K562-Derived Whole-Cell Vaccine Enhances Antitumor Responses of CAR-Redirected Virus-Specific Cytotoxic T Lymphocytes In Vivo

Ignazio Caruana1, Gerrit Weber1, Brandon C. Ballard1, Michael S. Wood1, Barbara Savoldo1,2, and Gianpietro Dotti1,3,4

Abstract

Purpose: Adoptive transfer of Epstein–Barr virus (EBV)–specific and cytomegalovirus (CMV)-specific cytotoxic T cells (CTL) genetically modified to express a chimeric antigen receptor (CAR) induces objective tumor responses in clinical trials. In vivo expansion and persistence of these cells are crucial to achieve sustained clinical responses. We aimed to develop an off-the-shelf whole-cell vaccine to boost CAR-redirected virus-specific CTLs in vivo after adoptive transfer. As proof of principle, we validated our vaccine approach by boosting CMV-specific CTLs (CMV-CTLs) engineered with a CAR that targets the GD2 antigen.

Experimental Design: We generated the whole-cell vaccine by engineering the K562 cell line to express the CMV-pp65 protein and the immune stimulatory molecules CD40L and OX40L. Single-cell–derived clones were used to stimulate CMV-CTLs in vitro and in vivo in a xenograft model. We also assessed whether the in vivo boosting of CAR-redirected CMV-CTLs with the whole-cell vaccine enhances the antitumor responses. Finally, we addressed potential safety concerns by including the inducible safety switch caspase9 (iC9) gene in the whole-cell vaccine.

Results: We found that K562-expressing CMV-pp65, CD40L, and OX40L effectively stimulate CMV-specific responses in vitro by promoting antigen cross-presentation to professional antigen-presenting cells (APCs). Vaccination also enhances antitumor effects of CAR-redirected CMV-CTLs in xenograft tumor models. Activation of the iC9 gene successfully induces growth arrest of engineered K562 implanted in mice.

Conclusions: Vaccination with a whole-cell vaccine obtained from K562 engineered to express CMV-pp65, CD40L, OX40L and iC9 can safely enhance the antitumor effects of CAR-redirected CMV-CTLs. Clin Cancer Res. 1–11. ©2015 AACR.

Introduction

Chimeric antigen receptor (CAR)–redirected T lymphocytes mediate HLA-independent cytotoxic activity against a variety of human malignancies in preclinical models (1, 2). In clinical trials, adoptively transferred CAR-T lymphocytes induce durable tumor regressions when CAR-T cells expand and persist in vivo (3, 4). Proliferation and survival of CAR-T cells are strictly dependent on their adequate costimulation (3, 5, 6). Antigen-presenting cells (APC), such as dendritic cells, that present MHC-restricted antigen epitopes to the T-cell receptor, and express costimulatory molecules in a spatially and temporally coordinated fashion, promote the most physiologic T-cell costimulation (7). We previously hypothesized that engrafting CARs in virus-specific cytotoxic T cells (VsCTLs), such as Epstein–Barr virus (EBV)-CTLs or cytomegalovirus (CMV)-CTLs, can recapitulate a physiologic T-cell costimulation of CAR-engineered T cells. VsCTLs expressing a CAR are indeed “dual specific” and can receive a proper costimulation by APCs processing and presenting viral epitopes to VsCTL native virus-specific T-cell receptors, while the CAR expression redirects their cytotoxic activity toward tumor cells (8–10).

We validated this strategy in clinical trials, in both the autologous and allogeneic settings. In patients with neuroblastoma, we described how autologous EBV-CTLs engineered with a first-generation (encoding only the ζ chain moiety) GD2-specific CAR have better initial engraftment compared with autologous polyclonal activated T lymphocytes expressing the same CAR (11). In the context of the allogeneic stem cell transplant, we also showed that donor-derived EBV-CTLs and CMV-CTLs engrafted with a second-generation CD19-specific CAR, encoding both the CD28 and ζ chain moieties, can produce antitumor and antiviral activity without causing graft versus host disease (12). However, there were some limitations in both autologous and allogeneic settings. For instance, in patients with neuroblastoma, although detectable long-term, autologous GD2-specific CAR-modified EBV-CTLs persisted at a very low frequency in vivo (13). This limited engraftment may indicate that the endogenous presentation of latent EBV antigens, in the absence of virus reactivation, does not promote robust and durable engraftment of the infused CAR-redirected EBV-CTLs. In the allogeneic setting, we found enhanced engraftment of the infused CD19-specific CAR-redirected VsCTLs only in patients who were infused relatively early posttransplant, when higher EBV or CMV viral loads can fully stimulate the infused CAR-redirected VsCTLs through their native T-cell receptors.

1Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas. 2Department of Pediatrics, Baylor College of Medicine, Houston, Texas. 3Department of Immunology, Baylor College of Medicine, Houston, Texas. 4Department of Medicine, 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: Gianpietro Dotti, Center for Cell and Gene Therapy, Baylor College of Medicine, 6621 Fannin St/MC 3-3320, Houston, TX 77030. Phone: 832-824-6891; Fax: 832-825-4732; E-mail: gdotti@bcm.edu

doi: 10.1158/1078-0432.CCR-14-2998

©2015 American Association for Cancer Research.
Caruana et al.

Translational Relevance

T cells recognizing viral antigens such as Epstein–Barr virus (EBV) and cytomegalovirus (CMV) acquire tumor specificity when genetically modified to express a chimeric antigen receptor (CAR). Prolonged expansion and persistence of adoptively transferred tumor-specific T cells in vivo are a critical step in achieving sustained clinical responses. Here, we provide data showing that a K562-based whole-cell vaccine generated to express the viral antigen CMV-pp65 and immune stimulatory molecules CD40L and OX40L enhances the antitumor effects of CMV-cytotoxic T cells (CTL) expressing a CAR by boosting their intrinsic virus specificity.

Isolation of peripheral blood mononuclear cells and generation of dendritic cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Gulf Coast Regional Blood Center) or blood donations from healthy donors (under Institutional Review Board-approved protocol, BCM) using Ficoll–Paque (Amersham Biosciences). Monocytes were obtained from PBMCs by positive magnetic selection with CD14 magnetic beads (Miltenyi Biotec). Dendritic cells (DCs) were generated from CD14+ cells cultured in DC media (CellGenix) supplemented with IL4 (1,000 U/mL) and GM-CSF (800 U/mL; R&D Systems). On day 5, DCs were matured with IL6 (1 µg/mL), TNFα (1 µg/mL), IL1β (1 µg/mL), and prostaglandin E (1 µg/mL; all from R&D Systems, Inc) for 48 hours.

K562-derived whole-cell vaccine

The vaccine was generated using the K562 cell line. These cells were transduced with lentiviral vectors encoding either human CD40L or OX40L or pp65/eGFP or the combination CD40L/pp65 or OX40L/pp65. After transduction, single cell clones were obtained. For selected experiments, K562 clones were also genetically modified with a retroviral vector to stably express the inducible caspase-9 suicide gene (iC9; ref. 28).

Generation of autologous phytohemagglutinin-activated T cells and lymphoblastoid cell lines

To generate PHA blasts, PBMCs were stimulated with the mitogen phytohemagglutinin-P (PHA-P, 5 µg/mL; Sigma-Aldrich). PHA blasts were then expanded in RPMI-1640 supplemented with 5% human serum (Valley Biomedical) and 2 mmol/L Glutamax, and in the presence of IL2 (100 U/mL; Teceleukin, Chiron Therapeutics). The lymphoblastoid cell lines (LCLs) were generated as previously described (29).

Activation of monocytes by K562-derived whole-cell vaccine

Monocytes were stained with the PKH26 red fluorescent cell linker compound and then cocultured at a ratio of 5:1 with irradiated K562 labeled with PKH2 green fluorescent cell linker compound (Sigma-Aldrich). After 72 hours, we analyzed the expression of activation/maturity markers in monocytes by flow cytometry, testing the level of expression of CD11c, CD80, CD83, and HLA-DR. Moreover, we monitored the coculture by a fluorescence microscope.

Materials and Methods

Cell line

K562, Raji, and A459 tumor cells were purchased from ATCC. K562 and Raji cells were cultured in RPMI-1640 (HyClone, Thermo Scientific) supplemented with 10% FBS (HyClone) and 2 mmol/L GlutaMax (Invitrogen). A459 tumor cell line was cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS and 2 mmol/L GlutaMax. A459 was single cell cloned based on the expression of the GD2 antigen. The neuroblastoma cell line CHLA-255 (ref. 27; kindly provided by Dr. Leonid Metelitsa, Baylor College of Medicine, Houston TX) was derived from a patient. CHLA-255 was cultured in IMDM (Gibco, Invitrogen) supplemented with 10% FBS and 2 mmol/L GlutaMax, and we verified that this line retains the surface expression of the target antigen GD2. Cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C. All cell lines were routinely tested to ensure that they were mycoplasma free and authenticated based on short tandem repeats (STR) at MD Anderson Cancer Center (Houston, TX) except for CHLA-255. For the coculture experiments, CHLA-255 and Raji cells were transduced with a retroviral vector encoding GFP (>98% GFP+ cells).

Process of CAR-virus reactivation

Receptors (12). In contrast, engraftment remains suboptimal if the cells are infused late after transplant when the probability of experiencing virus reactivations is rather low (12).

On the basis of the clinical evidence, we hypothesized that an intentional in vivo vaccine-mediated stimulation of adoptively transferred CAR-modified VsCTLs would produce enhanced engraftment and superior antitumor effect of these cells. We developed a whole-cell vaccine that promotes the cross-presentation of viral epitopes to the native virus-specific T-cell receptors of CAR-redirected VsCTLs. The proposed approach is preferable to vaccine-mediated stimulation of adoptively transferred tumor-specific cells. We envision further engineering K562 to express CD40L and OX40L immune stimulatory molecules to strengthen the T-cell costimulation.

A whole-cell vaccine approach based on the administration of irradiated allogeneic immortalized cell lines engineered to express immune-modulatory cytokines such as IL2 and GM-CSF to cross-present antigens to host APCs has been used in several clinical trials (14–18). On the basis of these clinical findings, we prepared a whole-cell vaccine by engineering the K562 cell line to stimulate, via antigen cross-presentation, the intrinsic virus-specificity of CAR-modified VsCTLs in vivo. As proof of principle, we selected to engineer the K562 cell line with the CMV-pp65 protein to stimulate CAR-redirected CMV-CTLs (CAR-CMV-CTLs) based on the high frequency of CMV seropositive individuals (19) and the robust evidence that CD8+ T cells specific for the CMV-pp65 protein play a dominant protective role in CMV infections (20).

We envisioned further engineering K562 to express CD40L and OX40L immune stimulatory molecules to strengthen the effect of our vaccine. CD40L promotes the maturation of APCs and directly activates CD8+ T cells (21–23), whereas OX40L promotes the recruitment of CD4+ T cells (24–26), which play an important role in controlling tumor growth in clinical trials of adoptive T-cell transfer (13). We then conducted experiments to show that the K562-derived whole-cell vaccine can safely and effectively stimulate CAR-CMV-CTLs in vitro and in vivo, enhancing their overall antitumor activity.

OF2 Clin Cancer Res; 2015
Generation of retroviral supernatant and transduction of VscTLs

Retroviral supernatants were produced in 293T cells, as previously described (30). Lentivirus supernatants were produced in 293T cells cotransfected with the lentiviral vector and separated plasmids encoding the HIV-G envelope, gag-pol, and REV (31). To generate CMV-CTLs, PBMCs from CMV seropositive donors were stimulated with DCs (20:1) loaded with the CMV-pp65 pepmix (HCMVA, JPT) at 5 μmol/L for 2 hours at 37°C in 5% CO₂. Cells were then plated in complete media containing RPMI-1640 45%, Clicks medium (Irvine Scientific) 45%, 10% human AB serum, and 2 mmol/L GlutaMax. After 10 days, T cells were restimulated with DCs loaded with the same pepmix. After the second round of stimulation, cells were expanded and fed with IL2 (50 μU/mL; Preludekin, Chiron). Three days later, cells were transduced with a retroviral vector encoding a CAR specific for the CD2 antigen and containing the CD28 endodomain (CAR-GD2) using retargeting-coated plates (Takara Bio Inc; ref. 12).

Stimulation of PBMCs and CMV-CTLs using K562-derived whole-cell vaccine

PBMCs from seropositive donors were incubated with irradiated K562 (80–100 Gy) at a ratio of 10:1 for 10 to 12 days in the absence of cytokines. Transduced CAR-CMV-CTLs were stimulated weekly with irradiated K562 and autologous CD3-depleted PBMCs at a ratio of 5:1:1 (CTLs:K562:PBMCs CD3-depleted) and fed with IL2 (50 μU/mL) twice/week.

IFNγ Enzyme-Linked Immunospot Assay (ELISpot)

The IFNγ ELISpot assay was performed as previously described (8). T cells were plated in triplicate at 10⁵ cells/well with 5 μmol/L of CMV-pp65 pepmix. In all experiments, T cells were also incubated with an irrelevant pepmix, as negative control, or stimulated with 25 ng/mL of phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 μg/mL of ionomicyn (Iono; Sigma-Aldrich) as positive control. In selected experiments, CAR-CMV-CTLs were tested in ELISpot plates coated with both IFNγ antibody and anti-idiotyp antibody (1A7) that induces cross-link of CAR molecules (11).

Flow cytometry

For phenotypic analysis, we used CD11c, CD80, CD83, HLA-DR, CD45, CD56, CD19, CD8, CD4, and CD3 mAbs (all from Becton Dickinson) conjugated with FITC, PE, PerCP, or APC fluorochromes. The expression of CAR-GD2 was detected using the 1A7 Ab. Samples were analyzed with a BD FACScalibur system equipped with the filter set for quadruple fluorescence signals and the CellQuest software (BD Biosciences). For each sample, we analyzed a minimum of 30,000 events. CTLs were also analyzed for binding of specific tetramers. Tetramers were prepared by the Baylor College of Medicine (Houston, TX) core facility. For each sample, a minimum of 100,000 cells were analyzed.

Chromium-release assay

The cytotoxic activity of T cells was evaluated using a standard 4-hour ⁵¹Cr-release assay, as previously described (9). Target cells were incubated in medium alone or in 1% Triton X-100 (Sigma-Aldrich) to determine spontaneous and maximum ⁵¹Cr-release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as follows: [(test counts – spontaneous counts)/(maximum counts – spontaneous counts)] × 100. The target cells tested included CHLA-255, Raji, and PHA blasts loaded with irrelevant or CMV-pp65 pepmixes.

Western blot analysis

Proteins were extracted from 5 × 10⁶ cells, using RIPA lysing buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Of note, 50 μg of protein were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad), and blocked with 5% (W/V) nonfat dry milk in TBS with 0.1% (V/V) Tween-20. Blots were stained with mouse anti-CMV-pp65 (1:200, clone 1-L-11; Santa Cruz Biotechnology) and mouse anti-human β-actin (1:10000, clone C4; Santa Cruz Biotechnology). Blots were washed with TBS containing 0.1% (V/V) Tween-20, stained with horseradish peroxidase-conjugated secondary Ab (1:5000, goat anti-mouse sc-2005; Santa Cruz Biotechnology), and incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Xenogenic SCID mouse models

Mouse experiments were performed in accordance with Baylor College of Medicine’s Animal Handlsy guide lines following Institutional Animal Care and Use Committee-approved protocols. In the first set of experiments, we tested the ability of the K562-derived whole-cell vaccine to stimulate CMV-CTLs from PBMCs collected from healthy CMV-seropositive donors. Figure 2A summarizes the design of the experiment. Eight- to 10-week-old NOG/SCID/γc⁻/⁻ mice (Jackson Lab) received three inoculations intraperitoneally (i.p.) and intravenously (i.v.) of 5 × 10⁶ PBMCs (32) and 10⁶ irradiated K562 and were euthanized by day 14 for analysis of immune responses. For the antitumor effects, two models were tested. In the first model, NOG/SCID/γc⁻/⁻ mice were implanted i.p. with CHLA-255 cells (2.5 × 10⁶), labeled with firefly luciferase, and resuspended in Matrigel (Becton Dickinson Biosciences). Tumor growth was measured by in vivo bioluminescence using the Luma IVIS imaging system (PerkinElmer; ref. 33). Five days after tumor inoculation, control and CAR-CMV-CTLs were injected i.p. (10 × 10⁶ cells/mouse). Mice were subsequently vaccinated according to the schedule illustrated in Fig. 2A. IL2 (1,000 U/mouse) was also administered i.p. twice a week for 2 weeks. In the systemic tumor model, NOG/SCID/γc⁻/⁻ mice were infused via tail injection with GD2⁺ A459 tumor cells labeled with firefly luciferase (6 × 10⁶ cells). On day 3, mice were injected i.v. with control or CAR-CMV-CTLs (8 × 10⁶ cells/mouse) and vaccinated with K562 as described in Fig. 2A. Tumor growth was monitored by using the Lumina IVIS imaging system. Mice were euthanized when signs of discomfort were detected by the investigator or as recommended by the veterinarian who monitored the mice three times a week or when luciferase signal reached 7.5 × 10⁷ p/sec/cm²/sr. For the validation of the iC9 suicide gene, mice were engrafted with K/CD40L/pp65 and K/OX40L/pp65 clones expressing iC9 and an enhanced firefly luciferase gene (34). After engraftment, mice were infused intra-peritoneally with the dimerizing drug AP20187 (50 μg/mouse; Clontech Lab) for 2 consecutive days. K562 growth was followed by in vivo bioluminescence.

Statistical analyses

Unless otherwise noted, data are summarized as mean ± SD. Student t test was used to determine statistically significant
differences between samples, with \( P \) value <0.05 indicating a significant difference. When multiple comparison analyses were required, statistical significance was evaluated by one-way ANOVA. Survival analysis was performed using the Kaplan–Meier method in GraphPad Software. The log-rank test was used to assess statistically significant differences between groups of mice. All \( P \) values <0.05 were considered statistically significant.

**Results**

**K562-derived whole-cell vaccine encoding CMV-pp65 and CD40L stimulates CMV-CTLs in vitro by mediating antigen cross-presentation**

To develop a whole-cell vaccine capable of boosting CMV-CTLs, we engineered the K562 cell line to express CMV-pp65, CD40L, and OX40L molecules as follows: CD40L/pp65 (K/CD40L/pp65), OX40L/pp65 (K/OX40L/pp65), CD40L (K/CD40L), OX40L (K/OX40L), or pp65 (K/pp65). K/pp65 also expressed GFP, as a marker of selection. Single cell clones of engineered K562 were used for all the experiments. The expression of CD40L and OX40L was confirmed by FACS analysis (Fig. 1A), whereas the expression of pp65 was assessed by Western blot analysis (Fig. 1B). To ensure in vitro that engineered and irradiated K562 cells promote antigen cross-presentation, we proved that apoptotic bodies derived from irradiated K562 were uptaken by monocytes. As shown in Fig. 1C, freshly isolated monocytes (stained with red fluorescent) were cocultured for 3 days with either irradiated K/pp65 or K/CD40L/pp65 (stained with green fluorescent). Monocytes engulfed K562-derived apoptotic bodies (stained with yellow fluorescent) and expressed CD80 and CD83, and showed more pronounced upregulation of CD11c and HLA-DR only in the presence of CD40L (Fig. 1D). In all experimental conditions T cells recovered from the spleen of mice vaccinated with detectable CMV-specific IFN\( \gamma \) production. However, the vaccination with combined K/CD40L/pp65 and K/OX40L/pp65 stimulated the highest CMV-specific response (101 ± 21 IFN\( \gamma \) SFU/10\(^5\) cells) compared with controls K/CD40L/OX40L (28 ± 6 IFN\( \gamma \) SFU/10\(^5\) cells; \( P < 0.001 \)), K/pp65 (53 ± 22 IFN\( \gamma \) SFU/10\(^5\) cells; \( P = 0.048 \)) and K/CD40L/pp65 (41 ± 14 IFN\( \gamma \) SFU/10\(^5\) cells; \( P = 0.033 \)). In contrast with the in vitro experiments, in vivo data supported a critical role for the combination of CD40L- and OX40L-mediated activation in stimulating CMV-CTLs.

**CD40L and OX40L expressed by K562-derived whole-cell vaccine cooperate in stimulating CMV-CTLs in vivo**

We assessed the capacity of the whole-cell vaccine to stimulate in vivo CMV-CTLs using NOG/SCID/γ\( \delta \)\(-/-\) mice. Animals were coinfused with freshly isolated PBMCs obtained from CMV-seropositive donors and vaccinated twice with irradiated whole-cell vaccines and PBMCs as a source of APCs. CMV-specific immune responses were measured 7 days after the last vaccination (Fig. 2A). At the time of analysis, human CD45\(^{+}\) cells engrafted in the spleen of mice from all groups, though engraftment was lower in mice vaccinated with K/pp65 as compared with mice vaccinated with K/CD40L/OX40L (\( P = 0.014 \)) or K/CD40L/pp65 and K/OX40L/pp65 (\( P = 0.033 \); Fig. 2B). Although the immunophenotype of engrafted human CD45\(^{+}\) cells isolated from the spleen showed a similar distribution in CD3\(^{+}\)CD4\(^{+}\), CD3\(^{+}\)CD8\(^{+}\), and NK cells (Fig. 2C), the antigen specificity of engrafted T cells was significantly different. As shown in Fig. 2D, in all experimental conditions T cells recovered from the spleen of mice vaccinated had detectable CMV-specific IFN\( \gamma \) production. However, the vaccination with combined K/CD40L/pp65 and K/OX40L/pp65 stimulated the highest CMV-specific response (101 ± 21 IFN\( \gamma \) SFU/10\(^5\) cells) compared with controls K/CD40L/OX40L (28 ± 6 IFN\( \gamma \) SFU/10\(^5\) cells; \( P < 0.001 \)), K/pp65 (53 ± 22 IFN\( \gamma \) SFU/10\(^5\) cells; \( P = 0.048 \)) and K/CD40L/pp65 (41 ± 14 IFN\( \gamma \) SFU/10\(^5\) cells; \( P = 0.033 \)). In contrast with the in vitro experiments, in vivo data supported a critical role for the combination of CD40L- and OX40L-mediated activation in stimulating CMV-CTLs.

**Virusespecificity of "dual-specific" CAR-CMV-CTLs is boosted in vitro by the K562-derived whole-cell vaccine**

To assess whether the whole-cell vaccines can be used to boost "dual-specific" CAR-CMV-CTLs, we generated CMV-CTLs as previously described (10, 12) and engrafted them with the CAR-GD2. The transduction efficiency of CMV-CTLs exposed to the retroviral supernatant encoding the CAR-GD2 ranged between 35% and 65%, as detected by flow cytometry. CAR-CMV-CTLs were then stimulated twice, one week apart, with engineered and irradiated K562 and autologous PBMCs (as a source of APCs), and assessed for phenotype and IFN\( \gamma \) production. CAR-CMV-CTLs stimulated with combined K/CD40L/pp65 and K/OX40L/pp65 showed a significant enrichment in specific precursors responding to the CMV-pp65 pepmix as assessed by IFN\( \gamma \) ELISPot assay (1,397 ± 212 IFN\( \gamma \) SFU/10\(^5\) cells) compared with CTLs stimulated with control K/CD40L/OX40L (749 ± 146 IFN\( \gamma \) SFU/10\(^5\) cells; \( P < 0.001 \); Fig. 3A). Similarly, CAR-restricted responses, measured after stimulation with the anti-idiotypic 1A7 Ab that cross-links CAR-GD2 molecules, significantly increased in CAR-CMV-CTLs stimulated with K/CD40L/pp65 and K/OX40L/pp65 (2,819 ± 452 IFN\( \gamma \) SFU/10\(^5\) cells) compared with CTLs stimulated with control K/CD40L/OX40L (1,610 ± 267 IFN\( \gamma \) SFU/10\(^5\) cells; \( P = 0.009 \); Fig. 3A). In HLA-A2\(^{+}\) donors, phenotypic analysis confirmed a significant enrichment in NLY-tetramer\(^{+}\) and CAR\(^{+}\) CTLs after stimulations with K/CD40L/pp65 and K/OX40L/pp65 (Fig. 3B).

We explored the retained effector function of CAR-CMV-CTLs stimulated in vitro with K/CD40L/pp65 and K/OX40L/pp65 against CMV-pp65\(^{+}\) target and neuroblastoma GD2\(^{+}\) cells.
K562-based whole-cell vaccine encoding CMV-pp65 and CD40L matures monocytes and stimulates CMV-CTLs in vitro. A, expression of CD40L and OX40L in engineered K562. Striped histograms indicate wild-type K562 cells. B, Western blot analysis showing the expression of CMV-pp65 in engineered K562. C, uptake of apoptotic bodies from irradiated K/pp65 and K/CD40L/pp65 by monocytes. Monocytes labeled with PKH26 red fluorescent cell linker compound. Analysis of fluorescence signals was performed after 72 hours of coculture using a fluorescence microscope (Olympus IX70). D, expression of CD80, CD83, CD11c and HLA-DR by monocytes 72 hours after coculture with irradiated K/pp65 (in blue) and K/CD40L/pp65 (in green). The red line represents the expression of CD80, CD83, CD11c, and HLA-DR before the stimulation. E, frequency of CMV-CTLs assessed by IFNγ ELISpot using the CMV-pp65 pepmix. Data represented mean ± SD of 11 CMV-seropositive donors. Stimulation with an irrelevant pepmix was used as a negative control.

Table 1. Phenotype of T cells collected by day 10 to 12 after coculture with K562-based whole-cell vaccine

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>CD3+/CD4+</th>
<th>CD3+/CD8+</th>
<th>CD3+/SD1c</th>
<th>CD3+/HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC/pp65 pepmix</td>
<td>48% ± 27%</td>
<td>45% ± 29%</td>
<td>5% ± 2%</td>
<td></td>
</tr>
<tr>
<td>K562 wild-type</td>
<td>46% ± 11%</td>
<td>17% ± 2%</td>
<td>29% ± 10%</td>
<td></td>
</tr>
<tr>
<td>K/pp65</td>
<td>33% ± 11%</td>
<td>14% ± 4%</td>
<td>47% ± 15%</td>
<td></td>
</tr>
<tr>
<td>K/CD40L</td>
<td>42% ± 7%</td>
<td>23% ± 7%</td>
<td>30% ± 5%</td>
<td></td>
</tr>
<tr>
<td>K/OX40L</td>
<td>40% ± 5%</td>
<td>20% ± 6%</td>
<td>33% ± 6%</td>
<td></td>
</tr>
<tr>
<td>K/CD40L/pp65</td>
<td>43% ± 11%</td>
<td>22% ± 3%</td>
<td>31% ± 13%</td>
<td></td>
</tr>
<tr>
<td>K/CD40L/pp65 + K/OX40L/pp65</td>
<td>42% ± 9%</td>
<td>22% ± 5%</td>
<td>31% ± 14%</td>
<td></td>
</tr>
</tbody>
</table>

Boosting Virus Specificity of CAR-Virus-Specific CTLs

through their native TCRs and CAR, respectively. In a standard 51Cr-release assay, CAR-CMV-CTLs showed cytotoxic activity against the GD2+ target (CHLA-255; 63% ± 14%) and pp65-pepmix loaded PHA blasts (59% ± 3%; at 20:1 E:T ratio), but not against the GD2+ target cell line (Raji) or PHA blasts loaded with an irrelevant pepmix (Fig. 3C and Supplementary Fig. S1). Control CMV-CTLs not expressing the CAR showed no activity against CHLA-255 (data not shown). Similar results were obtained by measuring IFNγ production in ELISpot assays. We plated CTLs and tumor cells at a ratio of 1:1, and after 24 hours, CAR-CMV-CTLs stimulated with K/CD40L/pp65 and K/OX40L/pp65 in response to CHLA-255 showed a trend for a higher IFNγ production (421 ± 21 IFNγ+ SFU/10^5 cells) as compared with CAR-CMV-CTLs stimulated with K/CD40L/OX40L (295 ± 81 IFNγ+ SFU/10^5 cells; P = 0.6; Fig. 3D). Reactivity against Raji cells (GD2+ targets) was low in all experimental conditions. In coculture experiments in which CTLs and tumor cells were plated at a 1:1 ratio and cultured for 4 days, CAR-CMV-CTLs retained their capacity to eliminate CHLA-255 but not Raji (Fig. 3E). Overall, these data indicate that CAR-CMV-CTLs stimulated with K/CD40L/pp65 and K/OX40L/pp65 retain their selective specificities for CMV-pp65 and GD2 antigens.

Vaccination with K562-derived whole-cell vaccine encoding CMV-pp65, CD40L, and OX40L increases the antitumor effect of "dual specific" CAR-CMV-CTLs

To assess whether vaccination with the K562-derived whole-cell vaccine increases the antitumor effects of CAR-CMV-CTLs,
NOG/SCID/γc−/− mice were implanted intraperitoneally with CHLA-255 cells labeled with firefly luciferase. Five days after tumor implant, mice received intraperitoneal control or CAR-CMV-CTLs followed by the vaccination schedule illustrated in Fig. 2A. Mice vaccinated with the K/CD40L/pp65 and K/OX40L/pp65 combination controlled tumor growth significantly better by day 50 than mice vaccinated with K/CD40L/OX40L (P = 0.011; Fig. 4A). Tumor control was CAR mediated, since tumors grew despite vaccinations with K/CD40L/pp65 and K/OX40L/pp65 in mice infused with control CMV-CTLs (Fig. 4A). We selected day 50 to stop the experiment and to assess macroscopically for the presence of tumor at the time of euthanasia. We found that although only 2 out of 17 (12%) mice vaccinated with K/CD40L/OX40L were tumor free, 8 out of 17 (47%) mice were tumor free in the group vaccinated with K/CD40L/pp65 and K/OX40L/pp65. In addition, tumors were significantly smaller in mice vaccinated with K/CD40L/pp65 and K/OX40L/pp65 compared with mice receiving control CMV-CTLs (P < 0.001) or CAR-CMV-CTLs and control K/CD40L/OX40L vaccine (P = 0.022; Fig. 4B). Human CD45+ T cells recovered from the spleen of mice vaccinated with combined K/CD40L/pp65 and K/OX40L/pp65 also showed the highest frequency of CMV-CTLs (85 ± 16 IFNγ SFU/10⁵ cells) compared with mice vaccinated with the control K/CD40L/OX40L (41 ± 11 IFNγ SFU/10⁵ cells; P = 0.035). We measured CAR-dependent immune responses after stimulation with the anti-idiotypic 1A7 Ab. Similar to above, responses were increased in T cells recovered from the spleen of mice vaccinated with combined K/CD40L/pp65 and K/OX40L/pp65 (71 ± 24 IFNγ SFU/10⁵ cells) compared with mice vaccinated with control K/CD40L/OX40L (23 ± 8 IFNγ SFU/10⁵ cells; P = 0.048; Fig. 4C).

We further validated the vaccination approach in a systemic model. For these experiments, NOG/SCID/γc−/− mice were infused intravenously with a single cell derived clone of the A459 tumor cell line that expresses GD2 and rapidly metastasizes upon lung engraftment. Tumor cells were labeled with firefly luciferase to measure tumor bioluminescence in vivo. In this model, control and CAR-CMV-CTLs were infused intravenously and the vaccination was performed as described in Fig. 2A. As illustrated in Fig. 4D, in this systemic model, vaccination with combined K/CD40L/pp65 and K/OX40L/pp65 also induced better control of tumor growth by CAR-CMV-CTLs, which translated into significantly improved overall survival (P = 0.016; Fig. 4E). Altogether, these data indicate that vaccination with combined...
K562-derived whole-cell vaccine K/CD40L/pp65 and K/OX40L/pp65 improves the antitumor effects of CAR-CMV-CTLs in xenograft models.

Activation of the iC9 suicide gene expressed by the K562-derived whole-cell vaccine abrogates their tumorigenicity

For a potential clinical application, we sought to ensure the safety of this approach *in vivo* as the whole-cell vaccine is derived from a tumor cell line. For these experiments, K/CD40L/pp65 and K/OX40L/pp65 were labeled with an enhanced firefly luciferase that allows visualizing fewer than 10 cells in a mouse (35). Irradiation of K/CD40L/pp65 and K/OX40L/pp65 before inoculation into NOG/SCID/γc−/− mice completely abrogated the cells' tumorigenicity. As shown in Fig. 5C, when K/CD40L/pp65 and K/OX40L/pp65 were irradiated at 80 to 100 Gy before infusion, tumor growth was completely prevented in mice observed for more than 90 days. As an extra precaution, and to guarantee the safety of the vaccination, we further engineered the K562 cell line with the inducible suicide iC9 that also expresses a truncated form of CD19 as a selectable marker (27). Single cell clones were selected based on the expression of CD19 (Fig. 5A and B). Cells were inoculated subcutaneously without irradiation (4 × 10^6 cells) into NOG/SCID/γc−/− mice. By day 15 after engraftment, mice received intraperitoneal AP20187 (50 μg/mouse) for 2 consecutive days. Mice monitored for more than 90 days did not develop the tumor (Fig. 5D–F). Overall, these data indicate that the safety of the vaccination with K/CD40L/pp65 and K/OX40L/pp65 can be further assured through the incorporation of the iC9 suicide gene.

Discussion

We previously reported that the infusion of EBV-CTLs and CMV-CTLs expressing a CAR promotes objective tumor regressions in clinical trials (11–13). However, *in vivo* expansion and persistence of these cells remain suboptimal likely because, in the absence of significant amounts of viral load, the costimulation provided by endogenous APCs processing and presenting latent
Vaccination with K562-derived whole-cell vaccine expressing CMV-pp65, CD40L, and OX40L enhances antitumor effects of CAR-CMV-CTLs in vivo. A, NOG/SCID/γc−/− mice engrafted i.p. with the neuroblastoma cell line CHLA-255 labeled with firefly luciferase were infused i.p. with control or CAR-CMV-CTLs and vaccinated. The graph summarizes tumor bioluminescence. Summary of CMV-CTL lines prepared from 4 donors: 15 mice (control CMV-CTLs plus K/CD40L/pp65 and K/OX40L/pp65), 17 mice (CAR-CMV-CTLs plus K/CD40L and K/OX40L), and 17 mice (CAR-CMV-CTLs plus K/CD40L/pp65 and K/OX40L/pp65) were used per group. B, mice euthanized were analyzed for the presence of macroscopic tumors. The graph summarizes the volume of the tumor collected in the different groups. C, enumeration of the CMV-CTLs in the isolated human CD45+ cells from the spleen as assessed by IFNγ ELISpot in response to CMV-pp65 and irrelevant pepmixes or the 1A7 Ab that cross-links the CAR-GD2. D, mice were inoculated intravenously with the GD2+ lung carcinoma cell line A459 labeled with firefly luciferase. Mice were then infused intravenously with control or CAR-CMV-CTLs and vaccinated. Tumor bioluminescence was then measured over time. E, Kaplan-Meier analysis of tumor-bearing mice.

Viral antigens is insufficient to promote robust engraftment of CAR-redireced VsCTLs once infused. Here, we developed a strategy that can achieve the necessary engraftment. We have generated a vaccination approach using a K562-derived whole-cell vaccine and demonstrated that the antitumor effect of adoptively transferred CAR-redireced CMV-CTLs is enhanced when these CTLs are boosted in vivo by the vaccine.

Vaccination is the most common modality to induce both humoral and cellular immune responses. In the absence of clinically approved vaccines to induce cellular immune responses to either EBV or CMV, several experimental vaccination approaches for a clinical translation can be envisioned. These include DNA-plasmids (36), peptides (37), and ex vivo expanded and antigen-loaded DCs (38). However, each of these approaches has limitations that are primarily due to low immunogenicity (DNA-plasmid vaccine; ref. 39), toxicity caused by the strong adjuvants included in the vaccine preparation (peptide vaccine; ref. 40), and significant variability of the biologic characteristics of the final product and manufacturing costs (DC-based vaccine). On the basis of these limitations, we elected to generate an off-the-shelf whole-cell vaccine to boost in vivo adoptively transferred CAR-CMV-CTLs.

Autologous and allogeneic whole-cell vaccines consisting of tumor cells genetically manipulated to express GM-CSF or other cytokines, chemokines and immune stimulatory molecules have been used in clinical trials to promote cross-presentation of tumor-associated antigen to APCs in vivo (14, 15, 17, 41–43). On the basis of this evidence, we proposed to engineer the very well-characterized tumor cell line K562 to express the highly immunogenic CMV-pp65 protein. We thus created a whole-cell vaccine to administer to patients infused with CMV-CTLs expressing a tumor-specific CAR.

Our data demonstrate that the ectopic expression of the viral protein pp65 by K562 can be efficiently used to transfer the protein, likely in the form of apoptotic bodies, to APCs that can then process and present pp65 epitopes in the context of the
appropriate MHC molecules. This approach, when applied directly in vivo to boost adoptively transferred CAR-CMV-CTLs, has the advantage of delivering preformed antigens to APCs without the need for the in vivo protein synthesis required by DNA-plasmid vaccines. In addition, such a cell-based vaccine easily can be further engineered to express other molecules to enhance immune responses. In our specific case, we selected CD40L and OX40L. We and others have used CD40L expression in the past to generate autologous vaccines for hematologic malignancies to induce the upregulation of the costimulatory molecules CD80 and CD86 in leukemic cells through the CD40-CD40L pathway (43–45). Here, we demonstrated that CD40L expressed in the whole-cell vaccine is essential in promoting the expression of CD80 and CD83 in monocytes engulfing apoptotic bodies. Control vaccine producing pp65 but lacking CD40L is indeed less efficient in that regard. Because CD80 and CD83 are upregulated in mature DCs to initiate immune responses (23, 46), CD40L expressed by the whole-cell vaccine seems to accomplish the crucial step of APC maturation upon antigen processing.

We also included OX40L in the K562-derived whole-cell vaccine. As illustrated by our data, OX40L does not play a role in inducing the expression of CD80 and CD83 by monocytes engulfing apoptotic bodies. As a consequence, K/OX40L/pp65 is not superior to control K/pp65 in boosting CMV-CTLs. We have previously combined CD40L and OX40L molecules/signaling showing that they mediate enhanced potency of an autologous leukemia vaccine (47). Consistent with that experience, we did not show an advantage in combining both CD40L and OX40L in short-term experiments in vitro, because OX40L mostly delivers critical late accessory signals that augment the proliferation and survival of memory CD4+ T cells (48, 49). However, the combination CD40L and OX40L within the whole-cell vaccine showed clear benefits in in vivo experiments. We found in mice a more profound increase of CMV-CTLs when both CD40L and OX40L were incorporated within the whole-cell vaccine, suggesting the critical role of CD4 in boosting CMV-CTL responses. As a consequence, when CMV-CTLs are expressing a CAR, boosting in vivo their native virus specificity with the combination K/CD40L/pp65 and K/OX40L/pp65 showed better antitumor effects in two models of xenogenic solid tumors. Because K562 is also known to stimulate the proliferation of natural killer cells (NKs), we found in vitro and in vivo that the boosting with K562-derived whole-cell vaccine induced the expansion of NKs in addition to CAR-CMV-CTLs. Although no description of increased NKs has been

Figure 5. Activation of the iC9 suicide gene eliminates engrafted K562-derived whole-cell vaccine in vivo. A, characterization of the clones by flow-cytometric analysis. Gray areas indicate wild-type K562 cells. B, Western blot analysis showing the expression of CMV-pp65 in the clones expressing the iC9 transgene. C and D, NOG/SCID/γc−/− mice were inoculated subcutaneously with irradiated (C) or nonirradiated (D) K562-derived whole-cell vaccine expressing the iC9 gene and labeled with an enhanced firefly luciferase. Tumor growth was measured by in vivo imaging. E–F, effects of the administration of the chemical inducer of dimerization (CID) AP20187 on the growth of engineered vaccine. Arrows indicate the time of the CID administration.

www.aacrjournals.org Clin Cancer Res; 2015 OF9
reported using K562/GM-CSF cells in patients (17), considering the antitumor effects of NKs, the in vivo boosting of this cell subset by the K562-derived whole-cell vaccine may be beneficial.

Finally, we also addressed the potential safety concerns raised by using tumor cells as a vaccine. Autologous and allogeneic tumor cell lines have been safely used in multiple large clinical trials, suggesting that irradiation before inoculation abrogates their growth. Despite this apparent safety, however, a lethal acute respiratory distress syndrome and severe eosinophilia were reported in a patient vaccinated with irradiated autologous myeloblasts admixed with GM-CSF secreting K562 (http://oba.od.nih.gov/oba/RAC/meetings/Dec2011/RAC_Minutes_12-11.pdf). We found in animals that irradiation abolishes the growth of our K562 engineered with pp65, CD40L, and OX40L. However, we also demonstrated that an additional safety mechanism can be implemented by further engineering these cells to express the iC9 suicide gene. Activation of iC9 by a small molecule halts the growth of live (deliberately nonirradiated) engineered K562 implanted in mice and since iC9 has been validated in a clinical trial, it can be used efficiently in the context of a vaccine approach (27).

In conclusion, we demonstrated that a K562-derived whole-cell vaccine can safely enhance the antitumor effects of adoptively transferred CAR-CMV-CTLs. Due to the high flexibility of the whole-cell vaccine, K562 can be properly engineered to express other immunogenic antigens derived from other viruses and provide other relevant molecules to activate the immune system.

Disclosure of Potential Conflicts of Interest
B. Savoldo and G. Dotti are investigators in a collaborative research grant between the Center for Cell and Gene Therapy and Celgene to develop genetically modified T cells. No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: I. Caruana, B. Savoldo, G. Dotti
Development of methodology: I. Caruana, B. Savoldo, G. Dotti
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Caruana, G. Weber, B. Savoldo, G. Dotti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Caruana, G. Weber, B. Savoldo, G. Dotti
Writing, review, and/or revision of the manuscript: I. Caruana, G. Weber, B. Savoldo, G. Dotti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Caruana, B.C. Ballard, M.S. Wood
Study supervision: I. Caruana, G. Dotti

Acknowledgments
The authors thank Dr. Brian Rabinovich from the MD Anderson Cancer Center (Houston, TX) for providing the enhanced firefly luciferase gene and Catherine Gillespie from the Center for Cell and Gene Therapy for editing the article.

Grant Support
This work was supported in part by R01 CA142636 NIH-NCl, W81XWH-10-10425 Department of Defense, Technology/Therapeutic Development Award. 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 November 20, 2014, revised January 20, 2015, accepted February 9, 2015; published OnlineFirst February 17, 2015.

References


K562-Derived Whole-Cell Vaccine Enhances Antitumor Responses of CAR-Redirected Virus-Specific Cytotoxic T Lymphocytes *In Vivo*


*Clin Cancer Res* Published OnlineFirst February 17, 2015.