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Multi-specific anti-HIV duoCAR-T cells display broad antiviral activity and potent elimination of HIV-infected cells *in vivo*

Kim Anthony-Gonda^{1,†}, Ariola Bardhi^{2,†}, Alex Ray², Nina Flerin², Mengyan Li², Weizao Chen³, Christina Ochsenbauer⁴, John C. Kappes^{4,5}, Winfried Krueger¹, Andrew Worden¹, Dina Schneider¹, Zhongyu Zhu¹, Rimas Orentas¹, Dimiter S. Dimitrov^{6,*}, Harris Goldstein^{2,*}, Boro Dropuli^{1,**}

¹Lentigen, a Miltenyi Biotec Company, Gaithersburg, MD 20878, USA

²Department of Microbiology and Immunology and Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461, USA

³Protein Interactions Section, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, Maryland, 21702, USA

⁴Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA

⁵Birmingham Veterans Affairs Medical Center, Research Service, Birmingham, AL 35294, USA

⁶Center for Antibody Therapeutics, University of Pittsburgh, Pittsburgh, PA 15261, USA

Abstract

Adoptive immunotherapy using chimeric antigen receptor gene-modified T cells (CAR-T) has made significant contributions to the treatment of certain B-cell malignancies. Such treatment modalities also show promise for the development of a single treatment for HIV/AIDS and obviating the need for long-term anti-retroviral drug therapy. Here we report the development of HIV-1 based lentiviral vectors that encode chimeric antigen receptors (CAR) 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 multi-specific anti-HIV duoCARs confer primary T cells with the capacity to potentially reduce cellular HIV infection by >99% *in vitro* and >97% *in vivo*, while simultaneously protecting them from

*Corresponding author. **Senior corresponding author. To whom correspondence should be addressed: boro.dropuli@lentigen.com; Dr. Boro Dropuli, Lentigen, a Miltenyi Biotec Company, 910 Clopper Rd, Gaithersburg, MD 20878, Phone: 301-527-4234; harris.goldstein@einstein.yu.edu; Dr. Harris Goldstein, Albert Einstein College of Medicine, 130 Morris Park Avenue, Bronx, NY 10461, Phone: 718-43-2156; dsd116@pitt.edu; Dr. Dimiter Dimitrov, University of Pittsburgh, 3550 Terrace Street, Pittsburgh, PA 15261, Phone: 412-647-9763.

[†]These authors contributed equally to the work.

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genetically diverse HIV-1 strains. Interestingly, the genetically modified CAR-T cells also potently eliminated PBMC infected with broadly neutralizing antibody-resistant HIV strains, including VRC01/3BNC117-resistant HIV-1. Furthermore, multi-specific 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. These data suggest that multi-specific anti-HIV duoCAR-T cells could be an effective approach for the treatment of patients with HIV-1 infection.

Introduction

Adoptive immunotherapy using chimeric antigen receptor modified T-cells (CAR-T) has shown unprecedented success for the treatment of refractory B-cell malignancies that express CD19, CD20, and 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).

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 envelope specific epitopes (8) and recurrence of viremia (9). First generation anti-HIV CAR approaches used the CD4 receptor as the targeting domain coupled with the CD3 ζ signaling domain to kill productively HIV-infected cells. However, later it was revealed that CD4-based CARs render the gene-modified T cells susceptible to HIV infection (10, 11). To overcome this limitation, several strategies to improve HIV-specific CAR-T cells were tested, including design of bispecific CAR-T cells (10), or CAR-T cells expressing a CD4-zeta CAR in combination with either a gp41-derived fusion inhibitor (11), or CCR5 ablation (12). Moreover, anti-HIV CARs have been re-engineered with 4-1BB or CD28 costimulatory signaling motifs to improve their *in vivo* persistence (13) and potency when combined with soluble broadly neutralizing antibodies (bNAbs) that recognize non-redundant gp120/gp41 epitopes (10, 12, 14, 15).

An alternative approach to using 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 (16); and unlike the CD4 receptor, a single bNAb cannot fully neutralize all HIV isolates (17, 18). Interestingly, recent clinical trials using bNAb monotherapies with VRC01, 3BNC117, or 10-1074 led to viral rebound upon ART interruption, but an antibody composed of multiple envelope-specific scFvs showed improved protection *in vivo* (19–23). 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 (24). 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* (25). Here we report the development of HIV-1 based lentiviral vectors (LV) encoding multi-specific anti-HIV CARs using a unique architecture, termed here duoCAR, where two CAR molecules consisting of multiple anti-HIV binders, including mD1.22, m36.4, and C46, are expressed on the surface of T cells from a single lentiviral vector. DuoCAR-modified T cells target multiple highly conserved sites on the HIV-1 envelope glycoprotein (Env) and effectively kill HIV-1 infected cells that express Env on their surface. In addition to their HIV envelope targeting and killing properties, we found that anti-HIV duoCAR-T cells were also simultaneously resistant to infection by several globally found HIV-1 strains.

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. The mD1.22 domain is derived from the human CD4 receptor and targets the highly conserved CD4 binding site on HIV gp120. Additionally, it 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 (26). The m36.4 domain is an affinity-matured, engineered human heavy chain only (V_H) antibody domain (27) 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 (28). When combined to form a multivalent, bispecific soluble antibody, these domains synergistically neutralized diverse HIV strains (24, 26, 27). The C46 peptide inhibits HIV infection at the level of viral fusion and is similar to the 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's membrane (29, 30).

Herein, we show potent *in vitro* and *in vivo* anti-HIV effects of multi-specific anti-HIV duoCAR-T cells that simultaneously kill productively HIV-infected cells 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 (31)), multi-specific 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 anti-retroviral therapy, with the potential to significantly delay or postpone disease progression. When combined with strategies that target the latent reservoir, anti-HIV duoCAR-T cells could offer a path towards functionally curing HIV infection.

Results

Design and functional characterization of multi-specific anti-HIV CAR-T cells

We generated HIV-1 based lentiviral vectors encoding mono-, bi-, and tri-specific anti-HIV CARs as shown in Fig. 1A. The structures of the resulting expressed anti-HIV CARs are depicted in Fig. 1B. Monospecific CAR lentiviral vector constructs were generated by fusing the mD1.22 (designated “1”) or m36.4 (designated “3”) domain in-frame to a single-molecule CAR architecture previously described by our group that consists of a CD8 ectodomain (EC) followed by the CD8 transmembrane domain (TM), 4-1BB costimulatory

domain to support CAR-T persistence, and a CD3 ζ T cell signaling chain (32). 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 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 (33)). However, the gp120 binding site for certain highly conserved neutralizing antibodies may not be exposed prior to 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 (27, 34, 35), we also designed lentiviral vectors 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 lentiviral vector to allow for simultaneous expression of both CARs in a single T cell. We therefore constructed a LV-encoding bispecific duoCAR that contained the mD1.22-CAR and the m36.4-CAR (D13).

To further potentiate multi-targeting, 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 (36, 37). For trispecific CARs, we considered that since 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 (10). Therefore, we tested a short (“S” for 3x G₄S linker) and long (“L” for 5x G₄S linker) flexible linker between the C46 peptide (4) and mD1.22 domain (1) in trispecific duoCARs to generate D413S and D413L, respectively. Lastly, 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 (D134) fused together in a different orientation and without the CD3 zeta signaling motif on the C46 peptide. Therefore, the membrane-anchored C46 peptide should lack effector function. The putative target site for each anti-HIV binder on the HIV Env trimer is illustrated in Fig. 1C and the general approach taken to generate anti-HIV CAR-T cells with the lentiviral vectors is shown in Fig. 1D (also refer to Materials and Methods).

Multi-specific anti-HIV duoCARs are efficiently expressed on the surface of primary human T-cells

Previously, we have shown that HIV-1 based lentiviral vectors safely and efficiently deliver a HIV-specific genetic payload to primary human T cells for the treatment of HIV-1 infected patients (5, 38–41). To test whether anti-HIV CARs could be efficiently delivered and expressed on the surface of primary T cells by lentiviral vectors, we transduced HIV-naïve

CD4⁺ and CD8⁺ T cells with the anti-HIV CAR lentiviral vectors and evaluated the cells for CAR expression by flow cytometry detecting either the mD1.22 domain (Fig. 2A) or C46 peptide (Fig. 2B). A figure exemplifying the gating strategy is provided in Fig. S1. As exemplified in Fig. 2A, 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. 2C). For monospecific and bispecific CAR-T cells, expression was determined via the increase in mD1.22 expression on the surface of transduced CD4⁺ and CD8⁺ T cells as compared to the untransduced control cells (UTD, refer to Fig. 2A). To detect trispecific CARs (D134, D413S, and D413L), we utilized the gp41-derived C46 peptide engineered into these constructs as a unique molecular tag recognized by the 2F5 antibody. As exemplified in Fig. 2B, trispecific CARs were detected by 2F5 on the surface of CD4⁺ and CD8⁺ T cells across multiple donors (Fig. 2D).

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/gp41_{ecto} derived from a clade B isolate (27)), as demonstrated by an increase in the amount of Env captured by total T cells containing CAR-modified T cells, indicating that the targeting domains were active on the surface of modified T cells and not altered by the binder architecture (Fig. 2E). We found that the m36.4-CAR (M3) was detectable on the surface of genetically-modified T cells by this method and bound to gp140 in the presence of the CD4 receptor, but to a lesser extent in its absence 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). This data indicates 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 (35). Taken together, we demonstrate that anti-HIV CARs are expressed by the modified T cells and capable of binding to the HIV envelope protein independently and sequentially which is consistent with our previous findings with the soluble binding domains (26, 27, 35).

Multi-specific 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 HEK 293T cells, designated 293T-Env-Luc (Env+), which was then shown to constitutively and highly express Env glycoprotein derived from a 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 non-functional 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 (i.e., lack of cytolytic activity) of CAR-T cells, which after transduction, contains a population of CAR-modified and unmodified T cells. We subsequently cultured the total anti-HIV CAR-T cell population with Env+ or Env- target cells at varying E:T ratios. Twenty-four hours later, co-cultures were lysed, and reduction of luciferase activity in the

presence of CARs was quantified to determine CAR-mediated cytotoxicity as described in Materials and Methods. As a control, we also constructed a CD4-CAR and compared its function to the mD1.22-CAR. As shown in Fig. 3B, the mD1.22-CAR was as active as the conventional CD4-CAR and demonstrated no off-target killing of Env⁻ targets (Fig. 3C). The specificity of CAR-mediated killing against Env⁺ targets was shown using untransduced T cells (UTD) or T cells modified to express a mCherry reporter, for which no cytotoxicity was observed. Comparative analysis revealed that among monospecific CARs, the mD1.22-CAR (M1) outperformed the m36.4-CAR (M3) (Fig. 3D). The conventional bispecific CAR (M13) was more potent than monospecific CARs (M1 or M3). Interestingly, the bispecific duoCAR (D13) was significantly more potent than effectors expressing a conventional single (M1, $P < 0.0001$) or bispecific single molecule CAR (M13, $P < 0.01$, Fig. 3D) despite lower transduction efficiency for this CAR among different donors. This is consistent with our previous work in which we demonstrate that m36.4 synergizes with the mD1.22 domain to bind to the HIV envelope (26, 27, 35). As shown in Fig. 3E, these CARs exhibited no off-target killing of targets that lacked HIV Env expression. Moreover, anti-HIV CAR-T cells did not kill Raji-Luc Env⁻ targets, which express MHC class II molecules, and therefore may interact with the mD1.22 domain, a mutant D1 domain derived from the CD4 receptor (Fig. 3F). The mD1.22 domain has been engineered for enhanced specificity for the HIV envelope and this result demonstrates the high specificity of mD1.22-based CAR-T cells. Lastly as shown in Fig. 3G, culturing anti-HIV CAR-T cells with Env⁺ target cells (E:T ratio 10:1) triggered IFN- γ secretion while no cytokine production was detected in the presence of untransduced T cells (UTD) or T cells modified to express the mCherry reporter. Minimal cytokine production was induced in the presence of Env⁻ targets and no killing was observed. Taken together, we demonstrate that monospecific and bispecific anti-HIV CAR-T cells exert powerful and specific anti-HIV activity.

Next, we screened trispecific CARs for their ability to specifically kill HIV Env⁺ targets. As shown in Fig. 3H, the trispecific duoCAR vector, D413S, was significantly more cytotoxic on target cells than trispecific CAR vectors, D134 and D413L, and bispecific CAR vectors, M13 and D13. As expected, bispecific and trispecific CARs maintained their specificity with no off-target killing observed on 293T-Luc and Raji-Luc Env⁻ targets, respectively (Fig. 3I, J). Trispecific anti-HIV duoCAR-T cells also produced significant amounts of IFN- γ when challenged with Env⁺ cells as compared to Env⁻ targets (Fig. 3K). Taken together, multi-specific CAR-T cells exert potent and specific cytotoxic effects against HIV Env expressing cells.

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 Th1 cytokine production. We hypothesized that the robust killing of HIV Env⁺ cells by anti-HIV duoCAR-T cells was due to its two-molecule CAR architecture. To test this hypothesis, we investigated CD107a and intracellular Th1 cytokine production upon exposure to HIV-infected target cells. To better assess the differential effects of the conventional CAR versus duoCAR architecture, we generated a more physiological relevant

target cell population by infecting human PBMC from a healthy donor with a HIV-1 replication competent infectious molecular clone (IMC) expressing the C.Du422.1 Env glycoprotein and *Renilla* luciferase reporter (24). Seven days after infection, infected PBMC were co-cultured 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, as 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 level 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 2-3-fold increase in IFN-gamma and IL-2 production as compared to either single molecule mono- or bispecific CAR-T cells (M1 or M13, respectively). Interestingly, M13 was inferior to M1 for CD107a expression, IL-2, and IFN-gamma production (Fig. 4B) even though 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, even though the D13 CAR showed similar binding as both M1 and M13 CARs, the level of CD107a, IFN-gamma, and IL-2 production was increased in T cells expressing the duoCAR. Although this data is observational, the result suggests that the differential expression of CD107a, IFN-gamma, and IL-2 are 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 level 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) may be characterized by the presence of multiple cell surface expressed co-inhibitory receptors (e.g., PD-1, Lag-3, and Tim-3) (42). We determined that duoCAR-T cells displayed no appreciable tonic signaling in the absence of antigen demonstrated by a lack of expression of exhaustion markers as compared to the conventional CAR-T cells (Fig. 4C). These data show that the duoCAR architecture does not impair T cell functionality and is similar to conventional CAR-T cells. Taken 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 PBMC 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 utilizing replication-competent infectious molecular clones (IMC) expressing different Envs 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 (43, 44); and, when co-cultured 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 PBMC at an E:T ratio of 1:1 for seven 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 multi-specific 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 as the CD4-CAR in our CTL assays, we used the mD1.22-CAR (M1) as a control for our *in vitro* HIV challenge assay to further interrogate our multi-targeting duoCAR approach. As shown in Fig. S3, anti-HIV CAR-T cells potentially eliminated donor PBMC targets infected with an IMC expressing Env from clade B viruses NL4-3 (X4-tropic), BaL (R5-tropic), and SF162 (R5-tropic). Overall, the mD1.22-CAR (M1) was less potent than a conventional bispecific CAR (M13). Interestingly, the bispecific duoCAR (D13) was consistently more potent than conventional monoCARs (M1 or M13) despite their valency. 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, clearly indicates that both domains are active and capable of triggering CAR-mediated cytolysis of HIV-infected PBMC. Conversely, the trispecific (D413S, D413L) and bispecific duoCAR-T cells (D13) exhibited similar potencies. Only modest improvements were seen for a trispecific CAR (D134) construct that is comprised of the membrane anchored C46 peptide and the bispecific M13 CAR.

To further interrogate multi-specific CAR constructs, we tested their anti-HIV effect against three IMC expressing Env from HIV-1 clade C viruses (Du422.1, Du172.17, and Cap45) that are resistant or partially-resistant to two broadly neutralizing antibodies 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 clearly 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, multi-specific duoCAR-T cells sustained their ability to eliminate HIV-infected target cells better than that of monoCAR-T cells (Fig. S4). More importantly, this 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 PBMC infected with IMC expressing Envs from other VRC01-resistant HIV strains, anti-HIV duoCAR-T cells nearly completely eliminated HIV-infected donor PBMC 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.

Finally, we investigated the ability of anti-HIV CAR-T cells to eliminate PBMC infected with IMC expressing Env from distinct 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 (45). As shown in Fig. S6 and S7, we demonstrate that anti-HIV duoCAR-T cells potentially eliminated HIV-infected PBMC infected with IMC expressing Envs from genetically distinct clades. The results from these studies are summarized as log HIV-1 inhibition relative to UTD control in Fig. 5C. 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 co-cultures makes it difficult to discern whether the potency or breadth of CAR-T cells may be further increased by trispecific duoCAR-T cells. Fig. S8 shows the percentage of HIV-1 inhibition via CAR-mediated elimination of infected cells by conventional monoCAR-T cells versus duoCAR-T cells. Interestingly, anti-HIV duoCAR-T cells comprising two functional CAR molecules, each with their own CD3 ζ signaling domain, were more effective at eliminating HIV-1 infection originating from PBMC infected with IMC expressing Env from distinct clades than CAR-T cells expressing a single CAR molecule, wherein the second polypeptide chain did not contain an intracellular CD3 ζ signaling domain (D413S or D413L versus D134 ; 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 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 PHA-activated PBMC with an Env-IMC-LucR virus encoding the *env* gene from a clade C strain, Du422.1, which was potentially inhibited by CAR-T cells in our standard *in vitro* HIV challenge assay shown in Fig. 5A. After four days of culture to enable initiation and spread of infection, we added the HIV integrase inhibitor raltegravir to some wells to prevent further spread of the infection followed by addition of autologous untransduced T cells (UTD) or a select group of CAR-T cells (M1, M13, and D13). Three days later, we quantified the LucR activity in the co-cultures treated with and without raltegravir and in the absence or presence of CAR-T cells. We postulated that if CAR-T cells were indeed eliminating infected cells, then we would observe a further reduction of LucR activity in wells containing infected cells treated with raltegravir plus CAR-T cell effectors below that of wells containing infected cells treated with raltegravir alone (no added effectors) or with raltegravir plus untransduced T cell (UTD) control; and, therefore should represent the elimination of cells already infected with HIV by CAR-T cells. In contrast to the added UTD control, the addition of D13 CAR-T cells to HIV-infected PBMC 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). Taken together, we show that multi-specific anti-HIV duoCAR-T cells potentially reduce cellular infection and eliminate cells infected with Env-IMC-LucR viruses encoding genetically distinct Env clades and the importance of duoCAR architecture functionality to enable these potent effects.

Protection of multi-specific 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 (10, 13), 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 addition of its substrate to lysed co-cultures. 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 multi-specific CAR-T cells from HIV infection. After seven 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 and significant in certain donors infected with Env-IMC-LucR viruses encoding BaL, NL4-3, SF162, Cap45, C.Du172.17, C.Du422.1, and AE.CNE55 HIV-1 Env proteins. 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 architecture-independent (M13 versus D13) as 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 indeed active in the bispecific CAR as well as 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 (10, 11), and our findings demonstrate that addition of the m36.4 domain appears to fully protect the mD1.22-CAR-T cells from infection by several IMC 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*. Previously, we described a humanized NSG mouse model (hu-spl-PBMC-NSG) that supports robust HIV infection after infection of intrasplenically injected PBMC with an Env-IMC-LucR virus that can be monitored by quantifying luciferase activity in the spleen of the mouse as early as one week after infection (46). We used this humanized mouse model to demonstrate that an antibody (LSEVh-LS-F) engineered with the same specificities as our CAR-T cells, mD1.22 and m36.4, mobilized ADCC activity and effectively eliminated cells infected with IMC expressing a broad range of Env glycoproteins *in vivo* via a NK cell-dependent mechanism (24). 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. Since CAR-T cells have been shown to persist in patients for years (4–7), we postulated that development of a CAR-T construct expressing the very broad specificities of mD1.22 and m36.4 would be a better therapeutic candidate for long-term control of HIV-1 infection in patients.

We adapted our hu-spl-PBMC-NSG mouse model to evaluate the *in vivo* efficacy of anti-HIV CAR-T cells (Fig. 7A). To evaluate the *in vivo* superiority of the two-molecule duoCAR architecture, 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 VRC01/3BNC117-resistant clade C *env* gene for our studies in the hu-spl-PBMC-NSG mice since it best distinguished the more potent *in vitro* activity of the duoCAR-T as compared to monoCAR-T cells. We hypothesized that the robust killing of HIV-infected PBMC by anti-HIV duoCAR-T cells *in vitro* would be recapitulated *in vivo* as a result of its more effective two-molecule CAR architecture. On day 0, the control untransduced T cells (UTD), monoCAR-T cells (M1 and M3), or duoCAR-T cells (D13) were intrasplenically co-injected into mice spleens with donor matched C.Du422.1-IMC-LucR virus-infected PBMC. Seven days later, the mice spleens were harvested and the *Renilla* luciferase activity was measured to quantify HIV infection and to evaluate the *in vivo* killing efficacy by the mono- and duoCAR constructs as compared to control untransduced T cells. 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 vs. M1 CAR and $p < 0.001$ for D13 vs. M13 CAR) as compared to 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 multi-specific duoCAR-T cells *in vivo*.

Immunophenotyping of the mice spleens by flow cytometry showed no adverse effects demonstrated by M1, M13 and D13 CAR-T cells in depleting the human CD4⁺ T cells (Fig. 7C) or CD8⁺ T cells (Fig. 7D). CAR-T cells persisted during acute HIV infection as detected by quantitative PCR specific to the lentiviral vectors 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. Since cells infected 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. Significantly, 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 5 out of 6 mice 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 (4 out of 5 mice for the M1 CAR and 4 out of 6 mice for the M13 CAR) (Fig. S11). These results combined with our extensive *in vitro* data further confirms the increased potency of multi-specific duoCAR-T cells, and their ability to kill HIV-infected cells, using the two-molecule duoCAR architecture as compared to 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* suppression of HIV infection as compared to the bispecific D13 duoCAR-T cells after 7 days and 30 days of HIV infection using our hu-spl-PBMC-NSG mouse model (Fig. 8A). The bispecific D13 duoCAR-T cells and trispecific D413L duoCAR-T cells or control UTD cells were co-injected into the mice spleens with donor matched C.Du422.1-IMC-LucR-

infected PBMC on day 0. Bispecific D13 duoCAR-T cells and trispecific D413L duoCAR-T cells potently suppressed HIV infection in the spleens evaluated 7 days (Fig. 8B) and 30 days (Fig. 8C) after intrasplenic injection as compared to the control mice intrasplenically injected with control untransduced T cells. Due to the potent *in vivo* activity of both CAR constructs, we could not discern an advantage of bispecific as compared to trispecific recognition. During acute HIV infection, the percentage of CD4⁺ T cells were maintained to a similar extent within the uninfected mice when treated with D13 bispecific duoCAR or D413L trispecific duoCAR-T cells (Fig. 8D), indicating the absence of off-target elimination of CD4⁺ T cells. One striking finding was that in untreated mice with persistent HIV infection, the human CD4⁺ T cells were almost completely depleted by the HIV-1 infection which was prevented in mice treated with D13 bispecific duoCAR ($P = 0.03$) or D413L trispecific duoCAR-T cells (Fig. 8E). The percentage of human CD8⁺ T cells were modestly elevated in acutely HIV-infected animals receiving CAR-T treatment in contrast to control treated mice (Fig. 8F). However, due to the significant loss of human CD4⁺ T cells in mice with persistent HIV infection, the UTD-treated mice yielded a higher percentage of human CD8⁺ T cells in contrast to a lower percentage of human CD8⁺ T cells in duoCAR-T cell treated mice (Fig. 8G). These results demonstrate that a single dose of duoCAR-T cells was highly efficacious in controlling HIV-1 infection even 30 days after the initiation of infection. This correlates with the persistence of anti-HIV duoCAR-T cells in the spleens of HIV-infected mice for 7 days (Fig. 8H) and 30 days after intrasplenic injection (Fig. 8I) as detected by quantitative PCR specific to the lentiviral vectors encoding the anti-HIV duoCARs. Taken together, we demonstrate that candidate multi-specific 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 while mitigating CD4⁺ T cell depletion during this period.

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 broadly neutralizing antibodies or engineered multivalent antibodies directed against the HIV envelope have shown improved neutralization, breadth, and protection *in vivo* (17, 23, 24, 47). Anti-HIV CAR-T cells 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. This requires the genetic modification of T cells to express CAR effectors that not only target diverse strains of HIV envelope glycoproteins on the surface of productively-infected cells, but do so without premature exhaustion, so to mediate long-term immunosurveillance of the HIV reservoir, killing HIV envelope expressing cells as they arise.

In this study, we developed a collection of lentiviral vectors encoding conventional and multi-specific anti-HIV duoCARs. The goal of our study was to identify the most favorable architecture where multi-specific anti-HIV CARs would have their most potent and broad anti-HIV effects for translation into the clinic. We interrogated several putative constructs expressed by primary T cells and identified candidates that potently eliminated HIV-infected cells (>99%) while conferring resistance to the same cells when challenged with IMC containing different HIV-1 clade-specific Env proteins. These studies identified that multiple

binders targeting the HIV envelope were better than single targeting. We demonstrated in several different *in vitro* assays that indeed engineering CARs with two major gp120 targeting domains, rather than a single domain, combined with a two-molecule architecture, led to synergistic anti-HIV effects and improved CAR-T functionality which was further corroborated *in vivo*.

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 co-expressed on the surface of multi-specific 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 to monoCAR-T cells containing one or more Env targeting domains.

We hypothesized that the duoCAR architecture was important for sequential binding of epitopes, such as the coreceptor binding site, that becomes exposed after initial binding to the HIV envelope protein. Of most interest was our demonstration that CAR-T cells have greater functional activity when they are comprised of two functional CAR molecules with transduction activity, rather than a single functional CAR and the second molecule lacking an intracellular CD3 zeta domain. The advantage of this approach was clearly 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*. In subsequent studies using a sensitive total HIV DNA qPCR assay, we found that the majority of 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.

Although multiple mouse models exist to study HIV-1, a major strength of our published intrasplenic HIV-PBMC-NSG mouse model (24, 46) is that it directly demonstrates the potency of CAR-T cells to eliminate HIV-infected cells populating in a lymphoid environment (e.g., spleen), which closely resembles what occurs in humans where lymphoid tissues are the major location of HIV replication. Furthermore, these CARs displayed very broad inhibitory activity against PBMC infected with IMC expressing Envs from different HIV-1 strains found worldwide. Finally, it's important to note that the HIV-1 Envs from the bNAb-resistant strains used in this study often contain mutations in their CD4-binding site, further supporting the broad reactivity of anti-HIV duoCARs to delay or prevent viral escape. While our *in vivo* studies were underway, two independent groups reported similar findings for the potency of multi-specific CAR-T cells against glioblastoma (48) and acute myeloid leukemia (49). In addition to these studies, we and others have reported that the multi-targeting CAR-T cell approach effectively mitigates immune escape due to antigen loss in B-cell malignancies when using dual-targeting (50), bivalent (51), and/or tandem CAR-T cells (32). Hence, these studies provide additional support for our multi-specific 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 multi-specific 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 CD4-zeta CAR or conventional bispecific CARs (10, 13, 52, 53). 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 binder, 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.

In our long-term *in vivo* study using a humanized mouse model, we demonstrated that one month after infection, mice treated with anti-HIV duoCAR-T cells, in contrast to UTD treated mice, effectively controlled HIV infection in 4 out of 5 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 an HIV resistant variant. 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. 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. 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 (26, 27). This is consistent with our previous work using a bispecific multivalent antibody fusion consisting of mD1.22 and m36.4, which together, synergize to improve neutralization, breadth, and protection from HIV infection. 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.

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 employ a single entry (10) or fusion inhibitor (11) 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. Although no clear advantages were observed, higher trending CD4⁺ T cells in mice infused with trispecific versus bispecific duoCAR-T cells during acute infection studies indicated a possible advantage for the trispecific duoCAR containing cells that additionally express the C46 peptide. We speculated that these CARs could be more advantageous during chronic viral infections, where the virus accumulates mutations over time and/or during high viral loads. In this scenario, loss of m36.4-mediated protection due to escape or high viral loads may be counteracted by the C46 fusion inhibitor peptide present on trispecific duoCAR expressing T cells. However, 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 tricistronic or trioCAR comprised of a three CAR molecule cassette could improve the functionality of the three anti-HIV binding domains. Additional studies are underway to further investigate various multi-specific anti-HIV CAR architectures that are greater than one or two molecules.

A significant finding of our study was the robustness of anti-HIV duoCAR-T cells to resist infection while inhibiting IMC expressing different HIV-1 clade-specific Env proteins. In this study, we demonstrated that anti-HIV duoCAR-T cells suppressed HIV infection by >99% with no significant loss of CD4⁺ T cells *in vitro* and *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-gamma and IL-2 production when compared to single molecule CARs even though 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 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 level of CD107a and cytokine production. Indeed, multi-specific 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.

Additional studies are needed to 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 anti-retroviral therapies. Similar to LRAs, anti-HIV CAR-T cells themselves may reactivate the latent HIV reservoir by secreting cytokines (52). This inherent feature of CAR-T cells may synergize with LRAs to reawaken the latent HIV reservoir (shock) leading to its subsequent eradication (kill). Further studies are planned to investigate the anti-HIV efficacy of duoCAR-T cells against LRA reactivated latently-infected T cells to model the “shock-and-kill” approach. 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 cytotoxic anti-HIV duoCAR-T cell effects. Studies are underway to investigate these potential synergistic effects.

The future direction of this work is aimed at developing a curative therapy for HIV/AIDS. Anti-HIV duoCAR-T cell therapy aims to decrease viral loads to levels that significantly 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 supports multi-

specific 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 multi-specific anti-HIV CAR construct that demonstrated the best potency, breadth, and resistance to HIV for translation into the clinic. We hypothesized that multi-specific duoCAR-T cells would demonstrate better potency and improved reactivity against diverse HIV viruses than conventional monoCAR-T cells, by virtue of its multi-targeting design and unique two-molecule CAR architecture, while simultaneously protecting the CAR-modified T cells from HIV infection. In our study, we screened multiple conventional and multi-specific anti-HIV CAR-T cell constructs for CAR expression, cytolytic activity, and IFN-gamma secretion followed by more comprehensive *in vitro* and *in vivo* HIV challenge studies using replication-competent infectious molecular clones of HIV that express different Env genes (Env-IMC-LucR viruses) to quantify HIV infection at the end of the study relative to untransduced T cells. The *in vitro* HIV challenge studies were conducted for six different CAR vectors, in at least three independent donors tested in triplicate to achieve statistical power. From the *in vitro* studies, 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 Envs represented worldwide. We further interrogated the *in vivo* efficacy of the multi-specific 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 PBMC (hu-spl-PBMC-NSG). The humanized mice were treated with anti-HIV CAR-T cells generated from multiple donors and 4-6 mice were selected randomly (non-blinded) per treatment cohort for statistical power. At the end of the study, the mice were sacrificed, and their spleens harvested for the following endpoint analyses: (1) *Renilla* luciferase activity to quantify the HIV infection, (2) flow cytometry detection of human CD4⁺ and CD8⁺ T cells to detect off-target toxicities and CD4⁺ T cell loss, (3) CAR-T cell persistence by qPCR, and (4) HIV persistence by detecting total cell-associated HIV DNA by qPCR in mice 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.

Characterization of cell lines used for Env⁺ and Env⁻ cytotoxicity assays

HEK 293T cells were engineered to constitutively express high amounts of gp120/gp41 envelope derived from a clade B isolate (293T-Env). Briefly, 293T-Env cells were grown in Dulbecco's modified eagle medium (DMEM) in the presence of 10% fetal bovine serum and 60 µg/ml of zeocin to maintain selection. To generate luciferase-expressing cells, 293T-Env cells were transduced with a lentiviral vector containing the firefly luciferase gene, single cell cloned, and tested for Env expression on the surface of HEK 293T cells by 2G12, b12, and 2F5 antibodies and luciferase activity. A single cell clone that expresses Env and has luciferase activity, designated 293T-Env-Luc, was isolated and used for cytotoxicity assays. For envelope-negative cell lines, Raji and HEK 293T cells were purchased from ATCC and

subsequently transduced with a lentiviral vector carrying firefly luciferase followed by single cell cloning and designated as Raji-Luc or 293T-Luc. Raji-Luc cells were maintained in RPMI media containing 10% fetal bovine serum. HEK 293T-Luc cells were maintained in DMEM media containing 10% fetal bovine serum. The absence or presence of HIV-envelope was determined by staining with recombinant human monoclonal antibodies 2G12, b12, or 2F5 (Polymun Scientific) added at a dilution of 1:1,000 for 30 minutes at 4°C followed by washing twice in MACS wash buffer. Cells were then incubated with FITC-labeled F(ab')₂ anti-human IgG (1:1000) for 30 minutes at 4°C, washed twice, and then resuspended in 0.2 mL MACS wash buffer (Phosphate-buffered saline containing 0.5% bovine serum albumin). Flow cytometry was carried out using a MACSQuant® VYB cytometer (Miltenyi Biotec) and analysis was performed using FlowJo software (Tree Star). Luciferase activity was confirmed for all cell lines by SteadyGlo reagent (Promega) followed by quantification of luminescence using an Enspire plate reader (Perkin Elmer).

Generation of lentiviral vector constructs encoding conventional and multi-specific anti-HIV CARs

CAR antigen-binding domain sequences were derived from previously published sequences (26, 27, 36) and synthesized. Synthesized gene fragments were subcloned in frame to a CD8 ectodomain, CD8 transmembrane domain, 4-1BB, and CD3ζ signaling domain to generate conventional CARs as previously described by our group³². For duoCARs and the trispecific D134 construct, a P2A site was placed downstream of the first CAR containing a single or two-linked antigen binding domains followed by a third antigen binding domain with or without the CD3ζ signaling domain. The mCherry reporter gene was sub-cloned into the same lentiviral backbone as CAR constructs.

Lentiviral vector production of conventional and multi-specific anti-HIV CARs

Lentiviral vectors expressing anti-HIV CAR transgenes were produced by transiently transfecting 293T suspension cells using a four-plasmid system. Briefly, suspension 293T were co-transfected with the CAR transfer plasmid, VSVg envelope, gag/pol, and rev plasmids. Supernatants (400 mL) containing packaged and pseudotyped lentiviral vectors were concentrated 48 hours later by overnight ultracentrifugation at 10,000 $\times g$ for at least 18 hours. Pellets were resuspended in 2 mL of resuspension buffer containing 6 mM HEPES and 100 mM NaCl until the pellet was easy to dissolve by gentle agitation. Re-suspended lentiviral particles were stored in aliquots at -80°C until further use.

PBMC isolation followed by CD4⁺ and CD8⁺ T cell enrichment by immunomagnetic bead selection

Buffy coats or leukapheresis products from healthy HIV naïve donors were purchased from Oklahoma Blood Institute or Key Biologics, respectively. Human PBMC were isolated using ficoll-paque gradient separation performed in a leucosep tube. CD4⁺ and CD8⁺ T cells were enriched by immunomagnetic bead selection using CD4 and CD8 microbeads according to manufacturer's protocol (Miltenyi Biotec). Isolated CD4⁺ and CD8⁺ T cells from HIV-naïve donors were cryopreserved in 70% FBS, 20% TexMACS™ medium, and 10% DMSO followed by storage in liquid nitrogen until further use.

Generation of anti-HIV CAR-T cells using lentiviral vector transduction

On day 0, CD4⁺ and CD8⁺ T cells were activated with MACS® Large-scale T Cell TransAct reagent (Miltenyi Biotec) in the presence of 40 IU/mL IL-2, GMP-grade (Miltenyi Biotec). On day 3, activated T-cells were transduced with lentiviral vectors encoding CAR constructs in the presence of 10 µg/ml protamine sulfate (Sigma-Aldrich) and 200 IU/mL IL-2. Cultures were expanded in TexMACS™ medium supplemented with 200 IU/ml IL-2 and cell culture density adjusted to 5 x 10⁵ cells/mL every two days until harvest on day 9-10 for functional analysis. In some experiments, untransduced T cells and CAR-T cells were cryopreserved in 70% FBS, 20% TexMACS™ medium (Miltenyi Biotec) and 10% DMSO and stored in liquid nitrogen until the day of the experiment.

Anti-HIV CAR detection on the surface of transduced primary T-cells

Approximately 1 x 10⁶ untransduced or CAR-transduced primary T cells containing both CD4⁺ and CD8⁺ T cells were washed in MACS wash buffer (Phosphate-buffered saline containing 0.5% bovine serum albumin). Vioblue-conjugated CD4 (clone VIT4, Miltenyi Biotec) and FITC-labeled CD8 (clone BW135/80, Miltenyi Biotec) antibodies were then added to the cells following the manufacturer's instructions. After a 30-minute incubation at 4°C, cells were washed twice in MACS wash buffer and resuspended in 0.2 mL of MACS wash buffer. For detection of CARs containing the C46 peptide, the recombinant human monoclonal antibody 2F5 (Polymun Scientific) was added at a dilution of 1:1,000 for 30 minutes at 4°C followed by washing twice in MACS wash buffer. 2F5 recognizes the epitope ELDKWA found within the C46 peptide. Cells were then incubated with PE-labeled F(ab')₂ anti-human IgG and FITC-conjugated CD8 antibodies for 30 minutes at 4°C, washed twice, and then resuspended in 0.2 mL MACS wash buffer. The m36.4-CAR was detected on the surface of mono- and duoCAR-T cells using an antibody developed by LTI against the m36.4 epitope. Briefly, untransduced T cells, control CAR-T cells (M1 CAR-T cells), or M13 and D13 CAR-T cells were stained with Vioblue conjugated anti-CD4 (detects the native CD4 and mD1.22-CAR), FITC-conjugated anti-CD8, and the anti-m36.4 detection reagent followed by secondary antibody staining with AlexaFluor 647 conjugated goat anti-human IgG Fc for 30 minutes at 4°C, washed twice, and then resuspended in 0.2 mL MACS wash buffer. Flow cytometry was carried out using a MACSQuant® VYB cytometer (Miltenyi Biotec) and analysis was performed using FlowJo software (Tree Star).

HIV envelope capture assay using flow cytometry

Briefly, 0.5-1 x 10⁶ untransduced or anti-HIV CAR transduced T-cells were washed twice in MACS wash buffer followed by incubation in 2 µg/mL of 6x his-tagged gp140 (clade B) for 30 minutes at 4°C. Cells were washed twice in MACS wash buffer and incubated with PE-conjugated anti-His (Miltenyi Biotec) and with or without FITC-conjugated CD8 antibody (Miltenyi Biotec) as per manufacturer's instructions. The capture of gp140 by a mixture of CAR-transduced CD4⁺ and CD8⁺ T cells (total) was determined relative to UTD control. To analyze the amount of gp140 captured on CD8-expressing T cells, the stained cells were gated on CD8-expressing T cells and the relative capture of gp140 analyzed as compared to the UTD control. Flow cytometry was carried out using a MACSQuant® VYB cytometer (Miltenyi Biotec) and analysis was performed using FlowJo software (Tree Star).

Infectious molecular clones of HIV-1 *Renilla* luciferase reporter viruses

We previously reported the generation of infectious molecular clones (IMC) of HIV-1 which express the *Renilla* luciferase (LucR) reporter gene in an NL4-3 derived isogenic proviral backbone encoding *env* gene sequence from heterologous HIV-1 strains, also referred to as Env-IMC-LucR viruses (43). Virus stocks were produced following 293T cell transfection with proviral plasmids, and titer (IU/ml) obtained on TZM-bl cells as previously described (43). The Env-IMC-LucR viruses utilized in this study were previously described and included NL-LucR.T2, NL-LucR.T2A-BaL.ecto, NL-LucR.T2A-SF162.ecto (43), NLLucR.T2A-AC.246F3.ecto, NL-LucR.T2A-AE.CNE8.ecto, NL-LucR.T2A-AE.CNE55.ecto, NL-LucR.T2A-BC.CH119.1.ecto (45), NL-LucR.T2A-C.Du172.17.ecto, NL-LucR.T2A-C.Du422.1.ecto (24) and NL-LucR.T2A-G.X1632_S2_B10.ecto, and LucR.T2A-C.CAP45.ecto. Viruses are referred to in the manuscript also by an abbreviated name (e.g., NL-LucR.T2A-C.Du422.1.ecto is referred to as C.Du422.1.LucR).

Firefly Luciferase-based cytotoxicity assay

The 293T-Env-Luc cell line highly expresses a clade B HIV Env glycoprotein and was used to screen more than 40 CARs and identify the most potent CARs for further analyses used in the study. Briefly, 5×10^3 target cells (293T-Env-Luc, Raji-Luc, or 293T-Luc) were co-cultured with or without UTD control, mCherry reporter expressing T cells, or anti-HIV CAR-T cells which contain a mixture of CAR⁺ and unmodified T cells generated from HIV-naïve donors (sample) at various effector to target ratios (10:1, 5:1, and 2.5:1) in a sterile 96-well plate and incubated overnight at 37°C in 200 µL of TexMACS™ medium without cytokines in the presence of 5% CO₂. Twenty-four hours later, 100 µL of SteadyGlo reagent (Promega) was added to each well and incubated for 10 minutes at room temperature followed by quantification of luminescence using an Enspire plate reader (Perkin Elmer). The luminescence was captured as counts per second (CPS) for each experimental well containing the indicated E:T ratio (sample CPS) and target cells alone (target CPS). The reduction in luciferase activity, which is shown on the graphs as percent specific lysis, was calculated as: $1 - (\text{sample CPS} / \text{target CPS}) \times 100\%$.

Cytokine release assay using sandwich ELISA

Supernatants from the cytotoxicity assay were collected (E:T ratio of 10:1), diluted 10fold, and assayed for IFN-γ by Ready-Set-Go ELISA as per the manufacturer's instructions (eBioscience).

Degranulation and intracellular cytokine staining

Healthy donor PBMC were activated with 100 U/ml IL-2 and 4 µg/ml PHA and cultured in RPMI media containing 10% FBS (R10) for 72 hrs. After activation, cells were washed, counted and plated at 100,000 cells/well in a 96-well U-bottom plate with 1×10^6 IU/ml of C.Du422.1.LucR virus and cells spininfected for 1 hour at 2500 RPM at 25°C, and then incubated for 7 days in culture. Then, HIV-infected PBMC and donor-matched CAR-T cells were co-cultured for 6 hours in the presence of monensin and brefeldin A to trap the cytokines being produced intracellularly. Each CAR-T cell to target cell condition was setup in triplicates, and then triplicates were pooled for staining at the end of the incubation. At

the endpoint, cells were washed, and then stained with live/dead viability dye, CD3-APC, CD4-FITC, and CD8-APC-Cy7 in addition to CD107a-PerCP-Cy5.5 or IL2-PerCP-Cy5.5 and IFN-gamma-Pacific Blue (Biolegend). Data was collected using a BD LSR II flow cytometer (BD Biosciences). FACS data was analyzed to identify the % of untransduced CD8⁺ T cells (UTD) or CAR⁺ CD8⁺ T cells expressing CD107a, IL-2, IFN-gamma, or double-positive IL-2/IFN-gamma expressing cells via FlowJo software (TreeStar). Graphs were generated using GraphPad 7.04.

Long-term co-culture assay for exhaustion marker analysis by flow cytometry

Cryopreserved CAR-T cells (effectors, Day 9 post-activation) were thawed and placed into culture for three days in TexMACS™ medium containing 200 U/mL of IL-2. On day 3, cells were washed extensively to remove cytokines and cultured for an additional 6 days in the absence of antigen stimulation to examine tonic signaling of duoCAR-T cells relative to conventional CAR-T cells. On day 6, the cells were stained with the following antibodies from Miltenyi Biotec unless otherwise stated: CD3-Vioblu, Tim3-PE, Lag3-APC, PD1-PE-Vio770, and 2G12 (Polymun Scientific) followed by secondary antibody staining with FITC-labeled F(ab')₂ anti-human IgG (ThermoFisher Scientific). Data was collected using a MACSQuant® Analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed using Flow Jo (Tree Star). Pie charts were generated using SPICE 6.0 software analysis tool (54).

In vitro HIV challenge assay to quantify CAR-T mediated killing of HIV-infected PBMC

Healthy donor PBMC were cultured in RPMI media containing 10% FBS (R10) and activated with 100 U/ml IL-2 and 4 µg/ml PHA for 72 hrs. After activation, the cells were washed, counted, and resuspended at 10⁶ cells/ml in R10 media with 100 U/ml IL-2 and 1 x 10⁶ IU/ml of Env-IMC-LucR virus (MOI = 1 based on TZM-bl titer). The cell-virus mixture was plated at 100 µl/well in 96-well U bottom plate, and spininfected for 1 hour at 2500 RPM at 25°C. Cells were incubated at 37°C overnight, and then 10⁶ donor-matched effector cells (T cells transduced with different CAR constructs) were added at an effector to target ratio of 1:1. Effectors were thawed and cultured in R10 media in the presence of 100 U/ml IL-2 one day prior to PBMC activation. All conditions were set up in triplicates. The assay was performed with 11 different Env-IMC-LucR viruses, described above, and 3 different donors. Seven days after the co-culture set up, cells were spun down, washed and lysed using the *Renilla* luciferase kit by Promega (Madison, WI). For the experiment shown in Fig. S9, PHA-activated PBMC were infected with an Env-IMC-LucR virus encoding the Du422.1 (clade C) *env* gene and four days post-infection, raltegravir (1 µM) was added some wells containing HIV-infected PBMC (1 x 10⁵ cells/well) to prevent HIV infection of new cells. Autologous untransduced T cells (UTD) or anti-HIV CAR-T cells (1 x 10⁵ total T cells) were then added to the infected PBMC cultures followed by quantification of the LucR activity (RLUs) three days later to quantify elimination of HIV-infected cells. Luciferase activity was quantified following the manufacturer's instructions. Data was presented as relative light units (RLU). Log inhibition of HIV-1 infection was calculated by the following formula: Log inhibition of HIV-1 infection = Log₁₀(CAR_{RLU}/UTD_{RLU}). Percent HIV-1 inhibition was calculated by 1-(CAR_{RLU}/UTD_{RLU}) x 100%.

***In Vivo* CAR-T efficacy using a hu-spl-PBMC-NSG mouse model of HIV infection**

We utilized our previously described hu-spl-PBMC-NSG humanized mouse model for *in vivo* studies (24, 55). On day 0, donor-matched PBMC were activated with 100 U/mL of IL-2 and 4 µg/mL of PHA and then spininfected on day 3 with Du422.1-IMC-LucR virus (~1x10⁶ IU/10⁷ PBMCs). One million of the indicated CAR⁺ effector T cells or untransduced T cells (UTD) were co-injected into each spleen together with ten million HIV-infected PBMC (0.1:1 E:T ratio). The dose of total CAR-T cells was adjusted to accommodate for differences observed in transduction efficiencies among the different CAR constructs. In later experiments using D13 and D413L, an E:T ratio of 0.5:1 was used to assess the *in vivo* efficacy of multi-specific duoCAR-T cells. A total of 5 million CAR-T cells (~30% transduction efficiencies for both constructs) was co-injected together with 10 million donor-matched HIV-infected PBMC. One week (7 days) or one month (30 days) after infection, mice were sacrificed, and spleens harvested for further analyses. The total splenocytes were separated into three groups to perform the following assays: 1) luciferase assay to measure infection, 2) flow cytometry analysis to detect CD4⁺ and CD8⁺ T cells, and 3) DNA extraction for precise detection of CAR-T cell persistence and total cell-associated HIV DNA at the end of the experiment by qPCR. The HIV-1 infection was quantified in the splenocytes using the *Renilla* luciferase assay system (Promega, Madison, WI) as previously described (24, 55).

Detection of total cell-associated HIV DNA in mice splenocytes using real-time qPCR

Total HIV DNA qPCR was performed by BioQual, Inc. Gag-specific primers and TaqMan probe were designed by BioQual to specifically target a region within the HIV-1 gag gene that is not present in the lentiviral backbone or CAR expression cassette. This assay detects all species of the HIV DNA (e.g., unintegrated, integrated, 1-LTR circle, 2-LTR circles). Briefly, the DNA was extracted from at least one million mouse splenocytes collected from *in vivo* studies and shipped on dry ice to BioQual, Inc for DNA isolation using the DNeasy Genomic DNA isolation kit (Qiagen). For each reaction, 10% input DNA was amplified using the primers/probe specific to the HIV-1 gag gene or human β-actin gene. The total HIV DNA per reaction (copies/well) was generated using a standard curve of known plasmid copies containing the amplicon of interest followed by normalization to the human β-actin gene (copies/well). The PCR efficiencies for both primer/probe sets were within the acceptable range (85-100%). The limit of detection for the assay was greater than or equal to 1 copy of total HIV DNA. Specificity controls confirmed the absence of amplification in LV-modified, HIV-negative T cell samples. The final results were calculated by BioQual, Inc and expressed as copies of total HIV DNA per 10⁶ β-actin equivalents.

Detection of CAR-T cell persistence using real-time c-Frag qPCR

Briefly, 1 million mouse splenocytes were harvested and genomic DNA extracted by ACGT, Inc. using the Promega Maxwell® 16 LEV Blood DNA Kit. To determine CAR-T persistence, a unique fragment (c-frag) contained within the lentiviral vector backbone of anti-HIV CARs, but not the HIV-1 virus, was used to assess copy numbers per 50 ng of genomic DNA isolated from mouse splenocytes. The following primers and probe were used to detect c-frag: Forward primer: 5'GGAGTTGAGACCAGTGTAGT-3', Reverse primer: 5'-

CCACTCCTGACAACTACTCT-3', Probe: 5'-FAM-CAGTAGGTGAAGGAGTCGTAGTTG-TAMRA-3'. The following primers and probe were used to detect the polypyrimidine tract binding protein 2 (PTBP2) gene to control for PCR inhibition: Forward primer: 5'-TCTCCATTCCCTATGTTTCATGC-3', Reverse primer: 5'-GTTCCCGCAGAATGGTGAGGTG-3', Probe: 5'-JOE-ATGTTCTCGGACCAACTTG-BHQ-1-3'. The PTBP2 gene was used as an internal control to assess PCR inhibition. The number of cFrag copies in splenic DNA was calculated using a standard curve consisting of known quantities of a plasmid DNA containing the c-frag genetic tag followed by normalization to input splenic DNA (0.05 µg) multiplied by the volume of the reaction added per well (12.5 µL).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 7.04. For CTL assays, a two-way ANOVA was performed followed by Bonferroni post-test. For *in vitro* HIV challenge assays, multiple comparison student's t-test was performed to evaluate differences between different CARs among different donors. 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 post-test analysis. Differences were considered statistically significant when $P < 0.05$.

Study approval

All the mouse studies were approved by the Institute for Animal 617 Studies and the Institutional Review Board at Albert Einstein College of Medicine in 618 compliances with the human and animal experimentation guidelines of the U.S. 619 Department of Health and Human Services and adherence to the NIH Guide for the 620 Care and Use of Laboratory Animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Illustration of the multi-specific anti-HIV CAR architecture.

(A) Gene structure of multi-specific anti-HIV CARs encoded by lentiviral vectors. Conventional and multi-specific CARs were designed as described in Materials and Methods. The term monospecific, bispecific (bi-), or trispecific (tri-), refers to the total number of binding specificities contained in the CAR molecule(s) within a single construct. For simplicity, each anti-HIV binder is assigned a number. For instance, mD1.22 = 1, m36.4 = 3, and the C46 peptide = 4. The term monoCAR (M, one-molecule CAR architecture) or duoCAR (D, two-molecule CAR architecture) refers to the number of CAR molecules

encoded by a lentiviral vector and expressed in a single cell. For instance, a monoCAR containing the mD1.22 domain is referred to as M1 or a duoCAR containing the mD1.22 and m36.4 domains is called D13. **(B)** Cartoon illustration of each anti-HIV CAR used in the study. Monospecific CARs contain either mD1.22 (1) or m36.4 (3) fused to a CD8 ectodomain (EC), CD8 transmembrane domain (TM), 4-1BB costimulatory domain, and CD3 ζ T cell signaling chain to form **M1** or **M3**, respectively. The conventional bispecific CAR contains mD1.22 fused to m36.4 via a 3x-G4S motif to form a one-molecule CAR construct (**M13**). The bispecific duoCAR contains the mD1.22-CAR and m36.4-CAR co-expressed in T cells using a two-molecule architecture (**D13**). In another iteration, the **D134** construct contains the bispecific CAR (M13) in addition to the C46 peptide that is anchored to the T cell membrane but does not contain a CD3 zeta chain (4). Lastly, the trispecific duoCARs contain three specificities across two CAR molecules within a single cell. The first CAR contains the C46 peptide (4) fused to the N-terminus of the mD1.22 (1) domain via a 3x- or 5x-G₄S linker (designated “S” for short or “L” for long) and a second m36.4 CAR (3) to form **D413S** or **D413L**, respectively. **(C)** The putative target sites for each anti-HIV binder on the HIV gp120/gp41 trimer (*not drawn to scale*; adapted image, credit: NIH/NIAID). Multi-specific CARs contain three distinct anti-HIV binders (mD1.22, m36.4, and/or C46 peptide) which target non-overlapping epitopes on the HIV envelope trimer (gp120/gp41). **(D)** Experimental design used to generate LV-modified anti-HIV CAR-T cells as described in Materials and Methods.

Fig. 2. LV-encoded multi-specific anti-HIV CARs expressed on the surface of CD4⁺ and CD8⁺ T cells capture HIV Env.

(A) Representative detection of anti-HIV CARs via the mD1.22 domain on the surface of activated primary T cells from one donor following transduction and expansion (n=6). (B) Flow cytometric detection of trispecific CARs via the C46 peptide using 2F5 Mab directed against the gp41 MPER region (representative figure, n = 5). Graphical representation of % CAR-modified T cells for multiple donors using either (C) anti-CD4 (n = 6) or (D) anti-C46 (2F5 Mab) flow cytometry (n = 5). The error bars represent mean $\pm$ SD of independent

samples. Statistical analysis was performed by one-way ANOVA followed by Bonferroni post-test analysis (*** $P < 0.0001$, **** $P < 0.00001$). (E) Multi-specific anti-HIV CARs capture soluble HIV envelope. Representative histogram shown ($n = 2$). 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 and CD19-CAR serve as a control for specificity. (F) The capture of HIV gp140 mediated by the m36.4-CAR was reduced in CD8⁺ T cells, likely due to less affinity in the absence of the CD4 receptor. Representative histogram shown ($n = 2$).

Fig. 3. Cytotoxicity of anti-HIV CAR-T cells against HIV-1 envelope-expressing target cells. (A) Detection of the HIV-1 envelope glycoprotein on the surface of cell lines used for cytotoxicity assays. The engineered 293T-Env-Luc (Env+) cell line constitutively expresses the HIV-1 envelope glycoprotein as detected by 2G12, b12, and 2F5 in contrast to envelope-negative cell lines, 293T-Luc and Raji-Luc. (B) Cytotoxicity of conventional CD4-CAR versus mD1.22-CAR-T cells against Env+ target cells (representative figure, $n = 2$). The mCherry reporter modified donor T cells serve as a negative control. (C) Specificity of the mD1.22-CAR as compared to the CD4-CAR shows no off-target effect on Env- target cells (293T-Luc). (D) Cytotoxicity of monospecific and bispecific anti-HIV CAR-T cells against Env+ target cells. Specificity of anti-HIV CARs against Env- targets (E) 293T-Luc and (F) Raji-Luc. (G) Monospecific and bispecific anti-HIV CAR-T cells elaborate IFN-gamma upon challenge with Env+ target cells. (H) Cytotoxicity of multi-specific anti-HIV CAR-T cells. Specificity of multi-specific CAR-T cells against Env targets (I) 293T-Luc, and (J) Raji-Luc. (K) Multi-specific anti-HIV CAR-T cells elaborate IFN-gamma in the presence of Env+ target cells ($n = 3$ for monospecific versus bispecific CARs; $n = 2$ for bispecific versus trispecific CARs). The error bars represent $\pm$ S.E.M of independent samples. Statistical analyses were performed by two-way ANOVA followed by Bonferroni post-test analysis performed for all comparisons (*** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$, ns = not significant).

Fig. 4. Anti-HIV duoCAR strongly induces T-cell activation without impairing T-cell functionality.

(A) Expression profile of the anti-HIV CARs used in the *in vitro* assay to determine CD107a and cytokine production in CAR-T cells upon challenge with HIV-infected donor-matched PBMC. (B) Bispecific duoCAR (D13) triggers T cells to produce a strong cytokine response against HIV-infected cells in comparison to conventional monoCAR-T cells (M1 and M13) containing one or two Env specificities, respectively. The graph shows the percentage of UTD control or CAR-T cells producing the indicated T cell activation marker in the

presence of PBMC infected with a Du422.1-IMC-LucR virus as described in Materials and Methods. The graph shows pooled data from a representative donor tested in triplicate. (C) DuoCAR-T cells (D13) are not prone to tonic signaling relative to conventional monoCAR-T cells (M1 and M13). Pie charts were generated using SPICE 6.051. 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 shown (n = 2).

Fig. 5. Broad and potent *in vitro* killing of HIV-infected PBMC by multi-specific duoCAR-T cells. (A) Schematic of the *in vitro* HIV-1.LucR PBMC killing assay. Infectious molecular clones (IMC) of HIV-1 encoding a heterologous HIV-1 envelope (Env) glycoprotein and a *Renilla* luciferase cassette (generically referred to as Env-IMC-LucR) were used to infect donor matched PBMC followed by co-culture with UTD control or CAR-T cells for seven days. After 7 days, the co-cultures were lysed, and luciferase activity assessed to quantify HIV-1 infection, and inhibition thereof, due to CAR-mediated elimination of Env-IMCLucR infected cells. (B) C.Du422.1.LucR, a VRC01/3BNC117-resistant clade C Env-encoding

HIV-1 virus, identifies bispecific and trispecific anti-HIV duoCARs as the most potent CAR architecture in the HIV-1.LucR PBMC killing assay. T = C.Du422.1-IMC-LucR infected PBMC (targets). Data shown is +/- SD of triplicate wells for four different donors. Statistical analysis was performed using a multiple comparison unpaired student's t-test. Significance is considered $P < 0.05$. (C) Anti-HIV duoCAR-T cells display broad and potent elimination of PBMC infected with Env-IMC-LucR viruses encoding genetically diverse *env* genes. The figure shows averaged log HIV-1 inhibition as a result of CAR-mediated elimination of HIV-infected cells relative to untransduced T cells (UTD) control ($n = 3$). Log inhibition of HIV-1 infection was calculated by the following formula using the relative light units (RLU) obtained for each sample: $\text{Log inhibition} = \text{Log}_{10}(\text{CARRLU}/\text{UTDRLU})$ and then averaged across donors.

Fig. 6. Multi-specific 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), (E) Du172.17 (clade C, resistant to VRC01), or (F) 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, and clade G (K) GX1632_S2_B10. The error bars shown are $\pm$ SD of three independent donors performed in triplicate. Statistical analysis was performed by multiple comparison unpaired student's t-test. Significance is considered $P < 0.05$.

Fig. 7. Anti-HIV duoCAR-T cells are more potent than monoCAR-T cells in suppressing bNAb-resistant HIV infection *in vivo*.

(A) Illustration of the intrasplenic HIV-1.LucR NSG mouse model and experimental design for acute HIV infection (also see Materials and Methods). (B) *In vivo* efficacy of monoCAR-T cells versus duoCAR-T cells against PBMC infected with C.Du422.1-IMC-LucR virus. DuoCAR-T cells eliminated >97% of HIV-1 infection relative to UTD control group as a result of CAR-mediated elimination of HIV-infected PBMC. This figure shows that the CAR two-molecule architecture is better than the one-molecule architecture containing one (mD1.22) or two Env specificities (mD1.22-m36.4): D13 >> M13 > M1. The relative HIV-1 inhibition was calculated using the *Renilla* luciferase activity detected in 1 million splenocytes by the formula: $1 - (RLU_{CAR}/RLU_{UTD}) \times 100\%$. The relative % HIV-1 inhibition is indicated on the bar graph above the corresponding CAR-T group. Statistics were performed as described in Materials and Methods. (C) The percentage of human CD4⁺ T-cells detected in the spleens of HIV-infected mice by flow cytometry during acute HIV infection. (D) The percentage of human CD8⁺ T-cells detected in the spleens of HIV-infected mice by flow cytometry during acute HIV-1 infection. (E) CAR-T cell persistence in the spleens of HIV-infected mice as detected by cFrag qPCR analysis. The plots represent n = 6 mice per group except for the HIV-negative PBMC control group (HIV- PBMC) which is n = 4. The error bars represent S.D.

Fig. 8. Multi-specific anti-HIV duoCAR-T cells display potent *in vivo* HIV-1 suppression and mitigate CD4⁺ T cell loss during persistent infection.

(A) Illustration of the intrasplenic HIV-1.LucR NSG mouse model and experimental design. *In vivo* efficacy of bispecific and trispecific anti-HIV duoCAR-T cells against PBMC infected with C.Du422.1-IMC-LucR after (B) 7 days and (C) 30 days of HIV-1 infection. The percentage of human CD4⁺ T-cells detected by flow cytometry in the spleens of HIV-infected mice treated with UTD control or duoCAR-T cells after (D) 7 days and (E) 30 days of HIV-1 infection. DuoCAR-T cells reduce and/or prevent CD4⁺ T cell depletion after one

month of HIV-1 infection. The percentage of human CD8⁺ T-cells detected by flow cytometry in the spleens of HIV-1 infected mice treated with UTD control or duoCAR-T cells after (F) 7 days and (G) 30 days of HIV-1 infection. *In vivo* persistence of duoCAR-T cells after (H) 7 days and (I) 30 days of HIV-1 infection. DuoCAR-T cells were detected in the spleens of HIV-infected cohorts by cFrag qPCR as described in Materials and Methods. The error bars represent $\pm$ SD n = 9 mice for HIV-infected groups and n = 4 mice for the uninfected, untreated PBMC group (7 days of HIV infection). For the 30-day HIV infection study, the error bars represent $\pm$ SD (n = 5 mice for the UTD and CAR-treated groups; n = 2 mice for the uninfected, untreated PBMC group). Statistical analysis was performed by ordinary one-way ANOVA followed by Tukey's post-test analysis. Significance is considered $P < 0.05$ and only significant P values are plotted.