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TCRs with Distinct Specificity Profiles Use Different Binding Modes to Engage an Identical Peptide–HLA Complex


The molecular rules driving TCR cross-reactivity are poorly understood and, consequently, it is unclear the extent to which TCRs targeting the same Ag recognize the same off-target peptides. We determined TCR–peptide–HLA crystal structures and, using a single-chain peptide–HLA phage library, we generated peptide specificity profiles for three newly identified human TCRs specific for the cancer testis Ag NY-ESO-1157–165–HLA-A2. Two TCRs engaged the same central peptide feature, although were more permissive at peripheral peptide positions and, accordingly, possessed partially overlapping peptide specificity profiles. The third TCR engaged a flipped peptide conformation, leading to the recognition of off-target peptides sharing little similarity with the cognate peptide. These data show that TCRs specific for a cognate peptide recognize discrete peptide repertoires and reconcile how an individual’s limited TCR repertoire following negative selection in the thymus is able to recognize a vastly larger antigenic pool. The Journal of Immunology, 2020, 204: 000–000.

The presentation of intracellularly processed peptides by class I HLA molecules on the surface of cancer or virally infected cells enables their direct recognition and elimination by CD8+ αβ T cells (1–3). The specificity of TCRs toward a given peptide–HLA (pHLA) complex ensures appropriately targeted natural T cell responses. However, for the ∼1 × 1017–8 unique TCRs present in the body to recognize the entire peptide repertoire, any given TCR must be capable of recognizing up to a million distinct theoretical peptides (4, 5). Thus, TCR cross-reactivity or polyspecificity is thought to be essential for adequate recognition of the potential pathogenic repertoire. TCRs must also simultaneously display remarkable specificity for distinguishing foreign Ag from the more limited HLA-specific self-peptide repertoire. To achieve this, TCRs typically engage a small number of exposed antigenic features or “hotspots,” whereas other peptide positions at the periphery of the TCR–pHLA interface show greater amino acid permissivity (6).

TCR specificity is driven by six CDR loops within the α- and β-chains of the receptor (2). The CDR1 and CDR2 sequences are germline encoded, whereas the CDR3 loops (ImMunoGeneTics database [IMGT] positions 105–117) have a much higher degree of sequence diversity because of somatic rearrangement, including the deletion and/or insertion of nucleotides as a consequence of V(D)J recombination (1–3). Although contacts between the germline-encoded CDRs and peptide are often observed, peptide specificity is most commonly associated with the central CDR3 residues (IMGT positions 108–113), which lie at the more flexible tip of the CDR loop, with flanking regions encoded by the germline V- and J-genes (7, 8).

TCRs that bind the same peptide have been reported to have restricted V-gene usage (9) and/or shared CDR3 sequences (7, 8). However, structural studies of multiple TCRs recognizing a common pHLA show that recognition motifs divergent in sequence but with common structural features can be used to target the same peptide hotspot. Examples include TCRs that recognize Tax–HLA-A2, HIV nef–HLA-A2 or human CMV–HLA-A2, and the MART-1–HLA-A2–specific TCRs DMF5 and Mel5 (10–15). Alternatively, TCRs can recognize different antigenic features on
a given peptide, for example, on EBV–HLA-B*08:01 (16, 17). This appears to be particularly common for TCRs recognizing longer class I peptides that are typically 11–14 aa, such as HIV–HLA-B*35:08 and NY-ESO–HLA-B*07:02, due to significant peptide flexibility (18, 19).

Sequence analysis of the Vβ repertoire of both natural and vaccine-induced TCRs recognizing the well-studied 9-mer peptide–loaded APCs (either monocyte-derived dendritic cells or CD40-L–amino acids (X-scanning). Plates were incubated overnight at 37˚C/5% CO2 culturing in R10 media.

Identification of NY-ESO-157–165–HLA-A2–specific T cell clones

NYE_S1 and NYE_S2 TCRs were generated from T cells isolated from HLA-A*02:01* healthy donors stimulated with NY-ESO-157–165(9V) peptide–loaded APCs (either monocyte-derived dendritic cells or CD40-L–activated B cells). Following multiple rounds of stimulation, polyclonal T cell lines were screened for peptide specificity by IFN-γ ELISPOT (BD Biosciences). T cell clones were established from polyclonal T cell lines by sorting activated T cells following peptide stimulation or tetramer binding using a BD FACSAria II. NYE_S1 TCR was identified by phage display using libraries derived from T cell isolated from HLA-A*02:01* healthy donors (25).

X-scanning mutagenesis IFN-γ ELISPOT assays

X-scanning mutagenesis IFN-γ ELISPOT assays were conducted according to the manufacturer’s instructions (BD Biosciences) and have been described previously (26). Briefly, TCR-transduced T cells were incubated at 5 × 10^5 cells per well with HLA-A*02:01* T2 cells pulsed at 5 × 10^5 cells per well with 10 μM NY-ESO-157–165–native peptide or with peptides substituted at each position with any of the 19 alternative naturally occurring amino acids (X-scanning). Plates were incubated overnight at 37˚C and quantified after development using an automated ELISPOT reader (Immunospot Series 5 Analyzer; Cellular Technology).

Protein production and biophysical measurements

Soluble disulphide-linked heterodimeric TCRs and biotinylated NY-ESO-157–165 (9V)–HLA–A2 were cloned into pGEMT7 vector encoding a C-terminal AviTag on the β-chain or H chain, respectively (27). Protein was expressed in the BL21 (DE3) Rosetta pl lysS strain, refolded from inclusion bodies and purified as previously described (28, 29).

In vitro site-specific biotinylation of the biotin ligase (BirA) tag was carried out prior to size exclusion chromatography using a ratio of 1 μg BirA per 100 μg TCR/pHLA–HLA-A2.

Surface plasmon resonance equilibrium-binding analysis was performed at 25˚C using a Biacore T200 instrument equipped with a CM5 sensor chip as previously reported (30). Approximately 900 response units of each pMHC were immobilized on a single CM5 sensor chip surface before all chip surfaces, including the negative control, were blocked with 1% BSA. Increasing concentrations of peptide were sequentially injected over the immobilized pHAs at 20 μl/min, and each TCR concentration series was repeated three times. Binding plots (equilibrium-binding response versus TCR concentration) were plotted in GraphPad Prism 8, and curves were fitted assuming 1:1 Langmuir binding (AB = B × AX_{max}/(K_{D} + B)) to obtain K_{D} estimates for each interaction.

Generation of single-chain HLA libraries

Single-chain HLA (scHLA) libraries were displayed on the surface of phage as disulphide-trapped single-chain trimers peptide–GCCGAS–(G4S)2–β2m–(G4S)_2–HLA-A2 Y84C (31). Briefly, to ensure the pHLa-recognition surface was unchanged by the addition of a C-terminal linker, a Y84C mutation was introduced into the HLA-A2 α chain. This mutation opens up the F-pocket, creating a groove where the linker can sit. The insertion of cysteine into the linker immediately following the peptide allows a disulphide bond to form with the introduced Y84C mutation, anchoring the linker to the HLA surface and additionally compensating for binding affinity lost by removal of HLA contacts to the peptide C terminus.

The scHLA construct was cloned into the phagemid pmid72 (32), based on the pE9022 vector (33), using SfiI-restriction enzyme. This inserts scHLA into an open reading frame additionally encoding an N-terminal pelB leader sequence and C-terminal coat protein phII. Diversity was encoded at the peptide level by introducing a flat distribution of 19 aa (excluding cysteine to avoid cyclic peptide formation). All 19 aa were represented at the MHC primary anchors, Pos2 and Pos9; however, to maximize the functionality of the library with peptide correctly bound in the Ag-binding groove, the amino acid distribution was biased toward known preferences for this allele, making the theoretical diversity of this library 3.22 × 10^11. The library sizes were determined postelectroporation by colony counting after limiting dilutions.

The phagemid library was introduced by electroporation into Escherichia coli TGL1 cells and grown in 2 × YT media containing 2% glucose. 100 μl were pelleted and resuspended in 2 × YT media containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and subsequently incubated at 26˚C with 16 h with shaking. Phage particles were isolated by polyethylene glycol (PEG) precipitation and 0.45 μM filtration. The use of PEG allowed higher-valence display such that each phage displayed ~5 copies of scHLA, enabling avidity-driven recognition of WT TCRs (34).

Panning

Two hundred nanomolar biotinylated TCRs NYE_S1, S2, and S3 were captured on streptavidin-coated paramagnetic beads (Thermo Fisher Scientific) and incubated at room temperature with gentle rotation with ∼1 × 10^10 phage particles per selection, preblocked in 1% BSA–PBS and 0.1% Tween. Phage particles were eluted with 10 μg/ml trypsin and used to infect early log phase TGL1 E. coli cells and plated onto 2 × YT (plus 2% glucose and 100 μg/ml ampicillin) plates at 30˚C for 2 h. Colonies were resuspended in 2 × YT containing 2% glucose, 100 μg/ml ampicillin, and 20% glycerol; frozen on dry ice; and stored at −80˚C for the next round of panning. Three rounds of selection were performed.

Deep sequencing and cluster analysis of pHLA libraries

DNA was isolated from each glycerol stock by Miniprep (27104; QIAGEN). Sequencing libraries were prepared with molecular indexing based on a method described in Turchaninova et al. (35). Briefly, phagedna library was amplified with primers containing unique molecular indexes. Purified PCR products were then sequenced with paired-end, 150 bp, Illumina MiSeq. Quality control was performed, and reads were filtered to remove adapter contamination and low-quality reads. Reads were clustered using the Merck Sequencing Tools (FASTQ files were merged using BBMerge (36); molecular index and peptide sequence were extracted from each read, and sequence logos were generated using iceLogo (37). Sampling 0.25% of the library indicated that 98.7% of the encoded peptides were unique.
Unique peptide sequences from sHLA libraries were clustered using 7-distributed stochastic neighbor embedding (tSNE) (38) on the basis of pairwise biochemical distance using the BLOSUM45 substitution matrix. The tSNE analysis was implemented using the Rtsne package in R version 3.4.4. tSNE hyperparameters were set to ensure cluster convergence, and consistency of peptide cluster identity between runs of the tSNE was ensured.

**Crystallization**

For NYE_S1, TCR–pHLA complex was prepared by mixing purified TCR and pHLA at equimolar ratios. Crystallization trials, using $100 \mul$ protein solution plus $100 \mul$ reservoir solution in sitting-drop vapor diffusion format, were set up in two-well MRC Crystallization plates using a mosquito (TPP Labtech) robot. Plates were maintained at 20°C in a Rock Imager 1000 (Formulatrix) storage system. Initial crystals grew in the PEGs II Crystallization Screen (QIAGEN), condition F6. Larger crystals were grown by cross-seeding into a grid of 10–25% PEG 4000, 0.1 M sodium citrate (pH 6.5–7.0), 0.2 M ammonium sulfate using a Seed Bead–generated seed solution and by increasing the drop size to $1 \mu l$ protein plus 1 $\mu l$ well solution. Crystals were cryoprotected by addition of 15% glycerol directly to the drop and then flash-cooled at 100 K. X-ray diffraction data were collected at Diamond Light Source on beamline ID22. Diffraction images were indexed, integrated, scaled, and merged using DIALS (39) and Aimless (40) through the xia2 package in Daresbury Laboratory (41). For NYE_S1, TCR–pHLA crystals, crystallization trials using $150 \mu l$ protein solution plus $150 \mu l$ reservoir solution in sitting-drop vapor diffusion setup were set up in two-well MRC Crystallization plates using a Crystal Gryphon (Art Robbins) robot. Plates were maintained at 20°C in a Rock Imager 1000 (Formulatrix) storage system. Diffraction quality crystals were grown in the following conditions: 0.02 M sodium/potassium phosphate, 20% PEG 3550 (NYE_S2 TCR–pHLA) and 0.2 M ammonium sulfate, 15% PEG 8000, and 0.1 M Tris (pH 7.5) (NYE_S3 TCR–pHLA). Crystals were cryoprotected using a 30% solution of ethylene glycol and then flash-cooled at 100 K. X-ray diffraction data were collected at Diamond Light Source (Oxfordshire, U.K.) at the I04-1 beamline at wavelength 0.91587 Å. Diffraction images were indexed, integrated, scaled, and merged using DLS and XSCALE (42, 43) through the xia2 package in Daresbury Laboratory (41). Quality control statistics were performed using XDS and XSCALE (42, 43) through the xia2 package in Daresbury Laboratory (41).

**Structure determination and refinement**

Molecular replacement was used to phase all crystal structures, using Protein Data Bank (pdb) entry 5e00 chains A and B (for HLA-A2 and β2m), pdb 3REV chain A (NYE_S2 TCRα), pdb 4DZB chain B (NYE_S2 TCRβ), 3QDJ chain D (NYE_S3 TCRα), and 5D2W chain E (NYE S3 TCRβ) as search models in Phaser (45). For NYE_S1 TCR, a higher resolution structure of a related complex was used as the search model (data not shown), which had been solved using 4FTV as a starting model. Three wild-type TCRs (NYE_S1, NYE_S2, and NYE_S3) rec–pHLA complexes were relaxed to medium resolution in final refinement rounds (excluding the following regions in NCS domain definitions: chains D, I, and K residues 108–115 and chains E, J, and L residues 65–71). Stereochemical properties of all models were assessed using the PDB Validation Suite (48). Ramachandran statistics are the following: NYE_S1 TCR–pHLA complex, 98% favored, 2% allowed, and no rotamer outliers; NYE_S2 TCR–pHLA complex, 97% favored, 3% allowed, and 1 rotamer outlier; NYE_S3 TCR–pHLA complex, 94% most favored, 6% additionally allowed, and a single rotamer outlier. Full data collection and refinement statistics are given in Table I.

Crystalllographic figures were created using PyMOL (Schrödinger). All structural alignments were performed using Superpose (49). Buried surface area and TCR docking geometry statistics based on those described previously (2) were generated using Molecular Operating Environment (Chemical Computing Group) (50). Briefly, TCR crossing angles are calculated from the eigenvector relating the vector through the midpoints of the Vα and Vβ domains’ disulfide bonds to the vector along the length of the MHC helices that defines the Ag-binding groove (V1). The tilt angle relates pseudo-2-fold TCR symmetry axis to the vector perpendicular to V1 lying in the plane of pHLA Ag-binding groove, where a negative value reflects a roll toward the HLA α1 helix, whereas a positive value reflects a roll toward the HLA α2 helix.

**Crystal structure composition**

The NYE_S1 TCR–pHLA crystal structure contains four copies of the TCR–pHLA complex per asymmetric unit: HLA, chain A aa 1–276 (lacking residues 225–228), chain F aa 1–276 (lacking residues 17–18), chain K aa 1–274 (lacking residues 195–198, 221–227, and 250–253), and chain P aa 1–275 (lacking residues 225–227); β2m, chains B, G, L, and Q aa 0–99; peptide, chains C, H, M, and R aa 1–9; TCRα, chain D aa 3–219 (lacking residues 3–141 and 225–227); TCRβ, chain E aa 3–256; TCRγ, chain P aa 1–275 (lacking residues 225–227); and TCRδ, chain T aa 2–256. TCR residue numbering was assigned according to IMGT conventions (51); despite sequence gaps between TCR residues 29–36, 58–63, 67–74, and 110–113 and TCRβ residues 29–37, 59–63, 72–74, 81–83, and 110–112, the TCRα and TCRβ-chains are continuous in the variable domains.

The NYE_S2 TCR–pHLA crystal structure contains one copy of the TCR–pHLA complex per asymmetric unit: HLA, chain A aa 1–276; β2m, chain B aa 1–99, peptide, chain C aa 1–9; TCRα, chain D aa 1–192 (lacking residues 145–147, 160–166, 180–184, and 193–222, which lie in disordered regions of the constant domain); and TCRβ, chain E aa 3–256 (lacking residues 1–252 (lacking residues 141–146), chain N aa 3–217 (lacking residues 139–146 and 181–182), chain S aa 3–217 (lacking residues 140–146 and 181); and TCRδ, chain E aa 3–256, chain J aa 3–256, chain O aa 3–254, and chain T aa 2–256. TCR residue numbering was assigned according to IMGT conventions (51); despite sequence gaps between chain D residues 29–36, 59–62, and 67–74 and chain E residues 29–37, 59–63, 81–83, and 110–112 plus a 2 aa insertion between chain D residues 111–112, the TCRα and TCRβ-chains are continuous in the variable domains.

The NYE_S3 TCR–pHLA crystal structure contains two copies of the TCR–pHLA complex and one apo TCR copy per asymmetric unit: HLA, chain A aa 1–276; β2m, chain B aa 1–99, peptide, chain C aa 1–9; TCRα, chain D aa 1–192 (lacking residues 145–147, 160–166, 180–184, and 193–222, which lie in disordered regions of the constant domain); and TCRβ, chain E aa 3–256 (lacking residues 1–252 (lacking residues 141–146), chain L aa 2–256 (lacking residues 1 and 257), and chain I aa 2–256 (lacking residues 1 and 257). TCR residue numbering was assigned according to IMGT conventions (51); despite sequence gaps between chains D residues 29–36, 59–62, and 67–74 and chain E residues 29–37, 59–63, 81–83, and 110–112 plus a 2 aa insertion between chain D residues 111–112, the TCRα and TCRβ-chains are continuous in the variable domains.

**Results**

**Characterization of three novel NY-ESO-1157–165–HLA-A2–specific TCRs**

Three wild-type TCRs (NYE_S1, NYE_S2, and NYE_S3) recognizing NY-ESO-1157–165 (9V)–HLA-A2, a heterocytic peptide variant of NY-ESO-1157–165–HLA-A2 (52), were identified and then expressed, purified, and tested to determine their biochemical characteristics (Table I). TCR sequences are reported then expressed, purified, and tested to determine their biochemical characteristics (Table I). TCR sequences are reported
K_{D} values are consistent with other immunologically productive TCRs, which have affinities in the 0.1–500-$\mu$M range (3).

**NYE_S1 and NYE_S2 TCRs adopt an overall canonical binding geometry toward NY-ESO-1{157-165}(9V)–HLA-A2**

We determined crystal structures of NYE_S1 and NYE_S2 TCR-NY-ESO-1{157-165}(9V)–HLA-A2 complexes to resolutions of 2.50 and 2.56 Å, respectively (Fig. 1A, 1D, Table I). The NYE_S1 structure contains four TCR–pHLA copies in the asymmetric unit, which are all in close overall structural agreement (root-mean-square deviation [RMSD], 0.35 Å between any two copies, comparing C_{a} positions for HLA residues 1–181, peptide 1–9, V_{a} 3–128, and V_{b} 3–128; copy 1 used for all further structural comparisons), whereas the NYE_S2 structure contains a single TCR–pHLA copy. For both structures, there is well-defined electron density at the TCR–pHLA interface (Fig. 1B, 1E). Both NYE_S1 and NYE_S2 TCRs display similar canonical pHLA-binding footprints in which the V_{a} and V_{b} domains and, in particular, the germline-encoded CDR1_{a} and CDR1_{b} lay over HLA helices \( \alpha_{2a} \) and \( \alpha_{1} \), respectively (Fig. 1C, 1F, Supplemental Table I).

The V(D)J recombination–derived CDRs, CDR3_{a} and CDR3_{b}, sit centrally above the HLA peptide-binding groove and dominate TCR contacts with the peptide (Fig. 1C, 1F).

The peptide conformations in both structures are very similar (RMSD, 0.306 Å across 9 C_{a} positions) and show relatively minor deviations from the conformations described for previously

**FIGURE 1.** NYE_S1 and NYE_S2 TCR-binding footprints on NY-ESO-1{157-165}(9V)–HLA-A2 and NY-ESO-1{157-165} peptide conformations. Overall cartoon representation of TCR–pHLA complexes for NYE_S1 (A) and NYE_S2 (D) TCRs. NY-ESO-1{157-165}(9V)–HLA-A2 peptide conformation within NYE_S1 (B) and NYE_S2 (E) TCR containing structures. CDR positions above pHLA surface for NYE_S1 (C) and NYE_S2 (F) TCRs. HLA H chain, wheat; \( \beta_{2m} \), brown; TCR CDR1_{a}, orange; TCR CDR2_{a}, yellow; TCR CDR3_{a}, maroon; TCR CDR1_{b}, dark blue; TCR CDR2_{b}, cyan; TCR CDR3_{b}, green; NY-ESO-1 peptide, pink. 2F_{o}–Fc maps contoured at 1σ and carved within 2 Å of the peptide are shown in light purple. Arrows above or below the peptide sequence indicate if each side chain is either exposed or buried respectively, relative to the HLA-peptide-binding groove.

Table I. NYE_S1, NYE_S2, and NYE_S3 TCR–pHLA data collection and refinement statistics

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<th>NYE_S3 Complex</th>
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<td>11.7 (99.9)</td>
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<td>( R_{free} ) (%)</td>
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\( R_{merge} \) (the precision-indicating R-value) = 1/(N−1) \times \sum_{i=1}^{N} r_{merge}^{i} \), where \( N \) is the redundancy. CC_{1/2} is the mean intensity correlation coefficient of half-data sets. Dashes (—) indicate that no waters were modelled for the NYE_S3 structure.

^\*Values in parentheses are for highest resolution shell.
determined NY-ESO-1 157–165–HLA-A2 structures (Fig. 1B, 1E, Supplemental Fig. 2). The large, hydrophobic side chains at positions Met\(^4\) and Trp\(^5\) point upwards, away from the peptide-binding groove. Peptide positions Leu\(^2\) and Val\(^9\) are the primary anchor residues, whereas Ile\(^6\) adopts a secondary anchor position, securing the peptide in the peptide-binding groove of the HLA.

**Specificity toward the peptide MW-peg motif is primarily driven by two germline CDR3\(\alpha\) residues**

For the 1G4 and NYE_S1 TCRs, the \(\alpha\)- and \(\beta\)-chains both contribute to the overall peptide-binding interface, whereas for NYE_S2, the \(\alpha\)-chain dominates the interface (Supplemental Table I). These three TCRs predominantly use the CDR3\(\alpha\) loop to engage the MW-peg motif, with the backbone of the central residues stacking around the hydrophobic Met\(^4\) and Trp\(^5\) side chains (Fig. 2A–C). NYE_S1 makes additional contacts to Trp\(^5\) via Gln\(^37\) in the CDR1\(\alpha\), whereas the longer CDR3\(\alpha\) for NYE_S2 versus NYE_S1 is able to wrap more closely around Met\(^4\) (Fig. 2D, 2F). \(\beta\)-Chain-mediated peptide contacts are dominated by the CDR3\(\beta\) loop that makes contacts to the exposed Ile\(^6\) backbone and Thr\(^7\), whereas NYE_S1 also makes additional contacts to side-chain Gln\(^8\), mediated by the CDR1\(\beta\) (Fig. 2G–I).

Aligning the NYE_S1 and NYE_S2 TCR sequences with those of previously identified NY-ESO-1 157–165-specific TCRs demonstrated significant diversity in CDR3\(\alpha\) length, amino acid sequence, and variable chain usage (Table II). However, of all residues contacting the peptide, the CDR3\(\alpha\) Arg\(^{107}/\text{Lys}^{107}\) and Tyr\(^{114}\) residues were the most highly conserved across multiple TRAV/TRBV chain pairings. From the crystal structures, we observed that the Arg\(^{107}/\text{Lys}^{107}\) side-chain carbon backbone stacks against the Trp\(^5\) side chain, whereas the Tyr\(^{114}\) side-chain hydrophobic ring stacks against both Met\(^4\) and Trp\(^5\) side chains, with its hydroxyl group making a putative hydrogen bond to the peptide backbone (to the Met\(^2\) carbonyl group) (Fig. 2G–I, Table II). This observation suggested that the \(\alpha\)-centric specificity displayed by the 1G4, NYE_S1, and NYE_S2 TCRs is driven by a selective pressure for these two CDR3\(\alpha\) amino acid positions, which, together, help to pin the loop around the MW-peg, highlighting the importance of this feature in shaping the TCR repertoire.

**NYE_S3 TCR binds to a flipped NY-ESO-1 157–165(9V)–HLA-A2 peptide conformation lacking an exposed MW-peg motif**

A crystal structure of the NYE_S3 TCR–pHLA complex was determined to a resolution of 3.12 Å (Fig. 3, Table I). This structure contains two TCR–pHLA copies, which are in close overall structural agreement (RMSD, 0.75 Å between the two copies, comparing C\(\alpha\) positions for HLA residues 1–181, peptide 1–9, V\(\alpha\)3–128, and V\(\beta\)3–128; copy 1 used for all further structural comparisons) and one apo TCR copy per asymmetric unit. The most striking observation from our NYE_S3 TCR–pHLA crystal structure was a novel peptide conformation with a pronounced rearrangement of the central peptide residues, Met\(^4\),

![Figure 2](http://www.jimmunol.org/) Structural characterization of NYE_S1, 1G4, and NYE_S2 TCRs binding to NY-ESO-1 157–165(9V)–HLA-A2. CDR3\(\alpha\)-mediated interactions with the MW-peg for NYE_S1 (A), 1G4 (B), and NYE_S2 (C) TCRs, respectively. CDR3\(\alpha\) and CDR3\(\beta\) stacking against the MW-peg motif for NYE_S1 (D), 1G4 (E), and NYE_S2 (F) TCRs. The areas depicted by dashed boxes are expanded in panels (G–I) to highlight CDR3\(\beta\)-mediated interactions. TCR CDR1\(\alpha\), orange; TCR CDR3\(\alpha\), maroon; TCR CDR3\(\beta\), green; peptide, gray sticks; MW-peg, blue sticks; HLA helix-\(\alpha\)-1, wheat cartoon; potential H-bonds, yellow dashed lines.
Trp^5, and Ile^6, relative to the conformations observed for pHLA alone (Fig. 3B, Supplemental Fig. 2G, 2H). In this structure, Ile^6 no longer acts as a secondary anchor as the orientations of Trp^5 and Ile^6 side chains are flipped to a down (Trp^5)–up (Ile^6) arrangement relative to the peptide-binding groove, rather than the usual up (Trp^5)–down (Ile^6) configuration observed in all previous structures. This disruption of the conserved MW-peg epitope results in a very distinct TCR–pHLA-binding interface for NYE_S3 versus the NYE_S1 and NYE_S2 TCRs (Fig. 3A, 3C, Supplemental Table I).

The NYE_S3 TCR binds with a typical TCR binding footprint, whereby TCR^a and TCR^b-mediated contacts are directed primarily to HLA helix-α2 and HLA helix-α1, respectively (Supplemental Table I). Despite shared Vα-chain usage between NYE_S1 and NYE_S3, the TRA12-2-germline–encoded CDRs do not bind to the same features of the pHLA. CDR1^a engages peptide in NYE_S1, whereas it engages the α2 helix of the HLA in NYE_S3. In contrast to the NYE_S1 and NYE_S2 TCRs, for NYE_S3, β-chain contacts dominate the peptide-binding interface (Fig. 3D–F, Supplemental Table I). Both the CDR3^a and CDR3^b loops of NYE_S3 contain central Pro–Gly motifs, which form rigid type II β-hairpin turns, potentially limiting the conformational flexibility of these CDRs (Fig. 3E). For CDR3^a, this tight turn sits directly above the central core of the peptide, stacking...
against exposed Met\textsuperscript{4} and Ile\textsuperscript{6} side chains. Further contacts to Ile\textsuperscript{6} come from the CDR3\textsubscript{\textit{b}} Ser\textsuperscript{108} and Gly\textsuperscript{111}, CDR1\textsubscript{\textit{b}} Val\textsuperscript{37}, and the CDR2\textsubscript{\textit{b}} Asn\textsuperscript{57} and Tyr\textsuperscript{58} residues (Fig. 3E, 3F). In addition, contacts to the Thr\textsuperscript{7} side chain and Gln\textsuperscript{8} backbone NH group as well as the Gln\textsuperscript{8} side chain are mediated by the CDR3\textsubscript{\textit{b}} backbone and CDR2\textsubscript{\textit{b}} Tyr\textsuperscript{58}, respectively (Fig. 3E, 3F).

Comparison of pHLA-bound and apo TCR copies within the NYE\textsubscript{S3} TCR–pHLA structure reveals possible conformational changes in the TCR upon engagement of pHLA (Supplemental Fig. 3). Alignment of either the V\textalpha or V\textbeta domains of NYE\textsubscript{S3} apo TCR (unbound) with the equivalent domain in NYE\textsubscript{S3} TCR–pHLA (bound) reveals close structural agreement, apart from the CDR3\textalpha loop (Supplemental Fig. 3A, 3B). In its unbound form, the CDR3\textalpha loop of the NYE\textsubscript{S3} TCR adopts an elongated conformation that is stabilized by an extension of the flanking \beta-strands. However, when bound to pHLA, flexion at Arg\textsuperscript{108} and Gln\textsuperscript{115} enables CDR3\textalpha to bend, without which the tip of the loop would clash with peptide Met\textsuperscript{4} (Supplemental Fig. 3C). This CDR3\textalpha conformation stacks between exposed peptide Met\textsuperscript{4} and Ile\textsuperscript{6} side chains. To accommodate the bent CDR3\textalpha conformation, a small twist in the TCR\textbeta-chain relative to TCR\textalpha also occurred in the NYE\textsubscript{S3} TCR–pHLA, compared with the NYE\textsubscript{S3} apo TCR (Supplemental Fig. 3D).

All three TCRs display unique peptide specificity profiles, enabling recognition of distinct off-target peptides

We performed peptide specificity profiling on the three identified TCRs using two complementary approaches. In the first cellular X-scan approach, each of the three NY-ESO-1\textsubscript{157–165}-specific TCRs were transduced into T cells. HLA-A*02:01–positive target cells were pulsed with NY-ESO-1\textsubscript{157–165} peptide in which each amino acid position was sequentially replaced with all 19 alternative naturally occurring amino acids (171 peptides in total), and IFN-\gamma release was measured relative to pulsing with cognate peptide by ELISpot (Fig. 4). Using this assay, we found Trp\textsuperscript{5} to be absolutely required for recognition by all three TCRs, as no IFN-\gamma release was observed when this residue was replaced (Fig. 4D–F). However, Met\textsuperscript{4} could be substituted more readily for NYE\textsubscript{S1}, as compared with NYE\textsubscript{S2} or NYE\textsubscript{S3}. Surprisingly, both NYE\textsubscript{S1} (Fig. 4D) and NYE\textsubscript{S2} (Fig. 4E) could also accommodate a Gln at position 4, potentially because of the similar length of the side chains and introduction of additional hydrogen bonding potential.

**FIGURE 4.** Cellular potency and X-scan TCR specificity profiles. Cellular potency was assessed using T cells transduced with NYE\textsubscript{S1} (A), NYE\textsubscript{S2} (B), and NYE\textsubscript{S3} (C) TCRs, exposed to HLA-A2\textsuperscript{+} T2 cells pulsed with increasing concentrations of NY-ESO-1\textsubscript{157–165} peptide. T cell activation was measured using an IFN-\gamma ELISpot assay at 24 h, and EC\textsubscript{50} values were determined (solid triangles). No response was seen at the highest concentration of peptide used for pulsing a nontransduced T cell control (open triangles) or irrelevant TAX peptide (open squares). (D–F) X-scanning mutagenesis of all NY-ESO-1\textsubscript{157–165} single amino acid variants. Data shown are from a representative donor (n = 2). An average of triplicate data points is presented for each of the 171 separate experiments normalized to the NY-ESO-1\textsubscript{157–165} peptide control highlighted in (A)–(C). Each amino acid is shown in one-letter code and colored according to functional similarity: positive (blue), aromatic uncharged (green), aliphatic (black), small nonpolar (orange), polar (pink), and negative (red). Outlined boxes highlight the cognate residues.
to the exposed CDR3α backbone. Consistent with its role as a secondary anchor, Ile<sup>6</sup> displayed limited selectivity for NYE_S1 and NYE_S2, tolerating substitutions to other hydrophobic amino acid side chains. However, the upward facing conformation of this residue in the NYE_S3 structure resulted in this TCR tolerating Arg in this position, a large charged residue that is not able to act as a downward-facing secondary anchor (Fig. 4F). In line with the structural observations, the NYE_S1 and NYE_S3 TCRs also showed restricted specificity for Gln at position 8, whereas this position was more permissive in NYE_S2 (Fig. 4D–F).

Because of the biased nature of X-scans toward the cognate peptide, we generated a more extensive, high throughput, and unbiased approach by profiling TCR specificity using scHLA phage libraries in which peptide diversity was encoded at all positions, including position 2 and position 9 (which were biased toward HLA-A2–specific anchor residues), and presented in a disulphide-linked single-chain trimer format (Fig. 5A). This method is a significant advancement for TCR specificity profiling over previous methods, as it enables the ability to simultaneously monitor the enrichment of millions of individual sequences through the use of next-generation DNA sequencing (6, 53, 54). Phage libraries encoding $5 \times 10^8$ variants were

**FIGURE 5.** Molecular analysis of TCR specificity for NY-ESO-1<sub>157-165</sub> peptide. (A) Schematic of multivalent disulphide trapped single-chain trimers (dsSCT) displayed on HLA-A2 phage libraries. (B) Schematic of phage display panning protocol. (C) Heatmaps and sequence logos displaying amino acid permissivity generated from next-generation DNA sequencing (NGS) workflow showing initial peptide diversity in which positions 2 and 9 are biased toward known HLA-A2 preferences, and Cys is excluded prior to TCR selection (left panel). The following three panels show peptide diversity following three cycles of panning using each of the three NY-ESO-1<sub>157-165</sub> specific TCRs. Outlined boxes highlight the cognate residues.
generated, and following three cycles of avidity-driven phage display panning (Fig. 5B), a peptide specificity profile was generated for each TCR (Fig. 5C).

Analysis of the sequence landscape revealed that, when given free choice of peptide, the NYE_S1 and NYE_S2 TCRs both recognized peptides shaped by the MW-peg: for both TCRs, more than 99% of the peptides \( n \) sequence counts) contained a tryptophan at position 5 (Fig. 5C). Cluster analysis showed that more than 99% of the high-confidence peptides enriched in response to the NYE_S1 TCR were found in the same sequence cluster as the NY-ESO-1\(_{157-165}\) peptide (Fig. 6). Approximately 40% of peptides enriched in response to the NYE_S2 TCR were also found in this cluster. The NYE_S2 TCR displayed a preference for peptides with Gln at position 4 and was relatively permissive at position 8, whereas the NYE_S1 TCR displayed a preference for Gln at position 8 and was relatively permissive at position 4 (Figs. 5C, 6). Within the cluster containing the NY-ESO-1\(_{157-165}\) peptide, it was possible to identify a subcluster with restricted specificity at Q4 and Q8. This overlapping region of the NYE_S1 and NYE_S2 specificity profiles accounted for 35.6 and 8.2% of the total TCR-specific peptides, respectively (Fig. 6C). Approximately 59% of peptides enriched by the NYE_S2 TCR recognized peptides belonging to a sequence cluster related to the NY-ESO-1\(_{157-165}\) peptide but displayed a preference for peptides with Pro at position 6 and an aromatic residue at position 3 (Fig. 6). Recognition of this cluster was restricted to the NYE_S2 TCR. Neither the NYE_S1 nor NYE_S2 TCR bound (Supplemental Fig. 1). Further an accurate \( K_D \) could not be determined for the NYE_S3 TCR binding to NY-ESO157–165-like peptides that are the consensus of peptides enriched by the NYE_S1 (estimated \( K_D > 350 \mu M \)) or NYE_S2 TCR. Assessment of pHLA-A2 stability at 25˚C was comparable for NY-ESO-1\(_{157-165}\), the heteroclytic 9V variant and SLYMLFPEV (data not shown).

**Discussion**

In this study, we show that TCRs binding the same peptide hotspot have partially overlapping specificity profiles, whereas those binding different hotspots have nonoverlapping specificity profiles. We observed that two TCRs (NYE_S1 and NYE_S2) bound peptide in which the MW-peg is a prominent feature (24). This binding configuration has previously been reported for the 1G4 TCR (22–24) (55) and, taken together, this provides further evidence that an antigenic feature within a peptide is able to shape the TCR repertoire.

The MW-peg is likely a dominant epitope because MW are the two least abundant amino acids in human proteins (only a single codon exists for each) and are thus rarely found in the self-peptidome. This premise is supported by the observation that only 0.69% of all HLA-A2 9-mer peptides have a tryptophan at position 5 (analysis of data from 24 publicly available large scale

**FIGURE 6.** Sequence cluster analysis of the most abundant peptides identified in response to the three NY-ESO-1\(_{157-165}\)-specific TCRs. **(A)** The 500 most enriched peptides for each NY-ESO-1\(_{157-165}\)-specific TCR were clustered by calculating BLOSUM 45 distance between all peptides, and unique sequences plotted with a two-dimensional tSNE analysis. Peptides are colored according to the TCR, to which binding has been observed. TCR NYE_S1 (orange), NYE_S2 (blue), and NYE_S3 (pink) and peptides recognized by both TCR NYE_S1 and NYE_S2 (green). The NY-ESO-1\(_{157-165}\) peptide is shown in black. **(B)** Sequence logos for each of the clusters, including a cluster that has no convergence and is presumed to be noise, are shown. **(C)** The number of unique peptide sequences and the percentage of peptides recognized by each TCR from each cluster are reported.
The T cell repertoire must maintain the ability to avoid recognizing the self-peptide repertoire. TCR specificity profiles do not typically involve all peptide positions, and restricted amino acid preferences are limited to a subset of the peptide (6). Therefore, targeting a limited number of peptide positions, particularly in which these antigenic features allow discrimination from the self-repertoire, enables T cells to survive negative selection in the thymus. Equally, the permissive nature of several peptide positions enables TCR recognition of a significant number of theoretical peptides, thereby providing the coverage required by the TCR repertoire to recognize a diverse and evolving pathogenic sequence landscape (13). However, Ag-driven expansion of TCR clonotypes skews the TCR repertoire such that an individual may have many TCRs that recognize a single Ag. In this study, we show that even when multiple TCRs have the capacity to engage a common Ag, they can, nevertheless, recognize distinct and, in some instances, almost completely nonoverlapping peptide repertoires.

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Disclosures
K.J.S. and S.G. are employees of GlaxoSmithKline. All other authors are or were employees of ImmunoCore, Ltd. The study was entirely funded by these organizations.

References
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### B

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**Table S1.** TCR-pHLA interface BSA and docking geometry analysis. A) Characterisation of NYE_S1, NYE_S2, NYE_S3 and 1G4 TCR α and β chain contributions to the TCR-pHLA interface measured by buried surface area (BSA). B) Characterisation of NYE_S1, NYE_S2, NYE_S3 and 1G4 TCR docking geometries on pHLA. For NYE_S1 and NYE_S3 TCR-pHLA structures, which contain more than one TCR-pHLA copy in the ASU, chains A-E (copy 1) was used for this analysis. 1G4; TCR-pHLA NY-ESO-1.157-165(9V) (PDB ID 2BNQ).
Figure S1. Biophysical analysis of NY-ESO-1 specific TCRs.

Equilibrium SPR experiments testing binding of NYE_S1, NYE_S2 and NYE_S3 TCRs to immobilised HLA-A2 containing either NY-ESO-1,157-165(9V) or peptides representative of the sequence clusters identified in Figure 6. (A) Summary table for all SPR data, listing the fold change in $K_d$ value for each pHLA-TCR interaction relative to the $K_d$ value measured in the same experimental set for that given TCR binding to NY-ESO-1,157-165(9V)-HLA-A2. Values highlighted in green indicate stronger and in orange indicate reduced TCR binding compared to binding to NY-ESO-1,157-165(9V)-HLA-A2, while interactions highlighted in red were unmeasurable (ND; not determined). Supporting data for all SPR experiments testing binding of NYE_S1, NYE_S2 and NYE_S3 TCRs to immobilised: (B) NY-ESO-1,157-165(9V)-HLA-A2 and SLYMLFPEV-HLA-A2, (C) NY-ESO-1,157-165(9V)-HLA-A2, KLMQWITQV-HLA-A2 and FLNQWVTAV-HLA-A2, (D) NY-ESO-1,157-165(9V)-HLA-A2 and YQWQWVPVAV-HLA-A2. All experiments were performed at 25°C in triplicate; a representative set of sensograms (top panel) and a binding curve fitted to all repeats (bottom panel) is shown for each interaction. Error bars for each binding curve data point correspond to standard deviation. Mean $K_d$ and $B_{\text{max}}$ values estimated from the binding curves are stated, together with 95% confidence interval in brackets (lower-upper limit). Legends provided for each TCR in the top sensogram apply to all other sensograms for that TCR within the same panel. No significant response was measured when testing the three TCRs against >14 irrelevant peptide HLA-A2 complexes (data not shown).
Figure S2. Comparison of NY-ESO-1<sub>157-165</sub> peptide conformations observed in crystal structures containing NY-ESO-1<sub>157-165</sub>-HLA-A2. (A) NY-ESO-1<sub>157-165</sub> conformations in apo pHLA structures: SLLMWITQC peptide (PDB ID 1S9W), SLLMWITQY (1S9X, 1S9Y), SLLMWITQL (3KLA), YLLMWITQV (4L29). Alignment on 1S9W chain A residues 57-84 (HLA helix-α1). (B) TCR bound NY-ESO-1<sub>157-165</sub> conformations: wildtype (PDB ID 2BNQ, SLLMWITQV peptide) and affinity enhanced (PDB ID 2BNR, 2F53, 2F54, 2P5E, 2P5W, 2PYE, all SLLMWITQC) 1G4 TCR-pHLA structures. Alignment on 2BNQ chain A residues 57-84 (HLA helix-α1). (C, E, G) NY-ESO-1<sub>157-165</sub> conformations TCR-pHLA structures (SLLMWITQV peptides coloured pink) compared to apo pHLA structure 1S9W (SLLMWITQC peptide grey) for NYE_S1, RMSD 0.955 Å, NYE_S2, RMSD 0.826 Å and NYE_S3, RMSD 1.36 Å over 9Ca atoms. Alignment to 1S9W chain A residues 57-84 (HLA helix-α1). (D, F, H) NY-ESO-1<sub>157-165</sub> conformation in 1G4 TCR-pHLA structure 2BNQ (SLLMWITQV peptide grey) compared to TCR-pHLA structures (SLLMWITQV peptides coloured pink) for NYE_S1, 0.262 Å 9Ca, NYE_S2, 0.261 Å 9 Ca and NYE_S3. Alignment to 2BNQ chain A residues 57-84 (HLA helix-α1). (I) ScFv bound NY-ESO-1<sub>157-165</sub> conformations (3M4E5, PDB ID 3GJF and 3M4F4, 3HAE, SLLMWITQV peptides coloured orange) compared to wildtype 1G4 TCR-pHLA structure (2BNQ, SLLMWITQV peptide grey). For all panels, peptides are shown in stick representation and the HLA helix-α1 as wheat coloured ribbons.
Figure S3. Structural comparison of NY-ESO-1\textsubscript{157-165}–HLA-A2 bound versus unbound NYE\_S3 TCR. (A) Unbound TCR V\textalpha{} domain aligned to bound TCR V\textalpha{} from TCR-pHLA copy 1 in S3 crystal structure (alignment based on Ca positions for residues 8-128). (B) Unbound TCR V\textbeta{} domain aligned to bound TCR V\textbeta{} from TCR-pHLA copy 1 in NYE\_S3 crystal structure (alignment based on Ca positions for residues 8-128). (C) Unbound TCR V\textalpha{} domain aligned onto TCR-pHLA copy 1 in S3 crystal structure (alignment as in (A)). Peptide, pink sticks; HLA helix-\textalpha{}1, wheat ribbon. (D) Unbound TCR V\textalpha{} domain aligned to bound TCR V\textalpha{} from TCR-pHLA copy 1 in NYE\_S3 crystal structure (alignment as in (A)). View shown looking down the symmetry axis of the TCR (indicated by the black circle), through the hydrogen bonded TCR\textalpha{} Q44-TCR\textbeta{} Q44 sidechains towards pHLA. N, V\textalpha{} or V\textbeta{} N-terminus; C, V\textalpha{} or V\textbeta{} C-terminus. (E) RMSD (Å) between TCRs in the two TCR-pHLA copies versus one unbound TCR copy in ASU for S3 (comparing Ca positions for either V\textalpha{} residues 8-128, V\textbeta{} 3-128, CDR3\textalpha{} 105-117 or V\textalpha{} (no CDR3\textalpha{}) 8-105 and 117-128).