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Selected Murine Residues Endow Human TCR with Enhanced Tumor Recognition

Gil Bialer,* Miryam Horovitz-Fried,* Shlomo Ya'acobi,* Richard A. Morgan,† and Cyrille J. Cohen*

TCR-gene transfer can mediate tumor regression in terminally ill melanoma patients. However, the formation of mix dimers between endogenous and transduced TCR chains may result in the surface dilution of the introduced TCR, which translates in poorer cellular avidity. Recently, we reported that murinization of human TCRs (i.e., the replacement of human C regions by murine ones) can improve TCR function. However, because xenogenic sequences may trigger immunogenicity, we sought to identify the essential murine residues that mediate this enhanced functional effect. We constructed murine/human chimeras of α- and β-chains and assessed for their surface expression and function. We identified an evolutionary-unique lysine residue in Cβ, central to murine TCR function. The mapping of Cα revealed that a few short stretches of amino acids play a role in enhancing TCR function, one of the most important ones being the SDVP sequence. This information led us to design improved and minimally murinized human TCR C regions that mediate increased tumor recognition. This also enabled us to suggest a structural model that could explain the role of the aforementioned residues in promoting the preferential pairing and stability of murinized TCRs. Overall, these findings could have implications for the treatment of malignant diseases using TCR-gene transfer. *The Journal of Immunology, 2010, 184: 6232–6241.

The tCR is an elaborate structure that conveys activation signals into T lymphocytes upon recognition of a specific Ag (1). Signaling through the TCR depends on the interaction of the highly variable TCRα- and β-chains with the invariant CD3 chains (2). The TCRα- and β-chains, linked by a disulfide bond, form the TCR that binds antigenic peptides presented by MHC molecules on APCs and dictates the specificity of the T cell. This recognition principle is central to therapeutic approaches based on the adoptive transfer of tumor-specific T cells for the treatment of metastatic melanoma (3). Although the infusion of ex vivo-expanded tumor-infiltrating lymphocytes can mediate the regression of large bulky tumors in up to 70% of the patients treated (4, 5), the applicability of this treatment has been limited by the need to isolate and expand tumor-specific T cells. Recently, using an alternative approach that relies on the gene modification of patient lymphocytes to express tumor-specific TCRs for the treatment of metastatic melanoma (3). Although the infusion of ex vivo-expanded tumor-infiltrating lymphocytes can mediate the regression of large bulky tumors in up to 70% of the patients treated (4, 5), the applicability of this treatment has been limited by the need to isolate and expand tumor-specific T cells. Recently, using an alternative approach that relies on the gene modification of patient lymphocytes to express tumor-specific TCRs, we showed it was possible to achieve objective clinical tumor regression in stage IV metastatic melanoma patients (6, 7). Although based on a limited number of patients, those studies also suggested that increasing the functional avidity of gene-transferred T cells using highly avid receptors (7, 8) may have improved the clinical response rate [from 13% (6) to up to 30% (7)].

In that regard, several strategies have been devised to improve the functional avidity of TCR-transduced cells. The latter is dependent on two main factors: the affinity of the transferred receptor and the number of TCR molecules expressed on the cell surface. To improve the first, attempts have been made to select high-affinity receptors (reviewed in Ref. 9) or to enhance the affinity of the transferred receptor by point mutations (10–12). Alternatively, various approaches have been devised to augment the number of TCRs on the surface of transduced cells, such as the engineering of the expression vector (9, 13), the use of codon-optimized TCR sequences (14, 15), the elimination of glycosylation sites (16), and the improved pairing of the introduced TCR chains. The latter is also of clinical importance to prevent or lower the risk for potential autoimmunity because of the generation of mixed-chains TCR dimers occurring between the introduced TCR chains and the endogenous ones (17, 18). Preferential pairing of the exogenous TCR chains can be achieved by molecularly altering the primary structure of both the C regions of the α and β TCR chains to different extent: by inverting residues in the CoCβ interface (knob into holes) (19), by fusing CD3-ξ domains to the C terminus of the TCR chains (20), or as we and others recently demonstrated, by introducing a second interchain disulfide bond (21, 22).

Recently, we also described an additional strategy based on the swapping of the human C regions with murine ones or “murinization” (23), because it was observed that murine TCRs are better expressed than (and can replace) human TCRs more efficiently on the surface of human lymphocytes (24–26). This also translated in improved cytokine release and cytotoxicity mediated by murinized TCRs (8, 15, 23, 27) and was associated with an improved pairing of the TCR chains as well as a stronger interaction with the CD3 complex (23).

Although this murinization strategy has been proven effective in enhancing the functional avidity of TCR-transduced lymphocytes,
the introduction of xenogenic sequences might potentially trigger immunogenicity, which could impair TCR-transduced cell function. Thus, we sought to identify the important murine amino acids responsible for the improved function of murinized TCRs. In this paper, we identified a single and evolutionary unique lysine residue, central to the increased tumor recognition mediated by the TCRβ C region. Correspondingly, the mapping of the α-chain revealed that a few short stretches of amino acids may play a role in the enhanced TCR function, one of the most important ones being the SDVP sequence. On the basis of this information, we designed improved and minimally murinized human TCR C regions that mediate increased antitumor recognition. We also attempted to define the molecular/structural basis for the improved pairing of murine TCR C region, and we are proposing, based on the solved murine TCR structure, that two oppositely charged murine residues might interact with each other and enhance the formation of TCR-interchain disulfide bond.

Overall, these results could have significant implications for the translation of TCR gene therapy to the clinical setting and may provide new insights into the biology of murine TCPR.

Materials and Methods

Patient PBMCs and cell lines

All of the PBMCs used in this study were from normal donors obtained from the Israeli Blood Bank (Sheba Medical Center, Tel-Hashomer, Israel). Melanoma cell lines, 526 (HLA-A2†), 624 (HLA-A2), 624.38 (HLA-A2†), 888 (HLA-A2†), and 938 (HLA-A2‡), were generated at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD) as described previously (28). JY is an HLA-A2 EbV-transformed B cell line. Jurkat RT3-T5.3 is a radiation-induced Jurkat mutant that is surface TCR negative (ATCC TIB-153). T2 cells are a lymphoblastoid cell line deficient in TAP function whose HLA/A2 protein can be easily loaded with exogenous peptides (29).

All cells were cultured in R10 medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FBS with exogenous peptides (29).

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MART-1/27L (6, 30), for gp100209–217 (31), and for p53264–272 (25) were designed, one in the sense direction and its reverse complement version. Two primers that incorporate the designed mutation (in the forward direction) and the 3'-primer (in the reverse direction) were purified and linked together in a subsequent PCR with both the flanking primers. The MART-α product was digested with XbaI and NotI. Subsequently, both were cloned separately into the pGEM-4Z/64A vector, providing new insights into the biology of murine TCPR.

Electroporation of PBLs

RNA electroporation enables the use of normalized levels of mRNA and is, unlike other common gene-transfer methods, not dependent on retroviral vector titer, insertion sites, or the influence of diverse transcription mechanisms (23, 32). In addition, we showed repeatedly that RNA electroporation recapitulates results obtained in retroviral transduction systems (6, 25, 32). This technique has been extensively described in our previous report (32). Briefly, in vitro-transcribed mRNA for both α and β TCR chains was generated using mMESSAGE mACHINE (Ambion, Austin, TX) and purified using RNAeasy mini kit (Qiagen, Valencia, CA). PBLs were collected by leukopheresis, and lymphocytes were separated by centrifugation on a Ficoll/Hypaque cushion, washed in HBSS, and then resuspended at a concentration of 1 × 10⁶ cells/ml in Biotarget supplemented with 5% human serum, 50 ng/ml OKT3, and 300 IU/ml IL-2. The lymphocytes were then plated at 1 × 10⁶ cells/ml in 24-well plates (Costar, Cambridge, MA) and cultured for ∼1 wk with the addition of new medium (without OKT3) as needed to maintain density of 10⁶ cells/ml. Electroporation was performed as follows: the lymphocytes were washed in OPTI-MEM (Invitrogen) and resuspended at 2 × 10⁵ cells/ml. Cells were transferred in 2-mm cuvettes chilled on ice and then electroporated at 400V/500 μs using an ElectroSquare Porator ECM 830 (BTX, San Diego, CA). The amount of in vitro-transcribed mRNA for each chain was 1 μg per 10⁶ PBLMs. Following electroporation, cells were transferred to 6-well plates containing fresh medium and cultured at 37°C.

FACS analysis and Abs

Cell surface expression of the DMF4 TCR was assessed by PE-labeled MART-1/27L tetramer (Beckman Coulter, San Jose, CA). Human V812 Ab (7E11) conjugated was supplied by Immunotech (Westbrook, ME). Anti-HA tag Ab was supplied by Miltenyi Biotec (Bergisch Gladbach, Germany) and FITC-labeled anti-CD107a (lyosomal-associated membrane protein 1) by Southern Biotechnology Associates (Birmingham, AL). FITC-labeled anti-human CD69, Cy5-labeled anti-human CD137, and Abs were purchased from BioLegend (San Diego, CA). Immunofluorescence, analyzed as the relative log fluorescence of live cells, was measured using a CyAn-ADP flow cytometer (Beckman Coulter). Approximately 1 × 10⁶ cells were analyzed. Cells were stained in a FACS buffer made of PBS, 0.5% BSA, and 0.02% sodium azide.

Cytokine release assays

PBL cultures were tested for reactivity in cytokine release assays using commercially available ELISA kits for IFN-γ and IL-2 (R&D Systems, Minneapolis, MN). As target cells, we either used melanoma cell lines and/or T2 and JY cells that were pulsed with peptide (1 μg/ml unless indicated otherwise) in medium for 2 h at 37°C, then washed before initiation of cocultures. For these assays, 1 × 10⁶ responder cells (PBL) and 1 × 10⁶ stimulator cells were incubated in a 0.2-ml culture volume in individual wells of 96-well plates. Stimulator cells and responder cells were cultured for 24 h. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay. As a control for T cell activity, we incubated the different TCR-electroporated cultures with the mitogen PHA at a concentration of 5 μg/ml.

Structural analysis and modeling

We analyzed the murine 2C-TCR–solved structure (PDB entry code: ITCR) (34), using the PyMol software.

Results

A comparison of the primary structure of murine and human C regions reveals that the α (Cα) and β-1 (Cβ1) chains share 64.3 and 78.5% identity, respectively (Fig. 1A, 1B). We decided to first generate human-mouse chimeras of the C region using the MART-1–specific DMF4 TCR that we described in the first TCR-gene transfer clinical trial (6) and that was enhanced by murinization as we showed recently (23).

Mapping of residues in the β-1 constant chain

The murine β-1 C region (Cβ1) was divided into three main regions according to its similarity with the human one: region 1 from the N terminus to residue 35, region 2 from residue 36 to residue 97, and region 3 from residue 97 to the C terminus (Fig. 1). Surprisingly, although regions 2 and 3 appear to be the most polymorphic regions
between human and murine chains, the introduction of those regions into the human constant chain did not yield any enhanced effect (data not shown). Alternatively, a human mutant termed 40β, in which the first 35 residues were replaced by murine ones (a total of 11 mutations), showed similar activity as the whole murine β region. More specifically, we electroporated OKT-3–stimulated human PBLs from three different donors with mRNA encoding the wild-type (wt) murine β1 C region, we synthesized two mutants, K1 and K2, in which the first 35 residues were replaced by murine ones (a total of 11 mutations), showed similar activity as the whole murine β C region. More specifically, we electroporated OKT-3–stimulated PBLs from four different donors.

In addition, those cells were cocultured with different melanoma cell lines (as indicated in the legend), and 16 h after the beginning of the coculture, we measured the IFN-γ secretion in the supernatant using an ELISA procedure. The combination Mα/Mβ (4372 versus 4791 pg/ml, respectively, equivalent to 3-fold increase in the percentage of tetramer-stained cells (mean fluorescence intensity [MFI] of 22) for Mα/40β versus 72% (MFI of 30) for the wt murine C region (Fig. 2).

FIGURE 1. Amino acid comparison of human and murine Cα (A) and Cβ1 (B) C regions.

In addition, those cells were cocultured with different melanoma cell lines (as indicated in the legend), and 16 h after the beginning of the coculture, we measured the IFN-γ secretion in the supernatant using an ELISA procedure. The combination Mα/Mβ (4372 versus 4791 pg/ml, respectively, equivalent to 3-fold increase in the percentage of tetramer-stained cells (mean fluorescence intensity [MFI] of 22) for Mα/40β versus 72% (MFI of 30) for the wt murine C region (Fig. 2).

Careful inspection of the amino acids alignment of human and mouse Cβ1s reveals that, among this 11-residue difference, there are two identical and radical permutations from glutamate (E) in the human sequence to lysine (K) in mouse C regions, at positions 10 and 18 (Fig. 1B). To study the effect of such a change from a negatively charged residue to a positively charged one in the human β-1 C region, we synthesized two mutants, K1 and K2, in which we replaced separately the original glutamate residue with a lysine at position 10 or 18, respectively. Although the K1 mutant did not exhibit any particular enhanced activity (alone or in combination with K2; data not shown), K2 seemed to be essential to the improved function of the chimeric C regions as the incorporation of this single mutation in the human β-1 C region induced a ~3-fold increase in the percentage of tetramer-stained cells (43 versus 15%) as well as in IFN-γ secretion (1380 versus 442 pg/ml; data not shown). We observed a provision of 69% positively stained cells, as well as the relative MFI (in parentheses), is shown. Background staining of mock-electroporated lymphocytes stained with MART-1 tetramer is shown as a black histogram. These results are representative of eight independent experiments. OKT-3–stimulated human PBLs were electroporated with mRNA encoding the different TCR combinations. The percentage of positive cells, as well as the relative MFI (in parentheses), is shown. Background staining of mock-electroporated lymphocytes stained with MART-1 tetramer is shown as a black histogram. These results are representative of eight independent experiments. OKT-3–stimulated human PBLs were electroporated with mRNA encoding the different TCR chains and cocultured in the presence of HLA-A2+/MART-1+ melanoma cell lines (as indicated in the legend), and 16 h after the beginning of the coculture, we measured the IFN-γ secretion in the supernatant using an ELISA procedure. The combination Mα/Mβ (4372 versus 4791 pg/ml, respectively, equivalent to 3-fold increase in the percentage of tetramer-stained cells (mean fluorescence intensity [MFI] of 22) for Mα/40β versus 72% (MFI of 30) for the wt murine C region (Fig. 2).

It should be noted that knocking down this lysine in the original murine sequence by replacing it with the human glutamate residue resulted in a significant (p < 0.05) decrease in TCR activity (60% of that of the wt murine CB; data not shown).

Interestingly, the addition of a second mutation (from serine to alanine at position 22) to the K2 mutant, to generate the KA mutant, improved both tetramer binding and IFN-γ secretion to 48% and 15% (9) (Fig. 1B) as well as in IFN-γ secretion (1380 versus 442 pg/ml; p = 0.02) (Fig. 2C). It should be noted that knocking down this lysine in the original murine sequence by replacing it with the human glutamate residue resulted in a significant (p < 0.05) decrease in TCR activity (60% of that of the wt murine CB; data not shown).

FIGURE 2. A, Sequence alignment of the Cβ1 N terminus C region of Hb, Mβ, KA, and K2 mutants. B, Comparison of the surface expression of the original TCRs and their hybrids. OKT-3–stimulated PBLs were electroporated with mRNA encoding the different TCR chains and assessed by FACS. Twenty-four hours after electroporation, we assessed MART-1 tetramer binding for the different TCR combinations. The percentage of positive cells, as well as the relative MFI (in parentheses), is shown. Background staining of mock-electroporated lymphocytes stained with MART-1 tetramer is shown as a black histogram. These results are representative of eight independent experiments. OKT-3–stimulated human PBLs were electroporated with mRNA encoding the different TCR chains and cocultured in the presence of HLA-A2+/MART-1+ melanoma cell lines (as indicated in the legend), and 16 h after the beginning of the coculture, we measured the IFN-γ secretion in the supernatant using an ELISA procedure. The combination Mα/Mβ (4372 versus 4791 pg/ml, respectively, equivalent to 3-fold increase in the percentage of tetramer-stained cells (mean fluorescence intensity [MFI] of 22) for Mα/40β versus 72% (MFI of 30) for the wt murine C region (Fig. 2).

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lymphocytes with HLA-A2\(^{-}\) melanoma cell lines 888 and 938 (data not shown) as a negative control did not yield IFN-\(\gamma\) secretion above the background for any of the TCR combination tested.

In conclusion, we discovered that a lysine—K2—at position 18 is essential to the improved function of the murine \(\beta\) C region, and that in conjunction with alanine 22, those only two mutations mediated a 4-fold increase (\(p = 0.02\) in activity when compared with the wt human \(\beta\)-1 sequence.

**Mapping of residues in the \(\alpha\) constant chain**

We turned to examine the relative contribution of different regions in the constant TCR\(\alpha\)-chain (C\(\alpha\)). Briefly, we divided it into three main regions (Fig. 1). Preliminary experiments demonstrated that regions 2a and 3 were not essential in mediating improved function of murine C\(\alpha\) (data not shown). Thus, we constructed a mutant—\(\alpha\) 1/2-SDVP—that incorporates regions 1, 2b, and 2c, which was able to function, in conjunction with murine C\(\beta\)1, in a similar fashion as the full murine C\(\alpha\) (3138 versus 3228 pg/ml IFN-\(\gamma\), respectively) (Fig. 3).

We then studied the effect of the 2c region, a short stretch of four amino acids (SDVP), and mutated it back into the human sequence (PESS) and generated the mutant \(\alpha\) 1/2-PESS. Replacement of the SDVP region with its corresponding human sequence caused a drop in tetramer-positive cells (46 versus 39\%) (Fig. 3A) but more importantly in IFN-\(\gamma\) secretion from 3138 to 1749 pg/ml (\(p < 0.001\)). Moreover, we attempted to modify this region by permuting one or two residues between the murine and human sequences and constructed three others mutants: \(\alpha\) 1/2-SESP, \(\alpha\) 1/2-PDVS, and \(\alpha\) 1/2-PEVS. None of those was able to mediate equally efficient tetramer staining or relative activity as the \(\alpha\) 1/2-SDVP mutant (Fig. 3A, 3B). Therefore, we identified the 2c region (SDVP) as the optimal sequence and as essential to the function of murinized TCRs.

To examine the role of region 1 in C\(\alpha\), we replaced the full murine region 1 with the corresponding human sequence (mutant \(\alpha\) 2-SDVP). On the basis of the latter, we generated two other mutants that contained either the murine region 1a (\(\alpha\) 1.20-SDVP) or region 1b (\(\alpha\) 1.30-SDVP) (Figs. 1, 4A). In coculture experiments and in tetramer staining assays (Supplemental Fig. 1), we noticed that region 1 was of a certain importance: its elimination caused a decrease in IFN-\(\gamma\) secretion from 1747 to 999 pg/ml (Fig. 4B). Both regions 1a and 1b were able to rescue part of the relative activity of the Co chimeric region, with region 1b being the most efficient (1513 pg/ml). This might be partially explained by the fact that the human sequence of region 1b at position 32 contains an N-linked glycosylation site (NVS) that is absent from the murine corresponding region (NVP). Knocking down this particular site has been demonstrated recently to enhance the functional avidity of TCR transduced (16). Still, partial mutants of region 1b, even when this glycosylation site was eliminated, did not recapitulate the full effect mediated by the entire region 1b (data not shown).

Last, we decided to study the effect mediated by the 2b region. This part was subdivided into two murine stretches: QDIFK and ETNATY (starting at positions 73 and 78, respectively) (Fig. 4C). Briefly, we generated two mutants, based on the \(\alpha\) 1/2-SDVP Co chimera, in which we replaced the QDIFK sequence with the human ANA (ANAFK mutant), and another one that also had an N residue (instead of K) at position 77 (ANAFN mutant) (Fig. 4C). ANAFN showed a marked decrease in IFN-\(\gamma\) secretion (913 pg/ml) (Fig. 4D), whereas ANAFK was able to function better when compared with the \(\alpha\) 1/2-SDVP mutant (1513 versus 1747 pg/ml, respectively). Again, one may postulate that by introducing an Asn (N) residue at position 77, this created an additional glycosylation site (ANA-FKET-NA), which might have hindered TCR function.

For the second half of the region 2b (ETNATY), we constructed two mutants DTFF and NATF based on the \(\alpha\) 1/2-SDVP Co chimeras (Fig. 4D). DTFF exhibited a significant loss of activity when compared with \(\alpha\) 1/2-SDVP (769 versus 1747 pg/ml, respectively). However, NATF functioned identically to \(\alpha\) 1/2-SDVP, mediating an IFN-\(\gamma\) secretion of 1825 pg/ml. A similar pattern of TCR expression was observed in MART-1 tetramer staining experiments (Supplemental Fig. 1B).

Overall, it seems that the QDIFK sequence is not essential to the improved chimeric TCR Co and that the murine tyrosine residue (at position 87) could be replaced with a human phenylalanine without a loss of activity.

**Function and expression of the combination of \(\alpha\) and \(\beta\) chimeras**

On the basis of the analysis of the Co region described in the previous section, we generated two additional \(\alpha\) mutants based on the human Co template: the first, termed ULT, contains subregion 1b (from region 1) and the sequences KETNATF (from region 2b) and SDVP (region 2c). The second mutant, B-ULT, is identical to ULT but with a full human region 1 (Fig. 5A). We tested the expression and function of these mutants in conjunction with the full murine C\(\beta\)1 (M\(\beta\)), mutant 40\(\beta\), and mutant KA.

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**FIGURE 3.** A, Comparison of the surface expression of mutants in the 2c region: OKT-3-stimulated PBLs were electroporated with mRNA encoding the different TCR chains and assessed by FACS. Twenty-four hours after electroporation, we assessed MART-1 tetramer binding for the different TCR combinations. The percentage of positive cells, as well as the relative MFI (in parentheses), is shown. Background staining of mock-electroporated lymphocytes stained with MART-1 tetramer is shown as a black histogram. These results are representative of four independent experiments. B, Previously described cells were cocultured in the presence of HLA-A2*\(\sim\)MART-1* melanoma line 624. IFN-\(\gamma\) secreted in the coculture supernatant was measured by ELISA (*\(p < 0.001\); calculated using a Student t test). No significant IFN-\(\gamma\) secretion was observed in coculture with control cell lines 888 and 938, and the differences in cytokine secretion by PHA-stimulated TCR-electroporated cultures were not statistically significant (control for general T cell activity). These results are representative of six independent experiments, performed with three different donors.
number of residues in Cα and Cβ) compared with wt human chains (Hα/Hβ) yielded a ~4-fold higher relative reactivity (based on IFN-γ secretion and relative to Mα/Mβ, 100%) than Hα/Hβ (52 versus 14%) (Table I). Accordingly, we also observed a greater functional avidity mediated by the full and minimally murinized receptors compared with the wt human DMF4 in MART-1 peptide titration experiments (Supplemental Fig. 2).

Those results were repeated with two other TCRs: a gp100–209-specific TCR, isolated from a highly avid anti-gp100 CTL clone (31), as well as with a p53-specific one (25). Although the relative IFN-γ secretion triggered by the wt gp100–209-specific TCR Ha/Hβ, compared with that mediated by Mα/Mβ, was higher (53% of Mα/Mβ) than that observed for the DMF4 TCR (14% of Mα/Mβ), the murinization of this TCR improved its function (Fig. 5). This suggests that the murinization-enhancing effect of TCR activity is also applicable to a high-affinity TCR, according to our previous observations (23). Nonetheless, a similar pattern of reactivity, as observed with the previous TCR tested, was mediated by the chimeric murine/human C regions, in which the ULT combinations functioned slightly better than the B-ULT ones (p < 0.05) (Fig. 5D). Similar results were also obtained when measuring the secretion of another cytokine, IL-2, using p53-specific TCRs that incorporated different C regions (Fig. 5E), demonstrating the general applicability of this strategy to different TCRs.

Additionally, we assessed for the expression activation markers such as CD69 and CD137 as well as for mobilization of CD107a—a marker of T cell degranulation correlated with cytotoxicity (35)—triggered by the wt and chimeric TCR chains. Although the fully murinized TCR (Mα/Mβ) was found to trigger the highest expression of the aforementioned markers (Fig. 6), partially murinized TCR combinations (ULT/40B and B-ULT/KA) were able to mediate the surface expression of CD69 (Fig. 6A), CD137 (Fig. 6B), and CD107a (Fig. 6C) in a higher proportion of cells than the Hα/Hβ (p < 0.05).

Overall, we were able to design minimally murinized TCR C regions that demonstrated an improved function compared with the original wt human Cα and Cβ.

**Minimally murinized enhanced TCR function is associated with preferential pairing**

We conducted several experiments to elucidate the mechanisms underlying the enhanced activity of partially murinized TCRs. To examine the relative contribution of TCR chain protein expression, we electroporated TCR-deficient Jurkat RT3-T3.5 cells with different MART-1–specific TCR combinations as indicated in Fig. 7A. The α-chains were previously modified to include an HA tag (33). Electroporated cells were stained with a Vβ12-specific Ab as well as with an anti-HA Ab (to detect the expression of the α-chain) and analyzed for TCR chain expression by flow cytometry (Fig. 7A). We did not observe any statistically significant difference in the expression of the mutant or wt TCRα-chain. Additionally, the expression of the tested TCRβ-chains was similar (n = 5 and p = 0.89; based on one-way ANOVA). This suggests that the enhanced function of murinized TCR is not due to a difference in protein expression or enhanced translation.

Because receptor internalization is an important feature of the TCR dynamics following antigenic stimulation, we sought to ascertain whether mutant TCRs would display a different level of endocytosis, which could explain their enhanced function. Consequently, Jurkat RT3-T3.5 expressing different MART-1 TCR combinations (as indicated in Fig. 7B) were cocultured in the presence of MART-126–35 peptide-pulsed T2 cells or mock pulsed as a control. We then stained them with a MART-1/HLA-A2 tetramer to assess the level of specific TCR surface expression and endocytosis, which could explain their enhanced function. Consequent...
calculated the level of TCR internalization relative to unstimulated lymphocytes. We repeated this experiment four times and did not observe a statistically significant difference in the level of TCR internalization mediated by either murinized or wt TCR (\(p = 0.79\); using one-way ANOVA).

Finally, we wanted to assess whether partially murinized TCRs will be less sensitive to the presence of a competitor human TCR than their human counterpart. Indeed, a potential hurdle in TCR gene transfer approaches is the pairing of the introduced TCR subunits with endogenous TCR chains. The immediate effect of this competition between exogenous and endogenous TCR subunits may result in the reduction of the cell surface density of the exogenous TCR (36–38). In contrast, we demonstrated an increased proportion of MART-1 tetramer-positive cells that expressed the fully and partially murinized form of the anti–MART-1 TCR rather than the original human version (Ha/Hβ) (Fig. 5B), which may suggest a preferential pairing of the murinized C regions with themselves. To test this hypothesis, we performed a competition experiment (23) by electroporating the TCR-deficient cell line Jurkat RT3-T3.5 with different combinations of DMF4 TCR and added another TCR (p53 Ha/Hβ, 87%; ULT/40B, 83%; and B-ULT/KA, 72% of relative...
TCR expression). In contrast, the expression of the native MART-1 TCR (Ha/Hb) was significantly reduced to 35% because of the presence of competitor TCRs. A similar pattern was observed when using gp100-specific TCRs as another competitor (data not shown).

Thus, the aforementioned data suggest that preferential pairing could account for the increased TCR expression and function mediated by partially murinized C regions.

Discussion
Murinization of human TCRs has been demonstrated to improve their function and expression (15, 23). However, the potential risk of immunogenicity that might be triggered by such xenogenic sequences might preclude their clinical use in TCR-gene transfer treatments. Therefore, we aimed in the present report, at determining the essential residues in murine TCR C regions that mediate the improved activity of murinized TCRs. To that end, we constructed and tested multiple chimeric murine/human C regions for both the a- and b-chains.

The mapping of Cb1 revealed that a single lysine (we termed K2, at position 18) was crucial in mediating superior TCR expression and function. Surprisingly, this lysine was found to be evolutionary unique in all the mammalian TCR b sequences we compared (Fig. 8A), in which it is replaced by an acidic glutamate residue, including in the human sequence.

![FIGURE 6.](image)

Increased expression of activation and degranulation markers mediated by minimally murinized TCRs. A and B, OKT-3–stimulated PBLs were electroporated with mRNA encoding the indicated MART-1–specific TCR chains and cocultured with the melanoma cell line 526. Sixteen hours after the beginning of the coculture, cells were analyzed by flow cytometry for CD69 (A) or CD137 (B) expression (gated on the CD8+ population). The percentage of positive cells for each marker is indicated. These results are representative of three independent experiments.

C, TCR competition assay. TCR-deficient Jurkat RT3-T3.5 cells were electroporated with the indicated MART-1–specific TCR chain combinations along with each chain of the competitor TCR (p53 Ha/Hb). Twenty-four hours after electroporation, the cells were stained with MART-1 tetramer, and the percentage of MART-1 TCR relative expression was calculated by dividing percentage of tetramer-positive cells of a given sample by that of the control sample (without competitor TCR). *p < 0.05, calculated using a Student t test.

![FIGURE 7.](image)

A. TCR-deficient Jurkat RT3-T3.5 cells were electroporated with the indicated TCR chain combinations and stained for anti-HA (specific for the a-chain) or anti-Vβ12. The difference of expression of the different TCR chains was found to be not statistically significant (p = 0.89, calculated using one-way ANOVA), based on five independent experiments. B, Internalization: TCR-deficient Jurkat RT3-T3.5 cells were electroporated with the indicated MART-1–specific TCR chain combinations and cocultured with MART-1 peptide-pulsed T2 cells or mock pulsed. Sixteen hours after the beginning of the coculture, cells were stained with MART-1 tetramer, and the percentage of Ag-induced TCR internalization is shown. These results are representative of four independent experiments. No statistically significant difference between the different TCR combinations was found, p = 0.79, calculated using one-way ANOVA. C, TCR competition assay. TCR-deficient Jurkat RT3-T3.5 cells were electroporated with the indicated MART-1–specific TCR chain combinations along with each chain of the competitor TCR (p53 Ha/Hb). Twenty-four hours after electroporation, the cells were stained with MART-1 tetramer, and the percentage of MART-1 TCR relative expression was calculated by dividing percentage of tetramer-positive cells of a given sample by that of the control sample (without competitor TCR). *p < 0.05, calculated based on a Student t test.
the addition of an alanine at position 22 was beneficial to TCR activity. Again, from an evolutionary standpoint, this murine residue is unique in all the mammals, but in the (evolutionary-related) rat sequence. These lysine and alanine residues are separated by three amino acids, and structural analysis revealed that both are part of the same α helix in AB loop located at the basis of the TCR structure and facing outward in the same direction (Fig. 8B). As we found that the SDVP sequence in Cβ was essential to TCR-improved activity, we also plotted it on the 2C (PDB: 1TCR) murine structure (34). To our surprise, the SDVP sequence is located in very close vicinity to K2. Moreover, careful inspection of the rotamers in the mouse TCR structure reveals that negatively charged Asp\(^{210}\) (SDVP) of the α-chain and positively charged Lys\(^{134}\) (K2) of the β-chain are sufficiently close to each other (2.6 Å) to form short-distance interactions that can result in the formation of a salt bridge between Cα and Cβ (Fig. 8B). In addition, the SDVP sequence is adjacent to the cysteine on Cα (SDVPC) (Fig. 8A), responsible for the interchain disulfide bond. The implications of these findings lead us to propose the following model to explain the improved function of murinized and chimeric human/murine TCR C regions: in this paper, we suggest that the formation of the TCR interchain disulfide bond, which would result in a more efficient pairing of the murine Cα and Cβ.

Although an acidic residue (glutamate) could also be found in the corresponding region in human Cα (PESS), it also seems that the integrity and the configuration of the SDVP sequence, and not only the presence of an acidic residue, is required as different mutants in this region did not function as efficiently (Fig. 3). One can only suggest the latter, based on experimental data as we were unable to find a solved human TCR structure that makes up the human 2c region (PESS). These findings and the abovementioned model might also explain why the expression of a TCR with human C regions in mouse lymphocytes is not necessarily enhanced when compared with a TCR with murine C regions (C. J. Cohen, unpublished observation) and may also raise questions in regard to the presence of evolutionary unique lysine and its possible link to murine TCR function.

Additionally, we also mapped two other regions in Cα that enhanced chimeric TCR function, namely regions 1 and 2b, which are respectively located in the C and F strands of the Cα IgG-like domain. The latter are highly polar and constitute one face of the Cα domain, which can form weak interactions with the bottom sheet, suggesting a mobility that may allow interactions with the CD3 heterodimers (34). Regardless of potential direct interactions with CD3 components, an improved TCR chain pairing ability has been suggested to enhance the association with CD3 components (39, 40), which is supported by our previous finding using murinized TCRs (23). Efforts are under way to better characterize those interactions looking at the relative stability of murine/human chimeric C regions with CD3 components.

From a functional perspective, we noticed that the murine Cα and Cβ1 combination (Mα/Mβ) reliably displayed superior surface expression and function compared with other murine codon combination assessed. This could suggest that additional murine residues may also play a limited role in the enhanced function of murinized TCRs. However, it could very well be that the structural integrity of the human/murine chimeras might be slightly altered in comparison with that of the native chains, even though there is a high degree of homology between human and murine TCR chains (Fig. 1). Nevertheless, an increase in the content of murine residues was associated with an enhanced TCR function (Table I). On the basis of the overall activity of a wt human TCR intended for clinical use and immunogenicity concerns, one may choose to define differently the optimal content of murine residues. Therefore, it might prove valuable to assess a few of the combinations we outlined in the present report. Yet, it would be possible to combine (partial) murinization and of other paring-promoting strategies (9), such as the addition of a second disulfide interchain bond as we recently demonstrated for wt murine C regions (21).

To better characterize the influence of partial murinization on the TCR protein dynamics, we performed further studies that revealed that the different TCR chains (either mutant or wt) exhibited a similar degree of expression and equivalent level of TCR internalization following Ag stimulation (Fig. 7A, 7B, respectively). Additionally, this would suggest that codon optimization cannot necessarily account for improved minimally murinized TCR function, especially as some of the murine codons introduced (such as the lysine K2 at position 18 on the β-chain or serine and valine residues at positions 86 and 88, respectively, on the α-chain) are not the optimal ones based on the human codon usage scale. However, we observed that fully and partially murinized TCRs were less influenced by the presence of competitor TCRs than their human counterpart, suggesting an increased capacity for correct pairing of the introduced TCR chains.

In that regard, preferential pairing is of importance to prevent the formation of mixed dimers that could potentially engender autoimmune manifestations (18) because of the generation nondesired specificity. Although this issue could be addressed using different types of cells or receptors (19, 20, 41–44), we postulate that partial...
murination, in light of our past observations (23) and the present report, can be also of interest in decreasing the formation of mixed TCR chain dimers.

One of our main motivations in the present report was to decrease the amount of murine residues in Cγ and Cβ1 that might prove immunogenic. Although immunogenicity is difficult to predict a priori, one can only assume that decreasing the murine residues content will lower the probability to trigger an immunological response against the murinized TCR. Nonetheless, preliminary analysis using an algorithm based on Kolaskar et al. (45) did not show an increased antigenic propensity of the partially murinized TCR chains when compared with their human counterpart. TCR/CD3 complex structure solution as well as the analysis of immune response in patients that were treated with the gp100−154-specific murine TCR (7) will be able to provide further insight in the immunogenic potential of minimally murinized TCRs.

In conclusion, we isolated key amino acids present in the murine TCR C regions that are crucial to the enhanced function of murinized TCRs. We showed that it was possible to engineer human C regions with selected murine residues, which resulted in improved TCR surface expression and function. These results could have significant implications for the use of murinized TCRs for human immunotherapy in the clinical setting.

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Disclosures

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References


