Research Article

Evaluation of Epstein-Barr Virus Latent Membrane Protein 2 Specific T-Cell Receptors Driven by T-Cell Specific Promoters Using Lentiviral Vector

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Transduction of latent membrane protein 2 (LMP2)-specific T-cell receptors into activated T lymphocytes may provide a universal, MHC-restricted mean to treat EBV-associated tumors in adoptive immunotherapy. We compared TCR-specific promoters of distinct origin in lentiviral vectors, that is, Vβ6.7, delta, luria, and Vβ5.1 to evaluate TCR gene expression in human primary peripheral blood monocytes and T cell line HSB2. Vectors containing Vβ 6.7 promoter were found to be optimal for expression in PBMCs, and they maintained expression of the transduced TCRs for up to 7 weeks. These cells had the potential to recognize subdominant EBV latency antigens as measured by cytotoxicity and IFN-γ secretion. The nude mice also exhibited significant resistance to the HLA-A2 and LMP2-positive CNE tumor cell challenge after being infused with lentiviral transduced CTLs. In conclusion, LMP2-specific CTLs by lentiviral transduction have the potential use for treatment of EBV-related tumors.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with many human malignancies including a subset of Hodgkin disease, Burkitt lymphoma, nasopharyngeal carcinoma (NPC), and some gastric carcinomas [1–5]. The malignancies associated with EBV can be grouped into 3 types according to the latency gene expression profiles [4–6]. In NPC, the EBV proteins expressed are EBNA1, latent membrane protein 1 (LMP1), and LMP2 [7]. It has been shown that all 3 antigens can induce CD8+ cytotoxic T lymphocytes (CTLs), which play roles in antitumor immune response [8, 9]. However, only weak responses against EBNA1 can be detected in some individuals and the phenotypic and functional analyses of these induced EBNA-1-specific T cells revealed that EBNA1 is presented to CD4+ T helper as well as Treg cells, which suppress the antiviral immune response. Moreover, the poor immunogenicity of EBNA1 has also been attributed to the presence of a Gly-Ala repeat (GAr) sequence, which prevents the presentation of EBNA1-derived antigenic peptides by MHC class I molecules. This GAr-mediated function has been linked to its capacity to prevent EBNA1 synthesis, as well as proteasomal degradation [10–13]. On the other hand, LMP-1 is the only EBV protein with recognized oncogenic activity that can transform normal cells into malignant ones, thereby limiting its application as a potential immunotherapeutic target. Additionally, the LMP1-specific CTL frequency is low, and the reactivation of LMP1-specific CTL lines has been shown very difficult, in part because LMP1 is toxic when expressed at high levels. In NPC, LMP2 offers the best opportunity for specific targeting since it is consistently expressed and
the T-cell determinants in LMP2 sequence have been well defined [14–17]. Many studies, including clinical trials, have proven LMP2 to be an ideal immunotherapeutic target and inducer, which so far has not shown oncogenicity [16, 18–20]. It has been shown that this antigen can be processed by a proteasome system. The peptides are engaged in the major histocompatibility complex (MHC) class I, then move to the cell surface, and migrate to the CD8+ T cells on the cell surface [21–24]. Many CD8+ T cell-recognizing epitopes have been identified and most of them are conserved in NPC cells among different populations. Low LMP2 is a widely used targeting molecule and antigen for the immunotherapy of type II EBV-associated malignancies [19, 21, 25–29].

Adoptive immunotherapy with CTLs holds great promise for the treatment of cancer. Among them, the treatment of EBV-associated tumors has by far shown the most success [26–28, 30, 31]. With the development of molecular and cellular biology, tumor-specific CTLs can be selected and the T cell receptor genes can be cloned into highly efficient viral vectors for transfer into the patient’s T cells. This concept has been utilized since 1999, when Clay et al. transferred lytic function by retroviral vectors encoding the α and β chains of the TCR against EBV-associated tumors [32]. Today many practitioners have designed and applied these engineered CTLs for the treatment of various human malignancies [33–37]. However, the efficacy and efficiency of this application still needs to be optimized, especially when using retro- or lentiviral vectors for TCR transduction. These vector systems can integrate transgenes into chromosomes that have the potential to “immortalize” a normal cell. Thus, a specific T-cell promoter that can be activated only in T cells becomes critical for safety concerns. At the same time, transducing efficiency should be considered when using both these viral vectors.

In our investigation, we used an HLA-A2-restricted EBV LMP2-specific TCR, TCR5.05, to compare 4 different kinds of T-cell-specific promoters: Luria, Delta [38], Vβ5.1 [39], and Vβ 6.7 [40]. Our results show that all these T-cell-specific promoters can drive the transcription of the TCR gene without changing the transduced T cell phenotypes. We also found that CTLs generated by a lentiviral vector containing specific promoters and TCR genes can lyse target cells specifically. We further evaluated the CTLs in vivo and found that they can retard the growth of EBV-LMP2 expressing tumors and prolong the life of tumor-bearing mice. We reported for the first time that Vβ 6.7 promoter is most efficient when using the lentiviral vector to transduce T cells for targeting HLA-A2-restricted EBV-LMP2 antigens. This study may be helpful in designing and developing novel TCR-based adoptive immunotherapy for the treatment of EBV-associated tumors.

2. Material and Methods

2.1. Animals and Cell Lines. Six- to 8-week-old nude mice were purchased and maintained in the SPF animal facility at Nanjing Medical University. All procedures used in this study complied with institutional policies of the Animal Care and Use Committee of Nanjing Medical University.

The cell lines used in these experiments included 293T, HSB2 (human leukemic T-cell line); HLA-A2 restricted, Epstein Barr virus-transformed B lymphoblastoid cell lines (LCLs); K562; CNE (nasopharyngeal carcinoma cell line); T2 cell lines (deficient in TAP but still express low amounts of MHC class I on the surface of the cells, kept in the author’s lab). In animal model, CNE cells stably expressing HLA-A2 and LMP2 were established by plasmid pIREs/HLA-A2/LMP2 transfection and selection. All cell lines were cultured in RPMI 1640 plus 10% fetal bovine serum, L-glutamine, nonessential amino acids, and penicillin-streptomycin (100 U/mL) (Invitrogen).

2.2. Construction of Lentiviral Vector Plasmids. TCR plasmid PL5.05 and 4 T lymphocyte-specific promoters (PSK-Delta/Vβ 5.1/Luria/Vβ 6.7) were kindly provided by Rimas Orentas from Medical College of Wisconsin. EBV LMP2-specific TCR cDNA we used was obtained from T-cell clone which was generated by incubating HLA-A2-restricted BMC with peptide (CLGGLL TMV , LMP2: 426–434) as described by Orentas et al. [36]. TCR PL5.05 α and β chains were amplified by PCR using PL5.05 as a template and cloned into 4 lentiviral vectors containing various 4 T-cell promoters constructed from the parent PWPT-GFP vector. The primers were α chain: Primer1A: CAACGGTCGAGAATTCAGGCTCTCTTTG; Primer2A-3A: GTCATGCCTTTTGATAGTGCGTTGAGGACACACACCCGC; CAGTGGAGGTCTCTCTTTGAGT; β chain: Primer1B: ACTACGCGTCACCATGGCTATAGT- GTCTCTTAGATGAAA; Primer2B-3B: TTCTGAGATGAG- TTTTTGGTCTCAAGGAAAGGAGCGGAGGAC; Primer3B: CTCAATTCAGATCCTCTTCTGAGATGAGTTT. The α and β were linked with Flag and Myc tag, respectively. All the amplicons were sequenced and cloned into Mlu I and Sal I sites of PWPT-GFP vectors.

2.3. Lentivirus Production. Lentiviruses were prepared by transient transfection of 293T cells, using a liposomal cotransfection method. To summarize, the 293 T cells were seeded at 1 × 10⁷ cells per 10-cm plate. The cells were transfected 12–16 hours later with 20 μg lentiviral transfer vector, 12 μg Delta 8.9, and 18 μg VSV-G envelope plasmids 8.91 (Delta 8.9 and VSV-G envelope plasmids are helper lentiviral plasmid which are used for packing lentivirus). Forty-eight to 72 hours later, the supernatant was collected, centrifuged to remove the cellular debris, and concentrated approximately 30-fold by ultracentrifugation.

2.4. Determination of Lentiviral Titer. Titers of concentrated lentiviruses encoding green fluorescent protein (GFP) were determined by serially diluting and infecting 293T cells by the polybrene transduction method as previously described [41]. Titers (transducing units (TUs) GFP-positive cell dilution) of the lentiviral vectors ranged from 10⁶ to 10⁷ TU/mL.

2.5. Transduction of PBMCs and T Cells. Peripheral blood monocytes (PBMCs) were from an HLA-A2, healthy human.
T cells were obtained from anti-CD3 conjugated magnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany). The PBMCs and T cells were cultured in AIM-V and interleukin-2 (IL-2; PeproTech, Rocky Hill, NJ, USA) at 300 IU/mL. For OKT3 stimulation, the cells were placed initially in either a medium with anti-CD3 antibody, OKT3 (Ortho Biotech, Bridgewater, NJ, USA) at 50 ng/mL or in an OKT3 medium after transduction at the initial changing of the culture medium in the presence of IL-2. For transduction of the PBMCs or T cells, 1 × 10^6 cells were adjusted to a final volume of 1 mL in a 24-well, tissue culture-treated plate with the viral supernatant and Polybrene (8 μg/mL; Sigma, St. Louis, MO, USA). The cells were transduced by centrifugation of the plates at 1000 g for 1.5 hours at 32°C. The plates were placed in a 37°C, humidified, 5% CO2 incubator overnight, and the medium was replaced the next day.

2.6. Flow Cytometric Analysis. CD3 expression on cell surface was measured with allophycocyanin-conjugated antibodies and the corresponding isotype controls (BD Biosciences). TCR PL5.05 staining was performed by using anti-TCR α chain antibody (prepared from our lab) followed by phycoerythrin (PE)-labeled second antibody. Cells were stained in a FACS buffer made of PBS (Invitrogen, Carlsbad, Calif, USA) and 0.5% bovine serum albumin. Cells were collected with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, Calif, USA) and analyzed using CellQuest software (BD Biosciences).

2.7. Real-Time PCR. After 3 days, total RNA was extracted from the HSB2 cells which have been infected with lentivirus containing EBV-LMP2-specific TCR α and β chain driven by Luria, Delta, Vβ 5.1, and Vβ 6.7 T-cell-specific promoters. cDNA was reverse transcribed by a high-capacity cDNA reverse transcription kit (ABI, Foster, Calif, USA) using random primers. For α chain SYBR forward primer: 5′-ctcttaacatctgctaggatgg, reverse primer: 5′-cagcttagcagcatttaacc. For β chain SYBR forward primer: 5′-ggccacctctggcagaac, reverse primer: 5′-agagccgctagaactgga. Real-time PCR with SYBR dyes was performed on an ABI 7900 real-time machine and analyzed by SDS2.4 software.

2.8. Western Blotting. Fifty micrograms of total protein from each sample was loaded for SDS-PAGE and subsequently transferred onto the PVDF membranes. After blocking, the membranes were hybridized with anti-Flag and Myc tag antibodies, respectively. The membranes were washed and incubated with secondary antibody, followed by developing.

2.9. Measurement of Lymphocyte Antigen Reactivity. Target cells were prepared by using T2 cells pulsed with peptides (10 ng/mL) in cell culture medium or tumor cell lines for 2 hours at 37°C and then washed twice in PBS. CD8+ T cells were isolated from lentiviral transduced PBMCs using anti-CD8 beads from Miltenyi Biotec according to the manufacturer’s protocol. For the assay, effector cells (CD8+ T cells) and target (peptide-pulsed T2 or tumor cells) were incubated in a 0.2-mL culture volume in the wells of a 96-well culture plate at E:T = 50:1, 25:1, and 5:1. The cells were cocultured for 18 hours, and the supernatant was harvested. The supernatants were analyzed for interferon (IFN)-γ secretion, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bender Medsystem, Vienna, Austria). The supernatants were also measured for LDH levels using a commercially available kit (Roche, Boehringer Mannheim, Germany).

2.10. Mouse Immunization and Tumor Challenge. Tumor-bearing model was established by injecting 1 × 10^6 HLA-A2 and LMP2-positive CNE cells subcutaneously in the flank of six- to 8-week-old nude mice. The mice were infused with transduced CTL via tail vein 1 week after tumor cell injection weekly for a total of two weeks. Mice immunized with the mock or saline were used as a control. Tumor diameter was measured by calipers twice per week and recorded as the mean of narrowest and longest surface length for each animal in the group. Mice were sacrificed when the tumor size reached a 20 mm average diameter. Each experiment was performed at least twice, and results were essentially similar unless described otherwise.

3. Results

3.1. Vβ 6.7 Promoter Is the Most Optimal for TCR Expression. The map of lentiviral vector pWPT-promotor-α/β chain and the schematic diagrams representing the structures of the lentiviral vectors are shown in Figure 1. The promoter-α/β chain was amplified by PCR and inserted between MluI and SalI sites. The mock vector contains TCR α/β chain without any T-cell-specific promoter. TCR expression under four T-cell-specific promoters was compared by using real-time PCR, as shown in Figure 2(a). The HSB2 cells were incubated for 24 hours in medium and then exposed to each vector at a multiplicity of infection (MOI) of 10. Three days aftertransduction, the T cells were analyzed by real-time PCR and Western blotting. We observed that all the lentiviral vectors were able to transduce the T cells, using the Luria, Delta, Vβ 5.1, and Vβ 6.7 promoter-containing vectors. The Vβ 6.7 promoter vector had the highest TCR at transcriptional level. When the normalized α and β chain mRNA levels of the Luria promoter group were set at 100.03 ± 21.09 and 68.45 ± 23.75, Delta was 46.15 ± 11.01 and 26.54 ± 6.86, Vβ 5.1 was 42.08 ± 6.78 and 28.76 ± 19.75, and Vβ 6.7 was 150.58 ± 32.02 and 102.564 ± 17.75, and mock was
Figure 1: The map of lentiviral vector pWPT-promotor-α/β chain and the schematic diagrams representing the structures of the lentiviral vectors. (a) The map of lentiviral vector pWPT-promotor-α/β chain. The promoter-α/β chain was amplified by PCR and inserted between Mlu I and Sal I sites. (b) The schematic diagrams representing the structures of the lentiviral vectors. All the α/β chains of the anti-LMP2 TCR PL5.05 were driven by individual T-cell-specific promoter except mock which contains only α or β chains without any promoter region. Promoter-α chains in diagram forms were the lentiviral vectors designed to express α chain driven by Vβ 5.1, Luria, Delta, and Vβ 6.7 promoters, respectively. Promoter-β chain used Vβ 5.1, Luria, Delta, and Vβ 6.7 promoters to produce the individual β chain.

4.89 ± 3.09 and 4.08 ± 2.98. The mRNA levels of TCR were consistent with the protein levels used in Western blotting to detect the protein levels of the TCR α and β chain. Protein levels of TCR were much higher in the Vβ 6.7 group than in the other groups (Figure 2(b)). The expression of TCR α and β chain on the HEK293T cells and HepG2 cells (human hepatocellular carcinoma cell line) were almost not detected (Data not shown). These results suggest that the lentiviral vectors can express TCR in the T cell lines and PBMCs. Four different promoters have different levels of capacity to drive TCR expression.

3.2. Lentiviral Vectors with Various Promoters Can Transduce T Cells Efficiently. HSB2 and PBMCs were infected with lentiviral vectors having various promoters expressing the TCR α and β chain at MOI = 1 or 10. Three days after infection, expression of the TCR α chain was detected in the CD3+ T cells by FACS with a Flag tag antibody. At MOI = 1, the TCR α chain positivities from CD3+ cells were 16.76 ± 4.62%, 34.15 ± 3.71%, 42.08 ± 6.03%, and 58.58 ± 5.02% under Luria, Delta, Vβ 5.1, and Vβ 6.7 promoters, respectively. At MOI = 10, the positive TCR α chain was 23.42 ± 10.63%, 47.14 ± 4.53%, 46.33 ± 2.96%, and 60.46 ± 5.41%, under Luria, Delta, Vβ 5.1, and Vβ 6.7 T-cell-specific promoters of CD3+ cells, respectively (Figure 3(a)). The Vβ 6.7 group had the highest transducing efficiency, as evidenced by means of 58.58% and 60.46% positive at MOI = 1 or 10. We next checked the LMP2-TCR expression by flow cytometric analysis. As shown in Figure 3(b), 51.3% or 62.1% of the HSB2 or PBMC cells, respectively, were
3.3. Transduced PBMCs Can Specifically Lyse HLA-A2/LMP2, Expressing Target Cells. To assess the recognition of tumor antigens by lentivirus-transduced PBMCs and CD8+ T cells, the cells were cocultured with the indicated tumor cell lines or T2 cells pulsed with LMP2426–434 (CLGGLLTMV) (CLGG). After sorting, the CD8+ cells were collected and incubated with target cells at effector-to-target-cell ratios (E: T) = 50:1, 25:1, and 5:1. As shown in Figure 4(a), the Vβ.6.7 group has the highest lytic activity when using all 3 E:T ratios. To test the specificity of cytotoxicity, we chose the Vβ 6.7 lentiviral vector infected with PBMCs and CD8+ groups against different targeting cells. The results showed that Vβ 6.7 lentiviral vector-infected PBMCs could lyse T2-CLGG and LCLs effectively moderately but could not lyse T2 cells, T2 cells loaded with nonrelated peptides (T2-LLWT), and K562 cells (Figure 4(b)).

We also measured the IFN-γ levels in the supernants of the transduced-PBMC cytotoxicity experiments. All 4 promoter-containing lentiviral vector groups which transduced PBMCs secreted high levels of IFN-γ (>500 pg/mL) when incubated with CLGG and LCLs but secreted very low levels of IFN-γ when incubated with T2, T2 LLWT, or K562 cells (Figure 4(c)). These results further confirmed that the lysis is specific.

We next tested the cytotoxicity of Vβ 6.7-transduced CD8+ T cells against the targeting cells described above. Similar to the result involving PBMCs, the transduced CD8+ T cells had a higher cytotoxicity against the T2-CLGG and LCL, but minimal effects on T2-LLWT, T2, and K562 cells (Figure 4(d)). LCLs are EBV-transduced B lymphocytes which belongs to type III infection, expressing nine EBV genes encoded by the virus including LMP2. The results indicated that Vβ 6.7 lentiviral transduced T cells can specifically lyse HLA-A2-restricted tumor cells expressing EBV-LMP2.

4. Transduced CD8+ Cells Can Slow the Growth Rate of LMP2-Expressing CNE Tumors in Mice

CNE tumor cells stably expressing HLA-A2 and LMP2 were inoculated subcutaneously at $5 \times 10^5$ cells per mouse to establish the tumor model. Ten days later, the peptide-pulsed, lentiviral vector-transduced CD8+ cells were infused via the tail vein. The tumors were monitored daily till the tumor reached 1 cm², when the mouse was sacrificed. Each group of the transduced CD8+ cells was shown to slow or abolish...
the established tumors in the mouse model (Figure 5(a)). There were no statistically significant differences between the antitumor effects of the 4 promoter groups. All immunized groups were significantly different when compared with the saline and mock groups (Figure 5(b)). The mice were deemed dead when the tumor reached 1 cm². None of the mice in the Vβ 6.7 group died, and only 1 mouse died in each of the Luria, Delta, and Vβ 5.1 groups. All the mice in the saline group died 36 days after inoculation. These results demonstrated the therapeutic effects of reinfused CTL transduced with lentiviral vectors containing the specific TCR.
5. Discussion

Adoptive T-cell immunotherapy remains an active area in the correction of birth defects and the treatment of malignancies [26–28, 30, 31]. Unlike traditional immunotherapeutic approaches such as use of vaccine or antitoxin, adoptive T-cell immunotherapy is specific, repeatable, and much more effective. Adoptive T-cell therapy has advanced from simple ex vivo expansion of therapeutic T cells to gene-modified T cells. As the most important functional molecule of T cells, specific TCR has been cloned from effective and specific T-cell clones and transduced into modified T cells, which may express a large quantity of cytokines or costimulating receptors to boost function of the T cells [42–47].

The EBV-associated tumor is a potential target for adoptive T-cell immunotherapy because of its latent antigen expression profile. Orentas et al. reported that, by using SAMEN retroviral vector, they could demonstrate the ability...
to transfer CTL activity from an LMP2 peptide-specific CTL clone to a stimulated PBMC population. These TCR-transduced PBMCs showed specific immunoactivity against LMP2 targets [36]. Here, we continued this work and attempted to develop an effective lentiviral-based TCR transduction system for future clinical practice. Compared with retroviral vectors, lentiviral vectors have many advantages including the ability to transduce minimally stimulated PBMCs, and they have a potentially safer integration site preference [48, 49]. Our results showed that lentiviral vectors can effectively transduce PBMCs and CD3+ cells with LMP2-specific TCRs using 4 different T-cell-specific promoters.

Using highly active T cell promoters to drive TCR α and β chains has been reported by many groups to evaluate different combinations of promoters. It has been shown to express that multiple protein subunits, viral vector, and promoters are required intensive optimization [50, 51]. We used LMP2-specific TCRs to compare 4 different promoters in lentiviral vectors. TCR α and β chains are driven by each promoter independently. Our results showed that, although lentiviral vectors of the various promoters express TCR α and β chains at different levels, all groups of transduced CD8+ cells dramatically slowed or abolished the growth of LMP2-positive tumors. These results indicate that the transducing efficiency of lentiviral vectors containing different promoters does not affect the antitumor activity of CTLs. In future studies, we hope to emphasize the expansion of functional CTLs after selection rather than switching promoters to achieve higher transduction efficiency.

We have demonstrated that, for a single promoter, Vβ 6.7 is relatively superior to other promoters. Since our work solely compared T-cell-specific promoters, we could not exclude the possibility that others may have more powerful functions. Jones et al. generally compared specific and nonspecific promoters, which gave a comprehensive picture of promoter selection and combination [51]. We believe that the trend of adoptive T-cell immunotherapy is to develop safer and more effective vectors to engineer T cells. The priority is still safety. A specific T-cell promoter can limit the expression of transgenes in a relatively small subset of cells, so it is theoretically safe. Our study provides suggestions for future designing of lentiviral vectors in adoptive T-cell immunotherapy.

**Conflict of Interests**

The authors have contributed significantly and declare that they have no conflict of interests.

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