ORIGINAL ARTICLE

Lentiviral vectors encoding human MUC1-specific, MHC-unrestricted single-chain TCR and a fusion suicide gene: potential for universal and safe cancer immunotherapy

Xiaochuan Chen · Wentao Gao · Andrea Gambotto · Olivera J. Finn

Abstract MUC1 tumor antigen is a target for immunotherapy of most human adenocarcinomas and some hematological malignancies. Expression of a MUC1-specific, MHC-unrestricted single-chain T cell receptor (scTCR) on cells of both innate and adaptive immune system through reconstitution of lethally irradiated mice by retroviral vector-transduced bone marrow cells, had been shown to effectively control the growth of MUC1^{+} tumors independent of their MHC type, suggesting that this receptor is a good candidate for broadly applicable gene therapy/immunotherapy. However, the translational application of this immuno-gene therapy modality was discouraged by the progressive transgene silencing in reconstituted T and B cells, as well as the potential of tumorogenesis intrinsic to oncoretroviral vectors. To overcome these problems and facilitate the future clinical use of this receptor, we have constructed a panel of novel self-inactivating lentiviral vectors (LVs) which harbor two independent internal promoters, one driving expression of the scTCR gene and the other of a fusion suicide gene, the HSV-TK–EGFP fusion gene, allowing the transduced cells to be destroyable by the pro-drug ganciclovir. Despite the large size of insert, these vectors were efficiently packaged into high titer virus that transferred the expression of transgene in both T cell lines and primary T cells. Sustained expression was maintained in a T cell line for over 4 months in vitro, suggesting its efficient resistance to transgene silencing. Both scTCR and HSV-TK–EGFP genes were functional in the transduced cells, as evidenced by their specific recognition of MUC1^{+} tumors and efficient eradication by ganciclovir.

Keywords MUC1 · T cell receptor (TCR) · Lentiviral vector · Cancer immunotherapy

Introduction

Epithelial cell mucin (MUC1) is overexpressed in an aberrantly glycosylated form on epithelial adenocarcinomas [1–4] as well as on leukemia, lymphoma and multiple myeloma [5–8]. Previously we reported the cloning of a unique T cell receptor (TCR) from a MUC1-specific cytotoxic T cell line derived from a breast cancer patient, which recognized the aberrant tumor form of MUC1 in an MHC-unrestricted manner [9–11]. This property enabled the TCR to target MUC1-positive tumors regardless of their HLA type, making it a "universal" therapeutic agent for all patients with different adenocarcinomas. We reported that a single-chain TCR (scTCR) consisting of the $V\alpha/V\beta/C\beta$ construct fused to a CD3ζ chain, in addition to T cells, can be expressed on the surface of other cells of both innate (macrophages, NK cells, granulocytes) and adaptive immune systems (B cells). Delivery of this scTCR gene into mouse bone marrow cells through the Moloney leukemia virus-based MFG

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oncoretroviral vector encoded its expression on the surface of cells of the innate and adaptive immune systems, and effectively controlled the growth of inoculated MUC1-positive tumors in the bone marrow transplanted mice [12]. However, we observed that the expression of the scTCR transgene in T and B cells was significantly reduced 3 months after bone marrow transplantation, while other cells continued high-level expression [12]. Problems with gene silencing in hematopoietic cells have been reported to be intrinsic to the native promoters of oncoretroviral vectors [13]. In addition, there has been an increasing concern about the safety of viral vectors in general that selectively integrate into transcription-active sites in the genome potentially leading to activation of proto-oncogenes [14–18].

In order to overcome transgene silencing in lymphocytes, and improve the safety and efficacy for clinical application of TCR gene therapy/immunotherapy, we chose HIV-derived lentiviral vectors (LVs). Although LVs can also be subject to transgene silencing, they are generally less susceptible than retroviral vectors [19]. Their biggest advantage, however, is that they can transduce a variety of slowly or nondividing cells, including unstimulated T cells [20–23], and clinical trials using LVs have been approved and conducted for HIV/AIDS gene therapy [24].

We have engineered these vectors to co-encode scTCR and a fusion suicide gene (HSV-TK–EGFP, TGL) [25–27], which makes the transduced cells trackable and destroyable. These two transgenes are under the control of two independent internal promoters, either EF1α [28] or MSCV U3 [29–30] controlling the scTCR gene expression, and PGK promoter [31] driving the expression of the TGL fusion suicide gene. These dual-promoter LVs were efficiently packaged to high-titer virus that transferred the expression of transgenes to mouse and human T cell lines as well as human primary T cells. All the transgenes were functional, suggesting they would be broadly applicable and highly safe reagents for cancer immunotherapy.

Materials and methods

Construction of lentiviral transfer vectors

The self-inactivating lentiviral vector system (transfer vector pTY linker (Fig. 1A), packaging plasmid pHP-dl-N/A, envelope plasmid pHEF-VSV-G, and accessory plasmid pCEP-tat) was kindly provided by Dr. Lung-Ji Chang at University of Florida through NIH AIDS Research & Reference Reagent Program (https://www.aidsreagent.org) [32, 33]. To increase the vector efficiency, the pTY linker was modified by cloning the Woodchuck hepatitis posttranscriptional regulatory element (WPRE) fragment [34, 35] (cut at the *Bam*HI and
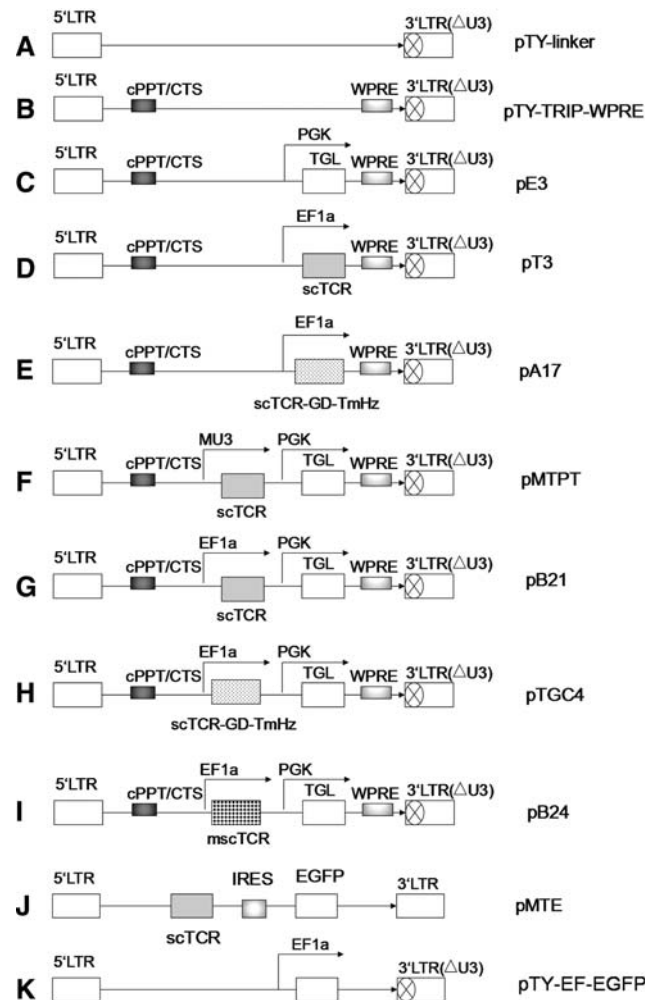

Fig. 1 Schematic representation of the new LVs. The pTY linker (*A*) was modified to construct pTY-TRIP-WPRE (*B*) that includes cPPT/CTS and WPRE elements. *C–E* Mono-promoter LVs, *F–I* dual-promoter LVs, *J* bicistronic retroviral vector (pMFG-scTCR-IRES-EGFP, pMTE) with wild-type LTR encodes scTCR and EGFP. *K* pTY-EF-EGFP, unmodified mono-promoter LV encoding EGFP, *LTR* long terminal repeat, *cPPT/CTS* HIV-1 central polypurine tract (cPPT) and the central termination sequence (CTS), *WPRE* Woodchuck hepatitis posttranscriptional regulatory element, *del U3* deletion of 3′U3, *scTCR* single-chain T cell receptor (scTCR) fused to mouse CD3ζ domain, *scTCR-AGD-TmHz* scTCR fused to human CD3ζ domain through the AGD linker. mscTCR mutated scTCR, *TGL* Herpes simplex virus thymidine kinase suicide gene (HSV-TK) and enhanced green fluorescence protein (EGFP) fusion gene, *EF1α* elongation factor 1α promoter, *PGK* human phosphoglycerate kinase promoter, *MU3* U3 promoter region of the murine stem cell virus long terminal repeat

*Kpn*I sites from pWOX-TGL, a gift from Dr. Jarmo Wahlfors, University of Kuopio, Finland) into the pTY linker at the corresponding sites to generate pTY-WPRE. To further increase the vector efficiency, the resulting pTY-WPRE was cut using *Nhe*I and blunt-ligated with the cPPT/CTS fragment TRIP [36–38] (cut from pLenti6-To-TRIP-EGFP at *Eco*RI and *Cla*I sites, kindly provided by Dr. Yukai He, University of Pittsburgh) to generate

pTY-TRIP-WPRE (Fig. 1A), which was used as a vector backbone for construction of a panel of LVs for current study and future clinical application. In order to construct vector pTY-TRIP-EF1a-scTCR-WPRE (pT3), the EF1a-scTCR fragment was cut from pEF4-scTCR [39] using *Mlu*I and *Not*I, and cloned into the pTY-TRIP-WPRE at *Sma*I site through blunt-ligation. To make the pTY-TRIP-TGL-WPRE, the HSV-TK–EGFP fusion gene (TGL) [25] was cut from pUC19-TGL, a kind gift from Dr. J Wahlfors of University of Kuopio, Finland, using *Xba*I, and then cloned into the pTY-TRIP-WPRE plasmid at *Sma*I site through blunt-ligation, to generate the vector pTY-TRIP-TGL-WPRE. The PGK promoter, cut from a commercial retroviral construct pMSCVpuro(Clontech) with *Hpa*I and *Hin*dIII, was cloned into pTY-TRIP-TGL-WPRE at *Sal*I site by blunt ligation to generate pTY-TRIP-PGK-TGL-WPRE (pE3). In order to make a dual-promoter lentiviral vector pTY-TRIP-EF1a-scTCR-PGK-TGL-WPRE (pB21), the EF1a-scTCR, which was cut from pEF4-scTCR using *Mlu*I and *Not*I, was cloned into pE3 at *Nhe*I site through blunt-ligation. In order to construct another dual-promoter lentiviral vector pTY-TRIP-MU3-scTCR-PGK-TGL-WPRE (pMTPT), MSCV U3 promoter (MU3) [28] was cut from pMSCVpuro (Clontech) using *Nhe*I and *Sma*I, and cloned into pTY-linker at the corresponding restriction sites to generate pTY-MU3. The pTY-MU3 was cut by using *Sma*I and blunt-ligated with scTCR fragment, which was cut from pEF4-scTCR with *Bam*HI and *Not*I. The resulting pTY-MU3-scTCR was cut using *Not*I and *Kpn*I, and the MU3-scTCR fragment was blunt-ligated with *Bam*HI-linearized pTY-TRIP-WPRE to generate pTY-TRIP-MU3-scTCR-WPRE, which was linearized with *Nhe*I and blunt-ligated with PGK-TGL fragment (obtained from pE3 by *Nhe*I/*Bam*HI digestin) to generate pTY-TRIP-MU3-scTCR-PGK-TGL-WPRE (pMTPT).

In order to construct a panel of LVs encoding the scTCR with human CD3ζ domain, EF1a-scTCR-AGD-TmHz fragment was cut from pEF6-scTCR-AGD-TmHz [39] using *Mlu*I and *Not*I and cloned into pTY-TRIP-WPRE at *Sma*I site to generate pTY-TRIP-EF1a-scTCR-AGD-TmHz-WPRE (pA17). The dual-promotor lentiviral vector pTY-TRIP-EF1a-scTCR-AGD-TmHz-PGK-TGL-WPRE (pTGC4) was constructed by cloning the EF1a-scTCR-AGD-TmHz fragment into pE3 at *Nhe*I site through blunt ligation.

In order to construct a dual-promoter LV encoding a site-mutated scTCR (mscTCR), which has shown an enhanced cell surface expression in human T cells (unpublished data), the EF1a-mscTCR was cut from pEF6-mscTCR using *Mlu*I and *Not*I, and cloned into pE3 at *Nhe*I through blunt ligation. The resulting plasmid is termed pB24.

Lentivirus production

293T cells (kindly provided by Dr. Yukai He, University of Pittsburgh) were cotransfected with four plasmids (the pTY transfer vector plus pHP-dl-N/A, pHEF-VSV-G, and pCEP-tat) [32, 33] using Lipofectamin 2000 kit (Invitrogen). All the plasmids were prepared using Qiagen endotoxin-free kit and quantitated by spectrophotometry at OD260/280. On day 1, transfected cells were placed in fresh medium and cultured for 24 h. Supernatants (first) were then harvested and fresh medium added for further culture for another 24 h, when the second supernatant was harvested and combined with the first. Virus containing supernatants were then filtered through a 0.45 μm Millex HV filter (Millipore, SLHV033RS) before used for transduction of target cells or stored at −80°C for later use.

Retrovirus production

293T cells were cotransfected with pMTE (pMFG-scTCR-IRES-EGFP)(12), helper plasmid pKAT [40] (a kind gift from Dr. Tao Cheng, University of Pittsburgh Cancer Institute) and envelope plasmid pVSV-G using the same protocol as in lentiviral vector production as described above. The viral supernatants were collected, filtered and stored at −80°C.

Lentivirus titration

Determination of viral titers was based on the expression of TGL or scTCR in transduced 293T cells. 293T cells were seeded in a six-well plate at 2×10^5 cells per well. After overnight culture, the medium was removed and 1 ml of serially diluted viral supernatants (1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, and mock), and polybrene (6 μg/ml, Millipore) added to the wells. 24 h later, supernatants were removed and 2 ml fresh medium were added. After further culture for 3 days, the cells were collected for FACS analysis. The titer was calculated using the following formula: $\text{TU/ml} = (P \times N/100 \times V) \times 1/DF$, where TU stands for transduction unit, P % GFP$^+$ cells, N number of cells at time of transduction $= 2 \times 10^5$, V volume of dilution added to each well $= 1$ ml, and DF dilution factor $= 1$ (undiluted), 10^{-1} (diluted 1/10), 10^{-2} (diluted 1/100), and so on [41].

Transduction of T cells

Human PBMCs isolated from healthy donors using Ficoll–Paque were activated for 3 days by coated anti-CD3 antibody (10 μg/ml) and soluble anti-CD28 antibody (1 μg/ml) in the presence of hIL-2 50 U/ml at 5–10 × 10⁶ cells per well in a six-well plate. The activated PBMC or T cell lines

(JRT3.5 or BWZ) were suspended in 1 ml of viral supernatant at 5×10^5 cells per ml in the presence of 6 µg/ml polybrene, in a six-well plate. The plate was centrifuged at 2,900 rpm for 50 min, followed by overnight incubation at 37°C, 5% CO_2. The viral supernatants were carefully removed and fresh medium was added for culture and expansion.

Flow cytometry

The HPAF pancreatic cancer cells and MCF-7 breast cancer cells were stained with PE-labeled MUC1 antibody 4H5 (a gift from Dr. Jo Hilgers, Amsterdam), which is specific to tumor-type hypoglycosylated MUC1(the epitope APDTRPAP in the VNTR region of MUC1) [42], followed by two washes with PBS (2.5% FBS and 0.09% NaN_3). Flow cytometry was used to measure the hypoglycosylated MUC1 expression on cell surface.

T cell functional assays

BWZ mouse T cells [12, 39] were transduced with LVs. After culture and expansion, 1×10^6 transduced cells were incubated at 37°C, 5% CO_2 with 1×10^6 MUC1$^+$ tumor cells, either HPAF pancreatic cancer cells, or MCF-7 breast cancer cells [12, 39], for 9 h. Brefeldin A (GolgiPlug, BD Bioscience) was added to the culture at the final concentration of 1 µg/ml. The cells were then permeabilized and fixed with BD Cytofix/Cytoperm kit (BD) following the instruction of manufacturer. After two times of washing with cytoperm buffer (BD), the cells were stained with APC-labeled anti-IL-2 antibody (BD) for 30 min at 4°C. The IL-2-producing cells were assessed by flow cytometry. The function of scTCR was also assessed by measuring the production of IL-2 in transduced T cells in response to the stimulation with BF1 antibody [12] using an ELISA kit (BD). A 24-well plate was coated with BF1 antibody (5 µg/ml in PBS) at 4°C overnight. The lentiviral vector transduced BWZ mouse T cells were then added to the wells and incubated at 37°C, 5% CO_2 for 48 h. After brief centrifugation of the plate, the supernatants were collected and the IL-2 concentration was measured by ELISA.

Cytotoxicity by ganciclovir

BWZ cells transduced with LVs encoding the HSV-TK–EGFP fusion gene (TGL) were cultured at 37°C, 5% CO_2 in RPMI1640 medium containing different concentration of ganciclovir or control medium. Every three to five days, the cells were split and resuspended in the same drug-containing medium or control medium. Two weeks later, cells were analyzed by flow cytometry to measure the disappearance of TGL-expressing cells. The selective

killing rate was calculated using the following formula: selective killing rate (%) = $(T_0 - T_n)/T_0 \times 100$, where T_n is the percentage of TGL-expressing cells after treatment with difference doses of ganciclovir, T_0 is the percentage of TGL-expressing cells after treatment with medium only.

Statistical analysis

Statistical significance was determined by multifactorial ANOVA using SAS9.1 with GLM Procedure. The EC_{50} of the selective killing efficiency of GCV and the 95% Confidence Interval were calculated using GraphPad Prism (GraphPad Software Inc.).

Results

Dual-promoter self-inactivating LVs co-encode scTCR and a fusion suicide gene

We constructed the new LVs based on a minimal self-inactivating lentiviral vector pTY system [32]. The pTY self-inactivating (SIN) vector has a maximal deletion of the 3′U3 region, with only 24 nt remaining, compared with 55 nt 3′U3 in other SIN LVs. The 3′U5 is replaced with a bGHpA signal, and the 5′U3 is replaced with a CMV TATA enhancer [32]. The maximal deletion of 3′U3 region would make it less likely to be mobilized in case of HIV super-infection or HIV *gag/pol* transfection, where the CMV TATA enhancer and 3′bGHpA poloy A signal could increase virus production.

To make this SIN vector more efficient, we included *cis* elements cPPT/CTS and WPRE in the vector backbone. The resulting construct, pTY-TRIP-WPRE, was used to produce a panel of SIN vectors, schematically represented in Fig. 1, that co-encode scTCR and HSV-TK–EGFP (TGL). We chose dual-promoter LV because it was reported to efficiently co-express multiple genes in transduced hematopoietic cells [43, 44], and appeared a better choice than bicistronic expression mediated by IRES, where the transgene is not reliably expressed in all cell types [43, 45]. In our vectors, the scTCR gene is under the control of EF1α promoter, and the TGL fusion suicide gene under the control of PGK promoter. Both EF1α and PGK promoters are physiological promoters, which were reported to be less genotoxic [46] and highly efficient in hematopoietic cells [29, 30].

High-titer virus production despite large packaging size of dual-promoter LVs

It has been reported that lentivirus titers decrease semi-logarithmically with increasing vector length [47, 48].

Although vectors as large as 18 kb could be packaged to give very low but still measurable viral titers, the optimum reported packaging size appears to be less than 5 kb, as the titer drops approximately 100-fold for inserts between 6 and 12.5 kb in length [48]. We had around 7 kb of inserts in the dual-promoter LVs, and 4.1 kb of inserts in the mono-promoter LVs. That raised concerns that the viral titer might be greatly compromised. This turned out to be not as big a problem as we anticipated. As shown in Fig. 2, the virus titer of three dual-promoter LVs, pB24, pTGC4, and pB21, all of which encode the TGL gene and have an insert of 6.7 kb, was 1–2 × 10^7 U/ml. This is only a four-fold reduction compared to the virus titer of the mono-promoter LV pE3, which also encodes TGL gene but with a smaller insert size of 3.4 kb. This titer is considered sufficiently high for efficient transduction of hematopoietic stem cells or primary T cells in vitro.

Transgene expression in T cell lines and primary T cells transduced by LVs
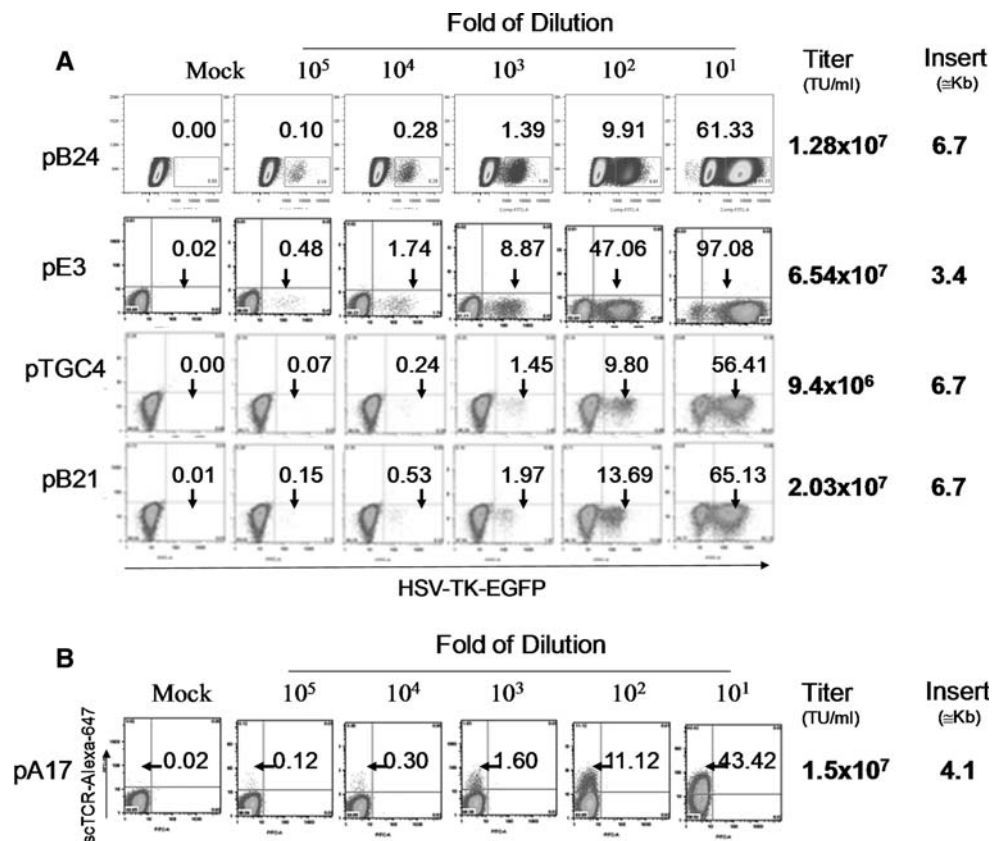
Transduction efficiency and transgene expression by mono-promoter LV (pE3 or pTY-EF-EGFP) or dual-promoter LV (pTGC4) were tested in T cell lines and primary T cells. As shown in Fig. 3, high transduction efficiency and expression level was achieved in T cell lines, for both mono-

promoter and dual-promoter LVs. In human primary T cells, while mono-promoter LV transferred high transduction efficiency (up to 40%) and transgene expression, the dual-promoter LV achieved lower performance, both in transduction efficiency (around 5%) and in transgene expression level. These percentage of antigen-specific cells is similar to levels observed in vivo in response to other antigens and thus would be expected to be therapeutic.

Resistance to gene silencing in dual-promoter LV transduced cells

The pMFG-scTCR-IRES-EGFP [pMTE, Fig. 1J] that we previously used in a preclinical gene therapy study [12] is Moloney murine leukemia virus (MMLV)-based oncoretroviral vector with wild-type LTR. Lethally irradiated mice that were transplanted with the pMTE oncoretroviral vector-transduced bone marrow cells successfully reconstituted the innate and adaptive immune system and efficiently controlled the growth of MUC1-positive tumors. However, the transgene expression in reconstituted immune cells, especially in T and B cells, was progressively reduced and silenced, which raised the concern for the long-term antitumor efficacy of this gene therapy strategy. Although SIN lentiviral vector was also reported to be subjected to gene silencing, it is generally agreed that

Fig. 2 High titer lentivirus production despite large packaging size of the LVs. 293T cells were transduced with indicated dilutions of viral supernatants produced from indicated vectors (see Fig. 1). Transduction efficiency was measured using flow cytometry. The titer was calculated by measuring either TGL (a) or scTCR positive cells (b) by flow cytometry. Numbers above arrows indicate percent positive cells. Calculation of virus titer is described in the Materials and Methods. TU stands for transduction unit. Insert size includes the cPPT/CTS element, internal promoter, transgene (scTCR and/or TGL), and WPRE
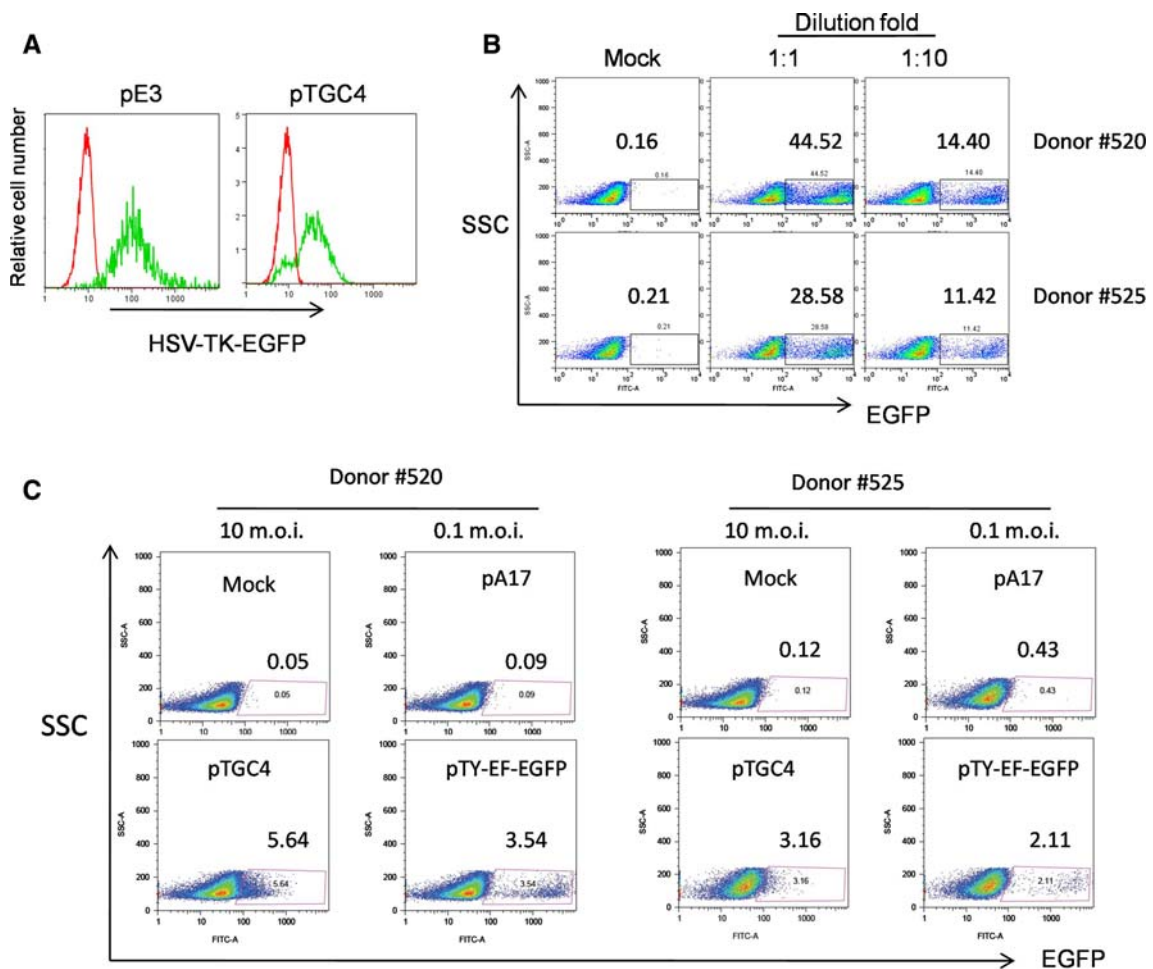
Fig. 3 Transduction efficiency of mono- and dual-promoter LVs in a T cell line and human primary T cells. JRT3.5 cells (**a**) were transduced with single-promoter LV pE3 encoding the TGL fusion gene, or dual-promoter LV pTGC4 that co-encodes scTCR and TGL (see Fig. 1). The CD3/CD28 activated human primary T cells were transduced with undiluted or tenfold diluted viral supernatants of mono-promoter LV pTY-EF-EGFP (**b**). The same primary T cells were also transduced with tittered mono-promoter LV pTY-EF-EGFP or dual-promoter LV (pTGC4) (**c**). Transgene expression was analyzed by flow cytometry

LV is more resistant to gene silencing than retroviral vector. Figure 4 shows that after a long-term in vitro expansion of transduced Jurkat cells, high expression level of the transgene was maintained in LV-transduced cells up to 4 months, and was always higher than in retroviral vector transduced cells (Fig. 4).

Recognition of MUC1-positive tumor cell lines by LV encoded scTCR

Our previous results showed that BWZ T cells transduced with pMFG-based vector encoding scTCR gene specifically recognized different human MUC1-positive tumor cell lines as measured by their IL-2 production using ELISA or intracellular cytokine staining. We first confirmed the ability of the scTCR expressed on the surface of the BWZ cells transduced with the dual-promoter LV pB21 to signal properly, by cross-linking it with the BF1 monoclonal antibody specific to human TCR β chain. As shown in Fig. 5a, transduced BWZ cells efficiently produced IL-2, while mock-transduced cells or control vector pE3 (not encoding scTCR) transduced cells failed to produce the cytokine. In order to confirm that the specificity of the scTCR was not altered during the construction of the new vectors, two human tumor cell lines, pancreatic cancer cell line HPAF and breast cancer cell line MCF-7, which express hypoglycosylated MUC1 (data not shown), were co-cultured either with BWZ cells transduced with two different LVs (mono-promoter LV pT3 and dual-promoter LV pB21) encoding scTCR, or with BWZ cells transduced with mock or mono-promoter LV encoding TGL gene only (pE3). The result shows that upon stimulation with two MUC1-positive tumor cells, a significantly higher percentage of scTCR-LV-transduced BWZ cells produced IL-2 compared to BWZ controls (Fig. 5b, c) ($P = 0.0019$, multiple factorial ANOVA analysis by SAS GLM
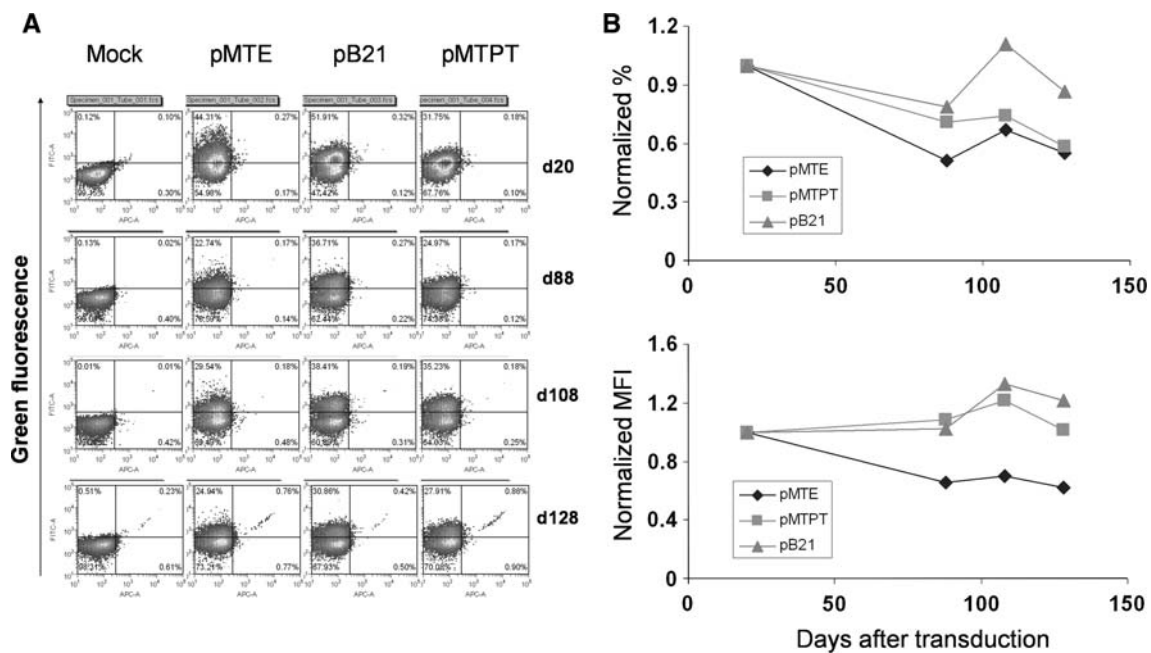
Fig. 4 Sustained transgene expression in T cells transduced with LVs. JRT3.5 cells were transduced with LVs pB21 or pMTPT (see Fig. 1) or the retroviral vector pMTE and transgene expression monitored by flow cytometry at indicated time points up to 4 months (**a**). **b** Percent positive cells and mean fluorescence intensity (MFI) normalized to the initial time point (day 20)

Procedure) There were no significant difference between the two MUC1$^+$ tumor cell lines ($P = 0.8728$), and no significant difference between the mono-promoter and dual-promoter LVs ($P = 0.4444$). Furthermore, no interaction between the tumor cell line and LV promoter was observed ($P = 0.8146$). This confirms the unaltered MHC unrestricted MUC1 specificity of this TCR.

Selective eradication by the pro-drug ganciclovir of LV-transduced T cells

In order to further improve the safety of the scTCR encoding LVs, we included an HSV-TK suicide gene allowing in vivo elimination of transduced cells. To test the function of this transgene, we treated transduced JRT3.5 cells with the prodrug ganciclovir. We took advantage of the EGFP gene fused to the HSV-TK to measure by fluorescence the percentage of surviving cells at different times after treatment and with different concentrations of the drug. As shown in Fig. 6, BWZ cells transduced with mono-promoter LV (pE3) or dual-promoter LVs (pB21 and pTGC4) are selectively eradicated by ganciclovir, with EC50 of 0.091 μM (95% confidence interval (CI): 0.060–0.137 μM, $R^2 = 0.9929$) in pE3-transduced cells, 0.102 μM (95% CI: 0.035–0.300 μM, $R^2 = 0.9261$) in pB21-transduced cells, and 0.037 μM (95% CI 0.022–0.063 μM, $R^2 = 0.9861$) in pTGC4-transduced cell, respectively.

Discussion

We have constructed several new dual promoter LVs for gene therapy/immunotherapy of MUC1$^+$ tumors. These vectors are self-inactivating and encode the MUC1-specific, MHC-unrestricted scTCR gene and the HSV-TK–EGFP fusion suicide gene that confers a high safety profile important for future clinical applications. These vectors efficiently transfer high-level and sustained expression of scTCR and HSV-TK transgenes to T cells. Transduced cells, either soon after or long after transduction, can be selectively eradicated by the prodrug ganciclovir. Furthermore, these SIN LVs have minimal mobilization activity adding to their safety (X. Chen et al. manuscript in preparation), as mobilization by HIV *gag/pol* was recently detected in SIN LVs and raised a new concern for LV safety profile [49–51].

In order for these vectors to be eventually used in the clinic, the virus titer should be high enough so the target cells (hematopoietic stem cells or primary T cells) can be efficiently transduced. Our results show that in spite of the large size of the inserts, the viral titers obtained with the dual-promoter LVs are unexpectedly high. This may be due to the unique design of the pTY SIN vector in that CMV/TATA enhancer was placed upstream of 5′LTR, and bGHpA signal was used to replace 3′U5. In addition, cPPT/CTS and WPRE were included in the vector to further enhance virus production. The other possible reason for the
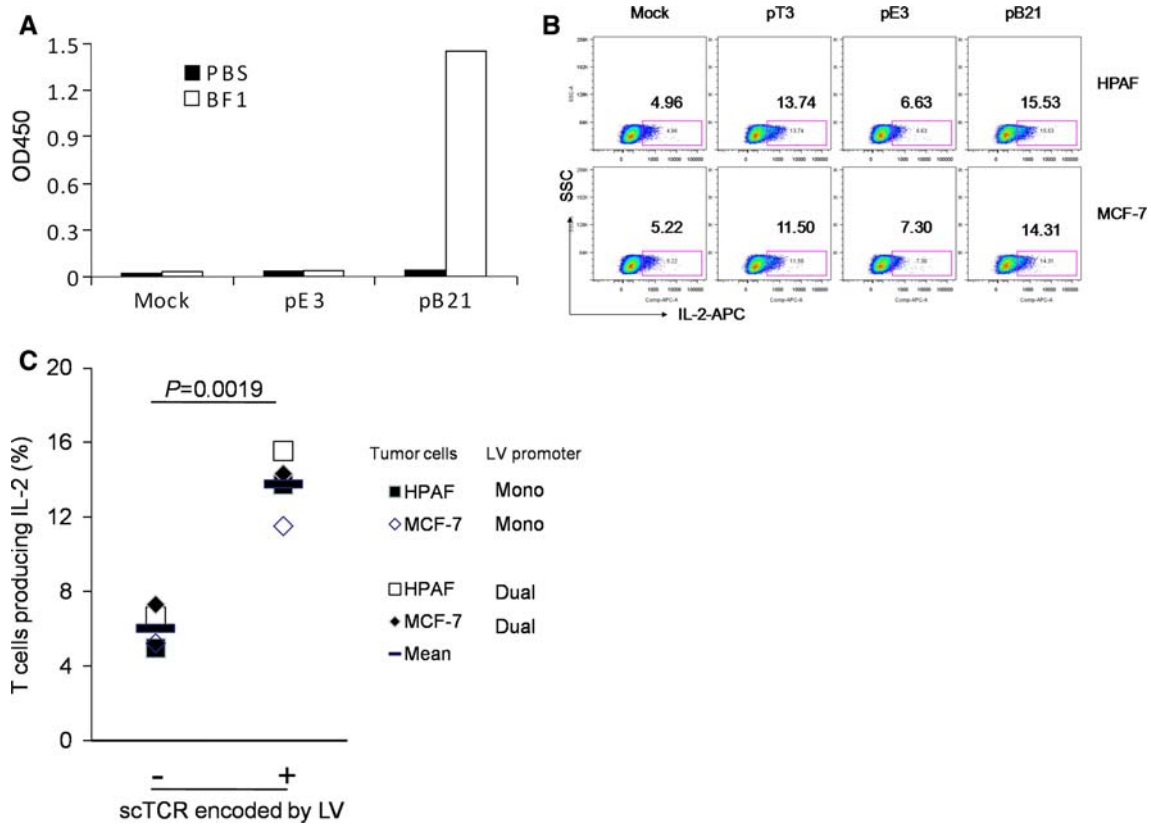
Fig. 5 LV encoded MUC1 specific scTCR signals in transduced T cells and specifically recognizes MUC1$^+$ tumor cells. Mouse BWZ T cells were transduced with indicated vectors or vector control (see Fig. 1). **a** The transduced cells were stimulated with plate-bound anti-TCR antibody BF1 for 48 h. IL-2 in the supernatants was measured by ELISA. **b** Transduced cells were co-cultured with MUC1$^+$ pancreatic cancer cell line HPAF or breast cancer cell line MCF7 for 9 h in the presence of GolgiPlug. Cells were permeabilized and fixed, followed by intracellular staining with anti-mouse IL-2 antibody. IL-2-producing cells were determined by flow cytometry. **c** Differences in recognition of different tumor cells by BWZ cells transduced with different LVs were analyzed by multifactorial ANOVA with SAS GLM procedure
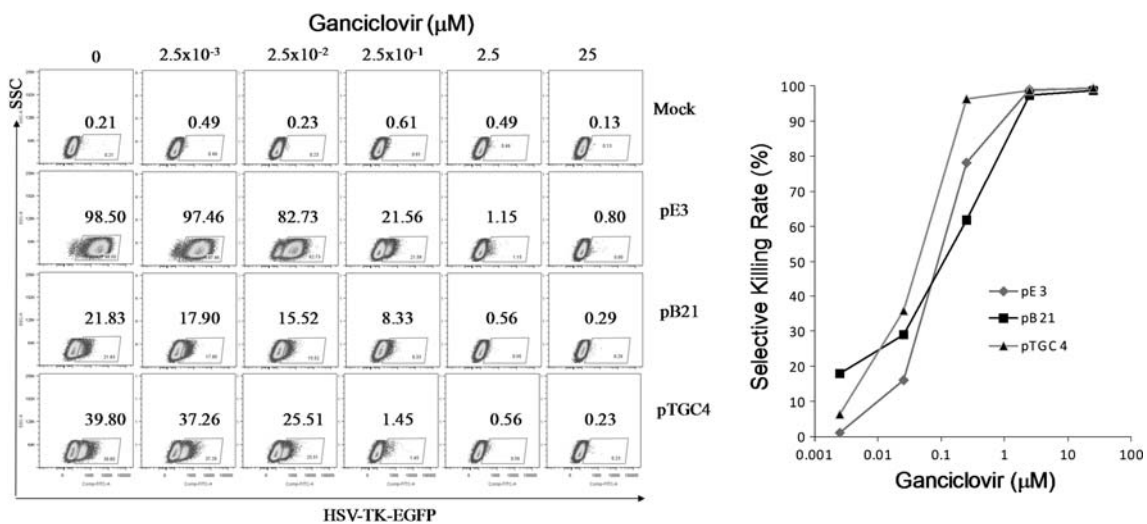

Fig. 6 Selective killing of LV-transduced cells by ganciclovir. Mouse BWZ T cells transduced with LVs pB21 and pTGC4 (see Fig. 1). After 2 weeks, remaining transduced (*green*) cells were measured by flow cytometry (**a**). Selective killing rate of ganciclovir was calculated using the formula described in M&M, and 50% effective concentration (EC$_{50}$) and its 95% confidence interval were determined giving the dose-effect curve (**b**)

high virus production is that we modified the protocol using 3:1 ratio of packaging plasmid to transfer plasmid instead of 1:1 ratio used by others. This high amount of packaging plasmid may have improved the encapsidation of large viral RNA, increasing the viral titers.

Since MUC1 is aberrantly over-expressed in over half of all human cancers [52], targeting this molecule through gene therapy/immunotherapy based on MUC1-specific, MHC-unrestricted scTCR would be applicable to a large number of cancer patients irrespective of their HLA types. More importantly, it would be applicable to the efforts of eradicating the minimal residual diseases, since cancer stem cells also express hypoglycosylated MUC1 [53] and are thus a potential target of this scTCR gene therapy strategy.

TCR-based gene therapy against cancer can be achieved through transduction of hematopoietic stem cells [12] or differentiated T cells [54, 55]. With our TCR, transduction of the hematopoietic stem cells resulted in not only antigen-specific T cells but also antigen specific B cells, NK cells, macrophages, granulocytes and other cells of the hematopoietic origin, directing innate and adaptive immunity to the tumor site simultaneously [12, 56]. Planned preclinical experiments in animal models and Phase I clinical trials will determine the benefit and toxicity of such an approach.

HSCs are highly desirable recipient cells for gene therapy because of their self-renewal and multilineage differentiation capacities. Stable engraftment of genetically modified HSCs leads to long term expression of transgene in stem and progenitor cells [57]. It was reported that oncoretroviral delivery of a tumor antigen-specific TCR gene into mouse hematopoietic stem cells provided long-term antigen-specific T cell immunity against cancer [58]. In our previous work, the MUC1-specific, MHC-unrestricted scTCR gene was delivered into mouse bone marrow cells using a MFG-based oncoretroviral vector, which resulted in the long-term scTCR transgene expression in repopulated progeny immune cells, both innate and adaptive, and effective control of the growth of MUC1-positive pancreatic cancer cells as well as mouse leukemia CLL in recepient mice [12]. This strategy represents a novel approach for cancer immunotherapy termed "instructive immunotherapy" [58] that re-programs the immune system to fight cancer by genetically engineering the hematopoietic stem cells.

Engineering and manipulation of lymphocytes through gene therapy strategy for adoptive transfer is another effective approach to eradicate tumor cells in vivo [59–61]. T cells transduced by oncoretroviral and lentivial vectors to express tumor-specific TCR can be expanded in vitro to a large number and adoptively transferred to animals and patients for killing tumor cells [54, 55, 60, 61]. The MHC-unrestricted feature of our MUC1-specific scTCR is particularly applicable to this strategy because tumors downregulate MHC expression as a means to evade host immunosurvailance [62]. This unique MHC-unrestricted function enables the immune cells expressing this scTCR to recognize and kill tumor cells irrespective of their MHC type and expression level, making this a "universal" TCR therapeutic gene for any individual with MUC1-positive tumor.

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