**Functional and Structural Characteristics of NY-ESO-1-related HLA A2-restricted Epitopes and the Design of a Novel Immunogenic Analogue**

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NY-ESO-1, a commonly expressed tumor antigen of the cancer-testis family, is expressed by a wide range of tumors but not found in normal adult somatic tissue, making it an ideal cancer vaccine candidate. Peptides derived from NY-ESO-1 have shown preclinical and clinical trial promise; however, biochemical features of these peptides have complicated their formulation and led to heterogeneous immune responses. We have taken a rational approach to engineer an HLA A2-restricted NY-ESO-1-derived T cell epitope with improved formulation and immunogenicity to the wild type peptide. To accomplish this, we have solved the x-ray crystallographic structures of HLA A2 complexed to NY-ESO (157–165) and two analogues of this peptide in which the C-terminal cysteine residue has been substituted to alanine or serine. Substitution of cysteine by serine maintained peptide conformation yet reduced complex stability, resulting in poor cytotoxic T lymphocyte recognition. Conversely, substitution with alanine maintained complex stability and cytotoxic T lymphocyte recognition. Based on the structures of the three HLA A2 complexes, we incorporated 2-aminoisobutyric acid, an isostereomer of cysteine, into the epitope. This analogue is impervious to oxidative damage, cysteinylatation, and dimerization of the peptide epitope upon formulation that is characteristic of the wild type peptide. Therefore, this approach has yielded a potential therapeutic molecule that satiates the hydrophobic F pocket of HLA A2 and exhibited superior immunogenicity relative to the wild type peptide.

Class I major histocompatibility complex (MHC) molecules play a crucial role in immune surveillance by selectively binding to intracellular peptide antigens (Ag) and presenting them at the cell surface to CD8+ T lymphocytes (TCD8), including cytotoxic T lymphocytes (CTL). Eradication of tumors is associated with a robust cytotoxic T cell response to antigens expressed by the tumor (tumor-associated antigens (TAA)). Because many TAA are self-proteins or closely related to self-proteins, they tend to be poorly immunogenic (1–5). Moreover, many TAA-derived peptides are not strong binders to class I molecules, making strategies that revolve around tumor Ag delivery poor inducers of CD8 T cell immunity (6). Synthetic peptide-based vaccines offer a flexible, relatively simple and cost-effective way to treat a variety of human diseases, including the immunotherapy of cancer. Moreover, synthetic peptides are easily engineered to improve the efficacy of the immunogen. Such engineering may include optimizing target MHC class I binding by substituting key residues with more appropriate anchor residues. In addition, peptide-based therapeutics can be engineered to improve formulation and storage properties, and strategies exist to protect labile peptide bonds by incorporating nonpeptidic structures (7–11). Several studies have incorporated nonnatural amino acids in peptidic structures to improve compound stability and maintain T cell cross-reactivity. For example, some studies have used nonnatural amino acids with modified side chains that approximate the natural amino acid (10, 11) or by modifying peptide bonds by introducing β-amino acids (12, 13), reducing peptide bonds from the natural amine bonds to aminomethylene (14, 15) or generation of partially modified retro-inverso pseudopeptides (8, 16, 17).

The search for appropriate TAA for vaccination and immunotherapy has extended to several classes of tumor antigens. Ideally such candidates are expressed solely in cancerous tissue and are essential for the malignant phenotype; however, few examples of such antigens exist. More often, TAA are self-proteins overexpressed in tumors or self-proteins that contain mutations that may or may not be discernible by the immune system. The risk of potential autoimmune complications in eliciting anti-tumor immunity requires strategies to minimize autoimmunity. One such strategy is to limit the immune response toward tumor-specific epitopes (e.g. in mu-
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Boon and colleagues cloned the first human tumor antigen capable of eliciting spontaneous CTL responses in melanoma patients (1). This antigen, known as MAGE-A1, is expressed only in normal testis yet is frequently found in many different cancers. This expression pattern has led to MAGE and related antigens being termed cancer-testis antigens. Because normal testis germ cells do not express class I MHC molecules, this family of antigens has been extensively studied by the tumor immunotherapy community. NY-ESO-1 is another cancer testis Ag, expressed in many different types of tumors, including melanoma, breast, lung, and bladder cancers. In addition to its widespread expression by different cancers, it is also immunogenic in patients with late stage disease, with evidence of spontaneous humoral and cellular immune responses toward this antigen (18). Both Class I and Class II restricted T cell determinants have been identified, making NY-ESO-1 or peptides derived from it potentially useful vaccine components (19–27). Clinical evidence suggests that CTL specific for NY-ESO-1 determinants can stabilize malignant disease and eradicate metastases. Peptide vaccination with NY-ESO-1 determinants has been very promising, but along the way these studies have highlighted problems of stability and bioavailability associated with peptide immunization and the frequent failure to elicit robust CTL that kill tumors (21, 23, 28).

Three peptides from an overlapping region of the NY-ESO-1 protein (residues 155–163, QLSLLLWIT; residues 157–165, SLLMWITQT; residues 157–167, SLLMWITQCF) have previously been reported as HLA A*0201-restricted determinants recognized by tumor-reactive TCD8 clones derived from melanoma patients (18). Despite poor binding to HLA A2, tumor-reactive TCD8 clones mainly recognize the NY-ESO-(157–165) determinant (21). The immunogenicity of these peptides was first evaluated in a trial vaccination of cancer patients in which a mixture of the peptides was administered intradermally to patients bearing NY-ESO-1+ tumors (28). A vigorous TCD8 response to NY-ESO-(157–165) was observed, whereas reactivity against NY-ESO-(157–165) determinant appeared later and at a lower level. The TCD8 response to NY-ESO peptide vaccination has also been examined by HLA A2/peptide tetramer analysis and revealed a heterogeneous response directed against several distinct overlapping epitopes, including cryptic determinants generated by aminopeptidase activity (24). Thus, only CTL recognizing the precise NY-ESO-(157–165) determinant also recognize the endogenously processed determinant on NY-ESO1 tumor cells, probably because it is the only constitutively presented determinant on tumor cells (20).

Analogs of NY-ESO-(157–165) where the C-terminal Cys residue has been replaced with more conventional anchor residues, namely Leu and Val analogs, have been generated (25). Whereas these analogs bind more efficiently to HLA A2 and are recognized by CTL raised against the natural NY-ESO-(157–165) peptide, they do not induce effective anti-tumor CTL. Indeed, the presence of the Cys at the C terminus seems critical for generating CTL that recognize endogenously processed NY-ESO determinants on tumor cells. The presence of this amino acid causes problems with formulation due to oxidative damage and dimerization, both of which reduce the efficacy of the peptide Ag as an immunogen (25). In this study, we have investigated the structure of NY-ESO-(157–165) complexed to HLA A*0201 and compared it with the C9A and C9S structures, which are more easily formulated and are potential vaccine candidates (see Table I). We have also examined the functional recognition of these analogs using a C6+ T lymphocyte lines derived from melanoma patients immunized with overlapping peptides spanning NY-ESO 155–167 (24) that respond to NY-ESO-(157–165). In our studies, we have been careful to pretreat all of the peptides including the Cys-containing peptides with a reductant to prevent dimerization or cysteinylnation of the peptides, which could mask the recognition of the wild type peptide relative to the analogs. This allowed for the first time a systematic analysis of relative antigenicity of the wild type peptide and analogues. Finally, we used structure guided design to test an analog that would satisfy the Cys requirement of anti-tumor CTL by substituting the Cys for a nonnatural isosteric analog of this residue 2-aminoisobutyric acid (Abu).

EXPERIMENTAL PROCEDURES

Peptides—All peptides were synthesized using standard FMOC synthesis and synthesized by Auspep Pty. Ltd. (North Melbourne, Victoria, Australia). All peptides were purified to >85% purity by preparative reverse phase high pressure liquid chromatography, and purity was determined by liquid chromatography-mass spectrometry using an Agilent 1100 LC-MSD SL ion trap instrument and a Stable Bond RP C18 column (100 × 0.5-mm inner diameter column) (see Table I). Peptides were dissolved in MeSO to a final concentration of 10–100 mg/ml.

Expression, Purification, Crystallization, and Structure Determination—A truncated HLA class I heavy chain, containing residues 1–274, was expressed as inclusion bodies (30) using the BL21 (RIL) strain of Escherichia coli. At an A600 of 0.6, cultures were induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside for 12 h, bacteria were lysed in 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 100 mM NaCl, and 10 mM dithiothreitol. Include bodies were isolated by centrifugation after washing with 50 mM Tris-HCl, 0.5% Triton X-100, 100 mM NaCl, 1 mM NaEDTA, 1 mM dithiothreitol, pH 8.0, and washing in 50 mM Tris-HCl, 1 mM NaEDTA, 1 mM dithiothreitol, pH 8.0, and then solubilized in 50 mM Tris, 8 mM urea, 10 mM NaEDTA, pH 8.0, with the protease inhibitors 1 µg/ml peptatin A and 200 µg/ml phenylmethylsulfonyl fluoride. Recombinant protein (30 mg of A2 heavy chain and 10 mg of β2-microglobulin) was refolded with 6 mg of peptide reconstituted in 3% guanidine HCl, 10 mM sodium acetate, pH 4.2, in a refolding buffer composed of 0.1 M Tris, 2 mM EDTA, 400 mM l-arginine-HCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, pH 8.0, at 4 °C for 72 h. Following refolding, protein was dialyzed overnight against MilliQ water (6,000–8,000-kDa molecular mass cutoff) and concentrated by ion exchange on a DE52 column (Whatman, Maidstone, Kent, UK) and subsequently purified by size exclusion on a Superdex 75pg gel filtration column (Amersham Biosciences) and a final ion exchange on a MonoQ HR 10/10 column (Amersham Biosciences). Quantitative analysis was based on comparisons with bovine serum albumin protein standards using SDS-PAGE. Protein was concentrated to 10 mg/ml for use in crystallization trials.

Crystallization—Large cubic crystals (0.3 × 0.3 × 0.3 mm) were obtained using the hanging drop vapor diffusion technique at room temperature. The crystals were grown within 3–5 days by mixing equal volumes of 10 mg/ml HLA A2-NY-ESO-1 peptide (and analogues thereof) with the reservoir buffer (2.0 M ammonium sulfate, 0.1 M sodium citrate, pH 6.5). The crystals belong to space group P212121 with unit cell dimensions a = b = c = 117.90 Å, α = β = γ = 90°. The crystals were flash-frozen prior to data collection using crystals that had been soaked in 15% glycerol. One 2.2 Å and two 2.5 Å data sets were collected for the NY-ESO-1 series and scaled using the HKL suite (31). For a summary of statistics, see Table I.

Structure Determination—The structure was solved by the molecular replacement method, using the program AmoRe within the CCP4 suite (32). The previously solved monomeric HLA A2 structure (Protein Data Bank code 1DUY) (33), minus the peptide, was used as the search probe. A clear peak in the rotation function yielded one clear solution in the translation function that placed well within the unit cell. Using rigid body fitting in AmoRe, the molecular replacement solution had an Rmerge and correlation coefficient of 68.2 and 38.1, respectively. Unbiased features in the initial electron density map, including that of the NY-ESO-1 peptide, confirmed the correctness of the molecular replacement solution. The progress of refinement was monitored by the Rfactor value (4% of the data) with neither a sigma, nor a low resolution cut-off being applied to the data. The structure was refined using rigid body fitting of the individual domains followed by the simulated annealing protocol implemented in CNS (version 1.0) (34), interspersed with rounds of model building using the program O (35). Tightly restrained...
individual B-factor refinement was employed, and bulk solvent corrections were applied to the data set. Water molecules were included in the model if they were within hydrogen-bonding distance to chemically reasonable groups, appeared in F* - F, maps contoured at 3.5σ, and had a B-factor less than 60 Å². See Table I for a summary of refinement statistics and model quality.

HLA A*0201 Assembly Assay—The cDNA encoding the ectodomain of HLA class I molecules HLA A*0201 (amino acids 1–276) was inserted into pET30 (Novagen) vector and verified by DNA sequencing. Inclusion of HLA A*0201 Assembly Assay—The cDNA encoding the ectodomain of HLA class I molecules HLA A*0201 (amino acids 1–276) was inserted into pET30 (Novagen) vector and verified by DNA sequencing. Inclusion 276) was inserted of HLA class I molecules HLA A*0201 (amino acids 1

RESULTS

Structure of NY-ESO-1-(157–165) Peptide Complexed to HLA A2—The HLA A2-NY-ESO complex and analogues thereof have been crystallized in the cubic space group P2_1_2_1, with one molecule per asymmetric unit, and diffracted to a resolution of 2.5 Å or better. The structures were determined via molecular replacement, using a previously determined HLA A2 structure as the search probe (Protein Data Bank number 1DUX (39)). The structure of HLA A2 complexed to peptide NY-ESO-1-(157–165) peptide has been refined to 2.2 Å to 2.5 Å to an R factor 27.9%, respectively (See Table II for a summary of the refinement statistics for each analogue). The three structures comprise residues 1–274 of the HLA A2 heavy chain, residues 1–99 of β_2-microglobulin, and nine residues of the bound peptide, one sulfate ion, and a variable number of water molecules.

The electron density for the bound NY-ESO peptide, and the two analogues (Fig. 1, A–D), as well as the interacting residues was unambiguous. The structure of the NY-ESO-1-(157–165) complex, the highest resolution complex, will be discussed initially, followed by the salient aspects of the analogue structures. The overall structure of the HLA A2 complex was very similar to those reported previously (e.g. see Refs. 30 and 40–46). Thus, our analysis focuses on the peptide conformation and cleft interactions of the NY-ESO peptides bound to HLA A2. The NY-ESO-1-(157–165) peptide is bound in an extended conformation, containing a centrally located bulge at P4-Met and P5-Trp, two prominent, surface-exposed hydrophobic residues (Fig. 1A). These two residues, along with the upward pointing side chains of P7-Thr and P8-Gln, are likely to contact the T cell receptor. The NY-ESO peptide is bound between the helical jaws of the antigen-presenting domain (Fig. 1A), making considerable polar contacts with the HLA A2 molecule along the length of the peptide (Table III, Fig. 2), with 12 hydrogen bonds and 12 water-mediated hydrogen bonds, as well as a number of van der Waals interactions.

The average temperature factor for the bound peptide is 34 Å², whereas the increased mobility of the P4-Met (B-factor 45 Å²) and P5-Trp (43 Å²) is consistent with the limited number of contacts these residues make with the HLA A2 heavy chain (Table III). The buried and anchor residues at positions P2, P3,
P6, and P9 are unlikely to interact with the T cell receptor. Conversely, P1-Ser is solvent-exposed and also a potential T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue. The peptide residues P4-Met, P5-Trp, P7-Thr, and P8-Gln have previously been implicated in T cell receptor contact residue.
the F pocket interactions are largely confined to the terminal functional group of each residue (R-CH₃, R-CH₂OH, R-CH₂SH). The methyl functionality of P9-Ala is in a similar position to the Cβ of P9-Ser and P9-Cys. Additional alterations occur to accommodate the more polar Ser functionality, with the P9-Ser Oγ making a direct hydrogen bond to Asp⁷⁷ resulting in small movement of the hydroxyl group relative to the thiol group of P9-Cys. As discussed below, these subtle changes in F pocket binding lead to substantial changes in complex stability, suggesting that the thiol group of the wild type peptide contributes further stabilizing influences.

Rational Design of a Peptidomimetic—Based on the observation that the Cysteine residue and closely related homologous substitutions (i.e. Ser and Ala) shared very similar structures and that the thiol of the cysteine was primarily involved in van der Waals interactions, we substituted the cysteine for Abu, a nonnatural amino acid that is isosteric for cysteine. We anticipated that the replacement of the thiol group with a methyl group would satisfy any stereochemical anchoring requirement and that indeed the more hydrophobic nature of this analog may be better suited to anchoring in the hydrophobic HLA A2 F pocket (41) (see Table I). This analog was synthesized using standard Fmoc chemistry and, unlike the wild type peptide, did not form dimers or become oxidized during synthesis, purification, and storage (data not shown).

Assembly and Stability of NY-ESO-(157–165) and Analogues Complexed to HLA A2—We used a newly developed HLA A2 assembly assay (37) to assess the binding of the wild type peptide and each analogue, including the C9Abu analogue, to HLA A2. This assay does not rely on cell surface stabilization of antibody determinants but rather utilizes an in vitro assembly reaction with quantitation by capture enzyme-linked immunosorbent assay (37, 47). As such, this assay is less influenced by cell culture-mediated oxidation and modification of cysteine-containing peptides. Over a peptide concentration range of 0.5–10 μM, each peptide drove assembly of HLA A2, with wild type and C9A mediating roughly equivalent assembly, C9V slightly better and C9Abu and C9S slightly worse than wild type (see Fig. 4). In order to further investigate the ability of these analogues to bind to and stabilize HLA A2, we also examined the thermostability of complexes formed by each analogue with HLA A2 by CD. All complexes gave similar spectra at 20 °C; however, the midpoint thermal denaturation revealed compelling differences in the stability of these complexes (Fig. 5). C9V was 4.5 °C more stable than the wild type, whereas C9A was of similar stability to the wild type peptide, with the new C9Abu analogue displaying modest improvement in thermostability of 1.5 °C. The C9S analog, however, was 10 °C less stable. The thermostability of complexes is related to the dissociation constant for the complexes (48) and the half-life of these complexes on the cell surface (49) and thus will impact on their immunogenicity.

Recognition of Analogues by a CD8⁺ NY-ESO-(157–165)-specific T Cell Lines—We next examined the ability of two independently derived T cell lines expanded in vitro by wild type NY-ESO-(157–165) pulsed APC to recognize each variant peptide. In order to rule out the effects of modification of the cysteine of the wild type NY-ESO-(157–165) peptide, all experiments were carried out in the presence of 500 μM tris-(2-carboxyethyl)-phosphine hydrochloride. At this concentration of reductant, no dimerization is observed in vitro (data not shown), and oxidation and cysteinylation are reduced without affecting T cell function or viability (38). As shown in Fig. 6, A and B, the C9Abu was recognized by T cells significantly better than the wild type peptide and other analogues for the two independent T cell lines derived from patients HH and M121. Moreover, C9Abu was able to expand cross-reactive CD8⁺ NY-ESO-(157–165)-specific T cells from peripheral blood mononuclear cells derived from immunized HLA A2+ patients (data not shown). A general pattern of reactivity was observed for both T cell lines such that C9Abu > C9A, C9V > wild type > C9S > C9L, which did not correlate directly with binding or stability of the complexes.

Fig. 1. Structures of HLA A2 complexed to NY-ESO (157–165) and analogues. HLA A*0201/NY-ESO-(157–165) complex 2.2-Å electron density omit map with a cut-away view of the peptide bound to the Ag binding cleft. The same view is presented for the 2.5-Å C9S complex structure and the 2.3-Å C9A complex structure. Very similar conformations were observed for all complexes, highlighting the exposed Met⁴, Trp⁵, Thr⁷, and Gln⁸ residues. The lower right is a view of the wild-type NY-ESO-(157–165) peptide in the cleft of HLA A2 as seen from above.
**Fig. 2. Image of the cleft contacts made between the HLA A2 heavy chain and the NY-ESO (157–165) peptide with hydrogen bond contacts only shown.** Numerous hydrogen bond and van der Waals contacts exist between the peptide and the HLA A2 cleft residues, including anchoring interactions between P2-Leu and B pocket residues and P9-Cys and F pocket residues. These interactions are summarized in Table III. A large number of peptide-main chain hydrogen bond interactions were observed for this complex relative to other HLA A2 complexes (45, 46), which tend to have more water-mediated hydrogen bonding networks.

**TABLE III**

| Peptide | HLA-A2 | Type of interaction |
|---------|--------|---------------------|
| Ser<sup>1</sup> | Trp<sup>167</sup> | van der Waals |
| Ser<sup>2</sup> Oy | Glu<sup>49</sup> Oy1 | Hydrogen bond |
| Ser<sup>3</sup> O | Tyr<sup>250</sup> Oy1 | Hydrogen bond |
| Ser<sup>3</sup> N | Tyr<sup>237</sup> Oy1 | Hydrogen bond |
| Leu<sup>4</sup> | Tyr<sup>1</sup>, Phe<sup>5</sup>, Met<sup>6</sup>, Val<sup>18</sup>, Tyr<sup>29</sup> | van der Waals |
| Leu<sup>5</sup> N | Glu<sup>145</sup> Oy1 | Hydrogen bond |
| Leu<sup>5</sup> | Tyr<sup>289</sup>, Gln<sup>156</sup>, Leu<sup>156</sup>, Tyr<sup>159</sup> | van der Waals |
| Leu<sup>6</sup> N | Tyr<sup>289</sup> Oy1 | Hydrogen bond |
| Met<sup>7</sup> | Lys<sup>86</sup> | van der Waals |
| Trp<sup>7</sup> | Gln<sup>135</sup> | van der Waals |
| Trp<sup>8</sup> O | Gln<sup>135</sup> Nye2 | Hydrogen bond |
| Ile<sup>9</sup> N | His<sup>70</sup>, Thr<sup>73</sup>, Arg<sup>97</sup> | van der Waals |
| Ile<sup>9</sup> | Wat<sup>19</sup> Mediates hydrogen bond to Asp<sup>77</sup> O | Hydrogen bond |
| Thr<sup>7</sup> O | Thr<sup>73</sup> Oy1 | van der Waals |
| Thr<sup>7</sup> N | Wat<sup>12</sup> | Mediates hydrogen bond to Thr<sup>73</sup> Oy1 |
| Thr<sup>7</sup> Oy1 | Wat<sup>14</sup> | Mediates hydrogen bond to Asp<sup>77</sup> Oy1 |
| Thr<sup>7</sup> O | Wat<sup>19</sup> | Hydrogen bond |
| Glu<sup>9</sup> N | Thr<sup>73</sup>, Val<sup>18</sup> | van der Waals |
| Glu<sup>9</sup> NE2 | Wat<sup>14</sup> | Hydrogen bond |
| Glu<sup>9</sup> O | Trp<sup>144</sup> N1 | van der Waals |
| Cys<sup>9</sup> | Asp<sup>77</sup>, Thr<sup>80</sup>, Leu<sup>81</sup>, Thr<sup>143</sup>, Trp<sup>147</sup> | Hydrogen bond |
| Cys<sup>9</sup> 5y | Asp<sup>77</sup>, Thr<sup>80</sup>, Leu<sup>81</sup> | van der Waals |
| Cys<sup>9</sup> N | Asp<sup>77</sup> Oey1 | Hydrogen bond |
| Cys<sup>9</sup> O | Wat<sup>111</sup> | Hydrogen bond |
| Cys<sup>9</sup> OXT | Thr<sup>49</sup> Oy1 | Hydrogen bond |
| Cys<sup>9</sup> | Lys<sup>81</sup> N<sub>F</sub> | Hydrogen bond |
| | Wat<sup>18</sup> | Mediates hydrogen bond to Asp<sup>77</sup> Oy1, Thr<sup>290</sup> Oy1 |

**DISCUSSION**

The structures of HLA A2 complexes to NY-ESO (157–165) and two C-terminally substituted analogue peptides have been solved to 2.5-Å resolution or better. Cysteine is an unusual anchor residue for a HLA A2 ligand, and prior to this study, the exact role of the Cys residue and in particular the potentially reactive thiol in providing anchor contacts with the hydrophobic F pocket of HLA A2 was unknown. In fact, the majority of HLA A2 structures encompass complexes in which the peptide ligand terminates in valine or leucine. One structure with an alanine-terminating peptide (a P9 leucine to alanine-substituted influenza virus matrix peptide, GILGFVFTFA) has been reported previously (44). The conformation of the HLA A2 heavy chain was conserved in all structures, and only small differences in peptide conformation were observed. A feature of each structure is the presence of a noncanonical P9 anchor residue that only partially satiates the hydrophobic HLA A2 F pocket. Despite their conserved structure, the analogues demonstrated remarkable differences in stability and functional recognition by two T cell lines derived from peptide vaccinated patients.

In this study, we were able to compare the relative immunogenicity of each analogue with the wild type peptide by minimizing oxidative damage of the peptide or cysteinylation of the P9-Cys residue by performing binding and stability assays in vitro and by treating the peptides with tris-(2-carboxyethyl)phosphine hydrochloride during Ag presentation assays. This was performed with two independent T cell lines (from patients HH (Fig. 6A) and M121 (Fig. 6B). The C9Abu analogue was consistently recognized more efficiently by the T cell lines, and as a general rule the following reactivity pattern was observed: C9Abu > C9A, C9V > wild type > C9S > C9L. This did not simply correlate directly with binding or stability of the complexes, as may be expected (6, 50–57), and is consistent with several other studies that show that the immunogenicity of some T cell determinants is influenced by additional factors (9, 58–62).

C9S bound to HLA A2 slightly less efficiently than the wild type peptide yet demonstrated drastically worse stabilization of the complexes. C9A and wild type bind and stabilize HLA A2 equally efficiently, suggesting that this analogue is equivalent to the wild type peptide in cross-sensitizing target cells for recognition. The C9V peptide exhibits superior binding and stabilization of HLA A2; the equivalent functional recognition...
of this determinant reflects somewhat diminished recognition on a mole for mole basis given that this peptide will generate a higher determinant density. C9Abu binds more weakly to HLA A2 than the wild type peptide, yet complexes of the two peptides with HLA A2 exhibit equivalent thermostability. Thus, although the Abu analogue is not the most stable or the best binder to HLA A2, it still demonstrates superior immunogenicity to the wild type and other analogues.

Based on the frequency of codons encoding for cysteine in the human genome, we have estimated that 14% of T cell epitopes potentially contain Cys residues (38), suggesting that immune responses to such antigens may frequently be masked by oxidation and cysteinylolation. Moreover, the seminal observations made by Meadows et al. (63) that a peptide originating from SMCY was only recognized by T cells following post-translational modification of a cysteine residue that involved attachment of a second cysteine residue via a disulfide bond highlight the importance of these types of reactions in immunity. Subsequent studies have indicated this type of modification has profound effects on T cell recognition (38) and that cysteine modification occurs in a number of different class I MHC-associated peptides including the epitope reported here. These observations support the notion that this form of modification has general importance as a mechanism of generating immunogenic T cell determinants. Finally, our strategy of substituting Abu for Cys in T cell epitopes may have general application, particularly for Cys-terminating epitopes (such as lymphocytic choriomeningitis virus glycoprotein determinants in C57BL/6 mice (64)).

It is a standard approach to engineer anchor residues to improve MHC binding characteristics in epitope-based vaccine strategies (6). Whereas this frequently imparts improved MHC binding, it does not always equate to improved immunity toward the naturally processed peptide in vivo. For example, our data clearly show that substitution for more appropriate P9 anchor residues for HLA A2 such as valine or leucine, while enhancing binding, do not increase T cell recognition, and in the case of C9L this substitution is detrimental for T cell recognition (Fig. 6). Interestingly, substitution of Cys with serine substantially affects complex stability and T cell recognition, which we hypothesize is due to the large reduction in complex stability. Given the close nature of these residues and the frequency with which Cys is substituted for by Ser in homologous substitution experiments, this highlights the requirement for more rational approaches for epitope engineering.

Because TAA are frequently related to self-proteins, the
available T cell repertoire may be diminished due to thymic and peripheral deletion of those clonotypes specific to the very immunogenic peptides with strong binding ability. As a result, many immunogenic tumor epitopes are relatively poor binders to their cognate class I molecule. Thus, many tumor epitopes have been engineered to produce heteroclitic responses as a result of improved MHC binding. Recent examples include substitution of subdominant anchor residues in an epitope in a B16 melanoma model (65) and identification of a HER-2/neu heteroclitic epitope that provides superior protection in mouse B16 melanoma model (65) and identification of a HER-2/neu substitution of subdominant anchor residues in an epitope in a result of improved MHC binding. Recent examples include those clonotypes specific to the very immunogenic TAA based on conservative or semiconservational amino acid substitutions. As such, this report is one of few studies to successfully incorporate nonnatural amino acids into T cell epitopes (9–11, 29) and highlights the path ahead for rational vaccine design.

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Functional and Structural Characteristics of NY-ESO-1-related HLA A2-restricted Epitopes and the Design of a Novel Immunogenic Analogue

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