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 LMP1166 (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 to EBV-LMP1-specific T-cells, such as the relatively long manufacturing time, limited availability, and comparably low avidity of effector T-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 (Schaf 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.

Peptides and reagents. Synthetic peptides representing CD8 T-cell epitopes WT1126 (RMFPNAPYL), LMP1101 (LLLALWNL), LMP1125 (YLLEMLWRL), LMP1159 (YLQQNWWTL), LMP1166 (TTLVDLLWL), LMP1176 (WLLLLFLAIL) 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 LMP1166-specific CD8 T-cells, HLA-A2 Tg mice were immunised intravenously with 2 × 10^6 dendritic cells (DCs) pulsed with 10 μg ml^{-1} LMP1166 for 18 h, and after 7 days, the mice received an intravenous TriVax-immunisation. TriVax consists of a mixture of 150 μg LMP1166, 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 LMP1166-specific cytokine-secreting CD8 T-cells. LMP1166-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} LMP1166 in 24-well plates. Seven days later, IFNγ-ElSpot assays were performed. Bulk cultured T-cells were cloned at single cells per well in U-bottom 96-well plates with 3 × 10^5 LMP1166-pulsed irradiated (10 000cGy) T2 and 1 × 10^5 irradiated (5000 cGy) splenocytes in medium containing 50 μU ml^{-1} IL-2 and 5 ng ml^{-1} IL-7. Proliferating T-cell clones were evaluated for responsiveness towards LMP1166 using intracellular IFNγ-staining.

Cloning of LMP1-specific, HLA-A*0201-restricted TCR. Total RNA from T-cell clones was isolated using an RNaseasy-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’-cttcagctggcagccagctttcagctgggaggctccgctgctctctctacagttcagctgctg-3’; TCRβ-reverse; 5’-tcttcctgctgagcagccgcgccagcgggaattgggggaggccgccgctgctctctctacagttcagctgctg-3’ encoding furin-sensitive spacer RVKRRGS-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 × 10⁵ 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 (PBLmock). 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 Biosciences, San Diego, CA, USA) was prepared; 1 µg dimer, 5 µg LMP1166, and 0.3 µg β2-microglobulin (Sigma-Aldrich) at 37 °C overnight. TCR-transduced PBLs (1 × 10⁶ cells) were incubated with LMP1166-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 × 10⁶ 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, Orogen, 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 × 10⁶ cells) were co-cultured with 5 × 10⁵ peptide-loaded K-A2B01/1-RBL, with 500 IU ml⁻¹ IL-2. The artificial APCs were loaded with either 5 µg ml⁻¹ LMP1166 or WT1126 for 6 h and irradiated (10000 Gy)/ C with 8 µg ml⁻¹ 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 × 10⁵ engineered cells were co-cultured with irradiated (10000 Gy)/ C 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 Gy) irradiated on day -1. Each mouse intravenously received 3 × 10⁶ K-Δ2LMP1166 tumour cells. Seven days later, each mouse was three times intravenously infused with 2 × 10⁷ engineered PBLs at every 2-day interval (on day 7, 9, and 11). Intraportal 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 LMP1166-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 LMP1166 (TLLVDLLW/L) using a novel vaccination strategy with LMP1166-loaded DCs followed by a mixture of LMP1166 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, 2015b). LMP1166-TriVax booster-immunisation after priming with LMP1166-loaded DCs yielded 0.5–1.5% IFNγ-producing CD8 T-cells in spleen (Figure 1A). To generate LMP1166-specific T-cell clones, purified CD8 T-cells were co-cultured with LMP1166-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-A2pos), where higher levels of T-cell responses were observed against un-pulsed or WT1126-pulsed LCL-A2pos than against LMP1166-pulsed HLA-A2-negative LCLs (LCL-A2neg). Subsequently, CD8 T-cells were cloned by limited dilution, and proliferative T-cell clones were examined for LMP1166-specific IFNγ-producing reactivity (Figure 1C). TCRx and TCRβ chains from each high level of IFNγ-secreting T-cell clones against LMP1166 were amplified. Since TCRs consist of heterodimers, isolated TCRx and TCRβ chains were linked with furin-spacer RKVRKGS-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 with 2-microglobulin (Sigma-Aldrich) at 37 °C overnight. TCR-transduced cells (1 × 10⁶ engineered cells) were co-cultured with irradiated (10000 Gy)/ C peptide-pulsed targets. 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.

**Functional specificities of HLA-A2-restricted LMP1166-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 PBLs4-12). 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 PBLmock (data not shown). Initially, the engineered PBLs4-12 were stained using LMP1166-loaded HLA-A2:lg 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 PBLs4-12 had high levels of LMP1166-specific TCR-transduced CD8 and CD4 T-cells (~9% per cell), whereas no significant LMP1166-specific staining was found in TCR S4-6-transduced PBLs (PBLs4-6), that was non-functional. Apparently, PBLs4-12 were not stained with control peptide (WT1126)-loaded HLA-A2:lg dimers (Supplementary Figure S2). To assess the specific reactivity, HLA-A2-restricted LMP1166-derived peptides were pulsed onto T2 cells, and co-cultured with the engineered PBLs4-12. Interestingly, PBLs4-12 were equipped to additionally recognise target cells pulsed with LMP1167 (LLVDLLWLW), which is one amino acid-shifted peptide from LMP1166, but not to other HLA-A2-restricted LMP1166-derived peptides including an irrelevant WT1126 (Figure 2B).

Moreover, PBLs4-12 were also stained with LMP1167-loaded HLA-A2:lg dimers, but in low frequency compared to that of LMP1166-loaded HLA-A2:lg dimers (Supplementary Figure S2).
Subsequently, peptides were serially diluted and pulsed onto T2 cells to evaluate the functional avidity of TCR S4-12. The engineered PBLS4-12 recognised LMP1166-pulsed targets, and secreted IL-2 in a dose-dependent manner, at a concentration as low as 1 ng ml⁻¹ of LMP1166, whereas PBLS4-12 recognition against LMP1167 was rapidly reduced by 100 ng ml⁻¹ of LMP1167 concentration, implying that LMP1166 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 WT1126 (Figure 2C). As such, antigen-specific recognition and cytolytic activity of PBLS4-12 were validated compared with that of PBLS4-6. As shown in Figure 2D and E, functional activity of PBLS4-12 was evident, which displayed high levels of cytotoxicity towards LMP1166-pulsed targets, whereas PBLS4-6 did not respond to the target cells.

Murine TCR S4-12 can recognise endogenous processed HLA-A2/LMP1166 complexes. One potential concern with TCR S4-12 was whether PBLS4-12 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/LMP1166 complexes (pHLA-A2/LMP1166) onto the cell surface by natural antigen-processing pathways. As shown in Figure 3A, high level of PBLS4-12 responses were observed against LMP1-RNA-electro-transferred DCs (DC/LMP1RNA), similar to those with LMP1166-pulsed DCs (DC/LMP1166) that showed the presence of saturated exogenous LMP1166. 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
the DC/LMP1\textsubscript{166} targets (Supplementary Figure S3), indicating that the functional reactivities of PBL\textsuperscript{S4-12} are due to endowing TCR S4-12, not due to endogenous activities of EBV-seropositive donor. Nonetheless, exogenous LMP1\textsubscript{166}-loaded LCL-A2\textsuperscript{pos} were recognised, resulting in production of a high level of IL-2, and were efficiently lysed by PBL\textsuperscript{S4-12}, whereas PBL\textsuperscript{S4-12} revealed relatively low responsiveness towards un-loaded and WT1\textsubscript{126}-loaded LCL-A2\textsuperscript{neg} though LMP1 is known to be naturally expressed in LCLs (Figure 3B and C). However, the functional reactivities of PBL\textsuperscript{S4-12} towards LCL-A2\textsuperscript{pos} target cells were significantly reduced by HLA-A2 blocking (Supplementary Figure S4), implying that the LMP1\textsubscript{166}-specific recognition of PBL\textsuperscript{S4-12} is HLA-A2-restricted. Apparently, LCL-A2\textsuperscript{neg} targets were not attacked while LMP1\textsubscript{166} loaded LCL-A2\textsuperscript{pos} were provided. To confirm the capability of TCR S4-12 to recognise endogenously processed pHLA-A2/LMP1\textsubscript{166} on target cells, we established K562-derived stable transfectants that expressed HLA-A2 alone (K-A2) and/or together with LMP1 (K-A2\textsubscript{LMP1}). After selection, LMP1 expression was examined in K-A2 and K-A2\textsubscript{LMP1} transfectants as well as LCL, results showed much lower level of LMP1 expression in LCL than K-A2\textsubscript{LMP1} cells (data not shown). Likewise, PBL\textsuperscript{S4-12} were effective in recognising K-A2\textsubscript{LMP1} pulsed either with or without LMP1\textsubscript{166} (Figure 4A), whereas TCR-non-transduced PBL\textsuperscript{mock} and TCR S4-6-transduced PBL\textsuperscript{S4-6} did not respond to the target cells (Supplementary Figure S5). In accordance, higher cytolytic activity of PBL\textsuperscript{S4-12} was observed against K-A2\textsubscript{LMP1} as compared to that with PBL\textsuperscript{S4-6} (Figure 4B). These data indicate that K-A2\textsubscript{LMP1} presents pHLA-A2/LMP1\textsubscript{166} on the surface through intrinsic LMP1-processing machinery.

We examined the composition of PBL\textsuperscript{S4-12} to clarify which subsets of T-cells have effector functions. As shown in Figure 4C, PBL\textsuperscript{S4-12} were mainly composed of CD8 T-cells (CD8 T\textsubscript{S4-12}; ~25%) but CD4 T-cells (CD4 T\textsubscript{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\textsubscript{S4-12} exhibited high levels of IL-2 production similar to those with that of purified CD8 T\textsubscript{S4-12} in response to LMP1\textsubscript{166}-loaded targets including K-A2\textsubscript{LMP1} (Figure 4D), as specific HLA-A2:lg 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...
lymphocytes exhibited higher level of IL-2 secretion similar to that of PBLs towards LMP1166-loaded and/or LMP1-expressing K-A280/160 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/LMP1166 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 PBLS4-12. Next, we assessed whether adoptive transfer of PBLS4-12 would offer a therapeutic benefit in vivo using a xenogeneic model, which was systemically engrafted with luciferase-expressing K-A280/160 (K-A2LMP1/LUC), and a bioluminescent imaging technique to monitor tumour growth. Mice that were intravenously engrafted with K-A2LMP1/LUC underwent adoptive infusion with PBLmock, PBLS4-6 or PBLS4-12 on day 7 (Figure 5A). Mice treated with PBLS4-12 had a reduced tumour progression as compared to those with PBLmock or PBLS4-6, drawing significantly lower bioluminescent signal by day 42 (Figure 5B and C). As such, the adoptive transfer of PBLS4-12 revealed significantly increased median survival of the mice by more than 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 PBLS4-12, correlated with the observed improved antitumour effects (Figure 5E), indicating that TCR-transduced PBLS4-12 have potential for substantial long-term engraftment in vivo after adoptive transfer.

Ex vivo expansion of CD8 T4-12 and PBLS4-12. 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-IBBL (K-A280/160/4-IBBL), which can increase the survival of activated T-cells. Subsequently, PBLS4-12 and CD8 T4-12 were expanded with LMP1166- or WT1126-loaded K-A280/160/4-IBBL and compared with conventional anti-CD3/CD28-coated beads (Figure 6A). In this setup, stimulation with LMP1166- or WT1126-loaded K-A280/160/4-IBBL revealed expansion of distinct clear homogeneous populations of CD8 T4-12 (Figure 6B), which resulted in higher absolute numbers of CD8 T-cells compared to those with anti-CD3/CD28-coated beads (~400-fold versus ~200-fold increase in PBLS4-12 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-A2LMP1). K562 expressing HLA-A2 alone (K-A2) and T2 cells without peptide (No pep) or with peptides (WT1126 and LMP1 166) were also included. IL-2-ELISA assay (A) and 51Cr-release assay (B) were performed as in Figure 3 including K-A2 and K-A2LMP1 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. 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. 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 WT1126-loaded K-A2 80/4-1BBL (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-Oakamura 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
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...
encoding multiple LMP1 epitopes (including LMP1166) 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 LMP1166-TCR could be specifically activated using a low concentration of IL-2.

Figure 6. Ex vivo expansion of the engineered CD8 T-cells and PBLs. (A) Schematic experimental plan. CD8 T S4-12 and PBL S4-12 were co-cultured with artificial K-A2 40/4-1BBL 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 S4-12 and PBL S4-12 stimulated with either anti-CD3/CD28-coated beads (upper panel) or LMP1 166-pulsed K-A2 40/4-1BBL 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 S4-12 and PBL S4-12 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 S4-12 and PBL S4-12 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.
peptides (Figure 2C), and these cells efficiently recognised peptide-loaded LCL-A2<sup>+</sup> and LMP1-transduced tumour cells (Figure 4). These results imply that LMP1<sub>166</sub>-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 native LMP1<sub>166</sub>-restricted LCL-A2<sup>+</sup> 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-CD3<sub>δ</sub> 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<sub>166</sub>-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 naive T-cell subsets (Frumento et al, 2013). We also observed that LMP1<sub>166</sub>-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<sup>+</sup>-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 (Mitsuysasu 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 subsequent 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<sub>166</sub>-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 (>10<sup>9</sup>) 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; Brinnes 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<sub>166</sub> epitope and provide the first evidence that LMP1<sub>166</sub>-TCR engineered T-cells allow efficient recognition to display potent cytolysis 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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