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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,*§ Timothy Cardozo,*† and Michelle Krosggaard*‡,¶

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: 000–000.

It is estimated that there are >10^8 αβ TCRs in the human naive T cell pool (13). However, this number is small when compared with the immense array of potential antigenic peptides (>10^15) (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 fewer 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 improve T cell responses against virus-infected cells or tumor cells that leads to T cell activation and target cell killing (1–3). Adoptive T cell therapy (ACT) with T cells transduced with Ag-specific TCRs has shown promise in cancer immunotherapy (2, 4–6). However, because of the low affinity of tumor-specific TCRs (micromolar range) for pMHC (7–11), the clinical efficacy of ACT remains suboptimal.

The diversity of TCRs is based on amino acid variability in the six CDRs (12). Theoretically, germline-encoded CDR1a, CDR1b, CDR2a, and CDR2β loops contact the germline element of the MHC molecule, whereas the non-germline CDR3α and CDR3β loops contact the variant peptide element. In practice, however, this convenient rule does no hold true for all of the crystallographic structures of TCR/pMHC complexes that have been solved to date (12).

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K.M. and M.K. designed the study; M.K. supervised the project; K.M. conducted most of the experiments, and K.M. and M.K. analyzed most of the data; M.K., K.M., A.G., and T.C. wrote the paper; K.M., A.G., S.Z., and W.J.G. generated DNA constructs and performed cell experiments; A.G. and T.C. predicted TCR point mutations; A.G. wrote scripts used in molecular modeling and analysis; A.G. and T.C. performed structure-based computational analyses of the mutated TCRs; and L.A.J. and S.A.R. isolated and cloned the TCR genes from patient samples at the Surgery Branch, National Cancer Institute (Bethesda, MD).

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The online version of this article contains supplemental material.

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.
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\(\alpha\) 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 transferred 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 \(\mu M\), 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 EAAGIGILTV) (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 protein HLA-A*0201 (4, 46, 47). In vitro functional assays indicated that DMF5 binds Mart-1 (27L)/HLA-A*0201 tetramer more efficiently than does DMF4 (48). Although not statistically significant, in a small clinical trial DMF5 (6 of 20 patients [30%]) showed indications of improving the rate of objective cancer regression compared with DMF4 (4 of 31 patients [13%]) (46). Furthermore, autoimmune toxicity was reported in the eye, ear, and skin for DMF5 but not DMF4 TCR (46, 49–51). Additionally, tyrosinase, gp100, and Melan-A/MART-1, normal self-proteins encoded for their ability to bind pMHC tetramer in a CD8-dependent and independent fashion. Based on these results we identified DMF5 TCR as the most potent TCR at low Ag concentrations. Given that the wild-type (WT) crystal structure of the DMF5/Mart-1 (27L)/HLA-A2 complex has been solved (47), we predicted affinity-enhancing point mutations of DMF5 using molecular modeling, which were subsequently analyzed for their T cell activation potential. We identified point mutations in critical TCR positions resulting in more potent T cell activation but maintaining overall specificity. 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 TCR\(\beta\) constant (clone H57-597) and PE anti-mouse CD3\(\epsilon\) (clone 145-2C11) Abs were from eBioscience. For Mart-1 (27L)/HLA-A2 TCR 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 \(\beta\)-microglobulin were purified as an inclusion body from Baculovirus-infected Sf9 cells. 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) according 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\(\kappa\) 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 \(\alpha\)\(\beta\) 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 as previously described (52, 53). The resulting construct was subcloned into the retroviral vector pMSGV (61). Amplification of the WT TCR DNA sequence with overlapping primers containing the mutant sequences generated the mutated TCR constructs. After several rounds of amplification, the entire mutated TCR sequence was assembled (34) and subcloned into retroviral vector pMSGV (61), which was also used for expression of the WT TCR.

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 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-TCR\(\beta\) 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 × 10^5; T2-A2/K6, M44, FM29) were loaded with different concentrations of Mart-1 (27L) peptide and were coincubated with 1 × 10^6 T hybridoma clones for 16 h at 37°C, 5% CO2. For self-peptide ELISA assays, 1 × 10^6 APCs (T2-A2/K6) were loaded with 10 µM of each of the self-peptides and were coincubated with 1 × 10^6 T hybridoma clones for 16 h at 37°C, 5% CO2. 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 tetramethylrhodamine 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 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 × 10^6 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-TCRβ 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 K0, subtracting, the initial time point as a background reference normalized determinations the MFI of individual samples and the resulting value was plotted on a logarithmic scale. The K0 was derived from the negative reciprocal of the slope of the line fit to Scatchard plot 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) (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 (h)CD8 only (no TCR) was expressed in 58K hybridoma cells (hCD8, 58K+) in the same cell line: 1) ΔA = integrated area (DMF5 TCR mutant) − integrated area (hCD8, 58K+); and 2) ΔA (WT DMF5 TCR) = integrated area (WT DMF5 TCR) − integrated area (hCD8, 58K+). Furthermore, the areas obtained for DMF5 TCR mutants were subtracted from the area obtained for the WT DMF5 TCR: 3) ΔA1 = ΔA − ΔA (WT DMF5 TCR). Positive ΔA1 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 × 10^6 s1Cr-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 58K (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 K0 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 hydrophilic 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-
lution (47). Inspection of the binding interface in this structure identified three candidate residues (αK96, βR31, and βT54) 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 αK96F, αK96E, αK96W, βR31F, βR31H, βT54M, βT54L, and βT54I (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 β-chain (Vβ 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 (αD26, αR27, and αS51) 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 (αD26W, αR27F, and αS51M) 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 (αD26Y and βL98W) (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+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 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.

To assess potency and specificity with a single quantitative score, we computed the AUC of cytokine production of the WT clones, as described in detail in Materials and Methods (Table II). Titration curves that did not reach background levels, such as αD26W/αR27F, were considered nonspecific clones (described below) and excluded from further analysis. As shown in Table II, the DMF5 mutations predicted by our structure-based approach (βT54M, βT54L, βT54I, αK96F, and αK96W in particular) exhibited superior differential AUCs (indicated as ΔAUC in Table II), suggesting affinity enhancement with preserved Ag specificity when compared with previously reported T cell activity enhancing mutations (35, 37). Taken together, these results indicate that selected individual mutated T cell hybridoma clones specifically enhanced T cell activation.

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 autoimmunity. 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+, and loaded with a different peptide such as gp100(2M) (Fig. 4C). The

![Figure 2](http://www.jimmunol.org/)
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-A22, HLA-A0101+, A2402+, B55+, B62+, Cw5+201+, Cw5+, DRbl*1502+, DRbl*1610+, DQbl*0601+, DRb5*0102+, DRb5*0203+) (71) (Fig. 4B).

Finally, we analyzed the combined mutant TCR (αR27F/αS51M and βT54I) 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 (αD26W/αR27F) (Supplemental Fig. 3). Again, the combined mutant TCR (αR27F/αS51M and βT54I) identified by our structure-based ICM approach showed less cross-reactivity compared with the αD26W/αR27F 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**

**βT54I/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 βT54I and βT54L were introduced decreased compared with the WT TCR/pMHC complex. Based on our calculations we found for the βT54I TCR mutant a SASA of 851.4 Å² and for the βT54L mutant a SASA of 763.2 Å², whereas for the WT TCR we found a SASA of 864.6 Å². For comparison, we evaluated SASA for the βT54A mutant introduced in (73), which our calculations show a SASA of 853.6 Å². 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 non-polar contacts with Q72, T73, and V76 of the MHC (Fig. 5A, 5B).

**αK96W/F mutations.** A similar critical structural evaluation of αK96W/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
obtained from phage display found significant cooperativity 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 most likely arises additively from each region independently, with the βT54I contributing to increased hydrophobic packing as discussed above and the αR27F/αS51M independently increasing the hydrophobic contact surface area at the pMHC/TCR interface.

Evaluation of combined TCR mutations in mouse primary T cell shows improved cytokine and effector function compared with the WT TCR

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ΔA</th>
<th>ΔA₁</th>
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<tbody>
<tr>
<td>FM29 cell line</td>
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<td></td>
</tr>
<tr>
<td>βR31F</td>
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Mutations highlighted in bold exhibited superior differential AUCs, suggesting activity enhancement with preserved Ag specificity when compared with previously reported T cell activity–enhancing mutations. ΔA = area of TCR mutant – area of cells without TCR; ΔA (WT) = area of WT – area of cells without TCR; ΔA = ΔA – ΔA (WT).

Mutations predicted in the present study (ICM-simulated mutations).

Mutations introduced by Hadar et al. (35).

Mutations introduced by Hawse et al. (37).

arginine. Cation–π interactions can contribute significantly to affinity at protein interfaces (74). For A6 TCR, the CDR3B loop contributes to cross-reactivity by adjusting in response to different ligands (75), and so one possible explanation for why αK96W/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 obtained from phage display found significant cooperativity within and between modules (79). A more recent study that combined mutations from phage display and structure-based design found that whereas many of the combinations exhibited cooperative and additive effects, three had a significant degree of anti–cooperative effects (also referred as negative cooperative effect) (76). To investigate whether combinations of the generated mutations enhancing T cell activation had additive or cooperative effects, we combined TCR individual mutations (αK96F, αK96W, βT54I, and αR27F/αS51M). Although most of the combinations did not enhance T cell function, the combination of αR27F/αS51M with βT54I produced TCR with improved T cell function against M44 (HLA-A2+3 melanoma cell line (Fig. 6). When functional differences were compared between the combination of αR27F/αS51M with βT54I TCR with either αR27F/αS51M TCR or βT54I TCR alone, the effect is additive (Supplemental Fig. 4B). The calculated AUC for change in IL-2 production also showed that αR27F/αS51M when combined with βT54I yield the highest value of AUC (i.e., 6.88) (Table III). In fact, the T cell hybridoma clones expressing combined mutations resulted in increased T cell function when analyzed against various melanoma cell lines, while retaining HLA-A2 specificity (Supplemental Fig. 4A). Surprisingly, the mutated TCRs did not show an increase in cross-reactivity, measured by T cell stimulation by APC without MART-1 Ag present (Supplemental Fig. 4B). Combined mutations (αR27F/αS51M and βT54I) showed a small increase in cross-reactivity (Supplemental Fig. 4B) when compared with the αR27F/αS51M or βT54I mutations alone (Supplemental Fig. 4B). These differences are not due to the off-rate, as indicated by tetramer decay assay determination (Supplemental Fig. 4C). The combined mutations (αR27F/αS51M βT54I) have a tetramer dissociation half-life of ~209 min, an off-rate similar to the WT (~201 min) (Supplemental Fig. 4C). Evaluation of the αR27F/αS51M and βT54I mutations when modeled into the TCR/pMHC structure showed that these positions are significantly located in three-dimensional space and thus the enhancement in T cell function most likely arises additively from each region independently, with the βT54I contributing to increased hydrophobic packing as discussed above and the αR27F/αS51M independently increasing the hydrophobic contact surface area at the pMHC/TCR interface.

Discussion

In this study, we sought to increase T cell potency by structure-based design while overcoming the inherent physiological plausibility of the TCR that potentially leads to cross-reactivity (6, 9, 14–17, 81). Unfortunately, effective tumor immunity requires the induction of the same responses that underlie autoimmunity. Hence, as the immune system is manipulated to treat cancer, the vital mechanisms that regulate self-tolerance are unbalanced. Therefore, a key scientific question can be posed: Is a more potent immunotherapy necessarily more destructive, or is there a window of opportunity to increase potency specifically?

Recently, we determined the TCR affinity threshold defining the optimal balance between effective antitumor activity and auto-
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 KD, 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...
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

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**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+ TCR2 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.
in the presence of 10 μM Mart-1 peptide for 20 h. IFN-cytokine production by incubating T cells with the M44 melanoma cell line expressing HLA-A2 loaded with the indicated concentrations of Mart-1 peptide.5

When compared with the WT but increased cross-reactivity. This TCR mutation is not expressed in the Gmel cell line. Notably, the Mart-1 (27L) peptide used in these studies altered the decamer epitope with a mutation in the second position (A27L) spanning residues 26–35 of the WT decameric epitope (ELAGIGILTV). 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. 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 (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 (EAAGIGILTV). 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.

However, activation differences between altered peptides are well documented in the literature. For instance, Cerottini and colleagues 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-

\[
\Delta A = \text{area of TCR mutant} - \text{area of hCD8}_{58}^{+/+}; \Delta A (\text{WT}) = \text{area of WT} - \text{area of hCD8}_{58}^{+/+}.
\]

**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 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 (EAAGIGILTV). 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. 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. For instance, Cerottini and colleagues 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-

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Table III. Evaluation of AUCs in IL-2 concentration when T cell hybridomas were stimulated with M44 cell line loaded with Mart-1 (26)
cific CTL clones. One class of T cell clones clearly preferred the decapeptide to the nanopetide, a second did not show any preference, and a third class preferred the nanopetide 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 \(\mu M\) versus nanomer 40 \(\mu M\)) (47), which demonstrates meaningful kinetic differences among these peptides.

Lastly, at low peptide concentrations (<0.1 \(\mu M\)), 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 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 8-fold difference in affinity between DMF5 TCR and each peptide/MHC class I molecule (decamer 5.6 \(\mu M\) versus nanomer 40 \(\mu M\)) (47), which demonstrates meaningful kinetic differences among these peptides.

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.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

Structural basis for the recognition of mutant self by a tumor-specific, MHC class II-restricted T cell receptor. Nat. Immunol. 8: 398–408.


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 TCRb-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.