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Astar Haga-Friedman, Miryam Horovitz-Fried, and Cyrille J. Cohen

TCR-gene transfer represents an effective way to redirect the specificity of T lymphocytes for therapeutic purposes. Recent successful clinical trials have underscored the potential of this approach in which efficient expression of the exogenous TCR has been directly linked to the efficacy of T cell activity. It has been also demonstrated that the TCR exhibits a lack of stability associated with the presence of positively charged residues in its transmembrane (TM) region. In this study, we designed an original approach selectively to improve exogenous TCR stability by increasing the hydrophobic nature of the TCRα TM region. Incorporation of hydrophobic residues at evolutionarily permissive positions resulted in an enhanced surface expression of the TCR chains, leading to an improved cellular avidity and anti-tumor TCR activity. Furthermore, this strategy was successfully applied to different TCRs, enabling the targeting of human tumors from different histologies. We also show that the combination of these hydrophobic mutations with another TCR-enhancing approach further improved TCR expression and function. Overall, these findings provide information regarding TCR TM composition that can be applied for the improvement of TCR-gene transfer-based treatments. The Journal of Immunology, 2012, 188: 5538–5546.

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The online version of this article contains supplemental material.

Abbreviations used in this article: HA, hemagglutinin; IRES, internal ribosome entry site; l-NGFR, low-affinity nerve growth factor receptor; MFI, mean fluorescence intensity; PI, propidium iodide; TM, transmembrane; wt, wild-type.

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dyes could compensate for the lack of TCR chain stability and increase TCR expression and function. We performed several modifications to the TCR TM region, and it appeared that unlike for other immune receptors (23), large changes had a detrimental effect on TCR expression and function. Nevertheless, by carefully selecting mutable amino acids based on an evolutionarily permissive approach, we identified three critical amino acids in the TCR TM region that when replaced with leucine or valine mediated an increased TCR expression and function. This was extended to other TCRs that were similarly modified and enhanced. The current work represents a novel approach that enables the enhancement of the anti-tumor activity of TCR-gene-transferred lymphocytes based on the manipulation of the TCR amino acid composition.

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 HLA-A2+/MART-1+ (526, 624, 624.38) and HLA-A2/ MAGE-A1+ (888, 938) were generated at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD) as described previously (24). SK-MEL23 is an HLA-A2+ melanoma cell line (25). Jurkat RT3-T3.5 is a radiation-induced Jurkat mutant that is surface TCR negative (ATCC/TIB-153). p53+/HLA-A2- cell lines were H2O87 (ATCC/CRL-5922) and Saos2p143 (4). T2 (ATCC/CRL-1992) is a lymphoblastoid cell line deficient in TAP function whose HLA-A2 protein can be easily loaded with exogenous peptides. Viral packaging line 293GP, which stably expresses GAG and POL proteins, has been previously described (26). Adherent cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% neglect med in BioTarget medium (Biological Industries) supplemented with 10% heat-inactivated FBS (Biological Industries, Bethesda, MD) and were maintained in 3% and 5% CO2 incubator. Lymphocytes were cultured in BioTarget medium (Biological Industries) supplemented with 10% heat-inactivated FBS and 300 IU/ml IL-2 and maintained at 37°C and 5% CO2.

Peptide

The sequences of the peptides used in this study are as follows: MART-126–35 (ELAIGILITV), gp100109A (IMDQFPFFSV), and p53264-271 (LLGRNSFEV).

TCRs

The α- and β-chains from the previously characterized TCRs specific for MART-126–35 termed F4 (or DMF4) (7, 27), for p53 (4), and for gp100 (28) were subcloned into the pGEM-4Z/64A vector as described previously (11). In addition, we created mutant TCRs in which we mutated residues in the TM region (20, 29) of the TCRα- and TCRβ-chains. These mutants, described in Fig. 1A, were created by overlapping PCR as described previously (12). To enable the detection of the specific TCRα-chain, we added a hemagglutinin (HA) tag (YPYDVPDYA) or a c-Myc tag (EQKLISEEDL) to its N terminus as described previously (30). The retroviral backbone used in this study, pMSGV1, is a derivative of the MSCV-based splice-gag vector (pMSGV), which uses an MSCV long terminal repeat and has been previously described (26). The sequences of the peptides used in this study are as follows: MART-126–35 (ELAIGILITV), gp100109A (IMDQFPFFSV), and p53264-271 (LLGRNSFEV).

Eлектroporation of PBLS

This technique has been extensively described in our previous reports (12, 33). Briefly, in vitro-transcribed mRNA for both TCR chains was generated using AmpliCap-Max T7 high yield message maker kit (Cellscript, Madison, WI) and purified using RNAcleanup kit (Norgen Biotek, Thorold, ON, Canada). PBLS were separated by centrifugation on a ficoll/hypaque cushion and were stimulated with 50 ng/ml OKT3 and 300 IU/ml IL-2. Electroporation was performed at 400 V/500 μs using an Eckliseedl Porator ECM 830 (BTX, San Diego, CA). The amount of in vitro-transcribed mRNA for each chain was 1–2 μg per 10^6 PBMCs.

Transduction of PBLS

For virus production, transfection of 2 × 10^6 293GP cells with 9 μg DNA of a MSGV1-F4/NGFR construct (Fig. 1B) and 4.5 μg envelope plasmid (VSV-G) was done using JetPrime transfection reagent (Polyplus, Illkirch, France). After 4 h, the medium was replaced. Rétroviral supernatant was collected 36 h after the DNA transfection. Freshly isolated PBLs were stimulated for 48 h in the presence of 50 ng/ml OKT3 before transduction. After stimulation, lymphocytes were transduced with retroviral vectors by transfer to nontreated tissue culture dishes (Nunc, Rochester, NY) that had been precoated with RetroNectin (Takara, Otsu, Japan) and retroviral vectors as previously described (26).

FACS analysis and Abs

Cell surface expression of the F4 TCR was assessed by PE-labeled MART-127/HLA-A2 tetramer (Beckman Coulter, San Jose, CA). Fluorescein-labeled anti-human CD8, CD25, CD69, CD137, 1-NGFR, and IFN-γ were purchased from BioLegend (San Diego, CA). FITC-labeled anti-CD107a (lyosomal-associated membrane protein 1) was supplied by Southern Biotechnology Associates (Birmingham, AL). PE-labeled anti-c-myc and anti-HA Abs were supplied by Miltenyi Biotec (Bergisch Gladbach, Germany). Immunofluorescence was analyzed as the relative log fluorescence of live cells, was measured using a CyAn-ADP flow cytometer (Beckman Coulter, Brea, CA). Approximately 1 × 10^6 cells were analyzed. Cells were stained in a FACS buffer made of PBS, 0.5% BSA, and 0.02% sodium azide.

CD4/CD8 separation

CD4+ and CD8+ populations were separated using a magnetic beads-based approach for both negative and positive selection of those subsets (Miltenyi Biotec, Auburn, CA).

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 lines and/or T2 cells that were pulsed with peptide (at 1 μg/ml, unless indicated otherwise) in medium for 2 h at 37°C. For these assays, 1 × 10^5 responder cells (PBL) and 1 × 10^3 stimulator cells were incubated in an 0.2-ml culture volume in individual wells of 96-well plates. Stimulator cells and responder cells were cocultured 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 T cell cultures with PMA/ionomycin at a concentration of 50 ng/ml and 1 μM, respectively.

Functional avidity

Curves were fitted to the data points using a nonlinear regression according to the equation \( y = \frac{\alpha_0}{\alpha_0 + \beta x} \), where \( \alpha_0 \) represents IFN-γ secretion, \( \alpha_0 \) is the concentration of the epitope, \( n \) is the slope of the curve at midpoint (n = 1), and \( \beta \) is the peptide concentration at which \( y = \frac{\alpha_0}{2} \) (representative of the functional avidity).

Western blot analysis

Jurkat RT3-T3.5 cells (2 × 10^6) were electroporated with either HA tag- or HA tag-Ho-LVL mRNA (5 μg). After 24 h, the cells were placed in lysis buffer (Pierce, Rockford, IL) and complete protease inhibitor (Roche, Indianapolis, IN) for 30 min on ice. After removal of nuclear debris by centrifugation, the resultant supernatants were quantitated using Coomassie (Bradford) Protein Assay (Pierce), and 20 μg of total protein from each lysate was subjected to immunoblotting with anti-HA tag peroxidase (Roche).

Cell-mediated cytotoxicity assay

Target cells were labeled with 2 μM CFSE (eBioscience, San Diego, CA) for 6 min and then cocultured with transduced lymphocytes at 37°C for 4 h, at an E:T ratio of 4:1. After the coculture, propidium iodide (PI) 1 μM (Sigma-Aldrich, Rehovot, Israel) was added for assigning the ratio of cell death. Samples were analyzed by flow cytometry.

Results

Isolation and characterization of TCR TM mutants with increased hydrophobicity

To study the impact of hydrophobic mutations in the TCR TM domain, we used the F4 TCR (27), specific for HLA-A2/MART-126–35, which was initially demonstrated to mediate tumor regression in patients (7). We generated the poly-Leu and poly-Lβ...
mutants in which we replaced selected amino acids (7 in TCRα and 10 in TCRβ) with either leucine/isoleucine residues (Fig. 1) while preserving the basic residues (R and K) responsible for the interaction with the CD3 components. As mentioned previously, such changes did not impact on surface expression for other immune receptors and, even to some extent, improved it (23). To test for the biological activity of the mutated receptors, OKT3-stimulated human primary PBLs were electroporated with mRNA encoding these mutants or the wt (Ho/Hβ) F4 TCR sequences and cocultured with the HLA-A2+/MART-1+ 624.38 tumor or with 938 (HLA-A2+/MART-1+ control). Neither the poly-La nor poly-Lβ mutants functioned better than their wt counterpart (Fig. 2A). Notably, the poly-Lβ showed a total loss of function.

We assumed that these changes were too radical, and thus we generated new mutants in which we modified only three to five residues. The mutated residues were selected based on a sequence alignment of the TCR chains from different mammalian species (Supplemental Fig. 1). The mutated residues were changed to either leucine or valine, which are among the energetically most favorable residues in the TM environment (34). Although none of the TCRβ mutants mediated tumor recognition, one of the TCRα mutants, namely Ho-LVL (in association with the wt Hβ-chain), triggered an improved IFN-γ secretion compared with the wt Ho sequence: 5034 versus 3354 pg/ml, respectively (Fig. 2A). This was associated with an improved surface expression of the TCR as measured by tetramer staining showing 45.6% of positive cells (mean fluorescence intensity [MFI] = 90) for the Ho-LVL/Hβ combination versus 25.6% (MFI = 55) for the wt receptor (Fig. 2B).

In an effort to study the impact of each of the three LVL mutations (Ser→Leu, Gly→Val, and Phe→Leu) on the biological activity of the receptor and to test whether it was possible to minimize the number of mutated residues in the LVL sequence, we generated mutant TCRα-chains with only single or dual permutations (as indicated in Fig. 1) and tested for their ability to mediate IFN-γ secretion in coculture with melanoma lines. As seen in Fig. 2C, the original combination of the three L-V-L

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

**FIGURE 1.** (A) Sequence alignments of the TM regions of the TCRα- and TCRβ-chains and their respective mutants. (B) Schematic representation of the MSGV-1 retroviral construct used in this work.

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**FIGURE 2.** Screening of TCR TM mutants. (A) OKT3-stimulated human PBLs were electroporated with mRNA encoding the different F4 TCR chains (as indicated) and cocultured in the presence of HLA-A2+/MART-1+ melanoma line 624.38. IFN-γ secreted in the coculture supernatant was measured by ELISA. No significant IFN-γ secretion was observed in coculture with control cell line 888, and the differences in cytokine secretion by PMA/ ionomycin-stimulated TCR-electroporated cultures were not statistically significant (control for general T cell activity). These results are representative of at least n = 10 independent experiments. A dashed line represents a ratio of 1. The average ratio, in parentheses is the average ratio of percentage of tetramer-positive cells expressing Ho-LVL/Hβ relative to Ho/Hβ calculated from n = 10 independent experiments. A dashed line represents a ratio of 1. The average ratio, indicated by a horizontal bar, was 1.96, and the difference between Ho-LVL/Hβ and Ho/Hβ was found to be statistically significant: p = 2 × 10⁻⁵ (Student t test). (C) Similarly as in (A), we tested for the activity of single and dual TM TCRα mutants (as indicated) in a coculture with 624.38 or SK-MEL23. We then calculated the percentage of IFN-γ secretion relative to the concentration obtained with the wt TCR combination (Ho/Hβ, 100%). These results are representative of at least n = 9 independent experiments performed with four different donors. *p < 0.05 (Student t test).
To delineate the mechanisms associated with the improved function of the LVL mutant, we first tested if this could be due to an increase in protein translation. To enable the detection of the F4 TCRα-chain, we added an HA tag (or a c-myc tag) to its N terminus as previously described (30). This addition did not impair TCR function (data not shown). Jurkat RT3-T3.5 cells were electroporated with identical amounts of mRNA encoding either the wt F4 TCR (Ha/Hβ) or the F4-LVL mutant (Ho-LVL/Hβ). Subsequently, samples of these cells were lysed and analyzed by Western blot for total TCRα-chain expression using an anti-HA Ab. In parallel, the remainder of these cells was tested for TCRα-chain surface expression using a fluorescent anti-HA Ab in flow cytometric assays. Whereas the total levels of TCRα-chain (normalized to GAPDH) were similar as seen in Western blot analysis, surface levels of TCRα-chain were markedly higher (Fig. 3A). This suggests that the LVL mutations do not influence levels of protein translation but rather facilitate TCR chain surface expression.

We then turned to compare the functional avidity of cells expressing the F4-LVL or the wt F4 TCR. The functional avidity of TCR-engineered cells is a function of mainly two biochemical parameters: the affinity of the TCR itself and the number of TCRs expressed by the transduced cell. Thus, we surmised that by enhancing TCR cell surface expression (Figs. 2B, 3A), this would increase the functional avidity of LVL-TCR expressing cells (35). To test this, human PBLs (electroporated with the wt or LVL F4-TCR) were cocultured with T2 cells pulsed with different concentrations of MART-1 epitope. IFN-γ concentrations were measured by ELISA, normalized to the maximum secreted concentration and plotted on a dose-response curve (Fig. 3B). We observed a difference of 0.589 between the values of log(EC50) for F4 and F4-LVL, which suggests an almost 4-fold increase in functional avidity displayed by the LVL-TCR compared with the wt TCR (p < 0.05).

After the stimulation by its cognate epitope, the TCR undergoes internalization. We set out to determine if F4-LVL displayed a different pattern of internalization and cocultured F4-expressing or F4-LVL—expressing T cells with MART-1-pulsed T2 cells. Cells were sampled every 2 h for 8 h and stained with MART-1 tetramer. The cells were analyzed by flow cytometry, and we calculated the percentage of TCR expression relative to t = 0 h (100%). As shown in Fig. 3C, we did not notice a statistically significant difference (p = 0.11) in the rate of TCR internalization for F4-LVL and F4 TCRs.

The data above suggest that F4-LVL is expressed at higher levels at the cell surface, which may be associated with a better stability of the TCRα-chain and which, in turn, could provide a better ability to compete with the endogenous TCR. To model this, we performed competition experiments in which we introduced myc-tagged (30) F4 TCRα-chain (either wt or LVL) simultaneously with a competing gp100 TCRα-chain (either wt or LVL) in human PBLs. The cells were then stained with an anti-myc Ab and analyzed by flow cytometry. As seen in Fig. 3D, the surface expression of Ho(myc)-F4 was more reduced when the

are representative of three independent experiments, performed with two different donors. *p < 0.05 (Student t test).
FIGURE 4. Anti-tumor activity mediated by the F4-LVL TCR. Human PBLs were transduced with a retroviral vector encoding either the F4 or the F4-LVL TCR followed by IRES–l-NGFR. (A) TCR and l-NGFR expression. The expression of the TCR (upper graphs) or the marker gene l-NGFR (lower graphs) was assessed by staining these cells with a MART-1/HLA-A2 tetramer or an Ab specific for l-NGFR after analysis by flow cytometry. The percentage of positive cells and the MFI (in parentheses) are shown. In the right-most panels, we display the ratio of percentage of tetramer-positive cells expressing F4-LVL relative to F4 calculated from at least n = 6 independent experiments. A dashed line represents a ratio of 1. For the tetramer staining, the average ratio, indicated by a horizontal bar, was 1.7, and the difference between F4-LVL and F4 was found to be statistically significant: \( p = 4 \times 10^{-6} \) (Student t test). For the l-NGFR staining (marker gene for transduction efficiency), the average ratio, indicated by a horizontal bar, was 1.02, and the difference between F4-LVL and F4 was not found to be statistically significant: \( p = 0.48 \) (Student t test). (B) Cytokine secretion. Transduced PBLs were cocultured with different melanoma lines as indicated. IFN-\( \gamma \) or IL-2 secreted in the coculture supernatant was measured by ELISA. The differences in cytokine secretion by PMA/ionomycin-stimulated cells were not statistically significant (control for general T cell activity). These results are representative of six independent experiments performed with two different donors, and the difference between F4-LVL and F4 was found to be statistically significant: \( p < 0.05 \) (Student t test). (C) Surface marker upregulation. TCR-transduced cells were cocultured with melanoma lines (526: CD25, CD69; and 624.38: CD137, CD107a) and analyzed by flow cytometry for marker expression (as indicated). The percentage of positive cells and the MFI (in parentheses) are shown. These results are representative of at least four independent experiments with at least three donors, and the difference between F4 and F4-LVL was found to be statistically significant: \( p < 0.05 \) (Student t test). (D) Cell-mediated cytotoxicity. TCR-transduced cells were (Figure legend continues)
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The redirection of CD8+ cells with a tumor-specific TCR might provide additional help to CD8+ when challenged by tumor cells (4, 36, 37). However, the lack of expression of the CD8 coreceptor by Th cells might impair their biological activity in this context as ordinary class I MHC-restricted receptors require CD8 molecules to stabilize binding (38). Because the avidity of a T cell is dictated by a combination of the affinity of its TCR for a defined MHC–peptide complex and the number of TCR molecules expressed on the surface, it might be possible to overcome the need for a coreceptor by augmenting the density of the transferred TCR. As F4-LVL is expressed at a higher density on the cell surface (Fig. 4A), in general, F4-LVL was able to trigger more cytokine secretion than the wt TCR (e.g., 1082 pg/ml versus 291 pg/ml of IFN-γ in coculture with 526, respectively). No significant cytokine secretion was noted in cocultures with control HLA-A2-938 melanoma line or by untransduced PBLs (data not shown).

Thus, compared with the wt TCR, F4-LVL exhibited a significantly higher anti-tumor activity by means of cytokine secretion, upregulation of activation markers, and cytotoxicity.

To evaluate better the potential of the LVL-mutant for future clinical application, we generated a retroviral vector encoding the MART-1–specific F4-LVL TCR (or the wt F4), in which TCRα and TCRβ were linked with a 2A sequence, based on the MSGV-1 backbone (31). Each TCR is followed by the control marker gene l-NGFR under an IRES (Fig. 1B) to control for transduction efficiency. We transfected human primary T cells with the retroviral supernatant encoding either F4 or F4-LVL and stained them with MART-1–HLA-A2 tetramer. We detected a higher proportion of F4-LVL–expressing cells compared with the wt F4 (58.2% [MFI = 122] compared with 33.1% [MFI = 82], respectively, Fig. 4A). In addition, to negate any bias in the results pertaining to transduction efficiency, these cells were stained with an anti–l-NGFR Ab. As seen in Fig. 4A, the levels of l-NGFR expression were similar for both F4 and F4-LVL (79.8% [MFI = 170] compared with 78.4% [MFI = 173], respectively) indicating that the increase in TCR surface expression of F4-LVL did not result from a difference in transduction efficiency. We determined whether the enhanced surface expression of F4-LVL was correlated with a higher biological activity by coculturing PBLs expressing F4 or F4-LVL with different human melanoma lines, HLA-A2+ melanoma tumors (526, 624, 624.38, and SK-MEL23) specifically stimulated F4-expressing T cells to secrete cytokines IFN-γ and IL-2 (Fig. 4B); in general, F4- or F4-LVL–expressing T cells that were cocultured with the melanoma line 526. Cells, gated on the CD4+ population, were analyzed for intracellular expression of IFN-γ and CD69 (Fig. 5A). Moreover, F4-LVL triggered higher levels of surface activation markers such as CD25 (49.3% [MFI = 49] versus 19.1% [MFI = 36]) and CD69 (19.1% [MFI = 36] versus 6.6% [MFI = 24]) (Fig. 5B, 5C). In each case, the difference between F4 and F4-LVL was found to be statistically significant: p < 0.03 (Student t test).

Increased anti-tumor activity mediated by the Ha-LVL TCR mutant

To determine whether the LVL mutations not only increase surface expression but also provide additional help to CD8+ when challenged by tumor cells (4, 36, 37). However, the lack of expression of the CD8 coreceptor by Th cells might impair their biological activity in this context as ordinary class I MHC-restricted receptors require CD8 molecules to stabilize binding (38). Because the avidity of a T cell is dictated by a combination of the affinity of its TCR for a defined MHC–peptide complex and the number of TCR molecules expressed on the surface, it might be possible to overcome the need for a coreceptor by augmenting the density of the transferred TCR. As F4-LVL is expressed at a higher density on the cell surface (Fig. 4A), in general, F4-LVL was able to trigger more cytokine secretion than the wt TCR (e.g., 1082 pg/ml versus 291 pg/ml of IFN-γ in coculture with 526, respectively). No significant cytokine secretion was noted in cocultures with control HLA-A2-938 melanoma line or by untransduced PBLs (data not shown).

Thus, compared with the wt TCR, F4-LVL exhibited a significantly higher anti-tumor activity by means of cytokine secretion, upregulation of activation markers, and cytotoxicity.


cocultured with the indicated CFSE-labeled cell lines at a ratio of 4:1 (E:T). After 4 h, PI was added, and the cells were analyzed by flow cytometry. The relative percentage of double-positive cells out of the CFSE-labeled population (tumor cells) is shown. These results are representative of three independent experiments with two different donors, p = 0.004 (Student t test).

FIGURE 5. Activity of F4-LVL in CD4+ cells. TCR-transduced T cells were cocultured with the melanoma line 526. Cells, gated on the CD4+ population, were analyzed for intracellular expression of IFN-γ (A) and surface expression of CD25 (B) and CD69 (C) by flow cytometry. The percentage of positive cells and the MFI (in parentheses) are shown. In the right-most panels, we display the ratio of percentage of positive cells expressing F4-LVL relative to F4, calculated from n = 3 independent experiments. A dashed line represents a ratio of 1. The average ratio, indicated by a horizontal bar, was 3.26 for IFN-γ, 2.71 for CD25, and 2.42 for CD69. In each case, the difference between F4 and F4-LVL was found to be statistically significant: p < 0.03 (Student t test).
Combining LVL mutations with other variable and constant region TCRs

To assess for the generality of the enhancement mediated by the LVL mutations, we incorporated the latter into two additional TCRs: a gp100-specific TCR and a humanized p53-specific TCR that were previously described (4, 28). PBls expressing the gp100-LVL and p53-LVL TCRs (or their wt versions) were subjected to an overnight coculture with gp100/HLA-A2+ or p53/HLA-A2+ cell lines, respectively. As seen in Fig. 6A, the LVL-modified TCRs mediated higher cytokine secretion than the wt TCRs; for example, we observed a secretion of 670 pg/ml (for gp100-LVL TCR) versus 329 pg/ml (for gp100 TCR) in coculture with the melanoma line 624.38, and 1357 pg/ml (p53-LVL TCR) versus 756 pg/ml (p53-TCR) in coculture with non-small cell lung cancer line H2087. Similar results were also observed for IL-2 secretion (data not shown). No significant cytokine section was observed using control cell lines (888 for gp100 and Saos2 for p53) or by mock-electroporated T cells (Fig. 6A). Thus, it is possible to enhance TCR activity generically using the LVL mutations, demonstrating the general applicability of this strategy to different TCRs.

Finally, we wanted to assess if it was possible to enhance murine TCR function using the LVL approach. We and others recently demonstrated that TCR in which the human constant regions were replaced with murine ones exhibited preferential chain-pairing and functioned better in human cells, leading to an increased sensitivity to tumor cells (11, 39, 40). We set out to test if it would be possible to combine both “murinization” and LVL mutations and incorporate the LVL mutations in the murine TM TCRα region (Fig. 6B). These murine or murine-LVL constant regions were fused to the variable regions of the F4 TCR. We introduced four different versions of this F4 TCR into primary human T cells: human (wt: Hu/Hb), human-LVL (Hu-LVL/Hb or F4-LVL), murinized (Mu/Mb), and murinized plus LVL (M0-LVL/Mb). These cells were analyzed for TCR expression with a MART-1/HLA-A2 tetramer: as seen in Fig. 6C, the LVL mutation improved percentage of positive cells expressing murinized-TCR from 61.7% (MFI = 54) for Mu/Mb to 82.4% (MFI = 72) for Mu-LVL/Mb (p < 0.05), the latter being considerably higher than what was observed for the wt human F4 TCR (17.6% [MFI = 22]). As expected, this gradual pattern detected for TCR surface expression translated into anti-tumor activity measured by means of cytokine secretion in coculture with melanoma cells. While human primary T cells expressing the wt TCR (Hu/Hb) secreted 527 pg/ml of IFN-γ, this rose to 1171 pg/ml for the human-LVL-TCR, then to 2020 pg/analyzing by flow cytometry. The percentage of positive cells and the MFI (in parentheses) are shown. Below the histograms, we display the ratio of the percentages of tetramer-positive cells expressing either Hu-LVL/Hb, Mu/Mb, or Mu-LVL/Mb relative to Hu/Hb (wt) calculated from n = 5 independent experiments. A dashed line represents a ratio of 1. The average ratio, indicated by a horizontal bar, was 2.03 (Hu-LVL/Hb), 4.21 (Mu/Mb), and 6.05 (Mu-LVL/Mb), and the difference between each of these combinations was found to be statistically significant: p = 1 × 10^{-3} (one-way ANOVA). (D) These cells were cocultured with melanoma lines as indicated. IFN-γ secretion in the supernatant was measured by ELISA. No significant IFN-γ secretion was observed in coculture with control cell line 888, and the differences in cytokine secretion by PMA/ionomycin-stimulated TCR-electroporated cultures were not statistically significant (control for general T cell activity). The differences between each version of the TCR were found to be statistically significant: p < 0.05 (one-way ANOVA). These results are representative of at least six independent experiments performed with four different donors.
ml for the murinized TCR, and finally to 3058 pg/ml for the murinized LVL-TCR, the latter representing an almost 6-fold improvement compared with the wt TCR (Fig. 6D). No significant IFN-γ secretion was noted for untreated T cells or in coculture with the HLA-A2 control cell line 888.

Thus, the combination of murinization and LVL mutations can noticeably improve TCR surface expression and function.

**Discussion**

TCR-gene transfer has emerged as a promising immunotherapeutic alternative for the treatment of multiple types of metastatic cancer (5). Current clinical data suggest that the increased functional avidity of the transduced cells may be associated with an improved clinical objective response (41). Previous studies have dealt with improving the functional avidity of TCR-transduced cells using structural modifications of mainly the extracellular parts of the TCR constant regions (10, 42). In this study, we focused essentially on the TCR TM region and aimed at enhancing TCR-engineered lymphocyte avidity by stabilizing and increasing the expression of the exogenous TCR. We found out that by mutating three TM residues into the hydrophobic amino acids leucine and valine, TCR surface expression can be augmented, translating into better anti-tumor function.

Notably, it was shown that large mutations in the TM region of diverse immune receptors did not preclude their assembly if the original charged residues (acidic or basic) were preserved. However, our current data do not support this assessment for the TCR. Indeed, this was clearly exemplified in the case of the TCRβ-chain: even a small number of mutations were found to abrogate almost completely TCR expression and function (Fig. 2A). This would suggest that the composition of the TCRβ TM is crucial, being also supported by the fact that it is highly conserved in many mammalian organisms (Supplemental Fig. 1B) and that it is more stable than the TCRα-chain (19). Although TCRα TM domain may seem more permissible to mutations (29), some of the TCRα mutants (besides the LVL-TCR) we examined had functioned worse than the wt sequence (Fig. 2). Thus, we conclude that unlike other immune receptors, the composition of the TM regions (in addition to their charged residues) is important for the proper function of the TCR.

The incorporation of the LVL mutations in the TCRα TM region resulted in an improved surface expression of the chain and a better capacity to compete with other TCR chains without altering other TCR properties such as the level of total protein expression or the rate of internalization (Fig. 3). A higher surface density of endogenous TCR may help to reach the critical threshold for T cell activation as previously demonstrated (35, 43), being particularly useful when using a low- to medium-affinity TCR to gene-modify CD4 cells (Fig. 5). Although the interaction of the TCR chains with the CD3 complex depends mainly on both their extracellular domains and their charged TM residues (1, 44, 45), we did not notice a preferred association of the LVL-TCR with the CD3 complex compared with the wt TCR (data not shown).

It is interesting to note that it is conceivable that the first replacement in the LVL sequence, namely of a serine residue by a leucine, might also have removed a glycosylation site present in the TCRα C region (N L S was replaced by N L L; Supplemental Fig. 1A). Glycosylation has been shown to favor TCR degradation and to diminish TCR motility in the membrane (46, 47), and it was recently demonstrated that the removal of glycosylation sites in the TCR constant regions may also increase TCR-gene-transferred cell avidity (18). However, this does not seem to account for the increased function of the LVL-TCR that we observed, as suggested by the modest performance of the LGF mutant (Figs. 1A, 2C; p = 0.32) or of another mutant (NGF, data not shown), compared with that of the wt TCR.

As shown in Fig. 6, the LVL strategy was generically applied to several TCRs leading to an improved function. Notably, we were able to extend this improvement also to murine TCRα C region (Fig. 6). The latter could be of interest when developing/studying TCR-gene transfer or TCR-transgenic mouse models, especially when using low-avidity TCRs. Moreover, the combination of murine constant regions and of LVL mutations led to a remarkable improvement compared with the wt TCR (greater than five times more cytokine secretion and increased tetramer staining), under-scoring the potential of combining multiple enhancement strategies to improve TCR-transduced function.

Although it is difficult to predict antigenicity a priori, preliminary analysis using an algorithm based on Kolaskar et al. (48) did not indicate the generation of novel Ab epitopes due to the addition of the LVL mutations. Moreover, it is reasonable to assume that TM regions should prove less accessible to neutralizing Ab. In this regard, a recent report by Davis et al. (49) showed that immunoreactivity triggered by TCR-modified cells was mainly directed toward TCR variable regions and not to constant regions, even when more radical mutations were introduced (in that case, the use of fully murine constant regions in human patients) than in the current study.

In conclusion, we demonstrated that it is possible to improve the expression and biological activity of TCRs by modulating the hydrophobicity of the TCRα TM region. Such biological improvement would have important implications for the treatment of viral diseases and cancer using TCR-gene transfer to reprogram the specificity of patient lymphocytes.

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**Disclosures**

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**References**


