

## Immunotherapy for hepatocellular carcinoma: From basic research to clinical use

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been proven efficient in HCC treatment, particularly for those patients not indicated for curative resection or transplantation. Immunotherapy has been developed for decades for cancer control and is attaining more attention as a result of encouraging outcomes of new strategies such as chimeric antigen receptor T cells and immune checkpoint blockade. Right at the front of the new era of immunotherapy, we review the immunotherapy in HCC treatment, from basic research to clinical trials, covering anything from immunomodulators, tumor vaccines and adoptive immunotherapy. The mechanisms, efficacy and safety as well as the approach particulars are unveiled to assist readers to gain a concise but extensive understanding of immunotherapy of HCC.

**Key words:** Interferon; Chemokine; Tumor vaccine; Adoptive immunotherapy; Chimeric antigen receptor; Checkpoint blockade

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**Core tip:** This paper supplies a comprehensive review of immunotherapy for hepatocellular carcinoma from basic experiments to clinical trials. The development of interferon, chemokines, tumor vaccines, adoptive immunotherapy, including natural killer, natural killer T and T cells armed with chimeric antigen receptor, as well as regulatory T cell is summarized.

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### Abstract

Hepatocellular carcinoma (HCC) is a common cancer worldwide with a poor prognosis. Few strategies have

### INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 95%

of primary liver cancer<sup>[1]</sup>, the second most common cause of cancer-associated death worldwide and estimated to be responsible for around 746000 deaths in 2012<sup>[2]</sup>. Only liver resection and liver transplantation are considered curative, with poor efficiency of other modalities such as radiofrequency ablation (RFA) and transarterial chemoembolization (TACE). However, very few patients are indicated for liver resection and donors of liver are usually exceptional. Postoperative recurrence frequently occurs, resulting in a dismal prognosis in most affected individuals<sup>[3]</sup>.

Immunotherapy has been explored in HCC for decades<sup>[4]</sup> and carries high expectations due to the recent progress in other malignancies such as melanoma. Different from other organs, liver shows its distinguished characteristics, such as an "immune organ", and patients with HCC present with unique anti- or pro-tumor responses during the development and progression of HCC<sup>[5]</sup>. Immunotherapy can be categorized into several types according to their distinct strategies. For instance, immunomodulators and tumor vaccines are used to enhance the immune response to HCC in an indirect way; adoptive immunotherapy introduces a great amount of effective immune cells to directly remove tumor cells. In this review, we summarize the critical immune characteristics of liver and cover the immunotherapy of HCC in animal models as well as clinical trials (Figure 1).

## ROLES OF THE IMMUNE SYSTEM IN CARCINOGENESIS AND PROGRESSION OF HCC

The inherent immune tolerance of liver hinders immune surveillance and therefore makes the carcinogenesis of HCC possible. Liver confronts abundant xenogenous antigens within blood from the gut *via* the portal vein. Specific mechanisms with regards to immune tolerance are activated to inhibit unneeded immune responses. Unfortunately, these mechanisms, such as recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells, as well as overexpression of inhibitory ligands such as programmed death ligand 1 (PD-L1), contribute to weaken anti-tumor immune responses<sup>[6]</sup>.

As an "immune privileged" organ, multiple pathways exist within liver to maintain its function. Not only can hepatocytes cause T cell anergy in certain conditions, but many other nonparenchymal cells, including stellate cells, hepatic dendritic cells and liver sinusoidal endothelial cells, also induce tolerance or apoptosis of effective or naive T cells. In particular, HCC lacks major histocompatibility complex (MHC) class II, the activator of CD4<sup>+</sup> T helper (Th) cells<sup>[6]</sup>.

Clinically, various cytokines dysregulate and contribute to HCC progression<sup>[7]</sup>. Increased immunosuppressed cells in patients are in parallel with a poorer prognosis. Th17 and its secretory product interleukin

17 (IL-17) promote angiogenesis of HCC and recruit neutrophils to enhance angiogenesis<sup>[8,9]</sup>. The effector function of CD8<sup>+</sup> T cells is prone to be impaired by increased Tregs, which predicts a poor prognosis of HCC patients<sup>[10]</sup>. In addition, the functional impairment of other cells like natural killer (NK) cells also contributes to tumor progression<sup>[11]</sup>.

## IMMUNOMODULATORS

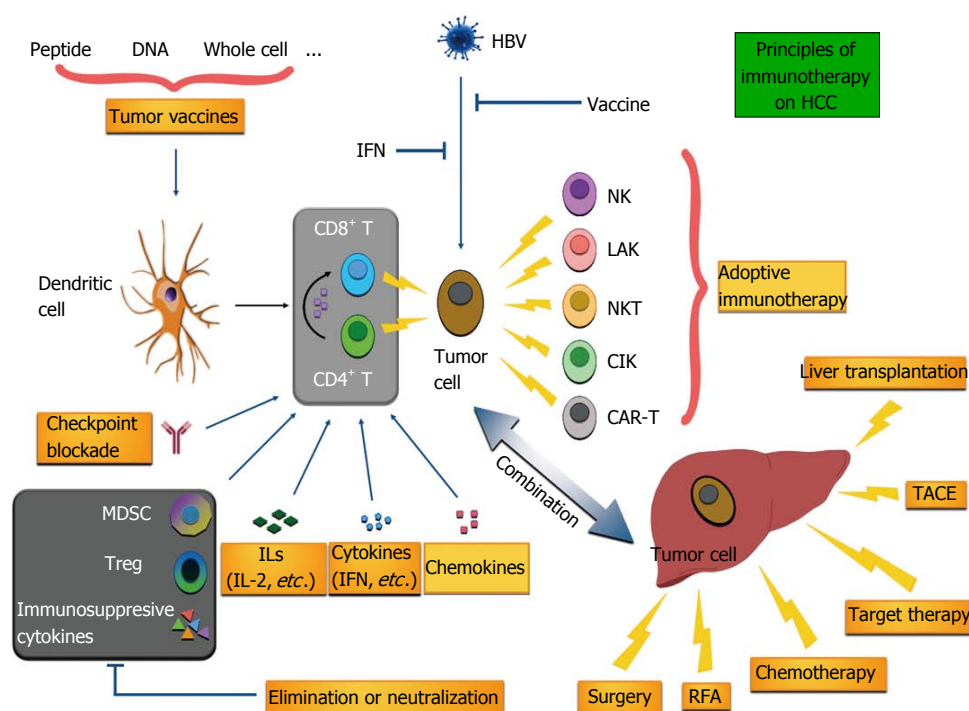
### Cytokines

Various cytokines are involved in immune responses. Certain cytokines can directly inhibit tumor cell growth or enhance the capacity of relevant immune cells to delay tumor development.

Interferon is well known for immunomodulation, anti-proliferation and anti-angiogenesis. They were found to be decreased in serum of patients with HCC. All three types of interferon (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ) have been proved to be effective in inhibiting HCC by inducing tumor cell apoptosis or autophagy<sup>[12-14]</sup>. However, the efficiencies among different types of interferon are under debate. Although some investigators have addressed that IFN- $\beta$  showed better anti-HCC effects<sup>[15]</sup>, the use of IFN- $\alpha$  in HCC treatment have been more frequently reported. However, IFN- $\alpha$  alone did not show satisfactory survival benefit in patients with unresectable HCC, confirmed by randomized controlled trials (RCTs)<sup>[16,17]</sup>. IFN- $\alpha$ -2b also failed to decrease the risk of postoperative recurrence<sup>[18]</sup>. In contrast, interferon showed some benefits when combined with other modalities such as chemotherapy, curative resection and TACE<sup>[19-22]</sup>. Two meta-analyses revealed that adjuvant interferon therapy after curative therapy for HCC could improve both overall survival and recurrence-free survival<sup>[23,24]</sup>. Combination of IFN- $\alpha$  with sorafenib was also reported to be efficient in a mouse model<sup>[25]</sup> but this has not been tested in humans. In addition, interferon treatment may gain further benefits for HCC patients with hepatitis B or C virus infection from removing the viruses<sup>[26,27]</sup>. Currently, two registered clinical trials regarding IFN- $\alpha$  are still recruiting participants. One multicenter RCT is planning to test IFN- $\alpha$  as an adjuvant therapy in HCC with low miR-26 expression (NCT011681446) and the other phase II trial is trying to combine IFN- $\alpha$  with fluorouracil to treat HCC patients who underwent liver resection (NCT01834963). Generally, IFN- $\alpha$  showed demonstrated equivocal effects and should only be applied to selected patients as supportive or adjuvant therapy within the assumption of the current evidence.

### ILs

ILs have been applied to enhance anti-tumor responses of the immune system. However, few studies concerning these ILs have been performed in humans. Small scale clinical studies evaluated the efficacies of IL-2 or IL-12 alone or combined together in HCC treatment but the results were inconclusive<sup>[28,29]</sup>. Other sorts of ILs were



**Figure 1** Principles of immunotherapy on hepatocellular carcinoma. NK: Natural killer; LAK: Lymphokine-activated killer; NKT: Natural killer T; CIK: Chemokine-induced killer; CAR-T: Chimeric antigen receptor-T; HBV: Hepatitis B virus; MDSC: Myeloid-derived suppressor cells; Treg: Regulatory T; IL-2: Interleukin 2; IFN: Interferon; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma.

also studied. For instance, IL-37 was found to selectively recruit NK cells to conduct anti-tumor activity in HCC patients<sup>[30]</sup>. IL-24 was reported to show *in vivo* anti-tumor activity in the presence of apoptin<sup>[31]</sup>. However, clinical trials are lacking to determine the therapeutic effects of ILs in human.

### Chemokines

Chemokines regulate activities and behavior of cells, including hepatocytes, immune cells and the tumor micro-environment. By mediating pro- and anti-inflammatory responses, chemokines can regulate leukocyte recruitment, angiogenesis and tumor progression<sup>[32]</sup>. Several chemokine-associated signaling, including CXCR4/CXCL12, CCR6/CCL20 axes, were evident in promoting HCC<sup>[33,34]</sup>. Blockade of these signalings by relevant receptors seems logical to control HCC.

Chemokines can regulate the function of immune cells by interacting with the receptors on the membrane of these cells. Tumor infiltrating anti-tumor cells, including T cells, NK cells and natural killer T (NKT) cells, showed enhanced expression of certain chemokine receptors<sup>[35]</sup>. Consistently, overexpression of certain chemokine genes, such as CXCL10, CCL5 and CCL2, in HCC tissue correlated with Th1, cytolytic T lymphocyte (CTL) and NK cells and predicted a better prognosis<sup>[36]</sup>. Regulated release of chemokines or genetic modification of chemokine receptors in immune cells may enhance anti-tumor immune response. Unfortunately, only preclinical data can be found in this field.

## IMMUNE CHECKPOINT BLOCKADE THERAPY

Due to the great achievements in melanoma, immune checkpoint blockade therapy sheds light on other solid tumors, including HCC. Co-inhibitory signals diminish the intensity of anti-tumor response even although HCC specific antigen has presented with MHC receptors. To overcome these, immune checkpoints should be a promising approach to restore anti-tumor function of immune cells. Many immune checkpoints have been identified in the lab. In addition to PD-1 and CTLA-4 which have been intensively studied, there are also other potential checkpoints, like TIM-3, OX40, VISTA, LAG-3 and BTLA<sup>[37]</sup>.

Anti-CTLA-4 antibody blocks the binding of CTLA-4 and CD80/86, which is a defunct antigen-presenting cell (APC) and results in suppressed anti-tumor immune responses mediated by T cells<sup>[38,39]</sup>. Basically, the efficacy of CTLA-4 blockade correlates with the immunogenicity of the tumor. A phase I trial of tremelimumab (anti-CTLA-4 monoclonal antibody) in HCC patients was reported in 2013<sup>[40]</sup>. The study enrolled 21 patients with advanced HCC not amenable to percutaneous ablation or TACE and showed that tremelimumab was well tolerated. Partial response was found in 17.6% of the patients and 45% of the cases had stable disease for more than 6 mo. Another phase I clinical trial of tremelimumab combined with RFA or TACE is now ongoing (NCT01853618; Table 1).

**Table 1** Information of clinical trials of checkpoint blockade on hepatocellular carcinoma after 2008

Interventions	Design	Start year	Main inclusion criteria	Primary outcomes	Registered No.	Status
CP-675,206: anti-CTLA-4 antibody	Phase II	2008	Unresectable disease not amenable to loco regional treatment, HCV chronic infection	Tumor response	NCT01008358	Completed
CT-011 (Pidilizumab): anti-PD-1 antibody	Phase I / II	2009	HCC not eligible for surgery, TACE, or other systematic treatments	Safety and tolerability	NCT00966251	Terminated because of slow accrual
Nivolumab: anti-PD-1 antibody	Phase I	2012	Advanced HCC, failed in previous one line therapy	Adverse events	NCT01658878	Recruiting
Tremelimumab: anti-CTLA-4 antibody, combined with TACE or RFA	Phase I	2013	Not amenable to curative resection, transplantation or ablation	Safety and feasibility	NCT01853618	Recruiting

HCV: Hepatitis C virus; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; HCC: Hepatocellular carcinoma; PD-1: Programmed death 1.

Anti-PD-1 and anti-PD-L1 antibodies interfere with the binding of PD-1 and PD-L1/2 which inhibits T cell proliferation and cytokine release<sup>[41]</sup>. Although CTLA-4 and PD-1 were found predominantly expressed in T cells with anti-tumor function<sup>[42,43]</sup> and showed similar effects when used alone, different mechanisms and indicated patients regarding the two pathways were suggested by clinical observations<sup>[44]</sup>. Unfortunately, the phase I / II trial (NCT00966251) of a new PD-1 blockade CT-011 was terminated because of slow accrual. Another phase I trial of nivolumab (anti-PD-1 monoclonal antibody) is ongoing. This trial plans to recruit three cohorts of patients stratified by viral infection hepatitis C virus, hepatitis B virus (HBV) and no viral infection.

With the satisfactory effects in animal models, more and more trials are being conducted to investigate the role of immune checkpoint blockade therapy in HCC treatment (Table 1). Immune checkpoint blockade therapy is considered to be a strategy with a bright future and is cast into the limelight by oncologists worldwide.

## TUMOR VACCINES

Although prophylactic vaccines for HCC such as HBV vaccine contributed to the decrease of HCC patients<sup>[45]</sup>, a therapeutic vaccine for HCC is still awaited. Numerous approaches have been investigated, seeking to trigger the host immune system to remove cancer cells. The most important constraint for progression of a tumor vaccine is the lack of tumor specific antigens or tumor associated antigens (TAA). With all the evolving understanding of tumor heterogeneity, it appears to be unprecedentedly challenging to exterminate cancer cells by a tumor vaccine alone. On the other hand, it could possibly be an effective approach to exhibit positive aspects in certain patients and play important roles in regimens.

### Peptide-based vaccine

Alpha-fetoprotein (AFP) and glypican-3 (GPC3) are two frequently used TAAs in HCC vaccines. GPC3 can be overexpressed in more than 80% of HCC and AFP can be positive in 60%-80% of HCC. Other HCC biomarkers

that might be candidates for a vaccine involving antigens include squamous cell carcinoma antigen, heat shock protein 70, NY-ESO-1b, *etc*<sup>[46,47]</sup>.

AFP is rare in healthy adults but can be highly expressed in HCC, making it an ideal target for anti-HCC immunotherapy. Actually, AFP is currently the most well studied target antigen for HCC immunotherapy. The earliest relevant clinical trial was more than a decade ago, testing AFP-specific T cell response to an AFP-derived peptide in six patients<sup>[48]</sup>. GPC3 (144-152) (FVGEFFTDV) and GPC3 (298-306) (EYILSLEEL) peptides were proven to induce specific CD8<sup>+</sup> CTLs in HCC patients with HLA-A2 and HLA-A24 restriction, respectively<sup>[49]</sup>. Encouraged by this result, a phase II trial of a GPC3-based vaccine as adjuvant therapy for patients after surgery or RFA was registered and is now ongoing (UMIN-CTR: 000002614).

Proper design of epitopes with cross-recognition of wild-type antigens can enhance immune responses. To overcome the limitation of weak immune responses induced by native TAAs, Hong *et al*<sup>[50]</sup> created a highly immunogenic AFP *via* computer-guided methodical epitope optimization. This genetic modified AFP vaccine showed amazing anti-tumor effects in xenograft and diethylnitrosamine-induced mouse model of HCC by means of activating CD8<sup>+</sup> T cells<sup>[50]</sup>. Polypeptide or fusion peptide was another method to amplify anti-tumor immune responses. A combination of full-length HBV core protein, HBV-X protein (HBX)<sub>52-60</sub>, HBX<sub>140-148</sub>, AFP<sub>158-166</sub> and melanoma antigen gene-A<sub>271-279</sub> is an example for a HCC vaccine<sup>[51]</sup>. Innovative creation of a fusion peptide containing different epitopes that involve multiple steps of the immune response was also proved to inhibit HCC in animals<sup>[52]</sup>.

For a peptide-based tumor vaccine, the choice of peptide is critical for clinical response. Not all proteins that contribute to tumor progression are suitable for vaccine development. For instance, although expression and activity of telomerase was found up-regulated in most HCCs<sup>[53,54]</sup>, telomerase peptide did not lead to any complete or partial responses in a phase II study on advanced HCC<sup>[55]</sup>. Additionally, the origin of the peptide



affects induction efficiency of CLTs and consequent anti-tumor effects. Peptides originated from endogenously presented antigen are thought to be sparse on tumor cells and inefficient in inducing CTLs<sup>[56]</sup>. Intratumoral peptide injection was thus developed to enhance tumor cell antigenicity<sup>[56]</sup>; however, this needs further investigation.

### **DNA-based vaccine**

A DNA-based vaccine assumes that DNA directly injected into the body undergoes transcription and translation in host cells and that the expressed peptide induces immune responses. Theoretically, all peptide vaccines can be transformed into DNA vaccines. AFP and GPC3 DNA vaccines were both developed and tested in the lab and showed tumor growth inhibition and survival improvement in mouse models<sup>[57-59]</sup>. In a recent small scale clinical observation, two HCC (stage II) patients after locoregional therapy underwent AFP DNA vaccine and adenovirus boost immunization. This approach was confirmed to be safe and well tolerated; however, both patients experienced HCC recurrence after a mere nine and eighteen months, respectively<sup>[60]</sup>. To our best information, no clinical trial regarding DNA vaccines on HCC has been reported or is currently ongoing.

### **Tumor vaccine using antigen-presenting cells**

APCs play a key role in anti-tumor function of immune responses. Dendritic cells (DCs) are the most potent APC and are closely related to HCC. Numerous studies have proved that DCs from peripheral blood and lymph nodes of HCC patients were decreased, with an immature phenotype and an impaired function<sup>[61-63]</sup>. A study revealed that the more DCs that were detected in HCC nodules, the better the prognosis would be. Infiltration of DCs in HCC nodules was strongly associated with the prognosis of HCC patients after surgical resection<sup>[64]</sup>. The composition of DCs in the hepatic lymph nodes of HCC patients was aberrant which may be one of the causes of the inadequate T cell response against HCC in these patients<sup>[65]</sup>. In addition, some tumor-derived factors, such as vascular endothelial growth factor, granulocyte macrophage colony-stimulating factor, IL-6 and IL-10, influence the differentiation, number and phenotype of DCs<sup>[66]</sup>.

Given the importance in cancer development, DCs are increasingly applied to vaccination in various cancers, including HCC. A DC-based vaccine was reported to not only induce tumor antigen-specific CTLs<sup>[67]</sup>, but also to activate NK cells and inhibit Tregs in HCC patients<sup>[51,68]</sup>. Logically, DCs pulsed with tumor tissue of an individual patient should be used. However, the tumor tissue is not always available. Therefore, peptides or cell line lysate was commonly used to substitute tumor tissue by many investigators. At least in HCC, this replacement strategy proved to be feasible and safe<sup>[69]</sup>. DCs infused with cancer cells, transfected

with total RNA of cancer cells or transfected with designed plasmids were all able to mature and prime Th1 cells and CTLs<sup>[69-71]</sup>.

Based upon these characteristics and success in preclinical studies, many clinical trials were carried out to evaluate the efficacy of DC-based immunotherapy to treat HCC patients. Two phase I studies showed immunization by tumor lysate pulsed DCs was feasible for end stage HCC patients<sup>[72,73]</sup>. Another clinical trial of a DC vaccine pulsed with autologous tumor lysate addressed that 12.9% of advanced HCC patients had partial response (PR) and 54.8% had SD<sup>[74]</sup>. Notably, a monthly boost vaccination resulted in a significantly better 1 year survival<sup>[74]</sup>. In another RCT on advanced HCC, DCs pulsed with HepG2 cell lysate resulted in 13.3% patients with PR and 60% with SD after 6 mo of treatment<sup>[75]</sup>. However, the proportion of clinical response with this therapy is relatively low. As an illustration, one phase II study using DCs pulsed with tumor lysate in HCC revealed only one out of 39 patients exhibited PR<sup>[76]</sup>. Furthermore, a phase I/II study using a multiple TAA-pulsed DC vaccine showed clinical response in only one out of five patients with advanced HCC<sup>[77]</sup>. Some studies also evaluated the efficacy of DC immunotherapy combined with local radiation<sup>[78]</sup> or TACE<sup>[79,80]</sup> but the results showed that DC infusion could not prevent HCC recurrence. Therefore, further studies are needed to increase the efficacy of this therapeutic approach. A new phase I trial on DC vaccine for HCC was registered last year and is now recruiting participants (NCT 01974661; Table 2).

## **ADOPTIVE IMMUNOTHERAPY**

Adoptive immunotherapy is now promising in the scenario of potential approaches for the treatment of solid tumors which are refractory to conventional therapies. An increasing amount of the literature discusses the efficacy of adoptive immunotherapy to control tumors. Meanwhile, many clinical trials have demonstrated that adoptive immunotherapy showed potentially promising anti-tumor effects on various cancers, including HCC.

### **Lymphokine-activated killer cells**

First reported in the early 1980s, lymphokine-activated killer (LAK) cells are cytotoxic effector lymphocytes whose cytolytic activities are not restricted by MHC and are capable of killing tumor cells as well as NK-resistant tumor cell lines. There are actually constrained studies concerning the effectiveness of LAK cells for HCC treatment. A report stated that dealing with LAK cells cultivated by IL-2 reduced the recurrence rate in postoperative HCC patients<sup>[81]</sup>. Another study found that LAK cell-based immunotherapy was not an ideal adjuvant strategy after hepatic resection<sup>[82]</sup>. Results from a clinical trial indicated that tumor-specific CTL therapy is more effective than LAK cell therapy in advanced HCC patients<sup>[83]</sup>. The enthusiasm of study on

**Table 2** Information of clinical trials of tumor vaccine on hepatocellular carcinoma after 2008

Interventions	Design	Start year	Main inclusion criteria	Primary outcomes	Registered No.	Status
AFP + GM-CSF plasmid prime and AFP adenoviral vector boost	Phase I / II	2008	Locoregionally treated HCC	Dose, toxicity, and immunological response rate	NCT00669136	Terminated because of poor accrual
DC loaded with autologous tumor	Phase II	2008	Metastatic HCC, available of tumor tissue	2-mo response rate	NCT00610389	Unknown
DC loaded with specific peptides of AFP	Phase I / II	2009	Patients with previous treatment, AFP $\geq$ 40 ng/mL, HLA A 0201 group	Adverse events	NCT01128803	Terminated
DEC-205-NY-ESO-1 fusion protein vaccine	Phase I	2012	After resection and TACE for HCC	Adverse events	NCT01522820	Recruiting
COMBIG-DC: allogeneic DC cancer vaccine	Phase I	2013	Not eligible for curative treatment or TACE, BCLC stage B and C	Adverse events	NCT01974661	Recruiting
<i>In-situ</i> therapeutic cancer vaccine	Phase I	2013	Refractory HCC, not eligible for or failed any treatment, AFP > 30	Safety	NCT01923233	Recruiting
V5 therapeutic vaccine	Phase III	2014	Advanced HCC	Changes in plasma AFP	NCT02232490	Not yet recruiting

AFP:  $\alpha$ -fetoprotein; DC: Dendritic cell; GM-CSF: Granulocyte macrophage colony-stimulating factor; HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; TACE: Transarterial chemoembolization; BCLC: Barcelona clinic liver cancer classification.

LAK for HCC treatment has significantly declined over the last decade.

### Cytokine-induced killer cells

Cytokine-induced killer (CIK) cells exhibit potent, non-MHC restricted cytolytic activities against susceptible tumor cells of both autologous and allogeneic origins<sup>[84]</sup>. CIK cells are characterized by expression of both T cell biomarker CD3 and NK cell biomarker CD56<sup>[85]</sup> and can be generated from the human peripheral blood mononuclear cells (PBMC) induced by IFN- $\gamma$ , anti-CD3 antibody and IL-2<sup>[84,86,87]</sup>.

Compared with other immune cells, CIK cells possess some advantages. Firstly, CIK cells have a higher proliferation rate and can be obtained from cancer patients by *in vitro* culture<sup>[88]</sup>. Secondly, CIK cells have strong cytolytic activities and cover a broad spectrum of targeted tumors, including those that are insusceptible to LAK cells or NK cells<sup>[89]</sup>. Finally, CIK cells show minimal toxicity and do not cause graft-vs-host disease<sup>[84,90]</sup>. These merits make CIK cells a preferential adoptive immunotherapy for selected cancer patients<sup>[91]</sup>.

Recently, escalating proof from clinical trials demonstrated that CIK cells adoptive transfer demonstrated a substantial anti-tumor effect in patients with solid tumors and hematological malignancies<sup>[92-94]</sup>. Some reports showed that CIK adjuvant immunotherapy significantly improved the outcomes of HCC patients<sup>[95-101]</sup>. In these studies, CIK cell transfusion reduced the relapse rate of HCC in patients after TACE and RFA therapy and prolonged the disease-free survival and OS for HCC patients after radical resection or TACE. A meta-analysis including 13 RCTs evaluated the efficacy of CIK immunotherapy in the treatment of HCC and revealed a significant superiority in prolonging the OS and progressive-free survival of patients<sup>[102]</sup>. Recently, another meta-analysis assessed the efficacy of CIK

therapy after TACE or TACE plus RFA and showed that CIK therapy combined with TACE plus RFA treatment was associated with a higher 1 year recurrent free survival rate and 1, 2 year OS rates<sup>[103]</sup>. However, due to the limited number of patients in this field, the efficacy of CIK immunotherapy for HCC is still not convincing.

CIK cells are an encouraging tool within cancer adoptive immunotherapy but more basic research and clinical trials with high quality are urgently desired. To date, we have witnessed at least four ongoing registered clinical trials regarding CIK therapy for HCC (Table 3).

### NK cells

NK cells belong to the innate immune system, predominantly reside in the liver and play a critical role in the host defense against tumorigenesis<sup>[104,105]</sup>. Carcinogenesis is under close surveillance of NK cells and other members of immune system. In addition to the capability of killing tumor cells directly, NK cells are able to release immunomodulatory cytokines which can activate leukocytes of both the innate and adaptive immune system<sup>[105]</sup>. Unfortunately, cytotoxicity of NK cells was significantly inhibited in patients with advanced HCC<sup>[104]</sup>. In line with this, NK cells derived from HCC patients displayed a reduced cytotoxicity against HCC cell lines after stimulation with IL-2 *in vivo*<sup>[106]</sup>. The authors suggested that functional defects of NK cells might be responsible for the failure of anti-tumor immune responses. The NK cells from HCC patients were also impaired in their IFN- $\gamma$  production and cytotoxicity and this functional impairment was found to be associated with increased Tregs<sup>[107]</sup>. Meanwhile, myeloid-derived suppressor cells inhibited NK cell cytotoxicity and cytokine secretion<sup>[108]</sup>. This evidence suggested that HCC patients could benefit from reactivation of NK cells. In a mouse model, administration of IL-12 and IL-18 increased NK cells in the liver and resulted in

**Table 3** Information of clinical trials of adoptive therapy on hepatocellular carcinoma after 2008

Interventions	Design	Start year	Main inclusion criteria	Primary outcomes	Registered No.	Status
Immunocell-LC: activated T lymphocyte	Phase III	2008	Stage I and II, complete resection within 12 wk	Efficacy and safety	NCT00699816	Completed
CIK	Phase III	2008	After radical resection of HCC, no prior anti-cancer therapy	Time to recurrence	NCT01749865	Recruiting
CIK	Phase III	2008	After radical resection, no prior anti-cancer treatment	Time to recurrence	NCT00769106	Recruiting
<i>Ex vivo</i> expanded autologous immune killer cell, combined with TACE	Phase II / III	2009	Never receive TACE treatment, BCLC stage B and C	2-yr reduction of tumor cells	NCT01024530	Unknown
NK cells, combined with liver transplantation	Phase I	2010	After liver transplantation for HCC	Side effect	NCT01147380	Ongoing, but not recruiting
Young TIL	Phase II	2010	Metastatic HCC with at least one lesion resectable	Tumor regression rate	NCT01174121	Recruiting
Autologous tumor infiltrating lymphocytes, combined with IL-2	Phase I	2011	Metastatic HCC	Safety and tolerability	NCT01462903	Unknown
CIK, combined with Licartin	Phase IV	2012	Postoperative patients	1-yr PFS	NCT01758679	Recruiting
Dendritic and cytokine-induced killer cells	Phase II	2013	After complete resection or TACE	PFS	NCT01821482	Not yet recruiting
CTL induced by DC loaded with multiple antigens	Phase I	2013	Complete tumor resection within 8 wk	2-yr PFS and adverse events	NCT02026362	Recruiting
DC incubated with irradiated autologous tumor stem cells + GM-CSF	Phase I	2013	Candidates for HCC resection	Vital signs, physical examinations and adverse events	NCT01828762	Completed
Cord blood-derived CIK	Phase I	2013	After radical resection	Adverse events	NCT01914263	Not yet recruiting
Autologous NKT cells	Phase I	2013	Advanced HCC, refractory to standard treatments	Adverse events	NCT01801852	Recruiting
Immunocell-LC, combined with Nexavar	Phase II	2013	Stage III and IV, receiving or ready for Nexavar treatment	2-yr PFS	NCT01897610	Recruiting
MG4101: <i>ex vivo</i> expanded allogeneic NK cell	Phase II	2013	Stage III, after curative resection	1-yr DFS	NCT02008929	Recruiting

CIK: Cytokine-induced killer; CTL: Cytolytic T lymphocyte; DC: Dendritic cell; DFS: Disease-free survival; NK: Natural killer; NKT: Natural killer T; PFS: Progression-free survival; TACE: Transarterial chemoembolization; TIL: Tumor infiltrating lymphocyte; HCC: Hepatocellular carcinoma; GM-CSF: Granulocyte macrophage colony-stimulating factor; IL-2: Interleukin 2; BCLC: Barcelona clinic liver cancer classification.

reduction of intrahepatic tumor nodules<sup>[109]</sup>. A similar outcome was obtained in additional research which established that activation of NK cells increased survival in a xenograft mouse model<sup>[110]</sup>. Thus, the approach of enhancing the function of NK cells could possibly be accomplished in human HCC treatment. Although much solid evidence showed the role of NK cells in an anti-tumor reaction, there are, however, insufficient clinical studies to corroborate the efficacy of NK cell immunotherapy in HCC. Recently, a study demonstrated that RFA could activate the peripheral blood circulating NK cells in HCC patients<sup>[111]</sup>. Two ongoing clinical trials are trying to assess NK cell therapy combined with liver resection (NCT02008929) or liver transplantation for HCC (NCT01147380; Table 3). In the future, it would be of great interest to investigate the efficacy of NK cells combined with other strategies to improve immunotherapy in HCC.

### NK T cells

NK T (NKT) cells are a heterogeneous group of T cells with a range of characteristics different from conventional T cells. Human NKT cells are found in small numbers in healthy liver (0.5%) and blood

(0.02%)<sup>[112,113]</sup>; however, they are critical players in the regulation of anti-tumor immunity<sup>[114-116]</sup>. NKT cells are best known for their immunosuppressive functions; however, they can interact with many other immune cells, such as DCs, macrophages as well as NK cells, and the outcome of NKT cell stimulation depends on these interactions and the cytokine milieu<sup>[115]</sup>. NKT cells can manifest anti-tumor effects mediated by their reactivation with exogenous cytokines or ligands but recently the natural role of NKT cells in anti-tumor immunity was reported<sup>[112,115]</sup>. A phase I trial using autologous NKT cells to treat advanced HCC is now ongoing (NCT01801852; Table 3). Further investigation is needed to elucidate the role of NKT cells in human HCC.

### Chimeric antigen receptor-T cells

One of the most important aims of T cell engineering is to generate tumor-targeted T cells through the genetic transfer of antigen specific receptors. T cell engineering consists of either physiological, MHC-restricted T cell receptors (TCRs) or non-MHC-restricted chimeric antigen receptors. The conception of the chimeric antigen receptor (CAR) originally generated from the

growing understanding of the barriers to effective immune therapy of various types of cancers. T cells armed with CARs (CAR-T cells) are able to recognize the cell surface antigens directly and are not blunted by tumor variations possessing lower surface expression of major MHC antigens which are considered a common mechanism of tumor immune escape<sup>[117,118]</sup>. The three basic elements of CAR are an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain.

To date, three generations of CARs have been developed. The first generation CAR only contains a T cell signaling domain that transmits the activation signal. It owns a feature of a single signaling domain most commonly derived from the CD3 $\zeta$  component of the TCR/CD3 complex. The full activation of T cells needs multiple signals and it is obvious that the signaling from these first-generation CARs only provided the so-called "signal one" that could drive T cell effector functions; however, due to the absence of further signals, the T cells are unable to fully engage their effector machinery<sup>[119]</sup>. Considering this modular nature of the CAR, later designs aimed to add additional signaling domains which would increase the potency of the CARs and the consequent effector function of T cells. The second generation CARs incorporate a single co-stimulatory molecule endodomain, such as the endodomain of CD28 or 4-1BB<sup>[120]</sup>. The third generation CARs incorporate at least two co-stimulatory molecule endodomains, such as the endodomains of CD28 and 4-1BB<sup>[121]</sup>. Ligation of CD28 on CAR-T cells through the expression of B7 co-stimulatory ligands on target cells or co-expression of the CD28 molecule together with the scFv (specific monoclonal antibody) and CD3 $\zeta$  domain of the CAR was shown to promote the proliferation of CAR-modified T cells and anti-tumor activity<sup>[122-124]</sup>.

Plenty of studies have evaluated the efficacy of CAR-T cell treatment in hematological malignancies such as lymphoid leukemia<sup>[125,126]</sup> and acute myeloid leukemia<sup>[127,128]</sup>. Nonetheless, the clinical efficacy of CAR-T cells continues to be marginal in solid tumors compared to leukemia. So far, as we have acknowledged, there are no reported clinical trials evaluating the efficacy of CAR-T in HCC. Only one research stated that genetically modified T cells could be used to reconstitute virus-specific T cell immunity in chronic HBV patients and target tumors in HBV-related HCC. They found TCR re-directed HBV-specific T cells produced from PBMC of hepatitis B related HCC patients were capable of recognizing HCC tumor cells<sup>[129]</sup>.

Besides the failed clinical trials of CAR-T cells in renal cell cancer and ovarian cancer<sup>[130-132]</sup>, the safety and tolerance of CAR-T cells likewise need further assessment since the trial ceased and the demise of patients was often documented<sup>[124,133,134]</sup>. In a clinical trial of renal cell cancer, a maximum of 10 infusions of a total 0.2 to 2.1  $\times 10^9$  CAR-T cells administered to patients manifested in liver enzyme disturbances and the treatment was finally stopped<sup>[133]</sup>. A patient receiving CAR-T cells based on a

Her2/neu-specific CARs died soon after the treatment<sup>[124]</sup>. These data from renal carcinoma seemed to indicate a dim future for CAR-T applications in solid tumors. To achieve a better clinical response, CAR-T cells need to overcome two major barriers, which are insufficient T-cell migration into the lesions and highly immunosuppressive microenvironments within the tumors<sup>[118,135,136]</sup>.

Considered collectively, the original studies highlighted two important lessons. To begin with, clinical anti-tumor responses of T cells seem to be proportional to T cell persistence. Consequently, we ought to enhance the T cell persistence and then try to efficiently traffic adequate quantities of CAR-T cells from the peripheral blood towards tumor tissues. Once there, these CAR-T cells must functionally respond against tumor cells within such an immunosuppressive context. Secondly, understanding and defining the specific target is crucial for the safety of CAR-T treatment. Increasing evidence demonstrates that a careful choice of target antigen, including an understanding of accessibility and expression level, must be under consideration for future CAR-T clinical trials.

### ***Treg-based immunotherapy***

Treg mediated immunosuppression is the essential mechanism accountable for tumor immune evasion and could be the primary hurdle of tumor immunotherapy<sup>[137]</sup>. Tregs, characterized by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>, play a critical role in immune homeostasis and suppress function of immune cells, such as CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells and NKT cells. Many reports have demonstrated that the amount of Tregs in solid tumor decreased and was inversely related to the prognosis of HCC patients<sup>[10,138-141]</sup>.

Targeting Tregs has been of great interest to change the immune suppression milieu and enhance tumor specific immune responses. Depletion of Tregs using anti-CD25 monoclonal antibody has been shown to have substantial anti-tumor effects in murine tumor models<sup>[142]</sup>. Nonspecific approach of CD25<sup>+</sup> T cell depletion by injection of PC61 antibody was also tested on an orthotopic HCC model and led to a significant protection against tumor development<sup>[143]</sup>. No such strategy has been performed on humans yet.

## **CONCLUSION**

An array of translational research and pilot clinical trials have revealed that immunotherapy is safe and tolerated by patients with cancers. The efficacies are also offered in some types of immunotherapy in selected patients. In HCC, more studies, including basic and clinical research, are urgently required to improve the outcomes of immunotherapy with best cost performance. Currently, immune checkpoint blockade and CAR-T strategies are specifically expected. In addition, it is become obvious that incorporating standard anti-tumor therapies with immunotherapy is the most likely effective alternative. Ultimately, more substantial randomized, controlled trials are required to authenticate the efficacy of



immunotherapy for HCC patients.

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