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 TCRβ 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

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HLA subtypes and presented LMP2A epitopes hugely vary in different individuals,¹⁸ it is essential to consider full length of LMP2A to screen LMP2A-reactive T cells for each patient regardless of the HLA types and corresponding epitopes. Hence, to obtain LMP2A-specific T cells for each patient, low-frequency LMP2A-specific T cells in peripheral blood were stimulated using autologous dendritic cells (DCs) pulsed with a pool of LMP2A peptides, consisting of 15-mer sequences with 11 amino acids overlap and covering the sequence of the human LMP2A protein. Followed by a weekly stimulation for 2 consecutive weeks, the frequency of LMP2A-specific T cells should significantly increase. Thus, by high throughput TCR β sequencing of prestimulated and poststimulated T cells as well as single-cell TCR sequencing of poststimulated T cells, we could identify candidate LMP2A-specific TCRs with significantly increased frequencies after two stimulations. And these LMP2A-specific TCRs were then introduced into their peripheral T cells to generate LMP2A-reactive TCR-Ts of which specific recognition and killing capacity against LMP2A presented by autologous DCs and lymphoblastoid cell lines (LCLs) in vivo and in vitro were observed.

This strategy provides a procedure to obtain individualized LMP2A-specific TCR-Ts for patients with EBV latency II malignancies, which might also be applied to generate other tumor antigen-reactive TCR-Ts.

METHODS

Donors and cell lines

Two healthy volunteers provided informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers by non-mobilized leukapheresis and Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) gradient centrifugation and used to generate T cells, DCs and LCLs. To generate monocytes, non-adherent cells of PBMC were removed following a 2-hour incubation at 37°C, and adherent cells were scraped from culture dishes. Monocytes were cultured in 6-well plates at 3×10^6 cells/well in X-VIVO 15 medium containing $1 \times$ GlutaMAX, 500 U/mL IL-4 and 1000 U/mL GM-CSF for 5 days to obtain immature DCs. All experiments used fresh immature DCs. LCLs were established by infecting PBMCs with B95-8 cell supernatant according to standard EBV transformation protocols.¹⁹ In brief, PBMCs were cultured with RPMI 1640 medium including 20% fetal bovine serum, streptomycin, penicillin and B95-8 cell supernatant. The cells were passaged every week until stable growth was exhibited. For LCL-based transfectants, EBV-LMP2A (from EBV B95-8 strain) and GFP cDNA were linked together using 2A sequence derived from foot-and-mouth disease virus and cloned into the pCDH-EF1-Luc2-T2A-TdTomato lentiviral vector. LCLs of donor 1 and donor 2 were transduced with this vector to generate LMP2A-overexpressing LCLs (LCL_{LMP2A}). HEK 293-FT (Life Technologies, USA), a packaging cell line used to produce lentivirus supernatants, were obtained from the American Type Culture Collection (Manassas, Virginia, USA).

HLA typing of donors

DNA of donors' peripheral blood was extracted with DNeasy 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 (RPAT4), 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 μ m 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 were 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 5×10^6 PBMCs (S0) were cocultured with 5×10^5 autologous irradiated (3000 rad) DCs pulsed with commercially available LMP2A peptide mix (Miltenyi Biotec, Germany) at a concentration of 10 μ g/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.²⁰ 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 C α and C β region primers. The TCR sequences were analyzed using IMGT/V-Quest tool (<http://www.imgt.org/>).

High-throughput sequencing of TCR β

S0 and S2 in each donor were harvested and used for high-throughput TCR β sequencing. Detailed information of TCR β sequencing has been described in our previous study.^{21 22} Briefly, the TCR β was amplified and sequenced using Multiplex PCR and Illumina HiSeq2500 platform (MyGenostics, Beijing, China) from >500 ng of genomic DNA for each sample. The TCR β 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.²³ Only productive reads that did not contain frameshifts or stop codons were used for statistical analysis. On average, 2,186,966 TCR templates were detected (range 1,678,257–2,695,675) from four samples of two donors, representing an average of 42,879 unique clonotypes (range 25,489–60,270) (online supplemental tables S2–S5). To quantify the diversity of TCR repertoire, we adopted clonality index that provides a diversity measurement as a function of distribution of TCR frequencies and is independent of sampling depth. The clonality is defined as 1—normalized Shannon's entropy with values ranging from 0 (most diverse) to 1 (least diverse).^{24 25}

IFN- γ ELISPOT assay

Human IFN- γ ELISPOT Kit (Abcam, USA) was performed as the manufacturer's protocols. Briefly, 2×10^4 T cells, rested in cytokine-free media overnight, were incubated with 1×10^4 PBS-washed autologous DCs pulsed with LMP2A peptide mix at a concentration of 10 μ g/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, 1×10^6 T cells were incubated with 1×10^5 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.²⁰ LCL_{LMP2A} were labeled with 5 μ M 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 μ g/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

TCR α/β chains were synthesized (GenScript) and cloned into our lentivirus vector.²⁰ TCRs were constructed in a β - α 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.^{20 26} To obtain TCR-T, T cells were transduced with lentivirus as previously described.²⁰ Briefly, PBMCs were stimulated in T-cell media supplemented with 50 ng/mL OKT3 and 1 μ g/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 40000 (Polysciences, USA).²⁰ 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.²⁰ And then activated T cells were transduced by concentrated lentivirus with 8 μ g/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 2×10^6 LCL_{LMP2A} tumor cells. Three days later, each mouse was intravenously infused twice with 3×10^6 TCR-Ts at 2-day interval (on days 3 and 5). Intraperitoneal administration of IL-2 (1000 IU per mouse) was given on days 3, 5, 7, 9 and 11. Calipers were used to measure tumors, and 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

Figure 1 Flowchart for isolation of individualized LMP2A-reactive TCRs and function validation of corresponding TCR-Ts. T cells were stimulated with autologous DCs pulsed with a pool of LMP2A peptides for twice, and we performed high-throughput TCRβ sequencing of prestimulated and poststimulated T cells (namely S0 and S2) as well as single-cell TCR sequencing of poststimulated T cells to obtain candidate LMP2A-reactive TCRs whose frequencies significantly increased after two stimulations. Subsequently, the potential LMP2A-reactive TCRs were cloned into lentiviral vector and introduced into peripheral T cells to generate LMP2A-reactive TCR-Ts. Finally, we evaluated whether these TCR-Ts could specifically recognize and kill LMP2A-positive cells in vivo and in vitro. DC, dendritic cell; EBV, Epstein-Barr virus; LCL, lymphoblastoid cell line; LMP2A, latent membrane protein-2A

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

Figure 2 Phenotype and functional screening of S0 and S2 in each donor. (A) Flow cytometry analysis revealed percentages of CD4+ and CD8+ T cells. (B) Flow cytometry analysis revealed percentages of naive, effect, effect memory (TEM) and central memory T cells (TCM). (C) IFN- γ ELISA analysis of S2 T cells cocultured autologous DCs pulsed with LMP2A peptides. (D) IFN- γ ELISPOT analysis of S2 T cells cocultured autologous DCs pulsed with LMP2A peptides or not. Column histogram summarized the number of positive spots (E). The mean values from each group are plotted. Error bars represent the SEM (** $p < 0.01$, ns, not significant, analyzed by one-way analysis of variance). NC, unstimulated peripheral T cells of each donor.

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, based on high-throughput TCR β sequencing results of prestimulated and poststimulated T cells (online supplemental tables 2–5). Based on these criteria, we found 36 and 57 eligible TCR β sequences for donor 1 and donor 2, respectively (online supplemental tables 6 and 7). To identify corresponding TCR α sequences for these TCR β 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 TCR β 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). To optimize expression of introduced TCRs in T cells to obtain candidate LMP2A-reactive TCR-Ts, TCRs were constructed in a β/α chain order and constant regions were replaced by mouse counterparts modified with hydrophobic substitutions and interchain disulfide bonds previously described (figure 3E). These TCRs were lentivirally transduced into peripheral T cells to manufacture TCR-Ts with transduction efficiency of more than 50% (online supplemental figure S2).

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

Figure 3 The high-throughput TCR β sequencing of S0 and S2. (A) The frequencies of specific V β -J β gene segment combinations in TCR β 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-EF1 α -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 TCR β and TCR α 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

Table 1 The top two candidate LMP2A-reactive TCRs for donor 1 and donor 2 based on high-throughput TCRβ sequencing and single-cell TCR sequencing

Origin	Name	TRAV	TRAJ	CDR3.aa	TRBV	TRBJ	TRBD	CDR3.aa	scPCR		TCRVβ deep sequencing			
									Freq	Rank	S0.Fre	S2.Fre	Rank	Fold
Donor 1	TCR1	V2*01	J4*01	AVEDGVSGGYNKLJ	V27*01	J2-5*01	No Result	ASSLSSMETQY	0.1702	2	3.50E-07	0.0242	1	69377
Donor 1	TCR2	V17*01	J11*01	ATEGNSGYSTLT	V6-5*01	J1-2*01	D1*01	ASSYQGGKDGYT	0.2979	1	6.99E-07	0.0193	2	27665
Donor 2	TCR3	V12-1*01	J3*01	VVTGYSS	V6-2*01	J2-1*01	D1*01	ASSDEGQYNEQF	0.0128	3	0	0.0786	1	∞
Donor 2	TCR4	V13-2*01	J13*02	AETPGGYQKVT	V27*01	J1-2*01	D1*01	ASSLYTPGTHNYGYT	0.0769	2	5.56E-05	0.0116	7	209

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 TCRβ sequences by high-throughput TCR β sequencing of prestimulated and poststimulated T cells and identified corresponding TCRα sequences for these TCRβ 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 TCRβ 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

A

B

C

Figure 4 Functional assay of potential LMP2A-reactive TCR-Ts. (A) IFN- γ ELISA assay on TCR-Ts cocultured with DCs loaded with LMP2A peptides. (B) IFN- γ ELISPOT assay on TCR-Ts cocultured with DCs pulsed with LMP2A peptides. (C) CD25 and CD137 staining on TCR-Ts cocultured with DCs loaded with LMP2A peptides. Error bars represent the SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant, analyzed by one-way analysis of variance). LMP2A, latent membrane protein-2A; NC, untransduced T cells.

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.¹⁵ 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 TCR β 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

Figure 5 Cytotoxic activity of TCR-Ts in vitro and in vivo. (A) IFN- γ ELISA assay and CD137 expression on TCR-Ts cocultured with LCL_{LMP2A}. NC, untransduced T cells. (B) Cytolytic activity of TCR-Ts against corresponding LCL_{LMP2A} at different E:T ratios. NC, untransduced T cells. (C) Schematic experimental plan. Each NOD/SCID mouse subcutaneously received 2 $\times 10^6$ LCL_{LMP2A} tumor cells on day 0. TCR-Ts or untransduced T cells (3 $\times 10^6$ cells per mouse) were infused twice and IL-2 was administrated five times with a 2-day interval, followed by weekly tumor monitoring. (D) Antitumor activity of TCR-Ts against LCL_{LMP2A} tumor cells in vivo. The tumor area is plotted on the y axis. Time after tumor cell injection is plotted on the x axis. The mean values from each group are plotted. Error bars represent the SEM (n=5 mice per group, *p<0.05, ***p<0.001, analyzed by one-way analysis of variance). NC, two intravenous injections of untransduced T cells and five intraperitoneal injection of IL-2 (1000 IU per mouse); TCR-T, two intravenous injections of TCR-Ts and five intraperitoneal injection of IL-2 (1000 IU per mouse). LCL, lymphoblastoid cell line; LMP2A, latent membrane protein-2A; NOD/SCID, non-obese diabetic severe combined immunodeficiency.

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 TCR β sequencing could identify rare TCR β sequences (frequency <0.01%), it is an optimal tool to obtain significantly increased TCR β sequences of candidate LMP2A-reactive TCRs by high-throughput TCR β sequencing of prestimulated and poststimulated T cells. To further identify corresponding TCR α sequences for these TCR β 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.^{27 28}

Although previous studies focused on specific HLA subtypes and corresponding LMP2A epitopes reported that LMP2A-specific TCR-Ts demonstrated promising results for LMP2A-expressing tumors and moreover these studies would consume less time to obtain LMP2A-specific TCR-Ts in the future clinical application,¹⁶ 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,¹⁸ our study attempted to provide a procedure to obtain individualized LMP2A-specific TCR-Ts 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 TCR-Ts, 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 TCR β 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 TCR-Ts 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 lentivector 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 corresponding 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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