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CAR T cells for infection, autoimmunity and allotransplantation

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Abstract

Chimeric antigen receptors (CARs) have shown remarkable ability to re-direct T cells to target CD19-expressing tumours, resulting in remission rates of up to 90% of individuals with paediatric acute lymphoblastic lymphoma. Lessons learned from these clinical trials of adoptive T cell therapy for cancer, as well as investments made in manufacturing T cells at commercial scale, have inspired researchers to develop CARs for additional applications. Here, we explore the challenges and opportunities of using this technology to target infectious diseases such as HIV and undesired immune responses such as autoimmunity and transplant rejection. Despite substantial obstacles, the potential of CAR T cells to enable cures for a wide array of disease settings could be transformational for the medical field.

ToC blurb

Taking CARs down new therapeutic roads. This Review explores the challenges and opportunities of developing chimeric antigen receptors (CARs) for treating infectious disease, autoimmunity and transplant rejection.

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Author contributions

All authors researched data for the article, discussed its content and wrote, reviewed and edited it before submission.

Author notes

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Competing interests

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Introduction

Two broad categories of T cells work together to ensure specific and long-term immunity against pathogens and tumours, whilst protecting the body from aberrant responses against self. The first subset is comprised of effector T cells, which eliminate pathogens and tumours; regulatory T (T_{reg}) cells make up the second subset and function to prevent an immune response against self. Although effector T cell responses are generally potent, a subset of infectious diseases and tumours have evolved a large variety of escape mechanisms to bypass T cell control[1]. Similarly, the incidence of autoimmune diseases, such as type 1 diabetes, highlights that T_{reg} cells are not always successful in preventing aberrant immune responses. Moreover, in organ transplantation, T_{reg} cells often fail to protect life-saving tissues from immune rejection.

Chimeric antigen receptor (CAR) technology has emerged as a promising approach to reprogramme T cells to overcome the barriers that confront naturally occurring T cells. Because CARs alter how T cells recognize antigen by directly binding to cell surface proteins without requiring peptide presentation by MHC molecules, there are fewer available targets for CARs to recognize relative to TCRs. However, CAR targeting has more specificity, no HLA restriction, and avoid many of the T cell escape mechanisms that are used by infectious agents and tumours are no longer effective against CAR T cells. Although clinical success is relatively new to the CAR T cell field, the concept first emerged in the 1990s when investigators showed that T cell specificity could be redirected by fusing a targeting moiety that recognizes a cell surface protein with a T cell activation domain such as the CD3ζ cytoplasmic tail. The first example of this technology fused CD4 to the CD3ζ chain (CD4ζCAR). When expressed in effector T cells, this construct redirected T cell specificity to HIV-infected cells by taking advantage of the interaction between HIV envelope protein (Env) and CD4[2]. This concept was brought to the clinic in the late 1990s; although it was shown to be safe and feasible, durable control of virus infection was not observed[3–6]. In the intervening years, our understanding of how to engineer potent effector CAR T cells to target tumours has flourished[7–9]. In addition, pharmaceutical companies have promoted the transition of effector CAR T cell therapy from a boutique Phase I single center clinical trial to a Food and Drug Administration (FDA)-approved therapy that is capable of treating thousands of patients across the United States and elsewhere[10].

As T cells have pivotal roles in controlling infectious disease and autoimmunity, many in the field are considering how CAR T cell therapy could provide long-term solutions to diseases outside of cancer in which traditional medical approaches have not provided a cure. We focus our discussion on both the progress and remaining challenges of making CAR T cell

therapy a reality for individuals suffering from chronic infectious disease (HIV), autoimmune disease and transplant rejection. Given the abundance of T cell subsets and heterogeneity[11], we refer to broad groups of T cell populations without delving into the possible of benefits or pitfalls of more nuanced T cell subsets. Moreover, we concentrate on the issues that are unique to treating HIV infection with effector CAR T cells, and the challenges facing the field before engineered CAR T_{reg} cells can be safely infused into patients. Without doubt, advances in these two areas will also fuel new ideas about how to enhance CAR T cell function in cancer and beyond.

CAR T cells for the clearance of HIV

Although HIV infection induces robust antiviral immunity, the immune system fails to clear all of the HIV-infected cells. In part, this is because a small fraction of infected cells avoid immunosurveillance by expressing low to no amounts of viral antigen. These latently infected cells (often referred to as the HIV reservoir) can remain dormant for many years only to sporadically start producing infectious virus, which necessitates lifelong anti-viral therapy. It is clear from several studies that although early initiation of combination antiretroviral therapy (cART) reduces the size of the HIV reservoir, it does not eliminate its formation as HIV can still emerge after years of undetectable viraemia[12,13]. In addition, other factors — such as virus escape, HIV-specific T cell dysfunction and/or exhaustion and the physical segregation between immune effectors and infected cells — contribute to the inability of endogenous immune responses to eradicate infection[14].

Together, these factors indicate that successful HIV cure strategies will require potent and persistent cellular immune surveillance that remains poised to suppress virus re-emergence for perhaps decades. The adoptive transfer of effector T cells genetically modified with a CAR may transcend the limitations of virus-specific cytotoxic T lymphocytes (CTLs) that develop during natural infection to control disease, and prevent latency reactivation from becoming a clinically significant event. The advantages of CAR T cells compared with naturally occurring HIV-specific CTLs are summarized in Table 1.

As noted above, between 1995 and 2005, several clinical trials investigated the safety and efficacy of using CD4ζCAR T cells in HIV-infected individuals. The outcomes of these studies reinforced the safety and feasibility of *ex vivo* adoptive T cell gene therapy, but ultimately, treatment failed to durably reduce the viral burden within blood and tissue reservoirs[4–6]. These findings raised concerns about the ability of first-generation CAR T cells, which only contain the CD3ζ signaling domain, to function *in vivo.* Following on from these initial clinical trials, the cancer immunotherapy field has advanced the design and manufacturing of effector CAR T cells to generate optimal antitumour responses[15,16]. A key improvement in design from first- to second-generation CARs has been the inclusion of costimulatory signaling domains, such as those from 4–1BB and CD28, that modulate T cell function, persistance and susceptibility to exhaustion[17,18]. Costimulatory domains can also influence memory development and metabolism of CAR T cells. For example, signaling from a CAR containing a 4–1BB domain promotes a central-memory phenotype in T cells and reliance on oxidative phosphorylation for energy, whereas signaling from a CAR containing a CD28 domain promotes an effector-memory phenotype in T cells and an

augmented rate of glycolysis[19,20]. These findings help to reconcile the differential persistence of CAR T cells that has been observed *in vivo*, whereby different types of costimulation can reprogramme T cell metabolism to generate long-lived memory cells or short-term effector cells.

Recently, we re-engineered the CD4ζCAR used in the original clinical trials by altering the vector backbone, promoter, and the structural and signaling domains. Optimized CD4-based CAR T cells containing the 4–1BB–CD3ζ signaling domain (Fig. 1a) were at least 50-fold more potent at suppressing HIV replication *in vitro* than were T cells expressing the original CD4ζCAR. Moreover, when the optimized CAR T cells were evaluated in a humanized mouse model of HIV infection, they preserved the $CD4^+$ T cell count, reduced the HIV burden, and expanded in response to HIV to a much greater extent than did first-generation CD4ζCAR T cells[21].

Several groups have explored targeting HIV-infected cells using second-generation CARs with alternative antigen-binding moieties. CARs containing single-chain variable fragments (scFvs) derived from broadly neutralizing antibodies (bNAbs) have been developed that target conserved sites within the Env protein, including the CD4-binding site, the gp41 membrane-proximal external region and variable region glycans[21–23] (Fig. 1b). Despite the antiviral capacity of scFv-based CAR T cells *in vitro*, several factors may limit their therapeutic potential in humans. To become a broadly applicable therapy, scFv-based CAR T cells must overcome HIV escape, be effective against the diversity of HIV strains, and be non-immunogenic so that they can persist for decades.

Furthermore, bi-specific CARs were recently developed that fuse a CD4 segment to either a bNab-based scFv[24] (Fig. 1c) or the carbohydrate recognition domain (CRD) of a human C-type lectin[25] (Fig. 1d). These bi-specific CARs have dual specificity for HIV through binding of the CD4 fragment to the gp120 subunit of Env and, in the case of the CRD, binding conserved glycans on Env. However, C-type lectins can bind endogenous cell components such as normal cell-associated glycans[26,27], which raises the possibility of on-target, off-tissue reactivity. Despite the advantages and potential drawbacks of each type of antigen-targeting moiety, it is clearly possible that highly potent HIV-specific T cells can be generated by improving CAR design. This will likely impact the durability and function of CAR T cells in HIV-infected individuals going forward into clinical trials.

[H2] Enhancing CAR T cell persistence in vivo

CD19CAR T cells can induce long-term remission in some patients with specific B-cell malignancies[28,29]. Importantly, the durability of remission has been shown to correlate with the maintenance of functional CAR T cells[30]. Several studies showed that total CD19 antigen burden in patients (from both malignant and nonmalignant cells) is a crucial factor driving the proliferation and persistence of CAR T cells *in vivo*. For example, individuals with a high level of $CD19^+$ cells in the bone marrow prior to CAR T cell therapy had a greater magnitude of CAR T cell persistence post-remission, which correlated with a reduced risk of $CD19^+$ disease relapse[31–33]. This suggests that a high antigen load upon CAR T cell infusion may be required to achieve durable remission.

HIV infection poses a unique challenge because the quantity of virus-infected cells in cART-treated individuals is substantially less than the number of cancer cells in patients with leukaemia. As a result, strategies such as therapeutic immunization and/or multiple infusions of HIV-specific CAR T cells should be implemented to augment the persistence of CAR T cells after infusion. This will be essential to ensure that a sufficient number of CAR T cells are ready to respond to HIV rebound after treatment interruption, and to maintain a persistent CAR T cell population that is poised to react when virus reappears from latently infected cells.

Therapeutic immunization.—The immunological memory generated by traditional vaccines mediates resistance to infection upon re-exposure. Often, booster immunizations are administered subsequently to maintain a sufficiently large population of antigen-specific memory cells[34]. The same rationale for vaccination against infection is actively being applied to CAR T cell therapy. For example, investigators have manufactured dual-specific T cells by transducing the CD19CAR into T cells specific for Epstein–Barr virus, adenovirus or cytomegalovirus (CMV)[35–37]. In this manner, vaccines expressing viral epitopes that are targeted by these T cells can be administered to reinvigorate CAR T cells through endogenous TCR signaling.

As a proof of concept, tumour-bearing mice were infused with CD19CAR-transduced, CMV-specific T cells and then vaccinated with CMVpp65 peptide alone or with peptide-loaded antigen-presenting T cells (T-APCs). Both vaccination regimens elicited robust CAR T cell proliferation and augmented antitumour activity *in vivo*[35]. Similar approaches are being applied in clinical trials using the CD19CAR[38–40]. However, repetitive TCR stimulation of virus-specific memory T cells induces terminal T cell differentiation and reduces their replicative capacity[34]. Thus, optimum re-stimulation may be achieved *in vivo* by directly stimulating through the CAR and by using less-differentiated T cells as source material. To this end, a pilot study is in progress to evaluate episodic administration of $CD19^+$ T-APCs, which are designed to increase the number of CD19CAR T cells after remission and hopefully reduce the incidence of disease relapse[32,41].

The HIV research community has developed numerous prophylactic vaccines that could be adapted for use in non-human primates or humanized mice to evaluate their impact on CAR T cell persistence *in vivo*[42]. Ultimately, CAR T cells for HIV cure will need to persist in environments with low antigen burden, and it is hoped that existing vaccination strategies or candidates in preclinical evaluation can be used to augment the long-term survival of functional CAR T cells.

CAR T cell resistance to HIV.—The ability of CAR T cells to mediate a functional HIV cure is likely to depend on T cell persistence following adoptive transfer. However, the persistence of CAR T cells will be limited if they become infected; thus, protecting these engineered T cells from infection will be crucial. Preventing viral entry is the most effective strategy for engineering HIV-resistant CAR T cells[43]. This approach blocks virus propagation, and importantly, precludes integration of the virus into the host genome where it could persist in a latent state. Several *ex vivo* gene-editing strategies have been clinically investigated to abrogate HIV entry, including targeted disruption of the gene encoding the

HIV coreceptor CC-chemokine receptor 5 (CCR5) using zinc-finger nucleases[44,45]. Recently, new gene-editing strategies have been developed that enable high rates of homology-directed repair (HDR) of gene cassettes into specific genomic loci[46–48]. One study simultaneously disrupted *CCR5* using site-directed megaTAL nuclease and drove HDR using an adeno-associated virus donor template encoding a scFv-based CAR[23]. This method produced functional HIV-specific CAR T cells lacking CCR5 expression that suppressed virus replication *in vitro* to a greater extent than did CAR T cells generated by lentiviral transduction that were not protected from infection. Furthermore, concurrent *CCR5* disruption and targeted CAR integration by HDR offer several advantages. For example, the efficiency of *CCR5* disruption using good manufacturing practice (GMP)-compatible approaches is modest[45,49], so by encouraging CAR integration into *CCR5*, the number of CAR T cells that are also CCR5 deficient increases. Also, although there have not been any reported oncogenic insertional events caused by lentivirus integration into the genome of T cells, HDR potentially adds another degree of safety due to its targeted integration into the genome[23].

Despite the potential advantages of HDR, this technology is relatively new and it remains unclear so far whether safe and sufficient editing of T cells can be achieved at the clinical scale. As an alternative method, other groups have incorporated protection from HIV infection into T cells by either co-transduction or the integration of resistance genes into the lentiviral vector containing the HIV-specific CAR. For example, constructs have co-expressed sequence variations of the gp41 heptad repeat 2 domain, which inhibits HIV fusion at the virological synapse, or small hairpin RNA (shRNA) molecules targeting CCR5 and the HIV long-terminal repeat (LTR) sequence[50–53]. CCR5-targeting shRNAs downregulate expression of CCR5 by CAR T cells to prevent virus entry, and as a secondary measure, the LTR-targeting shRNAs mediate HIV RNA degradation, thus blunting a productive infection within CAR T cells.

It is important to note that a majority of these HIV resistance strategies were initially developed to protect $CD4^+$ T cells from viral infection[43]. HIV-specific CAR T cells may prove to be a more difficult population to protect due to the ability of the CAR construct to bind and concentrate HIV on the T cell surface. In addition, many CAR T cells have a persistent, low level of activation due to tonic CAR signaling[17,21,54]. Given that T cell activation influences the rate of HIV infection[55–59], basal activation of CAR T cells may inadvertently increase their susceptibility to infection. Moreover, as new HIV resistance strategies are being developed, it is uncertain how additional T cell engineering affects the fitness of CAR T cells *in vivo*, and what sacrifice in antiviral function might be incurred to achieve resistance to virus infection.

HSPC-derived CAR T cells.—Haematopoietic stem/progenitor cell (HSPC)-based gene therapy may overcome the limited persistence of peripheral T cell-based products in environments with a low antigen burden. HSPCs have the inherent ability to self-renew, proliferate and produce mature, multilineage immune cells that egress into the blood and tissues[60]. As a result, HSPCs modified with an HIV-specific TCR or CAR could provide long-term production and maintenance of HIV-specific T cells and other immune effectors[61,62]. To evaluate this approach, immunodeficient mice were engrafted with first-

generation CD4ζCAR-modified HSPCs and human immune cell reconstitution was shown to occur[63]. After HIV challenge, CD4ζCAR T cells retained effector function, proliferated and suppressed virus replication to a greater extent than did T cells from control mice[64]. Interestingly, CAR-modified HSPCs underwent altered T cell development by suppressing endogenous TCR recombination. Although the implication of this finding remains unclear, the use of HSPC-based gene editing may therefore prevent the generation of cross-reactive T cells that maintain dual specificity imparted by the CAR and the mature TCR.

Subsequent to this study, the authors evaluated the persistence and function of HSPC-derived CAR-modified cells in a non-human primate model of HIV infection[52]. Similarly to the mouse study, CD4ζCAR-modified HSPCs engrafted and differentiated into multiple haematopoietic lineages that expressed the CAR, including natural killer cells, which have the cellular machinery to integrate signals through the TCR CD3ζ chain and may contribute to HIV-specific immunity[65]. However, in other lineages such as B cells, CAR expression has no obvious benefit and it may render these cells susceptible to HIV infection. Thus, the overall benefit of CAR expression by non-T cells remains unclear. To evaluate the protective effect of CAR-modified cells, animals were infected with a simian/human immunodeficiency virus (SHIV) variant for 24 weeks, followed by 28 weeks of cART before treatment interruption. Ultimately, CAR-modified cells failed to prevent SHIV replication in the absence of ART, but there was a marked reduction in the magnitude of rebound viremia following treatment cessation in CAR-expressing primates, which was concurrent with the expansion of CAR-modified cell populations[52]. Despite the absence of durable SHIV control, this study showed the safety of HSPC-based gene therapy in a preclinical animal model, and importantly, therapy resulted in the stable production of antiviral cells for nearly 2 years. These findings underscore the possibility that HSPC-based CAR therapy could overcome the poor persistence that is associated with peripheral-based effector CAR T cell products.

Purging the latent HIV reservoir

The central challenge to HIV cure efforts is the persistence of a latent viral reservoir despite effective cART[66]. The ‘shock and kill’ strategy aims to purge this reservoir by using latency-reversing agents (LRAs) to disrupt HIV quiescence[67]. It is hoped that reactivated cells will die as a result of virus-induced cytopathic effects and/or be lysed by immune effectors[68]. However, LRAs alone have not measurably reduced the size of the latent reservoir, potentially because of insufficient virus reactivation and/or because existing immunity fails to clear reactivated cells[69–71]. Recently, it has been suggested that additional mechanisms contribute to this phenomenon. $CD4^+$ T cells harboring intact replication-competent HIV provirus, which are the source of recrudescent viraemia after cART interruption, elude $CD8^+$ T cell-mediated clearance possibly by dysregulating antigen presentation through HIV Nef[72]. Unlike naturally occurring virus-specific CTLs, CAR T cells may be uniquely well equipped to target $CD4^+$ T cells harboring intact provirus as CAR T cells do not require antigen presentation by MHC molecules to elicit an immune response. Also, defective proviruses can be expressed and recognized by virus-specific CTLs, which may distract the immune response from targeting the latent reservoir[73]. However, defective proviruses often contain deletions in *env* that probably abrogate expression of the full-length protein[74]; this

could mean that CAR T cells are poised to specifically recognize and lyse infected $CD4^+$ T cells containing intact provirus. Despite these advantages over naturally occurring virus-specific CTLs, CAR T cells must still overcome several challenges to purge the latent reservoir.

Infected $CD4^+$ T follicular helper cells (TFH cells) in B cell follicles of lymphoid tissue are a major compartment for persistent virus replication during cART[75,76]. Although virus-specific CTLs have been detected in lymph nodes, they are largely absent from the B cell follicles because they lack expression of CXC-chemokine receptor 5 (CXCR5), which is responsible for the trafficking of cells into the B cell zone along a CXC-chemokine ligand 13 (CXCL13) concentration gradient[77,78]. As a result, the paucity of CXCR5 expression on virus-specific CTLs is one mechanism that promotes the persistence of infected $CD4^+$ T_{FH} cells within an immune privileged site[79]. In addition to the physical segregation of virus-specific CTLs from infected $CD4^+$ TFH cells, recent data suggest that these CTLs have markedly reduced cytotoxic potential in lymphoid tissue, characterized by low levels of expression of perforin and granzymes[80]. This finding implies the existence of an unknown phenomenon that blunts CTL-mediated immunopathology in lymph nodes, which may be important for the unimpeded development of adaptive immune responses, but creates a unique anatomical niche with immune privilege that can enable pathogens such as HIV to proliferate unrestricted by virus-specific CTLs. Consequently, CAR T cells must overcome the immune privilege of the B cell follicle and maintain cytolytic function to cure HIV infection.

One approach is to engineer effector CAR T cells to express the follicular homing receptor CXCR5, which will mediate the entry of CAR T cells into the B cell follicle where they can target HIV-infected $CD4^+$ TFH cells (Fig. 2). As a proof of concept, CXCR5 has been ectopically expressed in peripheral blood-derived $CD8^+$ T cells from SIV-infected rhesus macaques[81]. After infusion, these $CXCR5^+CD8^+$ T cells preferentially homed to B cell follicles in both spleen and lymph nodes, and colocalized with SIV-infected cells. This approach could be applied to effector CAR T cells alone or in conjunction with LRAs to promote virus reactivation from latently infected cells (Fig. 2). Recent data indicate that the IL-15 superagonist complex ALT-803 can reverse HIV latency and promote $CD8^+$ T cell entry into follicular regions[82,83]. Systemic treatment with ALT-803 in SIV-infected rhesus macaques induced the accumulation of virus-specific $CD8^+$ T cells within the B cell follicle. Infiltration of virus-specific T cells into the follicles of elite controller rhesus macaques was concurrent with a reduction in the number of SIV-infected cells[82]. Together, these findings highlight how additional T cell engineering and/or LRA treatment could synergize with effector CAR T cell therapy to alter the trafficking of CAR T cells into sites of cryptic virus replication to purge persistently infected cells (Fig. 2).

[H2] Summary

The field of cancer immunotherapy has made important advances in CAR technology, which have resulted in remissions of a subset of treatment-refractory malignancies. Recently, the HIV field has applied these advances to generate functional HIV-specific effector CAR T cells that could be used in an immunotherapeutic strategy to eradicate disease. Toxicity has

been, and will continue to be, a crucial issue for CAR T cell therapy in patients with cancer. It is not yet known to what extent toxicity induced by HIV-specific CARs will limit their clinical use. Fortunately, HIV Env is a non-self molecule, thus faithful targeting of HIV-infected cells by CAR T cells should be achieved. An additional concern of CAR therapy is the onset of a cytokine storm, which has been observed in patients with cancer, particularly in those with large tumour burden[84]. However, CAR T cells for the treatment of HIV infection will probably be infused into the body during cART, when minimal amounts of antigen are present; therefore, the initial threat of a cytokine storm is unlikely. In the event that HIV-specific CAR T cells fail to control viral rebound after treatment interruption, the cytokine storm may become a problem. As the excess cytokine production is driven by antigen, cART as well as IL-6-specific antibodies may be an effective approach to halt this adverse event[85]. Furthermore, the high cost of CAR T cell therapies is a concern in both cancer and HIV infection, but if durable remission can be achieved, we are confident that an economical way to administer these lifesaving, life-changing therapies will emerge. These issues of toxicity and cost are discussed in greater detail elsewhere[10,43,66].

The new generation of CAR T cells are equipped to overcome many of the failures of endogenous virus-specific CTLs to control infection. So far, second-generation CAR T cells have proven safe and have been shown to have antiviral activity in both mouse and non-human primate models of HIV infection. However, successful CAR T cell therapy in humans will likely depend on the long-term maintenance of functional T cells that remain poised to respond to latent HIV reactivation for months or years after infusion. Thus, research emphasis must be placed on augmenting the survival of CAR T cells in environments with a low antigen burden and developing strategies to protect CAR T cells from HIV infection. Furthermore, future investigation could examine the synergistic effects of CAR T cells with LRAs and other immunomodulatory drugs to eliminate the latent HIV reservoir. Together, these studies highlight the immense promise of CAR T cells to be used alone or in combination with other therapies to cure HIV infection in humans.

CAR T cells for autoimmune disease

Many state-of-the-art treatments for autoimmune diseases are not curative, have marked side-effects and do not treat all of the disease-related complications. Thus, disruptive therapies, such as CAR T cell-based therapies, are desperately needed. For example, effector CAR T cells could be directed to kill the pathological immune cells of an autoimmune disease. Alternatively, as many autoimmune diseases can be attributed to a combination of sub-optimal function, trafficking, stability and abundance of T_{reg} cells, CARs could be used to guide T_{reg} cells to the autoimmune milieu where they can be activated, proliferate and exert their suppressive function. We discuss both approaches below.

[H2] Chimeric autoantibody receptors

In chimeric autoantibody receptors (CAARs), the extracellular portion of the receptor consists of the protein target of self-reactive antibodies, which enables CAAR T cells to destroy autoimmune B cells in a manner analogous to the way in which CD19CAR T cells target and destroy B cell leukaemia cells. Thus, when the B cell receptor (BCR) of an

autoimmune B cell from the polyclonal pool encounters an effector CAAR T cell, it is destroyed and cannot produce autoantibodies. Preclinical proof of concept was obtained from a humanized mouse model of pemphigus vulgaris, in which autoimmune B cells target desmogleins causing skin and other mucous membranes to blister. Patients with this disease have traditionally been treated with corticosteroids and other broadly immunosuppressive agents that reduce whole-body immunosurveillance. Effector T cells expressing a CAAR that consists of desmoglein 3 fused to a second-generation 4–1BB–CD3ζ signaling domain interacted with cognate BCRs and induced the lysis of pathogenic B cells[86]. Because effector CAAR T cells function by killing their cognate cellular targets, the lessons learned from ongoing clinical and laboratory studies of effector CAR T cells for cancer therapy are likely to apply to effector CAAR T cell therapy also. The use of effector CAAR T cells could be extended to treat other B cell-mediated pathologies, such as systemic lupus erythematosus or rheumatoid arthritis. Furthermore, CAAR T cells targeting a CAR molecule could be used as a safety switch to eliminate autoimmunity caused by a previous infusion of effector CAR T cells[87].

[H2] Re-directing regulatory T cells

Recently, several Phase I clinical trials have been completed testing the safety and feasibility of using polyclonal T_{reg} cells to delay the progression of type 1 diabetes and prevent graft-versus-host disease (GVHD) after bone marrow transplantation[88–91]. These pioneering studies have shown that generating very large numbers of T_{reg} cells in a GMP-compliant manner is feasible[92,93], and that large T_{reg} cell infusions are well tolerated by patients with no evidence of global immunosuppression. Importantly, the incidence of acute GVHD observed in patients treated with expanded T_{reg} cells was reduced. Furthermore, in the study of patients with type 1 diabetes, T_{reg} cells were found up to a year after infusion, which indicates that infused T_{reg} cells can persist and thus may be capable of promoting long-term tolerance.

Introducing CARs into T_{reg} cells is an attractive way to generate antigen-specific T_{reg} cells. In addition to reducing the number of T_{reg} cells that are required for an effective response[94], antigen specificity should restrict the trafficking and off-target suppression of injected T_{reg} cells. However, there are key differences in the biology of T_{reg} cells and effector T cells, including their responses to TCR stimulation[95], co-receptor ligation[96] and cytokines[97], that question how applicable the axioms established from the use of effector CAR T cells in patients with cancer will be to CAR T_{reg} cells.

Relative to HIV and cancer therapy, the use of CAR T_{reg} cells to fight autoimmunity is a relatively new concept. In a landmark study, CAR T_{reg} cells specific for 2,4,6-trinitrobenzene sulfonic acid (TNBS) were used in a mouse model of TNBS-induced colitis[98]. The authors showed that CAR T_{reg} cells could: proliferate in an antigen-specific manner; traffic and accumulate at the target organ; prevent or ameliorate TNBS-induced colitis at suboptimal doses at which polyclonal T_{reg} cells had no effect; and promote bystander suppression of a different form of colitis in the presence of target antigen. Later studies built on these findings by showing the ability of CAR T_{reg} cells to prevent and/or ameliorate disease in other mouse models of colitis[99], colitis-associated cancer[100], and

experimental autoimmune encephalomyelitis[101]. These mouse studies provide a strong rationale to move CAR T_{reg} cell therapy into preclinical studies. In Box 1, we discuss why MHC-mismatched transplantation is an attractive indication to first test CAR T_{reg} cells in the clinic.

Target selection.—Similarly to the use of CAR T_{reg} cells in patients with cancer, the ideal target for CAR T_{reg} cells in autoimmune disease will be highly expressed on the cell surface and expression will be limited to the cell type or tissue of interest (see Table 2 for a comparison between the properties of effector CAR T cells and CAR T_{reg} cells). Unfortunately, the difficulty of identifying the ideal target for cancer-specific effector CAR T cells is also shared for CAR T_{reg} cells. However, the consequences of off-target recognition are very different. Reactivity of effector CAR T cells against on-target, off-tumour tissue can have serious effects. For example, HER2/neu-specific effector CAR T cells caused fatal acute respiratory distress syndrome in one patient owing to a low level of expression of the target antigen in the lung[102]. By contrast, the off-tissue reactivity of CAR T_{reg} cells will probably have less severe consequences, as it is known that the infusion of polyclonal T_{reg} cells does not cause opportunistic infection or cancer[88–91]. However, the tonic signaling that has been observed for some CAR constructs[54] may give CAR T_{reg} cells constitutive suppressive activity; thus, the safety profile of unmodified T_{reg} cells may not predict the safety profile of CAR T_{reg} cells. One concern with on-target, off-tissue recognition by CAR T_{reg} cells is that these cells may preferentially home to the off-tissue site at the expense of where they are needed, and thus these off-tissue sinks may limit the effectiveness of antigen-specific T_{reg} cell therapy. Moreover, the accumulation of T_{reg} cells in otherwise healthy tissue may create a milieu that is favorable for tumour formation or pathogen survival, but to the best of our knowledge this has not been experimentally addressed as yet.

Cell stability.—If an effector CAR T cell converts to either an exhausted T cell or T_{reg} cell, this is unlikely to raise any safety concerns. This conversion may decrease the efficacy of the therapy, and in theory if most of the effector CAR T cells were to convert to T_{reg} cells then this could hasten disease progression, but this has not been observed in the cancer trials so far. By contrast, significant safety concerns would be raised if CAR T_{reg} cells convert to effector T cells, as this has the potential to exacerbate disease progression.

Evidence from mouse studies shows that when T_{reg} cells are exposed to inflammatory conditions, some cells lose expression of Forkhead box protein P3 (FOXP3) and gain proinflammatory function[103]. Thus, the conversion of β cell-specific CAR T_{reg} cells into effector T cells will likely potentiate the killing of β cells and accelerate, rather than delay, type 1 diabetes. Another way in which effector T cells bearing CARs could arise is if they contaminate the isolation of T_{reg} cells that are used for source material. As stated earlier, T_{reg} cells are a rare population and achieving 100% purity will be near impossible using current GMP reagents on a clinical scale. As we do not fully understand how effector CAR T cells and CAR T_{reg} cells differentially proliferate and traffic *in vivo*, it is possible that a small population of effector CAR T cells could expand or traffic[104] much faster than CAR T_{reg} cells, with devastating consequences. The opposite is also a concern, in that T_{reg} cells

could contaminate effector CAR T cell infusion products. However, T_{reg} cells can be easily removed from the infusion product by selection on anti-CD25 beads prior to transduction, and the culture conditions by which effector T cells are proliferated *in vitro* do not favour T_{reg} cell expansion[105].

Safety.—Several strategies have been proposed to minimize the possibility that effector T cells will express CARs that are intended to be expressed by T_{reg} cells. First, the choice of initial starting material will be important. Engineered T_{reg} cells derived from cord blood, rather than from adult peripheral blood mononuclear cells (PBMCs), will likely be safest as a starting material because they lack effector T cells that arise later in life, are easily isolated relative to T_{reg} cells from peripheral blood, and have a naive phenotype that is associated with T_{reg} cell lineage stability and function[106–108]. In most scenarios for which a patient does not have cryopreserved autologous cord blood, third-party cord blood T_{reg} cells are a viable, safe alternative that is already being used in clinical trials for the treatment of GVHD[89,90,93,109]. However, for applications other than GVHD, it is unclear how potential MHC mismatches might affect the long-term persistence and function of infused T_{reg} cells. In the absence of a suitable source of cord blood cells, adult PBMCs could be sorted for naive T_{reg} cell markers[106,110,111] provided that a GMP-compatible sorter becomes commercially available.

To test the stability of an expanded T_{reg} cell product, methylation of the T_{reg} cell-specific demethylated region (TSDR) can function as a marker for effector T cell conversion potential[112]. This test can be carried out in fewer than 24 hours and has been included in the product release criteria of expanded T_{reg} cells for patient infusion[113]. Finally, we have shown that a TCR with too low affinity to function in effector T cells was able to confer potent, antigen-specific suppression when expressed in a T_{reg} cell[114], which suggests that the signal strength required to activate a T_{reg} cell is less than that required to activate effector T cells. Thus, one way in which the safety of CAR T_{reg} cell therapy could be improved would be to engineer the CAR so that it has the signal strength to function in a T_{reg} cell, but not an effector T cell.

Once the cell therapy has been administered to a patient, the ability to induce apoptosis of engineered cells could mitigate adverse effects. Several suicide switches **[G]** have been described, whereby administration of an otherwise inert drug causes controlled apoptosis of the infused CAR T cell product[115]. More complex switches could be envisioned in the future, such as ones that induce cell death autonomously when FOXP3 expression is lost by CAR T_{reg} cells or when expression of IL-17 and/or another proinflammatory cytokine is turned on.

Studies have shown that T_{reg} cells can induce effector T cells to become suppressive cells also, through a process known as infectious tolerance[116]. Thus, the CAR T_{reg} cells might not be necessary for all of the therapeutic effect, provided that they induce the generation of a durable oligoclonal population of local T_{reg} cells. Lastly, introduction of *FOXP3* into CAR T_{reg} cells under the control of a heterologous promoter may help to maintain FOXP3 expression and suppressive activity even if the natural *FOXP3* gene expression is lost[117–119]. At a minimum, this approach would help to ensure that if antigen-specific T_{reg} cells lost

suppressive activity, the ectopically expressed FOXP3 would minimize the activity of the resulting effector T cells.

[H3] Signalling.—Because effector T cells and T_{reg} cells have distinct costimulatory requirements, it is possible that the costimulatory domain that gives T_{reg} cells the most suppressive activity will be distinct from the costimulatory domain that yields the most potent effector T cell activity. Moreover, CAR T_{reg} cells might need to be uniquely designed for each targeted autoimmune disease, as the choice of costimulation domain might affect trafficking, metabolism and/or survival of the CAR T_{reg} cells. However, mounting evidence indicates that CD28-mediated costimulation will be necessary for CAR T_{reg} cells[120]. So far, each published CAR has included the CD28 signalling domain[98–101,121–125] as CD28 signalling is known to be essential for proper T_{reg} cell maintenance, proliferation and function[126,127]. No comprehensive study comparing CD28 with other costimulation domains has been carried out; thus, other costimulation domains alone or in combination with CD28 may be of benefit. For example, the intracellular domains of cytotoxic T lymphocyte antigen 4 (CTLA4)[128], CD27[129] and inducible costimulatory molecule (ICOS)[130] could be useful as part of a CAR based on their demonstrated roles in the proliferation and development of T_{reg} cells.

Dosing and persistence.—Understanding the optimal T cell dose to infuse into a patient specific for each application of CAR T cells will improve the safety, efficacy and economic feasibility of this approach[131,132]. However, CAR T cells are a ‘living’ drug whose half-life is difficult to ascertain, and as most of our knowledge of CAR T cell persistence comes from measuring their abundance in the peripheral blood not tissues, determining the optimal dose of T cells will at best be complicated and disease dependent. For initial studies that used expanded T_{reg} cell populations to prevent acute GVHD, patients who received a larger dose of expanded T_{reg} cells benefitted more than patients who received a smaller dose; 43% of patients developed low-grade GVHD in an earlier, low-dose trial whereas only 9% of patients developed GVHD in the higher-dose trial (compared with 63% for controls)[90,133]. As T_{reg} cells are a rare population of cells, highly efficient culture systems may be needed to reach the target dose for CAR T_{reg} cells[92,105]. For transplant applications, in which there is an abundance of antigen, a relatively small dose of CAR T_{reg} cells may be sufficient if the therapeutic population can be expanded in the patient.

A successful effector CAR T cell therapy is designed to kill every cancer or virus-infected cell in the patient, thereby eliminating the persistence of its target antigen. When target antigen becomes limiting, the pool of infused CAR T cells may retract to a size where it cannot then respond to a recurrence of cancer cells. By contrast, properly functioning CAR T_{reg} cell therapy will protect its target cells from elimination and these cells will function as a source of antigen to maintain CAR T_{reg} cell persistence. Thus, successful CAR T_{reg} cell therapy will positively support the maintenance of engineered T cells, and thus may have an advantage over effector CAR T cells in terms of generating a durable cure.

Summary

Ultimately, the adaptation of CAR technology to treat autoimmune diseases and to facilitate organ transplantation has shown promise in the laboratory and in small animal models, which sets the stage for organ transplant studies in MHC-mismatched non-human primates. Yet, we must determine the optimal extracellular binding domains, intracellular signalling domain(s) and manufacturing protocols for these CARs, before investigating cell dosage, timing and route of administration in the clinic to maximize the safety, efficacy and durability of a cure. Owing to inherent differences between the biology of T_{reg} cells and effector T cells, as well as disease-specific requirements, much work remains to be done in developing the prime therapeutic product of CAR T_{reg} cells.

Concluding remarks

T cells have a pivotal role in controlling cancer, infectious disease and autoimmunity. Thus, it seems likely that engineered, re-directed T cells will also be able to control these disease indications when naturally occurring T cells fail. Likewise, as we better understand the biology of naturally occurring T cells, these advances will help the field to engineer ‘better’ T cells to function in a wide array of disease areas. We have highlighted many hurdles that remain to broaden the scope of CAR T cell therapy from patients with cancer, which may give the impression that these other therapies will be a long time coming. However, it is important to keep in mind how the initial clinical success that was seen in just 3 patients receiving CD19CARs[84] ignited a firestorm of activity that led to FDA approval a mere 6 years later. If early clinical success of CAR T cells is observed in patients infected with HIV or with autoimmune disease, then the path to FDA approval may be quicker as a result of the path blazed by the approval of CD19CAR T cell therapies.

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Glossary

Chimeric antigen receptor
(CAR). A synthetic receptor engineered to be expressed on the surface of T cells or other immune cells to redirect cellular specificity.

Combination antiretroviral therapy
cART). Consists of two or more active drugs with different mechanisms of action that are used to subdue HIV replication.

HIV reservoir
Persistent HIV that remains transcriptionally silent as inactive provirus within infected $CD4^+$ T cells despite effective cART.

Exhaustion

A state of T cell dysfunction that develops over time with repeated exposure to cognate antigen, such as during chronic infection with a virus such as HIV.

Broadly neutralizing antibodies
(bNAbs). Antibodies that have the unique ability to neutralize and prevent infection against multiple and diverse strains of HIV.

Homology-directed repair
HDR). A mechanism in cells to repair double-stranded DNA breaks using a DNA donor template with homologous sequences flanking the break site.

MegaTAL nuclease
Sequence-specific endonuclease with a DNA binding domain that promotes efficient cleavage of genomic DNA with a high degree of fidelity.

Good manufacturing practice
(GMP). A series of guidelines enforced by the Food and Drug Administration in the United States, and other similar bodies elsewhere, regarding the manufacturing of safe biological therapeutic agents.

Latency-reversing agents
(LRAs). Pharmacological agents that induce HIV transcription from cells harboring HIV provirus.

T follicular helper cells
(T_{FH} cells). A specialized $CD4^+$ T cell subset that primarily resides in the B cell follicles of lymphoid tissue to aid the development of the humoral immune response.

Elite controller
A rare population of HIV-infected individuals who can spontaneously control HIV replication in the absence of cART.

Cytokine storm
The excessive production of pro-inflammatory cytokines often induced by the dramatic over activation of immune cells. **[Au:OK? Please expand as appropriate]**

Chimeric autoantibody receptors
(CAARs). A CAR-like receptor in which the extracellular domain consists of the protein target of a B cell-mediated autoimmune response.

Pemphigus vulgaris
An antibody-mediated autoimmune disease that causes blistering of the skin.

Desmogleins
Components of cell–cell adhesion complexes that form desmosomes under antibody-mediated attack in patients with pemphigus vulgaris.

Bystander suppression

The ability of a T_{reg} cell to suppress effector T cell responses directed at an antigen distinct from the antigen that stimulated the T_{reg} cell.

Tonic signaling
Low-level signaling caused by antigen-independent clustering of receptor molecules in the basal state of a cell.

T_{reg} cell-specific demethylated region
(TSDR). A conserved region of intron 1 of *Foxp3* that is demethylated in cells that are stably committed to the T_{reg} cell lineage.

Suicide switches
Engineered logic gates used as a safety mechanism that cause cells to undergo apoptosis when certain conditions are met. Many suicide switches activate in response to exogenous drugs.

Infectious tolerance
A phenomenon by which T_{reg} cell activation can impart suppressive activity to effector T cells.

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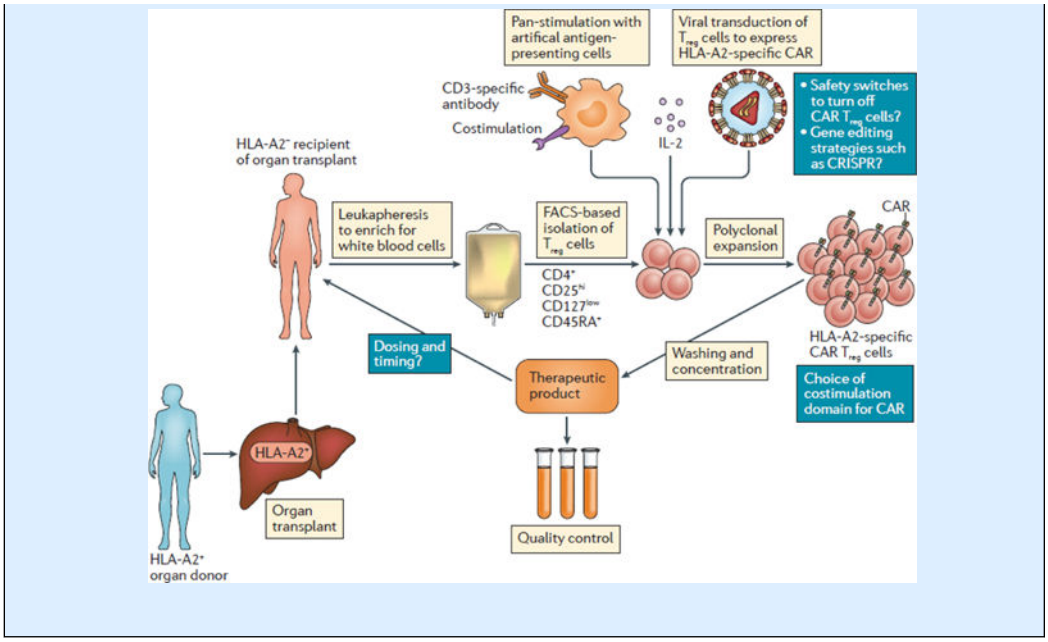
BOX 1 |

Towards the first clinical trial of a CAR T_{reg} cell therapy

Chimeric antigen receptor (CAR)-expressing regulatory T (T_{reg}) cells that recognize HLA molecules (allospecific T_{reg} cells) may provide the ideal scenario to test CAR T cell therapeutics for autoimmunity (see the figure for an idealized workflow. Human HLA molecules in the context of HLA-disparate transplantation are ideal targets for CARs, as the antigen is abundant and expressed solely on the transplanted organ. Moreover, the ligation of HLA molecules by CAR T_{reg} cells is unlikely to have any negative effect on graft cell function as these molecules have no signalling potential[134]. HLA-A2-specific CAR T_{reg} cells have been used to protect against graft-versus-host disease (GVHD) and skin transplant rejection in immunodeficient mice[59–61]. CAR T_{reg} cells that were transduced and expanded *in vitro* had normal levels of Forkhead box protein P3 (FOXP3) expression and demethylation of the T_{reg} cell-specific demethylated region (TSDR), and maintained the ability to expand into a suitable, therapeutic number of cells. Importantly, activation of the CAR caused minimal cytotoxicity of target cells[121]. In addition, the high levels of conservation between simian and human MHC molecules[135] raise the possibility of directly assessing human HLA-specific CAR constructs in primate-to-primate organ transplants. Hepatic transplantation is an attractive area in which to test allospecific T_{reg} cells. One-year graft survival rates are high, but long-term immunosuppression can reduce peripheral immunosurveillance and cause nephrotoxicity in graft recipients. Recent clinical studies have shown that some transplant recipients can be safely weaned off of drugs, creating a scenario in which CAR T_{reg} cell therapy could be safely tested for its ability to promote tolerance after immunosuppression is removed[136–138]. In those patients who experience acute rejection during the weaning process, immunosuppressive agents could be re-initiated to halt rejection. Furthermore, liver function tests enable non-invasive monitoring of graft rejection, and biopsies are routine if needed.



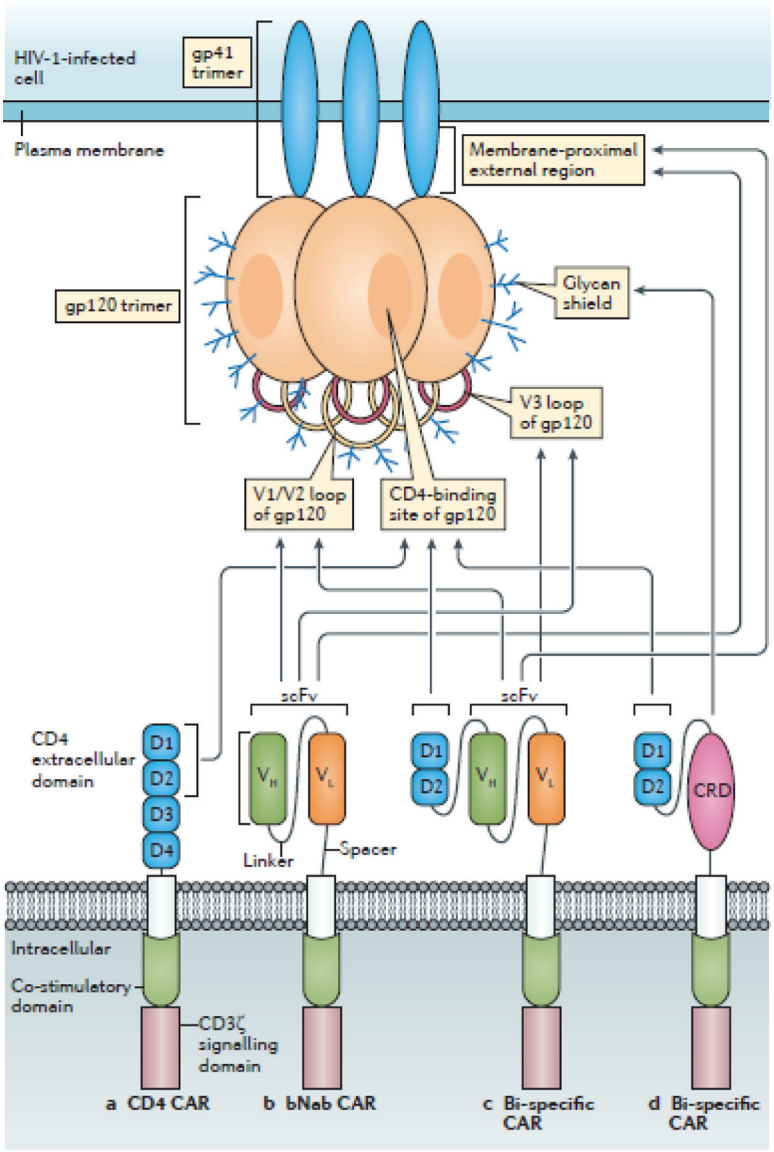


Figure 1. CD4-based chimeric antigen receptors (CARs) for HIV-1.

Extracellular antigen recognition domains of CARs determine their specificity for HIV-1 by targeting different regions of the HIV envelope protein (Env). **a** | The full-length extracellular domain of CD4 is comprised of four domains. Domains 1 and 2 are crucial for binding to the HIV gp120 component of the Env trimer. **b** | CARs containing broadly neutralizing antibody (bNab)-derived single chain variable fragments (scFVs) have been produced from antibodies such as VRCO1 and PG9, which differentially bind the Env trimer at the CD4-binding site and second variable (V2) loop, respectively. **c, d** | Bi-specific CARs confer dual specificity for HIV through the CD4–gp120 Env interaction, and either binding of the scFV to an alternative region in Env or binding of the carbohydrate recognition domain (CRD) of a C-type lectin to glycan motifs on Env.

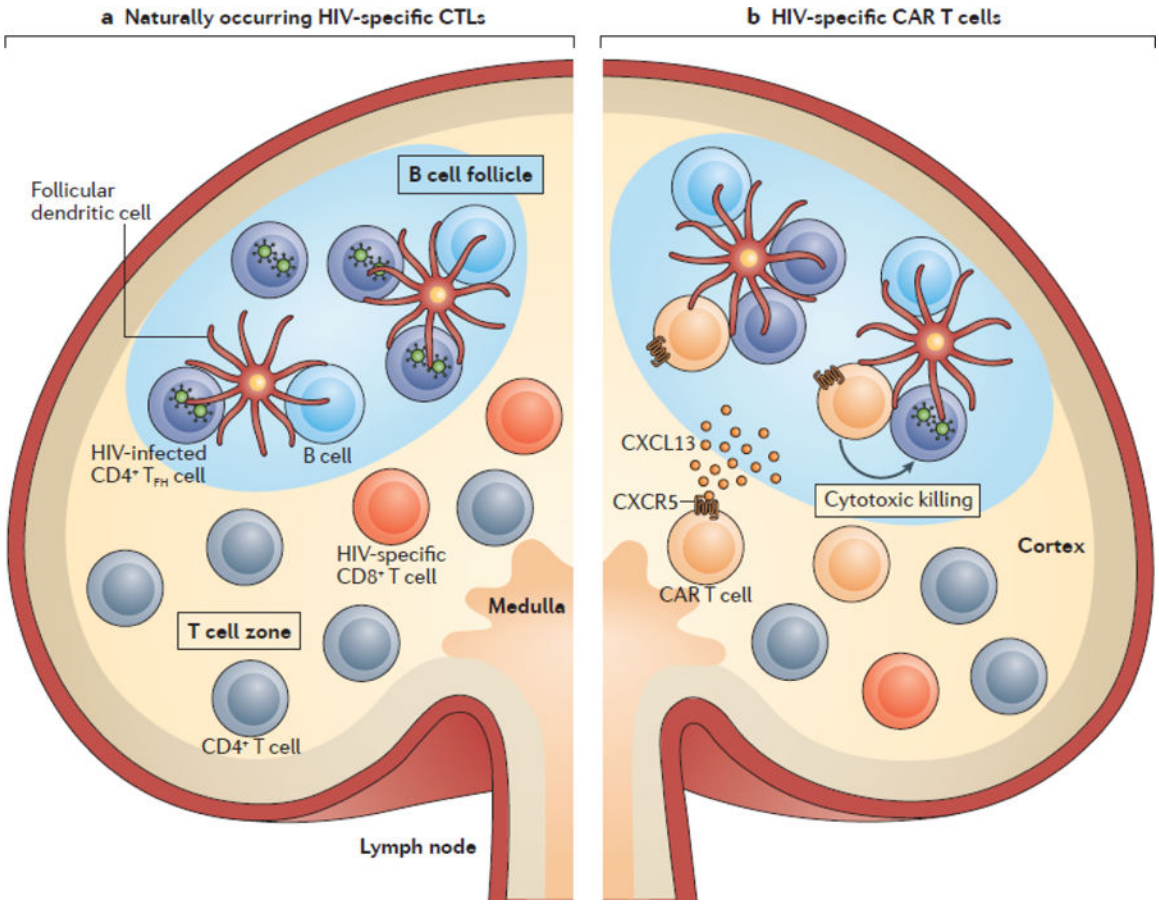


Figure 2. Engineering CAR T cells to traffic to B cell follicles.

The trafficking of chimeric antigen receptor (CAR)-expressing T cells into the B cell follicles of lymphoid tissue could facilitate the elimination of $CD4^+$ T follicular helper (TFH) cells persistently infected with HIV-1. Naturally occurring HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are present in the extrafollicular region of a lymph node. However, many CTLs fail to access the B cell follicle because they lack expression of the follicular homing receptor CXC-chemokine receptor 5 (CXCR5), which can mediate chemotaxis along a CXC-chemokine ligand 13 (CXCL13) concentration gradient. However, *CXCR5* gene engineering could enable HIV-specific CAR T cells to enter into the B cell follicle. Upon entry, CAR T cells could eliminate infected $CD4^+$ TFH cells and reduce the population size of cells that contribute to recrudescent viraemia after interruption of combination antiretroviral therapy (cART). In addition, before cART interruption, administration of latency-reversing agents, such as an IL-15 superagonist complex, could synergize with CAR T cells to eliminate the pool of infected $CD4^+$ T cells that exist in lymphoid and peripheral tissues.

Table 1 |

CAR T cells overcome the limitations of endogenous antiviral cytotoxic T lymphocytes

Properties	Naturally occurring HIV-specific CTLs	HIV-specific CAR T cells
Antigen recognition		
Virus escape	• Escape mutations within CTL-targeted epitopes render infected cells invisible to cell-mediated clearance	• Escape from CD4-based CARs is unlikely because it would impose a significant replicative fitness cost • Pre-existing escape mutations that abrogate recognition by bNabs may limit the efficacy of scFv-based CARs
Host protein dysregulation	• HIV accessory proteins down-regulate expression of HLA class I molecules and CD4, which promotes evasion from antiviral CTLs	• CAR T cells recognize virus-infected cells independently of HLA, and are thus insensitive to virus-mediated HLA downregulation
Targeting moiety	• HIV-specific TCR recognizes a single virus-derived peptide, so that virus escape and HLA downregulation counteract TCR-mediated recognition of virus-infected cells	• CAR targeting moieties engage HIV Env, which is expressed in an obligate manner on the cell surface during virus replication • CAR design can enable simultaneous recognition of Env in two or more distinct regions, which may enhance binding affinity of the CAR to Env
Functionality		
Exhaustion or persistence	• Persistent and large antigen burden in cART-untreated individuals induces exhaustion of antiviral CTLs that limits their function • Initiating cART during acute or chronic infection decreases viral antigen load, resulting in limited generation or reduced frequency of antiviral CTLs, respectively	• Quality of the infused CAR T cell product does not depend on the functional state of endogenous virus-specific CTLs, as the *ex vivo* T cell expansion and manufacturing process selects for the 'best-fit' T cells • Inclusion of costimulatory molecules into CAR design enhances in vivo function and persistence, and may prevent T cell exhaustion
$CD4^+$ T cell help	• HIV preferentially infects virus-specific $CD4^+$ T cells and impairs their ability to provide 'helper' signals to other arms of the immune system • Depletion of antiviral $CD4^+$ T cells contributes to immune dysfunction and disease progression	• CAR $CD4^+$ T cells can be engineered to be HIV-resistant, which would enable $CD4^+$ T cell-mediated help for CAR $CD8^+$ T cells and for endogenous antiviral immune responses • Inclusion of alternative costimulatory domains into the CAR could induce $CD4^+$ CAR T cell differentiation into various helper lineages, which could support different arms of the immune system.

bNab, broadly neutralizing antibody; CAR, chimeric antigen receptor; cART, combination antiretroviral therapy; CTL, cytotoxic T lymphocyte; Env, HIV-1 envelope protein; scFV, single chain variable fragment; TCR, T cell receptor.

Table 2 |

Unique challenges for therapy of autoimmune disease with CARs

Challenge	Effector CAR T cells	CAR T_{reg} cells
Stability	CAR T cells will lose function over time and may become exhausted. This may lead to a loss of efficacy but does not result in safety concerns.	CAR T_{reg} cells should be manufactured to be resistant to becoming ex-FOXP3' cells in an inflammatory microenvironment. CAR T_{reg} cells becoming effector CART cells is a major safety concern
Trafficking	CAR T cells must navigate to immunosuppressive cancer niches, through a dense network of stromal cells and collagen matrix, to reach the malignant cell types	$CD4^{+}T_{reg}$ cells typically function in secondary lymphoid organs together with APCs. CAR T_{re}, cells should be directed to tissue-specific locations, potentially through transgenic expression of chemokine receptors
Target antigens	The search for targets for CAR T cells has been focused on oncofetal antigens, tumour-associated antigens, protein overexpression or splice variants of normal proteins	The archetypal CAR T_{r}, cell antigen would be a normal self protein expressed exclusively on the target tissue under autoimmune attack. As disparate tissues can develop from a single multipotent progenitor, such antigens have proved to be elusive. The search should therefore be widened to include glycomic and lipidomic antigens

APCs, antigen-presenting cells; CAR, chimeric antigen receptor; FOXP3, Forkhead box protein P3; T_{reg} cells, regulatory T cells.