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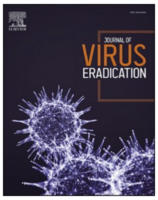

Original research

Advances in HIV-1-specific chimeric antigen receptor cells to target the HIV-1 reservoir

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ABSTRACT

Antiretroviral therapy (ART) for HIV-1 has dramatically improved outcomes for people living with HIV-1 but requires life-long adherence and can be associated with short and long-term toxicity. Numerous pre-clinical and clinical investigations are underway to develop therapies for immune control of HIV-1 in the absence of ART. The success of chimeric antigen receptor (CAR) cell therapy for hematological malignancy has renewed efforts to develop and investigate CAR cells as strategies to enhance HIV-1 immunity, enable virus control or elimination, and allow ART-free HIV-1 remission. Here, we review the improvements in anti-HIV-1 CAR cell therapy in the two decades since their initial clinical trials were conducted, describe the additional engineering required to protect CAR cells from HIV-1 infection, and preview the current landscape of CAR cell therapies advancing to HIV-1 clinical trials.

1. Introduction

Advances in antiretroviral therapy (ART) have significantly improved the longevity and quality of life for people with HIV-1 (PWH). The need for lifelong ART as well as its associated drug toxicities, cost, and the persistence of immune activation with accelerated ageing have inspired ongoing investigation of novel immunotherapies with the goal of sustained ART-free remission. Long-lived HIV-1 infected cells in blood and tissue reservoirs persist on ART and are the principal source of resurgent HIV-1 replication when ART is discontinued. Here, we review the recent preclinical and clinical efforts to enhance the HIV-1-specific immune responses with anti-HIV-1 chimeric antigen receptor (CAR) cells to allow long-term viral suppression or clearance.

CAR are antibody-based hybrid receptors designed to recognize specific ligands on the surface of target cells. All CAR constructs contain an ectodomain fused to a transmembrane region and intracellular immune cell activation domains. The ectodomain consists of either a single-chain variable fragment (scFv), usually derived from heavy and light chains of a monoclonal antibody, or for the CD4-based CAR, the extracellular portion of the CD4 molecule. The transmembrane domain anchors the CAR structure on the effector cell membrane. Once the CAR recognizes and is triggered by its specific antigen, its intracellular activation domain(s) will signal, resulting in downstream processes that facilitate the killing of target cells. CAR expression can thus direct or redirect T cells and natural killer (NK) cells to recognize and kill target cells expressing the antigen of interest.

In chronic HIV-1 infection, cytotoxic T lymphocytes (CTL) are functionally impaired due to exhaustion, viral escape, and major histocompatibility complex (MHC) downregulation on infected cells.[1] Attempts to replicate the success of immune checkpoint blockade from cancer therapy to HIV-1 to reinvigorate T cells responses have thus far struggled to advance through early phase human trials due to safety concerns.[2–5] Other strategies for T cell optimization for adoptive therapy can include *ex vivo* co-receptor editing, modifying HIV-1-specific T cell receptors (TCR), or CAR. One advantage of CAR cell is the ability of these synthetic receptors to redirect T cells to recognize viral proteins independent of antigen processing, TCR presentation, and MHC restriction.

CAR cell therapy for HIV-1 was developed almost 25 years ago; one containing an scFv derived from the anti-gp41 monoclonal antibody clone 98–6, and the second containing a CAR composed of the CD4 receptor fused to a CD3-ζ chain (CD4-ζ).[6,7] These CAR modified cells triggered T cell activation, proliferation, and cytokine production *in vitro* and were able to suppress diverse HIV-1 strains in primarylymphocytes. In early clinical trials, adoptive transfer of CD4-ζ gene-modified autologous $CD4^+$ and $CD8^+$ T cells demonstrated prolonged survival and tissue trafficking of the modified cells but no proliferation or antiviral activity, both in ART-suppressed aviremic and viremic PWH[8–10]

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[Table 1]. This CAR modified approach of adoptive immunotherapy was abandoned due to negligible efficacy which was likely due to susceptibility of CAR T cell to HIV-1 infection or perhaps a lack of visible HIV-1 antigen in suppressed individuals. These studies however demonstrated the safety of CD4-ζ CAR T cell in PWH and suggested that the presence of actively replicating virus is not required for *in vivo* maintenance of CD4-ζ-modified CD4$^+$ or CD8$^+$ T cells.

2. CAR cell therapy in cancer

The recent success of CAR T cells in B cell malignancies and advances in solid tumor CAR cell immunotherapy has renewed excitement for immunotherapy using advanced CAR technology towards the goal of ART-free remission or functional cure of HIV-1. Two CAR T cell products specific for B cell marker CD19, tisagenlecleucel and axicabtagene ciloleucel, were the first cellular therapeutic products with genetically modified autologous T cell immunotherapy to receive FDA approval for the treatment of B cell precursor acute lymphoblastic leukemia (B-ALL) and diffuse large B cell lymphoma (DLCBCL). The two autologous CAR T products differ in the gene-editing vector, the promoter and signaling domain but share toxicity concerns of cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome.[11] Subsequently, additional autologous CAR T cell therapies targeting the CD19 antigen and B cell maturation antigen have received FDA approval for hematological malignancies. Over 500 CAR T and more than 20 CAR NK clinical trials are ongoing including allogeneic induced pluripotent stem cell (IPSC)-derived off-the-shelf NK-CAR cells for hematologic and solid tumors. CAR T cell exhaustion, immunosuppressive tumor microenvironment, adequate access to antigen or antigen escape, and on-target/off-tumor binding with resultant off-target toxicity are challenges with current CAR therapeutics. Recent efforts utilize precise insertion of genes to circumvent graft-versus-host disease or employ a dual targeting approach[12] and adapter CAR to avoid therapy resistance due to antigen loss.[13]

Manipulation of the CAR construct has improved and focused their activity in both cancer and HIV-1 applications. The first-generation CAR contained only the intracellular CD3-ζ stimulatory domain of the TCR as the activation domain.[14] The second and third generations include one or two additional co-stimulatory domains, respectively (e.g. CD28 or CD137 (4–1BB)) which enhance the effector cells' proliferation, persistence, cytotoxicity, and sustained response.[15,16] Fourth-generation CAR T cells co-express key cytokines, such as interleukins and chemokines, or suicide genes that can significantly enhance the efficacy and safety of CAR T therapy[17–19] [Fig. 1].

3. HIV-1 CAR constructs

CTL mediate infected cell lysis through MHC-I molecules, but HIV-1 can downregulate the surface expression of MHC-I in infected cells to escape this immune response. CAR T cells could overcome this viral escape mechanism, as the CAR directly recognizes the antigen without MHC-I restriction and should not be affected by the HIV-1 nef-mediated down-modulation of MHC-1 in infected cells.[20] The HIV-1 envelope (env) expressing cell is the usual epitope targeted by anti-HIV-1 CAR constructs using either CD4 or anti-HIV-1 antibodies as the binding domain for HIV-1-infected cells. In contrast to CAR T cells targeting tumor-antigens such as CD19, which are also expressed in normal tissues, the HIV-1 CAR constructs would target only virus infected cells. CD4-based CAR are the most common strategy as they reduce the likelihood of viral escape due to the requirement for HIV-1 to bind CD4 for infection. However, the over-expression of the CD4 extracellular domain on the T cell make the gene-modified T cells vulnerable to HIV-1 infection. An alternative approach to using the CD4 receptor for targeting the HIV envelope glycoprotein uses a scFv derived from broadly neutralizing antibodies (bnAbs). However, unlike the CD4 receptor, a single bnAb cannot fully neutralize all HIV-1 isolates and requires further engineering. Modifications in HIV-1 CAR constructs since the initial clinical trials to improve CAR efficacy and engineer HIV-1 resistant anti-HIV CAR are discussed below.

3.1. *Optimized CAR constructs*

The original CD4-ζ CAR was expressed by a murine retroviral vector. Altering the viral vector to an HIV-based lentiviral vector resulted in higher CAR surface expression.[21] Similar to cancer targeting CAR, substituting a PGK promoter to promoter EF1, further augmented CAR expression. Other modifications in the non-signaling domain such as incorporation of a hinge domain contributed to CAR T expansion.[22] New

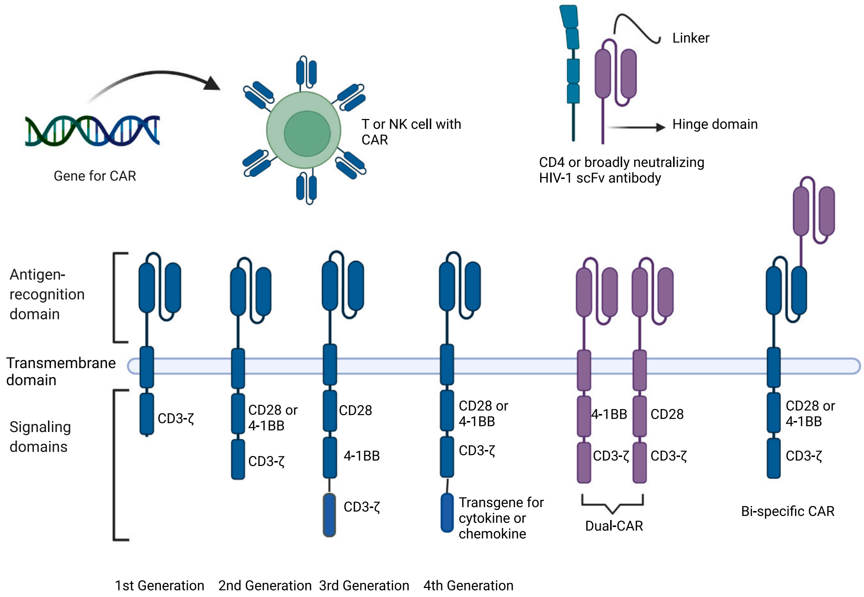

Fig. 1. Schematic Representation of anti-HIV-1 CAR Constructs. Created with BioRender.com.

generations of CD4-ζ CAR have been developed by including co-stimulatory signaling moieties in the intracellular structure, such as those in CD28[23], the tumor necrosis factor receptor (TNFR) family of genes (such as CD127, OX40, and/or 4-1BB molecules)[24], or the lymphocytic activation molecule (SLAM)-related receptor family (comprising CD244/2B4). T cell exhaustion is a primary factor limiting the antitumor efficacy of CAR T cells. In a chronic CAR signaling model using disialoganglioside GD2 CAR to study the effect of CAR structure on T cell exhaustion with persistent antigen exposure, CD28 endo-domain was noted to augment and the 4-1BB endo-domain in CAR ameliorated the development of exhaustion.[25]

Optimization of the vector backbone, promoter, HIV-1 targeting moiety, and transmembrane and signaling domains were systematically studied in a humanized mouse model to determine which components augmented the ability of T cells to control HIV-1 replication.[21] In this model, the re-engineered CD4 CAR was significantly better than the original CD4-ζ and controlled HIV-1 more effectively than bnAb-based CAR cells. Using a panel of CD4 CAR that incorporated a variety of costimulatory domains in conjunction with the CD3-ζ domain, CD4 CAR T cells containing the 4-1BB costimulatory domain controlled HIV-1 after ART removal better than analogous CAR T cells containing only the CD28 costimulatory domain. 4-1BB helps enhance proliferation and survival while CD28 improves effector function.[26] Clinical efficacy of these optimized CD4-based anti-HIV-1 CAR T therapies has not yet been evaluated.

Dual CAR T cell: Instead of adding 2 costimulatory domains to a single CD3-ζ endodomain (3rd generation CAR), a Dual CAR T cell was developed that simultaneously expressed both 4-1BB/CD3-ζ and CD28/CD3-ζ endodomains[27] [Fig. 1]. Dual-CAR T cells exhibit greater *in vivo* proliferation than 3rd generation CAR T cells. Dual CAR T cells exhibited both the cytotoxic potential and cytokine expression of CD28-costimulated CAR T cells and the proliferative capacity of 4-1BB-costimulated CAR T cells, suggesting concurrent contribution by both costimulatory signals. Dual CAR T cells when co-expressed with C34-CXCR4 fusion inhibitor significantly improved CAR T cell survival and effector function during early HIV-1 infection in a bone marrow, liver, thymus (BLT) humanized mouse model without durable antiviral suppression.[27]

bnAb scFv-based CAR: The previous clinically tested CAR against HIV-1 was based on the use of CD4 as the binding domain.[8–10] The growing availability of HIV-1 bnAbs affords the opportunity to evaluate novel CAR based on single-chain antibodies. HIV-1-specific bnAbs can be engineered into single-chain antibodies and then fused with the ζ domain with or without second- and third-generation CAR costimulatory domains to target HIV-1 infection. These bnAbs target conserved sites within the env protein, including the CD4-binding site, V1/V2 loop, V3 loop, the gp41 membrane-proximal external region (MPER), and the variable glycan regions.

Using sequences from well-defined bnAbs including 10E8, 3BNC117, PG9, PGT126, PGT128, VRC01, and X5, single-chain-antibody-based CARs were engineered which conferred potent antiviral activities to transduced CD8$^+$ T cells against HIV-1-infected cells *in vitro*.[28] A bnAb CAR construct using scFv of the broadly neutralizing HIV-1 specific antibody VRC01 to a third-generation CAR moiety transduced into primary CD8$^+$ T cells demonstrated antiviral activity against wild-type HIV-1 infected cells and reactivated latently infected T cells isolated from PWH receiving combined ART.[29] This third-generation anti-HIV-1 CAR molecule (VRC01-28BBz-shPTL CAR) with scFv region derived from the bnAb VRC01 with a combination of small hairpin RNAs (shRNAs) including sh-PD-1, sh-Lag-3, and sh-Tim-3, inserted into the vector for preventing exhaustion and increasing the *in vivo* persistence of CAR T cells, was evaluated in a phase 1 clinical trial with an analytic treatment interruption design[30] [Table 1]. This bnAb based CAR was safe, persisted *in vivo* for 48 weeks or more and modestly delayed virus rebound compared to historical control. The rebound viruses after adoptive transfer were CAR T cell resistant due to preexisting resistance or emergence of viral escape mutations suggesting the need for selecting specific scFvs targeting multiple conserved epitopes of HIV-1 env.

Bi- and Tri-specific anti-HIV-1 CAR: Bi- and tri-specific CARs target two or more distinct highly conserved non overlapping env determinants and allow combinational antigen sensing [Fig. 1]. Combinational targeting of 2 or 3 antigens in a single CAR T product has also shown to be advantageous and helps overcome antigen variability and enhances T cell effector function in acute myeloid leukemia and glioblastoma models, respectively.[31,32] A novel bispecific CAR was developed in which a CD4 segment is linked to a scFv of the 17b human monoclonal antibody recognizing a highly conserved CD4-induced epitope on gp120 (CD4-17b CAR).[33] A longer polypeptide linker between the CD4 and 17b moieties (CD4-35-17b CAR) was compared to a shorter linker (CD4-10-17b) with the number representing the lengths in amino acids of the linker joining the CD4 moiety to the scFv. CD4-10-17b CAR performed better than the CD4-35-17b CAR and is thought to be due to the ability to engage in serial antigen binding and permitting simultaneous binding of the two moieties to a single gp120 subunit to allow efficient T cell activation. This CD4-10-17b CAR also displayed increased potency compared to conventional CD4 CAR. These bispecific CD4–17b CARs were devoid of the unwanted property of CD4-ζ CAR rendering the transduced CD8$^+$ T cells susceptible to HIV-1 infection. Another bispecific CAR construct approach targets two distinct conserved regions on HIV-1 gp120; the primary receptor binding site and the dense oligomannose patch. In this CAR construct, the second moiety is the carbohydrate recognition domain (CRD) of a human C-type lectin which binds to the dense oligomannose patch present on envs of diverse HIV-1 isolates.[34] Potential advantage of CD4-CRD CAR over a CD4-17b or scFv second env binding moiety would be the avoidance of viral escape due to epitope mutation and anti-idiotypic immune responses against variable region.

Multi-specific anti-HIV duoCAR T cells: HIV-1 based lentiviral vectors encoding multi-specific anti-HIV CARs with a unique architecture using a two-molecule CAR architecture (duoCAR) have been developed.[35] DuoCAR molecules consist of multiple anti-HIV-1 binders which target three distinct non-overlapping highly conserved epitopes on the HIV-1 env trimer (mD1.22, m36.4, and/or C46 peptide), and are expressed on the surface of T cells from a single lentiviral vector. The hexavalent fusion protein consists 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, combined with gp41-derived C-peptide fusion inhibitor C46 peptide. The DuoCAR-modified T cells demonstrated potency *in vitro* and in humanized mice model and protected the CAR-modified T cells from infection. The engineered CAR with two major gp120 targeting domain combined with a two-molecule structure improved CAR T function.[35]

Universal CAR T platform: A newer approach to overcome antigen loss or antigen escape is the use of "universal" modular CAR designs where the scFv targeting the antigen of interest is fused to an intermediate soluble molecule or adaptor which can be bound by the construct containing the activation signal expressed by T cell. The 2 common modular CARs are split, universal and programmable (SUPRA) CARs and universal CARs (UniCAR) with the latter also allowing targeting more than one antigen and an on/off switch system in contrast to the traditional CAR which are always "on".[36,37] In HIV-1, this approach takes advantage of the natural binding of MHC 1 ligands to NKG2D expressed on CTL and NK cells to generate the components of a modular universal CAR T cell platform called convertibleCAR™-T (cCAR T)[38] [Fig. 2].The engineered extracellular domain of the NKG2D variant is tethered to the intracellular 4–1BB and CD3-ζ co-signaling domains to generate the CAR with the MIC ligand fused to broadly neutralizing HIV-1 antibodies to generate a bispecific molecule termed a MicAbody™. This platform is envisioned to allow multiplexing with several anti-HIV-1 antibodies yielding greater breadth and also allow control with ability of "on/off" of CAR T cells with the timing of administered HIV-1-specific

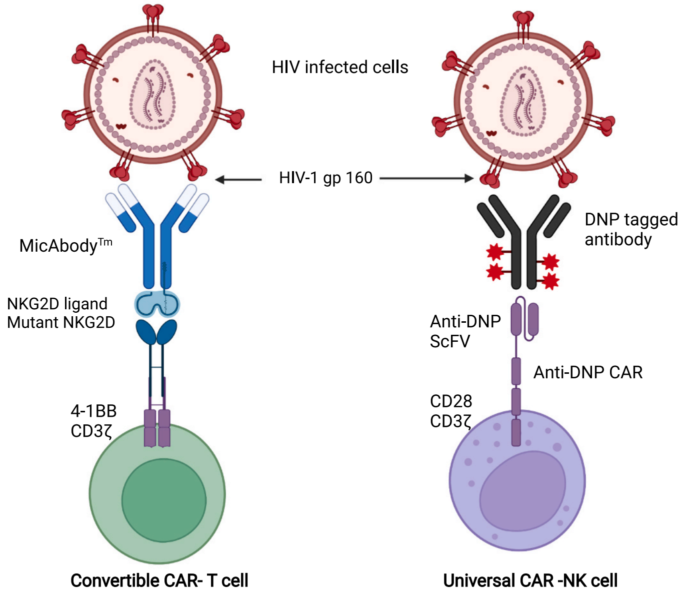

Fig. 2. Schematic representation of anti-HIV Universal CAR T and CAR NK Construct
Anti-HIV convertible CAR T consists of a mutant NK group 2D receptor (NKG2D) which recognizes mutant ligand domain fused to an anti-HIV antibody called MicAbody. Anti-HIV Universal CAR NK cell recognizes 2,4-dinitrophenyl (DNP) and is redirected to gp160 using DNP-conjugated antibodies as adaptor molecule. Created with BioRender.com.

MicAbody$^{\mathrm{Tm}}$, perhaps in conjunction with a latency reversal agent.

Hematopoietic Stem and/or Progenitor Cell (HSPC) CAR: In contrast to hematopoietic stem cell (HSPC)-based gene therapy, in which a high degree of stem cell replacement and ultimate peripheral cell reconstitution with the modified HSCs are necessary, HSPC-CAR if engrafted successfully, can offer long-term, stable, and continuous production of CAR cells, and circumvents the need for *ex vivo* expansion. CAR-bearing cells have been developed using HSPC that are modified with a protective CD4-ζ CAR that contains shRNAs against CCR5 and HIV-1 LTR (Triple CD4-ζ CAR). HSPC-derived cells were shown to differentiate into functional T or NK cells *in vivo* in humanized mice, to be resistant to HIV-1 infection and to suppress HIV-1 replication.[39] In a large animal preclinical model, HSPC-derived CAR T cells were capable of long-term engraftment and immune surveillance.[40] Autologous HSPC modified with a C46CD4CAR-expressing lentivirus were able to redirect HSPC-derived T cells against simian/human immunodeficiency virus (SHIV-1) infection in pigtail macaques with multi-lineage engraftment within tissue-associated viral reservoirs. Stem cell-derived CAR T cells trafficked to HIV-1 reservoirs in macaques and persisted for nearly 2 years in lymphoid germinal centers, the brain, and the gastrointestinal tract.[40,41]

4. CAR NK cells

4.1. Potential advantages of CAR-modified NK Cell immunotherapy

Alternatives to T cells have been explored to generate CARs, both for autologous and allogeneic CAR. NK cell-based immunotherapy is an attractive concept because, unlike T cells, NK cells do not require prior sensitization to kill susceptible virus infected cells or HLA matched antigen specific receptors. Adoptive immunotherapy with allogeneic NK cells therefore has had very low risk of GVHD[42] and a different cytokine profile with possibly lower risk of CRS was also suggested in an early CAR NK trial for CD19-positive cancer.[43]

NK cells isolated from HIV-1-infected individuals are impaired in their ability to kill HIV-1-infected autologous cells, as well as tumor cell lines.[44] HIV-1 infection leads to increased HLA-E expression resulting in impaired function of NK cells.[45] Like CTL, NK cells tend to undergo functional anergy and exhaustion during chronic HIV-1 infection.[46,47] Unlike B or T cells which possess a single activating receptor, NK cells possess a large array of receptors which recognize ligands on infected cells, usually by triggering two or more activating receptors. CTLs and NK cells express on their surface the NK group 2D receptors (NKG2D) which recognize a family of ligands overexpressed on cells stressed by viral infection or tumor.[48] Signaling of human NKG2D which is an important co-activating receptor for tumor recognition is critically dependent on disulfide-linked homodimer (DAP) 10.

Like CAR T cells, the CAR NK constructs used for cancer have used CD3-ζ with 4-1BB or CD28 as costimulatory domain. Sources of NK cells for CAR include autologous or allogenic peripheral blood, or cord blood or hematopoietic stem cell derived NK cells. In contrast to T cells, donor derived NK cells have limited transduction efficiency and there is heterogeneity in phenotypes and functional activity based on variations in NK subsets. Therefore off-the shelf immortal NK cell lines such as NK-92 derived from a human NK cell lymphoma are preferred. Irradiation of these cells prior to infusion and repeat administrations are required but use of cell lines provides a homogenous source of NK cells without variation in phenotype or cytotoxic activity and is amenable to genetic modifications.[49]

An initial approach to target HIV-1-infected CD4$^+$ T cells involved CD4-ζ CAR NK cells, derived from pluripotent stem cells.[50] These CAR NK cells effectively inhibited HIV-1 replication in CD4$^+$ T cells *in vitro* but did not suppress HIV-1 infection when compared to unmodified NK cells when infused in a humanized mouse model. This was possibly due to the lack of costimulatory molecules as have been used in 2nd and 3rd generation of CAR T cells. The optimal costimulatory molecules for NK cells signal transduction such as DAP 10 or CD28/4-1BB are not known.[51]

4.2. Universal CAR NK Cells

Anti-HIV CARs are usually limited by targeting a single epitope of the HIV-1 env gp160, but universal CAR NK cells use a tag-specific adaptor CAR to target various epitopes of gp160. Instead of targeting HIV-1 gp160 directly, NK-DNP CAR recognizes 2,4-dinitrophenyl (DNP), a small molecular tag, and are redirected to target HIV-1 gp160 by using DNP-modified bnAbs as adaptor molecules[52] [Fig. 2]. This anti-DNP CAR using the NK-92 cell line with CD28 co-stimulation was able to recognize and kill HIV-1-infected cell lines expressing gp160 from HIV-1 subtypes B and C. The gp160 epitope location affects the ability of

DNP-modified bnAb to redirect anti-DNP CAR NK cells. Antibodies targeting the membrane-distal epitopes such as V1/V2, V3, and CD4bs are more likely to activate universal CAR NK cells compared with those targeting MPER. If successful, this universal CAR NK platform approach can significantly expand HIV-1 epitope coverage.

Messenger RNA (mRNA) CARs

Historically, viral transduction has been used exclusively for generating CARs and the individualized *ex vivo* T/NK cell engineering process is extensive and costly. It also induces continuous and indefinite CAR expression once infused, with the risk for long-term adverse effects. To avoid or minimize CRS and other toxicities, adaptive expression systems for CAR T cells are being developed using mRNA encoded CARs which allow for transient CAR expression. CD5-targeted lipid nanoparticles containing mRNA instructions to encode a CAR *in vivo* yielded a transient therapeutic T cell population in a recent proof of principle study in a mouse model of heart disease.[53] An injectable nanocarrier that delivers *in vitro*-transcribed (IVT) CAR or TCR mRNA for transient reprograming of circulating T cells to recognize disease-relevant antigens has also been developed. Periodic infusions of CAR-encoding or TCR-encoding mRNA particles successfully induced antitumor responses with similar efficacies compared to virally transduced *ex vivo* conventional adoptively transferred T cells in mouse models of lymphoma, prostate cancer, and HBV-induced hepatocellular carcinoma.[54]

6. Preclinical novel strategies to protect CAR cells from HIV-1 infection

Expression of CD4 on gene modified T cells also renders them susceptible to HIV-1 infection and elimination.[55] Several strategies to protect the donor-derived CAR T cell products from HIV-1 infection are or have been evaluated in preclinical systems. First, co-expression of HIV-1 fusion peptides inhibitors, such as C34-CXCR4 have been engineered by conjugating a peptide from gp41 heptad repeat- 2 domain to the CXCR4 amino terminus[56], or membrane-anchored C peptide corresponding to a 46-amino acid sequence of gp41 that potently inhibits viral entry (mC46).[57] Next, co-expression of protective anti-HIV-1 shRNA targeting specific HIV-1 long terminal repeat (LTR) sequences (sh516) from the same lentiviral vector expressing the CAR have been developed, resulting in protection of the cell from direct infection through the CD4 extracellular domain.[58] Reduction or ablation of CCR5 expression on CD4-ζ CAR T cells or bnAb based CAR constructs has protected cells from infection using shRNA mediated knockdown of the HIV-1 co-receptor[59] using targeted disruption of the CCR5 gene locus by gene editing using zinc-finger nucleases (ZFN) or by non-homologous end joining (NHEJ) or homology-directed repair (HDR).[60] A CD4-CAR T cell modified by ZFN disruption of its CCR5 for HIV-1 resistance (ZFN CCR5 CD4 CAR) is being evaluated in an ongoing clinical trial (NCT03617198) [Table 1]. CCR5-edited CD4$^+$ T cells have been shown to augment HIV-1-specific immunity to enable post-rebound control of HIV-1 replication.[61] It is possible to protect HIV-1 CAR T cells by independent disruption of CCR5 by NHEJ or HDR with T cells engineered to express anti-HIV-1 CARs based on bnAbs.[60] Both methods produced functional CAR T cells that kill HIV-1-infected cells in the presence of ART, and HIV-1-resistant CAR T cells outperformed those without CCR5 disruption in live viral assays.

Another strategy to protect the donor-derived CAR T cell products from HIV-1 infection is co-expression of two shRNAs, one targeting CCR5 expression and one that downregulates HIV-1 expression by targeting the LTR region prevented CD4 receptor-based CAR T cells from becoming HIV-1 infected.[39] C46 can also be used in combination with the anti-CCR5 shRNA to prevent infection by X4 tropic or dual tropic viruses. A novel self-inactivating lentiviral vector, LVsh5/C46 which encodes shRNA for downregulation of CCR5, in combination with the HIV-1 fusion inhibitor C46 was stably expressed in the target cells and effectively protected gene-modified cells against infection with CCR5-and CXCR4-tropic strains of HIV-1[62] and was evaluated in a phase1/2 trial of viremic HIV-1 infected individuals with or without busulfan preconditioning (NCT01734850). The first two domains of the CD4 extracellular domains D_1D_2 domain, primarily mediate HIV-1 env binding to the CD4 molecule. Deleting D_3–D_4 domains of CD4-ζ CAR may prevent CD4-mediated HIV-1 infection of CD4-ζ CAR T cells while allowing env binding and signaling through the CD4 D_1D_2 domain, with varying results.[33,39] Finally, a novel approach to prevent viral reactivation in HIV-1 patient-derived CAR T cell products is a conditionally replication lentivirus (crLV)-derived CAR that parasitizes the HIV-1 machinery to encapsulate itself within the virion and confers a negative selective pressure on HIV-1 by acting as an interfering particle.[63] The crLV-derived CAR constructs functionally expanded T cells into anti-HIV-1 CARs *ex vivo*.

7. Anti-HIV-1 CAR cell therapy in clinical trials

Early generation CAR T cell trials demonstrated safety but no evidence of efficacy *in vivo*.[8–10] Numerous new CAR-based therapies developed to address specific challenges in HIV-1 therapy have shown promise in preclinical studies, with several products, including multiple lentivirus transduced CARs with modifications such as CCR5 gene deletion, advancing to early phase clinical trials [Table 1].

8. Challenges in anti-HIV-1 CAR cell therapy for immune surveillance and maintaining viral suppression

8.1. Antigen diversity and frequency of antigen expressing target cells

HIV-1 specific CAR T cells target HIV-1 env protein which is only expressed on the surface of virus-producing cells. In addition, the env glycoproteins can exhibit 35% amino acid diversity between subtypes and 20% within a subtype, leading to the expression of an extensively diversified gp160 proteins.[64] The rebound virus after ART interruption is genetically diverse, consistent with reactivation from many latently infected cells at multiple sites[65] and a CAR that can recognize various HIV-1 antigens would be required. Latency develops shortly after acute infection and the activated CD4$^+$ T cell can revert to a transcriptionally silent form, which results in minimal viral gene expression. HIV-1 remission trials will be attempted on PWH with durable suppression of viremia on effective ART where the frequency of HIV-1 env expressing cells will be very low. The level of antigen expression needed to enable recognition of infected cells by immunotherapies is not known.

A combination strategy of latency reversal agents (LRA) has been proposed to increase frequency of antigen expressing cells, however, clinical trials of LRAs to date have been disappointing and none sufficiently impacted the size of the latent viral reservoir.[66–69] Other LRAs, such as Toll-like receptor (TLR) agonists which have the potential for latency reversal and desirable immunomodulation are being explored in combination with bnAbs or vaccines.[70] Another study suggests that stimulation of HIV-1-specific CTL prior to reactivating latent HIV-1 using a potent LRA to enhance env protein expression is essential.[71]

Administration of allogeneic human peripheral blood NK cells delayed viral rebound following interruption of ART in humanized mice infected with HIV-1.[72] A kick and kill strategy comprised of the protein kinase C modulator SUW133 and allogeneic human peripheral blood NK cells during ART eliminated the viral reservoir in a subset of these mice. SMU-Z1, a novel small-molecule TLR1/2 agonist enhanced HIV-1 transcription and promoted NK cell-mediated inhibition of HIV-1 infected autologous CD4$^+$ T cells.[73] Exogenous cell-based antigen boosting using K562-env cell line combined with immune checkpoint blockade was successful in expanding anti-HIV-1 CAR T cells in a NHP model which is designed to recapitulate the low level of antigen present in ART suppressed patients.[74] Other considerations to increase antigen expression include combination with HIV-1 env vaccines, cell based artificial

Table 1
Completed, Ongoing, and Planned Clinical Trials of anti-HIV-1 CAR Cell Therapy.

CAR Construct	Virus vector and additional gene modification	Study Population	Preconditioning, ATI and/or Combination therapy	Trial Registry Identifier ([a]trial status) or publication reference number
Autologous CD4^{+} and CD8^{+} CD4-ζ CAR T cells co-stimulated with CD28	MMLV	ART suppressed (n = 40)		Deeks, Mol. Therapy, 2002[8]
Autologous CD4^{+} and CD8^{+} CD4-ζ CAR T cells	MMLV	HIV RNA 1000–100,000copies/ml (n = 24)	With and without exogenous IL-2 infusion	Mitsuyasu, Blood, 2000[9]
Syngeneic CD8^{+} CD4-ζ CAR T cells. Subsequent infusions CD4^{+} and CD8^{+} CD4-ζ co-stimulated with CD28	MMLV	HIV discordant twin pairs Both viremic and ART suppressed included (n = 30)	Subset with and without IL-2 infusion	Walker, Blood 2000[10]
Autologous CD4^{+} and CD8^{+} CD4-ζ CAR T cells	MMLV	ART suppressed (n = 17)	With and without exogenous IL-2 subcutaneous injection	NCT01013415 (Active, not recruiting)
Autologous T-cells stimulated with CD3 and CD28, encoding a broadly neutralizing HIV-1 scFv antibody	Lentivirus	ART suppressed (n = 8)		NCT04863066 (Not yet recruiting)
Autologous CD8^{+} T-cells encoding a broadly neutralizing VRC01 class antibody, co-stimulated with CD28 and 4-1BB (VRC01-28BBz-shPTL CAR)	Lentivirus Combination of shRNAs, including sh-PD-1, sh-Lag-3, and sh-Tim-3	ART suppressed (n = 15)	ATI in subset at least 3 weeks post infusion	Liu B. JCI 2021[30]
Autologous CD4^{+} CAR + CCR5 ZFN modified T cells	Zinc Finger nuclease-mediated disruption of the CCR5 gene	ART suppressed (n = 12)	Cohort 1: ATI 24 h after infusion Cohort 2: ATI 8 weeks after infusion	NCT03617198 (Active, not recruiting)
Autologous CD4^{+} and CD8^{+} T cells encoding Bispecific anti-GP 120 CAR molecules (LVgp120duoCAR T cells)	Lentivirus	ART suppressed (n = 18)	With and without non-ablative conditioning with cyclophosphamide. ATI immediately after infusion	NCT04648046 (Recruiting)
CAR T or TCR-T cellular therapy encoding broadly neutralizing HIV-1 scFv antibody		ART suppressed (n = 4)	In combination with latency reversal agent chidamide	NCT03980691 (Completed)

MMLV: Moloney Murine Leukemia Virus; ATI: Analytic treatment interruption; IL-2: Interleukin-2; TCR: T-cell receptor; n: number enrolled or planned.

[a] Clinical trial status as listed on ClinicalTrials.gov study record on March 11, 2022.

antigen presenting cells and leveraging mRNA for targeted protein expression or as an immunogen. A two-part "CARVac" strategy to overcome poor CAR T cell stimulation and responses *in vivo* is now in first in human clinical trial for solid tumors (NCT 04503278). This uses the tight junction protein claudin 6 (CLDN6) as a new CAR T cell target and a nanoparticulate RNA vaccine encoding the CAR directed toward CLDN6. This liposomal antigen-encoding RNA (RNA-LPX) vaccine promotes CLDN6 expression on the surface of dendritic cells, which in turn stimulates and enhances the efficacy of CLDN6-CAR T cells for improved tumor therapy.[75]

8.2. *Trafficking to tissue reservoirs and engraftment and persistence*

CAR cells must traffic to heterogeneous HIV-1 reservoir tissues and cell types and must engraft and persist to effectively survey, recognize, and kill infected cells. HIV-infected cells can persist during ART in anatomical sanctuary sites such as the brain and immune-privileged B cell follicular centers in the germinal centers of lymphoid tissue, and can contribute to viral rebound upon cessation of ART. A CD4-MBL CAR construct that enables the co-expression of CD4 and the carbohydrate recognition domain of mannose binding lectin (MBL) to target the follicular dendritic cells (FDC) reservoir did not respond to or eliminate FDC bound to HIV-1.[76] However, CXCR5 co-expression improved the concentration of CAR T cell in the B cell follicles in *ex vivo* tissues.[77] CAR T cells were detected in 98% of samples tested for at least 11 years post-infusion from three combined clinical trials of gammaretroviral vector engineered CD4-ζ T cells with stable engraftment and decay half-lives that exceeded 16 years.[78] Persistence of these first generation CAR cells did not translate to functional responses *in vivo*. Ten year follow-up of two participants with leukemia remission after CD19 CAR cell therapy provides some mechanistic clues for long-term remission. Both had persistence of CAR T cells with an initial CD8^{+} CAR T cells peak response phase followed by a long-persisting CD4^{+} CAR T cells with the latter thought to be primarily responsible for cytotoxic activity against CD19 expressing cells.[79] The role of the preconditioning regimen and its effect on CAR persistence and efficacy in HIV-1 is not well defined and needs to be carefully examined. A favorable cytokine profile induced by lymphodepletion was associated with durable remission in patients with aggressive non-Hodgkin lymphoma treated with CD19 CAR T cell therapy.[80] Further characterization of the use of different T cell subsets for the formulation of T cell products in CAR-based therapy is needed to optimize therapeutic strategies. CD19 CAR T cells derived from CD4^{+} naive and CD8^{+} central memory subsets conferred the strongest antitumor effects compared with CD19 CAR T cells derived from peripheral blood[81] and is being evaluated in CD19 CAR T cell manufacturing.

8.3. *Safety*

CD19 CAR T cell therapy for cancer can be associated with significant adverse events in patients, including CRS, macrophage activation syndrome, and B cell aplasia. Unlike cancer therapy, the threshold for tolerance of any adverse events in anti-HIV-1 CAR cell therapy will be very low. As part of the US FDA mandated long term follow-up for gene transfer studies using integrating vectors, CD4-ζ CAR has been found to be safe with three combined clinical trials providing over 500 patient years of clinical safety data and the recent bnAb based CAR trial also did not raise any safety concerns.[30,78] The risk of CRS and off target toxicities in HIV-1 CAR cell therapies is expected to be much lower as CRS is related to high antigen burden and env expression is restricted to target cells of interest. Newer gene edited techniques are less well evaluated. Additional genetic CAR modifications to achieve checkpoint blockade are in early clinical trials for malignancies, but the immune-related adverse events seen with checkpoint inhibitor immunotherapy, even if rare, would not be acceptable in trials for HIV-1 remission.

9. Conclusion

The remarkable progress in CAR cell technology and leveraging advances made in oncology raises hope of developing CAR as a "living drug" to enhance potent HIV-1 specific immune responses for long-term suppression of the reactivated latent viral reservoir without continuation of ART. Most people living with HIV-1 reside in geographical locations where autologous cell harvesting, *ex vivo* gene editing, and CAR cell product manufacturing for individualized cellular therapies is neither feasible nor scalable, and will need *in vivo* gene editing or "off-the shelf" CAR products for meaningful application. Advances in CAR technology and improved design demonstrate that potent HIV-1-specific T cells can be generated, and ongoing and planned clinical trials of anti-HIV-1 CAR cell therapy will provide additional insights into the amount of antigen required to sensitize cells, the role of CCR5, the *in vivo* potency and engraftment and persistence of these re-engineered CAR cells, and their effect on escape mutants. Continued progress toward an ART-free remission will require an iterative process including combinations of immune strategies that enhance antigen recognition in combination with CAR optimized HIV-1 control and eradication.

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