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Novel CD4-Based Bispecific Chimeric Antigen Receptor Designed for Enhanced Anti-HIV Potency and Absence of HIV Entry Receptor Activity

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ABSTRACT

Adoptive transfer of CD8 T cells genetically engineered to express "chimeric antigen receptors" (CARs) represents a potential approach toward an HIV infection "functional cure" whereby durable virologic suppression is sustained after discontinuation of antiretroviral therapy. We describe a novel bispecific CAR in which a CD4 segment is linked to a single-chain variable fragment of the 17b human monoclonal antibody recognizing a highly conserved CD4-induced epitope on gp120 involved in coreceptor binding. We compared a standard CD4 CAR with CD4-17b CARs where the polypeptide linker between the CD4 and 17b moieties is sufficiently long (CD4-35-17b CAR) versus too short (CD4-10-17b) to permit simultaneous binding of the two moieties to a single gp120 subunit. When transduced into a peripheral blood mononuclear cell (PBMC) or T cells thereof, all three CD4-based CARs displayed specific functional activities against HIV-1 Env-expressing target cells, including stimulation of gamma interferon (IFN-γ) release, specific target cell killing, and suppression of HIV-1 pseudovirus production. In assays of spreading infection of PBMCs with genetically diverse HIV-1 primary isolates, the CD4-10-17b CAR displayed enhanced potency compared to the CD4 CAR whereas the CD4-35-17b CAR displayed diminished potency. Importantly, both CD4-17b CARs were devoid of a major undesired activity observed with the CD4 CAR, namely, rendering the transduced $CD8^+$ T cells susceptible to HIV-1 infection. Likely mechanisms for the superior potency of the CD4-10-17b CAR over the CD4-35-17b CAR include the greater potential of the former to engage in the serial antigen binding required for efficient T cell activation and the ability of two CD4-10-17b molecules to simultaneously bind a single gp120 subunit.

IMPORTANCE

HIV research has been energized by prospects for a cure for HIV infection or, at least, for a "functional cure" whereby antiretroviral therapy can be discontinued without virus rebound. This report describes a novel CD4-based "chimeric antigen receptor" (CAR) which, when genetically engineered into T cells, gives them the capability to selectively respond to and kill HIV-infected cells. This CAR displays enhanced features compared to previously described CD4-based CARs, namely, increased potency and avoidance of the undesired rendering of the genetically modified CD8 T cells susceptible to HIV infection. When adoptively transferred back to the individual, the genetically modified T cells will hopefully provide durable killing of infected cells and sustained virus suppression without continued antiretroviral therapy, i.e., a functional cure.

Combination antiretroviral therapy (cART) (1) today offers the promise of near-normal life expectancy for HIV-infected individuals (2), most of whom would previously have succumbed to the lethal consequences of immune system demise. Nevertheless, even under conditions of plasma viral load suppression below the limits of detection, CD4 T-cell recovery is often incomplete. The pathogenic sequelae associated with chronically elevated inflammation (3) and significant drug-related side effects (4), coupled with high costs (5) and the adherence challenges of lifelong cART, have bolstered quests for an HIV cure in the form of either a "sterilizing cure" that completely eradicates all infectious virus from cells or a "functional cure" whereby durable remission is maintained in the absence of continued cART (6–10). Such efforts have been energized by the verified cure of HIV-1 infection in the "Berlin patient," achieved by hematopoietic stem cell transplantation from a CCR5-negative donor (CCR5-Δ32 homozygous) (11, 12). This was followed by the report of long-term viral remission after cART termination in a subset of subjects treated during primary infection (13), as well as by the apparent cure of an HIV-1-infected newborn (the "Mississippi baby") by aggressive cART very shortly after birth (14); however, in the latter case, the subsequent announcement of HIV rebound at 27 months after treat-

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ment cessation has raised questions about the potential for early cART alone to achieve a durable drug-free state of HIV remission (15). Similarly, the viral rebound after cART cessation in two HIV-infected patients who displayed long-term undetectable HIV in peripheral blood and rectal mucosa following allogeneic hematopoietic stem cell transplantation (16), as well as the emergence of CXCR4-using HIV-1 variants in a recently reported attempt to replicate the Berlin patient success (17), highlights the challenges to achieving sustained HIV suppression in the absence of cART.

Targeted cell-killing strategies, originally conceived for the treatment of cancer, suggest exciting potential applications in HIV cure efforts (18, 19). Durable selective killing of cancer cells can be achieved by adoptive transfer of autologous $CD8^+$ T cells genetically modified to express a T cell receptor (TCR) or a chimeric antigen receptor (CAR, also called a T-body) recognizing an intact surface antigen preferentially expressed on the surface of malignant cells (20–27). Early clinical successes with CARs against leukemia and lymphoma (28, 29) have garnered particular recognition (30), and the strategy has been proposed for use against viruses, including HIV (reviewed in references 31, 32, 33, and 34). Indeed, previous reports demonstrated favorable *in vitro* results with CARs targeted by single-chain variable-region antibody (Ab) constructs directed against the gp120 or gp41 subunits of HIV-1 Env (35–38) as well as with CARs employing extracellular CD4 domains (35, 36, 39–42). Clinical studies were conducted more than a decade ago with CD4 CARs (43–45; see also https://clinicaltrials.gov/ct2/show/NCT01013415), and subsequent analyses of peripheral blood samples verified long-term persistence as well as retention of CAR expression and the proliferative potential of adoptively transferred CAR-transduced T cells (CAR-T) (46). However, the antiviral effects were minimal. The CARs employed in these early clinical studies contained the extracellular and transmembrane domains of CD4 linked to the zeta chain intracellular domain of the CD3/T cell receptor complex. CARs containing only this single intracellular signaling motif are now considered "first generation"; improved "second-generation" and "third-generation" CARs containing additional intracellular motifs from costimulatory molecules such as CD28, 4-1BB (CD137), and OX40 (CD134) have shown promise in the cancer field (47) and could potentially enhance clinical efficacy against HIV-infected cells.

The focus of the present report is on design of a novel extracellular targeting domain for an anti-HIV CAR. CD4-based CARs have the inherent major advantage of recognizing the primary receptor-binding site on gp120 that must be retained on all clinical HIV-1 variants. However, it has long been known that, unlike the high soluble CD4 (sCD4) binding affinities observed for gp120 monomeric proteins from clinical HIV-1 isolates, the corresponding functional trimeric Env proteins on the surface of virions and infected cells bind significantly less strongly, contributing to the relatively poor sCD4 neutralization sensitivity of clinical strains (48, 49); this raises questions about the potential potency of a CD4 CAR, as well as about the means by which it might be improved. Moreover, the concern has been raised (37, 39, 40) that CARs employing CD4 as the targeting domain might render the transduced CD8 T cells susceptible to HIV infection, since the CCR5 coreceptor is also expressed abundantly on CD8 T cells in peripheral blood, gut, and lymphoid tissue of HIV-infected persons (50). These issues prompted us to design novel targeting domains for CD4-based CARs with enhanced potency and devoid of the undesired entry receptor activity. We report here a bispecific CD4-based CAR with these properties; moreover, our comparisons of related CAR constructs suggest mechanistic features associated with their different functional efficacies.

MATERIALS AND METHODS

PBMCs and cell lines. Peripheral blood mononuclear cells (PBMCs) (derived from healthy donors visiting the NIH blood bank) were isolated from buffy coats by Ficoll-Hypaque gradient separation and cultured in T cell medium (AIM-V medium [Life Technologies] containing 5% human AB serum [Valley Biomedical] and freshly added recombinant human interleukin-2 [IL-2; Chiron]). Excess PBMCs were cryopreserved until ready for use. For stimulation, PBMCs were washed once in T cell medium without IL-2 and then suspended at a concentration of 2×10^6/ml in T cell medium containing IL-2 and 50 ng/ml of the anti-CD3 monoclonal antibody (MAb) OKT3. A 2-ml volume of the cell suspension was added to each well in a 24-well plate. Cells were cultured in 5% CO_2 at 37°C for 2 days until retroviral transduction.

HEK293T cells (ATCC) and 293GP cells (BD Biosciences) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM glutamine, and 1% sodium pyruvate. SupT1-DCSIGNR cells (51) were grown in suspension in RPMI 1640 medium plus 10% FBS and 2 mM glutamine. Chinese hamster ovary (CHO) cells were cultured in complete DMEM containing 10% FBS, 2 mM glutamine, 1% nonessential amino acids, and 25 mM HEPES buffer. CHO-*env* cells constitutively expressing the Env protein from HIV-1 isolate III_B (52) were cultured in the same medium used for CHO cells, with the addition of 250 nM methotrexate (MTX). HeLa Tet-Off cells (Clontech) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 μg/ml Geneticin before transfection, and HIV Env expression was induced with 0 to 2 μg/ml doxycycline as previously described (53). All cell culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines and PBMCs were maintained in an environment of 37°C and 5% CO_2.

CAR-encoding retroviral constructs. Each of the CARs in this study contained the indicated targeting moiety linked to the hinge, transmembrane, and cytoplasmic signaling domains of CD28 followed by the signaling domain of the CD3 zeta chain; all were cloned into the pMSGV-1 gammaretrovirus vector as previously described (54). The starting construct, which we refer to here as the 139 CAR, contains as the targeting moiety a single-chain variable fragment (scFv) from human MAb 139 that recognizes a glioma-specific variant of the epidermal growth factor (EGF) receptor absent from normal cells (55). Recombinant plasmids containing codon-optimized sequences for the human CD4 (D1D2), CD4-10-17b, CD4-35-17b, and CD4-35-DDY3 targeting motifs were synthesized by Life Technologies; these sequences were excised and subcloned in frame to replace the 139 scFv to make the corresponding CAR-encoding gammaretroviral vectors. The 139 CAR served as a negative control in some experiments; as another negative control, we constructed the DDY3 CAR from the 139 CAR construct by replacing the 139 scFv with an scFv from the DDY3 MAb against the dengue virus E glycoprotein. The CAR protein constructs are shown schematically in Fig. 1, and the corresponding amino acid sequences are presented in Fig. S1 in the supplemental material.

Gammaretrovirus vector production and transduction of T cells. The gammaretroviruses carrying the CAR transgenes were made by transient transfection as described previously (56). Briefly, 293GP cells (BD Biosciences) were cotransfected with retroviral vector plasmid and the plasmid encoding RD114 envelope protein using Lipofectamine 2000 reagent (Life Technologies). Supernatants containing retrovirus were collected at 48 h posttransfection and stored at −80°C.

For transduction, non-tissue culture-treated 6-well plates were coated with 1.5 ml/well RetroNectin (TaKaRa) (10 μg/ml, diluted 1:100 in phosphate-buffered saline [PBS]) at 4°C overnight. The following day, retroviral supernatant was rapidly thawed and diluted 1:1 in serum-free RPMI

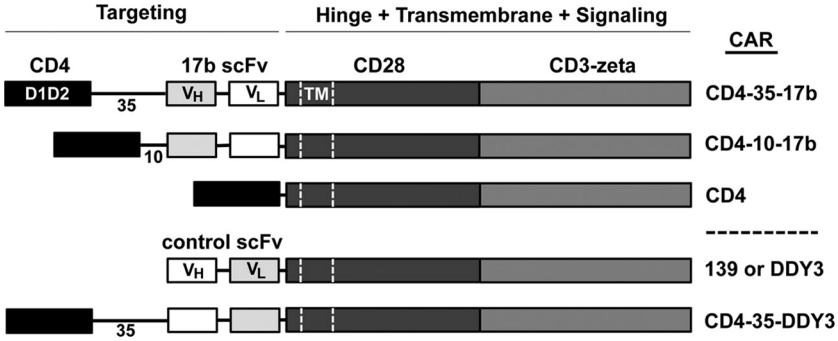

FIG 1 Schematic representation of CAR constructs. The extracellular targeting moieties consist of the indicated components. In each CAR construct, the targeting segment is linked via a tripeptide spacer (AAA) to the identical hinge, transmembrane (TM), and intracellular signaling domain of CD28 followed by the intracellular signaling domain of CD3-zeta. The numbers represent the lengths (in amino acids) of the linker joining the CD4 moiety to the scFv of the indicated CAR. The 139 and DDY3 CARs are controls containing scFvs against irrelevant antigens.

1640 medium. A 4-ml volume of retroviral supernatant (diluted 1:1) was added per well to RetroNectin-coated plates, which were then centrifuged at 2,000 × *g* for 2 h at 32°C. The supernatant was then aspirated from the wells, and 1.5 ml of activated PBMCs was added per well at a density of 0.5×10^6 cells/ml in T cell medium containing IL-2. The plates were then centrifuged for 10 min at 1,000 × *g* and incubated at 37°C overnight. The next day, the transduced PBMCs were given a second round of transduction following the same procedure. The cells were then cultured at 37°C in 5% CO_2 until analysis for CAR expression was performed.

Detection of CARs on transduced T cells. As recommended by BD Biosciences, approximately 1×10^6 cells were washed, suspended in fluorescence-activated cell sorter (FACS) buffer (PBS [pH 7.4] plus 0.4% bovine serum albumin [BSA] and 0.1% sodium azide), and incubated for 30 min at 4°C with the following reagents: for detecting CD4-based CARs, fluorescein isothiocyanate (FITC)-labeled anti-CD3 antibody (clone HIT3a), allophycocyanin (APC)-labeled anti-CD8 antibody (clone SK1), and phycoerythrin (PE)-labeled anti-CD4 antibody (clone RPA-T4); for detecting the 139 CAR, protein L-biotin followed by PE-streptavidin. After washing in FACS buffer was performed, flow cytometry acquisition was performed with a BD FACSCalibur or FACSCanto II system (BD Biosciences), and data analysis was performed with FlowJo (Treestar) (Fig. 2).

Regulated expression of HIV Env in HeLa Tet-Off cells. We employed the Tet-Off system to regulate Env expression in target cells (53). For Western blot analysis of Env expression (Fig. 3A), HeLa Tet-Off cells (ClonTech) were transiently transfected with plasmid pGL4.22-JRFL and incubated for 2 days in the presence of the indicated concentrations of doxycycline. Lysate samples (from 3×10^5 cells) were resolved under reducing conditions on a 4% to 20% gel using SDS-MOPS (SDS-morpholinepropanesulfonic acid) as the running buffer; a lysate from the same number of CHO-*env* cells served as a positive control. After transfer, the Western blot membrane was first subjected to a reaction with a 1:800 dilution of a polyclonal sheep anti-gp120 antibody (D7324; Aalto Bio Reagents) followed by incubation with rabbit anti-sheep IgG conjugated to horseradish peroxidase (HRP; Sigma). SuperSignal West Dura extended-duration substrate (Thermo Scientific) was used for final detection.

For analysis of Env activation of CAR-transduced T cells, HeLa Tet-Off cells were seeded at 1×10^4 per well in a 96-well plate overnight and then transfected with plasmid pGL4.22-JRFL using FugeneHD (Promega) according to the manufacturer's protocol and incubated in the absence or presence of the indicated concentrations of doxycycline. After 2 days, medium was aspirated from each well and replaced with 100 μl fresh medium containing 45×10^3 effector cells. Negative controls were prepared by using untransduced effector cells or effector cells expressing the 139 CAR. The plate was incubated overnight, and the media were diluted 1:5 the following day for a gamma interferon (IFN-γ) enzyme-linked immunosorbent assay (ELISA) (Thermo kit; catalog no. EHIFNG) (Fig. 3B).

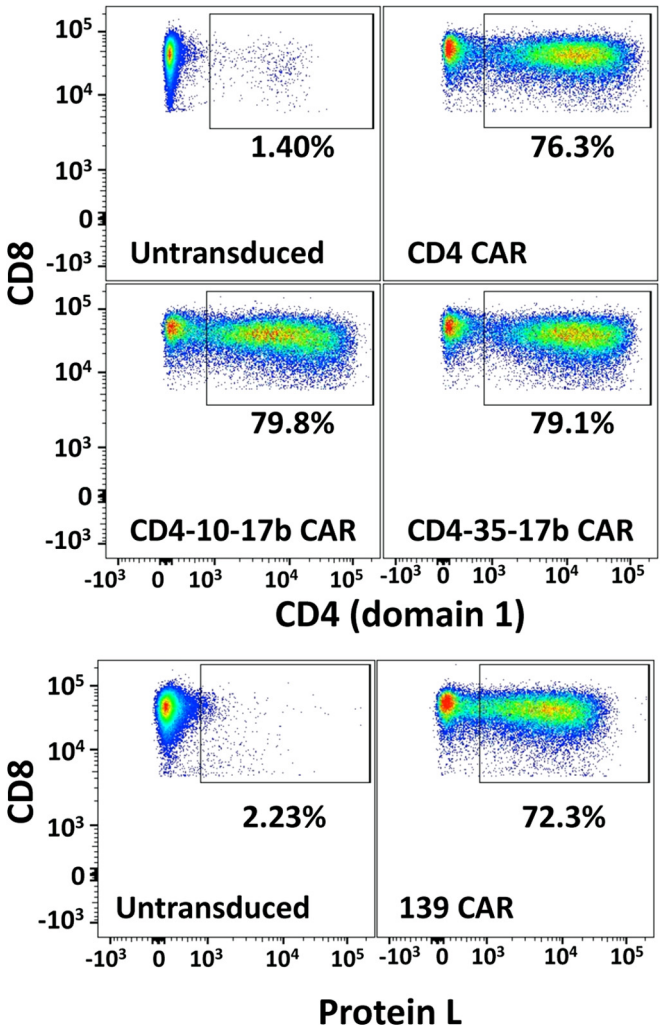

FIG 2 Detection of CARs on transduced T cells. $CD3^+$ cells (FITC-labeled anti-CD3 antibody, clone HIT3a) were gated on $CD8^+$ APC-labeled anti-CD8 antibody (clone SK1). The CD4 CAR (top four panels) was detected with PE-labeled anti-CD4 antibody (clone RPA-T4); the 139 CAR was detected with protein L-biotin followed by PE-streptavidin. Data on the *x* and *y* axes are based on relative fluorescence intensities (arbitrary units).

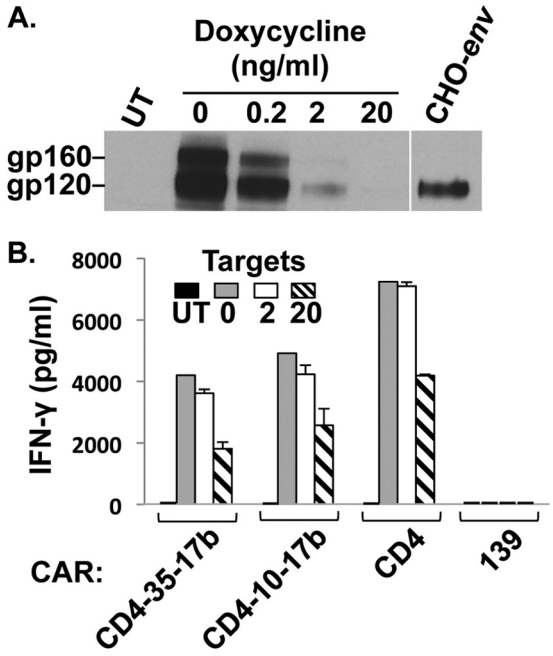

FIG 3 Regulated expression of JR-FL Env and its effect on activation of CAR-transduced PBMCs. (A) Western blot analysis of whole-cell lysates of HeLa Tet-Off cells showing the level of JR-FL Env expression in the presence of various concentrations of doxycycline. A lysate from untransfected cells (UT) served as a negative control; CHO-*env* cells, stably expressing HIV-1 III_B Env, served as a positive control. Only the relevant region of the blot is shown. (B) IFN-γ production in cocultures of Env-expressing HeLa target cells and CAR-transduced effector PBMCs. Three populations of target cells were tested that had been prepared by growing HeLa Tet-Off cells in the presence of 0, 2, or 20 ng/ml of doxycycline. The effector cells expressing the 139 CAR and untransfected (UT; Env-negative) target cells served as negative controls. The error bars represent standard deviations of the results derived from duplicate samples.

Cytotoxicity assay. Direct cytotoxicity assays (Fig. 4A) were performed using a radioisotope-free luminescence-based CytoTox-Glo kit (Promega) following the manufacturer's protocol to measure protease activity released from dead cells. Briefly, triplicate experiments using serial 2-fold dilutions of effector CAR-transduced T cells and a 96-well plate were performed, with the highest cell number being 1.87×10^5 per well in 50 μl. Equal volumes of target cell suspension, containing 1.87×10^4 cells, were added to all wells, and the cells were cocultured for 4 h at 37°C in 5% CO_2. CytoTox-Glo reagent was then added to each well followed by brief mixing on an orbital shaker and then incubation at room temperature for 15 min. Luminescence was measured using a Bio-Tek luminometer. Luminescence values determined from wells containing effector cells alone and target cells alone were combined and subtracted as the background from the values determined from the cocultures. The contents of wells containing target cells alone were mixed with a lysis reagent for 15 min at room temperature, and the resulting luminescence was considered to represent 100% lysis.

CAR-mediated inhibition of HIV-1 pseudovirus production. HEK293T cells were transfected with plasmids to generate the luciferase gene-carrying pseudovirus (Env from QH0692 primary isolate) as described previously (57). At 6 h posttransfection, 1×10^5 transfected cells (target cells) were mixed with CAR-transduced PBMCs (effector cells) at the indicated effector/target ratios. Cells were cocultured at 37°C for 2 days to allow pseudovirus release from target cells into the culture supernatant. Coculture supernatants were then collected, cleared by centrifugation at 2,000 rpm for 5 min, and assayed for infectious pseudovirus content. Pseudovirus infections of SupT1-DCSIGNR cells were performed in 96-well round-bottom plates in which 25 μl of pseudovirus-containing cleared culture supernatant was inoculated onto 2×10^5 cells per well in quadruplicate in the presence of 20 μg/ml of DEAE-dextran. At 48 h postinfection, infected cells were analyzed for luciferase content as previously described (57).

Inhibition of primary spreading HIV-1 infection by CAR-T cells. Frozen autologous PBMCs were rapidly thawed and suspended at a density of 2×10^6/ml in RPMI 1640 medium containing 20% FBS, IL-2, and 50 μg/ml phytohemagglutinin (PHA). A 2-ml volume of cell suspension was added to each well of a 24-well plate and incubated in 5% CO_2 at 37°C overnight. The next day, cells were collected and resuspended with fresh medium without PHA. After 2 to 3 days of culture, cells were resuspended at 5×10^6/ml in RPMI 1640 medium (containing 20% FBS and IL-2) and transferred to a T25 flask. A 1-ml volume of primary HIV-1 isolate stock (p24 titer of 50 to 150 ng/ml) was then added to each flask, and the cells were incubated at 37°C in 5% CO_2 overnight. Infected cells were spun down at $300 \times g$ for 10 min. After removal of the supernatant, cells were washed 3 times using 20 ml of medium per wash and then resuspended in complete medium (RPMI 1640, 20% FBS, and IL-2) at a density of 1.5×10^6 cells per ml in 96-well round bottom plates. Subsequently, 100 μl of infected PBMCs (targets [T]) was mixed in duplicate with 100 μl of serially diluted CAR-transduced T cells (effectors [generated from the same donor]) to obtain the various effector/target ratios indicated in the figures. Cocultures were performed at 37°C in 5% CO_2 for 8 days, at which time supernatants were collected and assayed for p24 content by ELISA

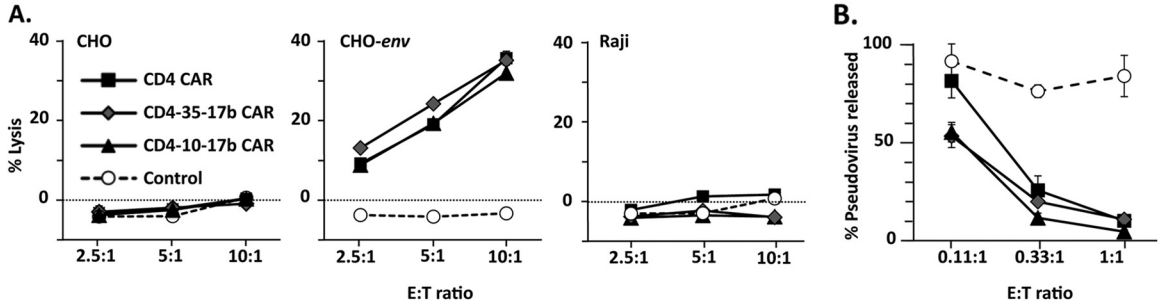

FIG 4 Targeted killing of Env-expressing cells by CD4-based CARs. (A) Direct killing of target cell lines. Direct cytotoxicity assays were performed using a CytoTox-Glo kit and, as targets (T), CHO, the CHO-*env* stable transfectant, and Raji cells. Cocultures were performed for 4 h with T cells expressing the indicated CARs (effector cells [E]); the 139 CAR served as a negative control. Error bars (representing negligible values) indicate standard deviations of the results from triplicate samples. (B) Suppression of HIV-1 pseudovirus production from producer target cells. HEK293T target cells (T) transfected with plasmids directing production of HIV-1 infectious pseudovirus particles (Env from the QH0692 primary isolate) were cocultured with CAR-transduced PBMCs (effector cells [E]) at the indicated effector/target (E:T) ratios. The amounts of infectious pseudovirus released into the supernatants were quantified by incubation with SupT1-DCSIGNR cells. Pseudovirus production in the absence of effector cells was defined as 100%. Untransduced effector cells served as a negative control. Error bars indicate standard deviations of the results from quadruplicate samples. For each panel, the number of target cells is defined as 1.

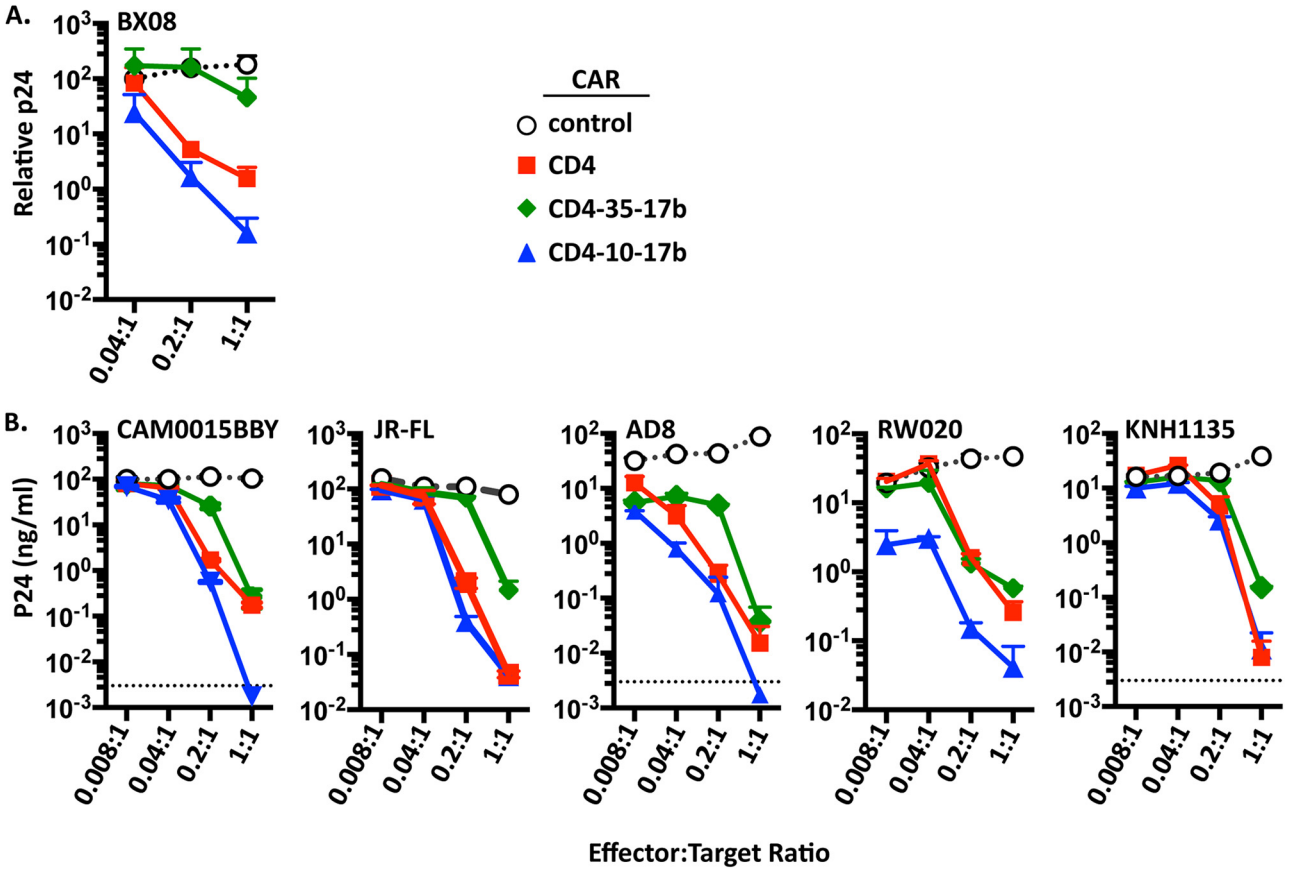

FIG 5 Suppression of spreading HIV-1 infection in PBMCs by T cells expressing various CD4-based CARs. (A) BX08 (clade B) data show the average level of infection of PBMCs from multiple donors, normalized to an infection obtained with effectors expressing the 139 CAR at a 0.04:1 E:T cell ratio (defined as 100). (B) CD4-based CARs were tested against the indicated HIV-1 isolates: CAM0015BBY, clade CRF02_AG; JR-FL and AD8, clade B; and RW020 and KNH1135, clade A. The 139 CAR was used as a negative control. In cases where points fell below detectable levels, horizontal dotted lines are shown to indicate the limits of detection.

(PerkinElmer). For comparisons of the relative CAR activities using cells from different donors (Fig. 5A), autologous PBMCs from 3 different healthy donors were used to generate HIV-1 (BX08 isolate)-infected target and CAR-transduced effector cells. For analysis of relative CAR activities using different HIV-1 isolates (Fig. 5B), single-donor autologous PBMCs were used to generate target and effector cells for coculture.

Epitope exposure on pseudovirus captured by HOS cells expressing CD4-based CARs. Human osteosarcoma (HOS) cells were transduced to express CD4-based or control CARs using the method described for human PBMC transduction; untransduced cells provided a negative control. CD4 CAR expression on HOS cells was measured with PE-labeled anti-CD4 (clone RPA-T4) following the method described for CAR detection on transduced T cells; equivalent expression levels were observed for all the CD4-based CARs (not shown). A total of 1×10^6 CD4-based CAR-expressing HOS cells were washed and suspended in 100 μl containing HIV-1 pseudovirus (Ba-L) for 30 min at 37°C; an equivalent incubation in the absence of pseudovirus served as a negative control. Cells were then washed three times with FACS buffer and incubated with either 2G12 or 17b human MAb for 30 min at 4°C. Cells were washed again three times and incubated with a goat anti-human (Hu)-IgG Alexa Fluor 488 conjugate. Flow cytometry acquisition was performed with a BD FACSCalibur system (BD Biosciences), and analysis was performed with FlowJo (Treestar) (Fig. 6).

Susceptibility of HOS-CCR5 cells expressing CD4-based CARs to pseudovirus infection. HOS-CCR5 cells were transduced with retroviruses encoding the various CD4-based CARs; their surface expression data were confirmed to be comparable by flow cytometry (>70% CAR positive). Equal numbers (10^4) of each transduced cell population were seeded overnight in quadruplicate in 96-well plates followed by incubation with 4-fold serial dilutions of Ba-L pseudovirus in the presence of 20 μg/ml DEAE-dextran. After 48 h, the media were aspirated and the cell lysates were assayed for luciferase activity as described previously (57) (Fig. 7A).

Susceptibility of CAR-expressing CD8 T cells to HIV-1 infection. CD8 T cells were isolated from PBMCs by magnet-activated cell sorting (MACS) negative selection (Miltenyi Biotec). The cells were activated and transduced with CAR retroviruses as described above. Cell-free HIV-1 (Ba-L isolate) was added to the cultures, and the cells were analyzed for infection by intracellular p24 staining (Ab clone KC57) 3 days later (Fig. 7B).

RESULTS

Design of CAR constructs. We previously reported a bispecific HIV-1-neutralizing soluble protein designated sCD4-17b containing the first two extracellular domains (D1D2) of human CD4 attached by a flexible polypeptide linker to an scFv of the human MAb 17b directed against a highly conserved CD4-induced epitope in the bridging sheet of HIV-1 gp120, involved in binding to the coreceptor (58, 59). The rationale was based on the ability of the sCD4 moiety to bind to native Env on virions and induce exposure and formation of the bridging sheet, thereby enabling the 17b scFv moiety to bind and neutralize infectivity. The breadth of activity against genetically diverse HIV-1 isolates derived from

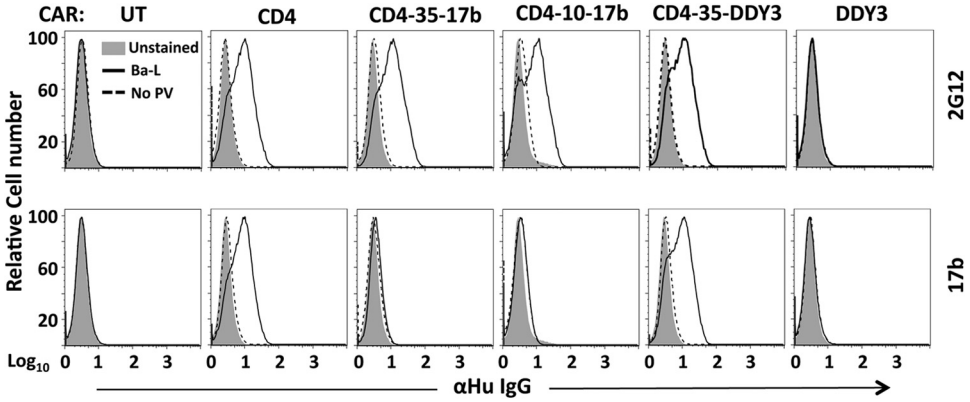

FIG 6 Accessibility of the 17b moiety on HIV-1 pseudovirions captured by HOS (CD4-negative) cells expressing the indicated CARs. Flow cytometry was performed to detect binding of anti-gp120 MAbs on captured virions; MAb 2G12 binding served as a positive control for virion capture; MAb 17b revealed accessibility to the CD4-induced 17b epitope. HOS cells without pseudovirions served as a control for staining specificity. Untransfected cells (UT) served as an additional negative control. Data on the *x* axes are based on relative fluorescence intensities (arbitrary units).

the high conservation of the CD4 and coreceptor binding sites; the strong neutralization potency was shown to be dependent on the presence of a linker of sufficient length (e.g., 35 or 40 amino acid [aa] residues) to enable simultaneous binding of both the sCD4 and 17b scFv moieties to a single gp120 subunit as determined from the atomic coordinates of the first reported X-ray crystallographic structures of a gp120 core complexed to CD4 D1D2 and the 17b Fab (60, 61). A sCD4-17b construct with a linker too short to enable simultaneous binding did not display potent neutralizing activity, as found for linkers of 5 aa (59) and 20 aa (L. A. Lagenaur and E. A. Berger, unpublished data). We considered the potential value of the bispecific sCD4-17b moiety as a targeting component for a CAR and designed variant CARs with a linker sufficiently long (CD4-35-17b CAR; 35 aa residues) or too short (CD4-10-17b CAR; 10 aa residues) for simultaneous binding of the two moieties to a single gp120 subunit. We performed comparisons with a "standard" CD4 CAR containing only the CD4 domains (D1D2) as well as with several control CARs containing an scFv (139 or DDY3) against an irrelevant antigen absent from the experimental systems examined, alone or linked to CD4 domains. In all cases, the targeting domains were linked to the same downstream second-generation signaling motifs consisting of continuous portions of CD28 (short extracellular hinge, transmembrane domain, and intracellular signaling domain) followed by the intracellular signaling domain of the CD3-zeta chain. These constructs, shown schematically in Fig. 1, were engineered into mouse stem cell virus-based splice-gag gammaretroviral vector MSGV-1.

CAR expression on transduced PBMC-derived T cells. PBMCs from a healthy donor were stimulated for 2 days with IL-2 and the anti-CD3 MAb OKT3; the resulting T cell-enriched population was then transduced (for 2 cycles) with pMSGV-1 gammaretroviral vectors encoding the designated CARs. Samples were analyzed by flow cytometry at 5 days after the second transduction. As shown in Fig. 2, transduction efficiencies were high for all CD4-based CARs as well as for the control 139 CAR (55). In multiple experiments with PBMCs from different donors, the transduction efficiencies typically were >70% and were similar for all CARs used in a given experiment.

HIV-1 Env stimulation of IFN-γ release from CAR-transduced T cells. Target cells transiently expressing various levels of HIV-1 Env (JR-FL isolate) were generated by transfecting HeLa Tet-Off cells with plasmid pGL4.22-JRFL encoding Env (53) and by culturing for 48 h in the presence of the indicated levels of doxycycline (a tetracycline analog that represses Env transcription by binding to the tetracycline transactivator and preventing its interaction with the upstream tetracycline response elements); untransfected cells served as negative controls. Western blot analyses (Fig. 3A) indicated high-level Env expression in the absence of doxycycline and marked suppression in response to increasing doxycycline concentrations (0.2 to 20 ng/ml); at much longer (overnight) exposure, only very faint gp120 bands were observed, with equivalent intensities for 20 and 2,000 ng/ml doxycycline (not shown), presumably reflecting "leakiness" of the Tet-Off system. Figure 3B shows that transduced effector T cells expressing all three CD4-based CARs were stimulated to secrete IFN-γ in specific response to coculture with Env-expressing target cells but not with control untransfected target cells lacking Env; further specificity was demonstrated by the absence of IFN-γ secretion with control effector cells expressing the 139 CAR. The potent sensitivity of the specific effector cell response is illustrated by the relatively high level of IFN-γ secreted (Fig. 3B; ~40% to ~60% of maximum), with target cells expressing extremely barely detectable Env levels (Fig. 3A) under conditions of suppression by high-dose (20 ng/ml) doxycycline.

Specific killing of Env-expressing cells by CD4-based CARs. We tested the CAR-transduced T cells for specific direct cytotoxic activity against Env-expressing target cells using a CytoTox-Glo kit (Fig. 4A). All three CD4-based CARs displayed robust cytotoxicity against the CHO-*env* cell line constitutively expressing Env from the T cell line-adapted IIIB isolate, at comparable efficacies over the indicated range of effector/target ratios. Specificity was confirmed by the absence of killing of the Env-negative parental CHO cells and by the lack of activity of effector cells transduced with the control 139 CAR. Importantly, the CD4-based CARs displayed no cytotoxicity against Raji cells, which express very high levels of major histocompatibility complex (MHC) class II (MHC-II) molecules (62), the natural binding partner for CD4; this finding parallels previous demonstrations of the absence of activity of CD4 CARs against MHC-II (35, 39) and presumably reflects the weak affinity of CD4 for MHC class II (63).

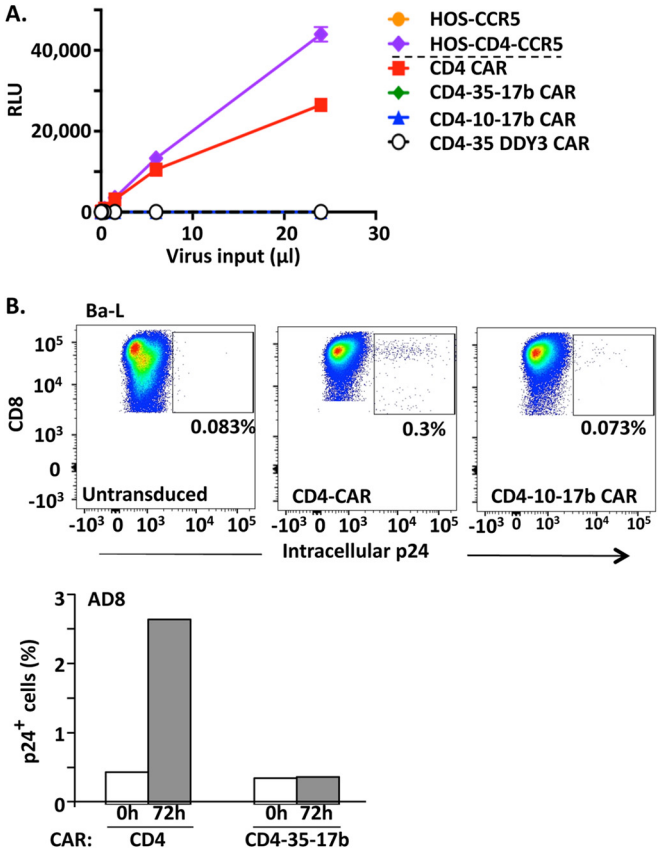

FIG 7 HIV-1 infection susceptibility of cells expressing CD4-based CAR variants. (A) Pseudovirus infectivity (Env from the Ba-L isolate) of HOS-CCR5 cells expressing CD4-based CARs. The error bars represent standard deviations of the results from quadruplicate samples. The extremely low relative light unit (RLU) values for both CD4-17b CARs and the CD4-35-DDY3 CAR were indistinguishable from background values as defined by value determined from the HOS-CCR5 negative-control cells (approximately 15 RLU). (B) Susceptibility of $CD8^+$ T cells expressing the different CD4-based CARs to infection by cell-free HIV-1. $CD8^+$ T cells obtained by negative selection and expressing the indicated CARs were challenged with cell-free HIV-1. Infection was analyzed by flow cytometry and staining for intracellular p24. (Upper panel) The $CD8^+$ T cells were challenged with the Ba-L isolate and analyzed 3 days later. Untransduced (CAR-negative) cells served as a negative control. The percent value in each dot blot indicates the p24-positive population. Data on the *x* axes are based on relative fluorescence intensities (arbitrary units). (Lower panel) CAR-expressing $CD8^+$ T cells were challenged with the AD8 isolate. Infection was assessed by the increase in intracellular p24 staining at h 0 (background) compared to h 72.

As an additional test of specific CAR-mediated cytotoxic activity, we analyzed suppression of release of infectious pseudovirus particles from 293T producer cells. This system enabled analysis of activity against the recombinant Env of choice. As shown in Fig. 4B with Env from the QH0692.42 primary isolate, all three CD4-based CARs strongly suppressed pseudovirus production over the indicated effector/target range, whereas the 139 control CAR had no effect.

Suppression of spreading HIV-1 infection in PBMCs. The CARs were tested against spreading HIV-1 infection in PBMCs, initiated by the input of cell-free HIV-1 and assay of supernatant p24 at day 8 postinfection (Fig. 5). In each assay, PBMCs from the same healthy donor were used as target cells for infection and to generate CAR-transduced T cells. Figure 5A summarizes data from experiments in which PBMCs from three different healthy donors were infected with the BX08 primary isolate (R5) and cocultured with T cells expressing the indicated CARs at various E:T ratios; p24 levels are expressed relative to cocultures with the control 139 CAR at the lowest E:T ratio (defined as 100). The small error bars indicate that comparable patterns were observed with PBMCs from all three donors. The results demonstrate various levels of suppressive activities by the three CD4-based CARs (CD4-10-17b > CD4 > CD4-35-17b); the control 139 CAR was ineffective. These analyses were extended to multiple HIV-1 primary isolates (all R5) representing different genetic subtypes, each tested with PBMC targets and T cell effectors from a single healthy donor (Fig. 5B). The CD4-based CARs suppressed infection by all HIV-1 isolates tested, with relative potencies similar to those described above (i.e., CD4-10-17b ≥ CD4 ≥ CD4-35-17b); again, the 139 control CAR did not suppress infection. The extreme potency of the CD4-10-17b CAR against all isolates (5- to 100-fold at a 0.2:1 ratio; 100- to 10,000-fold at a 1:1 ratio) is particularly notable.

Accessibility of 17b epitope upon Env engagement of CD4-based CARs. We predicted that binding of HIV-1 pseudovirus particles to cells expressing the CD4 CAR should induce exposure of the 17b epitope, which would be detectable by flow cytometry using the 17b MAb. In contrast, with cells expressing the CD4-35-17b CAR, binding of the CD4 moiety to a gp120 subunit should induce exposure of the 17b epitope, enabling the 17b scFv moiety of the same CAR molecule to bind; thus, the 17b epitope would not be accessible to exogenously added 17b MAb. With the CD4-10-17b CAR, the linker is too short to enable simultaneous binding of the CD4 and 17b moieties to the same gp120 subunit; therefore, binding of the CD4 moiety should expose the 17b epitope to exogenously added 17b MAb, unless the 17b moiety from another CAR molecule were to occupy the epitope. We analyzed this issue by measuring exposure of the 17b epitope on HIV-1 pseudovirus particles captured by CD4-based CARs expressed on the surface of HOS cells. Flow cytometry analyses (Fig. 6, upper panels) clearly demonstrated the capture of HIV pseudovirions by cells expressing the various CD4-based CARs, as revealed with MAb 2G12 directed against a glycan epitope on the gp120 outer domain; bound pseudovirions were not detected on control cells (untransduced cells or cells expressing the negative-control DDY3 CAR). This result indicates that pseudovirion capture is mediated by specific binding of pseudovirion Env to the CD4 moiety of the various CARs. A very different pattern was observed with MAb 17b, directed against the CD4-induced epitope on gp120 (Fig. 6, lower panels). As expected, the epitope was readily detected on cells expressing the CD4 CAR but minimally on cells expressing the CD4-35-17b CAR, consistent with the expected bispecific binding of the 17b moiety following epitope exposure induced by the CD4 moiety; this interpretation was supported by the prominent binding of the 17b MAb to pseudovirions captured by the CD4-35-DDY3 CAR. Interestingly, minimal binding of 17b MAb was also observed with the CD4-10-17b CAR, despite the inability of the short linker to enable simultaneous binding of the CD4 and 17b moieties of a single CAR molecule to the same gp120 subunit; we interpret this result to reflect binding of the 17b moiety of a different CAR molecule to the CD4-induced epitope.

Undesirable potential for CD4-based CARs to function as HIV-1 entry/infection receptors. As noted in the introduction, a major concern with respect to a CD4 CAR is that it might function

as an HIV entry receptor and render transduced CD8 T cells susceptible to infection, since these cells also express CCR5. As one test of this possibility, we expressed the CARs in the HOS-CCR5 stable transfectant cell line and tested infection by HIV-1 pseudotyped particles. As shown in Fig. 7A, expression of the CD4 CAR rendered the HOS-CCR5 cells highly permissive to infection, comparable to the level observed for the HOS-CD4-CCR5 stable transfectant cell line; in contrast, no infection was observed with the CD4-10-17b or CD4-35-17b CAR. To probe the mechanism by which the 17b scFv prevents the HIV entry/infection receptor activity of the CD4 moiety, we examined a CAR containing the CD4 moiety linked to an scFv against an irrelevant epitope (CD4-35-DDY3 CAR). Figure 7A shows that this CAR was also devoid of HIV entry/receptor activity. Thus, the scFv need not bind to gp120 in order to prevent the undesired entry receptor activity mediated by the CD4 moiety of the CAR.

As a second test, we directly exposed CAR-transduced purified $CD8^+$ T cells to cell-free HIV-1; 72 h later, the cells were fixed, permeabilized, and stained for intracellular HIV-1 p24. The flow cytometry analyses represented in Fig. 7B demonstrate that the CD4 CAR rendered CD8 cells susceptible to HIV-1 infection, as shown for both the Ba-L (upper panel) and AD8 (lower panel) isolates; this undesirable effect was not observed with the CD4-10-17b or CD4-35-17b CAR (or with untransfected control cells).

DISCUSSION

Adoptive immunotherapy with T cells genetically modified for targeted cytotoxic cell killing holds considerable promise for treatment of chronic viral infections (32–34). The CD4-10-17b bispecific CAR targeting domain described here displays important enhanced features compared to the monospecific CD4 moiety used in previous anti-HIV CARs, namely, enhanced suppressive potency for several primary HIV-1 isolates and absence of the undesired entry/infection receptor activity. The importance of the latter benefit is highlighted by the close intercellular contacts between the effector $CD8^+$ and infected $CD4^+$ T cells at the cytotoxic T lymphocyte (CTL) immunological synapse (64), which would likely foster highly efficient cell-cell infection (65) of $CD8^+$ T cells expressing a transgene that can function as an entry receptor. Our approach to preventing infection susceptibility is simpler and more direct than approaches requiring coexpression of an anti-HIV transgene (see, e.g., reference 42). The CD4-10-17b CAR is also favorable for minimizing virus escape potential, a critical feature for durable control of infection. All clinical HIV-1 variants are CD4 dependent, and the 17b epitope is highly conserved within the coreceptor binding domain of gp120. While 17b escape variants can potentially arise, the CAR would still provide suppressive activity via the CD4 moiety, although perhaps with less potency. Also noteworthy is the fact that both the CD4 and 17b scFv moieties are of human origin; only the linkers (minimally immunogenic Gly_4Ser repeats) and the novel junctions represent nonhuman sequences. Thus, the CD4-10-17b CAR is minimally prone to induction of the humoral and cellular responses that have been reported in clinical trials with CARs derived from murine MAbs (66, 67); however, an anti-idiotypic immune response to the variable region of the scFv is still possible (66).

The present findings reveal an intriguing contrast between the potencies of the sCD4-17b variants as soluble proteins for HIV neutralization and those of membrane-associated targeting motifs for HIV-suppressive CARs. Our previous studies revealed that soluble sCD4-17b constructs with linker lengths sufficiently long to enable simultaneous binding of the two moieties to a single gp120 subunit displayed HIV-1 pseudovirus neutralization potency greatly superior to that seen with equivalent bispecific constructs with linkers too short for simultaneous binding or to that seen with monospecific sCD4 (59). These results presumably reflected the enhanced binding affinity associated with the bispecific interaction. The CAR suppression data presented here (Fig. 5) show precisely the opposite pattern; i.e., the CD4-35-17b CAR (with a linker sufficiently long to enable bispecific binding) was consistently less potent than the CD4-10-17b CAR (with a linker too short for bispecific binding). These findings are consistent with the requirement for serial TCR/peptide-MHC engagement for optimal functionality, as originally proposed (68) and subsequently expanded to include influences of cytoskeletal interactions that modulate cellular dynamics at the immunological synapse (69, 70). Current models emphasize the importance of rapid dissociation and reformation of TCR-peptide/MHC bonds; moreover, they highlight the reduced sensitivity to low antigen densities associated with unnaturally high binding affinities. Accordingly, the bispecific interaction of the CD4-35-17b CAR would be expected to impair the rapid dissociation step, thereby reducing its potency relative to the CD4-10-17b CAR; as predicted, this distinction was found to be more evident for target cell killing in assays where Env surface densities were low (e.g., spreading HIV/PBMC infection; Fig. 5) than where they were high (e.g., Env-transfected cells; Fig. 4). These results suggest that the advantages of serial engagement initially proposed for TCR-peptide/MHC interaction can also apply to CARs, as has been proposed by others for CARs against cancer (71, 72, 73) as well as HIV (37).

The CD4-10-17b CAR displayed potency superior to that of the CD4 CAR for several of the isolates examined in the spreading HIV/PBMC infection assay (Fig. 5). Though the linker in the sCD4-10-17b CAR was too short to permit simultaneous binding of the two moieties to a single gp120 subunit, we found that the 17b epitopes were efficiently occupied when pseudovirions were captured by this bispecific CAR but not by the CD4 CAR (Fig. 6). This indicates that the 17b epitope induced on a gp120 subunit by binding to the CD4 moiety of one CAR must be occupied by the 17b moiety of a separate CAR molecule. Thus, a potential mechanistic explanation for the enhanced potency of the CD4-10-17b CAR over the CD4 CAR (and the CD4-35-17b CAR) is that in the former case, a single gp120 subunit engages (and presumably activates) two distinct CAR molecules.

In summary, the CD4-10-17b CAR displays special features that make it a particularly suitable candidate for genetic modification of T cells from HIV-1-infected individuals. The broad reactivity to genetically diverse HIV-1 isolates, high potency of virus suppression, minimal immunogenic potential, and freedom from HIV entry receptor activity all speak to the value of this CAR design. Further investigations will help assess its clinical potential in the quest for a functional cure of HIV infection.

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