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REVIEW

The promise of adoptive cellular immunotherapies in hepatocellular carcinoma

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ABSTRACT

Hepatocellular Carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide. Current systemic therapies result only in modest benefits and new therapeutic options are critically needed. Some patients show promising clinical responses to immune checkpoint inhibitors, however, additional immunotherapeutic approaches, such as adoptive cell therapies (ACT), need to be developed. This review summarizes recent ACT studies and discusses the promise and obstacles of this approach. We further discuss ways of improving the efficacy of ACT in HCC including the use of combination therapies and locoregional delivery methods.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer type and the third leading cause of cancer-related death worldwide. In the United States, the age-adjusted incidence of liver cancer has steadily increased over the last several decades and this year more than 42,000 new cases are expected in the United States alone.[1] This recent increase has been largely attributed to the spread of hepatitis C infection in this country, however increasing rates of metabolic syndrome and nonalcoholic fatty liver disease (NAFLD) are also suspected to be major contributory factors.[2]

In recent years, important advances have been made in the detection and management of HCC. The treatment of HCC is dictated by the size of the tumor, the underlying performance status of the patient (Child-Pugh), and the eligibility for transplant according to the Milan criteria, or more recently, the UCSF or Barcelona Clinic Liver Cancer (BCLC) criteria.[3] Patients with good underlying liver function (Child-Pugh A-B) and early stage disease defined as single tumors (up to 5cm) or small, multinodular tumors (<3 up to 2cm) can be cured through liver transplant and/or tumor resection/ablation. In the setting of intermediate stage disease, patients typically present with large and/or polynodular tumors that are less amenable to the curative treatments. In these patients, the intra-arterial approaches such as Transarterial Embolization (TAE), Transarterial Radioembolization (TARE) and Transarterial Chemoembolization (TACE) represent first line treatments according to most guidelines, although some patient subpopulations may benefit initially from hepatic resection or radiation therapy.[4] In patients with advanced disease and poor underlying liver function (Child-Pugh B-C), treatment options are limited and prognosis is poor with 5-year survival rates as low as 2%.[1] Currently, systemic therapy with sorafenib represents the mainstay of treatment for these patients, but it only extends median survival by 2–3 months obviating the need for additional therapeutic options.[5,6] Building on the successes of sorafenib, newer multi-kinase inhibitors including lenvatinib, regorafenib, cabozantinib, and ramucirumab have recently been tested as first line and second line agents. While these medications have led to modest survival benefits, they are not the therapeutic breakthrough many hoped for.[7] To this end, novel strategies are currently being investigated to improve survival in patients with this devastating cancer. In recent years, immunotherapy including checkpoint inhibition and adoptive cell transfer (ACT) have emerged as treatment options for a variety of different solid tumors, however, no standard immunotherapeutic approach has been defined for HCC.[8] This review will cover some future immunotherapeutic options for patients with HCC with an emphasis on ACT and methodologies for improving its efficacy in solid tumors.

The immune microenvironment of HCC

It has become increasingly recognized that tumors are not just a collection of malignant cells, but instead a complex mixture of enzymes, growth factors, signaling factors, and immune cells which together modulate tumor development and progression.[8] The microenvironment of HCC is thought to be highly immunosuppressive resulting in the production of a generally weak and inefficient anti-tumor immune response. As with many solid tumors, this is in part due to the high metabolic status of HCC. Rapid cellular growth and division creates a hypoxic, acidic, and nutrient-poor microenvironment which restricts T cell proliferation and cytokine production.[9] More specific to HCC, by virtue of the fact that the liver is continuously exposed to antigens from the gut microbiota, the organ itself has

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developed a tolerance to immune activation. This immunotolerance is imparted by a high concentration of immunosuppressive T-regulatory cells (Tregs) relative to effector T cells.[10] The role of a Treg is to dampen the local immune system directly and indirectly by increasing the content of inhibitory cytokines like TGF-B, IL-4, and IL-10 as well as other inhibitory signals like CTLA-4 and PD-1 (Figure 1a), which attenuate the proliferation of activated T-cells.[11] Furthermore, since the majority of HCCs develop in chronically inflamed and fibrotic livers secondary to viral hepatitis, alcohol abuse, and metabolic disease, these malignancies evade the immune system simply by undermining homing of immune cells into the tumor tissue (Figure 1b). The few immune effector cells that still arrive in the immediate tumor environment will face a dramatic downregulation of human leukocyte antigen (HLA) molecules presenting peptide fragments of tumor antigens (Figure 1c) and the co-stimulatory molecules CD80 and CD86.[12] However, as our understanding of the tumor microenvironment has evolved, approaches to modulate the local immune system to counter the immunosuppressive factors in HCC have emerged.

Spontaneous anti-tumor immune responses in HCC

The immune contexture of HCC includes a wide variety of cells that are necessary for innate and adaptive immune system activation. This includes amongst many others, the liver sinusoidal endothelial cells (LSECs) which function as APCs, $CD8^+$ cytotoxic T lymphocytes (CTLs), and $CD4^+$ T helper cells. Despite the aforementioned challenges, spontaneous and durable immune responses do occur in HCC and have been associated with improved survival and recurrence outcomes after surgical resection.[13,14] Such immune responses are predominantly $CD8^+$ T cell-driven and are directed against well-established tumor associated antigens (TAA) such as alpha-fetoprotein (AFP) and glypican-3 (GPC3).[15] These data suggest that all of the essential elements for local T cell activation are present in principle in HCC and that the immune system can hopefully be leveraged to attack the tumor.

Immunotherapies targeting HCC

Recent research efforts in cancer treatment have been geared toward mobilizing the patient's endogenous immune system to attack cancer cells. Immune checkpoint inhibitors (CKI), for example, are an antibody-based immunotherapy that effectively unleashes the immune system from a suppressed state by blocking cancer-mediated repressors of the immune system such as CTLA-4 and PD-1.[16] So far checkpoint inhibition has produced mixed results in advanced HCC disease.[17] In one of the first published trials (checkpoint 040), 47 patients with sorafenib-resistant HCC including 33 with extrahepatic metastasis and 6 with vascular invasion were treated with the anti-PD1 monoclonal antibody nivolumab. The response rate and disease control rates (DCR) were favorable with 19% of patients demonstrating disease regression and 67% of patients showing stable disease.[18] These data led to the accelerated FDA approval of nivolumab for HCC patients who failed sorafenib and served as the impetus for numerous subsequent trials exploring the expanded clinical use of nivolumab and other checkpoint inhibitors both as first-line monotherapy and as combination therapies in HCC.[19,20] Unfortunately, however, new unpublished data from these extension studies including the checkpoint 459 trial, which looked at nivolumab against sorafenib as a first line treatment, and the Keynote-240 trial, which investigated pembrolizumab against placebo as a second line treatment, both showed no statistically significant survival benefit leaving the door open for other immunotherapeutic approaches.[21,22]

Adoptive cell transfer (ACT), the focus of this review, encompasses cell-based immunotherapies that target cytotoxic T-cells to tumor cells using tumor-specific antigens.[23] Included in this class of immunotherapies are the tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-engineered T cells, and chimeric antigen receptor (CAR) T cells (Figure 2). Of these subtypes, TILs were the earliest development whereby a sub-population of endogenously produced tumor-specific T cells from the patient's blood or, preferentially, tumor tissue were enriched. Following *in vitro* expansion, the anti-tumor T-cells were then transfused into the host to target and destroy tumor cells. Although the results from this approach have been promising in a few specific cancers including one study on HCC,[24] the application of TILs is limited by the natural T cell repertoire of the host and technical difficulties in isolating and expanding these cells on an individual basis.[25] In light of these limitations, a second iteration of ACT came with the development of TCR-engineered T cells. Here, using genome engineering techniques, TCRs targeting specific tumor antigens are transduced into patient- or donor-derived T cells before expansion and transfusion. While this development has demonstrated considerable promise in pre-clinical studies (Table 1) targeting GPC3[26] and AFP,[27,28] these cells are dependent on the antigens being presented in an HLA context, the expression of which, as discussed above, is down-regulated in many cancers.[29] This downregulation may explain the unfortunate failure of HBV-TCR T-cell therapy as demonstrated in one patient with an extrahepatic HCC metastasis.[30]

To circumvent the HLA requirement, a third iteration of ACT came with the development of chimeric antigen receptor (CAR) T cells. In this technique, chimeric T cell receptors composed of an extracellular single chain variable fragment (scFv) antigen-specific target binding domain, a costimulatory transmembrane domain, and a CD3ζ-derived intracellular signaling and activation domain are artificially expressed in T cells. This approach in principle enables the same limitless targeting potential as TCR-engineered T cells without the dependence on HLA/TCR interactions.[31] CAR T cell therapy has proven effective in a variety of hematologic cancers, most significantly in the treatment of CD-19 expressing B cell acute lymphoblastic leukemia and B cell lymphomas.[32] The use of CAR T cell therapy in solid tumors including HCC, however, has been stalled by several obstacles that will be outlined below.[33]

Obstacles of ACT in HCC

The widespread use of ACT in solid tumor cancer treatment has been hampered by four major issues which together affect their *in vivo* efficacy and tolerability. This includes: (1) the lack of well-defined solid-tumor associated antigens (TAA) and, in the case of TCR-transduced T cells, the loss of HLA molecules on the tumor

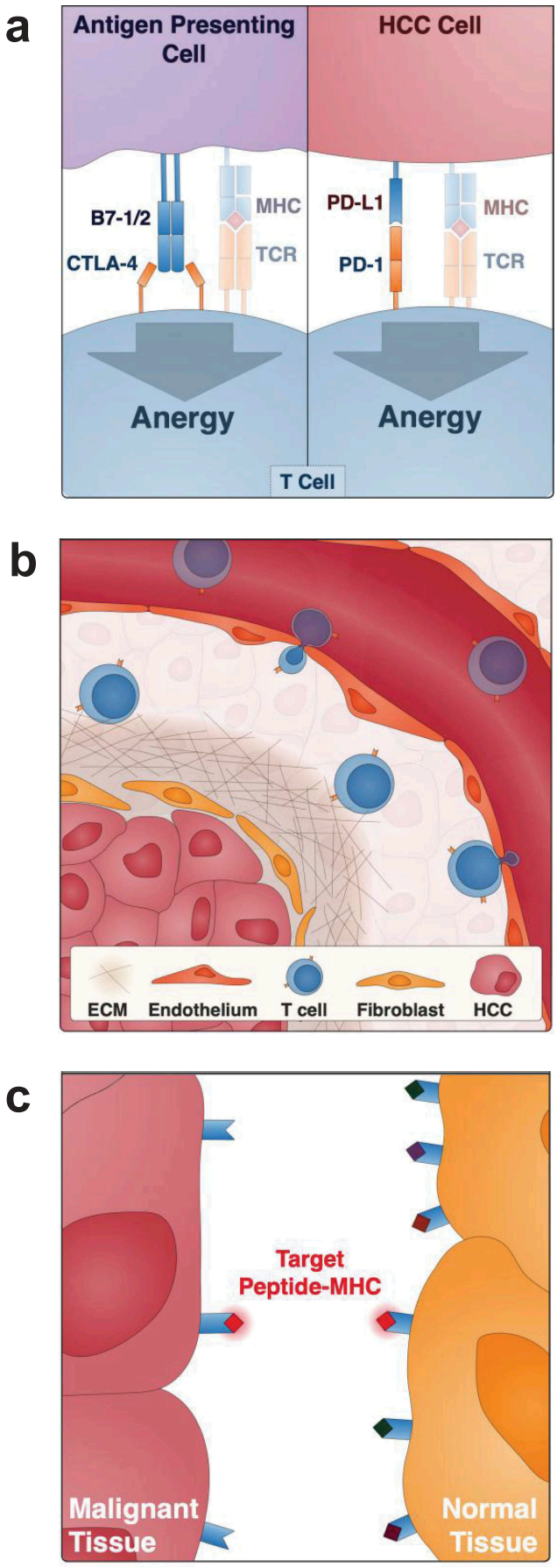

Figure 1. Obstacles to the use of ACT in HCC.

(a) Overexpression of inhibitory molecules CTLA-4 (left) and PD-1/PD-L1 (right) in patients with HCC. (b) Poor invasion of HCC by immune cells due to fibrous stroma. (c) Lack of immunogenic tumor antigens and loss of antigen-presenting HLA molecules on the HCC tumor cells.

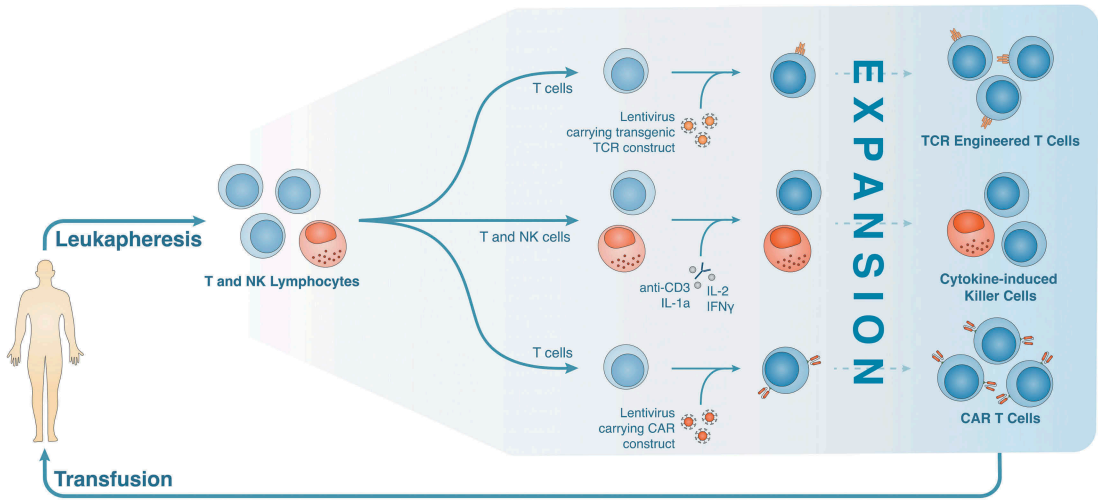

Figure 2. Production and optimization of TCR-engineered T cells, cytokine-induced killer cells, and CAR T cells.

Table 1. Clinical trials investigating systemic TCR/CAR T-cell delivery in HCC.

NCT Number	Phase	Target	Treatment Type
NCT01967823	Phase 2	NY-ESO-1	TCR T Cells
NCT02869217	Phase 1	NY-ESO-1	TCR T Cells
NCT03159585	Phase 1	NY-ESO-1	TCR T Cells
NCT03441100	Phase 1	Undisclosed CTA	TCR T Cells
NCT03175705	Phase 1	Multiple Antigens[a]	TCR T Cells
NCT03971747	Phase 1	AFP	TCR T Cells
NCT03132792	Phase 1	AFP	TCR T Cells
NCT03349255	Phase 1	AFP	CAR T Cells
NCT03965546	Phase 1	AFP	TCR T Cells
NCT02541370	Phase 1	CD-133	CAR T Cells
NCT03672305	Phase 1	c-Met	CAR T Cells
NCT03013712	Phase 1/2	EpCAM	CAR T Cells
NCT02729493	Phase 2	EpCAM	CAR T Cells
NCT03941626	Phase 1/2	EGFRvIII/DR5	CAR T Cells
NCT03638206	Phase 1/2	DR5	CAR T Cells
NCT02395250	Phase 1	GPC3	CAR T Cells
NCT03884751	Phase 1	GPC3	CAR T Cells
NCT02723942	Phase 1/2	GPC3	CAR T Cells
NCT03198546	Phase 1	GPC3	CAR T Cells
NCT03084380	Phase 1/2	GPC3	CAR T Cells
NCT03146234	Phase 1	GPC3	CAR T Cells
NCT02905188	Phase 1	GPC3	CAR T Cells
NCT02959151	Phase 1/2	GPC3	CAR T Cells
NCT02715362	Phase 1/2	GPC3	CAR T Cells
NCT03302403	Phase 1	GPC3	CAR T Cells
NCT02719782	Phase 1	HBV	TCR T Cells
NCT03899415	Phase 1	HBV	TCR T Cells
NCT02686372	Phase 1	HBV	TCR T Cells
NCT02587689	Phase 1/2	MUC1	CAR T Cells

[a]GPC3, NY-ESO-1, and AFP

cells, (2) the limited ability of exogenous T cells to traffic to the organ of interest and penetrate the fibrotic tumor stroma, (3) the intrinsically immunosuppressive tumor microenvironment and (4) off-target effects and toxicity (Figure 1a–c). Here, we review the evidence behind these limitations and highlight methodologies for overcoming these challenges to work toward the goal of expanding ACT to HCC.

Targeting the tumor: finding better antigens

A major barrier concerning the use of ACT in solid tumor immunotherapy is the identification of tumor-associated antigens (TAA). A preferable TAA is one that is universally highly expressed in tumor cells and negligibly expressed in normal tissues.[23] Unlike hematologic cancers, solid tumors often lack the expression of specific surface molecules necessary for CAR T cells. However, members of a unique class of intracellularly expressed proteins, cancer-testis (CT) antigens or cancer-germline (CG) antigens, have recently emerged as preferable immunotherapeutic targets in a number of solid tumor cancers, including HCC. More than 200 genes at present make up the CT antigen gene family. CT gene expression is normally restricted to male germ cells and early embryonic development, however, many CT genes are aberrantly expressed in human cancers including HCC.[34] In recent years, CT antigen gene function has been extensively studied with the hypothesis that embryonic or gametogenic transcriptional programs, which includes the CT genes, are reactivated in cancers and serve as a driving force for tumorigenesis. Through this work, we have learned that a number of CT antigens including MAGE-A, MAGE-C2, SSX, and PRAME withhold potent oncogenic properties by conferring resistance to apoptosis, invasive/metastatic potential, and uncontrolled growth.[34–36]

In addition to being cancer-specific, CT antigens are immunogenic meaning they are capable of evoking a robust spontaneous and vaccine-induced immune response. Several recent trials using CT antigens as immuno-targets have provided encouraging results. To this end, Odunsi et al. showed that treating advanced stage melanoma and ovarian carcinoma with an NY-ESO-1 vaccine induces a $CD8^+$ T cell response which correlates with progression free survival.[37] Likewise, using an NY-ESO-1 directed TCR-based therapy, multiple independent groups have succeeded in inducing durable anti-tumor responses and tumor regression in patients with metastatic melanoma and synovial sarcoma.[38,39] Most recently, Stevanovic et al. induced complete cancer regression in a patient with metastatic HPV-positive cervical carcinoma using TILs immunodominant for the CT antigen KK-LC-1.[40]

Although the expression and specificity of many different CT antigens have been confirmed in HCC, the targets with highest priority currently are NY-ESO-1 and MAGE-A1.[41]

NY-ESO-1 and MAGE-A1 are expressed in approximately 45% and 70% of HCC tumors, respectively, and have been associated with HCC tumor grade, metastasis, and recurrence post resection.[42,43] As with GPC3 and AFP, mentioned above, spontaneous immune responses against NY-ESO-1 and MAGE-A1 have also been described in a subset of HCC patients with advanced disease. Importantly, in these patients, the presence of naturally occurring CTA-specific $CD8^+$ cytotoxic T cells is associated with improved survival outcomes.[14] In line with these observations, multiple clinical trials are now underway targeting CTAs in HCC using TILs, TCR engineered T cells, and even CAR T cells (Table 1).

Improving T cell trafficking and tissue penetration: local delivery of cellular immunotherapies

Another factor regulating the efficacy of ACT transfer in HCC and other solid tumor malignancies is the relative inability of systemically infused T cells to migrate to and infiltrate the tumor stroma.[23] In part, the poor trafficking of adoptive T cells may be linked to a chemokine and chemokine-receptor mismatch between the tumor and the engineered cells. Innovative strategies, including the use of CAR-T cells co-expressing CCR2, the receptor for a functional tumor trafficking chemokine, have been developed but have led to only modest increases in anti-tumor activity indicating multiple additional barriers to successful trafficking.[44] One consideration is that many HCC tumors develop in the setting of cirrhosis (Figure 1b) which could make it difficult for systemically delivered T-cells to physically infiltrate the tumor and generate an efficacious response.[45] The extent to which this is true needs to be investigated as does the safety of this cytotoxic anti-tumor treatment in such a compromised organ.

With on-going advancements in imaging technology and intravascular techniques, a promising new strategy involves delivering ACT immunotherapy loco-regionally, either directly into the organ of interest or intratumorally if the surrounding fibrosis proves too tough for the cells to penetrate on their own (Figure 3). Not only would such an approach mitigate the trafficking issue, it could also decrease the well-known off-target and maybe even off-tissue toxicities associated with systemic infusion of manipulated T cells. Using a mouse model of human pleural malignancy, Adusumilli et al. demonstrated that intrapleural delivery of anti-mesothelin CAR-T cells outperformed intravenously delivered CAR T cells, which were dosed 30-fold higher, with respect to the magnitude and durability of tumor regression.[46] More recently, a pre-clinical mouse study of HER2+ breast cancer metastatic to the brain showed that intra-cranial delivery of anti-HER2 CAR T cells facilitated complete tumor regression whereas systemic delivery, even with higher doses of CAR-T cells, resulted in only partial tumor regression.[47] The first human trial investigating loco-regional delivery of immunotherapy to the liver was performed in metastatic colorectal cancer patients. In this study, anti-CEA CAR T cells were delivered directly to the liver through hepatic artery infusion (HAI).[48] In one patient, the CAR T cells were undetectable in the peripheral blood three days after delivery but they persisted in the liver for up to three months.[49] Clinically in these patients, localized and sustained CAR-T cell targeting coincided with increased tumor necrosis and fewer off-target effects compared to prior experiments using systemic infusion.[50] Based on these studies, multiple clinical trials are now underway looking at the efficacy of local T-cell delivery in primary and metastatic liver cancers (Table 2).

Combined ACT approaches to circumvent local immunosuppression

In theory, even if tumor-specific T cells can be effectively delivered to the liver, the immunosuppressive microenvironment

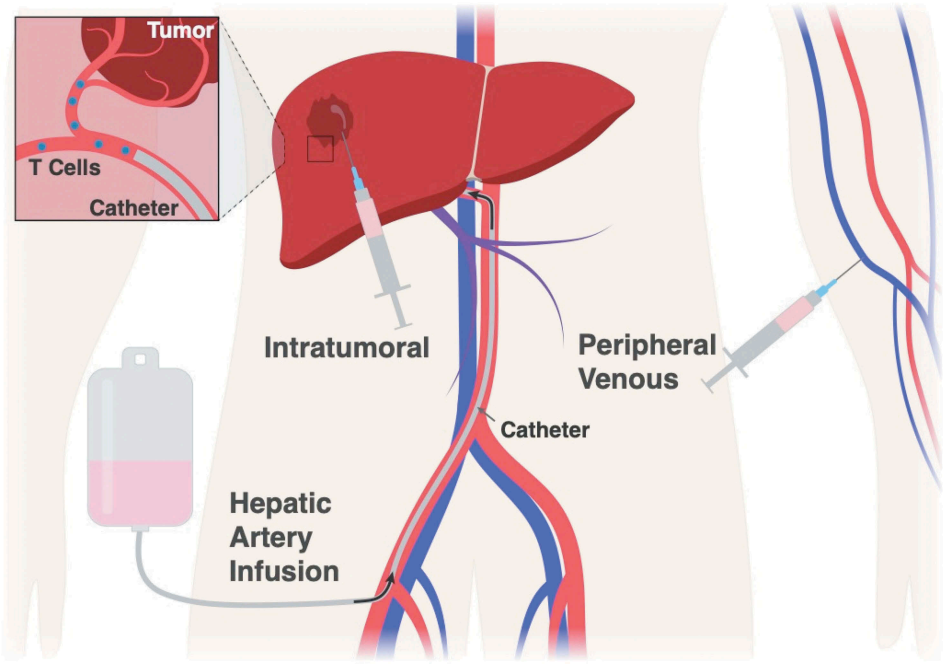

Figure 3. Different approaches for the local delivery vs. systemic administration of genetically engineered T cells for the treatment of HCC.

Table 2. Clinical trials investigating liver directed ACT.

NCT Number	Phase	Tumor Type	Origin	Target	Treatment Type
NCT03888859	Phase 1	Primary	HCC	AFP	TCR T Cells
NCT01373047	Phase 1	Metastatic	Adenocarcinoma	CEA	TCR T Cells
NCT02416466	Phase 1	Metastatic	Adenocarcinoma	CEA	CAR T Cells
NCT02850536	Phase 1	Metastatic	Adenocarcinoma	CEA	CAR T Cells
NCT02715362	Phase 1/2	Primary	HCC	GPC3	CAR T Cells
NCT03130712	Phase 1/2	Primary	HCC	GPC3	CAR T Cells
NCT02862704	Phase 1/2	Metastatic	GI Cancers	MG7	CAR T Cells
NCT02632201	Phase 1/2	Metastatic	Gastric Cancer	HER2	PIK-HER2 Cells/ DC-PMAT
NCT03370198	Phase 1	Metastatic	Colorectal Cancer	Multiple Antigens[a]	CAR T NKR-2 Cells
NCT03310008	Phase 1	Metastatic	Colorectal Cancer	Multiple Antigens[a]	CAR T NKR-2 Cells

[a]MICA, MICB, RAET1E/ULBP4, RAET1G/ULBP5, RAET1H/ULBP2, RAET1/ULBP1, RAET1L/ULBP6, and RAET1N/ULBP3

intrinsic to HCC must simultaneously be overcome. One strategy for doing so could be to strategically combine ACT with other systemic or locoregional treatments such as the tyrosine kinase inhibitors (e.g. sunitinib, sorafenib) or CKI (e.g. nivolumab, pembrolizumab, tremelimumab). Interestingly, in addition to its tumoricidal effects, sunitinib- but not sorafenib- has been shown to produce a strong immunomodulatory effect and suppress HCC progression when combined with immunotherapy.[51] A CKI combination would likely also enhance the local immune response thus improving T cell effector function, survival, and proliferation within a tumor. It is important to point out however that these combinations must be tailored to specific populations of HCC patient as there are marked differences in the endogenous immune response mounted in patients with HCC cancers caused by nonalcoholic steatohepatitis (NASH) compared to those caused by chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. For example, the $CD8^+$ T cells in NASH-related HCC predominantly express CTLA4 and thus would most likely benefit from combinations with ipilimumab whereas those in HBV-related cancers which express PD-1 would perhaps benefit more from nivolumab.[52] Another consideration is the mutation profile of the HCC which likely affects its immunogenicity. Tumors carrying the CTNNB1 mutation for example are poorly infiltrated by immune cells and tend to be resistant to CKI whereas tumors containing a TP53 mutation, which is associated with a high tumor mutational burden, are more likely to respond.[53,54] How the molecular features of a cancer affect the efficacy of ACT, if at all, remains to be studied. In addition to selecting specific checkpoint inhibitors based on the molecular and immune profile of the tumor, it is also conceivable that one could administer checkpoint treatment intratumorally to avoid the side-effects of systemic treatment. Along these lines, multiple clinical trials are currently open investigating the efficacy and toxicity of intratumoral ipilimumab in head and neck cancer (NCT02812524), glioblastoma (NCT03233152), colorectal cancer (NCT03982121), and melanoma (NCT02857569). Alternative approaches to combine ACT with checkpoint inhibition involve using CAR-T cells containing a genetically engineered mutant PD-L1 receptor, potentially preventing tumor-induced downregulation of effector T cell function. So far, these studies have demonstrated comparable efficacy while avoiding the common toxicities associated with checkpoint inhibition such as fatigue, rash, and diarrhea.[55,56]

Another strategy for enhancing the efficacy of CAR T-cells *in vivo* is to combine their delivery with tumor ablation or ionization radiation (IR) administered through external beam techniques (e.g. stereotactic body radiation therapy, SBRT) or with brachytherapy (i.e. Y90 radioembolization). Both ablation and IR affect the tumor microenvironment and the immune system in numerous ways. In addition to directly damaging tumor cell DNA, these treatments likely help provoke a productive immune response which may boost CAR-T cell function. In HCC, radiofrequency ablation (RFA) has been shown to increase the number of tumor antigen specific T-cells leading to improvements in recurrence-free survival.[57,58] Likewise, in a model of pancreatic cancer it was recently demonstrated that IR increases the susceptibility of antigen-negative tumor cells to CAR T cell therapy reducing the risk of tumor relapse.[59] One plausible explanation for this effect is that IR promotes antigen availability and presentation in otherwise antigen-negative tumor cells. A second and non-mutually exclusive explanation is that IR simply promotes a pro-inflammatory tumor microenvironment through tumor-intrinsic production of Type 1 Interferon (IFN) leading to the activation of M1 macrophages that drive tumor rejection.[60,61] This mechanism is thought to account for the rare anti-tumor response that occurs at distant disease sites after radiation therapy known as the abscopal effect.[62]

As IR can exacerbate underlying liver disease, alternative strategies for increasing antigen availability within the HCC tumor microenvironment should also be explored including local ablation and epigenetic inhibitors. One recently published study examined the use of epigenetic therapy consisting of small molecule inhibitors against DNMT1 and EZH2 in combination with CKI. Here, the authors were able to show that epigenetic therapy induces tumor cell expression of neoantigens like NY-ESO1 and stimulates better T cell trafficking and tumor cell apoptosis which together enhanced tumor regression compared to immunotherapy alone.[63] Perhaps the use of these inhibitors can be combined with ACT to improve trafficking and prevent antigen escape.

Conclusions and perspectives

The incidence of HCC continues to rise with limited treatment options currently available for many patients. Recent breakthroughs in cancer immunotherapy have led to renewed hope and enthusiasm for improved HCC treatments. Clinical responses have been achieved in a subset of patients with the use of immune checkpoint inhibitors. Clinical studies are already exploring the use of ACT such as CAR T cells and TCR-transduced T Cells with some promising preliminary findings. In addition, combination immunotherapies are emerging as

further strategies to further improve outcomes, particularly in the setting of an immunosuppressive microenvironment. Further studies to evaluate timing and locoregional delivery approaches of immunotherapies are necessary to identify the ideal approaches to improve treatment response and simultaneously reduce systemic toxicities.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PGH, DA and GCF reviewed the literature and wrote the manuscript. MLO wrote the manuscript and prepared the figures. TL and TH wrote the manuscript. SW wrote the manuscript and prepared the tables.

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