps.

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et al., 1993). Since then, DNA vaccines have remained an area of intense research interest, and vaccines targeting infectious disease and cancer have progressed into clinical trials. Advantages of the DNA vaccine platform include the remarkable safety profile of DNA vaccines, and ease of manufacture relative to proteins and other biologics (Donnelly *et al.*, 1997; Gurunathan *et al.*, 2000). Perhaps most important, however, is the flexibility and molecular precision of the platform, with the ability to genetically manipulate the encoded antigens, and/or incorporate other genes to amplify the immune response (Kutzler and Weiner, 2008). For instance DNA vaccines have been engineered to improve antigen expression (Lee *et al.*, 1997; Andre *et al.*, 1998), target dendritic cells (Trumpfheller *et al.*, 2006; Nchinda *et al.*, 2008), and/or coexpress molecular adjuvants capable of enhancing immune responses such as costimulatory molecules (Chan *et al.*, 2001), cytokines (Boyer *et al.*, 2005; Schadeck *et al.*, 2006; Chong *et al.*, 2007; Hirao *et al.*, 2008), or chemokines (Sumida *et al.*, 2004). Unfortunately, despite the dramatic preclinical success of DNA vaccines, and greater than 200 ongoing or completed human clinical trials, immune responses in non-human primates and humans have been disappointing (Calarota *et al.*, 1998; MacGregor *et al.*, 1998; Wang *et al.*, 1998), and no DNA vaccines have been approved for human use. One of the limitations of DNA vaccination is the requirement for intracellular processing and presentation of encoded antigens by MHC class I molecules through cross-presentation, a very inefficient process (Yewdell and Del Val, 2004). This is of particular concern in the context of cancer vaccine development as many immunodominant peptides derived from (self) tumor antigens are not presented efficiently at the plasma membrane (Chapatte *et al.*, 2006). This inefficiency results from the fact that surface expression of peptide-MHC complexes is influenced by a variety of factors including efficiency of antigen processing (Del Val *et al.*, 1991; Eisenlohr *et al.*, 1992), specificity of peptide translocation into the ER (Heemels and Ploegh, 1995), and the biogenesis and kinetic stability of the peptide-MHC complex itself (Deng *et al.*, 1997).

To address these limitations, we have engineered completely assembled peptide/MHC class I complexes, termed single chain trimers (SCT), whereby all three components of the complex (class I heavy chain, β_2m , and peptide) are attached by flexible linkers and expressed as a single polypeptide (Yu *et al.*, 2002). To date, over 30 different peptide-MHC allele combinations have been successfully constructed and validated, confirming the general applicability of this approach (Jaramillo *et al.*, 2004; Crew *et al.*, 2005; Huang *et al.*, 2005; Primeau *et al.*, 2005; Hung *et al.*, 2007a; Lilienfeld *et al.*, 2007; Zhang *et al.*, 2007). Of particular note, we and others have demonstrated that first generation SCT DNA vaccines are capable of generating antigen-specific T cell responses in a number of model systems (Yu *et al.*, 2002; Jaramillo *et al.*, 2004; Huang *et al.*, 2005; Hung *et al.*, 2007a). In addition to the obvious clinical potential of SCT as DNA vaccines, these reagents have begun to provide unique and significant insights into our understanding of the complex role of MHC class I molecules in lymphocyte development and activation (Choudhuri *et al.*, 2005; Hudrisier *et al.*, 2005; Kim *et al.*, 2005).

In this study, we tested the efficacy of various generations of SCT engineered to (1) enhance peptide binding, (2) enhance interaction with CD8 coreceptor, and (3) activate CD4⁺ helper T cells. Initial proof-of-principle experiments were performed using the ovalbumin antigen SIINFEKL (OVA_p), and the known altered peptide ligand, SIINYEKL (p5Y), which binds poorly to H-2K^b (Howarth *et al.*, 2004). Additional experiments were performed using SCT encoding a peptide from the human breast cancer antigen, mammaglobin-A (Watson and Fleming, 1996). Our data suggest that disulfide trap SCT DNA vaccines coexpressing a universal CD4 epitope are highly effective in generating immune responses to poorly processed cancer antigens.

Materials and Methods

DNA Constructs

SCT DNA constructs were generated using standard techniques as previously described (Yu *et al.*, 2002; Lybarger *et al.*, 2003; Mitaksov *et al.*, 2007; Truscott *et al.*, 2007) and were confirmed by DNA sequencing analysis. Mutations were introduced using the QuikChange II Site-Directed Mutagenesis System (Stratagene) following manufacturer's instructions. Table 1 summarizes the progressive engineering of SCT DNA constructs used in this study. Plasmid DNA encoding SIINFEKL (mini-OVA), SIINYEKL (mini-p5Y), and AKFVAAWTLKAAA (mini-PADRE) peptides, as well as full-length chicken ovalbumin cDNA (OVA cDNA and mutated OVAp5Y cDNA) were also used as DNA vaccines.

Animals

All animals were used between the age of eight and twelve weeks. C57BL/6 (H-2^b) mice were purchased from NCI-Frederick. OT-1 mice (CD45.1 congenic, JAX) were routinely screened for TCR V α 2/V β 5 expression. hCD8⁺/HLA-A2⁺ mice were generated by crossing human CD8 transgenic mice to HLA-A*0201 transgenic mice (both from JAX) and used as F1 generation. All protocols followed the guidelines approved by the Animal Studies Committee at Washington University School of Medicine.

Cell Lines and Antibodies

The E.G7 cell line, which expresses full-length OVA cDNA, and its parental thymoma cell line EL4 were provided by Dr. M Salem (Medical University of South Carolina). The CHO cell line was provided by Dr. T Murphy (Washington University). Cells were cultured in RPMI1640 or DMEM complete media (Invitrogen) supplemented with L-glutamine, 10% fetal bovine serum (AtlantaBiologicals), sodium pyruvate, non-essential amino acids, and penicillin-streptomycin. CHO cells were transfected with SCT using jetPEI transfection reagent (PolyPlus Transfection) according to the manufacturer's recommendations. Transfectants were selected by the addition of 0.6 mg/mL of Zeocin (InvivoGen). B8-24-3 is a monoclonal antibody that recognizes folded H-2K^b molecules. Monoclonal antibody 25-D1.16 (a gift from Dr. J. Yewdell, NIH, Bethesda, MD) recognizes K^b plus SIINFEKL peptide (Porgador *et al.*, 1997).

Flow Cytometry

Data from viable cells, gated by forward and side scatter, were acquired on a FACSCalibur (BD Biosciences) and analyzed using Flowjo software (TreeStar). Staining with 25-D1.16 culture supernatant was visualized using FITC-conjugated goat anti-mouse IgG (BD). The binding affinity of SCT to OT-1 transgenic T cells was determined by tetramer staining. SCT tetramers were prepared as described previously (Lybarger *et al.*, 2003). After the staining procedure, aliquots of the stained OT-1 cells were allowed to sit on ice and analyzed at different time points. The mean fluorescence was compared to that of $t = 0$ min and the half-life of the tetramer binding was determined using a nonlinear regression model.

CTL Assay

CHO cells transfected with the indicated class I constructs were labeled with Na⁵¹CrO₄ for 1 h. Activated OT-1 T cells were plated at various concentrations into 96-well microtiter plates and incubated with target cells for 4 h at 37°C in 5% CO₂. Radioactivity in supernatants was measured in an Isomedic gamma counter (ICN Biomedicals). The mean of triplicate samples was calculated, and specific lysis was determined as follows: specific lysis = $100 \times ((\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}))$, where experimental ⁵¹Cr release represents counts from target cells mixed with

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

DNA Vaccination and Tumor Challenge

DNA plasmids were amplified in *E. coli* DH5 α (Invitrogen) and purified using NucleoBond Maxi Plasmid DNA Purification kits (Macherey-Nagel). DNA concentration was determined by the optical density measured at 260nm. DNA vaccination was performed using a Helios gene gun (Bio-Rad) according to the protocol provided by the manufacturer. Each cartridge carries 0.5 mg of gold (1 μm diameter) coated with 1 μg of plasmid DNA. A total of 4 μg of DNA was delivered to non-overlapping shaved and depilated mice abdominal areas. The discharge helium pressure was set to 400 p.s.i. Vaccination was administered at 3-day intervals for a total of three doses (Bins *et al.*, 2005).

The tumor protection experiment was carried out as following. E.G7 cells were transplanted (*s.c.*) in the right flank of C57BL/6 mice four days before adoptive transfer of 2.5×10^6 splenocytes from OT-1 mice. Mice were vaccinated twice by a gene gun with 4 μg DNA at one day and one week after the OT-1 transfer. Tumors were measured using a caliper every other day after tumors became palpable. Tumor volumes were calculated by multiplying the two perpendicular diameters. 1×10^6 EL-4 cells were injected in the left flank as control.

ELISPOT

To measure primary CTL immune responses, IFN- γ ELISPOT assays were performed at five days after the final DNA vaccination. Erythrocyte-free single cell suspensions were prepared from spleens, inguinal lymph nodes, or peripheral blood. A MultiScreen 96-well PVDF filtration plate (Millipore) was coated overnight with 15 $\mu\text{g}/\text{mL}$ of capture antibody (clone AN18, Mabtech). After washing, the membrane was blocked with complete RPMI supplemented with 10% FBS for 1 hour at room temperature. Appropriate amounts of responder cells ($2 - 4 \times 10^5$) were added into wells in triplicate. Cells were incubated for 20 hr with or without the presence of 1 μM of OVAp or 40 $\mu\text{g}/\text{mL}$ of the mammaglobin-A-derived peptide, MamA2.1 (LIYDSSLCDL) (Jaramillo *et al.*, 2004) (both synthesized by AnaSpec). After extensive washes, 1 $\mu\text{g}/\text{mL}$ of biotinylated detection antibody (clone R4-6A2, Mabtech) was added and incubated at room temperature for 2 hr. Streptavidin-ALP (Mabtech) and BCIP/NBT (Moss Substrates) were then used for color development. The reaction was stopped by rinsing the plate extensively under tap water. The plates were left to dry before scanning and analysis on an ImmunoSpot ELISPOT Reader (C.T.L.).

Statistics

Data were analyzed using GraphPad Prism 4 software (GraphPad). The Mann-Whitney test was used to compare between vaccination groups. A *P* value equal or less than 0.05 is considered statistically significant.

Results

Disulfide trap SCT DNA vaccines enhance the immune response to low affinity peptides

In an effort to enhance the immunogenicity of first generation SCT, we have engineered SCT constructs to improve peptide binding (Figure 1A). The mutation (Y84C) creating a disulfide bond between K^b and an appropriately spaced cysteine residue in the peptide linker (disulfide trap SCT or dtSCT) was reported previously and showed enhanced peptide binding *in vitro* (Truscott *et al.*, 2007; Truscott *et al.*, 2008). It has been reported that improvements in the affinity of peptide/MHC interaction can trigger activation of naïve T cells against self antigens

(Dyall *et al.*, 1998; Gold *et al.*, 2003; Yu *et al.*, 2004). We hypothesized that the disulfide trap could increase the immunogenicity of SCT incorporating tumor antigen-derived peptides, most of which have low binding affinity to MHC class I molecules (Houghton and Guevara-Patino, 2004). We tested this hypothesis using the well-characterized OVA/K^b model system, in which the binding affinities of the immunodominant epitope SIINFEKL and its altered peptide ligands have been studied extensively (Howarth *et al.*, 2004). *In vitro* validation studies demonstrated that the disulfide trap does not affect the expression or assembly of the MHC complex, even when the low affinity peptide p5Y is incorporated, as determined by flow cytometry and ⁵¹Cr-release assays (Figure 1, B and C).

To test the ability of dtSCT to enhance the CD8 T cell response to low affinity peptide antigens *in vivo*, we vaccinated C57BL/6 mice with full-length cDNA or SCT constructs following adoptive transfer of OT-1 T cells. T cell responses were measured using IFN- γ ELISPOT assays. Conventional DNA vaccines (OVA cDNA) and disulfide-trap SCT DNA vaccines (OVAp/K^b dtSCT) generated comparable antigen-specific T cell responses to K^b/SIINFEKL, a high affinity peptide that is processed efficiently. However, for the low affinity peptide p5Y, the p5Y/K^b dtSCT vaccine was clearly superior to the p5Y cDNA vaccine (Figure 2A). The immune response generated through dtSCT DNA vaccination was able to suppress E.G7 tumor growth *in vivo* (Figure 2B). This antitumor response was antigen-specific as growth of the parental EL4 tumor was comparable among vaccination groups. The data strongly suggest that dtSCT are capable of inducing CD8⁺ T cell responses against weak binding epitopes and therefore are valuable for the development of tumor-specific DNA vaccines.

Q115E mutation enhances SCT interaction with CD8 co-receptor

SCT are recognized as intact molecules by the immune system, providing the ability to further engineer these constructs to enhance T cell activation. A critical component of the T cell receptor complex is the CD8 coreceptor. We have engineered an SCT to enhance interaction with the CD8 coreceptor by mutating a single amino acid residue (Q115E) in the $\alpha 2$ domain of the MHC class I heavy chain. This mutation has been shown to functionally enhance the interaction between the TCR and the pMHC complex (Wooldridge *et al.*, 2005; Wooldridge *et al.*, 2007). To test the affinity of the engineered SCT constructs, we created tetramers derived from wildtype and Q115E K^b molecules. The dissociation rates of the tetramers from OT-1 T cells were measured in a “tetramer decay” experiment (Holmberg *et al.*, 2003). The half-life of the wildtype and Q115E K^b SCT tetramers were 6.6 and 19.7 minutes, respectively (Figure 3A). This indicates that the Q115E mutation results in a higher affinity interaction between the SCT and TCR/CD8 complex. We then vaccinated C57BL/6 mice with wildtype and Q115E K^b SCT DNA vaccines. The data in Figure 3B demonstrate that the Q115E SCT DNA vaccine is associated with an enhanced CD8 T cell response compared to its wildtype counterpart.

Coexpression of the PADRE helper epitope increases immunogenicity of SCT

Concurrent activation of CD4⁺ T cells is critical for the induction and maintenance of cellular anti-tumor immune responses (Shedlock and Shen, 2003; Sun and Bevan, 2003). SCT vaccines have been optimized to enhance CD8 T cell responses, and were not initially engineered to elicit a CD4 T cell response. To address this potential deficiency, we have engineered a construct that drives simultaneous expression of the OVAp/K^b dtSCT and the universal pan DR epitope peptide (PADRE) (Figure 1A). PADRE is an artificial peptide sequence (AKFVAAWTLKAAA) engineered to achieve high binding affinity to most of all HLA-DR alleles and certain murine class II molecules (Alexander *et al.*, 1994). Previous studies have shown that PADRE is immunogenic *in vivo* and is able to elicit robust helper effect when delivered as either peptide (Alexander *et al.*, 1994) or DNA construct (Hung *et al.*, 2007b).

In vitro expression studies demonstrated that the expression levels of SCT with or without PADRE coexpression were comparable (data not shown). We tested the construct *in vivo* by gene gun immunization of mice followed by *in vitro* analysis of splenocytes. The frequency of IFN- γ producing cells in mice vaccinated with OVAp/K^b dtSCT PADRE were three- to five-fold higher than OVA cDNA or OVAp/K^b dtSCT vaccination, suggesting that PADRE coexpression dramatically increases the efficacy of the SCT DNA vaccine. The ELISPOT results from a representative experiment are shown in Figure 4.

SCT constructs targeting mammaglobin-A

Mammaglobin-A is a human breast cancer-specific antigen (Watson and Fleming, 1996; Watson *et al.*, 1999; Fleming and Watson, 2000) whose immunodominant epitopes have been identified (Jaramillo *et al.*, 2002; Manna *et al.*, 2003; Jaramillo *et al.*, 2004; Narayanan *et al.*, 2004). Generation of CD8 T cell responses to mammaglobin-A appears to be associated with anti-tumor activity, and there is evidence that mamA2.1 epitope is immunodominant in this response (Bharat *et al.*, 2008). In preclinical studies, we assessed the efficacy of an SCT construct in mice expressing both HLA-A2 and human CD8 transgenes (Figure 5A). This construct is identical to what would be used in human breast cancer patients. The SCT encode human β 2-microglobulin, HLA-A2 heavy chain, and the MamA2.1 peptide, and coexpress PADRE (MamA2.1 dtSCT PADRE). As shown in Figure 5B, vaccination with the MamA2.1 dtSCT PADRE induced stronger mammaglobin-A specific T cell responses than vaccination with MamA2.1 peptide or mammaglobin-A cDNA.

Discussion

Delivery of sufficient antigen into the class I antigen presentation pathway to elicit T cell activation is a major limitation in the clinical translation of DNA vaccines. To address this limitation, we have developed innovative SCT constructs for use as DNA vaccines. SCT encode completely assembled peptide/MHC class I complexes as single polypeptide chain, therefore bypassing the requirement for antigen presentation and/or cross-presentation. First generation SCT have proven to be highly effective DNA vaccines in selected preclinical models (Jaramillo *et al.*, 2004; Huang *et al.*, 2005; Hung *et al.*, 2007a; Zhang *et al.*, 2007). Unfortunately, immunodominant tumor-derived peptides usually have low binding affinity to MHC class I molecules, contributing to the difficulty in trying to induce immune responses against cancer cells (Houghton and Guevara-Patino, 2004; Chapatte *et al.*, 2006). We report in this study that engineering a disulfide trap into SCT constructs has a significant impact on the level of vaccine efficacy, particularly when a low affinity peptide is encoded. For proof-of-concept studies we chose the OVA/K^b model system because there are a number of reagents available that facilitate mechanistic studies in this system. These include the OT-1 TCR transgenic mouse, the K^b/OVA-specific mAb 25D1.16, and detailed experimental information available on altered peptide ligands. Our results provide critical insights into the development of SCT vaccines against tumor-associated antigens, which are commonly “self antigens” with relatively low binding affinity for MHC molecules.

The specificity of T cell activation depends upon TCR interaction with the peptide/MHC class I complex. However, the CD8 coreceptor is considered to be a key component of this interaction, by stabilizing the complex at the cell surface and facilitating TCR-mediated antigen recognition (Luescher *et al.*, 1995; Gao *et al.*, 1997; Arcaro *et al.*, 2001; Wooldridge *et al.*, 2005). We have previously reported that this interaction is particularly important for the generation of primary CD8 T cell responses to low affinity or poorly processed peptides, or by low avidity TCR (Alexander *et al.*, 1991). To directly enhance this interaction we engineered SCT to incorporate the Q115E mutation, previously shown to enhance CD8⁺ T cell responses *in vitro* (Wooldridge *et al.*, 2007). Our data demonstrate that this mutation leads to increased

half-life of the TCR-pMHC complex, which is associated with enhanced SCT vaccine efficacy *in vivo*.

CD4⁺ T cells play a critical role in the generation and maintenance of antigen-specific CD8⁺ T cell immune responses. It has been well documented that CD4⁺ T cells are required for the establishment of CD8⁺ T cell effector and memory responses (Shedlock and Shen, 2003; Sun and Bevan, 2003). Several models have been proposed to illustrate how CD4⁺ and CD8⁺ T cell are collaborating, although the exact mechanism is still unclear. In one model, antigen presenting cells (APC) deliver co-stimulatory signals to the CD4⁺ T cells. CD4⁺ T cells produce IL-2, which in turn activate CD8⁺ T cells (Mitchison and O'Malley, 1987; Bennett *et al.*, 1997; Xiang *et al.*, 2005). In another model, the engagement of CD4⁺ T cells with APC leads to APC maturation, which subsequently activate CD8⁺ T cells (Bevan, 2004; Xiang *et al.*, 2005). A recent article suggested an important role of IL-15 in promoting longevity of antigen-specific CD8⁺ T cells (Oh *et al.*, 2008). MHC class I SCT vaccines have been optimized to enhance CD8 T cell responses, and were not initially engineered to elicit a CD4 T cell response. One strategy explored by Dr T-C Wu's group involves the engineering of invariant chain (Ii), in which the CLIP region was replaced by antigen-specific or the universal helper epitope PADRE (Hung *et al.*, 2007b). Co-administration of the Ii-PADRE DNA and SCT vaccines encoding HPV E6 or E7 antigens resulted in more robust E6- or E7-specific CTL responses (Hung *et al.*, 2007b). The main drawback of this regimen is that the helper epitope and SCT are encoded in two separate plasmids. In the current study we employed a novel approach in which both SCT and the PADRE helper epitope are encoded in a single plasmid. Our data demonstrate that coexpression of PADRE in the same plasmid is able to enhance the efficacy of SCT DNA vaccines. This approach has the potential to facilitate the clinical translation of SCT DNA vaccines. With a single plasmid, vaccine manufacture, product release tests, and regulatory approval are streamlined.

We tested the engineered SCT in preclinical studies targeting the human breast cancer antigen mammaglobin-A. Mammaglobin-A is a novel breast cancer-associated antigen that is expressed exclusively in normal breast epithelium and breast cancer (Watson and Fleming, 1994). It is consistently overexpressed in breast cancer, and may ultimately prove to be a candidate for breast cancer prevention. Our data demonstrated that the MamA2.1 dtSCT PADRE DNA vaccine is superior to conventional cDNA and peptide vaccines. After thorough investigations of the basic biology/immunology of mammaglobin-A (Watson and Fleming, 1994; Watson and Fleming, 1996; Watson *et al.*, 1998; Watson *et al.*, 1999; Fleming and Watson, 2000; Manna *et al.*, 2003; Tanaka *et al.*, 2003; Goedegebuure *et al.*, 2004; Jaramillo *et al.*, 2004; Narayanan *et al.*, 2004; Viehl *et al.*, 2005; Viehl *et al.*, 2007; Bharat *et al.*, 2008) and the functional immunology of SCT DNA vaccines (Yu *et al.*, 2002; Lybarger *et al.*, 2003; Jaramillo *et al.*, 2004; Huang *et al.*, 2005; Mitaksov *et al.*, 2007; Truscott *et al.*, 2007), we are in a unique position to take the first step in the clinical translation of a rationally designed, innovative, and highly effective breast cancer DNA vaccine.

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Figure 1. dtSCT constructs encoding low affinity peptides generate functional MHC I complexes
 A. Schematic diagrams of progressive generations of SCT. Arrowheads indicate the translational start codon and asterisk, the stop codon. Also shown are positions of the disulfide bond formed between L1 and HC. LP, leader peptide (cleaved after ER membrane translocation); P, peptide; L1 and L2, flexible linkers (15 and 20-amino acids long) of G₄S repeat; β 2M, β -2 microglobulin; HC, MHC class I heavy chain. B. OVAp/K^b (solid line) and p5Y/K^b (dotted line) dtSCT expression on the surface of transfected CHO cells as measured by staining with monoclonal antibody 25-D1.16. Shaded histogram shows staining of parental CHO cells. C. CHO cells expressing the OVAp/K^b (circles) and p5Y/K^b (triangles) dtSCT constructs were tested as targets for activated OT-1 T cells in a 4-h ⁵¹Cr release assay. Untransfected CHO cells were used as controls (squares).

Figure 2. dtSCT DNA vaccines dramatically enhance the immune response to low affinity peptide antigens *in vivo*

A. 24 h after adoptive transfer of 2.5×10^6 OT-1 splenocytes, C57BL/6 mice were vaccinated by gene gun at day 0, 3 and 6 with the indicated DNA vaccines ($n = 5$ each vaccine group). At day 11, peripheral blood were collected and PBL were used to measure CD8⁺ T cell responses in an IFN- γ ELISPOT assay. Data were presented as numbers of spot forming units (SFU) per 10^5 PBL (mean $\pm$ SEM). Chicken ovalbumin (OVA) has an immunodominant peptide (SIINFEKL) with strong affinity to H-2K^b. p5Y (SIINYEKL) is an altered peptide ligand known to bind with low affinity to H-2K^b. ELISPOT analysis demonstrates that the OVA.p5Y dtSCT, but not the OVA.p5Y cDNA, is able to generate a robust antigen-specific T cell response. B. Tumor challenge with tumor cells expressing ovalbumin (EG.7) demonstrates that the OVA.p5Y dtSCT, but not p5Y DNA, is associated with a robust antitumor immune response. Tumor volumes (mean $\pm$ SEM) were measured every other day. There is no difference in the growth of the parental control cell line EL4 in mice vaccinated with these DNA constructs.

Figure 3. Q115E mutation increases SCT affinity for the TCR/CD8 complex

A. Wildtype and OVA/K^b Q115E SCT tetramers were created and used to stain OT-1 T cells in the presence of excess B8-24-3 antibody (specific for H-2K^b) to prevent tetramer rebinding. The mean fluorescence index (MFI) of FL2 channel was measured at different time points and presented as percentage of the initial MFI (t = 0 min). Dissociation rates of the tetramers were determined using a nonlinear regression curve fit model. The half-life of the wildtype (square) and OVA/K^b Q115E (triangle) SCT tetramers on OT-1 cells were determined to be 6.6 and 19.7 minutes, respectively. B. C57BL/6 mice (n = 5 each group) were vaccinated with the indicated SCT DNA constructs by gene gun after adoptive transfer of 2.0×10^6 OT-1 CD8⁺ T cells. Five days after final immunization, CD8⁺ T cells were isolated from draining lymph nodes. Vaccination with the Q115E SCT DNA vaccine was associated with a significant increase in the number of antigen-specific T cells by ELISPOT analysis (mean $\pm$ SEM). A SCT DNA vaccine encoding the irrelevant VSV8 peptide was used as control.

Figure 4. Coexpression of the universal CD4 helper epitope PADRE in the dtSCT construct dramatically enhances the efficacy of the SCT vaccine

C57BL/6 mice were immunized using gene gun as described in the text with the indicated DNA constructs. Five days after the final immunization, spleen cells from mice in each group ($n = 3$) were pooled and analyzed in a murine IFN- γ ELISPOT assay. Data from one representative experiment are shown (mean $\pm$ SEM). The frequency of antigen-specific IFN- γ secreting CD8⁺ T cells is 3- to 5-fold higher in the OVAp/K^b dtSCT PADRE vaccine group compared to OVA cDNA or OVA/K^b dtSCT. Representative ELISPOT images (2.0×10^5 cells/well) are also shown.

Figure 5. MamA2.1 dtSCT PADRE DNA vaccine is more robust than comparable peptide or conventional mammaglobin-A cDNA vaccines

A. Schematic design of the experimental time line. “Humanized” transgenic mice (n =3 each group) expressing human CD8 and HLA-A*0201 were used for this experiment. DNA vaccines were delivered by gene gun. For peptide vaccination, 45 μ g of MamA2.1 peptide (40 nmole) was mixed with 50 μ g of CpG oligo (synthesized by IDT) and an equal volume of Incomplete Freund’s Adjuvant (Sigma-Aldrich) before *s.c.* injection. Vaccinations were scheduled so that primary CD8⁺ T cell responses could be measure on the same day. B. ELISPOT assay was performed using splenocytes as described previously. The number of antigen-specific T cells (mean $\pm$ SEM) was higher in mice vaccinated with the optimized MamA2.1 dtSCT coexpressing PADRE compared to peptide vaccine and conventional cDNA vaccine.

Table 1

Progressive engineering of SCT constructs

Designation	Description
SCT	First generation SCT with varying linker lengths tested to maximize stable construction and T cell recognition. Linker length of 15/20 residues is optimal (Yu <i>et al.</i> , 2002).
Q115E SCT	The Q115E mutation in the $\alpha 2$ domain of the heavy chain enhances SCT interaction with the CD8 coreceptor (Wooldridge <i>et al.</i> , 2007). The CD8 coreceptor is a key component of the TCR-pMHC complex.
dtSCT	The Y84C mutation opens up the F pocket, and permits formation of a disulfide bond with a C residue engineered into the first flexible linker, "trapping" the antigenic peptide in the peptide binding groove of the class I molecule (disulfide trap) (Mitaksov <i>et al.</i> , 2007; Truscott <i>et al.</i> , 2007).
dtSCT PADRE	We have used positional cloning to coexpress the SCT and a helper epitope. Coexpression of a helper epitope effectively recruits CD4 help and dramatically enhances SCT DNA vaccine efficacy. CD4 help is considered essential for effector and memory CD8T cell responses (Bourgeois <i>et al.</i> , 2002; Janssen <i>et al.</i> , 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003).