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 CD19CAR 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:zEGFRt 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-redistributed 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
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 (Erbitux) which includes a cetuximab (Erbitux) binding domain but excludes the EGF-ligand binding and cytoplasmic signaling domains. A20 cells were purchased from the City of Hope pharmacy. The IFNγ Secretion Assay—Cell Enrichment and Detection Kit and CMVpp65 protein were purchased from Miltenyi Biotec. Phycoerythrin (PE)-conjugated CMV pp65 (NLVPMVATV)–HLA-A0201 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 (Miltenyi 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 Amixa Nucleofector kit T, OKT3-2A-Hygromycin_pEK plasmid was added to 5 × 10^7 cells, the cells were electroporated using the Amaxa 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 (FlucGFP/PLC), 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 NLVPMVATV (HLA-A0201 CMVpp65) at >90% purity was synthesized using automated solid phase peptide synthesis in the TVR (Beckman Research Institute of City of Hope). MP1 GIGFVFTL peptide (HLA-A0201 influenza) was synthesized at the City of Hope DNA/RNA Peptide Synthesis Facility (Duarte, CA), pepMix HCMVA (pp65; pp65pepmix) 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 on 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 (huEGFR), 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 CD19R:CD28ζ sequence to the huEGFR sequence, resulting in coordinate expression of both CD19R:CD28ζ and EGFR from a single transcript (CD19CARCD28EGFR; ref. 19). The CD19R:CD28EGFR DNA sequence (optimized by GeneArt) was then cloned into a self-inactivating (SIN) lentiviral vector pHIV7 (gift from Jiing-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

"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.

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cytokine, the PBMCs were stimulated with current good manufacturing practice (cGMP) grade CMVpp65 protein (Miltenyi Biotech) at 10 μL/10 × 10^6 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 5 U/mL IL2 and 10% human AB serum. CMV-specific T cells were selected using the IFNγ capture (Miltenyi Biotech) 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-D28EGFRt at MOI 3. Seven to 10 days after lentivirus-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 as 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 (10^5) were activated overnight with 10^5 LCL OKT3, LCL, or KG1a cells in 96-well tissue culture plates, and with 10^5 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 pp65U251T in 24-well tissue culture plates in the presence of 5 U/mL IL2 and 10% human AB serum. CMV-specific T cells were selected using the IFNγ capture (Miltenyi Biotech) technique according to the manufacturer's instructions.

**Cytotoxicity assays**

Cytotoxicity assays were performed using the LDH cytotoxicity assay (Promega) according to manufacturer's instructions.

**Cytokine production assays**

Cytokine production assays were performed using the Bio-Plex Human Cytokine 17-Plex Panel (Bio-Rad Laboratories) according to the manufacturer's instructions.

**Cytotoxicity assays**

Four-hour chromium-release assays (CRA) were performed as previously described (20) using effector cells that had been harvested directly after two rounds of CD19 stimulation.

**Xenograft models**

All mouse experiments were approved by the City of Hope Institutional Animal Care and Use Committee. Six- to 10-week-old NOD/Scid IL2Rγnull (NSG) mice were injected intravenously (i.v.) on day 0 with 2.5 × 10^7 flucGFPLCLs cells. Three days after tumor inoculation, recipient mice were injected i.v. with 2 × 10^6 bispecific T cells that had undergone two rounds of CD19 stimulation. To generate antigen-presenting T cells (T-APC) for vaccine, REM-expanded T cells from the autologous donor were pulsed (2 hours at 37°C in CM) with 10 μg/mL of either HLA-A2–restricted pp65 peptide (NLVPVMATV) or not (pp65 pepmix) depending on whether bispecific T-cell products are pp65 tetramer dominant (GIIFVFTL, donor 2) or not (pp65 pemix donor 1) or 10 μg/mL HLA-A2–restricted control peptide specific for MP1 (GIIFVFITL). Following one wash with phosphate-buffered saline (PBS), 5 × 10^6 T-APC that had been irradiated with 3,700 cGy were injected i.v into the T cell–treated mice. Tumor burden was monitored with Xenogen imaging twice a week. Human T-cell engraftment in peripheral blood, bone marrow, and spleen was determined by flow cytometry.

**Results**

Enrichment of CMV-specific T cells from PBMC of healthy donors after stimulation with cGMP grade CMVpp65 protein To demonstrate the consistency of this clinically feasible process, the selection was repeated five times using PBMC from three different donors. IFNγ-positive T cells were consistently enriched from a baseline mean of 3.8% (range, 1.8–5.6%) to a postculturative mean of 71.8% (range, 61–81) and contained polyclonal CD8+ (34%) and CD4+ T cells (37%) after selection (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 CD19RCD28EGFRt lentiviral construct containing the IgG4 Fc hinge region mutations (L235E; N297Q) that we have determined to improve potency. After CAR activation, using the second-generation CD19RCD28EGFRt lentiviral construct containing the IgG4 Fc hinge region mutations, the 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
Development of clinically feasible platform for derivation of bispecific T cells. A, CMV-specific T cells from CMV-immune HLA A2 donors were selected using IFNγ capture after overnight stimulation with cGMP grade CMVpp65 protein. After selection, the cells were stained with antibodies specific to IFNγ, CD4, and CD8. The frequency of each population is presented after exclusion of dead cells with DAPI. B, the selected cells were transduced with the second-generation CD19CAR with a double mutation in the spacer, 24 hours after the IFNγ capture. Seven to 10 days later, the transduced cells were stimulated with irradiated CD19-expressing NIH3T3 cells at 10:1 ratio (T cells:CD19 NIH3T3), and the stimulation was repeated 7 days after the first stimulation. CAR expression was defined by cetuximab–biotin and streptavidin (SA) APC-Cy7 staining. Percentages of CAR+ cells are indicated in each histogram (filled gray), and based on subtraction of that stained with SA-APC-Cy7 alone (black line), growth of total cell number was determined by Guava Viacount at different time points.

Figure 1: Development of clinically feasible platform for derivation of bispecific T cells. A, CMV-specific T cells from CMV-immune HLA A2 donors were selected using IFNγ capture after overnight stimulation with cGMP grade CMVpp65 protein. After selection, the cells were stained with antibodies specific to IFNγ, CD4, and CD8. The frequency of each population is presented after exclusion of dead cells with DAPI. B, the selected cells were transduced with the second-generation CD19CAR with a double mutation in the spacer, 24 hours after the IFNγ capture. Seven to 10 days later, the transduced cells were stimulated with irradiated CD19-expressing NIH3T3 cells at 10:1 ratio (T cells:CD19 NIH3T3), and the stimulation was repeated 7 days after the first stimulation. CAR expression was defined by cetuximab–biotin and streptavidin (SA) APC-Cy7 staining. Percentages of CAR+ cells are indicated in each histogram (filled gray), and based on subtraction of that stained with SA-APC-Cy7 alone (black line), growth of total cell number was determined by Guava Viacount at different time points.

cytotoxicity, cytokine production, and proliferation profiles. As expected, the expanded bispecific T cells specifically lysed CD19+ LCLs with the same maximum killing levels as the OKT3-expressing LCL used as positive controls. Likewise, specific killing was also observed when pp65U251T cells were used as targets as compared with parental U251T cells (Fig. 2B). Accordingly, after overnight stimulation, elevated IFNγ secretion was observed after either CD19 or pp65 antigen stimulation as compared with antigen-negative stimulators such as KG1a and U251T parental cells (Fig. 2C).

Although CMV-specific T cells were enriched prior to lentiviral transduction, the T-cell population is mixed, including CMV-specific T cells, CD19CAR+ T cells, bispecific T cells, and possibly a small percentage of T cells that are neither CMV-specific nor CD19CAR+. T-cell expansion following lentiviral transduction is predominantly CD19-driven through CAR stimulation, so we next investigated how CAR stimulation affects the composition of CMV-specific T cells. Using pp65 tetramer as an indicator of the CMV-specific population, we found that the percentage of CMVpp65 tetramer-positive cells increased from 0.5% to 6.6% by the end of the second CD19 stimulation, indicating bispecific T cells proliferated strongly with CD19 stimulation (Fig. 3A).

To further investigate that these effector functions were attributable to bispecific T cells rather than distinct CD19CAR+ and CMV-specific T-cell subsets in the population, we performed intracellular cytokine (ICC) assays. In response to pp65 antigen stimulation, 24% to 53% of the T cells in the population were CAR+ and able to secret IFNγ (Fig. 3B). Approximately 30% of T cells exhibited IFNγ 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 2 × 10^6 bispecific 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 cytokine release syndrome (CRS; 25) rather than tumor. More interestingly, augmented antitumor efficacy induced by pp65 vaccine was supported in a relapsed tumor model (Supplementary Fig. S3) and it is probable that the mice died of CRS.}

**Figure 2.**

Bi- and trispecific T cells exhibit specific effector function after engagement with CD19+ and CMVpp65+ tumors. A. 7 days after the second CD19 Ag stimulation, T cells were stained with HLA A2–restricted pp65 tetramer, cetuximab–biotin, anti-CD8, and antibodies specific to central memory T-cell surface markers. Percentage of positive cells is indicated after dead cell exclusion with DAPI, gating based on pp65 tetramer and cetuximab double-positivity, and isotype-matched stained samples. B, 4-hour 51Cr release assays were performed using the bispecific T cells and indicated 51Cr-labeled target cells at different effector:target (E:T) ratios. OKT3-expressing LCLs were used as positive controls, KG1A and U251T as negative controls. CD19+ LCL and engineered pp65U251T cells were used as targets for CD19 and CMV-specific T cells, respectively. Data from a representative donor are presented. C, bispecific T cells (10⁵) were activated overnight with 10⁵ LCL, OKT3, LCL, or KG-1A in 96-well tissue culture plates and 10⁵ U251T and engineered pp65-expressing U251T cells (pp65U251T) in 24-well tissue culture plates. Supernatants were collected after overnight coinubation of bispecific T cells and stimulators. Cytokine levels with indicated stimulators (means ± SEM of triplicate wells) were determined using cytometric bead array.

The levels of human T cells and bispecific T cells were well correlated with the tumor reduction based on GFP expression by FACS analysis (Fig. 6B). Furthermore, CAR+ and CMVpp65 tetramer+ bispecific T cells harvested from mice were more abundant in the pp65 peptide-challenged mice than in MP1 controls (Fig. 6C). However, pp65 Tetramer/CAR+ double-positive cells were only detected in the spleen, possibly indicating a unique homing characteristic of the population of bispecific T cells. In addition to the predefined viral TCR that can be used to boost antitumor activity in vivo 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 stimulate both types of T cells (Fig. 6A). These data suggested that bispecific T cells were able to proliferate and expand in vivo in response to stimulation of the TCR as well as the CD19 CAR.
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 and engineered pp65-expressing U251T cells (pp65U251T) in 24-well tissue culture plates. Cocultures were fixed and permeabilized using the BD 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.

**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 immunocompromised 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 CD19 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...
mouse studies. To further study the extent to which bispecific APC are present than in these immunocompromised mice, we performed a population analysis of tumor cells remaining in the peripheral blood, bone marrow, and spleen using flow cytometry. A few tumor cells remained after mice were treated with bispecific T cells and pp65 vaccine; in contrast, many more tumor cells were detected in the mice receiving only un-engineered CMV-specific T cells—"the same percentage as was seen in untreated controls, and in the mice that received bispecific T cells without pp65 vaccine (Fig. 6B). Consistently, expansion of bispecific T cells was much lower in mice that received an irrelevant MP1 vaccine compared with those that received pp65 vaccine (2% vs. 10%; Fig. 6C), further demonstrating the specificity of the response to vaccination in bispecific T cell–treated animals. Meanwhile, we noticed that the percentage of pp65+/CAR double-positive human cells harvested from mice were much decreased compared with the input human T-cell population. We speculate that the tetramer-negative population has disproportionately expanded in vivo compared with tetramer-positive cells, because this subset includes cells expressing mouse xenoreactive native T-cell receptors. It is also possible that another contribution to the decline in the proportion of pp65+/CAR+ cells from the input population could be a result of these double-positive cells undergoing AICD after killing tumor cells, due to their effector T-cell characteristics (Fig. 2A). AICD could be thought of as a deleterious effect of the vaccine on pp65+/CAR+, but could actually be a measure of effectiveness as demonstrated by decreased tumor burden (Fig. 6B). Ongoing studies on the functional responses to CMV vaccine of the different T-cell subsets of the infused product will further reveal the mechanisms of the enhanced antitumor activity.

Preclinical studies with engineered CAR T cells in different xenotransplant tumor models have demonstrated variable potency with some showing tumor eradication in the short window tested and some reporting eventual tumor relapse (17, 22, 32, 33). Several variables of these artificial systems, such as the aggressiveness of the tumor cell line, tumor burden at the time of CAR T-cell infusion, dose of CAR T cells may account for perceived differences in CAR potency, making it difficult 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 T 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 cytolitic 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 p53 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.
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+ 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+ 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 cells, which has been shown an effective immune-stimulation in our CMV peptide vaccine. There is also potential benefit of using the bispecific T cells preemptively postallologeneic HCT, both to eliminate minimal residual disease (MRD) and control CMV, potentially preventing reactivation of virus or undergoing expansion in response to latent CMV reactivation. 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,
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.

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