Title
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Journal
The Journal of Infectious Diseases, 222(5)

ISSN
0022-1899

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Publication Date
2020-08-04

DOI
10.1093/infdis/jiaa171

Peer reviewed

The Journal of Infectious Diseases

MAJOR ARTICLE

Chimeric Antigen Receptors Targeting Human Cytomegalovirus

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Human cytomegalovirus (CMV) is a ubiquitous pathogen that causes significant morbidity in some vulnerable populations. Individualized adoptive transfer of ex vivo expanded CMV-specific $CD8^+$ T cells has provided proof-of-concept that immunotherapy can be highly effective, but a chimeric antigen receptor (CAR) approach would provide a feasible method for broad application. We created 8 novel CARs using anti-CMV neutralizing antibody sequences, which were transduced via lentiviral vector into primary $CD8^+$ T cells. All CARs were expressed. Activity against CMV-infected target cells was assessed by release of cytokines (interferon-γ and tumor necrosis factor–α), upregulation of surface CD107a, proliferation, cytolysis of infected cells, and suppression of viral replication. While some CARs showed varying functional activity across these assays, 1 CAR based on antibody 21E9 was consistently superior in all measures. These results support development of a CMV-specific CAR for therapeutic use against CMV and potentially other applications harnessing CMV-driven immunotherapies.

Keywords. human cytomegalovirus; chimeric antigen receptor; cellular immunity.

Human cytomegalovirus (CMV) infection is highly prevalent, ranging from 60% to 100% across various demographics [1]. Although healthy infected adults generally contain the virus asymptomatically for life, CMV is the most common infectious cause of birth defects and is a significant pathogen of immunocompromised hosts. Disseminated infection is a serious and common complication in people living with AIDS and transplant recipients. Although several drugs are available for prophylaxis and treatment of CMV infection, these agents can have limiting toxicities such as bone marrow suppression and nephrotoxicity, and drug resistance is an increasing problem [2, 3].

Cellular immunity, particularly $CD8^+$ T lymphocytes (CTLs), comprises the critical arm of immunity that contains CMV in healthy individuals, and proof-of-concept studies have demonstrated the effectiveness of immunotherapy using adoptive transfer of ex vivo expanded autologous CMV-specific CTLs [4–6]. Clinical application of this approach is limited by the technically challenging and labor-intensive nature of expanding antigen-specific CTL, and human leukocyte antigen (HLA) restriction limits administration of CMV-specific CTL to HLA-matched recipients. A chimeric antigen receptor (CAR) gene therapy approach would address these issues by allowing rapid generation of autologous CMV-specific CTLs. To date there have been brief reports of 2 CARs both targeting the CMV cell surface protein glycoprotein B (gB) [7, 8], but to our knowledge these have not advanced to clinical testing, and it is not known whether CARs can successfully target other CMV proteins more effectively.

In addition to gB, CMV utilizes several other glycoproteins to infect cells. The pentameric complex (PC), composed of gH, gL, UL128, UL130, and UL131A, is essential for CMV entry into many host cell types, including epithelial cells, endothelial cells, and macrophages [9, 10]. It is a major target of potent anti-CMV neutralizing antibodies and is highly conserved among CMV strains, making it an attractive target for the development of a CMV-specific CAR T-cell therapy. Here we screen a panel of novel CARs based on previously reported neutralizing antibodies that target different proteins of the PC [11, 12].

MATERIALS AND METHODS

Anti-CMV Antibody Sequences

Neutralizing antibodies against CMV and their partial genetic sequences were previously described [11, 12]. Their full variable region sequences were utilized to create single chain antibody genes synthesized as codon optimized genes coding for the heavy chain and light chain variable regions (Table 1) separated

Received 11 February 2020; editorial decision 30 March 2020; accepted 10 April 2020; published online April 13, 2020.

Received 11 February 2020; accepted 30 March 2020.

Presented in part: Research meeting, University of California, Los Angeles, Department of Medicine, 12 October 2019.

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The Journal of Infectious Diseases® 2020;222:853–62

DOI: 10.1093/infdis/jiaa171

Table 1. Variable Chain Sequences From Anti-Cytomegalovirus Neutralizing Antibodies Utilized for Chimeric Antigen Receptor Construction

Parent Antibody	CMV Protein Target	VH Sequence	VL Sequence
1B2	UL128/UL130/UL131	EVQLVESGGVLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPE KRLEWVATISDDGNYTNYPDSVKGRFTISRDNAKNNLYLQ MSSLKSEDTAMYYCARGWLLPVFAYWGQGTLVTVSA	DIVLTQSPATLSVTPGDSVSLSCRASQSIGNNLHWYQQK SHESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLNINSV ETEDFGVYFCQQSNRWPWTFGGGTKLEIK
12E2	UL128/UL130/UL131	EVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMFWVRQT PEKKLEWVAYISNGGGSTYYPDTVKGRFTISRDNDKNTLY LQMSRLKSDDTALYYCVRPKRDFQYLYAMDYWGQGTSV TVSS	DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWY QQKPGQSPKLLIYLASNLESGVPARFSGSGSGTDFTL NIHPVEDEDAATYYCQHSRELPWTFGGGTKLEIK
13B5	UL128	QVTLKESGPGILKPSQTLSLTCSFSGFSLTTSGLGVGWIRQPS GKGLEWLAHIWWDDDKYFNPSLRNQLTISKDTSRNQVFL EITSVTTADTATYYCVRSLYDYDEGYYFDSWGQGTTLTVSS	EIVMIQSPATLSVNPGDRVSLSCRASQSISDYLHWYQQK SHESPRLLIKYASQSISGIPSRFSGSGSGSDFTLSINSVE PEDVGVYYCQNGHTFPPTFGGGTKLEIK
18F10	gH (UL75)	QVTLKESGPGILQPSQTLSLTCSFSGFSLSTYGIGIGWIRQPSG KGLEWLAHIWWNDNKNYNTALKSRLTISKDPSNNQVFLKI ASVDTADTATYFCARTGYFDVWGAGTTVTVSS	DVVLTQTPLSLPVSLGDQVSISCSSSQSLVHSNGNTYIH WYLQKPGQSPKLLIYTVSNRFSGVPDRFSGSGSGTDF TLKISRVEAEDLGLYFCSQSTHVPYTFGGGTKLEIK
2–80	gH (UL75)	QIQLVQSGPELKKPGETVKISCKASGYTFTNFGMNWVKQAPG KGLKWMGWINTYTGEPTYADDFKGRFAFSLETSASTASLQ INNLKNEDTATYFCARRGDGLYSMDYWGQGTSVTVSS	DIVLTQSPASLAVSLGQRATISCRASESIDSYGNSFMYWY QQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTI NPVEADDVATYYCQQSNEDPLTFGAGTKLELK
21E9	gH (UL75)	QIQLVQSGPELKKPGETVKISCKASGYTFTIYGMNWVKQAPG KGLKWMGWINTYTGEPTYADDFRGRFAFSLETSASTAYLQI NNLKNEDTATYFCARKGYYGSSGYFDYWGQGTTLTVSS	SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVSWYQQK PGQSPKLLIYYASNRYTGVPDRFTGSGYGTDFTFTISTV QAEDLAVYFCQQDYSSPWTFGGGTKLEIK
21F6	UL130/UL131	QIQLVQSGPELKKPGETVKISCKASGYTFTSYGMNWVKQAPG KGLKWMGWINTYTGEPTYADDFKGRFAFSLETSASTAYLQI NNLKNEDTATYFCAREHYYGINPLLGCWGQGTTLTVSS	DIQMTQTTSSLSASLGDRVTISCSASQGISNYLNWYQQK PDGTVKLLIYDTSSLHSGVPSRFSGSGSGTDYSLTISNL EPEDIATYYCQQYSKLPYTFGGGTKLEIK
62-11	gH (UL75)	QVQLQQPGAELVRPGASVKLSCKASGYTFTSYWMNWVKQR PGQGLEWIGMIDPSDSETHYNQMFKDKATLTVDKSSSTAY MQLSSLTSEDSAVYYCSNGYSSFAYWGQGTLVTVS	DIQMTQTTSSLSASLGDRVTISCSASQGISNYLNWYQQK PDGTVKLLIYDTSSLHSGVPSRFSGSGSGTDYSLTISNL EPEDIATYYCQQYSKLPYTFGGGTKLEIK

Abbreviation: CMV, cytomegalovirus.

by a GGGGSGGGGSGGGGS linker, additionally with an upstream leader sequence from granulocyte-macrophage colony-stimulating factor (MLLLVTSLLLCELPHPAFLLIP).

Cell Lines

The cell lines 293T and ARPE-19 were maintained with Dulbecco's modified essential medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 U/mL), and heat-inactivated fetal bovine serum (10%) as described previously [11, 13]. Primary $CD8^+$ T cells from healthy donors were generated from whole peripheral blood mononuclear cells (PBMCs) as previously described [13, 14]; in brief, they were purified with the MACS Column Separation Kit by positive selection according to the manufacturer's protocol (Miltenyi) and stimulated for 5 days with an anti-CD3 antibody in the presence of irradiated feeder PBMCs and 50 U/mL recombinant human interleukin 2 (IL-2; National Institutes of Health AIDS Reagent Repository), yielding > 95% pure $CD3^+$/$CD8^+$ cells. Experiments were repeated using PBMCs from 3 healthy donors provided by the University of California, Los Angeles AIDS Institute Virology Core Facility as institutional review board–exempt materials without any demographic information.

Chimeric Antigen Receptor Gene and Lentiviral Vector Construction

The lentiviral vector pCCLcMNDU3c-X2 [15], a gift of D. B. Kohn, was first modified to contain the sequence for the fixed regions (except the leader and single chain antibody sequences) of a previously reported human CAR, consisting of an IgG_4-based spacer (additionally containing a silent mutation creating a unique Apa I restriction site), CD8 transmembrane domain, co-signaling domain from 4-1BB (CD137), and signaling domain from the human CD3 complex ζ chain (CD247) as previously described in detail [14]. Additionally, sequences for the P2A ribosomal skip sequence with a furin cleavage site [16] followed by the heat-stable antigen murine CD24 reporter [17] were inserted immediately downstream of the CAR sequences. This modified vector was digested with Hpa I and Apa I restriction enzymes, and polymerase chain reaction–amplified products of the leader-single chain antibody sequences were inserted using the In-Fusion Cloning Kit (Takara), followed by sequence confirmation of the final vectors. Lentiviral stocks were produced by co-transfection of HEK-293T cells with these constructs with vesicular stomatitis virus envelope glycoprotein G protein and HIV-based packaging vectors as previously described [13, 14], and stored in aliquots at –80°C until use.

CAR Transduction of Primary $CD8^+$ T Cells

Cells were transduced with the CAR delivery lentiviral vectors as previously described [14]. In brief, polystyrene 24-well tissue culture plates were pre-coated with RetroNectin (Takara Bio). Lentiviral vector at a concentration of approximately 50 ng p24 antigen in 100 µL volume was added and centrifuged at 2000 g for 2 hours; 10^6 cells were then added for transduction, and maintained in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100

U/mL), heat-inactivated fetal bovine serum (10%), HEPES buffer (10 mM), and 50 U/mL recombinant human IL-2 (R10-50). These cells were maintained and enriched with periodic restimulations using a goat antihuman F(ab)2 antibody (catalog number 109-006-006, Jackson ImmunoResearch Laboratories) as previously described [14], to at least 70% purity before use in functional testing experiments.

Western Blot for CAR Expression

Transduced $CD8^+$ T cells were assessed for CAR expression by Western blot as previously described [14]. In brief, 2 million transduced cells were lysed and subjected to protein electrophoresis, followed by transfer onto a 0.45-μm polyvinylidene difluoride membrane (Millipore). The membrane was probed by using a mouse antihuman CD247 monoclonal antibody (BD Pharmingen, catalog number 551033) and the SuperSignal West Pico detection kit (Pierce), which yielded bands for both endogenous CD247 as well as higher molecular weight CD247-containing CAR proteins.

Flow Cytometric Analysis for Cell Surface CAR Expression

Detection of cell surface CAR expression on transduced cells was performed as previously described [14]. In brief, cells were stained with fluorescein isothiocyanate (FITC)–conjugated goat antihuman F(ab)2 antibody (catalog number 109-006-003, Jackson ImmunoResearch Laboratories) or isotype control antibody, fixed, and analyzed by flow cytometry (MACSQuantVYB, Miltenyi) for analysis of cell surface CAR expression (FlowJo).

CMV Strains and Production of Viral Stocks

Green fluorescent protein (GFP)–containing TB40/E and TR strains of human CMV that express a GFP reporter under the SV40 promoter were derived from bacterial artificial chromosome DNA [18, 19], a gift of T. E. Shenk and E. A. Murphy. CMV stocks were generated following viral propagation in ARPE-19 cells (American Type Tissue Culture Collection) by standard ultracentrifugation procedures as described previously [20, 21] and titrated on ARPE-19 cells by immunostaining using the IE1-specific monoclonal antibody p63-27 [22], gift of W. Britt, and the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions.

Flow Cytometric Analysis of Intracellular Cytokines and Cell Surface CD107a Expression by CAR-Transduced Primary $CD8^+$ T Cells`

ARPE-19 cells were seeded in 24-well plates at 2×10^5 cells/well, and 24 hours later were infected with CMV using multiplicity of infection (MOI) of 3, with mock-infected cells as a negative control. Five days later, when the cells were uniformly infected as reflected by GFP expression, 10^6 CAR-transduced primary $CD8^+$ T cells were added and incubated for an additional hour; stimulation with leukocyte activation cocktail (BD Biosciences) served as a positive control for activation. Brefeldin A and monensin (0.5 μL Golgi Plug and 0.5 μL Golgi Stop, BD Biosciences) and allophycocyanin-conjugated anti-CD107a antibody (catalog number 328620, Biolegend) antibody were then added. After 5 hours of co-incubation, the CAR-transduced $CD8^+$ T cells were removed from each well for analysis. Surface staining was then performed with antihuman antibodies including anti-IgG F(ab)2 conjugated with FITC (catalog number 109-096-003, Jackson ImmunoResearch Laboratories), anti-CD8 conjugated with phycoerythrin (catalog number 300908, Biolegend), followed by fixation and permeabilization (Cytofix/Cytoperm, BD Biosciences), followed by intracellular cytokine staining with antihuman antibodies including anti–interferon-γ conjugated with Alexa Fluor 647 (catalog number 506507, Biolegend) and anti–tumor necrosis factor–α (TNF-α) conjugated with Alexa Fluor 700 (catalog number 502928, Biolegend). Cells were analyzed by FACSCelesta (BD Biosciences) using FlowJo software (BD Biosciences).

Flow Cytometric Analysis of Cell Proliferation

ARPE-19 cells were used as stimulating cells to test for CMV-mediated proliferation of CAR-transduced $CD8^+$ T cells, as previously described with the following modifications [14]. In a 48-well plate, 10^5 ARPE-19 cells were seeded 1 day before infection with HCMV TB40/E or TR at an MOI of 1.5 and cultured for 3 days to obtain > 90% infection as seen by GFP fluorescence (or mock-infected as negative controls). CAR-transduced $CD8^+$ T cells were labeled with CellTrace Violet according to the manufacturer's directions (Thermo Fisher Scientific), and 5×10^5 CAR-transduced cells with 3×10^6 irradiated feeder PBMCs were added to each well and cultured in R10-50 for 7 days with a medium exchange at 3 days. Anti-CD3 antibody stimulation served as a positive control. For analysis, 0.5×10^6 cells were harvested from each well and co-stained for CD8 and human F(ab), fixed with 1% paraformaldehyde, and analyzed by flow cytometry gated on the $CD8^+$ population (MACSQuant VYB, Miltenyi Biotech; FlowJo software, BD Biosciences).

Chromium Release Assay of Cytolytic Activity Against CMV-Infected Cells by CAR-Transduced Primary CD8+ T Cells

Killing of CMV-infected target cells by CAR-transduced primary $CD8^+$ T cells was tested in ^{51}Cr release assays as previously described [13, 14, 23, 24], with the following modifications. ARPE-19 cells were plated at 10^6 per T25 tissue culture flask and infected the next day with CMV TB40/E or TR at a multiplicity of infection of 1.5 (or mock-infected as a negative control), then cultured for 4 days to achieve > 90% infection by GFP expression as previously described [11]. The cells were detached by incubation in 5 mM EDTA in phosphate-buffered saline for 15 minutes at 37°C, then labeled with ^{51}Cr for use in standard chromium release assays as described [13, 14, 23, 24].

Assessment of Suppression of CMV Replication by CAR-Transduced Primary CD8$^+$ T Cells

Suppression of CMV replication was assessed as previously reported [11] with modifications. ARPE-19 cells were seeded into 96-well plates at 2×10^4 cells/well, and infected following day with CMV TB40/E or TR with an MOI of 1. After 4 days, 2×10^5 CAR-transduced (or control mock-transduced) primary CD8$^+$ T cells were added to each well and imaged after 8 hours of co-incubation using an Axio Observer Z1 inverted fluorescence microscope equipped with a linear motorized stage (Carl Zeiss).

RESULTS

Genetic Construction of CARs Based on Neutralizing Antibodies Against CMV

The sequences of previously identified neutralizing monoclonal antibodies targeting different CMV PC proteins [11] were utilized as targeting regions for chimeric antigen receptor design (Table 1). These included antibodies targeting epitopes in the gH subunit (21E9, 2–80, 18F10, 62-11), conformational epitopes in the UL128/130/131A subunits (1B2, 12E2, 21F6), and a linear epitope in UL128 (13B5), all of which had previously been confirmed to bind CMV-infected cells (not shown). Genes for single chain versions of these antibodies were incorporated into the backbone that we previously described for HIV-1-specific CARs [14], consisting of the single chain antibody targeting region, a spacer based on the $IIgG_4$ constant region, the CD8 transmembrane domain, and cytoplasmic signaling domains from 4-1BB and CD3ζ (Figure 1). These novel CAR genes were inserted into a lentiviral vector for transduction of primary CD8$^+$ T cells.

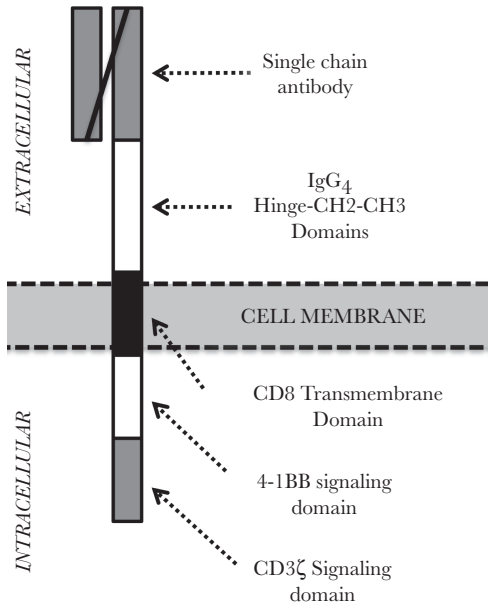

Figure 1. Schematic of chimeric antigen receptor structure. A targeting domain consisting of a single chain antibody against cytomegalovirus (Table 1) is fused to an IgG_4 (hinge, CH2, and CH3 domains of the heavy chain constant region)–based spacer, a CD8 transmembrane domain, and cytoplasmic signaling domains of 4-1BB and CD3ζ.

Anti-CMV Antibody-Based CARs Are Expressed by Transduced Primary CD8$^+$ T Cells

Primary CD8$^+$ cells from healthy donors were transduced and tested for expression of the novel CARs. Western blot analysis for expression of CD3ζ demonstrated detection of CAR expression as a larger CD3ζ-expressing protein in addition to the native CD3ζ protein (Supplementary Figure 1). Cell surface expression of the CARs was further confirmed by staining for the immunoglobulin domain (not present on native T cells) on the surface of the transduced cells and detection by flow cytometry (Figure 2). Both modes of detection revealed that all 8 novel CARs were expressed by primary CD8$^+$ T cells.

CAR-transduced primary CD8$^+$ T cells are specifically triggered to release cytokines and upregulate cell surface CD107a by CMV-infected target cells. To test whether the CARs recognize their target proteins on the surface of CMV-infected cells, CAR-transduced primary CD8$^+$ T cells were exposed to acutely CMV TR-infected ARPE-19 cells and assessed for specific production of interferon gamma (IFN-γ) and TNF-α. Nontransduced CD8$^+$ T cells demonstrated minimal cytokine production in response to the ARPE-19 cells regardless of CMV infection. In contrast, at least 2 CARS, 21E9 and 2–80 (both targeting gH), showed specific cytokine release in response to CMV-infected but not CMV-uninfected cells (Figure 3A and 3C). Simultaneous evaluation for cell surface expression of the degranulation marker CD107a correlated to cytokine release (Figure 3B and 3D), again demonstrating that CARs 21E9 and 2–80, and to a lesser extent 12E2 and 21F6 (targeting the UL128/130/131A subunit), mediated CMV-specific cell triggering. Further studies focused on these 4 CARs. Similar results were seen with target ARPE-19 cells infected with CMV TB40/E infected cells (data not shown).

CAR-Transduced Primary CD8$^+$ T Cells Proliferate in Response to CMV-Infected Target Cells

Further evaluating the function of CAR-transduced primary CD8$^+$ T cells, the proliferation of these cells in response to CMV-infected cells was tested (Figure 4). The 21E9 CAR mediated modest proliferation when exposed to CMV-infected cells, but did not exhibit proliferation in response to uninfected cells. The other 3 CARs (2–80, 12E2, 21F6) mediated no appreciable proliferation. Thus, at least 1 CAR conferred CMV-specific proliferation of transduced CD8$^+$ T cells.

CAR-Transduced Primary CD8$^+$ T Cells Mediate Modest Cytolysis of CMV-Infected Target Cells

It was previously reported that CMV-infected cells are intrinsically resistant to CD8$^+$ T-cell cytolysis [7]. The capacity of

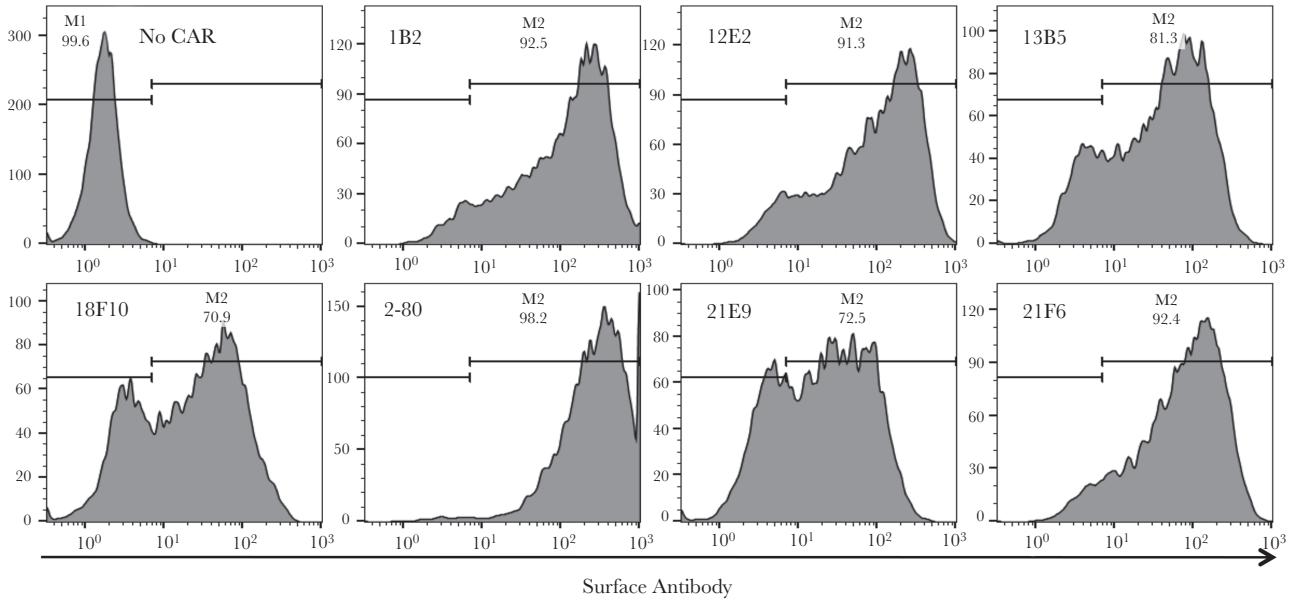

Figure 2. Chimeric antigen receptor (CAR) expression in transduced primary CD8$^+$ T cells by flow cytometry. Primary CD8$^+$ T cells were transduced with lentiviral vectors delivering genes for the indicated CARs. The cells were then stained for cell surface human antibody expression and analyzed by flow cytometry. Not shown: 62-11.

CAR-redirected primary CD8$^+$ T cells to mediate CMV-specific cytolysis was tested by ^{51}chromium release assays (Figure 5). The only CAR that mediated consistent specific killing of CMV-infected target cells in multiple experiments was 21E9, which mediated cytolysis at modest levels using high effector:target ratios. The other CARs yielded lower and inconsistent levels of killing across multiple experiments.

CAR-Transduced Primary CD8$^+$ T Cells Suppress CMV Replication

The antiviral activity of CAR-transduced CD8$^+$ T cells was evaluated by co-culture with acutely CMV-infected ARPE-19 cells using GFP-expressing CMV TR (Figure 6). The 4 tested CARs all exhibited quantifiable antiviral activity with reduction of the concentration of fluorescent CMV-infected cells, even after a short incubation period of 8 hours. Across multiple experiments, CAR 21E9 mediated the most consistently potent antiviral activity. Similar results were seen with CMV TB40/E-infected ARPE-19 cells (data not shown).

DISCUSSION

To date, only 1 CMV-specific CAR has been reported in detail [8]. That CAR targets CMV gB, and CTLs transfected with CAR-encoding RNA were shown to be CMV-specific by responding to infected cells by releasing IFN-γ and TNF-α and modestly upregulating surface CD107a. Although CAR-transfected cells also killed target cells expressing recombinant gB, it was subsequently shown that they do not kill CMV-infected cells [7], presumably due to viral escape mechanisms against cytolysis. One further CMV-specific CAR, also targeting gB, was reported in an abstract only to mediate killing of gB-expressing 293T-HEK cells [25]. Here we screened 8 candidate CARs targeting other viral proteins on infected cells. These antibodies were previously generated in mice using a modified vaccinia Ankara virus vector expressing PC sequences derived from TB40/E [21] and found to bind the PC with very high affinity [11, 12]. At least 1 of these CARs (based on the gH-specific antibody 21E9) was consistently active by multiple functional tests against cells infected with both CMV TB40/E and TR strains, which belong to 2 different gH genotypes [21, 26].

While all of these novel CARs exhibited adequate levels of expression on transduced cells, they varied in function. The 21E9-based CAR exhibited consistently superior activity in all functional tests, while the 2–80–based CAR showed activity in most assays, although it seemed to confer nonspecific background activity. The other CARs targeting other PC subunits demonstrated minimal activity overall. Whether this indicates that gH is a superior target for CARs than other PC proteins is unclear. 21E9 and 2–80 antibodies have about 10-fold less binding affinity than the other antibodies [11], but because the other 2 gH-specific antibodies 62-11 and 18F10 with similar affinity yielded poorly active CARs, affinity itself is probably not the major determinant of CAR activity. Target protein expression could be a factor; gH appears to be more abundant on CMV-infected cells than UL128, UL130, and UL131A [27]. Another potential element is that the gH-specific antibodies utilized here recognize gH in multiple contexts including monomeric gH, gH bound to gL, and gH associated with the PC, perhaps allowing a broader target for binding and recognition [11]. Finally, it is notable that 21E9 and 2–80 target a site on gH that is distinct from other gH-specific antibodies, suggesting

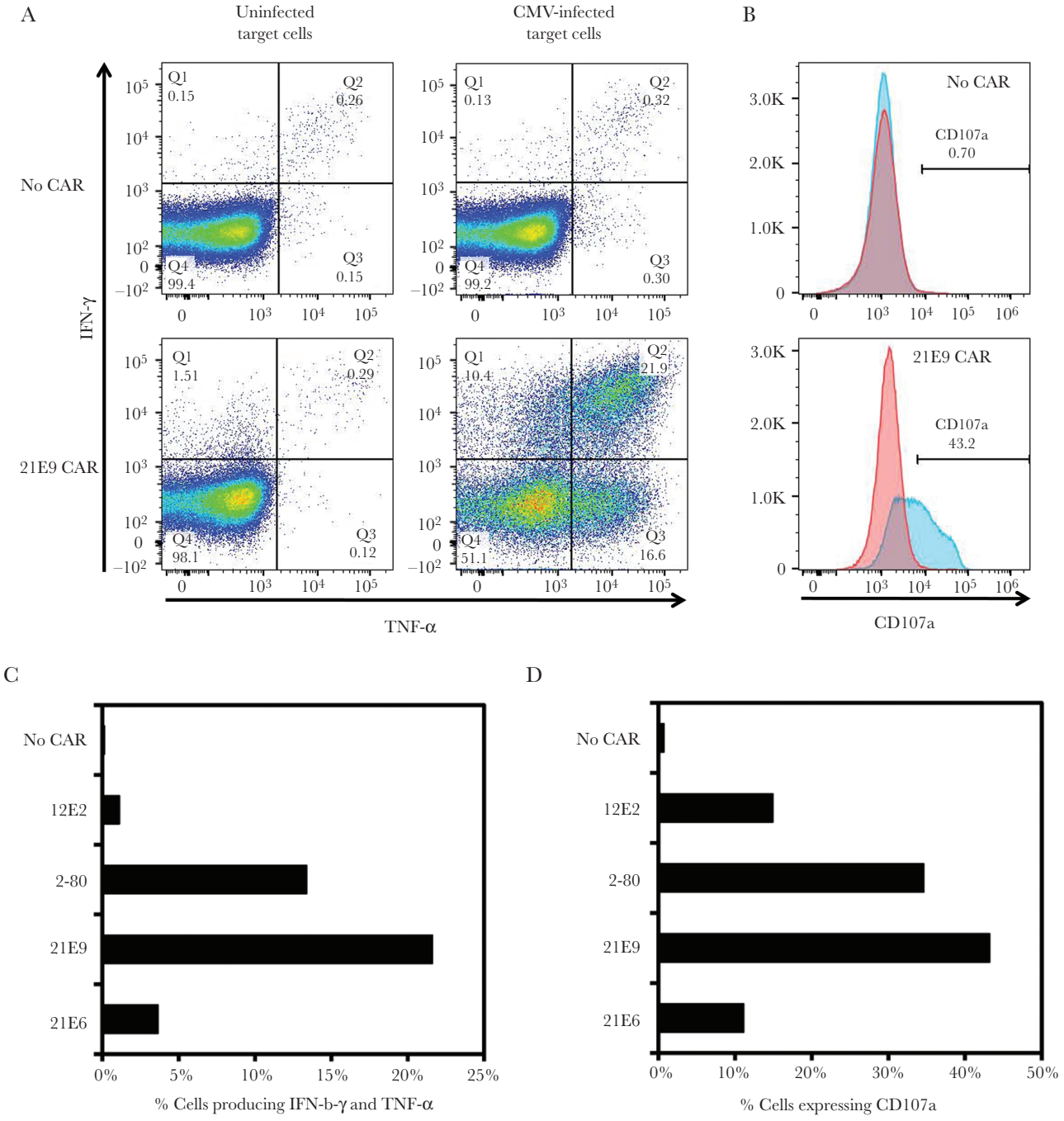

Figure 3. Intracellular cytokine and cell surface CD107a expression by chimeric antigen receptor (CAR)–transduced primary $CD8^+$ T cells upon exposure to cytomegalovirus (CMV)–infected cells. *A*, Representative dot plots are shown for production of both intracellular interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) by untransduced (top row) or CAR-transduced (bottom row) $CD8^+$ T cells exposed to uninfected (left column) or acutely CMV TR-infected (right column) ARPE-19 cells. *B*, Representative histograms are shown for cell surface expression of CD107a on untransduced (top) or CAR-transduced (bottom) $CD8^+$ T cells exposed to uninfected (red histogram) or CMV TR-infected (blue histogram) ARPE-19 cells. *C*, Net percentages of untransduced or CAR-transduced $CD8^+$ T cells producing both intracellular IFN-γ and TNF-α in response to CMV-infected target cells (after subtraction of the response to uninfected target cells) are plotted. *D*, Net percentages of untransduced or CAR-transduced $CD8^+$ T cells expressing cell surface CD107a after exposure to acutely CMV-infected ARPE-19 cells are plotted. Similar results were seen with CMV TB40/E-infected ARPE-19 cells (not shown). These results are representative of 3 experiments with 3 different $CD8^+$ T-cell donors; the other 4 CARs demonstrated minimal activity in 2 other experiments (not shown).

that this epitope region may be more accessible to antibody in the context of a CAR.

It is not clear why so few of these CARs mediated successful CMV-specific targeting of $CD8^+$ T cells as determined by our assays. Our prior constructions of CARs in the same backbone using 7 anti-HIV neutralizing antibody sequences all yielded highly functional HIV-specific CARs in the same assays [14]. Our previous attempts to generate CMV-specific CARs using 7

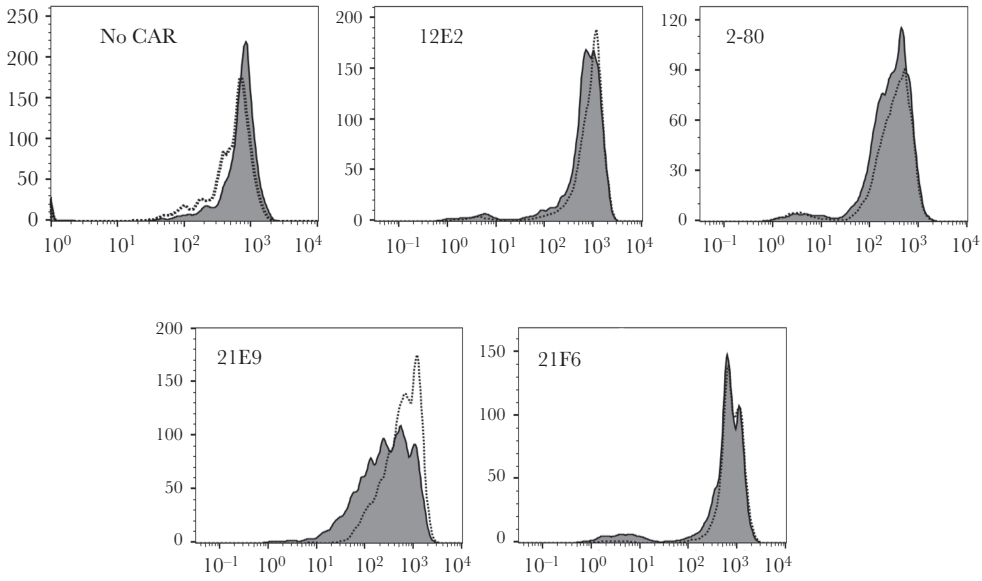

Figure 4. Proliferation of chimeric antigen receptor (CAR)–transduced primary CD8$^+$ T cells upon exposure to cytomegalovirus (CMV)–infected cells. Primary CD8$^+$ T cells transduced with the indicated CAR were labeled with CellTrace Violet dye and co-cultured for 6 days with uninfected (open gray histograms) or CMV TB40/E-infected (filled black histograms) ARPE-19 cells and analyzed by flow cytometry for dye expression after 7 days. These results are representative of 2 independent experiments with 2 different CD8$^+$ T-cell donors, each performed in biological duplicates.

previously reported neutralizing antibodies against gB [28–33], including AE11F, EV1-15, ITC33, ITC39, ITC48, ITC52, and ITC63, also failed despite adequate levels of expression (not shown). Thus, only 1 or 2 anti-CMV neutralizing antibodies of 15 tested yielded a functional CAR. A potential explanation could be that the binding of these CMV-specific antibodies is especially susceptible to conformational changes from conversion to single chain format, although this would require experimental confirmation. Additionally, we did not try the alternate orientation of heavy and light variable chains in our CAR constructs (light before heavy), which could potentially improve the function of some CARs.

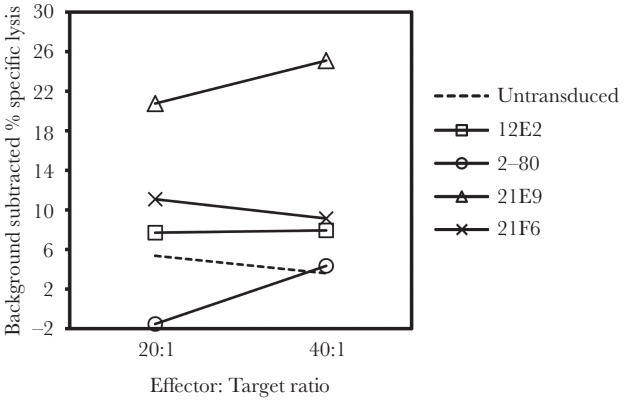

Figure 5. Killing of cytomegalovirus (CMV)–infected target cells by chimeric antigen receptor (CAR)–transduced primary CD8$^+$ T cells. Background-specific lysis of uninfected cells (<6%, except for 2–80 that had background levels of 20% and 32% at effector to target ratios of 20:1 and 40:1, respectively) was subtracted from specific lysis of CMV TB40/E-infected cells. In 3 independent experiments with 3 different CD8$^+$ T-cell donors, only 21E9 exhibited consistent targeted killing of CMV-infected cells.

Proff et al previously reported that CMV-infected cells are not lysed either by CAR-transduced or native class I–restricted CMV-specific CD8$^+$ T cells despite excellent activity against uninfected cells expressing the target protein alone [7]. They concluded that CMV renders infected cells intrinsically resistant to cytolysis, in addition to previously demonstrated interference with class I antigen processing [34], since CARs act independently of antigen processing. In contrast, our data showed modest but reproducible CAR-mediated CD8$^+$ T-cell killing of CMV-infected cells at levels approaching those observed by Rauser et al, who demonstrated cytolysis of infected cells by endogenous CMV-specific CD8$^+$ T cells [35]. It is unclear whether our discordant results might be related to methodological differences in CMV strains or target cell types, although Rauser et al did utilize the same virus and cells. Overall, the role of infected cell killing for in vivo efficacy of CMV-specific CARs may not be critical, since adoptive transfer of native CMV-specific CD8$^+$ T cells has clearly demonstrated their antiviral effects [4–6], suggesting that targeted noncytolytic mechanisms may be sufficient.

Beyond direct utility for anti-CMV therapy, a CMV-specific CAR may have other applications by harnessing the pathogenesis of chronic CMV infection. In normal immunocompetent hosts, this virus establishes a chronic lifelong infection that is mostly latent, but with frequent low-level subclinical reactivations that stimulate relatively high levels of persistently circulating functional anti-CMV T cells [36]. In this regard, CMV serves analogously to an endogenous vaccine that boosts and

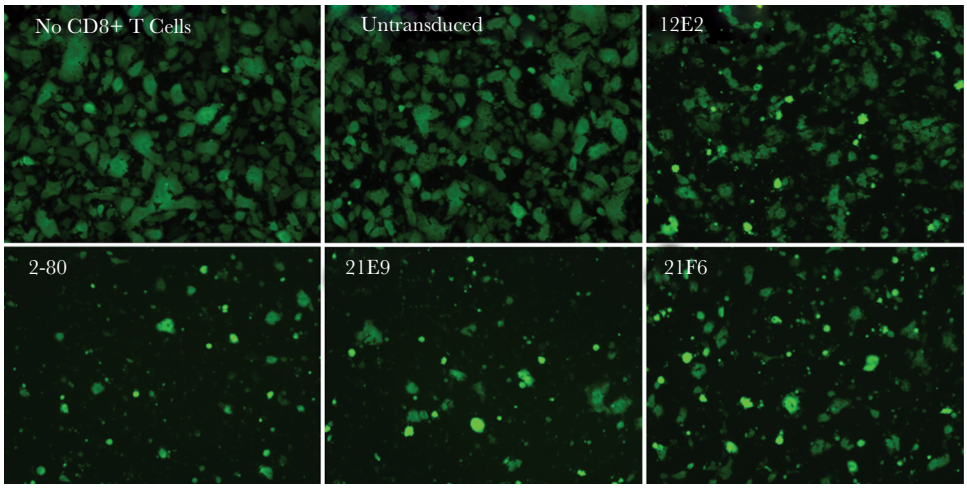

Figure 6. Suppression of cytomegalovirus (CMV) replication in cell culture by chimeric antigen receptor (CAR)–transduced primary $CD8^+$ T cells. ARPE-19 cells were acutely infected with CMV TR (green fluorescent protein [GFP]-expressing) for 4 days, and then co-cultured with no cells, untransduced primary $CD8^+$ T cells, or CAR-transduced $CD8^+$ T cells (at a ratio of 10 $CD8^+$ T cells per target cell), followed by imaging 8 hours later. Similar results were obtained with CMV strain TB40/E (not shown). These results are representative of 3 independent experiments with 3 different $CD8^+$ T-cell donors.

maintains cellular immunity against itself. A strategy being considered for cancer immunotherapy has been to harness this process by transducing CMV-specific T cells isolated from peripheral blood with an anti-CD19 CAR [37], thereby coupling the antitumor response to the anti-CMV response by creating bi-specific T cells recognizing both CMV and CD19. A functional anti-CMV CAR could achieve the same goal without the need to isolate CMV-specific T cells, via coexpression of the anti-CMV CAR with a T-cell receptor or CAR targeting another virus or tumor.

Overall, our data support the 21E9 CAR as a strong candidate for testing as immunotherapeutic intervention or prophylaxis for disseminated CMV infection and/or combination with T-cell receptor or CAR gene immunotherapy for other diseases, given that this specific CAR exhibited CMV-targeted function in terms of triggering to release cytokines, proliferation, infected cell killing, and suppression of viral replication.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH).

Financial support. This work was supported a grant from the AIDS Healthcare Foundation (to O. O. Y.); a seed grant from the University of California, Los Angeles (UCLA) AIDS Institute (to O. O. Y.); the National Institute of Allergy and Infectious Diseases of the NIH (grant number R0I AI103960 to D. J. D.); and the National Cancer Institute of the NIH (grant P30 CA033572 to D. J. D.). D. J. D. is partially supported by the National Cancer Institute (grant numbers R01 CA181045, P01 CA111412, and U19 AI128913). Recombinant human interleukin 2 was provided by the AIDS Reagent and Reference Repository of the NIH. Additional support was provided by the UCLA AIDS Institute and Center for AIDS Research (grant number P30 AI028697); the James B. Pendleton Trust; and the McCarthy Foundation.

Potential conflicts of interest. F. C., F. W., and D. J. D. hold US patent number 10487139 for the antibody sequences used to generate the chimeric antigen receptors. D. J. D. has received grant support and personal fees from Helocyte, Inc. All other authors report no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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