Improved Expression and Reactivity of Transduced Tumor-Specific TCRs in Human Lymphocytes by Specific Silencing of Endogenous TCR

Sachiko Okamoto,1 Junichi Mineno,1 Hiroaki Ikeda,2 Hiroshi Fujiwara,3 Masaki Yasukawa,3 Hiroshi Shiku, and Ikunoshin Kato1

1Center for Cell and Gene Therapy, Takara Bio, Inc., Shiga, Japan; 2Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie, Japan; and 3Department of Bioregulatory Medicine, Ehime University Graduate School of Medicine, Ehime, Japan

Abstract
Adoptive T-cell therapy using lymphocytes genetically engineered to express tumor antigen-specific TCRs is an attractive strategy for treating patients with malignancies. However, there are potential drawbacks to this strategy: mispairing of the introduced TCR α/β chains with the endogenous TCR subunits and competition of CD3 molecules between the introduced and endogenous TCRs can impair cell surface expression of the transduced TCR, resulting in insufficient function and potential generation of autoreactive T cells. In addition, the risk of tumor development following the infusion of cells with aberrant vector insertion sites increases with the vector copy number in the transduced cells. In this study, we developed retroviral vectors encoding both small interfering RNA constructs that specifically down-regulate endogenous TCR and a codon-optimized, small interfering RNA–resistant TCR specific for the human tumor antigens MAGE-A4 or WT1. At low copy numbers of the integrated vector, the transduced human lymphocytes exhibited high surface expression of the introduced tumor-specific TCR and reduced expression of endogenous TCRs. In consequence, the vector-transduced lymphocytes showed enhanced cytotoxic activity against antigen-expressing tumor cells. Therefore, our novel TCR gene therapy may open a new gate for effective immunotherapy in cancer patients. [Cancer Res 2009;69(23):OF1–9]

Introduction
Several groups have shown that TCR gene transfer using retroviral vectors is an attractive strategy for redirecting the antigen specificity of polyclonal primary T cells to create tumor- or pathogen-specific lymphocytes (1–6). This approach has been suggested as a way to overcome the limitations of current adoptive T-cell therapies, which rely on the isolation and expansion of antigen-specific lymphocytes that preexist in the patient (7–10). Recently, clinical trials for the treatment of metastatic melanoma patients reported objective cancer regression in up to 30% of patients using autologous lymphocytes that were retrovirally transduced with melanoma/melanocyte antigen-specific TCRs (11, 12). These data suggest that adoptive cell therapy with TCR gene-modified lymphocytes is a promising approach for immunotherapy in cancer patients and encourage the development of novel TCR gene therapy approaches that result in improved antitumor activity.

However, the limited efficacy of TCR gene therapy has been reported to be associated with inefficient surface expression of transduced TCRs (13–16). The existence of the endogenous TCR is one of the major reasons for inefficient cell surface expression of the introduced TCR heterodimers. The introduced TCR α and β chains have been reported to mispair with the endogenous TCR chains, resulting in insufficient formation of the introduced TCR heterodimers (16–19). In addition, endogenous TCRs compete with the introduced TCRs for CD3 molecules (16–18, 20, 21). Because the stable cell surface expression of TCRs requires TCR assembly with CD3 γ, δ, ε, and ζ chains, this mechanism can also reduce the cell surface expression of the introduced TCR.

The expression level of the transgene can be enhanced by increasing the vector copy number in the transduced cells (22). However, it has been suggested that restricting the proviral copy number per cell decreases the number of potential vector insertions into sites that may promote proto-oncogene activation, tumor suppressor gene inactivation, or chromosomal instability (23, 24). A low copy number is ideal for reducing the risk of insertion mutagenesis even when using mature T cells instead of stem cells (22). Therefore, it is necessary to determine the efficacy of retroviral vectors based on the proviral copy number in transduced cells to precisely evaluate the usefulness and safety of each vector. Thus, a strategy for achieving high expression of transduced TCRs with relatively low copy numbers is required. Moreover, it has been suggested that the mispairing of introduced TCRs with endogenous TCRs may result in the generation of T cells with unexpected specificities, including self-reactive T cells (16–18, 25).

Recently, we isolated rearranged TCR α and β genes from a human CD8+ T-cell clone that recognizes a tumor-specific antigen MAGE-A4–derived peptide in a HLA-A*2402–restricted manner. Polyclonal human lymphocytes retroviral transduced with these TCR genes showed stable expression of the transgenes and specific cytotoxicity against MAGE-A4–expressing tumor cells (26, 27).

In this study, we determined whether we could induce high expression of MAGE-A4–specific TCRs and enhance the biological activity of transduced human lymphocytes at a low proviral copy number by combining small interfering RNA (siRNA) gene silencing of endogenous TCRs with siRNA-resistant, codon-optimized, transduced TCR genes. After normalizing the proviral copy number in the transduced cells, we evaluated the newly developed retroviral vectors for expression of transduced TCRs, inhibition of endogenous TCRs, and specific cytotoxicity against MAGE-A4–expressing tumor cells. The effectiveness of this approach was further determined for another TCR specific for WT1 tumor antigen.
Materials and Methods

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors who gave their informed consent. PBMCs were cultured in GT-T503 (Takara Bio) supplemented with 1% autologous plasma, 0.2% HSA, 2.5 mg/mL fungizone (Bristol-Myers Squibb), and 600 IU/mL interleukin-2. The study was approved by the Ethics Committee of Takara Bio.

Cell lines for evaluating the specific inhibitory effects of siRNAs. The cDNAs of WT-MAGE-A4-specific TCR α and β chains with Cβ2 regions, codon-optimized TCR α and β chains, and WT-CEA-specific TCR β chain with a Cβ1 (28) region were individually cloned into the retroviral vector pMEI-5 (Takara Bio). VSVG-pseudotyped retroviruses were transiently obtained by conventional methods using HEK293T cells. HEK293 cells were transfected with VSVG-pseudotyped retroviruses and designated 293-A, 293-coa, 293-BCl, 293-BC2, and 293-cob. These HEK293 cell lines expressed WT-MAGE-A4-specific TCR α, codon-optimized MAGE-A4-specific TCR α, WT-CEA-specific TCR β, WT-MAGE-A4-specific TCR β and codon-optimized MAGE-A4-specific TCR β, respectively.

Construction of retroviral vectors and retroviral transduction. The sequences of TCR α and β cDNAs from the HLA-A*2402-restricted MAGE-A4-specific CD8+ CTL clone 2-28 (26, 27) were codon-optimized by GeneArt. We designed the siRNA sequences targeting TCRα-α and TCRβ-β to be mismatched to codon-optimized sequences. C-optiTCR and MS-bpa (27) retroviral vectors encoded the codon-optimized MAGE-A4-specific TCR α/β (Fig. 1A) and WT-MAGE-A4-specific TCR α/β, respectively. The multigene-expressing retroviral vectors were constructed by cloning the pol III promoter-driven short hairpin RNA into the C-optiTCR plasmid. The cluster sequences of human primary microRNA (pri-miRNA) were cloned into the C-optiTCR plasmid in which the mature miRNA sequences had been replaced by siRNA sequences (Fig. 1B and C).

The TCR genes of the HLA-A*2402-restricted WT1232-243-specific CD8+ CTL clone TAK-1 (29–31) were used to construct WT1-IT and WT1-CO retroviral vectors encoding wild-type and partially codon-optimized WT1-specific TCR α/β, respectively. Partial codon optimization was done by replacing the Cα and Cβ regions with codon-optimized TCR Cα and Cβ regions, respectively. The WT1-siTCR vector was also constructed by cloning the siRNA expression unit into the WT1-CO vector (Fig. 1D).

PBMCs were stimulated with 30 ng/mL OKT-3 (Janssen Pharmaceutical) and 600 IU/mL interleukin-2 and transduced using the RetroNectin-bound Virus Infection Method in which retroviral solutions were preloaded onto RetroNectin (Takara Bio)-coated plates, centrifuged at 2,000 × g for 2 h, and rinsed with PBS. Cells were applied onto the preloaded plate.

Transfection of siRNAs and TCR RNA quantification. The double-stranded siRNAs (negative control siRNA 5′-CCATTACGGTGTGGTACG-3′, siRNA-a01 5′-GTGAAGATTCTGGATATGA-3′, siRNA-b03 5′-CCACCATCTCATGTAGAT-3′, siRNA-a06 5′-CAAAATCTGACTTTTTCGA-3′, and siRNA-b02 5′-CCTTGGTACAGAGAAGAA-3′) were introduced using TransIT-TKO (Mirus Bio) or a Human T-Cell Nucleofector kit (Amaxa). To quantify the TCR RNA sequences, quantitative reverse transcription-PCR was done using SYBR PrimeScript Reverse Transcription-PCR kit (Takara Bio) with primer sets specific for the WT or codon-optimized TCR C regions. Primers for human glyceraldehyde-3-phosphate dehydrogenase were used for normalization. Primers were as follows: human glyceraldehyde-3-phosphate dehydrogenase 5′-GACGCCAGT-3′ and 5′-GTGCAAACGCCTTCAACAA-3′; 5′-GCCCTCAATGACTCCAGATAC-3′ and 5′-CA-3′; 5′-CAACCATCTCATTGAGA-3′ and 5′-GCACCGTCAAGGCTGAGAAC-3′; 5′-CAACCATCTCATTGAGA-3′ and 5′-CAACCATCTCATTGAGA-3′; 5′-CAACCATCTCATTGAGA-3′ and 5′-CAACCATCTCATTGAGA-3′; and 5′-CAACCATCTCATTGAGA-3′ and 5′-CAACCATCTCATTGAGA-3′.

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5'–TGAAGCGGCGACCTTTCCAG-3', and codon-optimized TCR β 5'-GGCTTCACCCAGGAGGCTA-3' and 5'-TCACCATGGCCATCAGCA-3'.

**Flow cytometric analysis.** PE-conjugated anti-human TCR α/β monoclonal antibody (mAb; BD Pharmingen), FITC-conjugated anti-CD8 mAb (Becton Dickinson), FITC-conjugated anti-human TCR Vβ2, Vβ3, and Vβ17 mAbs (Beckman Coulter), PE-conjugated MAGE-A4143-151/HLA-A*2402 tetramer (provided from Ludwig Institute for Cancer Research), and PE-conjugated WT1230-2405/HLA-A*2402 tetramer (provided by Dr. Kuzushima, Aichi Cancer Center Research Institute) were used. Stained cells were analyzed using Cytomics (Beckman Coulter) or FACS CantoII (Becton Dickinson).

**Measurement of proviral copy number of retrovirus-transduced PBMCs.** Genomic DNA was purified from transduced PBMCs, and the average proviral copy number per cell was quantified using the Cycleave PCR Core kit (Takara Bio) and the Proviral Copy Number Detection Primer Set (Takara Bio).

**Calcein-AM cytotoxicity assay.** The ability of the transduced PBMCs to lyse targets was measured using a calcein-AM (Dojindo) release assay as described previously (32).

**Statistics.** Student’s t test was used for comparison of the means. P values < 0.05 were considered statistically significant.

**Results**

**Codon-optimized TCR α and β cDNA and endogenous TCR α/β–specific siRNAs improve the expression of the introduced TCR.** Previously, we reported that genetic engineering of PBMCs using a retroviral vector MS-bPa that encodes MAGE-A4143-151–specific TCR α/β chains generated MAGE-A4–specific CD8+ T cells (26, 27). In the current study, we initially investigated the effect of codon optimization on the expression level of the MAGE-A4143-151–specific TCR by adapting the codon usage to the codon bias of Homo sapiens genes and avoiding cis-acting sequence motifs such as splice donor sites. The codon-optimized TCR α and β cDNAs were cloned into the retroviral vector and designated C-optiTCR (Fig. 1A). Codon optimization improved the expression of the MAGE-A4–specific TCR on the surface when compared with T cells transduced with MAGE-A4–specific WT-TCR (Fig. 2A) in agreement with previous reports (15, 33). Next, we designed several siRNAs specific for the TCR Cα region or the TCR Cβ1/Cβ2 regions to suppress only the endogenous WT-TCR α/β. We selected the best siRNAs based on their gene suppression efficiencies (data not shown). The selected siRNAs, siRNA-a04 and siRNA-b03, specifically inhibited the WT-TCR α and β genes but did not alter the expression of the codon-optimized TCR α or β (Fig. 2B). Finally, we confirmed that the combination of codon optimization and siRNAs was effective in increasing the MAGE-A4–specific TCR expression. PBMCs were transduced with the C-optiTCR retroviral vector and transfected with siRNA-a04 and siRNA-b03 (siRNA mixture) by electroporation. Introduction of the siRNA mixture reduced the WT-TCR α and β expression to 54% and 39% of the negative control siRNA-transfected PBMCs, respectively. The siRNA mixture did not affect the expression of the introduced codon-optimized TCR α or β chains (Fig. 2C). Tetramer staining
of MAGE-A4–specific TCRs showed that the siRNA mixture increased the cell surface MAGE-A4–specific TCR expression to levels ~1.6-fold greater than those observed with the control siRNA (Fig. 2D). These data indicate that the siRNA mixture specifically knocked down endogenous TCR RNA expression without inhibiting the introduced codon-optimized TCR, resulting in increased cell surface expression of the ectopic TCR.

Development of retroviral vectors that effectively express codon-optimized TCR and siRNAs targeting endogenous TCRs. We aimed to introduce lymphocytes with codon-optimized TCR α/β chains and siRNA targeting endogenous TCR using a single retroviral vector. To determine the most effective retroviral design, we constructed a total of 29 retroviral vectors and named them sITCR vectors (Fig. 1B and C). We generated constructs expressing siRNAs via short hairpin RNA transcription driven by pol III promoters (Fig. 1B) and constructs expressing pri-miRNA structures based on human miRNA miR-17/miR-18 clustered on the human genome and transcribed as a single transcriptional unit (34–36). This system uses the host processing system to generate mature siRNAs (Fig. 1C). A two-step selection process was done (Supplementary Fig. S1), and we selected the SH03, SH07, SH16, SH18, PM07, and PM11 sITCR vectors for further analysis (Supplementary Figs. S2 and S3).

Stable and effective expression of the ectopic TCR in lymphocytes transduced with sITCR retroviral vectors. We transduced PBMCs with the selected sITCR retroviral vectors and compared the cell surface expression of the ectopic TCR by staining with a specific tetramer. Transduction with SH16 and PM11 resulted in the highest percentage of tetramer-positive cells and the greatest mean fluorescent intensity (MFI; Fig. 3A and B). To select the best construct, we isolated tetramer-positive T cells and compared the endogenous TCR α and β gene knockdown efficiencies of SH16 and PM11. SH16 reduced the expression of endogenous TCR α and β to ~20% of the expression levels seen in C-optiTCR–transduced T cells. On the other hand, PM11 reduced the expression of both endogenous TCR α and β to ~20% of the expression levels seen in C-optiTCR–transduced T cells (Fig. 3C). These results suggest that the sITCR vector PM11 is the most effective. To confirm the long-term expression of the introduced TCRs and the inhibition of endogenous TCRs, MAGE-A4143–151 tetramer-positive T cells were separated from PM11 siTCR- and C-optiTCR–transduced PBMCs and cultured with interleukin-2 for 34 days after transduction. The proviral copy number, percentage of tetramer-positive cells, and TCR RNA expression levels were analyzed 4 and 34 days after transduction (Fig. 3D). In the PM11-transduced cells, all factors tested were sustained through day 34. Although the proviral copy number was slightly decreased at day 34, the percentage of tetramer-positive cells per proviral copy number was sustained through day 34. This shows that our sITCR constructs are able to maintain increased ectopic TCR expression and endogenous TCR α/β inhibition for >1 month.

Superiority of sITCR vectors in the expression of ectopic TCR with limited proviral copy number. We constructed an
additional siTCR retroviral vector, PM11-w (Fig. 1C). Whereas the PM11 siTCR retroviral vector has one pair of siRNAs targeting WT-TCR α and β (siRNA-a04 and siRNA-b03), the PM11-w siTCR retroviral vector has an additional pair (siRNA-a06 and siRNA-b02), the inhibitory effects of which against WT-TCR α and β were equivalent to those of siRNA-a04 and siRNA-b03, respectively (data not shown). PBMCs were transduced with serially diluted C-optiTCR, PM11 siTCR, and PM11-w siTCR retroviral vectors. Following transduction, the average proviral copy number was evaluated, and MAGE-A4 tetramer staining and quantification of TCR RNAs were done. We plotted the percentage of tetramer-positive cells and the MFI of the tetramer-positive cells against the average copy number (Fig. 4A, left and middle). PM11 and PM11-w siTCR-transduced PBMCs showed greater number of tetramer-positive cells and a higher MFI compared with the C-optiTCR–transduced PBMCs. Importantly, for the C-optiTCR–transduced cells, twice the average copy number was needed to obtain the same rate of tetramer positivity compared with the siTCR-transduced cells. Similarly, five times the average copy number was required for C-optiTCR to achieve the same MFI level of tetramer staining when compared with the siTCR. The inhibition of RNA expression was highly correlated with the average copy number, and despite the use of bulk-transduced PBMCs that contained nontransduced cells, we could effectively reduce endogenous WT-TCR α/β expression levels (Fig. 4A, right). PM11-w–transduced PBMCs expressed a similar level of endogenous TCR α/β RNA when compared with PM11-transduced PBMCs.

To confirm the reproducibility of the effects of the siTCR retroviral vectors, PBMCs were transduced with serially diluted (1×, 2×, 4×, and 8×) conventional WT-TCR–expressing MS-bPa, C-optiTCR, PM11 siTCR, and PM11-w siTCR retroviral vectors and stained with MAGE-A4 tetramer. A comparable proviral copy number (3.1, 2.0, 2.4, and 2.7 copies per cell for MS-bPa–, C-optiTCR–, PM11–, and PM11-w–transduced T cells, respectively) was achieved with each vector at 8-, 4-, 1-, and 2-fold dilutions, respectively. As reported previously (15, 33), codon optimization (C-optiTCR) increased the surface expression of the TCR by ~1.85-fold compared with conventional WT-TCR (MS-bPa) gene transfer. Furthermore, the siTCR vectors (PM11 and PM11-w) induced MAGE-A4–specific TCR surface expression that was twice as high as that with codon optimization (with similar proviral copy number integration). In PM11-w–transduced T cells, the proportion of tetramer-positive cells was 4.6 times higher than in conventional MS-bPa–transduced cells and 2.5 times higher than in C-optiTCR–transduced cells, PM11-w–transduced T cells also had MFI’s that were ~2.5 and 1.5 times higher than those of MS-bPa– and C-optiTCR–transduced cells, respectively (Fig. 4B).

To evaluate endogenous TCR α and β knockdown efficiencies, endogenous and exogenous MAGE-A4–specific TCR RNA expression levels were quantified in MAGE-A4143–151 tetramer-positive T cells isolated from C-optiTCR–, PM11–, and PM11-w–transduced PBMCs with the comparable proviral copy numbers mentioned above. The endogenous TCR α and β expression levels in PM11 and PM11-w gene-modified T cells were reduced to 30% to 35% of the levels in C-optiTCR–transduced T cells, respectively. Although there was little difference in the inhibitory effects of endogenous TCR (Fig. 4C, left), the introduced TCR α and β RNA expression level was markedly higher in PM11-w–transduced cells compared with PM11-transduced cells (Fig. 4C, right). As stem-loop structures in the untranslated regions have been reported to inhibit polyadenylation and protect mRNA from degradation (37–39), it is suggested that the additional short hairpin RNA stem-loops in the PM11-w siTCR vector may act to stabilize the ectopic TCR RNA to achieve the high RNA expression level of the transduced TCR (Fig. 4C, right), thereby inducing strong expression of the introduced MAGE-A4–specific TCR α/β heterodimers on the T-cell surface.

Knockdown of endogenous TCRs at the protein level with facilitated expression of ectopic TCR heterodimers on the cell surface. We evaluated endogenous TCR expression in TCR gene-transfected cells at the cell surface protein level. Using PM11-w– and C-optiTCR–transduced PBMCs with comparable proviral copy numbers, we stained transduced cells with PE-labeled MAGE-A4143–151/HLA-A*2402 tetramer and the mixture of FITC-labeled TCR Vβ antibodies (mixture of anti-Vβ2, Vβ5, and Vβ17 mAbs), none of which reacts with MAGE-A4–specific TCR. As shown in Fig. 4D (left), we found that staining with the mixture of anti-Vβ mAbs was markedly reduced in tetramer-positive cells compared with the tetramer-negative cells in both PM11-w– and C-optiTCR–transduced cells. Specifically, the MFI’s for FITC in TCR Vβ antibody-positive cells in the tetramer-positive cells were reduced compared with those in the tetramer-negative cells (2,595 versus 6,715 in PM11-w–transduced cells and 3,219 versus 5,519 in C-optiTCR–transduced cells for PBMCs from donor 1; 2,902 versus 7,382 in PM11-w–transduced cells and 3,600 versus 7,027 for C-optiTCR–transduced cells from donor 2). Moreover, the MFI’s for FITC in TCR Vβ antibody-positive cells in tetramer-positive cells were lower in PM11-w–transduced cells than in C-optiTCR–transduced cells for both donors. These results strongly suggest that TCR gene-modified T cells became tetramer-positive only when the cell surface expression of endogenous TCR was suppressed as a consequence of the competition for CD3 molecules between MAGE-A4–specific TCR α/β heterodimers and endogenous TCRs. We also performed single staining with a mixture of FITC-labeled TCR Vβ antibodies (Vβ2, Vβ5, and Vβ17) using PM11-w– and C-optiTCR–transduced PBMCs with comparable proviral copy numbers and mock-transduced PBMCs. As shown in Fig. 4D (right), the percentage of TCR Vβ–positive cells was reduced in TCR-transduced PBMCs with both constructs compared with mock-transduced PBMCs. Moreover, PM11-w–transduced PBMCs showed lower percentages of TCR Vβ–positive cells than C-optiTCR–transduced PBMCs.

Taken together with the finding that PM11-w–transduced cells showed a markedly increased number of tetramer-positive cells compared with C-optiTCR (Fig. 4A and B), these data suggest that the suppression of endogenous TCR by our siTCR retroviral vector facilitated increased expression of exogenous TCRs and yielded a larger population of MAGE-A4 tetramer-positive cells.

Enhanced antitumor cytotoxicity of lymphocytes transduced with the siTCR retroviral vector. We compared the function of T cells transduced with the C-optiTCR and PM11-w siTCR vector at similar proviral copy numbers. PBMCs were transduced with these retroviral vectors at several dilutions. Transduced T cells with comparable proviral copy numbers were selected for the CTL assay. In C-optiTCR– and PM11-w–transduced cells, the proviral copy numbers were 4.2 and 4.0 copies per cell, respectively, in the specific experiment shown in Fig. 5.

The lytic activity against the tumor cell line 11-18 (MAGE-A4+/HLA-A*2402+) differed between the two constructs: PM11-w–transduced PBMCs had greater lytic activity than C-optiTCR–transduced T cells. However, neither of the transduced cells showed lytic activity against the tumor cell line QG56, which
Figure 4. Superiority of siTCR vectors in the expression of an ectopic TCR and the suppression of endogenous TCRs with limited copy number. A, PBMCs were transduced with serially diluted PM11, PM11-w, or C-optiTCR retroviruses and used for proviral copy number analysis, tetramer staining, and quantification of endogenous TCR expression. Percentage of tetramer-positive cells among CD8+ cells (left), MFIs (middle), and RNA expression level as the percentage of mock-transduced cells (right) are plotted according to copy number. B, representative flow cytometric analysis of PBMCs that were transduced with PM11, PM11-w, MS-bPa, or C-optiTCR retroviruses with equivalent proviral copy number. The numerical value indicates the percentage of tetramer-positive cells in whole cells, the percentage among CD8+ cells (in parentheses), and MFI of tetramer-CD8 double-positive cells. C, tetramer-positive cells were collected. Left, endogenous TCR α and β mRNAs shown as the percentage of C-optiTCR(4×)–transduced PBMCs; right, RNA expression of ectopic TCR α/β in PM11(1×)- and PM11-w(2×)–transduced PBMCs was calculated as the percentage of PM11(1×)-transduced PBMCs. Representative of three independent experiments with PBMCs from two donors. D, activated PBMCs were transduced with PM11-w or C-optiTCR retroviruses. PBMCs with equivalent proviral copy numbers (donor 1: 3.4 copies per cell for PM11-w and 3.1 copies per cell for PM11; donor 2: 2.7 copies per cell for PM11-w and 2.5 copies per cell for C-optiTCR) were used for staining with tetramer and TCR Vβ mAb mixture (left) or for TCR Vβ mAb mixture single staining (right). The numerical value indicates the MFI for FITC in TCR Vβ-positive cells among tetramer-positive or tetramer-negative cells (left). Representative of three independent experiments. *, P < 0.05.
lacks the restriction MHC (MAGE-A4+/HLA-A*2402+; Fig. 5). These results indicate that the higher expression of MAGE-A4-specific TCR in cells transduced with PM11-w-siTCR resulted in increased cytotoxic function of these cells.

Effectiveness of the siTCR retroviral vector encoding WT1-specific TCRs. To confirm the effectiveness of the siTCR retroviral vector for other TCRs, PBMCs were transduced with retroviral expression vectors for TCR α/β genes specific for the tumor antigen WT1 at several dilution factors followed by staining with the WT1-11-18 (MAGE-A4+/HLA-A*2402+) or QG56 (MAGE-A4+/HLA-A*2402+) tetramer. The constructs of these retroviral vectors are shown in Fig. 1D. WT1-siTCR–transduced PBMCs showed a higher percentage of tetramer-positive cells and a higher MFI compared with the control WT1-11-18– or WT1-CO–transduced PBMCs with limited proviral copy number integration, showing the effectiveness of the siTCR vector on WT1-specific TCR α/β. When the transduced T cells with similar proviral copy number integration were compared, the proportion of tetramer-positive cells was 2.8 times higher, and the MFIs of tetramer-positive cells were 1.7 times higher than that of conventional WT1-11-18–transduced cells (Fig. 6B). These results indicate that the effectiveness of our siTCR vector is not specific for one TCR and that this approach is applicable for other TCRs.

Discussion

In the present study, we developed retroviral vectors to simultaneously express siRNAs to silence endogenous TCR gene expression and a codon-optimized (siRNA-resistant), tumor antigen-specific TCR. In transduced lymphocytes, these novel vectors efficiently expressed the introduced TCR, reduced the expression of the endogenous TCR, and enhanced the antigen-specific lysis of target cells at a relatively low proviral copy number. Therefore, this new approach to TCR gene therapy has potential clinical relevance for treating patients with malignancies or uncontrollable virus infections such as cytomegalovirus infection after bone marrow transplantation, and it may help to reach the threshold for clinical response via infusion of TCR-engineered T cells.

The TCR-constant region-specific siRNAs used in this study increased the cell surface expression of the codon-optimized MAGE-A4–specific TCR (Fig. 2D). Although the surface expression increased, codon-optimized MAGE-A4–specific TCR α and β mRNA expression did not change significantly when siRNAs were introduced (Fig. 2C). Therefore, we conclude that a decrease in TCR mispairing and reduced competition for CD3 molecules between endogenous and ectopic TCR mediated the increased cell surface expression of the ectopic TCR.

Several strategies have been reported to reduce TCR mispairing and enhance CD3 molecule association. The introduction of an additional cysteine residue, which promotes the formation of a second disulfide bond, facilitated the expression and pairing of an ectopic TCR in human lymphocytes (18, 19, 25). Alternatively, the replacement of human Cα and Cβ domains with corresponding murine C domains has been reported to improve the binding of ectopic TCR α and β chains and enhance the stability of the TCR/CD3 complex in human T cells (17, 19). Importantly, both methods resulted in increased reactivity against antigen-expressing tumor cells. However, the effectiveness of these methods was dependent on the variable region sequence of the TCR (19). It has been suggested that the enhanced association of the ectopic TCR chains disrupts “weak” TCR α/β combinations; however, this effect is minimized with “strong” TCR combinations in which the variable region sequences play an important role in efficient αβ pairing (16, 19, 20, 26). Despite the diversity of the variable region, the constant region consists of only one Cα region and two Cβ sequences (Cα1 and Cβ2). The simplicity of the constant regions allowed us to suppress diverse TCR α and β rearrangements with only two siRNAs directed against the Cα and the consensus Cβ1/Cβ2 sequence. Because the siRNAs used in this study were directed against the endogenous TCR constant regions, we believe that the effectiveness of the approach presented here can be universal for all TCR rearrangements, independent of the ectopic TCR V region sequence, as we showed the efficacy of the siTCR retroviral vector with WT1-specific TCR α/β (Fig. 6).

Several groups have shown the feasibility of retroviral TCR gene transfer to produce antigen-specific T cells for adoptive immunotherapy, and high retroviral transduction efficiency in T cells has been reported using the recombinant fibronectin fragment CH-296 (RetroNectin; refs. 1, 4–6). However, the use of retroviral vectors in gene therapy has raised safety concerns because of nonspecific insertion into the human genome (7, 45–47). Although no side effects related to insertional mutagenesis have been reported in clinical settings following the retroviral transduction of mature T cells instead of CD34+ bone marrow cells (48), continued follow-up will be required to assess the overall risk. Minimizing the viral vector copy number is preferable for any gene therapy approach even with mature cells (22, 24). Given this concern, our strategy is suitable for gene therapy as it can efficiently express an ectopic TCR with low proviral copy integration.

At a similar proviral copy number, transduction with PM11 and PM11-w resulted in 3.4- and 4.6-fold more tetramer-positive cells than the conventional WT-TCR vector and 1.8- and 2.5-fold more than the codon-optimized TCR vector, respectively. In addition, an average copy number of 4.86, 1.02, 0.60, and 0.58 copies per cell was required for the conventional WT-TCR vector, the codon-optimized TCR vector, the PM11 siTCR vector, and the PM11-w siTCR vector, respectively, to produce a similar percentage of tetramer-positive cells (16.0%, 14.5%, 13.6%, and 15.9%, respectively).
respectively; Fig. 4B; data not shown). These results were reflected in the biological function of the siTCR vector-transduced PBMCs: PM11-w-transduced cells showed greater cytotoxicity against antigen-expressing tumor cells than cell transduced with codon-optimized TCR vectors without siRNA (Fig. 5). To our knowledge, this study is the first to determine the efficiency of ectopic TCR expression based on the proviral copy number in the transduced cells. Our results clearly show the importance of this type of analysis for precise evaluation of the usefulness as well as the safety of each retroviral vector.

With regard to the clinical application of TCR gene therapy, cotransduction of several retroviral vectors may be challenging due to safety requirements, the cost of vector production and approval, and complications in their use, including the adjustment of retroviral vector titers. To overcome these problems, we aimed to develop constructs expressing multiple cassettes including a tumor antigen-specific TCR and two siRNAs targeting the endogenous TCR $\alpha/\beta$. We tested these constructs using a short hairpin RNA cassette driven by a pol III promoter, which is one of the most generally used methods for expressing siRNAs (49, 50). As an alternative approach, we generated an expression cassette in which the siRNAs were expressed similarly to miRNAs. miRNAs are noncoding RNAs that regulate a variety of biological processes through complementary binding to target miRNAs, resulting in the direct inhibition of mRNA translation and/or destabilization of the target mRNA. miRNAs are expressed as long transcripts (pri-miRNAs) that are processed into ~70-nucleotide stem-loop forms (pre-miRNAs). The pre-miRNAs are finally cleaved in the cytoplasm by Dicer to generate mature miRNAs (34–36). After screening of a variety of vector constructs (Fig. 3; Supplementary Figs. S2 and S3), we found that the PM11 vector that used a cluster of pri-miRNAs was the most effective vector for ectopic expression of the TCR in T cells (Fig. 3). This vector was further modified to produce PM11-w, which carried two pairs of siRNAs against each TCR $\alpha$ and $\beta$. We found that this vector more efficiently expressed the TCR on the cell surface with a low proviral copy number compared with PM11 (Fig. 4A and B). Comparing the cells transduced with PM11 and PM11-w, we observed a similar inhibitory effect on endogenous TCR but higher expression of ectopic TCR mRNA in cells transduced with PM11-w (Fig. 4C). These data suggested that the RNA stabilization effect mediated the superiority of PM11-w to PM11 (37–39). Although the off-target effects of siRNAs need to be examined in detail before this method can be applied clinically, these data suggest that retroviral constructs such as PM11-w may prove useful for TCR gene therapy in cancer patients.

In summary, we showed a novel approach to TCR gene therapy that satisfies the following requirements: enhancement of ectopic TCR heterodimer cell surface expression and enhanced biological function at low proviral copy number (which may reduce the risk of mutagenesis); reduction of endogenous TCR expression, which may effectively reduce TCR mispairing and decrease the risk of inducing self-reactive TCR $\alpha/\beta$ heterodimers; and a universal method that does not depend on TCR variation. Such a novel approach to TCR gene therapy using siTCR retroviral vectors may prove useful for the development of effective and safe TCR gene therapy.

Figure 6. Effective expression of WT1-specific TCR $\alpha/\beta$ with siTCR retroviral vector. A, PBMCs were transduced with serially diluted WT1-WT, WT1-CO, or WT1-siTCR retroviruses and used for proviral copy number analysis and tetramer staining. Percentage of WT1 tetramer-positive cells among CD8+ cells (left) and MFIs (right) are plotted according to copy number. Representative experiment of two donors’ PBMCs. B, representative flow cytometric analysis with equivalent proviral copy number (2.1 for WT1-WT, 2.0 for WT1-CO, and 2.5 for WT1-siTCR). The numerical value indicates the percentage and MFI of tetramer-positive cells among CD8+ cells. Representative experiment of two donors’ PBMCs.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Sachiko Okamoto, Junichi Mineno, Hiroaki Ikeda, et al.

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