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Specific Increase in Potency via Structure-Based Design of a TCR

Karolina Malecek,*† Arsen Grigoryan,‡,¹ Shi Zhong,*¹,² Wei Jun Gu,§ Laura A. Johnson,*³ Steven A. Rosenberg,*⁴ Timothy Cardozo,*⁺ and Michelle Krogsgaard*⁺,‖

Adoptive immunotherapy with Ag-specific T lymphocytes is a powerful strategy for cancer treatment. However, most tumor Ags are nonreactive “self” proteins, which presents an immunotherapy design challenge. Recent studies have shown that tumor-specific TCRs can be transduced into normal PBLs, which persist after transfer in ~30% of patients and effectively destroy tumor cells in vivo. Although encouraging, the limited clinical responses underscore the need for enrichment of T cells with desirable antitumor capabilities prior to patient transfer. In this study, we used structure-based design to predict point mutations of a TCR (DMF5) that enhance its binding affinity for an agonist tumor Ag–MHC (peptide–MHC [pMHC], Mart-1 (27L)-HLA-A2), which elicits full T cell activation to trigger immune responses. We analyzed the effects of selected TCR point mutations on T cell activation potency and analyzed cross-reactivity with related Ags. Our results showed that the mutated TCRs had improved T cell activation potency while retaining a high degree of specificity. Such affinity-optimized TCRs have demonstrated to be very specific for Mart-1 (27L), the epitope for which they were structurally designed. Although of somewhat limited clinical relevance, these studies open the possibility for future structural-based studies that could potentially be used in adoptive immunotherapy to treat melanoma while avoiding adverse autoimmunity-derived effects. The Journal of Immunology, 2014, 193: 2587–2599.

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Abbreviations used in this article: ACT, adoptive T cell therapy; AUC, area under the curve; h, human; MFI, mean fluorescence intensity; PDB, Brookhaven Protein Data Bank; pMHC, peptide–MHC; SASA, solvent-accessible surface area; WT, wild-type.

*Perlmutter Cancer Center, New York University University of Medicine, New York, NY 10016; †Program in Structural Biology, New York University School of Medicine, New York, NY 10016; ²Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY 10016; ³Department of Chemistry, New York University, New York, NY 10012; ⁴Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and ‖Interdisciplinary Melanoma Cooperative Group, New York University School of Medicine, New York, NY 10016

†A.G. and S.Z. contributed equally to this work.

‡Current address: Life Sciences Center, Xiangxue Pharmaceutical Co., Ltd., Guangzhou, China.

¹Current address: Perelman School of Medicine, University of Pennsylvania, Abramson Family Cancer Center, Philadelphia, PA.

Address correspondence and reprint requests to Dr. Michelle Krogsgaard, New York University School of Medicine, 522 1st Avenue, 13th Floor, Room 1311, New York, NY 10016. E-mail address: Michelle.Krogsgaard@nyumc.org

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It is estimated that there are >10⁸ Ag TCRs in the human naive T cell pool (13). However, this number is small when compared with the immense array of potential antigenic peptides (>10¹⁵) (14). Although TCRs do not undergo affinity maturation similar to B cell receptors in the form of somatic hypermutation, TCRs exhibit a measurable degree of promiscuity and potential cross-reactivity (14–17). Cross-reactive TCRs equip T cells with positive features such as polyclonal responses—temporally and spatially favorable interactions—as few T cells are needed to scan an infected cell and resources required to generate TCRs can be conserved (14–21). Alternatively, cross-reactivity can also be the basis for deleterious autoimmune responses (9, 15–17, 22).

Given that T cells have evolved to be cross-reactive to broaden immune recognition, TCR/pMHC interactions are likely to be of suboptimal affinity (23–26). Recent approaches for improving T cell potency by enhancing the affinity of the TCR for the pMHC have generally fallen in two categories: directed evolution and structure-based design. Directed evolution has been used to in-

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terrogate randomized TCR libraries via phage, yeast, or mammalian display systems to select strong binding T cell clones (27–34). However, these systems require large library sizes and can have inefficient protein folding and expression due to the specific nature of these expression systems (27–38). To overcome these challenges structure-based methods (35–37) have become widely used, being partly enabled by the growing database of TCR/pMHC crystallographic structures.

Previous studies analyzing the relationship between increasing TCR affinity, T cell functional outcomes, and cross-reactivity are controversial. Structural-based approaches have been used to increase TCR affinity, but their potential cross-reactivity has not been reported (35–37). Holler et al. (27, 38) used a yeast display system to engineer CDR3 variants with a higher affinity for the murine 2C TCR that retained their fine pMHC specificity, but they isolated some cross-reactive T cell clones as well. Recently, Greenberg and colleagues (39) demonstrated that murine TCRs with enhanced affinity for tumor/Ag transduced into peripheral CD8 T cells and transfected in vivo are safe and do not mediate autoimmune tissue infiltration or damage. Alternatively, June and colleagues (40) reported from a clinical trial that ACT of two patients with affinity-enhanced TCRs resulted in death by cardiac shock due to cross-reactivity to an unrelated peptide. We recently demonstrated that T cell antitumor activity and autoimmunity are closely coupled, but plateau at a defined TCR affinity of 10 μM, likely due to a diminished contribution of TCR affinity to avidity above this threshold (9). These results suggest that a relatively low-affinity threshold is necessary for the immune system to avoid self-damage given the close relationship between antitumor activity and autoimmunity. Therefore, the challenge for targeted T cell therapy remains to increase T cell potency and ensure on-target specificity.

Mart-1 (27L) is an MHC anchor-modified peptide of the Mart-1 melanoma Ag (MART-126–35 ELAGIGILTV) (41, 42) that binds HLA-A*0201 with increased stability and is more immunogenic than the unmodified peptide (43–45). Two TCRs specific for this Ag have been used in cancer gene therapy, that is, DMF4 and DMF5, which bind Mart-1 (27L) presented by class I MHC and suspended in water to 1 mM concentration. Allophycocyanin anti-mouse TCRβ constant (clone H57-597) and PE anti-mouse CD3ε (clone 145-2C11) Abs were from eBioscience. For Mart-1 (27L)/HLA-A2 tetramer production, an HLA-A2 H chain with a biotinylation sequence at the C terminus (provided from Dr. Cerundolo, John Radcliffe Hospital, Oxford, U.K.) and human β2-microglobulin were purified as an inclusion body from Escherichia coli. The complexes were refolded in vitro with Mart-1 (27L) peptide as previously described (9, 56, 57). The folded protein was concentrated and biotinylated with BirA biotin-protein Ligase (AviDity) accordingly to the manufacturer’s instructions. Protein purification and tetramer production were performed as previously described (58) by adding PE-labeled streptavidin (BD Pharmingen) in 1:10 volume aliquots to the biotinylated monomeric complexes in a 1:4 molar ratio. All the tetramer stains were done at 4°C.

Cell lines and cell cultures

The mouse 58/~– hybridoma cell line (54) (provided by Dr. D. M. Kranz, University of Illinois) with or without human CD8 and a chimeric HLA-A2–A2/Kb expressed on T2 cells (a gift from Dr. L. Sherman, Scripps Research Institute) were cultured in RPMI 1640 media supplemented with 10% FBS, GlutaMAX-I, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin. FBS was from Thermo Scientific HyClone, and all other cell culture reagents were from Invitrogen. Human recombinant IL-2 was from Novartis. The M44 (HLA–A2*) melanoma cell line and FM29 (HLA–A2*, Mart-1–), 888-mel (HLA–A2*, Mart-1–), and Gmel (HLA–A2*, Mart-1–) melanoma cell lines (59) (provided by Dr. Bhardwaj, Mount Sinai School of Medicine) were also cultured in RPMI 1640 media supplemented as described above.

For dendritic cell preparation, PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients (BioWhittaker) from healthy donor buffy coats (New York Blood Center). Cells were fed with additional GM-CSF/IL-4 on day 2 and later on day 4 of coculture and harvested for use on days 5–7. For all experiments, harvested dendritic cells were washed and equilibrated in serum-free X-Vivo 15 media (Lonza).

DNA constructs

The HLA-A2/Mart-1–specific TCR construct DMF5 (48) was cloned and expressed on hybridoma cells as previously described (9, 60). Human mouse chimeric αβ TCR constructs, consisting of human variable and murine constant TCR regions, were generated by PCR, and a self-cleavable 2A peptide was introduced for optimal TCR gene expression via structure-based design to achieve desirable T cell potency while avoiding increased cross-reactivity. When double and triple combination mutations were introduced, they exhibited an additive enhancement that further improved T cell activation while retaining specificity. Taken together, these results show that it is possible to manipulate human TCR affinity via structure-based design to achieve desirable T cell potency while avoiding increased cross-reactivity.

**Materials and Methods**

**Peptides, Abs, and pMHC tetramer production**

Mart-1 (27L) (ELAGIGILTV) peptide was synthesized by Bio-Synthesis. Ninety-six self-peptides (55) were also synthesized by Bio-Synthesis and suspended in water to 1 mM concentration. Allophycocyanin anti-mouse TCRβ constant (clone H57-597) and PE anti-mouse CD3ε (clone 145-2C11) Abs were from eBioscience. For Mart-1 (27L)/HLA-A2 tetramer production, an HLA-A2 H chain with a biotinylation sequence at the C terminus (provided from Dr. Cerundolo, John Radcliffe Hospital, Oxford, U.K.) and human β2-microglobulin were purified as an inclusion body from Escherichia coli. The complexes were refolded in vitro with Mart-1 (27L) peptide as previously described (9, 56, 57). The folded protein was concentrated and biotinylated with BirA biotin-protein Ligase (AviDity) accordingly to the manufacturer’s instructions. Protein purification and tetramer production were performed as previously described (58) by adding PE-labeled streptavidin (BD Pharmingen) in 1:10 volume aliquots to the biotinylated monomeric complexes in a 1:4 molar ratio. All the tetramer stains were done at 4°C.

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**Transduction of T cells and flow cytometry analysis**

Retroviral transduction of hybridoma and mouse primary CD8+ T cells was performed as described (60, 62). TCR expression on TCR-transduced hybridoma cells or mouse primary CD8+ T cells were assessed by dual color staining with PE-labeled HLA-A2/Mart-1 (27L) tetramer for 1 h at 4°C. TCR-transduced hybridomas were also stained for 30 min at 4°C with an allophycocyanin-labeled anti-TCRβ constant chain Ab (clone H57-597). Hybridoma cells were washed twice and then sorted at room temperature using a MoFlo cell sorter (Beckman Coulter).
Cytokine ELISA

APCs (1 × 10^3; T2-A2/Kb, M44, FM29) were loaded with different concentrations of Mart-1 (27L) peptide and were coincubated with 1 × 10^8 T hybridoma clones for 16 h at 37°C, 5% CO_2. For self-peptide ELISA assays, 1 × 10^3 APCs (T2-A2/Kb) were loaded with 10 μM of each of the self-peptides and were coincubated with 1 × 10^8 T hybridoma clones for 16 h at 37°C, 5% CO_2. Subsequently, a standard sandwich ELISA quantified IL-2 or IFN-γ production. All Abs and cytokine standards were from eBioscience. Streptavidin-HRP was from BD Biosciences and tetramethylbenzidine ELISA substrate was from Sigma-Aldrich. IL-2 and IFN-γ lower detection level for each assay was 9.5 pg/ml according to the manufacturer’s instructions.

Tetramer decay and mean fluorescence intensity determination

PE-labeled HLA-A2 streptavidin and tetramers bearing Mart-1 (27L) peptide were used for tetramer decay determinations. All stains and washes were done using FACs buffer (1× PBS, 5% FBS, and 0.1% sodium azide). For tetramer staining, 2 × 10^5 T hybridoma cells were washed and stained with tetramer for 2 h at 4°C. After incubation, cells were washed three times and stained with an anti-CTCRα mAb clone H57-597, BD Biosciences for 30 min. Following two washes cells were resuspended in 100 μl FACs buffer and 8-μl samples were taken before or after adding 5 μg (0.05 mg/ml final concentration) blocking Ab addition (HLA-A2 clone BB7.2, Genentech). Cells were immediately treated in 4% paraformaldehyde. Aliquots were taken at several time points and treated the same way. The mean fluorescence intensity (MFI) was determined based on gating of TCR+ cells. For Kt, subtracting the initial time point as a background reference normalized determinations the MFI of individual samples and the results were plotted on a logarithmic scale. The log MFI decay rate (k) was derived from the negative reciprocal of the slope of the line fit to Scatchard plots of bound tetramer/free tetramer versus bound tetramer (63).

Structural modeling of point mutations

All structural analysis as well as in silico mutagenesis and energy calculations/simulations were performed using ICM (Molsoft, La Jolla, CA) (64–66). A molecular model of the WT DMF5 TCR was generated using an x-ray structure of DMF5-Mart-1/HLA-A2 (Brookhaven Protein Data Bank [PDB] ID 3qdg) as input (47). Next, cavities/pockets at the DMF5-Mart-1/HLA-A2 binding interface were identified via a combination of the ICM pocket finder algorithm and visual inspection of the electrostatic molecular surface of each binding partner (TCR and pMHC) for hydrophobic patches. DMF5 TCR residues seem to be in close vicinity to these cavities/pockets with their side chains facing toward the pockets were selected for in silico mutagenesis. Amino acid mutations predicted to enhance DMF5 TCR binding affinity by increasing interface electrostatic complementarity and/or hydrophobic packing were individually introduced into the three-dimensional models of WT DMF5 TCR. For each mutation, only mutant residue side chain torsion angles were minimized by a systematic search procedure followed by energy calculation using electrostatic, solvation, and entropic terms for each conformation searched. The estimated binding energy of the complex was evaluated using the calcBindingEnergy macro implemented in ICM (67).

Solvent-accessible surface area calculation

The calcArea macro implemented in ICM was used to evaluate solvent-accessible surface area (Molsoft, La Jolla, CA) (64–66). The area is reported in square angstroms and the probe radius is assumed to be the radius of a water molecule.

Quantification of IL-2 expression and determination of cross-reactivity index

To analyze whether the introduced DMF5 TCR mutations resulted in enhancement of DMF5-Mart-1/HLA-A2 binding affinity, the area under the curve (AUC) for IL-2 cytokine production obtained for FM29 and M44 cell lines was calculated for each DMF5 TCR mutant as well as WT DMF5 TCR. All areas obtained for a given cell line were normalized by the AUC for IL-2 cytokine production obtained when human hCD8+ cells (Kd CD8=58 μM) in the same cell line: 1) ΔA = integrated area (DMF5 TCR mutant) – integrated area (hCD8=58 μM), and (2) ΔA (WT DMF5 TCR) = integrated area (WT DMF5 TCR) – integrated area (hCD8=58 μM). Furthermore, the areas obtained for DMF5 TCR mutants were subtracted from the area obtained for the WT DMF5 TCR. 3) ΔA = ΔA – ΔA (WT DMF5 TCR). Positive ΔA values indicate that the introduced DMF5 TCR mutations resulted in enhancement of DMF5-Mart-1 (27L)/HLA-A2 binding affinity.

To determine the specificity of each individual TCR, the values of IL-2 cytokine production when stimulated by a given cell line (FM29 and M44) in the absence of added peptide was established for each DMF5 TCR mutant and compared with the value of IL-2 cytokine production obtained for the WT DMF5 TCR when stimulated with the same APC. DMF5 TCR mutants that resulted in >4-fold increased IL-2 cytokine production when no peptide was added (>0.2 μg IL-2) compared with the WT DMF5 TCR would indicate loss of Ag specificity, and such clones were excluded from further analysis.

Cytotoxicity assay

Chromium release assays were performed by coculturing effector T cells with 5 × 10^4 51Cr-labeled M44 tumor cells at different E:T ratios for 5 h. Released 51Cr was determined with a beta counter (PerkinElmer Life Sciences) (70).

Results

Characterization of a panel of human melanoma Ag–specific TCRs indicates DMF5 TCR to be the most potent

To select the optimal TCR for our studies, we characterized a panel of six Mart-1–specific TCRs isolated from melanoma Ag–reactive tumor-infiltrating lymphocyte clones from the tumors of five patients (48). The TCRs were expressed on mouse T cell hybridoma by 58-α/β (54) (TCR+, CD3+), and because DMF5 TCR is less CD8 dependent (48), they were expressed with or without hCD8+ to enable meaningful comparison. DMF4 and DMF5 TCRs showed higher activation potential when compared with M7, M5, M17, and JKF6 TCRs, as measured by IL-2 cytokine production with or without the presence of coreceptor CD8 (Fig. 1A). Ten times higher peptide concentration (100 μM, x-axis) was used to obtain any activation for M7, M5, M17, and JKF6 TCRs expressing hybridoma when not expressing CD8 (Fig. 1A, top). Although DMF4 and DMF5 TCRs work equally well at high peptide concentrations (>10 μM for hCD8+ cells, Fig. 1A, top; >1 μM for hCD8- cells, Fig. 1A, bottom), DMF5 produced more cytokine at lower peptide concentrations (Fig. 1A), which is closer to the typical low and physiological Ag expression observed in vivo. Additionally, DMF5 has an apparent Kd of 27 nM versus 42 nM for DMF4 (Fig. 1C). These differences are largely due to the off-rate, as indicated by tetramer decay assay determination (Fig. 1B). DMF5 has a significantly slower tetramer dissociation half-life (~208 min) when compared with DMF4 and the remaining TCRs, which had a range of dissociation half-lives between 9.1 and 15.1 min (Fig. 1B). Lastly, DMF5 is less CD8–dependent than DMF4 as observed by binding pMHC tetramer in the absence of CD8 (Fig. 1D). Quantification of TCR and CD8 expression showed that the differences in activation potency and tetramer half-lives observed were not due to variation in TCR or CD8 expression levels (Supplemental Fig. 1). Based on these results, we identified DMF5 TCR as the most potent TCR from the panel of HLA-A2/MART-1–specific TCRs and selected it for further affinity enhancement by structure-based computational design.

Structure-based design of DMF5 TCR predicts mutant TCRs with enhanced interface complementarity

We used molecular modeling to generate DMF5 TCR variants with improved binding complementarity with the pMHC surface. Hydrophobic and salt–bridge interactions generally drive protein–protein affinity, and individual amino acids making hydrophobic contacts contribute considerably to Ab–Ag interactions (69). We reasoned that TCR amino acid substitutions to side chains with increased hydrophobic surface area or forming new electrostatic pairs at the TCR/pMHC interface could improve affinity without sacrificing specificity (70). The WT x-ray structure of the DMF5/ Mart-1 (27L)/HLA-A2 complex has been solved at 2.7 Å reso-
olution (47). Inspection of the binding interface in this structure identified three candidate residues (aK96, bR31, and bT54) on the DMF5 TCR that were adjacent to solvent cavities at the pMHC interface exhibiting hydrophobic patches or unoccupied polar regions on the pMHC surface of the cavities (Fig. 2A–C). To identify mutations that exploit the unoccupied hydrophobic area or electrostatic donor/acceptors adjacent to these three residue positions without introducing clashes or unfavorable entropic interactions, molecular modeling of all possible non–backbone perturbing mutations of these three residues was performed (see Materials and Methods). Briefly, each position was modified to 1 of 17 other amino acids (excluding backbone-altering amino acids glycine and proline) and the energy of the complex was minimized and recorded. The minimization step consisted of conformational search of the replaced side chain and calculation of van der Waals, electrostatic, entropy, and solvation energy terms for the whole complex. This energy calculation afforded the calculation of the estimated free energy change of the TCR/pMHC complex as a result of the mutation. This screen resulted in a ranked list of mutations from most favorable to least favorable free energy change. The eight top-ranked DMF5 TCR mutants were aK96F, aK96E, aK96W, bR31F, bR31H, bT54M, bT54L, and bT54I (Table I). These mutants were selected for further in vitro testing.

Previously, mutations were identified that improved the affinity of the Tax/HLA-A2–specific A6 TCR by nearly 100-fold (35). Because A6 TCR shares its TCR b-chain (Vb 6.4) with DMF5, we sought to use this prior study to derive additional DMF5 mutants to be used as positive controls for those we identified by structure-based design. We analyzed the amino acid residues mutated in the Tax TCR study and found that they were in a similar conformation compared with their corresponding residues in DMF5 TCR (Fig. 2D, 2E). We superimposed the crystal structures of A6-Tax/HLA-A2 (PDB ID 1qrn) and DMF5-Mart-1/HLA-A2 (PDB ID 3qdg) and found both chemical and structural equivalence between A6 and DMF5 TCRs at three (aD26, aR27, and aS51) out of four positions previously selected for mutagenesis (35) (Fig. 2A, 2D, 2E). To fully compare these previously reported mutants to our present mutants, we calculated the free energy change upon mutation of the complexes containing these three DMF5 mutants (aD26W, aR27F, and aS51M) and Mart-1/HLA-A2 by the same method we used to derive our mutants. Additionally, we calculated the free energy change of the TCR/pMHC complexes containing other previously identified DMF5 mutants (aD26Y and bL98W) (37). The results showed that the previously reported mutants all result in a favorable free energy change relative to the WT TCR/pMHC complex, but the mutants identified in this study consistently resulted in a greater favorable free energy change (Table I).

Mutated TCRs enhance T cell activation

To analyze the effect of DMF5 TCR mutations on T cell activation in vitro, we generated WT TCR (DMF5) and mutant variant retroviral constructs for transduction and expression in hCD8+2/2 TCR2, CD3+ T cell hybridoma cells. Whereas TCR and CD3 expression levels were comparable in individual mutant and WT
As shown in Table II, the DMF5 mutations predicted by our specific clones (described below) and excluded from further analysis. Ground levels, such as (Table II). Titration curves that did not reach back and Methods of mutated and the WT clones, as described in detail in between the area under the titration curves of cytokine production we computed the AUC of cytokine production as the difference different Ag concentrations.

T cell hybridoma clones (Supplemental Fig. 2), IL-2 cytokine production varied significantly among the T cell hybridoma clones. Analysis of BT54L, αK96F, αK96W, and αR27F/αS51M T cell hybridoma clones showed consistently enhanced T cell activation by two melanoma cell lines, F29 and M44 (Fig. 3), at T cell hybridoma clones showed consistency enhanced T cell activation.

To assess potency and specificity with a single quantitative score, we computed the AUC of cytokine production as the difference different Ag concentrations. To assess potency and specificity with a single quantitative score.

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<table>
<thead>
<tr>
<th>Mutant</th>
<th>Binding Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βR31P</td>
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<tr>
<td>βR31H</td>
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</tr>
<tr>
<td>βT54M</td>
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<tr>
<td>WT</td>
<td>-4.835</td>
</tr>
</tbody>
</table>

Calculated binding free energies of DMF5 TCR-Mart-1 (27L)/HLA-A2 complexes with indicated point mutations are shown. For each mutant, only the mutated side chain torsion angles were minimized energetically to achieve a stable conformation. The energy score includes terms for van der Waals energy, solvation, electrostatics, and entropy. For the binding energy, the estimated free energy (van der Waals, electrostatic, and internal energy terms) is according to ICM scoring function.

ICM-defined TCR mutations maintain specificity and exhibit limited cross-reactivity

An important feature of the T cell repertoire is the balance exhibited in reactivity with cancer cells and minimization of autoimmune. This would indicate that the specificity of recognition must be sufficiently high for cancer Ags with minimal cross-reactivity to self-proteins (pMHC or MHC). To investigate the specificity and potential cross-reactivity of the mutated T cell hybridoma clones, we measured IL-2 cytokine production of the T cell hybridoma clones stimulated with different APCs in the absence of Ag (Fig. 4A). No or very limited cross-reactivity was observed for the mutant T cell hybridoma clones that contain mutations generated by our structure-based ICM approach. In contrast, a significant increase in cytokine production in the absence of Ag was observed for previously reported TCR mutations αD26W/αS51M, αD26W/αR27F, αD26W/αR27F/αS51M, αD26Y, and αD26Y/BL98W (35, 37), which may be interpreted as increased cross-reactivity (Fig. 4A). All mutated T cell hybridoma clones activated strongly when stimulated with Gmel (HLA-A2*), Mart-1 melanoma cell line), which confirms the potency of the WT and mutant TCRs on Mart-1–expressing cells. Still, the mutant T cell hybridoma clones that contain mutations generated by our structure-based ICM approach showed lower activation compared with previously identified TCR mutants (Fig. 4A). However, the ICM-generated TCRs showed less cross-reactivity than previously reported TCR mutants (35, 37) when activated with human dendritic cells obtained from three different donors (HLA-A2*) not loaded with peptide (Fig. 4B) or loaded with a different peptide such as gp100(2M) (Fig. 4C). The
activation of the T cells expressing mutated TCR or WT was HLA-A2–specific, as none of the mutated T cell hybridoma clones reacted with the 888-mel cell line (HLA-A2\(^2\), HLA-A0101+, A2402+, B5\(^5\), B6\(^2\), Cw5201\(^6\), Cw55\(^5\), DRb1*1502\(^2\), DRb1*1610\(^1\), DQb1*0601\(^1\), DRb5*0102\(^2\), DRb5*0203\(^2\)) (71) (Fig. 4B).

Finally, we analyzed the combined mutant TCR (\(\alpha R27F/\alpha S51M\) and \(\beta T54I\)) for reactivity to a panel of peptides (55) that bind to HLA-A2 and compared these results against the results obtained for the WT TCR and a cross-reactive TCR that was identified in our initial screen (\(\alpha D26W/\alpha R27F\)) (Supplemental Fig. 3). Again, the combined mutant TCR (\(\alpha R27F/\alpha S51M\) and \(\beta T54I\)) identified by our structure-based ICM approach showed less cross-reactivity compared with the \(\alpha D26W/\alpha R27F\) mutant TCR and a minimal increase in cross-reactivity when compared with WT TCR.

Taken together, these results show that our structure-based design approach combined with careful functional analysis, in contrast to previous published work (Fig. 4), generated improved mutant TCRs that showed minimal cross-reactivity.

**Detailed structural changes associated with DMF5 TCR mutants**

\(\beta T54I/L/M\) mutations. In many published TCR/pMHC complexes, the interface between the TCR and the pMHC is populated by solvent cavities, and improvement of hydrophobic packing improves TCR/pMHC binding (12, 70, 72). Therefore, we evaluated the structural detail of packing efficiency of our WT and mutated TCR complexes. The solvent-accessible surface area (SASA) is a measure of the packing efficiency at a protein interface, and it is greater when there are more solvent cavities at the interface. The SASA at the TCR/pMHC interface where two hydrophobic substitutions \(\beta T54I\) and \(\beta T54L\) were introduced decreased compared with the WT TCR/pMHC complex. Based on our calculations we found for the \(\beta T54I\) TCR mutant a SASA of 851.4 Å\(^2\) and for the \(\beta T54L\) mutant a SASA of 853.6 Å\(^2\), whereas for the WT TCR we found a SASA of 853.6 Å\(^2\). For comparison, we evaluated SASA for the \(\beta T54A\) mutant introduced in (73), which our calculations show a SASA of 853.6 Å\(^2\). These results suggest that the improved interface resulting from the mutations generated in this study were associated with increased interface complementarity and hydrophobic packing by making new nonpolar contacts with Q72, T73, and V76 of the MHC (Fig. 5A, 5B).

\(\alpha K96W/F\) mutations. A similar critical structural evaluation of \(\alpha K96W/F\) mutations (Fig. 5C, 5D) predicted that both the W and F amino acids are within 6 Å of R65 of the MHC in a nearly parallel planar configuration. Based on this observation, one possibility is that these mutations create a new cation–π interaction with this...
arginine. Cation–π interactions can contribute significantly to affinity at protein interfaces (74). For A6 TCR, the CDR3β loop contributes to cross-reactivity by adjusting in response to different ligands (75), and so one possible explanation for why αK96/W/F mutations maintain specificity for the Ag could be that these mutations are in the CDR3α loop of the TCR.

**Evaluation of combined TCR mutations for changes in T cell function and cross-reactivity show additive effects**

Previous results have shown that combined TCR mutations can have additive (76–78) or cooperative (79, 80) effects on T cell function. A previous study of combinations of mutations on a TCR have additive (76–78) or cooperative (79, 80) effects on T cell function and cross-reactivity show additive effects. Evaluation of combined TCR mutations for changes in T cell function and cross-reactivity by adjusting in response to different ligands (75), and so one possible explanation for why αK96/W/F mutations maintain specificity for the Ag could be that these mutations are in the CDR3α loop of the TCR.
immunity (9). In that study, we used seven human melanoma gp100209–217-specific TCRs spanning physiological affinities (1–100 μM). We found that in vitro and in vivo T cell responses are determined by TCR affinity, except in one case that was compensated by substantial CD8 involvement. In the end, we concluded that T cell antitumor activity and autoimmunity are closely coupled but plateau at a defined TCR affinity of 10 μM, likely due to diminished contribution of TCR affinity to avidity above the threshold. Also, Corse et al. (82) demonstrated that pMHC binding to TCR with medium strength induced optimal in vivo CD4+ T cell activation. These and other studies (83–88) show that maximal T cell responses occur at an optimal TCR/pMHC off-rate ($k_{off}$) or $K_D$, whereas functional attenuation is observed above the natural range. Previous studies have used phage display to identify TCRs with affinities within the nanomolar range such as the wtc51m (81), which resulted in impaired function of human primary CD8+ T cells expressing this TCR (89).

Therefore, improving affinity via library display does not always have the desired outcome, and a more careful and specific design of mutations that enhance T cell function is often needed. Recently, Michielin and colleagues (88) used a structure-based rational design to generate a panel of HLA-A2/NY-ESO-1–specific TCRs with increased affinity of up to 150-fold from the WT TCR. These TCRs and others, including wtc51m mentioned above, were used to investigate the effect of TCR/pMHC binding parameters on CD8+ T cell responsiveness, which showed that maximal T cell responses occur at intermediate binding parameters (88). Because improving TCR affinity does not always correlate with improved T cell activation, other factors should be considered when improving TCRs. Previous studies have shown that the difference between degenerate and specific TCRs can rely on factors other than affinity or kinetics of binding to their pMHC, such as different binding topology (90, 91). Given that tetramer-based assays have limitations (expression level of TCRs, adhesion molecules or

**FIGURE 4.** Mutated T cell hybridoma clones show no cross-reactivity and maintain HLA-A2 specificity. Analysis of hCD8 58 hybridoma cells transduced with DMF5 WT TCR and mutated TCR variants is shown. (A) Identification of model-predicted mutations that do not cross-react with MHC without the presence of peptide. T cells were incubated with four different APCs (T2-A2/Kb, FM29, M44, Gmel) in the absence of peptide for 20 h. (B) T cells were incubated with dendritic cells from three different donors (HLA-A2+) in the absence of peptide. (C) T cells were incubated with dendritic cells from three different donors (HLA-A2+) and Gmel cell line with gp100/2M peptide. (D) T cells were incubated with T2-A2/Kb or the melanoma cell line 888-mel (HLA-A2+, Mart-1+) loaded with 10 μM Mart-1 (27L) peptide for 20 h. IL-2 cytokine production was quantified via an IL-2 sandwich ELISA assay. ICM identified mutations are shown in bold.
coreceptors, and changes in components of the signaling cascade as well as nonstandard distribution of oligomeric states in fluorescent MHC tetramer preparations (1), we only used tetramer based assays in Fig. 1 for ranking purposes and to select the most potent TCR for modification from our panel of TCRs. This initial selection strategy was necessary because crystal structures for all TCRs in the panel were not available. After selecting the most potent TCR where structural information was available for the pMHC complex (47), we focused on the novel structure-based design, which allowed us to use the atomic-detail contact interface of the TCR/pMHC complex to predict mutations with increased functional avidity without increasing cross-reactivity.

In the present study, we engineered a native TCR (DMF5) for increased binding to pMHC Mart-1 (27L)/HLA-A2 (4, 46, 47). We used a customized structure-based approach that allowed us to rationally design sequence substitutions in contact areas between the TCR and the pMHC. Some of these mutations improved the surface shape and energetic complementarity of the TCR with HLA-A2.

**FIGURE 5.** Models of structural changes associated with introduced DMF5 TCR mutants. HLA-A2 MHC is displayed in gray and cyan ribbons. WT αK96 and βT54 TCR residues are shown in orange. (A) βT54I (gray) versus (B) βT54L (gray) TCR mutants showing that I and L may both potentially increase interface complementarity and hydrophobic packing by making new nonpolar contact with Q72 on the α-chain of HLA-A2. (C) αK96W versus (D) αK96F TCR mutants. Both W and F are predicted to be within 6 Å of R65 of the MHC and potentially may be engaged in a new cation–π interaction with this arginine, which can contribute significantly to affinity at protein interfaces. Distances from the ring centroid to R65 NH1 are indicated.

**FIGURE 6.** Functional evaluation of the combined TCR mutations shows an additive effect of αR27F/αS51M and βT54I. Analysis of T cells transduced with the DMF5 TCR or the indicated combinations of the mutated TCR variants in hCD8 58−/− TCR+ hybridoma cells is shown. T hybridoma cells were incubated with the M44 melanoma cell line expressing HLA-A2 loaded with the indicated concentrations of Mart-1 (27L) peptide for 20 h. IL-2 cytokine production was quantified via an ELISA assay.
pMHC without increasing cross-reactivity with several melanoma cell lines in the absence of Ag presentation. In accordance with previous studies (81), we also found that high-affinity TCRs can be achieved through directed evolution of germline CDRs. Likewise, Kranz and colleagues (28) engineered high-affinity TCRs from libraries of CDR1, CDR2, and CDR3 and found that their TCRs retained remarkable peptide specificity. In this study, successful molecular modeling of various potency-enhancing mutants that retained Ag specificity suggests that the solvent cavities at the DMF5/Mart-1 (27L)/HLA-A*0201 interface are highly specific for that complex. Accordingly, precise atomic detail was used to change loosely associated spots into binding hotspots by increasing hydrophobic and electrostatic interactions that can improve potency without increasing cross-reactivity. Our molecular modeling mutations in the CDR3α (αK96) and CDR2β (βT54) loops produced the highest increase in T cell activation potency compared with WT DMF5 TCR without increasing cross-reactivity. These loops lie at a 31˚ angle diagonally across the MHC-bound peptide in the complex, suggesting that this is the most structurally variable area of the complex, and thus the area with the most opportunity for hydrophobic and electrostatic improvement. Moreover, a combination of mutations (αR27F/αS51M with βT54I) produced in TCR with improved T cell function while retaining specificity. Previously identified mutations inducing high-affinity TCR (43 nM) (a 250-fold increase in affinity compared with the WT) (37) did not fare well in our T cell activation assays, showing little improvement of activity when compared with the WT but increased cross-reactivity. This emphasizes the importance of measuring functional outcomes of engineered TCRs with very high affinity (above nanomolar range) when trying to improve T cell activation outcomes. Interestingly, the Gmel cell line activation data (Fig. 4A) highlight the remarkable specificity of this ICM structural-based design method and its limitations of identifying mutations that have a broad effect across MHC/TCR complexes presenting different peptides, as are present in the Gmel cell line. Notably, the Mart-1 (27L) (ELAGIGILTV) peptide used in these studies is the altered decameric epitope with a mutation in the second position (A27L) spanning residues 26–35 of the WT decameric epitope (EALAGIGILTV). The nanomer epitope (AAGIGILTV), which is not in this complex on which we based our design, does not include the first amino acid (glutamine) at the peptide N terminus of the decamer. This nanomer is thought to be the physiologically relevant epitope in HLA-A2* individuals (92–95). Therefore, it is important to emphasize that Gmel cell line is Mart-1* and to the best of our knowledge variably expresses the WT nanomer/decamer or both; however, it does not express Mart-1 (27L) peptide for which these TCRs were specifically optimized. The Gmel cell line activation data raise the point that the two peptides could have different activation capabilities and therefore the TCR optimized for one peptide might not outperform the other. However, activation differences between altered peptides are well documented in the literature (92, 96, 97). For instance, Cerottini and colleagues (96) performed titrations of both Mart-1 peptides (decamer versus nanomer) using T2 cells as targets to activate a large number of different Melan-A/Mart-1–specific T cell clones and determined that the two individual peptides allowed the Ag specificities to be distinguished among the Mart-1–spe-

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** TCR αR27F/αS51M and βT54I mutations show improved cytokine production and cytotoxicity when expressed in primary murine CD8+ T cells. (A) Primary CD8+ T cells transduced with the DMF5 TCR or the indicated combinations of the mutated TCR variants were analyzed for functional cytokine production by incubating T cells with the M44 melanoma cell line expressing HLA-A2 loaded with the indicated concentrations of Mart-1 (27L) peptide for 20 h. IFN-γ cytokine production was quantified via an ELISA assay. (B) To analyze the cytotoxic properties of the TCR mutations and compare them to the WT TCR, a standard chromium release assay was performed. S3Cr-labeled M44 cells and TCR-transduced primary CD8+ T cells were cultured in the presence of 10 µM Mart-1 (27L) peptide for 5 h. Cytotoxicity is shown as percentage of maximum S3Cr released after complete target cell lysis with Triton X-100.
cific CTL clones. One class of T cell clones clearly preferred the decapeptide to the nanopptide, a second did not show any preference, and a third class preferred the nanopptide to the decapeptide (96). Speiser and colleagues (93) compared the WT nanomer and decamer peptides and concluded that the two peptides should be regarded as distinct epitopes when analyzing tumor immunity and developing immunotherapy against melanoma. Additionally, a previous structural study has concluded that there are significant structural differences of how the nanomer versus the decamer bind HLA-A2 molecules (98). Compared with the nanomer, the additional amino acid in the decamer forces the peptide to bulge and zigzag in the HLA-A2 peptide-binding groove, resulting in the presentation of different surfaces to the T cell repertoire (98).

Surprisingly, DMF5 TCR engages both ligands almost identically using an open architecture apparently preformed in the free TCR (47). However, there is an 8-fold difference in affinity between DMF5 TCR and each peptide/MHC class I molecule (decamer 5.6 μM versus nanomer 40 μM) (47), which demonstrates meaningful kinetic differences among these peptides.

Lastly, at low peptide concentrations (<0.1 μM), as shown in Fig. 3, the ICM-optimized TCRs do not outperform WT TCR, as they both are close to the IL-2 detection range. In general, Mart-1 expression levels on melanoma cell lines (such as Gmel) range they both are close to the IL-2 detection range. In general, Mart-1 expression levels on melanoma cell lines (such as Gmel) range from low to moderate levels (95). Also, Renner and colleagues (94) showed that endogenous Ag processing of the decamer (Melan-A) protein or its A27L-mutated variant results in inefficient presentation of these peptides both WT and 27L mutant by HLA-A2+ tumor cell lines. Therefore, because the Gmel melanoma cell line in Fig. 4A most likely expresses low levels of WT Mart-1 peptide and was not loaded with any Mart-1 (27L) peptide, it was used as a measurement of cross-reactivity, not Ag specificity. Furthermore, we show (Supplemental Fig. 4) that when the Gmel cell line is loaded with Mart-1 (27L) peptide the mutated TCR outperforms DMF5 TCRs, which confirms the specific enhancement of the mutated TCR when stimulated with the exact peptide for which it is engineered in our structural-based studies.

Other factors besides the TCR/pMHC interface may influence potency. Recently, Rufer and colleagues (89) demonstrated that an increase in activity of negative regulators of T cell signaling such as PD-1 and SHP-1 can counteract the beneficial effect of an increased TCR affinity. This leads the way for future studies to elucidate the molecular mechanism underlying functional responses and differences between cross-reactive and specific high-affinity TCRs. This information could provide the rationale for improving T cell therapy using improved TCR variants in combination with treatments of other molecules such as anti-CTLA-4 and anti-CD-1/P-CD-L1 Abs (99, 100).

TCR-engineered T cells could cause off-target toxicity cross-reactivity through recognition of unrelated proteins expressed by normal tissue (40) or on-target toxicity through recognition of low levels of Ag expressed on normal tissue (9, 46, 49). Based on our results we generated TCRs that do not show off-target toxicity, even though this does not exclude the possibility of off-target toxicity in vivo or on-target toxicity as observed previously (9). Targeting of tumor-specific Ags (e.g., NY-ESO-1, SSX2) is generally thought to be the solution to avoid cross-reactivity, and the strategy used in the present study would nicely complement future studies focusing on such Ags. Even so, complete off-target toxicity can only fully be evaluated in human clinical trials (40). Applying a conditional suicide gene to insert a level of control in ACT, which in a previous clinical trial effectively ablated allogeneic T cells (101), may also improve the safety and application of ACT.

Our results reveal an optimal path to improve T cell potency without loss of specificity. A preliminary structure-activity map was generated, but its validity and detail can be greatly expanded by additional binding, kinetic analyses, and structural evaluation of these mutants in future studies, as well as their tumor rejection and cross-reactivity evaluation in vivo in animal models and hopefully in human clinical trials.

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Disclosures

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**Figure S1.** Quantification of TCR and CD3 expression on the surface of T cell hybridoma demonstrated comparable expression across hybridoma cell lines transduced with different mutated TCRs. T hybridoma clones were evaluated for CD3 (A) and TCR (B) expression using a biotin labeled anti-mCD3 antibody followed by staining with PE-labeled streptavidin or an APC-labeled anti-mouse-TCRCβ antibody and staining was evaluated by flow cytometry. MFI-values are plotted for individual transduced T hybridoma cell lines as indicated.
Figure S2. Analysis of ICM-simulated TCR mutants show equal TCRβ and CD3ε expression

T cells transduced with the DMF5 (WT) and mutated TCR variants when expressed in hCD8 58/- TCR-negative hybridoma cells were stained with anti-constant TCRβ-APC and anti-CD3ε-PE antibodies for 30 min at room temperature and evaluated via flow cytometry using a FACSCaliber instrument (BD Biosciences). Data was analyzed by Flowjo (TreeStar).
Figure S3. Combined TCR mutations show little cross-reactivity to a panel of HLA-A2 binding self-peptides.

Analysis of DMF5 TCR and the indicated combinations of mutated TCR variants when expressed in 58/- TCR-negative T cell hybridoma with hCD8. T cells were incubated for 20 hrs at 37°C with T2-A2/Kb cells expressing HLA-A2-Kb loaded with 10 µM each of a panel of self-peptides (1). IL-2 cytokine production was quantified via an ELISA assay.
**Figure S4. A.** Functional evaluation of αR27F/αS51M+βT54I TCR mutations show improved cytokine production. Analysis of DMF5 TCR and the indicated combinations of mutated TCR variants when expressed in 58-/- TCR-negative T cell hybridoma with hCD8. T cells were incubated for 20 hrs with FM29 (left) Gmel (middle) 888-mel (right) melanoma cell lines loaded with the indicated concentrations of Mart-1 (27L) peptide. FM29 and Gmel are HLA-A2 positive, while 888-mel is HLA-A2 negative. IL-2 cytokine production was quantified via an ELISA assay. **B.** Combined TCR mutations increase T cell function to a higher degree than individual mutations. Analysis of DMF5 TCR and the indicated combinations of the mutated TCR variants when expressed in hCD8 58-/- TCR-negative T cell hybridoma. T cells were incubated with T2-A2/Kb (left) FM29 (middle) and M44 (right) melanoma cell line expressing HLA-A2 loaded with 0.1 µM of Mart-1 (27L) peptide (top panel) or no peptide (bottom panel) for 20 hrs at 37°C. IL-2 cytokine production was quantified via an ELISA assay. **C.** Tetramer decay of TCR-transduced hybridoma cells with Mart-1-specific TCRs. Representative decay plots of the natural logarithm of the normalized fluorescence versus time. Calculated half-lives are shown in minutes.