

HIV-1-Specific Chimeric Antigen Receptors Based on Broadly Neutralizing Antibodies

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ABSTRACT

Although the use of chimeric antigen receptors (CARs) based on single-chain antibodies for gene immunotherapy of cancers is increasing due to promising recent results, the earliest CAR therapeutic trials were done for HIV-1 infection in the late 1990s. This approach utilized a CAR based on human CD4 as a binding domain and was abandoned for a lack of efficacy. The growing number of HIV-1 broadly neutralizing antibodies (BNABs) offers the opportunity to generate novel CARs that may be more active and revisit this modality for HIV-1 immunotherapy. We used sequences from seven well-defined BNABs varying in binding sites and generated single-chain-antibody-based CARs. These CARs included 10E8, 3BNC117, PG9, PGT126, PGT128, VRC01, and X5. Each novel CAR exhibited conformationally relevant expression on the surface of transduced cells, mediated specific proliferation and killing in response to HIV-1-infected cells, and conferred potent antiviral activity (reduction of viral replication in log₁₀ units) to transduced CD8⁺ T lymphocytes. The antiviral activity of these CARs was reproducible but varied according to the strain of virus. These findings indicated that BNABs are excellent candidates for developing novel CARs to consider for the immunotherapeutic treatment of HIV-1.

IMPORTANCE

While chimeric antigen receptors (CARs) using single-chain antibodies as binding domains are growing in popularity for gene immunotherapy of cancers, the earliest human trials of CARs were done for HIV-1 infection. However, those trials failed, and the approach was abandoned for HIV-1. The only tested CAR against HIV-1 was based on the use of CD4 as the binding domain. The growing availability of HIV-1 broadly neutralizing antibodies (BNABs) affords the opportunity to revisit gene immunotherapy for HIV-1 using novel CARs based on single-chain antibodies. Here we construct and test a panel of seven novel CARs based on diverse BNAB types and show that all these CARs are functional against HIV-1.

Recent years have seen a surge in immunotherapeutic approaches for treating malignancy, including numerous promising human trials of chimeric antigen receptor (CAR) gene therapy to generate tumor-specific T cells, based on the importance of CD8⁺ T lymphocytes (CTLs) in tumor surveillance and malignant cell clearance through cytotoxicity. The general approach has been to identify monoclonal antibodies that bind a tumor cell surface antigen and use a single-chain version of the antibody as an artificial T cell receptor by genetic fusion to the CD3 ζ chain signaling domain. As opposed to native T cell receptors (TCRs), CARs have the advantage of being major histocompatibility complex (MHC) unrestricted and therefore broadly applicable across human individuals and are also unaffected by tumor cell immune evasion through MHC downregulation.

Notably, one of the earliest tested clinical applications of CARs was for the treatment of HIV-1 infection. In 1994, Roberts et al. designed two virus-specific CARs using CD4 or a single-chain antibody as the binding domain for recombinant gp120 on the surface of cells (1), and these CARs were shown subsequently to have the direct capacity to kill HIV-1-infected cells and suppress viral replication at levels similar to those of HIV-1-specific CTL clones isolated from infected persons (2). Based on these data, the CD4-based CAR, consisting of the CD4 extracellular and transmembrane domains fused to the CD3 ζ intracellular signaling domain (CD4⁺ ζ), was advanced to clinical trials starting in the late 1990s, using retroviral transduction of autologous peripheral blood T lymphocytes and reinfusion. Unfortunately, this effort

was abandoned after these trials showed safety but no clear benefits: one study with viremic subjects showed no reduction in viremia, although there appeared to be decreased rectal tissue virus burden (3), while another study of antiretroviral drug-treated subjects with baseline undetectable viremia not surprisingly showed no change in the persisting blood viral reservoir in the form of proviral DNA (4). Follow-up of these studies after more than a decade showed low-level persistence of transduced cells without evidence of malignancy (5).

Several factors may have contributed to failure in these trials. The Moloney-based retroviral vector was relatively inefficient, and peripheral blood T cells were massively expanded *ex vivo* using supraphysiological levels of interleukin-2, likely contributing to the rapid loss of CAR expression and death of reinfused cells. The CAR itself may have been problematic: the CD4 domain may have allowed HIV-1 infection of transduced CTLs, or there could

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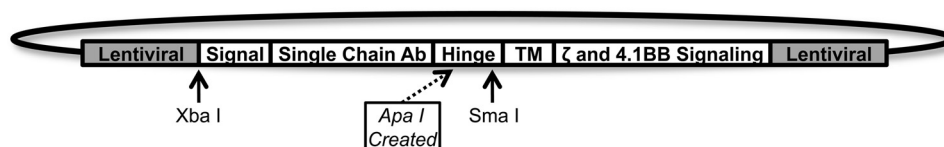


FIG 1 CAR structure and construction strategy. The parental vector contained a CAR based on a single-chain antibody. This vector was modified with a silent mutation to create an *Apa*I site (GGCCCT→GGGCCC; mutated nucleotides are underlined) in the hinge region of the CAR gene (within a sequence-confirmed *Xba*I-*Sma*I intermediate plasmid vector). New CAR genes were generated by the synthesis of single-chain antibody genes that were substituted into this vector via *Xba*I-*Apa*I restriction fragments.

have been selection for viral escape through reduced CD4 binding, which can vary greatly between different HIV-1 envelopes (6).

The identification of a growing number of broadly neutralizing antibodies (BNABs) against HIV-1 offers the possibility of creating new HIV-1-specific CARs with improved properties. These BNABs have high affinity and excellent reactivity against various HIV-1 strains, which could translate to efficient CARs with broad coverage of HIV-1 variation. Here we report the generation and testing of CARs based on seven BNABs that recognize diverse epitopes on the HIV-1 envelope.

MATERIALS AND METHODS

Cells and media. The immortalized HIV-1-permissive CD4-expressing cell lines T1 (7) and T2 (8) and Jurkat cells were maintained as previously described (9–11) in complete medium (R10) consisting of RPMI 1640 (Lonza, Allendale, NJ) supplemented with 2 mM L-glutamine (Mediatech, Manassas, VA), 100 U/ml penicillin (Mediatech, Manassas, VA), 100 U/ml streptomycin (Mediatech, Manassas, VA), 10 mM HEPES (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO). HEK 293T cells were maintained in Dulbecco's modified essential medium supplemented with L-glutamine, penicillin, streptomycin, and FBS as described above and previously (12). Primary CD8⁺ T lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy HIV-1-uninfected donors by using anti-CD8 antibody-coated magnetic beads according to the manufacturer's directions (MACS column separation kit; Miltenyi, San Diego, CA) and then cultured for 5 days in R10 supplemented with 50 U/ml recombinant human interleukin-2 (NIH AIDS Reagent Repository) (R10-50) in the presence of the anti-CD3 antibody 12F6 (13), yielding a purity of >99% CD3⁺/CD8⁺ cells by flow cytometry. All experiments were confirmed with cells from multiple donors and showed no significant donor-specific differences.

Construction of CAR vectors. The backbone for novel CAR constructions was the pTRPE-cMET-BBζ CAR plasmid, provided as a generous gift from Carl June. This lentiviral expression vector (Fig. 1) contained the gene for the CAR with a single-chain antibody against hepatocyte growth factor receptor (cMET) fused to the human IgG4 hinge sequence, the human CD8 transmembrane sequence, and cytoplasmic domains of human 4-1BB (CD137) and the human CD3 complex ζ chain (CD247). This vector was modified by creating a novel *Apa*I restriction site via a silent mutation in the hinge sequence (Fig. 1). This was accomplished by subcloning the *Xba*I-*Sma*I restriction fragment into pUC19, in which the mutation was created by point mutagenesis (QuikChange kit; Invitrogen, Carlsbad, CA). After sequencing of the entire fragment to ensure no PCR-induced errors, this restriction fragment was ligated into the parental vector. Single-chain antibody sequences of heavy chain-linker-light chain (Table 1) were synthesized as codon-optimized genes preceded by the signal sequence for granulocyte-macrophage colony-stimulating factor (MLLVTSLLLCELPHAPALLIP) and followed by the beginning of the hinge region, flanked by *Xba*I and *Apa*I restriction sites, allowing ligation into the parental vector after restriction digestion. The BNAB sequences used for this approach included 10E8 (14), 3BNC117 (15), PG9 (16), PGT126 (17), PGT128 (17), VRC01 (18), and X5 (19).

Production of lentiviral vectors and transduction of cells. Lentivirus was produced by cotransfection of HEK 293T cells with a CAR lentiviral vector construct plasmid (10 μg) in conjunction with packaging and pseudotyping vectors, including the lentiviral packaging plasmid pCMVDR8.2DVPR (7 μg) and the vesicular stomatitis virus envelope glycoprotein G expression vector pHCMVG (3 μg), using BioT transfection reagent according to the manufacturer's protocol (Bioland, Paramount, CA) with 5×10^6 HEK 293T cells that had been seeded into a T75 tissue culture flask 24 h previously. Supernatants were obtained 24 and 48 h after transfection, passed through a 0.45-μm filter, and concentrated by ultracentrifugation (26,000 rpm for 90 min at 4°C) (SW28 rotor; Beckman Coulter, Fullerton, CA). Aliquots containing ~50 ng HIV-1 p24 antigen in 50 μl were frozen at –80°C until use.

For transduction of primary CD8⁺ T lymphocytes, polystyrene 6-well plates (BD Biosciences, San Jose, CA) were coated with RetroNectin according to the manufacturer's instructions (TaKaRa, Mountain View, CA). An aliquot of the lentiviral vector was diluted to 500 μl in R10 and placed into a precoated well, followed by centrifugation at $2,000 \times g$ for 2 h at 32°C (Sorvall Legend RT; Thermo Fisher Scientific, Grand Island, NY). After aspiration of the medium, 10^6 recently stimulated CD8⁺ T lymphocytes were added per well in a total volume of 2 ml R10-50. After overnight incubation in a tissue culture incubator, the cells were transferred to fresh R10-50 and cultured for about 7 days before assessment of transduction efficiency (see below).

For Jurkat cells, 10^6 cells in log-phase growth were incubated with the lentiviral vector for 4 h with intermittent shaking, washed, and resuspended in fresh R10.

Western blotting for CD3 ζ. The cell lysate from 2×10^6 transduced cells was prepared by lysing the cells in lysis buffer (0.5% NP-40, 0.5% sodium deoxycholate, 50 mM NaCl, 25 mM Tris-HCl, 10 mM EDTA) containing 10 mM phenylmethyl sulfonyl (Sigma, St. Louis, MO) and $1 \times$ Halt protease inhibitors (Invitrogen, Carlsbad, CA). Proteins were separated by loading of 20 μl of the lysate onto a 10% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrophoresis, followed by blotting onto a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was probed by using a mouse anti-human CD247 monoclonal antibody (catalog number 551033; BD Pharmingen, San Jose, CA) and the SuperSignal West Pico detection kit (Pierce, Rockford, IL).

Flow cytometry for cell surface single-chain antibody expression. Transduced cells were washed, resuspended in 100 μl of wash buffer (5% bovine serum albumin [BSA] with 2 mM EDTA in phosphate-buffered saline [PBS]) containing either fluorescein isothiocyanate (FITC)-conjugated goat anti-human F(ab)₂ antibody (catalog number 109-006-003; Jackson ImmunoResearch Laboratories, West Grove, PA) or isotype control antibody, and incubated for 30 min at 4°C. After washing in fresh wash buffer, the cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry (LSR Fortessa II cytometer [BD Biosciences] and FlowJo software [FlowJo, Ashland, OR]).

Flow cytometry for CAR-mediated proliferation of transduced CD8⁺ T lymphocytes in response to HIV-1-infected target cells. HIV-1-infected T2 cells, which are MHC class I low due to a deletion in the transporter associated with processing (TAP) (8) and were previously shown to be suitable target cells for an HIV-1-specific CAR (20), served as

TABLE 1 Broadly neutralizing antibody sequences used for single-chain antibody CAR constructions^a

Specificity	Antibody	Chain	Sequence
CD4-binding site	VRC01	VL	ENVLTQSPGTLSSPGETAIISCRISQVYGLAWYQQRPQCAPRLVYSGSTRAAQIPDRFSGRWGPDDYNLTISNLESGDFGVYCCQOYEEFGGQTKVQVDIR
		VH	MLLVTSLLCELPHPAFELLPOVQLVQSGGQMKRPGESMRISCRASGYEHDCTLNWIRLAPGKRPEWMGWLKPRGAVNYPARPLQGRVTMTTRDVYSDTAFLRLS
	3BNC117	VL	LTVDDTAVFCTRGKNCQDYNWDEHHWGRGTPVIVSS
		VH	DIQMTQSPSSLSASVGDITVITTCQANGYLNWYQQRGKAPKLIYDGSKLERGVPSRFSGRRWGOEYVNLTNLQPEDIATYFCQYEFVVPGLRLDKRTVAAP
CD4-induced site	X5	VL	MLLVTSLLCELPHPAFELLPOVQLVQSGAAVTKPGASVRYSCASGYNIRDIYFIHWWRQAPGQGLQWVGWINPKTGQPNPQFQGRVSLTRHASWDEPDTSEY
		VH	MDLKAIRSDITAVYFCARQRSDYWDPEVWGSQTQVTVSSASTKGP
N-glycan	PGT126	VL	ELVLTQSPGTLSSLAGERATLSCRASQVSSGSLAWYQQKPGQAPRLIYGASTRATGIPDRFSGSGTDFTLTIGRLPEDLAIVYCCQYGTSPYTFGQGTKEI
		VH	MLLVTSLLCELPHPAFELLPOVQLVQSGGGLVEASETLSLCTVSGDSTACDYPFWGWRQPPGKGLEWIGLSHCAGYNTGTWYTHNPISLKSRLTISLDTPKNQV
V2 loop	PG9	VL	QSALTQPPSASGSPGQSISISCTGTNREVSWYQHPGKAPKLVYGVNKRPSGVDPDRFSGSKSGNTASLTVSGLQTDDEAVVYCSSLVGNWDVIFGGGTKLTVL
		VH	MLLVTSLLCELPHPAFELLPOVQLVQSGGLVEASETLSLCTVSGDSTACDYPFWGWRQPPGKGLEWIGLSHCAGYNTGTWYTHNPISLKSRLTISLDTPKNQV
MPER	10E8	VL	FLKNSVTAADTAIYTCARDEGLVYHDWPKPAWVDLWGRGTLTVTVSS
		VH	QSALTQPPSASGSPGQSITISCTGTNNRFSWYQQHAGKAPKLVYDVNKRPSGVDPDRFSGSKSGNTASLTVSGLQTDDEAVVYCGSLVGNWDVIFGGGTKLTVL
		VL	MLLVTSLLCELPHPAFELLPOVQLVQSGPTLVEASETLSLTCAVSGDSTACNSFWGWRQPPGKGLEWVGSLSHCASYNRGTWYTHNPISLKSRLTALDTPKNIV
		VH	FLKNSVTAADTAIYTCARDEGLVYHDWPKPAWVDLWGRGTLTVTVSS
		VL	QSALTQPPSASGSPGQSITISCTGTNDVGGYESVSWYQQHAPKLVYDVSKRPSGVSNRFSGSKSGNTASLTISGLQAEDEGDYVCKSLTSTRRYFGTGTKLTVL
		VH	MLLVTSLLCELPHPAFELLPOVQLVQSGGLVAPGSLRLSCASGDFDSRQGMHWWRQAPGQGLEWVAFIKYDGSSEKTHADSWGRLSISRDNSKDTLYIQMNSLR
		VL	VEDTATYFCVREAGGPPDRNGYNYDDYDGYNYHYMDVWGKGTITVTVSS
		VH	VEDTATYFCVREAGGPPDRNGYNYDDYDGYNYHYMDVWGKGTITVTVSS
		VL	SYELTQETGVSAALGRTVITTCRGLSLRSHVASWYQKKPGQAPILLFYGKNNRPSGVDPDRFSGSASGNRASLTISGAQAEDDAEYCCSSRDKSGSRLSYEGGTKLTVL
		VH	MLLVTSLLCELPHPAFELLPOVQLVQSGGLVAPGSLRLSCASGDFDSRQGMHWWRQAPGQGLEWVAFIKYDGSSEKTHADSWGRLSISRDNSKDTLYIQMNSLR
		VL	NLRMEDSGLYFCARTGKYDFWGSYPPGEEYFQDWGRGTLTVTVSS
		VH	NLRMEDSGLYFCARTGKYDFWGSYPPGEEYFQDWGRGTLTVTVSS

^a For each broadly neutralizing antibody, the specificity and heavy/light chain amino acid sequences are given. VL, variable region of light chain; VH, variable region of heavy chain.

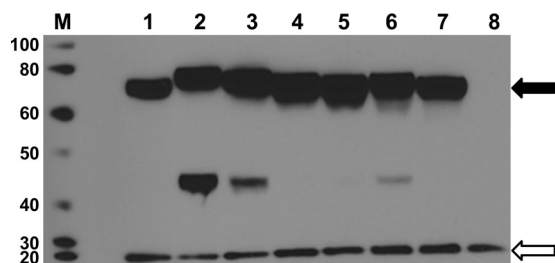


FIG 2 Confirmation of novel CAR expression via Western blotting of transduced Jurkat cells. Western blotting for CD3 ζ was performed on Jurkat cells after transduction with CAR expression lentiviral vectors. The open arrow indicates the expected size of the native CD3 ζ chain, and the closed arrow indicates the approximate expected size of the CAR (including the single-chain antibody, hinge, 4-1BB signaling, and CD3 ζ signaling domains). Lanes: M, marker; 1 to 8, CAR-10E8, CAR-3BN117, CAR-PG9, CAR-PGT126, CAR-PGT128, CAR-VRC01, CAR-X5, and a nontransduced Jurkat control, respectively.

target cells. These cells were infected with an excess of HIV-1_{NL4-3}-based reporter virus containing a gene for murine CD24 (mCD24) in the *vpr* locus (21) to yield >90% infected cells by 3 or 4 days after infection, as previously reported (9, 10, 12). These cells were irradiated immediately before use with 10,000 rads in a cesium irradiator, and peripheral blood mononuclear cells from a healthy donor were irradiated with 3,000 rads (feeder PBMCs). CAR-transduced primary CD8⁺ T lymphocytes were labeled with CellTrace Violet and washed according to the manufacturer's directions (Thermo Fisher Scientific, Grand Island, NY). In a 48-well plate well, 5×10^5 labeled transduced cells were added to 5×10^5 irradiated infected T2 cells and 2×10^6 irradiated feeder PBMCs and cultured in 1 ml R10-50 for 5 days with a medium change after 3 days. Flow cytometry (LSR Fortessa II cytometer; BD Biosciences) was then performed with costaining for human CD8 (peridinin chlorophyll protein [PerCP]-anti-human CD8, catalog number 30130; BioLegend, San Diego, CA), and proliferation was analyzed by using FlowJo software (FlowJo, Ashland, OR).

Chromium release killing assays for CAR-mediated killing of HIV-1-infected target cells. T2 cells infected with HIV-1_{NL4-3} as described above were used as target cells for CAR-transduced primary CD8⁺ T lymphocytes in standard ⁵¹Cr release assays as previously described (9, 10, 12). Briefly, infected and control uninfected T2 cells were ⁵¹Cr labeled for 1 h and incubated with or without effector CD8⁺ T lymphocytes for 4 h at various cell ratios in a 96-well U-bottom plate. Supernatants were then harvested for measurement of extracellular ⁵¹Cr by microscintillation counting in 96-well plates. Spontaneous release was measured on target cells lysed without effector cells, and maximal release was measured on target cells lysed with 2.5% Triton X-100. Specific lysis was calculated as follows: (experimental released chromium – spontaneous release)/(maximal release – spontaneous release).

Virus suppression assays. The ability of CAR-transduced CD8⁺ T lymphocytes to suppress the replication of HIV-1 was tested as previously described in detail (2, 9, 11, 12, 22–24). HIV-1 strains tested were obtained from the NIH AIDS Reference and Reagent Repository, including

94US_33931N (catalog number 11250), 90_US873 (catalog number 11251), 96TH_NP1538 (catalog number 11252), and 00TZ_A246 (catalog number 11256). In brief, T1 cells transduced with human CCR5 were infected at a multiplicity of 0.1 tissue culture infectious doses per cell and cocultured in a 96-well plate with CAR-transduced cells at a ratio of 5×10^4 to 1.25×10^4 cells, respectively, in 200 μ l of R10-50 or with no effector cells as a control. The effector cells had been confirmed to be >90% transduced. Experiments under each condition were run in triplicate, and viral replication was monitored by using a p24 quantitative enzyme-linked immunosorbent assay (ELISA) (XpressBio, Frederick, MD).

RESULTS

Genetic construction of chimeric antigen receptors based on broadly neutralizing antibodies against HIV-1. A set of BNABs was selected based on the binding of different HIV-1 Env domains and the availability of sequences (Table 1). These BNABs included seven antibodies targeting the CD4-binding site, the CD4-binding-induced site on gp120, the gp120 V2 loop, gp120 N-glycans, and the membrane-proximal region of gp41. Genes for single-chain versions of each antibody were created by the synthesis of codon-optimized sequences for the heavy and light chains, separated by a (GGGGS)₃ linker, and these genes were substituted for the single-chain antibody in a second-generation CAR vector containing the 4-1BB signaling domain fused to the CD3 ζ signaling domain (Fig. 1).

Confirmation of expression and potential functionality of the novel CARs. The CAR genes were delivered by lentiviral vectors to Jurkat cells for initial confirmation of expression and functionality. Western blotting for CD3 ζ confirmed that the transduced cells contained both native CD3 ζ and the expected larger CD3 ζ -containing CAR for all seven constructs (Fig. 2). Flow cytometry for cell surface CAR expression using a goat antibody against human Fab (antigen-binding antibody fragment) further demonstrated the cell surface expression of each CAR (Fig. 3). Primary CD8⁺ T lymphocytes were then transduced with the lentiviral vectors, and flow cytometry also confirmed cell surface CAR expression for each construct, although the transduction efficiency was lower than that for Jurkat cells. By using the goat anti-human Fab antibody as a stimulus, there was selective expansion and enrichment of the CAR-transduced cells within the bulk population (Fig. 4), indicating that cross-linking of CARs induced the proliferation of the transduced cells analogous to anti-CD3 antibody-induced proliferation of normal T lymphocytes.

CAR-transduced primary CD8⁺ T lymphocytes proliferate in response to HIV-1-infected cells. Enriched CAR-transduced ($\geq 90\%$) primary CD8⁺ T lymphocyte effector cells were tested for their capacity to proliferate in response to HIV-1-infected cells. After coculture with irradiated HIV-1_{NL4-3}-infected T2 cells or control uninfected T2 cells, all effector cells transduced with CARs exhibited HIV-1-specific proliferation to various degrees (Fig. 5). These results confirmed the retained specificity of the single-chain

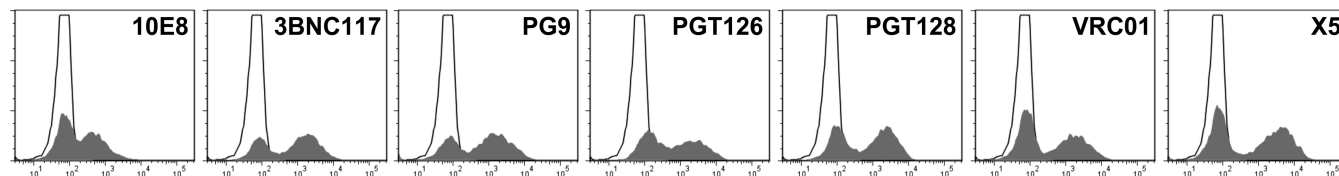


FIG 3 Confirmation of novel CAR expression via flow cytometry for cell surface immunoglobulin of transduced Jurkat cells. Transduced Jurkat cells were stained with goat antibody against human Fab and assessed by flow cytometry. Histogram negative gating was set on nontransduced control cells (not shown).

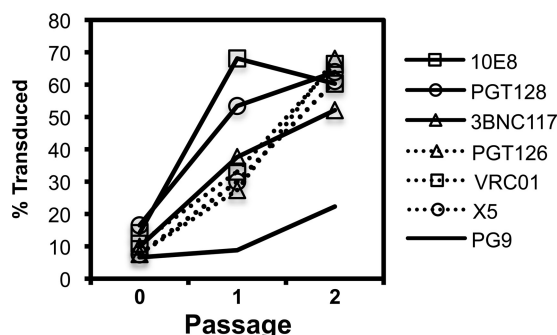


FIG 4 Selective functional expansion by binding of the single-chain antibody domain of the novel CARs. After transduction of primary CD8⁺ T lymphocytes with each of the seven CAR vectors, the cells were serially passaged (10 days each passage) by using stimulation with a goat anti-human Fab antibody with irradiated allogeneic feeder PBMCs and interleukin-2. The percentage of cells determined to express CAR was determined by flow cytometry as described in the legend of Fig. 3.

versions of the parental antibodies against HIV-1 envelope on the surface of infected cells.

All novel CARs mediate specific killing of HIV-1-infected target cells. The enriched CAR-transduced effector cells were tested for specific killing of HIV-1-infected CD4⁺ lymphocytes. They were assayed in standard chromium release assays against HIV-1_{NL4-3}-infected T2 cells or control uninfected T2 cells (Fig. 6). All CARs mediated substantial killing of infected versus uninfected target cells at effector-to-target cell ratios of 5:1, indicating specific targeting of HIV-1-infected cells.

All novel CARs exhibit antiviral activity. The enriched CAR-

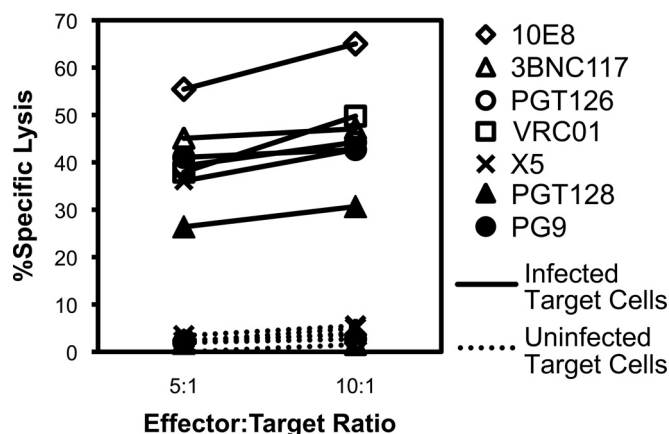


FIG 6 Specific killing of HIV-1-infected target cells mediated by novel CARs. CAR-transduced primary CD8⁺ T lymphocytes were cocultured with HIV-1-infected T2 cells in standard 4-h chromium release assays to assess killing mediated by the CARs. PGT128- and PG9-based CARs were tested for killing in an experiment separate from those for the other CARs. The relative efficiencies of the CARs varied between experiments, and no single CAR was consistently superior.

transduced effector cells were also tested for antiviral activity against infected CD4⁺ cells. T2-CCR5 cells were infected with a panel of HIV-1 strains, including primary R5-tropic isolates, and cultured in the absence or presence of the CAR-transduced effector cells. Virus replication was assessed by measurement of p24 antigen levels between days 7 and 10 of culture. Suppression of replication was calculated as the difference in log₁₀ units of p24

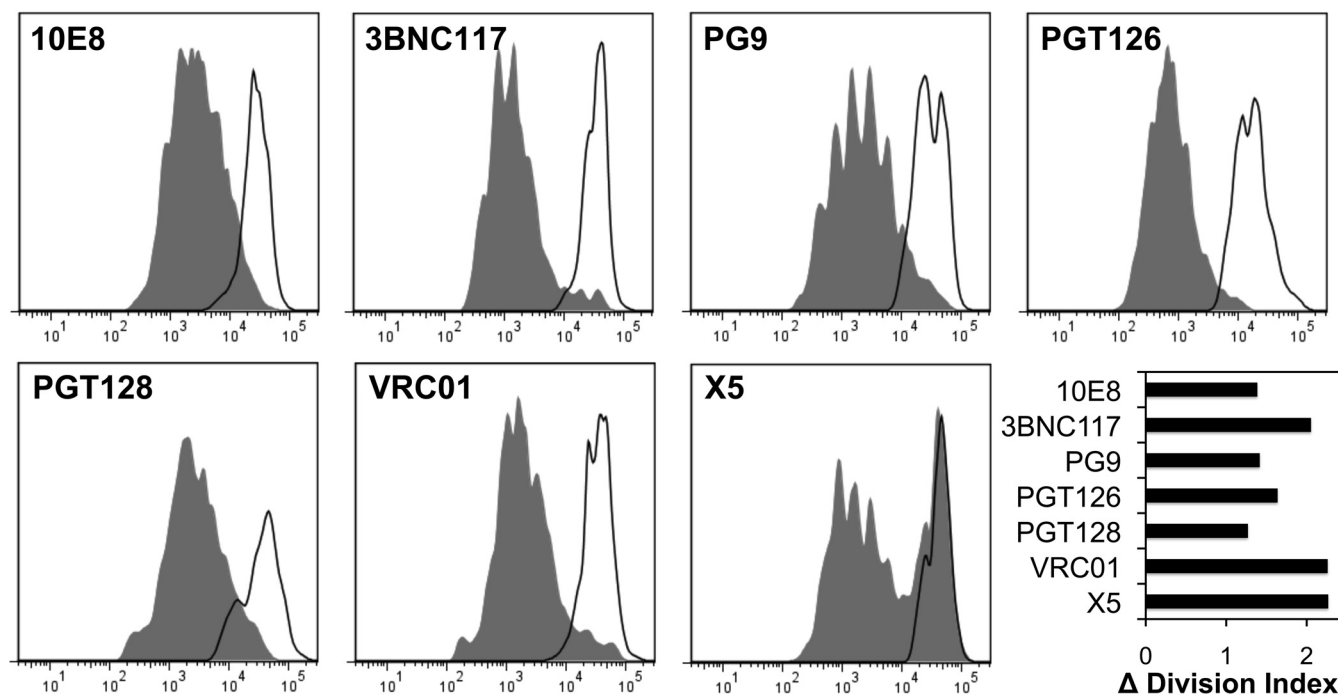


FIG 5 Proliferation mediated by novel CAR interactions with HIV-1-infected target cells. Primary CD8⁺ T lymphocytes transduced with the panel of CARs were enriched to >90% purity and labeled with CellTrace Violet and then cocultured with irradiated HIV-1 NL4-3-infected T2 cells. CellTrace Violet fluorescence was assessed by flow cytometry after 7 days. The open histograms indicate transduced cells exposed to control uninfected cells, while the shaded histograms indicate those exposed to infected cells.

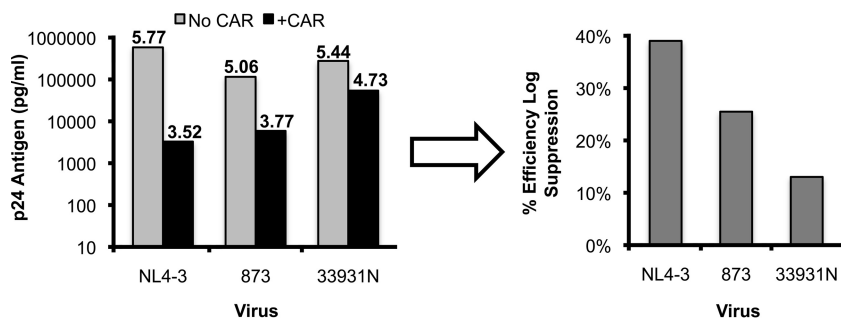


FIG 7 Sample calculation of percent log efficiency of suppression by CAR-transduced cells. T1-CCR5 cells were infected with the indicated viruses at a multiplicity of infection of 10^{-1} tissue culture infective doses/cell and cultured with or without CAR 10E8-transduced CD8⁺ T cells (>90% enriched) at an effector-to-target cell ratio of 1:4. (Left) The HIV-1 p24 antigen level was measured by an ELISA on day 7. Log units of p24 antigen (\log_{10} picograms per milliliter) are indicated above each bar. Virus suppression by CAR-transduced cells ranged from $5.44 - 4.73 = 0.71 \log_{10}$ units to $5.77 - 3.52 = 2.25 \log_{10}$ units (80.5% to 99.4%) for HIV-1_{33931N} and HIV-1_{NL4-3}, respectively. In general, replication without effector cells reached 3 to 6 \log_{10} pg/ml (10^3 to 10^6 pg/ml). (Right) Virus suppression is normalized to total replication without effector cells as the percent reduction in \log_{10} units of p24 antigen comparing cultures with and those without added effector cells: for HIV-1_{NL4-3}, $(5.77 - 3.52)/5.77 = 0.390 = 39.0\%$; for HIV-1₈₇₃, $(5.06 - 3.77)/5.06 = 0.255 = 25.5\%$; and for HIV-1_{33931N}, $(5.44 - 4.73)/5.44 = 0.130 = 13.0\%$.

between cultures without and those with effector cells, which was then normalized as the ratio to total replication without effector cells (Fig. 7). Across multiple experiments, each CAR exhibited consistent levels of antiviral activity against five HIV-1 strains, including four subtype B strains and one subtype C strain (Fig. 8). For this limited set of viruses, some CARs, such as the one based on PGT126, appeared to have broader coverage than others, such as the one based on 3BNC117.

DISCUSSION

Given the success of CARs for cancer immunotherapy and shortcomings of the prior attempt of this approach for HIV-1 treatment, revisiting CARs for HIV-1 infection is appropriate. We took advantage of the new generation of BNABs, which are remarkable for their affinity, potency, and breadth of HIV-1 neutralization. These BNABs were engineered as single-chain constructs to serve as binding domains for novel CARs.

Somewhat unexpectedly given the uncertain affinity of antibodies converted to single-chain versions, all seven CAR constructs showed HIV-1-specific functional activity. Each CAR construct demonstrated conformationally relevant cell surface expression (by binding of a goat anti-human Fab antibody) as well

as mediation of HIV-1-specific proliferation, killing, and suppression of viral replication. The generally high binding affinity of BNABs may have afforded the retention of enough affinity of the single-chain versions to meet the much-lower-affinity requirement of T cell receptors for signaling. Thus, each CAR conferred the functional properties that are likely important for transduced-cell expansion and clearance of HIV-1-infected cells *in vivo*.

Against HIV-1_{NL4-3}, these CARs had antiviral properties similar to those determined in preclinical *in vitro* antiviral tests of the CD4⁺ ζ CAR (2) that was previously advanced to clinical trials. Matching these novel CARs, that CAR mediated ~50 to 60% lysis of cells infected with HIV-1_{IIIB} (the source of the Env in HIV-1_{NL4-3}) and a ~40 to 50% log efficiency of virus suppression. In a separate previous study, the CD4⁺ ζ CAR also mediated HIV-1-specific proliferation of transduced T lymphocytes (1).

It is unclear as to which *in vitro* assays might predict the superiority of one CAR over the other for use *in vivo*. While proliferation and antiviral activity are likely to be the critical activities for efficacy *in vivo*, our *in vitro* assays are semiquantitative, and conditions may not reflect those *in vivo*. Our data suggest that some BNAB-based CARs have broader activity against HIV-1 variability

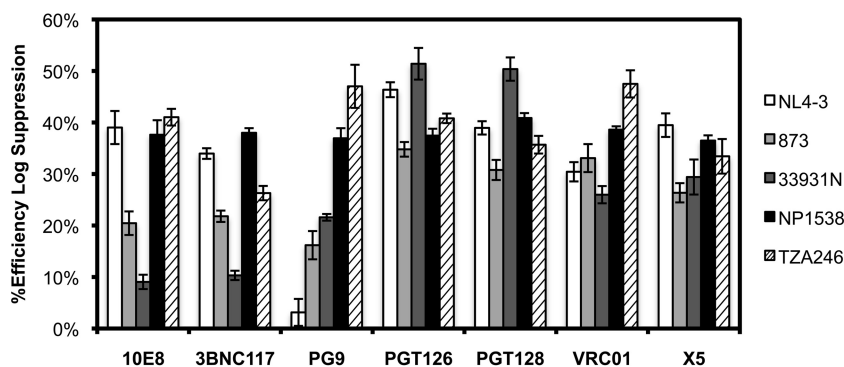


FIG 8 Efficiencies of novel CAR-transduced primary CD8⁺ T cells against a panel of HIV-1 isolates. CAR-transduced primary CD8⁺ T cells were tested against a panel of 4 subtype B viruses and one subtype C virus (TZA246) to determine the percent efficiency of log suppression, as shown in Fig. 7. For each virus, bars represent the medians for all replicates across 1 to 6 (mean, 2.9) independent experiments, each with triplicates, with standard-error bars. Note that a 30% log efficiency of suppression for a typical experiment with control viral replication of 5 \log_{10} pg/ml would correspond to a reduction of 1.5 \log_{10} units, or 96.8% suppression of viral replication.

than others, although more testing will be required. Given an optimal range of affinity for CARs against their target proteins, where higher or lower levels of affinity yield inferior activity (25), it may be that CARs will differ in their persistence depending on their affinity for the specific HIV-1 Env sequence encountered *in vivo*. In this regard, the demonstration by Webb et al. (26) that different BNABs have highly variable neutralization curve slopes might suggest that BNABs with a flatter slope would have a larger “sweet spot” of affinity across various HIV-1 Envs. Those authors reported that CD4-binding site (including VRC01 and 3BNC117) and V3-glycan (including PGT128) BNABs exhibit steeper slopes, while V2-glycan (including PG9) and membrane-proximal external region (including 10E8) BNABs exhibit flatter slopes. However, this appears to be contradicted by our observation that the PG9- and 10E8-based CARs seem to have less breadth of antiviral activity than the VRC01- and 3BNC117-based CARs, suggesting the influence of other potential factors such as the preservation of affinity in the single-chain form and/or a greater reserve of affinity. Further supporting this point, while X5 was originally considered broadly neutralizing, it has relatively poor neutralizing breadth against various HIV-1 isolates compared to the other antibodies tested here (27) yet demonstrates good breadth as a CAR. Regardless, because BNABs seem to be escaped by HIV-1 Env variation in their original hosts (28–30), viral variability and escape may remain a barrier to the therapeutic implementation of BNAB-based CARs.

In summary, we have demonstrated that seven BNABs with various epitope specificities all have activity as single-chain HIV-1 receptors in CARs. All constructs have the ability to recognize infected cells for proliferation, killing, and suppression of viral replication, although they may vary in their breadth of HIV-1 sequence diversity coverage. Additional studies will be necessary to understand and assay the properties important for transduced-cell proliferation and function for *in vivo* immunotherapies based on BNAB CARs.

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