T-cell Receptor-optimized Peptide Skewing of the T-cell Repertoire Can Enhance Antigen Targeting

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Background: Current peptide vaccines may select suboptimal antigen-specific T-cells from polyclonal populations. Rationally designed altered peptide ligands may enable the preferential selection of high quality, antigen-sensitive T-cell clonotypes.

Significance: This proof-of-principle study could facilitate the development of more effective peptide vaccination strategies.

Altered peptide antigens that enhance T-cell immunogenicity have been used to improve peptide-based vaccination for a range of diseases. Although this strategy can prime T-cell responses of greater magnitude, the efficacy of constituent T-cell clonotypes within the primed population can be poor. To overcome this limitation, we isolated a CD8+ T-cell clone (MEL5) with an enhanced ability to recognize the HLA A*0201-Melan A27–35 (HLA A*0201-AAGIGILTV) antigen expressed on the surface of malignant melanoma cells. We used combinatorial peptide library screening to design an optimal peptide sequence that enhanced functional activation of the MEL5 clone, but not other CD8+ T-cell clones that recognized HLA A*0201-AAGIGILTV poorly. Structural analysis revealed the potential for new contacts between the MEL5 T-cell receptor and the optimized peptide. Furthermore, the optimized peptide was able to prime CD8+ T-cell populations in peripheral blood mononuclear cell isolates from multiple HLA A*0201 individuals that were capable of efficient HLA A*0201+ melanoma cell destruction. This proof-of-concept study demonstrates that it is possible to design altered peptide antigens for the selection of superior T-cell clonotypes with enhanced antigen recognition properties.

The key aim of vaccination is to establish populations of memory T-cells and B-cells expressing antigen receptors that provide a rapid, robust targeted immune response. Antigen receptors on B-cells undergo affinity maturation through a process of somatic hypermutation that allows evolution toward more effective responses over time. In contrast, the T-cell receptor (TCR) is fixed on individual T-cell clonotypes.

Antigen-specific T-cells bearing heterodimeric αβ TCRs play a pivotal role in adaptive immunity to pathogens and cellular malignancies by recognizing short peptide fragments bound to major histocompatibility complex (MHC) molecules on the target cell surface (1, 2). It is estimated that there are <10⁸ αβ TCRs in the human naive T-cell pool (3), a number that is dwarfed by the immense array of potential antigenic peptides that could be encountered (4). Evolution appears to have solved this conundrum by endowing T-cells with an extremely high degree of cross-reactivity (4–6). A corollary of this hypothesis is that multiple TCRs can recognize individual peptide-MHC (pMHC) antigens. Indeed, there is now evidence that antigen-specific T-cell populations, although frequently skewed toward dominant clonotypes present at high frequency, can be highly polyclonal (7–9).

To date, vaccine strategies have aimed to induce the largest T-cell responses possible without specific consideration of the individual clonotypes that constitute each response. However, emerging evidence suggests that the quality of a T-cell response, determined at the clonotypic level, might be more important than quantity, defined as the overall magnitude of a specific T-cell population (10–12). For example, clonotype usage within two different codominant simian immunodeficiency virus-specific CD8+ T-cell responses in Mamu A*01+ rhesus macaques can determine patterns of viral escape by anti-
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gen mutation during acute infection and act as a molecular signature of subsequent disease course (13, 14). Adoptive transfer experiments further underscore the fact that T-cell clonotypes recognizing the same antigen should not be viewed on an equal basis as their individual sensitivity to antigen density at the target cell surface is a critical determinant of in vivo efficacy (15). Thus, it seems that the efficacy of an antigen-specific T-cell response and its clonotypic architecture are inexorably linked. Consequently, the best vaccines should aim to induce the most effective T-cell clonotypes.

In many cases, the most effective T-cells are those that recognize target cells bearing low densities of cognate pMHC on their surface (10). Consistent with early studies using altered peptide ligands (APLs) with weak binding affinities (16), our own experiments using biophysically defined T-cell antigens indicate that the best T-cell agonists are those that engage the TCR with the highest affinities and for the longest dwell times (17, 18). Similarly, transduction experiments using TCRs with different affinities for defined natural antigens (19) also show, across multiple systems, that T-cells expressing higher affinity TCRs are more sensitive to antigen (17). Thus, TCRs with relatively high affinities are preferable when a high level of sensitivity to low antigen density is required. This feature becomes all the more important in the case of neoplastic targets as pMHC class I (pMHCI) antigen copy number at the cell surface is often very low (20). In addition, TCRs that recognize “self” tumor-associated antigens strongly are likely to be culled during thymic selection; as a consequence, antitumor TCRs bind their cognate pMHC antigens relatively weakly when compared with pathogen-specific TCRs (21). The importance of clonotypic composition within antigen-specific T-cell populations should therefore be taken into consideration when attempting to design optimal vaccine strategies that aim to elicit effective T-cell immunity.

Here, we examined a system that has been popular for therapeutic vaccination and adoptive T-cell transfer for the treatment of malignant melanoma, the leading cause of skin cancer-related deaths worldwide. Melanoma immunotherapy efforts have largely focused on an 18-kDa melanocyte-specific transmembrane protein called melanoma antigen A (Melan A), also termed primary melanoma antigen recognized by T-cells (MART-1) (22, 23), which represents a good candidate target antigen because it is expressed by ~90% of melanomas (22, 23). HLA A*0201-restricted Melan A-specific CD8+ T-cells derived from melanoma patients primarily recognize the Melan A27–35 (AAGIGILTV) and Melan A26–35 (EAAGIGILTV) peptides (24, 25). The AAGIGILTV peptide is the primary epitope expressed on the surface of tumor-derived cells (26). There is a large HLA A*0201-restricted Melan A-specific naive T-cell pool available for manipulation in both melanoma patients and healthy donors (27, 28). However, the natural antigens AAGIGILTV and EAAGIGILTV are poorly immunogenic (29) and, consequently, this system has become widely used to champion the use of MHC anchor-modified “heteroclitic” peptides. Indeed, the heteroclitic peptide ELAAGIGILTV has been used in many published studies and several vaccination trials (30–33). The ELAAGIGILTV peptide variant binds to HLA A*0201 with increased stability when compared with the two natural peptides and exhibits heightened immunogenicity (29, 34, 35). The shorter LAGIGILTV peptide variant adopts a similar bulged confirmation to ELAAGIGILTV in the HLA A*0201 binding groove (36) and is also more immunogenic than its natural AAGIGILTV counterpart (37). We have recently demonstrated that TCRs specific for Melan A26–35 Can distinguish between the heteroclitic ELAAGIGILTV variant and the natural EAAGIGILTV peptide sequence (34). Furthermore, the ELAAGIGILTV variant primes CD8+ T-cells in vitro that bear different TCRs when compared with those primed in parallel with the natural EAAGIGILTV peptide (34) despite the fact that HLA A*0201-ELAAGIGILTV and HLA A*0201-EAAAGIGILTV adopt similar unligated structures (36). Indeed, recent work by Speiser et al. (33) has shown that vaccination with the natural decapeptide (EAAGIGILTV) induces T-cells with superior lytic activity against tumor cells when compared with those induced by the commonly used heteroclitic ELAAGIGILTV variant. Thus, the use of anchor-modified heteroclitic peptides requires careful re-evaluation to ensure that T-cells with the best specificity and sensitivity for the intended target are elicited (34).

The TCR repertoire of HLA A*0201-restricted Melan A-specific CD8+ T-cell populations is extremely diverse (34, 38–40) and encompasses a spectrum of antigen recognition properties that influences the ability of individual T-cells to recognize the naturally expressed Melan A epitopes (41). To design an analog peptide that stimulates functionally superior T-cell clonotypes, we studied multiple Melan A-specific CD8+ T-cell clones and selected a candidate, MEL5, that recognized the dominant natural epitope, AAGIGILTV, at the lowest antigen density (34). Unlike other Melan A-specific TCRs, the MEL5 TCR bound the natural melanoma antigen with a dissociation constant (Kd) of ~14 μM; this is one of the strongest affinities for TCR interactions with natural self tumor antigens (21). We hypothesized that CD8+ T-cells with identical or similar specificities to MEL5 might represent the most desirable targets for optimal therapeutic vaccination (34). Accordingly, we used this model system to design and test a methodology for priming high-quality Melan A-specific CD8+ T-cells from the human naive T-cell pool.

EXPERIMENTAL PROCEDURES

Melanoma Cell Lines—The HLA A*0201+ melanoma cell lines MEL 526 and MEL 624 have been described previously (42, 43).

Generation and Maintenance of CD8+ T-cell Clones and Lines Specific for the HLA A*0201-restricted Melan A26–35 Antigen—The MEL5 and MEL187.65 CD8+ T-cell clones were generated and restimulated as described previously (18, 34). CD8+ T-cell lines specific for HLA A*0201-restricted Melan A epitopes were generated by pulsing 6 × 10⁶ peripheral blood mononuclear cells from healthy HLA A*0201+ individuals with 100 μM peptide (ELAAGIGILTV, AAGIGILTV, EAAGIGILTV, or FAAGIGILTV) for 1 h at 37°C; cells were subsequently washed and resuspended in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 10% heat-inactivated fetal calf serum (all Invitrogen) (R10 medium). Lines were tested for specificity using pMHCI tetramer staining and subsequently maintained in R10 with 37°C.
2.5% Cellkines (Helveticah Healthcare), 20 IU/ml IL-2, and 25 ng/ml IL-15 (both PeproTech). Throughout this study, altered native sequence amino acid residues in antigenic peptides are indicated by bold underlined text.

**MIP-1β Release Assay**—MEL5 or MEL187.c5 CD8+ T-cell clones were incubated overnight at 37 °C with C1R A*0201 (C1R A2) cells (44) pulsed prior to assay for 1 h with peptide at various concentrations as indicated. After incubation, supernatant was harvested and assayed for MIP-1β by ELISA according to the manufacturer’s instructions (R&D Systems).

**Decamer Combinatorial Peptide Library Scan**—The decamer combinatorial peptide library (CPL) contained a total of 9.36 × 10¹² ((10 + 19) × 19⁵) different decamer peptides (45, 46) and was divided into 200 different positional scanning peptide pulsing, 3 and 21.3 (Beckman Coulter) and anti-human Vα antibodies for 30 min at 4 °C. Cells were stained with either allophycocyanin-conjugated anti-human CD8 (clone SK1, BD Biosciences) or fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 (clone RPA-T8; BD Biosciences) or fixable aqua amine-reactive fluorescent dye (Invitrogen) for 15 min at room temperature, washed once, and stained with allophycocyanin-conjugated tetramer (either HLA A*0201-restricted CD8+ T-cells-specific for Melan A26–35 were plated out in a final volume of 200 μl of R10 at E:T ratios of 25:1 to 0.1:1 with respect to each target; this ratio was determined by HLA A*0201-EAGGIGILTV tetramer staining immediately before assay to quantify the percentage of antigen-specific CD8+ T-cells in each line. The plates were then incubated for 4 h at 37 °C. For each sample, 20 μl of supernatant was harvested after incubation and mixed with 150 μl of OptiPhase scintillation mixture (PerkinElmer Life Sciences). Data were acquired using a liquid scintillator and luminescence counter (MicroBeta TriLux; PerkinElmer Life Sciences) with MicroBeta Windows Workstation software (PerkinElmer Life Sciences). Specific lysis was calculated according to the following formula: (experimental release – spontaneous release)/total release – spontaneous release) × 100.

**pMHCI Stability Assays**—SPR was used to determine pMHCI stability as described previously (34, 35). Circular dichroism measurements of thermal stability were performed with purified, soluble HLA A*0201-peptide using an Aviv 62DS spectrometer monitoring a wavelength of 218 nm as described previously (52). Solution conditions were 20 mm phosphate, 75 mm NaCl, pH 7.4. Protein concentrations were 10 μM. A temperature increment of ~0.3 °C/min was used. As unfolding is irreversible, data were normalized and fitted to a six order polynomial, and the Tm was taken as the midpoint of the fitted curve.
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Crystalization, Diffraction Data Collection, and Model Refinement—HLA A*0201-ELAGIGILTV and HLA A*0201-FLT7GIGILTV crystals were grown at 18 °C by vapor diffusion with the sitting drop technique, using a Phoenix robot (Alpha Biotech). Multiple attempts to cocrystallize MEL5 TCR/HLA A*0201-FLT7GIGILTV failed. Crystals of HLA A*0201-ELAGIGILTV and HLA A*0201-FLT7GIGILTV appeared in 0.1 M Mes, pH 7.0, 0.2 M ammonium sulfate, and 20% PEG 8000. Data were collected at 100 K on beamline IO3 at the Diamond Light Source (Oxfordshire, UK) using a wavelength of 0.976 Å with an Area Detector Systems Corporation Q315 CCD detector. Reflection intensities were estimated with XIA2 using the MOSFLM or XDS packages (53), and the data were scaled, reduced, and analyzed with SCALA and the CCP4 package (54). The structures were solved with Molecular Replacement using PHASER (55). Model sequences were adjusted with COOT (56), and the models were refined with REFMAC5 (57). Graphical representations were prepared with PyMOL (58). Data reduction and refinement statistics are shown in supplemental Table S1.

RESULTS

Melan A-specific CD8+ T-cells Exhibit Substantial Diversity in Their Ability to Recognize Natural Tumor Epitopes—It is established that the ELAGIGILTV and EAAGIGILTV peptides exhibit differential binding to HLA A*0201 (34, 59). The dominant natural peptide at the melanoma surface, AAGIGILTV (26), does not contain an optimal primary anchor residue for HLA A*0201 binding toward the N terminus; thus, the HLA A*0201-AAGIGILTV complex is likely to be unstable. To test this, we investigated the secondary structure of HLA A*0201-AAGIGILTV using circular dichroism over a range of temperatures (supplemental Fig. S1A). As expected, the HLA A*0201-AAGIGILTV complex was less stable than HLA A*0201-ELAGIGILTV and considerably less stable than HLA A*0201-ELAGIGILTV, with denaturation temperatures of 37, 39, and 55 °C, respectively. The enhanced stability of HLA A*0201-ELAGIGILTV when compared with HLA A*0201-EAAGIGILTV and HLA A*0201-AAGIGILTV (supplemental Fig. S1A) (34, 59) likely translates into substantially higher cell surface densities of HLA A*0201-ELAGIGILTV at any given exogenous peptide concentration relative to the natural antigens. Indeed, this difference in antigen density may contribute to the ability of some CD8+ T-cell lines to recognize ELAGIGILTV at lower exogenous concentrations when compared with the natural peptides (60).

Our recent work has shown that individual TCRs can exhibit substantially different binding to HLA A*0201-ELAGIGILTV and HLA A*0201-ELAGIGILTV, thereby demonstrating a preference for either type of antigen (34). The two extremes of variant recognition that we have observed with HLA A*0201-restricted CD8+ T-cell clones are depicted in supplemental Fig. S1, B and C. Most clonotypes exhibit a recognition pattern similar to MEL187.c5 (60), which recognizes the ELAGIGILTV heteroclitic variant at lower peptide concentrations than the natural sequences. Despite this preference for the ELAGIGILTV peptide at the population level, however, relatively rare individual clonotypes exist that do not conform to this pattern (61). The MEL5 clone epitomizes this incongruity and exhibits potent recognition of the dominant natural AAGIGILTV peptide. Indeed, this clone appears to compensate for the reduced HLA A*0201 binding of the natural EAA-GIGILTV and AAGIGILTV peptides (supplemental Fig. S1B), suggesting that the clonotypic TCR must bind with greater affinity to these variants than to HLA A*0201-ELAGIGILTV.

Consistent with these predictions, the MEL5 TCR bound to the natural epitope, HLA A*0201-EAAGIGILTV, with an affinity that lay within the range normally observed for TCRs that recognize pathogen-derived epitopes (KD ~6.4 μM) (Table 1) (34). To the best of our knowledge, this is the strongest affinity yet described for TCR binding to a non-MHC anchor-modified self-derived peptide. The MEL5 TCR bound to HLA A*0201-AAGIGILTV with a slightly weaker affinity (KD ~14 μM), but this was still stronger than that observed for the HLA A*0201-ELAGIGILTV complex (KD ~17 μM) (supplemental Fig. S1D and Table 1). Thus, although the MEL187.c5 and MEL5 TCRs bound to HLA A*0201-ELAGIGILTV with almost identical affinities (Table 1), very different interactions were apparent with the endogenous peptides, AAGIGILTV and ELAGIGILTV, which are presented on the melanoma cell surface. The MEL187.c5 TCR bound to HLA A*0201-AAGIGILTV and HLA A*0201-ELAGIGILTV with dissociation constants of 94 and 42 μM, respectively, compared with the corresponding values of 14 and 6.4 μM for the MEL5 TCR (supplemental Fig. S1, D and E and Table 1). Collectively, these data demonstrate that the MEL5 TCR is an outlier within the population of clonotypic TCRs that are able to engage Melan A27–35 and Melan A26–35 in the context of HLA A*0201 as it exhibits a preference for the natural antigens over the heteroclitic variant. We reasoned that T-cells bearing TCRs with specificities and binding affinities similar to MEL5, which can recognize the dominant natural antigen on the surface of melanoma cells efficiently, would represent the most effective clonotypes with which to target melanoma cells.

Generation of Optimized Melan A26–35 Analogues That Preferentially Activate CD8+ T-cells with Superior Natural Tumor Epitope Recognition Properties—CPL scan technology was used to design a peptide with two key properties necessary for the preferential induction of MEL5-like CD8+ T-cells from the naive pool. Specifically, we considered that the ideal TCR-optimized peptide (TOP) for priming purposes should exhibit: (i) enhanced binding to the MEL5 TCR and (ii) decreased binding

| Ligand                 | MEL5   | MEL187.c5 |
|------------------------|--------|-----------|
| HLA A*0201-ELAGIGILTV  | 17     | 18        |
| HLA A*0201-EAAGIGILTV  | 6.4    | 42        |
| HLA A*0201-AAGIGILTV   | 14     | 94        |
| HLA A*0201-ELAGIGILTV  | 3      | 35        |

TABLE 1

Binding affinities of the MEL5 and MEL187.c5 TCRs to Melan A peptide variants

Data are summarized from this study and Ref. 34.
to TCRs with similar specificities that mediate poor killing of melanoma cells. A CPL scan of the MEL5 CD8+ T-cell clone (Fig. 1A) showed that a restricted number of amino acid combinations were recognized in the central region of the peptide (residues 4–6), consistent with the fact that the TCR makes the majority of its peptide contacts with these residues (50). In contrast, recognition at peptide positions beyond this central core was highly degenerate. Indeed, when the results of four different individual CPL scans were combined, three of the top four activating sublibraries contained a fixed amino acid that was not in the central core and did not occur in the natural sequence (Fig. 1B). Overall, the MEL5 TCR showed a preference for amino acids phenylalanine, threonine, and isoleucine at positions 1, 3, and 8 of the antigenic peptide, respectively. These amino acids do not occur in the natural peptide (Fig. 1).

Single amino acid substitutions in the FLAGIGILTV peptide differentially altered recognition by the MEL187.c5 and MEL5 CD8+ T-cell clones (Fig. 2A). In contrast, a triple substitution (1F, 3T, and 8I) in the FLATGIGILTV peptide produced an analog (FLTGI GITV) that showed an enhanced ability to activate both of these clones (Fig. 2B). However, the same triple substitution (1F, 3T, and 8I) in the natural EAGAGILTV peptide produced an analog (FATGIGILTV) with different properties. The FATGIGILTV peptide acted as a superagonist for the MEL5 CD8+ T-cell clone, which preferentially recognizes naturally processed melanoma epitopes, but activated the MEL187.c5 CD8+ T-cell clone poorly (Fig. 2C). This difference between the FLTGI GITV and FATGIGILTV peptides was observed across several different effector functions (supplemental Fig. S2). Real time SPR-based analysis of HLA A*0201- peptide complex stability (34) and HLA A*0201 cell surface stability assays showed that HLA A*0201-FLTGI GITV and HLA A*0201-FATGIGILTV were more stable than the natural HLA A*0201-EAGAGILTV complex (\(t_1/2 = 25, 13, \text{and} \ 8\ 	ext{h, respectively}; \) Fig. 3). In accordance with the HLA A*0201 preferred binding motif XLXXXXY V/L (62, 63), the FLTGI GITV peptide was almost twice as stable in complex with HLA A*0201 when compared with the FATGIGILTV peptide (Fig. 3A). Similar results were obtained using a T2 surface binding assay (Fig. 3B).

The FATGIGILTV and FLTGI GITV Peptides Bind Differentially to the MEL5 and MEL187.c5 TCRs—The observation that the MEL5 and MEL187.c5 CD8+ T-cell clones exhibited differential recognition of the FATGIGILTV and FLTGI GITV peptides prompted us to examine the biophysical basis for these differences. Both the MEL187.c5 and the MEL5 TCRs bound similarly to the common heteroclitic peptide complex HLA A*0201-ELAGIGILTV (K\(_D\) = 18 \text{\mu M}; Table 1). The triple-substituted heteroclitic FLTGI GITV peptide in complex with HLA A*0201 displayed stronger affinity interactions with both the MEL5 (K\(_D\) = 5.1 \text{\mu M}; Fig. 4A) and the MEL7.c5 (K\(_D\) = 16 \text{\mu M}; Fig. 4B) TCRs, consistent with the observation that this peptide activated both of the corresponding CD8+ T-cell clones more potently than the ELAGIGILTV peptide (Fig. 2B and supplemental Fig. S2). In contrast, the triple-substituted natural FATGIGILTV peptide in complex with HLA A*0201 displayed a substantially stronger affinity interaction with the MEL5 TCR (K\(_D\) = 3 \text{\mu M}; Fig. 4C) and a weaker affinity interaction with the MEL7.c5 TCR (K\(_D\) = 35 \text{\mu M}; Fig. 4D), again consistent with the activation data (Fig. 2C and supplemental Fig. S2). These TCR binding properties at the monomeric level translated into consistent patterns with respect to pMHC tetramer staining (Fig. 5, A and B) and kinetics (Fig. 5, C–F) at the cell surface.

To investigate the structural mechanism governing the different biophysical and cellular effects of the FATGIGILTV, FLTGI GITV, and ELAGIGILTV peptides, we solved the atomic structures of HLA A*0201-FATGIGILTV to 2.75 Å and HLA A*0201-FLTGI GITV to 2.35 Å (supplemental Table S1). We compared the structure of HLA A*0201-FATGIGILTV to our recently solved structure of the MEL5 TCR in complex with HLA A*0201-ELAGIGILTV (Fig. 6A) (50). This comparison suggested that a GluP1 to PheP1 mutation could lead to an increase in both FATGIGILTV and FLTGI GITV peptide affinity for HLA A*0201 due to \(\pi-\pi\) stacking of the PheP1 side chain.
with that of Trp-167 in the MHC α2 domain, sandwiched into position by the side chain of Tyr-59 in the MHC α1 domain (Fig. 6C). A very similar result was seen with a modified version of the WT1 peptide, in which substitution of the P1 residue substantially enhanced HLA-A2 binding via \( \pi-\pi \) stacking (64). Additionally, there could be new contacts between the PheP1 side chain and Gly-29 of the MEL5 CDR1 loop. Interestingly, PheP1 in HLA A*0201-F\( \text{A} \)T\( \text{G} \)I\( \text{I} \)TV extended farther out of the groove when compared with HLA A*0201-FLT\( \text{G} \)I\( \text{I} \)TV (Fig. 6B). This difference, probably a consequence of extra flexibility allowed by the weaker interaction between AlaP2 and the B-pocket of HLA A*0201, could enable closer contacts between AlaP2 and the B-pocket of HLA A*0201, which could explain the stronger binding affinity when compared with MEL5 and HLA A*0201-FLT\( \text{G} \)I\( \text{I} \)TV. In both the HLA A*0201-F\( \text{A} \)T\( \text{G} \)I\( \text{I} \)TV and the HLA A*0201-FLT\( \text{G} \)I\( \text{I} \)TV structures, the AlaP3 to ThrP3 mutation enabled a new interaction between ThrP3 and HLA A*0201, which could explain the increased stability of the peptide in the binding groove (Fig. 6D). However, it is unlikely that the ThrP3 mutation could lead directly to new TCR contacts. The ThrP8 to IleP8 mutation could mediate a new nonpolar bridge between the HLA A*0201-peptide complex and the TCR, which would generate a genuine increase in affinity at the expense of exposing a nonpolar side chain on the surface of the unligated HLA A*0201-peptide structure (Fig. 6E).

The MEL5 TCR-optimized Analog Peptide \( \text{FA} \)T\( \text{G} \)I\( \text{I} \)TV Can Prime Large Populations of Melan A-specific CD8\( ^{+} \) T-cells from HLA A*0201 Peripheral Blood Mononuclear Cells—The commonly used Melan A\( _{26-35} \) heteroclitic peptide E\( \text{L} \)A-GIGILTV primes large populations of antigen-specific CD8\( ^{+} \) T-cells from the peripheral blood of healthy donors \textit{in vitro} (Fig. 7A) and has been widely used in the clinic (30–33). However, it has been recently shown that the natural decamer EAA-GIGILTV peptide can trigger human CD8\( ^{+} \) T-cells with stronger tumor reactivity when compared with the heteroclitic ELA-GIGILTV peptide (33). Using direct \textit{ex vivo} priming from healthy HLA A*0201 peripheral blood mononuclear cell preparations, we tested our hypothesis that the \( \text{FA} \)T\( \text{G} \)I\( \text{I} \)TV peptide might induce better quality CD8\( ^{+} \) T-cell responses. In 6/10 donors, the \( \text{FA} \)T\( \text{G} \)I\( \text{I} \)TV peptide primed a larger population of EAAGIGILTV-specific CD8\( ^{+} \) T-cells (supplemental Table S2). In the remaining four donors, the \( \text{FA} \)T\( \text{G} \)I\( \text{I} \)TV peptide primed a comparable or smaller population of EAAGIGILTV-specific CD8\( ^{+} \) T-cells (supplemental Table S2). Importantly, in...
contrast to CD8+ T-cells primed with either EAAGIGILTV or AAGIGILTV, the FATGIGITV peptide primed a population of CD8+ T-cells that stained with the HLA A*0201-EAAGIGILTV tetramer in all donors. The consistent priming potency of the ELAGIGITV peptide has made it the preferred ligand for immunotherapeutic modulation in vivo. In this regard, the observation that the FATGIGITV peptide also consistently primed antigen-specific CD8+ T-cells, in some cases in greater numbers than ELAGIGITV (although this difference was not statistically significant), was unanticipated and suggests that it could be a candidate for peptide vaccination.

Next, we tested the ability of FATGIGITV-primed CD8+ T-cells to kill tumor cells. Enhanced tumor lysis when compared with ELAGIGITV-primed CD8+ T-cells was observed in 3/7 donors with MEL 526 targets (Fig. 7B and supplemental Fig. S3, E and G) and in 2/7 donors with MEL 624 targets (Fig. 7C and supplemental Fig. S3F). Comparable tumor responses were observed in 3/7 donors with MEL 526 targets (supplemental Fig. S3, A, C, and I) and 3/7 donors with MEL 624 targets (supplemental Fig. S3, D, H, and J), and reduced tumor responses were observed in the remaining three instances (supplemental Fig. S3, B, K, and L).

Thus, the FATGIGITV peptide primed a larger population of CD8+ T-cells when compared with the ELAGIGITV tetramer in all donors. The consistent priming potency of the ELAGIGITV peptide has made it the preferred ligand for immunotherapeutic modulation in vivo. In this regard, the observation that the FATGIGITV peptide also consistently primed antigen-specific CD8+ T-cells, in some cases in greater numbers than ELAGIGITV (although this difference was not statistically significant), was unanticipated and suggests that it could be a candidate for peptide vaccination.
“industry standard” in 6/10 donors. In the majority of donors (79%), these primed CD8+ T-cell populations exhibited either an enhanced (36%) or a comparable (43%) ability to recognize and destroy melanoma cells.

CD8+ T-cell Populations Primed with the FATGIGILTV Peptide Are Clonotypically Distinct from Those Primed with the ELAGIGILTV Peptide—We used a panel of TCR Vβ antibodies to examine Vβ usage in HLA A*0201-EAAGIGILTV tetramer+ CD8+ T-cell populations primed with the ELAGIGILTV heteroclitic variant and those primed with the FATGIGILTV peptide. As might be expected, there was some overlap between ELAGIGILTV-primed and FATGIGILTV-primed CD8+ T-cell populations, with both showing preferential usage of Vβ 3, 13.2, and 14 TCRs (Fig. 8). Interestingly, an over-representation of Vβ 14 usage in HLA A*0201-Melan A26–35 tetramer+ CD8+ T-cells has been observed previously in ELAGIGILTV-primed populations (40). However, each peptide also induced some unique TCRs. For example, in donor 1 priming with FATGIGILTV produced an HLA A*0201-EAAGIGILTV tetramer+ population with 16.5% Vβ 1, 11.4% Vβ 9, and 2.1% Vβ 13.1 usage; in comparison, parallel priming with ELAGIGILTV generated a population with 0% Vβ 1, 3.2% Vβ 9, and 7.8% Vβ 13.1 usage.

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Similar results were observed in the corresponding HLA A*0201-ELAAGIGILTV tetramer population (supplemental Fig. S4). The ELAAGIGILTV-primed lines contained HLA A*0201-ELAAGIGILTV tetramer + CD8+ T-cells expressing Vβ 7.1 and 21.3 that were rare in CD8+ T-cell populations expanded by the FATGIGIITV peptide (Fig. 8). Collectively, these data show that subtle directed changes in the antigenic peptide can alter the clonotypic repertoire of a T-cell response.

**DISCUSSION**

It is possible to expand and maintain large numbers of functionally active tumor-specific CD8+ T-cells from the peripheral blood of cancer patients and healthy donors by repeated peptide-based vaccination. Despite this achievement, however, objective clinical response rates in peptide vaccine trials to date are low (<3%) and not significantly higher than spontaneous remission rates (65). One possible reason for this general lack of success is the inadvertent induction of ineffective CD8+ T-cell clonotypes with low levels of sensitivity for the natural antigenic target that are unable to control tumor growth in vivo. A popular approach for developing peptide candidates for vaccination is to introduce substitutions at MHC anchor residue positions that enhance the stability of MHC binding and, as a result, immunogenicity in vivo. The majority of tumor-derived HLA A*0201-restricted peptides do not contain an ideal MHC binding consensus, and the use of anchor-modified heteroclitic peptides in these systems is widespread (34). We have recently shown that TCRs can differentiate between natural and anchor-modified heteroclitic peptides, enabling T-cells to exhibit a strong preference for either type of antigen (34). Accordingly, MHC anchor-modified heteroclitic peptides can induce T-cell populations that are clonotypically distinct from those induced by natural tumor epitopes (34, 66). As a result, T-cells primed with MHC anchor-modified peptides can exhibit poor cross-recognition of the naturally occurring tumor antigen (33, 34). Thus, vaccination with MHC anchor-modified peptides may elicit T-cells that exhibit suboptimal recognition of the intended natural antigen and, consequently, impaired functional attributes in vivo (34). It is therefore important that the T-cell clonotypes induced by any APL-based immune intervention are carefully evaluated after ex vivo priming to ensure efficacy prior to studies in vivo.

An alternative approach to improve T-cell epitopes from tumor-associated antigens is to enhance their engagement with cognate TCRs. This approach has the advantage that the resultant APLs are highly likely to be viewed as “nonself” and may therefore have an increased chance of breaking immune tolerance. However, the use of any APL also runs the risk of inducing clonotypes that are ineffective at recognizing the native sequence. Several previous studies have attempted this approach and are therefore relevant to the current discussion. Walden and colleagues (67) demonstrated that CPL scans can be used to generate mimotopes for tumor-reactive CD8+ T-cells when the natural antigen is unknown. A CPL-selected mimotope that activated a cutaneous T-cell lymphoma-reactive HLA B*08-restricted CD8+ T-cell clone was able to induce populations of T-cells that killed tumor cells in vitro (67). Vac-
T-cell repertoire

The optimized HIV-1 p24 Gag-derived peptide TLNAWVKV (69). This mimotope failed to mobilize clonotypes that were more efficient at recognizing natural targets (69). A similar approach has been attempted to improve CD8+ T-cell responses to human carcinoembryonic antigen (CEA). CEA is expressed in the majority of colonic, rectal, gastric, and pancreatic tumors (70), 70% of lung carcinomas (71), and 50% of breast carcinomas (72), and there has been interest in a CEA-derived, HLA A*0201-majority of colonic, rectal, gastric, and pancreatic tumors (70), 70% of lung carcinomas (71), and 50% of breast carcinomas (72), and there has been interest in a CEA-derived, HLA A*0201-restricted epitope (YLSGANLN) known as CAP1 (73). Substitution of CAP1 at position 6, a TCR contact residue, with aspartic acid to generate the APL YLSGADLNl enhanced CAP1-specific CD8+ T-cell recognition in amplified lines by >100-fold when compared with the native CAP1 peptide (73). This superagonist variant, designated CAP1-6D, induced substantially larger responses in vitro when compared with the native sequence (73). Furthermore, CAP1-6D-generated CD8+ T-cell lines killed CEA-expressing human tumor lines efficiently (73). Subsequent studies in this system, however, demonstrated that the predominant CD8+ T-cells primed with the CAP-6D peptide displayed restricted TCR usage and lower levels of sensitivity for the natural antigen (74). Thus, previous studies with TOPs have failed to improve either the functional qualities of the selected clonotypes or the overall efficacy of the T-cell response in comparison with the native antigen (33, 69).

Unlike the process described herein, however, these previous approaches did not use improved ligands for the best T-cell clonotype. The optimized HIV-1 p24 Gag-derived peptide used by Kan-Mitchell and colleagues (69) was selected because it exhibited improved recognition by multiple polyclonal CD8+ T-cell lines. This approach does not optimize a ligand for a specific and beneficial functional phenotype, and so is equally likely to improve recognition by good and poor clonotypes alike. Such “universal” optimization may also combine with the selection of APL-specific clonotypes that recognize the natural sequence poorly to ensure that although the magnitude of the APL-induced response might far exceed that generated by the natural antigen, the overall quality of the mobilized T-cell population with respect to recognition of the intended natural target will be inferior.

The approach adopted here incorporates some fundamental differences to the attempts that preceded it. Most notably, we started by selecting a CD8+ T-cell clonotype with superior natural target antigen recognition properties. In this way, we hoped to skew the expanded CD8+ T-cell population toward clonotypes with optimal antitumor efficacy. We describe this approach as “TCR-optimized peptide skewing of the repertoire of T-cells” (TOPSORT). The primary natural Melan A-derived antigen on the surface of melanoma cells, AAGIGILTV (26), is an extremely poor immunogen. Accordingly, the highly immunogenic MHC anchor-modified peptide ELAGIGILTV has been widely adopted in the clinic (30–33). This approach has failed, however, and it has become apparent that HLA A*0201-ELAGIGILTV is recognized differently by TCRs when compared with the natural antigen (34). Consequently, HLA A*0201-ELAGIGILTV can induce populations of T-cells that bear TCRs with poor reactivity against the natural antigen (34). The naive Melan A-specific T-cell population is characterized by a highly diverse TCR repertoire and, as a result, exhibits a large degree of functional diversity. We selected a Melan A-specific CD8+ T-cell clone (MEL5) that displayed potent recognition of the natural tumor epitopes EAAGIGILTV and AAGIGILTV and then used CPL scan technology to identify analog peptides that exhibited improved recognition by this clonotype. The CPL scan data revealed that MEL5 exhibited a preference for phenylalanine, threonine, and isoleucine at positions 1, 3, and 8 of the antigenic peptide, respectively. Insertion of the triple mutation into the EAAGIGILTV peptide sequence produced the FATGIGIITV peptide. Biophysical data demonstrated that HLA A*0201-FATGIGIITV bound the MEL5 TCR with an affinity >10-fold stronger than that measured for the alternative MEL187.c5 TCR. These findings were confirmed with cell surface pMHC tetramer staining experiments. As a consequence, the FATGIGIITV peptide was a poor stimulator of the MEL187.c5 clone, which preferentially recognizes ELAGIGILTV, yet potently stimulated the MEL5 clone, which preferentially recognizes the natural peptide antigens. Indeed, the MEL5 TCR engaged the dominant natural antigen (HLA A*0201-AAGIGILTV) with a relatively high affinity ($K_d \sim 14 \mu M$), which explained the antitumor efficacy of the MEL5 clone.

The magnitude of the HLA A*0201-EAGIGILTV tetramer+ CD8+ T-cell population primed by FATGIGIITV exceeded that primed by ELAGIGILTV in 6/10 donors tested and was comparable or reduced in 4/10 donors. Furthermore, FATGIGIITV-primed CD8+ T-cells were clonotypically distinct from those induced by the ELAGIGILTV peptide and were capable of enhanced melanoma cell killing in 36% of donors. Notably, clonotypic analysis has revealed that the MEL5 TCR $\beta$-chain ($\beta_\beta 20.1$) was not ubiquitously expressed in all donors (75). Therefore, although FATGIGIITV improved antigen recognition by the Melan A-sensitive MEL5 clone, this clonotype might be absent in some donors, which likely explains why this approach was not universally superior when compared with priming with ELAGIGILTV. Thus, the availability of highly shared, or public, clonotypes may dictate the overall applicability of TOPSORT (76). It is not known whether there is an effective public clonotype for the HLA A*0201-AAGIGILTV antigen.

The observation that the FATGIGIITV peptide elicits CD8+ T-cell populations with a different clonotypic architecture when compared with ELAGIGILTV raises the possibility that some unwanted or autoreactive T-cell clones might be selected. This seems unlikely, however, because the primed clonotypes in both cases will have been through the same thymic editing process. Combined with peripheral tolerance mechanisms,
TCR-optimized Peptide Skewing of the T-cell Repertoire

there is therefore no a priori reason to suspect that FATGIGI-
TV-primed autologous T-cells will be more deleterious than
autologous T-cells primed with other natural or heteroclitic peptides.

The TOPSORT process used to generate the FATGIGI-TV sequence selected in this study differs in a number of key aspects from previous studies. These differences include: (i) identification of a high quality T-cell clonotype from the polyclonal population induced by the natural antigen; (ii) selection of a TOP superagonist for this clonotype; (iii) elimination of mutations that favor both “good” and “bad” clonotypes; and (iv) verification that superior functional qualities are induced in TOP-primed responses relative to those primed by the natural antigen. The work described here represents a proof-of-concept study for the use of TOPSORT, although confirmation of efficacy in vivo is still required. Nonetheless, combined with emerging next generation sequencing strategies for comprehensive TCR repertoire analysis, it can be envisaged that this approach might enable the rational design of individualized peptide-based vaccines.

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