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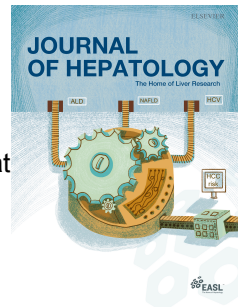

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Chimeric Antigen Receptor (CAR) T-cell Therapy: Engineering Immune Cells To Treat Liver Diseases

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Conflict of interest:

EJ is a co-founder, shareholder, and board member of Quell Therapeutics, London, UK

UP is a co-founder of SCG Cell Therapy Inc., Singapore, and a shareholder and board member. UP was a consultant for AATech, Aligos, Arbutus, GSK, Gilead, Leukocare, Roche, Sanofi, Vaccitech, and VirBio.

MH is listed as an inventor on patent applications and granted patents related to CAR technologies owned by the Fred Hutchinson Cancer Center, Seattle, WA, and the University of Würzburg, Würzburg, Germany, which have been partly licensed to industry. MH is a co-founder and equity owner of T-CURX GmbH, Würzburg, Germany. MH declares speaker honoraria from BMS, Janssen, Kite/Gilead, and research support from BMS.

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Abbreviations:

AIH: Autoimmune hepatitis

BCMA: B cell maturation antigen

CAR: Chimeric antigen receptor

CAAR: Chimeric autoantibody receptor

CCA: Cholangiocellular carcinoma

CD: Cluster of Differentiation

CRS: Cytokine release syndrome

ECM: Extracellular matrix

GPC3: Glypican3

HLA: Human leukocyte antigen

HCC: Hepatocellular carcinoma
HIT: HLA-independent TCRs
HLH: hemophagocytic lymphohistiocytosis
HSC: Hepatic stellate cell
ICI: Immune checkpoint inhibitor
ICANS: Immune Effector Cell-Associated Neurotoxicity Syndrome
MHC: Major histocompatibility complex
TAA: Tumor-associated antigen
TCR: T cell receptor
TME: Tumor microenvironment
TRAF: Treg with amplified suppressor function
Treg: regulatory T cell
TRUCK: T cells Redirected for Universal Cytokine Killing

Key points:

- A chimeric antigen receptor (CAR) is a synthetic receptor that recognizes an antigen on the surface of a target cell independent of the patient's HLA molecules.
- CAR-T cells are T cells engineered to express a CAR. The CAR activates the T cell upon binding to the target antigen.
- CAR-T cell transfer after lymphodepletion provided a breakthrough in treating hematologic malignancies but is increasingly used, e.g., in autoimmune diseases and fibrosis.
- CAR-T cells are an interesting, novel approach to treating liver diseases such as hepatobiliary cancers and autoimmune liver diseases, as well as hepatitis B and liver fibrosis.
- Regulatory T cells grafted with a CAR have the potential to support the acceptance of a liver graft after transplantation.
- Cytokine release syndrome, immune effector cell-associated neurotoxicity, and infectious complications are the most frequent but usually transient side effects of CAR-T cell therapies.

Abstract

Endogenous T cells recognize antigens through human leukocyte antigen (HLA)/peptide complexes. However, the polymorphism of HLA has posed significant challenges to the development of broadly applicable adoptive T-cell therapies. Engineered T cells can circumvent this barrier by targeting surface antigens independently from HLA through a synthetic chimeric antigen receptor (CAR) with an antibody-derived recognition domain fused to intracellular signaling motifs. CAR-T cell therapies have transformed the treatment of B-cell malignancies in hematology, and recent studies demonstrate therapeutic potential against solid tumors. This review presents an overview of CAR technology's fundamental principles and achievements, focusing on CAR-T cell applications in hepatic viral infections, autoimmune liver disease, and hepatobiliary tumors. Emerging senolytic therapies that target senescent cells and hepatic fibrosis are highlighted alongside regulatory CAR-T cells that induce liver-specific immune tolerance in transplantation. Future and ongoing research is reviewed that seeks to enhance the specificity, efficacy, and safety of CAR-based therapies as "living drugs" that facilitate targeted, sustained, and personalized interventions for liver diseases.

Introduction

T cells are crucial players in the adaptive immune system and have long been known to play a critical role in controlling HBV and HCV infections[1]. They also play an important role in attacking hepatocellular carcinoma (HCC), as indicated by the successful implementation of checkpoint inhibitors in HCC therapy[2]. Therefore, it seems intuitive to exploit T cells as the key substrate in a new generation of cellular immunotherapies that are genetically engineered to redirect and control their antigen-specificity and confer the attributes required for a reproducible and therapeutically beneficial immune response against liver diseases. Approved immune-based interventions for liver diseases or liver cancer include interferon alpha, immune sera containing neutralizing antibodies, and monoclonal antibodies inhibiting immune checkpoints and growth factors. With the current pace in translational research and clinical development of cell-based therapies, we expect to see engineered immune cells' approval to treat liver diseases in this decade. This review provides an overview of the principles of immune cell engineering, exemplified by T cells that are modified with a synthetic chimeric antigen receptor (CAR), with exemplary applications in viral hepatitis, hepatobiliary tumors, hepatic fibrosis, autoimmune liver diseases, and liver transplantation.

T cell therapy for liver diseases

Endogenous T cells recognize human leukocyte antigen (HLA)/peptide complexes through their T cell receptor (TCR). Although the adaptive T-cell response allows for a rapid and broad, but also particular immune reactivity against infections, the restriction of T-cells by the highly polymorphic HLA system complicates the development of adoptive T-cell therapies. TCRs recognizing hepatitis viruses with high specificity and avidity were cloned from donors with resolved infections and have become available for T cell engineering[3, 4]. TCR-engineered (TCR-) T cells follow the physiological path of antigen recognition and very sensitively recognize antigen-derived peptides in the context of an individual's HLA molecules. However, the fact that individual HLA subtypes vary significantly prevents applying the same TCR-T cells to all patients and requires careful pre-selection.

For cancer therapy, it has been difficult to reproducibly isolate T cells from the endogenous repertoire recognizing peptide epitopes derived from tumor-associated antigens (TAA) or mutated neoantigens expressed by a tumor with sufficient specificity and affinity to allow for

the recognition and elimination of primary tumor cells. A key challenge that has limited the application of TCR-T cells for HCC is that most TAAs are also expressed by "normal" developing or regenerating liver tissues. T cells that recognize such antigens with high affinity are eliminated during thymic selection in T-cell development[5]. An exception is virus-derived antigens, which vary significantly from the endogenous cellular antigens discussed below.

CARs are synthetic immune receptors that target antigens on the surface of cells.

A potential solution is the generation of CARs that recognize their antigen on their target cell's surface independently of HLA, first described in pioneering reports in the late 80's[6,7]. In 1991, *Irving et al.* showed that the intracellular CD3zeta domain of a TCR is sufficient to activate a T cell using a chimeric receptor[8]. However, developing clinically successful CAR-T cell products took over two decades of pre-clinical investigation.

In most cases, CARs carry an extracellular antibody-derived recognition domain consisting of a single-chain variable fragment (scFv). This scFv-based recognition domain is fused to an intracellular signaling moiety, consisting of CD3zeta and secondary costimulatory signals such as CD28 or 4-1BB co-stimulatory domains that enhance and expand CAR-T cell function.[9, 10] This secondary, costimulatory domain varies in different CAR constructs, and over the years, several generations of CARs have evolved. The latest developments included receptors in which the extracellular binding domains of CARs were fused to the physiological, intracellular signaling domains of a TCR (**Figure 1**).

Initially, the recognition domains that direct a CAR to its target were scFv from selected, murine or human monoclonal antibodies detecting the extracellular portion of a target antigen. Alternatively, scFv can be chosen from pre-existing libraries, or natural ligands or receptors can be used as binders. Nanobodies derived from camelidae provide another interesting alternative[11].

Clinical application of CAR-T cells

The use of gamma-retroviral or lentiviral vectors and the implementation of manufacturing schemes that enable the production of therapeutic CAR-T cells in sufficient amounts and within an acceptable time have supported clinical translation. Clinical observations informed subsequent iterations of CAR-T cell products on their efficacy and tolerability: using humanized CAR binding domains to reduce immunogenicity[12, 13], optimized CAR spacer and

transmembrane domains[14], and defined T cell subpopulations to confer consistent pharmacokinetic and pharmacodynamic attributes[15, 16]. This led to CAR-T cell therapies that have transformed treatments for hematologic malignancies and established the foundation for engineered T cell applications. CAR-modified T cells directed against the B-lineage molecules and cluster of differentiation (CD)19 are now routinely used to treat acute lymphoblastic leukemia[17, 18], B-cell lymphoma[19-21], and multiple myeloma[22, 23]. As of Spring 2025, seven autologous CAR T-cell products are FDA approved.

In some patients, CD19 CAR T-cells have still been detected more than 10 years after therapy, supporting the notion of CAR T-cells as a "living drug". Loss of CAR-T cell persistence has been attributed to T-cell intrinsic factors such as terminal differentiation or compromised "fitness" after prior chemotherapy in cancer patients or suboptimal stimulation with antigens that are either expressed at low density and therefore fail to induce a productive CAR T-cell response, or at too high density resulting in explosive CAR signaling and activation-induced cell death or T cell exhaustion[24, 25]. Primary and secondary cancer resistance mechanisms include antigen-downmodulation or loss, acquisition of mutations in apoptosis pathways, and cellular dormancy that results in cancer relapse. The rapid clinical development and success of CAR-T cell therapy in hematology spurred its application to treat solid tumors and applications in non-malignant diseases. In the following, we will discuss specific applications of CAR-T cells in various liver diseases.

Clinical safety and specific adverse reactions after CAR-T cell therapy

A Cytokine Release Syndrome (CRS) is the most prevalent and well-characterized toxicity. CRS occurs due to the massive release of pro-inflammatory cytokines, such as IL-6 and IL-1, mainly triggered by the activation of myeloid cells.[26] Symptoms range from mild fever and fatigue to severe manifestations, including hypotension, hypoxia, and multi-organ failure. Severe CRS necessitates interventions such as tocilizumab and corticosteroids, with early administration of these agents shown to significantly reduce fatal outcomes. Predictive biomarkers, including pre-infusion levels of C-reactive protein and ferritin, and scoring systems like "EASIX" have improved the stratification of patients at high risk for CRS.

Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS) is another critical toxicity with a diverse presentation, including confusion, aphasia, tremors, seizures, and, in severe

cases, cerebral edema.[26] The exact mechanisms involve the disruption of the blood-brain barrier and local cytokine surges in the central nervous system. Notably, ICANS often correlates with the severity of CRS, although it can also occur independently. Corticosteroids remain the cornerstone of ICANS management, with dexamethasone being commonly employed. *Anakinra*, an IL-1 receptor antagonist, has demonstrated efficacy in preclinical models and early clinical use, representing a potential adjunct therapy.

Long-term toxicities, such as prolonged cytopenia, pose significant challenges. Prolonged grade 3-4 neutropenia and thrombocytopenia can persist for months, increasing susceptibility to severe infections. Recent findings suggest that persistent bone marrow inflammation, mediated by IFN-γ-expressing T cells, may suppress hematopoietic recovery. Strategies under evaluation include thrombopoietin receptor agonists and autologous stem cell boosts, with growing evidence supporting their efficacy.

Emerging toxicities, such as movement disorders associated with anti-BCMA CAR-T cells, have added complexity to toxicity profiles. These delayed neurologic syndromes include Parkinsonism-like symptoms, such as rigidity and gait disturbances, which may result from CAR-T cell interaction with basal ganglia neurons. Enhanced monitoring and early interventions, such as bridging therapies to reduce pre-infusion tumor burden, have decreased their incidence.

Secondary hemophagocytic lymphohistiocytosis (HLH) represents a severe hyper-inflammatory state distinct from CRS. Characterized by hyperferritinemia, coagulopathy, and organ dysfunction, HLH often necessitates aggressive immunosuppressive therapy, including corticosteroids and IL-1 inhibitors. Its shared mechanisms with CRS highlight the need for differential diagnosis and tailored management strategies.

Infections and immunosuppression are pervasive concerns, driven by lymphodepletion regimens, B-cell aplasia, and hypogammaglobulinemia. Persistent infections, such as those caused by the reactivation of herpesviruses like cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human-herpesvirus-6, are particularly concerning, necessitating vigilant monitoring and prophylactic therapies. Immunoglobulin replacement or antiviral prophylaxis is increasingly used to mitigate these risks, especially in patients with prolonged B-cell depletion.

Obstacles to CAR-T cell therapy in liver disease

CAR-T cells are in clinical trials for HCC, hepatobiliary malignancies, and autoimmune liver disease, as well as to prevent rejection of liver grafts after transplantation. They are also promising approaches to treat viral hepatitis and liver fibrosis (**Figure 2**), but significant obstacles remain to CAR-T cell therapy of infectious and other non-malignant liver diseases. Lymphodepletion precedes T-cell therapy in almost all clinical trials because it creates a favorable immune environment for CAR-T cells, avoiding rejection and improving expansion, persistence, and clinical activity[27]. Lymphodepletion, however, has side effects and poses a particular problem for the endogenous control of viruses by T cells. Thus, clinical trials must develop strategies to avoid lymphodepletion in patients with infectious and non-malignant diseases[28].

In addition, CAR-T cells may face the exact immune modulatory mechanisms as endogenous T-cells (**Figure 3**). CAR-T cells reach the liver via the hepatic sinusoids lined by liver sinusoidal endothelial cells (LSEC). T cells can reach and kill hepatocytes through fenestrae in LSECs[29]. With increasing tissue disruption, however, this access is impaired, as a continuous endothelium is formed[30] and an extracellular matrix accumulates in the space of Disse that provides a physical barrier and hinders the contact of CAR-T cells with their target cells. The same hurdle applies to effector T and CAR-T cells trying to reach their target. This is particularly relevant for targeting HCC, stellate cells, or fibroblasts in advanced liver fibrosis or cirrhosis (**Figure 3B**).

CAR-T cells are also confronted with the exact immune regulatory mechanisms in the liver as "natural" T cells (summarized in [31]). Immunoregulatory cell populations, such as regulatory T (T_{reg}) or myeloid-derived suppressor cells (MDSCs), via methylglyoxal injection, prevent the local expansion of the CAR-T cells and restrict their cytotoxic activity (**Figure 3C**). Enzymes such as IDO, TDO, and arginase are expressed at high levels in the liver and metabolize amino acids essential for local CAR-T cell proliferation and function. Co-inhibitory signaling by binding PD1 on T cells to PDL1 on Kupffer cells, LSECs, stellate, and dendritic cells may restrict CAR-T cell effector functions, rendering them anergic or exhausted.

1. CAR-T cell therapy to treat viral hepatitis

Although the development of immunotherapies and proof-of-concept studies has been pursued mainly in hemato-oncology, infectious diseases, and cancers related to chronic infections are very appealing targets. Chronic viral infections may be an interesting application for T-cell therapy, as infected cells express specific virus-specific antigens, in contrast to TAAs that are also expressed in healthy tissue, which mainly differ from cancer cells in their antigen expression level.

Pioneering work with CAR-T cells directed against viral antigens

Very early on, CAR-T cells were exploited in clinical trials targeting HIV. First-generation CARs used the extracellular domain of human CD4, which targets the HIV envelope glycoprotein-120 (gp-120) expressed on the surface of infected cells, and later CARs used a scFv against gp-120 as a binder. However, only transient effects on viral load were observed in clinical trials, partially because the virus developed escape variants.[32] Herpesviruses like CMV or EBV threaten immunosuppressed patients. EBV is associated with lymphoma, as well as nasopharyngeal and gastric cancers. Thus, EBV also provides an interesting target for CAR-T cell therapy. The first CARs targeting EBV are currently in clinical trials targeting EBV-associated lymphoma and nasopharyngeal carcinoma, and results are eagerly awaited.

An essential limitation of targeting HIV, EBV, and other herpes- and coronaviruses is that these viruses undergo an early-late shift during their gene expression. Early genes express proteins that the viruses exploit to alter the cells and initiate viral genome replication. These early proteins are not displayed on the surface of infected cells and, therefore, are not suited as targets for CAR-T cells. Viral envelope proteins would be good targets. However, HIV, CMV, and EBV envelope proteins are only expressed late during the infection cycle, allowing CAR-T cells or antibodies targeting only for a short period before the cell releases newly formed viruses, often accompanied by cell lysis[33]. The situation is different for the hepatitis viruses, HBV, HCV, and HDV. These hepatitis viruses, unlike most other viruses, do not undergo an early-late shift. HBV and HCV express their envelope proteins continuously after productive infection of a hepatocyte.

CAR-T cell therapy for treating HBV infection

HBV expresses its small surface protein S constantly not only in infected cells but also from most viral genomes randomly integrated during long-term infection, which is a hallmark of premalignant cells, rendering it an interesting therapeutic target. The expression of HBV envelope proteins is clinically detected by a continuous secretion of the hepatitis B surface antigen (HBsAg). Because HBV envelope proteins are embedded in the endoplasmic reticulum and plasma membranes, they are displayed on the surface of infected cells, which can serve as targets for CAR-T cells[34] (**Figure 2**). This finding initiated the development of CARs and T-cell engager antibodies suitable to treat hepatitis B and hepatitis D.[35-38] A CAR has also been developed targeting the E2 envelope protein of HCV[39]. Still, with curative treatments using directly acting antivirals now available, CAR-T cell therapy for hepatitis C has not been further pursued.

Bohne et al. first attempted to re-target T cells against HBV-infected cells. They generated second-generation CARs, recognizing HBV S and large (L) surface proteins on the surface of replicating cells to target HBV-infected cells and efficiently kill HBV-positive hepatocellular carcinoma cells [35]. A scFv that recognizes a conformational epitope in the external loop of the HBV S protein of a broad range of HBV genotypes was selected as best suited[40]. Thus, these CARs recognize all three HBV envelope proteins, S, M, and L. T cells grafted with the S-CAR could clear HBV from infected, autologous primary hepatocyte cultures[35], infiltrated into the liver in immunocompetent HBV-transgenic animals, and controlled HBV replication in the liver[41]. In subsequent studies, new binders were cloned from human B cells able to optimize CAR-T cell function [37, 38], and a preS1-targeting CAR was shown to control HBV in HBV-infected humanized mice[37].

The CAR-T-cells described kill infected hepatocytes and secrete cytokines. These cytokines, including IFNγ, lymphotoxin, and TNFα derived from CAR T-cells inhibit virus replication in a non-cytopathic fashion[42] and activate endogenous CD4 and CD8 T-cell responses[43]. However, the effector function of virus-specific T cells, including the CAR-T cells, may be shut off by immune checkpoints (e.g., programmed cell death 1 (PD1) on T cells interacting with its ligand (PDL1)). In addition, a liver rheostat may influence the effector function of T cells, which, upon prolonged contact with LSEC, shut off their intracellular T-cell receptor signaling[44] (**Figure 3A**.

Because HBV causes about half of all HCC worldwide, T cells engineered with HBV-specific TCVRs have been used to treat HBV-associated HCC. In these HCCs, HBV genomic sequences are integrated, expressing the viral antigens HBs and HBx. HBs can serve as targets for TCR- or CAR-T cell therapy[45, 46]. HBsAg-targeting TCR-T cells applied as 2nd or 3rd line therapy were reported to reduce tumor load, slow down tumor progression[43, 46], and eliminate HBsAg, curing HBV and the underlying chronic infection.[46]

2. CAR-T cell therapy to treat hepatobiliary cancers

HCC is susceptible to antibody- and cellular immune recognition

The application of CAR-T cell therapy in solid tumors, including HCC, remains challenging due to the absence of true tumor-specific antigens, the underlying liver fibrosis or cirrhosis, and the highly inhibitory tumor microenvironment (TME)[47] (**Figure 3B, 3C**). The HCC TME is notably hostile to effective immune responses, characterized by a lack of metabolic factors necessary for immune cell function due to the activity of immune modulatory enzymes, the dominance of anti-inflammatory cytokines, the local enrichment of inhibitory immune cells such as Treg or MDSC, the overexpression of immune checkpoint molecules that inhibit T cell activity[47] and the lack of fenestrae in the endothelium (**Figure 3B**).

Initially, HCC was considered a low-immunogenic tumor, supported by the minimal efficacy observed in early immune checkpoint inhibitor (ICI) trials. However, this view changed significantly with the IMbrave150 trial, which demonstrated that combining an anti-VEGF agent (bevacizumab) with an antibody inhibiting PDL-1 (atezolizumab) could achieve substantial response rates, including long-term survival in a subset of patients[48, 49]. This pivotal study highlighted the importance of modifying the TME and activating the adaptive immune system to overcome HCC's inherent resistance to immune-mediated destruction.

Several TAAs have been identified in HCC that present viable targets for T-cells (**Table 1**). However, the endogenous T cell repertoire is often tolerant to these antigens, or T cells become exhausted due to chronic antigen exposure.[50] In addition, it is hard to identify

neoantigens because the mutation rate in HCC is relatively low compared to other cancers.[51, 52] Thus, CAR-T cells targeting TAA could have potent therapeutic effects, provided these engineered T cells can overcome the barriers imposed by the TME and the liver tissue alteration observed in most livers developing HCC (**Figure 3**). Ideally, T cell therapy could be used to target minimal residual disease after liver transplantation or HCC resection and prevent frequent HCC relapses.

Proof-of-concept with GCP3 CAR-T cells in HCC

A key target for CAR-T cell therapy in HCC is Glypican-3 (GPC3), a cell surface proteoglycan overexpressed in approximately 70% of HCCs (**Table 1**). GPC3 is minimally expressed in normal tissues, including normal and cirrhotic livers, making it an attractive target for immunotherapy. GPC3 plays a significant role in HCC pathophysiology by stimulating the Wnt signaling pathway, which is crucial for tumor growth and survival. Thus, the loss of GPC3 expression could reduce the malignant potential of HCC.

Gao et al. demonstrated the effectiveness of these CAR-T cells in vitro and humanized mouse models of HCC xenografts[53]. Given the challenges posed by the HCC TME, GPC3-specific CAR-T cells developed for clinical application were designed to resist the suppressive effects of the TME. For instance, GPC3-specific CAR-T cells engineered to secrete IL-7 and CCL19 achieved complete tumor disappearance in a single patient within 30 days of administration, demonstrating the potential of these advanced CAR designs[34].

Several larger phase I clinical trials have explored the efficacy of GPC3-specific CAR-T cells. In one study, fourth-generation IL-15-armored GPC3-specific CAR-T cells achieved a disease control rate of 66%, with an antitumor response rate of 33%[54]. Infusing these IL-15-enhanced CAR-T cells was associated with increased cytokine release syndrome, a common side effect of CAR-T cell therapy, which was rapidly controlled using an inducible caspase 9 safety switch. Another study utilized affinity-tuned GPC3-specific CAR-T cells co-expressing a dominant-negative TGF-β receptor II to neutralize the abundant TGF-β in the tumor microenvironment. This approach resulted in a disease control rate of 91%, with 42% of patients experiencing a tumor size reduction of more than 30%, even after failing 2-3 lines of prior therapy (http://www.clinicaltrials.gov/ct2/show/NCT05155189). Both studies highlight the critical

need for CAR-T cell therapies that target tumor-specific antigens and modulate the tumor microenvironment to enhance efficacy.

Expanding the target tumor antigen portfolio for CAR-T cells in hepatobiliary cancer

Beyond GPC3, other antigens have been identified as potential targets for CAR-T cell therapy in HCC and cholangiocarcinoma (CCC), such as the epithelial cell adhesion molecule (EpCAM), mucin 1 (MUC1), the HBsAg, CD147, alpha-fetoprotein (AFP), the hepatocyte growth factor receptor c-Met, and claudin-4 or its analog claudin-6. Their properties are summarized in **Table 1**. However, in most cases, solid clinical proof-of-concept is still missing (**Table 2**). In addition to these established targets, other potential antigens such as NKG2D, DLK1, and CEA are being investigated. However, the scientific data supporting their efficacy in CAR-T cell therapy for solid cancers is limited.

Combination therapies to augment the efficacy of CAR-T cells against HCC

T cell therapies against HCC showed limited efficacy when used as monotherapies, necessitating the development of combination therapies to enhance their therapeutic potential. One of the most frequently used combinations is the co-application of immune checkpoint inhibitors, which help to counteract the immunosuppressive TME and prevent T cell exhaustion. Combining CAR-T cell therapy with established HCC treatments has shown promise in improving tumor targeting. These combination strategies are tested alongside locoregional and systemic HCC therapies to reduce tumor burden and enhance CAR-T cell effectiveness.[55]

Another promising approach uses a fourth-generation CAR or a TCR fusion construct (TRuC) (**Figure 1**). Here, T cells are engineered to secrete cytokines upon antigen binding, such as IL-15, which enhances T-cell survival, and IL-12 or IL-18, which boosts anti-tumor immunity.[56] In vivo amplification of CAR-T cells using mRNA vaccines is a promising novel strategy.[57, 58] This method enables the expansion and activation of CAR-T cells directly within the patient's body. It can potentially reduce the need for prolonged *ex vivo* cell culture and enhance the overall efficacy of the treatment.

HCC is one of the few cancers that can be cured through liver transplantation. CAR-T cell therapy offers a unique advantage in this context, as the therapeutic goal may be partial

remission to enable downstaging to liver transplantation.[55] However, this combined approach depends on the availability of donor organs. CAR-T cell therapy for HCC after transplantation is challenged by maintaining T-cell function despite immunosuppression and avoiding alloreactive T-cell responses that could lead to transplant rejection.

3. CAR-T cell therapy to treat degenerative liver diseases

Targeting hepatic stellate cells to treat liver fibrosis

Fibrosis, a wound healing response during chronic liver injury, is associated with progressive accumulation of extracellular matrix (ECM) that ultimately impairs organ function and creates a stromal environment that confers a risk of cancer. The cellular source of ECM in the liver and other tissues is well established as resident pericytes that transdifferentiate or 'activate' into fibrogenic, contractile myofibroblasts[59]. In the liver, hepatic stellate cells (HSCs) are the origin of these fibrogenic cells. They are, therefore, an appealing target for clearance by CAR-T cells directed at cellular receptors expressed by the activated HSC. However, the targeting of HSC and fibrotic cells by CAR-T cells may be hindered by the loss of endothelial fenestration and the accumulation of ECM during the development of liver fibrosis (**Figure 3B**).

Single-cell sequencing studies highlight the remarkable heterogeneity and plasticity of HSCs in the liver[60, 61]. Among activated stellate cells, a subset with features of senescence is a particularly appealing target because this subset drives exuberant inflammation and tissue injury and promotes a carcinogenic milieu. *Amor et al.* sought to uncover cell surface markers of senescent HSCs using informatics, identifying urokinase plasminogen-activated receptor (uPAR) as an appealing candidate[62] (**Figure 2**). Administration of CAR-T cells targeting uPAR in two murine models of liver fibrosis significantly reduced ECM and improved liver function. In a subsequent study, the phenotype and ontology of senescence HSCs in mouse and human liver injury have been more thoroughly characterized[63]. While uPAR is restricted to HSCs in early experimental injury, its expression expands to other cell types as the disease progresses. This finding indicates that collateral clearance of uPAR-expressing macrophages may amplify the efficacy of this CAR-T strategy, a point discussed further below.

Targeting fibrotic tissue with FAP CAR-T cells

A related approach in cardiac fibrosis has utilized CAR-T cells directed at the cell surface protein fibroblast activation protein (FAP)[64] (**Figure 2**), which is restricted to fibrogenic cells in the heart as well as in other fibrotic tissues, including the liver[65, 66]. CAR-T cell-mediated clearance of FAP-expressing cells in the heart reduces fibrosis and improves cardiac function, reinforcing the appeal of this strategy in patients with fibrotic cardiac disease; in principle, this strategy should be effective in liver fibrosis, where HSCs express cell-surface FAP[65, 66]. More recently, CAR-T cell therapy is being explored for myelofibrosis by targeting a mutated surface protein, calreticulin, and additional fibrosis targets, likely to emerge in other tissues.

Whereas the long-term persistence of conventional CAR-T cells ensures ongoing surveillance of carcinogenesis, this durability may be less desirable in treating non-malignant diseases, including liver fibrosis. Unrestrained HSC clearance, for example, may be detrimental if the underlying disease is abrogated, for example, after the cure of hepatitis C infection. It may also be detrimental if the clearance of HSCs is too complete. This concern is underscored by a recent study in which >99% depletion of HSCs was accomplished in mice by administering recombinant CD8 T cells directed towards green fluorescent protein (GFP)[67], which was transgenically expressed in HSCs[68]. Complete HSC depletion dramatically impaired liver regeneration, pointing to a homeostatic role of HSCs that must be preserved when subsets of this cell type are depleted.

The concern about the impact of unchecked HSC depletion on liver homeostasis has been circumvented by developing a CAR-T cell strategy that generates target-specific CAR-T cells *in vivo,* whose long-term activity is constrained by the expression of a CAR through non-integrating mRNA instead of DNA. Specifically, lipid nanoparticles are administered to target T lymphocytes in the circulation, carrying instructions to reprogram T cells into CAR-T cells. Reprogrammed CAR-T cells then target FAP-expressing cells for clearance, yielding the same beneficial effects as conventional *ex vivo* CAR-T cells. This approach is exciting because: 1) The magnitude of CAR-T generation can be titrated based on the dose of LNP; 2) CAR-T cell-mediated clearance of target cells is self-limited because the effect of dosing with LNP-containing mRNAs is transient; 3) The approach can be scaled more readily than *ex vivo* CAR-

T cells, since frozen LNPs targeting FAP can be widely distributed, much like the LNP-mRNA vaccines that were used successfully for SARS-CoV-2.

CAR-T cells may combat aging

A remarkable study has raised the prospect of using CAR-T cells to prevent or treat aging-related metabolic dysregulation.[69] The same uPAR CAR-T cells used to clear senescent HSCs[62] and reduce fibrosis, described above, were administered to systemically clear senescent cells marked by uPAR expression. This approach improved glucose intolerance in naturally aged mice or animals fed high-fat diets. Even more impressively, a single prophylactic administration of uPAR CAR-T cells prevented features of age-dependent metabolic dysregulation.

The relevance of these findings to metabolic dysfunction-associated steatotic liver disease (MASLD) is compelling. With one-third of the world's population affected by MASLD associated with metabolic syndrome[70], this potential senolytic therapy links senescent cells directly to the pathogenesis of liver disease, affecting not only HSCs but also other cell types, including epithelial, immune, and other mesenchymal cells. Moreover, in this instance, the long-term activity of senolytic CAR-T cells could provide enduring benefits for aging and perhaps chronic diseases characterized by tissue injury and inflammation. While uPAR-directed CAR-T cells appear to have broad benefits, no universal signature for senescence is preserved in all cells. Thus, responsiveness to this treatment may vary across different tissues and cell types.

4. CAR-T Cells in Autoimmune Liver Diseases

The success of CAR-T-cell therapy in oncology has spurred its application in autoimmune diseases. Pioneering work by *Georg Schett* and *Andreas Mackensen* demonstrated the efficacy of autologous CD19 CAR-T cells in treating refractory systemic lupus erythematosus, with patients achieving long-term remission over three years[71-73]. CAR-T-cell therapy was well tolerated in lupus patients, with only mild cytokine release syndrome and no immune effector cell-associated neurotoxicity reported. Remarkably, B cells with a naïve, diverse repertoire re-emerged approximately 100 days post-therapy, as CAR-T cells became undetectable, suggesting a transient yet profound "deep tissue depletion" of B cells. This contrasts with the

persistent B-cell depletion observed in cancer treatments and highlights a unique therapeutic mechanism in autoimmunity.

Given these findings, similar CAR-T-cell therapies are now being explored in refractory cases of myasthenia gravis, systemic sclerosis, idiopathic inflammatory myositis, and multiple sclerosis[74], but also in autoimmune hepatitis (AIH) (**Figure 2**). Given the promising results of CAR-T-cell therapy in other autoimmune diseases, there is a strong rationale to explore profound tissue B-cell depletion with CAR-T cells in patients with advanced, refractory AIH, particularly those unable to achieve stable remission and facing progressive disease. AIH patients often exhibit strong humoral immune activation with elevated IgG levels, autoantibodies, and potentially misfolded polyreactive antibodies[75]. Rituximab has shown some success in treating AIH[76, 77], and therapies targeting the B-cell activating factor BAFF are currently under clinical investigation[78]. For these patients, CD19 CAR-T-cell therapy presents an interesting therapeutic option. This approach might also be extended to liver transplant patients with severe antibody-mediated rejection (ABMR), who typically have a poor prognosis.

An alternative CAR-T-cell strategy in autoimmunity involves ligand CARs called “chimeric autoantibody receptors” (CAARs, **Figure 2**), designed to specifically target and deplete autoantibody-producing B cells. In this approach, the extracellular domain of the CAR consists of a driver autoantigen, such as the desmoglein 3 in pemphigus vulgaris, rather than the traditional scFv. This allows the CAR-T cells to target and deplete only those B cells that produce antibodies against the specific autoantigen[79]. This approach has shown promise in preclinical models. Still, several challenges remain, including whether these CAAR-T cells would also target cells with bound autoantibodies and the impact on plasma cells lacking surface immunoglobulins. In AIH, where liver-specific driver autoantibodies are generally absent, the clinical application of ligand CAARs may be limited. However, primary biliary cholangitis PBC, characterized by a highly specific humoral immune response against PDC-E2, presents a potential target for this approach.

In summary, CAR-T-cell therapy has rapidly transitioned from an innovative cancer treatment to a promising therapeutic option for refractory autoimmune diseases. Its success in systemic lupus erythematosus, with minimal toxicity and profound tissue B-cell depletion, highlights

the potential of CAR-T cells to transform the treatment landscape for autoimmune conditions and autoimmune liver disease.

5. CAR-modified regulatory T cells to treat liver diseases

The concept of suppressor cells counteracting effector immune cells has been recognized for a long time. Yet, it wasn't until 1996 that Sakaguchi described regulatory T cells (Tregs) as a distinct and stable regulatory immune cell population[80] characterized by the expression of Foxp3 as a master transcription factor. It has since become evident that Tregs play crucial roles in various liver conditions.

Bluestone et al. demonstrated the safety and tolerability of large-dose adoptive Treg transfer in patients with recent-onset type 1 diabetes as an exemplary autoimmune disease. They showed the stability of the transferred Tregs' phenotype over a year[81], but the therapy had no clinical efficacy, underscoring the need for antigen-specific Tregs. Initial attempts to use Tregs as therapeutic agents in autoimmune liver disease[82, 83] and liver transplantation[84] revealed that polyspecific Tregs are significantly less potent than antigen-specific Tregs[85-87].

This poses a significant challenge, as only a few Tregs from the natural repertoire can recognize liver-specific target antigens. Even if 8-12% of Tregs are allospecific[88], transferring these cells has not been sufficient to induce tolerance after liver transplantation.[84] For autoimmune liver diseases, it is estimated that only one in a million Tregs can recognize autoantigens[89]. Therefore, transferring 400 million cells ($5x10^6$/kg) would yield only around 400 antigen-specific Treg cells, highlighting the urgent need to generate more Tregs specific for hepatic or biliary antigens by Treg engineering.

As for effector T cells, two primary strategies exist to engineer liver-specific Tregs. The first is a transfer of a liver-specific TCR. This method faces limitations due to HLA restriction with the highly diverse major histocompatibility complex (MHC) class II repertoire and the risk of mispairing with endogenous TCR chains. The second is the engineering of Treg with CARs, which can generate large amounts of liver-specific Tregs. While various intracellular signaling

domains are currently used for CARs in effector T cells, “classical” 2nd generation signaling domains using CD28 and CD3z seem best suited for Treg[90] (**Figure 3D**).

CAR-Treg after liver transplantation

The application of CAR-Tregs in liver diseases is being pioneered in liver transplantation. It was initially shown that CARs targeting the mismatched HLA A2 molecule expressed in the donor’s liver but not by the graft recipient could achieve allo-tolerance without immunosuppression in humanized mouse models.[91-93] This tolerance extended to highly immunogenic HLA A2-positive skin grafts transplanted onto HLA A2-negative recipients, leading to the development of clinical programs using HLA A2-directed CAR-Tregs for tolerance induction post-liver transplantation.[91] In the LIBERATE clinical trial, HLA A2-directed CAR-Tregs are administered to patients with minimal hepatic inflammation more than one year after liver transplantation (NCT05234190). The goal is to wean these patients off tacrolimus to everolimus mono-immunosuppression and eventually achieve operational tolerance without immunosuppression.

CAR-Tregs accumulate locally and persist long-term[91, 93], creating local immune tolerance without compromising the patient's overall immune competence. As living drugs, they can potentially survive for life. In mouse models, transferred Tregs have been observed for over 200 days post-transfer. Tregs can modulate multiple immune cells and suppress T cells recognizing various targets (cross-suppression). Tregs can also induce other T cells to become Tregs, creating new regulatory immune networks. While long-term survival of transferred Tregs might be beneficial, it is not yet clear if it is necessary.

CAR-Treg to combat autoimmune and metabolic liver diseases.

In autoimmune hepatitis (AIH), Tregs increase during active disease but are insufficient to control autoimmunity. Current steroid-based therapies may preferentially deplete Tregs, explaining the high relapse rates post-therapy. Therefore, liver-specific CAR-Treg therapies could re-establish local immune tolerance in AIH, potentially eliminating the need for chronic immunosuppressive therapy. *Oo* et al. were the first to demonstrate the safety of Tregs in AIH patients, paving the way for liver-specific CAR-Treg therapies[83]. In contrast, adoptive Treg

transfer increased metabolic inflammation and steatosis.[94] This discouraged the use of Treg for treating metabolic liver disease but encouraged its use for AIH.

The asialoglycoprotein receptor is being explored as a potential target for a pan-liver-specific CAR, which could be used in autoimmunity and liver transplantation. Still, proof of its efficacy in relevant liver-inflammatory models is lacking. In primary biliary cholangitis, the target antigen PDC-E2 is highly disease-specific but not organ-specific, complicating CAR-Treg generation. Similarly, generating CAR-Tregs for primary sclerosing cholangitis is challenging due to the lack of biliary epithelial cell-specific surface proteins. However, conventional CD19-CAR T cells may provide an option to treat these diseases.[95]

Current developments with CAR-Tregs

Future CAR-Treg products for inflammatory liver disease will focus on directing specificity and stabilizing the Treg phenotype. Overexpression of FOXP3 could stabilize the regulatory phenotype under inflammatory or low IL-2 conditions[96]. Making CAR-Tregs more independent of external IL-2 could stabilize their phenotype and enhance their suppressive function. Membrane-attached IL-2 molecules[97] or chimeric cytokine receptors activated by rapamycin might provide a benefit[98]. Liver-specific CAR-Tregs could be used as Trojan horses, delivering immune regulatory, regenerative, and anti-fibrotic molecules for AIH. Tregs with amplified effector functions ("TRAF" cells) might be an option to improve clinical efficacy in the future.

In summary, CAR-Tregs represent a promising new therapeutic option for achieving long-lasting tissue-specific tolerance without compromising overall immune competence. They offer potential benefits for local tissue regeneration and homeostasis, marking a significant advancement in treating inflammatory liver diseases and transplantation tolerance.

6. Improvements of CAR-T cell therapies

6.1. CAR T-cell specificity and sensitivity

Improving CAR-T cells' sensitivity without losing specificity is crucial to maximize their on-tumor effects while minimizing off-tumor toxicities. The affinity of the CAR for its target

antigen plays a significant role in this regard. A high-affinity binder enables CAR-T cells to recognize and bind to tumor cells even at low antigen densities. However, this may lead to trogocytosis, a process where the CAR-T cell strips the target antigen from the tumor cell. In contrast, lower-affinity binders in CARs require higher antigen densities for activation but exhibit less trogocytosis and exhaustion, resulting in prolonged T-cell survival.[99]

Another main avenue of investigation is dedicated to improving the intrinsic attributes of CAR-T cells. On the one hand, the desire is to prevent rapid, explosive CAR signaling and the ensuing clinical inflammatory side effects. On the other hand, there is an intention to endow T cells with the ability to persist long-term and unfold memory capable of protecting patients from relapse. Significant progress has been made in conferring these desired properties to T cells by modulating specific transcription factors such as c-Jun.[100, 101] Combining several transcription factors is currently being explored. This illustrates the need to fine-tune transcription factors' expression level and timing to achieve the desired T cell attributes.[102]

CAR recognition domains have been fused to other TCR/CD3 signaling complex molecules, generating TCR fusion constructs (TRuCs) (**Figure 1**), which are currently undergoing clinical testing.[103, 104] An exciting development in this field is the creation of HLA-independent TCRs (HIT receptors), which fuse the variable regions of the CAR recognition domains to the CD3 complex, i.e., the constant regions of the TCR alpha and beta chains (**Figure 1**). These HIT receptors reduced exhaustion, improved T-cell survival, and increased antigen sensitivity to < 200 target molecules per cell.[99] However, conventional CARs may also recognize target cells with < 100 target molecules[105], demonstrating that the binder plays a crucial role. An alternative concept to the HIT receptors is STAR receptors, which have recently been described. A scFv is fused to each TCR alpha and beta chain in STAR receptors. Hereby, using different scFv even allows the generation of bispecific receptors targeting two different antigens.[106]

Another area of interest is targeting cancer stem cells, as these cells are thought to play a crucial role in tumor recurrence and resistance. Targeting antigens expressed on cancer stem cells may achieve more durable tumor remissions. Lastly, artificial intelligence (AI) is used to improve CAR design and optimize known CAR binders or even generate entirely new binders that can be used in CARs.

6.2. CAR-T Cells targeting multiple targets

Multitarget CAR-T cells, which can recognize two or more antigens on their target cell, are being explored to prevent tumor escape due to antigen loss and to enhance the overall on-tumor effect. These multitarget CARs represent a logical OR gate (targeting target A OR B), activating the T cell if either of the target antigens is present on the tumor cell[107, 108]. Alternatively, they require both target antigens to be activated, representing a logical AND gate. These logical gating strategies are being developed to improve the specificity and safety of CAR-T cell therapies by ensuring that the engineered T cells target only tumor cells while sparing normal tissues.[109] They certainly represent the next frontier in the evolution of CAR-T cell therapy.

6.3. Fine-tuning CAR T cell efficacy

The intracellular activation domain of CAR-T cells is a critical determinant of their sensitivity, activation strength, in vivo survival, and susceptibility to exhaustion. Lower target molecule densities can activate CARs with CD28 co-stimulatory domains. This initially results in stronger T-cell activation and tumor killing, but fosters T-cell exhaustion[110]. In contrast, CARs with 4-1BB co-stimulatory domains require higher antigen densities but demonstrate slower exhaustion and more prolonged survival[109]. Therefore, further modifications to the signaling domains balancing initial activation with long-term survival are being investigated.

Strategies to prevent CAR-T cell exhaustion include modulation of CAR affinity, using early memory T cells for CAR-T cell production, and shortening in vitro manipulation times. Additionally, the provision of CD4+ T cell help, the use of on/off CARs to prevent tonic signaling, the modulation of T cell metabolism, and 4th and 5th generation CARs (**Figure 1**) are under investigation.

6.4. Homing of CAR-T Cells

The effective homing of CAR-T cells to the liver or into the tumor sites is essential for their success. This can be enhanced by various strategies, such as administering immune checkpoint inhibitors, expressing chemokines like CCL19 to attract other immune cells, and expressing chemokine receptors on CAR-T cells. Alteration of the liver tissue with the accumulation of ECM in the Space of Dissé and the continuous endothelium threatens CAR-T cell therapy in

liver fibrosis and HCC. An interesting approach is using a heparanase-secreting CAR to digest ECM and allow easier access to the CAR T cells[111]. Additionally, local delivery of CAR-T cells via intratumoral injection or arterial supply can improve their accumulation at the tumor site.

6.5. Safety

Although CAR-T cell therapies are generally safe, concerns remain regarding acute side effects such as cytokine release syndrome and immune effector cell-associated neurotoxicity (see the Excurse on side effects of CAR T-cells) and on-target, off-tumor effects. Additionally, there is ongoing debate about the potential risk of CAR-T cells inducing T cell lymphomas due to mutations caused by non-specific insertion of strong non-human promoters[112]. Genetic safety is being improved by using self-inactivating retroviral and lentiviral vectors with human promoters to introduce the CAR, modified CRISPR approaches for homologous recombination without double-strand breaks, and site-directed integration into safe harbors using integrases.[113, 114] CAR Insertion into the natural TCR locus also improves CAR-T cell functionality.[115]

Synthetic genetic switches are being evaluated to allow for the depletion of CAR-T cells if needed. These include suicide genes, such as inducible caspase 9 (iCAS9), depletion strategies via antibodies targeting a truncated receptor co-expressed on the cell surface, or using thymidine kinase inhibitors to mitigate CAR-T cell function.[33]

6.6. Alternative cells grafted with CARs

In addition to T cells, shorter-lived CAR natural killer (NK) cells are currently being investigated in clinical trials.[116] However, without further genetic modification to enhance potency, CAR-NK cells fall short in efficacy and longevity compared to CAR-T cells.[117] Rare T-cell subsets, such as gamma/delta T cells and invariant NKT cells, are under investigation to overcome tumor cells' primary and secondary resistance to conventional CAR-T cells. Currently, blended immune cell products of CAR-T plus other synergistic engineered immune cell products are of emerging interest.

To provide ready-to-use and affordable CAR-T cell products, allogeneic CAR-T cells derived from a healthy donor gene-edited to reduce immunogenicity have been intensively studied[118]. So far, however, the therapeutic potential is significantly below that of autologous CAR-T cell

products because of the toxicity associated with multiplexed gene-editing, and limited clinical efficacy is due to rejection by the host patient's immune system. Using stem cell-derived CAR-T cells from a perpetual source, such as induced pluripotent stem cells, provides an interesting alternative.[119]

6.7 Scaled manufacturing and in vivo gene transfer

An important ambition is to increase overall patient access and to ascertain the sustainability of engineered immune cell therapy for our healthcare systems. Accordingly, strategies for scalable, rapid, and affordable CAR-T cell manufacturing are in focus. Since 2020, the field has shifted towards reduced-expansion protocols, low-activation protocols, and point-of-care manufacturing, allowing for a rapid turnaround while providing less differentiated and exhausted T-cells.[120]

In vivo gene transfer into T cells may alleviate many of the logistical and infrastructural constraints of today's CAR-T cell therapy. To avoid handling blood and cells outside the patient's body, targeted lentiviral vectors[121] or lipid nanoparticles[122, 123] are administered that target T lymphocytes in the circulation, carrying instructions to reprogram them into CAR-T cells. These *in vivo* manipulations, however, face regulatory challenges as quality control is difficult. Most recent developments include imminently available methods of T-cell engineering that one can envision as modified hemodialysis machines. These would combine simplified T-cell engineering without requiring a specialized "good manufacturing process" laboratory, still allowing for the quality control needed to fully realize the clinical potential of CAR T-cell therapies.

Summary

CAR-T cell therapies have transformed the treatment of B-cell malignancies, and recent advancements in CAR technology are promising for application to solid tumors, including those in the liver. These developments open new avenues for targeted immune interventions in liver disease. CAR-T cells may allow for the local elimination of HBV-infected hepatocytes and hepatobiliary tumors, potentially avoiding the need for systemic chemotherapy and its associated side effects; initial clinical trials in these areas are underway. Meanwhile, preclinical research on CAR-T cells for removing senescent and fibrogenic liver cells is advancing. Additionally, CAR-Tregs can induce liver-specific immune tolerance in transplantation and autoimmune liver diseases, eliminating the need for lifelong immunosuppression. Current research aims to further refine the specificity, efficacy, and safety of CAR-based immune therapies. Ultimately, CAR-redirected immune cells represent “living drugs” that offer targeted, local, and sustained therapeutic solutions for the personalized treatment of hepatic diseases.

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References:

1. Chisari FV. Cytotoxic T cells and viral hepatitis. J Clin Invest 1997;99:1472-7.
2. Cabibbo G, Singal AG. The quest for precision oncology with immune checkpoint inhibitors for hepatocellular carcinoma. J Hepatol 2022;76:262-264.
3. Gehring AJ, Xue SA, Ho ZZ, et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. J Hepatol 2011;55:103-10.
4. Wisskirchen K, Metzger K, Schreiber S, et al. Isolation and functional characterization of hepatitis B virus-specific T-cell receptors as new tools for experimental and clinical use. PLoS One 2017;12:e0182936.
5. Lu L, Jiang J, Zhan M, et al. Targeting Tumor-Associated Antigens in Hepatocellular Carcinoma for Immunotherapy: Past Pitfalls and Future Strategies. Hepatology 2021;73:821-832.
6. Kuwana Y, Asakura Y, Utsunomiya N, et al. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. Biochem Biophys Res Commun 1987;149:960-8.
7. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci U S A 1989;86:10024-8.
8. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 1991;64:891-901.
9. Milone MC, Fish JD, Carpenito C, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther 2009;17:1453-64.
10. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. Nat Biotechnol 2002;20:70-5.
11. Nix MA, Wiita AP. Alternative target recognition elements for chimeric antigen receptor (CAR) T cells: beyond standard antibody fragments. Cytotherapy 2024;26:729-738.
12. Riddell SR, Sommermeyer D, Berger C, et al. Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. Cancer journal (Sudbury, Mass.) 2014;20:141–144.
13. Turtle CJ, Hanafi LA, Berger C, et al. Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. Sci Transl Med 2016;8:355ra116.
14. Sommermeyer D, Hill T, Shamah SM, et al. Fully human CD19-specific chimeric antigen receptors for T-cell therapy. Leukemia 2017;31:2191-2199.
15. Sommermeyer D, Hudecek M, Kosasih PL, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia 2016;30:492-500.
16. Terakura S, Yamamoto TN, Gardner RA, Turtle CJ, Jensen MC, Riddell SR. Generation of CD19-chimeric antigen receptor modified CD8+ T cells derived from virus-specific central memory T cells. Blood 2012;119:72-82.

17. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. N Engl J Med 2018;378:439-448.
18. Shah BD, Ghobadi A, Oluwole OO, et al. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. Lancet 2021;398:491-502.
19. Kamdar M, Solomon SR, Arnason J, et al. Lisocabtagene maraleucel versus standard of care with salvage chemotherapy followed by autologous stem cell transplantation as second-line treatment in patients with relapsed or refractory large B-cell lymphoma (TRANSFORM): results from an interim analysis of an open-label, randomised, phase 3 trial. Lancet 2022;399:2294-2308.
20. Morschhauser F, Dahiya S, Palomba ML, et al. Lisocabtagene maraleucel in follicular lymphoma: the phase 2 TRANSCEND FL study. Nat Med 2024;30:2199-2207.
21. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. N Engl J Med 2017;377:2531-2544.
22. San-Miguel J, Dhakal B, Yong K, et al. Cilta-cel or Standard Care in Lenalidomide-Refractory Multiple Myeloma. N Engl J Med 2023;389:335-347.
23. Munshi NC, Anderson LD, Jr., Shah N, et al. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. N Engl J Med 2021;384:705-716.
24. Feucht J, Sun J, Eyquem J, et al. Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. Nat Med 2019;25:82-88.
25. Salter AI, Rajan A, Kennedy JJ, et al. Comparative analysis of TCR and CAR signaling informs CAR designs with superior antigen sensitivity and in vivo function. Science signaling 2021;14.
26. Morris EC, Neelapu SS, Giavridis T, Sadelain M. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. Nat Rev Immunol 2022;22:85-96.
27. Amini L, Silbert SK, Maude SL, et al. Preparing for CAR T cell therapy: patient selection, bridging therapies and lymphodepletion. Nat Rev Clin Oncol 2022;19:342-355.
28. Lickefett B, Chu L, Ortiz-Maldonado V, et al. Lymphodepletion - an essential but undervalued part of the chimeric antigen receptor T-cell therapy cycle. Front Immunol 2023;14:1303935.
29. Guidotti LG, Inverso D, Sironi L, et al. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. Cell 2015;161:486-500.
30. DeLeve LD. Liver sinusoidal endothelial cells in hepatic fibrosis. Hepatology 2015;61:1740-6.
31. Ficht X, Iannacone M. Immune surveillance of the liver by T cells. Sci Immunol 2020;5.
32. Morte-Romea E, Pesini C, Pellejero-Sagastizabal G, et al. CAR Immunotherapy for the treatment of infectious diseases: a systematic review. Front Immunol 2024;15:1289303.
33. Lu L, Xie M, Yang B, Zhao WB, Cao J. Enhancing the safety of CAR-T cell therapy: Synthetic genetic switch for spatiotemporal control. Sci Adv 2024;10:eadj6251.
34. Pang N, Shi J, Qin L, et al. IL-7 and CCL19-secreting CAR-T cell therapy for tumors with positive glypican-3 or mesothelin. J Hematol Oncol 2021;14:118.
35. Bohne F, Chmielewski M, Ebert G, et al. T cells redirected against hepatitis B virus surface proteins eliminate infected hepatocytes. Gastroenterology 2008;134:239-47.

36. Quitt O, Luo S, Meyer M, et al. T-cell engager antibodies enable T cells to control HBV infection and to target HBsAg-positive hepatoma in mice. J Hepatol 2021;75:1058-1071.
37. Guo G, He W, Zhou Z, Diao Y, Sui J, Li W. PreS1- targeting chimeric antigen receptor T cells diminish HBV infection in liver humanized FRG mice. Virology 2023;586:23-34.
38. Schreiber S, Honz M, Mamozai W, et al. Characterization of a library of 20 HBV-specific MHC class II-restricted T cell receptors. Mol Ther Methods Clin Dev 2021;23:476-489.
39. Sautto GA, Wisskirchen K, Clementi N, et al. Chimeric antigen receptor (CAR)-engineered T cells redirected against hepatitis C virus (HCV) E2 glycoprotein. Gut 2016;65:512-23.
40. Zhao L, Chen F, Quitt O, et al. Hepatitis B virus envelope proteins can serve as therapeutic targets embedded in the host cell plasma membrane. Cell Microbiol 2021;23:e13399.
41. Krebs K, Bottinger N, Huang LR, et al. T cells expressing a chimeric antigen receptor that binds hepatitis B virus envelope proteins control virus replication in mice. Gastroenterology 2013;145:456-65.
42. Xia Y, Stadler D, Lucifora J, et al. Interferon-gamma and Tumor Necrosis Factor-alpha Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. Gastroenterology 2016;150:194-205.
43. Tan AT, Meng F, Jin J, et al. Immunological alterations after immunotherapy with short lived HBV-TCR T cells associates with long-term treatment response in HBV-HCC. Hepatol Commun 2022;6:841-854.
44. Bosch M, Kallin N, Donakonda S, et al. A liver immune rheostat regulates CD8 T cell immunity in chronic HBV infection. Nature 2024;631:867-875.
45. Qasim W, Brunetto M, Gehring AJ, et al. Immunotherapy of HCC metastases with autologous T cell receptor redirected T cells, targeting HBsAg in a liver transplant patient. J Hepatol 2015;62:486-91.
46. Wan X, Wisskirchen K, Jin T, et al. Genetically-modified, redirected T cells target hepatitis B surface antigen-positive hepatocytes and hepatocellular carcinoma lesions in a clinical setting. Clin Mol Hepatol 2024;30:735-755.
47. Guizhen Z, Guanchang J, Liwen L, et al. The tumor microenvironment of hepatocellular carcinoma and its targeting strategy by CAR-T cell immunotherapy. Front Endocrinol (Lausanne) 2022;13:918869.
48. Finn RS, Qin S, Ikeda M, et al. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. N Engl J Med 2020;382:1894-1905.
49. Cheng AL, Qin S, Ikeda M, et al. Updated efficacy and safety data from IMbrave150: Atezolizumab plus bevacizumab vs. sorafenib for unresectable hepatocellular carcinoma. J Hepatol 2022;76:862-873.
50. Philip M, Schietinger A. CD8(+) T cell differentiation and dysfunction in cancer. Nat Rev Immunol 2022;22:209-223.
51. Budczies J, Kazdal D, Menzel M, et al. Tumour mutational burden: clinical utility, challenges and emerging improvements. Nat Rev Clin Oncol 2024;21:725-742.
52. Lu L, Jiang J, Zhan M, et al. Targeting Neoantigens in Hepatocellular Carcinoma for Immunotherapy: A Futile Strategy? Hepatology 2021;73:414-421.

53. Gao H, Li K, Tu H, et al. Development of T cells redirected to glypican-3 for the treatment of hepatocellular carcinoma. Clin Cancer Res 2014;20:6418-28.
54. Steffin D, Ghatwai N, Montalbano A, et al. Interleukin-15-armored GPC3-CAR T cells for patients with solid cancers. Res Sq 2024.
55. Vogel A, Meyer T, Sapisochin G, Salem R, Saborowski A. Hepatocellular carcinoma. Lancet 2022;400:1345-1362.
56. Chmielewski M, Hombach AA, Abken H. Of CARs and TRUCKs: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma. Immunol Rev 2014;257:83-90.
57. Mackensen A, Haanen J, Koenecke C, et al. CLDN6-specific CAR-T cells plus amplifying RNA vaccine in relapsed or refractory solid tumors: the phase 1 BNT211-01 trial. Nat Med 2023;29:2844-2853.
58. Reinhard K, Rengstl B, Oehm P, et al. An RNA vaccine drives expansion and efficacy of claudin-CAR-T cells against solid tumors. Science 2020;367:446-453.
59. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. Nat Rev Gastroenterol Hepatol 2017;14:397-411.
60. Cogliati B, Yashaswini CN, Wang S, Sia D, Friedman SL. Friend or foe? The elusive role of hepatic stellate cells in liver cancer. Nat Rev Gastroenterol Hepatol 2023;20:647-661.
61. Filliol A, Saito Y, Nair A, et al. Opposing roles of hepatic stellate cell subpopulations in hepatocarcinogenesis. Nature 2022;610:356-365.
62. Amor C, Feucht J, Leibold J, et al. Senolytic CAR T cells reverse senescence-associated pathologies. Nature 2020;583:127-132.
63. Yashaswini CN, Qin T, Bhattacharya D, et al. Phenotypes and ontogeny of senescent hepatic stellate cells in metabolic dysfunction-associated steatohepatitis. J Hepatol 2024;81:207-217.
64. Aghajanian H, Kimura T, Rurik JG, et al. Targeting cardiac fibrosis with engineered T cells. Nature 2019;573:430-433.
65. Yang AT, Kim YO, Yan XZ, et al. Fibroblast Activation Protein Activates Macrophages and Promotes Parenchymal Liver Inflammation and Fibrosis. Cell Mol Gastroenterol Hepatol 2022;15:841-867.
66. Levy MT, McCaughan GW, Abbott CA, et al. Fibroblast activation protein: A cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis. Hepatology 1999;29:1768-1778.
67. Agudo J, Ruzo A, Park ES, et al. GFP-specific CD8 T cells enable targeted cell depletion and visualization of T-cell interactions. Nat Biotechnol 2015;33:1287-1292.
68. Trinh VQ, Lee TF, Lemoinne S, et al. Hepatic stellate cells maintain liver homeostasis through paracrine neurotrophin-3 signaling that induces hepatocyte proliferation. Sci Signal 2023;16:eadf6696.
69. Amor C, Fernandez-Maestre I, Chowdhury S, et al. Prophylactic and long-lasting efficacy of senolytic CAR T cells against age-related metabolic dysfunction. Nat Aging 2024;4:336-349.
70. Younossi ZM, Golabi P, Paik JM, Henry A, Van Dongen C, Henry L. The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review. Hepatology 2023;77:1335-1347.

71. Mougiakakos D, Kronke G, Volkl S, et al. CD19-Targeted CAR T Cells in Refractory Systemic Lupus Erythematosus. N Engl J Med 2021;385:567-569.
72. Mackensen A, Muller F, Mougiakakos D, et al. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. Nat Med 2022;28:2124-2132.
73. Muller F, Taubmann J, Bucci L, et al. CD19 CAR T-Cell Therapy in Autoimmune Disease - A Case Series with Follow-up. N Engl J Med 2024;390:687-700.
74. Schett G, Mackensen A, Mougiakakos D. CAR T-cell therapy in autoimmune diseases. Lancet 2023;402:2034-2044.
75. Taubert R, Engel B, Diestelhorst J, et al. Quantification of polyreactive immunoglobulin G facilitates the diagnosis of autoimmune hepatitis. Hepatology 2022;75:13-27.
76. Riveiro-Barciela M, Barreira-Diaz A, Esteban P, et al. Rituximab is a safe and effective alternative treatment for patients with autoimmune hepatitis: Results from the ColHai registry. Liver Int 2024.
77. Than NN, Hodson J, Schmidt-Martin D, et al. Efficacy of rituximab in difficult-to-manage autoimmune hepatitis: Results from the International Autoimmune Hepatitis Group. JHEP Rep 2019;1:437-445.
78. Arvaniti P, Giannoulis G, Gabeta S, Zachou K, Koukoulis GK, Dalekos GN. Belimumab is a promising third-line treatment option for refractory autoimmune hepatitis. JHEP Rep 2020;2:100123.
79. Ellebrecht CT, Bhoj VG, Nace A, et al. Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease. Science 2016;353:179-84.
80. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996;184:387-96.
81. Bluestone JA, Buckner JH, Fitch M, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med 2015;7:315ra189.
82. Hardtke-Wolenski M, Fischer K, Noyan F, et al. Genetic predisposition and environmental danger signals initiate chronic autoimmune hepatitis driven by CD4+ T cells. Hepatology 2013;58:718-28.
83. Oo YH, Ackrill S, Cole R, et al. Liver homing of clinical grade Tregs after therapeutic infusion in patients with autoimmune hepatitis. JHEP Rep 2019;1:286-296.
84. Tang Q, Leung J, Peng Y, et al. Selective decrease of donor-reactive T(regs) after liver transplantation limits T(reg) therapy for promoting allograft tolerance in humans. Sci Transl Med 2022;14:eabo2628.
85. Jaeckel E, von Boehmer H, Manns MP. Antigen-Specific FoxP3-Transduced T-Cells Can Control Established Type 1 Diabetes. Diabetes 2005;54:306-10.
86. Tsang JY, Tanriver Y, Jiang S, et al. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. J Clin Invest 2008;118:3619-28.
87. Tang Q, Henriksen KJ, Bi M, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 2004;199:1455-65.
88. Noyan F, Lee YS, Zimmermann K, et al. Isolation of human antigen-specific regulatory T cells with high suppressive function. Eur J Immunol 2014;44:2592-602.
89. Serr I, Furst RW, Achenbach P, et al. Type 1 diabetes vaccine candidates promote human Foxp3(+)Treg induction in humanized mice. Nat Commun 2016;7:10991.

90. Dawson NAJ, Rosado-Sanchez I, Novakovsky GE, et al. Functional effects of chimeric antigen receptor co-receptor signaling domains in human regulatory T cells. Sci Transl Med 2020;12.
91. Noyan F, Zimmermann K, Hardtke-Wolenski M, et al. Prevention of Allograft Rejection by Use of Regulatory T Cells With an MHC-Specific Chimeric Antigen Receptor. Am J Transplant 2017;17:917-930.
92. Boardman DA, Philippeos C, Fruhwirth GO, et al. Expression of a Chimeric Antigen Receptor Specific for Donor HLA Class I Enhances the Potency of Human Regulatory T Cells in Preventing Human Skin Transplant Rejection. Am J Transplant 2017;17:931-943.
93. MacDonald KG, Hoeppli RE, Huang Q, et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. J Clin Invest 2016;126:1413-24.
94. Dywicki J, Buitrago-Molina LE, Noyan F, et al. The Detrimental Role of Regulatory T Cells in Nonalcoholic Steatohepatitis. Hepatol Commun 2022;6:320-333.
95. Sokke Umeshappa C, Babu Kolla H, Hebbandi Nanjundappa R. Advancing CD19 CAR T Cell Therapy for Treatment of Primary Biliary Cholangitis. The Journal of Immunology 2024;212:0427_4710-0427_4710.
96. McGovern J, Holler A, Thomas S, Stauss HJ. Forced Fox-P3 expression can improve the safety and antigen-specific function of engineered regulatory T cells. J Autoimmun 2022;132:102888.
97. Kremer J, Henschel P, Simon D, et al. Membrane-bound IL-2 improves the expansion, survival, and phenotype of CAR Tregs and confers resistance to calcineurin inhibitors. Front Immunol 2022;13:1005582.
98. Yang SJ, Singh AK, Drow T, et al. Pancreatic islet-specific engineered T(regs) exhibit robust antigen-specific and bystander immune suppression in type 1 diabetes models. Sci Transl Med 2022;14:eabn1716.
99. Hamieh M, Dobrin A, Cabriolu A, et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. Nature 2019;568:112-116.
100. Ataide MA, Komander K, Knopper K, et al. BATF3 programs CD8(+) T cell memory. Nat Immunol 2020;21:1397-1407.
101. Lynn RC, Weber EW, Sotillo E, et al. c-Jun overexpression in CAR T cells induces exhaustion resistance. Nature 2019;576:293-300.
102. Blaeschke F, Chen YY, Apathy R, et al. Modular pooled discovery of synthetic knockin sequences to program durable cell therapies. Cell 2023;186:4216-4234 e33.
103. Helsen CW, Hammill JA, Lau VWC, et al. The chimeric TAC receptor co-opts the T cell receptor yielding robust anti-tumor activity without toxicity. Nat Commun 2018;9:3049.
104. Baeuerle PA, Ding J, Patel E, et al. Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response. Nat Commun 2019;10:2087.
105. Nerreter T, Letschert S, Gotz R, et al. Super-resolution microscopy reveals ultra-low CD19 expression on myeloma cells that triggers elimination by CD19 CAR-T. Nat Commun 2019;10:3137.
106. Simon S, Bugos G, Prins R, et al. Sensitive bispecific chimeric T cell receptors for cancer therapy. Res Sq 2024.

107. Furqan F, Shah NN. Multispecific CAR T Cells Deprive Lymphomas of Escape via Antigen Loss. Annu Rev Med 2023;74:279-291.

108. Shalabi H, Qin H, Su A, et al. CD19/22 CAR T cells in children and young adults with B-ALL: phase 1 results and development of a novel bicistronic CAR. Blood 2022;140:451-463.

109. Hamieh M, Mansilla-Soto J, Riviere I, Sadelain M. Programming CAR T Cell Tumor Recognition: Tuned Antigen Sensing and Logic Gating. Cancer Discov 2023;13:829-843.

110. Mansilla-Soto J, Eyquem J, Haubner S, et al. HLA-independent T cell receptors for targeting tumors with low antigen density. Nat Med 2022;28:345-352.

111. Caruana I, Savoldo B, Hoyos V, et al. Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. Nat Med 2015;21:524-9.

112. Ghilardi G, Fraietta JA, Gerson JN, et al. T cell lymphoma and secondary primary malignancy risk after commercial CAR T cell therapy. Nat Med 2024;30:984-989.

113. Pandey S, Gao XD, Krasnow NA, et al. Efficient site-specific integration of large genes in mammalian cells via continuously evolved recombinases and prime editing. Nat Biomed Eng 2024.

114. Yarnall MTN, Ioannidi EI, Schmitt-Ulms C, et al. Drag-and-drop genome insertion of large sequences without double-strand DNA cleavage using CRISPR-directed integrases. Nat Biotechnol 2023;41:500-512.

115. Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 2017;543:113-117.

116. Liu E, Marin D, Banerjee P, et al. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. N Engl J Med 2020;382:545-553.

117. Bachiller M, Perez-Amill L, Battram AM, et al. NK cells enhance CAR-T cell antitumor efficacy by enhancing immune/tumor cells cluster formation and improving CAR-T cell fitness. J Immunother Cancer 2021;9.

118. Chiesa R, Georgiadis C, Syed F, et al. Base-Edited CAR7 T Cells for Relapsed T-Cell Acute Lymphoblastic Leukemia. N Engl J Med 2023;389:899-910.

119. Themeli M, Kloss CC, Ciriello G, et al. Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy. Nature biotechnology 2013;31:928–933.

120. Agliardi G, Dias J, Rampotas A, Garcia J, Roddie C. Accelerating and optimising CAR T-cell manufacture to deliver better patient products. Lancet Haematol 2025;12:e57-e67.

121. Frank AM, Braun AH, Scheib L, et al. Combining T-cell-specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. Blood Adv 2020;4:5702-5715.

122. Hamilton JR, Chen E, Perez BS, et al. In vivo human T cell engineering with enveloped delivery vehicles. Nat Biotechnol 2024;42:1684-1692.

123. Smith TT, Stephan SB, Moffett HF, et al. In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers. Nature nanotechnology 2017;12:813–820.

124. Dai H, Tong C, Shi D, et al. Efficacy and biomarker analysis of CD133-directed CAR T cells in advanced hepatocellular carcinoma: a single-arm, open-label, phase II trial. Oncoimmunology 2020;9:1846926.

125. Zhang BL, Li D, Gong YL, et al. Preclinical Evaluation of Chimeric Antigen Receptor-Modified T Cells Specific to Epithelial Cell Adhesion Molecule for Treating Colorectal Cancer. Hum Gene Ther 2019;30:402-412.
126. Mao L, Su S, Li J, et al. Development of Engineered CAR T Cells Targeting Tumor-Associated Glycoforms of MUC1 for the Treatment of Intrahepatic Cholangiocarcinoma. J Immunother 2023;46:89-95.
127. Tseng HC, Xiong W, Badeti S, et al. Efficacy of anti-CD147 chimeric antigen receptors targeting hepatocellular carcinoma. Nat Commun 2020;11:4810.
128. Huang X, Guo J, Li T, et al. c-Met-targeted chimeric antigen receptor T cells inhibit hepatocellular carcinoma cells in vitro and in vivo. J Biomed Res 2021;36:10-21.
129. Xie C, Cecilia M, Mabry-Hrones D, et al. A phase I study of GPC3 targeted CAR-T cell therapy in advanced GPC3-expressing hepatocellular carcinoma (HCC). J Clin Oncol 2023;41.

Figure Legends

Figure 1: The Evolution of Chimeric Antigen Receptors

The first generation of CARs consists of an antibody recognition domain fused to an intracellular CD3-zeta activation domain, providing signal 1 only. The second generation combines signal 1 with a costimulatory signal 2. The third generation includes two costimulatory domains. The fourth generation combines the CAR with cytokine or antibody secretion to modify the tumor microenvironment. The fifth generation activates intracellular signaling independently of the TCR. Antibody recognition domains can also be directly fused to TCR components, forming HLA-independent TCR receptors (HIT) or T cell receptor fusion constructs (TRuC), which provide enhanced sensitivity but lack costimulatory signals.

Figure 2: Applications of CAR T Cells in Liver Diseases

CAR T cells can be directed against virus-infected hepatocytes, tumor cells, senescent cells, and fibrogenic cells. They can also deplete all B lymphocytes or specifically target autoantigen-specific B cells in autoimmune liver diseases.

Figure 3: The function of CAR-T cells in the liver

CAR-T cells can reach their target, hepatocytes, cancer or stellate cells, or fibroblasts, through the fenestrae in the sinusoidal endothelium. (A) Hepatocytes infected with HBV can be targeted by CAR-T-cells that kill infected hepatocytes and secrete cytokines (e.g., IFNγ, lymphotoxin, TNFα) that inhibit virus replication and activate endogenous CD4 and CD8 T-cell responses. However, the effector function of CAR-T cells may be altered by the liver microenvironment. (B) Over time, increasing tissue alteration is observed, and the extracellular matrix accumulates and hinders the contact of CAR-T cells with their target cells. CAR-T cells can target myofibroblastic cells, may be able to diminish extracellular matrix and revert liver fibrosis but also reduce aging processes in the liver. (C) CAR-T cells directed against cancer antigens kill cells and alter the tumor microenvironment by secreting cytokines. However, they are also confronted with immunoregulatory cell populations like regulatory T (T_{reg}) or myeloid-derived suppressor cells (MDSCs). Enzymes such as IDO, TDO, and arginase are expressed at high levels and may hinder CAR-T cell proliferation and function. Co-inhibitory signaling by binding of PD1 on T cells to PDL1 on Kupffer cells, LSECs, stellate, and dendritic

cells may restrict CAR-T cell effector functions, rendering them anergic or exhausted. (D) CAR-Tregs blunt the immune response against transplanted livers by snatching IL-2 and secreting IL-10 to inhibit NK cells, CD4, and CD8 T cells.

Figure 4: Overview of improvement options for CAR-T cell therapies

This figure illustrates advancements in improving antigen recognition, functionality, tumor and tissue accessibility, CAR-T cell persistence, and safety. The underlined mechanisms highlight promising modifications for clinical application.

Tables

Table 1 CAR targets in hepatobiliary malignancies

Target	% of hepatic cancer Tumor specificity	Expression in normal tissue	Involved in tumor pathogenesis	Effect in vitro/ mouse xeno-grafts	Clinical trials	Comments	Clinical trial No.
GPC3	70-80% HCC <10% CCC +++	Minimal	Proliferation Invasion stimulates wnt pathway	+/+	+	Combination with ICI or anti-PD1 secretion	Multiple NCT05155189 NCT05103631
CD133	10-40% +++	Stem/pro-genitor cells	Expressed in cancer stem cells	+/+	+	Disease control in 14/21 patients reported[124]	NCT02541370
EpCAM	15-50% HCC 60-80% CCC +	Epithelial cells	Cancer stem cell Growth Invasion	+/+	+	Also tested in colorectal cancer[125]	NCT02729493 NCT03013712 NCT05028933
MUC1	25-65% HCC 40-80% CCC (+)	Epithelial cells	Immune evasion	+/+	+	Target for CCC[126]	NCT02587689
HBsAg	50-60% HCC HBV-assoc 50% CCC +	HBV-infected hepatocytes	Indicates virus integration driving clonal cell proliferation	+/+	No CAR-, but TCT-T cell trials	Human trial with TCRs against HLA-A2/HBsAg [43, 46]	NCT06617000 NCT05339321 NCT06251115 NCT05195294 NCT02719782
CD147	80-90% HCC 50-70% CCC +++	Various tissues	Promotes tumor progression, invasion and metastasis	+/+	Preclinical [127]	Used in dual CARs (GPC3/CD147)	
AFP	60-70% +++	Regenerating hepatocytes	Immunosup-pression Apoptosis	+/+	Preclinical	CAR against peptide AFP158-166 presented on HLA*A02:01	
c-Met	20-50% HCC 50-60% CCC +	Hepatocytes	Receptor tyrosin kinase in HGF dependent proliferation	+/+	Preclinical	Proto-Oncogene[128]	
Claudin-4	80% CCC +++	Minimal	Tight junction formation	–/–	Preclinical	Clinical trials for solid cancers with mRNA vaccine against claudin-6[57, 58]	NCT04503278

Tab. 2 Clinical trials using CAR-T cells in liver diseases.

Indication	Immune cells	Phase	CAR target[Reference]	Amplification	Status	Start	Patients	Outcome	Location	NCT
Liver transplan-tation	CAR Treg	1/2	MHC A2	FOXP3	recruiting	2023	n=20	operational tolerance	UK, EU	NCT05234190
HCC	CAR T cell	1	GPC3[120]		completed	2019	n=13	2PR/2SD	Shanghai, China	NCT03884751, NCT02395250, NCT03146234
HCC	CAR T cell	case report	GPC3[49]				n=1	1 patient CR	Renji, China	NCT03146234
HCC	CAR T cell	1	GPC3[54]	IL-15 armored	completed	2019	n=121	PR 33%, 33%SD	Baylor, US	NCT02905188
HCC	CAR T cell	1	GPC3[102]	RUNX3	completed	2019	n=6	PR17%, SD33%	Zhejiang, China	NCT03980288
HCC	CAR T cell	1/2	GPC3[44]	dnTGFbRII	interim report	2025	n=24	ORR 75%	Zhengzou, China	NCT05155189
HCC	CAR T cell	1/2	GPC3[44]	dnTGFbRII	recruiting	2024	n=121	tumor response rate	Shanghai, China	NCT06590246
HCC	CAR T cell	1/2	GPC3		recruiting	2022	n=105	tumor response rate	Multicnter China	NCT05652920
HCC	CAR T cell	1	GPC3[129]		recruiting	2021	n=38	tumor response rate	NIH, USA	NCT05003895
HCC	CAR T cell	1	GPC3		recruiting	2024	n=48	tumor response rate	Multicenter, Korea/Australia	NCT06478693
HCC	CAR T cell	1/2	GPC3		recruiting	2023	n=94	tumor response rate	US multicenter	NCT06084884
HCC	CAR T cell	1	GPC3		recruiting	2023	n=12	tumor response rate	Korea	NCT05783570
HCC	CAR T cell	1	GPC3		recruiting	2024	n=15	tumor response rate	Zhejiang, China	NCT06461624
HCC	CAR T cell	1	GPC3		active, not recruiting	2021	n=3	tumor response rate	Tongji, China	NCT05070156
HCC	CAR T cell	1	GPC3	IL-15, IL_21	not yet recruiting	2026	n=21	tumor response rate	Baylor, US	NCT06198296
HCC	CAR T cell	1-2	B7H3/CD276		recruiting	2022	n=15	tumor response rate	Xuzhou, China	NCT03993743

HCC	CAR T cell	1	EPCAM		recruiting	2021	n=48	tumor response rate	Zhejiang, China	NCT05028933
HCC	Macrophages	1	HER2		active, not recruiting	2021	n=48	tumor response rate	Portland, US	NCT04660929
HCC	CAR T cell	1	IL1RAP		recruiting	2025	n=18	tumor response rate	Shanghai, China	NCT06757881
CCC	CAR T cell	1	CEA		recruiting	2023	n=36	tumor response rate	Nanchang, China	NCT06010862
CCC	CAR T cell	1	CEA		recruiting	2023	n=60	tumor response rate	Wanan, China	NCT06126406
CCC	CAR T cell	1	unknown		not yet recruiting	2024	n=60	tumor response rate	Hangzhou, China	NCT06196658
CCC	CAR T cell	1	CEA		recruiting	2023	n=30	tumor response rate	Hangzhou, China	NCT06043466
CCC	CAR T cell	1	Mesothelin		recruiting	2023	n=42	tumor response rate	Multicenter, USA	NCT05568680
CCC	CAR T cell	1	Mesothelin		recruiting	2024	n=24	tumor response rate	Bejing, China	NCT06256055

CR complete response; PR partial response; SD stable disease; ORR objective response rate. IL1RAP: interleukin-1 receptor accessory protein; GPC3: glypican-3; CEA: carcinoembryonic antigen; EpCAM: endothelial cell adhesion molecule; B7H3: B7 homolog 3protein; HER2: human epidermal growth factor receptor 2

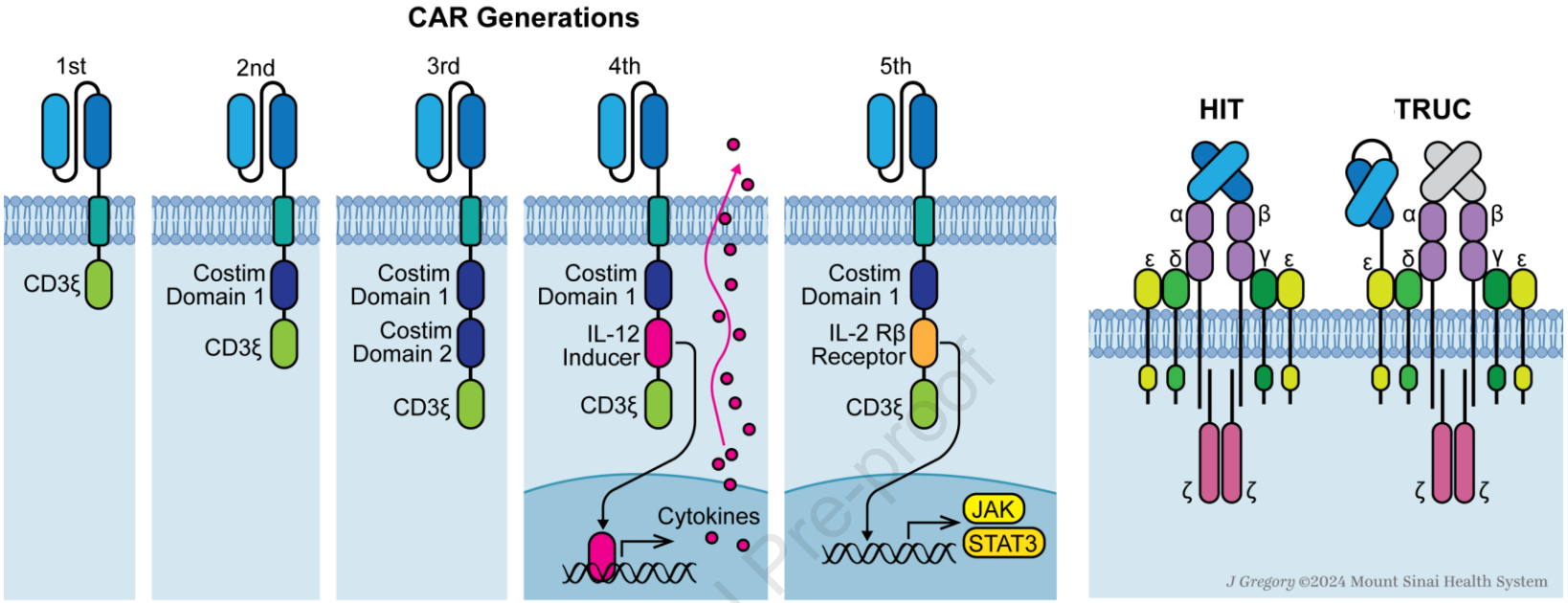


Fig. 1 The evolution of Chimeric Antigen Receptors (CARs)

The first generation of CARs consists of an antibody recognition domain fused to an intracellular CD3-zeta activation domain, providing signal 1 alone. The second generation combines signal 1 with a costimulatory signal 2. The third generation includes two costimulatory domains. The fourth generation combines the CAR with cytokine and antibody secretion to modify the tumor microenvironment. The fifth generation activates intracellular signaling independently of the TCR. Antibody recognition domains can also be directly fused to TCR components, forming HLA-independent TCR receptors (HIT) or T cell receptor fusion constructs (TRuC), which provide enhanced sensitivity but lack costimulatory signals.

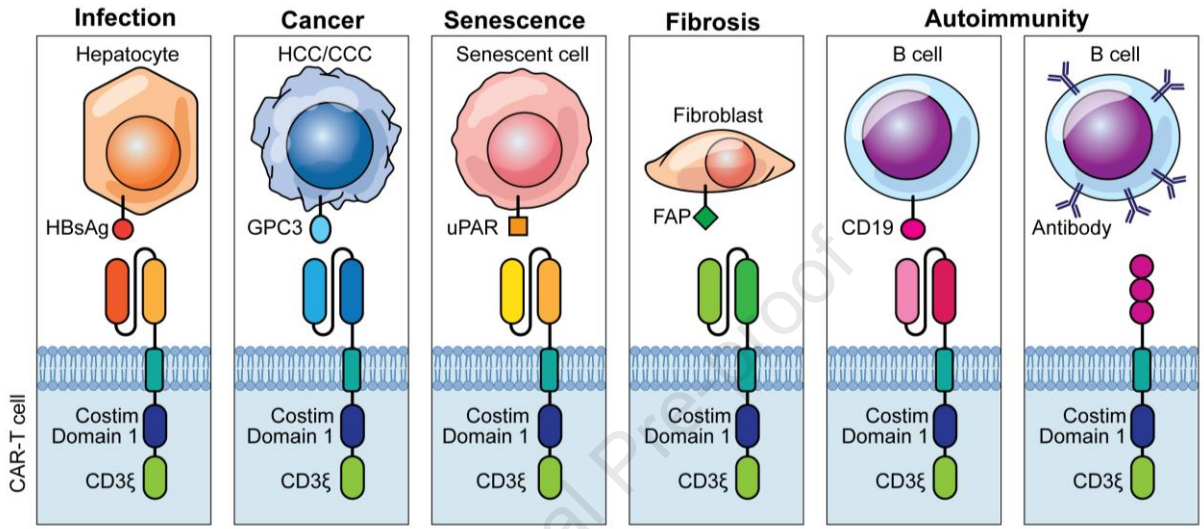


Fig. 2 Therapeutic application of CAR T cells in liver diseases

CAR T cells can be directed against virus-infected hepatocytes, tumor cells, senescent cells, and fibrogenic cells. They can also deplete all B lymphocytes or specifically target autoantigen-specific B cells in autoimmune liver diseases.

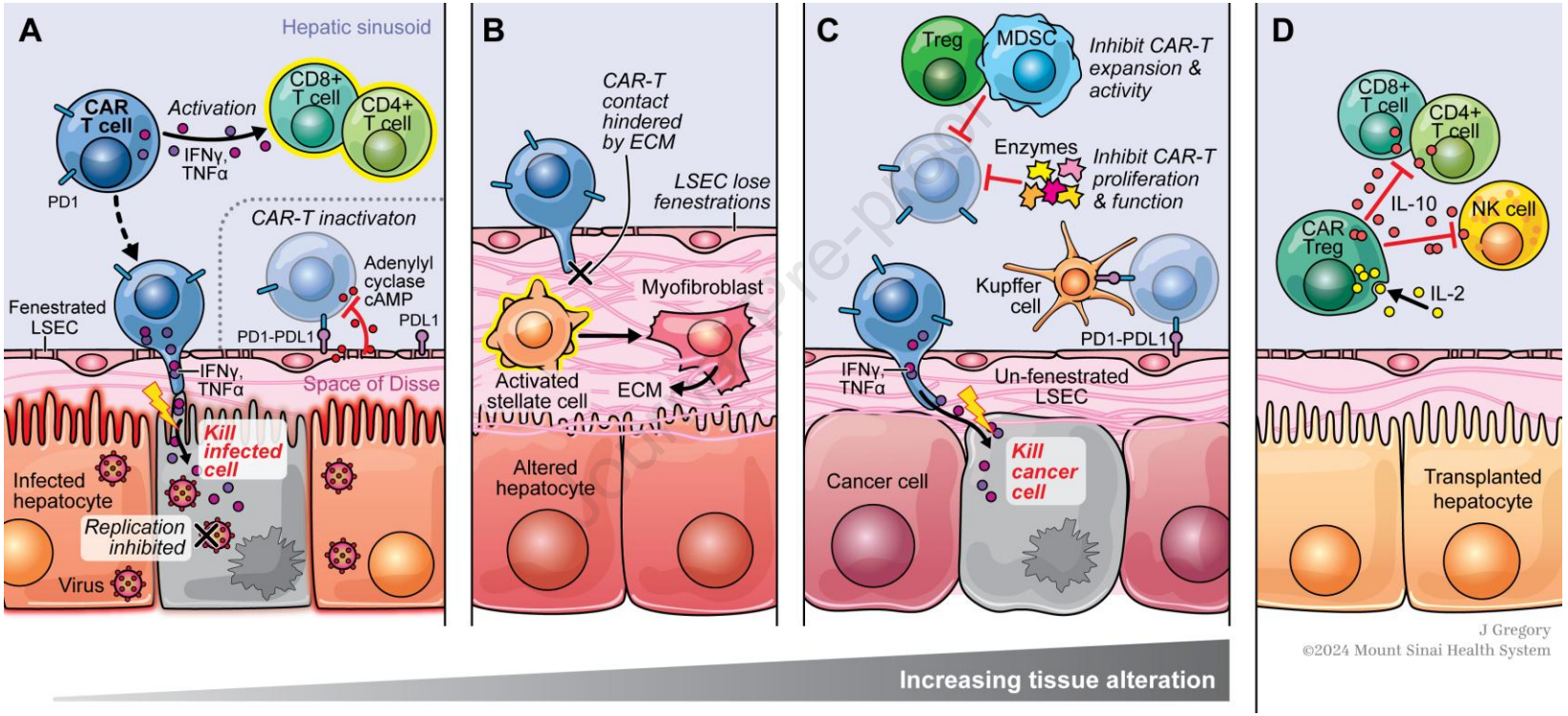

A
Hepatic sinusoid
CAR T cell
Activation
CD8+ T cell
CD4+ T cell
IFNγ, TNFα
PD1
CAR-T inactivaton
Adenylyl cyclase cAMP
PDL1
PD1-PDL1
Fenestrated LSEC
IFNγ, TNFα
Space of Disse
Kill infected cell
Infected hepatocyte
Replication inhibited
Virus
B
CAR-T contact hindered by ECM
LSEC lose fenestrations
Myofibroblast
Activated stellate cell
ECM
Altered hepatocyte
C
Treg
MDSC
Inhibit CAR-T expansion & activity
Enzymes
Inhibit CAR-T proliferation & function
Kupffer cell
PD1-PDL1
IFNγ, TNFα
Un-fenestrated LSEC
Kill cancer cell
Cancer cell
D
CD8+ T cell
CD4+ T cell
IL-10
NK cell
CAR Treg
IL-2
Transplanted hepatocyte
Increasing tissue alteration
J Gregory
©2024 Mount Sinai Health System

Fig. 3

CAR-T production

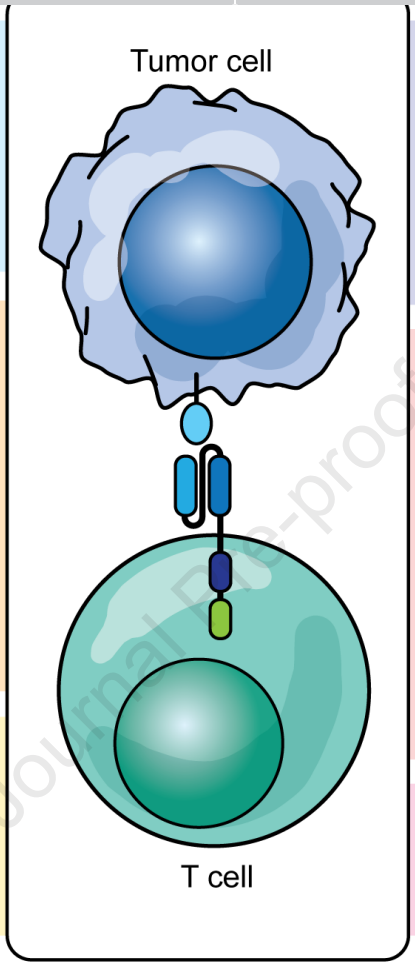
- **Allogeneic, off the shelf T cells**
- T-cell product with balanced CD4/CD8 ratio
- Limited in vitro expansion
- Non viral modification
- Stem cell-like/early memory T cells

Safety

- Short-lived CAR immune cells (NK)
- Suicide genes (iCAS9, RQR8)
- Modified CRISPR approaches
- Nanoengineering, RNA engineering
- Site-directed genomic integration into safe harbors (PASTE, PASSIGE)
- Non-viral CAR integration (sleeping beauty, minicircles)
- SIN viral vectors with eucaryotic promotors

Persistence

- **LNP-mRNA vaccines**
- Intracellular activation motifs
- Cytokines (IL-7, IL-15)
- Switch receptors, 5th gen CARs



Specificity

- TCR-complex based receptors
- Affinity tuned CAR
- Targeting cancer stem cells
- Logically gated CARs
- Dual-/multitarget CARs
- Induced CAR expression

Higher efficacy/TME barriers

- Add. costimulation, transcription factors
- **Cytokine armoured CAR (TRUCK)**
- **Combination with ICI**
- Prevention of exhaustion:
  - CD4 T cell help, TCR complex, on/off CARs
- Improved immune metabolism
- Block of inhibitory signals (e.g. PD1, TGFb)
- Immunoswitches (inhibitory into activating signals
- Induction of tertiary lymphoid structures

Homing of CAR-T cells

- Chemokine recptors
- Local application

Fig. 4 Overview of improvement options for CAR-T cell therapies
This figure illustrates advancements in improving antigen recognition, functionality, tumor and tissue accessibility, CAR-T cell persistence, and safety. The underlined mechanisms highlight promising modifications for clinical application.