

**Human Leukocyte Antigen (HLA) A\*1101-restricted Epstein-Barr virus-specific T-cell  
receptor gene transfer to target Nasopharyngeal carcinoma**

Yong Zheng<sup>1</sup>, Greg Parsonage<sup>1</sup>, Xiaodong Zhuang<sup>1</sup>, Lee R Machado<sup>2</sup>, Christine H. James<sup>1</sup>,  
Asmaa Salman<sup>1</sup>, Peter F. Searle<sup>1</sup>, Edwin P. Hui<sup>3</sup>, Anthony T.C. Chan<sup>3</sup>, Steven P. Lee<sup>1</sup>

<sup>1</sup>School of Cancer Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

<sup>2</sup>School of Health, University of Northampton, Boughton Green Road, Northampton NN2 7AL, United Kingdom

<sup>3</sup>Partner State Key Laboratory of Oncology in South China, Sir Y.K. Pao Centre for Cancer, Department of Clinical Oncology, Hong Kong Cancer Institute and Prince of Wales Hospital, The Chinese University of Hong Kong.

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Correspondence should be addressed to S.P.L. ([s.p.lee@bham.ac.uk](mailto:s.p.lee@bham.ac.uk)).

Corresponding author: Steven P. Lee (email [s.p.lee@bham.ac.uk](mailto:s.p.lee@bham.ac.uk); tel (44) 121 414 2803; fax (44) 121 414 4486). School of Cancer Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

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## **Abstract**

Infusing virus-specific T-cells is effective treatment for rare Epstein-Barr virus (EBV)-associated post-transplant 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 2-3 months of *in vitro* culture and favour outgrowth of T-cells targeting viral antigens expressed within EBV+ lymphomas but not 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 HLA A\*1101-restricted TCR, which would be widely applicable since 40% of NPC patients carry this HLA allele. Studying both wild-type and modified forms we have optimised expression of the TCR and demonstrated high avidity antigen-specific function (proliferation, cytotoxicity, cytokine release) in both CD8+ and CD4+ T-cells. The engineered T-cells also inhibited LMP2+ epithelial tumour growth in a mouse model. Furthermore, transduced T-cells from patients with advanced NPC lysed LMP2-expressing NPC cell lines. Therefore, using this approach, within a few days large numbers of high avidity LMP2-specific T-cells can be reliably generated to treat NPC, providing an ideal clinical setting to test TCR gene transfer without the risk of autoimmunity through targeting self-antigens.

## **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/100,000 men(1). Early stage disease responds well to radiotherapy (+/- chemotherapy) but a study of 2687 patients treated in Hong Kong reported that over half presented with advanced disease (Stage III-IV) with 5 year disease-specific survival rates 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 undifferentiated NPC patients and is strongly implicated in pathogenesis of this and other human tumours(4). Despite its oncogenic potential, EBV is ubiquitous in the human population where 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 tumour-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-positive lymphomas. Infusing EBV-specific polyclonal T-cell lines is highly effective as a therapeutic and prophylactic treatment for rare EBV-positive lymphomas that occur in transplant recipients(5). However, to extend this treatment to more common EBV-positive tumours such as NPC, two issues must be addressed. Firstly, 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 post-transplant EBV+ lymphomas), the virus expresses at least six nuclear antigens, EBNA1, 2, 3A, 3B, 3C, -LP, and two latent membrane proteins, LMP1 and LMP2. Of these, the EBNA 3 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 anti-tumour effects, but the procedure is clearly suboptimal since the majority of virus-specific T-cells targeted EBV genes not expressed in the tumour(7,9). Secondly, generating T-cells by LCL-reactivation takes over 2 months of *in vitro* culture to establish an LCL and then selectively expand EBV-specific effectors. This is labour 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 NPC-associated EBV 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 tumour-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 ( $>10^8\text{-}10^{10}$  cells/patient) with the desired specificity, regardless of the patient's pre-existing immune repertoire. TCRs are expressed on the surface of all T-cells and determine antigenic specificity. Having identified a tumour 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 patients' T-cells expressing the same TCR and targeting the same tumour 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 HLA-A\*1101, an allele carried by >50% of the Chinese population. This epitope comprises the sequence SSCSSCPLSK (referred to subsequently as "SSC"). Having cloned a SSC-specific TCR, we have studied both wild-type and modified forms of this receptor to determine their expression and function in transduced T-cells, and 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 (PBMCs) were isolated from heparinised blood by density gradient centrifugation on lymphoprep (Axis Shield, Oslo, Norway). 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 Prof. 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 lines were then cultured under drug selection using 20 $\mu$ g/ml Hygromycin or 50 $\mu$ g/ml G418 (Life technologies, UK) respectively. Though originally described as an NPC 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 $\mu$ g/ml Hygromycin, 600 $\mu$ g/ml G418 and 1 $\mu$ g/ml puromycin. All of the above lines were cultured in RPMI1640 (Sigma) containing 10% foetal bovine serum (FBS; PAA, Pasching Austria), 2mM glutamine, 100 IU/ml penicillin, and 100pg/ml streptomycin (standard medium). Fibroblasts were grown from a skin biopsy cultured in DMEM (Sigma, UK) supplemented as described above. All T, B and fibroblast 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 material for this study was approved by the National Research Ethics Service, U.K., 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 gave written informed consent.

### Synthetic peptides and recombinant vaccinia viruses

Peptides were synthesized using Fluorenylmethoxycarbonyl chemistry by Alta Bioscience, Birmingham, U.K. 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, UK) and reverse transcribed. TCR- $\alpha$  and - $\beta$  genes were then amplified with the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions using the following primers: TCR $\alpha$  constant region: 5'-agcacaggctgtttacaatcttgc-3'; TCR $\beta$ 2 constant region: 5'-ggacacagattgggaggcagg-3'. TCR genes were subcloned into pCR2.1 (Life Technologies) vector and sequenced. The TCR- $\alpha$  (TCRVA22) and - $\beta$  (TRBV4.01) chains were then cloned into the same retroviral pMP71-PRE vector(22) (kindly provided by C. Baum, Hannover, Germany) separated by a 2A peptide linker from porcine teschovirus. Modified TCR genes were designed and produced by GeneArt (Regensburg, Germany).

#### Retroviral transduction of human T-cells

Phoenix amphotropic packaging cells were transfected with pMP71 retroviral vector and pCL ampho (Imgenex) using FuGENE HD (Roche) according to manufacturer's instructions and retroviral supernatant harvested 48 hours later. PBMCs were pre-activated for 48 hours using anti-CD3 antibody (OKT3; 30ng/ml) and interleukin-2 (IL2; 600U/ml; Chiron, Emeryville, CA) in standard medium containing 1% human AB serum (TCS Biosciences, Buckingham,UK). These cells were then transduced with retroviral supernatant (or mock-transduced with conditioned supernatant from non-transfected phoenix cells) using retronectin-coated (Takara, Shiga, Japan) 6-well plates according to manufacturer's instructions. Cells were then maintained in standard medium containing 1% human AB serum and IL2 (100U/ml).

#### Flow cytometry

Cells were stained for 10 minutes at room temperature with a HLA-A\*1101/SSC pentamer (5 $\mu$ g/ml; ProImmune, Oxford, U.K.) according to the manufacturer's instructions. Cells were then washed

and stained on ice for 30 minutes with Pro5 Fluorotag (APC or R-PE-labelled; 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 two hours with T2-A11 cells prepulsed with or without SSC peptide (5 $\mu$ g/ml). Brefeldin A (10 $\mu$ g/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 permeabilisation buffers (E-bioscience, San Diego, CA) according to the manufacturer's instructions, cells were incubated for 30 minutes at 4°C with anti-cytokine antibodies (IL2-PE, IFN $\gamma$ -PECy7 and TNF $\alpha$ -APC) or an isotype- and concentration-matched control antibody (BD Pharmingen), then washed twice in PBS. Cells were analysed using an LSRII cytometer (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR).

#### CFSE labelling

T-cells were washed twice with PBS and incubated with 2.5 $\mu$ M Carboxyfluorescein succinimidyl ester (CFSE) for 10 minutes at 37°C. The labelling reaction was quenched by addition of RPMI-1640 containing 10% FBS. Cells were washed, resuspended in standard growth medium at 2x10<sup>6</sup> cells/ml, cocultured for 5 days with T2-A\*1101 cells pre-pulsed with SSC peptide (10 $\mu$ g/ml), then analysed by flow cytometry as described above.

#### IFN $\gamma$ release assay

Stimulator cells (5x10<sup>4</sup>/well) were co-cultured in triplicate with T-cells at responder:stimulator ratios indicated. Cells were incubated at 37°C/5% CO<sub>2</sub> in 100 $\mu$ l/well of Iscove's modified dulbecco's medium (Life Technologies) supplemented with 10% FBS and IL2 (25U/ml). After 18 hours, culture supernatant was tested for secreted IFN $\gamma$  using an ELISA (Pierce Endogen, Rockford, IL) 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 (2500 targets/well) and harvested after 5 or 8 hours. These have been described in detail previously(23).

*In vivo* tumour protection experiments

6-8 week old female NSG mice (Charles River Laboratories) were inoculated subcutaneously on the flank with MDA-MB-231 cells expressing A\*1101, LMP2 and luciferase ( $5 \times 10^6$  cells/mouse) in matrigel (BD Biosciences). One day later, mice received  $10^7$  TCR-transduced (or mock-transduced) T-cells intravenously. Intraperitoneal injections of  $10^4$  units IL2 were given on days 2, 4, 7, 9 and 11. Tumour growth was measured in a blinded fashion with callipers 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  $10^{-10}$ M peptide (Fig.1a). When tested for IFN $\gamma$  production in response to A\*1101-matched and mismatched LCL targets, a clear A\*1101-restricted response was observed (Fig.1b). Importantly, the clone recognised 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 most at risk of NPC.

Genes encoding TCR- $\alpha$  and - $\beta$  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 mock-transduced cells), but 3 days post 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.

Functional testing of this wild-type TCR began using transduced polyclonal T-cells exploring their ability to make IFN $\gamma$  in response to T2:A\*1101 cells pulsed with SSC peptide at titrating concentrations. TCR-transduced T-cells clearly recognised peptide-pulsed targets with only  $10^{-10}$ M

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 fibroblasts infected with the empty control vector (fig.2d).

#### Optimisation of the TCR gene construct.

Previous studies have suggested that function and/or expression of transduced TCRs can be improved by codon-optimisation 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 generating novel, potentially autoreactive TCRs. Therefore we generated two variants of our wild-type SSC-specific TCR, a codon-optimised version (coTCR) and a codon-optimised TCR in which amino acid residue 48 of the TCR- $\alpha$  chain and residue 57 of the TCR- $\beta$  chain were both changed to cysteine, thus introducing a second disulfide bond (coTCRcys)(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 seen with the coTCRcys construct (Fig.3a). Similar results were seen with CD4+ T-cells (data not shown). Not only was coTCRcys receptor expressed on a greater proportion of T-cells, but also levels of expression on individual cells were increased (fig.3b). These data are consistent with previous reports that introducing a second disulphide bond reduces mispairing with endogenous TCR chains(25). Staining transduced cells with an antibody to V $\beta$ 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  $\beta$ -chain and endogenous  $\alpha$ -chains. However an antibody was not available to stain the exogenous V $\alpha$ 22-chain, therefore it

is possible that increased expression of coTCRcys is a result of reduced mispairing between the exogenous  $\alpha$ -chain and endogenous  $\beta$ -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 optimisation alone (coTCR) affected neither surface expression nor functional activity (Fig.3), other studies have shown that despite such lack of *in vitro* effects, codon optimisation can nevertheless improve both frequency of TCR-modified T-cells detectable post-infusion and anti-tumour activity *in vivo*(26,27). Therefore the coTCRcys-construct was selected for further testing. Analysing the differentiation status of coTCRcys-transduced cells showed 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 optimised expression of the SSC-specific TCR, to explore its therapeutic potential we then determined the ability of coTCRcys-transduced T-cells to recognise LMP2 protein expressed at physiological levels in an LCL. For this we used cloned populations of TCR-transduced cells to study functional activity in CD8+ cells, which can have direct anti-tumour effects *in vivo*, and CD4+ cells, which can help generate and maintain effective CD8+ responses and 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 $\gamma$  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-labelling, 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 to T2-A\*1101 alone) (Fig.4b). 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 coTCRcys-transduced CD4+ T-cells can simultaneously produce multiple cytokines (IL2, IFN $\gamma$ , TNF $\alpha$ ) following antigen-specific stimulation (Fig.5).

#### In vivo studies with an LMP2+ epithelial tumour 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 tumour (MDA-MB-231) to co-express LMP2 and A\*1101 as well as luciferase for bioluminescence imaging. Immunodeficient mice carrying this tumour 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. Tumour growth in these mice was significantly reduced compared to 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 lines expressing LMP2. All NPC tumours are EBV-positive, but 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 IFN $\gamma$  production 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 T-cells were also

tested for cytotoxic activity towards NPC 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 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 pre-existing 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 $\gamma$ , TNF $\alpha$  and IL2), lysing target cells and inhibiting LMP2+ tumour growth in vivo. TCR-transduced T-cells from advanced NPC patients could also recognise 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, in a few days it should be possible to engineer  $>10^8$ - $10^9$  T-cells for infusion. 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 NPC by adoptive therapy with LCL-reactivated T-cells(7) where patients received only  $4 \times 10^7$ - $4 \times 10^8$  cells/m $^2$ , 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 they should persist and display greater anti-tumour responses in vivo(30).

We focussed on an A\*1101-restricted TCR because this HLA allele is very common in the populations most at risk of 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 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 tumours(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 affects antigenicity of the epitope(23).

T-cell-based therapies targeting a single epitope could lead to selection of tumour 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 tumour cells must be intact. Immunohistochemical analysis of NPC tissue has suggested critical components of the HLA class I antigen processing pathway may be downregulated in some NPC tumours(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, *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 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 tumour specific IFN $\gamma$ -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 $\beta$  receptor(42) may also help. If antigen presenting function is compromised, successful treatment may yet be possible by targeting stromal cells if they cross-present tumour antigens. Cross-presentation appears dependent on HLA binding affinity of the target epitope(43) which suggests SSC (predicted affinity (IC50) = 14nM based on the Immune Epitope Database Analysis Resource) should be readily cross-presented, thereby also reducing risk of tumour 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 with 22/87 patients treated. However, significant autoimmune reactions occurred in some patients where 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 since foreign (viral) rather than self-antigens can be targeted using naturally occurring high affinity TCRs. EBV is present in some normal lymphocytes, but only 1-50/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, and we found little evidence of mispairing at least with the exogenous  $\beta$  chain (Supplementary Fig.S1)), we have incorporated several approaches to reduce this risk with the coTCRcys receptor. Thus genes encoding the TCR  $\alpha$ - and  $\beta$ -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  $\alpha$ - and  $\beta$ -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 tumour growth(48,49) and the ability of our SSC-specific TCR to function in these cells is important for two reasons. Firstly, a concurrent antigen-specific CD4+ T-cell response aids expansion and efficacy of cytotoxic CD8+ T-cells(50). Indeed when NPC patients were immunised 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 they could help sustain coTCRcys-transduced CD8+ T-cells. Secondly, coTCRcys-transduced CD4+ T-cells were cytotoxic, indicating 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.

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## **Figure Legends**

### Figure 1

Characterising 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 $\gamma$  production. Target cells alone produced <100 pg/ml IFN $\gamma$ . Responder:stimulator ratio = 1:10. Results show mean+SD and are representative of 3 separate experiments.

### 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 PBMC from an advanced NPC case (TCR-T) compared with mock-transduced cells (Mock-T). Values shown refer to percentage of pentamer-positive 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 $\gamma$ -release. Mock-transduced T-cells (mock-T) were included as a control. Responder:stimulator ratio = 1:4. Results show mean+SD and are representative of 3 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 3 separate experiments.

### Figure 3

Optimising TCR gene construct.(a) SSC-specific TCR expression 3 days post-transduction with wild-type TCR (WT TCR), a codon optimised version (coTCR) or a codon optimised 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 $\gamma$  release. T-cell input numbers were adjusted to ensure equivalent numbers of transduced effectors were tested for each TCR

construct. Responder:stimulator ratio = 1:3. Results show mean+SD. Mock-transduced T-cells (Mock-T) were included as a control. All results shown are representative of at least 3 separate experiments.

#### 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 $\gamma$  production. Target cells alone produced <100pg/ml IFN $\gamma$ . Responder:stimulator ratio = 1:10. Results show mean+SD and are representative of 7 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 alone. Results show mean+SD and are representative of 4 clones for each subset.

#### 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 (grey area). (b) The percentage of these IL2-producing coTCRcys-T-cells that also produced TNF $\alpha$  and/or IFN $\gamma$ . 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 5 separate experiments.

#### Figure 6

coTCRcys-transduced T-cells control tumour growth in vivo. NSG mice were injected with A\*1101+LMP2+ MDA-MB-231 tumour cells then treated with T-cell infusions (6 mice per group). Tumour size, measured by calipers (a) or bioluminescence (b) showed significant

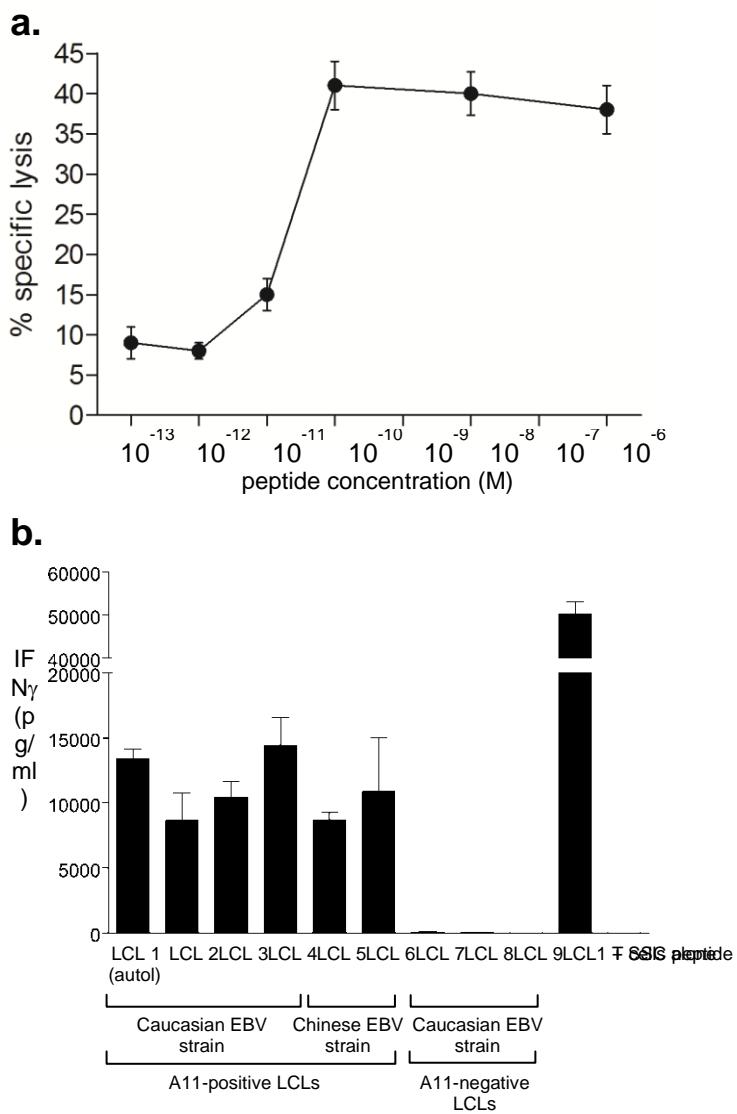
inhibition of tumour growth by coTCRcys-transduced T-cells compared with mock-T-cells.

Bioluminescence images were taken 17 days after T-cell infusion.

Figure 7

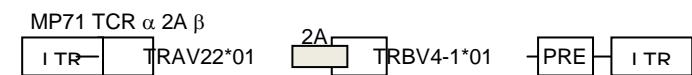
Functional testing of coTCRcys-transduced T-cells from advanced NPC patients. (a) IFN $\gamma$  production following stimulation with A\*1101+ targets infected with a recombinant modified vaccinia vector expressing LMP2 (MVA LMP2) or empty vector (MVA control). MVA LMP2-infected targets were also tested after pulsing with SSC peptide. Mock-transduced T-cells from the same donors acted as controls. Target cells alone produced <10pg/ml IFN $\gamma$ . (b) Cytotoxic activity of coTCRcys- (or mock-) transduced T-cells from an advanced NPC patient when co-cultured with NPC 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 empty vector (vacc control). Some vacc LMP2-infected targets were pre-pulsed with SSC peptide. All results shown represent mean+SD and are representative of 3-5 separate experiments.

**Figure 1.**

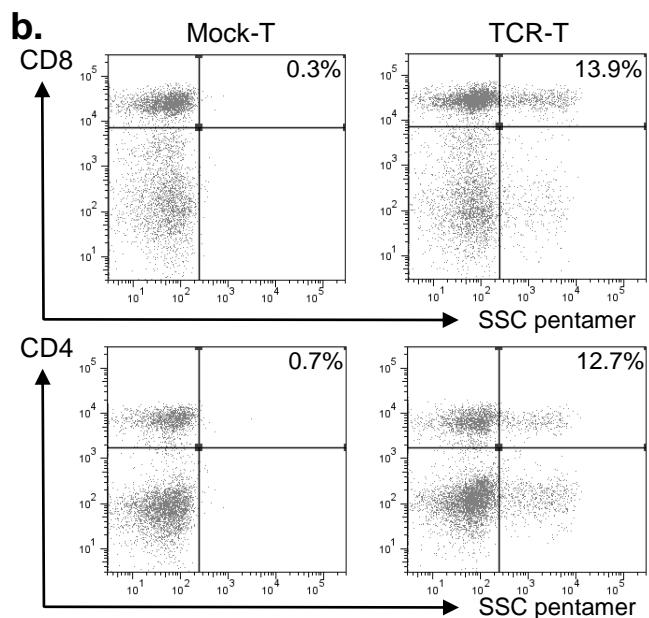


**Figure 2.**

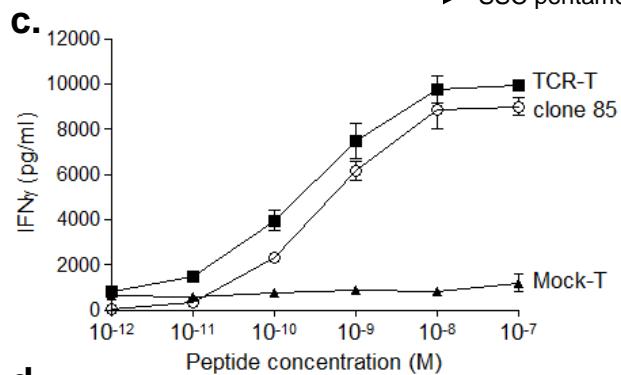
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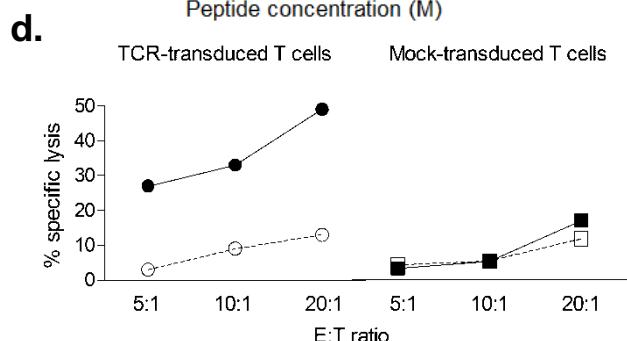
**b.**



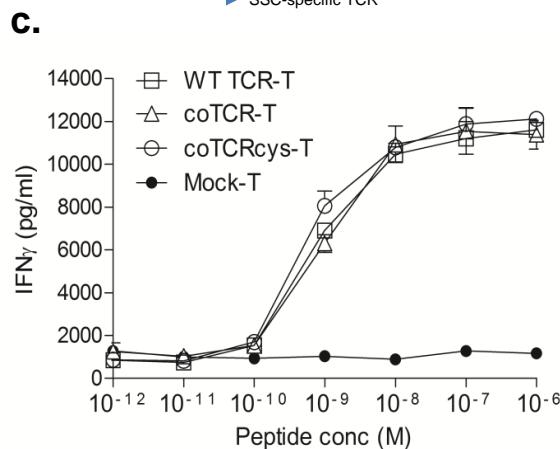
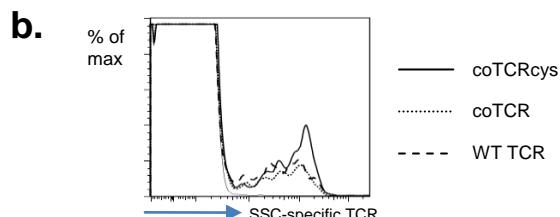
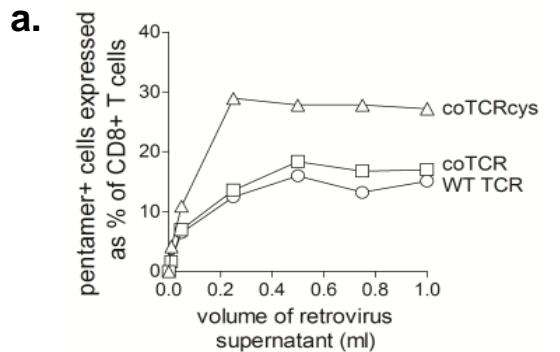
**c.**



**d.**

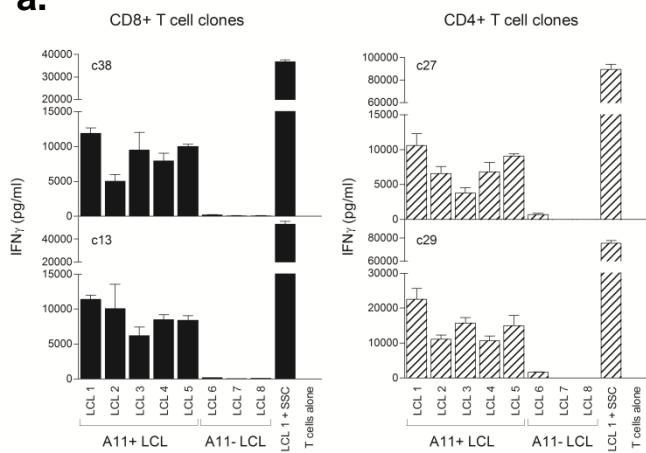


**Figure 3.**

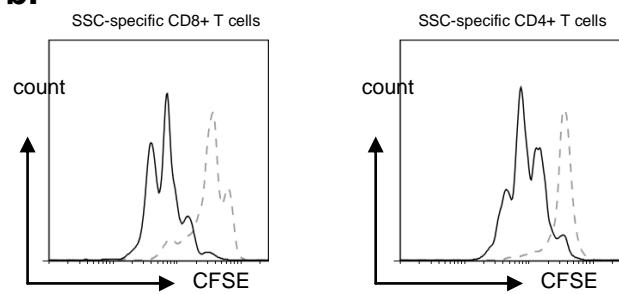


**Figure 4.**

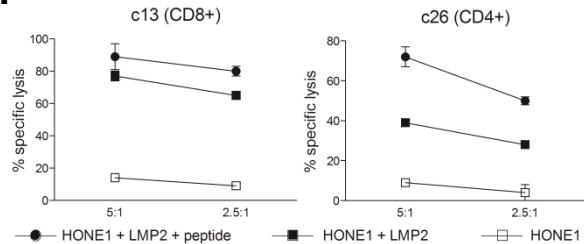
**a.**



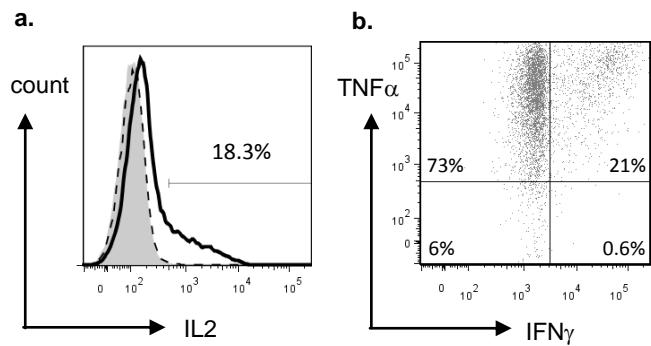
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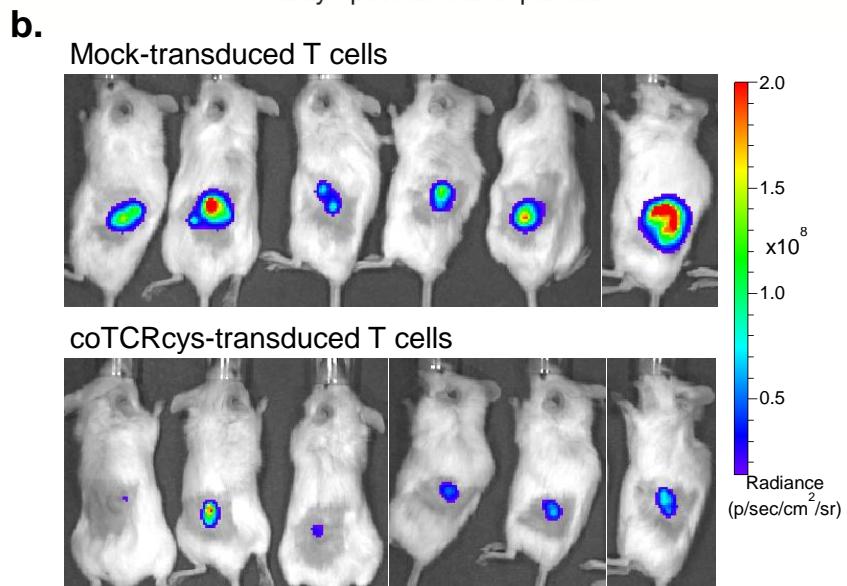
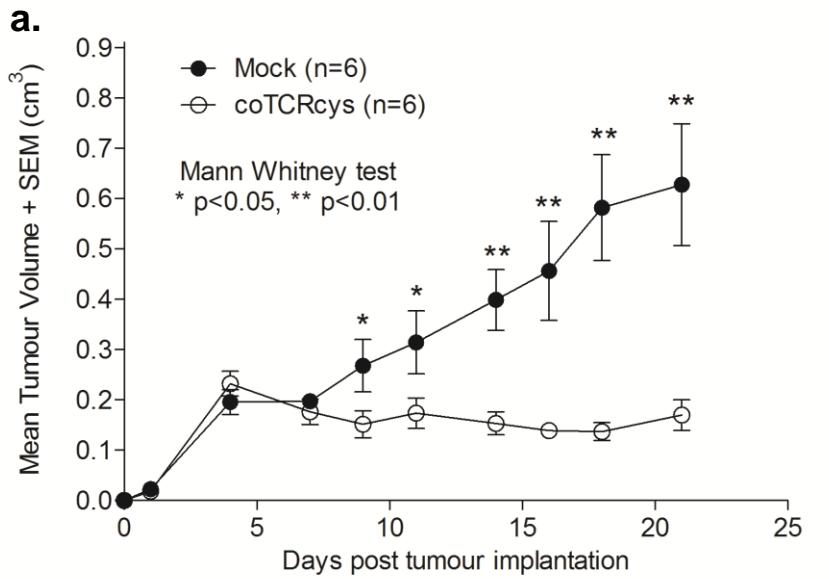
**c.**



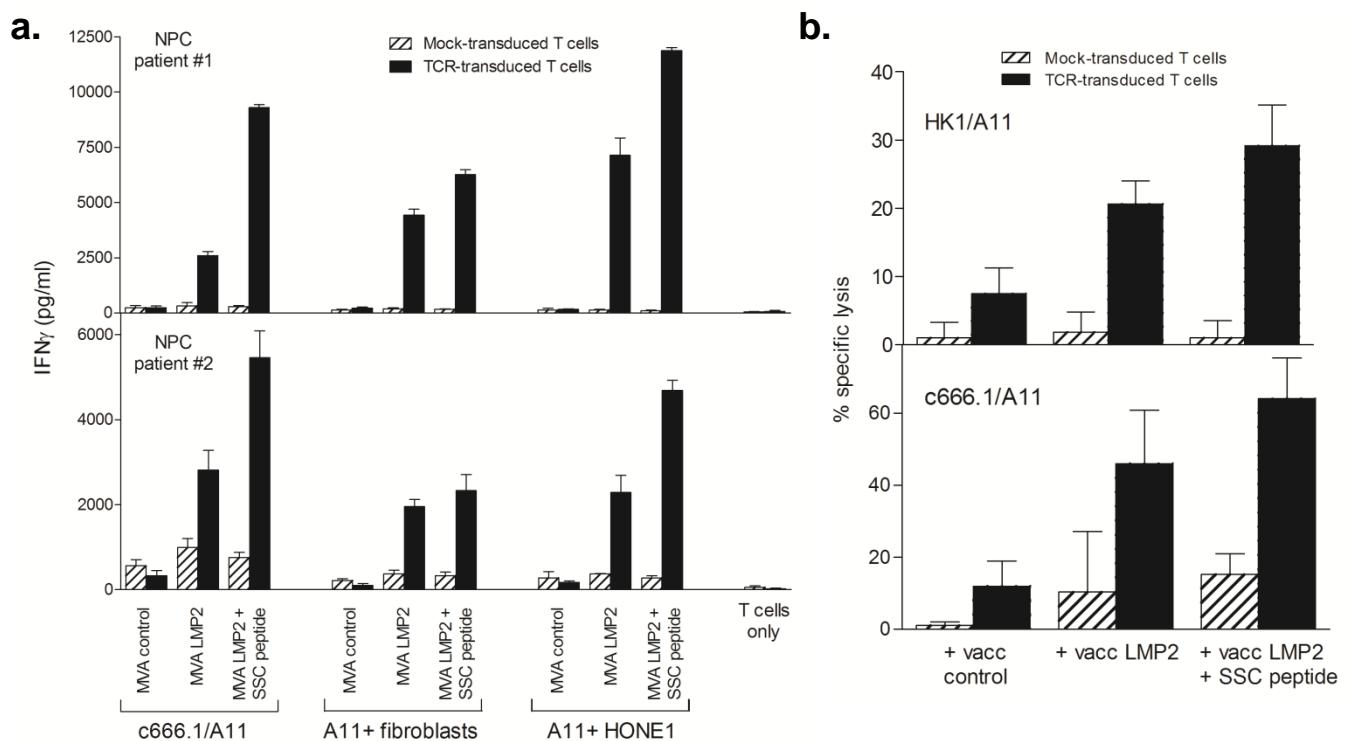
**Figure 5.**

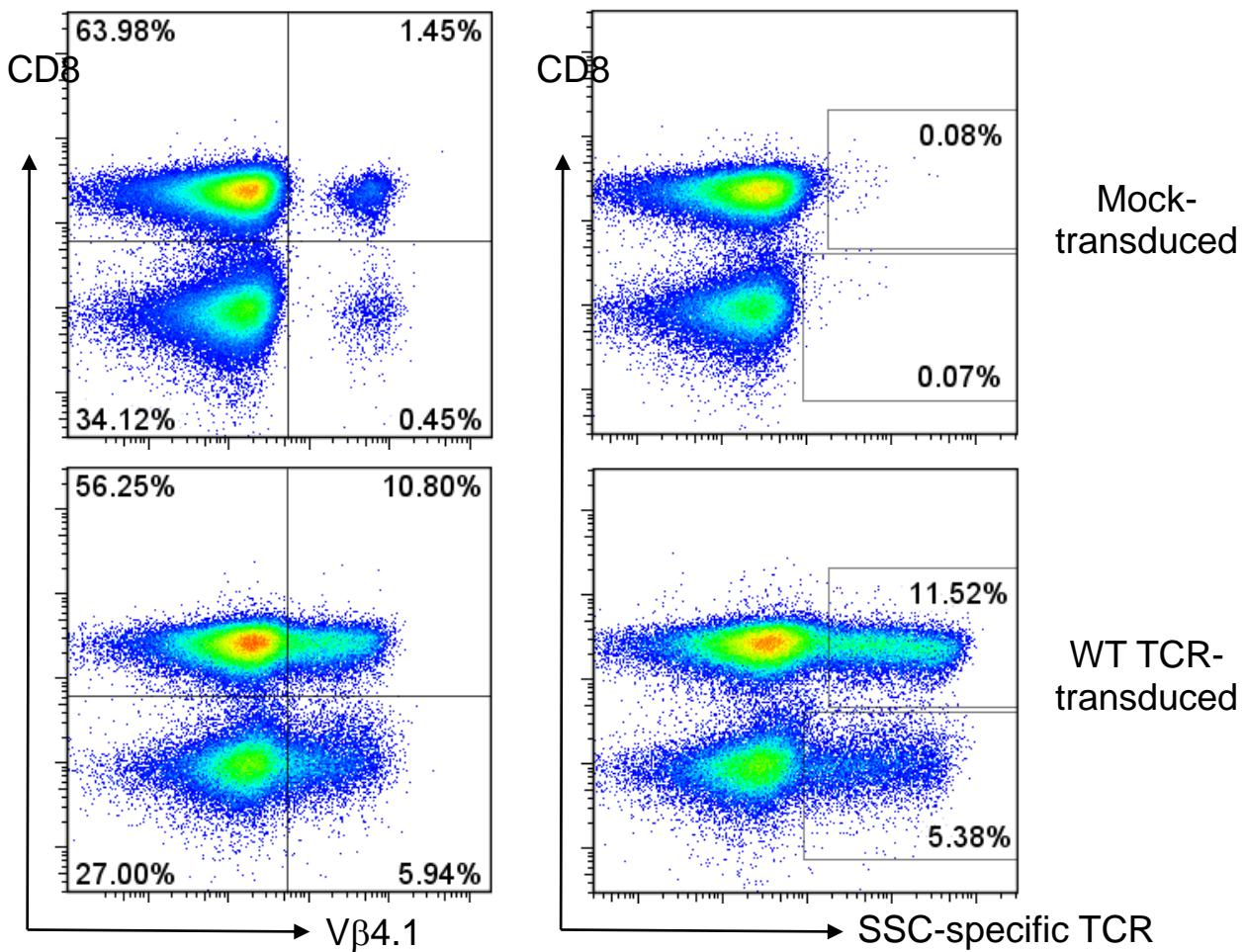


**Figure 6.**



**Figure 7.**

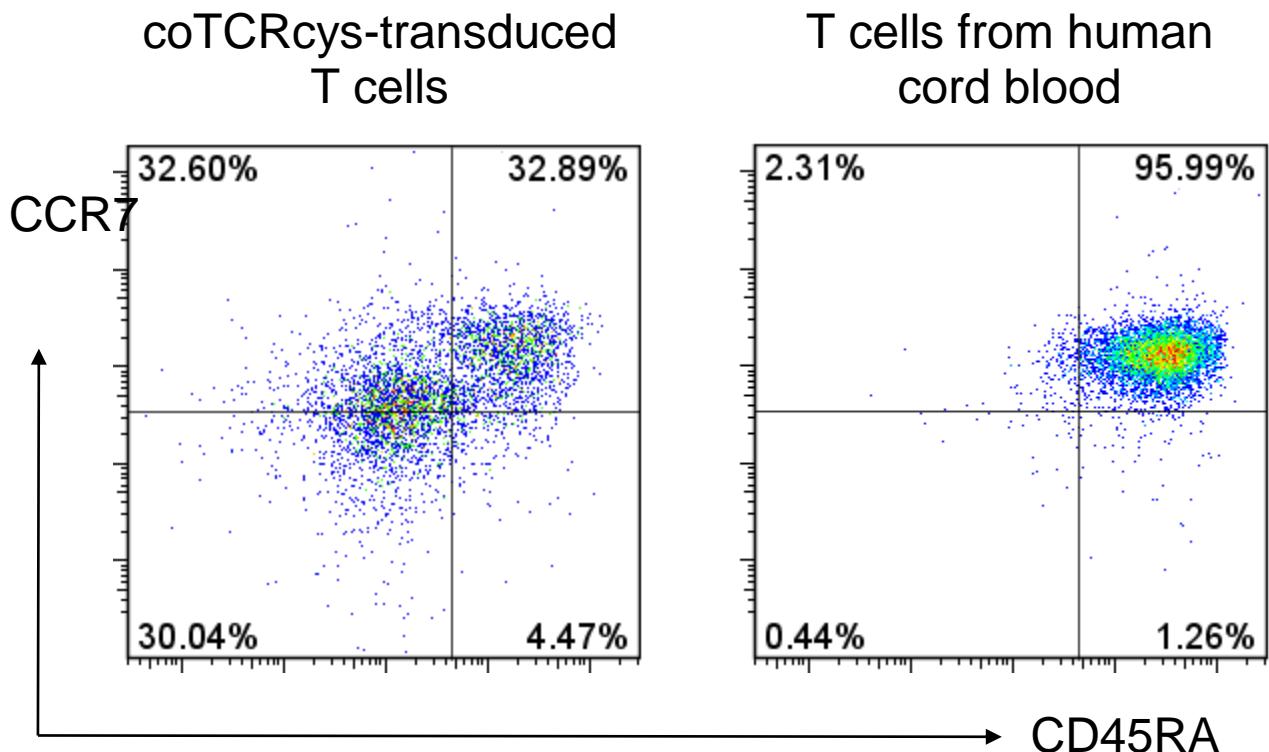




### Supplementary fig S1

Minimal mispairing with the exogenous TCR  $\beta$  chain was shown using Mock- and WT TCR-transduced T-cells from an NPC patient, where the percentage of cells that were engineered to express V $\beta$ 4.1 was similar to the percentage expressing the SSC-specific TCR. Data shown are gated on live CD3+ cells and are representative of that found with 3 patients using all three forms of the TCR (WT-TCR, coTCR and coTCRcys)

Staining for the SSC-specific TCR, CD3 and CD8 was conducted using a HLA-A\*1101/SSC pentamer, anti-CD3-FITC and anti-CD8-AmCyan (Pharmingen) as described in the methods section. Staining for V $\beta$ 4.1 was conducted on ice for 30 minutes with saturating concentrations of a PE-conjugated antibody (Immunotech). Dead cells were excluded using a viability dye (Life Technologies) according to the manufacturer's instructions.



### Supplementary fig S2

T cells from an NPC patient transduced 3 days earlier to express coTCRcys contain a mixture of mainly naïve (CCR7+ CD45RA+), central memory (CCR7+ CD45RA-) and effector memory (CCR7- CD45RA-) phenotypes. Data shown in the left panel were gated on CD3+ live lymphocytes that expressed the SSC-specific TCR and are representative of that obtained with two donors. Thresholds for positive CCR7 and CD45RA staining were determined with human cord blood (right panel) where the vast majority of CD3+ live lymphocytes are naïve (i.e. CCR7+ CD45RA+).