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Structure-Based Design of Altered MHC Class II–Restricted Peptide Ligands with Heterogeneous Immunogenicity

Shuming Chen,*1 Yili Li,†,1 Florence R. Depontieu,* Tracee L. McMiller,* A. Michelle English,‡ Jeffrey Shabanowitz,‡ Ferdynand Kos,§ John Sidney,¶ Alessandro Sette,* Steven A. Rosenberg,‖ Donald F. Hunt,† Roy A. Mariuzza,† and Suzanne L. Topalian*

Insights gained from characterizing MHC–peptide–TCR interactions have held the promise that directed structural modifications can have predictable functional consequences. The ability to manipulate T cell reactivity synthetically or through genetic engineering might thus be translated into new therapies for common diseases such as cancer and autoimmune disorders. In the current study, we determined the crystal structure of HLA-DR4 in complex with the nonmutated dominant gp100 epitope gp10044–59, associated with many melanomas. Altered peptide ligands (APLs) were designed to enhance MHC binding and hence T cell recognition of gp100 in HLA-DR4+ melanoma patients. Increased MHC binding of several APLs was observed, validating this approach biochemically. Nevertheless, heterogeneous preferences of CD4+ T cells from several HLA-DR4+ melanoma patients for different gp100 APLs suggested highly variable TCR usage, even among six patients who had been vaccinated against the wild-type gp100 peptide. This heterogeneity prevented the selection of an APL candidate for developing an improved generic gp100 vaccine in melanoma.

Our results are consistent with the idea that even conservative changes in MHC anchor residues may result in subtle, yet crucial, biochemical effects on peptide contacts with the TCR or on peptide dynamics, such that alterations intended to enhance immunogenicity may be unpredictable or counterproductive. They also underscore a critical knowledge gap that needs to be filled before structural and in vitro observations can be used reliably to devise new immunotherapies for cancer and other disorders. The Journal of Immunology, 2013, 191: 5097–5106.

Melanoma is an aggressive form of skin cancer that is curable in its early stages but carries a poor prognosis following distant organ metastasis (1). It is also highly immunogenic, as evidenced by endogenous antimelanoma T and B cell responses and the susceptibility of melanoma to drugs with a purely immunological mode of action, such as IL-2 (2), anti-CTLA-4 (3), anti–programmed cell death-1 (4), and anti–programmed cell death ligand-1 (5). Efficient vaccination with tumor-specific Ags can redirect the antitumor immune response and provide synergistic treatment effects when combined with systemic immune-enhancing agents (6–8). Thus, there is a need to develop optimal cancer vaccines and tumor Ag-specific detection methods for monitoring treatment outcomes in vitro. Rational chemical modification of tumor-specific peptide Ags to increase their immunogenicity, based on structural models, may facilitate this approach.

Gp100, a melanocyte lineage-specific transmembrane glycoprotein, is expressed in most melanomas and is involved in a multiple-step process of pigment production (9). Gp100 has been a widely used target for melanoma immunotherapy since the demonstration that tumor-infiltrating lymphocytes and circulating T cells from melanoma patients commonly recognize this Ag (10, 11). Despite the fact that the most gp100-directed melanoma therapies have focused on stimulating CD8+ T cell responses, CD4+ T cells play a central role in inducing and maintaining tumor-specific CD8+ T cells (12). Devising immunotherapies that can efficiently raise specific CD4+ T cell responses is therefore an important goal.

A gp100 MHC class II (MHC II)–restricted peptide, gp10044–59, was identified from HLA-DRB1*0401 (hereafter HLA-DR4)–positive melanoma cell lines (13) and was subsequently validated as a dominant epitope in a transgenic animal model (14). This peptide can generate melanoma-specific CD4+ T cells from the peripheral blood of melanoma patients following repetitive in vitro stimulation (14, 15). Nevertheless, in a clinical trial using gp10044–59 as a vaccine, no enhancement of gp100-specific reactivity

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Abbreviations used in this article: APL, altered peptide ligand; CLIP, collagen II; CM, complete medium; ETD, electron transfer dissociation; HA, influenza hemagglutinin; IGV, in vitro stimulation; LTQ, linear quadrupole ion trap; MART1, melanoma Ag recognized by T cells 1; MBP, myelin basic protein; MHC II, MHC class II; MS/MS, tandem mass spectrometry; WT, wild-type.
was detected in the peripheral blood of patients following vacci-
nation, dampening enthusiasm for its therapeutic potential (16).

Because gp100\textsubscript{144-59} is a nonmutated self-Ag with intermediate
binding affinity for HLA-DR4 (15), we hypothesized that altered
tooligopeptides (APLs) with single amino acid substitutions could
be designed to confer higher MHC binding affinity and hence
improved immunogenicity. Such APLs derived from gp100 MHC
class I-restricted epitopes have been employed as melanoma vac-
cines (17–20). Whereas unmodified HLA-A2-restricted gp100\textsubscript{209-217}
and gp100\textsubscript{380-388} peptides induced melanoma-reactive CTLs from
limited numbers of melanoma patients in vitro, and numerous
restimulations were required, the APLs gp100\textsubscript{209-217}(210M) and
gp100\textsubscript{280-288}(288V) with enhanced MHC affinity showed superior
immunogenicity in vitro and in vivo (17). Similarly, in mice,
a variant of gp100 that bound H-2D\textsuperscript{\textit{b}} with increased affinity indi-
cated high frequencies of melanoma-specific CTLs in the en-
dogenous CD8\textsuperscript{+} repertoire (21).

APLs based on MHC II-restricted epitopes have rarely been
explored, because these peptides are heterogeneous in length
and more degenerate in MHC binding specificity than class I-re-
stricted peptides (22), making it difficult to precisely define MHC
II-specific peptide binding motifs. However, combined informa-
tion from MHC II–peptide crystal structures, ligand sequencing,
and binding affinity determinations has enriched our knowledge
of the general chemical properties permitting optimal peptide binding
to HLA-DR4. A dominant large hydrophobic or aromatic residue
in the P1 binding position, a hydroxylated residue at P6, and a
hydrophobic or polar residue at P9 appear to be favored (22–25).

To obtain precise information about the binding characteristics
of gp100\textsubscript{144-59} to HLA-DR4 as a basis for designing optimal
melanoma vaccines and immunomonitoring tools, we determined
the crystal structure of this MHC–peptide complex. APLs based
on structural data were compared with the wild-type (WT) peptide
for their ability to detect gp100-specific reactivity in melanoma
patients vaccinated against the WT peptide or to raise melanoma-
specific T cells from prevaccination PBMCs.

Materials and Methods

Isolation and sequencing of native gp100 peptides complexed
to HLA-DR4

Peptide–HLA-DR complexes were isolated from cultured 1102-mel mel-
anoma cells on an anti–HLA-DR affinity column and peptides were eluted
as described (26). Peptides were then analyzed by nanoflow HPLC–
microelectrospray ionization coupled to either a hybrid linear quadrupole
ion trap (LTQ)–Fourier-transform ion cyclotron resonance mass spectrom-
trometer or an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific)
modified to perform electron transfer dissociation (ETD). Data were
acquired as previously described (27). In brief, a precolumn loaded with 1e7
cell equivalents of MHC peptides was connected with polytetrafluoro-
ethylene tubing (0.016-inch outer diameter and 0.012-inch inner diameter;
Zorbax SB C18, 300A) to the end of columns (500\textmu; m outer diameter and 50 \textmu;m inner diameter) containing 7-cm C18
reverse-phase packing material (5-\textmu;m particles; YMC). Peptides were
eluted into the mass spectrometer at a flow rate of 60 ml/min with a gra-
dient: A = 0.1 M acetic acid (Sigma-Aldrich) in water; B = 70% aceto-
nitrile (Malinckrodt) and 0.1 M acetic acid in water; and 0–60% B in 40
min and 60–100% B in 5 min. Parameters used to acquire ETD/tandem
mass spectrometry (MS/MS) spectra on a data-dependent mode on the
modified LTQ instrument have been described (28).

Sequence analysis was performed by searching MS/MS spectral data
downloading from http://www.jimmunol.org/ by guest on April 28, 2022
against a database consisting only of the gp100 protein using the Open Mass
Spectrometry Search Algorithm software to generate a list of candidate
spectra. A precursor mass tolerance of ±0.01 Da was used; MS/MS
spectra were searched using a monoisotopic fragment ion mass tolerance
of ±0.5 Da. Data were searched allowing variable modifications for
phosphorylation of serine, threonine, and tyrosine residues and oxidation
of methionine, with a total of 1024 variable modifications per peptide
being allowed. Other parameters used were: peptide charge range from +2
to +5, ±2 charge state products allowed for peptides of charge +3 and
above, and peptide size range 4–25 aa with no enzyme restriction. Peptide
sequence assignments were validated by manual interpretation of the
corresponding ETD and or collisionally activated dissociation MS/MS
spectra. Approximate copy numbers per melanoma cell for each peptide
were determined by comparing peak areas of the observed parent ions
to that of angiotensin I and vasoactive intestinal peptide (DRVYIHPFH
HisDavFDTNYT, 100 fmol; Sigma-Aldrich) spiked into the sample
mixture.

Synthesis of HLA-DR4–restricted peptides

Synthetic peptides used in this study were made with Fmoc chemistry,
isolated by HPLC to ≥90% purity, and validated with mass spectrometry
(GenScript). Peptide sequences were determined via mass spectrometry
(Table I); WT gp100\textsubscript{44-59} (WTWNRQLYPEWTEAQRLD);
gp100\textsubscript{44-59} APLs (Table III); influenza hemagglutinin (HA)\textsubscript{107-319}
(PKYVKQNTKLAT); and CDC27\textsubscript{569-772} (MNSFWMLDLPKGAN)
(30).

MHC–peptide binding affinity assay

Competition assays to quantitatively measure the binding affinities
of native and altered gp100 peptides for purified HLA-DR4 were performed
downloading from http://www.jimmunol.org/ by guest on April 28, 2022
essentially as detailed elsewhere (31–33). Briefly, purified HLA-DR4 molecules
(5–500 nM) were incubated with various concentrations of unlabeled peptide
inhibitors and 0.1–1 nM \textsuperscript{125}I-radioabeled probe peptide (human myelin
basic protein [MBP]\textsubscript{185-206}, sequence PVVHEFK/NIVTPRTPPY)
for 48 h in PBS containing 0.05–0.15% Nonidet P-40 in the presence of a pro-

assay inhibitor mixture. MHC binding of the radiolabeled peptide was
determined by capturing MHC–peptide complexes on L243 (anti–HLA-
DRA) Ab–coated Lumitect 600 plates (Greiner Bio-one, Frickhausen,
Germany) and measuring bound cpm using the TopCount microscintillation
counter (Packard Instrument, Meriden, CT). Peptides were typically tested
at six different concentrations covering a 100,000-fold dose range in
three or more independent assays. Under the conditions used, in which
[peptide] < [MHC] and IC\textsubscript{50} = [MHC], the measured IC\textsubscript{50} values are rea-
sonable approximations of the K\textsubscript{D} values (34, 35).

MHC–peptide protein preparation, crystallization, data
collection, and structure determination

The gp100\textsubscript{44-59}–HLA-DR4 complex was assembled in two steps to max-
imize the yield of recombinant protein. First, HLA-DR4 bearing CLIP\textsubscript{87-101}
peptide (PVSKMRMATPLMQMA) was prepared by in vitro folding from
bacterial inclusion bodies. Second, gp100\textsubscript{44-59} was loaded into CLIP–
HLA-DR4 using the peptide-exchange catalyst HLA-DM. Briefly, the
eXtreme portions of the HLA-DR α- and β-chains (residues 1–181 and 1–192,
respectively) were expressed separately as inclusion bodies in
Escherichia coli BL21 (DE3) cells (Novagen). Inclusion bodies were
dissolved in 8 M urea, 50 mM Tris-HCl (pH 8), and 10 mM DTT, followed
by purification on a Poros HQ20 anion exchange column (PerSeptive
Biosystems) in 50 mM Tris-HCl, 8 M urea, and 1 mM DTT at pH 8 (DR\textsubscript{a})
or pH 8.5 (DR\textsubscript{b}), using a linear NaCl gradient (36). For in vitro folding,
the purified subunits were diluted to a final concentration of 40 mg/ml each
in a folding solution containing 50 mM Tris-HCl, 30% (w/v) glycerol, 0.5 mM
EDTA, 3 mM reduced glutathione, and 0.9 mM oxidized glutathione (pH 8).
CLIP peptide (GenScript) was added to a final concentration of 5 μM,
and the folding mixture was kept for 2 wk at 4°C. The final folding solution
was concentrated and dialyzed against 50 mM Mes (pH 6). Purification
was carried out with sequential Superdex S-200 and Mono Q FPLC columns
 Görke Healthcare). The CLIP–HLA-DR4 complex was concentrated to 0.8 mg/ml
and loaded with gp100\textsubscript{44-59} by overnight incubation at 37°C in 100 mM
sodium citrate–HCl (pH 5.8) containing 200 μM gp100\textsubscript{44-59} and 0.2 mg/ml
soluble HLA-DM.

The gp100\textsubscript{44-59}–HLA-DR4 complex was crystallized at room temper-
ature in hanging drops by mixing equal volumes of the protein solution at
5 mg/ml and a reservoir solution of 14% (w/v) polyethylene glycol 8000,
0.2 M magnesium acetate, and 0.1 M HEPES (pH 7). For data collection,
crystals were transferred to a cryoprotectant solution (mother liquor con-
taining 30% [w/v] polyethylene glycol 8000), prior to flash-cooling in
a nitrogen stream. X-ray diffraction data were collected at beamline X29 of the Brookhaven National Synchrotron Light Source with
an ADSC Quantum–315 CCD detector (Area Detector Systems). All data
were indexed, integrated, and scaled with the program HKL 2000 (37).

Data collection statistics are shown in Table II.

The structure was solved by molecular replacement with the program
Phaser (38) using HLA-DR4 (Protein Data Bank accession code 1DSZ)
with HLA-DR4 + (1359 and 1102) or HLA-DR4 mAb. G7 cells are homozygous for HLA-DR4. (C) Specific recognition of HLA-DR4 + melanoma cells by G7 T cells, which were coincubated after every two vaccinations for in vitro immunologic monitoring studies. All proteins and structure factors have been deposited in the Protein Data Bank (accession code 4156 http://www.rcsb.org/pdb/home/home.do).

**Patients**

Patients with unresectable stage IV melanoma who expressed HLA-DRB1*0401 were treated with a synthetic gp100 peptide vaccine. Patients were vaccinated s.c. with 5 mg gp10044–59 peptide emulsified in IFA every 3 wk for a series of four inoculations (one treatment cycle), as described (16). PBMCs were collected by leukapheresis before treatment and 3 wk after each vaccination for in vitro immunologic monitoring studies. Patients underwent radiologic restaging after each treatment cycle. All patients were treated in the Surgery Branch of the National Cancer Institute, National Institutes of Health (Bethesda, MD), on a clinical trial approved by the Institutional Review Board of the National Cancer Institute, after signing informed consent (16). In addition to DRB1*0401, HLA-DR alleles expressed by the six patients reported in this study included the following: DRB1*0404, patient 6; DRB1*0701, patients 1, 3, and 5; DRB1*1104, patient 2; DRB1*1501, patient 4; DRB3*02, patient 2; DRB4*01, all patients; and DRB5*01, patient 4.

**T cell functional assays: ELISAs**

Peptide-specific CD4+ T cells at 1e5–3e5 cells per well were cocultured overnight in flat-bottom 96-well plates with 1e5 peptide-pulsed HLA-DR4+ allogeneic EBV-B cells or autologous PBMCs. Culture supernatants were harvested for measurement of GM-CSF and IFN-γ secretion by T cells using ELISA with commercially available kits (R&D Systems).

**In vitro stimulation and ELISPOT assay**

Cryopreserved PBMCs from vaccinated patients were thawed and suspended in complete medium (CM: RPMI 1640 plus 10% heat-inactivated human AB serum, 2 mM glutamine, 10 mM HEPES buffer, and antibiotics) at 1e6 cells/ml in 24-well plates with 20 μM peptide, 200 U/ml GM-CSF, and 100 U/ml IL-4. Parallel cultures were grown with IL-7 and IL-15 (25 ng/ml each) instead of GM-CSF and IL-4. Cells were incubated at 37°C. After 3 d, IL-2 was added at a final concentration of 10 IU/ml. At 9–12 d, some cells were harvested for ELISPOT assay, and the remaining cells were restimulated with peptide-pulsed irradiated autologous PBMCs and cultured in CM containing 150 IU/ml IL-2. At day 20, cells were harvested again for ELISPOT assay. One day prior to ELISPOT assays, approximately half of the culture volume was replaced with fresh CM (without IL-2).

ELISPOT assays were conducted in MultiScreen-IP Filter Plates coated overnight at 4°C with 50 μl/well 20 μg/ml mouse anti-human IFN-γ Ab (clone 1D1K; Mabtech). On the following day, the plates were washed and blocked with AB medium for 2 h at 37°C. For some assays, fresh cryopreserved PBMCs were thawed into ELISPOT media (RPMI 1640 with 10% heat-inactivated human AB serum, 2 mM glutamine, and 10 mM HEPES buffer) at 4e6 cells/ml. After 2 h incubation at 37°C and 5% CO2, 2e5 PBMCs were plated directly to each IFN-γ Ab-coated well. For ELISPOT assays using peptide-stimulated T cell cultures, 1e5 T cells were plated into each IFN-γ Ab-coated well. Then, 1e5 irradiated (5000 rad) autologous PBMCs were added to T cells in each well as APCs. GP100 peptides were added at 20 μM in 100 μl total volume. An unrelated HLA-DR4-restricted peptide, CDC2758-72, was used as a negative control. PMA/ionomycin stimulation provided a positive control. After overnight incubation at 37°C, cells were discarded, and the plates were washed with PBS/0.05% Tween 20 followed by PBS. A total of 100 μl biotinylated mouse anti-human IFN-γ Ab (clone 7B6-1; Mabtech) diluted at 2 μg/ml in PBS with 0.5% BSA was added to each well and incubated at 37°C for 2 h. The plates were washed, developed with avidin-peroxidase complex (Vector Laboratories), and stained with AEC substrate (Sigma-Aldrich). Spots were counted by a KS ELISPOT automated reader system (Carl Zeiss). The number of spots was averaged from triplicate wells. Peptide-specific T cells were defined as showing ≥20 spots per 1e6 fresh PBMCs or cultured T cells and greater than or equal to twice the numbers of spots observed in the negative control wells.

**Results**

**T cell recognition of native DR4-restricted gp100 peptides**

The DR4-restricted peptide gp10044–59 was originally described by Halder et al. (13) as a dominant peptide displayed on the surface of melanoma cells. The immunogenicity of this peptide was confirmed in DR4-transgenic mice and human in vitro studies.
leading to its clinical testing as a melanoma vaccine. Although many MHC II–restricted peptides occur as nested peptide sets, naturally occurring sequences overlapping gp10044–59 have not been previously identified. To identify native nested gp100 peptides with potentially enhanced immunogenicity, we searched peptides eluted from DR4 molecules displayed on cultured melanoma cells. A set of seven nested gp100 peptides spanning residues 40–59 was identified. Peptide abundance ranged from 100–4500 copies/ cell (Table I). We assessed the recognition of these peptides by measuring specific cytokine secretion from the CD4+ gp100-specific DR4-restricted G7 clone (Fig. 1). As shown in Fig. 2, recognition of gp100 44–59 exceeded all other peptides. Truncated peptides lacking the N-terminal residues Trp44 and Asn45, outside the peptide’s MHC core binding sequence, were not well recognized by T cells; this is reminiscent of our findings with another MHC II–restricted melanoma-associated peptide, phospho–melanoma Ag recognized by T cells 1 (MART-1), and highlights the

![Graph](image)

**FIGURE 2.** G7 clone recognition of gp10044–59 exceeds recognition of other members of a nested set of naturally processed gp100 peptides. APCs were pulsed with gp10044–59 or nested peptides at the indicated concentrations overnight. G7 cells were added and cultured overnight. Supernatants were harvested and tested for IFN-γ secretion by ELISA. Similar results were obtained for GM-CSF secretion (not shown). HA, HA307–319, an HLA-DR4–restricted peptide that was used as a negative control.

![Graph](image)

**FIGURE 3.** Structure of the gp10044–59–HLA-DR4 complex. (A) Electron density map for the bound gp10044–59 peptide. The $2F_o - F_c$ map at 2.5 Å resolution is contoured at 1σ. The peptide is drawn in stick representation with carbon atoms in yellow, oxygen atoms in red, and nitrogen atoms in blue. (B) Top view of the gp10044–59–HLA-DR4 complex, looking down on the peptide-binding groove. The molecular surface of HLA-DR4 is cyan (MHC a-chain) and green (MHC b-chain). (C) Conformation of high- and low-affinity peptides bound to HLA-DR4. The conformation of gp10044–59, CII1168–1180, and HA307–319 (blue) bind HLA-DR4 with higher affinity than MBP111–129 (pink) by superimposing the α1β1 domains of HLA-DR4 in the gp10044–59–HLA-DR4 and CII1168–1180–HLA-DR4 (Protein Data Bank accession code 2SEB) (47), HA307–319–HLA-DR4 (1J8H) (45), and MBP111–129–HLA-DR4 (3O6F) (48) complexes. The peptides are viewed from the side of the β1 helix; gp10044–59, CII1168–1180, and HA307–319 bind HLA-DR4 with higher affinity than MBP111–129.

![Graph](image)

**Table II.** Data collection and refinement statistics

<table>
<thead>
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<th>gp10044–59–HLA-DR4</th>
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<tr>
<td><strong>Data collection statistics</strong></td>
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| **Refinement statistics** |
| Resolution range (Å) | 30.0–2.50 |
| $R_{work}$ (%) | $26.9$ |
| $R_{free}$ (%) | $29.8$ |
| Protein atoms | 3,133 |
| Rms deviations from ideality |
| Bond lengths (Å) | 0.009 |
| Bond angles (°) | 1.47 |
| Ramachandran plot statistics |
| Most favored (%) | 86.2 |
| Additionally allowed (%) | 12.6 |
| Generously allowed (%) | 0.9 |
| Disallowed | 0.3 |

*Values in parentheses are statistics for the highest resolution shell (2.50–2.54 Å). $R_{work} = \sum I_{o} - I_{c} / \sum I_{o}$, where $I_{o}$ is the intensity of an individual reflection and $<I_{c}>$ is the average intensity of that reflection.

$R_{sym} = \sum |I_{o} - <I_{o}>| / \sum |I_{o}|$, where $F_{o}$ is the calculated structure factor. $R_{work}$ is as for $R_{work}$ but calculated for a randomly selected 10.0% of reflections not included in the refinement.
important effects that N-terminal residues outside the binding groove may have on nonmutated (self) epitope recognition (43). Thus, we were unable to identify a naturally processed overlapping peptide that was better recognized by the G7 clone than gp100_{44-59}. Furthermore, this peptide was the most abundantly expressed member of the nested peptide set. Therefore, all subsequent experiments were based on the gp100_{44-59} native peptide sequence.

Structure of gp100_{44-59} bound to HLA-DR4

The crystal structure of gp100_{44-59} complexed to HLA-DR4 was determined to 2.5 Å resolution (Table II). Continuous and unambiguous electron density was observed for gp100_{44-59} residues Arg_{56} to Leu_{58}; however, N-terminal residues Trp_{44} and Asn_{45} and C-terminal residue Asp_{59} were not defined in the electron density map (Fig. 3A), despite their importance for recognition by clone G7 (Fig. 2).

In the structure, the primary anchor residues for gp100_{44-59} bound to HLA-DR4 are Leu_{58} (P1) and Thr_{53} (P6); the secondary anchor residues are Glu_{51} (P4), Phe_{54} (P7), and Glu_{56} (P9). This result is consistent with the sequence motif for HLA-DR4 deduced from phage display and synthetic peptide libraries (24, 33, 44). Leu_{48} fulfills the requirement for a large nonpolar residue at P1 for efficient binding to HLA-DR4. Thr_{53} at P6 is a suitable primary anchor as well. By contrast, none of the secondary anchors (P4 Glu_{51}, P7 Phe_{54}, and P9 Glu_{56}) conform to the optimal binding motif for HLA-DR4. Thus, the P4 pocket of HLA-DR4, which is lined by β-chain residues Phe_{26}, Lys_{71}, Ala_{74}, and Tyr_{78}, is hydrophobic and prefers nonpolar residues (24, 33, 44). In the gp100_{44-59}-HLA-DR4 complex, the charged C-group of the P4 Glu51 side chain points toward the surface of HLA-DR4 instead of into the P4 pocket (Fig. 3B). The P7 pocket of HLA-DR4, formed by β-chain residues Trp_{73}, Trp_{74}, Leu_{75}, and Lys_{71}, is likewise hydrophobic, such that the side chain of P7 Glu_{54} adopts a conformation very similar to that of P4 Glu_{51}. The side chain of P9 Glu_{56} projects deep into the primarily nonpolar P9 pocket of HLA-DR4 (Fig. 4, left panel), which is formed by α-chain residues Asn_{69}, Ile_{72}, and Met_{73} and β-chain residues Tyr_{72}, Asp_{75}, and Trp_{73}. P9 Glu_{56} makes extensive van der Waals contacts with residues in this pocket, in addition to two side-chain–side-chain hydrogen bonds: P9 Glu_{56} Oε1–On DR4 Tyr_{72}β and P9 Glu_{56} Ne2–Oε1 DR4 Asp_{75}β (Fig. 4, right panel).

The conformation of gp100_{44-59}, which binds HLA-DR4 with intermediate affinity (15), was directly compared with those of three other peptides that bind HLA-DR4: 1) a self-peptide from collagen II (CHI_{1168-1180}) that binds HLA-DR4 with relatively high affinity (24, 44); 2) a foreign peptide from influenza virus HA_{307-319} that also binds HLA-DR4 with high affinity (45); and 3) a self-peptide from MBP_{111-129}, which binds weakly to HLA-DR4 (46). The main chain of gp100_{44-59} superposes very closely onto those of CHI_{1168-1180} and HA_{307-319} in complex with HLA-DR4 (45, 47), from residues P2 to P9 (Fig. 3C). The C terminus of gp100_{44-59} (residues P10 and P11), which is positioned ~1 Å higher than that of CHI_{1168-1180} or HA_{307-319}, is located outside the peptide-binding groove. However, all three peptides sit deeply in the binding groove. By contrast, the low-affinity MBP_{111-129} peptide diverges from gp100_{44-59}, CHI_{1168-1180}, and HA_{307-319} at anchor residues P6 and P7 (Fig. 3C), due to a longer side chain at P6 (MBP_{111-129}: Gln; gp100_{44-59}: Thr; CHI_{1168-1180}: Ala; and HA_{307-319}: Thr) (48). The consequent elevation of MBP_{111-129} results in fewer contacts to HLA-DR4 compared with gp100_{44-59}, CHI_{1168-1180}, or HA_{307-319}.

APLs and their affinity for DR4

According to the crystal structure of the DR4–gp100_{44-59} complex as well as published HLA-DR4 peptide binding motifs (22–24), we designed several APLs with substituted MHC anchor residues (Table III). The amino acid substitutions L48F (P1), E51Q and E51A (P4), E54L and E54T (P7), and Q56A (P9) are located at MHC anchor residues revealed by crystal structure and were designed to enhance MHC–peptide affinity. Indeed, these anchor-modified APLs were found to have 2–10-fold higher affinities for DR4 than the WT peptide. Y49M (P2), P50A (P3), W52A (P5), and A55G (P8), in which the substituted amino acids are positioned at potential TCR contact residues, had higher or similar MHC-binding affinities compared with WT peptide. As a negative control, Q56I was designed to reduce binding affinity to DR4 due to the large isoleucine residue in P9, incompatible with the corresponding shallow binding groove in HLA-DR4; the MHC binding affinity of this modified peptide was ~50% lower than WT (Table III).

Recognition of gp100_{44-59} APLs by gp100_{44-59}-specific CD4+ T cell clone G7

T cell recognition of gp100_{44-59} APLs was first characterized by cytokine secretion from the G7 clone. Peptides modified at putative TCR contact residues—Y49M (P2), P50A (P3), and W52A (P5)—were not recognized by the G7 clone, confirming the importance of these residues for T cell specificity (Fig. 5). In addition, a replacement of threonine by valine in anchor position 6 also abrogated T cell recognition (not shown), suggesting that P6 has high stringency for a hydroxylated residue. However, G7 secreted two to three times more IFN-γ and GM-CSF in response to Q56A than to WT peptide (Fig. 5), nominating the Q56A APL for further study. Other APLs showed equivalent or lower recognition by G7.

![FIGURE 4. Interaction of P9 Glu56 with HLA-DR4](http://www.jimmunol.org/)

Left panel: molecular surface of HLA-DR4 (MHC α-chain, cyan; MHC β-chain, green) showing the P9 pocket that accommodates the side chain of P6 Glu_{56}. Right panel: contact residues of HLA-DR4 are drawn and labeled. Hydrogen bonds are indicated by broken black lines.
Table III. gp10044–59 APLs and their affinity for HLA-DR4

<table>
<thead>
<tr>
<th>gp10044–59 and APLs</th>
<th>Amino Acid Sequence</th>
<th>Substituted Position</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>WT</td>
<td>WNRQLYPWEAQRQLD</td>
<td>NA</td>
<td>627</td>
</tr>
<tr>
<td>L48F</td>
<td>--------P------------</td>
<td>P1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>280</td>
</tr>
<tr>
<td>Y49M</td>
<td>--------N------------</td>
<td>P2</td>
<td>521</td>
</tr>
<tr>
<td>P50A</td>
<td>--------A------------</td>
<td>P3</td>
<td>189</td>
</tr>
<tr>
<td>E51A</td>
<td>--------A------------</td>
<td>P4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>199</td>
</tr>
<tr>
<td>E51Q</td>
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<td>P4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>200</td>
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<tr>
<td>W52A</td>
<td>--------A------------</td>
<td>P5</td>
<td>372</td>
</tr>
<tr>
<td>T33V</td>
<td>--------V------------</td>
<td>P6&lt;sup&gt;+&lt;/sup&gt;</td>
<td>489</td>
</tr>
<tr>
<td>E54L</td>
<td>--------T------------</td>
<td>P7</td>
<td>65</td>
</tr>
<tr>
<td>E54T</td>
<td>--------T------------</td>
<td>P7</td>
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<tr>
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<td>--------P------------</td>
<td>P8</td>
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<td>P9&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Q56I</td>
<td>--------I------------</td>
<td>P9&lt;sup&gt;+&lt;/sup&gt;</td>
<td>926</td>
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</tbody>
</table>

*Indicates MHC anchor residues according to crystal structure.

NA. Not applicable.

Compared with WT peptide (Fig. 5). The ability of G7 to cross-react with gp10044–59 and Q56A APL is easily understood in structural terms, because these peptides differ by only a single residue at anchor position P9. The Gln<sup>56</sup> side chain of gp10044–59 (and presumably the Ala<sup>56</sup> side chain of Q56A APL) is sequenced in the P9 pocket of HLA-DR4 and is therefore not exposed to TCR G7 (Fig. 3B).

Comparison of gp100 WT peptide versus the Q56A APL in detecting gp100-specific immunity in PBLs from vaccinated patients

In a prior study of patients with melanoma receiving the gp100 WT vaccine, no detectable immunity against the vaccinating peptide was detected in posttreatment PBMCs using a standard IFN-γ ELISPOT assay. Because the Q56A APL had higher affinity for DR4 and provoked greater recognition by the G7 clone, we assessed Q56A for its ability to detect gp100-specific reactivity in pre- and posttreatment PBLs from six patients previously reported in this clinical trial (16) (Fig. 6). Uncultured PBLs were assessed for reactivity against WT peptide or the Q56A APL using an IFN-γ ELISPOT assay. In addition, PBLs were in vitro stimulated (IVS) for 10–20 d with WT or Q56A peptide to amplify gp10044–59-specific T cells, prior to ELISPOT. With IVS, the CD<sup>+</sup> T cell population increased to 60–90% of cultured T cells (data not shown). We found reproducible evidence that new specific anti-gp100 responses developed after vaccination in two of six patients using uncultured or 10–20 d IVS T cells (patients 1 and 2). In patient 3, IVS cultures showed greater gp100-specific activity after four compared with two vaccines; pretreatment T cells did not proliferate in IVS culture and thus could not be assessed. There was pre-existing immunity against gp10044–59 that persisted after vaccination in uncultured PBMCs and IVS cultures in patient 4. Finally, two patients did not manifest evidence of a pre-existing or vaccination-induced anti-gp100 response (data not shown). Among the four patients with anti-gp100 immunity, only patient 3 had improved detection of responses using the Q56a APL, while also recognizing WT peptide. IVS cultures raised against WT or Q56A peptides showed equivalent recognition of the reciprocal peptide in patients 1, 2, and 4 (Fig. 6). In summary, IVS-enhanced ELISPOT techniques enabled the detection of gp100-specific immunity in four of six patients with melanoma (in three of four patients, immunity increased following vaccination). Unlike results with the G7 T cell clone, the Q56A APL was not consistently superior to WT peptide in revealing these responses.

T cells raised against WT peptide prefer a variety of APLs

We proceeded to evaluate a panel of gp100 APLs for their ability to detect gp100-specific CD<sup>+</sup> T cell responses in vitro by testing their recognition by T cells from vaccinated melanoma patients that were stimulated in vitro with WT peptide. Fig. 7A shows results from three patients, demonstrating that IVS T cells from each patient had a unique preference for recognizing various APLs. These results suggest heterogeneous TCR usage by gp100 WT-specific T cells from individual patients. Several APLs that were preferred by individual patients (L48F, E54L, and E54T) were assessed for their ability to generate gp100-specific CD<sup>+</sup> T cells from the prevaccination PBLs of the six patients with melanoma. None of these APLs was superior to WT or Q56A peptide in stimulating gp100-specific CD<sup>+</sup> T cells, and in some cases, APLs provoked APL-specific responses that did not cross-react with WT peptide (not shown). Finally, a peptide pool comprised of WT peptide plus the APLs L48F, E54L, E54T, and Q56A was evaluated for its ability to raise gp100-specific immunity in prevaccination T cells or to detect postvaccination gp100-specific immunity in melanoma patients 1, 2, and 4, similar to experiments shown in Fig. 6. The peptide pool was not superior to the WT peptide in raising or detecting gp100-specific immunity (Fig. 7B).

Discussion

Insights gained from the characterization of MHC–peptide–TCR interactions hold the promise that directed structural modifications can have predictable functional consequences. The ability to manipulate T cell reactivity synthetically or through genetic en-
not always be predictive of in vivo effects (51). These difficulties are amplified in the context of MHC II interactions, for which peptide binding motifs are permissive and not rigid, enabling binding of a single peptide to multiple MHC II alleles (52). An APL of an HLA-DR2–restricted MBP epitope containing modified TCR contact residues, designed to antagonize autoreactive T cells in patients with multiple sclerosis, caused disease exacerbation rather than alleviation in some vaccinated patients (53).

In the current study, APLs from a dominant HLA-DR4–restricted gp100 epitope were designed to enhance MHC binding and hence T cell recognition in patients with melanoma. APL design was based on precise structural definition of the WT peptide–MHC II complex, and increased MHC binding was validated by direct affinity measurements. Nevertheless, we observed heterogeneous preferences of CD4+ T cells from several HLA-DR4+ melanoma patients for different gp100 APLs, suggesting highly variable TCR usage, even among patients who had been vaccinated against the WT gp100 peptide. This could reflect heterogeneous responses of TCRs from different patients to APL–DRβ1*0401 complexes, but might also reflect immune responses to APLs bound to alternative DR alleles expressed by individual patients. This heterogeneity prevented the selection of an APL candidate for developing an improved generic melanoma vaccine.

Although we did not determine crystal structure of Q56A APL bound to HLA-DR4, it is unlikely that the conformation of this anchor-modified peptide differs significantly from that of WT gp10044–59, which could have explained loss of TCR reactivity. Q56A APL and gp10044–59 differ by only a single residue, at anchor position P9. The possible effect on the peptide backbone of replacing P9 Gln by Ala may be assessed by examining two other peptide–HLA-DR4 structures, one involving CII1168–1180, in which P9 is Gly (47), and the other involving HAA307–319, in which P9 is Leu (45). As shown in Fig. 3C, the main chain of gp10044–59 superposes very closely onto those of CII1168–1180 and HAA307–319, even though these three unrelated peptides have different residues at P9 (Gln, Gly, and Leu, respectively). This suggests that the P9 anchor modification in Q56A APL exerts subtle effects on peptide contacts with TCR or on peptide dynamics, such that an alteration intended to enhance immunogenicity may be counterproductive.

Indeed, there is growing evidence for an important role of peptide dynamics in modulating TCR recognition (54–56). In one especially relevant study (55), replacing suboptimal anchor residues of the HLA-A2–restricted MART-127–35 melanoma Ag unexpectedly abolished recognition by most MART-1–specific T cell clones, suggesting highly variable TCR usage, even among patients who had been vaccinated against the WT gp100 peptide. This could reflect heterogeneous responses of TCRs from different patients to APL–DRB1*0401 complexes, but might also reflect immune responses to APLs bound to alternative DR alleles expressed by individual patients. This heterogeneity prevented the selection of an APL candidate for developing an improved generic melanoma vaccine.

Engineering might thus be translated into new therapies for common diseases such as cancer and autoimmune disorders. In an early example with the HLA-A2–restricted gp100 epitope 209–217, an MHC anchor residue substitution improved MHC–peptide binding affinity, which translated into enhanced CD8 T cell recognition in vitro and increased immunogenicity in the clinic as a melanoma vaccine (8, 17). However, subsequent investigations on the same theme using HMC I–restricted melanoma-associated peptides and melanoma-specific TCRs have yielded variable results, demonstrating that structural modifications of MHC–peptide–TCR interactions may have unpredictable or undesirable functional consequences (49, 50), which may involve collateral damage to normal tissues when tolerance to nonmutated tumor Ags such as gp100 is alleviated. Furthermore, in vitro functional testing may not always be predictive of in vivo effects (51). These difficulties are amplified in the context of MHC II interactions, for which peptide binding motifs are permissive and not rigid, enabling binding of a single peptide to multiple MHC II alleles (52). An APL of an HLA-DR2–restricted MBP epitope containing modified TCR contact residues, designed to antagonize autoreactive T cells in patients with multiple sclerosis, caused disease exacerbation rather than alleviation in some vaccinated patients (53).
to reliably generate improved vaccine candidates, as we found in the current study.

In the gp10044–59–HLA-DR4 structure, N-terminal residues Trp44 and Asn45 lie outside the peptide-binding groove and are not defined in the electron density, suggesting flexibility. Nevertheless, these two N-terminal residues are required for efficient recognition by TCR G7, which is reminiscent of the way some autoreactive TCRs engage self- or altered self-peptides, including tumor Ags, presented by MHC II molecules (43, 58). For example, x-ray crystallographic studies of tumor-specific TCRs that recognize a somatically mutated human melanoma Ag (mutant triosephosphate isomerase [mutTPI]) have revealed substantial alterations in the topology of TCR binding to peptide–MHC compared with antiforeign TCRs (59). In these autotumor TCR–peptide–MHC complexes, the TCR is skewed toward the peptide N terminus relative to its central position in antiforeign TCR–peptide–MHC complexes, resulting in low-affinity binding (K_D >200 μM) that likely enabled escape from negative thymic selection. In another example, human autoimmune TCR Ob.1A12, which recognizes a self-peptide from MBP85–99 bound to HLA-DR2b, was found to only contact the N-terminal half of MBP85–99 (61). TCR Ob.1A12 cross-reacts with an E. coli peptide having limited sequence identity with MBP85–99. Cross-reactivity is due to structural mimicry of a binding hotspot at the N-terminal portion of the bacterial and self-peptide (62).

We attempted to measure the binding of TCR G7 to gp10044–59–HLA-DR4 by surface plasmon resonance (data not shown). However, we could not detect an interaction, even after injecting a high concentration (up to 200 μM) of rTCR G7 over immobilized gp10044–59–HLA-DR4, which precluded further analysis. This result is consistent with previous findings that autoreactive TCRs generally bind self-Ags, including tumor Ags, with very low affinity (58). For example, the human melanoma-specific TCR E8 binds mutTPI–HLA-DR1 with a K_D >200 μM (59). The low affinities of G7 and E8 for their self-peptide–MHC ligands likely enabled these autoreactive T cells to escape negative thymic selection (58).

Although an explosion of information in the field of molecular immunology over the past two decades has yielded extraordinarily precise and extensive data on molecular interactions and structure-function relationships in the immune system, there remains a critical gap in knowledge that hinders reliable clinical translation from structural and in vitro observations. As demonstrated in the current study, peptide modifications that improve binding to MHC II molecules do not necessarily translate to increased antigenicity. Confounding this strategy is the heterogeneity of human CD4+ T cell responses, as well as possible dynamical effects of peptide modifications. Predicting these effects will require the use of molecular dynamics simulations in conjunction with structural information. Such structure-guided computational design may eventually allow successful reorientation of immune responses against cancer with vaccines and other immunomodulatory therapies.

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**Disclosures**

The authors have no financial conflicts of interest.


