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A novel Epstein–Barr virus-latent membrane protein-1-specific T-cell receptor for TCR gene therapy

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Background: Adoptive transfer of genetically engineered T-cells to express antigen-specific T-cell receptor (TCR) is a feasible and effective therapeutic approach for numerous types of cancers, including Epstein–Barr virus (EBV)-associated malignancies. Here, we describe a TCR gene transfer regimen to rapidly and reliably generate T-cells specific to EBV-encoded latent membrane protein-1 (LMP1), which is a potential target for T-cell-based immunotherapy.

Methods: A novel TCR specific to LMP1 (LMP1-TCR) was isolated from HLA-A*0201 transgenic mice that were immunised with the minimal epitope $LMP1_{166}$ (TLLVDLLWL), and LMP1-TCR-transduced peripheral blood lymphocytes were evaluated for functional specificities.

Results: Both human CD8 and CD4 T-cells expressing the LMP1-TCR provoked high levels of cytokine secretion and cytolytic activity towards peptide-pulsed and LMP1-expressing tumour cells. Notably, recognition of these T-cells to peptide-pulsed cells was maintained at low concentration of peptide, implying that the LMP1-TCR has high avidity. Infusion of these engineered T-cells revealed remarkable therapeutic effects and inhibition of tumour growth in a preclinical xenogeneic model. We observed explosive *ex vivo* proliferation of functional TCR-transduced T-cells with artificial antigen-presenting cells that express co-stimulatory molecules CD80 and 4-1BBL.

Conclusions: These data suggest that the novel TCR-targeting LMP1 might allow the potential design of T-cell-based immunotherapeutic strategies against EBV-positive malignancies.

Significant progress in cancer immunology has been made in understanding the roles of tumour-reactive T-cells that can recognise and destroy malignant cells. Over the years, adoptive transfer of antigen-specific T-cells, mainly cytotoxic CD8 T-cells, has been applied as a safe and robust immunotherapeutic procedure in patients to eliminate malignant cells and extend survival without major complications (Riddell and Greenberg, 1995; Rosenberg *et al*, 2008).

Epstein–Barr virus (EBV) is associated with a broad range of malignancies that are distinguished by three distinct patterns of viral latency-related gene expression. Most successful clinical outcomes were obtained with EBV-specific cytotoxic T-cells against post-transplant lympho-proliferative disease, which expresses the complete array of EBV-latency-III antigens in transplant recipients (Gottschalk *et al*, 2005). However, EBV-positive nasopharyngeal carcinoma, Hodgkin's lymphoma (HL), and NK/T-cell lymphoma typically express more limited and weakly immunogenic EBV-latency-II antigens including latent membrane protein 1 (LMP1) and LMP2. Particularly, LMP1 is a transmembrane oncoprotein that mimics the tumour TNF receptor

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family members, capable of immortalising B-cells, and enhances cell survival by increasing bcl-2 activity (Graham *et al*, 2010; Pratt *et al*, 2012). Supported by this, although it is sometimes of low expression or absent (Kanemitsu *et al*, 2012), LMP1 has been proposed as an attractive target antigen for T-cell-based immunotherapy against EBV latency-II malignancies. Numerous reports have shown that T-cells specific for the EBV-latency-II antigens in patients are usually functionally impaired (Gandhi *et al*, 2007; Li *et al*, 2007) or suppressed in tumour microenvironments (Yamamoto *et al*, 2008; Fogg *et al*, 2013) and present in low frequency (Fogg *et al*, 2009), but also possessing the therapeutic potency and the capacity to be expanded with EBV-latency-II antigens-loaded antigen-presenting cells (APCs) *in vitro* (Straathof *et al*, 2005; Smith *et al*, 2006). Thus, several groups, including ours, have developed *in vitro* stimulation protocols to facilitate the generation of LMP1- and LMP2-specific T-cells and have demonstrated objective long-lasting clinical responses (Bollard *et al*, 2014; Cho *et al*, 2015b).

Despite their safety and apparent clinical effectiveness, there are significant drawbacks for *in vitro* expansion of EBV-specific T-cells, such as the relatively long manufacturing time, limited availability, and comparably low avidity of effector T-cells. Considering this, several groups have developed genetically engineered T-cells with an extrinsic antigen-specific T-cell receptor (TCR) or a chimeric antigen receptor (CAR) as an alternative approach to rapidly manufacture large numbers of potent tumour-reactive effector cells. Particularly, the clinical efficacy of TCR-engineered T-cells has been successfully demonstrated in patients with melanoma, synovial cell sarcoma, and multiple myeloma using MART1- and/or NY-ESO1-specific TCR (Morgan *et al*, 2006; Robbins *et al*, 2011; Rapoport *et al*, 2015), similar to CD19-targeted CAR-T-cells in patients with B-cell haematologic malignancies (Porter *et al*, 2011; Lee *et al*, 2015). Likewise, numerous groups have attempted to develop EBV-targeting engineered T-cells, either with CAR targeting CD30 (Savoldo *et al*, 2007) and CD70 (Shaffer *et al*, 2011), or with extrinsic TCR specific to EBV nuclear antigen 3 (Schaft *et al*, 2006) and LMP2a (Frumento *et al*, 2013; Xue *et al*, 2013; Zheng *et al*, 2015). However, although LMP1 is considered as an attractive target to treat EBV-positive malignancies, T-cells engineered with LMP1-specific TCR have not been developed. Here, we report the functionality and specificity of a novel murine TCR, which recognises an LMP1-derived epitope presented by HLA-A*0201 molecules. Mainly, we demonstrate that potent EBV-LMP1-specific T-cells can be efficiently generated by TCR gene transfer and exponentially expanded *in vitro* with artificial APCs regimen, suggesting potential applications in T-cell-based immunotherapy against EBV-associated diseases, including EBV-latency-II malignancies.

MATERIALS AND METHODS

Mice. Full-length HLA-A*0201-expressing transgenic (HLA-A2 Tg) mice (C57BL/6-Tg(HLA-A2.1)1Enge/J) and NSG mice (NOD.Cg-Prkd^{cscid} Il2rg^{tm1Wjl}/SzJ) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animal experiments were performed in accordance with our institutional animal care committee guidelines.

Cell lines. K562, Jurkat, T2, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). EBV-transformed B-lymphoblastoid cell lines (LCLs) were prepared with EBV B95-8 strain and HLA-A subtypes were determined with sequence-based typing. For K562-based transfectants, HLA-A*0201, CD80, 4-1BBL, EBV-LMP1 (from EBV B95-8 strain), and firefly-luciferase cDNA were cloned into the lentiviral vector pCDH-EF1 (System Bioscience, Palo Alto, CA, USA). K562 cells were first transduced with HLA-A*0201 (K-A2). Then, K-A2 cells were transduced with EBV-LMP1 (K-A2$_{LMP1}$) and sequentially firefly-luciferase (K-A2$_{LMP1/LUC}$). K-A2 cells were also transduced with human CD80 and 4-1BBL for artificial APCs (K-A2$_{80/4\text{-}1BBL}$). Stably viable clones were isolated with limiting dilutions, and gene expression was verified by immunohistochemical analysis or flow cytometry.

Peptides and reagents. Synthetic peptides representing CD8 T-cell epitopes WT1$_{126}$ (RMFPNAPYL), LMP1$_{32}$ (LLLALLFWL), LMP1$_{92}$ (LLLIALWNL), LMP1$_{125}$ (YLLEMLWRL), LMP1$_{159}$ (YLQ QNWWTL), LMP1$_{166}$ (TLLVDLLWL), LMP1$_{167}$ (LLVDLLWLL), and LMP1$_{173}$ (WLLLFLAIL) at $>85\%$ purity were purchased from A&A Labs (San Diego, CA, USA). Monoclonal anti-mouse CD40 (FGK45.5) was from BioXCell (West Lebanon, NH, USA). High molecular-weight Poly-IC was from InvivoGen (San Diego, CA, USA), and recombinant cytokines were from Peprotech (Rocky Hill, NJ, USA). Fluorescence-conjugated antibodies were obtained from eBioscience (San Diego, CA, USA).

Immunisation and T-cell clones. To generate LMP1$_{166}$-specific CD8 T-cells, HLA-A2 Tg mice were immunised intravenously with 2×10^6 dendritic cells (DCs) pulsed with 10 μg ml^{-1} LMP1$_{166}$ for 18 h, and after 7 days, the mice received an intravenous TriVax-immunisation. TriVax consists of a mixture of 150 μg LMP1$_{166}$, 50 μg poly-IC, and 100 μg anti-CD40 antibodies. Eight days after the booster-immunisation, intracellular IFNγ-staining was performed to measure the frequency of LMP1$_{166}$-specific cytokine-secreting CD8 T-cells. LMP1$_{166}$-specific T-cell cloning was carried out by following procedures with minor modification as described (Chinnasamy *et al*, 2011; Rosati *et al*, 2014). Briefly, 1.5×10^6 CD8 T-cells isolated from spleen were co-cultured with 5×10^5 irradiated (6000 cGy) DCs pulsed with 5 μg ml^{-1} LMP1$_{166}$ in 24-well plate. Seven days later, IFNγ-EliSpot assays were performed. Bulk cultured T-cells were cloned at single cells per well in U-bottom 96-well plates with 3×10^4 LMP1$_{166}$-pulsed irradiated (10 000cGy) T2 and 1×10^5 irradiated (5000 cGy) splenocytes in medium containing 50 IU ml^{-1} IL-2 and 5 ng ml^{-1} IL-7. Proliferating T-cell clones were evaluated for responsiveness towards LMP1$_{166}$ using intracellular IFNγ-staining.

Cloning of LMP1-specific, HLA-A*0201-restricted TCR. Total RNA from T-cell clones was isolated using an RNeasy-mini kit (Qiagen, Valencia, CA, USA), and TCRα/β genes were amplified using SMART-RACE cDNA-amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions with primers in the constant region of mouse TCRα and TCRβ chains (Chinnasamy *et al*, 2011) and sequenced. Amplified TCRα/β genes were linked using two-step overlapping-PCR with primers; TCRα-reverse; 5′-cttccacgtcgccggcctgcttcagcagggagaagttggtggcgccg ctgcctctcttcactctactggaccacagcctcagcgt-3′; TCRβ-reverse; 5′-ttctcc ctgctgaagcaggccggcgacgtggaagaaaaccctggcccc-3′ encoding furin-sensitive spacer RVKRGSG-P2A ribosomal-skip sequence ATNFSLLKQAGDVEENPGP. The full-length modified TCR-cDNA was cloned into pCDH-EF1 and sequenced.

Production and transduction of murine TCRs to peripheral blood lymphocytes. The use of human material was reviewed and approved by our Institutional Review Board. 293T-cells (8×10^6 cells) were seeded in a 100-mm plate. Twenty hours later, 12 μg cloned pCDH-EF1 and packaging plasmids (8 μg psPAX2, 4 μg pMD2G) were simultaneously transfected using 50 μl lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Two days later, lentiviruses were harvested and titrated into 293T cells. Peripheral blood lymphocytes (PBLs) were isolated from HLA-A2-positive healthy volunteers. For pre-activation, 1×10^6 PBLs were cultured with anti-CD3/CD28-coated beads (1 : 1 ratio; Invitrogen) and 300 IU ml^{-1} IL-2 for 2 days. Lentiviruses encoding murine TCR

(MOI = 0.5) were added to the activated 5×10^5 PBLs or Jurkat cells, and centrifugation was performed at 2500 rpm for 1 h at 25 °C with 8 μg ml^{-1} polybrene (Sigma-Aldrich, St Louis, MO, USA). Peripheral blood lymphocytes prepared identically without transducing murine TCR were referred as a mock-transduced control (PBL^{mock}). Two days later, PBLs were evaluated for surface expression and functional specificity of murine TCRs, and used in most experiments.

Flow cytometry analysis. For HLA-A2:immunoglobulin (Ig) dimer staining, Dimer-X reagent (BD Bioscience, San Diego, CA, USA) was prepared; 1 μg dimer, 5 μg LMP1$_{166}$, and 0.5 μg β2-microglobulin (Sigma-Aldrich) at 37 °C overnight. TCR-transduced PBLs (1×10^6 cells) were incubated with LMP1$_{166}$-loaded HLA-A2:Ig dimer for 40 min, and washed/stained with anti-human CD8a, CD4, and anti-mouse IgG1 for 20 min. For murine TCRs and *in vivo* persistence, 1×10^6 viable cells were stained with 0.5 μg indicated antibodies for 20 min. Fluorescence was measured using a FACS Calibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Otlen, Switzerland).

***Ex vivo* expansion and evaluation of TCR-transduced cells.** TCR-transduced CD8 and CD4 T-cells were isolated using MACS isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), with >90% purity. TCR-transduced cells (1×10^6 cells) were co-cultured with 5×10^5 peptide-loaded K-A2$_{80/4\text{-}1BBL}$ with 500 IU ml^{-1} IL-2. The artificial APCs were loaded with either 5 μg ml^{-1} LMP1$_{166}$ or WT1$_{126}$ for 6 h and irradiated (10 000 cGy)/washed before co-culturing. For comparison, cells were incubated with anti-CD3/CD28-coated beads (1 : 1 ratio). Growing cells were split every 3–4 days and re-stimulated after a 7-day interval under the same conditions. For cytokine-secretion, 5×10^5 engineered cells were co-cultured with irradiated (10 000 cGy) peptide-pulsed targets (1 : 1 ratio). After 2 days, cytokines in the supernatant were determined with an ELISA kit (eBioscience). For peptide-pulsing, target cells were incubated with 1 μg ml^{-1} peptide for 6 h at 37 °C. For antigen recognition, IFNγ-EliSpot assays were performed for peptide-pulsed T2 target cells (Cho *et al*, 2015b). For cytotoxicity determinations, conventional 5-h chromium-release assays were performed using various target cells.

***In vivo* therapeutic antitumour experiments.** NSG mice (6-8 weeks-old) were sublethally (300 cGy) irradiated on day -1. Each mouse intravenously received 3×10^6 K-A2$_{LMP1/LUC}$ tumour cells. Seven days later, each mouse was three times intravenously infused with 2×10^7 engineered PBLs at every 2-day interval (on day 7, 9, and 11). Intraperitoneal administration of IL-2 (1000 IU per mouse) was given on days 7, 9, 11, 13, 15, and 17. Tumour growth was monitored weekly by luciferase signal of bioluminescence imaging using Xenogen *in vivo* imaging system (Caliper Life sciences, Hopkinton, MA, USA). On day 20, peripheral blood samples were analysed to assess *in vivo* persistence of infused cells.

Statistical analyses. Statistical significance for tumour growth by bioluminescence intensity was determined using two-way ANOVA test, and survival analysis was established by Kaplan–Meier curves using log-rank tests. Results are representative of data obtained from at least two independent experiments. All analyses were performed and graphs were prepared using Prism 5.01 software (GraphPad).

RESULTS

Isolation of HLA-A2-restricted LMP1$_{166}$-specific murine TCR from CD8 T-cell clones. First, we generated EBV-specific CD8 T-cells in HLA-A2-Tg mice with the minimal epitope LMP1$_{166}$ (TLLVDLLWL) using a novel vaccination strategy with LMP1$_{166}$-loaded DCs followed by a mixture of LMP1$_{166}$ peptides, poly-IC adjuvant, and costimulatory anti-CD40 antibodies (TriVax). TriVax immunisation was highly efficient in stimulating and expanding antigen-specific CD8 T-cells that were primed with antigen-loaded DCs in mice (Cho *et al*, 2015a). LMP1$_{166}$-TriVax booster-immunisation after priming with LMP1$_{166}$-loaded DCs yielded 0.5–1.5% IFNγ-producing CD8 T-cells in spleen (Figure 1A). To generate LMP1$_{166}$-specific T-cell clones, purified CD8 T-cells were co-cultured with LMP1$_{166}$-loaded DCs for 7 days, and functional activity of *in vitro* stimulated T-cells was evaluated. As shown in Figure 1B, HLA-A2-restricted CD8 T-cell recognition was evident not only against peptide-pulsed T2 but also against HLA-A2-positive LCLs (LCL-A2^{pos}), where higher levels of T-cell responses were observed against un-pulsed or WT1$_{126}$-pulsed LCL-A2^{pos} than against LMP1$_{166}$-pulsed HLA-A2-negative LCLs (LCL-A2^{neg}). Subsequently, CD8 T-cells were cloned by limited dilution, and proliferative T-cell clones were examined for LMP1$_{166}$-specific IFNγ-producing reactivity (Figure 1C). TCRα and TCRβ chains from each high level of IFNγ-secreting T-cell clones against LMP1$_{166}$ were amplified. Since TCRs consist of heterodimers, isolated TCRα and TCRβ chains were linked with furin-spacer RVKRGSG-P2A element to express as a single open reading frame, and inserted into lentiviral vector. To examine the surface expression of murine TCRs, Jurkat cells and pre-activated PBLs were transduced with murine TCRs (named S4-1, S4-6, and S4-12). Although each TCR gene was isolated from highly antigen-responding T-cell clones, levels of transduced TCR expression varied. Above all, Jurkat cells and PBLs transduced with TCR S4-12, which comprises TRAV3D3*02 and TRBV26*01 (Supplementary Figure S1), exhibited more than 60% and 30% of surface expression, respectively (Figure 1D). To examine TCR functionality, engineered PBLs were co-cultured with LMP1$_{166}$-pulsed T2 and the concentration of cytokines in the supernatant was measured. TCR S4-12-expressing PBLs secreted high levels of IL-2 against LMP1$_{166}$-pulsed targets in comparison to PBL^{mock}, which were identically prepared without transferring murine TCR (Figure 1E). Jurkat cells transduced with TCR S4-12 also exhibited comparable LMP1$_{166}$-specific IL-2 production (data not shown). Hence, TCR S4-12 was chosen for further studies.

Functional specificities of HLA-A2-restricted LMP1$_{166}$-specific murine TCR S4-12. Subsequently, we further investigated the functional specificity of the novel murine TCR S4-12 after manipulating HLA-A2-positive PBLs to express the TCR (referred to PBL$^{S4\text{-}12}$). Levels of TCR S4-12 surface expression in the transduced PBLs were slightly different among donors, and elicited 25–35% transduction efficiency compared to that in TCR-non-transduced PBL^{mock} (data not shown). Initially, the engineered PBL$^{S4\text{-}12}$ were stained using LMP1$_{166}$-loaded HLA-A2:Ig dimers, which revealed comparable results with HLA-A2 tetramer assay for the immunologic monitoring (Schneck, 2000; Woll *et al*, 2004). The representative data presented in Figure 2A showed that PBL$^{S4\text{-}12}$ had high levels of LMP1$_{166}$-specific TCR-transduced CD8 and CD4 T-cells (~9% per each), whereas no significant LMP1$_{166}$-specific staining was found in TCR S4-6-transduced PBLs (PBL$^{S4\text{-}6}$), that was non-functional. Apparently, PBL$^{S4\text{-}12}$ were not stained with control peptide (WT1$_{126}$)-loaded HLA-A2:Ig dimers (Supplementary Figure S2). To assess the specific reactivity, HLA-A2-restricted LMP1-derived peptides were pulsed onto T2 cells, and co-cultured with the engineered PBL$^{S4\text{-}12}$. Interestingly, PBL$^{S4\text{-}12}$ were equipped to additionally recognise target cells pulsed with LMP1$_{167}$ (LLVDLLWLL), which is one amino acid-shifted peptide from LMP1$_{166}$, but not to other HLA-A2-restricted LMP1-derived peptides including an irrelevant WT1$_{126}$ (Figure 2B). Moreover, PBL$^{S4\text{-}12}$ were also stained with LMP1$_{167}$-loaded HLA-A2:Ig dimers, but in low frequency compared to that of LMP1$_{166}$-loaded HLA-A2:Ig dimers (Supplementary Figure S2).

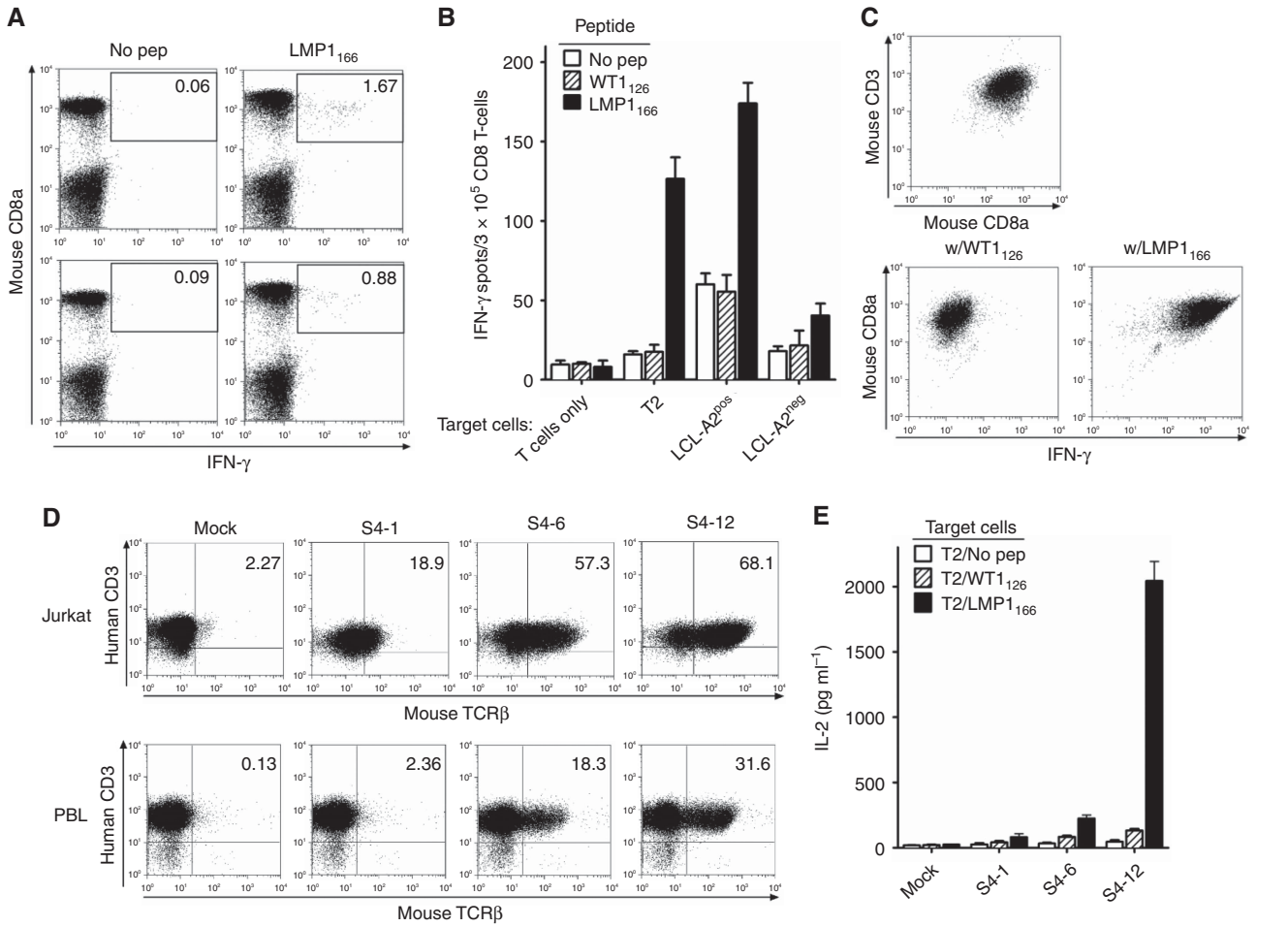

Figure 1. Isolation of murine TCR specific to LMP1$_{166}$ from CD8 T-cell clones. HLA-A2 Tg mice ($n = 2$) were immunised intravenously with 2×10^6 DCs loaded with LMP1$_{166}$ peptide for priming; 7 days later, the mice received a booster immunisation with TriVax. (**A**) Eight days after the booster, splenocytes were evaluated by intracellular IFNγ-staining after co-culturing with LMP1$_{166}$, or without peptide (No pep). Numbers in each rectangular gate represent the percentage IFNγ-positive cells of all CD8 T-cells. (**B**) After 7 days of *in vitro* stimulation with LMP1$_{166}$-loaded DCs, antigen-induced IFNγ-secretion of CD8 T-cells was evaluated. T2, and HLA-A2-positive and HLA-A2-negative LCLs (LCL-A2^{pos}, LCL-A2^{neg}, respectively) that were pulsed with LMP1$_{166}$ or WT1$_{126}$ were used as APCs. Peptides only without APCs (T-cells only) and un-pulsed APCs (No pep) were used as controls. Results represent the average number of spots from triplicate wells with s.d. (*bars*) of the means. These experiments of (**A**) and (**B**) were repeated thrice with similar results. (**C**) A representative T-cell clone established by limiting dilution cloning was verified for CD8 expression (*upper panel*) and antigen-specificity by intracellular IFNγ-staining (*lower panel*). (**D**) The *ex vivo*-activated PBLs and Jurkat cells were transduced with the murine TCRs isolated from LMP1$_{166}$-specific T-cell clones (S4-1, S4-6, or S4-12). Surface expression in the transduced Jurkat cells (*upper panel*) and PBLs (*lower panel*) was examined on day-2 post-transduction. TCR-non-transduced cells (mock) were used as control. Numbers in each rectangular gate represent the percentage TCR-expressing cells of all human CD8 T-cells. (**E**) Antigen-specific functionality of isolated murine TCRs. The TCR-transduced PBLs were co-cultured with peptide-pulsed T2 cells for 2 days, and the culture supernatants were measured for IL-2 production using ELISA assay. Results represent the average amount of cytokines from two independent experiments with s.d. (*bars*). LCL = lymphoblastoid cell lines; LMP = latent membrane protein; TCR = T-cell receptor.

Subsequently, peptides were serially diluted and pulsed onto T2 cells to evaluate the functional avidity of TCR S4-12. The engineered PBL$^{S4\text{-}12}$ recognised LMP1$_{166}$-pulsed targets, and secreted IL-2 in a dose-dependent manner, at a concentration as low as 1 ng ml^{-1} of LMP1$_{166}$, whereas PBL$^{S4\text{-}12}$ recognition against LMP1$_{167}$ was rapidly reduced by 100 ng ml^{-1} of LMP1$_{167}$ concentration, implying that LMP1$_{166}$ is a real or best antigenic epitope that can be recognised by TCR S4-12. Of note, the same was not observed for an irrelevant WT1$_{126}$ (Figure 2C). As such, antigen-specific recognition and cytolytic activity of PBL$^{S4\text{-}12}$ were validated compared with that of PBL$^{S4\text{-}6}$. As shown in Figure 2D and E, functional activity of PBL$^{S4\text{-}12}$ was evident, which displayed high levels of cytotoxicity towards LMP1$_{166}$-pulsed targets, whereas PBL$^{S4\text{-}6}$ did not respond to the target cells.

Murine TCR S4-12 can recognise endogenous processed HLA-A2/LMP1$_{166}$ complexes. One potential concern with TCR S4-12 was whether PBL$^{S4\text{-}12}$ could directly recognise endogenous LMP1-expressing target cells, rather than exogenously peptide-provided cells. DCs transfected with LMP1-RNA were applied as a target, which enabled presentation of HLA-A2/LMP1$_{166}$ complexes (pHLA-A2/LMP1$_{166}$) onto the cell surface by natural antigen-processing pathways. As shown in Figure 3A, high level of PBL$^{S4\text{-}12}$ responses were observed against LMP1-RNA-electro-transferred DCs (DC/LMP1$_{RNA}$), similar to those with LMP1$_{166}$-pulsed DCs (DC/LMP1$_{166}$) that showed the presence of saturated exogenous LMP1$_{166}$. In contrast, *in vitro* generated LMP1-specific cytotoxic T-cells that were stimulated with LMP1-RNA-transfected DCs from a same EBV-seropositive healthy donor did not respond to

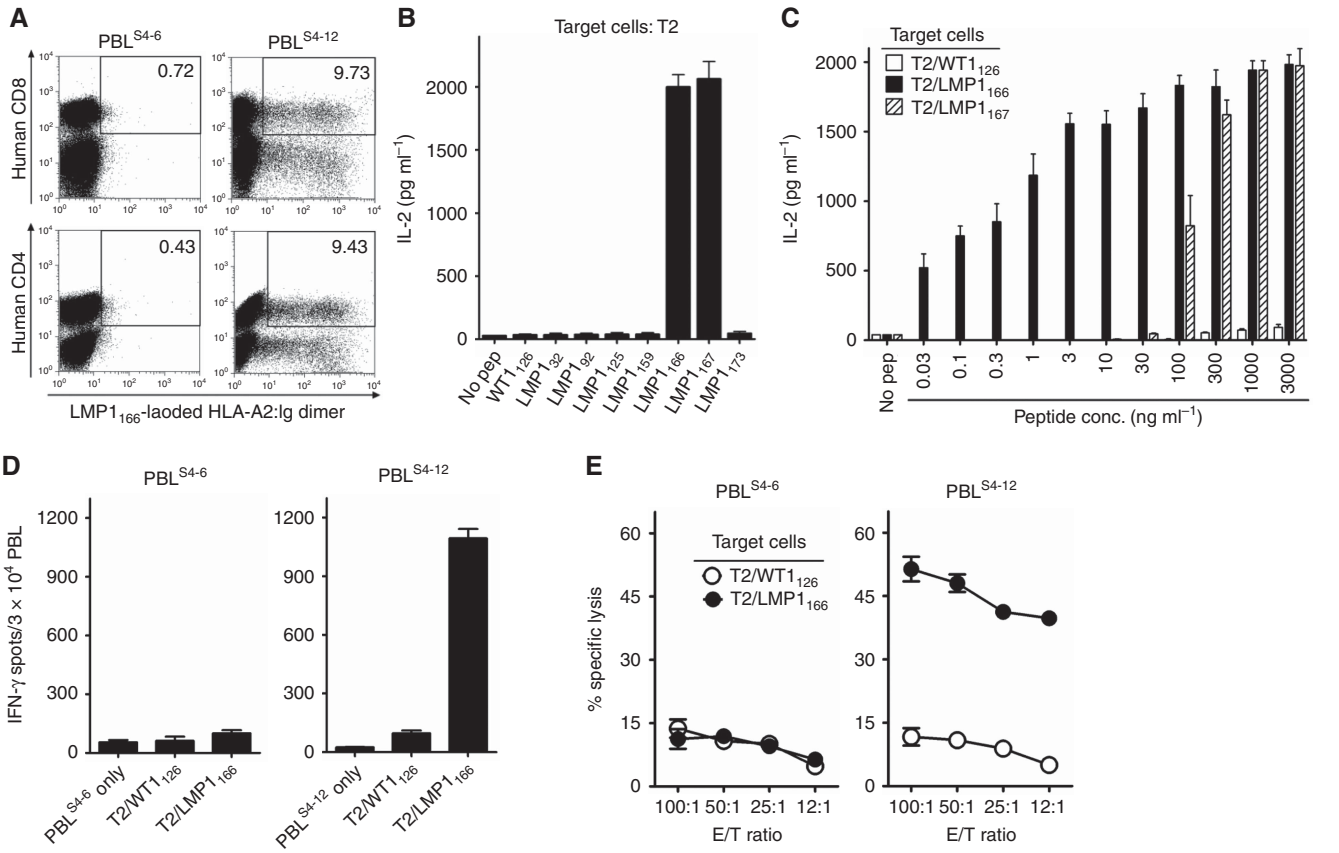

Figure 2. Evaluation of functional specificity of murine LMP1$_{166}$-specific TCR S4-12. (**A**) Murine TCR S4-6 and TCR S4-12-transduced PBLs (PBL^{S4-6} and PBL^{S4-12}, respectively) were stained with LMP1$_{166}$-loaded HLA-A2:Ig dimers on day 2 post-transduction and analysed by flow cytometry. Numbers in each rectangular gate represent the percentage peptide-dimer-positive cells of all human CD8 T-cells (*upper panels*) and CD4 T-cells (*lower panels*). These experiments were repeated two times with similar results. (**B** and **C**) Assessment of peptide specificity (**B**) and T-cell avidity (**C**) of TCR-transduced PBLs. PBL^{S4-12} were co-cultured with T2 cells that were pulsed with LMP1$_{166}$, with irrelevant peptides as indicated, or without peptide (No pep) for 2 days. IL-2-ELISA assay was performed with the culture supernatants. Results represent the average amount of cytokines from triplicate wells with s.d. (*bars*) of the means. (**D**) Antigen-induced IFNγ-secretion of PBL^{S4-6} and PBL^{S4-12} was evaluated using EliSpot assay. Results represent the average number of spots from triplicate wells with s.d. (*bars*) of the means. (**E**) Cytolytic activity of engineered PBLs against LMP1$_{166}$-pulsed T2 cells was assessed by a ^{51}Cr-release assay. Points represent the average values of cytotoxicity in different E:T ratio from triplicate wells with s.d. (*bars*) of the means. Engineered PBLs only and WT1$_{126}$-pulsed T2 cells were used as controls. These experiments were repeated twice with similar results. LMP = latent membrane protein; PBL = peripheral blood lymphocytes.

the DC/LMP1$_{166}$ targets (Supplementary Figure S3), indicating that the functional reactivities of PBL^{S4-12} are due to endowing TCR S4-12, not due to endogenous activities of EBV-seropositive donor. Nonetheless, exogenous LMP1$_{166}$-loaded LCL-A2^{pos} were recognised, resulting in production of a high level of IL-2, and were efficiently lysed by PBL^{S4-12}, whereas PBL^{S4-12} revealed relatively low responsiveness towards un-loaded and WT1$_{126}$-loaded LCL-A2^{pos} though LMP1 is known to be naturally expressed in LCLs (Figure 3B and C). However, the functional reactivities of PBL^{S4-12} towards LCL-A2^{pos} target cells were significantly reduced by HLA-A2 blocking (Supplementary Figure S4), implying that the LMP1$_{166}$-specific recognition of PBL^{S4-12} is HLA-A2-restricted. Apparently, LCL-A2^{neg} targets were not attacked while LMP1$_{166}$ were provided. To confirm the capability of TCR S4-12 to recognise endogenously processed pHLA-A2/LMP1$_{166}$ on target cells, we established K562-derived stable transfectants that expressed HLA-A2 alone (K-A2) and/or together with LMP1 (K-A2$_{LMP1}$). After selection, LMP1 expression was examined in K-A2 and K-A2$_{LMP1}$ transfectants as well as LCL, results showed much lower level of LMP1 expression in LCL than K-A2$_{LMP1}$ cells (data not shown). Likewise, PBL^{S4-12} were effective in recognising K-A2$_{LMP1}$ pulsed either with or without LMP1$_{166}$ (Figure 4A), whereas TCR-non-transduced PBL^{mock} and TCR S4-6-transduced PBL^{S4-6} did not respond to the target cells (Supplementary Figure S5). In accordance, higher cytolytic activity of PBL^{S4-12} was observed against K-A2$_{LMP1}$ as compared to that with PBL^{S4-6} (Figure 4B). These data indicate that K-A2$_{LMP1}$ presents pHLA-A2/LMP1$_{166}$ on the surface through intrinsic LMP1-processing machinery.

We examined the composition of PBL^{S4-12} to clarify which subsets of T-cells have effector functions. As shown in Figure 4C, PBL^{S4-12} were mainly composed of CD8 T-cells (CD8 T^{s4-12}; ~25%) but CD4 T-cells (CD4 T^{S4-12}; ~10%) were also present, which are crucial for the persistence of transferred CD8 T-cells and long-term immunologic memory T-cell responses. Notably, purified CD4 T^{S4-12} exhibited high levels of IL-2 production similar to those with that of purified CD8 T^{S4-12} in response to LMP1$_{166}$-loaded targets including K-A2$_{LMP1}$ (Figure 4D), as specific HLA-A2:Ig dimer bindings were observed in CD4 T-cells as well as CD8 T-cells (Figure 2A). Likewise, we manipulated cord-blood lymphocytes with TCR S4-12, which can also be used as a potential source of effector cells since the differentiation status of TCR-engineered T-cells is a factor influencing long-term *in vivo* persistence of infused cells. The engineered cord-blood

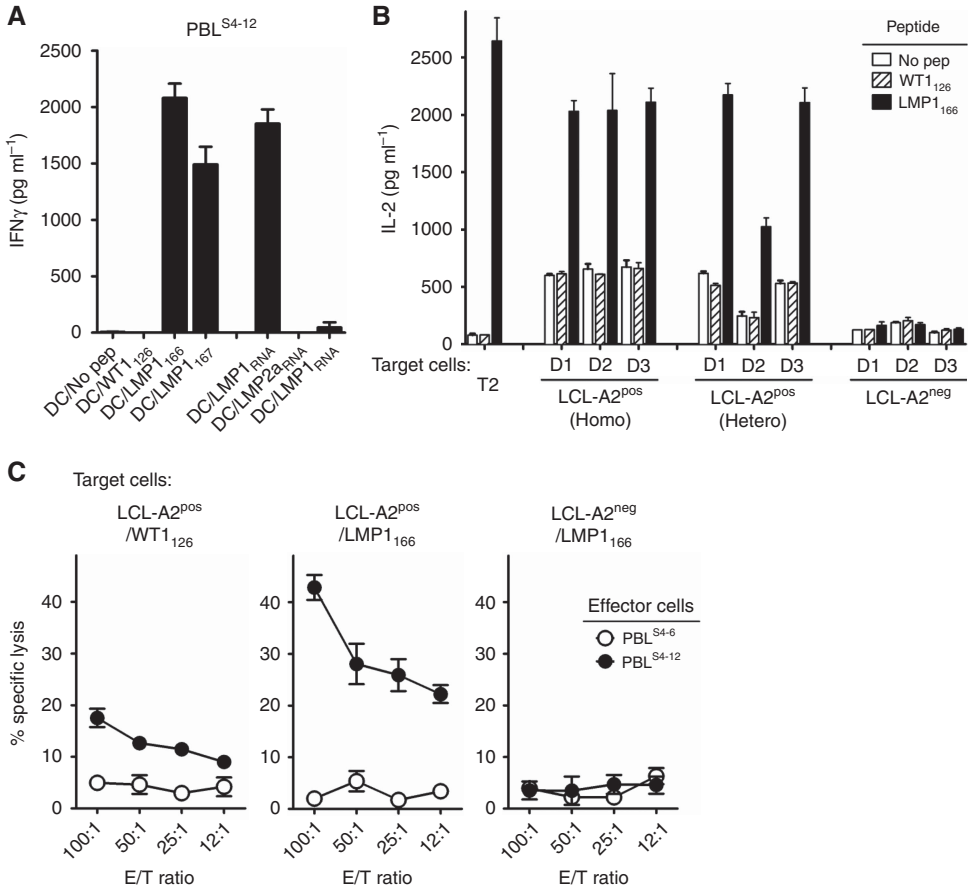

Figure 3. Antitumour activity of PBL$^{S4\text{-}12}$. (**A**) PBL$^{S4\text{-}12}$ were capable of recognising LMP1-expressing DCs. PBL$^{S4\text{-}12}$ were co-cultured with peptide-pulsed, or RNA-electro-transferred DCs as indicated for 2 days. DCs without peptide (No pep), irrelevant WT1$_{126}$, LMP2a- and WT1-RNA were used for controls. IFNγ-ELISA assay was performed with the culture supernatants. Results represent the average amount of cytokines from triplicate wells with s.d. (*bars*) of the means. (**B** and **C**) The functional specificity (**B**) and cytolytic activity (**C**) of PBL$^{S4\text{-}12}$ were evaluated towards HLA-A2-positive or HLA-A2-negative LCLs (LCL-A2^{pos} and LCL-A2^{neg}, respectively), which were pulsed with LMP1$_{166}$, WT1$_{126}$, or without peptide (No pep). (**B**) LCL-A2^{pos} were established from HLA-A2 homozygous (Homo) and/or HLA-A2 heterozygous (Hetero) donors, and three different donor-derived LCLs were used (D1, D2, and D3). T2 cells were also included. IL-2-ELISA assay was performed with the culture supernatants. Results represent the average amount of cytokines from two independent experiments with s.d. (*bars*). (**C**) PBL$^{S4\text{-}6}$ were used as control. Points represent the average values of cytotoxicity in different E:T ratio from triplicate wells with s.d. (*bars*) of the means. These experiments were repeated twice with similar results. LCL = lymphoblastoid cell lines; LMP = latent membrane protein; PBL = peripheral blood lymphocytes.

lymphocytes exhibited high level of IL-2 secretion similar to that of PBLs towards LMP1$_{166}$-loaded and/or LMP1-expressing K-A2$_{LMP1}$ and LCL target cells (Supplementary Figure S6). Overall, these results indicate that TCR S4-12 has high affinity for the recognition of the endogenously processed pHLA-A2/LMP1$_{166}$ target, and that functionality of TCR S4-12 is dependent on the degree of LMP1 expression in target cells.

***In vivo* therapeutic antitumour efficacy of PBL$^{S4\text{-}12}$.** Next, we assessed whether adoptive transfer of PBL$^{S4\text{-}12}$ would offer a therapeutic benefit *in vivo* using a xenogeneic model, which was systemically engrafted with luciferase-expressing K-A2$_{LMP1}$ (K-A2$_{LMP1/LUC}$), and a bioluminescent imaging technique to monitor tumour growth. Mice that were intravenously engrafted with K-A2$_{LMP1/LUC}$ underwent adoptive infusion with PBL^{mock}, PBL$^{S4\text{-}6}$, or PBL$^{S4\text{-}12}$ on day 7 (Figure 5A). Mice treated with PBL$^{S4\text{-}12}$ had a reduced tumour progression as compared to those with PBL^{mock} or PBL$^{S4\text{-}6}$, drawing significantly lower bioluminescent signal by day 42 (Figure 5B and C). As such, the adoptive transfer of PBL$^{S4\text{-}12}$ revealed significantly increased median survival of the mice by more than $\geqslant$2 weeks (Figure 5D), and the measurement of genetically modified (mouse TCR and human CD45 double-positive) cell numbers in blood at day 20 (2 weeks after cell infusion) showed sustained high numbers of PBL$^{S4\text{-}12}$, correlated with the observed improved antitumour effects (Figure 5E), indicating that TCR-transduced PBL$^{S4\text{-}12}$ have potential for substantial long-term engraftment *in vivo* after adoptive transfer.

***Ex vivo* expansion of CD8 T$^{S4\text{-}12}$ and PBL$^{S4\text{-}12}$.** Additionally, we investigated optimal conditions for *ex vivo* expansion conditions to obtain sufficiently high numbers of TCR-transduced cells for clinical applications, which is a prerequisite for the success of adoptive immunotherapy. For these experiments, we established K-A2-based APCs expressing co-stimulatory molecules CD80 and 4-1BBL (K-A2$_{80/4\text{-}1BBL}$), which can increase the survival of activated T-cells. Subsequently, PBL$^{S4\text{-}12}$ and CD8 T$^{S4\text{-}12}$ were expanded with LMP1$_{166}$- or WT1$_{126}$-loaded K-A2$_{80/4\text{-}1BBL}$ and compared with conventional anti-CD3/CD28-coated beads (Figure 6A). In this setup, stimulation with LMP1$_{166}$-loaded K-A2$_{80/4\text{-}1BBL}$ revealed expansion of distinct clear homogeneous populations of CD8 T$^{S4\text{-}12}$ (Figure 6B), which resulted in higher absolute numbers of CD8 T-cells compared to those with anti-CD3/CD28-coated beads ($\sim$400-fold versus $\sim$200-fold increase in PBL$^{S4\text{-}12}$ expansion); no significant T-cell expansion was found

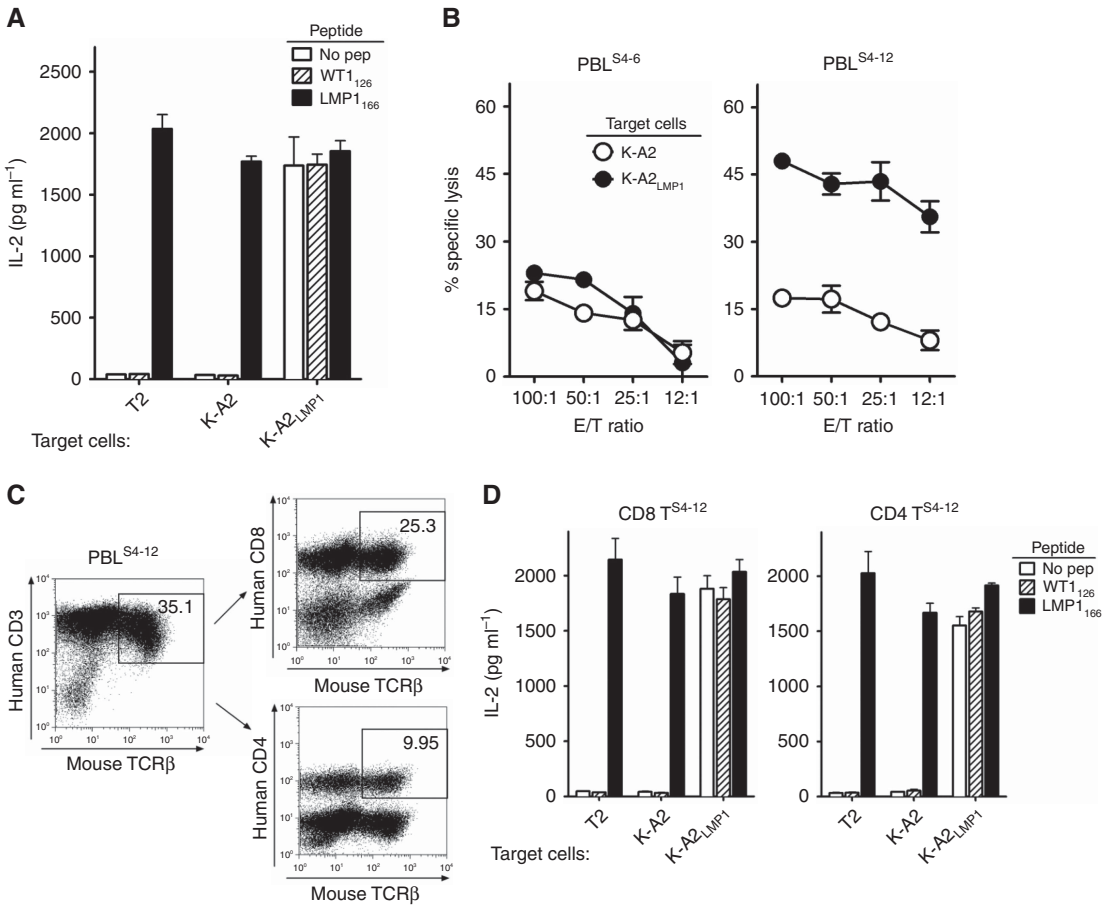

Figure 4. PBL^{S4-12} recognise endogenously processed LMP1$_{166}$/HLA-A2 complexes on tumour cells. (**A** and **B**) PBL^{S4-12} recognise HLA-A2 and LMP1 co-expressing K562 cells (K-A2$_{LMP1}$). K562 expressing HLA-A2 alone (K-A2) and T2 cells without peptide (No pep) or with peptides (WT1$_{126}$ and LMP1$_{166}$) were also included. IL-2-ELISA assay (**A**) and ^{51}Cr-release assay (**B**) were performed as in Figure 3 including K-A2 and K-A2 $_{LMP1}$ cells. (**B**) Effector PBL^{S4-6} were used as a control. Points represent the average values of cytotoxicity in different E:T ratio from triplicate wells with s.d. (*bars*) of the means. (**C** and **D**) Functional specificity of CD8 T^{S4-12} and CD4 T^{S4-12}. (**C**) Expression of TCR S4-12 in CD8 and CD4 T-cells was examined by flow cytometry. Numbers in each rectangular gate represent the percentage TCR S4-12-positive cells of all human T-cell subsets. (**D**) Antigenic specificity of purified CD8 T^{S4-12} and CD4 T^{S4-12} was evaluated as described above. (**A** and **D**) Results represent the average amount of cytokines from two independent experiments with s.d. (*bars*). These experiments were repeated twice with similar results. LCL = lymphoblastoid cell lines; LMP = latent membrane protein; PBL = peripheral blood lymphocytes; TCR = T-cell receptor.

with WT1$_{126}$-loaded K-A2$_{80/4\text{-}1BBL}$ (Figure 6C). The *ex vivo* expanded CD8 T-cells under all conditions for 3 weeks were mainly composed of effector-memory-like CD45RO$^+$/CD62L$^-$ phenotypes (Supplementary Figure S7). Moreover, antigen specificity of *ex vivo* expanded TCR-engineered cells was maintained during 28-day culture (Figure 6D), indicating that our artificial APCs can promote engineered cell proliferation with a homogeneously enriched population that maintained intact antigenic functional specificity.

DISCUSSION

EBV-specific T-cells have been successfully applied to restore EBV-specific immunity in patients with EBV-latency-III malignancies, whereas they have been in limited use for the treatment of EBV-latency-II malignancies, such as NK/T-cell lymphoma. Thus, extension of current adoptive immunotherapies toward EBV-latency-II malignancies demands more efficient immunotherapeutic strategies to generate sufficient numbers of T-cells specific to EBV-latency-II antigens, such as LMP1. Consequently, we and other have developed *ex vivo* expansion protocols capable of generating LMP1-specific T-cells using LMP1-expressing APCs that were transduced with mRNA (Demachi-Okamura *et al*, 2006; Cho *et al*, 2015b) or recombinant viruses (Gottschalk *et al*, 2003). However, in the clinical realm, there are significant drawbacks for reactivation of LMP1-specific T-cells because LMP1 is toxic when expressed at high levels (Hammerschmidt *et al*, 1989), and the precursor frequency of LMP1-specific T-cells is very low in healthy EBV-seropositive individuals (Khanna *et al*, 1998). Here, we explored the functional availability of genetically modified T-cells to endow antigenic specificity towards LMP1, which is a recent approach to rapidly manufacture large numbers of potent tumour-reactive effector cells. To our knowledge, this is the first report showing that T-cells engineered with LMP1-specific TCR enable them to recognise and elicit specific cytotoxicity towards LMP1-expressing tumour cells *in vitro* and in a xenogeneic allograft model *in vivo*.

Over a considerable period of time, adoptive transfer of *ex vivo*-engineered T-cells has been successful; particularly, anti-CD19-CAR-T-cells have demonstrated objective clinical responses towards B-cell malignancies, including complete remissions (Porter *et al*, 2011; Lee *et al*, 2015). Despite spreading and bypassing the HLA dependency, the CAR-T-cell-based approach requires surface expression of antigens and risks development of

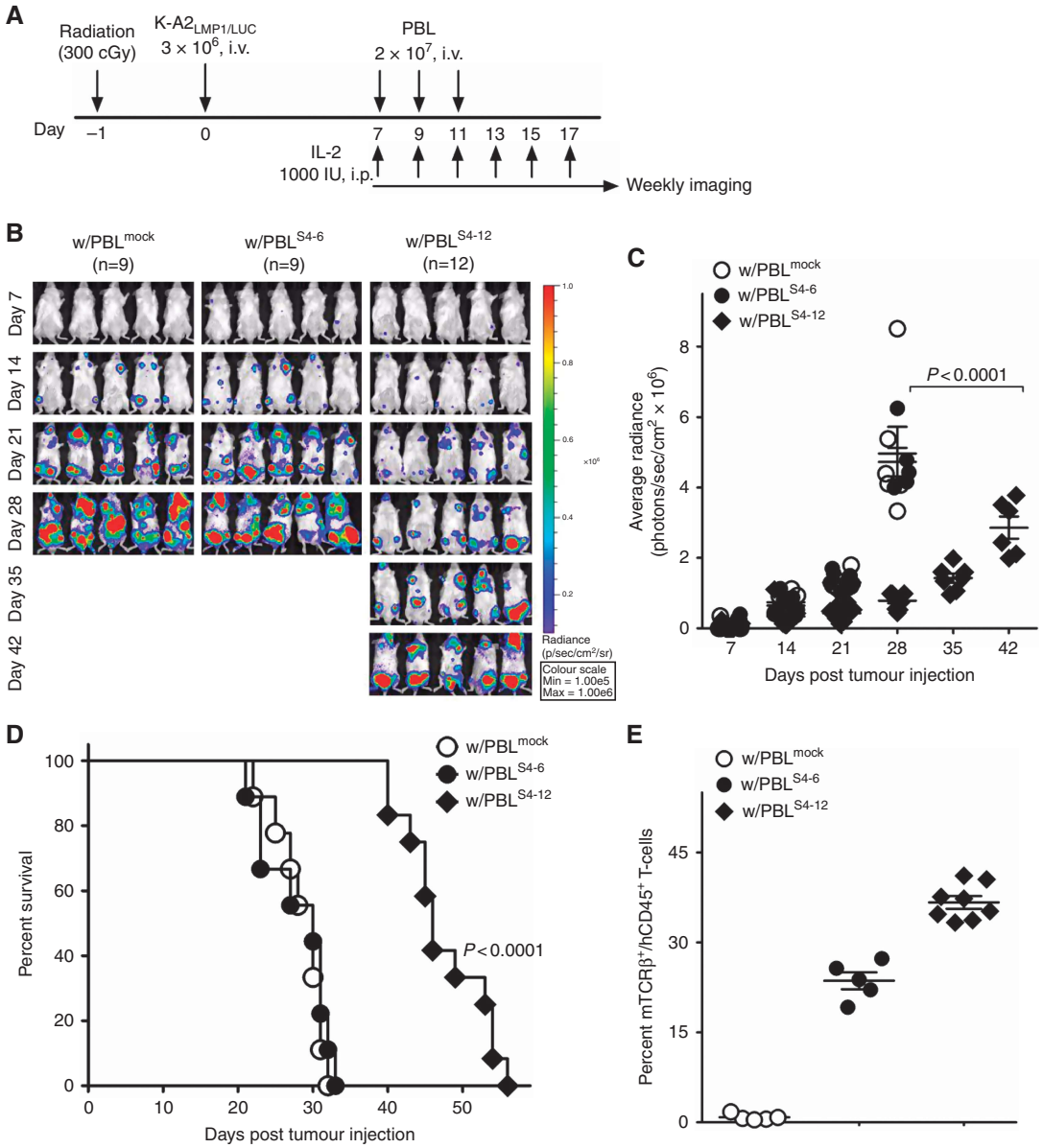

Figure 5. Adoptive transfer of PBL^{S4-12} prolongs survival of mice with LMP1-expressing tumours. (**A**) Schematic experimental plan. Sublethally irradiated (300 cGy) NSG mice intravenously received K-A2$_{LMP1/LUC}$ (3×10^6 cells per mouse) on day 0. Engineered PBLs (2×10^7 cells per mouse) were infused thrice and IL-2 was administrated six times with a 2-day interval, followed by weekly bioluminescence imaging. (**B**) Time course of *in vivo* bioluminescence imaging in representative individual mice treated with PBL^{S4-6} ($n = 9$) or PBL^{S4-12} ($n = 12$). A group treated with PBL^{mock} ($n = 9$) was included as control. Images were adjusted to the same pseudo colour scale to show relative bioluminescence changes over time. (**C**) Tumour growth was monitored by *in vivo* bioluminescence imaging. Average radiance per mouse in *B* was shown. Points represent the average values photons in mouse with s.d. (*bars*) of the means. *P*-values were calculated using two-way ANOVA test compared to the PBL^{S4-6}-infused group. (**D**) Kaplan–Mayer survival curves for all groups of mice. *P*-values were determined by log-rank tests compared to the PBL^{S4-6}-infused group. (**E**) Frequency of PBLs expressing murine TCR was evaluated on day 20 with blood samples from mice in (**D**). Points represent the percentage for each group of mice with s.d. (*bars*) of the means. These experiments were repeated twice with similar results. LMP = latent membrane protein; LUC = luciferase; PBL = peripheral blood lymphocytes; TCR = T-cell receptor.

tumour escape variants (Grupp *et al*, 2013; Anurathapan *et al*, 2014). In this respect, though it requires selection of patients with appropriate HLA alleles, TCR-engineered T-cells provide an effective therapeutic option for patients with CAR-T-induced tumour variants due to their high sensitivity for naturally processed antigenic peptides-HLA complexes (Corse *et al*, 2011; Caruso *et al*, 2015). Recent clinical trials using T-cells engineered with NY-ESO-1-specific TCR have shown objective responses in patients with melanoma, synovial cell carcinoma, and multiple myeloma (Robbins *et al*, 2011; Rapoport *et al*, 2015). In similar strategies to develop EBV-specific TCR-based therapies, numerous reports have shown objective EBV-specificities and *in vivo* therapeutic efficacies of genetically modified T-cells with isolated TCRs specific to HLA-A2- or HLA-A11-restricted LMP2 epitopes (Frumento *et al*, 2013; Xue *et al*, 2013; Zheng *et al*, 2015).

Selection of target T-cell epitopes is critical for the development of effective TCR-based T-cell immunotherapy. Here, we focus on an HLA-A2-restricted LMP1$_{166}$ epitope, which may be a subdominant epitope from LMP1, although it could be rather dominant in some situations. Khanna and colleagues have reported HLA-A2-restricted LMP1 epitopes (Khanna *et al*, 1998), and demonstrated that immunisation with recombinant viruses

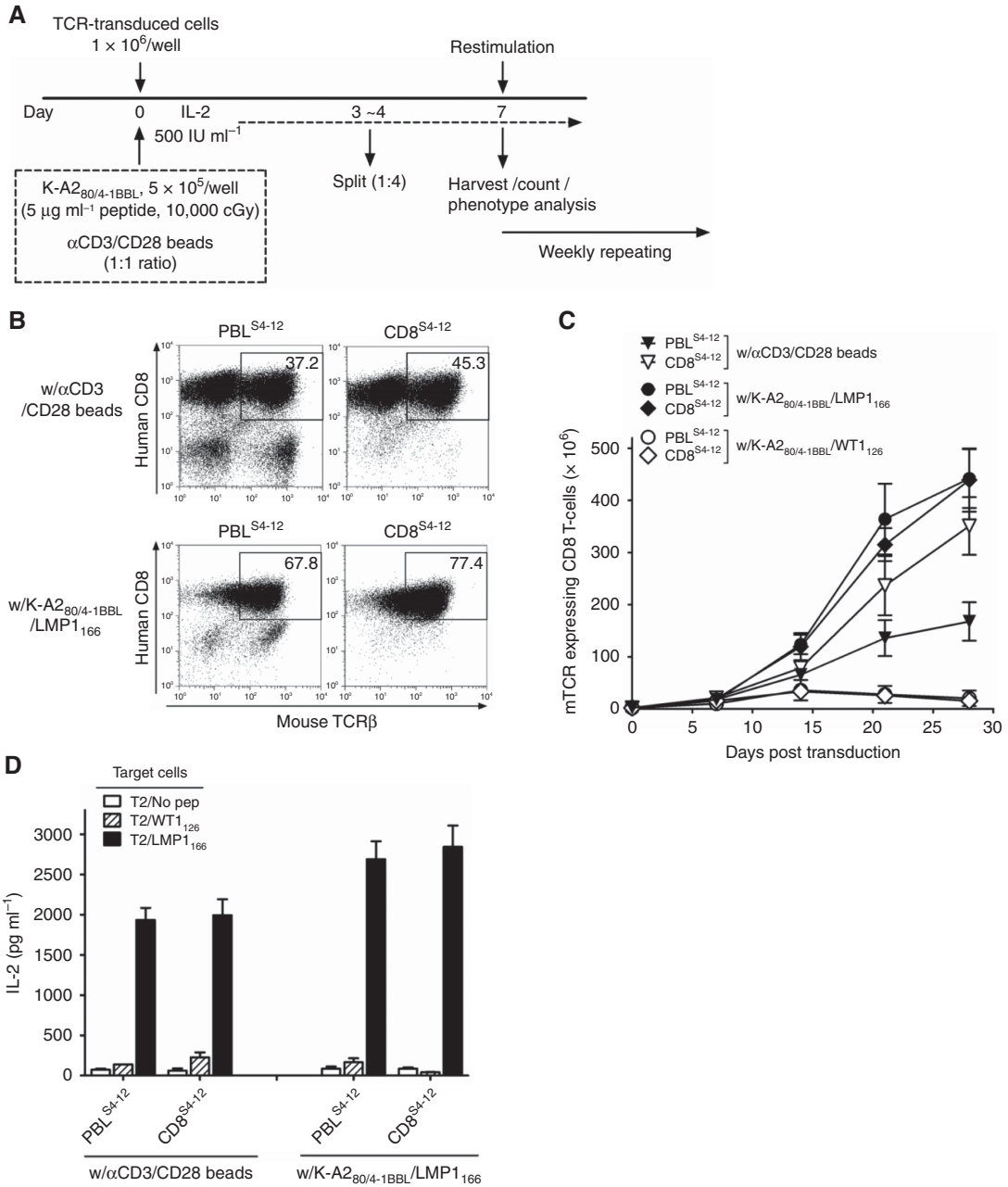

Figure 6. *Ex vivo* expansion of the engineered CD8 T-cells and PBLs. (**A**) Schematic experimental plan. CD8 $T^{S4\text{-}12}$ and $PBL^{S4\text{-}12}$ were co-cultured with artificial K-$A2_{80/4\text{-}1BBL}$ cells, which were pulsed with $LMP1_{166}$ or $WT1_{126}$, compared to anti-CD3/CD28-coated beads (1:1 ratio) with 500 IU ml^{-1} IL-2. (**B**) A representative analysis of murine TCR in *ex vivo* expanded CD8 $T^{S4\text{-}12}$ and $PBL^{S4\text{-}12}$ stimulated with either anti-CD3/CD28-coated beads (*upper panel*) or $LMP1_{166}$-pulsed K-$A2_{80/4\text{-}1BBL}$ cells (*lower panel*) for 21 days. Numbers in each rectangular gate represent the percentage TCR S4-12-positive cells of all human CD8 T-cells. (**C**) Proliferation of CD8 $T^{S4\text{-}12}$ and $PBL^{S4\text{-}12}$ in response to various conditions. Total numbers of murine TCR-expressing CD8 T-cells was calculated. *Points*, mean numbers of CD8 T-cell expansion over time; *bars*, s.d. Data are sum of single experiment on five donors. (**D**) Functional specificity of *ex vivo* expanded CD8 $T^{S4\text{-}12}$ and $PBL^{S4\text{-}12}$ was evaluated as in Figure 3B. Results represent the average amount of cytokines from two independent experiments with s.d. (*bars*). These experiments were repeated thrice with similar results. LCL = lymphoblastoid cell lines; LMP = latent membrane protein; PBL = peripheral blood lymphocytes; TCR = T-cell receptor.

encoding multiple LMP1 epitopes (including $LMP1_{166}$) induced potent T-cell responses against LMP1-expressing tumours (Duraiswamy *et al*, 2003). Although potential immune defects against EBV infection are not fully understood, it is generally accepted that persistent viral infection induces inefficient anergic T-cells eliciting immune tolerance and therefore fails to eliminate viral-infected cells in patients. Moreover, clonal T-cell anergy and the related adaptive tolerance is likely to remove high avidity T-cells specific for immune-dominant epitopes to a higher extent than those for subdominant epitopes, and subdominant T-cell epitopes are detected after immunisation with vaccines lacking immunodominant peptides (Rodriguez *et al*, 2001). Thus, isolation of TCRs specific for subdominant T-cell epitopes may be effective to manipulate genetic engineering of T-cell immunity. In view of this, our results show that T-cells engineered with $LMP1_{166}$-TCR could be specifically activated using a low concentration of

peptides (Figure 2C), and these cells efficiently recognised peptide-loaded LCL-A2^{pos} and LMP1-transfected tumour cells (Figure 4). These results imply that LMP1$_{166}$-TCR could have high avidity for antigen recognition and could be activated after a strong TCR stimulus capable of initiating a signal transduction cascade. Nonetheless, engineered cells exhibited relatively low responsiveness towards un-treated LCL-A2^{pos} targets (Figure 3), in line with previous reports that demonstrate no or low responsiveness of T-cells transduced with TCR specific to EBV and HIV antigens towards native antigen-positive cells (Orentas *et al*, 2001; Schaft *et al*, 2006) and virus-infected targets (Ueno *et al*, 2004). Particularly, CD28-CD3ξ domain-conjugate TCR specific to EBV antigen enhanced cytokine-secretion responses towards antigen-positive targets (Schaft *et al*, 2006), suggesting that properties of isolated TCRs such as signalling capacity may be responsible for TCR reactivity.

The clinical success of TCR-based therapies notwithstanding, potential concerns have been raised over the use of isolated TCR, because the pairing of transduced and endogenous TCR chains in TCR-gene-modified T-cells may induce unknown and possibly hazardous self-reactive side effects (Bendle *et al*, 2010; van Loenen *et al*, 2010). To this end, several groups have explored to improve transduced TCR pairing. Murine TCRs provide an alternative source for high-affinity TCRs because the murine TCR repertoire is non-tolerant to many human antigens (Chinnasamy *et al*, 2011; Rosati *et al*, 2014). Rosenberg and colleagues have reported that clinical trials using murine TCR-transduced cells demonstrated substantial antitumour responses (Johnson *et al*, 2009; Parkhurst *et al*, 2011; Morgan *et al*, 2013) and some patients with murine TCRs developed antibodies against TCR-variable regions with no effect on the clinical outcome (Davis *et al*, 2010). Furthermore, murine-human hybrid-TCRs produced by substituting the human constant region with the murine constant region showed a higher expression of the receptor, increased cytokine secretion, and enhanced antitumour activity mediated by improved TCR pairing and CD3 stability (Cohen *et al*, 2006; Goff *et al*, 2010). Overall, these studies support that the isolated murine LMP1$_{166}$-TCR could be applied in clinical trials with or without further genetic modification to treat EBV latency-II malignancies.

The clinical efficacy of infused T-cells correlates with their ability to sufficiently persist *in vivo* to exhibit substantial antitumour responses. Several studies have demonstrated that *in vivo* persistence and measurable antitumour immunity depends on the differential status of effector T-cells (Morgan *et al*, 2006; Hinrichs *et al*, 2009; Rosenberg *et al*, 2011). In view of this, a recent study reported that cord-blood T-cells can be used as a potential source for TCR-gene transfer because most of these cells belong to naïve T-cell subsets (Frumento *et al*, 2013). We also observed that LMP1$_{166}$-TCR-transduced cord-blood T-cells exhibit levels of cytokine secretion similar to that of peripheral CD8 T-cells towards LMP1-expressing targets (Supplementary Figure S6). Likewise, CD4 T-cells also play a crucial role in the persistence of transferred CD8 T-cells and generation of long-term memory T-cells, and significantly contribute to tumour prevention *in vivo* (Mitsuyasu *et al*, 2000). A previous report showed that TCR-engineered CD4 T-cells can confer functional specificity and subsequent antitumour immunity capable of preventing the tumour growth *in vivo* (Xue *et al*, 2013). Our results also showed the generation of TCR-engineered CD4 T-cells capable of recognising LMP1-expressing tumours (Figure 4), suggesting that pHLA-A2/LMP1$_{166}$-restricted CD4 T-cells could improve proliferation and memory development of adoptively transferred CD8 T-cells. The success of T-cell-based immunotherapy usually requires large numbers of cells ($\geqslant 10^9$) with intact effector functions. Nevertheless, long-term *ex vivo* cultured T-cells possess terminally differentiated properties, demonstrating low persistence *in vivo*. Numerous groups have developed *ex vivo* T-cell culturing protocols with anti-CD3/CD28-coated activator beads (Rasmussen *et al*, 2010; Brimnes *et al*, 2012). Particularly, Butler and colleagues have reported anti-CD3 antibody-expressing artificial APC-based system for *in vitro* expansion of CD8 T-cells under autologous assistance of CD4 T-cell (Butler *et al*, 2012). We have also developed *ex vivo* engineered T-cell expansion regimes with artificial APCs expressing HLA molecules and co-stimulatory CD80 and 4-1BBL (Figure 6). Notably, the exponentially expanded TCR-transduced T-cells could still maintain their functional specificity with a homogeneously enriched CD8 T-cell population.

In summary, we describe a novel HLA-A2-restricted TCR that specifically recognises LMP1$_{166}$ epitope and provide the first evidence that LMP1$_{166}$-TCR engineered T-cells allow efficient recognition to display potent cytotoxicity towards engineered LMP1-overexpressing tumour cells *in vitro* and *in vivo*. Additional studies for optimising the TCR avidity that affect the specificity of TCR-transferred T-cells could facilitate clinical applications in the treatment of EBV-associated diseases, including EBV latency-II malignancies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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