

Human Leukocyte Antigen (HLA) A*1101-Restricted Epstein-Barr Virus-Specific T-cell Receptor Gene Transfer to Target Nasopharyngeal Carcinoma

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Abstract

Infusing virus-specific T cells is effective treatment for rare Epstein-Barr virus (EBV)-associated posttransplant lymphomas, and more limited success has been reported using this approach to treat a far more common EBV-associated malignancy, nasopharyngeal carcinoma (NPC). However, current approaches using EBV-transformed lymphoblastoid cell lines to reactivate EBV-specific T cells for infusion take 2 to 3 months of *in vitro* culture and favor outgrowth of T cells targeting viral antigens expressed within EBV⁺ lymphomas, but not in NPC. Here, we explore T-cell receptor (TCR) gene transfer to rapidly and reliably generate T cells specific for the NPC-associated viral protein LMP2. We cloned a human leukocyte antigen (HLA) A*1101-restricted TCR, which would be widely applicable

because 40% of NPC patients carry this HLA allele. Studying both the wild-type and modified forms, we have optimized expression of the TCR and demonstrated high-avidity antigen-specific function (proliferation, cytotoxicity, and cytokine release) in both CD8⁺ and CD4⁺ T cells. The engineered T cells also inhibited LMP2⁺ epithelial tumor growth in a mouse model. Furthermore, transduced T cells from patients with advanced NPC lysed LMP2-expressing NPC cell lines. Using this approach, within a few days large numbers of high-avidity LMP2-specific T cells can be generated reliably to treat NPC, thus providing an ideal clinical setting to test TCR gene transfer without the risk of autoimmunity through targeting self-antigens. *Cancer Immunol Res*; 3(10); 1138–47. ©2015 AACR.

Introduction

Nasopharyngeal carcinoma (NPC) is unusually common throughout Southeast Asia especially in southern China where it is the third most common cancer in men with annual incidence rates of up to 28 cases per 100,000 men (1). Early-stage disease responds well to radiotherapy (\pm chemotherapy), but a study of 2,687 patients treated in Hong Kong reported that over half of these patients presented with advanced disease (stage III–IV) and have a 5-year disease-specific survival rate of only 72% (2). Survivors are also at risk of treatment-related toxicities, including secondary malignancies (3). Therefore, there is clear need to develop improved therapies for this cancer.

Epstein-Barr virus (EBV) is consistently detected in malignant cells of patients with undifferentiated NPC, and is strongly implicated in the pathogenesis of this and other human tumors (4). Despite its oncogenic potential, EBV is ubiquitous in the human population and it normally persists as an asymptomatic life-long infection under the control of virus-specific T cells (4). The presence of this virus within NPC, therefore, raises the possibility of a T-cell-based therapy for this disease.

Treatments based on infusing tumor-specific T cells have yielded impressive clinical responses in some cancers. Indeed some of the earliest data supporting this approach came from trials targeting EBV⁺ lymphomas. Infusing EBV-specific polyclonal T-cell lines is highly effective as a therapeutic and prophylactic treatment for rare EBV⁺ lymphomas that occur in transplant recipients (5). However, to extend this treatment to more common EBV⁺ tumors such as NPC, two issues must be addressed. First, polyclonal T-cell lines initially used to treat EBV⁺ lymphomas were reactivated *in vitro* using the autologous EBV-transformed lymphoblastoid cell line (LCL). Within an LCL (and most posttransplant EBV⁺ lymphomas), the virus expresses at least six nuclear antigens, EBNA-1, -2, -3A, -3B, -3C, -LP, and two latent membrane proteins, LMP1 and LMP2. Of these, members of the EBNA3 family are immunodominant antigens for CD8⁺ T cells. However, in NPC, EBV protein expression is restricted to EBNA1, LMP1 (variable), and LMP2. Nevertheless, attempts to treat NPC by infusing LCL-reactivated T-cell lines have yielded objective responses in a minority of patients (6–9). Low frequencies of LMP2-specific T cells were detectable within some infused cell preparations, and these may have mediated antitumor effects, but the procedure is

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clearly suboptimal because the majority of virus-specific T cells targeted EBV proteins not expressed in the tumor (7, 9). Second, generating T cells by LCL reactivation takes over 2 months of *in vitro* culture, including the time required to establish an LCL, and then the selective expansion of EBV-specific effector cells. This is labor intensive and does not always generate detectable T-cell responses specific for NPC-associated EBV antigens (7–9). More recently, selective reactivation of T cells targeting these antigens has been attempted using recombinant viral vectors or peptides (10–12), but again this requires several weeks of *in vitro* culture and/or often results in products with very low frequencies of tumor-specific T cells.

Therefore, we have explored the use of T-cell receptor (TCR) gene transfer, an approach that is rapid, reliable, and capable of generating large quantities of T cells ($>10^8$ – 10^{10} cells/patient) with the desired specificity, regardless of the patient's preexisting immune repertoire. TCRs are expressed on the surface of all T cells and determine antigenic specificity. Having identified a tumor antigen-specific T cell, by cloning the genes encoding its TCR into a retroviral vector it is then possible within a few days to engineer a patient's T cells expressing the same TCR and targeting the same tumor antigen. The efficacy of this approach to treat melanoma and synovial cell sarcoma has already been demonstrated in clinical trials (13, 14).

To ensure TCR gene transfer could be widely applicable to NPC patients, we focused on a T-cell response to an epitope derived from the NPC-associated EBV protein LMP2, presentation of which is restricted through human leukocyte antigen (HLA)-A*1101, an allele carried by >50% of the Chinese population. This epitope comprises the sequence SSCSSCPLSK (referred to subsequently as "SSC"). Here, we report the cloning of an SSC-specific TCR and studies to determine the expression and function of both the wild-type and modified forms of this receptor in transduced T cells. We thereby demonstrate that TCR transfer using this receptor offers a rapid and efficient means to generate T cells to target NPC.

Materials and Methods

Cells and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on lymphoprep (Axis Shield). LCLs were generated using Caucasian (B95.8) or Chinese (CKL) prototype 1 EBV strains (15). Phoenix amphotropic packaging cells were kindly provided by Gary Nolan (Stanford University). The T2 cell line transduced with HLA A*1101 gene was kindly provided by M. Masucci (Karolinska Institute, Stockholm, Sweden). NPC cell lines HK1 (16) and c666.1 (17) were transduced with retrovirus (pQCXIH and pQCXIN, respectively; Clontech, CA) into which we had cloned the gene encoding HLA A*1101. These cell lines were then cultured under drug selection using 20 μ g/mL hygromycin or 50 μ g/mL G418 (Life technologies), respectively. Though originally described as an NPC cell line, and used here because it naturally expresses HLA A*1101, HONE-1 now appears to be a HeLa-related somatic cell hybrid (18). The breast cancer cell line MDA-MB-231 (19) was transduced with three retroviruses (pQCXIH, pLXSN, and pMSCV) carrying genes encoding HLA A*1101, LMP2, and luciferase, respectively, and cultured under drug selection using 300 μ g/mL hygromycin, 600 μ g/mL G418, and 1 μ g/mL puromycin. All of the above cell lines were cultured

in RPMI-1640 (Sigma) containing 10% FBS (PAA), 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 pg/mL streptomycin (standard medium). Fibroblasts were grown from a skin biopsy cultured in DMEM (Sigma) supplemented as described above. All T, B, and fibroblast cell lines were derived from healthy donors or NPC patients of known HLA type. All cancer cell lines were authenticated by short tandem repeat analysis and passaged for fewer than 6 months before experiments. The use of human materials for this study was approved by the National Research Ethics Service, UK, and the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Work was conducted according to the declaration of Helsinki protocols and all donors provided written informed consent.

Synthetic peptides and recombinant vaccinia viruses

Peptides were synthesized using Fluorenylmethoxycarbonyl chemistry by Alta Bioscience, Birmingham, UK. Recombinant vaccinia and modified vaccinia Ankara viruses—expressing LMP2 and corresponding control vectors have been described previously (20, 21).

TCR gene cloning

RNA from the T-cell clone was isolated using an RNeasy mini kit (Qiagen) and reverse transcribed. TCR α and β genes were then amplified with the BD SMART RACE cDNA Amplification Kit (BD Biosciences) according to the manufacturer's instructions using the following primers: TCR α constant region: 5'-agcagcgtgtcttacaatcttgc-3'; TCR β constant region: 5'-ggacacagattggagcagg-3'. TCR genes were subcloned into the pCR2.1 (Life Technologies) vector and sequenced. The TCR α (TCRVA22) and TCR β (TRBV4.01) chains were then cloned into the same retroviral pMP71 vector (kindly provided by C. Baum, Hannover, Germany; ref. 22) separated by a 2A peptide linker from porcine teschovirus (Supplementary Fig. S3). The sequence has been deposited in GenBank, accession code KU163590. Modified TCR genes were designed and produced by GeneArt.

Retroviral transduction of human T cells

Phoenix amphotropic packaging cells were transfected with pMP71 retroviral vector and pCL amphi (Imgenex) using FuGENE HD (Roche) according to the manufacturer's instructions and retroviral supernatant harvested 48 hours later. PBMCs were preactivated for 48 hours using anti-CD3 antibody (OKT3; 30 ng/mL) and IL2 (600 U/mL; Chiron) in standard medium containing 1% human AB serum (TCS Biosciences). These cells were then transduced with retroviral supernatant (or mock transduced with conditioned supernatant from nontransfected phoenix cells) using retronectin-coated (Takara) 6-well plates according to the manufacturer's instructions. Cells were then maintained in standard medium containing 1% human AB serum and IL2 (100 U/mL).

Flow cytometry

Cells were stained for 10 minutes at room temperature with an HLA-A*1101/SSC pentamer (5 μ g/mL; ProImmune) according to the manufacturer's instructions. Cells were then washed and stained on ice for 30 minutes with Pro5 Fluorotag (APC or RPE-labeled; ProImmune) and saturating concentrations of anti-CD3 (PE-conjugated), anti-CD4 (FITC-conjugated; Pharmingen), and anti-CD8 (tricolor- or ECD-conjugated; Caltag) antibodies.

For intracellular cytokine staining T cells were stimulated for 2 hours with T2-A11 cells prepulsed with or without SSC peptide (5 $\mu\text{g}/\text{mL}$). Brefeldin A (10 $\mu\text{g}/\text{mL}$; Sigma) was then added and cells cultured for another 5 hours. Cells were then stained with pentamer and antibodies to surface markers (CD4-FITC, CD8-ECD; BD Pharmingen) as described above. After treatment with fixation and permeabilization buffers (E-bioscience) according to the manufacturer's instructions, cells were incubated for 30 minutes at 4°C with anti-cytokine antibodies (IL2-PE, IFN γ -PECy7, and TNF α -APC) or an isotype- and concentration-matched control antibody (BD Pharmingen), then washed twice in PBS. Cells were analyzed using an LSRII cytometer (Becton Dickinson) and FlowJo software (Tree Star).

CFSE labeling

T cells were washed twice with PBS and incubated with 2.5 $\mu\text{mol}/\text{L}$ Carboxyfluorescein succinimidyl ester (CFSE) for 10 minutes at 37°C. The labeling reaction was quenched by addition of RPMI-1640 containing 10% FBS. Cells were washed, resuspended in standard growth medium at 2×10^6 cells/mL, cocultured for 5 days with T2-A*1101 cells prepulsed with SSC peptide (10 $\mu\text{g}/\text{mL}$), then analyzed by flow cytometry as described above.

IFN γ release assay

Stimulator cells (5×10^4 /well) were cocultured in triplicate with T cells at responder:stimulator ratios as indicated. Cells were incubated at 37°C/5% CO $_2$ in 100 μL per well of Iscove's modified dulbecco's medium (Life Technologies) supplemented with 10% FBS and IL2 (25 U/mL). After 18 hours, culture supernatants were tested for secreted IFN γ using an ELISA (Pierce Endogen) according to the manufacturer's instructions.

Cytotoxicity assays

Chromium release assays, using vaccinia-infected or peptide-pulsed targets, were set up at known effector:target ratios (2,500 targets/well) and harvested after 5 or 8 hours. These protocols have been described in detail previously (23).

In vivo tumor protection experiments

Six- to 8-week-old female NSG mice (Charles River Laboratories) were inoculated s.c. on the flank with MDA-MB-231 cells (expressing A*1101, LMP2, and luciferase; 5×10^6 cells/mouse) in Matrigel (BD Biosciences). One day later, mice received 10^7 TCR-transduced (or mock-transduced) T cells intravenously. Intraperitoneal injections of 10^4 U IL2 were given on days 2, 4, 7, 9, and 11. Tumor growth was measured in a blinded fashion with calipers and bioluminescence imaging (IVIS Spectrum; Caliper Life Sciences). All experiments were performed under UK Home Office authorization.

Results

Expression and function of a wild-type HLA A*1101-restricted LMP2-specific TCR

EBV-specific T cells from a healthy Chinese donor were reactivated *in vitro* with the autologous LCL and cloned by limiting dilution as previously described (23). Clones were screened for reactivity to the A*1101-restricted LMP2 epitope SSC and clone 85 was selected. The avidity of this CD8 $^+$ clone for SSC peptide was determined using a cytotoxicity assay with A*1101 $^+$ targets pulsed with titrated concentrations of peptide. The clone displayed high avidity, with clear recognition of target cells pulsed with only

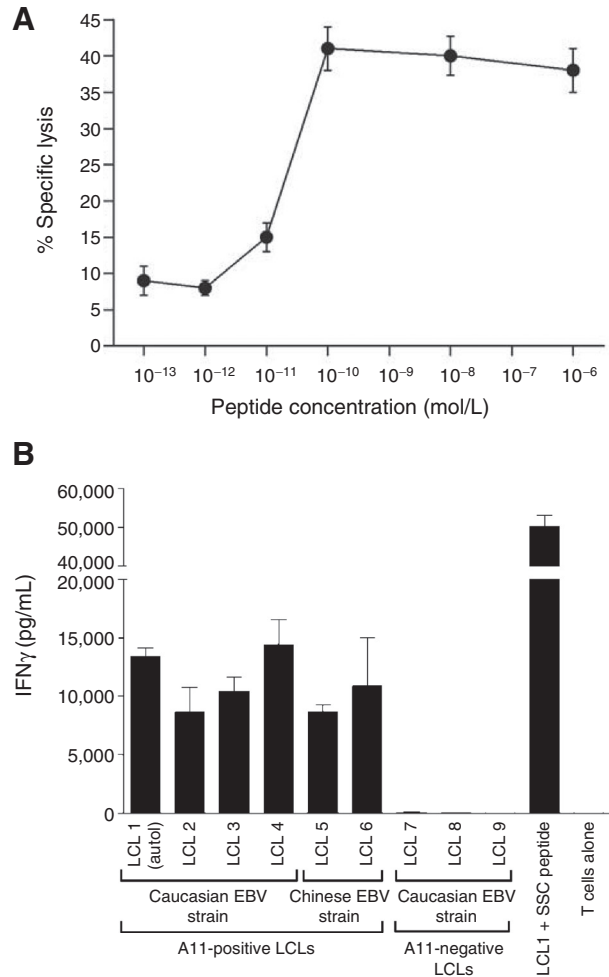


Figure 1.

Characterizing an A*1101-restricted SSC-specific CD8 $^+$ cytotoxic T-cell clone. A, avidity for SSC peptide was determined by cytotoxicity assay (E:T = 3:1). B, response to LMP2 expressed in A*1101-matched or -mismatched LCLs carrying EBV strains from Caucasian or Chinese populations was measured by IFN γ production. Target cells alone produced <100 pg/mL IFN γ . The responder:stimulator ratio = 1:10. Results show mean + SD and are representative of three separate experiments.

10^{-10} mol/L peptide (Fig. 1A). When tested for IFN γ production in response to A*1101-matched and -mismatched LCL targets, a clear A*1101-restricted response was observed (Fig. 1B). Importantly, this clone recognized not only A*1101 $^+$ LCLs carrying the standard EBV strain B95.8 (derived from a Caucasian population) but also those carrying EBV strains from the Chinese population, which is the most at risk of NPC.

Genes encoding TCR α and β chains from clone 85 were isolated and cloned into the same MP71 retroviral expression vector separated by a 2A peptide-linker from porcine teschovirus to ensure equimolar expression of these chains (Fig. 2A). Activated T cells from healthy donors and NPC patients were then transduced with the recombinant retrovirus and surface expression of SSC-specific TCR determined using an A*1101/SSC pentamer. Figure 2B shows results with T cells from a patient with advanced NPC. SSC-specific T cells are rare/undetectable in most NPC patients and healthy virus carriers (as indicated by

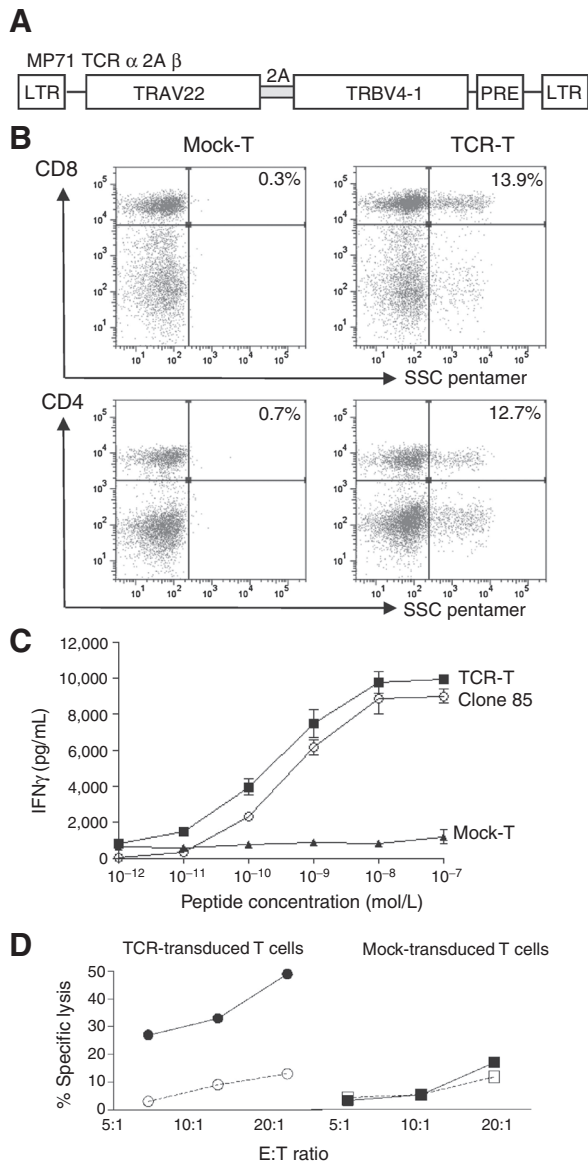


Figure 2.

Expression and function of wild-type SSC-specific TCR. A, design of the pMP71 retroviral expression vector. B, SSC-specific TCR expression on transduced PBMCs from a patient with advanced NPC (TCR-T) compared with that with mock-transduced cells (Mock-T). Values shown refer to the percentage of pentamer⁺ CD8⁺ or CD4⁺ cells. C, avidity for SSC peptide of TCR-transduced T cells (TCR-T) and T-cell clone 85 was measured by ELISA for IFN γ release. Mock-transduced T cells (mock-T) were included as a control. The responder:stimulator ratio = 1:4. Results show mean \pm SD and are representative of three repeat experiments. D, TCR-transduced (but not mock transduced) T cells lyse autologous fibroblasts expressing LMP2 from a recombinant vaccinia vector (closed symbols), but not fibroblasts infected with a control vaccinia vector (open symbols). Data representative of three separate experiments.

mock-transduced cells), but 3 days after transduction with recombinant retrovirus, surface expression of SSC-specific TCR was clearly detectable in 13.6% of CD8⁺ T cells. Note that 12% of CD4⁺ T cells also expressed this TCR following transduction. These data are representative of those from 9 healthy donors and 5 NPC patients.

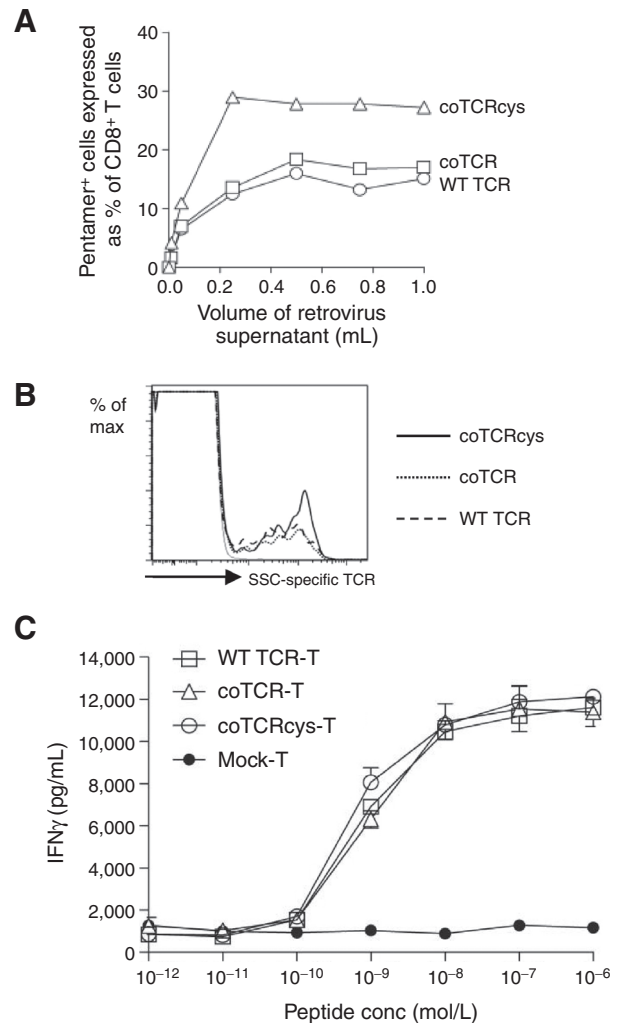


Figure 3.

Optimizing TCR gene construct. A, SSC-specific TCR expression 3 days after transduction with wild-type TCR (WT TCR), a codon-optimized version (coTCR) or a codon-optimized TCR incorporating an additional disulphide bond (coTCRcys). B, intensity of pentamer staining for the different TCR constructs. C, avidity for SSC peptide of T cells transduced with each of the TCR constructs was compared using an ELISA for IFN γ release. T-cell input numbers were adjusted to ensure equivalent numbers of transduced effectors were tested for each TCR construct. The responder:stimulator ratio = 1:3. Results show mean \pm SD. Mock-transduced T cells (Mock-T) were included as a control. All results shown are representative of at least three separate experiments.

Functional testing of this wild-type TCR began using transduced polyclonal T cells to explore their ability to produce IFN γ in response to T2:A*1101 cells pulsed with SSC peptide at titrating concentrations. TCR-transduced T cells clearly recognized peptide-pulsed targets with as low as 10^{-10} mol/L peptide, whereas mock-transduced T cells did not respond at any peptide concentration tested (Fig. 2C). Testing Clone 85, from which the TCR genes were derived, at the same input cell number as SSC-specific effectors within the transduced T cells yielded almost identical results (Fig. 2C). Transduced T cells also mediated specific cytotoxic function when tested against autologous fibroblasts expressing LMP2 protein from a recombinant vaccinia vector, compared

with that against fibroblasts infected with the empty control vector (Fig. 2D).

Optimization of the TCR gene construct

Previous studies have suggested that function and/or expression of transduced TCRs can be improved by codon optimization

to increase translation efficiency (24), and addition of a second disulphide bond in the TCR constant domains to aid preferential pairing of the introduced TCR chains (25). The latter also helps prevent exogenous TCR chains mispairing with endogenous TCR chains naturally expressed by T cells. Such mispairing not only reduces the number of SSC-specific TCRs expressed, but also risks

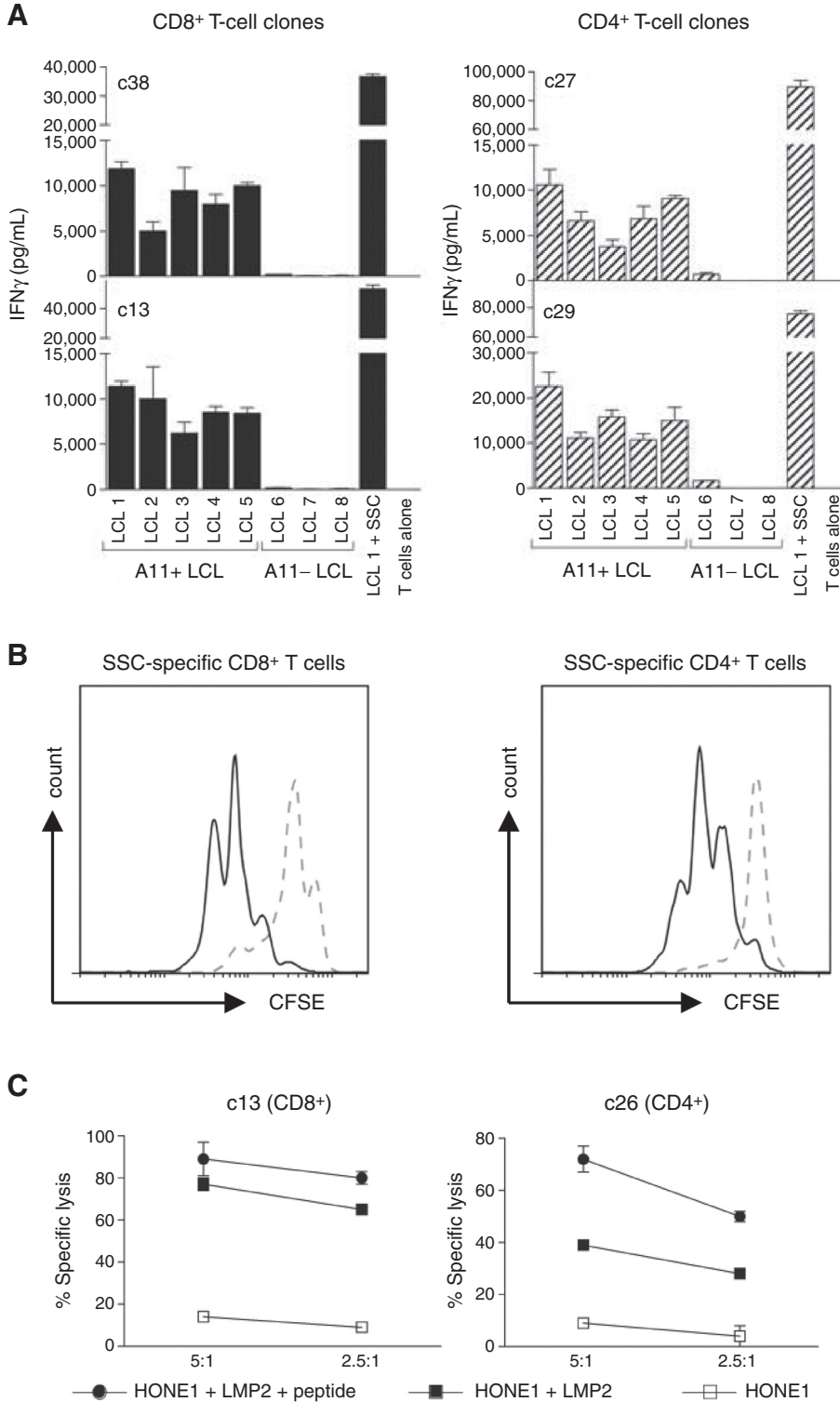


Figure 4. Function of coTCRcys-expressing CD8⁺ and CD4⁺ T cells. A, response of transduced T-cell clones to LMP2 expressed in A*1101-matched or -mismatched LCLs was measured by IFN γ production. Target cells alone produced <100pg/mL IFN γ . The responder:stimulator ratio = 1:10. Results show mean + SD and are representative of seven clones for each subset. B, proliferation of coTCRcys-expressing T cells measured by CFSE staining after stimulation with T2-A*1101 cells alone (dotted line) or T2-A*1101 cells pulsed with SSC peptide (solid line). C, cytotoxic activity of coTCRcys-transduced CD8⁺ and CD4⁺ T-cell clones against HONE1 cells expressing LMP2^{+/-} pulsed with SSC peptide, or HONE1 cells alone. Results show mean + SD and are representative of four clones for each subset.

generating novel, potentially autoreactive TCRs. Therefore, we generated two variants of our wild-type SSC-specific TCR, a codon-optimized version (coTCR) and a codon-optimized TCR in which amino acid residue 48 of the TCR α chain and residue 57 of the TCR β chain were both changed to cysteine, thus introducing a second disulfide bond (coTCRcys; refs. 25). A series of experiments then compared expression and function of these two variants with wild-type SSC-specific TCR (WT TCR). The main difference observed was TCR surface expression. Pentamer staining of CD8⁺ T cells, transduced with increasing volumes of the three retroviral supernatants produced in parallel, showed similar expression of WT TCR and coTCR, but a clear increase was observed with the coTCRcys construct (Fig. 3A). Similar results were obtained with CD4⁺ T cells (data not shown). Not only was the coTCRcys receptor expressed on a greater proportion of T cells, but the levels of expression on individual cells were increased (Fig. 3B). These data are consistent with previous reports that introducing a second disulfide bond reduces mispairing with endogenous TCR chains (25). Staining transduced cells with an antibody to V β 4.1 showed similar results to the same cells stained with the SSC pentamer (Supplementary Fig. S1), suggesting that there is little if any mispairing between this exogenous β -chain and the endogenous α -chains. However, an antibody was not available to stain the exogenous V α 22-chain; therefore, it is possible that increased expression of coTCRcys is a result of reduced mispairing between the exogenous α -chain and the endogenous β -chains. Although expression was improved with coTCRcys, when an equivalent number of transduced effectors were tested for each TCR construct, T-cell function was unaffected (Fig. 3C). Although codon optimization alone (coTCR) affected neither surface expression nor functional activity (Fig. 3), other studies have shown that despite such lack of *in vitro* effects, codon optimization can nevertheless improve both frequency of TCR-modified T cells detectable post-infusion and antitumor activity *in vivo* (26, 27). Therefore, the coTCRcys construct was selected for further testing. Analyzing the differentiation status of coTCRcys-transduced cells showed that they contained a mixture of mainly naïve, central-memory and effector-memory cells (Supplementary Fig. S2).

Functional analysis of coTCRcys in CD8⁺ and CD4⁺ T cells

Having optimized expression of the SSC-specific TCR, to explore its therapeutic potential, we then determined the ability of coTCRcys-transduced T cells to recognize LMP2 protein expressed at physiologic levels in an LCL. For this, we used cloned populations of TCR-transduced cells to study the functional activity in CD8⁺ cells, which can have direct antitumor effects *in vivo*, and CD4⁺ cells, which can help generate and maintain effective CD8⁺ responses and can also be cytotoxic. To ensure SSC-specific CD8⁺ clones had been engineered and were not naturally occurring effectors, we used PCR to detect the retroviral construct (data not shown). Both engineered CD8⁺ and CD4⁺ cells responded by IFN γ production in an A*1101-restricted manner when tested against a panel of A*1101-matched and -mismatched LCLs (Fig. 4A). Thus, this TCR can function in a CD8-independent manner.

Using CFSE-labeling, we explored the ability of coTCRcys-transduced T cells to proliferate following antigen encounter. Both engineered CD8⁺ and CD4⁺ T cells underwent several rounds of division following stimulation with SSC peptide-loaded T2-A*1101 cells (compared with T2-A*1101 alone; Fig. 4B).

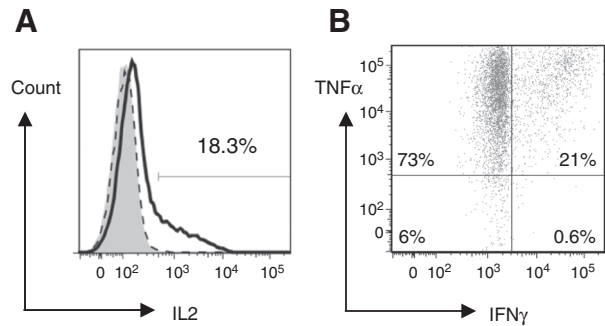


Figure 5. coTCRcys-expressing CD4⁺ T cells produce multiple cytokines following stimulation with T2-A*1101 cells prepulsed with SSC peptide. A, IL2 production by coTCRcys-T cells stimulated with T2-A*1101 + SSC (solid line) compared with coTCRcys-T cells stimulated with T2-A*1101 alone (dashed line), or mock-T cells stimulated with T2-A*1101 + SSC (gray area). B, the percentage of these IL2-producing coTCRcys-T cells that also produced TNF α and/or IFN γ . All data shown were gated on CD4⁺ T cells. Thresholds for positive cytokine staining were determined from coTCRcys-T cells stimulated with T2-A*1101 alone. Results are representative of five separate experiments.

Furthermore, both engineered CD8⁺ and CD4⁺ T cells were cytotoxic, lysing A*1101-positive HONE1 cells expressing LMP2 from a recombinant vaccinia vector with or without addition of the SSC peptide (Fig. 4C).

An increased frequency of CD4 T cells with multifunctional capacity for cytokine production is associated with improved control of some infections (28). Using intracellular staining, we showed that coTCRcys-transduced CD4⁺ T cells can simultaneously produce multiple cytokines (IL2, IFN γ , and TNF α) following antigen-specific stimulation (Fig. 5).

In vivo studies with an LMP2⁺ epithelial tumor model

Currently, there are no appropriate animal models of NPC to test the therapeutic potential of these T cells. Therefore, we engineered another human epithelial tumor (MDA-MB-231) to coexpress LMP2 and A*1101 as well as luciferase for bioluminescence imaging. Immunodeficient mice carrying this tumor were treated with coTCRcys-expressing T cells. Flow cytometric analysis showed the infused T cells contained a CD4:CD8 ratio of 3:2, with 50% CD4 and 60% CD8 T cells expressing the SSC-specific TCR. Tumor growth in these mice was significantly reduced compared with that in control mice that received mock-transduced T cells (Fig. 6).

TCR transduction of T cells from patients with advanced NPC and recognition of NPC cell lines

Finally, we sought to determine whether coTCRcys-transduced T cells from patients with advanced NPC could respond to NPC cell lines expressing LMP2. All NPC tumors are EBV⁺, with the exception of c666.1, NPC lines established *in vitro* have lost the EBV genome, and even c666.1 does not express LMP2 protein. Therefore, having introduced the restricting HLA allele into c666.1 by retroviral transduction (c666.1/A*1101), we expressed LMP2 from a recombinant modified vaccinia (Ankara) vector with or without addition of the SSC peptide. Transduced T cells from two advanced NPC patients clearly responded by producing IFN γ in an antigen-specific manner to LMP2-expressing c666.1/A*1101 cells. Similar levels of response were seen with antigen-loaded A*1101-matched fibroblasts and HONE1 cells (Fig. 7A). These

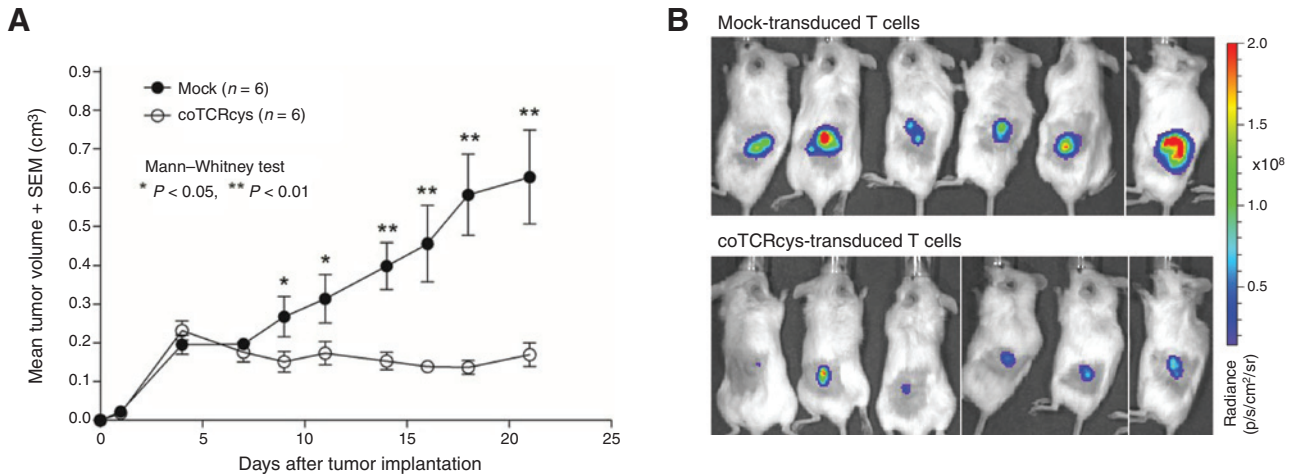


Figure 6.

coTCRCys-transduced T cells control tumor growth *in vivo*. NSG mice were injected with A*1101⁺ LMP2⁺ MDA-MB-231 tumor cells then treated with T-cell infusions (6 mice/group). Tumor size, measured by calipers (A) or bioluminescence (B), showed significant inhibition of tumor growth by coTCRCys-transduced T cells compared with mock-T cells. Bioluminescence images were taken 17 days after T-cell infusion.

T cells were also tested for cytotoxic activity toward NPC cell lines, and here, we included a second NPC line HK1, which again had to be transduced to express A*1101 (HK1/A*1101). Transduced (but not mock-transduced) T cells lysed both HK1/A*1101 and c666.1/A*1101 cells in an LMP2-specific manner (Fig. 7B).

Discussion

That NPC is responsive to EBV-specific T-cell–based therapies is apparent from studies using adoptive T-cell therapy (6–9). However, current approaches to generate such cells for infusion are both time consuming and unreliable. Therefore, we explored the use of TCR gene transfer, a technology that can reliably generate large quantities of specific T cells in a few days, regardless of the patient's preexisting immune response. Having identified a T-cell clone with high avidity for the HLA A*1101-restricted LMP2 epitope SSC, we cloned the genes encoding the TCR and through retroviral-mediated gene transfer expressed them in T cells from healthy donors and advanced NPC patients. T cells from healthy donors engineered to express a modified form of the TCR responded in an antigen-specific manner by proliferating, generating cytokines (IFN γ , TNF α , and IL2), lysing target cells and inhibiting LMP2⁺ tumor growth *in vivo*. TCR-transduced T cells from advanced NPC patients could also recognize NPC cell lines expressing the LMP2 protein.

As described in the methods, retroviral transduction requires only 48 hours of culture to preactivate T cells, and scaling up the process by starting with large numbers (10^9 – 10^{10}) of T cells available from leukapheresis of patients, it should be possible to engineer $>10^8$ – 10^9 T cells for infusion in a few days. Including a few days more for *in vitro* expansion, trials of TCR gene transfer have infused 10^9 – 10^{11} T cells per patient (13, 14). This greatly exceeds the dose used to successfully treat patients with NPC by adoptive therapy with LCL-reactivated T cells (7), in which patients received only 4×10^7 to 4×10^8 cells/m², and LMP-specific and SSC-specific T cells comprised $<1\%$ and $<0.05\%$ of this product, respectively (29). T cells transduced with the coTCRCys receptor contained a mixture of naïve, central–memory, and effector–memory cells (Supplementary Fig. S2). The presence

of less differentiated T cells suggests that they should persist and display greater antitumor responses *in vivo* (30).

We focused on an A*1101-restricted TCR because this HLA allele is very common in the populations most at risk for NPC. Indeed, approximately 40% of NPC patients are A*1101⁺ (31, 32), and are therefore available for treatment with an A*1101-restricted SSC-specific TCR. Encouragingly, several studies have also reported that A*1101 is associated with decreased risk of NPC (31, 32), supporting our hypothesis that SSC peptide is a good target for T-cell therapy. Furthermore, transiently boosting of T-cell responses to this epitope in A*1101⁺ NPC patients using SSC peptide-pulsed dendritic cells is safe and can induce partial clinical responses (33). The SSC epitope sequence, originally identified using standard laboratory strain B95.8, is largely conserved in EBV strains within the Southern Chinese population, including virus isolates from NPC tumors (23, 34). In Northern China an S–T mutation in residue 9 of the epitope has been detected in 50% of NPC patients (35). However, from our previous studies, we found no evidence that this mutation affects antigenicity of the epitope (23).

T-cell–based therapies targeting a single epitope could lead to selection of tumor cells carrying epitope-loss EBV variants. However, this could be avoided by using multiple TCRs targeting additional epitopes in NPC-associated EBV proteins. Indeed several epitopes have already been described, some of which are again restricted through HLA class I and II alleles present at relatively high frequency in the Chinese population (23, 36), thereby increasing the number of patients available for a TCR gene transfer-based therapy. Combining TCR gene transfer with vaccination (37) could also amplify and broaden the EBV-specific T-cell response *in vivo*.

If T-cell therapy is to be effective for NPC, antigen-presenting function in the tumor cells must be intact. Results from immunohistochemical analysis of NPC tissues have indicated that critical components of the HLA class I antigen-processing pathway may be downregulated in some NPC tumors (38). Furthermore, there is evidence for other potential immune evasion mechanisms in NPC, including the presence of regulatory T cells (39) and transforming growth factor beta (40). Nevertheless, results from

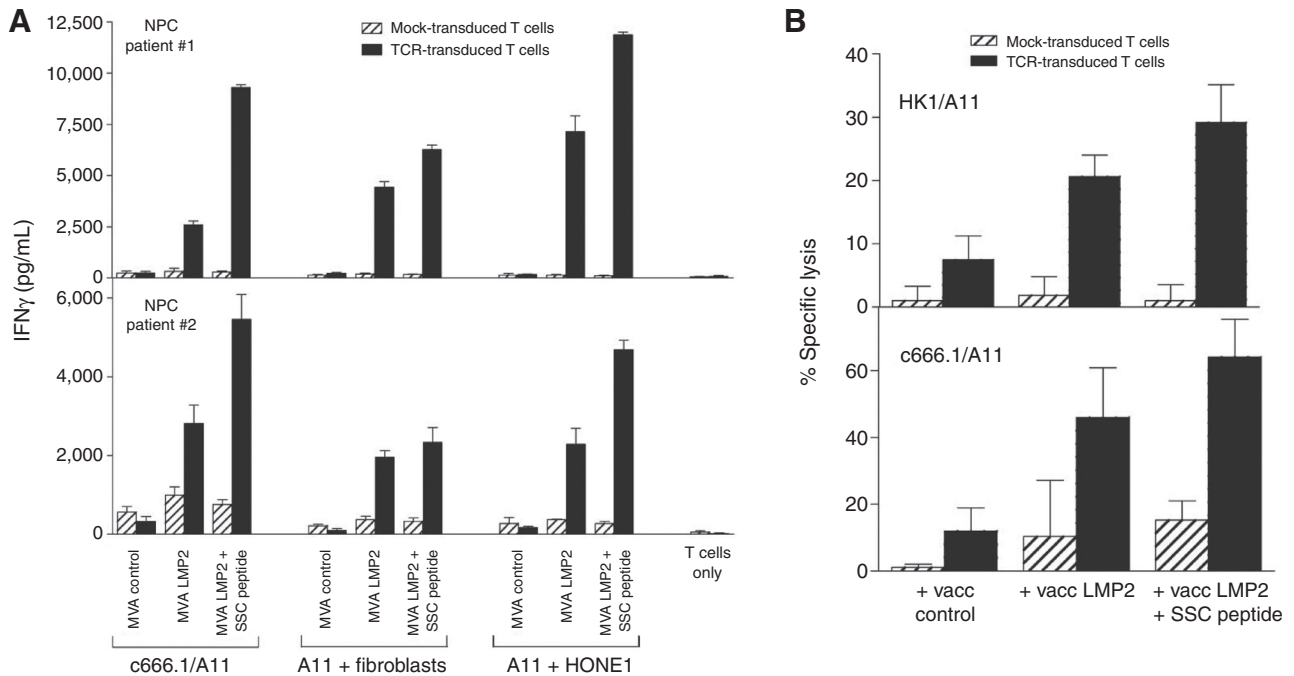


Figure 7.

Functional testing of coTCRCys-transduced T cells from patients with advanced NPC. A, IFN γ production following stimulation with A*1101⁺ targets infected with a recombinant modified vaccinia vector expressing LMP2 (MVA LMP2) or empty vector (MVA control). MVA LMP2-infected targets were also tested after pulsing with SSC peptide. Mock-transduced T cells from the same donors were used as controls. Target cells alone produced <10 pg/mL IFN γ . B, cytotoxic activity of coTCRCys- or mock-transduced T cells from a patient with advanced NPC when cocultured with NPC cell lines (HK1/A*1101 and c666.1/A*1101; effector:target = 6:1). Targets were infected with recombinant vaccinia vector-expressing LMP2 (vacc LMP2) or with empty vector (vacc control). Some vacc LMP2-infected targets were prepulsed with SSC peptides. All results shown represent mean + SD and are representative of three to five separate experiments.

in vitro studies on NPC cell lines (41), including data presented in this report, and the association of A*1101 with reduced risk for NPC (31, 32) suggest that the malignant cells can present antigen to T cells. More importantly, clinical responses following adoptive T-cell therapy (6–9) and vaccination (33) indicate that immune evasion mechanisms can be overcome at least in some patients. Indeed, effective delivery of large numbers of tumor-specific IFN γ -producing cytotoxic T cells may be sufficient to overwhelm immunosuppressive factors. Additional genetic modifications of infused T cells, such as expression of a dominant negative TGF β receptor (42) may also help. If the patient's antigen-presenting function is compromised, successful treatment may yet be possible by targeting stromal cells if they cross-present tumor antigens. Cross-presentation appears dependent on HLA binding affinity of the target epitope (43), which suggests that SSC [predicted affinity (IC₅₀) = 14 nmol/L based on the Immune Epitope Database Analysis Resource] should be readily cross-presented, thereby also reducing the risk of tumor relapse through escape variants.

TCR gene transfer has been tested in the clinic to treat advanced melanoma and synovial cell sarcoma (13, 14). Combining these studies, objective clinical responses were seen in 22 of 87 patients treated. However, significant autoimmune reactions occurred in some patients in whom TCRs targeted self-proteins expressed on normal cells (13). In this respect, NPC is an ideal setting to test the potential of TCR gene transfer because foreign (viral) rather than self-antigens can be targeted using naturally occurring high-affinity TCRs. EBV is present in some normal lymphocytes, but only 1 to 50 per million circulating B cells and most of these lack viral

protein expression (44). Therefore, the risk of on-target toxicity with an EBV-specific TCR is minimal.

TCR gene transfer carries a potential risk of off-target toxicity due to mispairing of TCR chains generating novel autoreactive receptor specificities (45). Although such toxicity has not yet been reported in clinical trials, we have incorporated several approaches to reduce this risk with the coTCRCys receptor. Thus, genes encoding the TCR α - and β -chains were cloned into a single retroviral vector with a 2A peptide-linker to ensure equimolar expression in the same T cell. Furthermore, we incorporated a second disulphide bond between the α - and β -constant domains, which also improved TCR surface expression. To reduce this risk further, it is possible to knockdown expression of endogenous TCR chains using shRNA (46). Nevertheless, it may be prudent to incorporate a suicide gene (47) for selective deletion of infused cells should autoimmunity develop.

Several studies have highlighted the importance of CD4⁺ T cells in controlling tumor growth (48, 49), and the ability of our SSC-specific TCR to function in these cells is important for two reasons. First, a concurrent antigen-specific CD4⁺ T-cell response aids expansion and efficacy of cytotoxic CD8⁺ T cells (50). Indeed, when NPC patients were immunized with dendritic cells expressing SSC peptide, CD8⁺ T-cell responses to this epitope were boosted but only temporarily (33). The implication was that boosting EBV-specific CD4⁺ T cells was also required. When stimulated with SSC peptide, CD4⁺ T cells transduced with coTCRCys produced cytokines, including IL2, indicating that they could help sustain coTCRCys-transduced CD8⁺ T cells. Secondly, coTCRCys-transduced CD4⁺ T cells were cytotoxic, indicating that

they might destroy NPC cells directly. Therefore, the ability of this TCR to function in both CD8 and CD4 T cells increases its potential for treating NPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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