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Molecular Design of the Caβ Interface Favors Specific Pairing of Introduced TCRαβ in Human T Cells

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A promising approach to adoptive transfer therapy of tumors is to reprogram autologous T lymphocytes by TCR gene transfer of defined Ag specificity. An obstacle, however, is the undesired pairing of introduced TCRα- and TCRβ-chains with the endogenous TCR chains. These events vary depending on the individual endogenous TCR and they not only may reduce the levels of cell surface-introduced TCR but also may generate hybrid TCR with unknown Ag specificities. We show that such hybrid heterodimers can be generated even by the pairing of human and mouse TCRα- and TCRβ-chains. To overcome this hurdle, we have identified a pair of amino acid residues in the crystal structure of a TCR that lie at the interface of associated TCR Ca and CB domains and are related to each other by both a complementary steric interaction analogous to a “knob-into-hole” configuration and the electrostatic environment. We mutated the two residues so as to invert the sense of this interaction analogous to a charged “hole-into-knob” configuration. We show that this inversion in the CaCB interface promotes selective assembly of the introduced TCR while preserving its specificity and avidity for Ag ligand. Noteworthily, this TCR modification was equally efficient on both a Mu and a Hu TCR. Our data suggest that this approach is generally applicable to TCR independently of their Ag specificity and affinity, subset distribution, and species of origin. Thus, this strategy may optimize TCR gene transfer to efficiently and safely reprogram random T cells into tumor-reactive T cells. The Journal of Immunology, 2008, 180: 391–401.

Central tolerance mechanisms in the thymus eliminate high-affinity TCR for self-Ag and thus diminish the frequency of potent tumor-reactive CTL in the Hu T cell repertoire (2). To bypass this restriction, we took advantage of HLA-A2-transgenic (Tg) mice to develop in vivo murine (Mu) high-affinity TCR (3, 4). Alternative strategies focus on in vitro techniques such as phage (5)/yeast (6)/TCR (7) display. The retroviral transfer of the related TCR genes into T cells preserved both effector efficacy and memory pool formation in vivo (8).

Upon transduction of T cells with TCR, mixed pairing of introduced and endogenous TCRα- and TCRβ-chains may generate a plethora of hybrid TCR of unknown specificities and potential autoimmune reactivities (9). In this regard, the size of the T cell repertoire is theoretically highly diverse in the order of 1016. However, it contracts several orders of magnitude depending on thymic output, aging, and homeostasis of the individual (10). Additionally, introduced TCR may have to compete with endogenous TCR for assembly into the CD3 complex and subsequent cell surface export (11).

To prevent the formation of hybrid TCR, three-domain single chain TCR were generated whose variable domains were covalently attached to each other by a linker. Effector function was improved by fusion to signaling molecules such as CD3ζ or Lck (12, 13). Additionally, chimerization of double chain TCR to CD3ζ resulted in preferential heterodimerization due to the transmembrane dimerization motif located in CD3ζ (13). Recently, the introduction of an artificial disulphide bond in the constant domains yielded stable TCR suited for both phage display (5) and gene therapy (14, 15). “Murinization” of Hu domains led to preferential pairing (16, 17). However, concerns associated with such chimeric TCR are their immunogenicity, interference with appropriate signaling, and residual pairing with endogenous TCR.

Analysis of the crystal structures of the alloreactive H-2Kb-restricted Mu 2C TCR (18) and a Hu HLA-A*0201-restricted TCR...
revealed a sterically and electrostatically complementary pair of interacting residues, which is located in the constant domains. We hypothesized that swapping their positions may favor pairing of the reciprocally mutated TCR and disfavor the formation of noncomplementary TCR chain combinations. According to ImMunoGeneTics information system (IMGT) nomenclature for the dedicated numbering of TCR constant domains (20, 21), the pair consisted of a glycine to arginine mutation at position 85.1 in the constant \( \alpha \) (ca) domain (Gly85.1Arg-88c) and an arginine to glycine mutation at position 88 in the constant \( \beta \) (cb) domain (Arg-88c-Gly). Initially, the feasibility of this approach was tested for a Mu V\( \beta \)6+ mouse TCRDM2(81–88) TAA-specific TCR (where \( \mathbb{V} \beta \) is the variable \( \beta \) domain and DM2 is the mouse double minute 2 oncprotein (3)). We extended this approach to a Hu V\( \beta \)14+ gp100(280–288) TAA-specific high-affinity TCR (22) and to the CD8\( ^{+} \) and CD4\( ^{+} \) subsets thereof. Structural avidity, i.e., native heterodimeric TCR\( \alpha \beta \) surface expression, as well as functional avidity, i.e., cytolytic and IFN-\( \gamma \)-secretory effector function (23), have been assessed in retrovirally transduced bulk Hu T cells for all wild-type and mutated TCR combinations. The presence of endogenous TCR did not compromise the results, because the favored behavior of chain pairing has been reproduced in the endogenous TCR-deficient hybridoma 58\( \alpha \)6\( \beta \)\( ^{+} \). This approach significantly inhibited the potential formation of an interspecies hybrid TCR of a mouse/human-mixed type. Tetrimer dissociation experiments revealed that this molecular design did not impair recognition of the cognate Ag.

**Materials and Methods**

**Graphic display of TCR structures and modeling**

The crystal structure atom coordinates of a Mu H-2K\( ^{b} \)-restricted TCR, ITCR (18), and a Hu HLA-A\(^2\)-0201-restricted TCR, IB2 (19), were fetched from the Research Collaboratory for Structural Bioinformatics ( RCSB) protein data bank (24) and visualized and analyzed with Swiss-PdbViewer version 3.7 (25); amino acids were mutated by taking advantage of the dedicated numbering of TCR constant domains (20, 21), the vector was modified by inserting an internal ribosome entry site (RES)-puromycin or RES-neomycin cassette (Takara/Clontech) as described in Ref. 27 to enable the selection of transduced PBMC for expression of TCR\( \alpha \) \( \beta \) in PBLu-RESneo. Amino acid replacements in the wild-type (Wt) DM2(81–88)-specific TCR sequences were done with the mutagenic primers 5'-gattcacaagacaagaggatctgcgtgct-3' and 5'-tactcttgagcctagctgctgtctgctgctg-3' to pair complementary counterparts to yield Gly85.1Arg-88c-Gly in Hu TCR\( \alpha \) and Arg-88c-Gly in TCR\( \beta \), respectively, using the QuickChange site-directed mutagenesis kit (Stratagene). For the chimeric TCR DM2 and full-length Hu TCR gp100, the mutagenic primers 5'-gattcacaagacaagaggatctgcgtgctgctg-3' and 5'-tactcttgagcctagctgctgtctgctgctg-3' yielded the reciprocal Ser-85.1Gca-Arg and Arg-88c-Gly. Nomenclature of the mouse and human CoC\( \beta \) was adopted from IMGT (20, 21).

**Retroviral transduction and drug selection**

The protocol of G. P. Nolan’s laboratory (28) was slightly modified as described in detail elsewhere (27). Selection of TCR\( \beta \)-transduced T cells was achieved by adding neomycin at a final concentration of 800 \( \mu \)g/ml (Invitrogen Life Technologies) for 7 days, and the selection of TCR\( \alpha \)-transduced T cells was accomplished by adding puromycin (Sigma-Aldrich) at a final concentration of 5 \( \mu \)g/ml for 2 days to RPMI 1640 medium supplemented with Hu AB serum. Surviving bulk T cells were cultured for at least 1 wk with CD3/CD28-labeled magnetic beads (Dynabeads; Invitrogen-Dynal) and 40–100 U of IL-2 (Proleukin, Chiron) before transgene expression and function analysis. For transduction of a Mu hybridoma, Phoenix-Eco and the amphotropic envelope vesicular stomatitis virus glycoprotein were used. TCR-introduced 58\( \alpha \)6\( \beta \) were sorted on FACSAria (BD Biosciences). Human T cells were assayed in a 4-h \( ^{51} \)Cr-release assay at various E:T ratios. The avidity, i.e., cytolytic and IFN-\( \gamma \)-secretoirstic behavior of chain pairing has been reproduced in the endogenous TCR-deficient hybridoma 58\( \alpha \)6\( \beta \)\( ^{+} \). This approach significantly inhibited the potential formation of an interspecies hybrid TCR of a mouse/human-mixed type. Tetrimer dissociation experiments revealed that this molecular design did not impair recognition of the cognate Ag.

**PBMC and cell lines**

Bulk CD4\( ^{+} \)CD8\( ^{-} \) T cells were obtained from buffy coats of Hu A2.1\(^{+} \) donor PBMC. For this, mononuclear cells were separated by ficoll (Bior- chrom) gradient centrifugation on Leucosep devices according to the manufacturer (Greiner) and cryopreserved. The Mu hybridoma 58\( \alpha \)6\( \beta \) CD3\( ^{+} \) lacks endogenous TCR\( \alpha \) and TCR\( \beta \)-chains and were cultured in histidinol (2 mg/ml) to mouse CD3\( ^{+} \) expression (26). The packaging cell lines 293T, Phoenix-Ampho (amphotropic), and Phoenix-eco (ecotropic) were obtained from American Type Culture Collection (ATCC). They were grown in HEPES-buffered (25 mM) DMEM supplemented with 1X glutamine, 1X penicillin-streptomycin, 1X nonessential amino acids, and 10% (v/v) FCS (Cultrex) and trypsinized with 1X trypsin-EDTA (Invitrogen Life Technologies).

**Flow cytometric analysis and evaluation**

Data acquisition was performed on a EPICS ALTRA HyPerSort (Beckman Coulter) or a FACSCalibur (BD Biosciences) device, and raw data analysis was conducted with the Expo32 software (Beckman Coulter). T cells were sorted on FACSAria (BD Biosciences). Human T cells were stained with anti-Mu V\( \beta \)6-FITC/PE (BD Biosciences), anti-Hu TCR\( \alpha \beta \)-PE (Beckman Coulter), anti-Hu V\( \beta \)14-PE (Beckman Coulter), anti-Hu CD8-PE, and anti-Hu CD4-FITC (Beckman Coulter) Ab. DM2(81–88)-PE and gp100(280–288)-PE-specific tetramers (0.4 mg/ml) were synthesized as published elsewhere (29). Tetramers (0.5 \( \mu \)g) were added to 0.1 \( \times \) 10\(^{9} \) T cells in 50 \( \mu \)l (0.4 \( \mu \)g/ml) for 45 min at 8°C.

**Chromium-release assay**

Transduced T cells were assayed in a 4-h \(^{51} \)Cr-release assay at various E:T ratios for lytic activity against TAP-deficient T2, with or without antigenic peptide, or tumor cell lines (2). To correct for varying TCR expression and size of CD8\( ^{+} \) population in retrovirally transduced bulk Hu T cells, V\( \beta \)6\( ^{+} \)CD8\( ^{-} \) : T and gp100 tetramer \( ^{+} \)CD8\( ^{-} \) : T ratios, respectively, were calculated from contemporary flow cytometry analysis (3). EC\( _{50} \) as a measure of ligand concentration yielding half-maximal lysis was calculated from peptide titration cytotoxicity experiments and dose-response sigmoidal regression analysis according to \( \gamma = \log_{2}(1+10^{(logEC_{50}-X)}) \) with GraphPad Prism version 3.0 software.

**Competitive tetramer dissociation assay**

The experiments were performed at 4°C to increase cell viability and minimize residual tetramer binding due to capping and tetramer internalization (30). The protocol was as outlined in Ref.31 using a FITC-labeled anti-V\( \beta \)6 Ab as competitor. Briefly, 10 \( \times \) 10\(^{9} \) TCR-introduced T cells were stained in a volume of 2500 \( \mu \)l with 25 \( \mu \)l of PE-Cy5 (PC5)-labeled tetramer (0.4 \( \mu \)g/ml) at 4°C for 45 min in the presence of 0.5% sodium azide to reduce the internalization of TCR/tetramer complexes. All subsequent steps were performed in the presence of NaN3. After washing two times with ice-cold PBS, the T cells were sorted on a FACSARia device (BD Biosciences) to equal and high tetramer geometric mean fluorescence (GMF) intensities for different TCR-transduced T cell populations. Sorted and viable T cells (0.5 \( \times \) 10\(^{5} \)) were resuspended in 150 \( \mu \)l and tetramer dissociation was initiated by the addition of 12.5 \( \mu \)l (0.5 mg/ml) V\( \beta \)6 Ab.
As a control, $0.2 \times 10^6$ bulk Wt TCR MD2-transduced T cells were stained with an irrelevant gp100(280–288)-PC5-specific tetramer displaying a very low signal over time. Twelve aliquots each corresponding to 40,000 cells were withdrawn between 0 min, i.e., before competitor addition, and 1–90 min after addition and immediately pipetted into ice-cold paraformaldehyde-containing PBS (1%; w/v) to stop the reaction. Three thousand viable cells were gated and analyzed for residual tetramer binding intensities were done with GraphPad Prism version 3.0 software.

**Results**

**Designing reciprocal complementarity at the CaB interface in Mu TCR**

To favor the interaction of introduced TCRα- and TCRβ-chains in Hu T cells, we focused on the CaB interface in the associated TCR heterodimer. We used as model the crystal structure of the Mu 2C TCR (Ref. 18 and the RCSB protein data bank). We concentrated on the invariant constant domains so as to generalize the approach to the majority of TCR subfamilies. This is particularly relevant for the transduction of bulk T cell populations, as this is the favorable setting in a clinically adoptive transfer protocol. A successful strategy was the reciprocal substitution of two facing residues, differing in size, to yield sterically complementary “knob-into-hole” mutations as previously shown for Abs (32). However, a characteristic of the TCR structure is the pronounced charge asymmetry between the Ca- and Cβ-domains (18). Because electrostatic forces exert a long-ranging effect in protein structures (33), we hypothesized that the combined reciprocal substitution of both side chain volume and charge would enhance preferential TCR chain pairing.

The TCR Ca- and Cβ-domains were structurally related (Fig. 1A). The β-sheets comprising β-strands ABED of each domain were juxtaposed to each other while the β-sheets GFC were staggered behind. According to IMGT numbering, a pair of interacting residues, Arg-88c in the constant domain of TCRβ (Arg-88cβ) and Gly-85.1c in the constant domain of TCRα (Gly-85.1αc), turned out to be candidate residues for reciprocal exchange (Fig. 1B). They were both located midway in the inner β-strand E of their respective C-domain. The Arg-88cβ side chain centrally protrudes from its twisted β-sheet and points to Gly-85.1αc, which is nestled at the base of its concave-shaped β-sheet.

The CaB interface was endowed with polar amino acids, hydrophobic patches, and a skewed distribution of acidic residues in Ca and basic residues in Cβ. The side chain of Arg-88cβ was stacked between the hydrophobic residues Leu-24c (strand B), Leu-84.2c (strand D), Ile-86c, Val-22c, Gly-88c, and partly Arg-90cβ (strand E). Arg-88cβ was not thoroughly in a trans conformation because the dihedrals $\chi_1$, $\chi_2$, and $\chi_3$ equal $-73.8^\circ$, $-63.3^\circ$ and $149^\circ$, respectively, so as not to conflict with the opposite β-strand E of Ca. The positively charged guanidinium group of Arg-88cβ did not form a salt bridge but was predominantly surrounded by polar residues.

The reciprocal substitutions Gly-85.1αc→Arg-85.1αc and Arg-88cβ→Gly-88cβ (Fig. 1C) elicited no steric hindrance for that particular arginine conformer available from the empirical database in the Swiss-PdbViewer ($\chi_1$ = $-175^\circ$, $-175^\circ$, $71.1^\circ$, $176^\circ$, and $179.9^\circ$) that yielded the lowest score for steric distortion. Although the charged guanidinium group was slightly shifted toward the Cβ domain, the Arg-85.1αc mutant is predicted to be similarly stacked between the same hydrophobic residues such as in the Wt. Correspondingly, interactions of the guanidinium group were restricted to polar residues. However, the empirical structure of the Arg-85.1αc conformer was not available from crystallization and x-ray diffraction analysis. In 2C TCR, the coordination of water molecules at the interface, such as H2O 15 centered between Wt Arg-88cβ and Arg-90cβ, indicated hydrophilic accessibility and allowed some stereochemical freedom, although low temperature factors for Arg-88cβ (Cβ: 24.07 Å$^2$) and its proximate residues predicted a rather rigid structure.

From a structural point of view, we hypothesized a positive effect on chain pairing for the prominent Arg-88cβ in analogy to binding “hot spots” that dictate the association of proteins by contributing substantially to the binding free energy (34). Human TCR bear also a small residue, Ser-85.1αc, and large residue, Arg-88cβ, as a consequence of the high homology in primary and tertiary structure. This may suggest a species-independent generalization of our approach.

![FIGURE 1](http://www.jimmunol.org/)
Introduce TCRβ needed heterodimerization to TCRα for export to the cell surface

Initially, we needed an assay to determine the impact that the mutations would have on TCR chain pairing. For this, we assessed whether surface expression of the introduced TCR β-chain served as a readout for effective chain pairing of either the introduced TCR α- and β-chains among themselves or a single introduced TCR β-chain with an arbitrarily endogenous TCRα counterpart to form a hybrid TCR (11). The rationale was that pairing stabilizes the TCR and subsequently promotes its integration into the multimeric CD3 complex as a prerequisite for export to the cell membrane. The TCRα could not be assessed due to the lack of Abs. Moreover, a construct with an octamer histidine tag in front of the variable domain failed to allow flow cytometric identification of surface TCRα (data not shown). Introduced TCRβ were detected with subfamily-specific Abs. The presence of both Ag-specific TCRα and TCRβ was detected with soluble fluorescent tetramer molecules, which are composed of streptavidin/biotin-linked TAA peptide/HLA-A2 multimers (29). But, as shown below, the correlation of tetramer with any TCRα expressed either as double (Mu Wt TCR p53; Ref. 4), and a Hu gp100(280–288)-specific TCR (Mu Wt TCR MDM2 or Mu chimeric TCR MDM2; Ref. 6) was probably due to some structural instability of the chimerized elbow region. Although Wt TCRβ expression was substantially reduced in the absence of its TCRα counterpar (drop from 92.4 to 33.9%), transduction of the single chimeric TCR β-chain construct resulted in expression at a level as high as that of the heterodimeric TCR (82.3%; Fig. 2C). Noteworthily, the Hu TCR was able to associate with Mu CD3ξ. Tetramer staining of CD8-deficient hybridoma succeeded only for introduced CD8-independent TCR such as TCR gp100 and p53, not for the CD8-dependent TCR MDM2.

Point mutations at the interface of TCRβ affected export to the T cell surface

Next, we examined the chain pairing of introduced TCR chains in antibiotic-selected bulk Hu T cells. We anticipated that retrovirally transduced Mu and even more Hu TCR chains were able to interact with endogenous TCR. As opposed to the hybridoma model (Fig. 2A), the pairing of TCRβ with any endogenous TCRα should rescue its surface expression. The point mutation candidate Arg-88cβ→Gly-88cβ in TCRβ was expected to be poorly expressed in the absence of its complementary TCRα counterpart in Hu T cells. We tested this concept with the Wt and partially humanized MDM2-specific TCR. We observed a marked expression for the heterodimeric Wt TCR MDM2 irrespective of the CD4+CD8+ T cell subset (92.4%; Fig. 2B). Reduction of Vβ6 positivity for chimeric TCR MDM2 (82.3%; Fig. 2C) was probably due to some structural instability of the chimerized elbow region. Although Wt TCRβ expression was substantially reduced in the absence of its TCRα (drop from 92.4 to 33.9%), transduction of the single chimeric TCR β-chain construct resulted in expression at a level as high as that of the heterodimeric TCR (82.3% to 80.4%). This may be explained by its higher propensity to associate with endogenous Hu TCRα. The residual Vβ6 staining for single Wt TCRβ reflected some competence to bind to Hu TCR. Eventually, as already shown for the

pBullet-IRESpuromycin. Transfectants were selected with the appropriate antibiotics (see Materials and Methods and Fig. 2A). Thus, introduced TCRαβ expression follows a Gaussian-like random distribution on hybridoma and bulk T cell cultures. Essentially, in all cases the TCR β-chain could be detected at the cell surface by FACS analysis only when TCRα was coexpressed. Likewise, we observed tetramer binding only for the heterodimeric Hu TCR gp100 in hybridoma, proving that the pairing of TCRα with TCRβ is a necessity for TCR export (see Fig. 3A).

FIGURE 2. Single TCRβ vs heterodimeric TCRαβ surface expression. Flow cytometry analysis of retrovirally transduced and antibiotic-selected Mu hybridoma 58αβ CD3ξ (A) or bulk Hu T cells (B and C). The Wt or Mu TCR constructs of Mu or Hu origin comprised both or merely the single TCRα or TCRβ-chain as indicated from left to right: A. Overlay histogram for surface expression of a heterodimeric Mu Wt MDM2(81–88)-specific TCR, its partially humanized (Chim, chimeric) construct, a Mu Wt p53(264–272)-specific and a Hu Wt gp100(280–288)-specific TCR (dotted line) vs that of the corresponding single TCRβ-chain (bold line). B. Dot plots for surface expression of introduced TCRβ in the CD4+CD8+ subsets of PBMC monitored by anti-Hu CD8 and anti-Mu Vβ6. It aligns from left to right the heterodimeric Mu Wt MDM2(81–88)-specific TCR, its single TCRβ, a point-mutated TCRβ (Arg-88cβ→Gly; Fig. 1), and a single TCRα as negative control. C. As in B, but documents the expression of the partially humanized TCR in CaCβ.
Replacement of Arg-88β for Gly-88β to generate a TCRβ mutant unmatched to endogenous TCR α-chains abolished surface expression (Fig. 2B, from 33.9 to 3.7%) and substantially diminished that of the single chimeric TCRβ mutant (Fig. 2C, from 80.4 to 39.4%). The higher residual Vβ6 positivity (39.4% vs 3.7%) was explained by the higher potency of a Hu TCRβ C-domain to pair with its endogenous counterparts and thus compensates for the local residue mismatch.

It was highly unlikely that the reduced staining of the TCRβ mutants was merely an artifact of a disabled recognition by the Vβ6 Ab, because the mutation and the epitope were distantly located in different domains. Furthermore, chimerization to a Hu constant domain preserved Vβ6 staining (Fig. 2, B and C; Mu Wt TCR MDM2 vs Mu chimeric TCR MDM2). Additionally, a decrease in surface expression correlated with an increase in cytoplasmic detection (data not shown). However, the total intracellular recovery was decreased for the TCRβ mutants, indicating an increased instability of those unmatched and therefore largely unpaired chains. Collectively, TCR Vβ6 staining closely reflected TCR surface expression on CD8− and CD4+ T cell subsets and thus proposes a mechanism for TCR export depending on effective chain pairing.

**Reciprocal mutations in CaCB of introduced TCR restored surface expression in hybridoma**

The results from Fig. 2 suggested, that the rebuilding of complementarity at the interface of TCRαβ would restore surface expression. Mutant TCR bearing the reciprocal mutations as described in Fig. 1 were coexpressed after drug selection in hybridoma 58αβCD3ζ+ in the absence of endogenous TCR. As opposed to the CD8-dependent TCR MDM2, the presence of both TCRα and TCRβ of the gp100-specific TCR was detectable via tetramer staining in a CD8-deficient hybridoma. Hence, we chose the Hu TCR gp100 to study the combinations of Wt and mutated (Mt) chains of its own species (Fig. 3A). Wt TCR gp100 achieved the highest expression when quantified by a tetramer (mean fluorescence intensity (MFI) 29.2), followed by the reciprocally mutated TCR (also referred to as matched TCR; i.e., Arg-85.1ca/Gly-88bc, MFI 6.5). Both unmatched Mta/Wtβ (i.e., Arg-85.1ca/Arg-88bc; MFI 0.3) and Wto/Mtβ (i.e., Ser-85.1ca/Gly-88bc; MFI 2.9) TCR combinations substantially decreased in surface expression (Fig. 3A). The same ranking was observed for a Vβ14-staining (data not shown). The presence of two bulky and charged arginine residues that triggered steric hindrance and electrostatic repulsion seemed to be more detrimental to chain association than that of small and neutral residues, which led to withdrawal of steric complementarity and charge.

**The formation of hybrid TCR of an interspecies mouse/human-mixed type does occur**

The generation of a novel high-affinity, tumor-specific TCR in an A2-Tg mouse model (3, 4) necessitated us to assess the propensity of Mu TCR chains to interact with Hu TCR. Human TCR gp100 was combined with murine TCR MDM2 chains to yield a Hu/Mu mixed type (Fig. 3, B and C). Monitoring the surface expression of Hu TCRβ (Fig. 3B; Hu Vβ14) allowed us to indirectly quantify the interaction of Mu TCRα with Hu TCRβ, while monitoring Mu TCRβ (Fig. 3C; Mu Vβ6) allowed us to directly quantify the interaction of Mu TCRβ with Hu TCRα. In either setting, interspecies Wt TCR combinations displayed a strong propensity to heterodimerize (dark gray-shaded histograms in Fig. 3: MFI 21.5 for Mu Wto/Mu MDM2/Hu Wtβ gp100 and 18.4 for Hu Wto gp100/Mu Wtβ MDM2) somewhat behind that of the related intraspecies Wt/Mu TCR combinations displayed a strong propensity to het-
TCR (bold lines: MFI 25.2 in Fig. 3B and 33.4 in Fig. 3C). Interspecies TCR are able to interact, at least for the Hu Vα8/Mu Vβ6 and Mu Vα10/Hu Vβ14-subfamily combinations examined here, and may also take place in Hu PBMC as a disfavored side reaction. Importantly, either one of the unmatched combinations of a Mu Mtoα- or Mfβ-chain with a Hu Wt counterpart were substantially reduced in surface expression (light gray-shaded histograms: MFI 2.8 for Mu Mtoα MDMD2/Hu Wt β gp100 in Fig. 3B and MFI 1.0 for Hu Wtα gp100/Mu Mtβ MDMD2 in Fig. 3C). The reciprocal mutations also tended to restore pairing of interspecies-matched TCR (dotted lines) better than the single mutation of the other Mu Wt/Hu Mt unmatched TCR (dashed lines). Although the latter TCR combinations do not have any relevance for gene therapy, this result in principle may argue for the envisaged structural manipulation. Thus, a single residue substitution on either side of the Mu CaCβ interface disfavors, as designated, the pairing of introduced and endogenous TCR and may diminish the formation of hybrid TCR.

Reciprocal mutations in introduced Mu TCR MDMD2 restored structural avidity in Hu T cells

All intraspecies combinations of wild type and mutant Mu TCR MDMD2 were then analyzed in antibiotic-selected bulk Hu PBMC (Fig. 4) where endogenous TCR might interfere with chain pairing. First, we stained for Vβ6 + as a surrogate for overall TCR expression (Fig. 4A). The frequency of Wt TCR MDMD2-positive T cells was high for both the larger CD8 + (92.5%) and the smaller CD4 + (3.2%) T cell subsets, whereas introducing a single mutation as outlined previously in either TCRα (Mtoα/Wtβ) or TCRβ (Wtα/Mtβ) decreased the surface density of TCR. Because the single Wt or Mt β-chain (Wtβ and Mtβ) only elicited TCR Vβ6 + in a minor fraction of T cells (Fig. 2B), the observed en bloc Vβ6 positivity for unmatched Mtoα/Wtβ TCR MDMD2 (52.7% for CD8 + and 6.4% for CD4 +) or Wtα/Mtβ
was likely due to residual interaction with the introduced counterpart. T cells transduced with the matched Mta/Mtβ TCR MDM2 exhibited a significant recovery of V6 positivity (78.4 and 10.3%), indicative of the restoration of steric and electrostatic complementarity. Conclusively, Hu endogenous TCR did not counteract the desirable effect of preferential chain pairing despite a pronounced propensity for interspecies hybrid TCR formation (Fig. 3, B and C).

A more comprehensive readout for functional TCR expression was provided by tetramer staining, because HLA-A2 binding requires the dual presence of TCRα and TCRβ in a native conformation and thus gives an estimate for structural avidity (Fig. 4B and Ref. 23). The unmatched TCR pairs failed to confer tetramer staining (4.4 and 2.6%, respectively). In contrast, the results from Fig. 4A indicated a gradual decline for the unmatched TCR, implying that expression of heterodimeric TCR with improper structure at their interface was not abolished but had a severe impact on Ag recognition. Most importantly, coexpression of the complementary TCR chain pair restored tetramer positivity and hence structural integrity to more than one-half of that of the Wt TCR (29.7 vs 54%). In general, we did not observe >70% tetramer-positive CD8+ T cells for the CD8-dependent Wt TCR MDM2 in any experiment (4).

Introduced TCR have to compete with endogenous TCR for integration into the CD3 complex (11). Retroviral overexpression and favorable pairing of Mu TCR among themselves promoted their incorporation into the signaling machinery and the down-regulation of endogenous TCR on the cell surface of Hu T cells (16, 17). The amount of introduced TCR-dependent down-regulation of endogenous TCR correlated with the competitive strength and stability of a particular introduced TCR construct (Fig. 4C). The unmatched TCR did not suppress endogenous TCR (6.1 and 10.1%) as opposed to the matched TCR. From flow cytometry analysis, apparently 26.2% of the T cells have lost endogenous TCR surface expression. With respect to its diminished Vβ6 positivity (CD8+ and CD4+: 88.7 vs 95.7%; Fig. 4A) this is in line with a higher competitive strength for Wt TCR MDM2 (33.8%, Fig. 4C). Successful pairing corresponded to introduced TCR surface expression and inversely related to endogenous TCR recovery. This equally applied to CD8+ and CD4+ T cell subsets (not shown).

These differences in the cellular phenotype were not caused by genetic modifications at the RNA level. Semiquantitative RT-PCR according to (36) demonstrated the equal abundance of mRNA for all TCR combinations (data not shown). Furthermore, intracellular
FIGURE 7. Half-lives of competitive tetramer dissociation for Wt and matched TCR MDM2 were indistinguishable. A and B, CD8⁺ Mu Wt TCR MDM2 (A, C, E, and F)-introduced or Mu Mtx/Mtβ TCR MDM2 (B, D, E, and F)-introduced T cells were stained with tetramer (TetMDM2-PC5) and were sorted on a FACS Aria flow cytometer (BD Biosciences) to equal and high fluorescence intensities (gate B in the left-hand density plot). All incubations were done in the presence of 0.5% NaN₃ to minimize tetramer-complexed TCR internalization and cell toxicity (left-hand forward scatter (FSC)/side scatter (SSC) dot plot). Reanalysis of the sorted cells demonstrated a reasonable 80% survival (right-hand scatter dot plot) and almost equal tetramer signal intensity (right-hand density plot; MFI 3.5 and 3.2, respectively). Bound tetramer dissociated somewhat during sample preparation as shown by the modest shift out of gate B. C and D, Sorted T cells were submitted to Vβ6 Ab-mediated competition immediately after sorting. Inset numbers give GMF intensities of tetramer signals (upper right) for gates B or C, and time intervals in minutes (lower right). For clarity, decrease of GMF intensities are only shown for 0 min, i.e., before competitor addition, and 1, 10, and 90 min after addition. A horizontal black bar inside gates B or C refers to GMF for t = 0 min and helps visualize the subsequent tetramer decay. E, Chart of tetramer dissociation over time for Mu Wt TCR MDM2 (○) and the matched TCR MDM2 (●). GMF intensities were submitted to nonlinear regression using a one-phase exponential decay equation. The inset demonstrates a closely related tetramer dissociation for the normalized data. F, Due to residual tetramer internalization, a more reliable evaluation of tetramer decay kinetic is confined to the initial 10 min (31); the runs test confirms a linear relationship for both TCR (p = 0.5 and 0.2, respectively). The related half-lives equal ln2/slope; t₁/₂ = 11.4 ± 0.7 min and t₁/₂ = 9.6 ± 0.8 min. The difference of their slopes was statistically not significant (p = 0.2, according to GraphPad Prism version 3.0 software). The 95% confidence intervals (curved dashed lines) support this notion.
staining demonstrated an almost equivalent TCR distribution indicative for comparable protein translation.

**Reciprocal mutations in Mu TCR MDM2 restored functional avidity in Hu T cells**

The functional avidity of transduced T cells was determined in chromium-release assays on targets sensitized with decreasing amounts of the antigenic peptide (Fig. 5A). T cells transduced with the matched TCR were almost as efficient as those transduced with the Wt TCR; EC50 values were 0.28 nM for the Wt TCR and 0.58 nM for the matched Mta/Mtβ TCR. T cells transduced with the unmatched TCR displayed a 10-fold reduction in functional avidity (2.94 nM for Mta/Wtβ TCR and 2.41 nM for Wta/Mtβ). Interestingly, the lower but still detectable cytolytic efficacy did not correspond to the lack of tetramer staining (Fig. 4B). However, this is in line with other reports (23).

Subsequently, the ability of transduced T cells to recognize and lyse target cells presenting naturally processed MDM2(81–88) peptide was determined (Fig. 5B and Ref. 3). An MDM2-secretant of the osteosarcoma Saos-2 was efficiently recognized by T cells transduced with the Wt TCR (82% for a corrected E:T ratio of 5 for CD8+ Vβ6+:T) and, to a lower extent, by T cells expressing the matched TCR (52% for CD8+ Vβ6+:T = 4). Accordingly, the tumor cell lines EU-3, IM-9, and BV-173 were lysed more efficiently by the Wt than by the matched TCR MDM2 (45/28% for CD8+ Vβ6+:T = 15/13). T cells transduced with the unmatched TCR pair did not detectably recognize any target cell line.

**Reciprocal mutations in Hu TCR gp100 restored structural and functional avidity in Hu T cells**

Analogous to the experiments performed with the Mu MDM2-specific TCR, we determined the function of a high-affinity Hu gp100(280–288)-specific TCR and its mutants bearing the same design of reciprocal mutations, Ser-85.1c-Arg and Arg-88c-Gly (Fig. 6). More likely than Mu TCR, the Hu TCR will form a hybrid TCR with endogenous TCR that would be counterproductive to the aim of preferential pairing. As shown before for a partially humanized TCR MDM2 (Fig. 2C), the weakening of the interface interaction by the mutation Gly-88cβ seemed to be vastly antagonized by the multitude of interactions along the large C-domain’s surface area. Despite this, the matched TCR yielded more than one-half of the Wt tetramer positivity for the CD8+ T cell subset (56.4 vs 81.3%; Fig. 6A) while the unmatched TCR failed (9.6 and 2.3%). This could also be demonstrated for the TCR gp100-transduced as opposed to the TCR MDM2-transduced CD4+ T cell subset (Fig. 4B).

Next, peptide titration was performed for all Wt and mutant TCR combinations (Fig. 6B). Although the EC50 value of the Wt TCR (0.01 nM) was 28-fold lower than that of the Mu Wt TCR MDM2, the reduction in cytolytic efficiency for all mutant TCR (Mta/Mtβ TCR gp100, factor 2.1; Wta/Mtβ, factor 6.6; Mta/Wtβ, factor 46) followed the same order and roughly the same range. Again, unmatched TCR were predominantly affected. Mta/Wtβ TCR gp100 was particularly compromised in function. In the native structure Ser-85.1cα contributes with a polar side chain interaction in contrast to a glycine in Mu TCR. When mutated to charged Arg-85.1cα, it may more severely distort protein structure. This corresponded to the weakest structural avidity (Fig. 6A). Substantial recognition of an A2 gp100+ melanoma was apparent with only the Wt and matched TCR (Fig. 6C).

IFN-γ cytokine secretion was tested by ELISPOT analysis for a homogenous CD4+ population. Again, recognition of gp100(280–288)-pulsed T2 was accomplished down to the nanomolar range exclusively for the Wt and matched TCR (data not shown). Collectively, the reciprocal mutations affected structural and functional avidity equally for both the CD8+ and CD4+ T cell subsets. The feasibility of the reciprocal mutation concept was proven for a Hu tumor-reactive TCR despite a putatively higher propensity to form hybrid TCR.

**Reciprocal mutations did not affect molecular recognition of Ag**

There are several lines of evidence indicating that dissociation rates rather than equilibrium binding to peptide/MHC ligands reflect the functional avidity of T cells (37, 38). First-order dissociation kinetics were accomplished for Wt and reciprocally mutated TCR MDM2 by anti-TCR (Vβ6-antibody (Fig. 7) in analogy to Ref. 31)- or anti-class I (Ref. 39 and data not shown)-based competition to tetramer-saturated T cells. To yield comparable introduced TCR surface densities, we stained bulk T cells with the MDM2(81–88)-specific tetramer on a preparative scale and sorted them for equal and high MFI (Fig. 7, A and B). Spontaneous tetramer dissociation after sorting (Fig. 7, A vs B) and washing (Fig. 7, C vs D) may account for the slight differences in their MFI values. Nevertheless, sorting greatly improved the normalization of the tetramer signal when compared with TCR MDM2-transduced bulk T cell populations (Figs. 4B and 7, A vs B). Because rapid tetramer internalization (30) deprives the competitor of the accessible TCR on the cell surface, we initially observed competition efficiencies of at best 50% (not shown). We then determined 0.5% sodium azide as an optimal concentration to combine reduced tetramer internalization with low cell toxicity (Fig. 7, A and B). The kinetic analysis revealed a very similar dissociation for both TCR constructs (Fig. 7, C and D) that proved to be nearly indistinguishable after nonlinear regression (Fig. 7E). Transformation of the GMF intensities to the natural logarithm (39) revealed a substantial nonlinear relationship for the plateau phase (not shown) presumably due to residual tetramer internalization. Hence, the evaluation was confined to the initial 10 min that obeyed a linear relationship (Fig. 7F (runs test) in analogy to Ref. 31); the half-lives of tetramer binding were t1/2 = 11.4 ± 0.7 min for the Wt TCR MDM2 and t1/2 = 9.6 ± 0.8 min for the matched TCR MDM2. The difference of their slopes was statistically not significant (p = 0.2). From this we conclude that the reciprocal mutations did not perturb Ag recognition.

**Discussion**

The main findings of this work are: 1) both Hu and Mu TCR chains can readily form hybrid TCR upon transduction into T lymphocytes, thus revealing a marked propensity to pair with unrelated TCR chains being synthesized in the same lymphocyte; and 2) we have identified by visual inspection of the 2C TCR crystal structure a pair of residues buried in the CαCβ interface that are critical for αβ pairing. These findings have important practical implications for future immunotherapy strategies based on gene transfer of high avidity TCR directed against viral or tumor Ags (1).

Cell surface expression reflects the intrinsic stability of a membrane protein such as the stabilization of HLA molecules that depends on peptide binding (39). As shown here, the expression of single TCR β-chains, irrespective of being of Mu or Hu origin, is conferred by the presence of their counterpart in a Mu hybridoma lacking endogenous TCR. Detection of a Mu TCR β-chain in Hu T cells is further increased by its humanization in Cβ and suggests a stabilizing effect by pairing to endogenous TCR α-chains. This reveals chain pairing as one of the major functions of TCR C-domains (40) and implies that heterologous expression of Hu or humanized TCR in Hu T cells causes the random combination of chains (11) leading to a plethora of hybrid TCR with unknown and potentially autoimmune specificities (9).
We further observed an inverse relationship between the level of Mu TCR MDM2 expression relative to endogenous Hu TCR expression. Correspondingly, murinization of a Hu TCR gp100 in Caβ triggered down-regulation of endogenous TCR (Ref. 16 and data not shown). Thus, we assume that the Mu TCR α- and β-chains favorably associate to each other, provoking its thermal and/or proteolytic stabilization. This confers efficient competition with endogenous TCR for binding to the Hu CD3 complex, the shuttle for export to the cell membrane (16, 17). However, we cannot rule out a mechanism governed by a stretch of conserved residues in Mu C-domains to favor interaction to Hu CD3εγ (41).

Although different mechanisms of TCR/CD3 chain assembly prevail for introduced Hu and Mu TCR as outlined previously, the outcome of the reciprocal mutations for structural and functional avidity was the same: the complementarity matched TCR retain an efficiency comparable to that of Wt TCR while that of the unmatched TCR was highly reduced. This may be explained by, first, the importance of the chosen residues for the overall integrity of the TCR structure. Even intraspecies combinations of a Hu introduced and endogenous TCR C-domain, for which one may assume a high interchain affinity, were drastically affected by the replacement of either a small (Ser-85.1ceα) or a bulky charged residue (Arg-88β). Second, we assume that, despite the favorable incorporation of Mu TCR chains into the Hu CD3-complex (16, 17), the assembly of Mu TCR chains resembles that of Hu TCR due to their homology in primary and tertiary structure (18, 19). This is supported by our finding that the interchain affinity of a Mu and a Hu TCR is sufficiently high to form interspecies hybrid TCR.

The rational design of interfaces of Ig-like folded domains have been formerly studied in detail for Abs in P. Carter’s laboratory. The reciprocal introduction of a pair of specifically interacting “knob-into-hole” residues Thr-366-Trp/Tyr’-407-Ala at the interface of the invariant Cα3 domains favored the generation of an anti-CD3/CD4-IgG heterodimer that has been further improved by “phage display” (32, 42). In this study we aimed at minimizing the impact on TCR structure by simply swapping the positions of native Gly-85.1ceα or Ser-85.1ceα and Arg-88β between the invariant Cα- and Cβ-domains, thus rendering both stericly (i.e., Van der Waals surface of side chains) and electrostatically (i.e., charge of arginine) unchanged except for their orientations.

The Cα-Cβ interface of the 2C TCR is characterized by high complementarity and polarity skewed by a predominant location of basic residues in Cβ and acidic and polar residues in Cε (18). The asymmetric charge distribution at the interface raises the question of the relative contributions of steric vs electrostatic interactions to chain pairing. In most experiments we observed a much more pronounced reduction in avidities for the Mto/Wttβ combination than for the Wto/Mtβ combination. Obviously, steric hindrance and electrostatic repulsion of two bulky and charged arginines facing each other is more detrimental to structural avidity than the lack of interaction and withdrawal of charge. From this we draw the conclusion that reciprocal mutations do not lead to preferential chain pairing in terms of an improved fitting but to prohibitive pairing to unmatched endogenous TCR. This shifts the heterodimerization equilibrium toward reciprocally designed TCR.

However, the poor recovery of single mutant TCRβ MDM2 in T cells is consistent with the idea that the withdrawal of charge also exerts a severe effect on chain pairing. The reduction of Van der Waals interactions by omission of the Arg-88cβ side chain should be compensated somewhat by the multitude of interactions along the vast buried Cεββ-surface area of ~2049 Å² (18). In light of the charge asymmetry in TCR C-domains (18), we propose that the charged guanidinium group of Arg-88cβ, as one of a few basic key amino acids (Fig. 1B), contributes to long-ranging electrostatic forces and a fast kinetic of C-domain association (33). Subsequently, the variable domains associate mainly governed by hydrophobic interactions (43) and may demand more time for proper assembly due to their TCR subfamily-related sequence degeneracy.

Hence, for the functional optimization of TCR chain pairing, the hypothesized two-step mechanism suggests that the last event should not be manipulated (i.e., chain pairing of the variable domains) because most of the decisive interactions, located in the C-domains, may have already formed at that time. This notion is consistent with the observations that the formation of heterodimeric constant domains of a truncated TCR is sufficient for stable surface expression (40) and that the large surface area of the Cεββ interface (2049 Å²) overwhelms that of the variable domains (1160 Å²; Ref. 18). These aspects account for the pivotal function of C-domains in heterodimerization. Moreover, the sequence degeneracy of the core V-domains (43), their hydrophobicity, and their close juxtaposition to the Ag recognition site complicates the identification of ideally suited (un)charged “knob-into-hole”-residues to apply to a subfamily-independent approach.

However, the tiny shift of the charged guanidinium group in mutated Arg-85.1ceα toward the “basic” surface of Cβ may cause a structural alteration responsible for the slight decline in efficacy of the matched TCR. Currently, we are trying to optimize the reciprocal configuration by changing the size, charge, and potential hydrogen bonding of the involved residues. In an effort to endow each chain with a repulsive and charged knob, we introduced into the matched Mt Arg-85.1ceα/Mt Gly-88cβ TCR vice versa an Ile-86ceα-Gly/Ser-86cβ-Arg mutation in a juxtaposed position. Even the reduction of all bulky arginines to lysines in these 4-fold mutated heterodimers yielded nonfunctional TCR (data not shown). Hence, TCR structure turns out to be very susceptible to local alterations at its interface.

Recently, TCR have been stabilized by introducing an artificial disulfide bridge into the constant domain (14, 15). This approach may benefit from preferential pairing due to the formation of an irreversible covalent linkage but disregards the risk of hybrid TCR formation. Serine, threonine, and cysteine are chemically homologous and will hardly initiate steric repulsion or lack of interaction. This notion is supported by the fact that disulfide bridges are not necessary for TCRεβ heterodimerization (40). As an objective, one may attempt to combine the advantageous effect of the reciprocal mutations with that of the artificial disulfide bridge.

Although the Wt TCR MDM2- and gp100-introduced T cells differed by a factor of 28 in cytotoxic efficiencies, the EC50 values revealed approximately a proportional reduction in peptide recognition for their unmatched TCR MDM2 (factor of 9–11) and TCR gp100 (factor of 7–46). Although they are still able to recognize titrated Ag in a low nanomolar range, recognition of tumor cell lines or transfectants was nearly abolished. Thus, the reciprocal mutation approach may be generalized to tumor-reactive TCR with a broad range of Ag affinities. Because the resulting avidity of T cells for their Ag equals the product of TCR cell surface density and its molecular affinity to the cognate peptide/MHC ligand, we conclude from first-order dissociation experiments that it is not a decreased TCR affinity but its diminished surface expression that causes the observed differences in structural and functional avidities between the Wt and matched TCR. Ag recognition needs a minimal avidity that, besides TCR affinity, also depends on heterodimerization for sufficient TCR expression (44).

In summary, we describe the successful rational design of TCR interfaces to promote specific chain pairing. This approach paves the way for the molecular refinement of TCR mutants that favorably interact by noncovalent modifications leaving the overall topology unchanged for optimal avidity and maximal reduction of potential immunogenicity.
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Disclosures

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References