CMVpp65 Vaccine Enhances the Antitumor Efficacy of Adoptively Transferred CD19-Redirected CMV-Specific T Cells

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Abstract

Purpose: T cells engineered with chimeric antigen receptors (CAR) recognizing CD19 can induce complete remission of B-cell malignancies in clinical trials; however, in some disease settings, CAR therapy confers only modest clinical benefit due to attenuated persistence of CAR T cells. The purpose of this study was to enhance persistence and augment the antitumor activity of adoptively transferred CD19 CAR T cells by restimulating CAR+ T cells through an endogenous cytomegalovirus (CMV)-specific T-cell receptor.

Experimental Design: CMV-specific T cells from CMV seropositive healthy donors were selected after stimulation with pp65 protein and transduced with clinical-grade lentivirus expressing the CD19R:CD28:EGFRt CAR. The resultant bispecific T cells, targeting CMV and CD19, were expanded via CD19 CAR-mediated signals using CD19-expressing cells.

Results: The bispecific T cells proliferated vigorously after engagement with either endogenous CMVpp65 T-cell receptors or engineered CD19 CARs, exhibiting specific cytolytic activity and IFNγ secretion. Upon adoptive transfer into immunodeficient mice bearing human lymphomas, the bispecific T cells exhibited proliferative response and enhanced antitumor activity following CMVpp65 peptide vaccine administration.

Conclusions: We have redirected CMV-specific T cells to recognize and lyse tumor cells via CD19CARs, while maintaining their ability to proliferate in response to CMV antigen stimulation. These results illustrate the clinical applications of CMV vaccine to augment the antitumor activity of adoptively transferred CD19CAR T cells in patients with B-cell malignancies. Clin Cancer Res; 21(13); 2993–3002. ©2015 AACR.

Introduction

Human studies of cancer and infectious diseases demonstrate that adoptive transfer of T cells of defined antigen specificity can establish or augment immunity to eradicate targeted malignant or infected cells. Adoptive transfer of in vitro expanded, chimeric antigen receptor (CAR)–redirected CD19-specific T cells can induce dramatic disease regression in patients with leukemia and lymphoma (1–4). However, the full potential of this emerging modality is hampered in some cancer settings by a significant rate of therapeutic failure arising from the attenuated engraftment and persistence of CAR-directed T cells following adoptive transfer. In contrast, the adoptive transfer of native virus-specific T cells efficiently prevents progressive viral infections and exhibits longer-term persistence in patients (5–7).

The mechanisms for the differential persistence of adoptively transferred virus-specific T cells in hematopoietic cell transplantation (HCT) recipients versus tumor-reactive T cells in cancer patients is not fully understood, but possibly reflects both the environment into which the T cells are infused and qualitative attributes of the T cells that are isolated and expanded for adoptive transfer. In attempts to improve the efficacy of CAR T cells for tumor eradication, adoptive T cells with dual specificity have been created: isolated Epstein–Barr virus (EBV)–specific T cells modified to express GD2 or CD30 CARs recognizing tumors of neural crest origin (8–10), and isolated influenza A matrix protein 1 (MP1)–specific T cells modified to express CD19 CARs recognizing B-cell malignancies (11). These virus and CAR bispecific T cells demonstrate superior survival and antitumor activity compared with CAR T cells alone, possibly due to a more potent costimulation of virus-specific T cells after engagement of their native receptors. Recent studies demonstrate that adoptively transferred EBV × CMV × CD19CAR bi (tri)–specific T cells proliferate in patients as a result of CMV reactivation (12).

Cytomegalovirus (CMV) is a common virus for which 75% of adults in the United States test positive (13, 14) and it was the first virus targeted by adoptive transfer strategies. Pioneering immunotherapy trials (5–7) show that adoptive transfer of
Adoptive transfer of chimeric antigen receptor (CAR)–redirected CD19-specific T cells can induce durable regression in patients with leukemia and lymphoma. However, in some disease settings, CAR therapy confers only modest clinical benefit due to attenuated persistence of CAR T cells. We have improved the existing CAR technology by engrafting CD19CAR into CMV-specific T cells and investigating the effects of restimulating CAR+ T cells through an endogenous cytomegalovirus (CMV)-specific T-cell receptor. This study has demonstrated that the antitumor activity of CMV/CD19 bispecific T cells can be enhanced as a consequence of proliferation following CMV peptide vaccination. These results illustrate the clinical applications of CMV vaccine to augment the antitumor activity of adoptively transferred CD19CAR T cells in patients with B-cell malignancies. This CMV vaccine strategy has the potential to profoundly affect the general field of T-cell therapy by substituting a CAR specific to any individual tumor.

**Translational Relevance**

Adoptive transfer of chimeric antigen receptor (CAR)–redirected CD19-specific T cells can induce durable regression in patients with leukemia and lymphoma. However, in some disease settings, CAR therapy confers only modest clinical benefit due to attenuated persistence of CAR T cells. We have improved the existing CAR technology by engrafting CD19CAR into CMV-specific T cells and investigating the effects of restimulating CAR+ T cells through an endogenous cytomegalovirus (CMV)-specific T-cell receptor. This study has demonstrated that the antitumor activity of CMV/CD19 bispecific T cells can be enhanced as a consequence of proliferation following CMV peptide vaccination. These results illustrate the clinical applications of CMV vaccine to augment the antitumor activity of adoptively transferred CD19CAR T cells in patients with B-cell malignancies. This CMV vaccine strategy has the potential to profoundly affect the general field of T-cell therapy by substituting a CAR specific to any individual tumor.

**Materials and Methods**

**Antibodies and flow cytometry**

Fluorochrome-conjugated isotype controls, anti-CD3, anti-CD4, anti-CD8, anti-CD28, anti-CD45, anti-CD27, anti-CD62L, anti-CD127, anti-IFNγ, and streptavidin were obtained from BD Biosciences. Biotinylated cetuximab was generated from cetuximab purchased from the City of Hope pharmacy. The IFNγ secretion assay—Cell Enrichment and Detection Kit and CMVpp65 protein were purchased from Millenyi Biotec. Phycoerythrin (PE)-conjugated CMV pp65 (NLVPMATV)–HLA-A2*0201 iTAg MHC tetramer, PE-conjugated multi-allele negative tetramer was obtained from Beckman Coulter. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen. All monoclonal antibodies, tetramers, and CFSE were used according to the manufacturer’s instructions. Flow cytometry data acquisition was performed on a MACSQuant (Millenyi Biotec) or FACScalibur (BD Biosciences), and the percentage of cells in a region of analysis was calculated using FCS Express V3 (De Novo Software).

**Cell lines**

EBV-transformed lymphoblastoid cell lines (LCL) were made from peripheral blood mononuclear cells (PBMC) as previously described (16). To generate LCL OKT3, allogeneic LCLs were resuspended in nucleofection solution using the Amaxa NucleoFector kit T, OKT3-2A-Hygromycin, pEK plasmid was added to 5 μg/10⁷ cells, the cells were electroporated using the Amaza Nucleofector I, and the resulting cells were grown in RPMI-1640 with 10% FCS containing 0.4 mg/mL hygromycin. To generate firefly luciferase+ GFP+ LCLs (FlucGFPPLCLs), LCLs were transduced with lentiviral vector encoding eGFP-fluc. Initial transduction efficiency was 48.5%, so the GFP+ cells were sorted by FACS for >98% purity. To generate CD19 NIH3T3 cells, parental NIH3T3 cells (ATCC) were transduced with a retrovirus encoding CD80, CD54, and CD58 (17). The established cell line was further engineered to express CD19GFP by lentiviral transduction. GFP+ cells were purified by FACS sorting and expanded for the use of stimulation of bispecific T cells. To generate pp65 stimulator cells, U251T cells derived from human glioblastoma cells from an HLA A2 donor (ATCC) were transduced with a lentiviral vector encoding full-length pp65 fused to green fluorescent protein (GFP). pp65U251T cells were purified by GFP expression using flow cytometry. Banks of all cell lines were authenticated for the desired antigen/marker expression by flow cytometry prior to cryopreservation, and thawed cells were cultured for less than 6 months prior to use in assays.

**Peptides**

The pp65 peptide NLVPMATV (HLA-A 0201 CMVpp65) at >90% purity was synthesized using automated solid phase peptide synthesis in the TVR (Beckman Research Institute of City of Hope). MP1 GIGFVTLP peptide (HLA-A 0201 influenza) was synthesized at the City of Hope DNA/RNA Peptide Synthesis Facility (Duarte, CA), pepMix HCMVA (pp65; pp65epmɪx) was purchased from JPT peptide Technologies (GmbH). All peptides were used according to the manufacturer’s instructions.

**Lentivirus vector construction**

The lentivirus CAR construct was modified from the previously described CD19-specific scFvFcζ chimeric immunoreceptor (18), to create a third-generation vector. The CD19CAR containing a CD28ζ costimulatory domain carries mutations at two sites (L235E; N297Q) within the CH2 region of the IgG4-Fc spacers to ensure enhanced potency and persistence after adoptive transfer (Supplementary Fig. S1). The lentiviral vector also expressed a truncated human epidermal growth factor receptor (huEGFRt), which includes a cetuximab (Erbitux) binding domain but excludes the EGF-ligand binding and cytoplasmic signaling domains. A T2A ribosome skip sequence links the codon-optimized CD19RCD28ζ sequence to the huEGFRt sequence, resulting in coordinate expression of both CD19R-CD28ζ and EGFRt from a single transcript (CD19RCARCD28EGFRt; ref. 19). The CD19RCARCD28EGFRt DNA sequence (optimized by GeneArt) was then cloned into a self-inactivating (SIN) lentiviral vector pHIV7 (gift from Jinding Kuan Yee, Beckman Research Institute of City of Hope, Duarte, CA) in which the CMV promoter was replaced by the EF-1α promoter.

**Enrichment of CMV-specific T cells after CMVpp65 protein stimulation**

PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech) from peripheral blood of consented healthy, HLA-A2 CMV-immune donors under a City of Hope Internal Review Board–approved protocol. PBMCs were frozen for later use. After overnight rest in RPMI medium containing 5% Human AB serum (Gemini Bio Products) without...
cytokine, the PBMCs were stimulated with current good manufacturing practice (cGMP) grade CMV pp65 protein (Miltenyi Biotec) at 10 μL/10 x 10⁶ cells for 16 hours in RPMI-1640 (Irvine Scientific) supplemented with 2 mmol/L L-glutamine (Irvine Scientific), 25 mmol/L N-2-hydroxyethylpiperezine-N-2-ethanesulfonic acid (HEPES; Irvine Scientific), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Irvine Scientific) in the presence of N 1/μL IL2 and 10% human AB serum. CMV-specific T cells were selected using the IFNγ capture (Miltenyi Biotec) technique according to the manufacturer's instructions.

**Derivation and expansion of bispecific T cells**

The selected CMV-specific T cells were transduced on day 2 after IFNγ capture with lentiviral vector expressing CD19CAR-D28EGFR at MOI 3. Seven to 10 days after lenti-transduction, the bispecific T cells were expanded by stimulation through CAR-mediated activation signals using 8,000 cGy-irradiated CD19-expressing NIH 3T3 cells at a 10:1 ratio (T cells:CD19 NIH3T3) once a week described previously (17) in the presence of IL2 50 U/mL and IL15 1 ng/mL. After two rounds of expansion, the growth and functionality of the bispecific T cells was evaluated in vitro and in vivo.

**Intracellular cytokine staining**

Bispecific T cells (10⁵) were activated overnight with 10³ LCL OKT3, LCL, or KG1a cells in 96-well tissue culture plates, and with 10⁵ U251T and engineered pp65-expressing U251T cells (pp65U251T) in 24-well tissue culture plates in the presence of Brefeldin A (BD Biosciences). The cell mixture was then stained using anti-CD8, cetuximab, and streptavidin to analyze surface expression of CD8 and CAR, respectively. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences). After fixation, the T cells were stained with an anti-IFNγ antibody.

**CFSE proliferation assays**

Bispecific T cells were labeled with 0.5 μmol/L CFSE and cocultured with stimulator cells LCL OKT3, LCLs, and pp65 U251T for 8 days. Cocultures with U251T and KG1a cells were used as negative controls. Proliferation of CD3- and CAR-positive populations was determined using multicolor flow cytometry.

**Cytokine production assays**

T cells (10⁵) were cocultured overnight in 96-well tissue culture plates with 10³ LCL OKT3, LCL, or KG1a cells and in 24-well tissue culture plates with 10³ U251T and engineered pp65-expressing U251T cells. Supernatants were then analyzed by cytometric bead array using the Bio-Plex Human Cytokine 17-Plex Panel (Bio-Rad). The selected CMV-specific T cells, such as CD62L, CD28, and IL7Ra (Fig. 2A and Supplementary Table S1 and Fig. 1A). Moreover, the selected CMV-specific T cells included both CD4 and CD8 subsets and represented the entire spectrum of CMV-specificity, showing responsiveness to CMVpp65 pepmix stimulation with broad recognition.

**Genetic modification of enriched CMV-specific T cells to express CD19 CAR and in vitro expansion of the bispecific T cells**

In the clinically adaptable procedure, IFNγ-captured CMV-specific T cells were transduced 2 days after the selection, without OKT3 activation, using the second-generation CD19RCD28EGFR lentiviral construct containing the IgG4 Fc hinge region mutations L235E; N297Q that we have determined to improve potency due to distortion of the FcR-binding domain (21, 22). Starting 7 days after lenti-transduction, the cells were stimulated on a weekly basis with 8,000 cGy-irradiated, CD19-expressing NIH3T3 cells at a 10:1 ratio (T cells:CD19NIH3T3). The percentage of CAR+ cells detected by cetuximab increased from 8% after transduction to 46% after two rounds of stimulation with a 120- to 150-fold total cell increase (Fig. 1B and C).

**Bispecific T cells exhibited specific effector function after stimulation through predefined viral TCR and CD19CAR**

Recapitulating our previous studies (23), the ex vivo–expanded CMV-specific T cells possessed an effector phenotype and no longer expressed the central memory markers of the originally selected cells, such as CD62L, CD28, and IL7Ra (Fig. 2A and Supplementary Fig. S2). However, levels of CD27 remained high, suggesting a greater proliferative potential that has been associated with greater clinical efficacy (24). To investigate bispecific T-cell effector function via signaling by both the endogenous CMV-specific TCR and the introduced CD19CAR, we evaluated response to engineered pp65-expressing U251T cells from HLA-A2 donors, and also allogeneic CD19+LCLs, based on
After selection, the cells were stained with antibodies specific for CMV pp65, and CMV-specific T cells were analyzed by intracellular cytokine (ICC) assays. In response to pp65 antigen stimulation, 24% to 53% of the T cells in the population were CMV-specific, and able to secret IFN-γ (Fig. 3B). Approximately 30% of T cells exhibited IFN-γ secretion upon stimulation with CD19+ LCL cells.

To assess the ability of bispecific T cells to proliferate in response to CD19 or pp65 antigen stimulation, T cells were labeled with CFSE and cocultured with different stimulators, and then evaluated for CFSE dilution 8 days later. Unlike the cultures stimulated with CD19-negative KG1a and U251T cells, cell division was more robust after stimulation through either the CD19 CAR+ (LCL cells) or the CMV-specific TCR (pp65U251T cells; Fig. 4). Building on these findings, we next performed in vivo experiments to examine the effects of CMV peptide vaccine on the expansion and antilymphoma efficacy of adoptively transferred bispecific T cells.

**Antilymphoma activity of adoptively transferred bispecific T cells was augmented in vivo by vaccination with CMVpp65 peptide antigen**

Our preliminary studies have demonstrated that engineered CD19CAR T cells can target and lyse CD19-positive lymphoma in vivo. However, the antitumor efficacy is suboptimal and tumor reduction represents a transient event followed by eventual tumor progression (data not shown) unless high doses of CAR T cells were infused (21). In this study, we wanted to tease out the differences between the targeted and control vaccines. Therefore, we chose a suboptimal T-cell dose (2 × 10^6 T cells), which is 5 times lower than the curative dose we used previously (10 × 10^6; ref. 21). We attempted to augment antitumor efficacy using a CMV peptide vaccine boost (Fig. 5A). As expected, as few as 10^6 CMV-specific T cells were able to induce a specific tumor reduction as compared with untreated and CMV-monospecific T cell–treated mice. We observed augmented antitumor activity after vaccination with pp65-peptide-pulsed T-APC of two
different formulations [pp65pepmix (Fig. 5B) and HLA A2-restricted CMVpp65 peptide (Fig. 5C)], but not in mice that were vaccinated using T-APC loaded with the irrelevant peptide MP1 (HLA A2-restricted). Interestingly, mice that received bispecific T-cell treatment had to be euthanized around the same time as control mice even though the tumor signals were dramatically lower (Fig. 5). Our further studies indicated that there were highly elevated levels of human-specific IFNγ and IL6 in the mouse serum (Supplementary Fig. S3) and it is probable that the mice died of exposure to CD19 antigen in vivo. This was supported by the finding that there were lower levels of engraftment of CMV-specific T cells as compared with bispecific T cells in tumor-bearing mice, even though the same pp65 peptide vaccine was used to boost antitumor activity through peptide vaccine, functional bispecific T cells are also expected to proliferate upon exposure to CD19 antigen in vivo. This was supported by the finding that there were lower levels of engraftment of CMV-specific T cells as compared with bispecific T cells in tumor-bearing mice, even though the same pp65 peptide vaccine was used to boost antitumor activity through peptide vaccine, functional bispecific T cells are also expected to proliferate upon exposure to CD19 antigen in vivo. This was supported by the finding that there were lower levels of engraftment of CMV-specific T cells as compared with bispecific T cells in tumor-bearing mice, even though the same pp65 peptide vaccine was used to boost antitumor activity through peptide vaccine, functional bispecific T cells are also expected to proliferate upon exposure to CD19 antigen in vivo. This was supported by the finding that there were lower levels of engraftment of CMV-specific T cells as compared with bispecific T cells in tumor-bearing mice, even though the same pp65 peptide vaccine was used to boost antitumor activity through peptide vaccine, functional bispecific T cells are also expected to proliferate upon exposure to CD19 antigen in vivo. This was supported by the finding that there were lower levels of engraftment of CMV-specific T cells as compared with bispecific T cells in tumor-bearing mice, even though the same pp65 peptide vaccine was used to boost antitumor activity through peptide vaccine.
Adoptively transferred bispecific T cells are efficiently ablated by cetuximab-mediated antibody-dependent cell-mediated cytotoxicity in vivo

The impressive clinical efficacy of CAR T-cell therapy and the frequently associated on/off-target toxicities, such as CRS, have highlighted the need for T-cell ablation strategies (1, 3, 4, 26). Taking advantage of the properties of the EGFRt receptor translated from the same transcript as the CD19CAR, we tested the anti-EGFR monoclonal antibody cetuximab for its ability to ablate CAR⁺ T cells. Fourteen days after engrafting mice with bispecific T cells, cetuximab was administered intraperitoneally at 1 mg/day for 4 consecutive days. CAR⁺ T cells in the bone marrow were significantly decreased as compared with untreated mice. Fifty percent to 60% of human T cells are CAR⁺ in the bone marrow of untreated controls; however, less than 10% of the human T cells in cetuximab treated mice are CAR⁺ (Fig. 6D), suggesting successful ablation (81% CAR T-cell elimination) based on antibody binding to the EGFRt.

Discussion

Adoptively transferred CMV-specific T cells can control latent CMV infection during HCT, are efficiently expanded in response to vaccine, and are able to control viral reactivation in immuno-compromised patients (5–7). CAR-engineered adoptive T-cell therapy is a developing area in cancer immunotherapy, with the potential for augmentation of antitumor efficacy by manipulating the processes prior to (proximal) and after (distal) T-cell infusion. One strategy to improve the persistence of in vitro-expanded effector cells is to isolate defined T-cell subtypes with an intrinsic capacity to persist during in vitro manipulation. Our studies have shown that central-memory–derived viral- or tumor-specific T cells exhibit superior persistence upon adoptive transfer compared with effector-memory–derived T cells (23, 27). We now seek approaches to magnify the potency of tumor-specific T cells after adoptive transfer.

Initially the major obstacle to the clinical application of CMV-specific T cells was the lengthy process required for their generation and selection (28, 29). In this study, our use of IFNγ capture of CMV-specific T cells consistently and efficiently enriched CMV-specific T cells while preserving the broad spectrum of CMV repertoires. Moreover, these cells remained amenable to gene modification after a brief CMVpp65 stimulation, avoiding the need for CD3/CD28 bead activation prior to transduction, which can cause activation-induced cell death (AICD) of CMV-specific T cells (30). Engineering the bulk IFNγ-captured T cells with the CD19CAR lentivirus followed by stimulation with CMV antigen resulted in 50% to 70% of the CAR⁺ T cells responding to pp65 stimulation, representing the subset of functional bispecific T cells. The bispecific T cells exhibited specific cytolytic activity and secreted IFNγ, as well as proliferating vigorously after engagement of endogenous CMVpp65 T-cell receptors or engineered CD19 CARs. Upon transfer into tumor-bearing mice, the bispecific T cells mediated CRS, which has been found to correlate with antitumor efficacy in the clinic (2, 31). We have demonstrated the IFNγ-capture platform for derivation of bispecific T cells to be clinically feasible, and were able to generate therapeutic doses of 

Figure 3.

Bispecific T cells exhibit bieffector function after stimulation through TCR and CAR. A, pp65 tetramer analysis of expanded bispecific T cells was performed before and after each CD19 Ag stimulation by flow cytometry. Percentages of pp65 tetramer and CD8 double-positive cells are indicated on the basis of negative tetramer and isotype gating. B, bispecific T cells (10⁷) were activated overnight with 10⁵ of LCL OKT3, LCL, and KG1a in 96-well tissue culture plates and 10⁵ U251T and engineered pp65–expressing U251T cells (pp65U251T) in 24-well tissue culture plates. Cocultures were fixed and permeabilized using the BD Cytofix/Cytoperm Kit according to the manufacturer’s instructions. After fixation and permeabilization, the T cells were stained with anti-IFNγ. Before fixation, anti-cetuximab–biotin and anti-CD3 staining was used to analyze surface expression of CAR and T cells. Percentages of positive cells on gated CD3 T cells are presented on the basis of that stained with isotype antibodies.
A professional APC are present than in these immunocompromised
marrow, and spleen using 
analysis of tumor cells remaining in the peripheral blood, bone 
cells eradicate tumors in NSG mice, we performed a population 
ther espo ns efo b i s p e c i 
cty compared with irrelevant MP1 challenge. We anticipate that 
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peci pepti de or pp65 pepmix. Both approaches elicited bis-
The effects of vaccination were indistinguishable whether using 
T cells with either pp65 peptide or a full-length pp65pepmix. 
mouse models, we generated APC by loading autologous 
for 8 days. A, CFSE retention on gated live T cells is shown. B, quanti 
CD19 Ag were labeled with CFSE and cocultured with indicated stimulators 
CFSE retention of CAR c T cells isolated by IFN 
c T cells to vaccine will be even more 
further demonstrating the speci 
f in vivo 
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function in bispeci 
to compare between xenograft models. Optimal 
growth signals are required for efficient and sustained expansion 
of transfused effector T cells in vivo. These signals encompass 
T-helper cell interactions, native TCR/CD3 complex 
signaling, and the activation of costimulatory signals. Although 
the CAR is designed to mimic the TCR and transmit activation 
signaling, the lack of in vivo persistence of some CAR T cells has 
been attributed to incomplete stimulation after engagement of 
the CAR (8, 10). This study suggests that the interaction of CAR 
cells with tumor cells is inadequate to completely eradicate 
the transplanted tumor. This could be a result of insufficient 
growth signal transmission through the CAR for T-cell expansion 
and activation, or insufficient cytolytic activation of T 
cells to kill tumor targets. T-cell activation through viral TCRs 
has several advantages over self-antigen TCR in promoting 
robust T-cell expansion. Signaling through a viral TCR is 
generally far more robust than through a self-antigen–specific TCR, 
because the viral-specific TCR affinity to antigen has not been 
dampened by the effects of tolerance and negative selection 
(34). A recent study is emblematic of the contrast in T-cell 
activation caused by stimulation through a self-antigen such as 
p33 and the immune response to antigens expressed from a 
viral vector (35). Because the viral TCR is expressed from the 
same cell as the CAR, the robust T-cell activation caused by an 
antiviral TCR could lead to enhanced antitumor activity as a 
consequence of the expansion of CMV-specific CAR T cells.

Figure 4.
Bispecific T cells proliferate after restimulation through TCR and CAR. 
Bispeci T cells isolated by IFNy capture and stimulated with two cycles of 
CD19 Ag were labeled with CFSE and cocultured with indicated stimulators 
for 8 days. A, CFSE retention on gated live T cells is shown. B, quantification 
of CFSE retention of CAR c T cells. Subtractions of percentages and mean 
fluorescence intensity (MFI) of CFSE expression of negative control KG1a to 
LCL and U251T to pp65U251T are depicted.

CD19CAR/C MV Bispecific T Cells

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Efficiently controlling proliferation to avoid cytokine storm and off-target toxicity is an important hurdle for the success of T-cell immunotherapy. The EGFRt incorporated in the CD19CAR lentiviral vector will serve not only as a marker for detection and selection of CAR T cells, but may also act as suicide gene to ablate the CAR ts T cells in cases of treatment-related toxicity. In this study, bispecific T-cell-engrafted mice were treated with cetuximab daily for 4 days. Consequently, more than 68% of the persistent CAR ts T cells were ablated in NSG mice as a result of antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and direct killing by cetuximab (36), despite the lack of professional ADCC effectors such as NK and B cells in the NSG mouse model. More efficient ablation is expected in humans, in the presence of a full panel of effector cells.

This study has demonstrated that the antitumor activity of bispecific CMV/CD19 T cells can be enhanced as a consequence of proliferation following CMV peptide vaccination. We hypothesize that the cell dose of bispecific T cells could be significantly decreased as compared with conventional CD19CAR T cells, due to their potential to proliferate in vivo in response to vaccine, avoiding prolonged culture times and the risk of terminal differentiation. Potential on/off-target toxicity can potentially be controlled by ablation of infused CAR T cells using cetuximab. These results illustrate the clinical applications of CMV vaccine to augment the antitumor activity of adoptively transferred CD19CAR T cells in several scenarios: (i) to salvage patients not achieving complete remission or relapsing after CAR T-cell therapy, (ii) vaccine boost when CD19 CAR T cells are failing to persist regardless of tumor responses at that time, (iii) planned vaccination on days 28 and 58 post-CD19 CAR T-cell therapy, (ii) vaccine boost when CD19 CAR T cells are failing to persist regardless of tumor responses at that time, (iii) planned vaccination on days 28 and 58 post-CD19 CAR T-cell therapy, (iii) planned vaccination on days 28 and 58 post-CD19 CAR T-cell therapy, (iii) planned vaccination on days 28 and 58 post-CD19 CAR T-cell therapy. City of Hope is currently accruing patients on clinical trials of two CMV vaccines (NCT01588015 and NCT01941056; ref. 15), enabling us to advance this combined therapy to the clinic rapidly, because we already hold INDs for both the CD19CAR T-cell vector and the CMV vaccines.

Moreover, this CMV vaccine strategy has the potential to profoundly affect the general field of adoptive T-cell therapy,

Figure 5.
Antitumor activity of adoptively transferred bispecific T cells is enhanced by CMVpp65 vaccination. A, NSG mice were injected i.v. on day 0 with $2.5 \times 10^6$ GFPfluc+C LCL cells. Three days after tumor inoculation, recipient mice were injected i.v. with $2 \times 10^6$ bispecific cells that underwent two rounds of CD19 stimulation. Vaccine was given by i.v. injection of peptide pulsed autologous T cells. Fourteen to 17 days after T-cell infusion, $5 \times 10^6$ pp65pepmix (B) or pp65 peptide (C; or MP1) loaded autologous T cells were irradiated and injected (i.v.) into T cell-engrafted mice as vaccine. pp65 vaccine was also supplemented to the mice that were treated with $2 \times 10^6$ CMV-specific T cells from the same donor, and untreated mice were used as another type of control. Tumor growth was evaluated by Xenogen imaging. $N = 5$ for each group in the experiments. The Mann–Whitney test was used for statistical analysis.
**Figure 6.** Adoptively transferred bispecific T cells can be expanded via CMVpp65 vaccine and ablated by cetuximab. A total of 2 x 10^6 CMV-specific or bispecific T cells from the same donor were adoptively transferred into CD19 tumor-bearing NSG mice. Two weeks after T-cell infusion, mice received either pp65 vaccine or MP1 vaccine. A, percentages of human T cells pooled from blood, bone marrow, and spleen from multiple mice (N = 4) and (B) GFP^+ tumor cells in the mouse spleen were determined by flow cytometry. The Mann–Whitney test was used for statistical analysis. C, CMVpp65 tetramer and CAR double-positive cells in the spleen of mice were analyzed by flow cytometry after labeling with antibodies specific to human CD45, pp65 tetramer, and EGFR, 28 days after bispecific T-cell infusion. The percentages of CMVpp65 tetramer^+ CAR^+ T cells in the human T-cell population of a representative mouse are presented. D, 1 x 10^6 bispecific T cells were adaptively transferred into CD19 tumor-bearing NSG mice. Two weeks after T-cell engraftment, mice received cetuximab (Erbitux) 1 mg/day i.p. injection for 4 days. One day after the last injection, CD45^+ GFP human T cells and CD45^+ CAR^+ T cells in the bone marrow were analyzed by flow cytometry after staining with antibodies specific to human CD45 and cetuximab-biotin. Representative FACS data from cetuximab-treated and untreated mice are depicted on the left, and percentages of CAR^+ T cells in the mouse bone marrow from multiple mice are presented on the right.

Because by transducing a variety of tumor-directed CARs into our CMV-specific T cells, we have the potential to tailor this strategy to a wide range of malignancies and tumor targets.

**Disclosure of Potential Conflicts of Interest**

M.C. Jensen reports receiving commercial and other research grants from, has ownership interest (including patents) in, and is a consultant/advisory board member for Juno Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, C.W. Wong, R. Urak, A. Mardiros, L.E. Budde, C.E. Brown, R. Nakamura, S.J. Forman

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References


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Xiuli Wang, ChingLam W. Wong, Ryan Urak, et al.