INTRODUCTION
Adoptive immunotherapy using chimeric antigen receptor–modified T cells (CAR-T) has shown extraordinary success for the treatment of refractory B cell malignancies that express CD19, CD20, or CD22 antigens (1–3). In contrast, past attempts using first-generation HIV–specific CAR-T cells for the treatment of HIV/AIDS were unsuccessful in humans despite demonstration of long-term persistence of gene-modified T cells in HIV-positive patients (4–7). HIV infection of susceptible cells such as CD4+ T cells or macrophages is initiated by binding of the gp120 HIV envelope (Env) glycoprotein to the CD4 receptor, which triggers conformational changes in Env that enable it to bind to CCR5 or CXCR4 proteins expressed on the surface of susceptible cells, penetrate the cell, and activate the HIV replication program (8).

The application of immunotherapeutic strategies to treat HIV infection has been limited by factors unique to HIV infection including the high mutation rate of reverse transcriptase, which enables the rapid emergence of immune escape variants mutated in HIV envelope–specific epitopes (9) and recurrence of viremia (10). First-generation anti-HIV CAR approaches used the extracellular region of the CD4 receptor as the targeting domain coupled with the CD3ζ signaling domain to kill productively HIV-infected cells. However, it was revealed later that CD4-based CARs render the gene-modified T cells susceptible to HIV infection (11, 12). To overcome this limitation, several strategies to improve HIV-specific CAR-T cells were tested, including design of bispecific CAR-T cells (11), or CAR-T cells expressing a CD4ζ CAR in combination with either a gp41-derived fusion inhibitor (12) or CCR5 ablation (13). Moreover, anti-HIV CARs have been reengineered with 4-1BB or CD28 costimulatory signaling motifs to improve their in vivo persistence (14) and potency when combined with soluble broadly neutralizing antibodies (bNAbs) that recognize nonredundant gp120/gp41 epitopes (11, 13, 15, 16).

An alternative approach to using the extracellular region of the CD4 receptor for targeting the HIV envelope glycoprotein is a single-chain variable fragment (scFv) derived from bNAbs. However, one major drawback to developing bNAb-based CARs has been that their scFv antigen-binding domain generally requires further engineering to account for reduced therapeutic effectiveness (17), and unlike the CD4 receptor, a single bNAb cannot fully neutralize all HIV isolates (18, 19). Recent clinical trials using bNAb monotherapies with VRC01, 3BNC117, or 10-1074 led to viral rebound upon antiretroviral therapy (ART) interruption in people with chronic HIV infection, but an antibody composed of multiple envelope-specific scFvs showed improved protection in a simian/human immunodeficiency virus macaque model (20–24). We recently reported that a hexavalent fusion protein consisting of an scFv-derived heavy chain–only domain, m36.4, which targets the highly conserved CD4-induced (CD4i) gp120 co-receptor binding site, and mD1.22, an engineered mutant of the D1 extracellular domain of CD4, mediates potent and broad in vitro and in vivo suppression of HIV infection in a humanized mouse model of HIV infection, where human HIV–infected and CAR-T cells are

Multispecific anti-HIV duoCAR-T cells display broad in vitro antiviral activity and potent in vivo elimination of HIV-infected cells in a humanized mouse model

Kim Anthony-Gonda 1*, Ariola Bardhi 2*, Alex Ray 2, Nina Flerin 2, Mengyan Li 3, Weizao Chen 2, Christina Ochsenbauer 4, John C. Kappes 1,5, Winfried Krueger 1, Andrew Worden 1, Dina Schneider 1, Zhongyu Zhu 1, Rimas Orentas 1†, Dimitr S. Dimitrov 6‡, Harris Goldstein 2, Boro Dropulic 1†

Adoptive immunotherapy using chimeric antigen receptor–modified T cells (CAR-T) has made substantial contributions to the treatment of certain B cell malignancies. Such treatment modalities could potentially obviate the need for long-term antiretroviral drug therapy in HIV/AIDS. Here, we report the development of HIV–1–based lentiviral vectors that encode CARs targeting multiple highly conserved sites on the HIV–1 envelope glycoprotein using a two-molecule CAR architecture, termed duoCAR. We show that transduction with lentiviral vectors encoding multispecific anti-HIV duoCARs confer primary T cells with the capacity to potently reduce cellular HIV infection by up to 99% in vitro and >97% in vivo. T cells are the targets of HIV infection, but the transduced T cells are protected from genetically diverse HIV–1 strains. The CAR-T cells also potently eliminated PBMCs infected with broadly neutralizing antibody-resistant HIV strains, including VRC01/3BNC117-resistant HIV–1. Furthermore, multispecific anti-HIV duoCAR-T cells demonstrated long-term control of HIV infection in vivo and prevented the loss of CD4+ T cells during HIV infection using a humanized NSG mouse model of intrasplenic HIV infection. These data suggest that multispecific anti-HIV duoCAR-T cells could be an effective approach for the treatment of patients with HIV–1 infection.
directly injected into the spleens of animals (25). In addition, it has been demonstrated that further combining the mD1.22–m36.4 hexavalent fusion protein with T20, which is a gp41-derived C-peptide fusion inhibitor similar to the C46 peptide, enhances its inhibitory effect against HIV-1 in vitro (26).

Here, we report the development of HIV-1–based lentiviral vectors (LVs) encoding multispecific anti-HIV duoCARs. The architecture of duoCAR has two CAR molecules consisting of multiple anti-HIV binders, including mD1.22, m36.4, and C46, expressed on the surface of T cells from a single LV. Primary human T lymphocytes were engineered with up to three functionally distinct HIV envelope-binding domains (mD1.22, m36.4, and C46) to form bispecific and trispecific targeting anti-HIV duoCAR-T cells. We hypothesized that CD8+ T cells engineered with LVs expressing multiple HIV envelope-binding CAR molecules would target and eliminate productively HIV-infected cells while protecting cooperating CD4+ CAR-T cells from HIV infection. The mD1.22 domain targets the highly conserved CD4-binding site on HIV gp120. In addition, the mD1.22 domain has been engineered to be molecularly compact with enhanced specificity and higher affinity for the HIV envelope glycoprotein and thus serves as an improved CD4-derived binding domain (27). The m36.4 domain is an affinity-matured, engineered human heavy chain–only (VH) antibody domain (28) that binds to a discontinuous CD4-induced (CD4i) epitope on gp120 in the vicinity of the co-receptor binding site, similar to other co-receptor bNAb-binding sites, such as 17b (29). When combined to form a multivalent, bispecific soluble antibody, these domains synergistically neutralized diverse HIV strains in vitro (25, 27). The C46 peptide inhibits HIV infection at the level of viral fusion and is similar to the U.S. Food and Drug Administration (FDA)–approved fusion inhibitor enfuvirtide or T20. When expressed on the surface of T cells as an anchored membrane-associated molecule, it potently abrogates HIV fusion to the T cell membrane (30, 31).

Previously, we described a humanized NSG mouse model (hu-spl-PBMC-NSG) that supports robust HIV infection after infection of intrasplenically injected peripheral blood mononuclear cells (PBMCs) with an Env-IMC-LucR virus that can be monitored by quantifying luciferase activity in the spleen of the mouse as early as 1 week after infection (32). We used this humanized mouse model to demonstrate that the LSEvH-LS-F fusion protein, which contains the same specificity as the CAR-T cells described herein, mobilized antibody-dependent cell-mediated cytotoxicity activity and effectively eliminated cells infected with different infectious molecular clone (IMC) constructs expressing a broad range of Env glycoproteins in vivo via a natural killer cell–dependent mechanism (25). Despite its potency, this antibody displayed a short half-life, which renders it unsuitable as a therapeutic agent for long-term maintenance of viral suppression. Because gene-modified T cells including CAR-T cells have been shown to persist in patients for years (4–7), we postulated that development of a CAR-T LV construct expressing the very broad targeting specificities of mD1.22 and m36.4 would be a better therapeutic candidate for long-term control of HIV-1 infection in patients.

Here, we show potent in vitro and in vivo anti-HIV effects of multispecific anti-HIV duoCAR-T cells that simultaneously kill productively HIV-infected cells in our hu-spl-PBMC-NSG mouse model and protect the CAR-modified T cells from infection using HIV-1 viruses expressing diverse Env proteins. Coupled with the known long-term persistence and immunosurveillance properties of CAR-T cells [reviewed in (33)], multispecific anti-HIV duoCAR-T cells could represent a viable approach for controlling viral loads and eliminating HIV-infected cells in HIV-positive patients in the absence of ART.

RESULTS
Design and functional characterization of multispecific anti-HIV CAR-T cells

We generated HIV-1–based LVs encoding mono-, bi-, and trispecific anti-HIV CARs as shown in Fig. 1A. These CARs target three putative sites on the Env trimer, which include the gp120 CD4-binding site (mD1.22), gp120 co-receptor–binding site (m36.4), and gp41 near the membrane-proximal external region (MPER) (C46 peptide). The structures of the resulting expressed anti-HIV CARs are depicted in Fig. 1B. Monospecific CAR LV constructs were generated by fusing the mD1.22 (designated “1” for mD1.22) or m36.4 (designated “3” for m36.4) domain in-frame to a single-molecule CAR architecture previously described by our group that consists of a CD8 EC followed by the CD8 TM domain, the 4-1BB costimulatory domain to support CAR-T persistence, and a CD3ζ T cell signaling chain (34). For simplicity, the single-molecule CAR architecture was designated monoCAR (M) followed by the binders used to generate the CAR construct. For instance, monoCARs containing the mD1.22 or m36.4 domain were designated M1 or M3, respectively. Conventional bispecific CARs were also generated in a monoCAR format by linking both mD1.22 and m36.4 domains together (M13) using a 3xG4S motif.

CAR-T cell therapies currently in the clinic for cancer treatment use a conventional monoCAR design whereby a single molecule with one or more binding domains targets exposed epitopes on antigens that are located on the surface of target cells [reviewed in (35)]. However, the gp120-binding site for certain highly conserved neutralizing antibodies may not be exposed before the conformational change that is induced by the interaction between CD4 and gp120. To facilitate sequential targeting, first, the mD1.22 domain to its CD4-binding site and, subsequently, the m36.4 domain to its conformationally exposed binding site (28, 36, 37), we also designed LVs encoding a unique two-molecule CAR architecture designated duoCAR (D). The duoCAR can be engineered with two or more Env specificities via a bicistronic P2A comprising LV to allow for simultaneous expression of both CARs in a single T cell. We therefore constructed an LV-encoding bispecific duoCAR that contained the mD1.22-CAR and the m36.4-CAR (D13).

To further potentiate multitargeting and protection from HIV infection, we engineered the anti-HIV CARs with a third anti-HIV domain based on the FDA-approved fusion inhibitor enfuvirtide or T20. As previously reported, T20 and similar gp41-derived C peptides (e.g., C46) block HIV infection by binding to the gp41 six-helix bundle locking the HIV-1 envelope glycoprotein into a fusion-incompetent state (38, 39). For trispecific CARs, we considered that because the C46 peptide targets gp41 and mD1.22 targets gp120, it would be more logical to fuse the C46 peptide to the N terminus of the mD1.22 domain using different linker lengths. As previously reported by Liu et al., bispecific anti-HIV CAR function is influenced by the spatial separation between two antigen-binding domains (11). Therefore, we tested a short (“S” for 3xG4S linker) and long (“L” for 5xG4S linker) flexible linker between the C46 peptide (designated as “4” for C46) and mD1.22 domain (1) in trispecific duoCARs to generate D413S and D413L, respectively. Last, to determine the optimal location of the C46 peptide, we constructed an additional trispecific construct containing a bispecific CAR and a membrane-anchored C46 peptide
Multispecific anti-HIV duoCARs are efficiently expressed on the surface of primary human T cells

Previously, we have shown that HIV-1–based LVs safely and efficiently deliver an HIV-specific genetic payload to primary human T cells for the treatment of HIV-1–infected patients (5, 40–43). To test whether anti-HIV CARs could be efficiently delivered and expressed on the surface of primary T cells by LVs, we transduced HIV-naïve CD4+ and CD8+ T cells with the anti-HIV CAR LVs and evaluated the cells for CAR expression by flow cytometry detecting either the mD1.22 domain (Fig. 2A) or the C46 peptide (Fig. 2B). A figure exemplifying the gating strategy is provided in fig. S1. For monospecific and bispecific CAR-T cells, expression was determined by guest on May 2, 2021 http://stm.sciencemag.org/ Downloaded from
via the increase in mD1.22 expression on the surface of transduced CD4+ and CD8+ T cells as compared with untransduced (UTD) T cells. The CD4 antibody used for flow cytometry experiments recognizes the D1 domain of the native CD4 receptor expressed by CD4+ T cells and the mD1.22-CAR, which contains a modified D1 domain. UTD T cells are used as a biological control to set gates. The x axis shows CD4+ T cells (Q4) and mD1.22 expression on CD4+ T cells in the quadrant labeled CAR+ CD4+. The y axis shows CD8+ T cells (Q1), mD1.22 expression on CD8+ T cells is shown in the quadrant labeled CAR+ CD8+. Graphical representation of % CAR-modified T cells from multiple donors using either (C) anti-CD4 (n=7 donors) or (D) anti-C46 (2F5 Mab) flow cytometry (n=5 donors). The error bars represent mean ± SD of independent donors. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni posttest analysis (***P < 0.0001 and ****P < 0.00001). IgG, immunoglobulin G; PE, phycoerythrin; FACS, fluorescence-activated cell sorting. (E) Measurement of soluble HIV envelope (Env) capture by all T cells. Representative histogram is shown (n=2 donors). Specific binding of anti-HIV CARs to soluble, His-tagged gp140 (clade B) relative to the endogenous CD4 receptor expressed on CD4+ T cells in the total T cell population. UTD T cell control and CD19-CAR-T cells serve as a control for specificity. (F) Measurement of soluble HIV envelope capture by CD8+ T cells. Representative histogram is shown (n=2 donors).
peptide engineered into these constructs as a unique molecular tag recognized by the 2F5 antibody. As shown in Fig. 2B, trispecific CARs were detected by 2F5 on the surface of CD4+ and CD8+ T cells across multiple donors. LV-modified primary CD4+ and CD8+ T cells expressed anti-HIV CARs with transduction rates up to 60% across different donors after a single round of transduction (Fig. 2, C and D).

The ability of anti-HIV CARs to bind to Env was demonstrated by their capacity to capture soluble gp140 [His-tagged soluble version of gp120/gp41ecto derived from a clade B isolate (28)], indicating that the targeting domains were active on the surface of modified T cells and not altered by the linker architecture (Fig. 2E). The m36.4-CAR (M3) was detectable on the surface of genetically modified T cells but bound more gp140 when expressed on CD4+ T cells than on CD8+ T cells. The capture of gp140 by the m36.4-CAR on CD8+ T cells was increased by the presence of the mD1.22-containing CARs (Fig. 2F). These data indicate that the ability of the m36.4-CAR to bind to the HIV envelope is enhanced in the presence of the CD4 receptor as well as the CD4-like mD1.22 domain and supports a sequential targeting mechanism as previously described for the soluble m36 domain (37).

Multispecific duoCAR-T cells exert strong and specific cytotoxic effects against HIV Env+ targets

The major goal of our study was to identify the most favorable CAR construct and architecture having the most potent and broad anti-HIV effects. To screen for functional anti-HIV CARs, we generated a surrogate HIV-1–infected cell line from human embryonic kidney (HEK) 293T cells, designated 293T-Env-Luc (Env*), which constitutively and highly expresses Env glycoprotein derived from an HIV-1 clade B isolate on its surface, as detected by the 2G12, b12, and 2F5 antibodies using flow cytometry (Fig. 3A). Using this cell line, we screened more than 40 different anti-HIV CAR iterations, which enabled us to quickly rule out nonfunctional and suboptimal CARs to select the most potent CARs to evaluate within this study and further interrogate in later described HIV-1 challenge assays. Envelope negative HEK 293T-Luc or Raji-Luc cells (Env*) were used as negative controls to test the specificity of CAR-T cells, which, after transduction, contain a population of CAR-modified and unmodified T cells. We subsequently cultured the total CAR-T cell population with Env+ or Env− target cells at varying effector-to-target (E:T) ratios. Twenty-four hours later, cocultures were lysed, and reduction of luciferase activity in the presence of CARs was quantified leading to high CD107a expression and a two- to threefold increase in IFN-γ and interleukin-2 (IL-2) production (29).

Anti-HIV duoCAR molecules induce potent CD107a expression and Th1 cytokine production from primary human T cells

We next compared the relative potency of anti-HIV conventional and duoCAR-T cells by measuring expression of CD107a, a functional marker of T cell degranulation, and intracellular T helper type 1 (Th1) cytokine production. To better assess the differential effects of the conventional CAR versus duoCAR architecture, we generated a more physiologically relevant target cell population by infecting human PBMCs from a healthy donor with an HIV-1 replication-competent IMC expressing the C.Du422.1 Env glycoprotein and Renilla luciferase reporter (25). Seven days after infection, infected PBMCs were cocultured with M1-, M13-, or D13-engineered donor-matched CAR-T cells, followed by flow cytometry analysis of the above-mentioned T cell activation markers in CAR+ CD8+ T cells. We selected these three constructs, because they differ from each other by the number of binders (M1: mD1.22 versus M13: mD1.22-m36.4) or by the number of CAR polypeptide molecules expressed within the CAR-T cell (M13: bispecific monoCAR versus D13: bispecific duoCAR). Given the similar magnitude of cell surface expression (Fig. 4A) and binding of these three constructs to Env, we could effectively differentiate the effect of binder and CAR architecture on T cell activation. As shown in Fig. 4B, we observed that the bispecific duoCAR (D13) strongly induced T cell activation, leading to high CD107a expression and a two- to threefold increase in IFN-γ and interleukin-2 (IL-2) production as compared with either single-molecule mono- or bispecific CAR-T cells (M1 or M13, respectively). M13 was inferior to M1 for CD107a expression, IL-2, and IFN-γ production (Fig. 4B), although the binding of M13 and M1 CAR to soluble HIV gp140 protein was very similar. This shows that placing the mD1.22- and m36.4-binding domains on a single CAR resulted in diminished CD107a and Th1 cytokine expression and, therefore, some loss of functionality. In contrast, although the D13 CAR showed similar binding to both M1 and M13 CARs, the expression of CD107a, IFN-γ, and IL-2 production was increased in T cells expressing the duoCAR. Although these data are observational, the results suggest that the differential expression of CD107a, IFN-γ, and IL-2 is not due to differences in Env binding on infected cells but may be due to their ability to transmit two signals independently
via simultaneous or sequential gp120 engagement. Given the high amount of cytokine production, we tested whether the duoCAR architecture induced any potential deleterious tonic signaling or exhaustion effects that could potentially affect the ability of these cells to persist long term in the body. Typically, antigen-independent CAR-T signaling (tonic signaling) is characterized by the presence of multiple cell surface expressed co-inhibitory receptors (e.g., PD-1, Lag-3, and Tim-3) (44). We determined that duoCAR-T cells displayed no appreciable tonic signaling in the absence of antigen demonstrated by the lack of multiple coexpressed exhaustion markers on these cells (Fig. 4C). These data show that the duoCAR architecture does not impair T cell functionality and is similar to conventional CAR-T cells. Together, these data indicate that the two-molecule architecture of anti-HIV duoCAR-T cells affords superior effector functionality over similar single-molecule CAR-T cells.

Anti-HIV duoCAR-T cells eliminate PBMCs infected with broad Env-IMC-LucR viruses

We evaluated the anti-HIV activity of duoCAR-T cells against several different HIV-1 strains to assess their ability to broadly eliminate productive HIV-infected cells. To do so, we adapted a previously described neutralization assay using replication-competent IMCs expressing different Env that also encode a luciferase reporter (hereafter referred to as Env-IMC-LucR). The Env-IMC-LucR viruses allow for a highly quantitative readout of HIV-1 infection in primary target cells (45, 46), and when cocultured with CAR-T cell effectors, the luciferase activity can be used to monitor the inhibitory activity of different CAR constructs. We cultured anti-HIV CAR-T cells in the presence of donor-matched HIV-infected PBMCs at an E:T ratio of 1:1 for 7 days followed by quantification of luciferase activity as a measurement of HIV infection and assessment of CAR-mediated elimination of HIV-infected target cells (Fig. 5A). These CAR-T effectors were generated from different donors enriched for both CD4+ and CD8+ T cells, with the composition being slightly more CD4+ T cells (fig. S2). We hypothesized that multispecific anti-HIV CAR-modified CD4+ T cells in combination with CD8+ T cells would eliminate HIV-infected cells and protect the CAR-T cells from HIV infection. Given that the mD1.22-CAR exhibited similar cytotoxic effects to the CD4-CAR in our cytotoxic T lymphocyte (CTL) assays, we used the mD1.22-CAR (M1) as a control for our in vitro HIV challenge assay to further interrogate our multitargeting duoCAR approach. As shown in fig. S3, anti-HIV CAR-T cells potently eliminated donor PBMC targets infected with an IMC expressing Env from clade B viruses NL4-3 (X4-tropic), BaL (R5-tropic), or SF162

Fig. 3. Cytotoxicity of anti-HIV CAR-T cells against HIV-1 Env-expressing target cells. (A) BnAb detection of the HIV-1 Env glycoprotein on the surface of cell lines used for cytotoxicity assays. (B) Cytotoxicity of conventional CD4-CAR versus mD1.22-CAR-T cells against Env+ target cells (representative figure, n = 2 donors) and (C) Env- target cells (293T-Luc). The mCherry reporter–modified donor T cells serve as a negative control. (D) Cytotoxicity of monospecific and bispecific anti-HIV CAR-T cells against Env+ and Env- target cells, (E) 293T-Luc, and (F) Raji-Luc. (G) IFN-γ secretion from monospecific and bispecific anti-HIV CAR-T cells upon challenge with Env+ target cells. (H) Cytotoxicity of multispecific anti-HIV CAR-T cells against Env+ and Env- target cells, (I) 293T-Luc, and (J) Raji-Luc. (K) IFN-γ secretion from multispecific anti-HIV CAR-T cells in the presence of Env+ target cells. n = 3 donors for monospecific versus bispecific CARs; n = 2 donors for bispecific versus trispecific CARs. The error bars represent ±SEM. Statistical analyses were performed by two-way ANOVA followed by Bonferroni posttest analysis performed for all comparisons (***P < 0.0001, **P < 0.001, and *P < 0.01; n.s., not significant).
Overall, the mD1.22-CAR (M1) was less potent than a conventional bispecific CAR (M13). To account for donor variability, we compared the CAR constructs within each donor group. The bispecific duoCAR (D13) was consistently more potent than conventional monoCARs (M1 or M13) despite their valency \((P < 0.05,\) pairwise Student’s t test). Given that the potency of the mD1.22-CAR increased upon the addition of the m36.4 domain as demonstrated by the M13 or D13 vectors indicates that both domains are active and capable of triggering CAR-mediated cytolysis of HIV-infected PBMCs. Conversely, the trispecific (D413S and D413L) and bispecific duoCAR-T cells (D13) exhibited similar potencies. Only modest improvements were seen for a trispecific CAR (D134\(\Delta\)) construct that is composed of the membrane-anchored C46 peptide and the bispecific M13 CAR.

**Fig. 4.** Anti-HIV duoCAR strongly induces T cell activation without impairing functionality. (A) Expression profile of the anti-HIV CARs used to determine CD107a and cytokine production upon challenge with HIV-infected donor-matched PBMCs. (B) CAR-T cell activation profile 6 hours after challenge with HIV-infected PBMCs. The graph shows the percentage of UTD T cell control or CAR-T cells producing the indicated T cell activation marker (CD107a) or intracellular cytokine (IL-2, IFN-γ, or double-positive for IL-2 and IFN-γ) in the presence of donor-matched PBMCs infected with Du422.1-IMC-LucR virus. The graph shows pooled data from combined triplicate wells of a single donor. (C) Exhaustion marker profile of anti-HIV CAR-T cells in the absence of antigen stimulation. The coexpression of Lag-3, PD-1, and Tim-3 on the surface of anti-HIV CAR-T cells in the absence of antigen stimulation was quantified by flow cytometry and analyzed in SPICE 6 software. The pie slices show the proportion of cells expressing 0, 1, 2, or 3 inhibitory receptors. The arc above the pie slice indicates the inhibitory receptor(s) expressed by effector T cells. Representative data are shown \((n = 2\) donors).
To further interrogate multispecific CAR constructs, we tested their anti-HIV effect against three IMCs expressing Env from HIV-1 clade C viruses (Du422.1, Du172.17, and Cap45) that are resistant or partially resistant to two bNAbs that target the CD4-binding site, VRC01 and 3BNC117, a potential limitation for mD1.22-based CARs. Among the CARs tested, the IMC expressing the VRC01/3BNC117-resistant Du422.1 Env discriminated the relative anti-HIV activity of duoCAR-T cells from other CAR constructs across four different donors. Statistical analysis was performed using a pairwise Student’s t test for each donor. Significance is considered $P < 0.05$.

In vitro HIV challenge of anti-HIV CAR-T cells with PBMCs infected with Env-IMC-LucR viruses expressing Env found worldwide. The figure shows averaged log HIV-1 inhibition relative to UTD T cells ($n$ = at least 3 donors tested in triplicate; $n$ = 2 donors tested in triplicate for AE.CNE55). Log inhibition of HIV-1 infection was calculated by the following formula using background-corrected relative light units (RLUs) obtained for each sample: log inhibition $= \log_{10}\left(\frac{RLU_{CAR}}{RLU_{UTD}}\right)$. Statistical analysis was performed by pairwise comparison of CAR constructs across multiple donors via a fixed-effects ANOVA model of background-corrected log inhibition of HIV-1 infection adjusting for donor and treatment. To account for multiple comparisons, we chose a more conservative $P$ value of $≤0.01$ for statistical significance.

Fig. 5. Broad and potent in vitro killing of HIV-infected PBMCs by multispecific duoCAR-T cells. (A) Schematic of the in vitro HIV-LucR PBMC killing assay. IMCs of HIV-1 encoding a heterologous HIV-1 Env glycoprotein and a Renilla luciferase cassette (generically referred to as Env-IMC-LucR or HIV-LucR) were used to infect donor-matched PBMCs followed by coculture with UTD T cell control or CAR-T cells for 7 days. After 7 days, the cocultures were lysed, and luciferase activity was assessed to quantify HIV-1 infection. (B) In vitro HIV challenge of anti-HIV CAR-T cells with PBMCs infected with Du422.1-IMC-LucR virus (VRC01/3BNC117-resistant Env, clade C). T, targets (Du422.1-IMC-LucR–infected PBMCs). Data shown are ±SD of triplicate wells for four different donors. Statistical analysis was performed using a pairwise Student’s t test for each donor. Significance is considered $P < 0.05$. (C) In vitro HIV challenge of anti-HIV CAR-T cells with PBMCs infected with Env-IMC-LucR viruses expressing Env found worldwide. The figure shows averaged log HIV-1 inhibition relative to UTD T cells ($n$ = at least 3 donors tested in triplicate; $n$ = 2 donors tested in triplicate for AE.CNE55). Log inhibition of HIV-1 infection was calculated by the following formula using background-corrected relative light units (RLUs) obtained for each sample: log inhibition $= \log_{10}\left(\frac{RLU_{CAR}}{RLU_{UTD}}\right)$ and then averaged across donors. Statistical analysis was performed by pairwise comparison of CAR constructs across multiple donors via a fixed-effects ANOVA model of background-corrected log inhibition of HIV-1 infection adjusting for donor and treatment. To account for multiple comparisons, we chose a more conservative $P$ value of $≤0.01$ for statistical significance.

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To further interrogate multispecific CAR constructs, we tested their anti-HIV effect against three IMCs expressing Env from HIV-1 clade C viruses (Du422.1, Du172.17, and Cap45) that are resistant or partially resistant to two bNAbs that target the CD4-binding site, VRC01 and 3BNC117, a potential limitation for mD1.22-based CARs. Among the CARs tested, the IMC expressing the VRC01/3BNC117-resistant Du422.1 Env discriminated the relative anti-HIV activity of duoCAR-T cells from other CAR constructs across four different donors (Fig. 5B). Further CAR-T titration studies revealed that even at lower E:T ratios, multispecific duoCAR-T cells sustained their ability to eliminate HIV-infected target cells better than monoCAR-T cells (Fig. S4). These data effectively demonstrated the synergistic effect of the mD1.22 and m36.4 domains within the bispecific monoCAR and duoCAR-T cells. In the presence of PBMCs infected with an IMC expressing Env from other VRC01-resistant HIV strains, anti-HIV duoCAR-T cells nearly completely eliminated HIV-infected donor PBMCs as measured by virus-encoded reporter gene expression relative to other CARs (Fig. S5), indicating that duoCAR-T cells, irrespective of their valency, are the most potent anti-HIV CAR architecture.

Last, we investigated the ability of anti-HIV CAR-T cells to eliminate PBMCs infected with different IMCs expressing Env from diverse HIV-1 clades representing the genetic diversity of the HIV Env found in different regions of the world. We selected a global Env representative for each clade tested based on the phylogenetic characterization of its envelope and neutralization profile as previously...
described (47). As shown in figs. S6 and S7, we demonstrate that anti-HIV duoCAR-T cells potently eliminated HIV-infected PBMCs infected with an IMC expressing Env from genetically distinct clades. The results from these studies are summarized as averaged log HIV-1 inhibition relative to UTD T cells from multiple donors in Fig. 5C. To determine statistical significance across multiple PBMC donors, we evaluated CAR constructs via ANOVA and chose a stricter P value cutoff of ≤0.01 to account for multiple pairwise comparisons. Overall, we found that D13 duoCAR-T cells were more potent than monoCAR-T cells for most Env-IMC-LucR viruses tested at the more stringent P value cutoff and consistent with the Student’s t test analysis for individual donors. Notably, the bispecific duoCAR-T cells (D13) were as potent as the trispecific duoCAR-T cells (D413L). However, the capacity of bispecific duoCAR-T cells to eliminate >99% of HIV infection in PBMC cocultures makes it difficult to discern whether the potency or breadth of CAR-T cells may be further increased by trispecific duoCAR-T cells. Figure S8 shows the percentage of HIV-1 inhibition via CAR-mediated elimination of infected cells by conventional monoCAR-T cells versus duoCAR-T cells. Anti-HIV duo-CAR-T cells comprising two functional CAR molecules, each with its own CD3ζ T cell signaling domain, were more effective at eliminating HIV-1 infection originating from PBMCs infected with an IMC expressing Env from distinct clades than CAR-T cells expressing a single CAR molecule, where the second polypeptide chain did not contain an intracellular CD3ζ T cell signaling domain (D413S or D413L versus D134ζ; Fig. 5C and fig. S8). This further supports the added impact of the two-molecule architecture to provide improved effector functionality over conventional CAR designs and the notion that both major gp120 targeting domains are active.

To better distinguish between suppression of infection and elimination of cells already infected with HIV by the anti-HIV CAR-T cells, we infected phytohemagglutinin-activated PBMCs with an Env-IMC-LucR virus encoding the env gene from a clade C strain, Dru242.1, which was potently inhibited by CAR-T cells in our standard in vitro HIV challenge assay shown in Fig. 5A. After 4 days of culture to enable initiation and spread of infection, we added the HIV integrase inhibitor Raltegravir to the test cell cultures to prevent further spread of the infection followed by the addition of autologous UTD T cells or a select group of CAR-T cells (M1, M13, and D13). Three days later, we quantified the LucR activity in the cocultures treated with and without Raltegravir and in the absence or presence of CAR-T cells. We postulated that if CAR-T cells were eliminating infected cells, then we would observe a further reduction of LucR activity in cell cultures containing infected cells treated with Raltegravir plus CAR-T cell effectors as compared to the cell cultures containing infected cells treated with Raltegravir alone (no added effectors) or with Raltegravir plus UTD T cells. In contrast to the added UTD T cell control, the addition of D13 CAR-T cells to HIV-infected PBMCs also treated with Raltegravir resulted in marked further reduction in LucR activity in contrast to single-domain monoCAR-T cells (M1) and slightly better than bispecific monoCAR-T cells (M13), demonstrating its capacity to eliminate HIV-infected cells (fig. S9). Together, we show that multispecific anti-HIV duoCAR-T cells not only reduce HIV infection but also can additionally target and eliminate HIV-infected cells.

Protection of multispecific anti-HIV duoCAR-T cells from HIV-1 infection

Next, we sought to understand whether the genetically modified CAR-T cells, which are CD4-enriched, were susceptible to HIV-1 infection. In contrast to reports that use flow cytometry to detect intracellular p24 as a measure of HIV-1 infection within CAR-T cells (11, 12), the intracellular Renilla luciferase that is produced upon infection of T cells by Env-IMC-LucR viruses is an extremely sensitive indicator of HIV infection upon the addition of its substrate to lysed cocultures. In addition, the Renilla luciferase reporter has a relatively short half-life in cells (~3 hours) and allows for quantification of HIV infection in CAR-T cells before cell killing occurs. Therefore, we directly challenged anti-HIV CAR-T cells with several Env-IMC-LucR viruses expressing Env from different HIV-1 clades to assess protection of multispecific CAR-T cells from HIV infection. After 7 days of infection, we quantified luciferase activity in the effector cells. Direct challenge of anti-HIV CAR effectors (both CD4+ and CD8+ T cells) revealed that in some donors, the mD1.22-CAR (M1) failed to fully suppress viral infection (Fig. 6). This loss of suppression was most pronounced in certain donors infected with Env-IMC-LucR viruses expressing BalL, NL4-3, SF162, Cap45, C.Du172.17, C.Du422.1, and AE.CNE55 HIV-1 Env glycoproteins. In contrast, HIV infection was not detected in all anti-HIV CAR-T cells that additionally contained m36.4 and/or the C46 peptide. This protective effect was essentially architecture independent (M13 versus D13) because the additional presence of the m36.4 domain was sufficient for CAR-T protection from infection in both constructs. This result proves that the m36.4 domain is active in the bispecific CAR and the duoCAR as demonstrated by its consistent ability to protect mD1.22-CAR-T cells from HIV-1 infection. Furthermore, we observed no immediate additional benefit with the addition of the C46 peptide, at least for the period of this short-term assay.

These data support other reports showing that CAR-T cells engineered with the CD4 receptor and domains such as the mD1.22 domain are susceptible to HIV infection (11, 12), and our findings demonstrate that the addition of the m36.4 domain appears to fully protect the mD1.22-CAR-T cells from infection by several IMCs containing Env found in different regions of the world.

Anti-HIV duoCAR-T cells display more potent suppression of in vivo HIV infection than anti-HIV monoCAR-T cells

After demonstrating the efficacy of anti-HIV duoCAR-T cells in vitro, we sought to assess their therapeutic potential by evaluating their efficacy in vivo. We adapted our hu-spl-PBMC-NSG mouse model to evaluate the in vivo efficacy of anti-HIV CAR-T cells (Fig. 7A). We selected three constructs that differ from each other by the number of binders (monospecific M1: mD1.22 versus bispecific M13: mD1.22-m36.4) or by the number of different CAR polypeptide molecules expressed within the bispecific CAR-T cells (M13: monoCAR versus D13: duoCAR). Of the 11 Env-IMC-LucR viruses tested in vitro, we chose the C.Du422.1-IMC-LucR virus, which encodes a VRC01/3BCNC117-resistant clade C env gene for our studies in the hu-spl-PBMC-NSG mice because it best distinguished the more potent in vitro activity of the duoCAR-T as compared with monoCAR-T cells. On day 0, the control UTD T cells, monoCAR-T cells (M1 and M3), or duoCAR-T cells (D13) were intrasplenically co-injected into nude mice spleens with donor-matched PBMCs infected with C.Du422.1-IMC-LucR. Seven days later, the mouse spleens were harvested, and the Renilla luciferase activity was measured to quantify HIV infection and to evaluate the in vivo killing efficacy. As shown in Fig. 7B, HIV infection in the hu-spl-PBMC-NSG mouse spleens was significantly more suppressed by the D13 duoCAR-T cells (97.5%, P = 0.004 for D13 versus M1 CAR and P < 0.001 for D13 versus M13 CAR) as
compared with the spleens of mice treated with the M1 monoCAR-T cells (42%) and M13 monoCAR-T cells (61%). Because both domains of the anti-HIV mono- and duoCAR constructs, mD1.22 and m36.4, are simultaneously expressed on the surface of the same CAR-T cell (fig. S10), and the anti-HIV activity increases with the addition of the m36.4 domain in both M13- and D13-treated mice, we can conclude that both domains contribute to the overall improved anti-HIV efficacy demonstrated by multispecific duoCAR-T cells in vivo. Immunophenotyping of the mouse spleens by flow cytometry revealed ample CD4+ T cells (Fig. 7C) and CD8+ T cells (Fig. 7D) during acute HIV infection in all groups despite the differential inhibitory effects upon HIV infection by the CAR-T cells. Furthermore, CAR-T cells were present in mouse spleens during acute HIV infection as detected by qPCR specific to the LVs encoding the anti-HIV CARs (Fig. 7E). To further investigate the ability of anti-HIV CAR-T cells to kill target cells, as well as look for signs of HIV persistence, we analyzed the mouse spleens for the presence of cell-associated total HIV DNA by real-time qPCR. Because cells infected

Fig. 6. Multispecific anti-HIV CAR-T cells are protected from HIV-1 infection. CD4-enriched anti-HIV CAR-T cells were directly challenged with Env-IMC-LucR viruses encoding env from strains (A) Bal (clade B, R5-tropic), (B) NL4-3 (clade B, X4-tropic), (C) SF162 (clade B, R5-tropic), (D) CAP45 (clade C, partially resistant to VRC01), (E) C.Du172.17 (clade C, resistant to VRC01), or (F) C.Du422.1 (clade C, resistant to VRC01/3BNC117). Anti-HIV CARs were also challenged with Env-IMC-LucR viruses expressing Env from representative HIV-1 clade AC (G) AC.246-F3, clade AE (H) AE.CNE8, a second clade AE (I) AE.CNE55, clade BC (J) BC.CH119.10 (partially resistant to VRC01), and clade G (K) GX1632_S2_B10. Donor-matched HIV-negative PBMCs (HIV− PBMC) and HIV-infected PBMCs (HIV+ PBMC) serve as negative and positive controls for the assay, respectively. The error bars shown are ±SD of three independent donors tested in triplicate. Statistical analysis was performed by pairwise comparison of CAR constructs across multiple donors via a fixed-effects ANOVA model of log-transformed RLU values adjusting for donor and treatment. Significance is considered P ≤ 0.01.
with HIV are injected in the animals in excess of anti-HIV CAR-T cells, lack of detection of HIV DNA would indicate CAR-T cell–mediated elimination of HIV-infected cells located in the mouse spleens. The more potent in vivo suppressive activity of the D13 duoCAR-T cells was also supported by the lack of detectable total HIV DNA in five of six mouse spleens, which is in contrast to the M1 and M13 monoCAR-T cell–treated mice where most animals harbored detectable cell-associated total HIV DNA in their spleens (four of five mice for the M1 CAR and four of six mice for the M13 CAR) (fig. S11). These results combined with our extensive in vitro data further confirm the increased potency of multispecific duoCAR-T cells and their ability to kill HIV-infected cells using the two-molecule duoCAR architecture as compared with CAR-T cells using the monoCAR architecture, even when recognizing the same epitopes.

Next, we tested whether trispecific D413L duoCAR-T cells displayed more potent in vivo suppressive activity of the D13 duoCAR-T cells was also supported by the lack of detectable total HIV DNA in five of six mouse spleens, which is in contrast to the M1 and M13 monoCAR-T cell–treated mice where most animals harbored detectable cell-associated total HIV DNA in their spleens (four of five mice for the M1 CAR and four of six mice for the M13 CAR) (fig. S11). These results combined with our extensive in vitro data further confirm the increased potency of multispecific duoCAR-T cells and their ability to kill HIV-infected cells using the two-molecule duoCAR architecture as compared with CAR-T cells using the monoCAR architecture, even when recognizing the same epitopes.

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duoCARs. Together, we demonstrate that candidate multispecific anti-HIV duoCAR-T cells persist and continuously suppress in vivo HIV infection for up to 30 days in mice after the initiation of infection during persistent infection.

**DISCUSSION**

The major obstacles for developing curative therapies for HIV/AIDS are the HIV latent reservoir and viral escape. Passive immunotherapies using a cocktail of bNAbs or engineered multivalent antibodies directed against the HIV envelope have shown improved neutralization, breadth, and protection in vivo (18, 24, 25, 48) but lack long-term effects. Genetic modification of T cells by LVs (5, 40–43) that express molecules to generate anti-HIV CAR-T cells (11–16) represents an approach that may enable long-term control of viral load in the absence of ART, due to the known long-term persistence and immunosurveillance properties of T cells (33).

In this study, we developed a collection of LVs encoding conventional and multispecific anti-HIV duoCARs. The goal of our study was to identify the most favorable architecture where multispecific anti-HIV CARs would have their most potent and broad anti-HIV effects for translation into the clinic. We hypothesized that the duoCAR architecture was important for sequential binding of epitopes, such as the co-receptor binding site, that becomes exposed after initial binding to the HIV envelope protein. We interrogated several putative constructs expressed by primary T cells using the duoCAR architecture and identified candidates that potently eliminated HIV-infected cells (up to 99% or more) in vitro. These studies identified that multiple binders targeting the HIV envelope were better than single targeting. The advantage of the duoCAR approach was demonstrated when gene-modified primary T cells were challenged with bNAb-resistant viruses, where anti-HIV duoCAR-T cells consistently displayed increased HIV inhibitory activity as a result of elimination of HIV-infected cells as compared to conventional monoCAR-T cells in vitro and in vivo. Furthermore, these CARs displayed very broad
inhibitory activity against PBMCs infected with IMCs expressing Env from different HIV-1 strains found worldwide. This is most likely attributed to the improved anti-gp120 binders targeting highly conserved epitopes used to engineer the CARs, mD1.22 and m36.4, which is consistent with our previous work using a bispecific multivalent antibody consisting of mD1.22 and m36.4, which, together, synergize to improve neutralization, breadth, and protection from HIV infection (27). A major advantage of using the mD1.22 and m36.4 domains is that in contrast to the wild-type CD4 receptor, these domains have been engineered for improved specificity and higher affinity for gp120 (27, 28). In the context of CARs, these improved binders exhibited no off-target cytotoxicity of MHC II expressing Raji cells and no apparent in vivo toxicities, signifying their safety for clinical use.

One limitation of our study is the lack of formal proof that CAR-T cells are targeting multiple epitopes. However, multiple targeting is highly likely given that the Env targeting domains are coexpressed on the surface of multispecific duoCAR-T cells, and these CAR-T cells consistently demonstrate more potent in vitro and in vivo HIV suppression and elimination of HIV-infected cells in multiple donors as compared with monoCAR-T cells containing one or more Env targeting domains. In the current study, we did not evaluate the capacity of anti-HIV CAR-T cells to suppress established HIV infection in our humanized mouse model. Adding the CAR-T cells at the time of the infection likely facilitated the capacity of the multispecific duoCAR-T cells to potently inhibit HIV infection. Clinical studies are needed to determine the effects of anti-HIV duoCAR-T cells in established HIV infection in humans.

Although multiple mouse models exist to study HIV-1, a major strength of our published hu-spl-PBMC-NSG mouse model of intrasplenic HIV infection (25, 32) is that it directly demonstrates the potency of CAR-T cells to eliminate productive HIV-infected cells populating in a lymphoid environment (e.g., spleen), which closely resembles what occurs in humans where lymphoid tissues are a major location of HIV replication. Last, it is important to note that the HIV-1 Env s from the bNAb-resistant strains used in the in vivo study often contain mutations in their CD4-binding site, further supporting the broad reactivity of anti-HIV duoCARs to delay or prevent viral escape. In subsequent studies using a sensitive total HIV DNA qPCR assay, we found that most of the duoCAR-T cell–treated mice had no detectable total cell-associated HIV DNA in their spleens, which was consistent with the potent in vivo anti-HIV activity demonstrated by the duoCAR-T cells. This result further suggests that duoCAR-T cells may be able to reduce the HIV reservoir size by eliminating HIV-infected cells as they arise. In our long-term in vivo study, we demonstrated that 1 month after infection, mice treated with anti-HIV duoCAR-T cells, in contrast to UTD–treated mice, effectively controlled HIV infection in four of five mice as determined by the extremely sensitive detection of luciferase activity. The long-term suppression of HIV infection in the mice treated with anti-HIV duoCAR-T cells, but not UTD–treated mice, indicates that the anti-HIV duoCAR-T cells did not select for a resistant strain of HIV. Moreover, the preservation of CD4+ T cells in duoCAR–treated groups with persistent HIV infection (30 days), but not the UTD–treated group, further supports this conclusion.

While our studies were under way, two independent groups reported similar findings for the potency of multispecific CAR-T cells against glioblastoma (49) and acute myeloid leukemia (50). In addition to these studies, we and others have reported that the multitargeting CAR-T cell approach effectively mitigates immune escape due to antigen loss in B cell malignancies when using dual-targeting (51), bivalent (52), and/or tandem (34) CAR-T cells. Hence, these studies provide additional support for our multispecific anti-HIV duoCAR-T cell design, which may be leveraged by others in the field to further improve the therapeutic effectiveness and capacity of HIV-specific CAR-T cells to prevent immune escape. Overall, we show that multispecific anti-HIV duoCARs demonstrated significantly increased functional activity as compared to previously described CARs that contain a single targeting domain such as the mD1.22-CAR, or which are similar to the reported CD4E CAR or conventional bispecific CARs (11, 14, 53, 54). Notably, duoCAR-T cells were found to be consistently superior to monoCAR-T cells in controlling HIV infection in vitro, including CAR-T cells that solely expressed mD1.22, which was further corroborated by in vivo studies using the hu-spl-PBMC-NSG mouse model. The superiority of duoCAR-T cells is an interesting finding and could have implications for CAR design beyond targeting HIV.

A major finding of this study is the demonstration that anti-HIV duoCAR-T cells are protected from HIV-1 infection. However, it was clear from the data that mD1.22-CAR-T cells were not highly protected from HIV-1 challenge. In contrast, bispecific and trispecific CAR-T cells were highly protected from HIV infection during direct challenge of CAR-T effector cells with the same Env-IMC-LucR viruses used for in vitro killing assays. Our results suggest that the m36.4 domain is a potent entry inhibitor and sufficient to protect CAR-T cells from infection. This is consistent with other reports in the field that use a single entry (11) or fusion inhibitor (12) to protect CD4-based CAR-modified immune cells. However, this domain serves two functions when placed in the context of a duoCAR: gp120-mediated killing and protection. We found that bispecific duoCAR-T cells (D13) were sufficient to control HIV infection long term and prevent CD4+ T cell depletion better than trispecific duoCAR-T cells (D413L). It is possible that a triscistronic or trioCAR composed of a three CAR molecule cassette could improve the functionality of the three anti-HIV binding domains.

Another finding of our study was the robustness and breadth of anti-HIV duoCAR-T cell suppression of HIV infection from 11 different Env-IMC-LucR viruses expressing globally represented Env glycoproteins. We demonstrated that anti-HIV duoCAR-T cells suppressed HIV infection by up to 99% or more for most viruses tested in vitro with no significant loss of CD4+ T cells in vivo. The reason for the potency of the anti-HIV duoCARs could be due to their unique architecture over conventional CARs. We found that the D13 CAR strongly induced T cell activation, leading to high CD107a expression and increased IFN-γ and IL-2 production when compared to single-molecule CARs, although the binding of other CAR types, independent of architecture, was similar. This strongly suggests that the higher differential expression of CD107a and cytokine secretion was due to the duoCAR architecture and not binding affinity per se. Examination of tonic signaling by assaying for the presence of multiple coexpressed exhaustion markers in the absence of antigen stimulation demonstrated that anti-HIV duoCAR-T cells maintained their functionality like conventional CAR-T cells, despite their increased CD107a and cytokine production. Multispecific duoCAR-T cells persisted in mice that effectively controlled HIV infection long term in vivo. These results suggest that such potent anti-HIV CAR-T cell effectors have the potential to persist long term in vivo in humans and maintain their anti-HIV effects.
Our study did not evaluate the ability of anti-HIV duoCAR-T cells to effectively migrate to sites of active HIV infection and target HIV latently infected cells after their reactivation. One of the current HIV eradication approaches, "shock and kill," exploits latency-reversing agents (LRAs) to reactivate the latent HIV reservoir and then expose HIV-infected cells to ARTs. Similar to LRAs, anti-HIV CAR-T cells themselves may reactivate the latent HIV reservoir by secreting cytokines (53). This inherent feature of CAR-T cells may be combined with LRAs to reawaken the latent HIV reservoir (shock), leading to its subsequent eradication (kill), which may offer a path toward functionally curing HIV infection. One could also consider armored anti-HIV duoCAR-T cells that secrete cytokines or other factors to further potentiate activation and killing of latently HIV-infected cells as well as increase the ability of CAR-T cells to migrate to distal anatomical sites of HIV infection. Another approach is the use of neutralizing antibodies to complement the cytopoxic anti-HIV duoCAR-T cell effects.

The goal of this work is to develop a curative therapy for HIV/AIDS. Anti-HIV duoCAR-T cell therapy aims to delay or indefinitely postpone the development of symptomatic disease. Moreover, such a therapeutic strategy could potentially eliminate HIV-infected cells including reactivated latent infected cells and thereby target the HIV reservoir, when used alone or in combination with the approaches described above. In conclusion, our data strongly support multispecific anti-HIV duoCAR-T cells as powerful new therapeutic candidates in which further clinical studies are needed to evaluate their safety and efficacy.

MATERIALS AND METHODS

Study design

The goal of our study was to identify a multispecific anti-HIV CAR construct that demonstrated the best potency, breadth, and resistance to HIV for translation into the clinic. We hypothesized that multispecific duoCAR-T cells would demonstrate better potency and improved reactivity against diverse HIV viruses than conventional monoCAR-T cells, while simultaneously protecting the CAR-modified T cells from HIV infection. In our study, we screened multiple conventional and multispecific anti-HIV CAR-T cell constructs for CAR expression, cytolytic activity, and IFN-γ secretion followed by more comprehensive in vitro and in vivo HIV challenge studies using replication-competent IMCs of HIV that express different env genes (Env-IMC-LucR viruses) to quantify HIV infection at the end of the study relative to UTD T cells. The in vitro HIV challenge studies were conducted for six different CAR vectors, in cells from at least three independent donors (except for AE.CNE55.LucR, n = 2 donors) tested in triplicate to achieve statistical power. We identified the best CAR constructs for in vivo studies based on their in vitro killing efficacy and resistance to HIV infection for 11 different Env-IMC-LucR viruses expressing clade-specific EnvRS represented worldwide. We further interrogated the in vivo efficacy of the multispecific duoCAR and control monoCAR vectors using a humanized NSG model of HIV infection that supports robust and rapid HIV infection within 7 days after intrasplenic injection of HIV-infected human PBMCs. All the mouse studies were approved by the Institute for Animal Studies and the Institutional Review Board at Albert Einstein College of Medicine in compliance with the human and animal experimentation guidelines of the U.S. Department of Health and Human Services and in adherence to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The humanized mice were treated with anti-HIV CAR-T cells generated from different donors (Fig. 7, n = 1 donor; Fig. 8, 7 days of HIV infection, n = 2 donors; Fig. 8, 30 days of HIV infection, n = 1 donor), and four to six mice per treatment cohort were selected (nonblinded) for statistical power. At the end of the study, the mice were euthanized, and their spleens were harvested for the following endpoint analyses: (i) Renilla luciferase activity to quantify the HIV infection, (ii) flow cytometry detection of human CD4+ and CD8+ T cells, (iii) CAR-T cell persistence by qPCR, and (iv) HIV persistence by detecting total cell-associated HIV DNA by qPCR in mouse spleens (data collected only for Fig. 7). Data outliers were excluded when appropriate. Primary data for the in vitro and in vivo HIV challenge assays are reported in data file S1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 7.04 or SAS version 9.4. For CTL assays, a two-way ANOVA was performed followed by Bonferroni posttest. For in vitro HIV challenge studies, a pairwise Student’s t test was performed to evaluate differences between different CAR constructs for individual donors as follows: M1 versus M13, M1 versus D13, M13 versus D13, D13 versus D413S, and D13 versus D413L. P < 0.05 was considered significant. For Fig. 5C, CAR constructs were evaluated by pairwise comparison of select CAR groups (M1 versus M13, M1 versus D13, M13 versus D13, D13 versus D413S, and D13 versus D413L) across multiple donors via a fixed-effects ANOVA model of background-corrected log inhibition of HIV-1 infection adjusting for donor and treatment. For Fig. 6, CAR constructs were evaluated by pairwise comparison across multiple donors via a fixed-effects ANOVA model of log-transformed RLU values adjusted for donor and treatment. To account for multiple comparisons, we chose a more conservative P value of ≤0.01 for statistical significance. For in vivo studies in Fig. 7, overall and pairwise group differences in log-transformed values were evaluated via ANOVA and Dunnett’s test. For in vivo studies in Fig. 8, statistical significance was determined using a one-way ANOVA with Tukey’s posttest analysis. Differences were considered statistically significant when P ≤ 0.01 unless stated otherwise.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. 1. Flow cytometry gating strategy for detection of anti-HIV CARs on the surface of CD4+ and CD8+ T cells.

Fig. 2. Ratio of CD4+ and CD8+ T cells for different donors used in the study.

Fig. 3. In vitro killing efficacy of multispecific anti-HIV CAR-T cells against PBMCs infected with an IMC expressing Env from HIV-1 clade B viruses.

Fig. 4. Multispecific anti-HIV CAR-T cells exhibit superior in vitro killing efficacy at low E:T ratios.

Fig. 5. In vitro killing efficacy of multispecific anti-HIV CAR-T cells against PBMCs infected with an IMC expressing Env from two HIV-1 clade C viruses.

Fig. 6. In vitro killing efficacy of multispecific anti-HIV CAR-T cells against PBMCs infected with an IMC expressing Env from HIV-1 clade AC, BC, and G viruses.

Fig. 7. In vitro killing efficacy of multispecific anti-HIV CAR-T cells against PBMCs infected with an IMC expressing Env from HIV-1 clade AE virus.

Fig. 8. Percentage of HIV-1 inhibition for conventional and multispecific anti-HIV CAR-T cells tested against PBMCs infected with 11 different Env-IMC-LucR viruses encoding genetically diverse env genes.

Fig. 9. In vitro elimination by anti-HIV CAR-T cells of PBMCs infected with an IMC expressing Env from HIV-1 clade C virus.

Fig. 10. Simultaneous expression of the mD1 22 and m36.4 domains on the surface of mono- and duoCAR-T cells.
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Multispecific anti-HIV duoCAR-T cells display broad in vitro antiviral activity and potent in vivo elimination of HIV-infected cells in a humanized mouse model

Kim Anthony-Gonda, Ariola Bardhi, Alex Ray, Nina Flerin, Mengyan Li, Weizao Chen, Christina Ochsenbauer, John C. Kappes, Winfried Krueger, Andrew Worden, Dina Schneider, Zhongyu Zhu, Rimas Orentas, Dimiter S. Dimitrov, Harris Goldstein and Boro Dropulic

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Driving a new HIV therapy forward

CAR T cells, which are now associated with cancer therapy, were previously investigated as a treatment for HIV almost 30 years ago. Now wielding new technology and biological knowledge, Anthony-Gonda et al. report a series of multispecific anti-HIV CARs. These CARs target different portions of the HIV envelope protein and were able to eliminate diverse strains of HIV in vitro, even those that are resistant to potent broadly neutralizing antibodies. The CAR T cells are resistant to HIV infection and were able to control HIV in a humanized mouse model. The persistent surveillance capabilities of CAR T cells suggest that this therapy may one day be able to eradicate HIV in an infected person.