Clinical Cancer Research

Vaccination Targeting Native Receptors to Enhance the Function and Proliferation of Chimeric Antigen Receptor (CAR)-Modified T Cells

Miyuki Tanaka[1], Haruko Tashiro[1], Bilal Omer[1,2], Natasha Lapteva[1], Jun Ando[1], Minhtran Ngo[1,3], Birju Mehta[1], Gianpietro Dotti[1,2,4], Paul R. Kinchington[5], Ann M. Leen[1,2,6], Claudia Rossig[7], and Cliona M. Rooney[1,2,3,6,8]

Abstract

Purpose: The multiple mechanisms used by solid tumors to suppress tumor-specific immune responses are a major barrier to the success of adoptively transferred tumor-specific T cells. As viruses induce potent innate and adaptive immune responses, we hypothesized that the immunogenicity of viruses could be harnessed for the treatment of solid tumors if virus-specific T cells (VST) were modified with tumor-specific chimeric antigen receptors (CAR). We tested this hypothesis using VZV-specific T cells (VZVST) expressing a CAR for GD2, a disialoganglioside expressed on neuroblastoma and certain other tumors, so that the live-attenuated VZV vaccine could be used for *in vivo* stimulation.

Experimental Design: We generated GMP-compliant, GD2.CAR-modified VZVSTs from healthy donors and cancer patients by stimulation of peripheral blood mononuclear cells with overlapping peptide libraries spanning selected VZV antigens, then tested their ability to recognize and kill GD2- and VZV antigen–expressing target cells.

Results: Our choice of VZV antigens was validated by the observation that T cells specific for these antigens expanded *in vivo* after VZV vaccination. VZVSTs secreted cytokines in response to VZV antigens, killed VZV-infected target cells and limited infectious virus spread in autologous fibroblasts. However, while GD2.CAR–modified VZVSTs killed neuroblastoma cell lines on their first encounter, they failed to control tumor cells in subsequent cocultures. Despite this CAR-specific dysfunction, CAR-VZVSTs retained functional specificity for VZV antigens via their TCRs and GD2.CAR function was partially rescued by stimulation through the TCR or exposure to dendritic cell supernatants.

Conclusions: Vaccination via the TCR may provide a means to reactivate CAR-T cells rendered dysfunctional by the tumor microenvironment (NCT01953900). *Clin Cancer Res; 23(14); 3499–509. ©2017 AACR.*

Introduction

T cells of any native specificity can be made tumor-specific by modification with tumor-specific chimeric antigen receptors (CARs). CD19-CARs incorporating costimulatory endodomains have had great success for the treatment of B-cell malignancies (1). However, CAR-T cells directed to solid tumors have been less effective, (2, 3) due in part to their highly immunosuppressive microenvironment and to their inhibitory phenotype, which contrasts with the costimulatory phenotype of B cells that provides professional costimulation to CD19-directed CAR-T cells.

In most clinical studies, CARs are introduced into polyclonally activated T cells whose native antigen specificities are unknown. Hence, *in vivo* CAR-T-cell proliferation is dependent on the CAR ligation as well as lymphodepletion of the patient to provide homeostatic cytokines. Our group evaluated a different strategy to enhance CAR-T-cell persistence: to test the hypothesis that endogenous Epstein–Barr virus (EBV)-infected B cells would provide *in vivo* stimulation of CAR-T cells via the native TCR, we infused EBV-specific T cells (EBVST) modified with a first-generation GD2-CAR into patients with neuroblastoma (4). Indeed GD2.CAR-EBVSTs circulated with higher frequency than similarly modified CD3-activated T cells (ATC) in the first 6 weeks after infusion. Five of 11 children with measurable disease had tumor responses, including 3 complete remissions (CR), illustrating the therapeutic potential of CAR-modified virus-specific T cells (VST) for solid tumors (5, 6). However, even in responders, GD2.CAR-EBVST expansion could not be detected in peripheral blood, as measured by PCR for the transgene, and patients with bulky disease did not

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital and Houston Methodist Hospital. [2]Department of Pediatrics, Division of Hematology and Oncology, Baylor College of Medicine, Houston, Texas. [3]Program of Translational Biology and Molecular Medicine, Baylor College of Medicine, Houston, Texas. [4]Department of Medicine, Baylor College of Medicine, Houston, Texas. [5]Departments of Ophthalmology, and Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania. [6]Department of Pathology, Division of Immunology, Baylor College of Medicine, Houston, Texas. [7]University Children's Hospital Muenster, Pediatric Hematology and Oncology, University of Pittsburgh, Pittsburgh, Pennsylvania. [8]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Cliona M. Rooney, Center for Cell and Gene Therapy, Baylor College of Medicine, 1102 Bates St. Suite 1770.01, Houston, TX 77030. Phone: 832-824-4693; Fax: 832-825-4732; E-mail: crooney@bcm.edu

doi: 10.1158/1078-0432.CCR-16-2138

Translational Relevance

We have generated GMP-compliant T cells that are specific for varicella zoster virus (VZV) via their native TCR and for the disialoganglioside, GD2, via a chimeric antigen receptor (GD2.CAR), so that *in vivo* expansion and persistence can be induced by VZV vaccination in patients with GD2-positive tumors, such as neuroblastoma or sarcoma. The VZV vaccine should overcome the lack of immunogenicity of solid tumors by providing potent extratumoral stimulation of CAR-modified VZV-specific T cells (CAR-VZVST) and should promote their function despite the inhibitory tumor environment. CAR T cells may become dysfunctional on exposure to tumors and fail to kill tumor cells after their first encounter *in vitro*. We show that GD2.CAR-VZVSTs rendered dysfunctional by exposure to neuroblastoma cell lines can be partially rescued by stimulation via the TCR.

attain CRs. The lack of proliferation of CAR-EBVSTs in patients with neuroblastoma contrasted with the exponential proliferation of EBVSTs after infusion into hematopoietic stem cell transplant (HSCT) recipients. Important differences between these patient groups include the lymphopenic state and high EBV load of many HSCT recipients (7–9). In contrast, neuroblastoma patients were not lymphopenic and did not have elevated EBV loads.

Routine vaccination provides a minimally toxic means to break the homeostatic control of memory T cells and produce antigen-specific T-cell expansion *in vivo*, even under normal homeostatic conditions. As there is no commercially available vaccine for EBV, we considered the use of the VZV, another human herpesvirus for which successful vaccines have been licensed for widespread use. VARIVAX is a live attenuated VZV vaccine that has been used safely in millions of children (10) and Zostavax, a 14-fold higher dose of the same strain combination has been used as a booster vaccine in VZV-seropositive adults (11, 12). These safe vaccines should provide a potent strategy to induce *in vivo* activation and proliferation of adoptively transferred VZVSTs that are genetically modified with a tumor-specific CAR. T cells specific for a number of VZV protein antigens have been identified, most notably for the immediate early (IE) proteins 62 and 63 and the glycoprotein gE (13–23).

We previously demonstrated that memory VZVSTs could be reactivated and expanded using VZV lysate–pulsed dendritic cells (DC) and modified to express first- and second-generation CARs for GD2 and CD19 (24). We have now developed an improved, targeted, GMP-compliant approach by activating VZVSTs with overlapping peptide libraries spanning immediate early (IE) and abundant virion proteins of VZV. Here, we show that the frequency of T cells specific for five VZV proteins (gE, IE61, IE62, IE63, and ORF10) increase in response to both primary and booster vaccination, and that these cells can be genetically modified with a third-generation GD2.CAR and expanded to clinically relevant numbers *in vitro*. CAR-modified VZVSTs recognized and killed both $GD2^+$ tumor cells and VZV-infected cells, and limited the spread of infectious VZV *in vitro*. This ability to control viral spread may be an important safety feature for cancer patients who receive the live-attenuated VZV vaccine to boost their CAR-modified VZVSTs. Finally, we show that stimulation through the TCR, which can be achieved *in vivo* by vaccination, is able to partially rescue the antitumor activity of GD2.CAR-VZVSTs that are rendered dysfunctional by serial coculture with neuroblastoma cell lines.

Materials and Methods

Generation of antigen-presenting cells

Dendritic cells (DC) were derived from adherent PBMCs and cultured in CellGro DC medium (CellGenix) in the presence of IL4 (1,000 U/mL) and GM-CSF (800 U/mL; both R&D Systems). On day 5, immature DCs were matured for 48 hours using a cytokine cocktail consisting of IL4, GM-CSF, IL6 (10 ng/mL), TNFα (10 ng/mL), IL1β (10 ng/mL; all R&D Systems), and PGE2 (1 μg/mL; Sigma-Aldrich).

Activated T cells (ATC) for use as APCs were generated from PBMCs by stimulation on nontissue culture–treated 24-well plates coated with 1 μg/mL OKT3 (Ortho Biotech) and 1 μg/mL anti-CD28 (Becton Dickinson) antibodies in T-cell medium [50% RPMI1640 (Hyclone), 45% EHAA (Life Technologies), 2 mmol/L glutamax, and 5% human AB serum (Valley Biomedical Inc, Winchester, VA)] in the presence of human IL2 (50 IU/mL, from the NCI Biological Resources Branch, Frederick MD) for 8 to 14 days. ATCs were restimulated with CD3 and CD28 antibodies to upregulate HLA and costimulatory molecules 2 days prior to use as APCs (25).

Generation of VZVSTs

PBMCs were pulsed with pepmixes (10 ng of each peptide per 10^6 PBMCs) for 60 minutes, and then cultured in the presence of 400 U/mL of IL4 and 10 ng/mL of IL7 (R&D Systems; ref. 26) for 9 days in 24-well plates. On days 9 and 16 of culture, effector T cells were restimulated with autologous, irradiated (30 Gy), pepmix-pulsed ATCs (ATCpx; 10 ng of each peptide per 10^6 ATCs) and irradiated K562cs cells (VZVSTs:ATCpx:K562cs of 1:1:5) in 2-mL wells at 7×10^5 total cells per well with 5 ng/mL of IL15 (R&D Systems). ATCs upregulate CD80, CD86, and HLA class I and class II molecules after TCR stimulation and are able to present peptides to both $CD4^+$ and $CD8^+$ T cells, while K562cs cells provide costimulation *in trans* (25, 27). We termed this combination of stimulator cells KATpx for simplicity.

GD2.CAR retroviral vector and transduction of VZVSTs

The third-generation GD2.CAR retroviral vector (GD2.CAR3) has been described previously (4, 28) and comprises the 14g2a ScFv (29), a short spacer from the CH2 domain of IgG1, a CD28 transmembrane and intracellular signaling domains from CD28, OX40, and CD3 ζ. For generation of GD2.CAR-VZVSTs, PBMCs were activated with pepmix-pulsed DCs in the presence of IL4 and IL7 and on day 2 transduced with the GD2.CAR retroviral vector on retronectin (Takara Bio) coated plates, in the presence of IL4 and IL7. On day 4, cells were returned to regular culture, as described above (30).

VZV infection of fibroblasts

VZV expressing the EGFP protein fused to the N terminal end of the ORF66 protein kinase (31) was maintained as infected cell stocks by culture with fresh MRC-5 cells and used to prepare VZV when cytopathic effects were observed. Cell-free VZV was released by brief sonication and stored at −80°C until further use (32, 33). For infectious spread assays, dermal fibroblasts were infected with cell-free VZV at 2×10^3 PFU per 1×10^6 cells. When cytopathic effects were observed in approximately 75% of cells (2–3 days),

cells were harvested by trypsinization and added to uninfected cells to propagate the virus or to test the ability of VZVSTs to limit virus spread.

VZVST inhibition of virus spread in VZV-infected fibroblasts

VZVSTs, PBMCs, or autologous ATCs were cocultured with GFP-VZV–infected fibroblasts and uninfected fibroblasts at a ratio of 1:7:20 in T-cell medium in the presence of recombinant human IL2 (50 IU/mL) in a 12-well plate at 5.6×10^5 total cells per well. Three days later, cultures were collected and stained with CD45 antibodies (BD Biosciences), and analyzed by FACS. Absolute numbers of events for GFP-positive infected cells are compared using CountBright Absolute Counting Beads (Invitrogen).

Coculture experiments

GD2.CAR-VZVSTs or nontransduced (NT)-VZVSTs were cocultured with LAN-1 cells at the ratio of 1:1 in T-cell medium without cytokines. Five days later, cultures were collected and stained with CD3 and 14g2a (anti-GD2) antibodies, and analyzed by flow cytometry. For serial killing experiments, GD2.CAR-VZVSTs were cocultured with GFP-ffluc-LAN1 cells at 1:1 ratio in the presence of IL2 (20 U/mL; first coculture). On day 7, GD2.CAR-VZVSTs were harvested from the coculture plates and then were left unstimulated or were stimulated with autologous pepmix-pulsed DCs for 30 minutes at a 10:1 ratio of VZVSTs: DCs. After three days, T cells were plated at a 1:1 ratio with fresh GFP-ffluc-LAN1 cells in the presence of IL2 (20 U/mL; second coculture). Cocultures without DCs were used as unstimulated controls. T cells and tumor cells were counted by flow cytometry using CountBright beads.

Additional methodology can be found in the Supplementary Data.

Results

VZV pepmixes and an antigen-presenting complex activate and expand VZVSTs

To determine whether we could reactivate and expand VZVSTs from both naturally infected and vaccinated donors using overlapping peptide libraries (pepmixes), we stimulated PBMCs from 9 naturally infected (median age, 34 years; range 22–55 years) and 5 vaccinated donors (median age, 17 years; range 3–21 years, including three children aged 3, 5, and 17 years) with combined pepmixes spanning IE61, IE62, IE63, gE, and ORF10 in the presence of IL4 and IL7. These antigens were chosen because they should be processed and presented to T cells before immune evasion genes of VZV can be expressed and because they had previously proved immunogenic (15, 16, 19, 21). Responder cells

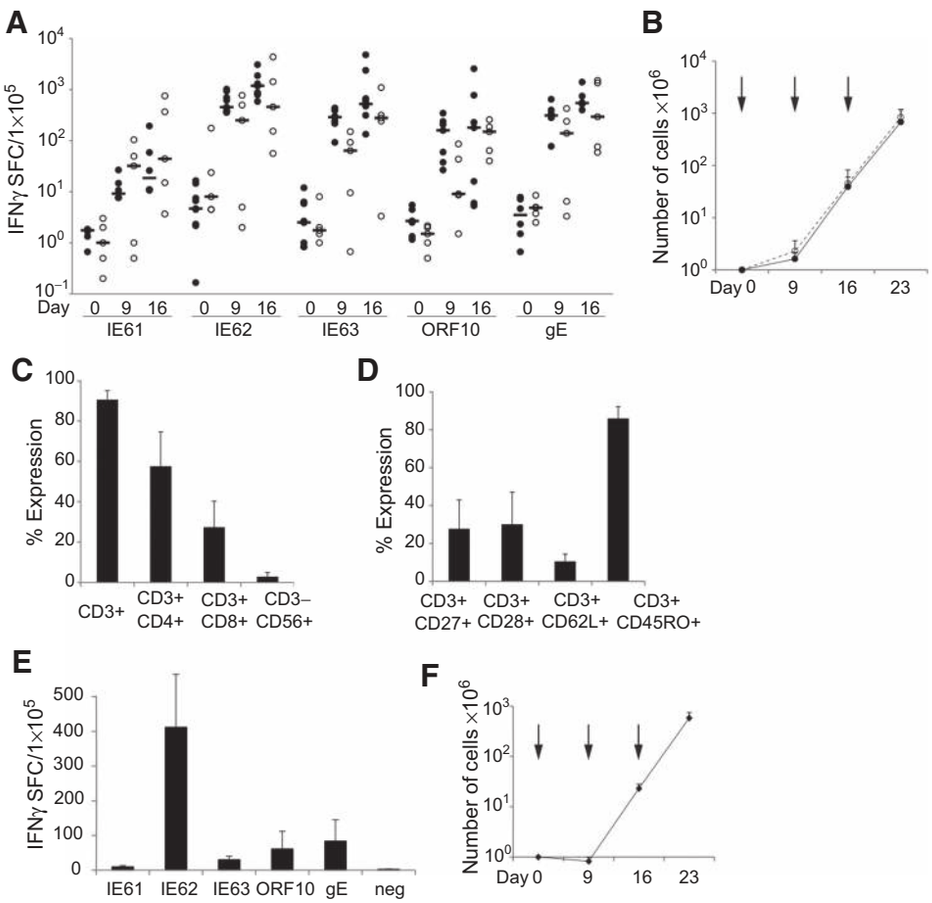

Figure 1.
VZVSTs can be reactivated and expanded from the peripheral blood of healthy donors and cancer patients. **A,** VZVSTs were generated from donors immunized by natural infection ($n = 9$) or by VZV vaccination without history of natural infection ($n = 5$). The frequency of antigen-specific T cells in peripheral blood was determined by IFNγ release ELISPOT assay after overnight stimulation of PBMCs with VZV antigen–spanning pepmixes (day 0) and again on days 9 and 16. Closed circles (●) represent naturally infected donors and open circles (○) are vaccinated donors. **B,** T-cell expansion was evaluated by viable cell counting using Trypan blue exclusion. Solid line represents naturally infected donors ($n = 9$) and dashed line, vaccinated donors ($n = 5$). Results are shown as mean cell numbers ± SEM. Each arrow indicates stimulation. **C** and **D,** The expression of surface markers as a percentage of the live cells was assessed on day 16 ($n = 6$). Results are shown as mean ± SD. **E** and **F,** PBMCs from 6 cancer patients, including three pediatric patients, were stimulated with VZV pepmix–pulsed mature DCs. Results from the five responders are shown. **E,** Specificity on day 16 was measured by IFNγ release ELISPOT assay (mean ± SEM, $n = 5$). **F,** Cell expansion was evaluated by counting using Trypan blue exclusion. Results are shown as mean cell numbers ± SEM ($n = 5$). Each arrow indicates each stimulation.

were restimulated on day 9 using pepmix-pulsed, irradiated autologous ATCs combined with irradiated HLA-negative K562 cells genetically modified with CD80, CD86, 41BB-ligand, and CD83 (K562cs) to provide costimulation *in trans* (25), an antigen-presenting complex we termed KATpx. IFNγ ELISPOT assays were performed on fresh PBMCs at the end of each stimulation cycle on days 9 and 16. Low frequencies of VZVSTs, usually less than 10 spot-forming cells (SFC) per 10^5 cells were detected in fresh PBMCs from both naturally infected and vaccinated donors, (Fig. 1A). However, these frequencies increased rapidly over the next 9 to 16 days in response to peptide stimulation (Fig. 1A). The majority of individuals showed some response to all five antigens, but the strongest responses were to IE62 (median 1184 SFC/1 × 10^5 cells, range 584–3,073), IE63 (median 521 SFC/1 × 10^5 cells, range 133–4,771), and gE (median 542 SFC/1 × 10^5 cells, range 399–1,400 on day 16), while IE61 induced weaker responses (median 19 SFC/1×10^5 cells, range 11–194). VZVSTs increased not only in frequency, but also in absolute numbers as total cell numbers increased more than 3 logs over 23 days (Fig. 1B). We observed no obvious differences in the specificity or proliferation of VZVSTs expanded from the 9 donors who had been naturally infected and the 5 who had been vaccinated. Expanded cells were predominantly $CD3^+$ T cells (90.6% ± 4.3%), with a majority of $CD4^+$ T cells (57.6% ± 16.9%) as reported previously (13, 15–17, 19, 34, 35) and a minority $CD8^+$ T-cell component (27.4% ± 12.9%; Fig. 1C). The majority of $CD3^+$ T cells expressed markers that were transitional between central memory and effector memory cells expressing $CD45RO^+/CCR7^-/CD62L^+$ or $CD45RO^+/CCR7^-/CD28^+$ at the end of the third stimulation (Fig. 1D). These data demonstrate our ability to manufacture T cells specific for all five of the selected VZV antigens using the strategy proposed.

VZVSTs can be generated from cancer patients

As our ultimate goal was to generate autologous GD2.CAR-VZVST for clinical use, we generated VZVSTs from 6 patients with osteosarcoma (median age 19.5, range 10–53) and measured the frequency of VZV antigen-specific cells on day 16 in IFNγ ELISPOT assays. In 5 of the 6 patients, we successfully demonstrated VZV antigen specificity (Fig. 1E). The expanded T cells from only one patient lack VZV antigen specificity as determined by IFNγ ELISPOT assay (data not shown); we do not know the VZV serostatus of this patient. Over 23 days of culture, patient-derived VZVSTs expanded by 581 ± 372-fold (Fig. 1F). Although this rate of expansion was lower than for healthy adults, sufficient T cells for multiple doses on our highest clinical dose level (10^8 per m^2) could readily have been achieved from 5 of the 6 patients, suggesting that the manufacturing strategy used is sufficiently robust to produce VZVSTs for clinical use in cancer patients (6).

Circulating VZVSTs increase after VZV booster vaccination

As we plan to use VZV vaccination to boost the frequency of GD2.CAR-VZVSTs in patients, it was important to show that T cells specific for the chosen antigens expand *in vivo* in response to VZV vaccination. Therefore, we compared the frequency of

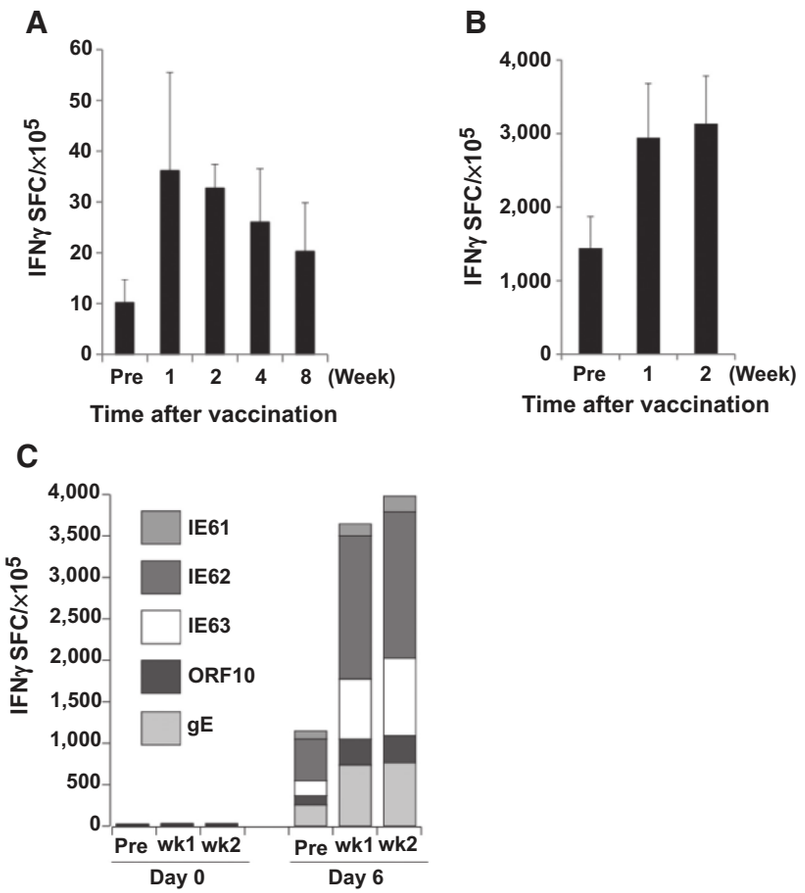

Figure 2.
Circulating VZVSTs are increased in response to VZV booster vaccination. PBMCs drawn before and at intervals after immunization of seropositive adults with the VZV booster vaccine, Zostavax, were stimulated with VZV antigens. The frequency of antigen-specific T cells in peripheral blood was determined by IFNγ release ELISPOT assay. Frequency of VZVSTs shown after 24 hours of stimulation (**A**) and after restimulation (**B**) with pepmixes 4 days later in one donor and 6 days later in 2 donors. The background frequency of IFNγ-secreting T cells was subtracted from the specific responses. Results are shown as mean ± SD ($n = 3$). **C,** The response to each antigen of one of three vaccinated donors.

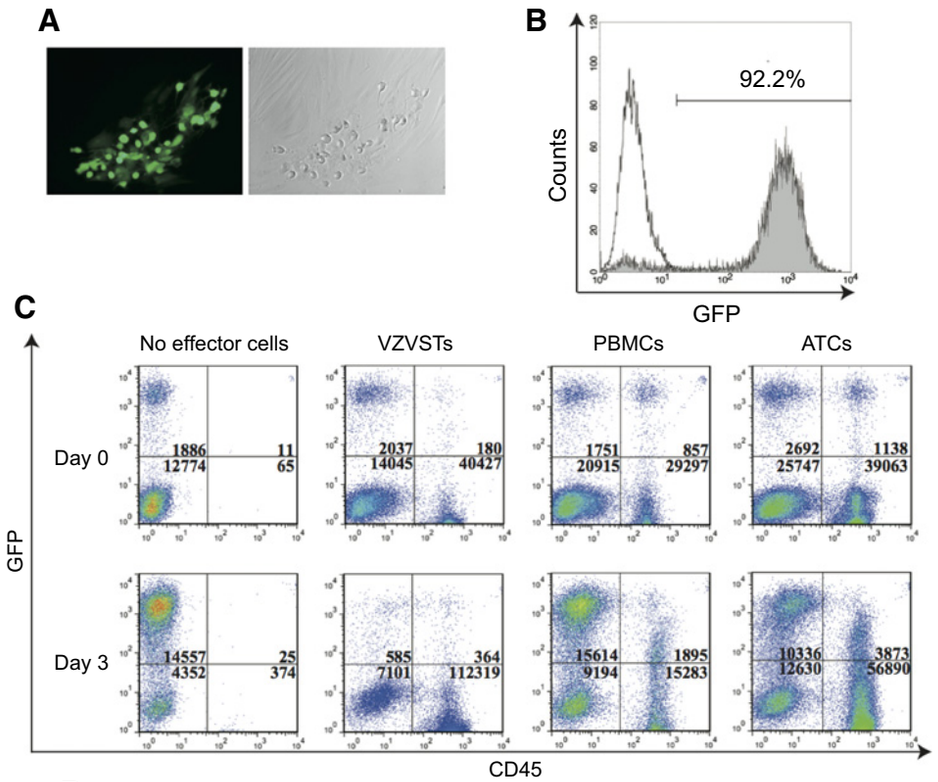

Figure 3.
VZVSTs prevent infectious virus spread. **A,** GFP-tagged, VZV-infected dermal fibroblasts were used as targets. Infected fibroblasts were GFP positive and a fraction show cytopathic effect. **B,** Frequency of infected fibroblasts as measured by GFP expression. **C,** GFP-tagged VZV-infected fibroblasts, fresh uninfected fibroblasts, and effector cells were cocultured at the ratio of 1:7:20. Three days later, cultures were collected and stained with CD45 antibodies, and analyzed by FACS. Indicated are the absolute numbers of events for GFP-positive VZV-infected cells. **D,** Infectious center assay. % Inhibition was calculated as (number of infectious center in each test condition/number of infectious center in controls without effectors) × 100. VZVSTs prevent infectious spread, whereas PBMCs and ATCs had little protective capacity.

D

Effectors	Number of infectious center / coculture well	% Inhibition
No effector cells	7.75×10^4	
VZV STs	6.00×10^2	99.23
PBMCs	6.80×10^4	12.26
ATCs	1.80×10^4	76.77

VZVSTs in PBMCs before and after administration of the VZV booster vaccine Zostavax to 3 seropositive adults. While the frequencies of VZVSTs before VZV vaccination were low (mean 10.2 ± 4.6 SFC/1×10^5 PBMCs, $n = 3$), frequencies increased to 36.1 ± 19.3 SFC/1×10^5 PBMCs within one week and remained elevated for over 8 weeks (Fig. 2A). Although responses observed after direct stimulation of PBMC were low, after 4 to 6 days of *in vitro* expansion with VZV pepmixes in the presence of IL4 and IL7, clear differences between pre- and postvaccination were discerned and persisted for at least 8 weeks (Fig. 2B and C). The responses from individual donors are shown in Supplementary Fig. S1. These data show that VZV vaccination increases the *in vivo* frequency of T cells specific for the chosen VZV antigens.

VZVSTs prevent infectious VZV spread in fibroblasts

To determine whether expanded VZVSTs had biological activity against live VZV, we established a VZV infectious spread cell model using a GFP-tagged VZV (GFP-VZV) in dermal fibroblasts. VZV spreads almost exclusively by cell-to-cell spread involving membrane fusion, and is normally propagated by coculture of infected and uninfected cells. Although the complete VZV replication cycle takes from 9 to 12 hours, cytopathic effects may not appear for up to two days. However, GFP-VZV infection can be readily be detected by monitoring GFP expression early after infection (Fig. 3A). After two passages in which uninfected fibroblasts and fresh medium were added to the infected cultures, over 90% of cells were GFP^+ (Fig. 3B). To determine whether VZVSTs could inhibit virus spread within the cocultures, VZV-infected dermal fibroblasts were cocultured with uninfected autologous fibroblasts and autologous VZVSTs at a ratio of 1:7:20. $CD56^+$ cells were depleted from VZVSTs using CD56 microbeads prior to coculture to ensure that any activity was derived from T cells. Autologous PBMCs and autologous CD3/28-activated ATCs were used as control effector cells. After 3 days of culture, the degree of VZV spread within each coculture well was quantified by flow cytometry in the presence of counting beads. Effector cells (VZVSTs, PBMCs, and ATCs) were distinguished from fibroblasts by CD45 expression. In VZV-infected cocultures without effector cells, the number of GFP^+-infected fibroblasts increased about 7.7-fold (Fig. 3C). Virus spread was unaffected by the presence of PBMC (8.9-fold increase) and was only partially inhibited by CD3/28-activated T cells (3.8-fold increase). However, in the cocultures with VSVSTs, the number of infected fibroblasts decreased by over 70% compared with cultures without effector

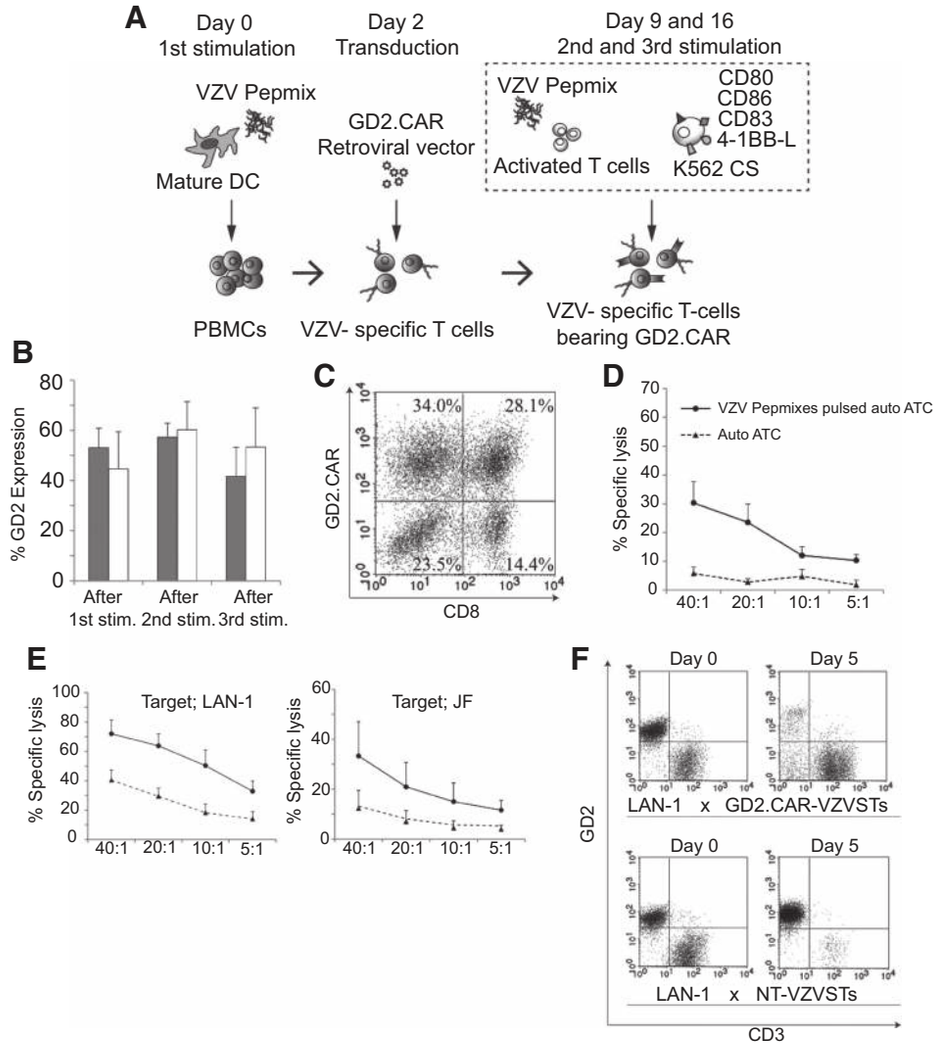

Figure 4.

Chimeric antigen receptors (CAR) can be expressed in VZVSTs, maintained over three stimulations and have functional specificity for both VZV and GD2. **A,** Diagram of GD2.CAR-VZVST generation. **B,** GD2.CAR expression on transduced VZVSTs was measured using the 14g2a-specific anti-idiotype antibody (1A7) after the first, second, and third stimulations. T-cell lines from donors either naturally infected with VZV (gray bars, $n = 3$) or immunized by vaccination (white bars, $n = 3$) were assessed. Results are shown as mean % of expression ± SD. **C,** One representative dot blot showing expression of the GD2.CAR. **D,** VZV pepmix–pulsed autologous ATCs were labeled with ^{51}Cr and cultured with the transduced VZVSTs for 6 hours at the effector:target ratios shown (solid lines). Control targets were autologous ATCs (dotted lines). Results are shown as mean % specific lysis ± SEM ($n = 4$). **E,** GD2-positive NB cells (LAN-1 and JF) were labeled with ^{51}Cr and cultured with GD2.CAR-transduced VZVSTs (solid lines) or nontransduced VZV-specific T cells (dotted lines) for 6 hours at the effector target ratios shown. Results are shown as mean of % specific lysis ± SEM ($n = 3$). **F,** GD2.CAR-VZVSTs and NT-VZVSTs were cocultured with LAN-1 cells at the ratio of 1:1. Five days later, cultures were stained with CD3 and GD2 antibodies, and analyzed by flow cytometry.

cells. To quantify this virus inhibition, we measured the number of infected cells in each coculture in an infectious center assay. As shown in Fig. 3D, VZVSTs prevent infectious spread effectively, while PBMCs and ATCs had little protective capacity. Of note, 11% of PBMCs and 6% of ATCs became infected with VZV during the coculture, as measured by GFP expression in CD45$^+$ cells, while VZVSTs remained uninfected (0.3%; Fig. 3C). Furthermore, VZVSTs expanded by 2.8-fold during the culture with autologous VZV-infected fibroblast, whereas PBMCs decreased in number (0.6-fold) and ATC number increased by 1.5-fold.

VZVSTs can be transduced with CARs and stably express the transgene during expansion

VZVSTs generated from donors either naturally infected with VZV ($n = 3$) or immunized by vaccination and without history of natural infection ($n = 3$), were transduced with a retroviral vector encoding a third-generation GD2.CAR two days after the first stimulation in the presence of IL4 and IL7. This previously described CAR includes costimulatory endodomains from CD28 and OX40, and is currently under evaluation in clinical trials. The efficiency of transduction was increased when pepmix-pulsed DCs were used for the first stimulation (not shown). The second and third stimulations were performed on days 9 and 16 using KATpx (as illustrated in Fig. 4A). Transgene expression was evaluated by flow cytometry using the GD2.CAR (14g2a)-specific anti-idiotype antibody (1A7) after the first, second, and third stimulations. The GD2.CAR was expressed in 53.1% ± 7.7% of VZVSTs from naturally infected donors and 44.6% ± 14.8% of VZVSTs from immunized donors on day 9 after the first stimulation. CAR expression was maintained over the second and third stimulations (Fig. 4B). CD8-positive and -negative T cells were transduced with similar efficiency (Fig. 4C).

GD2.CAR-VZVSTs kill both VZV- and GD2-expressing target cells

To demonstrate that GD2.CAR-modified VZVSTs have functional specificity for both VZV and GD2 antigens, transduced-VZVSTs were cocultured for 6 hours with ^{51}Cr-labeled GD2-positive and VZV antigen–expressing target cells. GD2.CAR-VZVSTs killed both GD2-positive NB cells (LAN-1 and JF) and

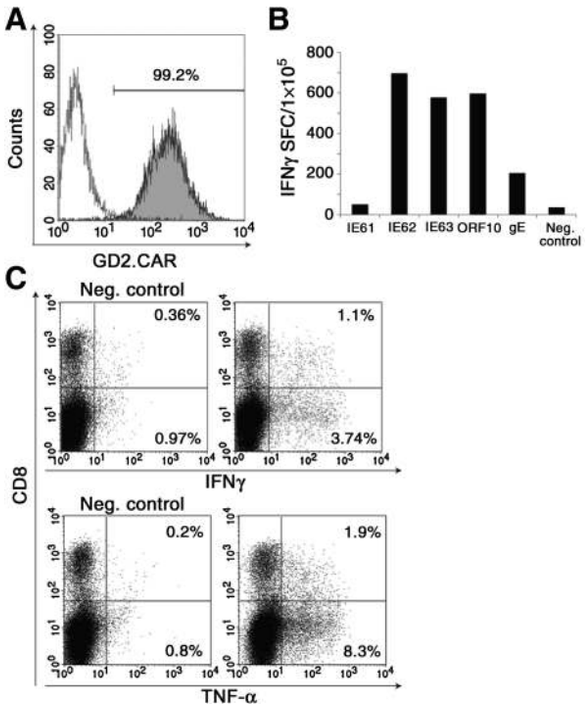

Figure 5.

Transduced T cells are bispecific. GD2.CAR-VZVSTs were stained with the 14.g2a-specific idiotype antibody 1A7 (mouse IgG1) and sorted with anti-mouse IgG1 MicroBeads. Sorted cells were rested 48 hours in incubator. **A,** Purity of sorted cells was tested after 48 hours incubation. **B,** The VZV antigen specificity of the sorted cells from one of the two donors tested is shown and was determined using IFNγ release ELISPOT assays. **C,** Sorted cells were stimulated with VZV antigen pepmixes and IFNγ or TNFα production was evaluated by intracellular cytokine staining. One representative of two donors is shown.

VZV pepmix–pulsed autologous activated T cells (auto ATC), but not unpulsed autologous ATCs (Fig. 4D and E). NT-VZVSTs showed relatively low levels of killing of GD2-positive, VZV-negative NB cells. Longer term cocultures of GD2.CAR-VZVSTs and neuroblastoma cell lines were also performed to evaluate the effects of the GD2.CAR on T-cell proliferation in response to GD2. GD2.CAR-VZVSTs cocultured with LAN-1 cells without cytokines at the ratio of 1:1 expanded in numbers and eliminated the majority of LAN-1 cells by day 5, while LAN-1 cells grew out in cocultures with NT-VZVSTs, and NT-VZVST numbers decreased (Fig. 4F).

To ensure that individual VZVSTs modified with GD2.CARs were bispecific and retained functional specificity for VZV, GD2.CAR-positive cells were selected from the bulk population using the 14.g2a idiotype-specific antibody 1A7 (mouse IgG1) and anti-mouse IgG1 microbeads. After resting for 48 hours, the purity of the sorted GD2.CAR-positive cells was confirmed by FACS; over 99% of VZVSTs expressed the GD2.CAR (Fig. 5A). VZV antigen specificity was then determined using IFNγ ELISPOT assays and intracellular cytokine staining. Sorted GD2.CAR$^+$ T cells that were stimulated with VZV pepmixes produced IFNγ in ELISPOT assays (>2,000 SFCs per 10^5 T cells; Fig. 5B). IFNγ and TNFα-secreting cells were also observed in both CD8$^+$ and CD8$^-$ populations by intracellular cytokine staining after stimulation with VZV pepmixes (Fig. 5C). These results showed that GD2.CAR-modified cells were functionally bispecific.

CAR-VZVSTs exposed to tumor cells become dysfunctional via their CAR, but retain responsiveness to their VZV-specific TCR

GD2.CAR-VZVSTs killed LAN-1 tumor cells on their first encounter in coculture assays (Fig. 4F), but if the cocultured GD2.CAR VZVSTs were harvested and exposed to fresh LAN-1 cells, they were still able to expand, but no longer able to control tumor cells ($P = 0.03$; Fig. 6A; Supplementary Fig. S2A). IFNγ and IL2 secretion was also significantly diminished after the second coculture compared with the first coculture, as measured by ELISA (Fig. 6B). This phenomenon was not unique to VZVSTs; GD2.CD28OX40z CAR–modified CD3/28-activated T cells also lost their ability to secrete IFNγ after the second coculture (Supplementary Fig. S2B). When we evaluated VZVSTs modified with GD2.CARs expressing other endodomains (GD2.z, GD2.CD28z, and GD2.41BBz), we observed the same phenomenon, with decreased IFNγ secretion and loss of tumor cell killing after the second coculture (Supplementary Fig. S2C). Lack of responsiveness to the CAR could not be explain by loss of the CAR, as VZVSTs cocultured with tumor cells retained CAR expression as determined by flow cytometry. Indeed, as illustrated for one representative donor, the frequency of CAR-modified VZVSTs increased from 48.9% to 84.5% and the mean fluorescence intensity increased from 951 to 3,430 after coculture (Supplementary Fig. S2D). GD2.CAR VZVSTs expressed high levels of TIM 3 compared with nontransduced cells, even prior to coculture and did not change after coculture with tumor cells, while expression of PD1 increased. GD2.CAR proliferation in response to the second CAR stimulation was reduced compared to the first stimulation (Fig. 6A), but the T cells were not exhausted because they remained responsive to TCR stimulation (Fig. 6D). Thus, the T cells displayed dysfunction via their CAR, but retained a functional response to the TCR.

To determine whether the antitumor activity of GD2.CAR-VZVSTs could be restored by stimulation through their TCR, they were first cocultured with GFP-modified LAN-1 cells for 7 days (first coculture) and then restimulated with DCs pulsed with VZV or irrelevant pepmixes for 3 days in the presence of IL2 (20 U/mL) prior to coculture with fresh GFP-ffluc LAN-1 cells (second coculture). During the first coculture, LAN-1 cells were eliminated. Although subsequent stimulation with DCs did not fully restore the antitumor function of GD2.CAR-VZVSTs, GD2.CAR-VZVSTs restimulated with VZV pepmix–pulsed DCs showed significantly greater tumor control compared with GD2.CAR-VZVSTs that received no interim stimulation ($P < 0.01$; Fig. 6E). However, DCs alone or pulsed with irrelevant an pepmix also partially rescued LAN-1–cultured GD2.CAR-VZVSTs, as did supernatants from VZV pepmix–pulsed DCs and CD3 antibody alone or combined with CD28 antibody (Supplementary Fig. S2E). These results suggest that restoration of the antitumor activity of GD2.CAR-T cells could be produced by a range of proinflammatory signals. VZV vaccination might therefore restore the function of CAR-T cells rendered dysfunctional by the tumor, whether VZV-specific or not. However, only VZV-specific T cells should accumulate at the site of VZV vaccination where they will be exposed to proinflammatory conditions.

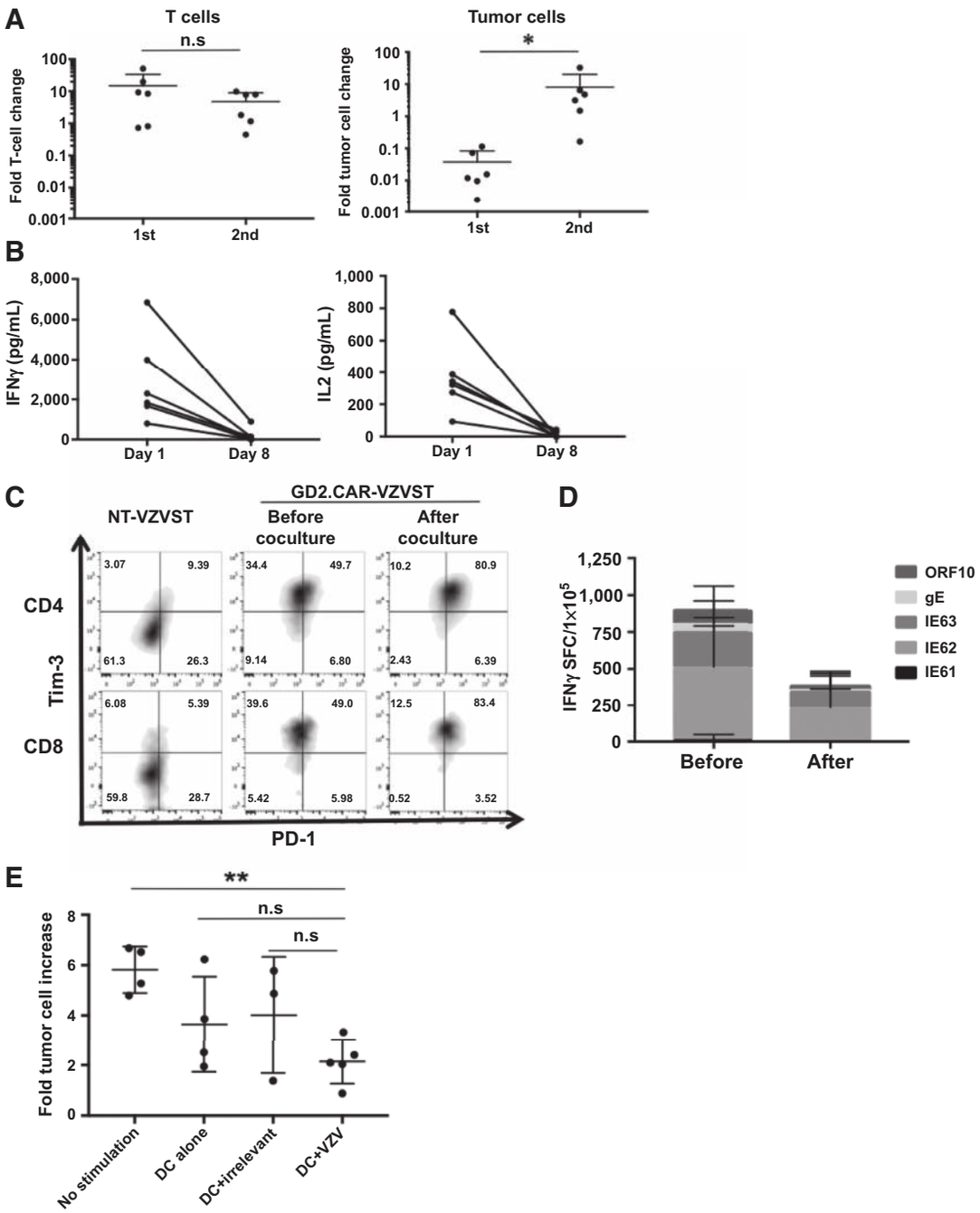

Figure 6.

CAR-VZVSTs became exhausted by stimulation through the CAR but the TCRs remained functional. **A,** GFP-ffluc-LAN1 (GFP$^+$) and T cells (CD3$^+$) were analyzed quantitatively after the first and second cocultures. The fold change of T-cell or tumor cell numbers between the end of first coculture and the end of second coculture are shown. ($n = 6$, mean ± SD). **B,** Cytokine secretion from GD2.CAR-VZVST after encountering LAN1 cells. T cells and LAN1 cells were plated at 1:1 ratio and after 24 hours supernatants were collected for ELISA. On day 7, T cells were harvested and replated with LAN1 cells at the same effector: target ratio. Supernatants were collected on day 8 (24 hours after second coculture) and then IFNγ and IL2 concentrations were measured. ($n = 6$). **C,** PD-1 and Tim-3 expression on nontransduced VZVST, GD2.CAR-VZVST before or 7 days after coculture with LAN1 cells are shown. GD2.CAR VZVSTs were gated on CAR$^+$ and CD4$^+$ or CD8$^+$ cells. Dot plots from one representative donor of two are shown. **D,** The frequency of antigen-specific T cells before and after coculture with LAN1 was determined by IFNγ release ELISPOT assay after overnight stimulation with VZV antigen-spanning pepmixes. Data denote mean ± SD ($n = 3$). **E,** After the second coculture, T cells were harvested and cultured with VZV pepmix–loaded DCs, irrelevant pepmix–loaded DCs, DCs alone, or no stimulation for 3 days and then cocultured with fresh GFP-ffluc-LAN1 cells. The fold tumor cell increase on day 7 to day 0 are shown. Data denote mean ± SD ($n = 4$, no stimulation, DC alone; $n = 3$, DC + irrelevant pepmix, $n = 5$, DC + VZV pepmix).

Discussion

The VZV antigens, gE, IE62, IE63, IE61, and ORF10 are known immunodominant antigens of VZV (34), and we have shown that T cells specific for these antigens can be expanded to clinically relevant numbers from healthy donors and cancer patients, whether vaccinated or naturally infected. T cells specific for all five antigens increased in frequency in response to vaccination and should therefore be suitable carriers for tumor-specific CARs in clinical trials that evaluate the ability of VZV vaccination to boost the frequency and function of CAR-modified VZVSTs. GD2.CAR expression was stable over at least three *in vitro* stimulations and we demonstrated dual specificity of transduced T cells for both GD2 and VZV. As shown by other groups, the majority of the VZVSTs were $CD4^+$ T cells (34), but they were able to kill both $GD2^+$ tumor cells and VZV-infected cells and inhibited the spread of infectious VZV in autologous fibroblast cultures. Culture of GD2.CAR VZVSTs (or CD3/28-activated T cells) with live tumor cells resulted in T-cell dysfunction, so that the T cells lost their ability to secrete cytokines and kill tumor cells on re-exposure to fresh tumor cells. However, GD2.CAR-VZVSTs remained responsive to stimulation via the TCR, and APCs pulsed with VZV peptides could restore the ability of GD2.CAR-VZVSTs to eliminate neuroblastoma cells in serial cocultures. Partial reversal of CAR dysfunction could also be induced by DCs alone and antibodies to CD3 and CD28, suggesting that a proinflammatory milieu may be able to restore tumor-specific T-cell dysfunction. These data establish many of the parameters for developing a therapy using GD2.CAR-modified VZVSTs for patients with GD2-positive tumors (NCT01953900).

This work expands upon earlier studies that demonstrated the potential of harnessing the potent immune activity of VSTs for CAR therapy. First-generation GD2.CAR-modified EBVSTs circulated with a higher frequency in patients with neuroblastoma than similarly modified CD3-activated T cells, producing 3 complete tumor responses among 11 patients with relapsed disease. However, in these patients, none of whom showed EBV reactivation, there was no expansion of CAR.EBVSTs in blood and patients with bulky disease did not enter remission. This lack of evident expansion may stem from the fact that patients were not lymphodepleted and EBV was well controlled, limiting the supply of viral antigens for T-cell expansion. Herpes virus reactivation *in vivo* is an uncontrollable and possibly rare event, while T-cell activation by vaccination allows for precise and potent timing of T-cell stimulation. The use of VZVSTs instead of EBVSTs allows us to take advantage of the commercially available VZV vaccine to provide extratumoral T-cell boosting in a proinflammatory environment, and may perhaps obviate the need for cytotoxic lymphodepletion. An attractive feature of this live attenuated viral vaccine is that it replicates in infected cells and continues to produce viral proteins in an inflammatory environment for some time, in contrast to any subunit vaccine. In this study, we used a third-generation CAR (28), that should also enable intratumoral proliferation upon CAR engagement and provide resistance to tumor-derived inhibitory factors.

CAR-modified VZVSTs used in combination with VZV vaccination for the treatment of patients with cancer must fulfill several requirements: CAR-VZVSTs must increase in frequency in response to VZV vaccination in a majority of individuals, they must be able to kill tumor cells expressing the CAR ligand, and they should be able to protect cancer patients, who may be immunocompromised as a result of prior chemotherapy or their immunosuppressive tumors, from the live-attenuated VZV vaccine. Therefore, it is important to choose viral antigens that induce protective T cells. Such VZV antigens must be processed and presented by infected cells before viral immune evasion proteins take effect, and mediate the killing of infected target cells before new infectious virus particles are produced. This is particularly important for VZV, which is able to infect and kill T cells and DCs (35). Candidate antigens would be abundant virion proteins, such as gE and ORF10, which are introduced into the cell during the infection process, IE61 and IE62, which are the earliest proteins to be expressed after infection, and IE63, which is expressed on reactivation of VZV from latency in neurons and, together with IE62, is present in virions. T cells specific for all of these proteins have already been identified in seropositive individuals (15, 16, 36, 37), and all of our donors, whether they achieved their immunity naturally or after vaccination, reacted with most of these viral proteins. Although ELISPOT assays indicated that the frequency of T cells in VZVSTs that secreted IFNg in response to VZV stimulation was only around 2% to 5%, this assay underestimates the true frequency of activated antigen-specific by about 10- to 100-fold, as can be determined if T cells specific for a specific peptide epitope are quantified by both ELISPOT and tetramer assays.

T cells specific for all five VZV antigens increased in frequency after vaccination of healthy seropositive adult donors who had been infected naturally during childhood, with the most robust increases seen in T cells specific for IE62, IE63, and gE. Over 100-fold VZVST expansion, representing 6 to 7 antigen-specific T-cell doublings, was seen in all three vaccinated donors within 6 days of *ex vivo* stimulation. Hence, T cells specific for these antigens should be suitable as carriers of tumor-specific CARs. The ability of pepmix-activated VZVSTs to prevent the spread of infectious virus in autologous fibroblasts suggests that they should provide antiviral protection to recipients with cancer who are immunosuppressed as a result of their tumor or their prior cytotoxic chemotherapy. Antiviral protection is an important factor, as VARIVAX is contraindicated in individuals with hematopoietic malignancies or who are immunosuppressed. Although our group has shown that pepmix-activated VSTs can control reactivations of CMV, EBV, adenovirus, and BK virus in immunosuppressed HSCT recipients (38), the ability of VZVSTs to protect against VZV infection or reactivation *in vivo* has yet to be demonstrated.

GD2.CAR-VZVSTs were able to kill neuroblastoma cell lines in coculture, but if harvested from the coculture and added to fresh tumor cells, they lost their ability to kill tumor cells and secrete cytokines in response to CAR stimulation. Long and colleagues (39) reported that 14g2a-CARs spontaneously dimerize via the ScFv resulting in tonic signaling and leading to cell differentiation and exhaustion (40). Our GD2.CAR construct differs in spacer, transmembrane, and costimulatory endodomains and GD2.CAR-VZVSTs did not become exhausted, as they remained responsive to TCR stimulation and did not become dysfunctional unless exposed to GD2. This phenomenon was previously demonstrated by Stotz and colleagues, who showed that two different TCRs expressed in a murine hybridoma functioned independently, so that "anergy" in one TCR did not affect signaling through the other (41). Importantly, even if CAR dysfunction was induced via GD2 stimulation, GD2.CAR-VZVSTs continued to respond to VZV pepmix–pulsed DCs and partially regained their ability to kill $GD2^+$ tumor cells. This rescue did not require cognate antigen and

as shown in Supplementary Fig. S1, DCs alone and supernatants from mature DCs were able to increase the function of the CAR on subsequent culture with tumor cells. Vaccination with a live virus such as VZV can provide prolonged exposure to professional antigen-presenting cells by inducing innate signals for T-cell recruitment to the vaccine site, followed by strong antigen-specific stimulation and costimulation. Our results also demonstrate that T cells can be unresponsive to a CAR, while remaining responsive to their TCR.

Our serial coculture experiment revealed a common problem with CAR T cells, which are apparently functional in their ability to proliferate and kill after a single exposure to tumor. Unless these T cells are reexposed to tumor, their dysfunction may never become apparent. In our first attempts to rescue dysfunctional GD2.CAR-VZVSTs, we used VZV-infected autologous fibroblasts to provide TCR stimulation in cocultures with neuroblastoma cell lines. However, VZV rapidly infected and destroyed the neuroblastoma cells. Therefore, VZV vaccination of patients with relapsed tumors may have an additional benefit as an oncolytic virus. Use of VZV in this manner was suggested by Leske and colleagues who showed that VZV replicated efficiently in primary malignant gliomas and glioma cell lines (42). VZV is also known to infect and replicate in T cells and although VZV infected PBMCs and nonspecifically activated T cells in the virus spread assay, VZVSTs did not become infected. Either activated VZVSTs are resistant to infection or, as suggested by our results, they killed VZV-infected cells before they could release infectious virus. This likely explains our observation that nonspecifically activated T cells were susceptible to virus infection in cocultures, while VZVSTs were not.

Our proposed strategy for combining GD2.CAR-VZVSTs with VZV vaccination would likely not be effective for VZV-seronegative patients, as it is difficult, to generate VSTs from seronegative donors. Although most children in the United States are vaccinated between the ages of 12 to 24 months, about one-third of children are diagnosed with neuroblastoma before the age of one (43). However, strategies for reactivating VSTs from seronegative individuals have been published (44, 45), and these T cells have been evaluated in clinical trials (46). In addition, our CAR-modified antigen-specific T-cell combination strategy would be applicable for any T-cell antigen for which there is a vaccine and could be adapted to CARs of any specificity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Tanaka, H. Tashiro, N. Lapteva, M. Ngo, C. Rossig, C.M. Rooney

Development of methodology: M. Tanaka, H. Tashiro, M. Ngo, G. Dotti, P.R. Kinchington, C.M. Rooney

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Tanaka, H. Tashiro, J. Ando, B. Mehta

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Tanaka, H. Tashiro, P.R. Kinchington, C.M. Rooney

Writing, review, and/or revision of the manuscript: M. Tanaka, H. Tashiro, B. Omer, G. Dotti, P.R. Kinchington, A.M. Leen, C. Rossig, C.M. Rooney

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Omer

Study supervision: C.M. Rooney

Acknowledgments

We thank Malcolm Brenner and Catherine Gillespie for expert editing and Kathan Parikh for technical support.

Grant Support

This work was supported in parts by a grant from the Japanese Foundation for Pediatric Research (to M. Tanaka), NIH-NCI PO1 CA94237, Alex's Lemonade Stand Foundation, and the V-Foundation. P.R. Kinchington was supported by PHS awards NS064022, EY08098, and unrestricted funds from Research to Prevent Blindness Inc. and the Eye & Ear Institute of Pittsburgh.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 31, 2016; revised January 1, 2017; accepted January 13, 2017; published OnlineFirst February 9, 2017.

References

1. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371:1507–17.
2. Wang LC, Riese MJ, Moon EK, Albelda SM. Overcoming intrinsic inhibitory pathways to augment the antineoplastic activity of adoptively transferred T cells: re-tuning your CAR before hitting a rocky road. Oncoimmunology 2013;2:e26492.
3. Gilham DE, Debets R, Pule M, Hawkins RE, Abken H. CAR-T cells and solid tumors: tuning T cells to challenge an inveterate foe. Trends Mol Med 2012;18:377–84.
4. Rossig C, Bollard CM, Nuchtern JG, Rooney CM, Brenner MK. Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. Blood 2002;99: 2009–16.
5. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 2008;14:1264–70.
6. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood 2011;118:6050–6.
7. Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 1998;92:1549–55.
8. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 2010; 115:925–35.
9. Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, et al. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther 2012;20:1622–32.
10. Galea SA, Sweet A, Beninger P, Steinberg SP, Larussa PS, Gershon AA, et al. The safety profile of varicella vaccine: a 10-year review. J Infect Dis 2008;197:S165–9.
11. Gershon AA, Gershon MD. Pathogenesis and current approaches to control of varicella-zoster virus infections. Clin Microbiol Rev 2013;26:728–43.
12. Gagliardi AM, Gomes Silva BN, Torloni MR, Soares BG. Vaccines for preventing herpes zoster in older adults. Cochrane Database Syst Rev 2016;3:CD008858.
13. Bergen RE, Sharp M, Sanchez A, Judd AK, Arvin AM. Human T cells recognize multiple epitopes of an immediate early/tegument protein (IE62) and glycoprotein I of varicella zoster virus. Viral Immunol 1991;4:151–66.
14. Sadzot-Delvaux C, Arvin AM, Rentier B. Varicella-zoster virus IE63, a virion component expressed during latency and acute infection, elicits humoral and cellular immunity. J Infect Dis 1998;178:S43–7.

15. Jones L, Black AP, Malavige GN, Ogg GS. Phenotypic analysis of human CD4+ T cells specific for immediate-early 63 protein of varicella-zoster virus. Eur J Immunol 2007;37:3393–403.
16. Malavige GN, Jones L, Black AP, Ogg GS. Varicella zoster virus glycoprotein E-specific CD4+ T cells show evidence of recent activation and effector differentiation, consistent with frequent exposure to replicative cycle antigens in healthy immune donors. Clin Exp Immunol 2008;152:522–31.
17. Malavige GN, Jones L, Black AP, Ogg GS. Rapid effector function of varicella-zoster virus glycoprotein I-specific CD4+ T cells many decades after primary infection. J Infect Dis 2007;195:660–4.
18. Black AP, Jones L, Malavige GN, Ogg GS. Immune evasion during varicella zoster virus infection of keratinocytes. Clin Exp Dermatol 2009;34: e941–44.
19. Frey CR, Sharp MA, Min AS, Schmid DS, Loparev V, Arvin AM. Identification of CD8+ T cell epitopes in the immediate early 62 protein (IE62) of varicella-zoster virus, and evaluation of frequency of CD8+ T cell response to IE62, by use of IE62 peptides after varicella vaccination. J Infect Dis 2003;188:40–52.
20. Arvin AM, Koropchak CM, Williams BR, Grumet FC, Foung SK. Early immune response in healthy and immunocompromised subjects with primary varicella-zoster virus infection. J Infect Dis 1986;154:422–9.
21. Arvin AM, Sharp M, Smith S, Koropchak CM, Diaz PS, Kinchington P, et al. Equivalent recognition of a varicella-zoster virus immediate early protein (IE62) and glycoprotein I by cytotoxic T lymphocytes of either CD4+ or CD8+ phenotype. J Immunol 1991;146:257–64.
22. Asanuma H, Sharp M, Maecker HT, Maino VC, Arvin AM. Frequencies of memory T cells specific for varicella-zoster virus, herpes simplex virus, and cytomegalovirus by intracellular detection of cytokine expression. J Infect Dis 2000;181:859–66.
23. Giller RH, Winistorfer S, Grose C. Cellular and humoral immunity to varicella zoster virus glycoproteins in immune and susceptible human subjects. J Infect Dis 1989;160:919–28.
24. Landmeier S, Altvater B, Pscherer S, Eing BR, Kuehn J, Rooney CM, et al. Gene-engineered varicella-zoster virus reactive CD4+ cytotoxic T cells exert tumor-specific effector function. Cancer Res 2007;67:8335–43.
25. Ngo MC, Ando J, Leen AM, Ennamuri S, Lapteva N, Vera JF, et al. Complementation of antigen-presenting cells to generate T lymphocytes with broad target specificity. J Immunother 2014;37:193–203.
26. Gerdemann U, Vera JF, Rooney CM, Leen AM. Generation of multivirus-specific T cells to prevent/treat viral infections after allogeneic hematopoietic stem cell transplant. J Vis Exp 2011;27.
27. Suhoski MM, Golovina TN, Aqui NA, Tai VC, Varela-Rohena A, Milone MC, et al. Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. Mol Ther 2007;15:981–8.
28. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 2005;12: 933–41.
29. Mujoo K, Kipps TJ, Yang HM, Cheresh DA, Wargalla U, Sander DJ, et al. Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal antiganglioside GD2 antibody 14.18. Cancer Res 1989;49:2857–61.
30. Sun J, Huye LE, Lapteva N, Mamonkin M, Hiregange M, Ballard B, et al. Early transduction produces highly functional chimeric antigen receptor-modified virus-specific T-cells with central memory markers: a Production Assistant for Cell Therapy (PACT) translational application. J Immunother Cancer 2015;3:5.
31. Eisfeld AJ, Yee MB, Erazo A, Abendroth A, Kinchington PR. Downregulation of class I major histocompatibility complex surface expression by varicella-zoster virus involves open reading frame 66 protein kinase-dependent and -independent mechanisms. J Virol 2007;81:9034–49.
32. Diaz PS, Smith S, Hunter E, Arvin AM. T lymphocyte cytotoxicity with natural varicella-zoster virus infection and after immunization with live attenuated varicella vaccine. J Immunol 1989;142:636–41.
33. Sadzot-Delvaux C, Kinchington PR, Debrus S, Rentier B, Arvin AM. Recognition of the latency-associated immediate early protein IE63 of varicella-zoster virus by human memory T lymphocytes. J Immunol 1997;159: 2802–06.
34. Arvin A, Abendroth A. VZV: immunobiology and host response. In: Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, Chapter 39. Ann Arvin, Gabriella Campadelli-Fiume, Edward Mocarski, Patrick S. Moore, Bernard Roizman, Richard Whitley, Koichi Yamanishi, editors. Cambridge: Cambridge University Press; 2007.
35. Sen N, Mukherjee G, Sen A, Bendall SC, Sung P, Nolan GP, et al. Single-cell mass cytometry analysis of human tonsil T cell remodeling by varicella zoster virus. Cell Rep 2014;8:633–45.
36. Eisfeld AJ, Turse SE, Jackson SA, Lerner EC, Kinchington PR. Phosphorylation of the varicella-zoster virus (VZV) major transcriptional regulatory protein IE62 by the VZV open reading frame 66 protein kinase. J Virol 2006;80:1710–23.
37. Milikan JC, Kinchington PR, Baarsma GS, Kuijpers RW, Osterhaus AD, Verjans GM. Identification of viral antigens recognized by ocular infiltrating T cells from patients with varicella zoster virus-induced uveitis. Invest Ophthalmol Vis Sci 2007;48:3689–97.
38. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med 2014;6:242ra83.
39. Long HM, Orentas RJ, Mackall CL. Synthetic chimeric antigen receptors (CARs) rapidly induce exhaustion and augmented glycolytic metabolism in human T cells and implicate persistent CD28 signaling as a driver of exhaustion in human T cells. Blood 1912;122:192.
40. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4–1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med 2015;21: 581–90.
41. Stotz SH, Bolliger L, Carbone FR, Palmer E. T cell receptor (TCR) antagonism without a negative signal: evidence from T cell hybridomas expressing two independent TCRs. J Exp Med 1999;189:253–64.
42. Leske H, Haase R, Restle F, Schichor C, Albrecht V, Vizoso Pinto MG, et al. Varicella zoster virus infection of malignant glioma cell cultures: a new candidate for oncolytic virotherapy? Anticancer Res 2012;32: 1137–44.
43. Schmidt ML, Lal A, Seeger RC, Maris JM, Shimada H, O'Leary M, et al. Favorable prognosis for patients 12 to 18 months of age with stage 4 nonamplified MYCN neuroblastoma: a Children's Cancer Group Study. J Clin Oncol 2005;23:6474–80.
44. Hanley PJ, Cruz CR, Savoldo B, Leen AM, Stanojevic M, Khalil M, et al. Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. Blood 2009;114:1958–67.
45. Metes D, Storkus W, Zeevi A, Patterson K, Logar A, Rowe D, et al. Ex vivo generation of effective Epstein-Barr virus (EBV)-specific CD8+ cytotoxic T lymphocytes from the peripheral blood of immunocompetent Epstein Barr virus-seronegative individuals. Transplantation 2000;70:1507–15.
46. Hanley PJ, Melenhorst JJ, Nikiforow S, Scheinberg P, Blaney JW, Demmler-Harrison G, et al. CMV-specific T cells generated from naive T cells recognize atypical epitopes and may be protective in vivo. Sci Transl Med 2015;7:285ra63.