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Targeting the Wilms Tumor Antigen 1 by TCR Gene Transfer: TCR Variants Improve Tetramer Binding but Not the Function of Gene Modified Human T Cells

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We have previously described the functional activity of a human TCR specific for an HLA-A2-presented peptide derived from the Wilms tumor Ag 1 (WT1). Recent studies showed that the expression and function of human TCR was improved by the introduction of an additional disulfide bond between the α- and β-chains or by the exchange of the human constant region for murine sequences. In this study, we analyzed the functional activity of WT1-TCR variants expressed in Jurkat cells and in primary T cells. The introduction of cysteine residues or murine constant sequences into the WT1-TCR did not result in a global reduction of mispairing with wild-type TCR chains. Instead, the level of mispairing was affected by the variable region sequences of the wild-type TCR chains. The analysis of freshly transduced peripheral blood T cells showed that the transfer of modified TCR constructs generated a higher frequency of Ag-responsive T cells than the transfer of the wild-type TCR. After several rounds of peptide stimulation this difference was no longer observed, as all transduced T cell populations accumulated ~90% of Ag-responsive T cells. Although the Ag-responsive T cells expressing the modified TCR bound the HLA-A2/WT1 tetramer more efficiently than T cells expressing the wild-type TCR, this did not improve the avidity of transduced T cells nor did it result in a measurable enhancement in IFN-γ production and cytotoxic activity. This indicated that the enhanced tetramer binding of modified WT1-TCR variants was not associated with improved WT1-specific T cell function. The Journal of Immunology, 2007, 179: 5803–5810.

In the past years, several groups have demonstrated that retroviral TCR gene transfer is an attractive strategy to redirect the Ag specificity of primary T cells (1–15). The feasibility of TCR gene therapy was recently demonstrated in the first clinical trial in melanoma patients (16). The retroviral transfer of a MART1-specific TCR efficiently generated MART1-specific CD8+ lymphocytes that were used for adoptive T cell therapy. Infused T cells expanded in vivo and engrafted at high levels in most melanoma patients. Compared with the impressive clinical response rate of conventional adoptive T cell therapy with expanded tumor-infiltrating lymphocytes (~50% response rate), the anti-melanoma activity of the TCR-transduced lymphocytes was relatively inefficient with only two of 15 patients showing tumor regression (16, 17). This indicated that the efficiency of TCR gene therapy should be further improved to achieve better tumor protection in vivo.

The inefficient expression of introduced TCR α- and β-chains in T lymphocytes can be one of the rate-limiting steps for TCR gene therapy. Because TCR surface expression requires association with CD3 γ-, δ-, ε-, and ζ-chains, the introduced TCR competes with the endogenous TCR for a limited number of CD3 molecules. In addition, the introduced TCR chains may mispair with endogenous chains, thus further reducing the expression of relevant TCR αβ heterodimers on the surface of transduced T cells. Recently, two strategies to reduce TCR mispairing and enhance the association with CD3 molecules have been described. The introduction of an additional disulfide bond, which was originally used to produce soluble recombinant TCR molecules, facilitated TCR pairing and expression in human T cells (18–20). Similarly, hybrid TCR chains in which the human constant region was exchanged for murine sequences displayed improved TCR pairing and enhanced association with the CD3 molecules in human T cells (21).

We have previously isolated from the allogeneic repertoire a TCR that is specific for a peptide epitope of Wilms tumor Ag 1 (WT1) presented in the context of HLA-A2 class I molecules (15, 22). We demonstrated that TCR-transduced human T cells efficiently killed human tumor cells in vitro and were able to inhibit the growth of a human leukemia cell line in NOD/SCID mice. However, WT1-TCR expression in freshly transduced human T cells was generally lower than the expression levels of endogenous TCR chains, and several rounds of in vitro stimulation with WT1 peptides were required to selectively expand the Ag-responsive T cells.

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Abbreviations used in this paper: WT1, Wilms tumor Ag 1; MFI, mean fluorescence intensity.
The goal of this study was to explore whether cysteine modifications or the insertion of murine constant region sequences can improve the function of the WT1-TCR when transduced into human T cells. In one TCR variant, the native disulfide bond between the α- and β-chains was removed and a new disulfide bond was introduced. This TCR construct was no longer able to form heterodimers, and the TCR β-chain was expressed on the cell surface in the absence of the α-chain. In another TCR variant, a new disulfide bond was introduced without changing the native bond. This TCR modification reduced mispairing and enhanced correct pairing between the modified chains. Similar results were obtained with a variant TCR containing the murine constant region sequences. Surprisingly, although human T cells expressing the cysteine-modified and hybrid TCR displayed enhanced tetramer binding, this did not result in higher functional avidity or enhanced killing activity.

Materials and Methods

**Media, cells, Abs, tetramer, and peptides**

Unless otherwise stated, all culture media were RPMI 1640 (Cambrex) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 1% penicillin/streptomycin (Invitrogen Life Technologies), and 1% l-glutamine (Invitrogen Life Technologies). The cells lines used were the human TCR-negative Jurkat 76 cell line, the HLA-A2-positive leukemia cell line K562-A2 that expresses endogenous WT1 protein, and the HLA-A2-positive lymphoblastoid cell line C1R-A2 that is WT1 negative. The HLA-A2-positive T2 cell line is deficient in TAP (transporter associated with Ag processing) and can be efficiently loaded with exogenous peptides. PBMCs were obtained from volunteer donors from the National Blood Service, Colindale, London, U.K. Flow cytometry Abs were anti-human PE αβ TCR, allophycocyanin CD3 ε, allophycocyanin IFN γ (BD Biosciences) and PE Vβ2.1 (ImmunoTech). PE-labeled HLA-A2/WT126 tetramers were obtained from Beckman Coulter and used at 3.3 μg/ml. The peptides used in this study were the HLA-A2 binding peptides pWT126 (RMFNPAYPL) and pWT235 (CMTWNQMNL) and were synthesized by ProImmune as described previously (15).

**Retroviral TCR constructs**

All TCR α- and β-chain constructs were cloned separately into retroviral pMP71 vectors. The pMP71 vector containing the WT1-TCR genes was described previously (15). Cysteine-modified α and β TCR chains were generated by PCR mutagenesis. For the introduction of the disulfide bond in the constant domain, residue 48 of the Vα1.5 TCR chain was changed from a threonine to a cysteine and residue 57 of the Vβ2.1 TCR chain was changed from a serine to a cysteine. For the removal of the endogenous disulfide bond, residue 95 of the Vα1.5 TCR chain and residue 131 of the Vβ2.1 TCR chain were changed from cysteines to serines. To generate the hybrid TCR α-chain, a fragment encoding the first 141 residues of the WT1-specific TCR α-chain was joined to the C-terminal 130 residues of the murine MDM2-specific TCR α-chain (23). The hybrid α-chain was subsequently cloned upstream of an IRES-GFP (where IRES is internal ribosome entry site) element in the pMP71 vector using the restriction sites NotI and SalI. The hybrid TCR β-chain was similarly produced by fusing the N-terminal 136 residues of WT1-βTCR β-chain to the C-terminal 168 residues of a murine MDM2-specific TCR β-chain. The fragment was then transferred into the pMP71 vector using restriction sites NotI and BsiGI. The HLA-A2-restricted Tax-TCR (Vα12.2 and Vβ13.1) (24) specific for the peptide sequence LLPGTNYV of HTLV-1) was provided by Dr. B. Jakobsen (MediGene) and the HLA-A2-restricted LMP2 TCR (Vα3.1 and Vβ13.1; specific for the peptide sequence CLGGLTLMV of the LMP2 protein of EBV) was provided by Dr. B. Wilcox (University of Birmingham, Birmingham, U.K.).

**Transduction of retroviral TCR constructs into Jurkat cells and primary T cells**

For retroviral transduction, 2 × 10^6 Phoenix amphotropic packaging cells were cultured in 10-cm culture plates for 24 h at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, and 1% l-glutamine. The culture medium was changed and the cells were transfected with the vector constructs and pCL-ampho using calcium phosphate precipitation (Invitrogen Life Technologies). After culturing for 24 h at 37°C with 5% CO₂, the DMEM culture medium was replaced with RPMI 1640 culture medium and incubated for another 24 h. The viral supernatant was then harvested. Jurkat cells were split 24 h before retroviral transduction and PBMCs were activated for 48 h using the anti-CD3 Ab OKT3 at 30 ng/ml and IL-2 (600 U/ml; Chiron). For retroviral transductions, retrocoating-coated (Takara) 24-well plates were seeded with cells at 1 × 10^5 per well in 1 ml, cultured for 30 min, and then transduced with 500 μl of the TCR α-chain viral supernatant and 500 μl of the TCR β-chain viral supernatant. For PBMCs the transductions were conducted in culture medium supplemented with IL-2 at 200 U/ml. After 24 h at 37°C with 5% CO₂, the culture medium for Jurkat cells was replaced and for PBMCs the replaced medium was supplemented with IL-2 at 100 U/ml. Flow cytometry analysis was conducted on a BD LSR II flow cytometer (BD Biosciences) after a further 48-h culture period. FACS data were analyzed using FACSDiva or WinMDI version 2.9 software.

**Immunoblotting**

For the immunoprecipitation experiments Jurkat cells were transduced with the various TCR α- and β-chains and then subsequently cloned by limiting dilution to produce populations of cells that were >95% TCR positive. For each immunoprecipitation experiment 3 × 10⁷ Jurkat cells were lysed in 1 ml of Brij 96 lysis buffer containing protease inhibitors (1% Brij 96, 150 mM NaCl, 10 mM Tris-HCl (pH 7.8), 10 mM iodoacetamide, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Protein A-Sepharose together with the anti-Vβ2.1 Abs were added to the lysates and incubated for 4 h at 4°C. The immunoprecipitates were resolved in a 7–17% SDS-polyacrylamide gel, immunotransferred to a nitrocellulose membrane and incubated sequentially with anti-TCR α (clone 9F11; Endogen), anti-CD3 ε (clone M20; Santa Cruz Biotechnology), and anti-CD3 γ (clone 448; Ref. 25). Abs. The membranes were afterward hybridized with streptavidin HRP (Amersham Biosciences) and developed by ECL (Bio-Rad Laboratories).

**Ag-stimulation of TCR-transduced T cells**

Transduced primary T cells were stimulated and expanded every 8–10 days. The stimulations were conducted in 24-well plates in 2 ml of culture medium containing 10% nonheat-inactivated FCS and 10 U/ml IL-2 (Roche) at 37°C with 5% CO₂. Each well contained 5 × 10⁵ transduced cells, 2 × 10⁵ irradiated T2 cells loaded for 2 h with 100 μM of the pWT126 (stimulator cells), and 2 × 10⁵ irradiated PBMCs as feeder cells.

**IFN-γ secretion assays**

TCR transduced T cells (1 × 10⁵) were stimulated with 1 × 10⁵ irradiated T2 cells loaded for 2 h with pWT126 (relevant peptide) or pWT235 (irrelevant peptide). Assays were conducted in triplicates in round-bottom 96-well plates in 200 μl of culture medium. After 18 h of incubation at 37°C with 5% CO₂, the supernatant was harvested and tested for secreted IFN-γ using a human ELISA kit (BD Biosciences) as per the manufacturer’s instructions. The data was analyzed using Excel software.

**Intracellular IFN-γ detection assays**

This assay was performed in 96-well round-bottom plates. TCR-transduced T cells and T2 stimulator cells loaded with relevant (pWT126) or irrelevant (pWT235) peptide were added at 4 × 10⁶/well in 200 μl of culture medium containing brefeldin A (Sigma-Aldrich) at 1 μg/ml. After an incubation period of 4 h at 37°C with 5% CO₂, the cells were first stained for surface CD8 and then fixed, permeabilized, and stained for intracellular IFN-γ using the Fix & Perm kit (Caltag) according to the manufacturer’s instructions. Samples were acquired on a LSR II flow cytometer and the data was analyzed using FACSDiva (BD Biosciences).

**CTL assays**

For the CTL assays, T2 cells, K562-A2 cells or C1R-A2 cells were labeled with ⁵¹Cr for 1 h at 37°C with 5% CO₂ in culture medium and washed three times. ⁵¹Cr-labeled T2 cells were then loaded with pWT126 peptide or pWT235 peptide at decreasing concentrations for 1 h at 37°C with 5% CO₂ in culture medium. The CTL assays were conducted in round-bottom 96-well plates in 200 μl of culture medium at 37°C with 5% CO₂. For different E:T ratios, peptide-loaded ⁵¹Cr labeled T2 cells were added to 2-fold dilutions of TCR-transduced T cells. For the peptide titration assays, TCR-transduced cells and peptide-loaded ⁵¹Cr labeled T2 cells were cultured at a ratio of 5:1 (E:T). After an incubation period of 4 h at 37°C with 5% CO₂, 50 μl of supernatants was harvested, diluted with 150 μl of scintillation fluid, and counted using a Wallac 1450 Microbeta Plus counter. Percentage specific killing = experimental ⁵¹Cr-release − spontaneous ⁵¹Cr-release/maximum ⁵¹Cr-release − spontaneous ⁵¹Cr-release.
Results

Expression of wild-type, cysteine mutant, and hybrid TCR constructs

We have generated three variants of the WT1-specific TCR (Fig. 1). In the cysteine variant 1, position 48 in the constant region of the TCR α-chain was changed from threonine to cysteine and from serine to cysteine at position 57 in the constant TCR β-chain as described in recent studies (18–20). Cysteine variant 2 was identical to variant 1, except that the cysteine residues responsible for the formation of the natural disulfide bond between the TCR α- and β-chains were removed in an attempt to prevent pairing with wild-type TCR chains. Like the parental TCR, variant 2 could only form one disulfide bond between the α- and β-chain. Finally, the constant regions of the human WT1 TCR α- and β-chains were replaced with murine sequences to generate the third variant construct.

Wild-type and variant TCR α and β genes were inserted separately into the retroviral vector MP71 for the transduction of human T cells. The TCR constructs were introduced into CD3-positive, TCR-negative Jurkat 76 T cells followed by FACS analysis using Abs specific for the Vβ2.1 variable segment used by the WT1-TCR and anti-CD3 ε Abs. As expected, control Jurkat cells did not stain with these Abs whereas cells transduced with the wild-type WT1-TCR, the cysteine-1 TCR, and the hybrid TCR double-stained with anti-TCR and anti-CD3 ε Abs (Fig. 2A), suggesting that the introduced TCR was assembled with endogenous CD3 components. Surprisingly, Jurkat cells transduced with the cysteine-2 TCR construct stained with the anti-TCR Abs but not with anti-CD3 ε Abs (Fig. 2A), indicating that this cysteine-modified TCR was expressed on the cell surface without CD3 ε. Staining of permeabilized cells showed that the cysteine-2 TCR was expressed intracellularly at similar levels as those of the wild-type and cysteine-1 TCRs (Fig. 2B).

We performed immunoprecipitation with anti-Vβ2.1 Abs followed by Western blotting to analyze whether the cysteine-2 TCR β-chain was associated with TCR α, CD3 ζ and CD3 ε. As expected anti-Vβ2.1 immunoprecipitates of the wild-type and cysteine-1 TCRs coprecipitated TCR α, CD3 ζ, and CD3 ε (Fig. 3). In contrast, anti-Vβ2.1 precipitates of Jurkat cells expressing the cysteine-2 TCR failed to coprecipitate TCR α, CD3 ζ or CD3 ε, suggesting that the TCR β-chain was expressed without the α-chain and CD3 molecules. This was confirmed by the demonstration that Jurkat cells transduced with only the cysteine-2 β-chain stained with anti-Vβ2.1 Abs as efficiently as Jurkat cells transduced with the cysteine-2 TCR αβ combination (Fig. 2A). As expected, the cysteine-2 TCR was nonfunctional (data not shown) and was omitted from subsequent experiments.

Analysis of TCR mispairing

We used Jurkat cells to determine whether the cysteine-1 and the hybrid WT1-TCR were able to pair with the wild-type chains of the WT1-TCR or two unrelated TCRs, one specific for a Tax peptide of human T cell leukemia virus type 1 (HTLV-1) and the other for a LMP2 peptide of EBV. The same batches of retroviral vector preparations for the α and the β TCR genes were used for these mispairing experiments. Coinfection of Jurkat cells with the vectors containing the “matched” α and β genes resulted in the surface expression of the wild-type TCR, the cysteine-1 TCR, and hybrid

![FIGURE 1. Schematic representation of the WT1-TCR variants used in this study. The cysteine-1 variant TCR and the cysteine-2 variant TCR contain an introduced cysteine bond at residues 48 and 57 of the α and β TCR chains, respectively. The cysteine-1 variant TCR also contains the endogenous bond that has been removed in the cysteine-2 variant TCR. In the hybrid variant TCR, the human constant region has been replaced with murine sequences.](http://www.jimmunol.org/)

![FIGURE 2. WT1-TCR variants are expressed in transduced Jurkat cells. Jurkat cells, negative for endogenous TCRs, were transduced with vectors encoding the α- and β-chains of the wild-type TCR, cysteine-1 TCR, cysteine-2 TCR, and hybrid TCR. In addition, Jurkat cells were also transduced with only the β-chain of the cysteine-2 TCR. A. Transduced cells were Ab stained with anti-Vβ2.1 and anti-CD3 ε Abs, followed by FACS analysis to determine cell surface coexpression of TCR and CD3 ε. B. Transduced cells were permeabilized followed by staining with anti-Vβ2.1 Ab and FACS analysis to determine cell surface and intracellular TCR β expression.](http://www.jimmunol.org/)

![FIGURE 3. Biochemical analysis of TCR-transduced Jurkat cells. Jurkat cells transduced with three variants of the WT1 TCR were lysed in 1% Brij 96 and immunoprecipitated with the specific Ab Vβ2.1. The immunoprecipitates were subjected to 7–17% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with either anti-α mAb (clone αF1), anti-CD3 ε mAb (clone M20), or anti-CD3 ζ polyclonal Ab 448. The specific chains are shown by arrows.](http://www.jimmunol.org/)
FIGURE 4. WT1-TCR variants can mispair with wild-type TCR chains. A, Jurkat cells were transduced with vectors encoding the α- and β-chains of the wild-type (WT) TCR, the cysteine-1 (Cys-1) TCR, and the hybrid (Hyb) TCR. Jurkat cells were also transduced with vectors encoding the α- and β-chains of the Tax-TCR and the LMP2-TCR. B, Jurkat cells were cotransduced with vectors encoding the α-chain of the wild-type TCR and the β-chains of the Tax-TCR or the LMP2-TCR. Alternatively, Jurkat cells were cotransduced with vectors encoding the β-chain of the wild-type TCR and the α-chains of the Tax-TCR or the LMP2-TCR. C, Jurkat cells were cotransduced with vectors encoding the α-chain of the cysteine-1 variant TCR and the β-chain of the wild-type TCR, the Tax-TCR, or the LMP2-TCR. Alternatively, Jurkat cells were cotransduced with vectors encoding the β-chain of the cysteine-1 variant TCR and the α-chain of the wild-type TCR, the Tax-TCR or the LMP2-TCR. D, Jurkat cells were cotransduced with vectors encoding the α-chain of the hybrid variant TCR and the β-chain of the wild-type TCR, the Tax-TCR, or the LMP2-TCR. Alternatively, Jurkat cells were cotransduced with vectors encoding the β-chain of the hybrid variant TCR and the α-chain of the wild-type TCR, the Tax-TCR, or the LMP2-TCR. Cells were stained with anti-Vβ2.1 and anti-CD8 Abs and then FACS analyzed to determine cell surface expression of TCR and CD3 ε. E, Primary human PBMCs were mock transduced or transduced with vectors encoding the β-chains of the wild-type TCR, cysteine-1 variant TCR, or hybrid variant TCR. Cells were stained with anti-Vβ2.1 and anti-CD8 Abs followed by FACS analysis.

Together, these data show that the alterations in the WT1-TCR constructs reduced pairing with some wild-type TCR chains while maintaining the pairing efficiency with other wild-type chains.

Finally, we analyzed the level of mispairing of the WT1-TCR β-chain and the cysteine-1 and hybrid versions with the repertoire of endogenous TCR α-chains present in primary human T cells. Activated human T cells were transduced with the retroviral TCR β constructs only, followed by anti-Vβ2.1 staining to detect cells expressing the introduced β-chains. Flow cytometry of transduced T cells revealed that a similar percentage of human T cells expressed the introduced wild-type, cysteine-1, and hybrid β-chains (Fig. 4E). This indicated that the modified β-chains paired with the endogenous repertoire of α-chains as efficiently as the wild-type WT1-TCR β-chain.

Due to the lack of anti-Vα1.5 Abs, similar experiments could not be performed with the modified WT1-TCR α-chains.

Analysis of TCR-transduced primary T cells

The retroviral vectors encoding the wild-type WT1-TCR, the cysteine-1, and the hybrid versions were used to cotransfer the α and β genes into primary human T cells. We measured the ability of transduced bulk T cells to produce IFN-γ after stimulation with WT1 peptides and control peptides. Consistently, early bulk cultures transduced with the modified TCR constructs produced more
IFN-γ than cultures transduced with the wild-type TCR (Fig. 5). This is consistent with recent reports demonstrating the enhanced effector function of human T cells transduced with cysteine-1 and hybrid TCR constructs (18, 19, 21).

We explored further the reason for the enhanced Ag response after transduction with the WT1-TCR variants. It was possible that individual T cells expressing the TCR variants mounted stronger peptide-specific effector functions than T cells expressing the wild-type WT1-TCR. Alternatively, it was possible that the TCR variants assembled more efficiently in transduced bulk T cells and thus generated a higher frequency of Ag-responsive T cells, which may account for the improved peptide-specific response compared with bulk T cells transduced with the wild-type TCR.

Monitoring the accumulation of Ag-responsive T cells

Staining with anti-Vβ2.1 Abs and HLA-A2/WT1 tetramers was used in an attempt to monitor the numbers of T cells expressing the introduced WT1-TCR β-chain, and the αβ heterodimer, respectively. Primary human T cells transduced with the wild-type, cysteine-1 and hybrid TCRs contained similar percentages of CD8+ cells expressing the Vβ2.1 TCR chain (4.5–4.7%), indicating similar transduction efficiency (Fig. 6A). A small number of tetramer-positive T cells were clearly detectable after transduction with the cysteine-1 and hybrid TCRs, but not after transduction with the wild-type TCR (Fig. 6A). This initial observation suggested that the wild-type TCR was unable to assemble detectable levels of functional αβ heterodimers in primary T cells.

However, repeated peptide-stimulation of T cells transduced with the wild-type TCR led to the selective accumulation of
CD8$^+$ Vβ2.1$^+$ T cells that remained largely tetramer negative, suggesting that tetramer-negative T cells expressed functional WT1-TCR heterodimers that were able to respond to stimulation with WT1 peptides (Fig. 6B). After three rounds of peptide stimulation larger numbers of CD8$^+$ Vβ2.1$^+$ T cells accumulated in cultures transduced with the cysteine-1 and hybrid TCRs (57 and 66% CD8$^+$ Vβ2.1$^+$ T cells, respectively) compared with cultures transduced with the wild-type TCR (35% CD8$^+$ Vβ2.1$^+$ T cells; Fig. 6B). In addition, a large proportion of the T cells expressing the cysteine-1 and hybrid TCR-bound tetramers (22 and 21%), whereas only 1.4% of T cells expressing the wild-type TCR were tetramer positive. Additional experiments (data not shown) demonstrated that the differences in the staining profiles between T cells expressing the wild-type, cysteine-1, and hybrid TCRs were also detectable using a 10 times higher concentration of tetramer. After six rounds of peptide stimulation, all cultures transduced with the different TCR constructs accumulated a large percentage (88–95%) of CD8$^+$ Vβ2.1$^+$ T cells (Fig. 6C). As seen after 3 wk, many of the CD8$^+$ Vβ2.1$^+$ T cells expressing the cysteine-1 and hybrid TCRs bound HLA-A2/WT1 tetramers, while most CD8$^+$ Vβ2.1$^+$ T cells expressing the wild-type TCR remained tetramer negative (Fig. 6C).

Similar observations were made with transduced T cells from different donors in independent experiments. Consistently, during the first three rounds of peptide stimulation CD8$^+$ Vβ2.1$^+$ T cells accumulated rapidly after transduction with the cysteine-1 and hybrid TCRs, and many of these T cells bound WT1 tetramers. The accumulation of CD8$^+$ Vβ2.1$^+$ T cells occurred more slowly after transduction with the wild-type TCR, and most of the T cells were tetramer negative. Continued Ag selection beyond three rounds of stimulation resulted in the accumulation of similar high numbers of CD8$^+$ Vβ2.1$^+$ T cells in cultures expressing the wild-type, cysteine-1, or hybrid TCR, but accumulation of tetramer-positive T cells only occurred in cultures expressing modified TCRs.

**CD8$^+$ Vβ2.1$^+$ T cells expressing wild-type, cysteine-1, or hybrid TCR are functionally equivalent**

We used Ag-selected T cell lines containing mostly CD8$^+$ Vβ2.1$^+$ T cells to test whether we could detect functional differences between the wild-type, cysteine-1, and hybrid TCRs. Intracellular IFN-γ staining demonstrated that the majority of T cells, independently of the TCR they expressed, produced IFN-γ when stimulated with the WT1 peptides (Fig. 6D). In these T cell lines, the percentage of IFN-γ-positive T cells was nearly the same as the percentage of Vβ2.1$^+$ T cells, showing that the majority of Vβ2.1$^+$-expressing T cells were peptide specific. Furthermore, the mean fluorescent intensity (MFI) of the IFN-γ staining was similar in cells expressing the wild-type, cysteine-1, and hybrid TCRs (MFI of 1982, 2014, 2020, respectively), suggesting that the three TCR constructs triggered comparable levels of intracellular IFN-γ. An ELISA of the culture supernatant was used to measure the amount of IFN-γ that was secreted by the Ag selected T cell lines. The results indicated that the T cell lines expressing the wild-type, cysteine-1, and hybrid TCRs secreted similar amounts of IFN-γ (Fig. 7A), confirming the results of the intracellular staining.

Next, the cytotoxic activity of T cell lines expressing the different TCR constructs was determined. Using target cells coated
with the WT1 peptides, similar levels of peptide-specific cytotoxicity were observed for all TCR constructs even at low effector T cell to target cell ratios (Fig. 7B). Although this showed that T cells expressing the wild-type, cysteine-1, and hybrid TCRs all displayed similar killing activity, these experiments did not analyze the triggering threshold for the three TCR constructs. To explore this, cytotoxicity assays were performed against target cells coated with decreasing concentrations of the WT1 peptide. The results revealed a nearly identical dose-response for the T cell lines expressing the wild-type, cysteine-1, and hybrid TCRs (Fig. 7C). Similarly, the T cells expressing wild-type TCR killed the leukemia cell line K562-A2, which expresses WT1 endogenously, as efficiently as T cells expressing the cysteine-1 and hybrid TCRs (Fig. 7C). The WT1-negative cell line C1R-A2 was used as a negative control in these experiments.

Together, these results showed that although T cell lines expressing the cysteine-1 and hybrid TCRs consistently contained much higher numbers of tetramer binding CD8\(^+\) cells than T cell lines expressing the wild-type TCR, this was not associated with a measurable increase in IFN-\(\gamma\) production, T cell avidity, or killing activity against WT1-expressing tumor cells.

**Discussion**

We have analyzed the expression and function of three variants of the WT1 TCR. The introduction of a new disulfide bond and removal of the native bond produced TCR chains that were unable to pair as heterodimers. The cysteine-2 TCR \(\beta\)-chain not only failed to pair with the \(\alpha\)-chain but also with the CD3 \(\varepsilon\) and \(\xi\)-chains. Despite this lack of pairing, the cysteine-2 \(\beta\)-chain was expressed on the cell surface, presumably as a homodimer. We noted that the human/murine hybrid \(\beta\)-chain displayed similar properties, as it was able to reach the cell surface without the TCR \(\alpha\)- and the CD3 \(\varepsilon\)-chain. This was apparent in Jurkat cells transduced with the hybrid \(\beta\) gene together with TCR \(\alpha\) genes where a population of cells that expressed TCR \(\beta\) but not CD3 \(\varepsilon\) was detectable by FACS analysis (see Fig. 4A). Furthermore, this TCR \(\beta^+\)CD3 \(\varepsilon^+\) population was also apparent in Jurkat cells transduced with only the hybrid TCR \(\beta\)-chain. This highlights the fact that genetic TCR modifications can result in unexpected alterations of TCR assembly and CD3 association.

A detailed analysis of mispairing showed that the introduction of murine constant region sequences or the addition of a single cysteine (without removal of the native cysteine) decreased pairing with only some wild-type TCR chains. At present, the molecular mechanisms that determine the efficiency of TCR pairing are not known. Recent elegant studies with human TCR genes showed that “strong” TCR pairs were efficiently expressed while “weak” TCR pairs were inefficiently expressed on the surface of T cells (26, 27). As the constant regions of human TCR \(\alpha\) and \(\beta\) genes are identical, except for possible differences due to the usage of constant \(\beta\) 1 and 2, these studies showed that the variable region sequences play a major role in determining the efficiency of TCR expression (26). It is likely that efficient \(\alpha\)-\(\beta\) pairing and formation of stable heterodimers are features of a “strong” TCR, whereas inefficient pairing is a feature of a “weak” TCR. We postulate that the TCR constant region modifications explored here more readily disrupt “weak” TCR \(\alpha\beta\) combinations, whereas little effect is seen with “strong” TCR combinations where the variable region sequences drive efficient \(\alpha\beta\) pairing that can proceed despite modifications in the constant region.

Similarly as in previous studies, we observed that T cells transduced with the hybrid and the cysteine-1 TCRs bound tetramers more efficiently than T cells transduced with the wild-type WT1 TCR (18, 19, 21). Ag-stimulation of T cells transduced with the modified TCRs resulted in the expansion of V\(\beta\)2.1 \(^+\)tetramer\(^+\) T cells as well as V\(\beta\)2.1 \(^+\)tetramer\(^-\) T cells, indicating that both were Ag responsive. Ag stimulation of T cells transduced with the wild-type TCR resulted in the expansion of T cells that were largely unable to bind tetramer. The expression levels of V\(\beta\)2.1, as determined by MFI, was generally lower in cells expressing the wild-type TCR compared with cells expressing the modified TCRs (e.g., Fig. 6, B and C). Although this difference was relatively modest, it might be sufficient to alter tetramer staining, which is based on low affinity interactions that might be susceptible to small changes in the density of the TCR ligand. In contrast, T cell activation involves multiple receptor/ligand interactions, including ligation of the TCR, the CD8 coreceptor, costimulatory molecules such as CD28, and accessory molecules such as LFA1, which may render this activation pathway less susceptible to small reductions in the amounts of TCR expressed by the responding T cells. This could explain our observation that, despite differences in tetramer staining, T cells expressing the wild-type, hybrid, and cysteine-1 TCRs showed similar levels of tumor cell killing and displayed comparable avidity as determined by peptide titration in cytotoxicity assays (Fig. 7C) and IFN-\(\gamma\) production (not shown). This observation that the lack of tetramer binding was not associated with a reduction in T cell avidity is similar to the demonstration that hepatitis B-specific CD8\(^+\) T cells of chronically infected patients showed reduced tetramer binding while retaining high avidity T cell function (28).

This functional similarity of the TCR constructs seems to contrast with previous studies where hybrid and cysteine-modified TCR displayed increased cytokine production and cytotoxicity when introduced into human T cells. In these studies, T cells were functionally analyzed immediately after RNA transfection (18, 21) or after retroviral transduction followed by expansion with CD3/CD28 beads (19). Importantly, the T cells were not selected using Ag-stimulation before the functional analysis. In our study, bulk T cells transduced with the modified TCR constructs showed stronger peptide-specific IFN-\(\gamma\) production than bulk T cells transduced with the wild-type TCR. However, after several rounds of peptide stimulation all T cell lines accumulated a high percentage of Ag-responsive T cells, at which stage functional differences between the modified and the wild-type TCR were no longer detectable.

These data suggest that the TCR modifications provide a major advantage by increasing the frequency of freshly transduced T cells expressing sufficient levels of the introduced TCR \(\alpha\beta\) heterodimer to mount peptide-specific immune response. This is most likely due to the recently demonstrated reduction of mispairing (18, 19, 21), the improved \(\alpha\beta\) pairing between the modified TCR chains (19), and the stable association with CD3 molecules (21) that together would be expected to increase the frequency of successful competition with the endogenous TCR chains. In contrast, the wild-type TCR chains succeeded less frequently in this competition, thus reducing the frequency of Ag-responsive T cells present in a bulk population of transduced PBMCs.

**Disclosures**

The authors have no financial conflict of interest.

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