Induction of EBV latent membrane protein-2A (LMP2A)-specific T cells and construction of individualized TCR-engineered T cells for EBV-associated malignancies

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ABSTRACT

Background Latent membrane protein-2A (LMP2A)-specific TCR-engineered T cells could be a promising treatment approach to Epstein–Barr virus-associated malignancies. However, previous studies mainly reported LMP2A-reactive TCRs only focusing on specific HLA subtypes and corresponding epitopes, and thus, they were only suitable for patients with specific HLA.

Methods Due to hugely varied HLA subtypes and presented LMP2A epitopes in different individuals, our study attempted to develop an individualized approach, based on the weekly in vitro stimulation of peripheral T cells for 2 weeks with autologous dendritic cells (DCs) pulsed with a pool of LMP2A peptides covering LMP2A whole protein and combination analysis of high throughput TCR β sequencing of prestimulated and poststimulated T cells and single-cell TCR sequencing of poststimulated T cells, and to identify LMP2A-specific TCRs of which poststimulated frequencies significantly increased than corresponding prestimulated frequencies.

Results Following this approach, multiple LMP2A-reactive TCRs were identified, optimized and cloned into lentiviral vector, and then transduced into peripheral T cells. These engineered T cells were demonstrated to specifically recognize the LMP2A presented by autologous DCs and lymphoblastoid cell lines in vitro and in vivo.

Conclusions This approach provides an efficient procedure to isolate individualized LMP2A-specific TCRs for basic and translational research, as well as for clinical applications.

BACKGROUND

Epstein–Barr virus (EBV) is associated with multiple malignancies, which are distinguished by three different patterns of viral latency-associated gene expression.1 2 Most remarkable clinical outcomes were achieved with EBV-specific T cells against post-transplant lymphoproliferative disease expressing the complete array of EBV latency III antigens.3 4 By contrast, EBV-positive Hodgkin’s lymphoma, NK/T-cell lymphoma and nasopharyngeal carcinoma typically express more restricted and weakly immunogenic EBV latency II antigens including EBNA-1, LMP1 and LMP2A.5 Since EBNA-1 is processed and presented poorly to CD8+ T cells,6 7 LMP1 demonstrates significant sequence variability among different viral strains8 and by contrast, LMP2A is consistently expressed and moreover its epitopes are conserved.9 LMP2A could be an attractive target antigen to generate EBV-specific T cells for patients with EBV latency II tumors.

Since most individuals have a low but measurable frequency of LMP2A-specific T cells in peripheral blood, several groups developed in vitro stimulation protocols to enrich LMP2A-specific T cells for immunotherapy.10–12 Although clinical effectiveness of adoptively transferred LMP2A-specific T cells were intermittently observed in patients with relapsed or resistant cancer, the overall objective response rates were low.4 12–14 Due to low frequency of circulating LMP2A-specific T cells in most individuals, most infused LMP2A-reactive T cells were extensively expanded and terminally differentiated with limited replicative capacity and could not persist long-term in vivo, which could mainly result into low response rates.15

Considering this, numerous groups attempted to obtain LMP2A-specific TCR-engineered T cells (TCR-Ts) as an alternative approach to rapidly obtain large numbers of LMP2A-reactive T cells for immunotherapy.16 17 Although previous studies reported that LMP2A-specific TCR-Ts demonstrated promising results for LMP2A-expressing tumor,16 they only focused on specific HLA subtypes and corresponding epitopes, and thus, they were only suitable for patients with specific HLA subtypes. Since...
HLA typing of donors

DNA of donors’ peripheral blood was extracted with DNAeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer’s protocol. Genotypes of HLA alleles were performed using high-resolution, high-throughput HLA genotyping with deep sequencing (BGI Diagnosis, Shenzhen, China). The HLA types of donors were outlined in online supplemental table S1.

Cell staining and flow cytometry

Staining steps were performed at room temperature for 15 min with PBS washes between steps. Flow cytometry antibodies were used (all anti-human; clone IDs are given in parentheses): Fixable Viability Stain 780 (FVS780), CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD45RA (HI100), CCR7 (150503), CD25 (M-A251), CD137 (4B4-1), CD107A (H4A3), CD107b (H4B3). All antibodies were from BD Biosciences, except for anti-mouse TCR-β constant region (clone H5-597, eBioscience, USA).

Immunohistochemical staining

Fresh biopsy samples from Patient-Derived Xenograft (PDX) model were fixed in 10% formalin and embedded in paraffin tissue blocks. Then, tissues were sectioned at 5 μm and prepared according to standard methodology for routine histology. For immunohistochemistry, the investigated antibodies included mouse monoclonal anti-CD3 (clone LN10; ZSGB-BIO, Beijing, China) and mouse monoclonal anti-PD1 (clone UMAB199; ZSGB-BIO). Counterstaining was performed using H&E and bluing reagent from ZSGB-BIO; slides were mounted with coverslips and air-dried.

Generation and isolation of LMP2A-reactive T cells after repeated stimulation with LMP2A peptide mix

PBMCs was incubated for 7 days in T-cell media consisting of X-VIVO 15 (Lonza, USA), Glutamax (Life Technologies), IL-2 (50 U/mL, Perprotech, USA), IL-7 (10 ng/mL, Perprotech) and IL-15 (10 ng/mL, Perprotech). And then 5x10^6 PBMCs (S0) were cocultured with 5x10^5 autologous irradiated (3000 rad) DCs pulsed with commercially available LMP2A peptide mix (Miltenyi Biotec, Germany) at a concentration of 10 μg/mL in T-cell medium for 7 days, after which they were re-stimulated with autologous irradiated (3000 rad) DCs pulsed with commercially available LMP2A pepmixes one more time to generate LMP2A-reactive T cells (S2). Furthermore, CD3+ T cells of S2 were sorted into 96-well PCR plates by single-cell sorting using BD FACS Aria II (Special Order System). And then, 96-well PCR plates were placed into liquid nitrogen and conserved at −80°C prior to performing single-cell TCR sequencing.

Single-cell TCR sequencing and analysis

TCRs present in CD3+ T cells of S2 were identified using single-cell TCR sequencing as described in our previous study. Briefly, TCR sequences from the sorted single cells were obtained using a series of two nested PCR reactions. PCR products were purified and sequenced by Sanger
sequencing method with Cα and Cβ region primers. The TCR sequences were analyzed using IMGT/V-Quest tool (http://www.imgt.org/).

**High-throughput sequencing of TCRβ**

S0 and S2 in each donor were harvested and used for high-throughput TCRβ sequencing. Detailed information of TCRβ sequencing has been described in our previous study. Briefly, the TCRβ was amplified and sequenced using Multiplex PCR and Illumina HiSeq2500 platform (MyGenostics, Beijing, China) from >500 ng of genomic DNA for each sample. The TCRβ information was identified based on the definition established by the International ImMunoGeneTics collaboration, and the V, D, J segments were discerned by a standard algorithm. Only productive reads that did not contain framemaps or stop codons were used for statistical analysis. On average, 2,186,966 TCR templates were detected with values ranging from 0 (most diverse) to 1 (least diverse).

**IFN-γ ELISPOT assay**

Human IFN-γ ELISPOT Kit (Abcam, USA) was performed as the manufacturer’s protocols. Briefly, 2×10⁶ T cells, rested in cytokine-free media overnight, were incubated with 1×10⁵ PBS-washed autologous DCs pulsed with LMP2A peptide mix at a concentration of 10 μg/mL for approximately 20 hours in the absence of exogenous cytokines. The number of colored spots was calculated by ImmunoSpot plate reader and associated software (Cellular Technologies, USA).

**IFN-γ ELISA assay**

For approximately 20 hours, 1×10⁶ T cells were incubated with 1×10⁵ autologous LCL_LMP2A or LMP2A peptide pulsed DCs in the absence of exogenous cytokines. IFN-γ level in coculture supernatant was measured using human IFN-γ ELISA kit (ExCell Bio, China) as the protocol’s procedure.

**Cytotoxic assay**

CFSE-based cytotoxicity assay was performed as shown in our previous study. LCL_LMP2A were labeled with 5 μM CFSE (BD Biosciences) for 15 min at 37°C and then cocultured with T cells at room temperature for 4 hours, at E:T ratio of 0.5:1, 2:5:1 and 10:1. After the coculture, 1 μg/mL propidium iodide (BD Biosciences) was added to evaluate the ratio of target cell death, and then the samples were analyzed by flow cytometry.

**Construction of lentivirus vectors and transduction of T cells**

TCRα/β chains were synthesized (GenScript) and cloned into our lentivirus vector. TCRs were constructed in a β-γ chain order and their constant regions were replaced by mouse counterparts modified with hydrophobic substitution and added disulfide bond as previously described, which not only was convenient for detection of TCR-T but also improved TCR pairing and TCR/CD3 stability. To obtain TCR-T, T cells were transduced with lentiviruses as previously described. Briefly, PBMCs were stimulated in T-cell media supplemented with 50 ng/mL OKT3 and 1 μg/mL anti-CD28 for 2 days before transduction. TCR lentivirus were obtained by cotransfection of 293-FT cells with both lentivector and packaging plasmids using PEI MAX 4000 (Polysciences, USA). The lentiviral supernatants were harvested at 48 and 72 hours after transfection and concentrated using ultracentrifugation method with 20,000 g, 90 min at 4°C. And then activated T cells were transduced by concentrated lentivirus with 8 μg/mL polybrene (Sigma-Aldrich, USA). Two days later, the transduction efficiency was evaluated using mouse TCR-β chain constant region staining by flow cytometry.

**In vivo antitumour experiments**

Each NOD/SCID mouse subcutaneously received 2×10⁶ LCL_LMP2A tumor cells. Three days later, each mouse was intravenously infused twice with 3×10⁶ TCR-T cells. To evaluate the ratio of target cell death, and then the products of the perpendicular diameters were recorded.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism V.7.0 (GraphPad Software, California, USA) and Stata V.11.0 (Stata Corp). Statistical comparison was conducted with unpaired two-tailed Student’s t test and one-way analysis of variance with Bonferroni post-test. All tests were two-sided and p value <0.05 was considered statistically significant.

**Data access**

Raw sequencing data were submitted to the Sequence Read Archive (BioProject No. PRJNA642688).

**RESULTS**

**Generation of LMP2A-specific T cells**

To obtain LMP2A-specific T cells, we first enrolled two healthy donors and obtained their peripheral T cells and DCs, and then peripheral T cells of each donor were stimulated with autologous irradiated DCs pulsed with a pool of LMP2A peptides for twice (figure 1). Flow cytometry showed that phenotypic characteristics of prestimulated and poststimulated T cells (namely S0 and S2) hugely varied in each donor, and per cent of CD8+ T cells and effect memory T cells significantly increased (figure 2A,B). In addition, ELISA and ELISPOT assays...
demonstrated that poststimulated T cells could specifically identify LMP2A peptides (figure 2C,D).

**Identification and isolation of LMP2A-specific TCRs and construction of corresponding TCR-Ts**

Since frequencies of LMP2A-specific T cells should significantly increase after stimulation, high-throughput TCRβ sequencing of prestimulated and poststimulated T cells could identify significantly increasing TCRβ sequences, which could be β chains of LMP2A-specific TCRs, but corresponding TCRα sequences were unknown. Hence, poststimulated T cells were also sorted into 96-well plates and were amplified using single-cell PCR to obtain their TCRs, which could identify corresponding TCRα of significantly increasing TCRβ (figure 1). High-throughput TCRβ sequencing of prestimulated and poststimulated T cells demonstrated that more clonal VJ usage in S2 than S0 T cells (figure 3A and online supplemental figure S1A). In addition, we plotted the Lorenz curve for S0 and
S2 to assess any potential skewing and diversity of the TCR repertoire composition, which demonstrated that diversity of TCR repertoires in S0 cells was higher than that in S2 cells (figure 3B and online supplemental figure S1B). Indeed, hyperexpanded T-cell clonotypes (HEC) of S0 cells occupied a median of 13.6% of the total TCR repertoire; reciprocally, the HEC of S2 cells contributed to a median of 30.5% of the total TCR repertoires (figure 3C).

To systematically and quantitatively assess the repertoire clonality of S0 and S2 cells, we found that the clonality of S2 cells was higher than that of S0 cells (figure 3D). In general, these findings suggested that frequencies of LMP2A-reactive T cells could significantly increase after repeated stimulation with LMP2A peptide mix. Furthermore, to identify candidate LMP2A-reactive T cells, LMP2A-reactive TCRs could meet two criteria: frequencies of TCRs in poststimulated T cells were >0.1% and meanwhile frequencies of TCRs in poststimulated T cells was >10-fold higher than those in prestimulated T cells (online supplemental tables 2–5). Based on these criteria, we found 36 and 57 eligible TCRβ sequences for donor 1 and donor 2, respectively (online supplemental tables 6 and 7). To identify corresponding TCRα sequences for these TCRβ sequences, we performed single-cell TCR sequencing of poststimulated T cells for donor 1 and donor 2, respectively (online supplemental table 8). Hence, we integrated high-throughput TCRβ sequencing and single-cell TCR sequencing results and found two and five candidate LMP2A-reactive TCRs for donor 1 and donor 2, respectively (online supplemental table 9). In final, top two candidate LMP2A-reactive TCRs were used for function validation for each donor (table 1).

**Functional validation of LMP2A-reactive TCR-Ts**

To evaluate the ability of these TCR-Ts to specifically identify and mediate effector functions in response to LMP2A in vitro, ELISA and ELISPOT assays both showed high IFN-γ secretion in all four TCR-engineered T cells on coculture with LMP2A-pulsed DCs (figure 4A,B). Furthermore, gating on CD3+ T cells, CD25 and CD137 upregulation was also observed in all four TCR-engineered T cells cocultured with LMP2A-loaded DCs (figure 4C).

To verify that the LMP2A peptides are endogenously processed and presented on the cells with corresponding HLA molecules, we transfected LMP2A-encoding lentivector into LCLs to generate LMP2A-overexpressing LCLs (LCL LMP2A) for each donor. As expected, IFN-γ...
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Figure 3  The high-throughput TCRβ sequencing of S0 and S2. (A) The frequencies of specific Vβ-Jβ gene segment combinations in TCRβ CDR3 sequences of S0 and S2 in donor 1. (B) Lorenz curve is used to graphically demonstrate TCR clonality of S0 and S2 in donor 1. (C) Bar plot shows frequency distribution of TCR clones of S0 and S2 in each donor. (D) The comparison of TCR clonality of S0 and S2 in each donor. (E) Sketch map of pCDH-EF1α-TCR lentiviral vector. The construct employed the β-α chain order, added disulfide bond (presented as black dots), α chain hydrophobic substitutions (presented as red dot) and murine constant region. Leader, leader sequences of TCRβ and TCRα chains, respectively.

Production and CD137 upregulation were observed in all four TCR-engineered T cells cocultured with LCL

LMP2A

(figure 5A). In addition, all four TCR-engineered T cells revealed cytotoxic activity against LCL

LMP2A

(figure 5B). These findings indicated that the LMP2A epitopes were
expectedly processed and presented on the cell surface and could be specifically recognized by LMP2A-reactive TCR-Ts.

Next, since all four TCR-engineered T cells of two donors demonstrated in vitro recognition and cytotoxicity against LCL_{LMP2A}, showing feasibility and effectiveness of screening individualized LMP2A-reactive TCR approach, to simplify in vivo function validation of LMP2A-reactive TCR-Ts, we only evaluated therapeutic benefit in vivo of TCR1 and TCR2-engineered T cells using a xenogeneic model subcutaneously engrafted with LCL_{LMP2A} NOD/SCID mice that were subcutaneously engrafted with LCL_{LMP2A} underwent adoptive infusion with untransduced T cells, TCR1-Ts, TCR2-Ts on day 3 (figure 5C). Mice treated with TCR1-Ts or TCR2-Ts had a reduced tumor progression compared with those with untransduced T cells, drawing significantly lower tumor area by day 40 (figure 5D). In addition, we found that infused TCR-T cells were infiltrated into tumors but mock T cells did not, when mice were killed (online supplemental figure S3A). Although infused TCR-T cells were infiltrated into tumor, we did not find upregulation of exhaustion marker, for example, PD-1 (online supplemental figure S3B). However, this might be due to lower number of infiltrated TCR-T cells. Unexpectedly, TCR2-Ts demonstrated more stronger in vivo antitumor ability than TCR1-Ts, although TCR1-Ts and TCR2-Ts showed similar in vitro cytotoxicity against LCL_{LMP2A} (figure 5D).

**DISCUSSION**

LMP2A-specific TCR-Ts could be a promising treatment approach to EBV-associated malignancies. However, since specific recognition and killing of TCR-Ts is based on both epitopes and HLA types, it is essential to develop an approach to identify individualized LMP2A-specific TCRs due to hugely varied HLA subtypes and presented LMP2A epitopes in different individuals.18

In our study, we stimulated peripheral T cells with LMP2A-pulsed autologous DCs for 2 weeks, obtained significantly increased TCR\(\beta\) sequences by high-throughput TCR\(\beta\) sequencing of prestimulated and poststimulated T cells and identified corresponding TCR\(\alpha\) sequences for these TCR\(\beta\) using single-cell TCR sequencing of poststimulated T cells. We found two and five candidate LMP2A-reactive TCRs from donor 1 and donor 2 by integration of high-throughput TCR\(\beta\) sequencing with single-cell TCR sequencing data, respectively. The top two candidate LMP2A-reactive TCR-engineered T cells in each donor demonstrated specific identification and cytotoxic ability against LMP2A presented by autologous DCs and LCLs in vitro and in vivo.

Although several previous studies reported in vitro stimulation protocols to enrich LMP2A-specific T cells for immunotherapy,10–12 the overall objective response rates were low.4 12–14 Since frequencies of circulating LMP2A-specific T cells were low in most individuals, infused T cells in most previous studies were derived...
from extensive expansion of peripheral low-frequency LMP2A-reactive T cells, and thus, most of them were terminally differentiated with limited replicative capacity and could not persist long-term in vivo. However, our study not only obtained LMP2A-reactive T cells by stimulating with a pool of LMP2A peptides but identified and isolated corresponding LMP2A-reactive TCRs by high-throughput TCRβ sequencing and single-cell TCR sequencing. In addition, T cells genetically engineered to express TCRs identified in our approach demonstrated LMP2A-specific cytotoxic ability and could show stronger antitumor response compared with extensively expanded
LMP2A-reactive T cells. Moreover, we found that LMP2A-reactive TCR-Ts with similar in vitro cytotoxic ability demonstrated significantly different in vivo antitumor ability.

Besides our study, several groups also attempted to generate LMP2A-specific TCR-engineered T cells for immunotherapy.\(^{16,17}\) Although these studies reported that LMP2A-specific TCR-Ts demonstrated promising results for LMP2A-expressing tumor,\(^{16,17}\) they only focused on specific HLA subtypes and corresponding epitopes, and thus, they were only suitable for patients with specific HLA. In our study, LMP2A-specific T cells were stimulated using autologous DCs pulsed with a pool of LMP2A peptides, consisting mainly of 15-mer sequences with 11 amino acids overlap and covering the whole sequence of the LMP2A protein, and thus we could obtain LMP2A-specific T cells and corresponding TCRs genetically engineered T cells for each patient regardless of specific HLA types and epitopes.

Since frequencies of peripheral LMP2A-reactive T cells could significantly increase after 2-week LMP2A...
stimulation and moreover high-throughput TCRβ sequencing could identify rare TCRβ sequences (frequency <0.01%), it is an optimal tool to obtain significantly increased TCRβ sequences of candidate LMP2A-reactive TCRs by high-throughput TCRβ sequencing of prestimulated and poststimulated T cells. To further identify corresponding TCRα sequences for these TCRβ sequences, we sorted single T cells of poststimulated T cells into individual wells of 96-well plates by flow cytometry, followed by PCR amplification and Sanger sequencing. Although our approach detected multiple candidate LMP2A-reactive TCRs for each donor, the number and percentage of single cells captured and analyzed in each experiment were still low, which could be overcome by high-throughput single-cell TCR-sequencing technology in spite of high expense.

Although previous studies focused on specific HLA subtypes and corresponding LMP2A epitopes reported that LMP2A-specific TCRs demonstrated promising results for LMP2A-expressing tumors and moreover these studies would consume less time to obtain LMP2A-specific TCRs in the future clinical application, they were only suitable for a small proportion of patients with specific HLA subtypes. Since HLA subtypes and presented LMP2A epitopes hugely vary in different individuals, our study attempted to provide a procedure to obtain individualized LMP2A-specific TCRs for each patient with EBV latency II malignancies. Although this was a proof-of-concept study and would consume more time to obtain LMP2A-specific TCRs, it could be potentially feasible for future clinical applications. It takes 2–3 weeks to generate LMP2A-specific T cells and LCLs. Further, it takes 2–3 weeks to obtain LMP2A-reactive TCRs by high-throughput TCRβ sequencing and single-cell TCR sequencing, and meanwhile generate LCL_{LMP2A}. In final, it takes 2–3 weeks to generate LMP2A-reactive TCR-T and verify their effect function. Therefore, it takes 6–9 weeks to complete the whole process, which could be potentially feasible for future clinical applications.

Our study attempted to provide a procedure to obtain individualized LMP2A-specific TCRs for patients with EBV latency II malignancies. Since antigen identification of TCRs depends on both antigens and HLA subtypes and additionally enormous HLA subtypes hugely vary in different individuals, it is difficult to find a tumor cell line that has the same HLA types as the donor and thus we transfected LMP2A-encoding lentivirus into LCLs to generate LMP2A-overexpressing LCLs (LCL_{LMP2A}) for each donor just to evaluate whether individualized LMP2A reactive TCR-T could identify LMP2A endogenously processed and presented on the cells with correponding HLA molecules. Of course, if the donors were patients with EBV latency II tumors, autologous tumor cells of patients should be more appropriate target cells. In the future studies, we would enroll patients with EBV latency II tumors to validate our approach.

In conclusion, we successfully isolated four LMP2A-specific TCRs and validated cytotoxic ability of corresponding TCRs genetically engineered T cells against LMP2A in vivo and in vitro from two donors. This pilot study supports the feasibility and effectiveness of our approach to screen individualized LMP2A-reactive TCRs in patients with EBV-associated malignancies, which could also be used to screen neoantigen-specific TCRs in other solid tumors.

CONCLUSION

In summary, our approach provides an efficient procedure to isolate individualized LMP2A-specific TCRs for basic and translational research, as well as for clinical applications.

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Funding This work was supported by Natural Science Foundation of China (Grant No 81972880 to ZL, Grant No 82003246 to CZ, Grant No 81963515 to YL; Capital’s Funds for Health Improvement and Research (Grant No 2020-1028 to ZL; Beijing Municipal Natural Science Foundation (Grant No 72020233 to YL); Beijing Hospitals Authority Youth Program (Grant No GML20131105 to CZ); Open Project funded by Key laboratory of Carcinogenesis and Translational Research, Ministry of Education/Beijing(Grant No 2019 Open Project-4 to ZL); Peking University Medicine Fund of Fostering Young Scholars’ Scientific & Technological Innovation (Grant No BMU2020PYB017 to CZ); the Fundamental Research Funds for the Central Universities.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was approved by the Institutional Review Board of the Peking University School of Oncology, China.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplemental information. The ORCID iD associated with luzheming@bjmu.edu.cn is: 0000-0003-3775-9435

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REFERENCES

1. Shannon-Lowe C, Rickinson A. The global landscape of EBV-associated tumors. 
   Front Oncol 2019;9:713.

2. Münz C. Latency and lytic replication in Epstein-Barr virus-associated oncogenesis. 
   Nat Rev Microbiol 2019;17:691–700.

3. Doubrovina E, Ofiazo-Sozomen B, Prokop SE, et al. Adoptive immunotherapy with unselected or 
   EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell 
   transplantation. 
   Blood 2012;119:2644–56.

4. Tashiro H, Brenner MK. Immunotherapy against cancer-related viruses. 
   Cell Res 2017;27:59–73.

5. Young LS, Yap LF, Murray PG. Epstein-Barr virus nuclear antigen 1 peptides to CD8+ T lymphocytes. 
   J Exp Med 2004;199:459–70.

6. Levitskaya J, Sharipo A, Leontchiks A, et al. Inhibition of Ubiquitin/proteasome-dependent protein degradation 
   by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. 
   Proc Natl Acad Sci U S A 1997;94:12616–21.

7. Khanim F, Yao QY, Niedobitek G, et al. Analysis of Epstein-Barr virus gene polymorphisms in 
   normal donors and in virus-associated tumors from different geographic locations. 
   Blood 1996;88:3491–501.

8. Busson P, Edwards RH, Tursz T, et al. Sequence polymorphism in the Epstein-Barr virus 
   latent membrane protein (LMP)-2 gene. 
   J Gen Virol 1995;76(Pt 1):139–45.

9. Sing AP, Ambinder RF, Hong DJ, et al. Isolation of Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes 
   that lyse Reed-Sternberg cells: implications for immune-mediated therapy of EBV+ Hodgkin's disease. 
   Blood 1997;89:1978–86.

10. Gerdemann U, Keirman JM, Kataru UL, et al. Rapidly generated multivirus-specific cytotoxic T 
    lymphocytes for the prophylaxis and treatment of viral infections. 
    Mol Ther 2012;20:1622–32.

11. Bellard CM, Goel S, Salokhin V, et al. Sustained complete responses in patients with lymphoma receiving 
    autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. 
    J Clin Oncol 2014;32:798–808.

12. Huang J, Fogg M, Wirth LJ, et al. Epstein-Barr virus-specific adoptive immunotherapy for recurrent, 
    metastatic nasopharyngeal carcinoma. 
    Cancer 2017;123:2642–50.

13. Chia W-K, Teo M, Wang W-W, et al. Adoptive T-cell transfer and chemotherapy in the first-line 
    treatment of metastatic and/or locally recurrent nasopharyngeal carcinoma. 
    Mol Ther 2014;22:132–9.

14. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function in vitro paradoxically 
    impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. 
    J Clin Invest 2005;115:1616–26.

15. Zheng Y, Parsonage G, Zhuang X, et al. Human leukocyte antigen (HLA) A*1101-Restricted Epstein-Barr virus-specific 
    T-cell receptor gene transfer to target nasopharyngeal carcinoma. 
    Cancer Immunol Res 2015;3:1138–47.

16. Xue S-A, Gao L, Ahmad M, et al. Human MHC Class I-restricted high avidity CD4+ T cells 
    generated by co-transfer of TCR and CD8 mediate efficient tumor rejection in vivo. 
    Oncoimmunology 2013;2:e22590.

17. Zhou F, Cao H, Zuo X, et al. Deep sequencing of the MHC region in the Chinese population 
    contributes to studies of complex disease. 
    Nat Genet 2016;48:740–6.

18. Neitzel H. A routine method for the establishment of permanent growing lymphoblastoid cell lines. 
    Hum Genet 1986;73:320–6.

19. Tan Q, Zhang C, Yang W, et al. Isolation of T cell receptor specifically reactive with autologous 
    tumour cells from tumour-infiltrating lymphocytes and construction of T cell receptor engineered 
    T cells for esophageal squamous cell carcinoma. 
    J Immunother Cancer 2019;7:232.

20. Chen Z, Zhang C, Pan Y, et al. T cell receptor [beta]-chain repertoire analysis reveals 
    intratumor heterogeneity of tumour-infiltrating lymphocytes in esophageal squamous cell carcinoma. 
    J Pathol 2016;239:450–8.

21. Lu Z, Zhang C, Sheng J, et al. T cell receptor [beta]-chain repertoire analysis reveals the association 
    between neoantigens and tumour-infiltrating lymphocytes in multifocal papillary thyroid carcinoma. 
    Int J Cancer 2017;141:877–82.

22. Youssi Monod M, Giudicelli V, Chaume D, et al. IMGT/JunctionAnalysis: the first tool for the analysis of the 
    immunoglobulin and T cell receptor complex V- [beta]-gene. 
    Bioinformatics 2004;20 Suppl 1:i379–85.

23. Zhang C, Huang H, Miao Y, et al. TCR repertoire intratumor heterogeneity of CD4+ and CD8+ 
    T cells in centers and margins of localized lung adenocarcinomas. 
    Int J Cancer 2019;144:818–27.

24. Zhao J, Zhang C, Huang H, et al. Identification of TCR repertoire heterogeneity in 
    non-small cell lung cancer. 
    J Immunother Cancer 2018;6:37.

25. Zhang C, Huang H, Miao Y, et al. Clonal distribution and intratumor heterogeneity of the 
    B-cell repertoire in esophageal squamous cell carcinoma. 
    J Pathol 2018;246:323–30.

26. Jin BY, Campbell TE, Draper LM, et al. Engineered T cells targeting E7 mediated regression of human 
    papillomavirus cancers in a murine model. 
    JCI Insight 2018;3: doi:10.1172/jci.insight.99488. 

27. Grotz A, Tran E, Parkhurst MR, et al. Recognition of human gastrointestinal cancer neoantigens 
    by circulating PD-1+ lymphocytes. 
    J Clin Invest 2019;129:4992–5004.

28. Olsen TK, Baryawno N. Introduction to single-cell RNA sequencing. 
    Curr Protoc Mol Biol 2018;122:e57.